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Scrapie Pathogenesis: The Role of Complement C1q in Scrapie Agent Uptake by Conventional Dendritic Cells

Adriana Flores-Langarica,* Yasmine Sebti,* Daniel A. Mitchell,‡ Robert B. Sim,† and Gordon G. MacPherson2*

Mice lacking complement components show delayed development of prion disease following peripheral inoculation. The delay could relate to reduced scrapie prion protein (PrPSc) accumulation on follicular dendritic cells (DCs). However conventional DCs (cDCs) play a crucial role in the early pathogenesis of prion diseases and complement deficiency could result in decreased PrPSc uptake by cDCs in the periphery. To explore this possibility, we cultured murine spleenic or gut-associated lymph node cDCs with scrapie-infected whole brain homogenate in the presence or absence of complement. Uptake decreased significantly if the serum in the cultures was heat-inactivated. Because heat inactivation primarily denatures C1q, we used serum from C1q−/− mice and showed that PrPSc uptake was markedly decreased. PrPSc internalization was saturable and temperature-dependent, suggesting receptor-mediated uptake. Furthermore, uptake characteristics differed from fluid-phase endocytosis. Immunofluorescence showed colocalization of C1q and PrPSc, suggesting interaction between these molecules. We evaluated the expression of several complement receptors on cDCs and confirmed that cDCs that take up PrPSc express one of the C1q receptors, calreticulin. Our results show that C1q participates in PrPSc uptake by cDCs, revealing a critical role for cDCs in initial prion capture, an event that takes place before the PrPSc accumulation within the follicular DC network. *The Journal of Immunology, 2009, 182: 1305–1313.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of infectious neurodegenerative diseases that affect humans as well as domestic and wild animals. TSE pathology is characterized by neuronal loss, glial activation, and amyloid accumulation of the (abnormal) prion protein (PrP), scrapie PrP (PrPSc), in the brain. The host (normal) cellular PrP (PrPc) is a broadly expressed, protease K-sensitive, detergent soluble, membrane glycoprotein with which presence is crucial for prion disease susceptibility. Intriguingly the difference between PrPSc and PrPc is mainly conformational because PrPSc has high β-sheet content in contrast to PrPc, which has a high α-helix content. This conformational change modifies the biochemical properties of the molecule confering characteristics such as amyloid formation, detergent insolubility, and protease K resistance (1). The “prion hypothesis” postulates that PrPSc constitutes the infectious component, which acts as a “seed” to facilitate the conversion of PrPc into PrPSc (2).

Mucosal or epithelial exposure (intestine or skin) to TSE agents has been implicated in the transmission of disease, after which accumulation of PrPSc is detected in lymphoid organs, in particular associated with nodal dendritic cells (FDC) in B cell follicles (3, 4). In humans, Kuru represents the classical example of this transmission (5); a similar mechanism occurs in scrapie and bovine spongiform encephalopathy. Neuroinvasion occurs later in the infection and involves the translocation of PrPSc via peripheral nerves and its accumulation in the CNS, after which the characteristic pathology of the disease develops.

A role for complement in prion diseases was first recognized in animal models that lacked (genetically or temporally) complement components, in which delayed onset of CNS disease was observed after i.p. inoculation of TSE agents (6, 7). Interestingly this delay was observed only after peripheral inoculation of the TSE agent, suggesting a crucial role for complement at early time points of infection. The mechanisms responsible for this delay are currently undefined. One possibility is reduced PrPSc retention by FDC. FDC are stromally derived cells, located within B cell follicles, which express high levels of both complement and Fc receptors, permitting them to retain (complement-containing) immune complexes on their surfaces (8). After oral inoculation of TSE agents, PrPSc accumulates on FDCs in Peyer’s patches (PP) and mesenteric lymph nodes (MLN), this accumulation is essential for the ensuing neuroinvasion (9). Recently the role of CD21/35 in Ag retention by FDCs has also been addressed, showing that these complement receptors mediate the early retention and accumulation of PrPSc on FDCs (10).

An alternative or additional mechanism underlying the delayed development of prion disease in the absence of complement components may involve conventional dendritic cells (cDCs). The cDCs are the most efficient APC and constitute a link between innate and adaptive immune responses. Widely and strategically distributed in nonlymphoid tissues, immature cDCs efficiently acquire Ags, migrate constitutively to regional lymph nodes, and...
present Ags to lymphocytes initiating adaptive immune responses (11). After oral inoculation of TSE agents, PrPSc must not only cross the intestinal epithelium but also be transported to the B cell follicles where FDCs reside. It is likely that cDCs participate in these events because they would rapidly interact with PrPSc after oral inoculation. The cDCs may directly capture PrPSc from the gut lumen or after translocation by M cells, and transport it to lymphoid organs. We have shown that cDCs can acquire and transport TSE agents from the intestinal lumen (12). In addition, in the absence of cDCs in the intestinal mucosa, the early accumulation of PrPSc in PP is blocked (13), highlighting the crucial role of cDCs in the early stages of the infection.

The complement component C1q is the recognition protein of the classical pathway. When C1q binds to a target surface, the serine proteases Clr and Cls, which are bound to C1q, are activated. C1s then cleaves C4 and C2, forming the C3-convertase complex, C4b2a. When C3 is activated, it binds covalently to the target surface, and is subsequently degraded to iC3b and C3dg. C3b and iC3b are major opsonins (14). C1q binds via its globular heads to a wide range of foreign or altered self-targets. These include Gram-negative bacteria, several viruses, apoptotic cells and products of tissue damage, and certain aggregates such as IgG- and IgM-containing immune complexes, and also amyloids (15, 16). Additionally C1q can act as an opsonin (17). In relation to TSE pathogenesis, the interaction between C1q and recombinant PrP is dependent on conformational modifications of PrP (18), and density of packing of PrP (19), suggesting that this interaction might be enhanced after PrPSc conversion. PrPSc can also activate the complement classical pathway (19, 20), suggesting again that the PrPSc and C1q interact directly, but whether this phenomenon occurs in vivo is unclear.

In this study we have assessed the role of complement in the endocytosis of PrPSc by cDCs. PrPSc uptake was evaluated after in vitro coculture of cDCs with whole brain homogenate (WBH) from terminally ill mice infected with the ME7 scrapie strain as a source of PrPSc. We show that in the absence of C1q, there is a significant decrease in PrPSc uptake by cDCs and that C1q-mediated uptake is very probably receptor-mediated. Finally, we confirmed the expression of calreticulin, a C1q receptor, on cDCs. Our results demonstrate that C1q participates in PrPSc uptake by cDCs, suggesting an important role in initial capture by intestinal cDCs, which will then ferry the protein to secondary lymphoid tissues where it accumulates at early time points of infection.

Materials and Methods

Mice and reagents

Specific pathogen-free, adult (6–8 wk) C57BL/6 mice were obtained from Harlan Sprague-Dawley. Scrapie ME7-infected mouse brains were provided by Dr. N. Mabbott (Roslin Institute, Edinburgh, U.K.). Scrapie RML-infected C1q−/− mouse brains were provided by Dr. A. Aguzzi (Institute of Neuropathology, Zürich, Switzerland). The 10% WBH (10% w/v) from terminally ill mice infected with the ME7 scrapie strain as a source of PrPSc. We show that in the absence of C1q, there is a significant decrease in PrPSc uptake by cDCs and that C1q-mediated uptake is very probably receptor-mediated. Finally, we confirmed the expression of calreticulin, a C1q receptor, on cDCs. Our results demonstrate that C1q participates in PrPSc uptake by cDCs, suggesting an important role in initial capture by intestinal cDCs, which will then ferry the protein to secondary lymphoid tissues where it accumulates at early time points of infection.

Uptake assays

The cDCs (1 × 10^6 in 1 ml of serum-free medium) were incubated with 1 mg/ml 10% (w/v) brain homogenate from terminally ill ME7 scrapie-infected C57BL/Dk mice. Normal C57BL/6 mouse brain homogenate was used as a control. Cultures were supplemented with 10% v/v untreated mouse serum, 10% heat-inactivated (56°C, 30 min) mouse serum, or 10% C1q−/− mouse serum as stated. Where indicated, FITC-dextran (500 μg/ml) was also added to the cultures. Incubations were maintained for the indicated times at 37°C in 5% CO₂.

Immunofluorescence

After incubation, cells were washed and cytospins prepared (~1 × 10^5 cells per cytospin). Cells were fixed in cold acetone for 10 min, and Fc receptors blocked with anti-CD16/32 (1 μg/ml) for 1 h at room temperature. Samples were treated with 3% guanidine isothiocyanate (GdnSCN/10 mM Tris-HCl (pH 7.8)) for 10 min at room temperature. Primary mAbs at optimal concentrations were incubated for 2 h at room temperature. PrP was detected using an Alexa 488-conjugated anti-PrP mouse mAb (POM2). Double staining was performed with biotin-conjugated anti-CD11c mAb, and detected with Alexa Fluor 555-conjugated streptavidin. After incubation, cells were washed with PBS/0.1% BSA and mounted using Vectashield (DakoCytomation). Images were acquired with a Zeiss microscope (Axioplan 2).

Flow cytometry

After incubation, cells were washed with cold RPMI 1640. To eliminate any free WBH, CD11c⁺ cells were enriched by MACS according to the manufacturer’s instructions (Miltenyi Biotec). After enrichment, cells were washed in FACS buffer (PBS/2% FBS/0.01% sodium azide) and processed for multicolor FACS analysis. Before staining, cells were incubated with anti-CD16/32 for 15 min at 4°C. Surface staining was performed first; primary mAbs were added for 20 min at 4°C. Intracellular staining was performed according to the manufacturer’s instructions (BD Biosciences) using an anti-PrP mAb (H9262) for 15 min at room temperature as primary Ab, and an Alexa Fluor 488- or 555-conjugated anti-mouse IgG1 mAb as the secondary Ab. Appropriate isotype controls and nonpermeabilized samples were included in every set of experiments. Cells were analyzed using a FACSCalibur cytometer and FlowJo software.

Detection of PrPSc by immunoblot

After incubation, the cells were washed in 0.1 M Tris (pH 7.4) and lysed with 2% (w/v) Sarkosyl (Sigma-Aldrich) dissolved in PBS (pH 7.4), as positive and negative controls 0.5 mg of ME7-infected WBH and uninfected WBH were used. Samples were divided into two, and one set was treated with proteinase K (100 μg/ml; Roche) for 1 h at 37°C with constant agitation. The reaction was stopped with PMSF (5 mM final concentration; Sigma-Aldrich). PrPSc was precipitated from untreated and proteinase K-treated samples using 4% sodium phosphotungstic acid (Sigma-Aldrich). Samples were run in a 4–12% acrylamide Invitrogen SDS-PAGE gel and treated with proteinase K (100 μg/ml) for 1 h at room temperature. PrP was detected using an Alexa Fluor 488-conjugated anti-mouse IgG (0.5 μg/ml diluted in 1% w/v BSA in PBS) for 1 h at room temperature. PrP was also detected using an Alexa Fluor 555-conjugated anti-mouse IgG (0.5 μg/ml diluted in 1% v/v BSA in PBS) for 1 h at room temperature. The blot was then washed three times for 15 min with 0.2% v/v Tween 20, 0.5 mM EDTA in PBS. A HRP-conjugated goat anti-mouse IgG Ab (0.5 μg/ml diluted in 1% w/v BSA in PBS) was used as the secondary reagent. Finally, chemiluminescence was developed using the ECL reagent (Pierce).

Statistical analysis

Data are expressed as the mean ± the SD. Statistical significance comparing multiple experimental groups was analyzed using the one-way ANOVA test, followed by the Bonferroni post hoc test.

1306 C1q PARTICIPATES IN PrPSc UPTAKE BY cDCs
Complement enhances PrPSc uptake by cDCs

To evaluate uptake of PrPSc by cDCs, purified MLN cDCs were cultured with ME7-infected WBH, uninfected WBH, or medium alone either in the presence of 10% normal mouse serum (right) or with 10% heat-inactivated mouse serum (left) for 12 h. After incubation cDCs were labeled for CD11c (red) and PrP (green) on cytospins. Scale bar represents 5 μm.

FIGURE 1. Complement enhances PrPSc uptake by MLN cDCs. MLN cDCs were enriched by negative selection as described and cocultured with ME7-infected WBH, uninfected WBH, or medium alone either in the presence of 10% normal mouse serum (right) or with 10% heat-inactivated mouse serum (left) for 12 h. After incubation cDCs were labeled for CD11c (red) and PrP (green) on cytospins. Scale bar represents 5 μm.

Results

Complement enhances PrPSc but not PrPc uptake by cDCs

To evaluate uptake of PrPSc by cDCs, purified MLN cDCs were cultured with ME7-infected WBH, uninfected WBH or medium alone, in the presence of normal mouse serum or heat-inactivated (complement-inactivated) mouse serum. After incubation, cells were washed, cytospins prepared and PrP uptake by CD11c+ cells evaluated by immunofluorescence. As previously described, cDCs express endogenous PrP (21); however, a clear increase in the PrP (green) signal was observed in cDCs that had been incubated with ME7-infected WBH, in comparison with those cells incubated with uninfected WBH or the medium control.

Uptake of PrP from infected-WBH was greater in the presence of normal mouse serum than with heat-inactivated WBH, showing that PrP uptake by cDCs is an active process (Fig. 1, bottom panels), whereas uptake of PrP from uninfected WBH was not affected by heat treatment of serum. Traces of free WBH remained in our preparations, as shown in the samples cultured with uninfected WBH.

To quantify PrP uptake by cDCs we used flow cytometry as a readout. We confirmed our previous results using spleen cDCs and performing intracellular staining for PrP in permeabilized and nonpermeabilized samples. Importantly, to eliminate any free WBH, CD11c+ cells were enriched by MACS after culture. Cells cultured in medium alone expressed PrP, but a significant increase in PrP signal was revealed by the mean fluorescence intensity (MFI) in cells cultured with either normal or infected WBH (Fig. 2A, top panels). Again, uptake of PrP from ME7-infected WBH but not uninfected WBH decreased when heat-inactivated serum was used (percentage of CD11c+/PrPhigh cDCs) (Fig. 2B). To demonstrate that the increase in PrP signal represented internalized PrP, samples were labeled without permeabilization (Fig. 2A, bottom panels), in which case no increase in PrP signal was detected. To confirm that cDCs had endocytosed proteinase K-resistant PrPSc, cells were lysed after incubation and samples were untreated or treated with proteinase K before immunoblotting. Fig. 2C shows that proteinase K-resistant PrP was present in cDCs incubated with ME7-infected WBH, confirming that cDCs have endocytosed PrPSc (the single band present in the proteinase K-treated medium and WBH samples represents background due to the secondary Ab). Together these data show that cDCs can endocytose PrPSc and suggest that uptake of PrPSc but not PrPc is increased in the presence of complement.

Complement C1q enhances PrPSc uptake by cDCs

We have shown that uptake of PrPSc by cDCs is decreased in the presence of heat-inactivated serum as compared with normal serum. The complement component primarily denatured by heat treatment is C1q (22), although Factor B and mannose-binding lectin are also inactivated at 56°C. To directly address the role of C1q in the uptake of PrP we incubated cDCs with uninfected WBH, ME7-infected WBH or medium alone, in the presence of normal serum or serum from C1q−/− mice and analyzed PrP uptake by flow cytometry. In the absence of C1q uptake of PrP from ME7-infected WBH was clearly diminished (Fig. 3A). In contrast, the uptake of PrP from normal brain was not affected by the absence of C1q.

Because the brain samples may contain small amounts of blood, some free C1q could be present in the WBH. In addition C1q mRNA is up-regulated in scrapie-infected mouse brains (23). To exclude a role for endogenous C1q during uptake, we used WBH from C1q−/− mice infected with RML scrapie. When cDCs were incubated with RML-infected WBH in the presence of normal serum a significant increase in PrP uptake was observed (Fig. 3B, top). Conversely, when C1q−/− serum was used, PrP uptake was markedly decreased. When cDCs were incubated with C1q−/− RML-infected WBH in the absence of C1q−/− serum, PrP uptake was almost abolished, strongly suggesting that uptake of PrP in this setting is largely C1q-dependent. From these results we conclude that C1q is directly involved in PrPSc uptake by cDCs.

Complement-dependent PrPSc uptake is primarily receptor-mediated

To show that complement-mediated PrP uptake is an active process we incubated cDCs with ME7-infected WBH in the presence of normal mouse serum for 4 h at 37°C or at 4°C. Cells were then permeabilized and in nonpermeabilized samples, had been incubated with ME7-infected WBH, in comparison with those cells incubated with uninfected WBH or the medium control. Uptake of PrP from infected-WBH was greater in the presence of normal mouse serum than with heat-inactivated serum (Fig. 1, bottom panels), whereas uptake of PrP from uninfected WBH was not affected by heat treatment of serum. Traces of free WBH remained in our preparations, as shown in the samples cultured with uninfected WBH.

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and enriched for CD11c⁺ cells (to eliminate any free WBH) and cultured for a further 2 h at 37°C. Cells were then stained for intracellular PrP. We observed internalization of the surface-bound PrP (cells cultured first at 4°C) when the samples were subsequently cultured at 37°C (Fig. 4B). As we reported previously, there is a statistical difference between the samples cultured with ME7-infected WBH and the control samples. If PrPSc uptake is receptor-mediated, surface binding should be saturable. To test this

![FIGURE 2](image)

**FIGURE 2.** Complement enhances PrPSc uptake by splenic cDCs. Splenic cDCs were enriched by negative selection and cocultured with ME7-infected WBH, uninfected WBH, or medium alone either in the presence of 10% normal mouse serum or with 10% heat-inactivated mouse serum for 12 h. PrP staining was performed on samples with and without permeabilization. A, FACS analysis of cDCs after incubation. CD11c⁺ cells were gated and the PrP signal analyzed using ME7-infected WBH (thick line histogram), uninfected WBH (thin line histogram), and medium alone (dotted line histogram). Data show MFI. ***, p < 0.001. B, Quantification of the frequency of CD11c⁺/PrP⁺ cells. Results are mean ± SD of five independent experiments. ***, p < 0.001. Normal mouse serum (□) and heat-inactivated mouse serum (◆) are used. C, Proteinase K resistance of PrP was evaluated by Western blot analysis. CD11c⁺ cells were enriched after culture to eliminate any traces of free WBH. The cells were lysed and treated with (+) or without (−) proteinase K, and PrPSc was precipitated by sodium phosphotungstic acid (NaPTA) before electrophoresis. The equivalent of 5 × 10⁵ cells was loaded per lane, as positive and negative controls ME7-infected WBH, and uninfected WBH samples were loaded on the same gel (lanes 6–10). The characteristic PrPSc migration shift after proteinase K treatment is present in (lanes 6 and 10). The single band (lanes 2 and 4) represents nonspecific binding of the secondary Ab.

![FIGURE 3](image)

**FIGURE 3.** Complement component C1q enhances PrPSc uptake by cDCs. Splenic cDCs were incubated with ME7-infected WBH, uninfected WBH, or medium alone in the presence of normal mouse serum or C1q⁻/C1q⁺ mouse serum for 12 h. PrP uptake was evaluated by FACS. A, Frequency of CD11c⁺/PrP⁺ cells after incubation using normal mouse serum (□) and C1q⁺ serum (◆) is shown. Results are mean ± SD of five independent experiments. ***, p < 0.001. B, cDCs were cultured with RML-infected C1q⁺ WBH (thick line histogram), uninfected WBH (thin line histogram), or medium alone (dotted line histogram), in the presence of normal mouse serum or C1q⁺ mouse serum for 12 h. After incubation, PrP uptake was analyzed by multicolor FACS analysis. Top panels show permeabilized samples, whereas bottom panels show samples without permeabilization. Data show MFI of PrP signal. ***, p < 0.001.
FIGURE 4. Complement-dependent PrPSc uptake by cDCs is mainly receptor-mediated. A, Splenic cDCs were incubated with ME7-infected WBH (thick line histogram), uninfected WBH (thin line histogram), or medium alone (dotted line histogram) for 4 h at either 37°C or 4°C, in the presence of normal mouse serum. Cells were washed after incubation and stained for PrP and CD11c. Samples were either permeabilized or not before PrP labeling. Results show the frequency of cells within the indicated gate. *** p < 0.001. B, Cultures were performed as described in A at either 37°C or 4°C during the initial 4 h. Cells were washed and CD11c+ cells were enriched by MACS before culturing them for a further 2 h at 37°C. Finally cDCs were labeled for PrP and CD11c. The frequency of CD11c+/PrPhigh cells is indicated as a percentage. Normal mouse serum (□) and heat-inactivated mouse serum (■) are used. Results are mean of three independent experiments ± SD. *** p < 0.001. C, cDCs were cultured with increasing concentrations of ME7-infected WBH in the presence of normal mouse serum for 12 h. PrP uptake was evaluated by FACS. Results are mean of three independent experiments ± SD. D, cDCs were cultured with ME7-infected WBH (thick line histogram), uninfected WBH (thin line histogram), or medium alone (dotted line histogram), in the presence of normal mouse serum or heat-inactivated mouse serum, in all conditions 0.5 mg/ml FITC-dextran was also added. After 12 h, cells were washed and labeled for multicolor FACS analysis. The PrP signal (top panels) and FITC-dextran uptake (bottom panels) by the same CD11c+ cells are shown. The filled histogram shows CD11c+ cells cultured in medium alone. Data show MFI of the PrP or FITC-dextran signal, respectively. *** p < 0.001.

possibility, we incubated cDCs with increasing concentrations of ME7-infected WBH in the presence of normal mouse serum and assessed PrP internalization by the increase in PrP MFI. Fig. 4C shows that uptake is saturable and plateaus at a concentration of 1 mg/ml.

To investigate the relationship of receptor-mediated PrPSc uptake with other internalization pathways, we used FITC-dextran as a marker for fluid phase internalization (24). Cocultures were performed as previously described, but in addition, FITC-dextran was included in the cultures. After 12 h in culture, cells were washed and CD11c+ cells analyzed by FACS for both PrP and FITC-dextran uptake. Fig. 4D (bottom) shows that in the presence of normal mouse serum or heat-inactivated serum (and independently of the coculture condition), cDCs internalize similar amounts of FITC-dextran (cDCs cultured in medium alone were also used) evaluated by the MFI. When PrP uptake was evaluated on the same cells, the same pattern of PrP uptake was observed (increased PrP uptake in the presence of normal mouse serum, which is statistically significant). Taken together these results suggest that complement-dependent PrPSc uptake is receptor-mediated and that this process is distinct from fluid phase internalization.

PrP and C1q colocalize during endocytosis

We have shown that the increased PrP uptake by cDCs in the presence of normal serum is C1q-dependent. To determine whether PrP and C1q are endocytosed together, cytospins were prepared after coculture and cells stained for PrP and C1q. Colocalization was observed in cells that were cultured with ME7-infected WBH (Fig. 5A), in most cases colocalization was seen in intracellular vesicles. Heat inactivation denatures C1q; accordingly no C1q signal was detected on the samples incubated in presence of heat-inactivated serum. Because WBH may contain traces of serum or FCS to evaluate the participation, of any mouse C1q population cocultures cultured with ME7-infected WBH. Colocalization was only observed when cells were cultured with mouse serum, strongly suggesting that the internalized C1q originates from serum added to the culture. These results suggest that PrP and C1q are being cointernalized by cDCs.

Complement receptors are expressed by different cDCs populations

We showed that not all cDCs endocytose PrP following in vitro culture with ME7-infected WBH. To show that this result was not concentration-dependent, we incubated splenic cDCs with increasing concentrations of ME7-infected WBH in the presence of normal mouse serum. The proportion cDCs that endocytosed PrP showed a dose-dependent increase that plateaued at 1 mg/ml (Fig. 6A). To determine whether uptake was associated
expression of CD103.

receptor calreticulin at high levels and it correlates with the ex-

markers was evaluated in splenic and MLN cDCs (Fig. 6

receptor-expressing cDCs that internalized PrP was evaluated by FACS. B, cDCs were enriched from spleen and MLN by negative selection with MACS and further processed for multicolor FACS analysis. Results show the expression of complement receptors (black line histogram) on CD11chigh cells in the defined gate (gray line histogram, isotype control). C, Coexpression of several cDCs markers and calreticulin on splenic and MLN CD11chigh cells. The frequency is expressed as a percentage shown for dot plots.

with a particular cDC subset we analyzed cDCs for coexpression of CD8α and PrP. Similar proportions of CD11c⁺/CD8α⁺ and CD11c⁺/CD8α⁻ cDCs endocytosed PrP (data not shown) showing that uptake was not restricted to one of the well-defined cDCs subsets in the spleen.

Because the previous results suggested that internalization was receptor-mediated, we used FACS to screen expression of complement receptors on different populations of spleen and MLN cDCs. Fig. 6B shows histograms of complement receptor expression on CD11c⁺ cDCs, which is itself the α-chain of the iC3b receptor, CR4. Although cell surface calreticulin is expressed on cDCs from both spleen and MLN, it is important to note that MLN cDCs have a higher proportion of calreticulin-positive cells and also a higher level of expression. In contrast, CD21/CD35 (receptor for C3b, C3dg, and C4b) was expressed only on splenic cDCs. Finally, CD11b (part of the CD11b/CD18 CR3 complex that recognizes iC3b) is highly expressed on cDCs, especially on splenic CD11c⁺CD8α− cells. To characterize in more detail the phenotype of the calreticulin-positive cDCs coexpression of several markers was evaluated in spleen and MLN cDCs (Fig. 6C). We conclude that several splenic and MLN cDCs express several candidate receptors for C1q. Nevertheless cDCs populations relevant to PrPSc transmission in vivo, such as MLN cDCs, express the C1q receptor calreticulin at high levels and it correlates with the expression of CD103.

The cDCs that take up PrPSc express calreticulin

Finally, to address the participation of calreticulin as a candidate receptor in the complement-mediated uptake of PrPSc by cDCs, cultures supplemented with normal serum were set up as previously described (WBH, ME7-infected WBH, or medium alone). After culture, cDCs were stained to evaluate PrP uptake by FACS, additionally the surface expression of calreticulin was evaluated. Fig. 7A shows the increase in the PrP signal from cDCs cocultured with ME7-infected WBH. Interestingly ~70% of cDCs that had taken up PrPSc (CD11c⁺/PrPhigh, population within the indicated gate) express cell surface calreticulin. Conversely, cells cultured in presence of uninfected WBH that take up PrP do not express calreticulin.

Because the direct participation of calreticulin during the uptake of PrPSc will result in the internalization of both molecules, we evaluated by microscopy the colocalization of PrP, calreticulin, and C1q. Fig. 7B shows representative of the triple staining, where colocalization was always observed as intracellular vesicles. As we showed previously in Fig. 5, PrP and C1q clearly colocalize only in cDCs cultured with ME7-infected WBH. Importantly we show that calreticulin also colocalizes in the same vesicles. From these results we conclude that calreticulin is directly involved in the C1q-mediated uptake of PrPSc by cDCs.
expression were evaluated. The PrP signal and extracellular calreticulin
infected WBH, uninfected WBH or medium alone, in the presence of 10%
MLN cDCs were enriched by negative selection and cocultured with ME7-
expression was evaluated on the CD11chighPrPhigh (indicated
gate). B, Triple labeling for PrP (green), calreticulin (red), and C1q
A role for complement in prion diseases was first revealed by
delayed disease development in animal models lacking comple-
ment components (6, 7). This development could represent a pri-
mary defect in accumulation/retention of PrPSc by FDCs, but could
also be a consequence of decreased uptake and transport from the
periphery by cDCs. It is known that migratory cDCs can acquire
and transport intestinal PrPSc (12) and temporary depletion of
cDCs because the former are likely to be the cells that would
up-regulation of extracellular PrP over the culture period (Fig. 2C).
Our results show that C1q will interact with the globular portion of C1q.
study using bone marrow-derived cDCs (25), splenic cDCs (26), and Langerhans-
cells in the intestine (36). Thus in vivo, free C1q from
scrapie as a source of prions, RML. In this case a similar uptake
pattern with the participation of C1q was observed, confirming that
our observation is not strain-dependent (Fig. 3A). In particular, in
this setting we observed a complete abrogation of PrPSc uptake by
cDCs in the absence of C1q. This could represent a strain-specific
phenomenon because we cannot rule out the possibility that some
PrPSc is being taken up by fluid phase endocytosis (see Figs. 2
and 3).
PrPSc uptake by cDCs has been previously studied using bone
marrow-derived cDCs (25), splenic cDCs (26), and Langerhans-
like cells (27). In all cases PrPSc internalization has been observed after
time periods (30 min and 2 h); however, the frequency of the cDCs that take up PrPSc was not addressed. An
important feature of these reports is that cultures were supple-
mented with heat-inactivated serum, which would exclude a role for complement. We chose to use cDCs obtained from lymphoid tissues in the steady state instead of bone marrow-derived
cDCs because the former are likely to be the cells that would
relate most closely to in vivo events. Our results show that both
MLN and splenic cDCs internalized PrPSc after culture in the
presence of normal mouse serum (Figs. 1 and 2). However,
whereas only 30–40% of the CD11c\textsuperscript{high} cells were able to in-
ternalize PrPSc even after 18 h of culture, all CD11c\textsuperscript{high} cDCs
dendocytosed FITC-Dextran after 2 h in culture, suggesting that
only a subpopulation of cDCs is capable of complement-medi-
ated PrPSc uptake. Additionally, that cDCs internalized similar
amounts of FITC-Dextran under all culture conditions suggests
that WBH was not inducing cDC activation during the culture
periods (Fig. 4D).
The cDCs cultured with uninfected WBH also showed uptake of PrPSc but importantly no difference was observed when heat-
inactivated rather than intact mouse serum was used (Fig. 2). These
results indicate that PrPSc but not PrP\textsuperscript{+} is internalized by a
complement-dependent mechanism. C1q is known to interact
with conformationally modified PrP\textsuperscript{+} (18), suggesting that it
may only interact with PrPSc. Moreover, PrPSc can activate the
classical complement pathway (19, 20), which strongly sug-
gests that PrPSc will interact with the globular portion of C1q.
We used FACS analysis to evaluate PrPSc uptake and compared
staining in cells with and without permeabilization. In the nonper-
meabilized samples, only extracellular PrP was detected (i.e., natu-
relly occurring protein and surface-bound protein). The expression
of PrP on cDCs is well documented (21, 28, 29) and is up-regu-
lated on mature cDCs (30). In our system we did not observe up-regulation of extracellular PrP over the culture period (Fig. 2A);
confirming first that the difference in PrP signals between perme-
abilized and nonpermeabilized samples represents the actual up-
take, and secondly that the maturation stage of the cDCs is not
modified during the culture. Some PrP\textsuperscript{+} may be intracellular (31,
32), which would explain why the expression of PrP in the medium
control differs between permeabilized and nonpermeabilized
samples.
FIGURE 7. Calreticulin expression by cDCs that have taken up PrPSc.
MLN cDCs were enriched by negative selection and cocultured with ME7-
infected WBH, uninfected WBH or medium alone, in the presence of 10%
normal mouse serum, for 12 h. The PrP signal and extracellular calreticulin
expression were evaluated. A, FACS analysis of cDCs after incubation.
CD11c\textsuperscript{high} cells were gated and the PrP signal analyzed. ME7-infected
WBH (thick line histogram), uninfected WBH (thin line histogram), and
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Several potential receptors have been described for C1q. These include CR1/CD35 (also a receptor for C3b and C4b), C1qRp/CD93, calreticulin (37, 38), and recently the αβ2 integrin (39). CR1/CD35 binds immune complexes and transports them to the liver and spleen mediating their clearance, and can also bind C1q (40). However, although CR1/CD35 is expressed by splenic cDCs (41), it is not expressed on MLN cDCs thus excluding it as a candidate receptor in our system. C1qRp is now known to act as an adhesion receptor and does not interact with C1q (42). Calreticulin, also known as the collectin receptor, is expressed in the cytoplasm and the endoplasmic reticulum by all nucleated cells. However, when expressed on the cell surface it forms the collagenous portion of C1q and mediates its internalization in association with CD91 (34, 43).

Extracellular expression of calreticulin has been described on macrophages and cDCs, particularly immature cDCs (44). We showed surface expression of calreticulin by cDCs from spleen and MLN (Fig. 6A). In both cases, receptor-expressing cells represent only ∼30–40% of the CD11c+ population, as has also been shown for human cDCs (45). Importantly in MLN cDCs calreticulin is expressed by the CD103+ cells, which are most likely derived from the intestine (Fig. 6B). By evaluating the expression of CD80 and CD11b, calreticulin does not appear to be expressed by a discrete population of cDCs. Additionally, calreticulin is expressed by MHC class IIhigh cells, which suggests that the molecule is not exclusively expressed on immature cDCs, as it has been proposed in studies using human cDCs (44). Importantly we confirm that a high proportion of the cDCs taking up PrPSc expressed C1q and mediates its internalization in association with CD91 (34, 43).

In conclusion efficient uptake of PrPSc by cDCs is C1q-dependent. Our results therefore suggest an alternative or complementary mechanism to explain the delay of prion disease development in the absence of C1q. Altogether our results provide new evidence that highlights the role of cDCs and complement at very early time points in prion disease pathogenesis, particularly after oral inoculation.

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Disclosures

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References


