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ORIGINAL PAPER

Spreading of prions from the immune to the peripheral nervous system: a potential implication of dendritic cells

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Abstract The implication of dendritic cells (DCs) in the peripheral spreading of prions has increased in the last few years. It has been recently described that DCs can transmit prions to primary neurons from the central nervous system. In order to improve the understanding of the earliest steps of prion peripheral neuroinvasion, we studied, using an in vitro model, the effect of exposing primary peripheral neurons to scrapie-infected lymphoid cells. Thanks to this system, there is evidence that bone marrow dendritic cells (BMDCs) are in connection with neurites of peripheral neurons via cytoplasmic extensions. BMDCs are competent to internalize prions independently from the expression of cellular prion protein (PrP^{C}) and have the capacity to transmit detergent-insoluble, relatively proteinase K-resistant prion protein (PrP^{Sc}) to peripheral neurons after 96 h of coculture. Furthermore, we confirmed the special status of the peripheral nervous system in front of prion diseases. Contrary to central neurons, PrP^{Sc} infection does not disturb survival and neurite outgrowth. Our model demonstrates that PrP^{Sc}-loaded dendritic cells and peripheral nerve fibers that are included in neuroimmune interfaces can initiate and spread prion neuroinvasion.

Keywords Prions · Dendritic cells · Peripheral nervous system · Neuroinvasion

Introduction

Prion leads to severe neurodegenerative diseases in a large number of animals as well as in humans. At the biochemical level, these diseases are characterized by the conversion of a normal cellular prion protein, PrP^{C}, into an abnormal isoform, PrP^{Sc}, which is enriched by β-structures and is partially resistant to proteinase K (Cohen and Prusiner 1998). Prion-affected nervous tissues show accumulation of PrP^{Sc}, neuronal vacuolization and cell death in the central nervous system (CNS) (Bruce et al. 1989).

Following peripheral infection in various animal models, the sequential detection of abnormal prion protein in lymphoreticular tissues and subsequently along neural projections to the CNS has led to the conclusion that these tissues are implicated in the spread of prion agent to the CNS (Kratzel et al. 2007).

In natural and experimental diseases, the first neural tissues in which PrP^{Sc} can be highlighted both in sheep and mice scrapie, cattle bovine spongiform encephalopathy (BSE) and deer chronic wasting disease (CWD) is the enteric nervous system (ENS) of the gut (Andreoletti et al. 2000; van Keulen et al. 2000; Sigurdson et al. 2001; Iwata et al. 2006). This intramural nervous system is composed of two networks, the submucosal and the myenteric plexi,

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which contain the neuronal cell bodies and their processes. It is likely that prion infection of the ENS occurs within fine nerve fibers directly underneath the villous or crypts epithelium (Jeffrey et al. 2006). It is also possible that transmissible spongiform encephalopathy (TSE) agents are transported through the epithelium of the Peyer's patches (Defaweux et al. 2005). Then, prions migrate asymptomatically along the peripheral nervous system (PNS), by axonal anterograde and retrograde transport mechanisms via the vagus and splanchnic nerve (van Keulen et al. 2002), and reach the CNS, where they induce the clinical signs of the disease.

Up to now, the mechanism responsible for ENS infection has not been defined yet. Recent studies have pointed out the potential implication of dendritic cells, a highly migrating cell type localized in the gut lamina propria and mesenteric lymphoid tissues, in the uptake of PrP^{Sc} (Kelsall and Rescigno 2004). The role of DCs in prion disease appears to be ambiguous. Some authors have shown, through in vitro models, that DCs can impair prion dissemination and that they are able to process PrP^{Sc}, by cysteine protease, and to decrease the amount of infectivity associated with scrapie (Luhr et al. 2002, 2004) or BSE (Rybner-Barnier et al. 2006). Several studies have demonstrated that DCs act in the spreading of prions. They appear to be implicated in the uptake of PrP^{Sc}. Peyer's patches dendritic cells of the suprafollicular dome seem capable of capturing pathogenic prions, especially the CD11c/CD11b DC subset (Dorban et al. 2007b), either by direct uptake across the follicular-associated epithelium, or after that PrP^{Sc} has been internalized by M cells (Heppner et al. 2001; Huang et al. 2002).

The DCs are also implicated in the neuroinvasion process. The first convincing evidence appeared in a study stressing that PrP^{Sc}-loaded DCs isolated from infected mice are able, and sufficient, to induce scrapie in Rag mice, without any accumulation of prions in the spleen, after an intraperitoneal challenge (Aucouturier et al. 2001). Moreover, DCs seem to disseminate PrP^{Sc} to peripheral sites of neuroinvasion such as mesenteric lymphoid tissues (Huang and MacPherson 2004) and the spleen (Blattler et al. 1997). Recent studies have demonstrated that the temporary depletion of dendritic cell impaired neuroinvasion following oral inoculation (Raymond et al. 2007) and intraperitoneal infection (Cordier-Dirikoc and Chabry 2008). An up-to-date in vitro study has shown that infected dendritic cells are able to transmit prions to primary neurons from the CNS by the intermediary of tunneling nanotubes (TNT) (Gousset et al. 2009). Previously we have also demonstrated that CD11c-DCs established a lot of contacts with the nerve fibers inside the mouse Peyer's patches in physiological conditions and following a scrapie challenge (Defaweux et al. 2005; Dorban et al. 2007a). Similar results have been obtained within sheep Peyer's patches (Marruchella et al. 2007; Chiocchetti et al. 2008). The molecular basis of these neuroimmune interfaces between DCs and PNS could implicate a ligand-receptor connection via the muscarinic acetylcholine M2 receptor (Ma et al. 2007) or involve TNT (Gousset et al. 2009).

The aim of this study, using an infectious coculture system, focused on the ability of dendritic cells and autonomic neurons to accumulate prions and the impact of prion infection on the development of neurites by primary neurons isolated from the PNS. We also investigated the capacity of BMDCs and peripheral nerve fibers to establish cell-to-cell contacts and the proficiency of dendritic cells in transmitting PrP^{Sc} to the PNS. These findings give an insight concerning the implication of DCs in the earliest steps of prion neuroinvasion.

Materials and methods

Generation of bone marrow dendritic cells (BMDCs)

DCs were generated from BMDCs isolated from C57Bl/6 mice or $PrP^{-/-}$ mice, as previously described (Berthier et al. 2000). In short, bone marrow (BM) cells were isolated by flushing cells from the mouse femurs. Erythrocyte cells and $Gr1^{+}$ cells were removed by magnetic cell sorting. Negatively selected cells were cultured at 5×10^5 cells/mL in complete Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with 1% of the GM-CSF-transfected J558 cell line supernatant, 40 ng/mL recombinant Flt3-lg, and 5 ng/mL mouse recombinant IL-6. 5×10^5 cells/mL were resuspended in complete IMDM every 3 days. By day 6, IL-6 was removed and Flt3-lg was used at 20 ng/mL. By day 8–9 of culture, 90% of cells demonstrated characteristic DC morphology and phenotype. BMDCs were exploitable from day 9 of the primo-culture up to day 18. During this period of time, the culture was mainly made up of immature BMDCs with the phenotype of differentiated DCs representative of the in vivo population. Cells were deposited on coverslips coated with 50 µL (0.1 mg/mL) of PORN (Sigma) at 37°C for 45 min and were rinsed twice before introducing 50 µL (10 µg/mL) of laminin (ICN Biomedicals) at 4°C for 24 h. The development of contacts between BMDCs cultured on coverslips was checked twice a day from the 6th to the 96th hour.

Isolation of peripheral neurons

Vertebral columns of C57Bl/6 mice were dissected and the dorsal root ganglia (DRGs) were further processed, as described below, to dissociate sensory neurons. Spines were sliced and fragments were conserved in PBS-glucose (1.5 mL at 30% with 100 mL of sterile-PBS). Nervous ganglia were dissected twice under sterile conditions and incubated

with 1 mL (5 mg/mL) of collagenase A (Roche) at 37°C for 45 min. 1 mL of trypsin (Bio-Whittaker) at 0.25% was added for 30 min. Enzymatic digestion was stopped with 500 µL of FCS (Perbio Hyclone) and cells were centrifuged at 755×*g* for 5 min. The supernatant was removed and the pellet was suspended in a *neuron complete medium* containing: RPMI 1640 + L-glutamine (Gibco), 1% Na pyruvate (Cambrex), 15 µL/mL of glucose 30% (Merck), 2.5 µL/mL of insulin (Sigma) and 5 µL/mL of N2 (Invitrogen). After two successive mechanical dissociations using glass Pasteur pipettes, suspended cells were introduced into an equal quantity of Percoll (Sigma) at 26% and were centrifuged at 2,096×*g* for 12 min. The cell pellet was diluted in 500 µL of medium and conserved at 4°C. The growth of neurites from peripheral neurons cultured on coated coverslips was checked twice a day from the 6th to the 96th hour.

Coculture of BMDCs with neurons

The number of each cell type, introduced into the coculture, was calculated to obtain the morphology closest to the in vivo situation. Thus, on each coverslip, 200 neurons and 10,000 BMDCs were cultured in 50 µL of medium at 37°C for 60 min. Then 400 µL of *the coculture complete medium*, based on the specific medium of BMDCs and enriched with nutrients essential for neurons (91.4% of IMDM (Gibco), 6% of SVF, 1% of NEAA, 1% of Na pyruvate, 0.1% of β-mercaptoethanol, 0.5% of penicillin–streptomycin, 60 µL/mL of GM-CSF, 20 ng/mL of Flt3-lg, 15 µL/mL of glucose 30%, 2.5 µL/mL of insulin at 500 µg/mL and 5 µL/mL of N2), were added and the incubation was prolonged for 48 or 96 h. The development of neurons-BMDCs interfaces was checked twice a day from the 6th to the 96th hour. Cocultures on coverslips were prolonged for at least 48 h to allow for a sufficient growth of neurites necessary for the installation of neuroimmune interfaces. At the end of the coculture, BMDCs were separated from neurons by a treatment of 0.2% EDTA (Sigma) at 37°C for 10 min. Finally, coverslips were rinsed three times in PBS, air-dried, fixed in acetone at 4°C for 10 min, and stored at −20°C until use.

Infection of cells in culture

Scrapie-infected tissues were dissected from the brain of C57Bl/6 mice, which had been inoculated with the scrapie strain ME7, and which had developed the clinical signs of the disease. Phosphate buffer (Lonza) and phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma) at 10 mM were added to obtain 10% w/v homogenate. An equal volume of sterile PBS with 4% sarkosyl (Fluka) was added before incubation under agitation at 37°C for 10 min; 50 U/mL of Benzonase (Sigma) was introduced into the homogenate followed by incubation at 37°C for 30 min. Preheated Phosphotungstic acid at 4% (diluted in H_2O with 170 mM of $MgCl_2$) was added to obtain a final solution at 0.3%. The homogenate was incubated at 37°C for 30 min. Samples were centrifuged at 656×*g* for 30 min. The PrP^C-containing supernatant was removed and the pellet containing PrP^{Sc} was suspended in PBS-0.1% sarkosyl. Homogenates (ME7-PTA) were kept at −80°C before utilization. Neurons, BMDCs, lymphocytes and fibroblasts were incubated with ME7-PTA, corresponding to 500 µg of homogenate/mL, for 72 h, and were then rinsed three times and centrifuged at 480×*g* for 3 min. Cells, in culture or in coculture inoculated with ME7-PTA, were controlled after 6, 12, 24, 48 and 96 h for PrP^{Sc} expression, cell-to-cell interactions and survival.

Immunofluorescence

After re-hydration, cultures on coverslips were incubated with a range of primary antibodies directed against DCs and neurons. These antibodies were directly bound to a fluorochrome or were revealed with a species-specific secondary antibody bearing a fluorescent label. Incubation with antibodies was performed at room temperature in the dark for 1 h. DCs were detected with an anti-CD11c (1/400) (eBioscience) antibody directly labeled with fluorescein isothiocyanate (FITC) or with an anti-MHCII (1/100) (Chemicon) labeled with FITC or with an antibody directed against CD1d (1/1,500) (eBioscience) coupled with biotin. Neurons and nerve fibers were labeled with an antibody directed against the intermediate neurofilaments: rabbit anti-mouse NF M 1/800 (200 kDa) (Chemicon). Prion proteins were detected with SAF 32 antibody (1/100) (J. Grassi). Nuclei were stained with ToPro3 (1/100 Invitrogen), a monomeric cyanine stain which allows ultrasensitive detection of double-stranded nucleic acids.

To test the general specificity of the antibodies used, samples were incubated with irrelevant antibodies, conjugated with fluorescein (FITC) and tetramethylrhodamine (TRITC). Negative controls were obtained by incubating samples with only secondary antibodies.

All samples were observed with a Leica SP2 confocal microscope. Cocultures were scanned on their best fluorescent zone (5–7 µm), which was divided into 10–15 sections. Each section was analyzed. Virtual colors were attributed for detection channels (green for immune cells and red for nerve fibers) and the merge (yellow). Two analytical methods were used to highlight neuroimmune interfaces: image analysis and spectral analysis of the color channels. These combined approaches allowed us to confirm contacts.

Neurites outgrowth quantification

The quantification of neurites outgrowth was evaluated with the *neurites outgrowth quantification assay kit* (Chemicon).

Prior to initiation of neurites outgrowth the inserts were coated with 10 µg/mL of laminin (ICN Biomedicals) at 4°C for 24 h. After removing, without rinsing, inserts were placed into well containing 200 µL of neurons-complete medium enriched with or without ME7-PTA, corresponding to a concentration of 500 µg of homogenate/mL. Freshly isolated peripheral neurons were suspended in the insert. Following a neurites extension period of 96 h, inserts were removed, gently rinsed in excess PBS and fixed in cold acetone at 4°C for 10 min. Neurites were labeled with a rabbit anti-mouse NF M 1/800 (200 kDa) (Chemicon) revealed with an anti-rabbit Alexa 488 1/6,000 (Invitrogen). Standardized pictures (8.1×10^3 µm² at a resolution of 1,024 × 1,024 pixels) of neurites (virtual color: green) in each condition, obtained with a TCS SP2 confocal microscope, were treated by an image analyzer (QWIN, Leica) following this method: [number of green pixels]/[1,024² pixels]. Values obtained with control medium and infected medium were compared with a parametric *t* test as implemented in the Statistica 8.0 software. Statistical significance was fixed at $p < 0.05$. The experiment was repeated four times.

Electron microscopy

Cell pellets were incubated for 1 h at 4°C, with primary SAF 32 (1/100) antibody diluted in phosphate buffer saline (PBS) and then washed in PBS. The cells were then incubated for 1 h in a biotinylated anti-mouse antibody (1/1,000). The secondary antibody was revealed for 1 h at 4°C by a streptavidin–gold (10 nm gold particles, Aurion) complex diluted 1/10 in PBS. Following a wash in PBS, cells were fixed for 1 h at 4°C in a glutaraldehyde solution (2.5%) in 0.1 M cacodylate buffer (pH 7.4) buffer and then washed in the same buffer. The specimens were postfixed for 1 h in a mixture of 1% OsO_4 and 1.5% $K_4Fe(CN)_6$ in 0.1 M cacodylate buffer (pH 7.4), dehydrated in a graded series of alcohols and embedded in Epon 812 (Fluka, Belgium). The resin specimen blocks were trimmed, and 70- to 90-nm sections of selected area were cut on an ultramicrotome (Reichert, Ultracut E; Leica). Ultrathin sections were collected on 6,200 copper grids, stained with uranyl acetate solution in advance, with a Zeiss EM910 transmission electron microscope at 80 kV.

Immunoblotting

Tissue and cell homogenates (10%, w/v) were prepared on ice in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, 0.1 mM PMSF) at pH 7.4. Samples were treated at 37°C with Proteinase K (20 units/mg, Roche Diagnostics), using various concentration combinations for 1 h (concentration range for PK, 20–150 g/mL). PK stock solutions (10 mg/mL or higher) were prepared in storage buffer (50% glycerol, 10 mM Tris, pH 7.5, 2.9 mg/mL $CaCl_2$). Small aliquots were prepared and stored at −20°C. Protease digestion was terminated by the addition of 2 mM PMSF.

10% tissue/cell homogenates were suspended in sample buffer (final concentration: 3% SDS, 4% β-mercaptoethanol, 10% glycerol, 2 mM EDTA, 62.5 mM Tris, pH 6.8) and boiled for 8 min before loading. Protein samples, concentrated twice for immune cells, were separated within 12 or 15% SDS-polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) by a gel electrophoresis apparatus holding running minigels (5.5 cm) (Bio-Rad). Proteins were transferred to Immobilon P (Millipore) at 65 V for 2 h, blocked with 10% non-fat milk in Tween Tris-buffered saline, pH 7.5, and probed with the appropriate antibody. The monoclonal antibody SAF 32 (1:1,500) was used as the primary antibody. Immunoreactivity was visualized by enhanced chemiluminescence (ECL advance Bio-Rad) using the Bio-Rad ChemiDoc EQ system.

Results

Peripheral neurons and dendritic cells are connected by cytoplasmic extensions

We found that BMDCs in culture readily interact by the use of very thin cytoplasmic projections. These experiments indicate that large numbers of observed DCs could efficiently form membrane bridges of different lengths and diameters. Dendritic cells are connected by one to several membrane bridges containing MHCII molecules (Fig. 1) and actin-f (data not shown). In order to analyze the presence of in vitro neuroimmune interfaces, involving BMDCs and peripheral nerve fibers, cocultures were immunolabeled with an anti-CD11c-FITC and an antibody directed against neurofilament M subunit (NF M) coupled with TRITC. By confocal analysis, we observed numerous interfaces between DCs and nerve fibers or perikaryon (Fig. 2), subsequently confirmed by spectral analysis. In the coculture of at least 2 days, we observed cytoplasmic bridges between the two cell types. Further labeling with anti-MHCII-FITC antibody demonstrated that BMDCs and peripheral neurons are connected by extensions arising from dendritic cells (Fig. 3).

Dendritic cells and peripheral neurons express cellular PrP in culture

To confirm that both BMDCs and peripheral nerve cells conserve the same phenotype of PrP^C expression in our model as in lymphoid organs and in order to verify that isolation from their microenvironment does not alter their PrP^C

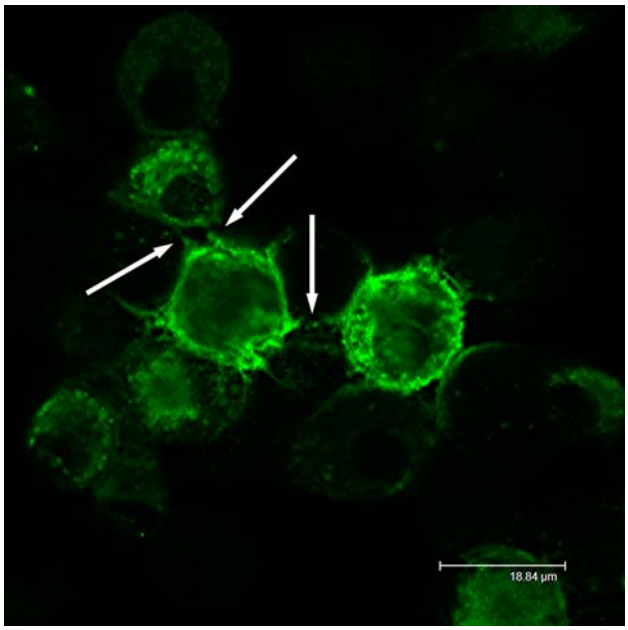

Fig. 1 Cell-to-cell connections between dendritic cells via cytoplasmic extensions. Confocal analysis and Z-stack snapshot of a network of cellular bridges in BMDCs labeled with anti-MHCII antibody coupled with FITC. The confocal analysis performed with Leica confocal software showing that the tubes are situated at different depths

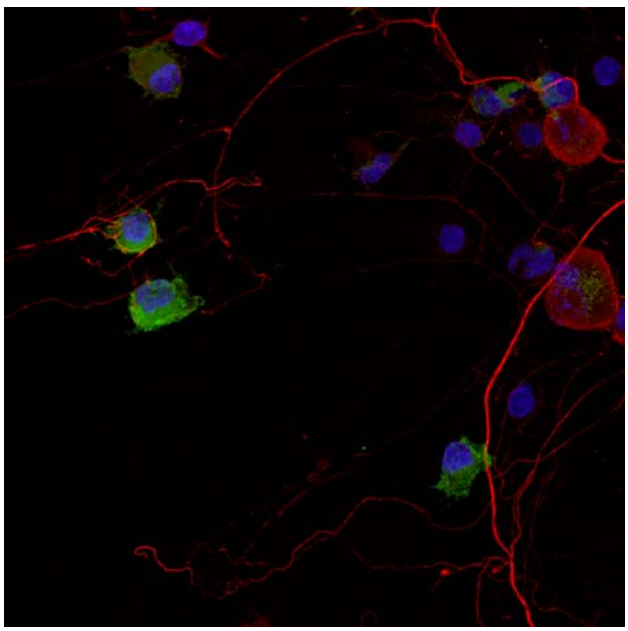

Fig. 2 Coculture of dendritic cells and peripheral neurons incubated for 48 h. A triple immunolabeling analyzed by confocal microscopy allowed to identify cell nuclei in *blue* (labeled with ToPro-Alexa 633®), BMDCs in *green* (labeled with anti-CD11c-FITC) and primary neurons in *red* (labeled with anti-NF M). Cytoplasmic connections between dendritic cells and peripheral nerve fibers appear in *yellow*. The image results from the overlapping of a series of 15 three-channel photographs

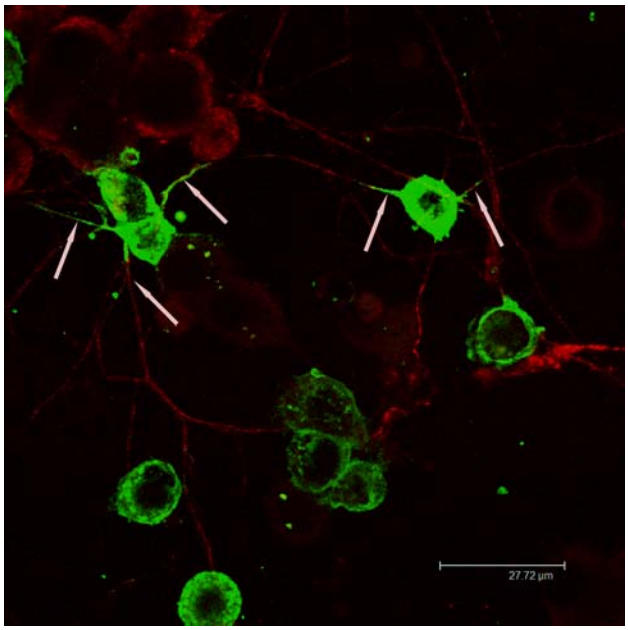

Fig. 3 Cell-to-cell connections between BMDCs and peripheral neurons via cytoplasmic extensions. Confocal analysis and Z-stack snapshot of a network of cellular bridges between BMDCs in *green* (MHCII) and primary peripheral neurons in *red* (NF M). Each dendritic cell emits several variable-sized extensions which connect neurites (*arrows*). The image results from the merging of a series of 12 two-channel photographs

expression, we studied the PrP^C profile of the two cell types. The distribution of cytoplasmic and anchored PrP^C was analyzed through immunofluorescence. Expression of cellular prion protein and MHCII molecules appeared simultaneously in BMDCs (Fig. 4). It suggests that specifically maturing DCs express PrP^C, as demonstrated in vivo (Martinez del et al. 2006; Dorban et al. 2007b). Freshly isolated peripheral neurons cultured for 48 h expressed PrP^C within the perikaryon and neurites (Fig. 5). Membrane expression vanished during the enzymatic digestion and was recovered after the adherence on coverslip. Neurite outgrowth and PrP^C expression within nervous extension appeared at once. The pattern of PrP^C glycosylation expressed by cultured dendritic cells and peripheral neurons was studied through immunoblotting analysis. The BMDCs mainly showed a pattern of PrP^C glycosylation with a monoglycosylated band, while peripheral neurons mostly expressed a diglycosylated PrP^C. Finally, the analysis of cell homogenates, containing the same amount of protein (25 mg/mL), showed that the level of PrP^C expression in peripheral neurons is higher than in BMDCs (Fig. 6).

Detection of PrP^{Sc} in infected BMDCs and peripheral neurons in culture

Furthermore, to determine whether DCs and neurons could acquire and retain prions, an ME7-PTA sample was

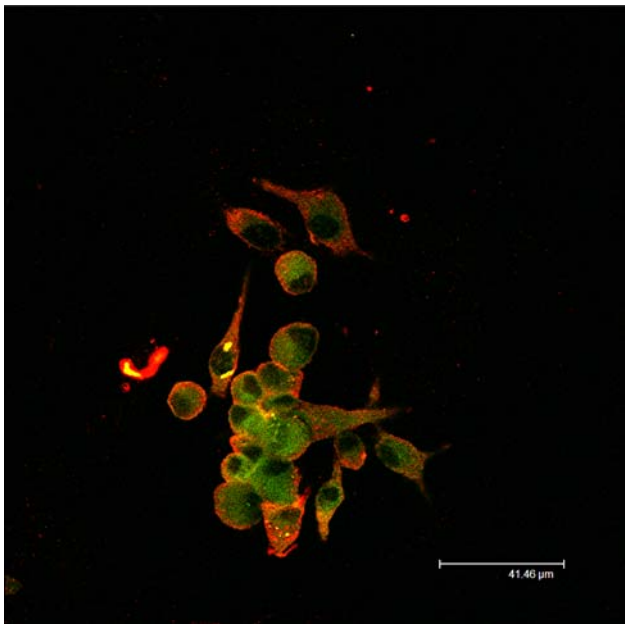

Fig. 4 Double immunolabeling of dendritic cells differentiated from bone marrow precursors with GM-CSF and Flt3-lg on the 11th day of the primo-culture. SAF 32 antibody directed against PrP^{C} (*red*) and anti-MHCII antibody (*green*) were used

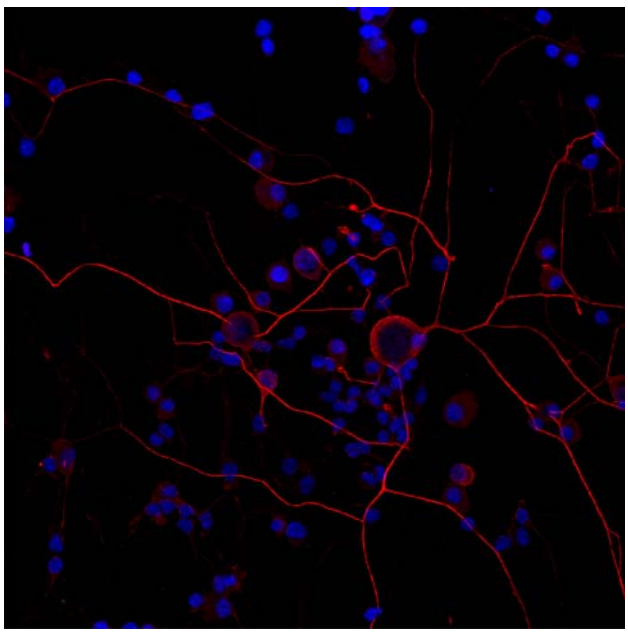

Fig. 5 Immunolabeling of neurons isolated from dorsal root ganglia and cultured on coverslip for 48 h. PrP^{C} (*red*), expressed in the perikaryon and in the neurites, was labeled with the SAF 32 antibody. Cells nuclei were stained with ToPro-Alexa 633® (*blue*)

incubated with each cell type. Detergent-insoluble, relatively proteinase K-resistant PrP^{Sc} accumulations were detected in BMDCs and peripheral neurons lysates a few hours after the culture with the scrapie agent. Peripheral neurons were cultured for 48 h in the prions-free medium before the introduction of the scrapie sample. The detection of PrP^{Sc} in neuron lysates showed no fluctuation up to 72 h of incubation (Fig. 7). Prions were also introduced in BMDC culture on day 9 of the primo-culture. Conversely to peripheral neurons, the level of PrP^{Sc} detected in BMDC homogenates showed fluctuation between 24 and 48 h of culture: before 24 h of culture with ME7-PTA, the level of PrP^{Sc} detected declined moderately and then increased from 24 to 48 h. Stability reached from the 48th hour (Fig. 8). No PrP^{Sc} accumulations were detected at any time in lysates from BMDCs and neurons treated with uninfected brain-PTA. Neither was uptake identified when lymphocytes (freshly isolated from murine spleen) or fibroblasts (3T3 cell line) were incubated with prions in vitro (Fig. 9).

Influence of PrP^{Sc} upon neurites outgrowth

Given the suspected ability of PrP^{Sc} and its peptides to influence the survival and the homeostasis of central neurons, the effect of prions was evaluated on their capacity of disturbing neurites outgrowth in a specific assay. The measure of axon and dendrites network constitutes a simple and consistent parameter to evaluate the health of the culture. Consequently, primary neurons isolated from the PNS were cultured within insert for 96 h and the development of neurites was continually observed. Adherence of the cells on coverslip started at the same time for both conditions and the growth of neurites was not delayed in the scrapie-infected medium. It stood out from the morphometry that no neurotoxicity or neurites outgrowth disorder was observed after comparing the culture of peripheral neurons with or without PrP^{Sc} (Fig. 10). The absence of a negative effect of prions upon the neurites network is in accordance with in situ observations made on different species (Dorban et al. 2007a; Marruchella et al. 2009).

Transmission of prions from infected BMDCs to neurons

To examine the capacity of PrP^{Sc}-BMDCs to transmit prions, scrapie-infected BMDCs and peripheral neurons were cocultured for a period of 48 to 96 h. The medium was replaced twice a day to avoid prion contamination by apoptotic BMDCs or by exosomes release. Aiming at obtaining a PrP^{Sc}-specific signal from the peripheral neurons, BMDCs and nervous cells were separated at the end of the coculture. The efficiency of the partition was confirmed by subsequent immunoblotting analysis under each cell homogenate, where the DC population expressed CD1d but not NF M. By contrast, neurons expressed NF M but no CD1d (data not shown). After 48 h of coculture with PrP^{Sc}-loaded BMDCs, peripheral neurons pretreated with PK were analyzed by Western blotting. Although the results showed that PrP^{C} was successfully eliminated, PrP^{Sc} remained undetectable

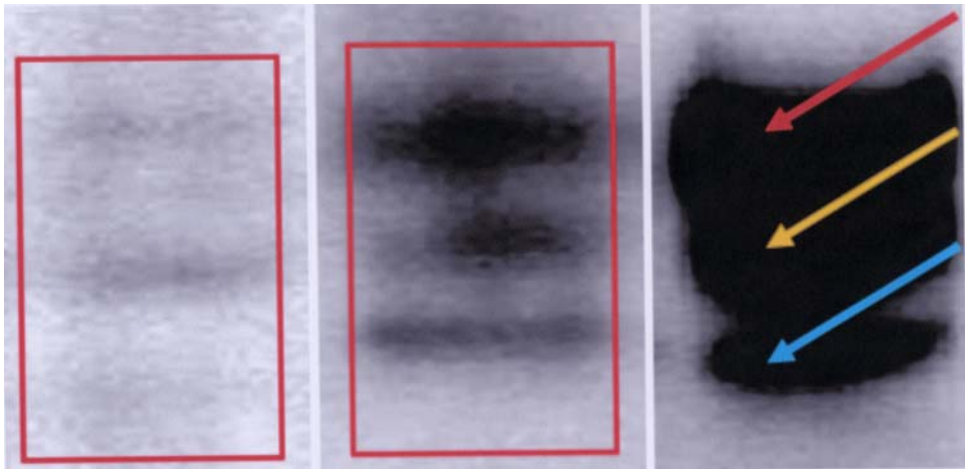

Fig. 6 Immunoblotting analysis of cellular prion protein expression by bone marrow dendritic cells, peripheral neurons and brain. PrP^C from murine cell and tissue samples (25 mg/mL) were revealed with SAF antibody. *Left* BMDCs homogenate; *center* peripheral neurons homogenate and *right* brain homogenate. *Red arrow* diglycosylated PrP^C, *yellow arrow* monoglycosylated PrP^C and *blue arrow* unglycosylated PrP^C

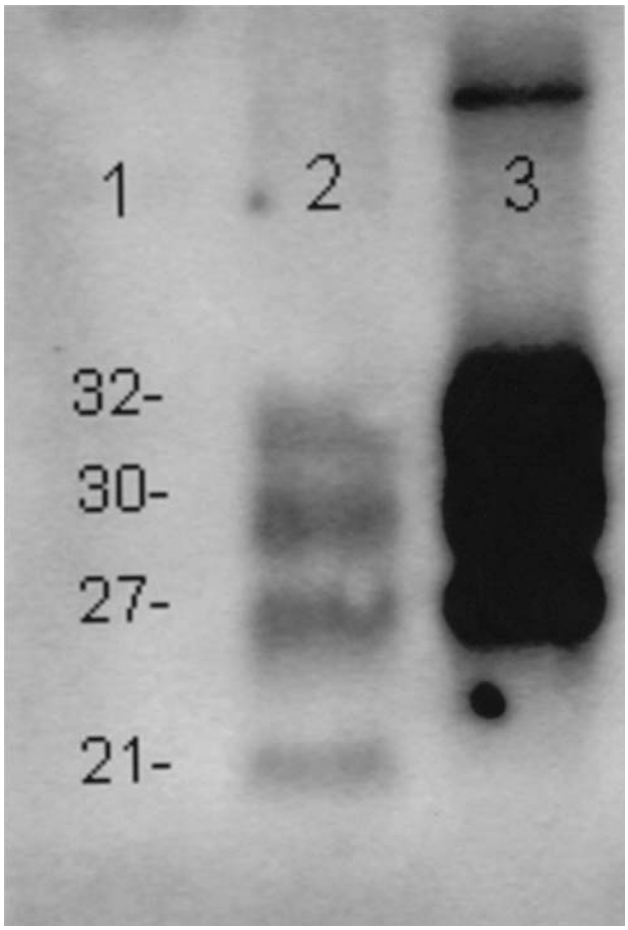

Fig. 7 Proteinase K-resistant PrP accumulation in peripheral neurons incubated with ME7-PTA for 72 h. Peripheral neuron samples were analyzed by immunoblotting and the membrane was revealed with SAF antibody. Homogenate in *lane 2* was treated with proteinase K and showed a typical PrP^{Sc}-band at 21 kDa. Untreated homogenate in *lane 3* exhibited only the three bands at 32, 30 and 27 kDa. *1* molecular weight in kDa, *2* $Neurons^+$ PK^+ and *3* $Neurons^+$ PK^-

(Fig. 11). It suggested that neurons were uninfected by prions. Therefore, the coculture was extended to 96 h. Subsequent to this lengthened time of incubation, proteinase-K

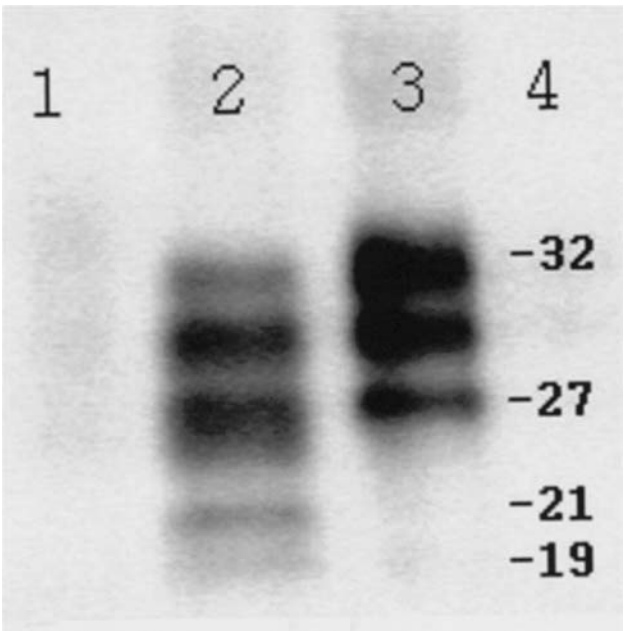

Fig. 8 PrP^{Sc} accumulation in dendritic cells incubated with ME7-PTA for 72 h. BMDCs samples were analyzed by immunoblotting and the membrane was revealed with SAF antibody. Homogenate in *lane 2* was treated with proteinase K and showed PrP^{Sc}-bands at 21 and 19 kDa. Untreated homogenate in *lane 3* exhibited only the three bands at 32, 30 and 27 kDa. *1* and *4* molecular weight in kDa, *2* $BMDC^+$ PK^+ and *3* $BMDC^+$ PK^-

resistant accumulations were detected in neurons, according to the band of PrP^{Sc} at 21 kDa (Fig. 12). These results were qualitatively confirmed by cell-blotting (data not shown). Neurons cultured for 96 h with PrP^{Sc}-BMDCs or neurons directly incubated with ME7-PTA displayed the same electrophoretic profile after PK treatment.

PrP^C expression is dispensable for the uptake of PrP^{Sc} by dendritic cells

Primary cultures of dendritic cells were generated from PrP^{nul} mice to bear out that the presence of membrane-PrP^C

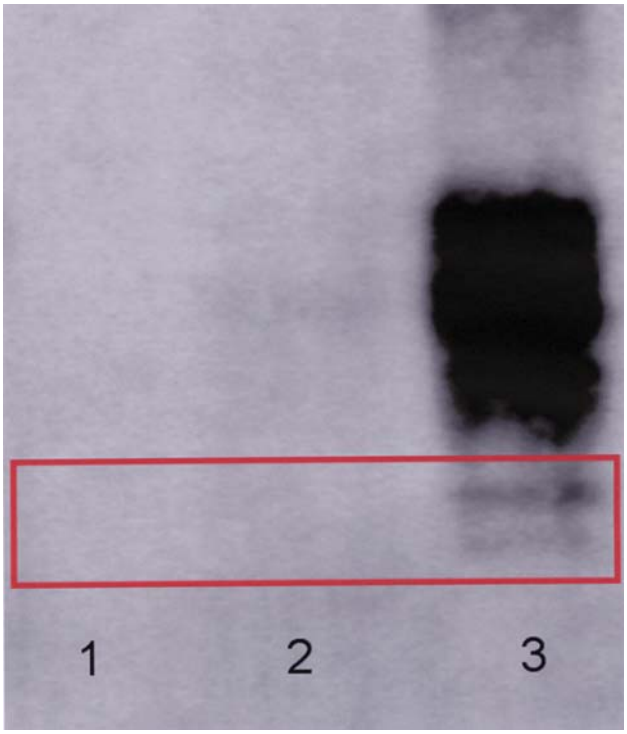

Fig. 9 Absence of PrP^{Sc} retention by fibroblasts and lymphocytes cultured with scrapie homogenates for 72 h. All samples were pretreated with proteinase K before the electrophoresis to detect PrP^{Sc} deposit. Neither fibroblast samples nor lymphocyte samples analyzed by immunoblotting exhibit PrP^{Sc}-band (21 and 19 kDa) detected in scrapie-infected brain homogenate (*red rectangle 1* Fibroblasts cultured with ME7-PTA (PK^+). *2* Lymphocytes cultured with ME7-PTA (PK^+). *3* ME7-infected brain (PK^+)

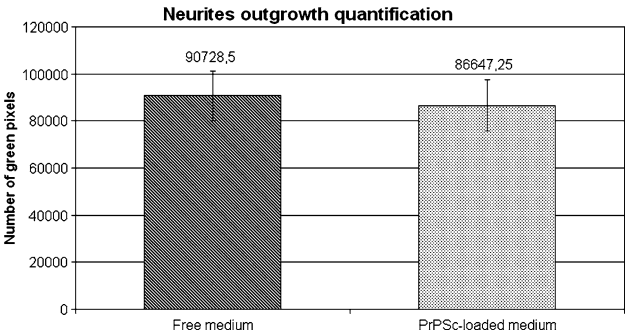

Fig. 10 Prions introduced in the neurons medium do not influence neurites outgrowth. The development of the neurites network, in scrapie and control conditions, was evaluated by a specific in vitro assay. The number of green pixels, which highlights NF M immunolabeling, was counted on standardized images obtained by confocal analysis (as described in "Methods"). No significant statistical difference appeared after comparing the values acquired in the two experimental conditions. The average is indicated on the top of each histogram. SD: 10,641.04 (free medium) and 10,786.49 (PrP^{Sc}-loaded medium). The experiment was repeated four times

is needless to load BMDCs with PrP^{Sc}. In this condition neither immature nor mature BMDCs express PrP^{C}. The ME7-PTA was introduced in BMDC culture on day 9 of the

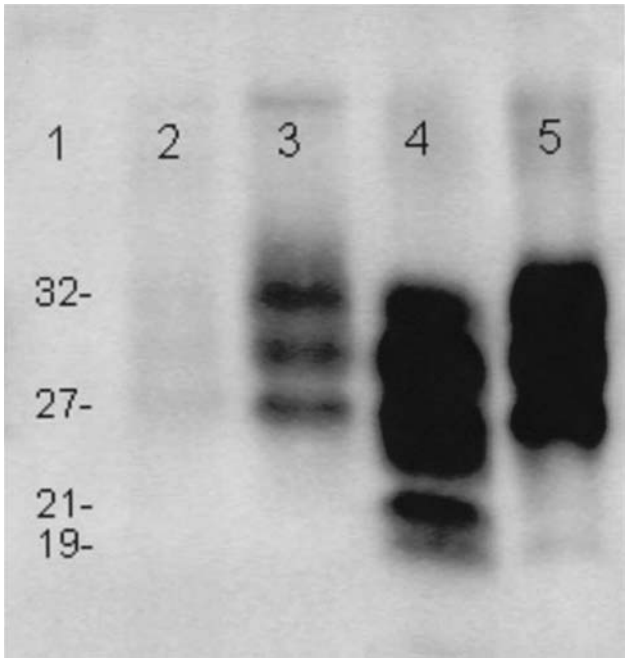

Fig. 11 Absence of prion accumulation in peripheral neurons from dorsal root ganglia cocultured in the presence of infected-BMDCs for 48 h. Samples in *lanes 2* and *4* were treated with proteinase K. Neurons sample in *lane 2* was treated with proteinase K and showed no PrP-band. Infected-brain sample in *lane 4* was treated with proteinase K and showed typical PrP^{Sc}-bands at 21 and 19 kDa. Untreated neurons sample in *lane 3* and infected brain sample in *lane 5* exhibited only the three bands at 32, 30 and 27 kDa. *1* molecular weight in kDa, *2* Neurons (cultivated with infected-BMDCs) PK^+, *3* Neurons (cultivated with infected-BMDCs) PK^-, *4* ME7-infected brain PK^+ and *5* ME7-infected brain PK^-

primo-culture for 48 h. Firstly, the ability of dendritic cells to take up PrP^{Sc} was confirmed by the detection of ME7-PTA deposits by immunoblots (Fig. 13i). Secondly, we analyzed the cellular localization of PrP^{Sc} within BMDCs. No membrane deposits of PrP^{Sc} were detected using Nomarski interference and fluorescence immunocytochemistry (Fig. 13f). This last result was attested with a better sensitivity, using pre-embedding immunogold cytochemistry, by electron microscopy analysis (Fig. 13c). On the contrary, PrP^{Sc} accumulations were detected in the intracellular compartment of BMDCs. Immunoblots show that BMDCs have internalized PrP^{Sc} (Fig. 13i).

Discussion

The hypothesis of the implication of DCs-peripheral nerve fibers interfaces in the pathogenesis of prion diseases originally resulted from in vivo studies that described neuroimmune connections in scrapie-infected lymphoid organs (Defaweux et al. 2005; Ma et al. 2007; Dorban et al. 2007a). In contrast to other models based on neuroblastoma

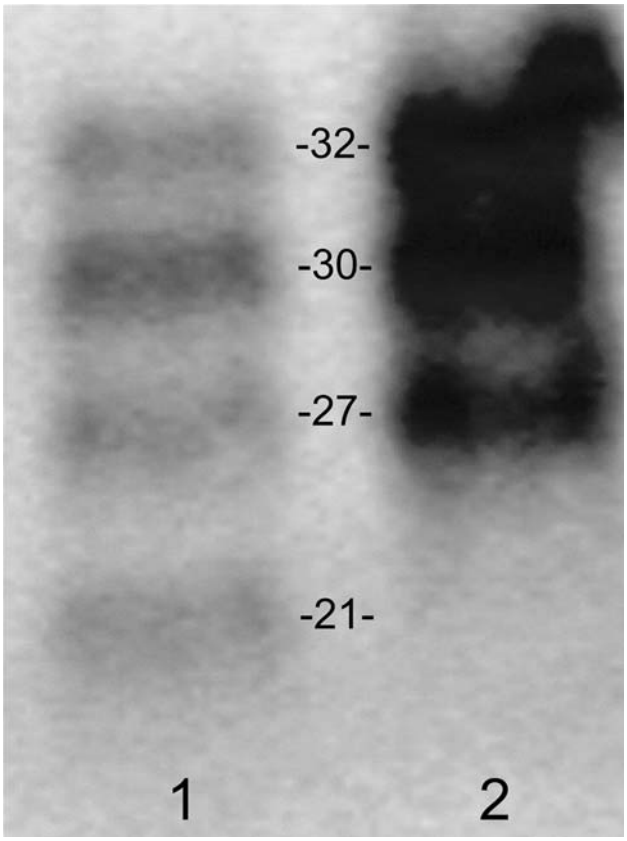

Fig. 12 PrP^{Sc} accumulation in peripheral neurons cocultured with scrapie-infected BMDCs for 96 h. Neurons sample in *lane 1* was treated with proteinase K and showed a typical PrP^{Sc}-band at 21 kDa. Untreated homogenate in *lane 2* exhibited only the three bands at 32, 30 and 27 kDa

(Luhr et al. 2002, 2004) our model used peripheral primary neurons that were infected in vivo after oral scrapie challenge (Beekes and McBride 2007). Thus our model specifically targeted a cell-to-cell transmission of PrP^{Sc} (Caughey and Baron 2006) from the immune system to the PNS, hypothetically at the genesis of the prion peripheral neuroinvasion (Kimberlin and Walker 1989). In this purpose, the dissemination of prions via infected apoptotic cells or cellular fragments was limited by frequent washing. Therefore, most of the potentially infected exosomes (Fevrier et al. 2004; Couzin 2005) generated by BMDCs were removed in order to support cell-to-cell infection.

The molecular composition of neuroimmune interfaces throughout close contacts and their implications in prion pathogenesis remains unclear. In addition to synaptic and ligand–receptor interaction, a new type of inter-cellular communication was discovered a few years ago, that is based on de novo formation of membranous nanotubes between cells. These structures, referred to as tunneling nanotubes (TNT) were shown to facilitate the transport of various cellular components (Rustom et al. 2004). Some authors have hypothesized that some small pathogens, such as prions, can be disseminated by these exchange processes (Caughey and Baron 2006; Gousset et al. 2009). Several arguments attest of the implication of TNT in prions spreading. (1) Gousset et al. demonstrated that BMDCs can transmit prions to primary neurons from the CNS (Gousset et al. 2009). (2) Peripheral neurons are particularly competent to generate TNT (Rustom et al. 2004). (3) In this study we have showed thin cytoplasmic extensions, arising from BMDCs, which connected dendritic cells and primary neurons.

In our model we demonstrate that, after 96 h of co-culture with scrapie-infected BMDCs, neurons have accumulated PrP^{Sc}. Before this point in time, immuno-blotting analysis did not reveal any PrP^{Sc} in nerve cells. This insufficient amount of prion protein could result from a slow rate of transfer. As a result, after 48 h the contacts between BMDCs and neurons were recent and the quantity of prions accumulated in neurons was undetectable by Western-blotting. This assumption implicates that the proteins detected in peripheral neurons after 96 h were ME7-PTA prions previously introduced into BMDCs medium. In this case no replication of PrP^{Sc} occurred. Alternatively the infection of neurons is rapid and requires a small number of prions but PrP^{Sc} has to be replicated by nerve cells. Following this hypothesis the proteins highlighted after 96 h were newly synthesized prions.

Despite cellular PrP expression is necessary for the replication of prions (Bueler et al. 1993), it has been previously demonstrated that the retention of PrP^{Sc} is facilitated when host PrP^{C} and the infectious strain share a similar glycosylation profile (DeArmond et al. 1999). In this study we infected both peripheral neurons and BMDCs with a strongly diglycosylated prion strain (Kuczius et al. 1998). Peripheral neurons, displaying a mainly diglycosylated PrP^{C}, and ME7 scrapie strain share a similar pattern of glycosylation. Therefore, this accumulation appears to be in agreement with the theory of DeArmond et al. On the contrary, BMDCs with a monoglycosylated PrP^{C} profile can also be loaded with a diglycosylated prion strain. The infection of dendritic cells suggests alternative explanations. As it has been earlier described in neurons (Nielsen et al. 2004), prion inoculation in the BMDCs medium could stimulate diglycosylation of newly synthesized PrP^{C}, which could enhance PrP^{Sc} uptake. Our results, based on PrP^{nul} strategy, prove that BMDCs can retain prions random mechanism, by endocytosis, or via a specific mechanism, such as specific lectins or receptors, which are both independent of PrP^{C} expression. It has been showed that C1q participates in PrP^{Sc} uptake and prion-loaded DCs express one of the C1q receptors, calreticulin (Mitchell et al. 2007; Flores-Langarica et al. 2009). The 37/67-kDa laminin receptor expressed at the cell surface can also act in

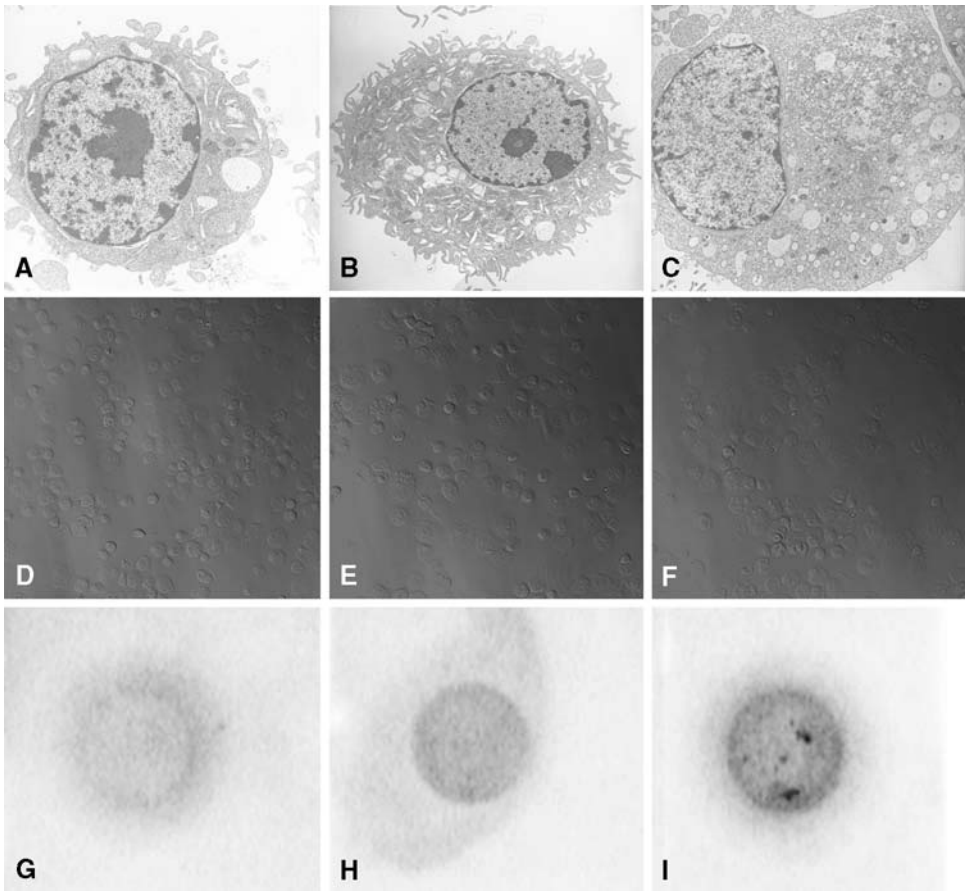

Fig. 13 PrP^{Sc} accumulation in BMDCs, differentiated from PrP^{nul} mice, incubated with ME7-PTA for 72 h. Dendritic cells deprived of cellular prion protein were exposed to medium only (**a**, **d**, **g**), uninfected-PTA (**b**, **e**, **h**), or ME7-PTA (**c**, **f**, **i**) and labeled with SAF 32 antibody and analyzed by electron microscopy (**a**–**c**). Nomarski interference and fluorescence (**d**–**f**), and immunoblotting (**g**–**i**). BMDCs exhibit no membrane PrP^{Sc} when incubated with prions for 72 h (**c**, **f**). As well as in control conditions (**a**, **b** and **d**–**e**) no gold 10 nm-particle was detected on the surface of dendritic cells (**c**) and no red dye was highlighted by immunofluorescence (**f**). Conversely PrP^{Sc} was identified when intracellular proteins were analyzed (**i**). Control conditions (**g**, **h**) were PrP-negative

PrP^{Sc} retention (Gauczynski et al. 2006). Recently, evidences have been demonstrated that the interactions of PrP^{Sc} and laminin receptor occur on the cell surface and also in perinuclear compartments suggesting a putative role of LRP/LR in the trafficking of PrP^{Sc} molecules (Nikles et al. 2008). An up to date study has beard out that lipids rafts are involved in PrP internalization and are associated with prion protein in the cytoplasm (Sarnataro et al. 2009).

Although prions cause neuronal death in the CNS, we showed here that prions introduced in the culture of peripheral neurons did not induce neurotoxicity or neurites outgrowth disturbance. Various explanations can be put forward for this. (a) Microglial cells seem to be implicated in the process of neurodegeneration via IL-1β and IL-6 secretion (Priller et al. 2006; Pasquali et al. 2006) and they are associated with amyloid accumulation (Rezaie and Lantos 2001). Our system did not contain any microglial cells and could therefore have been protected from neurotoxicity. (b) Within the neurons of the CNS, PrP^{Sc} was localized in two different compartments: LAMP-1 and flotillin1 vesicles, whereas in the PNS prions were only located in LAMP-1 vesicles (Pimpinelli et al. 2005; Leblanc et al. 2006). This supplementary compartment in the CNS could explain the lack of neurotoxicity in our system using peripheral neurons. Indeed flotillin1 interacts with neuroglobin, which protects neurons from oxidative stress (Wakasugi et al. 2004). PrP^{Sc} could disturb the protective effect of neuroglobin and expose central neurons to oxidative stress. (c) Fatty-acid components of the plasma membrane influence apoptosis after prions inoculation. Neuroblastoma pretreated with palmitic acid and infected with the $PrP_{106–126}$ were found to be much more sensitive than untreated cells (Dupiereux et al. 2006). A possible variation in the composition of membrane fatty-acids and their relative percentage in peripheral and central neurons could explain the variation in PrP^{Sc} sensitivity.

Our system of coculture demonstrated that infected DCs are competent to transmit PrP^{Sc} to primary peripheral neurons by cell-to-cell cytonemes and is additional of the study of Gousset et al. (Gousset et al. 2009). Besides, this system could be use to investigate the species barrier using BSE and vCJD strains. In order to highlight molecular actors responsible for prion capture and transmission, our model could also be used with knockout DCs for complement factors. Finally, our model could be a powerful system to test molecules that are potential candidates for reducing or stopping prion propagation.

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