Characterization of the role of dendritic cells in prion transfer to primary neurons

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TSEs (transmissible spongiform encephalopathies) are neurodegenerative diseases caused by pathogenic isoforms (PrPSc) of the host-encoded PrPc (cellular prion protein). After consumption of contaminated food, PrPSc deposits rapidly accumulate in lymphoid tissues before invasion of the CNS (central nervous system). However, the mechanisms of prion spreading from the periphery to the nervous system are still unclear. In the present study, we investigated the role of DCs (dendritic cells) in the spreading of prion infection to neuronal cells. First, we determined that BMDCs (bone-marrow-derived DCs) rapidly uptake PrPSc after exposure to infected brain homogenate. Next, we observed a progressive catabolism of the internalized prion aggregates. Similar experiments performed with BMDCs isolated from KO (knockout) mice or mice overexpressing PrP (tg2a0) indicate that both PrPSc uptake and catabolism are independent of PrPc expression in these cells. Finally, using co-cultures of prion-loaded BMDCs and cerebellar neurons, we characterized the transfer of the prion protein and the resulting infection of the neuronal cultures. Interestingly, the transfer of PrPSc was triggered by direct cell–cell contact. As a consequence, BMDCs retained the prion protein when cultured alone, and no transfer to the recipient neurons was observed when a filter separated the two cultures or when neurons were exposed to the BMDC-conditioned medium. Additionally, fixed BMDCs also failed to transfer prion infectivity to neurons, suggesting an active transport of prion aggregates, in accordance with a role of TNTs (tunnelling nanotubes) observed in the co-cultures.

Key words: cerebellar granule neuron, dendritic cell, intercellular transfer, prion infection, tunnelling nanotube.

INTRODUCTION

TSEs (transmissible spongiform encephalopathies) as variant of Creutzfeldt–Jakob disease, scrapie or chronic wasting disease can be acquired from the consumption of contaminated food. Following oral exposure, prions enter the host organism through the gut before invasion of the draining lymphoid tissues, where the first prion amplification takes place [1–3]. Prions subsequently spread to the CNS (central nervous system), where a characteristic neurodegeneration process is engaged concomitantly with the prion aggregate deposition in the brain [4–6]. Prior to prion neuroinvasion, PrP (prion protein) deposits are mainly visualized in tangible body macrophages and FDCs (follicular DCs (dendritic cells)) of the secondary lymphoid tissues (Peyer’s patches, mesenteric lymph nodes, spleen) [7–12].

Although a number of studies suggest that FDCs could play an important role in prion replication, the mechanisms of prion spreading from the gastrointestinal tract to the FDCs and from lymphoid tissues to the CNS are still undetermined [13–15]. Prion neuroinvasion is initiated in the enteric nervous system and followed by retrograde transport along the sympathetic and parasympathetic nerve fibres [16–18]. Because of the absence of neuroimmune synapses between resident FDCs and nerve fibres, direct prion transfer mechanisms between these two cell types can be excluded [19–22]. Based on in vitro studies of intercellular prion transfer mechanisms, different hypotheses have been suggested. FDCs might passively transfer prion to proximal cells or nerve endings through exosomes or vesicle secretion [21,23]. Alternatively, mobile haematopoietic DCs might transfer PrPSc (pathological form of PrP) from the gut to FDCs, or possibly directly to nerve fibres. Indeed, different studies have characterized the role of DCs in the prion infection process [24–27]. DCs are mobile cells, which can directly uptake antigens by insertion of dendrites through the tight junctions of the intestinal epithelium cells [28] or after prion transpethelial migration through microfold cells [29,30]. Following antigen capture, DCs can retain proteins in native form for a sufficient time to facilitate their subsequent migration to the targeted lymphoid tissues [31]. Furthermore, as TSE infection by the oral route, PrPSc deposits have been identified in DCs from Peyer’s patches, mesenteric lymph nodes or spleen [32,33]. Finally peripheral prion infection performed in mice devoid of DCs failed to accumulate PrPSc in lymphoid tissues and the subsequent neuroinvasion was partially impaired [24,27,34]. Overall, these data strongly point to DCs as potentially important candidates in prion transport from the gut to the lymphoid tissues, even though the subsequent neuroinvasion mechanisms are still undetermined. In addition, in contrast with FDCs, DCs can theoretically promote prion transfer to nerve cells by direct contacts with nerve fibres [32,33,35,36] or through TNT (tunnelling nanotube)-like structures [37,38]. Indeed BMDCs (bone-marrow-derived DCs) are able to form TNT-like structures in vitro when co-cultured with primary neurons, and can transfer PrPSc and infection to these cultures [37,38].

In the present study, we have characterized the role of BMDCs in the transfer of prions to primary neurons using an in vitro approach. First, we analysed the uptake and the fate of scrapie...
homogenate in BMDC cultures. We demonstrated that BMDCs rapidly internalize PrPSc aggregates and retain them for several hours, independently of the PrPc (cellular PrP) expression levels. Next, we characterized the transfer of PrPSc from prion-loaded BMDCs to primary neurons using short-time co-cultures. We found that BMDCs begin to transfer PrPSc as early as 4 h after cell cultures have been established and that this transfer is triggered by cell–cell contact. Furthermore, we show that PrPSc transfer results in prion infection (e.g. prion replication) of primary neurons. Overall, the present study demonstrates that DCs can discharge prions to target cells upon direct cell–cell contact, and confirms that TNTs could be a major transfer mechanism in the passage of prions from the periphery to the CNS.

**EXPERIMENTAL**

**Mouse lines**

Primary cultures were established from C57BL/6J mice provided by Charles River Laboratories or the transgenic mouse lines PrP0/0 [PrP-KO (knockout) mice] (Zurich I) [39] and tga20 (mouse Prnpa allele) [40] provided by the CDTA (Cryopréservation, Distribution, Typage et Archivage animal). All experiments were performed according to national guidelines.

**Cell culture**

CGNs (cerebellar granule neurons)

Primary cultures of CGNs were established as described previously [41]. Briefly, CGNs were extracted from brains of 6-day-old C57BL/6 mice by enzymatic and mechanical dissociations. They were plated at a density of 800 000 cells/well in 12-well plates coated with 10 μg/ml poly-D-lysine (Sigma) to avoid proliferation of astrocytes. As negative controls, CGN cultures were established from PrP0/0 mice.

**BMDCs**

BMDCs were differentiated from bone marrow cells from 6–8-week-old C57BL/6 mice according to a method adapted from Méderlé et al. [42]. Briefly, bone marrow cells were seeded at 5 × 10⁶ cells per 100 mm diameter Petri dish (Falcon, Becton Dickinson Labware) in 10 ml of Iscove’s modified Dulbecco’s medium (RPMI 1640; Gibco) supplemented with 10% FCS (fetal calf serum), 20 mM KCl, penicillin (50 units/ml), streptomycin (50 μg/ml; Gibco) and 2-mercaptoethanol. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. On day 3, 10 ml of complete RPMI 1640 was added. On day 6, cells in suspension and loosely adherent cells were harvested. The recovered cells were further cultured under the same conditions as described above. On day 10, cells were harvested with EDTA as above and distributed in CellBIND six-well plates (Corning) at a concentration of 1 × 10⁶ cells/well in 3 ml of complete RPMI 1640.

moRK13 cells were provided by Dr Andrew Hill (Bio21 Institute, University of Melbourne, Melbourne, Australia). Cells were maintained at 37°C in 5% CO₂ in Opti-MEM medium (Gibco) supplemented with 10% FCS, penicillin (50 units/ml) and streptomycin (50 μg/ml).

**Prion loading of BMDCs**

Brain homogenates were prepared from the brains of mice terminally affected with the mouse 139A strain, from an original 139A-affected brain provided by Dr M. Baier (Robert Koch Institute, Berlin, Germany). Homogenates were diluted to a final concentration of 20% (w/v) in a 5% (w/v) glucose solution, sonicated in RPMI 1640 medium and a suspension equivalent to 2.5 mg of infected brain tissue was added to the wells of BMDCs for the times indicated.

**BMDC–CGN co-cultures**

BMDCs were loaded with the equivalent of 2.5 mg of infected brain tissue for 18 h. At 2 days after plating, CGNs were co-cultured with prion-loaded BMDCs overnight (CGN/BMDC, 4:1). BMDCs were removed from the CGN cultures by extensive washing before analyses of PrPres (prion-resistant PrP) in CGNs after short times (30 min–4 h) or after 2 and 3 weeks post-co-culture. Then, 50 μg of protein was treated with 0.5 μg of PK (proteinase K) for 30 min at 37°C before methanol precipitation. Samples were then subjected to SDS/PAGE and Western blot analysis with the Sha31 anti-PrP antibody (SPI-Bio). The same amounts were methanol-precipitated without PK treatments and analysed by SDS/PAGE and Western blot analysis using an M5/114 antibody (directed against MHC class II proteins specifically expressed in BMDCs) or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody.

Imaging prion uptake in BMDCs

At 10 days post-dissection, 1 × 10⁶ BMDCs were plated overnight on Ibidi dishes (Biovalley) coated with fibronectin (Sigma). Cells were then exposed to 2.5 mg of 139A scrapie brain homogenate for the times indicated, washed thoroughly in RPMI 1640 and fixed in 4% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100, treated with 3 M guanidium thiocyanate to expose the PrPSc epitopes and labelled with the Sha31 anti-PrP antibody.
anti-PrP antibody and with the cytosolic dye HCS CellMask Blue (1:5000) (Invitrogen). The cells were washed and mounted with Aqua-Poly/Mount (Polysciences). Images were acquired with an epifluorescence microscope (Zeiss Axiovert 200M) controlled by Axiovision software. Random mosaics (3 × 3 fields) were obtained using a 63× objective Plan-Apochromat objective (1.4 NA (numerical aperture)). All Z-stacks were acquired with Z-steps of 0.4 μm. Representative tiles are presented. For higher-magnification representations of the internalization process, a confocal microscope Andor Revolution Nipkow spinning-disc imaging system was used. The Andor technology was installed on a Zeiss Axiovert 200M microscope, equipped with an Andor EMCCD DV885 camera, three diode-pumped solid-state lasers with excitation at 405, 488 and 560 nm, a piezo mono-objective for fast three-dimensional acquisitions, and a confocal head spinning-disc Yokogawa CSU22. Images were acquired with an oil 63× Plan-Apochromat objective (1.4 NA). All Z-stacks were acquired at maximum speed of the microscope with Z-steps of 0.250 μm.

For wide-field analysis, cells were fixed with 4% paraformaldehyde for 10 min. Phase-contrast images were then acquired by high-resolution wide-field microscope Marianas (Intelligent Imaging Innovations) using a 63× oil objective. All Z-stacks were acquired with Z-steps of 0.4 μm.

PK digestion

Prion detection in BMDCs

Following incubation for the times indicated, cells were washed in PBS before lysis in TL1 buffer [50 mM Tris/HCl (pH 7.4), 0.5% sodium deoxycholate and 0.5% Triton X-100]. After a short centrifugation (3000 g for 5 min), 50 μg of cell lysates were treated with 2 μg of PK for 30 min at 37 °C. Next, the proteins were methanol-precipitated for 1 h at −20 °C before centrifugation at 13 000 g for 30 min. Pellets were resuspended in sample buffer before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP (horseradish peroxidase). Immunoreactivity was visualized by ECL (enhanced chemiluminescence; Amersham).

Prion detection in CGNs

The accumulation of PrPSc was analysed in neuronal cultures at different times post-infection. Neuron lysates performed in TL1 buffer were pre-cleared by centrifugation at 3000 g for 5 min. Then, 50 μg of cell lysates were treated with 0.5 μg of PK for 30 min at 37 °C before stopping the digestion with 5 mM PMSF. Proteins were methanol-precipitated for 1 h at −20°C before centrifugation at 13 000 g for 30 min. Pellets were resuspended in sample buffer and denatured before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP. Immunoreactivity was visualized by ECL.

Prion detection in moRK13 cells

The accumulation of PrPSc was analysed in moRK13 cells after 18 h of co-culture. Lysates were performed in TL1 buffer after pre-clearing by centrifugation at 3000 g for 5 min. Then, 20 or 200 μg of cell lysates were treated with 0.5 μg or 5 μg of PK for 1 h at 37°C before methanol precipitation (1 h at −20°C). Proteins were then centrifuged at 13 000 g for 30 min. Pellets were resuspended in sample buffer and denatured before analysis by SDS/PAGE (12% acrylamide gels) and Western blotting with the Sha31 antibody and secondary anti-mouse antibody coupled to HRP. Immunoreactivity was visualized by ECL.

RESULTS

Characterization of prion uptake in BMDCs

We first analysed the rate of internalization of PrPSc by BMDCs after in vitro exposure to infected brain homogenate. At 10 days post-plating, BMDCs were exposed to 139A infected brain homogenate for 30 min, 1 h, 2 h or 18 h, fixed, treated with guanidium and labelled with the Sha31 antibody to detect PrPSc. Mosaics of different fields were obtained to analyse the overall spreading and endocytosis of PrPSc aggregates in BMDCs. Z-stacks were acquired to encompass all of the homogenate signals (see the Experimental section). In contrast with control BMDCs where no signal was observed after guanidium treatment (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310189add.htm), large fields of view show that PrPSc aggregates are well spread and associated with the majority of the BMDCs exposed to the infected brain homogenate (Figure 1A). Whereas most of the aggregates were outside the cells at the early time points (Figure 1A; 30 min–1 h), over time PrPSc aggregates were progressively internalized (Figure 1A; 2–18 h). After 18 h, the PrPSc aggregates were found inside the cells, as shown by the perfect focus of both BMDCs and PrPSc aggregates (Figure 1A; 18 h). Detailed confocal analyses and three-dimensional reconstructions of the PrPSc aggregates associated with BMDCs confirmed their localization at the cell surface and outside the cells after 30 min or 1 h of exposure (Figure 1B; 30 min–1 h, Supplementary Movies S1–S4 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). After 2 h, some PrPSc was still visualized at the level of the plasma membrane, but could also be detected in the cytosol of most cells (Figure 1B; 2 h, Supplementary Movies S5 and S6 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Finally, after 18 h of exposure, the localization of PrPSc was drastically shifted and entirely restricted to the cytosol of the BMDCs (Figure 1B; 18 h, Supplementary Movies S7 and S8 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). At this time point, no free PrPSc aggregates could be detected outside the BMDCs (Figure 1).

These data demonstrate the rapid uptake of PrPSc homogenate by BMDCs after in vitro exposure. The kinetics of internalization observed are in accordance with the results previously described in rat BMDCs and Langherans cells [43,44]. Additionally, biochemical analyses of PrPSc internalization performed on BMDCs derived from KO or PrP-overexpressing mice (tg20) showed a similar increase in PrPSc internalization up to 18 h post-exposure, indicating that prion uptake is independent of the levels of PrPc (Supplementary Figure S2 at http://www.BiochemJ.org/bj/431/bj4310189add.htm).

Characterization of PrPSc degradation in BMDCs

Next, we wanted to investigate the fate of PrPSc once internalized by BMDCs. In vivo studies have identified DCs as important candidates during prion spreading from the periphery to the peripheral nervous system [24,43]. However, subsequent in vitro experiments have indicated that the rapid uptake of PrPSc is progressively followed by prion degradation in various subsets of DCs [43–46]. Because a rapid degradation of PrPSc would be inconsistent with a role of DCs in prion spreading, we investigated...
whether and how PrPSc was processed in BMDCs by analysing the levels of PrPres over time following the uptake of prion homogenate (Figure 2A).

At 10 days post-plating, $10^6$ BMDCs were subjected to 2.5 mg of brain homogenate (obtained from terminally infected mice injected with the 139A scrapie strain) for the duration of the experiment (Figure 2A). Alternatively, the cells were first allowed to internalize PrPSc for 18 h, then washed and replated before performing the PK assay (Supplementary Figure S3 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Following prion capture, BMDCs isolated from wild-type mice progressively degraded 139A prion aggregates as determined by the decrease in PrPres signal between 24 and 168 h (C57Bl/6) (Figure 2A and Supplementary Figure S3). These results show a progressive clearance of prion aggregates by DCs. However, it is also clear that, following prion uptake, BMDCs are able to carry infectious PrPSc for up to 4 days. This is consistent with a dual role of DCs both in the transfer of prions to other cells and in prion
PrPSc aggregates were localized exclusively inside the cytosol that at the time of the co-cultures with the primary neurons the membrane-associated PrPSc aggregates, since we demonstrated PrPSc had been transferred from the BMDCs to the neurons removed from the co-cultures, indicating that a large amount of PrPres was detected only in the neurons and not in the BMDCs blotting after PK treatment. Interestingly, under these conditions neuronal cells were analysed for the presence of PrPres by Western extensive washes. The lysates of both removed BMDCs and incubation, BMDCs were removed from the CGN cultures by 4:1 ratio between neuronal cells and DCs [37]. After overnight washed before addition to the CGN primary cultures.

PrPSc internalization (see Figure 1). Cells were then extensively cultures. To detect PrPSc transfer, BMDCs were loaded with 139A after its uptake (Figure 2A), we further investigated their ability having established that BMDCs retained PrPSc for at least 96 h to understand whether PrPc had a role in PrPSc catabolism, we repeated the same experiments using BMDCs isolated either from KO mice or from tga20 mice, which express 10-fold more PrPc than wild-type mice (Figure 2 and Supplementary Figure S3; tga20 and KO). Western blot quantification of the PrPres signal indicated that 139A brain homogenate was catabolized over time with similar kinetics as BMDCs isolated from KO, wild-type or tga20 mice (Figure 2B). Overall, these data indicate that both PrPSc uptake and degradation are independent of PrPc expression.

BMDCs transfer PrPSc to neuronal cells

Having established that BMDCs retained PrPSc for at least 96 h after its uptake (Figure 2A), we further investigated their ability to transfer PrPSc to primary cultures of neurons using in vitro cocultures. To detect PrPSc transfer, BMDCs were loaded with 139A scrapie brain homogenate for 18 h in order to allow complete PrPSc internalization (see Figure 1). Cells were then extensively washed before addition to the CGN primary cultures.

As described previously, co-cultures were established at a 4:1 ratio between neuronal cells and DCs [37]. After overnight incubation, BMDCs were removed from the CGN cultures by extensive washes. The lysates of both removed BMDCs and neuronal cells were analysed for the presence of PrPres by Western blotting after PK treatment. Interestingly, under these conditions PrPres was detected only in the neurons and not in the BMDCs removed from the co-cultures, indicating that a large amount of PrPSc had been transferred from the BMDCs to the neurons (Figure 3A, co-culture). We could exclude prion transfer from membrane-associated PrPSc aggregates, since we demonstrated that at the time of the co-cultures with the primary neurons the PrPSc aggregates were localized exclusively inside the cytosol of BMDCs (see Figure 1, 18 h). Therefore transfer could have occurred either through the secretion of PrPSc in the medium or through direct passage from the cytosol of BMDCs to the cytosol of the neurons, possibly via TNTs as we had suggested previously [37].

To evaluate the possible role of the secretory pathway, and more specifically of exosomal release [47–50], we examined whether prion transfer could occur through filters, which would allow the passage of secretory vesicles and exosomes. Quantification of the PrPres signals demonstrated that the transfer efficiency is reduced by more than 98% when filters were used to separate the cultures, compared with direct co-cultures (Figure 3A). This suggested that PrPSc secretion was not involved in the transfer (Figure 3A, filter). However, to rule out the possibility that the filters could trap PrPSc aggregates, we analysed whether prion transfer could be mediated by the supernatant of the scrapie-loaded BMDCs. To this aim, neurons were exposed to the supernatant of BMDCs loaded with 139A brain homogenate collected after 24 h. After 18 h of exposure to the conditioned medium, neurons were washed and analysed for PrPres. Similar to the filter conditions, neurons exposed to the supernatant of BMDCs did not contain high PrPres signals as compared with the signal obtained from the direct coculture experiments (Figure 3B; supernatant), further suggesting that PrPSc secretion was not the main transfer mechanism.

Finally, to ensure that the PrPres signal observed in CGNs was not the result of BMDCs left in the co-cultures, we analysed by Western blotting, the presence of BMDCs in the CGN co-cultures using the BMDC-specific MHC class II antibody. BMDCs and non-exposed CGN cell extracts were used as positive and negative controls respectively (Figure 3C). As expected, a very strong signal for MHC class II proteins was detected in BMDC extracts, but not in non-exposed CGNs (NI) or in CGNs exposed to BMDCs through filter (filter) (Figure 3C). Interestingly, only a very faint signal was detected in CGNs directly exposed to BMDCs (co-culture) (Figure 3C), indicating that the BMDCs were efficiently removed from the CGNs. This also excluded the possibility that the PrPres signals detected in CGN post co-cultures could be derived from prion-loaded BMDCs.

Figure 2  Time course of PrPSc degradation in BMDCs

BMDC cultures were established from C57BL/6 (left-hand panel), tga20 (middle panel) or KO (right-hand panel) mice. At 10 days post-plating, cells were exposed to 139A brain homogenate for the times indicated. (A) Cells were lysed and PK-treated before analysis of PrPres expression by immunoblotting using the Sha31 antibody. Western blot analysis indicated a progressive decrease in the PrPres signal between 24 and 96 h of exposure preceding total disappearance of the signal at 168 h. The molecular mass in kDa is indicated on the left-hand side of the blots. (B) PrPSc degradation follows similar kinetics in BMDCs isolated from C57BL/6, tga20 or KO mice, suggesting that the PrPSc catabolism we observed is independent of PrPc expression. The relative degradation of PrPSc in prion-loaded BMDCs was quantified from two independent experiments for C57BL/6 and tga20 and from one experiment for KO cells.
Figure 3  Characterization of PrPSc transfer from prion-loaded BMDCs to CGNs

(A and B) Cell–cell contact is required for PrPSc transfer from BMDCs to neurons. BMDCs were exposed in vitro to 139A brain homogenate for 18 h. (A) Prion-loaded BMDCs were co-cultured with neurons directly (co-culture) or through filters (filter). After 18 h BMDCs were removed from the CGNs with extensive washes. The lysates of both the removed BMDCs and of the CGNs were PK-treated to evaluate PrPSc transfer by immunoblotting using the Sha31 antibody. PrPres is only detected in neurons after direct co-culture, suggesting that intercellular prion transfer cannot occur in the absence of cell–cell contact. On the other hand, prion protein is only visualized in BMDCs removed from filters, suggesting that direct contact triggers the prion discharge from BMDCs. The PrPSc signal in CGNs and BMDCs was quantified from Western blot analysis from three different experiments and are presented as relative percentages (lower panels). (B) To determine the impact of PrPSc present in the supernatant (e.g. exosomal release, vesicle secretion), prion-loaded BMDCs were co-cultured with neurons directly (co-culture), through filters (filter) or the neurons were exposed to the conditioned medium of loaded BMDCs (supernatant). Similar to what was found in (A), PrPres was only detected in neurons after direct co-cultures. (C) To evaluate the efficiency of removal of BMDCs in CGN cultures, we evaluated the presence of MHC class II proteins in CGN lysates after co-cultures through filters (filter), direct exposure to BMDCs (co-culture) or in non-exposed CGNs (NI). Protein (50 μg) from BMDCs or CGNs were analysed by Western blot with MHC class II and GAPDH antibodies for normalization. Whereas a strong MHC class II signal is detected in BMDC cell extracts, a faint signal is observed in CGNs only after direct exposure. (D) To assess the types of contact between the two cell populations in our co-cultures, prion-loaded BMDCs were co-cultured with CGNs 2 days post-plating for 18 h before fixation. Wide-field acquisitions were performed using a Marianas microscope (Triplet) and selected frames of two different 3-stack acquisitions (0.4 μm steps) are shown. BMDCs (indicated by an asterisk) are in close contact with dendrites (arrow, upper panel) or neuronal cell bodies (arrow, lower panel). The scale bar represents 10 μm. In (A and C), the molecular mass in kDa is indicated on the left-hand side of the blot.

Overall, these data demonstrate that efficient PrPSc transfer from BMDCs requires cell–cell contact and does not appear to be associated with PrPSc secretion. In order to quantify the amount of PrP discharged by BMDCs, we analysed the amount of PrPres remaining in BMDCs co-cultured directly or through filters with primary neurons (Figure 3A). Interestingly, different levels of PrPres could be detected in an equivalent number of BMDCs from the different co-culture conditions (Figure 3A). Indeed, by normalizing the gel loading to 50 μg of protein of the different BMDC lysates, we found that BMDCs seeded on to filters contained much higher levels of PrPres compared with BMDCs seeded directly on top of CGNs (Figure 3A). Quantification of PrPres signals indicated that direct co-cultures triggered 97% of PrPSc release as compared with filter conditions.
(Figure 3A). These data clearly indicate that the transfer of PrPSc from BMDCs to the primary neurons was triggered by direct cell–cell contact. Similar experiments were performed with epithelial moRK13 cells, which can be infected after prion transfer from BMDCs [37]. As in the case of primary neurons, moRK13 cells contained PrPres after 18 h of direct co-culture with loaded BMDCs, but not if the cells were separated by filters or exposed to the supernatants of BMDCs (Supplementary Figure S4 at http://www.BiochemJ.org/bj/431/bj4310189add.htm). Interestingly, the amount of PrPres found in moRK13 was equivalent to one-quarter of the total amount of PrPres found in loaded BMDCs, and was comparable with the amount left in the BMDCs that were co-cultured through a filter. Therefore these data highlight the important role of direct cell contact in the stimulation of prion discharge by BMDCs.

Furthermore, we observed that, upon co-culture, BMDCs were able to interact with both the dendrites (Figure 3D, upper panels) and the cell bodies (Figure 3D, lower panels) of neuronal cells. These observations are in agreement with in vivo results [20,32], which show close contact between DCs and nerve fibres in lymph nodes of scrapie-infected animals.

**BMDCs efficiently transfer prion infectivity to neuronal cells**

Because we have previously shown that PrPSc transfer from BMDCs could result in de novo infection of primary neurons [37], we decided to further characterize the cellular mechanisms involved in the transfer of infectivity.

To this aim, after 12 h of co-culturing with loaded BMDCs, we analysed the evolution of PrPres signals in primary neurons over time, up to 3 weeks of culture. Direct co-cultures established between 139A-loaded BMDCs and CGNs from wild-type C57BL/6 mice gave rise to neuronal infection, as demonstrated by the progressive increase in PrPres signal observed from 7 to 21 days post-co-culture (Figure 4A). On the other hand, similar experiments performed with CGNs derived from PrP KO mice did not show any PrPres signal even after 3 weeks of culture (Figure 4A). Since PrPSc transfer is similar in KO neurons compared with wild-type neurons (results not shown), these data show that the PrPres signal observed in wild-type neurons derives from PrPSc neo-synthesis and is not the result of remnant PrPSc from BMDCs. Next, we analysed whether there was transfer of infectivity from loaded BMDCs to neurons in co-cultures separated through filters. As expected from the observed lack of transfer under these conditions (Figure 3A and Supplementary Figure S4), we were unable to detect newly synthesized PrPSc in primary neurons maintained in culture up to 3 weeks after overnight filter co-culture with loaded BMDCs (Figure 4B). Finally, in order to analyse whether infection was due to an active transfer mechanism, we decided to alter our co-culture experiments in order to inhibit membrane remodelling. To this aim, loaded BMDCs were fixed (with paraformaldehyde/glutaraldehyde solutions) before exposure to neuronal cultures. These treatments strongly inhibited the plasma membrane plasticity, blocking both TNT formation and PrPSc secretion. Similar to the results obtained after co-culture through filters, fixation of BMDCs prior to the co-cultures did not result in neuronal infection, as indicated by the absence of PrPres signals in the co-cultured neurons (Figure 4B). These data indicate that PrPSc infection results from an active process, which cannot occur upon fixation in a short 12 h co-culture and requires cell–cell contact. Because in our experiments we excluded that prion transfer could occur via secretion, all our data are consistent with a role of TNT in intercellular spreading from BMDCs to neurons, as we have proposed previously [37] (see also Supplementary Movie S9 at http://www.BiochemJ.org/bj/431/bj4310189add.htm).

**DISCUSSION**

In the present study, we investigated the role of BMDCs in the processing and spreading of prions. To this aim, we developed an in vitro approach in which prion-loaded BMDCs were cocultured with cerebellar primary neurons. First, we characterized the prion uptake by BMDCs exposed to scrapie brain homogenate over time by immunofluorescence analyses and three-dimensional reconstructions (Figure 1 and Supplementary Figure S2). While prion aggregates were mainly associated with the cell surface up to 2 h post-incubation, prion internalization was detected between 2 and 18 h post-exposure resulting in a progressive shift of localization of PrPSc from the plasma membrane to the cytosol.

Following scrapie uptake, we also demonstrated that BMDCs progressively degraded PrPSc between 24 and 72 h post-exposure. After 72 h, we observed higher PrPSc catabolism leading to the rapid disappearance of PrPSc signal between 96 and 168 h,
consistent with what was previously determined in other models [43,44,46,51].

The fact that we have been able to detect consistent amounts of PrPres up to 72 h post-loading indicates that, after uptake, BMDCs could present native prion proteins to other cell types during this time frame, before starting massive protein degradation. Furthermore, complementary experiments performed in BMDCs isolated from KO or PrP-overexpressing mice indicated that neither the uptake, as recently shown [38], nor the degradation of PrPSc was influenced by PrPc expression. Interestingly, similar experiments performed with different prion strains (22L and Me7) showed similar kinetics of uptake and catabolism of PrP (results not shown), suggesting that both mechanisms are not influenced by the different prion strains.

Next, we analysed whether and how BMDCs transferred PrPSc to primary neurons. In these experiments, neurons were co-cultured with BMDCs 2 days post-plating, a stage of differentiation that we found facilitates the establishment of TNTs between neurons and BMDCs. We characterized our co-cultures by microscopic approaches and showed that after overnight co-cultures BMDCs were either in close contact with dendrites or directly linked to neurons via TNTs (Supplementary Movie S9). Recently, similar connections have been observed between BMDCs and peripheral neurons isolated from the dorsal root ganglia [38]. Having established the presence of such cell–cell contacts, we turned our attention to the characterization of intercellular prion transfer mechanisms. According to previous models of prion transfer, prion-loaded BMDCs could transfer prions to neuronal cells by excreting PrPSc in the medium [41,52–54], by secretion of membrane exovesicles [43–46] or by direct cell–cell transfer [37,55].

Direct co-cultures from BMDCs and neurons established for 18 h allowed us to detect prion transfer to neuronal cells. However, when cells were co-cultured through filters, no detectable transfer was observed, arguing against the involvement of secreted PrPSc. To rule out the possibility that prion aggregates were retained on the filters, we also exposed neuronal cultures to medium conditioned by prion-loaded BMDCs. These experiments did not show significant prion transfer, as PrPres signal observed in neurons was much lower compared with the signal observed in the cases of direct cell–cell contact (Figure 3). We also determined the kinetics of prion transfer establishing short time co-cultures (from 30 min to 4 h), and demonstrated that efficient transfer required as little as 4 h of co-culture, which is consistent with the time necessary for the establishment and transfer via TNTs in cell cultures (Supplementary Figure S5 at http://www.BiochemJ.org/bj/431/bj4310189add.htm) [37,56].

Overall, our results indicate that prion-loaded BMDCs are able to transfer PrPSc to neuronal cells upon direct and relatively short cell–cell contact. Although BMDCs could secrete prion-enriched exosomes, we have been unable to show the involvement of the secretory pathway in the PrPSc transfer to neuronal cells under our particular culturing conditions (e.g. short incubation time and one-quarter cell dilution). Thus although we cannot rule out other manners of transfer, our data indicate that the transfer mediated by direct cell–cell contact is very efficient.

Interestingly, no PrPres signal can be detected in BMDCs removed from direct contact with the neuronal cells, whereas PrPres was still present in BMDCs exposed to neurons through filters. These data strongly support the hypothesis that prion transfer from BMDCs to neurons is strongly induced upon cell–cell contact. Furthermore, because at the time of co-cultures (after 18 h of uptake), all of the PrPSc aggregates are in the cytosol of BMDCs (Figure 1) and not at the cell surface, these data suggest a transfer from the cytosol possibly via TNTs, excluding a transfer through plasma membrane to neighbouring cells. Interestingly, the transfer of PrPSc from Me7-loaded BMDCs to dorsal root ganglion neurons has recently been examined [38]. In this study, experimental conditions also suggested prion transfer through TNT-like structures shown to connect BMDCs to dorsal root ganglia and excluded the involvement of PrPSc secretion.

Since we have previously shown that co-cultures with prion-loaded BMDCs results in infection of primary neurons, we next followed up the cultures to determine the requirements for prion infection of the targeted neurons. Consistent with the transfer experiments, we found that prion infection is only detected after direct co-culture conditions and does not occur if cells are separated by filters or when co-cultures were performed with aldehyde-fixed BMDCs. These experiments indicate that the transfer is an active mechanism requiring remodelling of the plasma membrane. Interestingly, a similar experiment performed by Kanu et al. [55] had shown a reduction of 75% in the efficiency of transfer of infection from fixed scrapie SM (Scrapie mouse brain) cells co-cultured with targeted HMH (cells expressing a chimaeric mouse and hamster PrPc) cells, as opposed to live cell co-cultures. These data are in agreement with our conclusion that efficient transfer requires an active membrane remodelling, although it is clear that infection can be acquired via different mechanisms in less efficient manners (e.g. long co-cultures with fixed cells) [47–50,55].

In the present study, using a number of restrictive experimental conditions such as short co-culture times, low BMDC/CGN ratios, physical separation and pre-fixation of cells, we were able to show that direct cell–cell transfer of PrPSc between these two cell types occurs in a PrPc-independent manner. Interestingly, having excluded transfer from the cell surface and by secretion, all of our data point towards a role of TNT-like structures in the intercellular transfer of PrPSc from BMDCs to CGNs, similar to what was recently shown with dorsal root ganglion neurons [38]. Finally, our system of co-cultures suggests that DCs could be important players during prion spreading in vivo and will allow further characterization of prion spreading from the periphery to the nervous system of different scrapie strains, which could lead to a better understanding of the species barrier phenomenon.

**AUTHOR CONTRIBUTION**

Chiara Zurzolo conceived the project. Christelle Langevin planned and performed most of the scrapie degradation experiments, co-incubation assays and analysed the data. Karine Gousset and Maddalena Costanzo planned and performed the uptake experiments, and analysed the data. Christelle Langevin and Odile Richard Le Goff prepared the BMDCs. Christelle Langevin, Karine Gousset and Chiara Zurzolo wrote the manuscript. All authors discussed the results and manuscript text.

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ral neuronal cells are highly permissive to sheep prion infection. J. Virol. 78, 482–490


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SUPPLEMENTARY ONLINE DATA

Characterization of the role of dendritic cells in prion transfer to primary neurons

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Figure S1 No PrPSc signal is detected in control BMDCs

BMDCs plated on fibronectin-coated Ibidi dishes were washed, fixed and immunolabelled with the Sha31 anti-PrP antibody and Alexa-Fluor®-546-conjugated secondary antibody. HCS CellMask Blue was used to label the cytosol of the BMDCs (blue). No PrPSc punctate (red) can be detected. Mosaics (3×3 fields) were acquired by wide-field microscopy. The scale bar represents 10 μm.

Figure S2 PrPSc endocytosis in BMDCs is independent of PrPc expression

BMDC cultures isolated from KO or tga20 mice, were exposed to 139A brain homogenate for the times indicated. Cells were washed, lysed and 50 μg of proteins were treated with PK prior to immunoblot analysis with the Sha31 antibody. PrPres is progressively internalized by BMDCs with a peak between 6 and 18 h. Identical kinetics of internalization were observed for KO and tga20 mice. The molecular mass in kDa is indicated on the left-hand side of the blots.

Figure S3 Time course of PrPSc degradation in BMDCs

BMDC cultures were established from C57BL/6 (left-hand panel) or tga20 (right-hand panel) mice. At 10 days post-plating, cells were exposed to 139A brain homogenate for 18 h. They were washed three times by centrifugation and plated for the times indicated. Cells were lysed and PK-treated before analysis of PrPres expression by immunoblotting using the Sha31 antibody. Western blot analysis indicates a progressive decrease in the PrPres signal between 24 and 96 h of exposure. The molecular mass in kDa is indicated on the left-hand side.

Figure S4 Cell–cell contact is required for PrPSc transfer from BMDCs to moRK13

Similar to CGNs, prion-loaded BMDCs were co-cultured with moRK13 cells directly (co-culture), through filters (filter) or moRK13 cells were exposed to the conditioned medium of BMDCs (supernatant). After 18 h BMDCs were removed and the moRK13 cells were extensively washed. Then a PK assay was performed on BMDCs 24 h post-loading (input). Prion transfer was evaluated by detection of PrPres in moRK13 cells and prion-loaded BMDCs after the co-cultures. Similar to CGNs (Figure 3A of the main text), PrPres is only detected in moRK13 cells after direct co-culture, whereas it stays in BMDC cultures separated by a filter, confirming that cell–cell contact is a prerequisite for the discharge of BMDCs and prion transfer to the recipient cells. Several dilutions of the original input show that one-quarter of the original PrPSc is discharged from BMDCs to the recipient cells. The molecular mass in kDa is indicated on the left-hand side of the blot.

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Figure S5  Kinetics of PrPSc transfer from prion-loaded BMDCs to neurons

Following BMDC exposure to 139A brain homogenate, co-cultures were established with CGNs for the times indicated. BMDCs were removed and neuronal cell extracts were analysed to detect PrPres by immunoblot using the Sha31 antibody. PrPres was detected as early as 4 h of co-culture, suggesting a rapid mechanism of transfer. The molecular mass in kDa is indicated on the left-hand side.