Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis

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Summary

The transfer of antigens from oligodendrocytes to immune cells has been implicated in the pathogenesis of autoimmune diseases. Here, we show that oligodendrocytes secrete small membrane vesicles called exosomes, which are specifically and efficiently taken up by microglia both in vitro and in vivo. Internalisation of exosomes occurs by a macropinocytotic mechanism without inducing a concomitant inflammatory response. After stimulation of microglia with interferon-γ, we observe an upregulation of MHC class II in a subpopulation of microglia. However, exosomes are preferentially internalised in microglia that do not seem to have antigen-presenting capacity. We propose that the constitutive macropinocytotic clearance of exosomes by a subset of microglia represents an important mechanism through which microglia participate in the degradation of oligodendroglial membrane in an immunologically 'silent' manner. By designating the capacity for macropinocytosis and antigen presentation to distinct cells, degradation and immune function might be assigned to different subtypes of microglia.

Keywords: Macropinocytosis, Microglia, Myelin, Oligodendrocytes, Exosomes

Introduction

A crucial step in the induction of an autoimmune response against myelin as occurs in multiple sclerosis (MS) is the transfer of autoantigens to accessory cells (Sospedra and Martin, 2005; Steinman, 2001; Weiner, 2004). How these encephalitogenic antigens are transported to an immunogenic environment is an important and unresolved question. One attractive possibility is that membrane vesicles have a role in the transfer. In fact, cells not only use vesicular carriers to transport cargo within a cell, but also release a range of different vesicles into the extracellular space (Court et al., 2008; Schorey and Bhatnagar, 2008; Simons and Raposo, 2009; Thery et al., 2009). Some of these vesicles are generated by direct shedding from the plasma membrane (Bianco et al., 2009; Cocucci et al., 2009), others are formed within the endosomal system by the budding of the limiting endosomal membrane into its lumen inducing the formation of multivesicular endosomes (van Niel et al., 2006). Following fusion of these multivesicular endosomes with the plasma membrane, the intraluminal vesicles are released into the extracellular space as exosomes. Exosomes are small (50–100 nm) membrane vesicles that contain proteins involved in membrane transport or vesicle biogenesis as well as different heat shock proteins, tetraspanins and integrins (Thery et al., 2009). In addition, exosomes contain distinct proteins, depending on the cell type from which they are derived. For example, oligodendrocytes secrete relatively large amounts of exosomes enriched in the proteolipid protein (PLP), a major protein of central nervous system (CNS) myelin and a candidate autoantigen in MS (Krämer-Albers et al., 2007; Trajkovic et al., 2008; Hsu et al., 2010).

Thus, different mechanisms of how exosomes interact with their target cells have been described. Exosomes can attach to the cell surface or are internalised into the recipient cell by receptor–ligand interactions (Thery et al., 2009). Consequently, depending on their origin and their target, many different functions have been attributed to exosomes. One function of exosomes is to remove obsolete protein and lipids and to secrete them into a drainage system, such as the gut or the urine (Gonzales et al., 2009; Keller et al., 2007; Pan and Johnstone, 1984). Most studies have focused on the role of exosomes in modulating the function of the immune system. For example, antigen-presenting cells such as dendritic cells (DCs) or B cells can release exosomes containing preformed peptide–MHC complexes and costimulatory molecules that are able to induce T-cell activation (Raposo et al., 1996). In addition, numerous MHC-independent roles of exosomes in regulating immune responses have been described. DC-derived exosomes can induce a specific T-cell-mediated antitumor response and tumor-cell-derived exosomes modulate immune reactions, subsequently resulting in decreased tumor growth (Zeelenberg et al., 2008; Zitvogel et al., 1998).

In this study, we analysed the extracellular fate of oligodendroglia-derived exosomes and addressed the question of how exosomes are transferred from the donor to the target cell. We found that exosomes are selectively transferred from oligodendrocytes to microglia by macropinocytosis. Internalisation of exosomes occurred predominantly in a subpopulation of unstimulated and MHC-class-II-negative cells in an immunologically 'silent' manner. Our result suggest that the macropinocytotic clearance of exosomes is an important mechanism

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through which microglia participate in the degradation of oligodendroglial membrane.

Results

Internalisation of oligodendroglia-derived exosomes by microglia

To obtain exosomes in large quantities, we isolated exosomes from the culture medium of the mouse oligodendroglial precursor cell line Oli-neu. Sequential centrifugation steps with increasing centrifugal forces up to 100,000 ***g*** yielded a pellet, which we have previously shown to contain small membrane vesicles with a diameter of about 50–100 nm (Trajkovic et al., 2008). Further analysis of the 100,000 ***g*** pellet with continuous sucrose density gradients revealed that PLP and myelin oligodendrocyte glycoprotein (MOG) are recovered from fractions with the characteristic density of exosomes that contain the exosomal marker proteins Alix (ALG-2-interacting protein 1), TSG101 and flotillin-2 (Fig. 1A). Hence, we refer to the 100,000 ***g*** membrane fractions as exosomes.

To follow the fate of exosomes, suspensions of purified exosomes were labelled with the dye PKH67 and added to primary cultures of mouse oligodendrocytes, cortical neurons, astrocytes and microglia. After incubating the exosomes for 2 hours at 37°C, cells were fixed and analysed by confocal microscopy. We observed efficient internalisation of exosomes by microglia, whereas uptake by astrocytes, neurons or oligodendrocytes was almost absent (Fig. 1B). By confocal microscopy, we confirmed that exosomes were inside microglia rather than attached to the cell surface. In addition, we observed that exosomes colocalised with Lamp1, a marker for late endosomes or lysosomes, verifying that exosomes had been endocytosed and transported to endosomal organelles (Fig. 2A). When exosomes were added to mixed brain cultures that contained all major glial cell types, we again observed that exosomes were almost exclusively internalised by Iba-1-positive microglia (supplementary material Fig. S1). Similar results were obtained when exosomes prepared from primary cultures of mature rat oligodendrocytes were added to microglia (supplementary material Fig. S2). These purified exosomes from mature oligodendrocytes contained several exosomal marker proteins such as Alix and flotillin-2, but not the ER protein, calnexin (supplementary material Fig. S2). Efficient uptake of exosomes was also detected when the microglial cell line EOC-20 was used (supplementary material Fig. S3).

To rule out unspecific transfer of the dye PKH67 to microglia, we performed several control experiments. The 100,000 ***g*** pellet was labelled with PKH67 and further purified on a sucrose density gradient. All fractions of the gradient were pelleted at 100,000 ***g*** and used for transfer experiments. We observed transfer of PHK67 into microglia mainly from fractions with the characteristic density of exosomes (supplementary material Fig. S3). As an additional control, we added the PKH67-labelled 100,000 ***g*** pellet to living

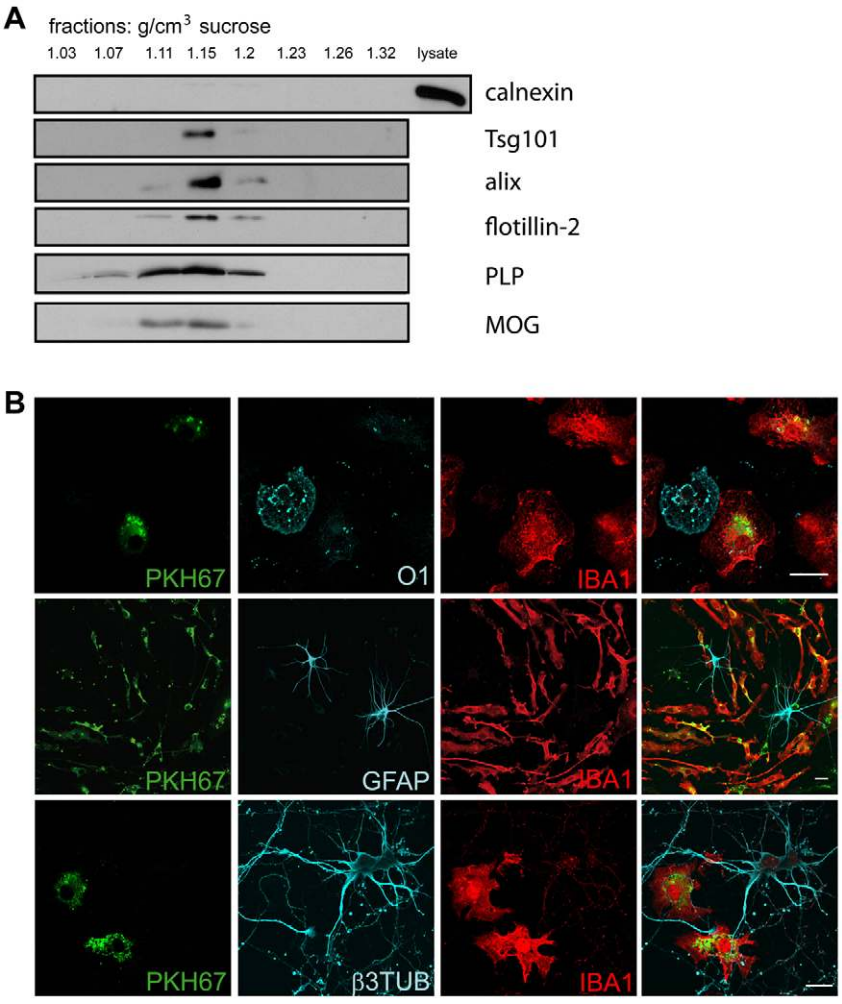

Fig. 1. Oligodendroglial exosomes are selectively internalised by microglia. (**A**) Exosomes were purified from the culture medium of the oligodendroglial precursor cell line, Oli-neu, by sequential centrifugation steps with increasing centrifugal forces up to 100,000 ***g***. The 100,000 ***g*** pellet was loaded onto continuous sucrose density gradients and centrifuged for 16 hours. Fractions were collected, pelleted at 100,000 ***g*** and subjected to western blot analysis. After transient transfection of the cells, PLP and MOG are recovered from fractions with the characteristic density of exosomes that contain the exosomal marker proteins, Alix, TSG101 and flotillin-2. (**B**) Purified exosomes (100,000 ***g*** pellet) were labelled with the dye PKH67 and added to primary cultures of mouse oligodendrocytes, astrocytes, cortical neurons and microglia. After incubation with exosomes for 2 hours at 37°C, cells were fixed and analysed by confocal microscopy. Oligodendrocytes, astrocytes, neurons and microglia were recognised by antibodies against GalC (O1), GFAP, β3-tubulin and Iba1, respectively. Exosomes are taken up by Iba1-positive microglia. Scale bars: 20 µm.

and fixed cells. We only observed transfer of PKH67 into living, but not fixed microglia showing that transfer is not due to passive diffusion, but to an active uptake process (supplementary material Fig. S4). Furthermore, we used the dye carboxytetramethylrhodamine (TAMRA), which was covalently attached to proteins of the exosomal membrane fraction. TAMRA-labelled exosomes were readily taken up by primary microglial cells (Fig. 2A). Finally, exosomes containing PLP–GFP were internalised into microglia (Fig. 2A).

To verify that exosomes are indeed cleared from the extracellular space by microglia, we used an alternative approach. Cell culture medium from primary mature oligodendrocytes was centrifuged at 3000 ***g*** and twice at 4000 ***g*** to clear the medium from cellular debris. This cleared conditioned culture medium – still containing

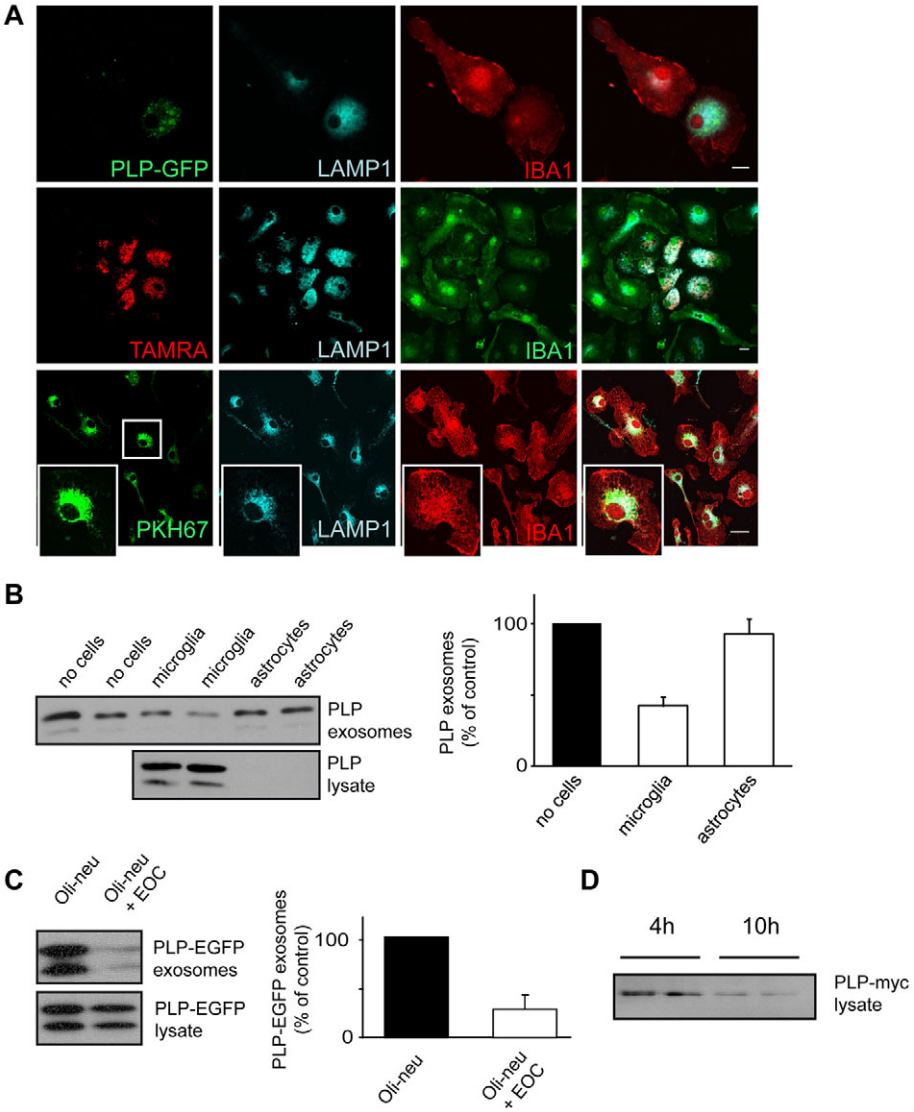

Fig. 2. Oligodendroglial-derived exosomes are transferred to late endosomes and lysosomes. (**A**) Exosomes were purified from Oli-neu cells expressing PLP–GFP or from wild-type cells and subsequently labelled with the lipophilic dye PKH67 or through the crosslinking of carboxytetramethylrhodamine (TAMRA) to proteins of the exosomal membrane fraction. PLP–GFP-positive or fluorescent-labelled exosomes were added to primary microglia cultures and incubated for 2 hours at 37°C. For microscopic analysis, microglia were visualised using anti-Iba1 antibody. Internalised exosomes colocalise with Lamp-1, a marker for late endosomes and lysosomes in primary microglia. Scale bars: 10 µm. (**B**) Culture medium of primary cultures of mature oligodendrocytes was added to dishes without cells (no cells) or to an equal number of primary microglia or astrocytes (two independent experiments as shown). After 8 hours of incubation, exosomes were purified from the culture medium and the amount of PLP was determined. After culture with primary microglia, less PLP was found in the medium and more PLP accumulated in the microglial cell lysate, compared with cultures with primary astrocytes. Only a fraction (1/5) of the total culture medium was loaded on to the gel. Values are given as the mean ± s.d. (n=3). (**C**) The oligodendroglial cell line, Oli-neu stably expressing PLP–Myc–EGFP was (co) cultured with or without the microglial cell line, EOC-20 for 24 hours. Exosomes were subsequently isolated from the culture medium and the amount of PLP–Myc–EGFP determined by western blotting with primary antibodies against the Myc epitope. Equal amounts of proteins (~20 µg) from the cell lysates were loaded on to the gel. In the presence of microglia, a significant reduction of PLP–Myc–EGFP in the culture medium was observed. Values are means ± s.d. (n=3). (**D**) To analyse whether PLP–Myc is degraded after its uptake in microglia, we added purified exosomes to primary microglia, allowed uptake to occur for 4 hours and harvested the cells after 4 and 10 hours. PLP–Myc signals on the western blot were revealed using anti-Myc antibodies. PLP decreases with time in the microglial cell lysate. Results are representative of two independent experiments.

exosomes – was then added to cultured astrocytes or microglia and incubated for 8 hours. Subsequently, the medium was removed from the cultures and exosomes were purified. We found that, in the presence of microglia, PLP-containing exosomes were cleared from the medium and, instead, accumulated in the microglial cell lysate (Fig. 2B). The uptake efficiency was estimated and these calculations revealed that under cell culture conditions 3×10^5 microglia clear within 8 hours ~40% of the PLP-containing exosomes produced by 1.5×10^6 oligodendrocytes.

Contrasting results were obtained when astrocytes were used for the experiments. Here, PLP remained in the culture medium and was not detected in the lysate of astrocytes (Fig. 2B). The conclusion that microglia internalise exosomes was further strengthened by experiments, in which microglial and oligodendroglial cell lines were kept together in a coculture system for 24 hours. In coculture with the microglial cell line EOC-20, PLP-containing exosomes were efficiently cleared from the culture medium (Fig. 2C).

To analyse whether PLP is degraded after its uptake in microglia, we added purified exosomes to primary microglia, allowed internalisation to occur and harvested the cells at different time points. We found that PLP decreases with time in the microglial cell lysate, illustrating its degradation over time (Fig. 2D). Together, these data clearly demonstrate the transfer of membrane via exosomes from oligodendrocytes to microglia.

Because all these findings relied on cultured microglia, we wanted to verify that exosomes are also taken up by microglia in vivo. For these experiments, we used transgenic mice showing specific expression of enhanced green fluorescent protein (EGFP) in resident microglia of the CNS by insertion of the EGFP reporter gene into the Cx3cr1 locus (Jung et al., 2000). Exosomes were injected into the spinal cord and images acquired by two-photon microscopy 30 minutes after injection. As observed in cell culture, time-lapse imaging revealed that exosomes were taken up by EGFP-marked microglia and moved in vesicular structures along the cellular processes (Fig. 3A). In addition, accumulation of labelled exosomes was found within Lamp-1-positive late endosomal or lysosomal compartments, demonstrating the intracellular localisation within microglia (Fig. 3B). At 400 μm from the injection sites, ~50% of the EGFP-marked microglia showed colocalisation with PKH26-labelled exosomes. The life-imaging approach thus confirmed our conclusion that oligodendroglia-derived exosomes are cleared by microglia.

Exosomes are internalised by macropinocytosis

Next, we wanted to determine by which endocytic pathway microglia use to take up exosomes. Microglia are capable of different modes of endocytosis, ranging from receptor-mediated, clathrin-dependent to independent endocytosis. In contrast to many other cells, microglia are also capable of constitutive macropinocytosis and phagocytosis (Napoli and Neumann, 2009). Macropinocytosis and phagocytosis share many common features, such as the formation of large vacuoles, and molecular factors as part of the respective cellular machinery (Kerr and Teasdale, 2009). However, there are some important differences. Phagocytosis is particle driven and depends on the tight interaction of ligands on the particle with receptors on the entire surface of the enclosing cup. Macropinocytosis can occur constitutively and is associated with the actin-dependent formation of membrane ruffles, resulting in the uptake of fluid into large vacuoles. Membrane ruffling and closure is dependent on Rac1 GTPase activity, Na^+/H^+ exchanger function and, in some cells, on dynamin (Kerr and Teasdale, 2009; Mercer and Helenius, 2009).

To determine whether exosome use macropinocytosis for entry into microglia, we treated cells with various pharmacological inhibitors, including dynasore, to inhibit the function of dynamin; NSC23766, a small molecule inhibitor of Rac1; cytochalasin D, to interfere with the function of the actin cytoskeleton; and amiloride, to block the activity of the Na^+/H^+ exchanger. When uptake assays were performed in the presence of these drugs, we observed an inhibition of exosome internalisation into microglia, which was consistent with a macropinocytic mechanism (Fig. 4A).

Previous studies have shown that vacuolar acidification is required for macropinocytosis (Mercer and Helenius, 2008).

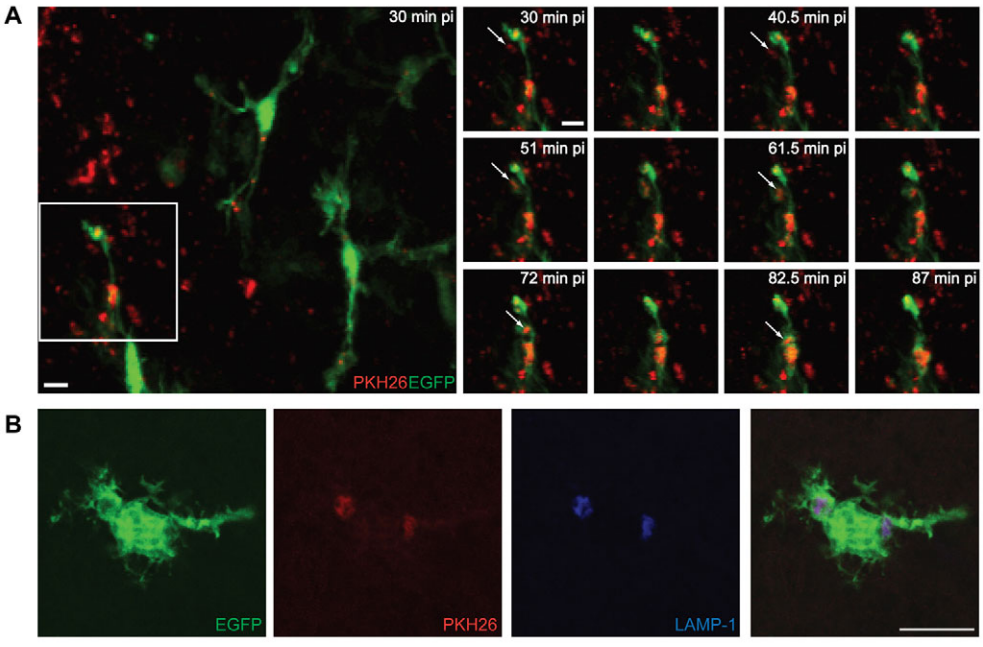

Fig. 3. Exosomes are internalised by microglia in vivo. (**A**) Purified oligodendroglial exosomes were labelled with the dye PKH26 (red) and injected into the spinal cord of anaesthetised CX3CR1–GFP mice. Time-lapse imaging was performed by two-photon microscopy. The boxed area in the left image is shown as a time-lapse series. Fluorescently labelled exosomes moving into and within microglia are indicated by arrows at different time points. The elapsed time after injection of exosomes (post injection, pi) is shown in the images. Scale bars: 10 μm. (**B**) Immunohistochemistry of spinal cord sections of CX3CR1–GFP mice injected with PKH26-labelled exosomes (red) 2 hours before perfusion. Confocal microscopy images of microglia expressing EGFP and internalised exosomes. Sections were stained for LAMP-1 (blue). Scale bar, 10 μm.

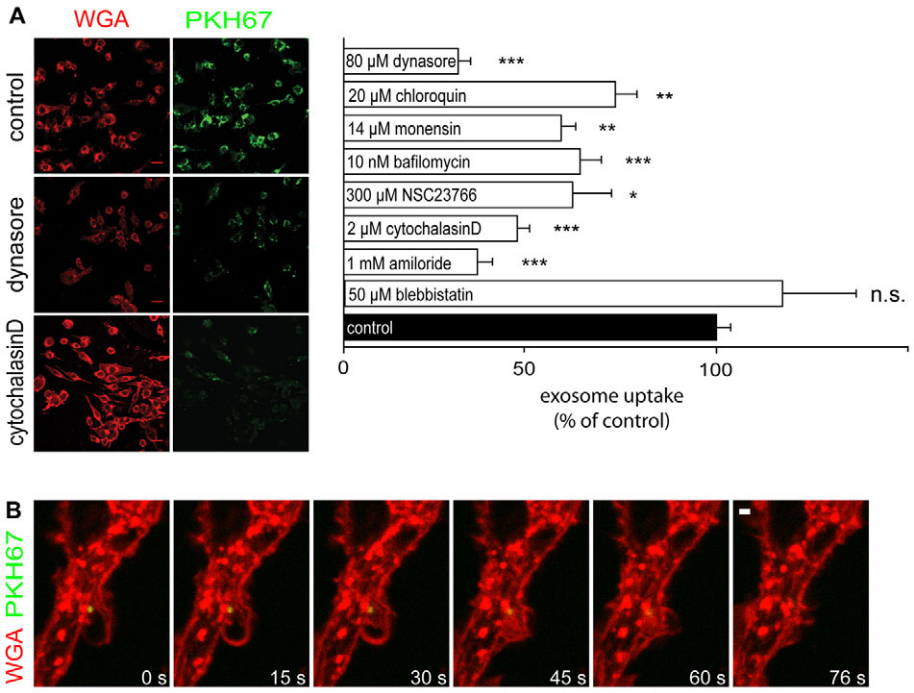

Fig. 4. Microglia internalise exosomes by macropinocytosis. (**A**) Exosomes were purified from the oligodendroglial cell line, Oli-neu, labelled with the dye PKH67 (green) and incubated for 2 hours at 37°C together with the microglial cell line, EOC-20. Cells were incubated with the indicated inhibitors (dynasore, chloroquine, monensin, bafilomycin, NSC23766, cytochalasin D, amiloride, blebbistatin) for 30 minutes before and during the 2 hour incubation. Cells were subsequently fixed, stained with Rhodamine-conjugated wheat germ agglutinin (WGA) (red) and uptake was determined by microscopy and image analysis. Changes in exosome internalisation were quantified from three independent experiments. Values represent means ± s.e.m. (n.s., not significant; **P*<0.05; ***P*<0.01; ****P*<0.001). (**B**) Purified exosomes labelled with PKH67 (green) were incubated with EOC-20 cells labelled with WGA (red) and examined by confocal live microscopy at 37°C. An image sequence spanning 76 seconds showing engulfment of exosomes by large lamellipodia-like ruffles is displayed. Scale bar: 1μm.

Treatment of cells with the alkalinising drugs bafilomycin A, monensin and chloroquine, resulted in a significant inhibition of exosome internalisation (Fig. 4A). Different modes of macropinocytosis have been described. The formation of macropinosomes can occur by lamellipodia-like ruffles, or as recently shown, by blebbing of the plasma membrane (Mercer and Helenius, 2008). To test whether blebbing was needed for exosome uptake, we also used blebbistatin, an inhibitor of myosin-II-dependent blebbing (Mercer and Helenius, 2008), but we did not observe any inhibition of uptake (Fig. 4A). Additionally, we tested whether PKH26-labelelled exosomes cointernalise with the fluid-phase marker FITC–dextran during 10 minutes of internalisation. We found that ~26% of the vesicles that contain PKH26-labelled exosomes also contained FITC–dextran (data not shown).

Finally, we added purified labelled exosomes to microglia and performed time-lapse experiments on the living cells. We were unable to observe bleb formation, but in some cases, exosomes were engulfed by large lamellipodia-like ruffles (Fig. 4B). By live microscopy, large vesicles were also observed in the peripheral region of the cell from where they moved towards the centre (data not shown).

In contrast to uptake by phagocytosis, macropinocytosis does not necessarily depend on receptor–ligand interaction (Kerr and Teasdale, 2009). To study the involvement of receptors, we used specific inhibitors of known cell surface receptors in microglia. Fucoidan inhibits interactions of both scavenger receptor class A and B, but had no influence on exosome internalisation in microglia (Fig. 5A). Moreover, lectins have been implicated in phagocytosis – but again, specific inhibitors, such as mannosamine and N-acetylglucosamine, did not influence the internalisation of exosomes. An inhibition was also not observed when all three drugs were combined. Likewise, treatment with receptor-associated protein (RAP) and low-density lipoprotein (LDL) receptor-related protein 1 (LRPAP1) to block uptake through the LDL receptor or with the RGD peptide to interfere with integrin-mediated internalisation, had no effect (Fig. 5A). Furthermore, we were unable to detect an appreciable amount of binding of exosomes to the surface of microglia at low temperature (supplementary material Fig. S5), which is consistent with our conclusion that uptake occurs by macropinocytosis, which is not necessarily dependent on a close interaction of the cargo with a receptor.

The membrane lipid, phosphatidylserine, has a key role in initiating the removal of apoptotic bodies by phagocytosis (Ravichandran and Lorenz, 2007) and clearance of certain viruses by macropinocytosis (Mercer and Helenius, 2008). Phophatidylserine is normally found within the inner leaflet of the plasma membrane, but can under certain conditions, such as apoptotic cell death, get exposed to the extracellular environment where it serves as an engulfment signal for phagocytes. Exosomes have a membrane topology that is similar to the plasma membrane, but previous studies have shown that exosomes contain phosphatidylserine facing the extracellular environment (Fomina et al., 2003; Laulagnier et al., 2004; Miyanishi et al., 2007; Morelli et al., 2004). We confirmed this finding using annexin-V labeling of purified exosomes of primary oligodendrocytes (Fig. 5B,D). To test the involvement of phosphatidylserine in exosome internalization, we prepared liposomes containing a mixture of phosphatidylcholine and phophatidylserine. Exosome uptake was reduced in the presence of phosphatidylserine-containing liposomes. However, this also occurred, albeit to a lesser extent, with liposomes only composed of phosphatidylcholine (Fig. 5C). These findings show that exosomes compete with liposomes for their macropinocytic uptake and that this competition is more efficient if the liposomes contain phosphatidylserine.

Downregulation of exosome internalisation by inflammatory stimuli

To test whether the internalisation of exosomes by microglia resulted in the release of immunoregulatory mediators, we determined the secretion of several cytokines and chemokines by microglia. For this purpose, different concentrations of purified exosomes were added to microglia and release was measured by

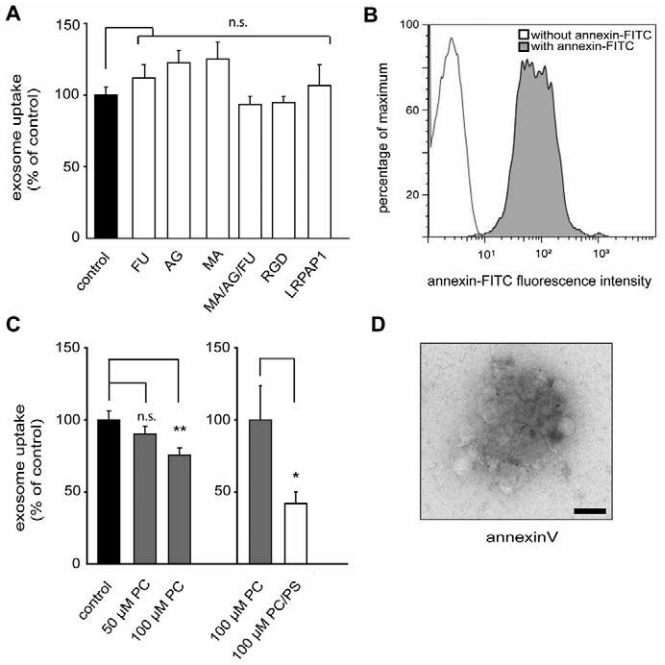

Fig. 5. Uptake of exosomes is reduced in the presence of phosphatidylserine-containing liposomes. (**A**) Purified exosomes were incubated with the indicated substances for 30 minutes before and during the 2 hours of incubation. Exosome uptake was determined by microscopy and image analysis. Treatment with fucoidan (FU), N-acetyl-D-glucosamine (AG), D-mannosamine-hydrochloride (MA), a combination of AG, MA and FU, the peptide GRGDSP (RGD) and LRPAP1 did not influence the internalisation of exosomes. (**B**) Exosomes were bound to the surface of latex beads, either stained or not with annexin-V and analysed by FACS. (**C**) Liposomes containing phosphatidylcholine (PC) or a mixture of phosphatidylcholine and phosphatidylserine (PC/PS) were coincubated with exosomes for 2 hours and uptake of exosomes was quantified. Values are given as the mean ± s.e.m. (n=3; *P<0.05; **P<0.01). (**D**) Surface-exposed phosphatidylserine (PS) was detected with annexin-V–biotin and streptavidin-coupled gold particles on purified primary exosomes by electron microscopy. Scale bar: 200 nm.

ELISA after exosomes had been internalised. When analysing the proinflammatory cytokines (TNFα, IL-6 and IL-12) exosomes did not appear to induce any release, whereas stimulation by lipopolysaccharide (LPS) resulted in a robust induction (Fig. 6A). The same was true for the proinflammatory CXC chemokine KC. Macrophage inflammatory protein 1α (MIP1α) and RANTES were slightly induced, but only with high concentrations of exosomes and to a much lower degree than that observed after stimulation with LPS (Fig. 6A). In addition, secretion of the anti-inflammatory cytokine IL-10 was unaffected by exosomes. We also evaluated whether exosomes were able to influence the cytokine and chemokine release by microglia as induced by LPS. However, exosomes did not affect the LPS-triggered secretion of cytokines or chemokines (Fig. 6A). Additionally, and in contrast to stimulation with IFNγ, the level of MHCII surface expression on microglia as determined by FACS analysis was not modulated by exosomes (Fig. 6B).

Furthermore, labelled exosomes injected into the central nervous system of *Cx3cr1/Egfp* transgenic animals did not induce morphological changes of microglial cells, as observed with two-photon time-lapse imaging (data not shown). Cells also did not increase their motility towards the injection site, as they did, for example, when a laser lesion was introduced (data not shown).

As a result of all these experiments, we conclude that the uptake or presence of exosomes does not activate microglia. Exosomes secreted by oligodendrocytes potentially carry candidate autoantigens implicated in MS such as MOG. To test whether exosomes are able to carry antigens to microglia for presentation to $CD4^+$ T cells, we took advantage of the MOG-specific T cell receptor (TCR) transgenic mice, which express a TCR that recognises MOG35–55 peptide (2D2 mice) (Bettelli et al., 2003). The activation of MOG-specific $CD4^+$ T cells was measured by the release of IL-2 into the culture medium. In contrast to activation assays performed with splenocytes, we found that the incubation of microglia with high doses of MOG35–55 peptide did not result in the activation of MOG-specific $CD4^+$ T cells (Fig. 6C). However, when microglia were stimulated with IFNγ before adding the MOG35–55 peptide, a significant release of IL-2 was detected (Fig. 6C). The response was comparable with the response obtained with splenocytes. A robust stimulation of 2D2 T cells was also observed when recombinant full-length MOG was added to IFNγ-stimulated microglia. To analyse the response to exosomes of MOG-specific $CD4^+$ T cells, purified exosomes from oligodendrocytes or Oli-neu cells overexpressing MOG were added to IFNγ-stimulated microglia. Exosomes did not induce a MOG-specific T cell activation (Fig. 6C). One possible interpretation of this result is that antigen levels were not high enough. An alternative explanation for the lack of T cell activation derives from exosome uptake experiments with IFNγ-stimulated microglia. We observed a decrease in exosome internalisation when microglia were treated with IFNγ before performing the internalisation assay (Fig. 7A). Because exosomes use macropinocytosis for their uptake, we also quantified the endocytosis of the fluid-phase marker dextran after IFNγ treatment. Again, FACS analysis revealed a lower rate of dextran internalisation in stimulated cells (Fig. 7A). Similar results were obtained after stimulation with LPS (Fig. 7B). These results show that macropinocytosis is downregulated by inflammatory signals. Additionally, we observed that stimulation with IFNγ resulted in an upregulation of MHCII in only a fraction of the microglial cells. Likewise, the levels of the costimulatory molecules B7-1 and B7-2 were only upregulated in a subpopulation of microglia after IFNγ treatment (supplementary material Fig. S6). Virtually all cells (>98%) were identified as microglia by their immunoreactivity to Iba-1 and lectin (supplementary material Fig. S7). Interestingly, exosomes were preferentially internalised into microglia with low levels of MHC II (Fig. 7C). In other words, exosome clearance would preferentially be carried out by microglia with low(er) T cell interaction capacity. This subpopulation could thereby serve as an immunologically silent sink of exosomal antigen cargo.

Taken together, our results show that exosomes are preferentially internalised into microglia with high rates of macropinocytosis, a process that is modulated by inflammatory signals. These finding raise the intriguing possibility of identifying heterogeneity in microglia based on their macropinocytotic capacity.

Discussion

The aim of this work was to follow the fate of exosomes secreted by oligodendrocytes. We found that microglia efficiently internalise exosomes by a macropinocytotic mechanism. Uptake of exosomes occurs in an immunologically silent fashion, i.e. without signs of microglial activation and without microglia supporting a MOG-

specific CD4$^+$ T cell response. Activation of microglia by IFNγ leads to an upregulation of MHC class II in a subpopulation of microglia. However, internalisation of exosomes occurs predominantly in a subpopulation of unstimulated and MHCII-negative cells. Removal of exosomes from the extracellular space might occur in microglia that are mainly engaged in the clearance

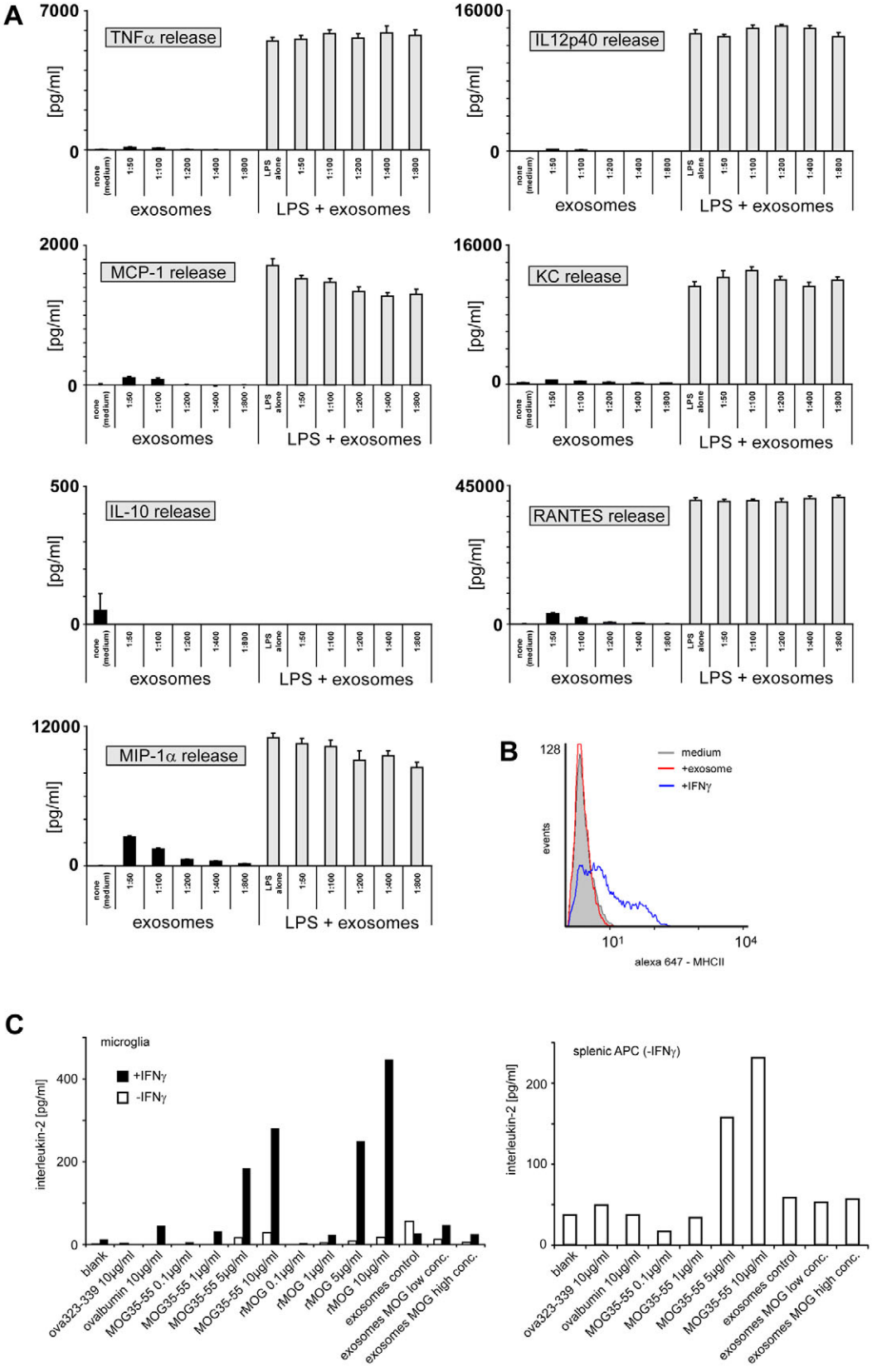

Fig. 6. Uptake of exosomes by microglia occurs in an immunologically silent manner. (**A**) Purified exosomes (from 2×10^7 cells, resuspended in 100 µl PBS; diluted as indicated) were incubated with primary cultures of microglia (15,000 cells/well) in the presence or absence of LPS (100 ng/ml) for 16 hours and the cytokine and chemokine release profile was determined. Data are means ± s.e.m. of triplicates of two independent experiments. (**B**) Cell culture medium alone or containing either purified exosomes or IFNγ was added to microglial cultures and MHCII expression was determined on the surface of microglia by FACS analysis. (**C**) Recombinant extracellular MOG (MOG 1–125), MOG peptide 35–55, ovalbumin, ovalbumin peptide 323–339 and purified exosomes in low (from 1.5×10^6 cells) or high (from 3×10^6 cells) concentration were added to primary microglia prepared from newborn mice (5×10^4 cells/well) or splenocytes (2×10^5 cells/well) together with purified MOG-specific 2D2 CD4$^+$ T cells (1×10^5 cells/well) and incubated for 72 hours. Microglia were either prestimulated with IFNγ (overnight) or not. A MOG-specific T cell response was determined by IL-2 secretion in the culture supernatants using enzyme-linked immunosorbent assay system. Data are displayed as the mean of duplicates showing one representative out of three independent experiments.

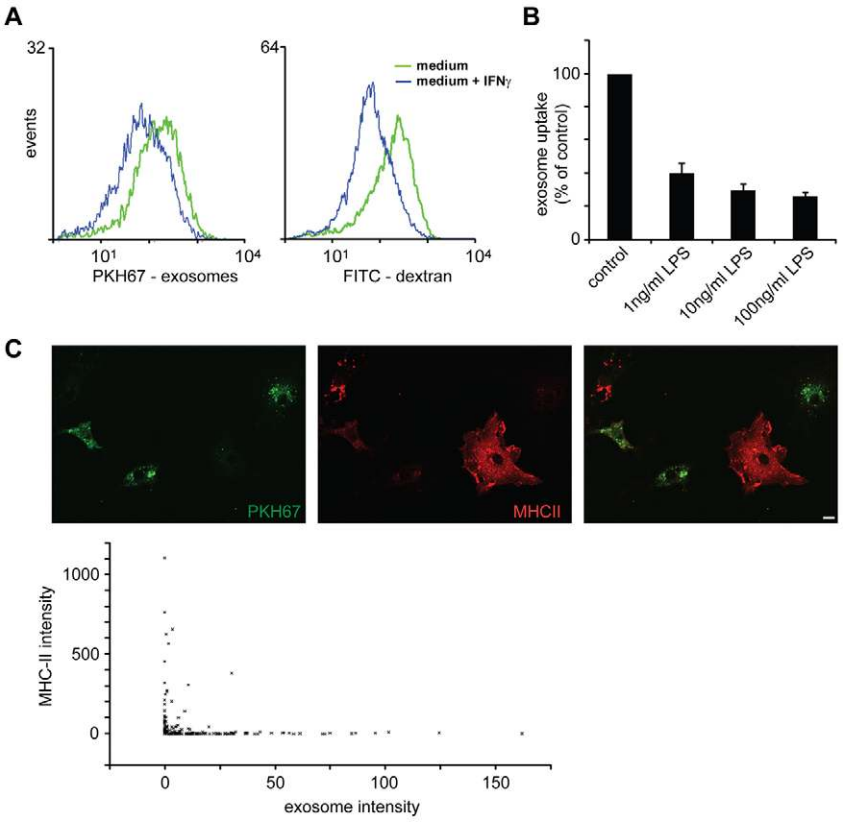

Fig. 7. Inflammatory stimuli downregulate macropinocytosis of exosomes in microglia. (**A**) Primary microglia were treated for 24 hours with IFNγ or left untreated. Purified exosomes labelled with the dye PKH67 (green) or 40 kDa FITC-conjugated Dextran were incubated for 2 hours with primary microglia (exosomes from 1×10^7 Oli-neu cells per well) and internalisation was determined by FACS. (**B**) Primary microglia were treated for 24 hours with different concentration of LPS and uptake of purified exosomes was determined by microscopy and image analysis. Values represent means ± s.e.m. of three independent experiments (**P*<0.05; ***P*<0.01; ****P*<0.001). (**C**) Microglia were treated for 8–12 hours with IFNγ before adding purified labelled exosomes to the cells for 2 hours. Microglia were fixed, permeabilised and stained for MHCII. Scale bar: 10 µm. Average fluorescence intensities were determined for exosomes (green) and MHC II (red) by image analysis from two independent experiments, as depicted in the graph.

of material. We found that activation of microglia by IFNγ or LPS resulted in a decrease of the macropinocytotic activity. One possible explanation of this finding is that the inflammatory activation converts a subpopulation of microglia to cells that are no longer active in macropinocytotosis. It is also feasible that the inflammatory activation reduces macropinocytosis in all microglia as it has been described for dendritic cells (Sallusto et al., 1995).

Traditional concepts of microglia are currently changing (Hanisch and Kettenmann, 2007). Recent in vivo time-lapse imaging of microglia especially challenged the view of ‘resting’ and ‘activated’ microglia (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Microglia are not ‘resting’ or functionally dormant, as previously thought. They exert a highly active scanning behaviour of their motile processes. One important function of these resting or surveying microglia is the patrol of brain parenchyma to detect invasion of pathogens and other foreign material (Hanisch and Kettenmann, 2007). Surveying microglia also have another important function. They remove damaged structures such as apoptotic cells and obsolete synaptic connections from the extracellular space (Kreutzberg, 1996). Whereas the uptake of foreign material leads to microglial activation to engage an immunological response, ingestion of apoptotic bodies is associated with an anti-inflammatory reaction (Magnus et al., 2001). By contrast, endogenous molecules derived from injured or necrotic cells serve as danger signals to microglia (Hanisch et al., 2008; Lehnardt et al., 2008). Phagocytosis of neuronal or glial debris by microglial cells results in upregulation of MHCII expression (Beyer et al., 2000). The basic concept is that a receptor–ligand interaction determines how microglia will respond. For example, when the families of pattern recognition receptors, such as the Toll-like receptors, detect foreign structures on pathogens downstream, pro-inflammatory cascades are triggered. Induction of a pro-inflammatory reaction can also be induced by serum components, such as immunoglobulin–antigen complexes or opsonising complement (Hanisch and Kettenmann, 2007).

However, there is an alternative model to explain the response of microglia. It is possible that subpopulations of microglia exist: one pool that is able to shift into a proinflammatory, activated state and another pool that lacks this ability. We found that stimulation by IFNγ triggers the expression of MHCII only in one fraction of microglia. Exosome internalisation preferentially associated with unresponsive microglia which presented with low levels of MHCII. We propose that the mode of uptake could represent a mechanism that determines the response of internalised material. It is possible that macropinocytosis is particularly active in cells with a low capacity to mediate immunological responses. Exosomes are well disposed to use such a pathway because they transport potential autoantigens. We do not know why oligodendrocytes release part of the membrane in form of exosomes, but we favour the idea that this is a convenient way to remove excess membrane. Exosomal release instead of lysosomal processing might be an advantage to cells that have poor degradative capacities or that are located towards a drainage system, such as the tubules of the kidney or the gut (Simons and Raposo, 2009). Oligodendrocytes are, in fact, cells with extremely high and efficient membrane synthesis capacity (Pfeiffer et al., 1993). Membrane turnover, however, might pose a problem to a cell that is able to form up to 50 different myelin internodes, but only contains one cell body where degradation usually occurs. It is possible that nature solved this problem by dividing these different tasks of myelin membrane synthesis and degradation into separate cell types. We suggest that microglia partially take care of the degradation of oligodendroglial membrane. This might occur by transferring membrane from oligodendrocytes in the form of exosomes or related vesicular carriers to microglia.

If such a process, indeed, constantly occurs, it is important to prevent an immune response in microglia. Recently, it has been proposed that macropinocytosis directs the incoming cargo away from compartments with pro-inflammatory receptors (Mercer and Helenius, 2009). We currently do not know whether the distinct microglial subpopulations are uniformly distributed throughout the CNS or preferentially recruited from, for example white versus grey matter. Within a culture, the anatomical origin of the cells cannot be identified. We will, therefore, address this question by using stable microglial cultures prepared from adult CNS regions with different white matter index.

The vaccinia virus might exploit this pathway by using phosphatidylserine at the outside of its membrane for the targeting to macropinosomes and to escape an immunological response (Mercer and Helenius, 2008).

Prevention of an unwanted immune reaction by maintaining macropinocytosis at a low level

Macropinocytosis is characterised by the constitutive or growth-factor-activated formation of membrane ruffles that form dynamic protrusions involved in the uptake of fluid and solid particles into large vacuoles. This pathway might be particularly prominent in resting or surveying microglia that contain highly motile filopodium-like protrusion in their processes to constantly scan the extracellular environment in the CNS. Consistent with a macropinocytic mechanism, we find that exosomes are taken up in an actin- and Rac1-GTPase-dependent manner. In addition, Na^+/H^+-exchanger function, dynamin and a low pH are required. It is interesting to compare the phagocytosis of myelin debris released from damaged oligodendrocytes with the uptake of exosomes. Several important differences can be noted. Myelin debris phagocytosis is particle-driven and depends on the tight interaction of different ligands with various receptors, such as the Fc receptors, complement receptor-3 or scavenger receptor-AI/II (Cohen et al., 2006; DeJong and Smith, 1997; Reichert and Rotshenker, 2003; Smith, 2001). In addition, LRPAP1 has been implicated in myelin uptake (Gaultier et al., 2009). Furthermore, myelin phagocytosis by microglia associates with a highly ordered secretion of a profile of cytokines and chemokines that might localise macrophages, neutrophils and Th2 cells (van Rossum et al., 2008). Suppression of proinflammatory activities in microglia has also been observed after myelin phagocytosis (Liu et al., 2006). Uptake of damaged myelin might induce signals that are required to promote tissue repair and remyelination in a pathological condition, whereas exosome uptake is likely to be part of a physiological process which takes place on a daily basis. Microglia could handle fibrillar Aβ peptide and soluble Aβ peptide in a similar fashion. Fibrillar Aβ formed in the course of Alzheimer's disease is taken up by phagocytosis via receptors that trigger the release of inflammatory factors (Floden and Combs, 2006; Koenigsknecht and Landreth, 2004; Liu et al., 2005), whereas soluble Aβ produced under physiological conditions is cleared by macropinocytosis and rapidly degraded (Mandrekar et al., 2009).

In DCs, the most professional of all antigen-presenting cells, macropinocytosis is also required to concentrate soluble and particular antigen in MHCII-positive compartments (Sallusto et al., 1995; Savina and Amigorena, 2007). In response to inflammatory stimuli or infectious agents, the stored peptide–MHCII complexes are transferred to the cell surface and presented to T cells (Cella et al., 1997; Pierre et al., 1997). Maturation induces major changes in the endosomal–lysosomal system of DCs (Garrett et al., 2000; West et al., 2000). Immature cells have a high capacity for antigen capture and storage, whereas mature cells are effective in antigen processing and presentation. Future cell-biological studies need to determine, whether microglia are able to undergo similar phenotypical changes. We propose that exosomes are preferentially internalised in microglia with a high degradative capacity to digest the lipid-rich membrane of oligodendrocytes. DCs, by contrast, seem to use exosomes mainly as a source of antigens for the presentation to T cells to mediate immune responses (Thery et al., 2009).

Importantly, cells infected by certain pathogens might secrete pathogen-derived components in association with exosomes. For example, macrophages infected with *Mycobacteria* incorporate some of their antigens into exosomes by which they are released into the extracellular space (Bhatnagar and Schorey, 2007). The same holds true for the cytomegalovirus and retrovirus (Booth et al., 2006; Gould et al., 2003; Walker et al., 2009). It is possible that exosomes containing viral or bacterial proteins are redirected to other cells and endocytic pathways. They could also have proinflammatory properties that could result in the activation of antigen-presenting cells. By such a mechanism, self-antigens might trigger an autoimmune response. In this respect, the association of MS with infectious agents is intriguing. Whether viral components do integrate into exosomes from oligodendrocytes – dragging along autoantigens – and how microglia respond to such particles needs to be evaluated in the future. Regardless of the direct link to infection, identification of extracellular carriers for candidate autoantigens, such as those involved in the pathogenesis of MS, opens new avenues to approach the questions of how myelin autoantigens are transferred to an immunogenic environment.

Materials and Methods

Materials

Antibodies against the following antigens were used: lysosomal-associated membrane protein 1 (Lamp-1, 1:50, CD 107a, rat monoclonal; BD Biosciences), Myc (1:2000, mouse; Cell Signaling), glial fibrillary acidic protein (GFAP, 1:200, rat polyclonal; Promega), β3-tubulin (1:1000, mouse monoclonal; Promega), ionised calcium binding adaptor molecule 1 (iba1, 1:200, rabbit polyclonal; Wako), galactosylceramide (GalC/O1, 1:50, mouse monoclonal), proteolipid protein (PLP/3F4, 1:200, mouse monoclonal), major histocompatibility complex 2 (MHC-2, 1:100, rat, clone M5/114.15.2), B7-1 (Pharmingen, 1:50, mouse monoclonal), B7-2 (Pharmingen, 1:15, mouse monoclonal). Secondary antibodies were purchased from Dianova (Hamburg, Germany). Rhodamine-conjugated wheatgerm agglutinin (WGA, 1:500) was used for general membrane staining. Annexin-V FITC and FITC-isolectin B4 were from Sigma.

The following inhibitors, sugars and peptides were used: bafilomycin A1 (Sigma), blebbistatin (Calbiochem), chloroquine diphosphate crystalline (Sigma), cytochalasin D from *Zygosporium manonsii* (Sigma), fucoidan from *Fucus vesiculosus* (FU, Sigma), N-acetyl-D-glucosamine (Sigma), D-mannosamine-hydrochloride (MA, Sigma), the peptides GRGDSP (RGD, Calbiochem), and GRADSP (RAD, Calbiochem), NSC23766 (Calbiochem), LDL-receptor-related protein-associated protein 1 (RAP/LRPAP1, recombinant human; Enzo life science), recombinant mouse interferon-γ from (IFNγ, Sigma), lipopolysaccharide (LPS, Sigma), FITC-dextran 40 kDa (Molecular Probes)

Cell culture

Primary oligodendrocytes were prepared as described previously (Trajkovic et al., 2006). After shaking oligodendrocytes from a mixed glial culture, cells were plated onto poly-L-lysine-coated coverslips or dishes and cultured in DMEM containing B27 supplement, 1% horse serum, L-thyroxine, tri-iodo-thyronine, glucose, glutamine, gentamycin, pyruvate and bicarbonate. The remaining cell monolayer was further incubated in DMEM with 10% FCS and 15% of MCSF-enriched cell culture supernatant from L929 cells. After 4 days, microglia were shaken off and plated in dishes or onto coverslips. Astrocyte cultures were prepared by trypsinisation of mixed glial cultures after oligodendrocytes or microglia were shaken off and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS).

For FACS and cytokine release assays, primary microglial cells were separated from the mixed glial culture by shaking and placed in 96- or 12-well plates at densities of 1.5×10 and 3.0×10^5 cells per cavity, respectively (van Rossum et al.,

2008). After a 30 minute attachment period, cells were extensively washed with DMEM containing 10% FCS and kept in culture for 1 to 3 days before being used. Cultures routinely consisted of >98% microglial cells as determined by staining with *Griffonia simplicifolia* isolectin B4 (Vector Laboratories, Burlingame, CA).

The oligodendroglial precursor cell line, Oli-neu (provided by Jacqueline Trotter, University of Mainz, Germany) was cultured as described previously (Trajkovic et al., 2006). The microglial cell line EOC-20 (provided by Hannelore Ehrenreich, MPI for Experimental Medicine, Göttingen, Germany) was cultured in DMEM with 10% FCS, 1% pyruvate, 1% L-glutamate and 100 ng/ml macrophage colony stimulating factor (MCSF, Sigma). L929 cells were cultured in DMEM with 10% FCS for 2 weeks. The supernatant was used for stimulation of microglial cultures.

Liposome preparation

Liposomes were prepared as described previously (Mui et al., 2003). For internalisation assays, the phospholipids L-α-phosphatidylcholine (PC) and L-α-phosphatidylserine (PS) (Avanti Polar Lipids) were either used separately or in a 50:50 ratio. Briefly, liposomes were prepared by resuspension of phospholipids in HBS (100 mM NaCl, 20 mM HEPES-NaOH buffer, pH 7.5). Subsequently, vesicles were repeatedly passed through a polycarbonate filter to obtain uniformly sized, unilamellar vesicles ~100 nm in diameter. For internalisation assays, exosomes from Oli-neu cells were mixed with liposomes containing either PC or PC-PS in a final concentration of 100 μM.

Immunofluorescence

Immunofluorescence and immunohistochemistry was performed as described previously (Trajkovic et al., 2006). Briefly, cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 in PBS and incubated in blocking solution for 30 minutes for subsequent staining. For WGA staining, cells were fixed, washed and directly incubated with WGA (1:500 in PBS) for 15 minutes at room temperature.

Exosome purification and labelling

Exosomes from Oli-neu cells and primary oligodendrocytes were prepared as described previously (Fevrier and Raposo, 2004). One day before preparation of exosomes, culture medium was replaced by serum-free medium. Cell culture supernatants were collected after 12–24 hours and centrifuged for 10 minutes at 3000 ***g***, twice for 10 minutes at 4000 ***g***, 30 minutes at 10,000 ***g*** and 1 hour at 100,000 ***g***. For sucrose gradient analysis, the exosome suspension was loaded onto a sucrose gradient (1.03–1.32g/cm^3 sucrose in 10 mM HEPES) and centrifuged for 16 hours at 200,000 ***g***. Subsequently, fractions were collected, diluted 1:3 in PBS and centrifuged at 100,000 ***g*** for 1 hour. Pellets were resuspended in PBS. Subsequently, exosome fractions were subjected to western blot and ECL. Western blots were quantified using imageJ.

For immunofluorescence analysis, the exosome pellet was resuspended in PBS and stained with PKH67 or PKH26, respectively (Sigma) (Morelli et al., 2004). The staining reaction was stopped after 5 minutes with exosome-free FCS. Exosomes were then washed in PBS and pelleted by ultracentrifugation (100,000 ***g***, 1 hour). Alternatively, exosomes were labelled by crosslinking TAMRA-NHS (Carboxytetramethylrhodamine succinimidyl ester, Biotium) to exosomal proteins as described previously (Tian et al., 2010).

Internalisation assay

Glial or neuronal cells were plated on coverslips and incubated for 12 hours in the respective growth medium. Before the uptake assay, exosomes were purified, labelled and diluted in growth medium. The uptake was performed by incubating cell cultures with exosome dilutions in a humid chamber for 2 hours (37°C, 7% CO_2). For inhibition experiments, cell cultures were pre-incubated with the inhibitor for 30 minutes before performing the uptake in its further presence.

For live imaging of exosome uptake, coverslips containing EOC-20 cells were placed in a tempered live cell imaging chamber (The cube/The box, Live Imaging Services) and observed under imaging medium containing PKH26-labelled exosomes (HBSS, 10 mM HEPES, 1% horse serum, pH 7.4, 37°C) using a Leica confocal microscope DMIRE2. Images were required at ~2 second intervals using sequential line excitation at 488 and 543 nm and appropriate band pass emission filters. The pinhole was set to 1.12 airy units.

Microscopy and image analysis

Confocal images were acquired on a Leica (Nussloch, Germany) DMIRE2 microscope or a Zeiss (Jena, Germany) LSM 510 confocal microscope with a 40× or a 63× oil-immersed objective. Wide-field images were acquired on a Leica DMRXA (Nussloch, Germany) microscope, with a 40× oil objective.

For internalisation assays, 10–20 viewing field images of each sample were randomly captured. Integrated fluorescence intensity was determined with a module of Meta Imaging Series 6.1 software (Universal Imaging Corporation, West Chester, PA). A fixed threshold was used to eliminate the low-intensity background signal in both channels. Integrated fluorescence intensity of exosome signals was normalised with the respective integrated WGA surface stain intensity. For correlation of MHC class II staining with exosome uptake, intensities of single cells were measured and compared. Data presented in the figures were acquired in at least two independent experiments. Normal distribution of data was analysed with Kolmogorov–Smirnov test and significance was tested using Student's *t*-test or Mann–Whitney U-test, respectively.

Electron microscopy

Ultrathin sections were cut using a Leica Ultracut S ultramicrotome (Leica) and stained with aqueous 4% uranylacetate, followed by lead citrate. Glutaraldehyde-fixed exosomes were stained with annexin-V (Sigma) and labelled with immuno gold. Observations were made with an electron microscope (Leo EM912AB; Zeiss), and images were acquired using an on-axis 2048×2048 charge-coupled device camera (Proscan; Schering).

Cytokine release assays

For release studies on homogeneous cultures, microglia were plated in 96-well plates. After 1 day, cells received either fresh medium (basal release values) or medium containing exosomes alone or in combination with LPS (at 1 ng/ml, *E. coli* R515) TLR ligand set 1 (APO-54N-018; Axxora/Appotech). Cells were incubated for 18 hours and supernatants analysed. Cells were rinsed and given new medium with WST-1 agent for viability assay (Roche Diagnostics). Measurements of cytokines and chemokines in culture supernatants were performed with factor- and species-specific complete ELISA systems or sets of capture and detecting antibody pairs as well as standard proteins (R&D Systems, Biolegend) as described earlier (van Rossum et al., 2008). The colour reaction was measured at 450 nm on a microplate reader (Bio-Rad, Munich, Germany). Adaptations were made to reduce sample size and assay volume. Factors included TNFα, IL-6, IL-10, IL-12p40 (covering the monomeric, homodimeric and heterodimeric versions, including IL-12p70 and IL-23), CXCL1 (KC, the mouse equivalent of GROα), CCL2 (MCP1), CCL3 (MIP-1α), CCL5 (RANTES) and CCL22 (MDC).

Fluorescence-activated cell sorting

For FACS analysis, microglia were placed in 12-well plates. After 1 day, the cells received either new medium, medium containing exosomes in various dilutions and/or IFNγ (mouse recombinant, R&D 485-MI/CF) at a final concentration of 10 ng/ml. After 2 days of incubation, cells were stained with MHC class I (KH 114 Alexa Fluor 647) or MHC class II (KH116 Alexa Fluor 647) antibodies, processed for FACS analysis (FACSCalibur, Becton Dickinson, Germany), and analysed with WinMDI software (Purdue University, West Lafayette, IN). For FACS analysis, exosomes were bound to the surface of beads (aldehyde-sulfate latex, 4% w/v, 4 μm; Invitrogen) (Ostrowski et al., 2010). Briefly, exosomes and beads were incubated overnight, washed and blocked with BSA in PBS and subsequently transferred to annexin-V binding buffer. Loaded beads were incubated for 15 minutes with FITC-labelled annexin-V (1:20) and washed four times in annexin-V binding buffer. Beads were acquired on a FACSCalibur (BD).

T cell activation assay

In co-culture experiments, indicated numbers (5×10^4, 1×10^5 or 2×10^5/well) of primary microglia isolated from brains of C57BL/6 mice were plated in 96-well round-bottom plates. Cells were stimulated overnight with IFNγ (10 ng/ml), or left without stimulation, and then gently washed twice with DMEM containing 10% FCS. Myelin oligodendrocyte glycoprotein (MOG)-specific CD4$^+$ T cells were isolated from TCR-transgenic 2D2 mice (Bettelli et al., 2003) by negative selection using magnetic beads (R&D Systems) as previously described (Krishnamoorthy et al., 2006). 1×10^5 purified CD4$^+$ T cells were mixed with microglial cells and recombinant MOG protein (rMOG), MOG peptide 35–55 (MOG35–55), recombinant ovalbumin, ovalbumin peptide 323–339 (ova323–339) or exosomes were added at indicated concentrations. Three days after stimulation, supernatants were collected and to determine IL-2 concentrations by ELISA. IL-2 concentration was determined using a set of capture and detection antibodies against IL-2 (BD Biosciences) as well as purified standard cytokine. 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS; Sigma) was used as a substrate for detection and plates were read at 405 nm.

In vivo imaging of microglia

In vivo experiments were carried out with CX3CR1/EGFP-transgenic mice (Nimmerjahn et al., 2005) at ages between 70 days and 120 days. General anaesthesia was initiated by pentobarbital sodium i.p. and continued with methohexital sodium (i.v.). Body temperature and electrocardiogram were monitored throughout the experiment.

Spinal cord segments L4 and L5 were exposed by laminectomy of L1 and L2 spines for imaging of the dorsal white matter. To avoid movement caused by active respiration, the mice were paralysed with pancuronium bromide. The spinal column was rigidly fixed by custom-made clamps and the dura mater was carefully removed. A region of interest was selected and a microcapillary positioned. With a delay of 30–60 minutes, PKH26-labelled exosomes derived from 1.5×10^5 Oli-neu cells were injected into the respective area.

In vivo imaging was performed by a custom-made 2P-LSM equipped with a fs-pulsed titanium-sapphire laser (Chameleon Ultra II; Coherent, Glasgow, UK) and a Zeiss 20×/1.0 water immersion objective. Uniformly spaced (2 μm) planes of 250×250 to 500×500 μm regions of the spinal dorsal columns were recorded and processed to obtain *z*-stacks of images (512×512 pixels in size). Image processing

was performed using Matlab (version 7, MathWorks, Ismaning, Germany) and ImageJ (http://rsbweb.nih.gov/ij).

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