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IMMUNOLOGY

Exosomes Secreted by Bacterially Infected Macrophages Are Proinflammatory

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Exosomes are small vesicles that are secreted from cells. They are derived from multivesicular endosomes that fuse with the plasma membrane, thereby releasing their internal vesicles into the extracellular environment. Exosomes from antigen-presenting cells contain a range of immunostimulatory molecules that activate T cells, which suggests that they may have an important role in the propagation of immune responses. Of considerable interest is the finding that exosomes derived from bacterially infected macrophages carry bacterial coat components and use these to stimulate bystander macrophages and neutrophils to secrete proinflammatory mediators, including tumor necrosis factor-α, the chemokine regulated upon activation, normal T cell–expressed and –secreted (RANTES, also known as CCL5), and inducible nitric oxide synthase. Here, we address these studies in relation to other findings on dendritic cell–derived exosomes that are also powerful immunoregulators.

Exosomes are small (50 to 100 nm in diameter) vesicles that are secreted by a range of cell types, including antigen-presenting cells (APCs) such as dendritic cells (DCs), B lymphocytes, and macrophages. The release of exosomes by APCs offers a mechanism for cells to release proteins, lipids, and nucleic acids into the extracellular environment. These small vesicles develop by the inward invagination and budding off of the limiting membrane of endosomal compartments, thus forming multivesicular endosomes (MVEs), which also appear to be major histocompatibility complex II (MHC-II)–rich compartments in DCs (*1*–*3*). When MVE membranes fuse with the plasma membrane, exosomes are expelled into the extracellular space, where they can interact with other cells. Exosomes produced by DCs and B lymphocytes have generated considerable interest because they carry a range of immunostimulatory molecules that are also found on the cell surface of APCs, including MHC-I, MHC-II, and costimulatory molecules such as CD86 and CD80 (*2*–*5*). Exosomes act as immunostimulatory vesicles because they activate T cells, which leads to T-cell proliferation and the development of an antigen-specific immune response (Fig. 1).

Studies by Bhatnagar *et al*. (*6*, *7*) now report that exosomes that are produced by macrophages are proinflammatory, which heralds a new and important role for exosomes in early or innate immunity. Exosomes released by bacterially infected, but not uninfected, macrophages stimulated neighboring macrophages and neutrophils to release inflammatory mediators. These proinflammatory agents are thought to be bacterially derived coat components, possibly glycolipids and glycopeptidolipids acquired from phagocytosed bacteria that entered late endosomes, which were incorporated into the membranes of exosomes (*6*–*8*). The importance of this finding lies in the ability of infected macrophages to release exosomes that incorporate bacterial molecules that contain pathogen-associated molecular patterns (PAMPs). PAMPs represent a range of molecules that act as Toll-like receptor (TLR) ligands, which activate cells that express TLRs. After binding to TLRs, macrophage-derived exosomes activate uninfected macrophages, which leads to their release of proinflammatory factors (*6*–*8*). The production of these factors represents a very effective innate immune response that has the potential to limit the spread of bacterial infection.

Without detracting from the importance of macrophage-derived exosomes, the story that is developing about DC-derived exosomes is very noteworthy. DC-derived exosomes function in antigen-specific T cell activation, in some cases leading to T cell effector function and vaccination that can protect against reinfection or recurrence of disease (*1*, *3*, *9*). Exosomes produced by DCs directly activate T cells because they carry a range of immunostimulatory molecules such as MHC-I, MHC-II, CD86, intercellular adhesion molecule 1 (ICAM-1), CD63, and CD82 (*2*, *3*, *10*). DC-derived exosomes carry antigenic peptides derived by proteolysis of antigens that were endocytosed by the parent DC. These peptides are presented as MHC-peptide complexes that are necessary for antigen-specific T cell activation (*1*, *9*, *11*, *12*). The role of exosomes in indirect activation of T cells depends on the transfer of MHC-peptide complexes to bystander DCs, which then activate T cells (*11*, *13*, *14*) (Fig. 1). Indeed, DC-derived exosomes have been investigated for their potential use in vaccination (*3*, *11*, *15*). As well as a range of immunostimulatory molecules, DC-derived exosomes contain a unique set of molecules associated with endocytic compartments (*16*). This makes exosomes readily distinguishable from apoptotic vesicles that are released by dying cells, because exosomes lack those molecules that are associated with the blebbing of membranes that exposes the internal membrane components of apoptotic cells (*16*, *17*).

The story for macrophage-derived exosomes also portends the development of important clinical therapies. The exosomes produced within the MVEs of infected macrophages acquire molecules derived from bacteria after their phagocytosis or replication within the cell. Macrophages infected with *Mycobacterium bovis* BCG release exosomes that contain several mycobacterial lipids and immunostimulatory molecules that are commonly associated with exosomes (*8*). Using *M. avium* to infect macrophages, Bhatnagar *et al*. (*6*) monitored the localization of pathogen-associated glycopeptidolipids (GPLs) with X-labeled antibodies. GPLs were found on the bacterial cell surface within phagosomes and in cell compartments separated from the phagosome. These endocytic compartments resembled late endosomes and were identified as MVEs because of the detection of lysosome-associated membrane

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protein (LAMP)–1, LAMP-2, MHC-I, and MHC-II (*6*). Bacterial GPLs that had trafficked into MVEs became incorporated into exosomes and were expelled from infected cells. The exosomes were then able to move from infected to nearby uninfected cells, where they interacted with TLRs. Exposure of uninfected macrophages to exosomes carrying GPLs led to their activation and the secretion of proinflammatory factors such as tumor necrosis factor-α (TNF-α), regulated upon activation, normal T cell–expressed and –secreted (RANTES, also known as CCL5), and exosome release in cellular communication and interactions.

However, there are still questions that need to be addressed. Although Bhatnagar *et al.* (*6*) confirmed

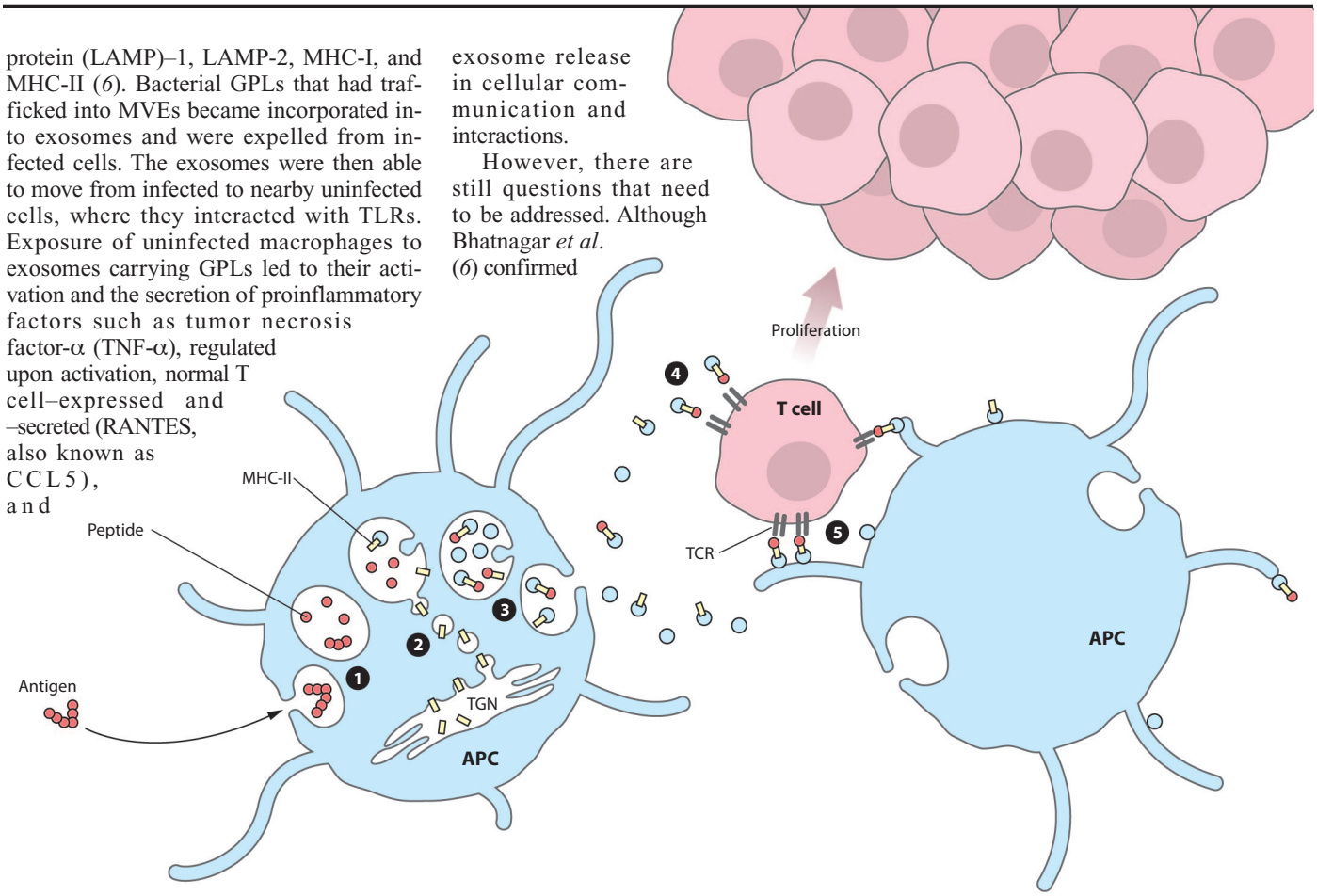

Fig. 1. Production of immunostimulatory exosomes by antigen-presenting cells. Protein antigens taken up by antigen-presenting cells (APCs) enter the endosomal pathway and are degraded in late endosomes to give peptides (1). Here, they interact with MHC-II molecules transported from the trans-Golgi network (TGN) (2). The membranes of these MHC-II–rich compartments invaginate and bud off, thus expelling vesicles into the lumen and forming a multivesicular endosome (2 and 3). The intraluminal vesicles contain molecules derived from the membrane of the MHC-II–rich compartment, including MHC-II, MHC-I, and CD86. (Only MHC-II is shown as an example.) Multivesicular endosomes fuse with the plasma membrane, which results in the release of the internal vesicles, called exosomes, into the extracellular space (3). The exosomes interact with and stimulate T cells through the interaction of their MHC and costimulatory molecules with antigen-specific T cell receptors (TCRs) on the T cells. Activation and proliferation of T cells by exosomes occurs either directly (4) or indirectly (5) after display of antigen-carrying exosomes on the surface of bystander APCs.

inducible nitric oxide synthase (iNOS) through a myeloid differentiation marker 88 (MyD88)–dependent pathway (*6*) (Fig. 2). Only those exosomes that were derived from infected macrophages were proinflammatory. The involvement of exosomes in trafficking bacterial components was confirmed when bacterial GPLs were found associated with exosomal particles and not with any contaminating mycobacteria or cell debris. As for DC-derived exosomes, the repertoire of molecules carried by macrophage-derived exosomes was distinct from that of apoptotic vesicles (*6*, *16*). The combined evidence for DC-derived and macrophage-derived exosomes serves to emphasize the biological importance of the transport of GPLs by exosomes, they did not definitively identify the bacterial component that was the effective macrophage stimulant, nor did they confirm that exosomes were indeed the carriers of such PAMPs. In fact, exosomes derived from macrophages infected with a GPL-deficient strain of *M. avium* were equally effective as those from wild-type *M. avium*–infected macrophages in their ability to activate bystander macrophages (*6*). We therefore await further characterization of *M. avium* PAMPs and their derivation from intracellular bacteria to rule out the possibility of potential contaminants, discussed below. Interestingly, this phenomenon has been described for a range of bacteria that replicate within phagosomes, including *M. bovis* BCG, *M. avium*, *M. tuberculosis*, *BCG*, and *Salmonella typhimurium* (*7*). It has also been claimed for exosomes produced by macrophages infected with *Toxoplasma gondii*. Although this pathogen does not replicate within phagosomes, the *Toxoplasma*-containing vacuole is connected to the endosome-lysosome pathway through a microtubule-mediated pathway (*6*–*8*). Studies also showed that exosomes from DCs that were infected in vitro with either *M. bovis* BCG or *M. tuberculosis* stimulated the activation and recruitment of macrophages and neutrophils in vivo, which confirmed the proinflammatory power of exosome

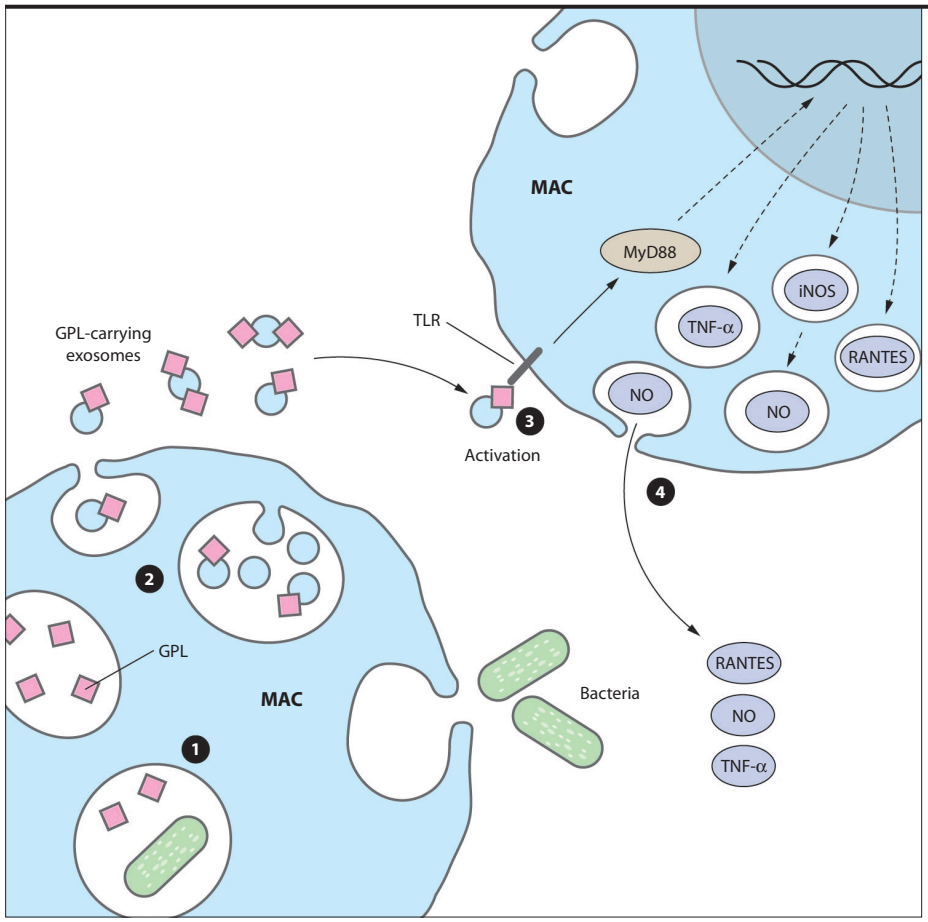

Fig. 2. Production of proinflammatory exosomes by infected macrophages. Bacteria, such as mycobacteria, are taken up by macrophages (MAC) and shed various coat components such as glycopeptidolipids (GPLs) that associate with membranes within the lumen of the endosome (1). Intraluminal vesicles formed by invagination of the limiting membrane within these endosomes would then carry the bacterial GPLs on their surface (2). When these multivesicular endosomes fuse with the plasma membrane, the vesicles or exosomes expressing the GPLs or bacterial coat components are released extracellularly. These released particles bind to PAMP receptors (for example, TLRs) on nearby macrophages, which leads to MyD88-dependent macrophage activation and changes in gene expression in the nucleus (3). Exosome-mediated activation of macrophages leads to the production of proinflammatory mediators, which include TNF-α, RANTES, and iNOS, the latter resulting in production of NO (4). Extracellular release of these factors constitutes an innate immune response.

preparations in a physiological setting. However, these studies still failed to definitively identify those bacterial components that were the specific proinflammatory agent(s) (*7*). Thus, it appears that macrophages and DCs infected with a number of different bacteria secrete exosomes that express a range of TLR ligands in the form of PAMPs, and as such they are very powerful proinflammatory regulators of innate immunity. Transport of bacterial components by exosomes could provide a very effective means of spreading PAMPs to effector cells of the innate immune system. Moreover, aside from providing the signal necessary to activate phagocytes such as macrophages and neutrophils, exosomes could also potentially carry protein antigens to DCs. By delivering both antigen and activation signals to DCs, exosomes could then help with the propagation of T cell responses and thereby the adaptive immune response.

There is, however, one potential problem yet to be fully investigated in studies of macrophage-derived exosomes, which became apparent in our own investigations of exosomes from DCs. Exosomes produced by DCs and macrophages may carry very small infectious agents such as viruses, retroviruses, or mycoplasma, which may have been introduced in the bacterial preparations used to infect the cells. Indeed, mycoplasma contaminate cultured cell lines with very little effect on cell growth, and they contain many potentially mitogenic glycolipids (*18*, *19*). Contamination of exosomes is possible given that myoplasma are found throughout the host cell and that exosomes could thus incorporate mycoplasmal components or be copurified with them. Because most immune response studies of exosomes involve cells cultured in vitro, it is difficult to eliminate the possibility that contaminating infectious agents are involved or that molecules derived from such agents are in the exosome membrane (*20*). Indeed, our previous studies identified mycoplasma as contaminants of exosome preparations (*20*), and these contaminants were very strong polyclonal activators of B lymphocytes (Fig. 3). Mycoplasmal glycolipids represent powerful immunoregulators that induce a mitogenic response in B lymphocytes even in the presence of an antigen-specific response in T lymphocytes, which is dependent on immunostimulatory molecules such as CD86, MHC-I, and MHC-II (*1*, *20*). Mycoplasmal components are also powerful activators of macrophages; *M. arthriditis* and *M. fermentas* signal through TLR2, which leads to the activation of macrophages and DCs (*21*, *22*). The problem of accidental copurification of mycoplasma with exosomes was also addressed in our studies. We were unable to separate exosomal vesicles from infectious mycoplasma with common procedures for exosome purification such as differential centrifugation and filtration (*20*).

An emerging characteristic of DC-derived exosomes is that they reflect the state of differentiation and activation of the DCs from which they are generated. For example, exosomes with immunogenic capacity are commonly released by DCs that are generated by culture of bone marrow (BM) precursors with factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (*3*, *9*). Exosomes derived from immature DCs, or from DCs that develop under the influence of suppressive factors such as transforming growth factor-β (TGF-β) and interleukin 10 (IL-10), tend to exhibit properties of tolerogenic DCs. These DCs and their exosomes are immunosuppressive for T cells (*23*, *24*), and indeed give therapeutic benefits in T cell–induced disease states (*25*, *26*). This tight functional association between the DC and its exosomes raises the possibility that some of the

assumed functions of DCs in vivo may be mediated by exosomes secreted by the DCs rather than by the DCs themselves. Indeed, exosomes present molecules that determine the ability of a DC to either activate or suppress T cells. For example, immunostimulatory molecules such as CD86 are found in immunogenic DC–derived exosomes (*3*), whereas the apoptosis-inducing molecule Fas ligand (FasL) is found in immunosuppressive or tolerogenic DC-derived exosomes (*23*, *26*). Indeed, DC-derived exosomes with immunosuppressive properties induce antigen-specific tolerance in disease states and in transplantation models (*23*, *27*). A study by Kim *et al*. (*25*) now demonstrates how to genetically engineer DCs to produce exosomes of defined functional potential. For example, BM-derived murine DC precursors genetically engineered to express the gene that encodes IL-4 became immunosuppressive DCs that were effective in reversal of the autoimmune disease collagen-induced arthritis and in the control of delayed-type hypersensitivity (*25*). The effectiveness of DCs as immunosuppressive agents was independent of the production of IL-4 as either a soluble or a membrane-bound molecule, but dependent on the secretion of exosomes with immunosuppressive potential. IL-4, like IL-10 and TGF-β, appeared to have a similar suppressive effect on DC development, and this was reflected in the composition and functional property of the exosomes produced. The therapeutic agent responsible for the reversal of collagen-induced arthritis was a DC-produced exosome that presents FasL and MHC-II (*25*), although the actual molecules involved have not yet been defined.

Exosomes are also powerful immunogens. The usefulness of DC-derived exosomes for immunomodulation is due to their acellular nature and their capacity to present antigens to T cells in an immunogenic manner. In terms of immunization, it is also desirable if the antigen is introduced in a form that gives sustained induction of T cell activation. Indeed, for immunization by adoptive transfer, DC-derived exosomes are far more effective than the original DC. This is attributed to the much higher turnover rate of DCs compared with that of exosomes (*28*). Adoptively transferred DCs undergo apoptosis soon after interaction with T cells and are then lost from draining lymph nodes within 2 or 3 days. In contrast, exosomes that present MHC-I-peptide complexes are resistant to recognition and elimination by cytotoxic T cells (CTLs) and, even in the face of an ongoing CTL response, continue to activate naïve T cells (*28*).

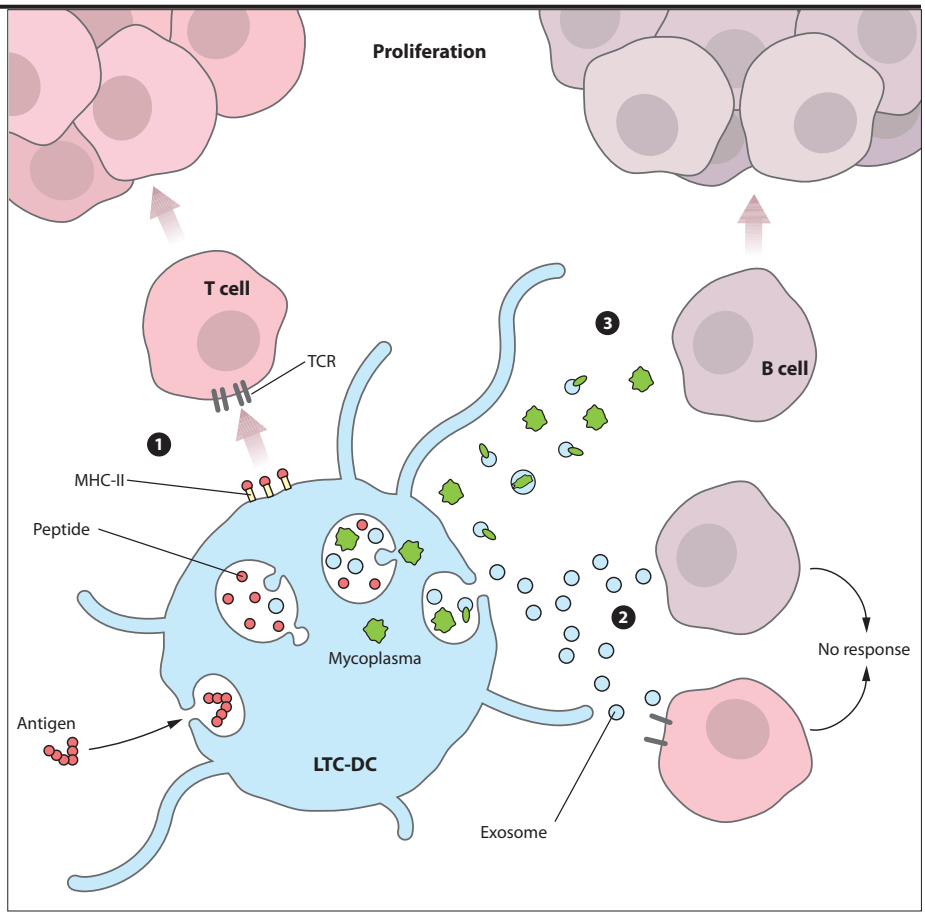

Fig. 3. Mycoplasma contaminants of dendritic cell–derived exosomes are mitogenic for B lymphocytes. Our studies have demonstrated the copurification of mycoplasma contaminants in DC lines with exosome preparations. DCs grown in long-term cultures (LTC-DC), which had the capacity to stimulate T lymphocytes in their own right (1), also secreted exosomes (2). Exosome preparations were initially found to be potent B cell mitogens. However, further analysis revealed that the mitogenic effect on B lymphocytes was the result of contaminating mycoplasma that copurified with exosomes (3). In fact, exosomes cannot stimulate B lymphocytes in the absence of mycoplasma (2). Whole, viable mycoplasma were present in the exosome preparations based on the ability of the contamination to be transferred to other cells by exosome fractions alone. However, whether mycoplasma alone or exosomes carrying mycoplasmal components are responsible for the stimulation of B lymphocytes is not clear and still remains a possibility (3).

Macrophage-derived exosomes have not yet been investigated for their effectiveness as immunogens. Their potential to transfer proinflammatory molecules to other cells propagates an innate immune response, and this is important for triggering further events leading to lymphocyte activation. Bhatnagar *et al*. (*7*) confirmed that exosomes derived from bacterially infected macrophages activated nearby uninfected macrophages, which led to their release of inflammatory factors. Exosomes carrying TLR ligands might also activate DCs, thus increasing their immunogenic capacity. Indeed, exosomes derived from bacterially infected macrophages appear to be important adjuvants for immunization. Biotechnology strategies that lead to the incorporation of PAMPs into antigen-carrying immunostimulatory exosomes could provide an immunogen that ensures the development of an immunogenic rather than a tolerogenic immune response. Antigen-carrying exosomes could be prepared from cells that have been genetically engineered to express appropriate TLR ligands so that an inflammatory response and the activation of macrophages or DCs could enhance

the immunization outcome. Although the power of exosomes as an acellular delivery vehicle that carries immunomodulatory molecules is well established, their safety, in terms of the delivery of pathogen-derived toxins or infectious pathogens themselves, still needs to be confirmed. If this cannot be achieved, liposomes or genetically engineered exosomes may be a necessary alternative.

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