Immunomodulation by Ectosomes

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Table of Contents

General Summary

Considerable progress has been made in recognizing microvesicles as important mediators of intercellular communication rather than irrelevant cell debris. Microvesicles released by budding directly from the cell membrane surface either spontaneously or in response to various stimuli are called ectosomes. Of particular interest is that ectosomes released by polymorphonuclear neutrophils (PMN-Ect) down regulate the inflammatory potential of macrophages. This finding was a real surprise since PMNs are known to be proinflammatory cells *par excellence*. Thus the very same cells known for their destruction capacity have the property of releasing ectosomes, which inhibit further macrophage activation, although various cytokines released by PMNs are chemotactic for macrophages. This might be a powerful regulatory mechanism responsible for the control of excess inflammation. In my thesis I wanted to

(1) see whether the same ectosomes have also the property to down regulate dendritic cells (DCs), so as to prevent the activation of the acquired immune system (T cells), when it is not required (section 1).

(2) see whether ectosomes from other cells would have similar properties. For this we investigated ectosomes released by erythrocytes (section 2).

(3) define the signaling pathways induced by ectosomes (section 3).

(1) PMN-Ect were recently shown to induce an anti-inflammatory response on human monocyte-derived macrophages (HMDMs). I found that PMN-Ect also inhibited the LPS-induced maturation of human monocyte-derived DCs (MoDCs). This effect was evidenced by reduced expression of cell surface markers (CD40, CD80, CD83, CD86 and HLA-DP DQ DR), inhibition of cytokine release (IL-8, IL-10, IL-12, and TNFα), and new morphological and functional characteristics (reduced phagocytic activity, and increased TGF-β1 release). Importantly, the skewed MoDC differentiation resulted in a reduced capacity to activate T-cells, suggesting an active role for PMN-Ect in adaptive immunity as well.

(2) Microvesicles derived from erythrocytes during storage had all the properties of ectosomes. E-ecto revealed an inhibitory potential on zymosan A and LPS activated HMDMs as shown by down-regulation of IL-8 and TNFα release. However, different from PMN-Ect, E-ecto did not enhance the release of TGF-β1. In addition, the effect of E-ecto was found to be long lasting. Thus, E-ecto transfused with erythrocytes may account for some of the immunosuppressive effects seen after blood transfusions

(3) The receptors and signaling pathways involved in ectosome-induced down-modulation are unknown. I showed that the encounter of PMN-Ect with HMDMs induced an immediate calcium flux. Mer receptor as well as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway were activated, and NFκB translocation and phosphorylation were blocked. Consequently, the transcription of many proinflammatory genes in zymosan A activated HMDMs were reduced. Finally, my data revealed that TGF-β1 release induced by PMN-Ect was not related to a modification in its transcription.

Taken together these results suggest that ectosomes have a profound effect on the innate immune system, here macrophages, as well as on the induction of the adaptive immune system, here DCs, globally reprogramming these cells toward an immunosuppressive and possibly tolerogenic phenotype.

General Introduction

Various eukaryotic cell types release membrane-derived microvesicles under specific physiologic conditions. Whereas this fact was relatively unknown, and was not considered important until recent years, latest studies point toward a common mechanism that involves these vesicles in intercellular cross-talk (1). Interestingly, this phenomenon seems conserved during evolution, since even bacteria are described to release microvesicles that are important components of biofilms, and a major signal trafficking system (2, 3).

Vesiculation is a physiological mechanism that is used in cell growth, protection and activation. For example, in the mechanism of mineral formation in cartilage, bone, and predentin, calcification is initiated by matrix vesicles released by chondrocytes, osteoblasts, and odontoblasts (4). Vesicle formation is also an important autodefense mechanism protecting against complement attack, by allowing the removal of the C5b-9 attack complex from the cell surface as shown for polymorphonuclear neutrophils (PMNs), oligodendrocytes, platelets and erythrocytes (5-10). It is reported that microvesicle-release increase upon pathological conditions like inflammation, injury, vascular dysfunction, or cancer (1, 11-13).

A major problem in microvesicle literature is the rather confusing nomenclature. Various names have been used including particles, microparticles, vesicles, microvesicles, nanovesicles, exosomes, dexosomes, argosomes and ectosomes. Whereas their formation mechanisms, size and effects are different, one common point between all is the fact that their protein and lipid compositions are similar to that

of the cell membrane from which they originate (1, 14). A more rigorous differentiating nomenclature awaits to be established. In the meantime, the major point in distinguishing them is the budding mechanism (budding into an intracellular compartment vs. surface budding).

Exosomes are defined as small membrane vesicles formed by inward budding of endosomal membranes, called multivesicular bodies. When multivesicular bodies fuse with the plasma membrane, exosomes are released extracellularly (15, 16). Exosomes that are between 30 to 100 nm in diameter, are secreted from various cell types, including reticulocytes (17), mast cells (18), dendritic cells (DCs) (19), platelets (20), B-lymphocytes (21), T-lymphocytes (22), epithelial cells (23), and tumor cells (24). Dendritic cell-derived exosomes are also called dexosomes (25). The biological functions of exosomes largely depend on their surface proteins and the cell types from which they originate. The two prominent functions are to eliminate obsolete proteins during cell maturation, and to mediate intercellular communication by transferring material among cells (15, 26, 27). However, exosomes are best described in the immune system, where they are capable to present specific antigens to T-lymphocytes, and therefore have strong immunostimulatory activities (14-16, 26). Indeed, exosomes were considered as potential candidates for cancer vaccines, and several clinical trials have been established (28-30). In contrast with this theory, recent studies show that exosomes could also exhibit immune suppressive effects (31-33).

The other category of vesicles includes ectosomes and microvesicles that are released by budding directly from the cell membrane surface (i.e. ectocytosis) (10, 34-36).

Many eukaryotic cells, including tumor cells, release ectosomes, either spontaneously or in response to various stimuli (10, 34, 37-40). Commonly, ectosomes are rightside-out vesicles with cytosolic content, and expose phosphatidylserine (PS) in the outer leaflet of their membrane (10, 35). Depending on their cellular origin, ectosomes have been associated with a broad spectrum of biological activities. However, the effects observed were mainly procoagulant and proinflammatory (37, 39, 41-44).

Characteristics and molecular properties of human polymorphonuclear neutrophil-derived ectosomes (PMN-Ect) have recently been described (34, 35). PMN-Ect have a diameter of 50-200 nm, and express a specific set of receptors (complement receptor 1 (CD35), CD11a, CD11b, CD-16, L-selectin (CD62L), HLA class I), enzymes (myeloperoxidase, elastase, metalloproteinase-9, proteinase-3), complement proteins (CD46, CD59), and a marker of the cells they originate (CD66b). The absence of CD14, CD32, CD55, CD63 and CD87 was described as evidence of selection during sorting (34, 35). Gasser et al. reported that PMN-Ect block inflammatory response of human monocyte-derived macrophages (HMDMs) to zymosan A and lipopolysaccharide (LPS) by inhibiting the release of TNFα, and reducing the release of IL-8 and IL-10 (45). PMNs having a major role in defense against pathogens and in inflammatory process, these results were unexpected (46). PMNs phagocytose, and eventually eliminate invading microorganisms by means of potent antimicrobial agents released during the process of degranulation. This microbicidal weaponry, because of the lack of specificity, can lead to severe tissue damage if not controlled (46, 47). Early release of immunosuppressive PMN-Ect might have an essential role in counterweighing these proinflammatory mechanisms (45).

Gout is a disease caused by the deposition of monosodium urate monohydrate (MSU) crystals in articular and periarticular tissues. The acute inflammation manifests as massive infiltration of PMNs into the joints, and inflammatory cell activation leading to dramatic clinical signs and symptoms. However even in the absence of clinical intervention, acute gouty arthritis undergoes self-resolution within a few days (48). The underlying responsible for the resolution of the inflammation still remains unknown. Recent observations imply that macrophages attracted by PMNs to the site of inflammation might play a major role in its resolution (49-51). Indeed, Yagnik and colleagues have demonstrated that macrophages do not release proinflammatory cytokines like IL-1β, IL-6 and TNFα, in contrast release the anti-inflammatory cytokine TGF-β1, in presence of MSU crystals or in human cantharidin-induced skin blisters (52). These results are similar with the ones obtained with macrophages that have encountered PMN-Ect in presence of stimuli (45). Whether PMN-Ect are involved in the resolution of inflammation in acute gout remain to be tested.

An important property of PMN-Ect is the PS exposed on the outer membrane leaflet (35). Similarly, cells undergoing apoptosis start to loose their membrane asymmetry, and PS appears on the surface (53). Apoptotic cells (ACs) and the exposure of PS are the subject of numerous studies. The clearance of ACs by phagocytes like macrophages and DCs occurs in a non-inflammatory manner, and there is growing evidence that their phagocytosis results in powerful anti-inflammatory or even immunosuppressive effects (54). Interestingly, PMN-Ect and ACs not only share PS exposure on their surface, but also their anti-inflammatory effects on macrophages (45, 55).

Thus, we were interested to investigate the effects of PMN-Ect on human monocyte-derived DCs (MoDCs), and this is the subject of the first section. For this purpose, we analyzed the morphology, maturation and function of non-stimulated and LPS-stimulated MoDCs in presence of PMN-Ect.

In the meantime, we were also interested in ectosomes released by other cells than PMNs. The essential cells to be transfused are erythrocytes, and during blood storage erythrocyte-derived ectosomes (E-ecto) are formed in large quantities. Moreover, although generally not accepted, clinical studies suggest that transfusions might be immunosuppressive (56-58). A recent study reported that transfusions of erythrocytes might be responsible for a diminished survival in cancer patients (59). The properties of E-ecto, and their effects on HMDMs are the topics of the second section.

Having the results of the effects of PMN-Ect on HMDMs and MoDCs, and E-ecto on HMDMs, we started to study the receptors and signaling pathways involved in ectosome-induced immunosuppression. In the third section, we report the inhibitory mechanisms used by PMN-Ect in HMDMs.

References

1. Ratajczak, J., M. Wysoczynski, F. Hayek, A. Janowska-Wieczorek, and M. Z. Ratajczak. 2006. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 20:1487-1495.
2. Schooling, S. R., and T. J. Beveridge. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. *J Bacteriol* 188:5945-5957.
3. Mashburn, L. M., and M. Whiteley. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437:422-425.
4. Anderson, H. C. 2003. Matrix vesicles and calcification. *Curr Rheumatol Rep* 5:222-226.
5. Campbell, A. K., and B. P. Morgan. 1985. Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature* 317:164-166.
6. Iida, K., M. B. Whitlow, and V. Nussenzweig. 1991. Membrane vesiculation protects erythrocytes from destruction by complement. *J Immunol* 147:2638-2642.
7. Morgan, B. P., J. R. Dankert, and A. F. Esser. 1987. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J Immunol* 138:246-253.
8. Scolding, N. J., B. P. Morgan, W. A. Houston, C. Linington, A. K. Campbell, and D. A. Compston. 1989. Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature* 339:620-622.

9. Sims, P. J., and T. Wiedmer. 1986. Repolarization of the membrane potential of blood platelets after complement damage: evidence for a Ca++ -dependent exocytotic elimination of C5b-9 pores. *Blood* 68:556-561.

10. Stein, J. M., and J. P. Luzio. 1991. Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem J* 274 (Pt 2):381-386.

11. Diamant, M., M. E. Tushuizen, A. Sturk, and R. Nieuwland. 2004. Cellular microparticles: new players in the field of vascular disease? *Eur J Clin Invest* 34:392-401.

12. Freyssinet, J. M. 2003. Cellular microparticles: what are they bad or good for? *J Thromb Haemost* 1:1655-1662.

13. Lynch, S. F., and C. A. Ludlam. 2007. Plasma microparticles and vascular disorders. *Br J Haematol* 137:36-48.

14. Johnstone, R. M. 2006. Exosomes biological significance: A concise review. *Blood Cells Mol Dis* 36:315-321.

15. Li, X. B., Z. R. Zhang, H. J. Schluesener, and S. Q. Xu. 2006. Role of exosomes in immune regulation. *J Cell Mol Med* 10:364-375.

16. Thery, C., L. Zitvogel, and S. Amigorena. 2002. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2:569-579.

17. Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 262:9412-9420.

18. Raposo, G., D. Tenza, S. Mecheri, R. Peronet, C. Bonnerot, and C. Desaymard. 1997. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* 8:2631-2645.

19. Zitvogel, L., A. Regnault, A. Lozier, J. Wolfers, C. Flament, D. Tenza, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 4:594-600.

20. Heijnen, H. F., A. E. Schiel, R. Fijnheer, H. J. Geuze, and J. J. Sixma. 1999. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 94:3791-3799.

21. Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 183:1161-1172.

22. Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, and C. Hivroz. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* 168:3235-3241.

23. van Niel, G., G. Raposo, C. Candalh, M. Boussac, R. Hershberg, N. Cerf-Bensussan, and M. Heyman. 2001. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 121:337-349.

24. Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Thery, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, E. Angevin, S. Amigorena, and L. Zitvogel. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 7:297-303.

25. Le Pecq, J. B. 2005. Dexosomes as a therapeutic cancer vaccine: from bench to bedside. *Blood Cells Mol Dis* 35:129-135.

26. Fevrier, B., and G. Raposo. 2004. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* 16:415-421.

27. Mack, M., A. Kleinschmidt, H. Bruhl, C. Klier, P. J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, and D. Schlondorff. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* 6:769-775.

28. Li, X., Z. Zhang, T. Beiter, and H. J. Schluesener. 2005. Nanovesicular vaccines: exosomes. *Arch Immunol Ther Exp (Warsz)* 53:329-335.

29. Chaput, N., J. Taieb, N. Schartz, C. Flament, S. Novault, F. Andre, and L. Zitvogel. 2005. The potential of exosomes in immunotherapy of cancer. *Blood Cells Mol Dis* 35:111-115.

30. Chaput, N., C. Flament, S. Viaud, J. Taieb, S. Roux, A. Spatz, F. Andre, J. B. LePecq, M. Boussac, J. Garin, S. Amigorena, C. Thery, and L. Zitvogel. 2006. Dendritic cell derived-exosomes: biology and clinical implementations. *J Leukoc Biol* 80:471-478.

31. Valenti, R., V. Huber, P. Filipazzi, L. Pilla, G. Sovena, A. Villa, A. Corbelli, S. Fais, G. Parmiani, and L. Rivoltini. 2006. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res* 66:9290-9298.

32. Valenti, R., V. Huber, M. Iero, P. Filipazzi, G. Parmiani, and L. Rivoltini. 2007. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res* 67:2912-2915.

33. Iero, M., R. Valenti, V. Huber, P. Filipazzi, G. Parmiani, S. Fais, and L. Rivoltini. 2008. Tumour-released exosomes and their implications in cancer immunity. *Cell Death Differ* 15:80-88.

34. Hess, C., S. Sadallah, A. Hefti, R. Landmann, and J. A. Schifferli. 1999. Ectosomes released by human neutrophils are specialized functional units. *J Immunol* 163:4564-4573.

35. Gasser, O., C. Hess, S. Miot, C. Deon, J. C. Sanchez, and J. A. Schifferli. 2003. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* 285:243-257.

36. Pilzer, D., O. Gasser, O. Moskovich, J. A. Schifferli, and Z. Fishelson. 2005. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. *Springer Semin Immunopathol* 27:375-387.

37. Nomura, S., N. N. Tandon, T. Nakamura, J. Cone, S. Fukuhara, and J. Kambayashi. 2001. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis* 158:277-287.

38. Dolo, V., A. Ginestra, D. Cassara, S. Violini, G. Lucania, M. R. Torrisi, H. Nagase, S. Canevari, A. Pavan, and M. L. Vittorelli. 1998. Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells. *Cancer Res* 58:4468-4474.

39. Satta, N., F. Toti, O. Feugeas, A. Bohbot, J. Dachary-Prigent, V. Eschwege, H. Hedman, and J. M. Freyssinet. 1994. Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. *J Immunol* 153:3245-3255.

40. Lee, T. L., Y. C. Lin, K. Mochitate, and F. Grinnell. 1993. Stress-relaxation of fibroblasts in collagen matrices triggers ectocytosis of plasma membrane vesicles containing actin, annexins II and VI, and beta 1 integrin receptors. *J Cell Sci* 105 (Pt 1):167-177.

41. MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North, and A. Surprenant. 2001. Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15:825-835.

42. Mesri, M., and D. C. Altieri. 1999. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 274:23111-23118.

43. Mesri, M., and D. C. Altieri. 1998. Endothelial cell activation by leukocyte microparticles. *J Immunol* 161:4382-4387.

44. Sabatier, F., V. Roux, F. Anfosso, L. Camoin, J. Sampol, and F. Dignat-George. 2002. Interaction of endothelial microparticles with monocytic cells in vitro induces tissue factor-dependent procoagulant activity. *Blood* 99:3962-3970.

45. Gasser, O., and J. A. Schifferli. 2004. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 104:2543-2548.

46. Witko-Sarsat, V., P. Rieu, B. Descamps-Latscha, P. Lesavre, and L. Halbwachs-Mecarelli. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 80:617-653.

47. Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317-1327.

48. Akahoshi, T., Y. Murakami, and H. Kitasato. 2007. Recent advances in crystal-induced acute inflammation. *Curr Opin Rheumatol* 19:146-150.

49. Yagnik, D. R., P. Hillyer, D. Marshall, C. D. Smythe, T. Krausz, D. O. Haskard, and R. C. Landis. 2000. Noninflammatory phagocytosis of monosodium urate monohydrate crystals by mouse macrophages. Implications for the control of joint inflammation in gout. *Arthritis Rheum* 43:1779-1789.

50. Landis, R. C., D. R. Yagnik, O. Florey, P. Philippidis, V. Emons, J. C. Mason, and D. O. Haskard. 2002. Safe disposal of inflammatory monosodium urate monohydrate crystals by differentiated macrophages. *Arthritis Rheum* 46:3026-3033.

51. Dalbeth, N., and D. O. Haskard. 2005. Inflammation and tissue damage in crystal deposition diseases. *Curr Opin Rheumatol* 17:314-318.

52. Yagnik, D. R., B. J. Evans, O. Florey, J. C. Mason, R. C. Landis, and D. O. Haskard. 2004. Macrophage release of transforming growth factor beta1 during resolution of monosodium urate monohydrate crystal-induced inflammation. *Arthritis Rheum* 50:2273-2280.

53. Zwaal, R. F., and A. J. Schroit. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89:1121-1132.

54. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol* 2:965-975.

55. Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109:41-50.

56. Tartter, P. I. 1995. Immunologic effects of blood transfusion. *Immunol Invest* 24:277-288.

57. Vamvakas, E. C. 2002. Possible mechanisms of allogeneic blood transfusion-associated postoperative infection. *Transfus Med Rev* 16:144-160.

58. Vamvakas, E. C. 2004. White blood cell-containing allogeneic blood transfusion, postoperative infection and mortality: a meta-analysis of observational 'before-and-after' studies. *Vox Sang* 86:111-119.

59. Jensen, L. S., E. Puho, L. Pedersen, F. V. Mortensen, and H. T. Sorensen. 2005. Long-term survival after colorectal surgery associated with buffy-coat-poor and leucocyte-depleted blood transfusion: a follow-up study. *Lancet* 365:681-682.

Section 1:

Polymorphonuclear Neutrophil-Derived Ectosomes Interfere with the Maturation of Monocyte-Derived Dendritic Cells

Abstract

Polymorphonuclear neutrophils (PMNs) are a key component of the innate immune system. Their activation leads to the release of potent antimicrobial agents through degranulation. Simultaneously, PMNs release cell surface-derived microvesicles, so-called ectosomes (PMN-Ect). PMN-Ect are rightside-out vesicles with a diameter of 50–200 nm. They expose phosphatidylserine in the outer leaflet of their membrane and down-modulate monocyte/macrophage-activation in vitro. In this study, we analyzed the effects of PMN-Ect on maturation of human monocyte-derived dendritic cells (MoDCs). Intriguingly, exposing immature MoDCs to PMN-Ect modified their morphology, reduced their phagocytic activity, and increased the release of TGF-β1. When immature MoDCs were incubated with PMN-Ect and stimulated with the TLR4 ligand LPS, the maturation process was partially inhibited as evidenced by reduced expression of cell surface markers (CD40, CD80, CD83, CD86, and HLA-DP DQ DR), inhibition of cytokine-release (IL-8, IL-10, IL-12, and TNFα), and a reduced capacity to induce T cell proliferation. Together these data provide evidence that PMN-Ect have the ability to modify MoDC maturation and function. PMN-Ect may thus represent an as yet unidentified host-factor influencing MoDC maturation at the site of injury, thereby possibly impacting on downstream MoDC-dependent immunity.

Introduction

Many eukaryotic cells release small vesicles by ectocytosis (i.e., ectosomes), either spontaneously or in response to various stimuli (1, 2). Data on the function(s) of ectosomes have accumulated recently. Depending on their cellular origin, ectosomes have been associated with a broad spectrum of biological activities. Ectosomes derived from endothelial cells have been described to bind monocytic cells and to induce procoagulant activity (3), whereas ectosomes derived from platelets and monocytes were shown to directly promote hemostasis and induce inflammation by activating endothelial cells (4, 5). As for monocyte-derived ectosomes, their proinflammatory potential has been linked to their potential to mediate the rapid secretion of IL-1β and to express tissue factor (6, 7, 8).

Activated human polymorphonuclear neutrophils (PMNs) release ectosomes at the time of degranulation. PMN-Ect have been well characterized (9, 10, 11). They are rightside-out vesicles with cytosolic content and a diameter of 50–200 nm and expose phosphatidylserine (PS) in the outer leaflet of their membrane. Contrasting other ectosomes, PMN-Ect have recently been shown to inhibit the inflammatory properties of human monocyte-derived macrophages in vitro. Induction of TGF-β1 secretion by macrophages and the exposure of PS on the surface of PMN-Ect were shown to contribute independently to this effect (11).

Dendritic cells (DCs) function as sentinels of the immune system, bridging innate and acquired immunity. In their tissue of residence, immature DCs (iDCs) internalize and proteolytically process self- and non-self antigens (Ags). When Ag uptake and processing occurs under inflammatory conditions, for example, conditions characterized by concomitant pattern recognition signals delivered to iDCs via

pathogen-derived products, iDCs change their morphology, shut down phagocytosis, and increase expression of costimulatory molecules and secretion of cytokines. Simultaneously, DCs migrate into secondary lymphoid organs (i.e., spleen or lymph nodes). DCs activated and induced to mature under inflammatory conditions are then capable of priming and fully activating naive $CD4^+$ and $CD8^+$ T cells. By contrast, partially and/or "inappropriately" activated iDCs are thought to induce immunological tolerance to Ags presented on their surface (12, 13, 14, 15). The precise factors determining immunogenic vs. tolerogenic DC-mediated priming remain to be defined.

PMN-Ect share important biological properties with apoptotic cells, including the expression of PS (9, 10, 11, 16). Apoptotic cells have been identified as major regulators of DC function both in vitro and in vivo (12, 13, 14, 15, 17, 18, 19, 20). PS, both on apoptotic cells as well as when incorporated into artificial liposomes, has been identified as a major factor influencing monocyte-derived dendritic cell (MoDC) maturation and function (21, 22, 23, 24). Furthermore, vesicles expressing PS released by tumor cells have recently been shown to down-regulate the activation of DCs, thus impairing the possible immune response against tumor Ags (25).

Although it is plausible that during the early phase of an immune response PMN-Ect interact with DCs, no data characterizing such interactions exist. In this study, we investigated the impact of PMN-Ect on MoDCs. Specifically, we examined the maturation of MoDCs in the presence/absence of PMN-Ect and the functional activity of MoDCs that were matured in the presence of PMN-Ect.

Materials and Methods

Collection of PMN-Ect

To isolate PMNs, a fresh buffy coat was diluted 1/1 (v/v) with PBS-EDTA (2 mM), mixed with 0.25 vol of 4% dextran T500 (GE Healthcare Bio-Sciences), and left for 30 min for erythrocyte sedimentation. Leukocyte-rich supernatant was aspirated and centrifuged for 10 min at 200 x *g*. The pellet was resuspended 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 for 10 min at 350 x g and resuspended in 20 ml of PBS-EDTA. This suspension was layered over 20 ml of Histopaque-1077 (Sigma-Aldrich) and centrifuged for 30 min at 350 x *g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA. All manipulations were performed at 4°C, thus minimizing PMN activation (10, 11).

For stimulation, pooled PMNs (1 x 10^7 cells/ml) from healthy blood donors were diluted 1/1 (v/v) in prewarmed (37°C) RPMI 1640 (Invitrogen Life Technologies) with 1 μM fMLP and incubated for 20 min at 37°C. PMNs were removed by centrifugation (4000 x *g* for 15 min at 4°C), and PMN-Ect contained in the supernatant were concentrated with Centriprep centrifugal filter devices (molecular mass 10,000 MW cut-off; Millipore) and stored in aliquots at –80°C until use (10, 11).

Isolation, culture, and maturation of MoDCs

MoDCs were derived from monocytes isolated from fresh buffy coats. A buffy coat was diluted 1/1 (v/v) with HBSS (Invitrogen Life Technologies), layered over Histopaque-1077, and centrifuged for 30 min at 350 x *g*. PBMCs were washed and cultured in complete medium (RPMI 1640, 1% L-glutamine, 1%

penicillin/streptomycin, and 10% FCS) for 1 h at 37°C in 6-well plates or in 75-ml flasks. After incubation, nonadherent cells were removed by washing twice with prewarmed RPMI 1640. The remaining adherent cells were then cultured in complete medium supplemented with 50 ng/ml GM-CSF and 50 ng/ml IL-4 (ImmunoTools). On days 2 and 5, the media including the supplements were replaced. On day 6, nonadherent immature MoDCs (iMoDCs) were harvested, counted, and plated in 6- or 24-well plates (1 x 10^5 cells/ml) in fresh medium containing GM-CSF and IL-4 (50 ng/ml each) (26, 27). On day 6, LPS (10 ng/ml final concentration; Sigma-Aldrich) and/or PMN-Ect was added. MoDCs and supernatants were collected 24 h later.

Flow cytometric analysis

Flow cytometric analyses of cell surface markers were performed using the following mouse mAbs conjugated with FITC: CD14, CD40, CD80, CD83, CD86, and HLA-DP DQ DR (Serotec) and with PE: CCR7 (BD Biosciences/BD Pharmingen). In each experiment, parallel stainings with isotype-matched controls IgG1-FITC, IgG2a-FITC (Serotec), and IgG2a-PE (BD Biosciences/BD Pharmingen) were performed. After each incubation, cells were spun down, resuspended in PBS/1% BSA, and labeled for 30–45 min at 4°C with appropriate Abs. After labeling, cells were washed twice in PBS/1% BSA and data were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed using Summit software (DakoCytomation). A minimum of 10,000 events were collected per dataset.

Detection of apoptosis

Detection of apoptotic and/or necrotic cells was performed using FITC-conjugated annexin V (AnV; BD Biosciences/BD Pharmingen) and Via-Probe (BD

Biosciences/BD Pharmingen), a nucleic acid dye (7-aminoactinomycin D) used for the exclusion of nonviable cells. MoDCs were washed twice with cold PBS and resuspended in AnV-binding buffer (BD Biosciences/BD Pharmingen) at a concentration of 1 x 10^6 cells/ml. Aliquots of 100 μl were stained with 5 μl of AnV-FITC and 2 μl of Via-Probe and incubated for 15 min at room temperature in the dark. Samples were then diluted in 400 μl of binding buffer and analyzed by flow cytometry.

Endocytic activity

Endocytic activity of MoDCs was measured by assessing uptake of FITC-conjugated dextran (molecular mass, 40,000 kDa; Molecular Probes) (27). To that end, cells were incubated with 0.5 mg/ml FITC-conjugated dextran in complete medium for 15, 30, or 45 min at 37 and 4°C to measure specific uptake vs. nonspecific binding, respectively. MoDCs were then washed three times and analyzed by flow cytometry.

Quantitation of cytokines by ELISA

Relevant supernatants were collected and spun for 10 min at 500 x *g* at 4°C to remove cellular debris. The concentrations of IL-8, IL-10, IL-12p70, TNFα, and TGF-β1 were measured using OptEIA ELISA kits (BD Biosciences) according to the manufacturer's instructions. All samples were measured in duplicates.

T cell proliferation assay

iMoDCs incubated for 24 h with 1) medium alone, 2) medium and PMN-Ect, 3) medium and LPS, and 4) medium and LPS and PMN-Ect were collected and washed twice to remove excess PMN-Ect and LPS. $CD3^+$ T cells, obtained by positive

magnetic selection with $CD3^+$ microbeads (Miltenyi Biotec), were labeled with 0.25 mM CFSE at room temperature for 10 min in the dark. The reaction was stopped by adding cold complete medium. Cells were then washed twice with cold medium and seeded at a 1:1 ratio (if not stated otherwise) with MoDCs. After 5 days of culture, proliferation of CFSE-labeled T cells was assessed by flow cytometry.

PS blocking assay

On day 6, before coincubation with MoDCs, PMN-Ect were preincubated for 30 min at 4°C with recombinant AnV (50 μg/ml final concentration; BD Biosciences/BD Pharmingen) and then washed. MoDCs and supernatants were collected after 24 h.

Statistical analysis

Datasets were tested for normality. For normally distributed data, parametric analysis (two-tailed paired Student's *t* test) and for non-normally distributed data nonparametric analysis (Wilcoxon-matched pairs test) were performed using GraphPad Prism software. Data are expressed as mean ± SEM. A $p < 0.05$ was considered statistically significant.

Results

PMN-Ect modified the morphology of MoDCs

We first assessed the effects of PMN-Ect on MoDC morphology. Before LPS exposure, iMoDCs were round, whereas after 24 h of LPS maturation the name-giving dendritic morphology became evident (Fig. 1*A*). The major finding was that the formation of dendrites was inhibited by PMN-Ect when MoDCs were matured with LPS. In line with the literature, there were no significant scatter modifications between iMoDCs and LPS-matured MoDCs (mMoDCs) (Fig. 1*B*: Ø vs. LPS) (21, 28). Intriguingly, the incubation of MoDCs with PMN-Ect, exposed or not to LPS, produced a shift of the scatter of the MoDCs (Fig. 1*B*), indicating that the PMN-Ect had a direct effect on MoDC morphology. We could not detect a modification of PMN-Ect on MoDC viability, tested both via AnV and Via-Probe binding (data not shown).

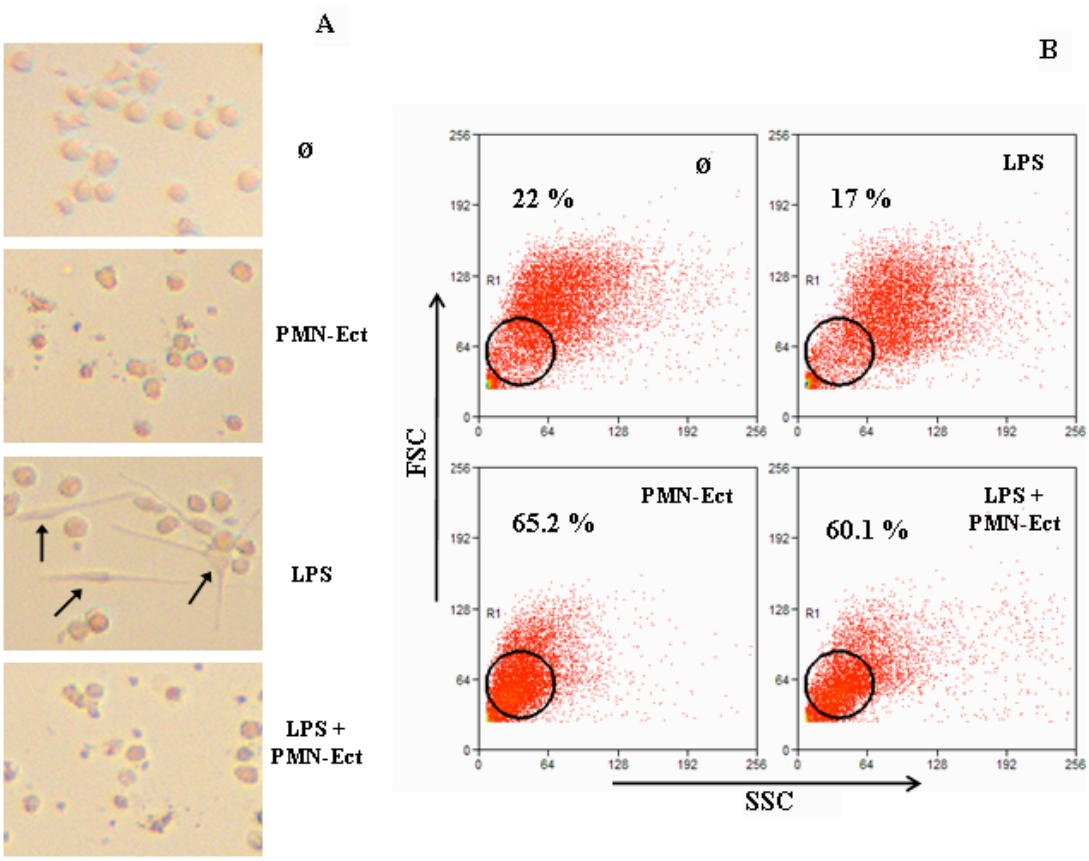


Figure 1. PMN-Ect change the morphology of human MoDCs. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + LPS (10 ng/ml), and 4) medium + LPS + PMN-Ect. *A*, When observed by light microscopy (original magnification, x20), iMoDCs were round, whereas after LPS activation dendrites became apparent in a large fraction of the cells (indicated by arrows). The appearance of dendrites by mMoDCs was largely abolished by PMN-Ect. *B*, Forward scatter (FSC)/side scatter (SSC) characteristics were used as a quantitative readout of changes in MoDC morphology. To define the modifications of FSC/SSC, the percentage of gated cells in a arbitrary circle are indicated. The FSC/SSC characteristics of MoDCs were significantly modified when exposed to PMN-Ect for both iMoDCs and mMoDCs (for iMoDCs, $p = 0.024$; for mMoDCs, $p = 0.013$; $n = 5$).

PMN-Ect down-regulated the endocytic activity of MoDCs

We next examined whether PMN-Ect have an impact on the endocytic activity of MoDCs. MoDCs were incubated with FITC-conjugated dextran at 37°C to measure specific uptake and at 4°C to quantify nonspecific binding. As expected, mMoDCs lost partially their capacity to phagocytose dextran particles (Fig. 2). PMN-Ect significantly reduced the endocytic capacity of iMoDCs (Fig. 2*A*) as well as mMoDCs (Fig. 2*B*). Strikingly, PMN-Ect reduced iMoDC phagocytosis to the level of phagocytosis observed in mMoDCs. At 4°C no incorporation of FITC-conjugated dextran by MoDCs was observed (Fig. 2). These data indicate that PMN-Ect alter the endocytic capacity of MoDCs.

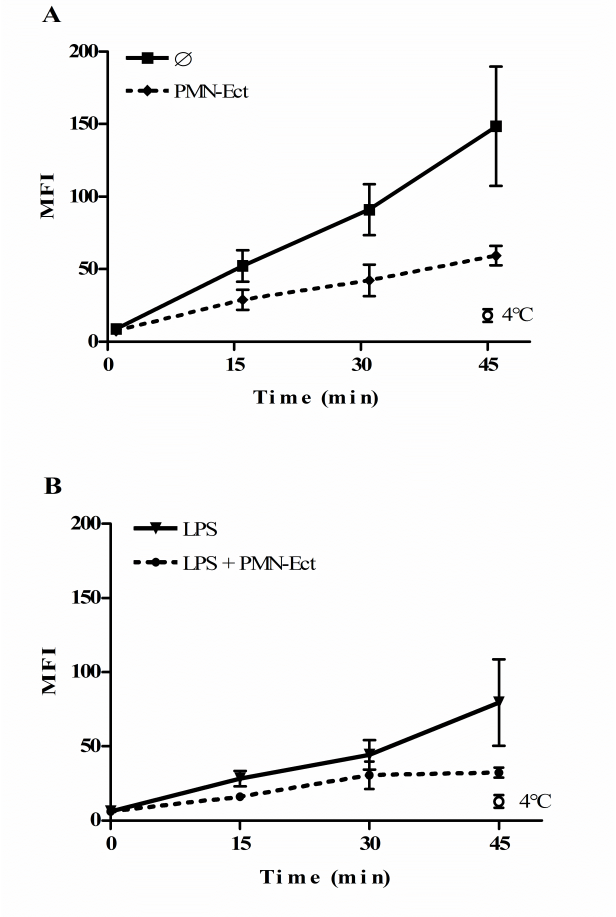


Figure 2. PMN-Ect reduce MoDC phagocytosis. MoDCs were incubated with FITC-conjugated dextran at 37°C and examined at different time points by flow cytometry to measure specific uptake. *A*, The phagocytosis level of iMoDCs plus PMN-Ect was significantly lower than iMoDCs ($p = 0.002$). *B*, PMN-Ect reduced significantly the phagocytosis level of mMoDCs ($p = 0.005$). The results are presented as mean MFIs ± SEM of five independent experiments.

PMN-Ect down-regulated the phenotypic maturation of MoDCs

Having shown that PMN-Ect influence MoDC morphology and endocytic capacity, we next asked the question whether PMN-Ect impact on expression of surface markers of nonactivated vs. mMoDCs as well. The data of six independent experiments were analyzed using the two-tailed paired Student *t* test. Of interest, iMoDCs exposed to PMN-Ect consistently expressed less CD40, CD86, and HLA-DP DQ DR than iMoDCs incubated without PMN-Ect (illustrated in Fig. 3), although these differences did not reach statistical significance.

As expected, coincubation of iMoDCs with LPS induced significant up-regulation of surface markers indicative of MoDC maturation (mMoDCs): CD40 (mean fluorescence intensity (MFI), 72.05 ± 10.02 vs. 175.9 ± 36.74; $p = 0.026$), CD83 (6.308 ± 0.681 vs. 17.48 ± 2.991; $p = 0.007$), CD86 (104.5 ± 18.11 vs. 121 ± 20.01; $p = 0.021$), HLA-DP DQ DR (1049 ± 62.92 vs. 1402 ± 74.28; $p < 0.001$). Up-regulation of CD80 was evident as well, but did not reach statistical significance (7.795 ± 1.019 vs. 9.487 ± 1.282; $p = 0.14$; Fig. 3).

mMoDCs coincubated with PMN-Ect, in contrast, expressed significantly less CD40 (MFI, 175.9 ± 36.74 vs. 127.1 ± 40.73; $p = 0.027$), CD80 (9.487 ± 1.282 vs. 7.665 ± 0.9681; $p = 0.019$), CD83 (17.48 ± 2.991 vs. 11.74 ± 1.932; $p = 0.042$), CD86 (121 ± 20.01 vs. 78.93 ± 19.23; $p = 0.002$), and HLA-DP DQ DR (1402 ± 74.28 vs. 1014 ± 124.8; $p = 0.029$) than mMoDCs alone (Fig. 3).

Together these data are evidence that PMN-Ect have the potential to modify MoDC maturation as judged by the expression pattern of various cell surface markers.

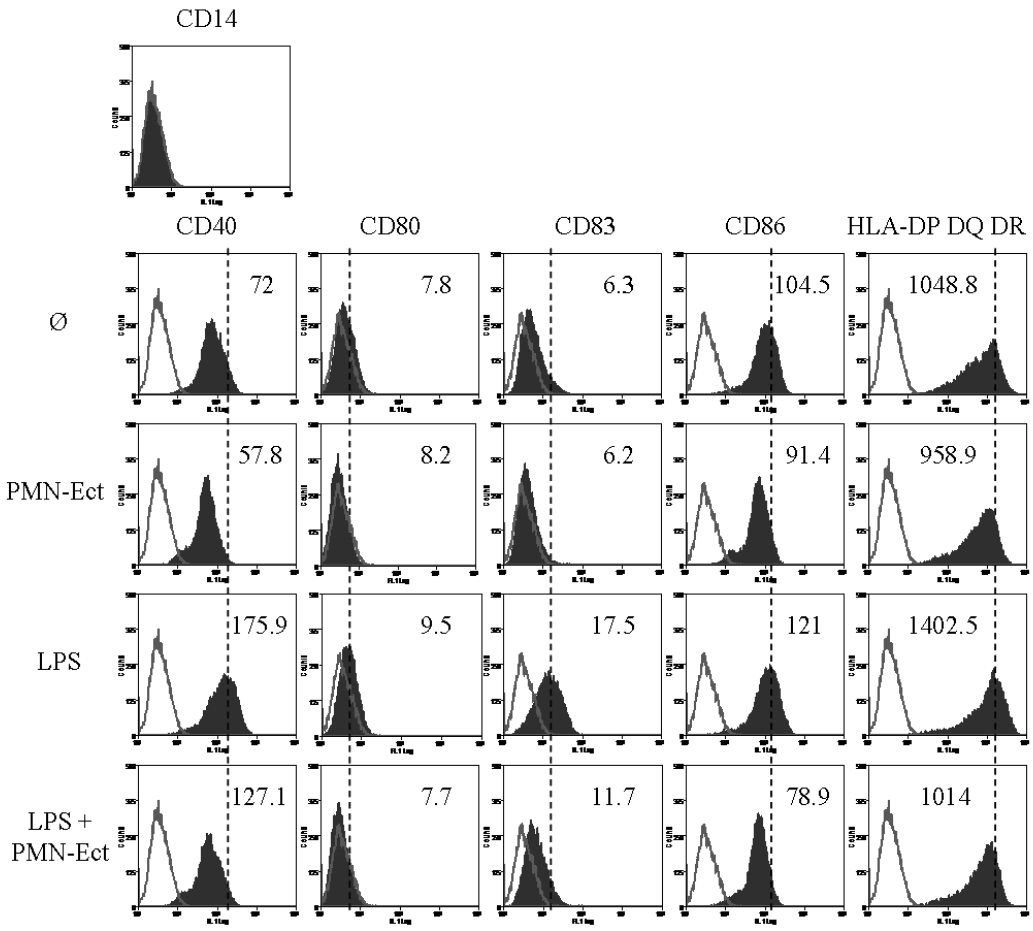


Figure 3. PMN-Ect inhibit up-regulation of key surface markers of human MoDCs. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + LPS (10 ng/ml), and 4) medium + LPS + PMN-Ect. Cells were then collected, washed, and analyzed by flow cytometry for cell surface expression of CD14, CD40, CD80, CD83, CD86, and HLA-DP DQ DR (filled histograms). Open gray lines represent staining with matched control Abs. The results shown are from one representative experiment. The indicated numbers represent the mean MFIs of six independent experiments.

PMN-Ect inhibited the cytokine release of MoDCs

We next assessed whether the effect of PMN-Ect on MoDC phenotype was accompanied by changes in their release of cytokines (IL-8, IL-10, IL-12p70, and TNFα). Levels of IL-8, IL-10, IL-12p70, and TNFα remained unchanged when iMoDCs were incubated with PMN-Ect as compared with iMoDCs alone (Fig. 4). Compared with iMoDCs alone, secretion of each of these cytokines was up-regulated when iMoDCs were matured with LPS (IL-8: 274.8 ± 44.39 vs. 24 346 ± 9410 pg/ml; $p = 0.002$; IL-10: 30.27 ± 12.98 vs. 241.5 ± 51.72 pg/ml; $p = 0.002$; IL-12p70: 3.71 ± 0.52 vs. 23.8 ± 3.14 pg/ml; $p = 0.002$; TNFα: 107 ± 24.83 vs. 3168 ± 912.9 pg/ml; $p = 0.002$).

Importantly, and in line with the effect of PMN-Ect on cell surface maturation markers, coincubation of mMoDCs with PMN-Ect strongly down-modulated the release of IL-10 (241.5 ± 51.72 vs. 144.4 ± 56.56 pg/ml; $p = 0.002$), IL-12p70 (23.80 ± 3.140 vs. 9.053 ± 1.629 pg/ml; $p = 0.014$), and TNFα (3168 ± 912.9 vs. 983.8 ± 361.9 pg/ml; $p = 0.002$) and slightly but significantly reduced the release of IL-8 (24 346 ± 9410 vs. 17 237 ± 7071 pg/ml; $p = 0.01$).

Of note, variability in the absolute concentrations of cytokines was important and somewhat unpredictable. This variability might originate largely from the fact that for each experiment cells and PMN-Ect from different donors were used. For example, as shown in Fig. 4, cells from one donor reacted very strongly to LPS and, compared with other donors, released huge amounts of IL-8, IL-10, and TNFα. Importantly, however, also in this donor a relative reduction was observed when iMoDCs were matured in the presence of PMN-Ect.

Because our results might have been modified by the fact that the PMN-Ect were from different donors than the MoDCs, we repeated the same studies using an autologous system. However, similar differences in the expression of surface markers and cytokine release were observed using PMN-Ect and MoDCs from the same donor (four independent experiments, data not shown).

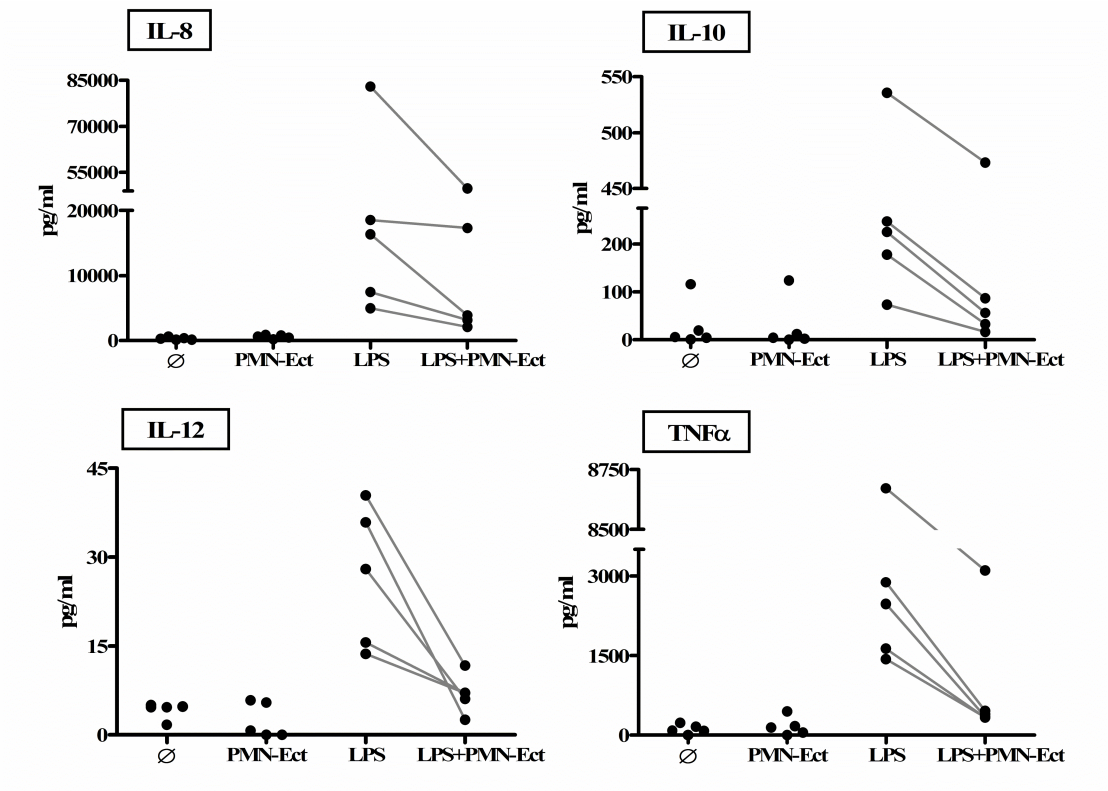


Figure 4. PMN-Ect inhibit the release of inflammatory cytokines by LPS-matured human MoDCs. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + LPS (10 ng/ml), and 4) medium + LPS + PMN-Ect. Concentrations of IL-8, IL-10, IL-12, and TNFα were analyzed in supernatants. The results of five experiments done in duplicates are shown.

MoDCs exposed to PMN-Ect released TGF-β1 and expressed less CCR7

Since TGF-β1 is known to be a central down-regulator of DCs, we measured its release when these cells were exposed to PMN-Ect. Strikingly, PMN-Ect increased the release of TGF-β1 from MoDCs in all experiments performed, whether the cells were immature (43.42 ± 19.64 vs. 217 ± 46.61 pg/ml; $p = 0.002$) or LPS matured (90.63 ± 24.43 vs. 260.3 ± 59 pg/ml; $p = 0.002$). Identical results were found whether the MoDCs and the PMN-Ect were from the same donor or not (Fig. 5*A*).

Since the expression of the chemokine receptor CCR7 in DCs is inhibited by TGF-β1 (29), we measured surface expression of CCR7 in different cells and found that it was reduced when MoDCs were LPS matured in the presence of PMN-Ect (21.13 ± 4.25 vs. 12.7 ± 4.67% positive cells, $p = 0.003$; Fig. 5*B*).

MoDCs exposed to PMN-Ect stimulated T cell proliferation poorly

Given that PMN-Ect were found to impact on phenotypic maturation and the amount of inflammatory cytokines released by MoDCs, we next examined the immunostimulatory capacity of MoDCs exposed to PMN-Ect. When MoDCs were incubated with PMN-Ect without LPS, no significant effect on T cell proliferation was observed. By contrast, MoDCs coincubated with PMN-Ect at the time of LPS exposure induced significantly less T cell proliferation than their non-PMN-Ect-exposed counterparts (mean decrease in percent proliferating cells: 20.4%; $p < 0.001$; Fig. 6). The effect was seen using MoDCs suppressed by autologous and allogeneic PMN-Ect. The results observed in a MoDC:T cell ratio of 1 was also observed at a ratio of 1:10 (data not shown).

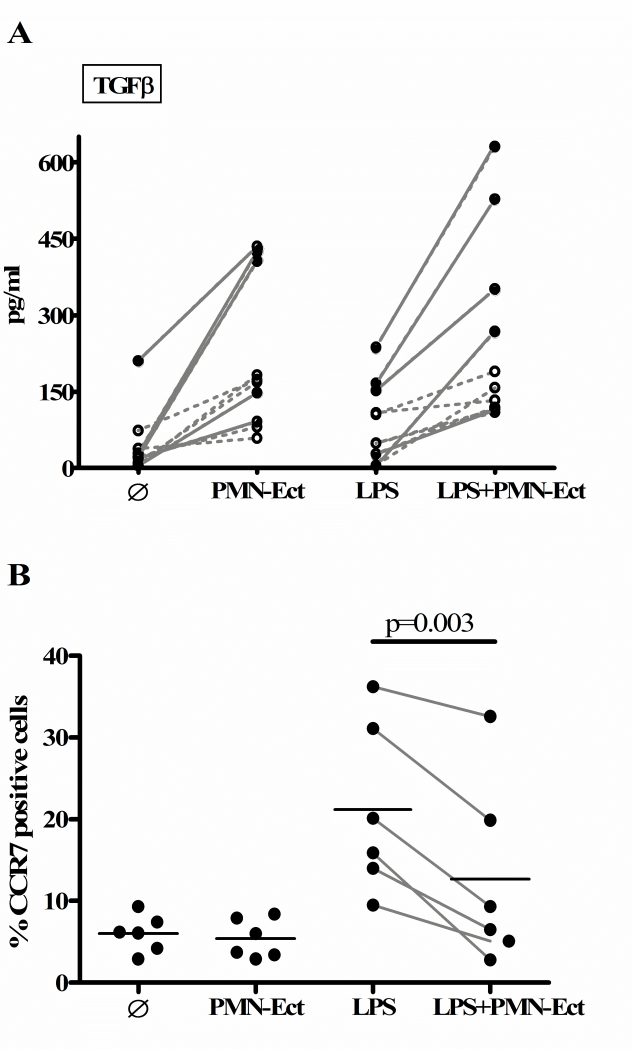


Figure 5. PMN-Ect increase the release of anti-inflammatory cytokine TGF-β1 and decrease the chemokine receptor CCR7 by human MoDCs. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + LPS (10 ng/ml), and 4) medium + LPS + PMN-Ect. *A*, Concentrations of TGF-β1 were analyzed in supernatants (n = 10). •, Allogeneic experiments (n = 5) and ○ and dotted lines autologous experiments (n = 5). *B*, Surface expression of CCR7 was analyzed by flow cytometry and results are indicated in percent positive cells. CCR7 was significantly reduced when MoDCs were LPS-matured in the presence of PMN-Ect (n = 6).

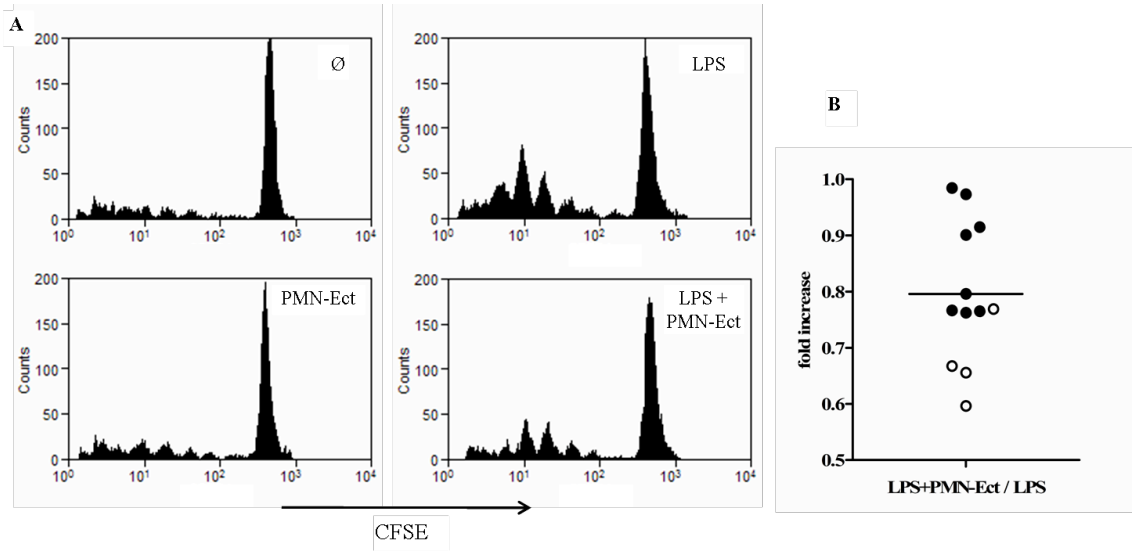


Figure 6. Human MoDCs matured in the presence of PMN-Ect are less efficient inducers of T cell proliferation. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + LPS (10 ng/ml), and 4) medium + LPS + PMN-Ect. Cells were washed and incubated with CFSE-stained $CD3^+$ T cells. *A*, Proliferation of T cells was assessed by flow cytometry after 5 days of coculture (representative of $n = 12$). *B*, T cells proliferated significantly less ($p < 0.001$) when exposed to MoDCs coincubated with PMN-Ect at the time of LPS activation. •, Allogeneic experiments ($n = 8$) and ○, autologous experiments ($n = 4$).

The activities of PMN-Ect were reversed by AnV binding

PS exposure on PMN-Ect has previously been shown using AnV binding (10). Since PS might be involved in the functional property of PMN-Ect to down-regulate the maturation of MoDCs, we coated first the PMN-Ect with recombinant AnV before adding them to the MoDCs. For mMoDCs, this coating reversed the inhibitory effects of PMN-Ect on expression of surface markers (for CD40, CD83, CD86, and HLA-DP DQ DR) (Fig. 7, cf LPS plus PMN-Ect and LPS plus PMN-Ect/AnV). We could not analyze the release of cytokines, because AnV per se induced an activation of MoDCs.

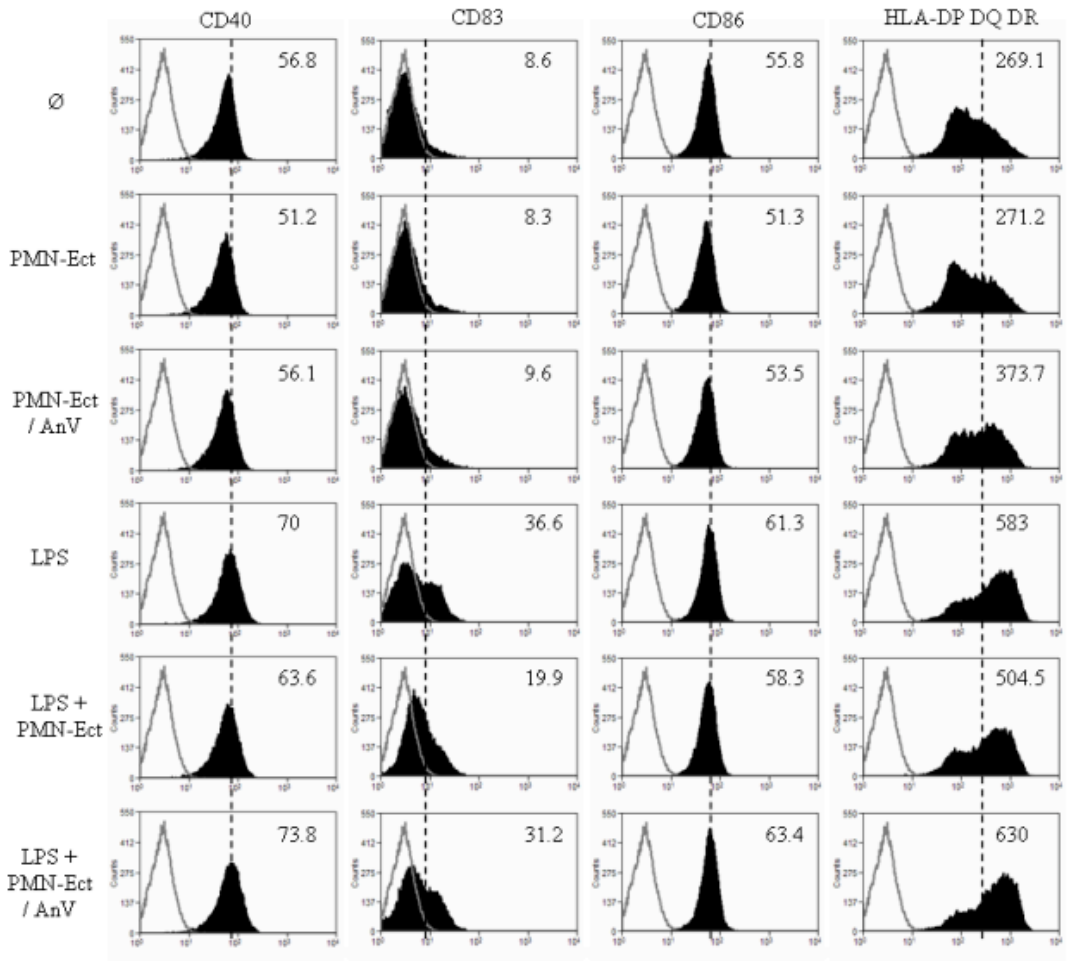


Figure 7. The effects of PMN-Ect were reversed by AnV binding. iMoDCs were incubated for 24 h with 1) medium alone (Ø), 2) medium + PMN-Ect, 3) medium + PMN-Ect preincubated with AnV (PMN-Ect/AnV), 4) medium + LPS (10 ng/ml), 5) medium + LPS + PMN-Ect, and 6) medium + LPS + PMN-Ect/AnV. Cells were then collected, washed, and analyzed by flow cytometry for cell surface expression of CD40, CD83, CD86, and HLA-DP DQ DR (filled histograms). Open gray lines represent staining with matched control Abs. The results shown are from one representative experiment (n = 4). The indicated numbers represent the MFI for CD40, CD86, and HLA-DP DQ DR and the percentage of positive cells for CD83.

Discussion

In the present study, we identified a new pathway by which activated human polymorphonuclear leukocytes, through the release of ectosomes, skew DC differentiation. It is likely that PMN-Ect released in vivo interact with tissue-resident iDCs at the site of injury or infection, i.e., when iDCs are exposed to maturation-inducing substances released from bacteria (14, 30). LPS and zymosan trigger, respectively, TLR4 and TLR2 receptors on iDCs (13, 31, 32) and induce maturation with release of specific cytokines (TNFα, IL-12, etc.) and increased expression of costimulatory molecules such as CD80 and CD86, CD40, CD83, and HLA class II molecules (12, 15, 33). In this study, we showed that PMN-Ect inhibited the maturation of iMoDCs by LPS and induced new morphological and functional characteristics with a resulting reduced capacity to activate T cells. In addition, iMoDCs exposed to PMN-Ect released TGF-β1 and it might well be that to some or a larger extent TGF-β1 was responsible for the down-regulation of TLR4-mediated maturation of iDCs, as observed by others (15, 34), and of surface expression of CCR7 (29). During DC maturation, the up-regulated CCR7 is responsible for directing the migration of DCs to the lymph nodes. It has been shown that CCR7 controls the cytoarchitecture, the rate of endocytosis, the survival, the migratory speed, and the maturation of the DCs (35), so that a reduced expression of CCR7 might interfere with normal immune response. PMN-Ect have already been shown to induce the release of TGF-β1 from macrophages, a release, which was responsible in part for the inhibition of macrophage activation by LPS and zymosan (11). Hence, it appears that PMN-Ect have down-regulating properties at different levels in the inflammatory process, which lead to T cell activation. Interestingly, similar properties have been ascribed to cells

undergoing apoptosis. Good evidence indicates that exposure of iDCs to apoptotic cells induces a tolerogenic – as opposed to an immunogenic-DC phenotype (12, 13, 15, 18, 20).

Together these observations highlight the complexity of the inflammatory process, which on one hand has to be amplified so as to trigger a specific immune response, but also has to limit excessive inflammation and prevent autoimmunity. For instance, apoptotic PMNs may have a protective role in allowing the termination of acute inflammation due to their overexpression of CCR5, which may adsorb CCL3 and CCL5 away from their targets, and thus act as "terminators" of chemokine signaling during the resolution of inflammation (36, 37). Ectosomes are released at the early phase of PMN activation, when much phagocytic and inflammatory activity is still needed at the site of injury, whether this injury is related to cell necrosis and/or infection. But such local inflammation requires control as well and does not need systematically DCs to provoke T cell stimulation and an acquired immune response. Our results indicate that such early down-regulation is a property of PMN-Ect, which in the local context may participate in the control of autoimmune responses, similarly to what has been suggested for apoptotic cells (15, 19, 20, 22). However and by contrast to apoptotic cells, PMN-Ect have the particularity to be involved very early in inflammation, a time point, which might be crucial for determining later aspects of the cascade responsible for acquired immunity, in that sense not terminator of inflammation, but responsible for controlling the immune response.

Two aspects merit attention. First the effects of PMN-Ect on resting iMoDCs and then those on the maturation process of MoDCs induced by LPS. The morphological and phenotypic changes of iMoDCs cocultured with PMN-Ect were subtle. There was a

minimal change in the forward and side scatters seen by FACS analysis and a nonsignificant but repeatedly observed very slight reduction of the expression of costimulatory molecules (CD40, CD86) and HLA class II molecules. However, iMoDCs that were exposed to PMN-Ect lost their phagocytic activity as indicated by a clearly reduced uptake of dextran particles. Thus, PMN-Ect may actively change the biological behavior of iDCs. Neither PS liposomes (a model of apoptotic cells) nor apoptotic primary cells (keratinocytes, monocytes, T and B cells) or cell lines were reported to induce similar functional changes in iDCs (21, 38). Recently, βig-h3, a protein expressed by iDCs and macrophages, has been shown to be involved in phagocytosis and suppressed during maturation of DCs. Whether PMN-Ect suppress the expression/production of βig-h3 remains an intriguing hypothesis (39). The comparisons between the expression of costimulatory and HLA class II molecules on iDCs induced by either apoptotic PMNs or PMN-Ect show divergent trends as well. Whereas both down-modulate CD40, CD80, and CD86 on iDCs, apoptotic PMNs increase CD83 and HLA class II, whereas PMN-Ect did the opposite (40). From the foregoing it seems evident that whereas there might be similarities in the responses induced by Ect and apoptotic cells, these responses are not identical.

Compared with their influence on iMoDCs, the impact of PMN-Ect on the LPS-induced maturation process of MoDCs was more obvious, significantly affecting phenotype, release of proinflammatory cytokines, and their immunogenicity vis-à-vis allogeneic T cells. Effects of PMN-Ect on LPS-induced maturation are analogous to those obtained with apoptotic cells and PS liposomes (19, 21, 24, 33, 38, 41, 42). Specifically, DCs exposed to PS liposomes before LPS maturation expressed significantly lower levels of CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR, secreted significantly less IL-10 and IL-12, and had impaired ability to activate

allogeneic T cells (21). These data might indicate that PS per se plays a major role in interfering with the maturation process of DCs.

The mechanisms underlining the biological effects of PMN-Ect on DCs remain speculative. In many respects, they may be similar to those proposed for the down-regulation of DCs by apoptotic cells, including the high expression of PS, which in multiple experiments has been shown to allow specific binding of apoptotic cells to macrophages or DCs (16, 22, 43, 44, 45, 46). AnV is known to interfere with the binding of PS-expressing cells/particles to macrophages (11). In the experiments performed here, we could reverse the down-regulation of surface markers of MoDCs by PMN-Ect by incubating first the PMN-Ect with AnV, suggesting that the expression of PS on PMN-Ect was responsible for their property to modify MoDCs. However, PMN-Ect, which had bound AnV, induced by themselves the release of TNFα by iDCs, indicating that AnV might have induced/blocked other interactions not directly related to PS as well.

Apoptotic cells and ectosomes released by the same type of cells express different sets of proteins. For instance, PMN-Ect express high levels of complement receptor 1 and CD66b (9, 10), whereas these molecules are down-regulated on apoptotic PMNs (47). Apoptotic cells express on their surface nuclear components (48), which will not be present on ectosomes released by live, activated cells. Thus, it is likely that diverse sets of proteins on dying cells vs. ectosomes will allow different functional activities, although many of the basic properties might be very similar.

For instance, the specific release of TGF-β1 induced by the binding of PMN-Ect to iMoDCs is most likely one of the essential mediators reprogramming the DCs so that it has a lower reactivity to LPS. Indeed TGF-β is a major player in modulating the

activity of iDCs and their maturation (15, 25, 49, 50). It is produced by iDCs exposed to apoptotic cells as well and under such conditions reprograms the DCs to become tolerogenic (15, 20). Whether iDCs exposed to PMN-Ect have similar properties, i.e., become tolerogenic, remains to be tested.

In conclusion, we suggest that, in addition to regulating macrophage activation (11), PMN-Ect may have the potential to influence the outcome of Ag-specific immunity by playing an active role in shaping DC-dependent immunity. In vivo models of inflammation/infection will now have to test the relevance of the here-proposed activities of PMN-Ect.

References

1. Stein, J. M., J. P. Luzio. 1991. Ectocytosis caused by sublytic autologous complement attack on human neutrophils: the sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem. J.* 274: 381-386.

2. Pilzer, D., O. Gasser, O. Moskovich, J. A. Schifferli, Z. Fishelson. 2005. Emission of membrane vesicles: roles in complement resistance, immunity, and cancer. *Springer Semin. Immunopathol.* 27: 375-387.

3. Sabatier, F., V. Roux, F. Anfosso, L. Camoin, J. Sampol, F. Dignat-George. 2002. Interaction of endothelial microparticles with monocytic cells in vitro induces tissue factor-dependent procoagulant activity. *Blood* 99: 3962-3970.

4. Satta, N., F. Toti, O. Feugeas, A. Bohbot, J. Dachary-Prigent, V. Eschwege, H. Hedman, J. M. Freyssinet. 1994. Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. *J. Immunol.* 153: 3245-3255.

5. Nomura, S., N. N. Tandon, T. Nakamura, J. Cone, S. Fukuhara, J. Kambayashi. 2001. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis* 158: 277-287.

6. Mesri, M., D. C. Altieri. 1998. Endothelial cell activation by leukocyte microparticles. *J. Immunol.* 161: 4382-4387.

7. Mesri, M., D. C. Altieri. 1999. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J. Biol. Chem.* 274: 23111-23118.

8. MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North, A. Surprenant. 2001. Rapid secretion of interleukin-1β by microvesicle shedding. *Immunity* 15: 825-835.

9. Hess, C., S. Sadallah, A. Hefti, R. Landmann, J. A. Schifferli. 1999. Ectosomes released by human neutrophils are specialized functional units. *J. Immunol.* 163: 4564-4573.

10. Gasser, O., C. Hess, S. Miot, C. Deon, J. C. Sanchez, J. A. Schifferli. 2003. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp. Cell Res.* 285: 243-257.

11. Gasser, O., J. A. Schifferli. 2004. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 104: 2543-2548.

12. Banchereau, J., R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.

13. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* 20: 621-667.

14. Gogolak, P., B. Rethi, G. Hajas, E. Rajnavolgyi. 2003. Targeting dendritic cells for priming cellular immune responses. *J. Mol. Recognit.* 16: 299-317.

15. Wallet, M. A., P. Sen, R. Tisch. 2005. Immunoregulation of dendritic cells. *Clin. Med. Res.* 3: 166-175.

16. Zwaal, R. F., A. J. Schroit. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89: 1121-1132.

17. Banchereau, J.. 1997. Dendritic cells: therapeutic potentials. *Transfus. Sci.* 18: 313-326.

18. Steinman, R. M., S. Turley, I. Mellman, K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* 191: 411-416.

19. Savill, J., I. Dransfield, C. Gregory, C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2: 965-975.

20. Erwig, L. P., P. M. Henson. 2007. Immunological consequences of apoptotic cell phagocytosis. *Am. J. Pathol.* 171: 2-8.

21. Chen, X., K. Doffek, S. L. Sugg, J. Shilyansky. 2004. Phosphatidylserine regulates the maturation of human dendritic cells. *J. Immunol.* 173: 2985-2994.

22. Fadok, V. A., D. L. Bratton, P. M. Henson. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J. Clin. Invest.* 108: 957-962.

23. Wu, Y., N. Tibrewal, R. B. Birge. 2006. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol*. 16: 189-197.

24. Shi, D., M. Fu, P. Fan, W. Li, X. Chen, C. Li, X. Qi, T. Gao, Y. Liu. 2007. Artificial phosphatidylserine liposome mimics apoptotic cells in inhibiting maturation and immunostimulatory function of murine myeloid dendritic cells in response to 1-chloro-2,4-dinitrobenze in vitro. *Arch. Dermatol. Res*. 299: 327-336.

25. Valenti, R., V. Huber, P. Filipazzi, L. Pilla, G. Sovena, A. Villa, A. Corbelli, S. Fais, G. Parmiani, L. Rivoltini. 2006. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-β-mediated suppressive activity on T lymphocytes. *Cancer Res*. 66: 9290-9298.

26. Sallusto, F., A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med*. 179: 1109-1118.

27. Means, T. K., F. Hayashi, K. D. Smith, A. Aderem, A. D. Luster. 2003. The toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J. Immunol*. 170: 5165-5175.

28. Mack, M., A. Kleinschmidt, H. Bruhl, C. Klier, P. J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, D. Schlondorff. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles:

a mechanism for cellular human immunodeficiency virus 1 infection. *Nat. Med.* 6: 769-775.

29. Ogata, M., Y. Zhang, Y. Wang, M. Itakura, Y. Y. Zhang, A. Harada, S. Hashimoto, K. Matsushima. 1999. Chemotactic response toward chemokines and its regulation by transforming growth factor-β1 of murine bone marrow hematopoietic progenitor cell-derived different subset of dendritic cells. *Blood* 93: 3225-3232.

30. Serhan, C. N., J. Savill. 2005. Resolution of inflammation: the beginning programs the end. *Nat. Immunol.* 6: 1191-1197.

31. Beutler, B., K. Hoebe, X. Du, R. J. Ulevitch. 2003. How we detect microbes and respond to them: the toll-like receptors and their transducers. *J. Leukocyte Biol.* 74: 479-485.

32. Sallusto, F., A. Lanzavecchia. 2002. The instructive role of dendritic cells on T-cell responses. *Arthritis Res.* 4: *(Suppl. 3)*:S127-S132.

33. Urban, B. C., N. Willcox, D. J. Roberts. 2001. A role for CD36 in the regulation of dendritic cell function. *Proc. Natl. Acad. Sci. USA* 98: 8750-8755.

34. Mou, H. B., M. F. Lin, H. Cen, J. Yu, X. J. Meng. 2004. TGF-β1 treated murine dendritic cells are maturation resistant and down-regulate Toll-like receptor 4 expression. *J. Zhejiang Univ. Sci.* 5: 1239-1244.

35. Sanchez-Sanchez, N., L. Riol-Blanco, J. L. Rodriguez-Fernandez. 2006. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J. Immunol*. 176: 5153-5159.

36. Ariel, A., G. Fredman, Y. P. Sun, A. Kantarci, T. E. Van Dyke, A. D. Luster, C. N. Serhan. 2006. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nat. Immunol*. 7: 1209-1216.

37. Ariel, A., C. N. Serhan. 2007. Resolvins and protectins in the termination program of acute inflammation. *Trends Immunol*. 28: 176-183.

38. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med*. 191: 423-434.

39. Cao, W., P. Tan, C. H. Lee, H. Zhang, J. Lu. 2006. A transforming growth factor-β-induced protein stimulates endocytosis and is up-regulated in immature dendritic cells. *Blood* 107: 2777-2785.

40. Clayton, A. R., R. L. Prue, L. Harper, M. T. Drayson, C. O. Savage. 2003. Dendritic cell uptake of human apoptotic and necrotic neutrophils inhibits CD40, CD80, and CD86 expression and reduces allogeneic T cell responses: relevance to systemic vasculitis. *Arthritis Rheum*. 48: 2362-2374.

41. Gallucci, S., M. Lolkema, P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med*. 5: 1249-1255.

42. Stuart, L. M., M. Lucas, C. Simpson, J. Lamb, J. Savill, A. Lacy-Hulbert. 2002. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J. Immunol*. 168: 1627-1635.

43. Gilbreath, M. J., D. L. Hoover, C. R. Alving, G. M. Swartz, Jr, M. S. Meltzer. 1986. Inhibition of lymphokine-induced macrophage microbicidal activity against *Leishmania major* by liposomes: characterization of the physicochemical requirements for liposome inhibition. *J. Immunol*. 137: 1681-1687.

44. Fadok, V. A., A. de Cathelineau, D. L. Daleke, P. M. Henson, D. L. Bratton. 2001. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J. Biol. Chem*. 276: 1071-1077.

45. Huynh, M. L., V. A. Fadok, P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J. Clin. Invest*. 109: 41-50.

46. Verhoven, B., S. Krahling, R. A. Schlegel, P. Williamson. 1999. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. *Cell Death Differ*. 6: 262-270.

47. Hart, S. P., J. A. Ross, K. Ross, C. Haslett, I. Dransfield. 2000. Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Differ*. 7: 493-503.

48. Radic, M., T. Marion, M. Monestier. 2004. Nucleosomes are exposed at the cell surface in apoptosis. *J. Immunol*. 172: 6692-6700.

49. Tiao, M. M., L. Lu, L. T. Huang, C. D. Liang, C. L. Chen, R. Tao, J. J. Fung, S. Qian. 2007. Cross-tolerance of recipient-derived transforming growth factor-β dendritic cells. *Transplant Proc*. 39: 281-282.

50. Rutella, S., S. Danese, G. Leone. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 108: 1435-1440.

Section 2:

Erythrocyte-derived Ectosomes have Immunosuppressive Properties

Abstract

Several clinical studies have suggested that blood transfusions are immunosuppressive. Whereas there have been reports describing immunosuppression induced by leukocytes or fragments thereof, the possibility that microparticles released by erythrocytes during storage are also involved was not investigated. We present here evidence that such microparticles have all the properties of ectosomes including size, the presence of a lipid membrane, and the specific sorting of proteins. These erythrocyte-derived ectosomes (E-ecto) fixed C1q, which was followed by activation of the classical pathway of complement with binding of C3 fragments. Similarly to ectosomes released by polymorphonuclear neutrophils, they express phosphatidylserine on their surface membrane, suggesting that they may react with and down-regulate cells of the immune system. In vitro, they were taken up by macrophages, and they significantly inhibited the activation of these macrophages by zymosan A and LPS, as shown by a significant drop in TNFα and IL-8 release (respectively, 80% and 76% inhibitions). In addition, the effect of E-ecto was not transient but lasted for at least 24 hours. In sum, E-ecto may interfere with the innate immune system/inflammatory reaction. Therefore, E-ecto transfused with erythrocytes may account for some of the immunosuppressive properties attributed to blood transfusions.

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 after fusion of the multivesicular bodies with the cell membrane [1]. Many hematopoietic cells, including reticulocytes, platelets, and leukocytes secrete exosomes, which have different functions. For example, for reticulocytes, exosomes mediate the clearance of obsolete proteins such as the transferrin receptor [2]. Beside the release of preformed vesicles, many cells shed small membrane vesicles, which are budding directly from the cell membrane [3]. This reaction was initially described as providing protection against complement attack, as it allows the removal of the C5b-9 attack complex from the cell surface of polymorphonuclear neutrophils (PMNs), oligodendrocytes, and even erythrocytes [4-6]. Stein and Luzio coined the term ectocytosis for the release of right-side-out oriented vesicles (ectosomes) from the surface of PMNs attacked by complement [7]. 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. Thus, ectocytosis is different from exocytosis, which describes the release of preformed vesicles. Despite these clear differences, it is often difficult to distinguish between the two types of vesicles, particularly when they are harvested from *in vivo* materials, supernatants of cell cultures or blood prepared for transfusion [8-10]. Most authors described such material as “microparticles” or “microvesicles”, even “nanovesicles”.

Stored blood for transfusion in humans contains microparticles formed by exo- and ectocytosis from leukocytes, platelets and erythrocytes. The number of such particles

increases over time during storage, so that large quantities of microparticles are transfused together with erythrocytes. Since the use of leukocyte- and platelet-depleted erythrocyte transfusions, it is likely that mostly erythrocyte-derived microparticles are transfused, probably ectosomes or possibly cell debris that accumulated during storage. Erythrocytes release ectosomes (E-ecto) after complement attack and *in vitro* when ATP is depleted. The importance of the progressive loss of lipid membrane during blood storage was initially described by Haradin et al. [11] and shown later to result from the formation of large numbers of haemoglobin-containing vesicles (E-ecto). Interestingly, spectrin is absent from E-ecto despite being an abundant protein in the cell-membrane cytoskeleton. This selective absence of spectrin might provide indications about the mechanisms responsible for the budding of the cell membrane and release of E-ecto. By contrast, E-ecto are enriched in several membrane proteins, in particular in glycophosphatidylinositol-linked proteins such as decay accelerating factor (DAF) and acetylcholine esterase [12]. The expression of phosphatidylserine (PS) in the outer-membrane leaflet of the E-ecto indicates that the lipid asymmetry of the two membrane leaflets has been lost, at least in part. The expression of PS varies according to the stimulus used to produce E-ecto in vitro; e.g. powerful stimuli such as Ca^{2+} ionophores lead to high level PS expression [12-19].

The exposure of antigen presenting cells to PS-expressing particles induces transient tolerance for danger signals with ensuing down-regulation of the immune response, as suggested by the literature regarding cells undergoing apoptosis [20], PMN-derived ectosomes [21], and PS-expressing liposomes [22]. Many clinical studies suggest that transfusions might be immunosuppressive, although these observations are not

generally accepted [23-25]. However, a recent clinical study indicated that transfusions of erythrocytes might be responsible for a diminished survival in cancer patients [26].

In the present study, we analyzed the pool of microparticles released by stored leukocyte-depleted erythrocytes; after having demonstrated that they had all the properties of ectosomes, we tested their capacity to interfere with the activation of macrophages by zymosan A and LPS. The main finding was that they down-regulated the capacity of these Toll-like ligands to activate macrophages.

Materials and Methods

Antibodies and reagents

All antibodies used were mouse anti-human monoclonal antibodies (mAb): glycophorin A (GPA), CD35/ CR1, CD55/DAF, CD59. The following mAb directed against GPA, CD45, CD41 were phycoerythrin (PE) coupled. We also used mAb fluorescein isothiocyanate (FITC) directed against GPA, C1q, C3d, and Annexin V (AnV) FITC; they were all purchased from BD Biosciences (San Jose, CA, USA). Zymosan A from *Saccharomyces cerevisiae* and Cytochalasin D were from Sigma (St Louis, MO, USA); lipopolysaccharide (LPS) and human C1q were from Calbiochem (San Diego, CA, USA); PMSF and EDTA from Serval Feinbiochemicals (Heidelberg, Germany). Human TGF-β1 Duoset ELISA development system were from R&D Systems (Minneapolis, MN, USA). OptEIA ELISA kits for IL-8, IL-10, and TNFα were from Becton Dickinson (San Diego, CA, USA).

Erythrocytes

Human erythrocytes were purified from fresh blood of healthy volunteers using dextran sedimentation. Briefly, the blood was centrifuged at 680 x g for 7 min, and plasma and buffy coat were removed. Remaining blood cells were mixed with 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and PBS and left on ice for 40 min. After sedimentation, the supernatant (SN) was removed with the upper layer of the erythrocytes, and the rest of the purified red blood cells (RBC) were washed five times with DMEM medium, each time removing the upper layer of the cells with the SN. The final contamination with leukocytes was reduced to less than one leukocyte / 10,000 RBC. We added an excess of erythrocytes, ratio

(100 erythrocytes for one macrophage) to activated human monocyte-derived macrophages (HMDM) in the experiment described below.

Erythrocyte supernatants

Blood units were obtained from healthy volunteer donors. Blood units were submitted to standard procedure preparation and storage of packed erythrocytes. Briefly, whole blood (450 mL) was collected in plastic bags (triplicate bag system with an integrated whole blood filter Leucoflex Sang Total 1, Macopharma, Tourcoing, France) with 63 mL citrate phosphate dextrose in the primary bag. Bags were stored at room temperature (RT), and filtration took place 3 hours following donation. After centrifugation (10 min, 1500 x g, 20°C), packed leuko-depleted erythrocytes (LD-E) were separated from plasma and transferred into the satellite bags containing 100 mL saline-adenine-glucose-mannitol. Storage time for packed LD-E before tests was 25 days. The SNs of packed LD-E were obtained through a centrifugation (10 min, 1000 x g, 4°C), which of the SNs, was repeated in the same conditions to clear all residual erythrocytes. The SNs containing E-ecto were concentrated with Centriprep centrifugal filter devices (10,000 MW cut-off; Millipore) and stored in aliquots at -80°C until use.

Staining and FACS of erythrocyte-derived ectosomes

After concentration, E-ecto were incubated with 10 μl of antibodies for 30 min at RT in the dark into tubes preloaded with a known density of fluorescent TruCount™ bead lyophilized pellets (BD Biosciences). We used to identify E-ecto anti-GPA FITC, and for leukocytes- and platelet-derived microparticles respectively, anti-CD45-PE and anti-CD41-PE.

In a separate experiment, E-ecto were incubated for 30 min at RT in the dark with 5 μl of FITC-conjugated AnV and 10 μl of PE-conjugated GPA in the absence or presence of 2.5 mM $CaCl_2$. The mixture was then diluted in PBS containing 2.5 mM $CaCl_2$ and analyzed by flow cytometry within 30 min.

Analysis of E-ecto was performed using a FACSort flow cytometer (BD Biosciences). Data from 20,000 events were acquired and analyzed with the use of CELLQuest™ software (BD Biosciences).

E-ecto and TruCount beads were identified by their size, as assessed by the logarithmic amplification of their forward scatter (FSC) and side scatter (SSC) signals. E-ecto were localized within R1 region. The TruCount beads were localized in R2 region (see Fig 1A).

Transmission electron microscopy of ectosomes/ negative staining

After concentration, E-ecto were resuspended in PBS and then fixed in 1% glutaraldehyde (final concentration) for 20 min at RT. E-ecto 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 80 kV.

SDS-PAGE, Silver Stain and Western blot

Concentrated E-ecto were ultracentrifuged (60 min at 200,000 x g at 4°C), and the pellet was resuspended in sample buffer containing 1% SDS and DTT. Erythrocyte membranes were prepared as described previously [27]. The erythrocytes were washed in 0.9% NaCl and hemolyzed, and their respective membranes were solubilized with 0.5% Triton X-100 in the presence of 5 mM EDTA and 1 mM PMSF. The same amount of erythrocytes and E-ecto-protein quantified using a Bradford

protein assay (Pierce, Rockford, IL, USA) was run on gradient 5-20% polyacrylamide gels. After SDS-PAGE, silver stain, or Western blotting with mouse anti-human GPA clone JC159, anti-CR1 (CD35) clone 3D9, anti-DAF (CD55) clone BRIC216, or anti-CD59 clone MEM-43/5 was performed. As control, we used a monoclonal mouse anti-human factor D. All of the monoclonals were used at 1:1000 dilution. The proteins were detected, respectively, with silver stain solutions or anti-mouse IgG biotinylated followed with HRP-streptavidin (Milan Analytica, LaRoche, Switzerland) and ECL (Amersham Pharmacia Biotech).

Binding of C1q on ectosomes and complement activation

E-ecto were isolated as described above. E-ecto were then ultracentrifuged and incubated after gentle resuspension for 30 min at 4°C in 100 μL 0.9% NaCl alone or 0.9% NaCl supplemented with 1 μg purified human C1q (Calbiochem), normal human serum (NHS; 30% final dilution), or heat-inactivated NHS (30% final dilution); serum heat inactivation was achieved after its exposure of 30 min at 56°C. The binding of C1q was then detected using an anti-C1q FITC antibody. The deposition of C3 fragments was detected using an anti-C3d FITC antibody.

Membrane labeling of ectosomes

An amphiphilic cell linker dye kit (PKH67, Sigma Chemical Co.) was used, as described by the manufacturer. Briefly, E-ecto resuspended in 200 μL Diluent C/dye solution (dye diluted 1/200) were incubated with gentle shaking for 1 min at room temperature. Then, RPMI 1640 (1 mL; without phenol red) was added to stop the reaction. Labeled E-ecto were separated from the unbound dye by ultracentrifugation (20 min, 160,000 x g at 4°C) and washed with 0.9% NaCl.

Confocal fluorescence microscopy

HMDM were generated on eight-well culture slides (Falcon, Becton Dickinson). After 7-10 days of culture, macrophages were washed several times with serum-free DMEM medium and incubated with fluorescently labeled E-ecto for 30 min. For some experiments, HMDM were pretreated with 0.5 μM cytochalasin D in DMEM for 60 min at 37°C prior to washes and incubation with ectosomes. Analysis was performed on an Axiovert confocal laser scanning microscope (LSM 510) from Zeiss AG (Feldbach, Switzerland). The lens used was a Zeiss Plan-Neofluar 100x. The acquisition software was LSM 510 (Zeiss AG). The imaging medium was Vectashield fluorescence mounting medium

Isolation and culture of human monocyte-derived macrophages

Monocytes were isolated from fresh buffy coats as described previously [28]. Briefly, a buffy coat was diluted 1/1 (vol/vol) with HBSS, layered over Ficoll-Hypaque (Sigma Chemical Co.), and centrifuged for 30 min at 350 g. Monocytes were recovered, washed twice in HBSS, and layered over a Percoll gradient. Percoll was prepared by mixing 1 vol 1.5 M NaCl with nine vol Percoll (Sigma Chemical Co.). The Percoll gradient was done by mixing 1.5/1 (vol/vol) isosmotic Percoll with PBS/citrate (NaH2PO4 1.49 mM, NaH2PO4 9.15 mM, NaCl 139.97 mM, C6H5Na3O7.2H2O 13 mM, pH 7.2). Isolated monocytes were resuspended at $2x10^6$ cells/mL in DMEM supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, and 2 mM L-glutamine. Monocytes were then allowed to adhere for 1 h at 37°C on culture plates. Adherent monocytes were washed three times with prewarmed DMEM and finally incubated for 7 days in DMEM supplemented with 10% NHS (pooled from 40 healthy donors). The culture was maintained in 5% CO2 at

37°C, and the medium was changed at days 3 and 7. Macrophages were used between days 7 and 10.

Activation of human monocyte-derived macrophages

HMDM were washed several times with prewarmed DMEM without NHS. Subsequently, each well was filled with 250 μL (final volume) fresh DMEM without NHS. Zymosan A (5 μg /mL final concentration) or LPS (10 ng/mL final concentration) and/or E-ecto were added, and SN were collected 15 h later (if not stated otherwise). The amount of ectosome-protein used in our experiments was quantified by using a Bradford protein assay, and assays were performed in triplicate. Results are representative of six independent experiments.

Collection of supernatants and analysis of cytokines

HMDM SNs were collected and spun for 10 min at 800 x g at 4°C (Mikro 24-48R centrifuge, Hettich, Bäch, Switzerland) to remove cell debris. Cytokine concentrations were determined by ELISA according to the manufacturer's instructions.

ATP depleted erythrocyte-derived ectosomes

ATP depletion of fresh-packed LD-E was carried out as described previously by Pascual et al. [15] with small modifications. In brief, LD-E were incubated under sterile conditions in 50 mL Falcon tubes at 20% hematocrit and 300 mOsM in a medium consisting of 50 mM glycylglycine, 5 mM KCI, 130 mM NaCl, 0.5 mM EDTA, and antibiotics (200 U/ml penicillin G, 0.2 mg/ml streptomycin) at pH 7.4. After incubation for 24 h at 37°C, the erythrocytes were pelleted through two successive centrifugations for 7 min at 480 x g. The SN containing ectosomes was collected and ultracentrifuged for 60 min at 4°C and 200,000x g. The ultracentrifugation was repeated to wash the ectosomes.

Ca^{2+} Ionophore erythrocyte-derived ectosomes

Ectosomes were prepared according to Allan et al. [29] with minor modifications. Briefly, erythrocytes were resuspended in 3 vol TBS containing 1 mM CaCl2 and 5 μM ionophore A23187 (Sigma Chemical Co.) and incubated at 37°C for 30 min. After addition of EDTA to a final concentration of 5 mM, the erythrocytes were pelleted through two successive centrifugations for 7 min at 480 x g. The SN containing ectosomes was then ultracentrifuged for 60 min at 4°C and 200,000 x g. The ultracentrifugation was repeated to wash the ectosomes.

PMN-derived ectosomes

PMNs were isolated from fresh buffy coats of normal donors, according to the technique described previously [30]. Briefly, a fresh buffy coat was diluted 1/1 (v/v) with 2 mM PBS-EDTA, mixed gently with 0.25 vol 4% Dextran T500, and left for 30 min for erythrocyte sedimentation. The leukocyte-rich SN was aspirated and centrifuged for 10 min at 200 x g. The pellet was resuspended for 1 min in 9 mL ultrapure water to lyse erythrocytes. Isotonicity was restored by addition of 3 mL 0.6 M KCl and 40 mL 0.15 M NaCl. Cells were then centrifuged 10 min at 350 x g and resuspended in 20 mL 2 mM PBS-EDTA. This suspension was layered over 20 mL Ficoll-Hypaque and centrifuged for 30 min at 350 x g. The PMN-rich pellet was recovered and washed twice in 2 mM PBS-EDTA. All manipulations were performed at 4°C. For stimulation, PMNs (10^7 cells/mL) were diluted 1/1 (v/v) in prewarmed (37°C) RPMI 1640 (Life Technologies, Basel, Switzerland) with 1 μM fMLP from Sigma Chemical Co. and incubated for 20 min at 37°C. PMNs were removed by centrifugation (4000 x g at 4°C), and the SNs were concentrated with Centriprep centrifugal filter devices (10,000 MW cut-off, Millipore) and stored in aliquots at -80°C until use.

Statistical analysis

For the comparison of the cytokine levels released by zymosan activated HMDM, in the absence or presence of ectosomes, we performed statistical analysis using paired *t*-test. $p<0.05$ was considered significant.

Results

Erythrocytes are known to release microparticles during storage before being transfused. We first characterized the properties of such microparticles isolated from leukocyte-depleted erythrocytes stored for 25 days in the blood bank (300 ml packed erythrocytes).

FACS analysis of erythrocyte-derived microparticles (ectosomes) released during conventional blood storage

Microparticles were isolated by differential centrifugation of leukocyte-depleted, packed erythrocytes (see Materials and Methods) and analyzed by FACS. They were identified by their size, as assessed by the logarithmic amplification of FSC and SSC signals (Fig. 1A). Ectosomes were localized within the R1 region. Flow cytometry analysis demonstrated that they were derived from erythrocytes, as >95% reacted with an antibody against GPA, a known, specific marker of erythrocytes (Fig. 1, B and C). There was no contamination with platelet-derived microparticles or leukocyte-derived ectosomes, as assessed by the absence of reaction with a specific marker of platelets CD41 and leukocytes CD45 (Fig. 1, B and C). Using an amphiphilic membrane dye, we observed the incorporation of the lipid dye, indicating the existence of a lipid membrane (Fig. 1D). This latter observation confirmed that microparticles of erythrocytes are vesicles with a membrane, most likely deriving from the erythrocyte membrane by ectocytosis [7]. Thus, we will refer to these vesicles as E-ecto.

PS expression is a marker for the loss of lipid membrane asymmetry of a cell and is found on cells undergoing apoptosis. Ectosomes released by PMNs express PS as well, indicating that ectocytosis is accompanied by a scrambling of PS in the cell membrane. By flow cytometry, we found that AnV bound specifically to E-ecto

(Fig. 1F). Thus, the E-ecto expressed PS similarly to other ectosomes and apoptotic cells.

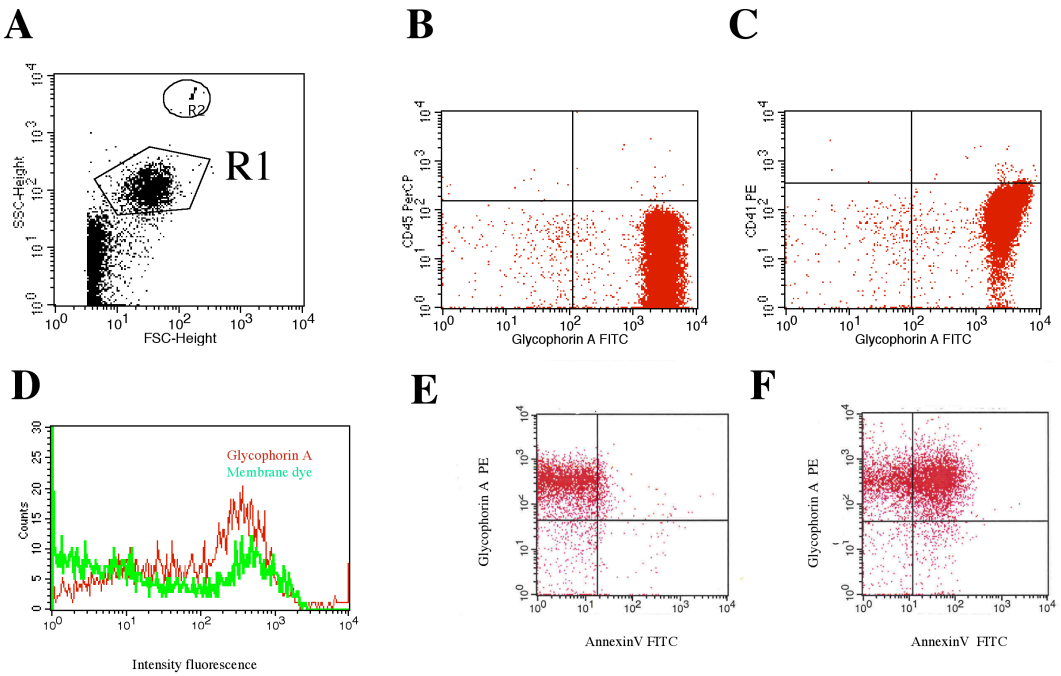


Figure 1. FACS analysis of E-ecto. (A) Representative flow cytometric dot plot of E-ecto. The region R1 represents FSC/SSC light-scatter gate of E-ecto. The region R2 represents the known density TruCount beads. (B and C) E-ecto were identified as GPA-positive events in the lower-right quadrant. The double-staining with anti-CD45 (leukocyte marker) showed the absence of contamination with leukocyte-derived ectosomes (B). The same holds true for the anti-CD41 staining (platelet marker), where no platelet-derived ectosome contamination was observed (C). (D) The double-staining with anti-GPA and a membrane dye showed that a large population of E-ecto incorporated the lipid dye, confirming the existence of a lipid membrane. (E and F) Flow cytometric dot plot of E-ecto double-stained with AnV-FITC and anti-GPA-PE. In the presence of binding buffer (F), a large part of the GPA-positive population binds AnV-FITC, attesting for the expression of PS on E-ecto. When incubated in calcium-depleted binding buffer, no binding of AnV-FITC was observed (E).

Electron microscopy analysis of erythrocyte-derived ectosomes

Electron microscopy showed that E-ecto represented a heterogeneous population of vesicles with sizes between 50 nm and 500 nm, the smaller vesicles corresponding almost to the nanovesicles released by erythrocytes, activated by Ca^{2+} ionophores, as described recently by Salzer et al. [31] (Fig. 2).

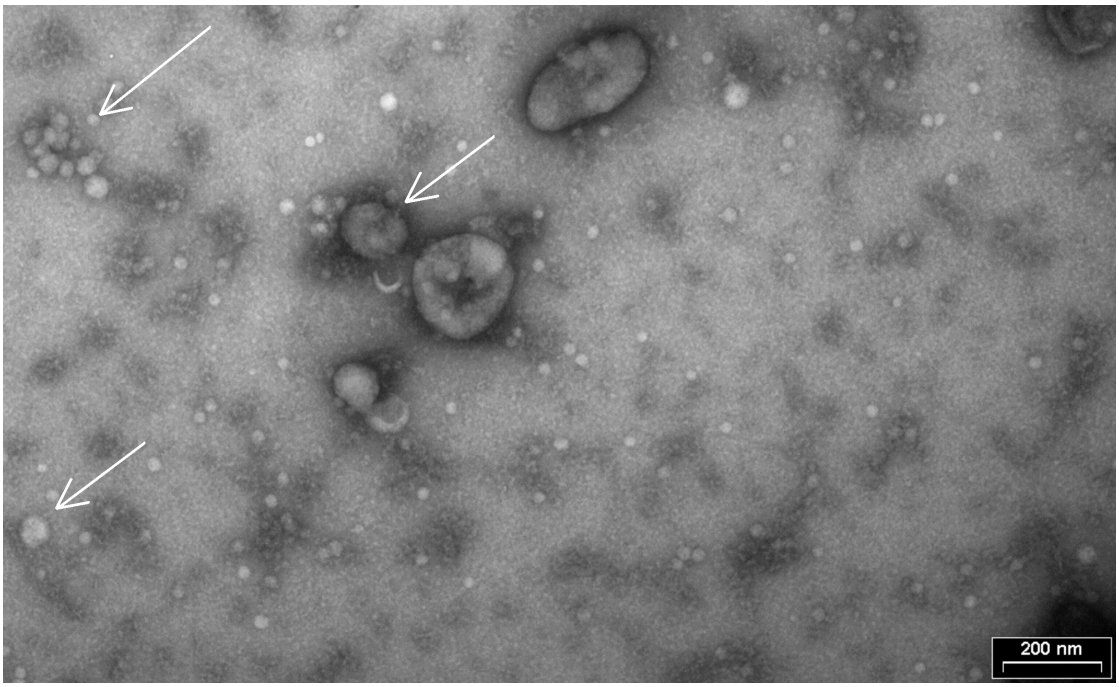


Figure 2. Electron microscopy of E-ecto. Picture of a standard E-ecto preparation showing heterogeneity in size (arrows), which ranged from 50 to 500 nm.
Original size bar: 200 nm.

Proteins expressed by the membrane of ectosomes

One of the general characteristics of ectosomes is that they do not express membrane proteins in the same ratios than the cells from which they derive. By SDS-PAGE analysis, it was evident that this was also the case for E-ecto (Fig. 3A). Although many proteins were present on erythrocyte and ectosome membranes, some were found predominantly on erythrocytes or E-ecto, indicating a specific sorting of proteins into and out of the membrane patch, which buds out of the cell (Fig. 3A). We did not specifically analyze any protein enrichments. As GPA was used as a marker, we controlled for its expression. GPA was present as dimers and monomers on erythrocytes and E-ecto and at a similar ratio (Fig. 3B). In addition, E-ecto expressed the three major complement regulators of erythrocytes, CD35 (CR1), CD55 (DAF), and CD59 (Fig. 3C), which is in line with previous work analyzing ectosomes released by ATP-depleted erythrocytes. CD35, as transmembrane protein, and CD55, a GPI-anchored protein, have been shown to be present and enriched slightly (i.e., 1.3-fold) on ectosomes [15]. In other words, it is unlikely that the E-ecto are particularly sensitive to complement attack.

Having established the general structure of E-ecto, we turned to define their properties.

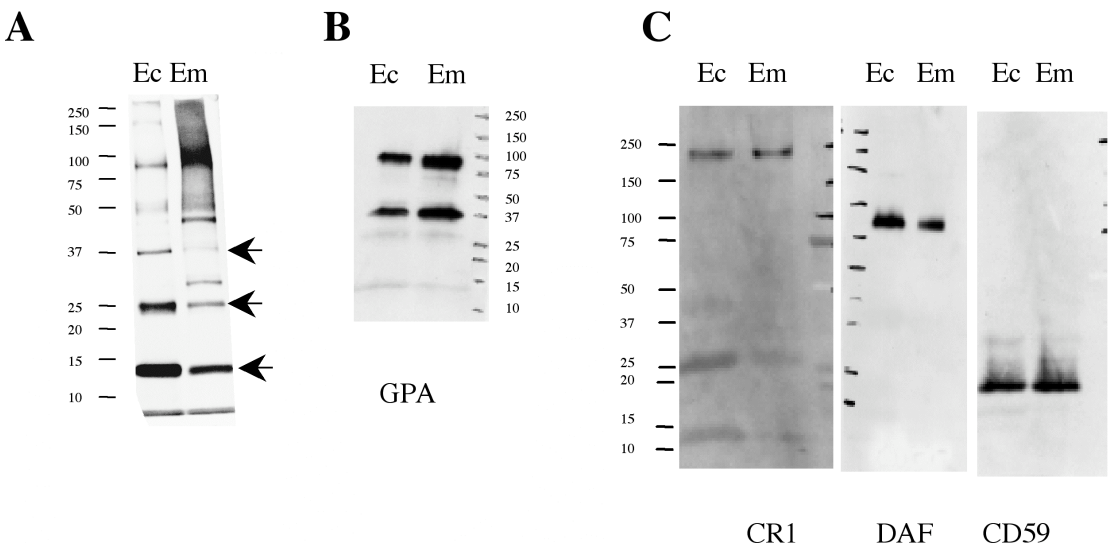


Figure 3. Protein expression of erythrocyte membranes and E-ecto. (A) The erythrocyte membranes (Em) and E-ecto (Ec) were fractionated by SDS-PAGE, followed by staining with silver stain reagents. The pattern of proteins of E-ecto showed that many proteins were present on erythrocyte membranes and ectosomes, with some found predominantly on erythrocyte membranes or E-ecto, indicating a specific sorting of proteins into and out of E-ecto at the time of their formation. The arrows show some proteins expressed predominantly on E-ecto. The molecular weight is indicated on the left. (B and C) The erythrocyte membranes and the E-ecto proteins were subjected to SDS-PAGE. After transferring to a nitrocellulose membrane, the membrane was reacted with antibodies, (B) anti-GPA, which showed that GPA was present as monomers and dimers on erythrocyte membranes and E-ecto. The molecular weight is indicated on the right. (C) Anti-CD35, -CD55, and -CD59 confirmed the expression of complement regulatory proteins on erythrocyte membrane proteins and E-ecto. The molecular weight is indicated on the left.

Binding of C1q to E-ecto and activation of complement

An important feature of apoptotic cell death is the specific recognition and removal of apoptotic cells by professional phagocytes. The binding of C1q to apoptotic cells/bodies is of critical importance for their removal by phagocytes. To find out whether C1q would also bind to E-ecto, we incubated them with purified C1q or directly with NHS. C1q was detected on E-ecto after incubation with purified C1q (Fig. 4A) and more interestingly, after incubation in whole serum, in which C1q is part of the macromolecular C1 complex (Fig. 4B). No signal was detected in the absence of C1q (Fig. 4A) and in the negative control using heat-inactivated human serum (Fig. 4B).

To investigate whether once bound to E-ecto, C1q had the potential to activate complement, we analyzed binding of complement C3 fragments. We incubated E-ecto with NHS and analyzed for the presence of C3 fragments with an anti-C3d mAb. As shown in Figure 4C, C3 fragments were bound after incubation in normal serum but not so when the serum was heat-inactivated. Thus, similarly to apoptotic cells, E-ecto fix complement C1q and allow complement activation and binding of C3 fragments.

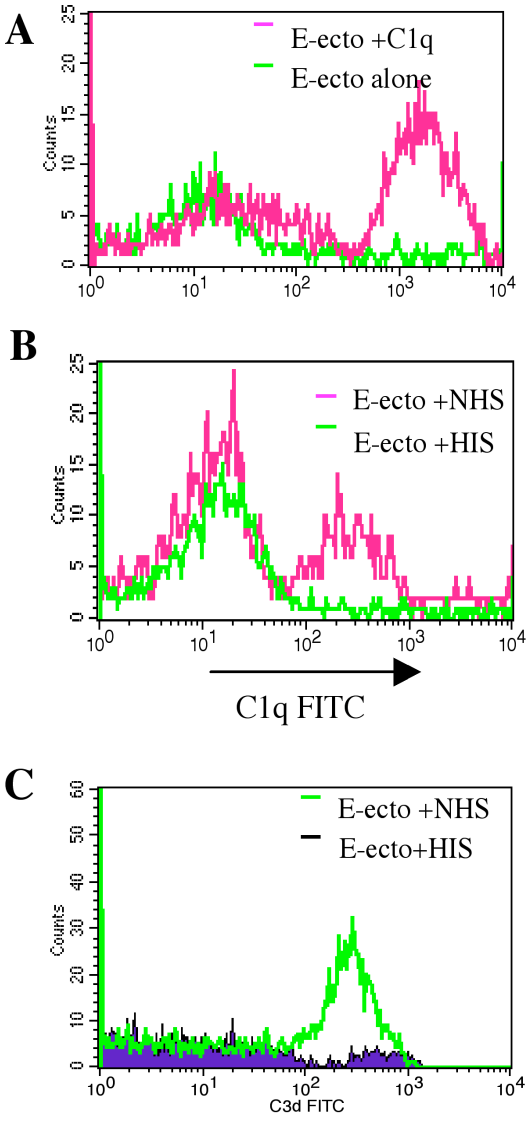


Figure 4. C1q binds to E-ecto and activates complement. FACScan histogram of ectosomes incubated for 30 min at 4°C (A) in the absence or presence of purified C1q or (B and C) in the presence of NHS or heat-inactivated NHS (HIS). In the presence of C1q or NHS, anti-C1q mAb showed the binding of C1q on ectosomes (A and B). This binding did not occur in the presence of heat-inactivated NHS (B). In the presence of NHS, C3 fragments bound E-ecto, as detected by the anti-C3d mAb but not in the presence of heat-inactivated NHS (C).

HMDMs bind and ingest ectosomes

The expression of PS and possibly other undefined changes in the membrane structure is likely to induce macrophages to recognize, bind, and phagocytose E-ecto. This was evident when studied by confocal microscopy. Fluorescently labeled E-ecto incubated for 30 min with HMDMs were phagocytosed, as reflected by the intense fluorescent staining of the cells (Fig. 5A). In contrast, when HMDMs were preincubated with cytochalasin D, a potent inhibitor of phagocytosis, they showed almost no fluorescence and hence, no uptake of E-ecto (Fig. 5B).

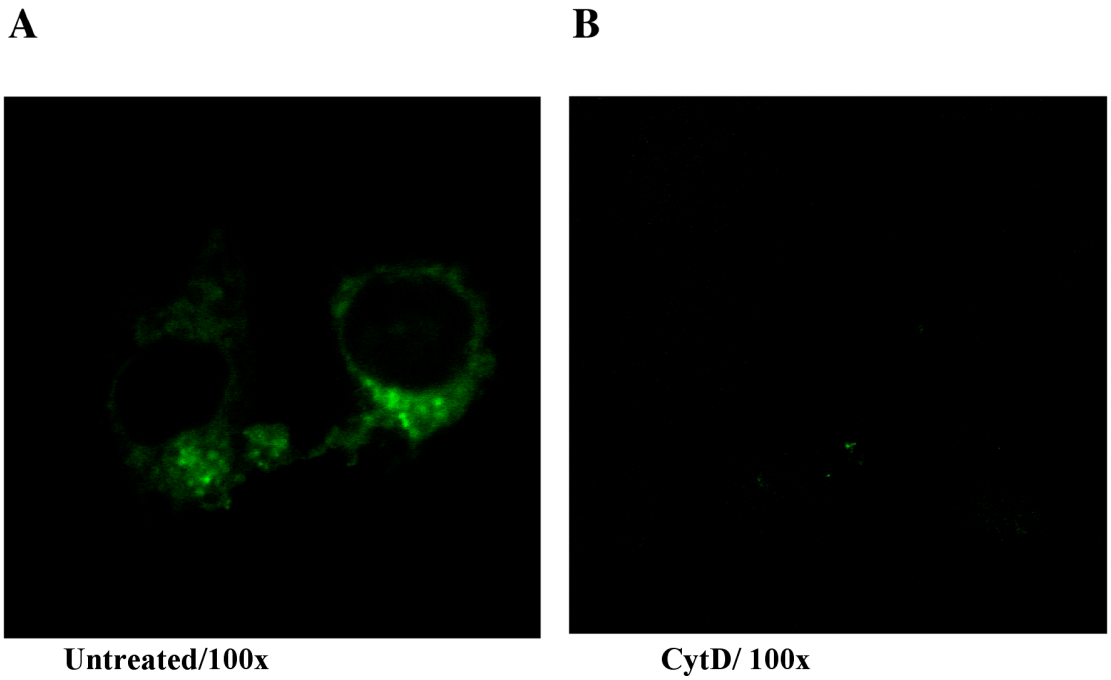


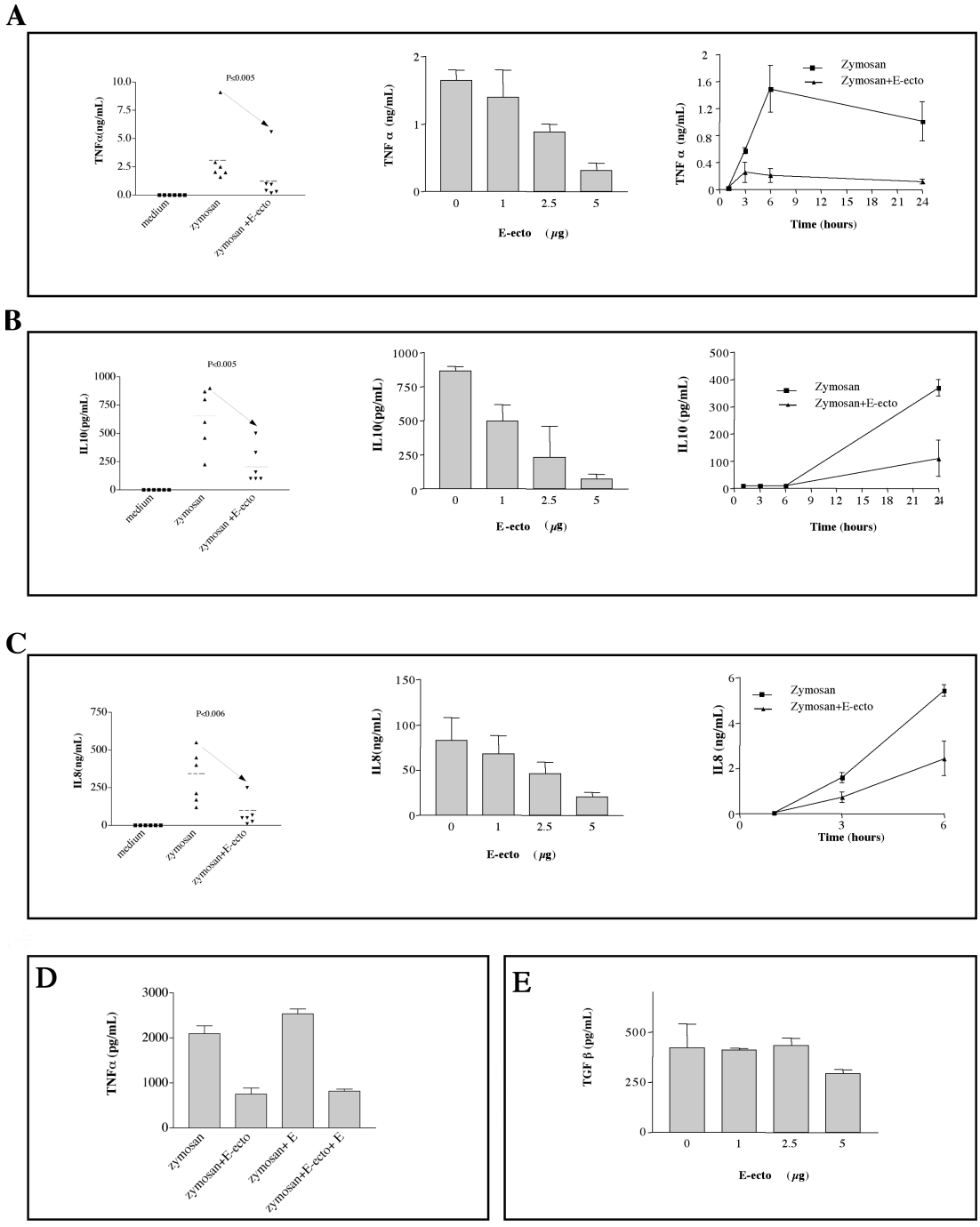
Figure 5. Confocal microscopy of phagocytosis. HMDM were incubated with fluorescently labeled ectosomes for 30 min, fixed, and analyzed by confocal laser microscopy. (A) HMDM bind and ingest E-ecto in the absence of cytochalasin D. (B) Alternatively, macrophages were preincubated with cytochalasin D (CytD) prior to the addition of ectosomes. The lens used was a Zeiss Plan-Neofluar 100x from Zeiss AG.

E-ecto have immunosuppressive effect on macrophages

Apoptotic cells as well as PMN-derived ectosomes have anti-inflammatory and immunosuppressive properties [20, 21, 32]. This may be related to the expression of PS [22]. Accordingly, it was of interest to test whether E-ecto that express PS as well could affect HMDM when these cells were activated. Thus, we coincubated E-ecto with HMDM in the presence or absence of zymosan A or LPS for 15 h. SN were analyzed for TNFα, IL-10, IL-8, and TGF-β1. First and as a control, we did not observe any modification in cytokine release by resting HMDM when exposed to E-ecto (not shown), but E-ecto inhibited the activation of HMDM by zymosan A. Whereas zymosan A induced release of TNFα, IL-10, and IL-8 by HMDM, the simultaneous addition of E-ecto was responsible for a significant decrease in the release of all three cytokines (Fig. 6, A–C). The inhibition was dose-dependant (Fig. 6, A–C). We next investigated the time-dependent effect of E-ecto on cytokine secretion by zymosan-activated HMDM. E-ecto induced a rapid and sustained, inhibitory effect on the release of TNFα, IL-10, and IL-8 (Fig. 6, A–C). In this experiment, we also tested as control the effect of erythrocytes alone and in the presence of E-ecto. Erythrocytes in excess by themselves had no effects on the release of TNFα by zymosan A-activated macrophages (Fig. 6D) nor did their addition to E-ecto; i.e., the E-ecto effect was not modified by the presence of erythrocytes. These effects were similar to those observed previously with PMN-ectosomes, with a major difference; i.e., E-ecto did not induce the release of TGF-β1 (Fig. 6E). Indeed, PMN-ectosomes induce on their own the release of TGF-β1 by HMDM, a release that is not modified by activating agents such as zymosan A or LPS [21]. The induction of TGF-β1 release was thought to explain the down-regulation of HMDM so that they react only weakly or not at all when exposed to zymosan A. As we could find no TGB-β1

release induced by E-ecto, we repeated the experiments in parallel with PMN-ectosomes prepared as described previously (Fig. 6F). The results confirmed the differences between the two types of ectosomes. To see whether these differences were intrinsically a result of differences in the cell origin or possibly to level of PS expression, we repeated the experiments with E-ecto, formed by in vitro aging of erythrocytes or (ATP depletion, not shown) by incubating erythrocytes with Ca^{2+} ionophores (Fig. 6F). Again, PMN-derived ectosomes induced TGF-β1 release, whereas none of the E-ecto could do so (Fig. 6F). By contrast, all three types of ectosomes had inhibitory effects on the release of TNFα, IL-10, and IL-8 (Fig. 6F).

LPS induced the release of TNFα, IL-8, and IL-10 but had no effect on TGF-β1 release. When HMDM were stimulated with LPS, E-ecto produced an inhibition of TNFα, IL-8 (Fig. 6G), and IL-10 (not shown) release similar to that induced on the zymosan A-activated HMDM. In a control experiment, LPS-stimulated HMDM released no TGF-β1 when exposed to E-ecto (not shown).


A
P≤0.005
TNFα(ng/mL)
10.0
7.5
5.0
2.5
0.0
medium
zymosan
zymosan+E-ecto
TNF α (ng/mL)
2
1
0
0
1
2.5
5
E-ecto (μg)
Zymosan
Zymosan+E-ecto
TNF α (ng/mL)
2
1.6
1.2
0.8
0.4
0
0 3 6 9 12 15 18 21 24
Time (hours)
B
P≤0.005
IL10(pg/mL)
1000
750
500
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medium
zymosan
zymosan+E-ecto
IL10(pg/mL)
1000
750
500
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E-ecto (μg)
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Zymosan+E-ecto
IL10 (pg/mL)
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Time (hours)
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zymosan
zymosan+E-ecto
IL8(ng/mL)
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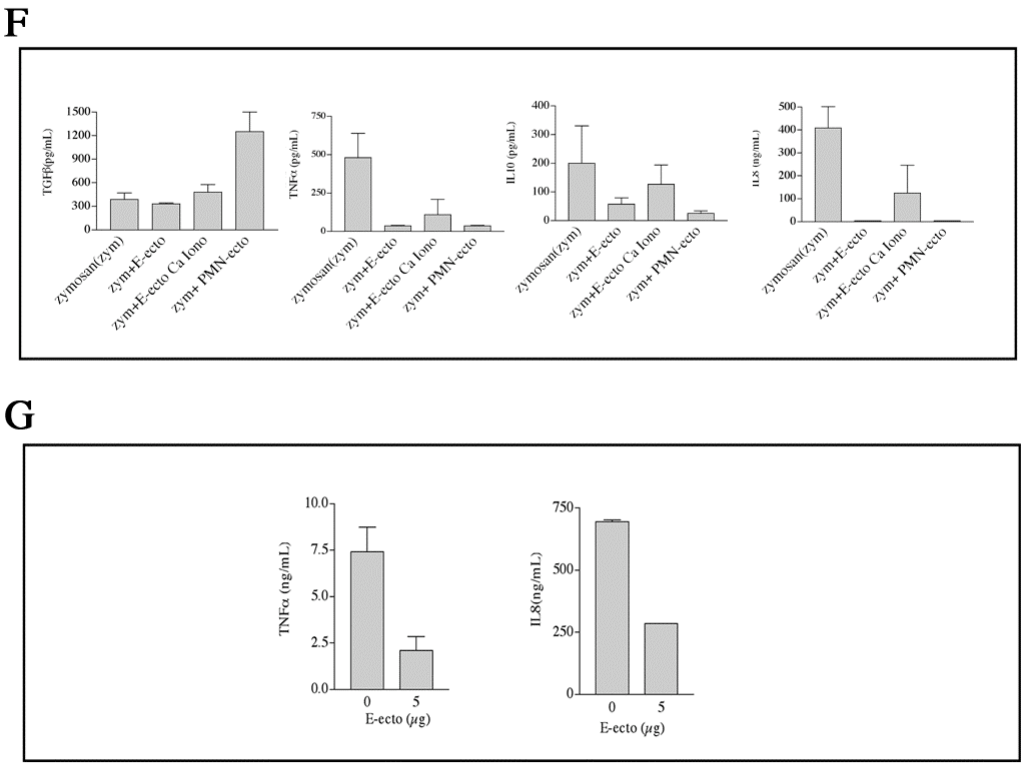

Figure 6. E-ecto have immunosuppressive properties. (A–C, left) HMDM were incubated in medium alone, medium with zymosan A, and medium with zymosan A and ectosomes overnight. SN were harvested and analyzed by ELISA, respectively, for (A) TNFα, (B) IL-10, and (C) IL-8. (Each point corresponds to one experiment.) (Center) HMDM were incubated in medium with zymosan A in the absence of ectosomes (0), medium with zymosan A, and different concentration of ectosomes (1, 2.5, 5 μg/mL) overnight. SN were harvested and analyzed for (A) TNFα, (B) IL-10, and (C) IL-8. (Right) HMDM were incubated in medium with zymosan A, medium with zymosan A, and ectosomes for up to 24 h. SN were harvested after 1, 3, 6, and 24 h for A and B and analyzed for (A) TNFα, (B) IL-10, and (C) IL-8. (D) HMDM were incubated overnight in medium with zymosan A, medium with zymosan A and E-ecto, medium with zymosan A and erythrocytes (E), and medium with

zymosan A + E-ecto and erythrocytes. SN were harvested and analyzed for TNFα. (E) HMDM were incubated in medium with zymosan A in the absence of ectosomes (0), medium with zymosan A, and different concentration of ectosomes (1, 2.5, 5 μg/mL) overnight. SN were harvested and analyzed for TGF-β1. (F) HMDM were incubated in medium with zymosan A, medium with zymosan A and E-ecto, zymosan A, E-ecto, and Ca^{2+} ionophore (Ca Iona), and zymosan A and PMN-ecto overnight. SN were harvested and analyzed, respectively, for TGF-β1, TNFα, IL-10, and IL-8. (G) HMDM were incubated in medium with LPS and medium with LPS and ectosomes overnight. SN were harvested and analyzed, respectively, for TNFα and IL-8. Assays were performed in triplicate. Results are representative of six independent experiments. Error bars indicate SEM.

The inhibition of macrophages by erythrocyte-derived ectosomes is long-lasting.

Macrophages were preincubated with E-ecto for 60 min and next washed with prewarmed medium to remove the remaining ectosomes. Macrophages were then left to recover (1 h, 24 h) before overnight stimulation with zymosan A. The release of TNFα, IL-8, and IL-10 by zymosan A-activated macrophages was inhibited up to 24 h after the contact between ectosomes and macrophages (Fig. 7, A–C), indicating a lasting alteration of the macrophage signaling machinery. The release of TGF-β1 was not modified (not shown).

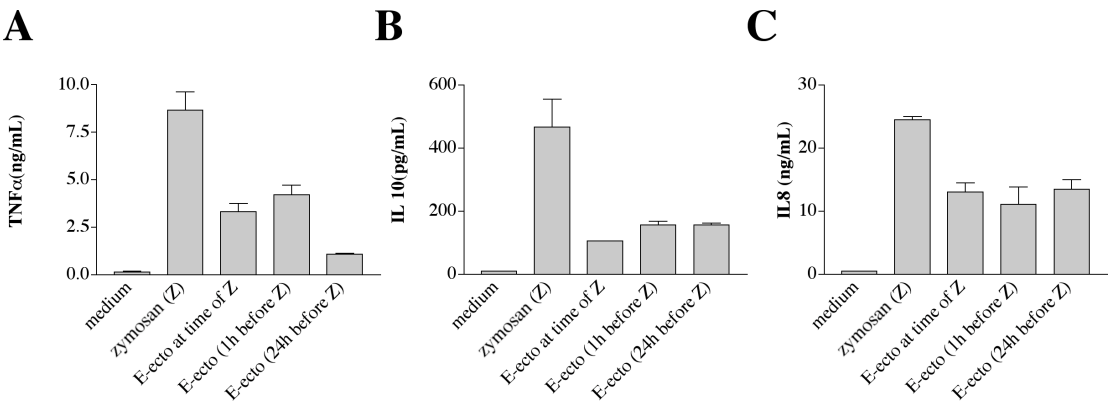

Figure 7. E-ecto have a lasting effect. HMDM were incubated in medium with E-ecto for 1 h, unbound ectosomes were washed, and HMDM were let to settle down during 24 h and 1 h before exposing them to zymosan A overnight. SN were harvested and analyzed for (A) TNFα, (B) IL-10, and (C) IL-8. The effects of E-ecto only on HMDM are identical to medium only; i.e., without zymosan A stimulation, no measurable cytokine amounts are released. Assays were performed in triplicate. Results are representative of three independent experiments. Error bar indicates SEM.

Discussion

In the present study, we could demonstrate that ectosomes derived from erythrocytes during storage have inhibitory potential on activated macrophages, indicating that they are active players in the regulation of inflammation.

A major aspect in cell biology is communication, which occurs through a direct contact between cells or by means of soluble substances, which react with cells. The fact that microvesicles released by cells influence other cells is a rather new concept but is a basic mechanism to deliver a message in a highly concentrated manner (multiple epitopes/costimulation) or to add a missing molecule. The vesicles released by dendritic cells (exosomes) can trigger an immune response, and the proteins of a vesicle can be incorporated in the cellular membrane to which it has bound [33, 34]. Because of the numerous functions of microvesicles, we can no longer have multiple, unclear definitions. Therefore, we propose, as suggested years ago by Stein and Luzio [7], that we define them as ectosomes when they are formed by budding out of a cell membrane, have a lipid membrane, and express a selection of membrane proteins. The release of preformed vesicles formed in multivesicular bodies has been defined as exosomes from the start. Undefined terminology such as "vesicles, particles, microparticles" should be used only when the origin of these vesicles remains undefined.

According to this definition, the microparticles found in stored erythrocyte concentrates are clearly ectosomes, as evidenced here and by other groups [16, 17, 35–38]. We showed here that in stored erythrocyte concentrates, more than 95% of the microparticles originate from erythrocytes, as shown by the expression of GPA on their surface. The presence of GPA by FACS indicated as well that they are

outside-out vesicles, thus formed by budding. They were not contaminated by leukocyte or platelet-derived particles, as they did not express CD45 or CD41, which allowed us to define their function more precisely. They had a lipid membrane, and they expressed a selective series of membrane proteins, which we did not characterize further, as the absence of given proteins, such as spectrin, and the enrichment of others, including GPI-anchored proteins (e.g., acetylcholinesterase), have already been demonstrated several times. The presence of the three complement regulators and inhibitors (CR1/CD35, DAF/CD55, CD59) indicates, however, that they might be resistant to complement attack and thus, would not be lysed easily [12, 15].

The expression of PS was also confirmed by the evident calcium-dependent binding of AnV. The exposure of PS has been suggested to induce and enhance thrombogenic activity [39, 40]. However, the expression of PS may be linked to other properties, such as the binding of C1q of the complement cascade [41]. Apoptotic cells and bodies express PS, bind C1q, and activate complement [20]. Ectosomes derived from PMNs have similar properties [42, 43]. Whether this binding is a general property of cells that have rearranged their membrane by scrambling the lipid molecules remains to be demonstrated, as the expression at the membrane of specific intracellular proteins such as calreticulin might be involved as well [44, 45]. Here, we demonstrated the binding of C1q, not only when purified C1q was added to E-ecto but also directly from whole serum. It may well be that C1q binds to PS or another lipid component of the membrane. C1q binding was followed by complement activation and fixation of C3 fragments, which are likely to favor binding to specific receptors on cells, particularly the newly described C3b receptor on fixed phagocytes [46, 47].

A “light” complement activation occurs on apoptotic cells as well, and C1q appears to be important, not only for the uptake of apoptotic cells but also for the prevention of

autoimmunity [48]. PS expression on dying cells in itself is also linked with recognition by phagocytes in down-regulating inflammation and preventing the immunization against autoantigens [20]. Thus, we have tested whether E-ecto have the capacity to down-regulate the reactivity of macrophages in an assay of macrophage activation. The E-ecto had evident capacity to inhibit the release of TNFα by macrophages exposed to zymosan A or LPS, known to react with TLR-2 and TLR-4, respectively. IL-8 and IL-10 were down-regulated as well, indicating that the E-ecto-induced down-regulation of macrophages was a global reaction not restricted to one cytokine only. Whereas IL-8 and TNFα are known, proinflammatory cytokines, the reduction in IL-10 was unexpected in this context, as IL-10 is in general considered as an anti-inflammatory/ immunosuppressive cytokine. However, when the kinetics of the cytokine releases were analyzed, it became clear that the release of IL-10 occurs after that of IL-8 and TNFα and may represent another mechanism to limit inflammation that may have been induced by the proinflammatory cytokines. If that is the case, it would not be surprising that less release of TNFα and IL-8 in the presence of E-ecto is by itself directly responsible for less IL-10 release.

Differently to ectosomes released by PMNs and vesicles released by different tumor cells, E-ecto did not enhance the release of TGF-β, suggesting that ectosomes originating from different cells, despite their common PS expression, have additional and specific interactions with macrophages. PS-liposomes are known to promote TGF-β secretion [49, 50], which could be an indication of the role of PS alone, when no other interactions occur. E-ecto might express other molecules capable of modulating the role of PS and/or having direct inhibitory activities on macrophages such as CD47 [51].

We specifically studied E-ecto found in stored blood. Erythrocytes release ectosomes under other experimental conditions, such as in vitro aging (ATP depletion) or Ca^{2+} ionophore exposure. Although all vesicles are formed by ectocytosis, there is no evidence that the ectosomes released by different stimuli express the same molecules and have similar properties [31]. Here, by correcting for the amount of protein, we found that all had similar inhibitory activity (i.e., TNFα IL-8, and IL-10 suppression, no TGF-β stimulation), although the strengths of these activities differed slightly, and Ca^{2+} ionophore-derived ectosomes were the least inhibitory. As a cautionary note, it has to be mentioned that even if our Ca^{2+} ionophore-ectosomes were washed before incubation with macrophages, a part of the Ca^{2+} ionophores probably remained trapped into the Ca^{2+} channel of the E-ecto and may have been released in the phagocyte, which has taken up the E-ecto. Thus, it is difficult to be certain that the reaction of the phagocytes exposed to Ca^{2+} ionophore-produced ectosomes is "ionophore"- independent.

Of particular interest were the lasting effects of E-ecto on activated macrophages. Indeed, the endocytosis of E-ecto had modified the macrophage for at least 24 h so that it did not react to further stimulation. This observation suggests that the signaling processes related to TLR-2 stimulation were downregulated or inhibited by an active, lasting process. Thus, the phagocytosis of E-ecto does not produce only a direct, anti-inflammatory signal to macrophages but also keeps the proinflammatory potential of macrophages down. How long this effect is lasting, particularly in vivo, remains an intriguing question.

It is known that every blood transfusion contains large amounts of E-ecto, most of which are probably removed efficiently by the fixed phagocytes, possibly with C3 fragment fixation being involved in the rapid recognition of the E-ecto. The property

of E-ecto to down-regulate the activity of macrophages may be relevant for those that travel to the spleen. Whether a related down-regulation on specific immune cells might occur in vivo is certainly speculative; however, considering the disputed, immunosuppressive activity of blood transfusion, investigations in this field should be pursued.

References

1. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., Geuze, H. J. (2000) Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci*. 113, 3365–3374.
2. Johnstone, R. M. (2006) Exosomes biological significance: a concise review. *Blood Cells Mol. Dis*. 36, 315–321.
3. Zwaal, R. F., Schroit, A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89, 1121–1132.
4. Campbell, A. K., Morgan, B. P. (1985) Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature* 317, 164–166.
5. Scolding, N. J., Morgan, B. P., Houston, W. A., Linington, C., Campbell, A. K., Compston, D. A. (1989) Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature* 339, 620–622.
6. Iida, K., Whitlow, M. B., Nussenzweig, V. (1991) Membrane Vesiculation protects erythrocytes from destruction by complement. *J. Immunol*. 147, 2638–2642.
7. Stein, J. M., Luzio, J. P. (1991) Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem. J*. 274, 381–386.
8. Navabi, H., Croston, D., Hobot, J., Clayton, A., Zitvogel, L., Jasani, B., Bailey-Wood, R., Wilson, K., Tabi, Z., Mason, M. D., Adams, M. (2005)

Preparation of human ovarian cancer ascites-derived exosomes for a clinical trial. *Blood Cells Mol. Dis.* 35, 149–152.

9. Liu, C., Yu, S., Zinn, K., Wang, J., Zhang, L., Jia, Y., Kappes, J. C., Barnes, S., Kimberly, R. P., Grizzle, W. E., Zhang, H. G. (2006) Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J. Immunol.* 176, 1375–1385.

10. Chen, W., Wang, J., Shao, C., Liu, S., Yu, Y., Wang, Q., Cao, X. (2006) Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. *Eur. J. Immunol.* 36, 1598–1607.

11. Haradin, A. R., Weed, R. I., Reed, C. F. (1969) Changes in physical properties of stored erythrocytes relationship to survival in vivo. *Transfusion* 9, 229–237.

12. Palek, J., Liu, A., Liu, D., Snyder, L. M., Fortier, N. L., Njoku, G., Kiernan, F., Funk, D., Crusberg, T. (1977) Effect of procaine HCLl on ATP: calcium-dependent alterations in red cell shape and deformability. *Blood* 50, 155–164.

13. Wagner, G. M., Chiu, D. T., Yee, M. C., Lubin, B. H. (1986) Red cell vesiculation—a common membrane physiologic event. *J. Lab. Clin. Med.* 108, 315–324.

14. Wagner, G. M., Chiu, D. T., Qju, J. H., Heath, R. H., Lubin, B. H. (1987) Spectrin oxidation correlates with membrane vesiculation in stored RBCs. *Blood* 69, 1777–1781.

15. Pascual, M., Lutz, H. U., Steiger, G., Stammler, P., Schifferli, J. A. (1993) Release of vesicles enriched in complement receptor 1 from human erythrocytes. *J. Immunol.* 151, 397–404.

16. Dumaswala, U. J., Bentley, N. L., Greenwalt, T. J. (1994) Studies in red blood cell preservation. 8. Liquid storage of red cells in a glycerolcontaining additive solution. *Vox Sang*. 67, 139–143.

17. Dumaswala, U. J., Dumaswala, R. U., Levin, D. S., Greenwalt, T. J. (1996) Improved red blood cell preservation correlates with decreased loss of bands 3, 4.1, acetylcholinestrase, and lipids in microvesicles. *Blood* 87, 1612–1616.

18. de Jong, K., Beleznay, Z., Ott, P. (1996) Phospholipid asymmetry in red blood cells and spectrin-free vesicles during prolonged storage. *Biochim. Biophys. Acta* 1281, 101–110.

19. Greenwalt, T. J. (2006) The how and why of exocytic vesicles. *Transfusion* 46, 143–152.

20. Savill, J., Dransfield, I., Gregory, C., Haslett, C. (2002) A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol*. 2, 965–975.

21. Gasser, O., Schifferli, J. A. (2004) Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 104, 2543–2548.

22. Hoffmann, P. R., Kench, J. A., Vondracek, A., Kruk, E., Daleke, D. L., Jordan, M., Marrack, P., Henson, P. M., Fadok, V. A. (2005) Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J. Immunol*. 174, 1393–1404.

23. Tartter, P. I. (1995) Immunologic effects of blood transfusion. *Immunol. Invest*. 24, 277–288.

24. Vamvakas, E. C. (2002) Possible mechanisms of allogeneic blood transfusion-associated postoperative infection. *Transfus. Med. Rev*. 16, 144–160.

25. Vamvakas, E. C. (2004) White blood cell-containing allogeneic blood transfusion, postoperative infection and mortality: a meta-analysis of observational "before-and-after" studies. *Vox Sang*. 86, 111–119.

26. Jensen, L. S., Puho, E., Pedersen, L., Mortensen, F. V., Sorensen, H. T. (2005) Long-term survival after colorectal surgery associated with buffycoat- poor and leukocyte-depleted blood transfusion: a follow-up study. *Lancet* 365, 681–682.

27. Lach-Trifilieff, E., Marfurt, J., Schwarz, S., Sadallah, S., Schifferli, J. A. (1999) Complement receptor 1 (CD35) on human reticulocytes: normal expression in systemic lupus erythematosus and HIV-infected patients. *J. Immunol*. 162, 7549–7554.

28. de Almeida, M. C., Silva, A. C., Barral, A., Barral Netto, M. (2000) A simple method for human peripheral blood monocyte isolation. *Mem. Inst. Oswaldo Cruz* 95, 221–223.

29. Allan, D., Thomas, P., Limbrick, A. R. (1980) The isolation and characterization of 60 nm vesicles ("nanovesicles") produced during ionophore A23187-induced budding of human erythrocytes. *Biochem. J*. 188, 881– 887.

30. Hess, C., Sadallah, S., Hefti, A., Landmann, R., Schifferli, J. A. (1999) Ectosomes released by human neutrophils are specialized functional units. *J. Immunol*. 163, 4564–4573.

31. Salzer, U., Hinterdorfer, P., Hunger, U., Borken, C., Prohaska, R. (2002) Ca(++)-dependent vesicle release from erythrocytes involves stomatinspecific lipid rafts, synexin (annexin VII), and sorcin. *Blood* 99, 2569– 2577.

32. Eken, C., Gasser, O., Zenhaeusern, G., Oehri, I., Hess, C., Schifferli, J. A. (2008) Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J. Immunol*. 180, 817–824.

33. Mack, M., Kleinschmidt, A., Bruhl, H., Klier, C., Nelson, P. J., Cihak, J., Plachy, J., Stangassinger, M., Erfle, V., Schlondorff, D. (2000) Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat. Med*. 6, 769–775.

34. Sloand, E. M., Mainwaring, L., Keyvanfar, K., Chen, J., Maciejewski, J., Klein, H. G., Young, N. S. (2004) Transfer of glycosylphosphatidylinositolanchored proteins to deficient cells after erythrocyte transfusion in paroxysmal nocturnal hemoglobinuria. *Blood* 104, 3782–3788.

35. Willekens, F. L., Roerdinkholder-Stoelwinder, B., Groenen-Dopp, Y. A., Bos, H. J., Bosman, G. J., van den Bos, A. G., Verkleij, A. J., Werre, J. M. (2003) Hemoglobin loss from erythrocytes in vivo results from spleenfacilitated vesiculation. *Blood* 101, 747–751.

36. Willekens, F. L., Werre, J. M., Kruijt, J. K., Roerdinkholder-Stoelwinder, B., Groenen-Dopp, Y. A., van den Bos, A. G., Bosman, G. J., van Berkel, T. J. (2005) Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. *Blood* 105, 2141– 2145.

37. Werre, J. M., Willekens, F. L., Bosch, F. H., de Haans, L. D., van der Vegt, S. G., van den Bos, A. G., Bosman, G. J. (2004) The red cell revisited—matters of life and death. *Cell. Mol. Biol. (Noisy-le-grand)* 50, 139–145.

38. Dumaswala, U. J., Greenwalt, T. J., Bryan, D. J. (1984) Human erythrocytes shed exocytic vesicles in vivo. *Transfusion* 24, 490–492.

39. Shapira, S., Friedman, Z., Shapiro, H., Presseizen, K., Radnay, J., Ellis, M. H. (2000) The effect of storage on the expression of platelet membrane phosphatidylserine and the subsequent impact on the coagulant function of stored platelets. *Transfusion* 40, 1257–1263.

40. Chung, S. M., Bae, O. N., Lim, K. M., Noh, J. Y., Lee, M. Y., Jung, Y. S., Chung, J. H. (2007) Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler. Thromb. Vasc. Biol.* 27, 414–421.

41. Pittoni, V., Valesini, G. (2002) The clearance of apoptotic cells: implications for autoimmunity. *Autoimmun. Rev.* 1, 154–161.

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

43. Gasser, O., Schifferli, J. A. (2005) Microparticles released by human neutrophils adhere to erythrocytes in the presence of complement. *Exp. Cell Res.* 307, 381–387.

44. Alloisio, N., Texier, P., Denoroy, L., Berger, C., Miraglia del Giudice, E., Perrotta, S., Iolascon, A., Gilsanz, F., Berger, G., Guichard, J. (1996) The cisternae decorating the red blood cell membrane in congenital dyserythropoietic anemia (type II) originate from the endoplasmic reticulum. *Blood* 87, 4433–4439.

45. Subramanian, S., Tsai, R., Discher, D. E. (2006) The "metabolon," CD47, and the "phagocytic synapse": molecular co-localization and species divergence. *Transfus. Clin. Biol.* 13, 31–38.

46. Wiesmann, C., Katschke, K. J., Yin, J., Helmy, K. Y., Steffek, M., Fairbrother, W. J., McCallum, S. A., Embuscado, L., DeForge, L., Hass, P. E., van Lookeren Capagne, M. (2006) Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature* 444, 217–220.

47. Helmy, K. Y., Katschke Jr., K. J., Gorgani, N. N., Kljavin, N. M., Elliott, J. M., Diehl, L., Scales, S. J., Ghilardi, N., van Lookeren Campagne, M. (2006) CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* 124, 915–927.

48. Botto, M., Dell'Agnola, C., Bygrave, A. E., Thompson, E. M., Cook, H. T., Petry, F., Loos, M., Pandolfi, P. P., Walport, M. J. (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet.* 19, 56–59.

49. Fadok, V. A., de Cathelineau, A., Daleke, D. L., Henson, P. M., Bratton, D. L. (2001) Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J. Biol. Chem.* 276, 1071–1077.

50. Huynh, M. L., Fadok, V. A., Henson, P. M. (2002) Phosphatidylserinedependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *J. Clin. Invest.* 109, 41–50.

51. Oldenborg, P. A., Zheleznyak, A., Fang, Y. F., Lagenaur, C. F., Gresham, H. D., Lindberg, F. P. (2000) Role of CD47 as a marker of self on red blood cells. *Science* 288, 2051–2054.

Section 3:

Polymorphonuclear Neutrophil - Derived Ectosomes inhibit NFκB activation in zymosan activated human macrophages in a Mer tyrosine kinase dependent manner

Abstract

At the earliest stage of activation, human polymorphonuclear neutrophils release vesicles, which bud from the cell surface. These vesicles, called ectosomes (PMN-Ect), expose phosphatidylserine in the outer membrane leaflet. They inhibit the inflammatory response of human monocyte-derived macrophages and dendritic cells to LPS and zymosan, and induce TGF-β1 release, suggesting a reprogramming towards a tolerogenic phenotype. The receptors and signaling pathways involved have not yet been defined. Here, we demonstrated that PMN-Ect induced an immediate calcium flux in macrophages indicating that signaling processes were immediately activated. PMN-Ect interfered with zymosan A activation of macrophages via inhibition of NFκB translocation and NFκB p65/RelA phosphorylation. Mer tyrosine kinase receptor and phosphatidylinositol 3-kinase (PI3K)/Akt pathway played a key role in this immunomodulatory effect as shown by using specific Mer blocking antibodies and LY 294002, a PI3K inhibitor. As a result, PMN-Ect reduced the transcription of many proinflammatory genes in zymosan A activated macrophages. Of interest, the TGF-β1 release induced by PMN-Ect was not related to a modification in its transcription. In sum, PMN-Ect modulated the inflammatory response of macrophages by different means including rapid signaling possibly

responsible for the release of TGF-β1, and via Mer receptor regulated PI3K/Akt and NFκB pathways.

Introduction

In general, intercellular communication occurs via membrane contact, soluble mediators (e.g. hormones, cytokines, chemokines), and small molecular mediators (e.g. nucleotides, bioactive lipids, nitric oxide ions) (1-3). However, recent evidence emphasizes the transfer of information by small vesicles released by one cell to its target cell (4-6). In the literature such vesicles got various names including microvesicles, nanovesicles, particles and microparticles. Specific vesicles released from multivesicular bodies (preformed vesicles) from various cell types have been named exosomes, and best described in the immune system, where they are capable to present specific antigens to T-lymphocytes (7-10). Others, released at the time of cell activation by budding directly from the cell surface, i.e. by ectocytosis, have been named logically ectosomes (11, 12).

Ectosomes are released by human polymorphonuclear neutrophils (PMN-Ect) at the time of cell activation. Interestingly, such ectosomes did not increase inflammatory processes; to the contrary they were shown to down-modulate cellular activation of macrophages (13) and dendritic cells (DCs) (14). In human monocyte-derived macrophages (HMDM), PMN-Ect could inhibit the release of TNFα, and reduce the release of IL-8 and IL-10 induced by zymosan A and lipopolysaccharide (LPS) (13). When immature monocyte-derived dendritic cells (MoDCs) were exposed simultaneously to PMN-Ect and LPS, their morphology was modified, their phagocytic activity and the expression of cell surface markers (CD40, CD80, CD83, CD86, and HLA-DP DQ DR) were reduced, the cytokine-release (IL-8, IL-10, IL-12,

and TNFα) was inhibited, and their capacity to induce T cell proliferation was impaired (14). These data suggested that ectosomes induce a tolerogenic phenotype in immature dendritic cells, similarly to what has been described for apoptotic cells (ACs).

So far, the mechanisms responsible for the biological effects of PMN-Ect remain speculative. The phosphatidylserine (PS) exposed in the outer membrane leaflet of PMN-Ect may be a major factor influencing macrophages and DCs, as shown for ACs and liposomes (15-18). Similarly to ACs, PMN-Ect have been shown to induce the release of TGF-β by macrophages and dendritic cells, which might be responsible for the down-regulation of their TLR-mediated maturation (19-22).

Recent studies revealed that TAM (Tyro3, Axl, Mer) receptor tyrosine kinases bind PS, and APC-produced TAM ligands are bridging these interactions in a calcium dependent manner (e.g., growth-arrest-specific 6 (GAS6) and Protein S) (23, 24). TAM receptors, expressed in a large variety of cells, including macrophages and DCs, are shown to have the capacity to down-modulate the inflammatory response (25, 26). *In vivo* and *in vitro* studies demonstrate that these receptors are required for AC clearance and homeostatic regulation of the immune system (27-30). Most of the analyses concerning ACs and TAM receptors have been carried out with the Mer receptor. Recently, ACs have been proposed to inhibit DC activation via the induction of Mer which activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and inhibits NFκB (31).

The transcription factor NFκB has a major role in regulating many aspects of cellular activity, in inflammation, cell proliferation, differentiation and cell survival (32). The mammalian NFκB protein family consists of five proteins p50, p52, p65 (RelA), c-Rel and RelB found in homodimers and heterodimers (32-34). In the cytoplasm,

NFκB is regulated by a class of inhibitor proteins, called IκBs (34, 35). Upstream activating signals cause the phosphorylation of IκBs, their ubiquitination and subsequent degradation, allowing NFκB dimers to enter the nucleus (32, 34-36). Once translocated in the nucleus, NFκB undergoes post-translational modifications, like phosphorylation of the p65 subunit at serine 276 and serine 536, that affect its transcriptional activation (32). Whether PMN-Ect interfere with the NFκB signaling pathway is currently undefined.

We have previously shown that PMN-Ect have immediate inhibitory effects on HMDMs and DCs (13, 14). In the present study, we investigated whether PMN-Ect modify the NFκB signaling pathways of HMDMs. We also analyzed the early transcriptional responses of HMDMs to PMN-Ect with or without TLR-2 stimulation.

Materials and Methods

Isolation, culture and maturation of HMDMs

HMDMs were derived from monocytes isolated from fresh buffy coats. A buffy coat was diluted 1/1 (v/v) with Hanks balanced saline solution (HBSS; GIBCO, Invitrogen, UK), layered over Histopaque-1077 (Sigma, St Louis, MO, USA), and centrifuged for 30 min at 350 x g. Monocytes were recovered, washed and cultured in Dulbecco modified essential medium supplemented with 1% penicillin/streptomycin and 1% L-glutamine ($DMEM^+$) for 1 h at 37°C. After incubation, non-adherent cells were removed by washing twice with pre-warmed $DMEM^+$. The remaining adherent cells were then cultured in $DMEM^+$ supplemented with 10% normal human serum (NHS). The culture was maintained in 5% CO_2 at 37°C, and the medium was replaced at days 4 and 7. On day 7 to 10, HMDMs were washed, and PMN-Ect and/or zymosan A (5 μg/ml final concentration; Sigma) were added in fresh $DMEM^+$ without NHS (13).

Collection of PMN-Ect

To isolate PMNs, a fresh buffy coat was diluted 1/1 (v/v) with PBS-EDTA (2 mM), mixed with 0.25 vol 4% Dextran T500 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and left for 30 min for erythrocyte sedimentation. Leukocyte-rich supernatant was aspirated and centrifuged for 10 min at 200 x g. The pellet was resuspended in 9 ml ultrapure water to lyze erythrocytes. Isotonicity was restored by addition of 3 ml KCl (0.6 M) and 40 ml NaCl (0.15 M). Cells were then centrifuged 10 min at 350 x g and resuspended in 20 ml PBS-EDTA. This suspension was layered over 20 ml Histopaque-1077 and centrifuged for 30 min at 350 x g. The PMN-rich

pellet was recovered and washed twice in PBS-EDTA. All manipulations were performed at 4°C, thus minimizing PMN activation (11-14).

For stimulation, pooled PMNs ($1x10^7$ cells/ml) from healthy blood donors were diluted 1/1 (v/v) in prewarmed (37°C) RPMI 1640 (GIBCO, Invitrogen) with 1 μM fMLP, and incubated for 20 min at 37°C. PMNs were removed by centrifugation (4000 x *g* for 15 min at 4°C), and PMN-Ect contained in the supernatant were concentrated with Centriprep centrifugal filter devices (molecular mass 10,000 MW cut-off; Millipore) and stored in aliquots at -80°C until use (11-14).

Calcium measurement

For calcium (Ca^{2+}) imaging, glass coverslip-grown HMDMs were loaded with the fluorescent Ca^{2+} indicator fluo-4 AM (Invitrogen; 5 μM final concentration) for 60 min at 37°C. Cells were then washed with Krebs Ringer containing 1 mM $CaCl_2$ and the coverslips were mounted on a thermostated perfusion chamber. Experiments were started by adding PMN-Ect or zymosan A. $DMEM^+$ medium or lanthanum, a nonspecific cellular Ca^{2+} channel blocker, were used as negative controls. Changes in fluo-4 fluorescence were monitored with a Nikon Eclipse TE2000-E fluorescent microscope equipped with a CFI APO TIRF 60x objective. Changes in fluorescence were detected by exciting at 488 nm and recording the emission at 510 nm via an electron multiplier C9100-13 Hamamatsu CCD camera. Image analysis was performed with the Openlab imaging system.

Detection of NFκB activation

HMDMs were cultured in 96 well plates for 7 days. The phosphorylation level of NFκB p65/RelA was quantified with Cellular Activation of Signaling ELISA (CASE) (SABiosciences, MD, USA), according to the manufacturer's instructions. In some

experiments, HMDMs were treated with 20 μg/ml of αMerTK antibody (AF891; R&D Systems, MN, USA) or with 50 μM of the PI3K inhibitor LY 294002 (SABiosciences) for 1 h at 37°C prior to PMN-Ect and/or zymosan A incubation. All samples were measured in duplicates or triplicates and the results are given as an absorbance ratio of phosphorylated NFκB p65/RelA protein / total p65/RelA protein.

Fluorescence microscopy

HMDMs were generated on 8-well culture slides (Falcon, Becton Dickinson). After 7 to 10 days of culture, HMDMs were washed with DMEM$^+$ without NHS and incubated 20 min with or without zymosan A in absence/presence of CD66b-FITC (GeneTex Inc., TX, USA) labeled PMN-Ect. Then, HMDMs were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Staining was performed by incubation with anti-NFκB primary antibody (RelA, sc-109) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) followed by Cy5 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Labeled cells were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Analysis was performed on an Olympus BX61 microscope from Olympus Schweiz AG (Volketswil, Switzerland).

RNA extraction, labeling, and microarray hybridization

For microarray experiments, we cultured monocytes isolated from fresh buffy coats of 4 healthy donors (2 males and 2 females between the ages of 20 to 45) for 7 days at 37°C. At day 7, HMDMs were cultured for another 3 h alone, in presence of PMN-Ect, with zymosan A, and with zymosan A and PMN-Ect. Then, total RNA was extracted using the Trizol method (Invitrogen), and cleaned up with RNeasy MinElute Cleanup Kit (Qiagen Inc., Valencia, CA). RNA quantity was measured with

NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, DE, USA), and integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). The GeneChip expression two-cycle target labeling kit (Affymetrix, CA, USA) was used for all samples according to manufacturer's instructions. Labeled cRNA was hybridized to GeneChip Human GenomeU133A 2.0 Array (Affymetrix) for 16 hours at 45°C. Then, the arrays were washed and stained with streptavidin-phycoerythrin, and fluorescent images were recorded on a GeneChip Scanner 3000 (Affymetrix).

Microarray analysis

Microarray data were summarized using gcrma (as implemented in the Bioconductor package). Data were then median-centered on a per-gene and per-patient basis. These normalized data were then visualized using hierarchical clustering (Euclidean distance metric, complete linkage).

Quantitative real-time RT-PCR

At day 7, HMDMs were cultured for 3 h alone, in presence of PMN-Ect, with zymosan A, and with zymosan A and PMN-Ect. After 3 h, total RNA was isolated with the Nucleospin RNA/Protein (Macherey-Nagel, Düren, Germany) and was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc.) in the presence of random hexamers (Promega) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70°C and then for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. SYBR-PCR was performed based on SYBR green fluorescence (SYBR green PCR master mix; Applied Biosystems, Foster City, CA). Primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), IL-1β, IL-6, IL-8, IL-12, TNFα, TGF-β, TXNIP, and CXCR4 are shown in Table 1. The difference in the cycle

threshold (ΔCT) value was derived by subtracting the CT value for GAPDH, which served as an internal control, from the CT value for transcripts of interest. All reactions were run in duplicate by using an ABI 7500 sequence detection system (Applied Biosystems). mRNA expression levels of the transcripts were calculated relative to GAPDH from the ΔCT values using the formula 2-ΔCT.

GAPDH	fwd ATTGCCCTCAACGACCACT rev GCACAGGGTACTTTATTGATGG
IL-1β	fwd GGGCCTCAAGGAAAAGAATC rev AGCTGACTGTCCTGGCTGAT
IL-6	fwd CAGTTCCTGCAGAAAAAGGC rev ATCTGAGGTGCCCATGCTAC
IL-8	fwd ACATACTCCAAACCTTTCCACCC rev CAACCCTCTGCACCCAGTTTTC
IL-12	fwd CATAACTAATGGGAGTTGCCTGGC rev AACGGTTTGGAGGGACCTCG
TNFα	fwd GAGTGACAAGCCTGTAGCCCATGTTGTAGC rev GCAATGATCCCAAAGTAGACCTGCCCAGAC
TGF-β1	fwd TCCGCAAGGACCTCGGCTGGA rev ATCATGTTGGACAGCTGCTCC
TXNIP	fwd CTAAGCAGCAGAACATCCAG rev CGGTGATCTTCAGGAATGAC
CXCR4	fwd GCCTGAGTGCTCCAGTAGCC rev TGGAGTCATAGTCCCCTGAGC

Table 1. Primers for quantitative real-time RT-PCR.

Quantitation of cytokines by ELISA

HMDM supernatants were collected and spun for 10 min at 500 x g at 4°C to remove cellular debris. The concentrations of TNFα and TGF-β1 were measured using OptEIA ELISA kits (Becton Dickinson) according to the manufacturer's instructions. All samples were measured in duplicates.

Statistical analysis

Data sets were tested for normality. For normally distributed data, parametric analysis (two-tailed paired Student t-test), for non-normally distributed data non-parametric analysis (Wilcoxon matched pairs test) were performed using GraphPad Prism software (GraphPad Software Inc.). Data are expressed as mean ± SEM. A p value less than 0.05 was considered statistically significant.

Results

PMN-Ect induce immediate Ca^{2+} release from intracellular stores in HMDMs

Calcium (Ca^{2+}) is a universal intracellular signal that mediates various cellular processes (37, 38). We were interested in investigating whether Ca^{2+} signaling is involved in HMDM activation upon encounter with PMN-Ect or zymosan A. HMDMs were loaded with the fluorescent Ca^{2+} indicator Fluo-4, and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored. As shown in Fig.1A the addition of PMN-Ect was accompanied by repetitive $[Ca^{2+}]_i$ oscillations, which occurred only seconds after their addition. Interestingly, addition of zymosan A was also accompanied by an increase in the $[Ca^{2+}]_i$, however, in contrast to the pattern observed after addition of PMN-Ect, the signal obtained with zymosan A was a single peak, which subsequently declined back to resting levels (Fig.1B). Interestingly, when both PMN-Ect and zymosan A were added simultaneously, they did not give rise to synergistic signals but both the oscillations and peak increase in the $[Ca^{2+}]_i$ were maintained (Fig.1C). These results indicate that the HMDM Ca^{2+} signaling patterns generated by PMN-Ect and zymosan A are different. Since amplitude and frequency modulations of Ca^{2+} signals activate differential gene transcription (39-41), we next investigated the major transcription factors controlling the proinflammatory genes, Nfκ B.

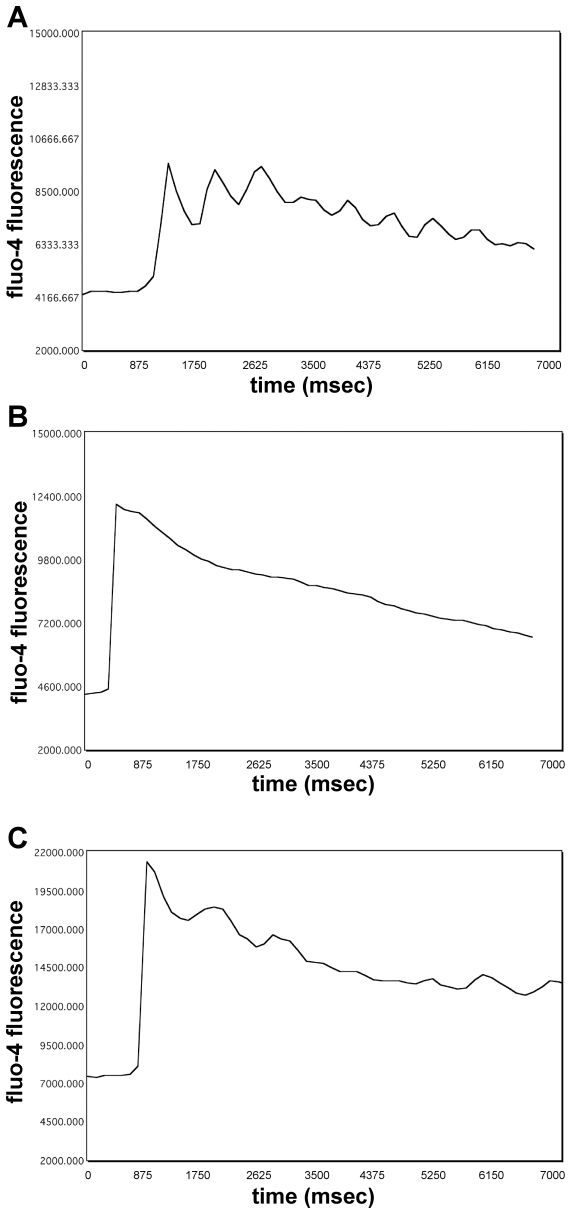

Figure 1. Single-cell intracellular calcium imaging on human macrophages. HMDMs grown on glass coverslips for 7 days were loaded with fluo-4 AM (5 μM final concentration) for 60 min at 37°C. Traces show changes in fluorescence detected by exciting at 488 nm and recording the emission at 510 nm. Experiments were performed in Krebs Ringer containing 1 mM $CaCl_2$. Experiments were started by adding (A) PMN-Ect, (B) zymosan A, or (C) both. DMEM medium or lanthanum were used as negative controls. DMEM medium made no changes, and in the presence of lanthanum, intracellular Ca^{2+} was released, but further signals were blocked. Image analysis was performed with the Openlab imaging system. Results are representative of multiple HMDMs analyzed from different donors.

PMN-Ect inhibit NFκB activation in HMDMs

NFκB dimer p50/p65 is typically sequestered in the cytoplasm by its interaction with IκB. Upon stimulation, IκB is phosphorylated, ubiquitinated, and degraded, allowing NFκB dimer to enter the nucleus (32, 34-36). Since phosphorylation of NFκB p65 at serine 536 (Ser536) is reported to enhance its transcriptional activation (32), we measured phosphorylation of NFκB p65 in resting or zymosan-stimulated HMDMs in absence/presence of PMN-Ect, after 20 and 45 min (Fig.2). PMN-Ect alone had no effect on NFκB p65 Ser536 phosphorylation. In contrast, zymosan A alone induced a significant Ser536 phosphorylation of NFκB p65 compared to resting HMDMs. Strikingly, the effect of zymosan A was to a large extent suppressed by PMN-Ect (at 20 min, $p=0.04$; at 45 min, $p=0.04$; $n=3$). These results demonstrate that PMN-Ect have the capacity to interfere with NFκB p65 Ser536 phosphorylation at the time of HMDM stimulation, suggesting an NFκB transactivation inhibitory effect of PMN-Ect.

To see whether this reduced phosphorylation was related to interference with the translocation of NFκB to the nucleus, we performed immunofluorescence microscopy. We first labeled PMN-Ect with CD66b-FITC, and incubated HMDMs with labeled PMN-Ect, and zymosan alone, or zymosan and labeled PMN-Ect for 20 min. We then probed all samples with anti-NFκB antibody. In resting HMDMs and in presence of PMN-Ect, NFκB was mainly in the cytoplasm (Fig. 3 A, C). In contrast, in zymosan-stimulated HMDMs, NFκB was translocated to the nucleus (Fig. 3 B). When co-incubated with zymosan and PMN-Ect, the translocation of NFκB to the nucleus was clearly blocked (Fig. 3 D). This inhibition of nuclear translocation is probably responsible for the reduced phosphorylation of NFκB.

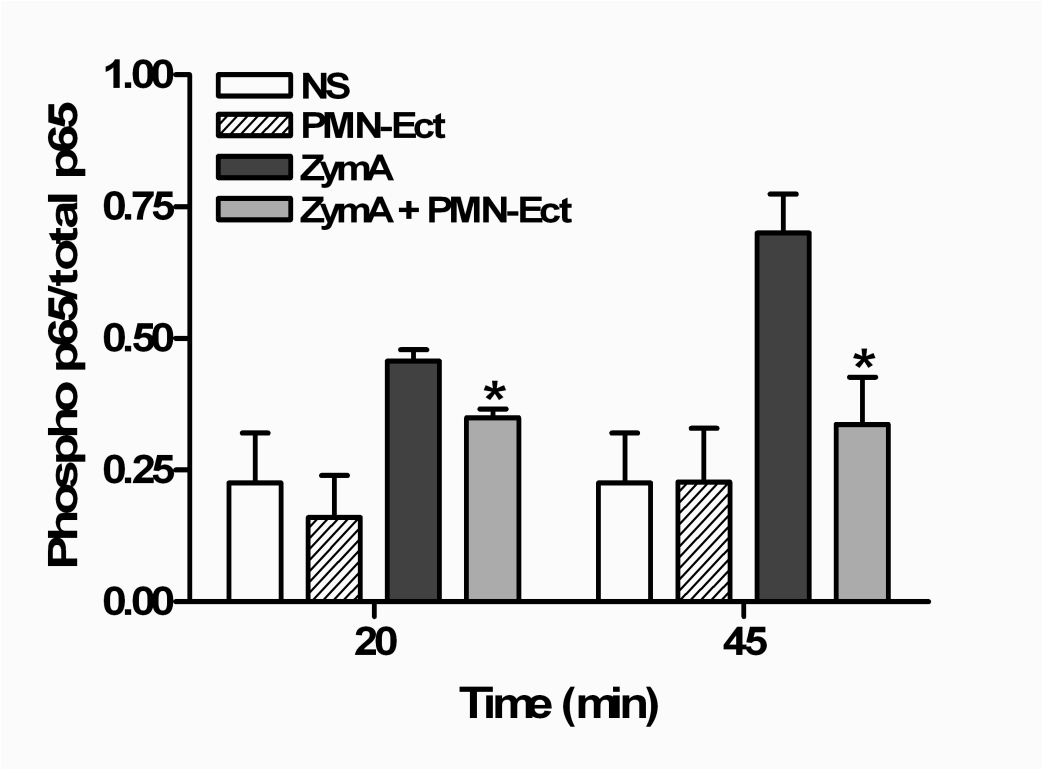

Figure 2. Inhibition of NFκB p65 phosphorylation in zymosan A-stimulated human macrophages by PMN-Ect. HMDMs were incubated with medium alone (NS) with or without PMN-Ect, and with medium supplemented with zymosan A with or without PMN-Ect. At 20 and 45 minutes, the phosphorylation of NFκB p65 was analyzed. *, ZymA + PMN-Ect vs. ZymA $p < 0.05$. Results are the mean ± SEM of three independent experiments.

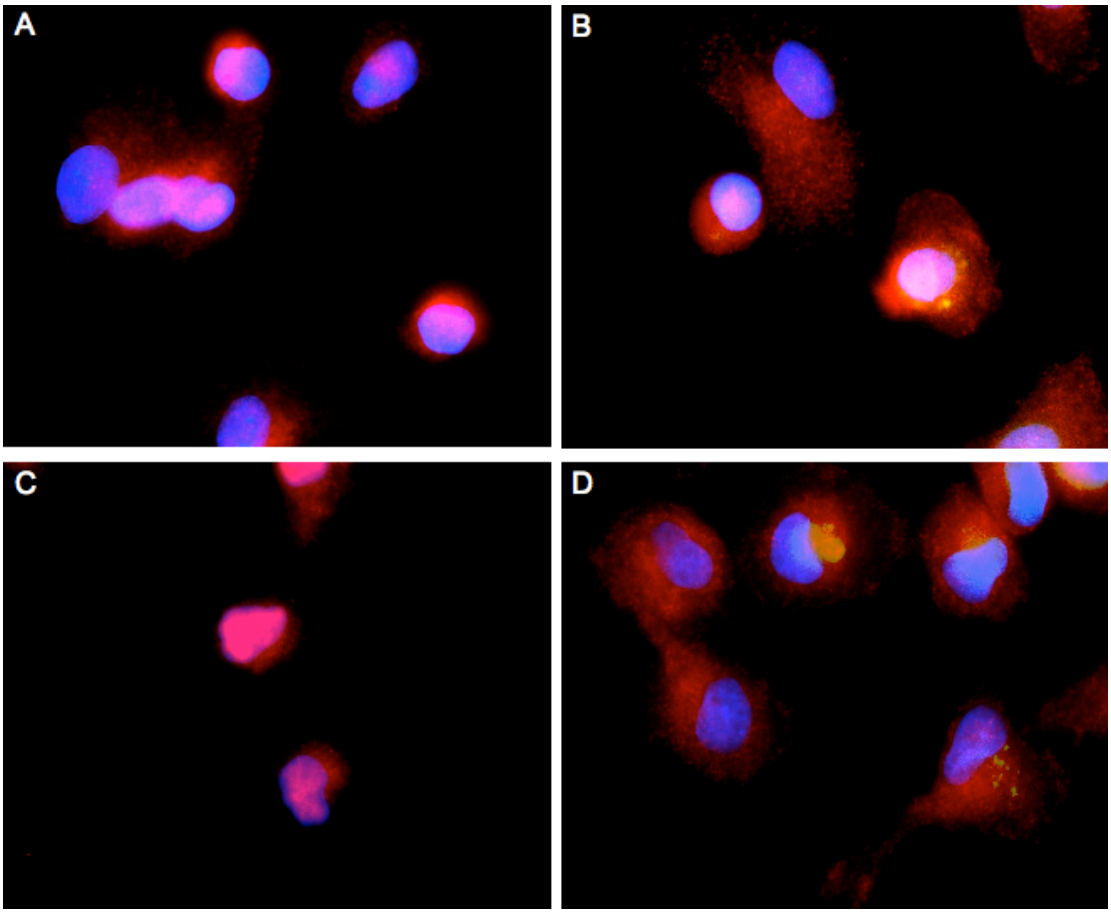

Figure 3. Immunofluorescence microscopy of the inhibition of NFκB translocation in the nucleus by PMN-Ect in zymosan A-stimulated human macrophages. HMDMs were incubated with (A) medium alone, (B) medium + PMN-Ect, (C) medium + zymosan A, and (D) medium + zymosan A + PMN-Ect, for 20 min. Cells were then fixed, stained with anti-NFκB primary antibody (RelA, sc-109) followed by Cy5 donkey anti-rabbit secondary antibody, and analyzed by immunofluorescence microscopy. In B and D, green dots correspond to CD66b-FITC pre-labeled PMN-Ect that are present in the cytoplasm. The imaging medium was Vectashield fluorescence mounting medium containing DAPI, and the analysis was performed on an Olympus BX61 microscope.

PMN-Ect induce Mer and PI3K dependent inhibition of NFκB

TAM receptors, and more specifically Mer, are involved in the uptake of AC by APCs, and in the ensuing immunosuppression (23, 25-30). Despite many structural differences, PMN-Ect share important biological properties with ACs, including the surface expression of PS, which might be bridged to Mer (12-14). To determine whether PMN-Ect activate downstream signaling via Mer, we pre-treated HMDMs with αMerTK antibody for 1h, then incubated the cells with zymosan A and/or PMN-Ect for 45 min and measured NFκB p65 phosphorylation. As shown in Fig. 4 and expected, NFκB p65 phosphorylation was highly increased in non-treated HMDMs stimulated with zymosan A, an effect that was blocked by PMN-Ect. By itself αMerTK had no effect on the phosphorylation of NFκB in resting or zymosan A activated cells. The pre-incubation with αMerTK however abolished the inhibitory activity of PMN-Ect on zymosan A activated cells, suggesting that Mer is required for the biological activity of PMN-Ect.

In a recent study, ACs inhibition of mouse DC activation by Mer was mediated by the activation of the PI3K/Akt pathway, which was directly responsible for blocking NFκB signaling (31). Moreover, activation of PI3K/Akt pathway is shown to negatively regulate NFκB in human monocytic cells (42). So, we investigated whether PI3K have a role in the inhibitory effects of PMN-Ect. We pre-treated HMDMs with LY 294002, a PI3K inhibitor, for 1 h, and measured NFκB p65 phosphorylation after 45 min incubation with zymosan A and/or PMN-Ect. Resting or zymosan A-stimulated HMDMs treated with LY 294002 showed similar NFκB p65 phosphorylation when compared to their non-treated counterparts. In contrast, LY 294002 abolished the inhibitory effect of PMN-Ect on zymosan A-stimulated HMDMs (Fig. 4).

Together, these results demonstrate that PMN-Ect have immediate inhibitory effects on zymosan A-stimulated HMDMs by Mer tyrosine kinase and the PI3K/Akt pathway mediated inhibition of NFκB signaling.

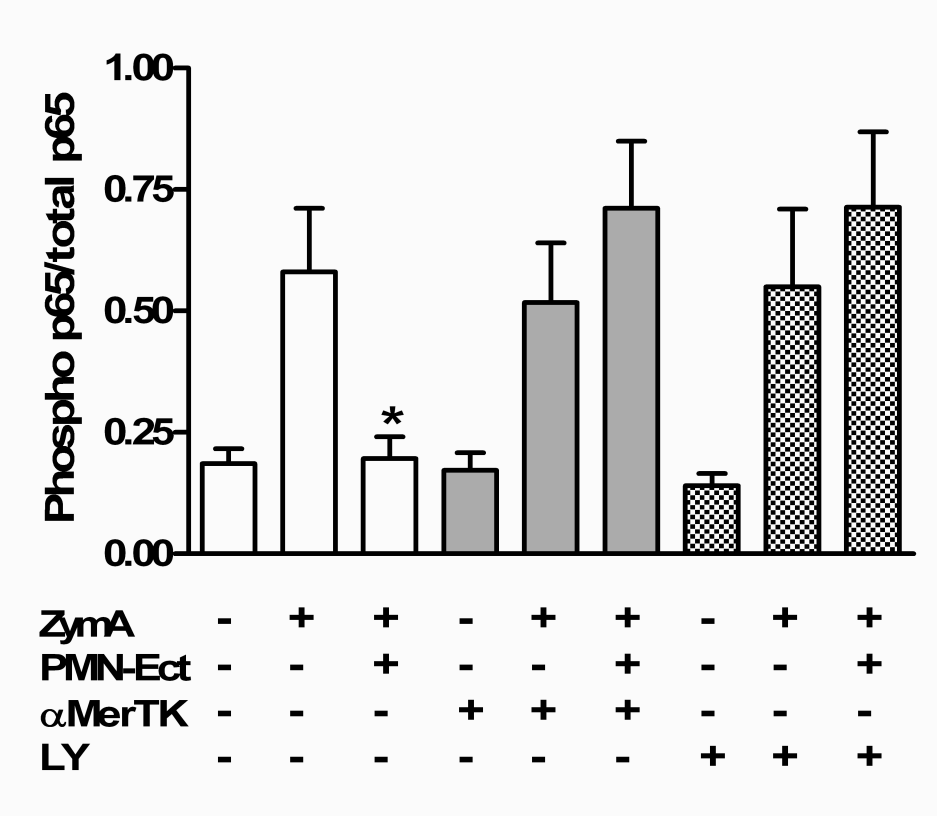

Figure 4. Roles of Mer and PI3K in PMN-Ect-induced inhibition of NFκB p65 phosphorylation in zymosan A-stimulated human macrophages. HMDMs were pretreated with 20 μg/ml of αMerTK antibody, or with 50 μM of the LY 294002, and then incubated with medium alone (NS), or with medium supplemented with zymosan A with or without PMN-Ect. At 45 minutes, the phosphorylation of NFκB p65 was analyzed. *, ZymA + PMN-Ect vs. ZymA $p < 0.05$. Results are the mean ± SEM of three independent experiments.

Gene expression profiling in presence of PMN-Ect

Little is known about the immediate effects of zymosan A and/or PMN-Ect on the gene expression profiling of HMDMs, which contrasts with their known effects on the release of various proinflammatory cytokines. In order to further define the inhibitory activity of PMN-Ect, we investigated the early gene expression profiles of resting and zymosan A-stimulated HMDMs in the absence/presence of PMN-Ect. Monocytes isolated from fresh buffy coats of 4 donors (2 males and 2 females between the ages of 20 and 45) were cultured for 7 days at 37°C. The resulting HMDMs were cultured for another 3 h alone, in presence of PMN-Ect, zymosan A, or both, before extracting their mRNAs to perform microarray analyses with Affymetrix GeneChip HGU133A 2.0 that determines the expression level of about 14500 human genes. The complete data will be deposited in NCBIs Gene expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo). The first observation was the important differences in the gene expression profile between the different individuals, with the first donor being the most distant to the other three as shown in the dendrogram (Fig. 5). However, the observations for zymosan A and the effects of PMN-Ect were the same for all 4 individuals. Zymosan A produced a massive shift in the gene expression, including many proinflammatory mediators, whereas the modulation induced by PMN-Ect on resting and zymosan A activated cells was limited but present in all samples. Despite the fact that there were quite strong inter-individuals differences in the strength of the responses induced by zymosan A and/or PMN-Ect, the trend included always the same general set of genes. Whereas zymosan A induced a clear increase in proinflammatory gene expression such as IL-1β, IL-6, IL-8, IL-12 and TNFα, it was surprising that PMN-Ect did not reduce in a measurable way the expression of these genes, although many others were modified independently of the

presence of zymosan A. At that stage we felt that the sensitivity of our gene profiling might have been limited and missed small differences in the expression of proinflammatory genes.

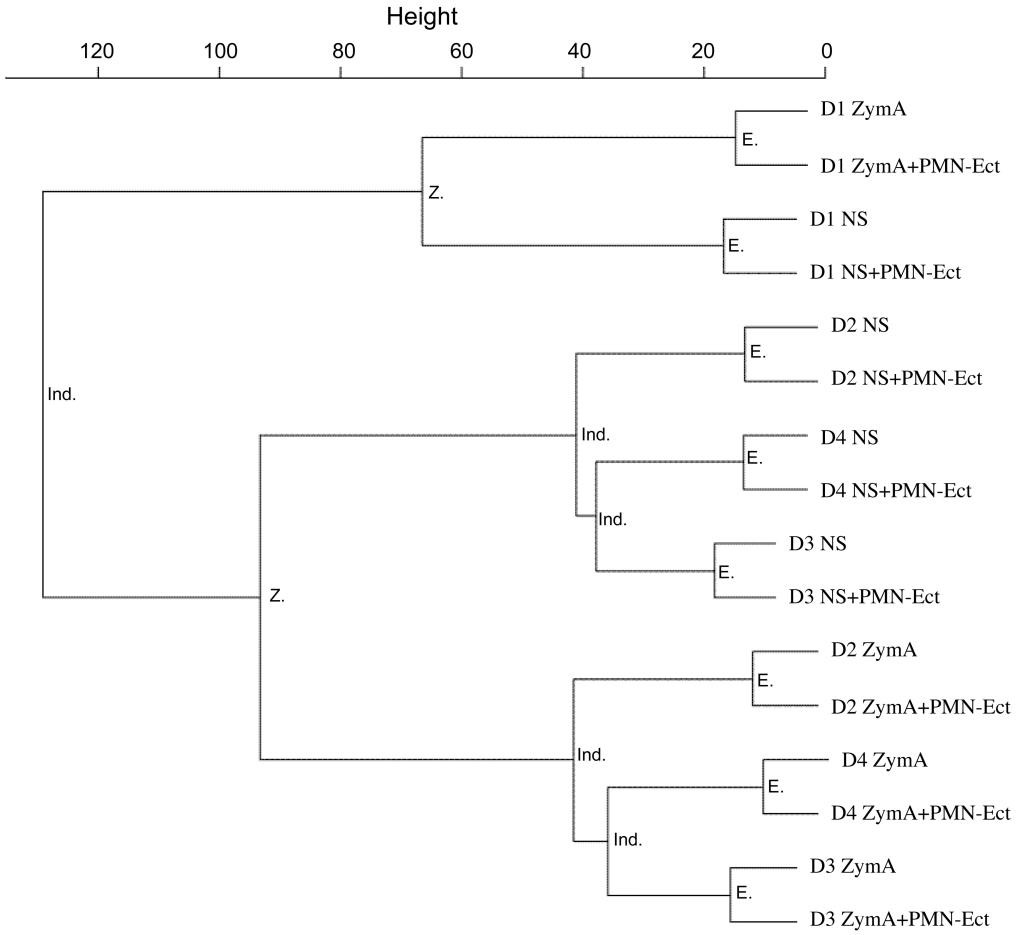

Figure 5. Dendrogram of microarray samples. The dendrogram constructed using hierarchical clustering (Euclidean distance metric, complete linkage) shows that the differences induced by zymosan A were clearly more important than those produced by PMN-Ect in all 4 individuals. Ind.: individual differences, Z.: zymosan A-induced differences, E.: PMN-Ect-induced differences.

Analysis of gene expressions by real-time RT-PCR

Thus, we selected 8 genes of interest, IL-1β, IL-6, IL-8, IL-12, TNFα, TGF-β, TXNIP and CXCR4 associated with inflammation and NFκB signaling pathway, and measured their mRNA levels after 3h in resting or stimulated HMDMs in absence/presence of PMN-Ect by real-time RT-PCR. Levels of IL-1β, IL-6, IL-8, IL-12, and TNFα mRNA were highly increased when HMDMs were stimulated by zymosan A with increases going from 14 to almost 4500 folds (Fig. 6). In the presence of PMN-Ect, the increases of all 5 gene-mRNA were evidently reduced, however the reduction was only 2-4 fold. These results might explain the data obtained by the gene profiling, which provided evidence only for major shifts. To confirm that the observations made by RT-PCR corresponded to the release of cytokines, we measured the TNFα in the supernatants of the previous experiment. TNFα was very low, or even under the detection limits under resting conditions, increased up to 1400 fold when the cells had been exposed to zymosan A for 3 h, and the suppression activity of PMN-Ect reduced TNFα release by 2-3 fold but still many log fold higher than the resting conditions (Fig. 7A). Similar results have been obtained in the past for the other cytokines as well (13), thus confirming that our RT-PCR results corresponded to protein synthesis and release.

Interestingly, we observed that the mRNA levels of TXNIP and CXCR4 decreased when HMDMs were stimulated with zymosan A, whereas in the presence of PMN-Ect we observed constantly a slight increase in both, stimulated as well as non-stimulated cells (Fig. 6). Importantly, TGF-β mRNA levels showed no significant changes under all conditions (Fig. 6).

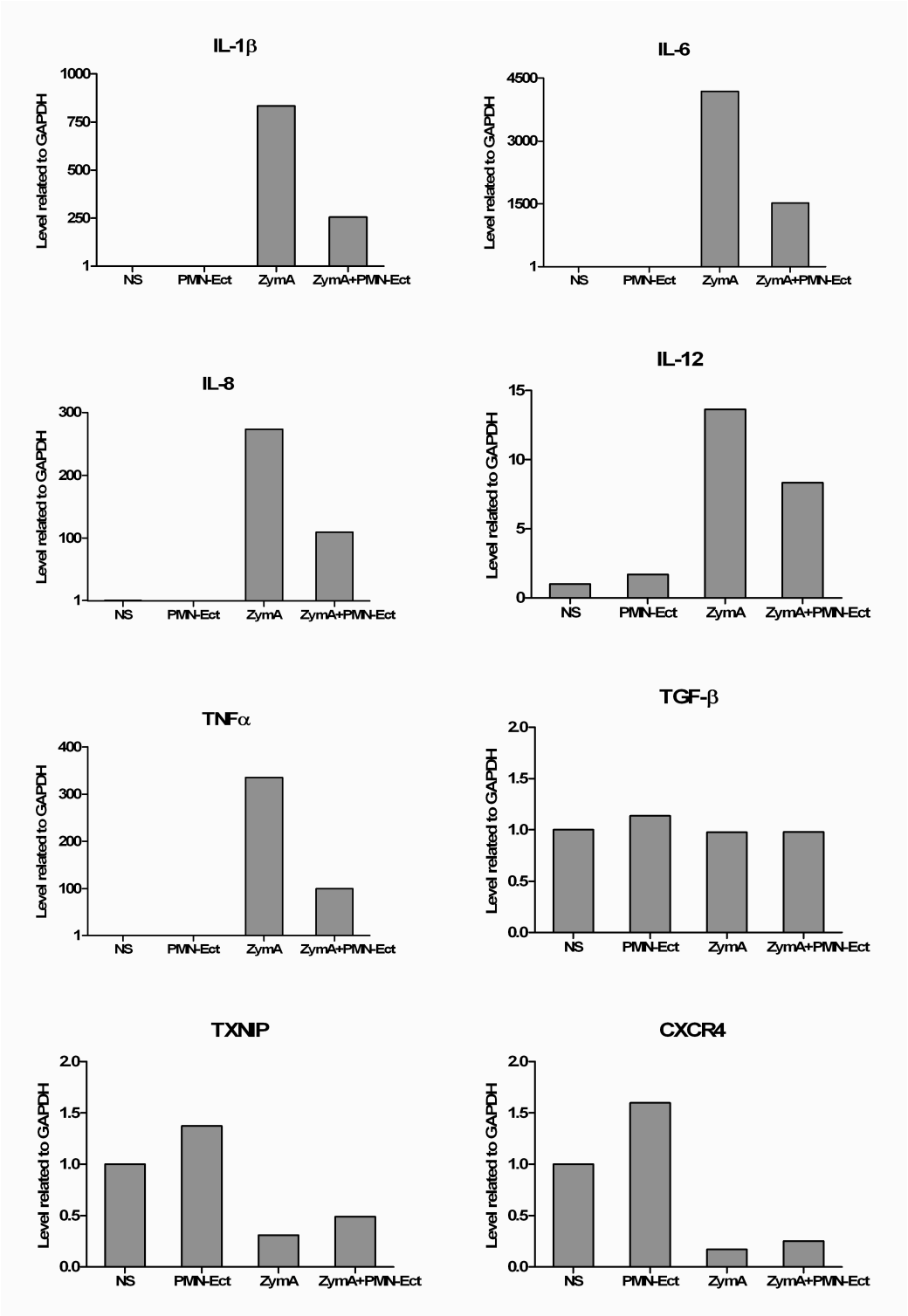

Figure 6. Analysis of genes associated with inflammation and NFκB signaling pathway by quantitative real-time RT-PCR. HMDMs were incubated for 3 h with medium alone (NS), medium + PMN-Ect, medium + zymosan A, and medium + zymosan A + PMN-Ect. The differential expression of IL-1β, IL-6, IL-8, IL-12, TNFα, TGF-β1, TXNIP and CXCR4 were measured by quantitative real-time RT-PCR. Results were normalized to NS=1.

PMN-Ect induce rapid TGF-β1 release

Since TGF-β1 mRNA levels of the macrophages were unaffected by exposure to PMN-Ect, and/or zymosan A stimulation, we measured its release in the supernatants of HMDMs used for the microarray experiments (3h of incubation). Confirming previous data (13), PMN-Ect increased the release of TGF-β1 from HMDMs in all 4 donors, whether the cells were exposed or not to zymosan A. On its own, zymosan A had no effects on TGF-β1 release (Fig. 7B). Thus and contrary to the effects of PMN-Ect on the transcription of zymosan A induced proinflammatory mediators, the TGF-β1 release is an early and direct effect of PMN-Ect independently of the presence of zymosan A.

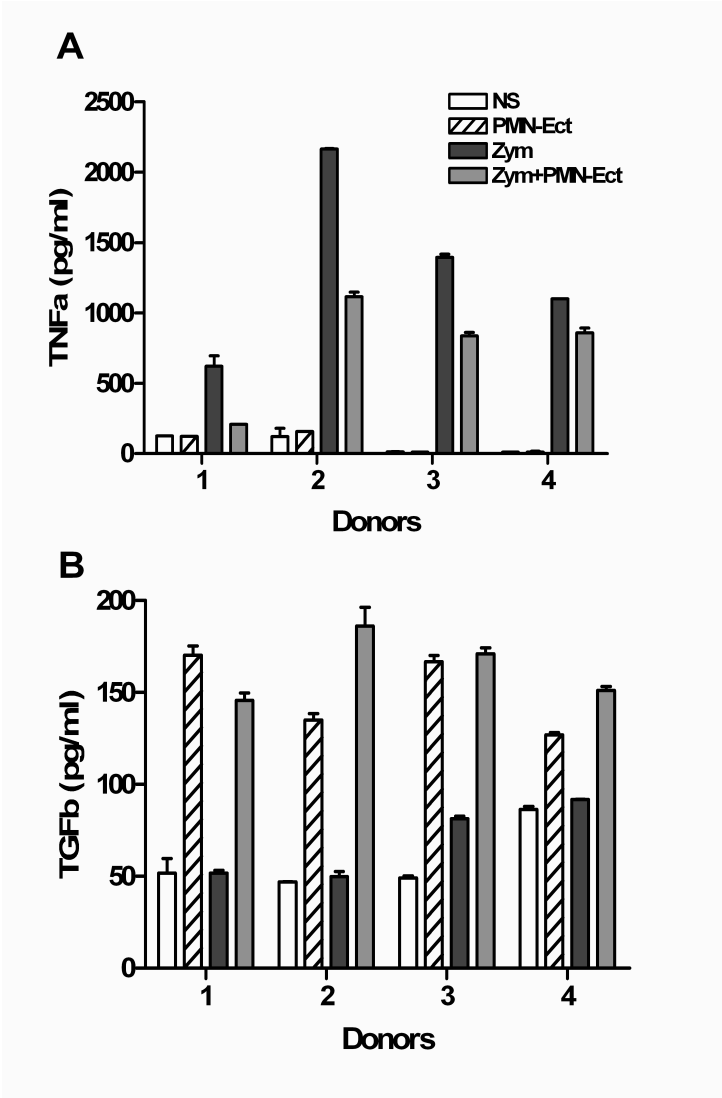

Figure 7.

Cytokine measurements in the supernatants of human macrophages cultured for microarray experiments. HMDMs were incubated for 3 h with medium alone (NS), medium + PMN-Ect, medium + zymosan A, and medium + zymosan A + PMN-Ect. Concentrations of (A) TNFα and (B) TGF-β1 were analyzed in supernatants. Errors bars indicate SEM of measurements done in triplicates.

Discussion

Whereas the involvement of PMNs in enhancing inflammation by the release of multiple mediators and enzymes has been extensively studied, the mechanisms responsible for the control of this reaction are less well understood. That activated PMNs release ectosomes possessing anti-inflammatory and immunosuppressive properties is a new *in vitro* finding, which suggests that the very same cells known to be central in inflammation are involved in controlling it as well (13, 14). In the current study, we deepened these findings by defining essential signaling pathways responsible for the biological properties of PMN-Ect and the consequences on the transcriptional machinery of HMDMs.

After their first contact with HMDMs, PMN-Ect induce in a few seconds a specific Ca^{2+} flux pattern (Fig. 1). Ca^{2+} is a major player in cellular information processing, and different Ca^{2+} signals can trigger various transcriptional responses (37, 39-41). The complexity of the Ca^{2+} fluxes does not allow to further speculate on what signaling pathways have been activated, but it is of particular interest that the oscillation of Ca^{2+} produced by PMN-Ect are preserved even in the presence of zymosan A. These immediate effects might be responsible for the rapid release of TGF-β1 which might be involved in PMN-Ect induced HMDM down-modulation. Indeed TGF-β1 released upon AC uptake plays an important role in modulating macrophages towards an anti-inflammatory phenotype (22, 43). When we analyzed the TGF-β1 mRNA levels of HMDMs at 3h after incubation with PMN-Ect in absence or presence of stimulation, we could not detect any significant changes. Thus, TGF-β1 was released as immediate-early response of HMDMs to PMN-Ect (Fig. 7B and (13)).

The key and central finding of our study is that PMN-Ect inhibited the NFκB p65 phosphorylation seen in zymosan A activated HMDMs. Corresponding to this finding, the NFκB translocation to the nucleus was blocked by PMN-Ect. This inhibition of NFκB was responsible for the down-regulation of major proinflammatory genes although in mathematical terms this inhibition was much less pronounced that the activation due to zymosan A. Indeed zymosan A induced a manifold (14 to 4500) increase of mRNA expression of proinflammatory cytokines, versus a 2-4 fold inhibition of this high activation by PMN-Ect. However, a 2-4 fold drop in the production of cytokines may well be significant in immunological terms, particularly if one adds the specific signals induced by PMN-Ect directly such as TGF-β1 release (Fig. 7B). Interestingly there are several studies on ACs down-modulation of macrophages, which report that NFκB is unaffected (44), or translocated to the nucleus but inactive (45). These results emphasize that despite similarities between PMN-Ect and ACs, there are also major differences in the pathways used to down-regulate inflammatory signals. Of note, in some experimental setups testing for the effects of ACs or PS expressing liposomes, macrophages or dendritic cells had to be pre-incubated sometimes several hours with ACs or PS liposomes in order to see an inhibitory effect on inflammatory stimuli (15, 46). In the present study the biological effects of PMN-Ect were observed immediately and were not dependent on a first phase of protein synthesis indispensable for ACs or PS-liposomes effects. This again differentiates ectosomes form ACs and might suggest that the fluctuations in Ca^{2+} fluxes induced by PMN-Ect were an indication that specific signaling pathways were activated and responsible for immediate inhibition.

Mer is centrally involved in the inhibition of NFκB by PMN-Ect as demonstrated by using a blocking antibody. Recent *in vivo* and *in vitro* studies showed that Mer is

required for the "tolerogenic" clearance of ACs by macrophages and DCs (25, 27, 28, 47). PMN-Ect share PS exposure with ACs (12), and PS may play a key role in many of the immunomodulatory effects of both PMN-Ect and ACs. However we do not know how the binding occurs, and beside PS many other cell surface components or soluble factors released by the target cells might be involved. For instance Gas6 secreted by HMDMs might be an efficient link to the PS exposed on PMN-Ect (23). Evidently, other possibilities will have to be explored, including the recently described Tim receptors (48), which might induce additional signaling pathways responsible for the modulation of the cellular response.

Our results showed that PMN-Ect induced inhibition of HMDMs by Mer-mediated activation of the PI3K/Akt pathway, with a resulting blockade of NFκB (Fig. 4). Sen et al. demonstrated similar results with AC-induced inhibition of mouse DCs (31). Consistent with our findings, activation of PI3K/Akt pathway in LPS-stimulated human monocytes regulated negatively NFκB, and limited TNFα and tissue factor expression (42). In contrast, TLR2-mediated induction of the PI3K/Akt pathway has been reported to up-regulate the NFκB p65 transactivational activity (49). These differences might be explained by the experimental models and protocols used (human vs. mouse cells, different cell types and stimuli).

Whether the cell modifications induced by PMN-Ect are long-lasting is unknown, although the real-time RT-PCR data of TXNIP and CXCR4 might suggest a reprogramming of the cell. For instance, thioredoxin-interacting protein (TXNIP) blocks thioredoxin, which is a transcriptional co-activator through interaction with transcription factors such as NFκB (50, 51). PMN-Ect induced upregulation of TXNIP might be a complementary mechanism for the inhibition of NFκB pathway. Upregulation of CXCR4 might have been induced by TGF-β1 (52, 53). High CXCR4

expression increase HMDM susceptibility to its specific chemokine SDF-1, and thus leading HMDMs to migrate away from PMNs (52). PMN-Ect have been shown to have long lasting effects on human immature DCs by modifying their morphology and phagocytic properties (14). In addition, HMDM activation is inhibited by a short pre-exposure to ectosomes derived from erythrocytes (for 1 h, 24 h before testing HMDM activation) (54). Thus, part of the biological effect produced by PMN-Ect may require protein synthesis in the target cells. The released TGF-β1 might be involved in these changes (22), although not directly via NFκB inhibition as seen in Fig. 4. In addition, this cannot explain the durable effects of erythrocyte-derived ectosomes on macrophages, since such ectosomes do not induce TGF-β1 (54) .

Although the present study demonstrates that PMN-Ect "reprogram" HMDMs by inhibiting NFκB via Mer and PI3K/Akt pathways, other complementary mechanisms might still exist. Recent studies report that microvesicles (i.e. ectosomes) may contain mRNAs that can be delivered to other cells (55-57). Based on this suggestion, Valadi et al. found that mast cell exosomes contain and transfer mRNAs and microRNAs (miRNAs), and proposed to call this RNA " exosomal shuttle RNA" (58). miRNAs are a class of evolutionarily conserved, small, non-coding RNA molecules that play important regulatory roles in various biological processes (59, 60). PMN-Ect contain small RNAs as well (C.E. unpublished data), which may be involved in late effects of PMN-Ect.

In summary, we showed that, when PMN-Ect bind the target macrophages, a specific Ca^{2+} flux signal is induced. Mer receptor as well as the PI3K/Akt pathway is activated, and NFκB translocation and phosphorylation are blocked so that the proinflammatory gene transcription is reduced (Fig. 8). The role of PMN-Ect in reprogramming macrophages merits further attention.

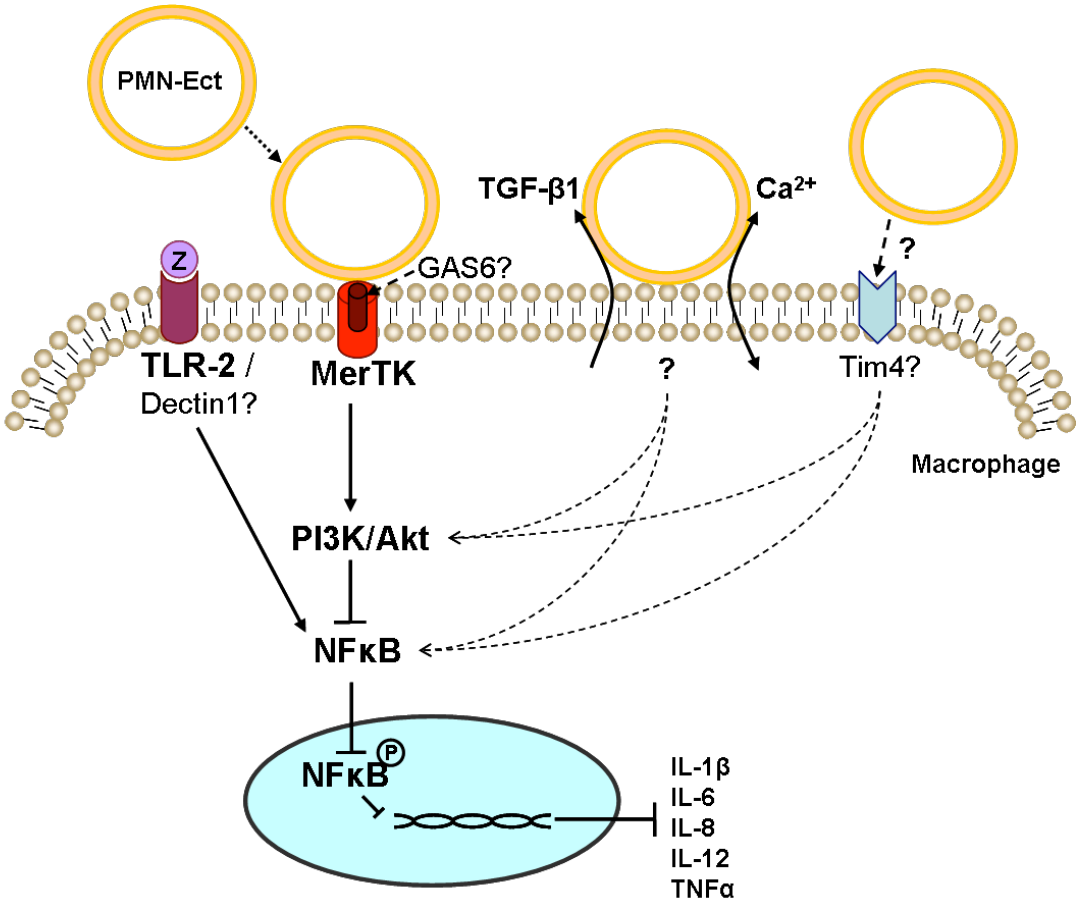

Figure 8. Model to explain the inhibitory effect of PMN-Ect on zymosan A activated human macrophages. Binding of zymosan A (Z) to TLR2/Dectin1 activates the NFκB pathway. PMN-Ect via Mer tyrosine kinase receptor (MerTK) down-regulate NFκB by activating PI3K/Akt pathway, which inhibits the translocation and transactivation of NFκB. PMN-Ect induce an immediate calcium flux in HMDMs, and a rapid release of TGF–β1. ⟶: activation or release; ⊣: inhibition; - -> : interactions not known.

References

1. Pawson, T. 1995. Protein modules and signalling networks. *Nature* 373:573-580.
2. Pawson, T., and P. Nash. 2000. Protein-protein interactions define specificity in signal transduction. *Genes Dev* 14:1027-1047.
3. Bowers-Morrow, V. M., S. O. Ali, and K. L. Williams. 2004. Comparison of molecular mechanisms mediating cell contact phenomena in model developmental systems: an exploration of universality. *Biol Rev Camb Philos Soc* 79:611-642.
4. Ratajczak, J., M. Wysoczynski, F. Hayek, A. Janowska-Wieczorek, and M. Z. Ratajczak. 2006. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 20:1487-1495.
5. Mack, M., A. Kleinschmidt, H. Bruhl, C. Klier, P. J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, and D. Schlondorff. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* 6:769-775.
6. Koppler, B., C. Cohen, D. Schlondorff, and M. Mack. 2006. Differential mechanisms of microparticle transfer toB cells and monocytes: anti-inflammatory propertiesof microparticles. *Eur J Immunol* 36:648-660.
7. Johnstone, R. M. 2006. Exosomes biological significance: A concise review. *Blood Cells Mol Dis* 36:315-321.

8. Thery, C., L. Zitvogel, and S. Amigorena. 2002. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2:569-579.

9. Fevrier, B., and G. Raposo. 2004. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* 16:415-421.

10. Katzmann, D. J., G. Odorizzi, and S. D. Emr. 2002. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* 3:893-905.

11. Hess, C., S. Sadallah, A. Hefti, R. Landmann, and J. A. Schifferli. 1999. Ectosomes released by human neutrophils are specialized functional units. *J Immunol* 163:4564-4573.

12. Gasser, O., C. Hess, S. Miot, C. Deon, J. C. Sanchez, and J. A. Schifferli. 2003. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* 285:243-257.

13. Gasser, O., and J. A. Schifferli. 2004. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 104:2543-2548.

14. Eken, C., O. Gasser, G. Zenhaeusern, I. Oehri, C. Hess, and J. A. Schifferli. 2008. Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J Immunol* 180:817-824.

15. Chen, X., K. Doffek, S. L. Sugg, and J. Shilyansky. 2004. Phosphatidylserine regulates the maturation of human dendritic cells. *J Immunol* 173:2985-2994.

16. Fadok, V. A., D. L. Bratton, and P. M. Henson. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest* 108:957-962.

17. Wu, Y., N. Tibrewal, and R. B. Birge. 2006. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol* 16:189-197.

18. Shi, D., M. Fu, P. Fan, W. Li, X. Chen, C. Li, X. Qi, T. Gao, and Y. Liu. 2007. Artificial phosphatidylserine liposome mimics apoptotic cells in inhibiting maturation and immunostimulatory function of murine myeloid dendritic cells in response to 1-chloro-2,4-dinitrobenze in vitro. *Arch Dermatol Res* 299:327-336.

19. Valenti, R., V. Huber, P. Filipazzi, L. Pilla, G. Sovena, A. Villa, A. Corbelli, S. Fais, G. Parmiani, and L. Rivoltini. 2006. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res* 66:9290-9298.

20. Tiao, M. M., L. Lu, L. T. Huang, C. D. Liang, C. L. Chen, R. Tao, J. J. Fung, and S. Qian. 2007. Cross-tolerance of recipient-derived transforming growth factor-beta dendritic cells. *Transplant Proc* 39:281-282.

21. Rutella, S., S. Danese, and G. Leone. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 108:1435-1440.

22. Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109:41-50.

23. Lemke, G., and C. V. Rothlin. 2008. Immunobiology of the TAM receptors. *Nat Rev Immunol* 8:327-336.

24. Nagata, K., K. Ohashi, T. Nakano, H. Arita, C. Zong, H. Hanafusa, and K. Mizuno. 1996. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J Biol Chem* 271:30022-30027.

25. Camenisch, T. D., B. H. Koller, H. S. Earp, and G. K. Matsushima. 1999. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccharide-induced endotoxic shock. *J Immunol* 162:3498-3503.

26. Lemke, G., and Q. Lu. 2003. Macrophage regulation by Tyro 3 family receptors. *Curr Opin Immunol* 15:31-36.

27. Scott, R. S., E. J. McMahon, S. M. Pop, E. A. Reap, R. Caricchio, P. L. Cohen, H. S. Earp, and G. K. Matsushima. 2001. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* 411:207-211.

28. Cohen, P. L., R. Caricchio, V. Abraham, T. D. Camenisch, J. C. Jennette, R. A. Roubey, H. S. Earp, G. Matsushima, and E. A. Reap. 2002. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J Exp Med* 196:135-140.

29. Behrens, E. M., P. Gadue, S. Y. Gong, S. Garrett, P. L. Stein, and P. L. Cohen. 2003. The mer receptor tyrosine kinase: expression and function suggest a role in innate immunity. *Eur J Immunol* 33:2160-2167.

30. Wallet, M. A., P. Sen, R. R. Flores, Y. Wang, Z. Yi, Y. Huang, C. E. Mathews, H. S. Earp, G. Matsushima, B. Wang, and R. Tisch. 2008. MerTK is required for apoptotic cell-induced T cell tolerance. *J Exp Med* 205:219-232.

31. Sen, P., M. A. Wallet, Z. Yi, Y. Huang, M. Henderson, C. E. Mathews, H. S. Earp, G. Matsushima, A. S. Baldwin, Jr., and R. M. Tisch. 2007. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* 109:653-660.

32. Graham, B., and S. B. Gibson. 2005. The two faces of NFkappaB in cell survival responses. *Cell Cycle* 4:1342-1345.

33. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16:225-260.

34. Ghosh, S., and M. Karin. 2002. Missing pieces in the NF-kappaB puzzle. *Cell* 109 Suppl:S81-96.

35. Baldwin, A. S., Jr. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 14:649-683.

36. Gilmore, T. D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 25:6680-6684.

37. Hardingham, G. E., and H. Bading. 1999. Calcium as a versatile second messenger in the control of gene expression. *Microsc Res Tech* 46:348-355.

38. Berridge, M. J., P. Lipp, and M. D. Bootman. 2000. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1:11-21.

39. Berridge, M. J. 1997. The AM and FM of calcium signalling. *Nature* 386:759-760.

40. Dolmetsch, R. E., R. S. Lewis, C. C. Goodnow, and J. I. Healy. 1997. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature* 386:855-858.

41. Dolmetsch, R. E., K. Xu, and R. S. Lewis. 1998. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392:933-936.

42. Guha, M., and N. Mackman. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 277:32124-32132.

43. Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 101:890-898.

44. McDonald, P. P., V. A. Fadok, D. Bratton, and P. M. Henson. 1999. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J Immunol* 163:6164-6172.

45. Cvetanovic, M., and D. S. Ucker. 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol* 172:880-889.

46. Lucas, M., L. M. Stuart, A. Zhang, K. Hodivala-Dilke, M. Febbraio, R. Silverstein, J. Savill, and A. Lacy-Hulbert. 2006. Requirements for apoptotic cell contact in regulation of macrophage responses. *J Immunol* 177:4047-4054.

47. Lu, Q., and G. Lemke. 2001. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science* 293:306-311.

48. Miyanishi, M., K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, and S. Nagata. 2007. Identification of Tim4 as a phosphatidylserine receptor. *Nature* 450:435-439.

49. Arbibe, L., J. P. Mira, N. Teusch, L. Kline, M. Guha, N. Mackman, P. J. Godowski, R. J. Ulevitch, and U. G. Knaus. 2000. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat Immunol* 1:533-540.

50. Schenk, H., M. Klein, W. Erdbrugger, W. Droge, and K. Schulze-Osthoff. 1994. Distinct effects of thioredoxin and antioxidants on the activation of

transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci U S A* 91:1672-1676.

51. Nishiyama, A., M. Matsui, S. Iwata, K. Hirota, H. Masutani, H. Nakamura, Y. Takagi, H. Sono, Y. Gon, and J. Yodoi. 1999. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* 274:21645-21650.

52. Wang, J., E. Guan, G. Roderiquez, V. Calvert, R. Alvarez, and M. A. Norcross. 2001. Role of tyrosine phosphorylation in ligand-independent sequestration of CXCR4 in human primary monocytes-macrophages. *J Biol Chem* 276:49236-49243.

53. Chen, S., D. L. Tuttle, J. T. Oshier, H. J. Knot, W. J. Streit, M. M. Goodenow, and J. K. Harrison. 2005. Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology* 114:565-574.

54. Sadallah, S., C. Eken, and J. A. Schifferli. 2008. Erythrocyte-derived ectosomes have immunosuppressive properties. *J Leukoc Biol* 84:1316-1325.

55. Baj-Krzyworzeka, M., R. Szatanek, K. Weglarczyk, J. Baran, B. Urbanowicz, P. Branski, M. Z. Ratajczak, and M. Zembala. 2006. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. *Cancer Immunol Immunother* 55:808-818.

56. Bess, J. W., Jr., R. J. Gorelick, W. J. Bosche, L. E. Henderson, and L. O. Arthur. 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. *Virology* 230:134-144.

57. Ratajczak, J., K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak, and M. Z. Ratajczak. 2006. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 20:847-856.

58. Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee, and J. O. Lotvall. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9:654-659.

59. Sonkoly, E., M. Stahle, and A. Pivarcsi. 2008. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin Cancer Biol* 18:131-140.

60. Lodish, H. F., B. Zhou, G. Liu, and C. Z. Chen. 2008. Micromanagement of the immune system by microRNAs. *Nat Rev Immunol* 8:120-130.

Conclusion and Future Perspectives

PMN-Ect were shown to induce the release of TGF-β1 by human monocyte-derived macrophages (HMDMs) *in vitro*, and additionally block their inflammatory response to zymosan A and LPS by down-regulating the release of IL-8 and TNFα. Importantly, ectosome-to-cell contact was sufficient for this immunomodulatory function (1). We further investigated the role of PMN-Ect in the immune system, and showed that they have the potential to influence not only the innate but also the adaptive immunity by playing an active role in shaping dendritic cell (DC)-dependent response. We reported that PMN-Ect inhibit the LPS-induced maturation of human monocyte-derived DCs, and induce new morphological and functional characteristics which result in a reduced capacity to activate T-cells (2).

In addition, we confirmed that erythrocytes release also ectosomes (E-ecto) that revealed an inhibitory potential on activated HMDMs, indicating an active role in the regulation of inflammation as well (3). Importantly, E-ecto are released in large amounts during blood storage (4), which might explain the immunosuppressive effects seen after transfusions, although these observations are not generally accepted (5-8).

A very interesting property of E-ecto was their lasting effects on activated macrophages. The phagocytosis of E-ecto had modified the macrophage for at least 24 hours so that it did not react to further stimulation (3). Preliminary results obtained with PMN-Ect showed similar effects (Fig. 1). These observations suggest that the signalling processes related to TLR-2 stimulation were down-modulated by an active

lasting process. Thus, the phagocytosis of ectosomes seems not only to produce a direct anti-inflammatory signal to HMDMs, but also to keep their proinflammatory potential down. How long this effect lasts, and what are the cellular mechanisms involved, particularly *in vivo*, remain intriguing questions.

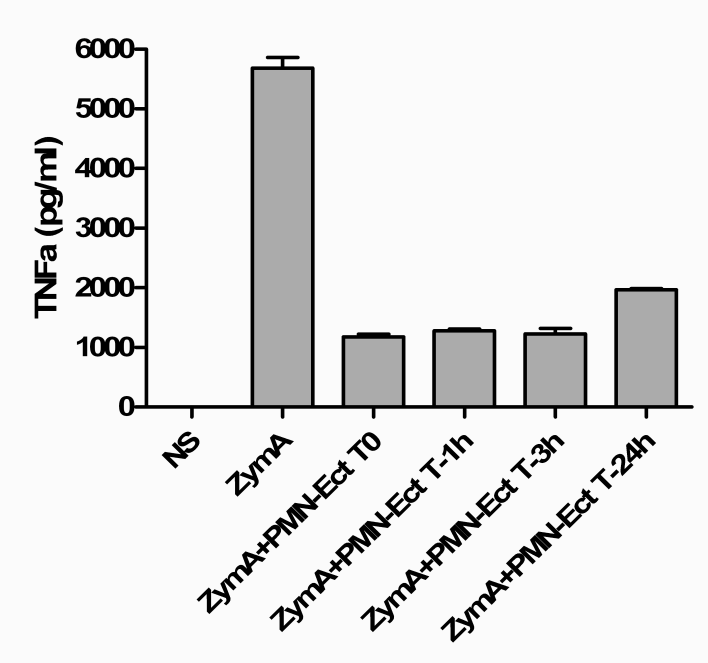

Figure 1. Long lasting effect of PMN-Ect on zymosan A-stimulated human macrophages. 24, 3 and 1 hours before overnight zymosan A stimulation, HMDMs were incubated with PMN-Ect for 1 hour, and then unbound PMN-Ect were washed away. At T0, PMN-Ect were not removed from the medium. The proinflammatory cytokine TNFα was analyzed in supernatants. In presence of PMN-Ect without stimulation no measurable amounts of cytokines was released. Assays were performed in duplicates. Results are representative of 3 similar experiments. Error bar indicates standard error of the mean.

In vivo models of inflammation/infection will now have to test the relevance of the activities of ectosomes (the project will start in 2009). A model of acute peritoneal inflammation induced by thioglycollate or zymosan A should allow us to gain insight into the effects of E-ecto on the kinetics and extent of zymosan A-induced peritoneal inflammation, and inhibition thereof. An alternative is to study the immune response to i.p. injected human E-ecto (or human PMN-Ect) as compared to the proteins obtained from the same solubilized ectosomes. The expectation would be that ectosome as such are not inducing an immune response as opposed to the protein extract. Indeed the PS of ectosomes might down-regulate directly the immune response.

In many respects, ectosomes may be similar to apoptotic cells (ACs), including the high expression of PS, which in multiple experiments has been shown to allow specific binding of ACs to macrophages or dendritic cells (9-14). In a recent study, AC-induced inhibition of mouse DCs was shown to depend on Mer-mediated activation of PI3K/Akt pathway, with a resulting inhibition of NFκB (15). Similar to these findings, we could demonstrate that PMN-Ect induced modulation of the inflammatory response of HMDMs via Mer tyrosine kinase receptor regulated PI3K/Akt and NFκB pathways (*Eken et al., submitted*). As a next study, it would be of great interest to define if Mer tyrosine kinase receptor, and PI3K/Akt and NFκB pathways are also responsible for the immunoregulatory effects of PMN-Ect in MoDCs. If such, a common regulatory mechanism would suggest that PMN-Ect have indeed a defined role in downmodulating major antigen presenting cells.

Whereas there might be similarities in the responses induced by ectosomes and ACs as well, these responses are not identical. By contrast to ACs, ectosomes have the particularity to be involved very early in inflammation, a time point, which might be crucial for determining later aspects of the cascade responsible for acquired immunity. In that sense, ectosomes might not terminate the inflammation, but control the immune response. However, ectosomes originating from different cells do not have the same effects as well. For example, unlike PMN-Ect, E-ecto did not induce the release of TGF-β1 from HMDMs (3). And interestingly, platelet-derived ectosomes seems to have down-regulatory effects, and those, similar to PMN-Ect, induce TGF-β1 release from HMDMs (unpublished data, Sadallah, Eken et al.). Furthermore, microvesicles released by tumor cells have recently been shown to skew monocyte differentiation into DCs toward the generation of a myeloid immunosuppressive cell subset, thus impair the possible immune response against tumor (25). These findings suggest that ectosomes originating from different cells might have additional and specific characteristics. Thus, it would be interesting to investigate the effects of E-ecto and platelet-derived ectosomes on MoDCs, and tumor-derived ectosomes on HMDMs, and the cellular pathways of human macrophages and DCs involved in their immunosuppressive effects. A comparison between all might help us generate a more precise definition of ectosomes.

Although we demonstrated that PMN-Ect down-modulate HMDMs by inhibiting NFκB via Mer and PI3K/Akt pathways, other complementary mechanisms might still exist. Microvesicles (i.e. ectosomes) contain mRNAs that can be delivered to other cells (16-18). Recently, Valadi et al. reported that mast cell exosomes contain and transfer mRNAs and microRNAs (miRNAs) that can be functional in the recipient

cells, and proposed to call this RNA "exosomal shuttle RNA" (esRNA) (19). Of note, miRNAs are a class of evolutionarily conserved, small, non-coding RNA molecules that play important regulatory roles in various biological processes (20, 21). During microarray experiments, we found that also PMN-Ect contain small RNAs (Fig. 2), which might be involved in their "reprogramming" effects. However, further research is needed to confirm these results.

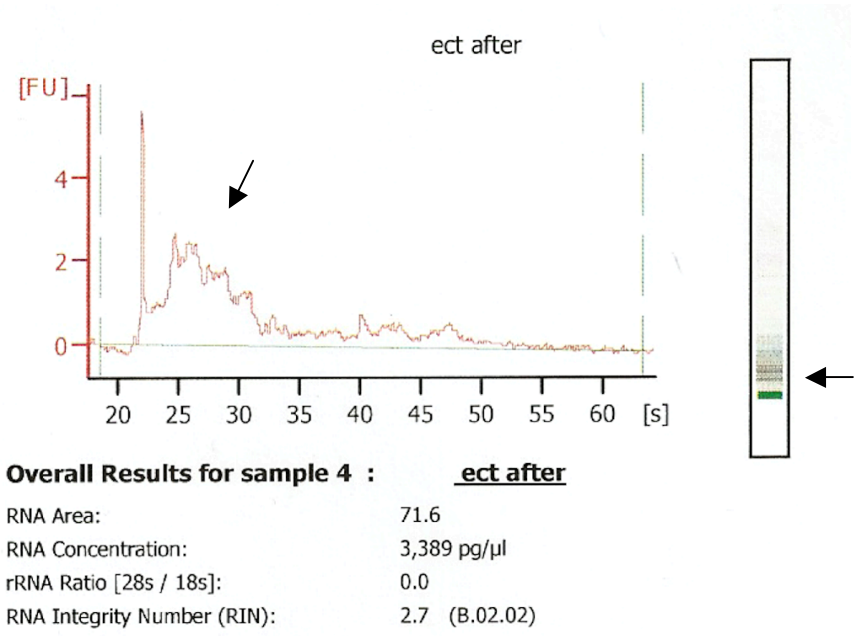

Figure 2. Bioanalyzer analysis of PMN-Ect. The graph and the electrophoresis lane show that PMN-Ect contain small RNAs (arrows).

Another complementary mechanism might be the peroxisome proliferator-activated receptor (PPARs) induced regulation. PPARs (PPARα, PPARβ/δ, PPARγ) are a family of nuclear receptor proteins that function as transcription factors regulating the expression of target genes (22). PPARs are implicated in major metabolic and

inflammatory regulations (23). *In vitro* and *in vivo* studies has showed that PPARγ mediate anti-inflammatory effects on several immune cell types (24). Additionally, a recent study demonstrated that activated human platelets shed PPARγ in microparticles (25). Whether ectosomes have PPARγ, or activate PPARγ of encountered cells remain to be tested.

In the light of all these results, we suggest that ectosomes have the potential to reprogram macrophages and dendritic cells toward an immunosuppressive phenotype. Further investigations in "ectosome biology" should help in understanding the regulation of the innate and adaptive immunity.

References

1. Gasser, O., and J. A. Schifferli. 2004. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 104:2543-2548.
2. Eken, C., O. Gasser, G. Zenhaeusern, I. Oehri, C. Hess, and J. A. Schifferli. 2008. Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J Immunol* 180:817-824.
3. Sadallah, S., C. Eken, and J. A. Schifferli. 2008. Erythrocyte-derived ectosomes have immunosuppressive properties. *J Leukoc Biol* 84:1316-1325.
4. Haradin, A. R., R. I. Weed, and C. F. Reed. 1969. Changes in physical properties of stored erythrocytes relationship to survival in vivo. *Transfusion* 9:229-237.
5. Tartter, P. I. 1995. Immunologic effects of blood transfusion. *Immunol Invest* 24:277-288.
6. Vamvakas, E. C. 2002. Possible mechanisms of allogeneic blood transfusion-associated postoperative infection. *Transfus Med Rev* 16:144-160.
7. Vamvakas, E. C. 2004. White blood cell-containing allogeneic blood transfusion, postoperative infection and mortality: a meta-analysis of observational 'before-and-after' studies. *Vox Sang* 86:111-119.
8. Jensen, L. S., E. Puho, L. Pedersen, F. V. Mortensen, and H. T. Sorensen. 2005. Long-term survival after colorectal surgery associated with buffy-coat-poor and leucocyte-depleted blood transfusion: a follow-up study. *Lancet* 365:681-682.

9. Zwaal, R. F., and A. J. Schroit. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89:1121-1132.

10. Gilbreath, M. J., D. L. Hoover, C. R. Alving, G. M. Swartz, Jr., and M. S. Meltzer. 1986. Inhibition of lymphokine-induced macrophage microbicidal activity against Leishmania major by liposomes: characterization of the physicochemical requirements for liposome inhibition. *J Immunol* 137:1681-1687.

11. Fadok, V. A., A. de Cathelineau, D. L. Daleke, P. M. Henson, and D. L. Bratton. 2001. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 276:1071-1077.

12. Fadok, V. A., D. L. Bratton, and P. M. Henson. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest* 108:957-962.

13. Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 109:41-50.

14. Verhoven, B., S. Krahling, R. A. Schlegel, and P. Williamson. 1999. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. *Cell Death Differ* 6:262-270.

15. Sen, P., M. A. Wallet, Z. Yi, Y. Huang, M. Henderson, C. E. Mathews, H. S. Earp, G. Matsushima, A. S. Baldwin, Jr., and R. M. Tisch. 2007. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* 109:653-660.

16. Baj-Krzyworzeka, M., R. Szatanek, K. Weglarczyk, J. Baran, B. Urbanowicz, P. Branski, M. Z. Ratajczak, and M. Zembala. 2006. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. *Cancer Immunol Immunother* 55:808-818.

17. Bess, J. W., Jr., R. J. Gorelick, W. J. Bosche, L. E. Henderson, and L. O. Arthur. 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. *Virology* 230:134-144.

18. Ratajczak, J., K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak, and M. Z. Ratajczak. 2006. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 20:847-856.

19. Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee, and J. O. Lotvall. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9:654-659.

20. Sonkoly, E., M. Stahle, and A. Pivarcsi. 2008. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin Cancer Biol* 18:131-140.

21. Lodish, H. F., B. Zhou, G. Liu, and C. Z. Chen. 2008. Micromanagement of the immune system by microRNAs. *Nat Rev Immunol* 8:120-130.

22. Kota, B. P., T. H. Huang, and B. D. Roufogalis. 2005. An overview on biological mechanisms of PPARs. *Pharmacol Res* 51:85-94.

23. Feige, J. N., L. Gelman, L. Michalik, B. Desvergne, and W. Wahli. 2006. From molecular action to physiological outputs: peroxisome proliferator-

activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog Lipid Res* 45:120-159.

24. Straus, D. S., and C. K. Glass. 2007. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends Immunol* 28:551-558.

25. Ray, D. M., S. L. Spinelli, S. J. Pollock, T. I. Murant, J. J. O'Brien, N. Blumberg, C. W. Francis, M. B. Taubman, and R. P. Phipps. 2008. Peroxisome proliferator-activated receptor gamma and retinoid X receptor transcription factors are released from activated human platelets and shed in microparticles. *Thromb Haemost* 99:86-95.

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Eken, C., P. Martin, S. Sadallah, S. Treves, V. Reddy, M. Schaller, and J. A. Schifferli. Polymorphonuclear Neutrophil - Derived Ectosomes inhibit NFκB activation in zymosan activated human macrophages in a Mer tyrosine kinase dependent manner. *Submitted*.

Sadallah, S., **C. Eken**, and J. A. Schifferli. 2008. Erythrocyte-derived ectosomes have immunosuppressive properties. *J Leukoc Biol* 84:1316-1325.

Eken, C., O. Gasser, G. Zenhaeusern, I. Oehri, C. Hess, and J. A. Schifferli. 2008. Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J Immunol* 180:817-824.

Gasser, O., A. Missiou, **C. Eken**, C. Hess. 2005. Human CD8+ T cells store CXCR1 in a distinct intracellular compartment and up-regulate it rapidly to the cell-surface upon activation. *Blood* 106(12):3718-24.

Scientific Meetings and Abstracts

22nd International Complement Workshop, 28 Sept - 2 Oct 2008, Basel - Switzerland.
Sadallah, S., **C. Eken**, and J. A. Schifferli. 2008. Ectosomes released by erythrocytes, polymornuclear leukocytes and platelets have some common properties and react with complement.

2nd International Conference on Crossroads between Innate and Adaptive Immunity, 17-22 June 2007, Crete – Greece
Eken, C., O. Gasser, C. Hess, J. A. Schifferli. 2007. PMN-ectosomes interfere with the maturation of monocyte-derived dendritic cells.

4th International Conference on Innate Immunity, 4 - 9 June 2006, Corfu – Greece
Eken, C., O. Gasser, C. Hess, J. A. Schifferli. 2006. PMN-derived ectosomes – a host factor influencing maturation of monocyte-derived dendritic cells.