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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. MacPherson**

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 (FDCs). 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 splenic 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.


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Abbreviations used in this paper: TSE, transmissible spongiform encephalopathies; C1q, complement component 1q; PrP, prion protein; PrPSc, scrapie prion protein; FDC, follicular dendritic cell; MFI, mean fluorescence intensity.

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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 readily 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 Cl1s, which are bound to C1q, are activated. Cls then cleaves C4 and C2, forming the C3-converter complex, C4b2a. When C3 is activated, it binds covalently to the target surface, and is subsequently degraded to iC3b and C3d. 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. A. Aguzzi (Institute of Neuropathology, Zürich, Switzerland). The 10% WBH (10% v/v) were prepared by weighing the brains and adding 9 mlg/kg of sterile (endotoxin-free) PBS. C1q−/− mouse serum was a gift from Dr. M. Botto (Imperial College, London, U.K.).

The following mAbs were purchased from BD Biosciences: purified anti-CD16/32, biotinylated anti-CD11c, FITC-conjugated CD11c, and PE-conjugated anti-CD11c, anti-CD11b, anti-CD21/CD23. Polyclonal Ab anti-calreticulin was purchased from AbD Serotec. Anti-mouse C1q mAb was purchased from Cedarlane Laboratories. Anti-PrP mAb 8H4 was purchased from Acris, and anti-PrP mAb POM2 was provided by Dr. A. Aguzzi. The following secondary mAbs were purchased from Molecular Probes: Alexa Fluor 488-conjugated anti-mouse IgG1, Alexa Fluor 555-conjugated anti-mouse IgG, Alexa Fluor 555-conjugated anti-rabbit IgG, Alexa Fluor 555-conjugated streptavidin, and Alexa Fluor 647-conjugated streptavidin. HRP-conjugated goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratories. FITC-dextran was purchased from Sigma-Aldrich.

Cell isolation

MLN and spleens were harvested in cold RPMI 1640 and single-cell suspensions were prepared after enzymatic digestion with 400 U/ml collagenase D (Roche), for 25 min at 37°C. The cDCs were enriched by negative selection using the following MACS beads (Miltenyi Biotec): CD19, CD5, and DX5. Cells were kept in MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) during enrichment. Purity after enrichment was > 75%, most contaminants being granulocytes, which were excluded during the analysis.

Uptake assays

The cDCs (1 × 10^6 in 1 ml of serum-free medium) were incubated with 1 mg/ml 10% (v/v) brain homogenate from terminally ill ME7 scrapie-infected C57BL/DKj 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 (35°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 μM 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 (DakoCytometry). 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/0.05% 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 (8H4) 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 FACS Calibur cytomter 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% (v/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 protease 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 protease K-treated samples using 4% sodium phosphotungstic acid (Sigma-Aldrich). Samples were run in a 4–12% acrylamide Invitrogen SDS-PAGE gel and then transferred to an Immobilon membrane. Blots were blocked for 1 h with 2% w/v BSA in PBS at room temperature. PrP was detected using the 8H4 mAb (0.5 μg/ml diluted in 1% w/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 antibody (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 ± SD. Statistical significance comparing multiple experimental groups was analyzed using the one-way
were washed, cytospins prepared and PrP uptake by CD11c
alone, in the presence of normal mouse serum or heat-inactivated
cultured with ME7-infected WBH, uninfected WBH or medium
alone, in the presence of normal mouse serum for 4 h at 37°C or at 4°C. Cells were then
permeabilized or left intact before labeling for PrP. As shown (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<sup>c</sup>/PrP<sup>high</sup>
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
dendocytosed proteinase K-resistant PrP<sup>Sc</sup>, 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 PrP<sup>Sc</sup> (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 PrP<sup>Sc</sup> and suggest that uptake of PrP<sup>Sc</sup> but not PrP<sup>c</sup> is increased in the presence of complement.

**Complement C1q enhances PrP<sup>Sc</sup> uptake by cDCs**

We have shown that uptake of PrP<sup>Sc</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> serum was used, PrP uptake was markedly decreased. When cDCs were incubated with C1q<sup>−/−</sup>-RML-infected WBH in the presence of C1q<sup>−/−</sup> 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 PrP<sup>Sc</sup> uptake by cDCs.

**Complement-dependent PrP<sup>Sc</sup> 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 or left intact before labeling for PrP. As shown (Figs. 2 and 3), cDCs incubated with ME7-infected WBH at 37°C endocytosed PrP<sup>Sc</sup>, indicated by the increased MFI value (medium, 206; WBH, 220; ME7-WBH, 250; p < 0.001). However, when cultures were maintained at 4°C there was a clear reduction of the PrP uptake due to a reduction of the frequency to cells that internalized it (as shown in the indicated gate, additionally a reduction in the MFI value for the whole population was observed: medium, 198; WBH, 204; ME7-WBH, 215) compared with cultures at 37°C, showing that PrP uptake by cDCs is an active mechanism (Fig. 4A, top panels). Importantly, we observed a similar frequency of cells within the indicated gate in permeabilized and in nonpermeabilized cells when cultured at 4°C, showing that PrP was surface-bound, also suggesting a receptor-mediated mechanism.

To ascertain whether we could induce uptake of surface-bound PrP, cells were cultured with WBH at 4°C or 37°C, then washed
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. 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⁺/PrP⁺ 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. Normal mouse serum (□) and heat-inactivated mouse serum (■) are used. 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. 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⁻/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.
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 PrP<sup>Sc</sup> 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<sup>+</sup> 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<sup>-</sup>/PrP<sup>-</sup> cells was 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<sup>+</sup> cells are shown. The filled histogram shows CD11c<sup>-</sup> cells cultured in medium alone. Data show MFI of the PrP or FITC-dextran signal, respectively. *** p < 0.001.

**FIGURE 4.** Complement-dependent PrP<sup>Sc</sup> 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<sup>+</sup> 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<sup>-</sup>/PrP<sup>+</sup> 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<sup>+</sup> cells are shown. The filled histogram shows CD11c<sup>-</sup> cells cultured in medium alone. Data show MFI of the PrP or FITC-dextran signal, respectively. *** p < 0.001.

**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, cytofilms were prepared after coculture and cells stained for PrP and C1q. Colocalization was observed (increased PrP uptake in the presence of normal 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.

**FIGURE 5.** Pairs of representative pictures of samples cultured with ME7-infected WBH. A) C1q and PrP colocalize in cells 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 and as C1q expression may be up-regulated on ME7-infected WBH, the cultures were supplemented with mouse serum or FCS to evaluate the participation, of any mouse C1q present in the WBH. B) shows representative pictures of samples 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...
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 cDC 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 splenic 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% normal mouse serum, for 12 h. The PrP signal and extracellular calreticulin expression were evaluated. A, FACS analysis of cDCs after incubation. CD11c<sup>high</sup> cells were gated and the PrP signal analyzed. ME7-infected WBH (thick line histogram), uninfected WBH (thin line histogram), and medium alone (dotted line histogram) are used. Data show MFI. Extracellular calreticulin expression was evaluated on the CD11c<sup>high</sup>PrP<sup>high</sup> (indicated gate). B, Triple labeling for PrP (green), calreticulin (red), and C1q (blue) was performed.

FIGURE 7. Calreticulin expression by cDCs that have taken up PrP<sub>Sc</sub>. 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<sup>high</sup> cells were gated and the PrP signal analyzed. ME7-infected WBH (thick line histogram), uninfected WBH (thin line histogram), and medium alone (dotted line histogram) are used. Data show MFI. Extracellular calreticulin expression was evaluated on the CD11c<sup>high</sup>PrP<sup>high</sup> (indicated gate). B, Triple labeling for PrP (green), calreticulin (red), and C1q (blue) was performed.

Discussion

A role for complement in prion diseases was first revealed by delayed disease development in animal models lacking complement components (6, 7). This development could represent a primary defect in accumulation/retention of PrP<sub>Sc</sub> 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 PrP<sub>Sc</sub> (12) and temporary depletion of cDCs before feeding infected WBH greatly slows down the accumulation of PrP<sub>Sc</sub> on FDCs and the development of disease (13). How cDCs acquire PrP<sub>Sc</sub> is not clear and the current experiments were designed to test the hypothesis that complement may be involved in the uptake.

PrP<sub>Sc</sub> uptake by cDCs has been previously studied using bone marrow-derived cDCs (25), splenic cDCs (26), and Langerhans-like cells (27). In all cases PrP<sub>Sc</sub> internalization has been observed after early time points (between 30 min and 2 h); however, the frequency of the cDCs that take up PrP<sub>Sc</sub> was not addressed. An important feature of these reports is that cultures were supplemented 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 PrP<sub>Sc</sub> after culture in the presence of normal mouse serum (Figs. 1 and 2). However, whereas only 30–40% of the CD11c<sup>high</sup> cells were able to internalize PrP<sub>Sc</sub> even after 18 h of culture, all CD11c<sup>high</sup> cDCs endocytosed FITC-Dextran after 2 h in culture, suggesting that only a subpopulation of cDCs is capable of complement-mediated PrP<sub>Sc</sub> 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 PrP<sub>P</sub> but importantly no difference was observed when heat-inactivated rather than intact mouse serum was used (Fig. 2). These results indicate that PrP<sub>Sc</sub> but not PrP<sub>P</sub> is internalized by a complement-dependent mechanism. C1q is known to interact with conformationally modified PrP<sub>P</sub> (18), suggesting that it may only interact with PrP<sub>Sc</sub>. Moreover, PrP<sub>Sc</sub> can activate the classical complement pathway (19, 20), which strongly suggests that PrP<sub>Sc</sub> will interact with the globular portion of C1q.

We used FACS analysis to evaluate PrP<sub>Sc</sub> uptake and compared staining in cells with and without permeabilization. In the nonpermeabilized samples, only extracellular PrP<sub>P</sub> is detected (i.e., normally occurring protein and surface-bound protein). The expression of PrP on cDCs is well documented (21, 28, 29) and is up-regulated on mature cDCs (30). In our system we did not observe up-regulation of extracellular PrP<sub>P</sub> over the culture period (Fig. 2A); confirming first that the difference in PrP signals between permeabilized and nonpermeabilized samples.

The direct role of C1q in PrP<sub>Sc</sub> uptake by cDCs was confirmed by the reduced uptake of PrP<sub>Sc</sub> in cultures in the presence of C1q<sup>−/−</sup> serum (Fig. 3A). C1q represents the first element of the classical complement cascade, and participates in several functions besides complement activation such as removal of apoptotic cells (17, 33, 34) and immune complexes (35). Importantly to evaluate the PrP<sub>Sc</sub> uptake in a C1q free assay we used a different strain of 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. 3B). In particular, in this setting we observed a complete abrogation of PrP<sub>Sc</sub> uptake by cDCs in the absence of C1q. This could represent a strain-specific phenomenon because we cannot rule out the possibility that some PrP<sub>Sc</sub> is being taken up by fluid phase endocytosis (see Figs. 2 and 3).

Much of our evidence suggests that C1q-dependent PrP<sub>Sc</sub> uptake is receptor-mediated. The reduced internalization observed in cultures maintained at 4°C is indicative of an active process. Nevertheless, we detected an increased PrP signal in nonpermeabilized cells cultured at 4°C, showing the presence of surface-bound PrP (Fig. 4A). By using a two-step incubation system, we confirmed that the FACS signal observed at 4°C in the nonpermeabilized samples was surface-bound PrP<sub>Sc</sub> (Fig. 4B). Furthermore, saturation of PrP<sub>Sc</sub> uptake by culturing cDCs with increasing concentrations of ME7-infected WBH strongly suggests that the uptake mechanism is receptor mediated (Fig. 4C).

We also showed by immunofluorescence that cDCs that had endocytosed PrP<sub>Sc</sub> showed clear colocalization with C1q in the form of intracellular vesicles. Importantly colocalization of PrP and C1q was not observed when the cells were cultured with uninfected WBH, confirming C1q only interacts with PrP<sub>P</sub>. As our results show that C1q plays an important role in the PrP<sub>Sc</sub> uptake by cDCs, an important element to consider is the availability of C1q in the intestinal mucosa. In contrast with most complement components, which are synthesized by the liver, C1q is synthesized by macrophages, cDCs and, importantly, by epithelial cells in the intestine (36). Thus in vivo, free C1q from any of the mentioned sources could be available to interact with PrP<sub>Sc</sub>.
Several potential receptors have been described for C1q. These include CR1/CD35 (also a receptor for C3b and C4b), C1qRp/CD93, calreticulin, and the α/β2 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 binds 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 CD8α and CD11b, calreticulin does not appear to be expressed by a discrete population of cDCs. Additionally, calreticulin is expressed by MHC class II+ 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 calreticulin, which suggests the direct participation of this receptor. Alternatively other molecules such as scavenger receptors might be involved because the molecular properties of the C1q-PrPSc aggregates could change, affecting the affinity and the avidity to the described receptors.

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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