

# Immunologically Induced, Complement-Dependent Up-Regulation of the Prion Protein in the Mouse Spleen: Follicular Dendritic Cells Versus Capsule and Trabeculae<sup>1</sup>

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The expression of the prion protein (PrP) in the follicular dendritic cell network of germinal centers in the spleen is critical for the splenic propagation of the causative agent of prion diseases. However, a physiological role of the prion protein in the periphery remains elusive. To investigate the role and function of PrP expression in the lymphoid system we treated naive mice i.v. with preformed immune complexes or vesicular stomatitis virus. Immunohistochemistry and Western blot analysis of the spleen revealed that 8 days after immunization, immune complexes and vesicular stomatitis virus had both induced a strong increase of PrP expression in the follicular dendritic cell network. Remarkably, this up-regulation did not occur in mice that lack an early factor of the complement cascade, C1q, a component which has been shown previously to facilitate early prion pathogenesis. In addition to the variable PrP level in the germinal centers, we detected steady and abundant PrP expression in the splenic capsule and trabeculae, which are structural elements that have not been associated before with PrP localization. The abundant trabeculo-capsular PrP expression was also evident in spleens of Rag-1-deficient mice, which have been shown before to be incapable of prion expansion. We conclude that trabeculo-capsular PrP is not sufficient for splenic prion propagation. Furthermore, our observations may provide important clues for a physiological function of the prion protein and allow a new view on the role of complement and PrP in peripheral prion pathogenesis. *The Journal of Immunology*, 2003, 170: 6040–6047.

Prion diseases or transmissible spongiform encephalopathies (TSEs)<sup>4</sup> belong to a group of fatal neurodegenerative diseases in humans and animals (1, 2). Peripheral prion pathogenesis is dependent upon components of the host immune system. Follicular dendritic cells (FDCs) of splenic germinal centers and lymph nodes play an important role in the peripheral pathogenesis. There is strong evidence that expression of the cellular PrP by these cells is involved in the peripheral accumulation or replication of the infectious agent. First, prion infectivity is early and abundantly detected in the spleen and in lymph nodes following infection (3). Second, the pathological form of PrP, which is associated with prion infectivity, accumulates in the FDC network (4–7). Third, the absence of FDCs expressing the normal cellular PrP interferes with the fatal course of peripherally initiated TSE (8). Further analysis on the role of FDCs in the pathogenic process resulted in the discovery that the complement system facilitates splenic prion propagation and neuroinvasion of prions (9,

10). Despite these recent advances, the exact role of PrP-expressing FDCs in prion diseases has not yet been determined. Its role might well be related to an immunological function of PrP under nonprion pathogenic conditions. However, a clear function for PrP in lymphoid and nonlymphoid tissues has remained elusive (11).

The aim of the present study was to find a clue to the immunological role of PrP, which in turn may be linked to the function of FDCs in the maturation and maintenance of a humoral immune response (12, 13). We examined whether the PrP expression pattern or level might be altered in spleens of experimental mice following involvement of FDCs in humoral immune responses. For immune stimulation, we treated mice with either preformed immune complexes (ICs) of HRP and mouse-monoclonal anti-HRP IgG, or vesicular stomatitis virus (VSV), a member of the Rhabdoviridae family. Treatment with preformed ICs has been reported to lead to IC trapping on the surface of FDCs and presentation to germinal center B cells (14). An infection with VSV, in contrast, is defeated primarily by a neutralizing IgM response, but also elicits a germinal center reaction that is characterized by long-time persistence of VSV Ag on FDCs and the maintenance of a memory IgG titer (15).

The results from our experiments show that prominent PrP expression in the immunologically naive mouse spleen is not limited, as suggested in numerous previous reports, to the germinal center but is predominant in the splenic capsule and trabeculae. Following immune stimulation of mice with preformed ICs or VSV, we observe a strong increase of PrP expression in the FDC network of germinal centers, surpassing the unaltered level in the capsule and trabeculae. We estimate that PrP up-regulation in the FDC network can be in the order of 6-fold or more. In addition, we demonstrate that up-regulation of PrP upon immune stimulation involves the complement system, as mice deficient for C1q, an early component of the classical arm of the complement system, did not show this phenomenon under equal experimental conditions. These results lead to considerations about the normal function of PrP as well as

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<sup>4</sup> Abbreviations used in this paper: TSE, transmissible spongiform encephalopathy; FDC, follicular dendritic cell; PrP, prion protein; IC, immune complex; VSV, vesicular stomatitis virus; LCMV, lymphocytic choriomeningitis virus; C<sub>T</sub>, cycle number at detection above threshold; CR1, complement receptor 1; wt, wild type.

consequences of immune stimulatory effects for the course of prion diseases.

## Materials and Methods

### Mice and virus

Wild-type (wt) and Rag-1<sup>-/-</sup> (C57BL/6) mice were purchased from the Institute for Laboratory Animals (University of Zürich, Switzerland). C1qA<sup>-/-</sup> mice on a C57BL/6 background were a gift from M. Botto (Imperial College of Science, London, U.K.). *Prnp*<sup>Tga20</sup> and *Prnp*<sup>0/0</sup> mice were provided by A. Aguzzi (Institute of Neuropathology, University Hospital, Zürich, Switzerland). Experiments were performed according to institutional animal care guidelines, and mice were used at the ages of 9–12 wk. VSV Indiana (Mudd-Summers isolate) was originally obtained from D. Kolakovsky (University of Geneva, Geneva, Switzerland). For virus stock production, BHK21 cells were infected at a multiplicity of infection of 0.01. After 2 h of incubation at room temperature, the initial inoculum was discarded and replaced by fresh medium. The virus was harvested after 22 h of incubation at 37°C from the second supernatant.

### Immune stimulation of mice and tissue harvesting

A single bolus of 100 µg of soluble complexes of HRP and anti-peroxidase mouse monoclonal IgG1 (PAP mouse clone P6-38; Sigma-Aldrich, St. Louis, MO) or of 2 × 10<sup>6</sup> PFU of VSV Indiana was administered to mice by tail vein injection. Four, 8, or 12 days after injection mice were sacrificed and spleens removed, apportioned and immediately frozen in liquid nitrogen for later analyses by immunohistochemistry, Western blotting, and real-time RT-PCR. Successful VSV infection of C1qA<sup>-/-</sup> mice was confirmed by ELISA of spleen samples for anti-VSV IgM and IgG titers.

### Immunohistochemistry

Spleen tissue sections of 5-µm thickness were cut with a cryostat, placed on glass slides, air-dried for 1 min at 40°C and stored at room temperature. Immunostaining was conducted at the same day of sectioning, starting with brief rehydration in PBS and preincubation in PBS supplemented with 1% BSA and 0.1% saponin for 5 min. Primary Abs directed against PrP were diluted in PBS/BSA/saponin 1/600 (rabbit sera XN; Ref. 9) and 1B3 (16) and to 10 µg/ml (mouse monoclonal 6H4; Prionics, Zurich, Switzerland). Rabbit serum against lymphocytic choriomeningitis virus (LCMV) was diluted 1/500 (hyperimmune rabbit serum; provided by R. Zinkernagel, Institute of Experimental Immunology, University Hospital, Zürich, Switzerland). An Ab specific for mouse complement receptor 1 (CR1) (CD35) was used at a concentration of 1 µg/ml (rat monoclonal 8C12; BD Pharmingen, San Diego, CA). Incubation with primary Abs was conducted for 1 h at room temperature, as was the incubation with Alexa fluorochrome-conjugated secondary Abs (Molecular Probes, Eugene, OR). After washing the sections with PBS, coverslips were mounted with Moviol (Hoechst, Frankfurt am Main, Germany).

### Cell culture and immunocytochemistry

Mouse fibroblasts of the 3T3-Swiss albino cell line (CCL-92; American Type Culture Collection, Manassas, VA) were grown in DMEM/F12 supplemented with 10% FBS, 1% glutamax, and 0.5% sodium pyruvate. For PrP-specific immunofluorescence, staining cells were briefly washed with warm PBS and warm water and then completely dried with the warm air jet from a hair dryer. The subsequent staining procedure was the same as described for the air-dried spleen sections.

### Western blot analysis

Spleen tissue samples were processed to 10% homogenates in 100 mM TBS, pH 7.5, supplemented with 1% Tween, 1% Nonidet P-40, 0.5% sodium deoxycholate, and a protease inhibitor mixture (Complete; Roche, Basel, Switzerland). To obtain capsular and core fractions of a spleen, a frozen piece of spleen was placed onto a block of dry ice. With the tip of a scalpel, the capsular surface of the spleen was warmed and the thawed capsular region carefully peeled off and placed into buffer for homogenization. The remaining core portion was further trimmed to remove residual capsular fragments before homogenization.

Fibroblasts in culture were harvested by rinsing and detaching them with PBS/EDTA. The number of collected cells was determined by counting in a Neubauer chamber. Following sedimentation, cells were lysed in the homogenization buffer and the protein concentration was determined by colorimetry (Bio-Rad protein assay; Hercules, CA). Protein concentration, cell number, and actin staining were all used to control gel loading.

For each sample, 15 µl of spleen homogenate or cell lysate was loaded on a 12% SDS-PAGE gel. Following electrophoresis, proteins were trans-

ferred to nitrocellulose by wet blotting. Membranes were blocked with PBS/4% nonfat milk/0.2% Tween and incubated overnight at 4°C with Abs specific for PrP (1B3), β-actin (mouse monoclonal clone AC-74; Sigma-Aldrich), or Rab4 (rabbit serum, kindly provided by I. Mellman (Yale University, New Haven, CT)). Primary Ab binding was revealed with suitable secondary Abs and alkaline phosphatase-catalyzed color reaction.

### Densitometric quantification

Immunostained Western blot membranes were scanned and the resulting digital duplicates were analyzed with densitometry software (WinCam 2.1; Cybertech, Berlin, Germany). To monitor the accuracy of the densitometric reading, standard rows with defined dilution steps were repeatedly analyzed.

To quantitatively analyze the immunofluorescence signal on spleen sections, digital images were recorded with a fluorescence microscope equipped with a digital camera and subsequently analyzed with the densitometry software.

### RNA quantification by real-time RT-PCR

Total RNA from spleens or cells was obtained by direct lysis of the samples in TRI reagent (MRC, Cincinnati, OH) and further processing according to the manufacturer's protocol. The concentration and purity of the RNA samples were determined by spectrophotometry.

To perform real-time RT-PCR, a TaqMan RT-PCR One Step Master-Mix (Applied Biosystems, Foster City, CA) was used. For each reaction (40-µl volume) 100 ng of total RNA served as template. The PrP-specific primer-probe set was: forward 5'-ttggtggctacatgctggg-3', reverse 5'-cccagctgtgcccattg-3', probe FAM-5'-agccgatgagcaggccca-3'-TAMRA. For GAPDH-related RNA, the internal standard, the set was: forward 5'-actggcatggcctccg-3', reverse 5'-caggcggcagctcagatc-3', probe FAM-5'-ttctaccaccaatgtgtccctcgt-3'-TAMRA. All RT-PCRs were controlled and the data were recorded real-time with an ABI PRISM 7700 Sequence Detector (Applied Biosystems). For all the reactions, the cyclor conditions were as follows: reverse transcription at 48°C for 30 min; DNA amplification in 50 cycles: 95°C for 15 s and 60°C for 1 min. Resulting cycle number at detection above threshold (C<sub>T</sub>) values were used to calculate the relative abundance of PrP-specific RNA.

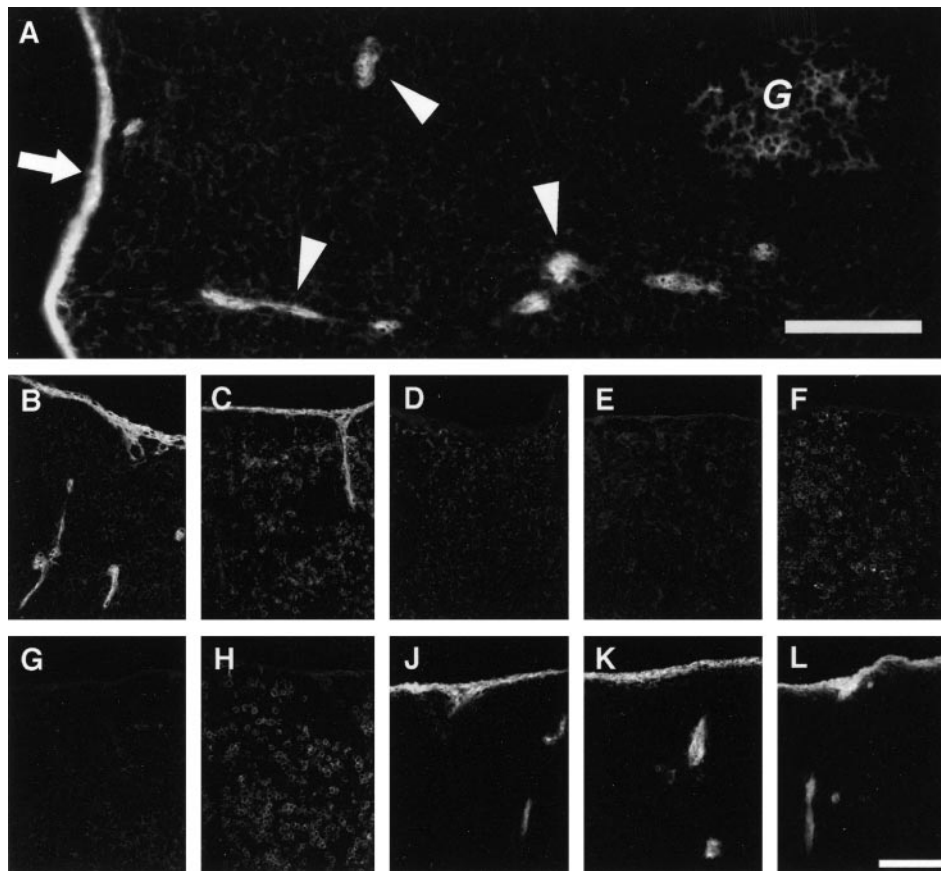
## Results

### Expression of the prion protein is most abundant in the capsule and trabeculae in control mouse spleens

Processing of mouse spleen tissue for PrP-specific immunohistochemistry was conducted in a way that kept the degree of physicochemical modifications as low as possible. In particular, any treatment like aldehyde, acetone, or alcohol fixation before or after cryosectioning was avoided. Cryostat sections of shock-frozen tissue were thawed and air-dried on glass slides and immediately used for immunohistochemistry.

To characterize the normal PrP expression in spleen, we examined the tissue from control C57BL/6 mice, which had been bred and housed under specified pathogen-free conditions. Immunostaining of PrP revealed a strong signal in the splenic capsule and trabeculae (Fig. 1). This was an unexpected finding because, to our knowledge, earlier reports on PrP in the spleen never mentioned a capsular or trabecular PrP expression pattern (5, 8, 11, 17), although the present results show that the trabeculocapsular signal may normally be much more prominent than the well-established signal in the germinal center areas. Because the connective tissue structures of capsule and trabeculae are prone to unspecific staining, a series of experiments was performed to rule out any possibility of a staining artifact. First, three different Abs directed against PrP, and established by other researchers for specific detection of PrP in mouse tissue samples, were used (9, 16, 18). All three Abs revealed a similar trabeculocapsular PrP expression (Fig. 1, A–C). In contrast, the corresponding secondary Abs alone did not produce a comparable signal in the capsule or trabeculae (Fig. 1, G and H). Second, the staining of capsule and trabeculae with the three PrP-specific Abs was extended to spleen tissue of PrP-deficient (*Prnp*<sup>0/0</sup>) mice. As illustrated in Fig. 1, D–F, no capsular

**FIGURE 1.** Immunohistochemistry reveals PrP localization in the splenic capsule and trabeculae. Cryostat sections of nonfixed spleens from naive wt (A–C, G, and H), *Prnp<sup>0/0</sup>* (D–F), and *Rag-1<sup>-/-</sup>* mice (J–L), respectively, were stained with different PrP-specific Abs and corresponding fluorochrome-conjugated secondary Abs (A–F and J–L), or with secondary Abs alone against rabbit IgG (G) or mouse IgG (H). The primary Abs were rabbit serum XN (A, D, and J), rabbit serum 1B3 (B, E, and K) and mouse monoclonal IgG 6H4 (C, F, and L). In the wt and *Rag-1<sup>-/-</sup>* spleens each primary Ab strongly stained the splenic capsule (arrow in A) and trabecular structures (arrowheads). Variable but generally weak staining was detected in germinal centers (G) of wt spleens. In *Prnp<sup>0/0</sup>* spleens with primary and secondary Abs, or in the wt spleens with only secondary Abs, a weak and non-PrP-specific immunofluorescence in widely dispersed cells was detected. This background was absent in *Rag-1<sup>-/-</sup>* spleens. Bars = 100  $\mu$ m.

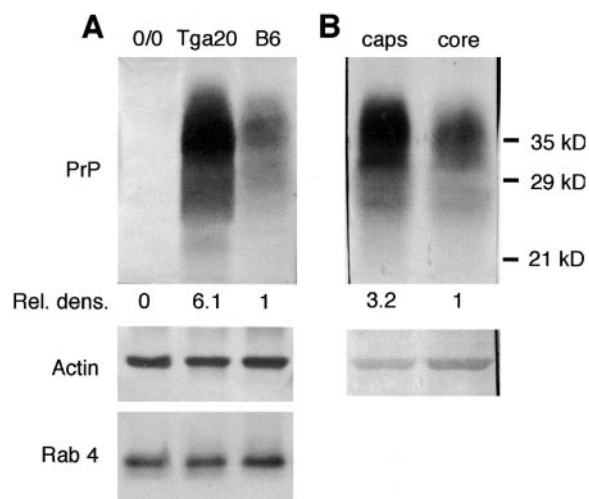


or trabecular staining occurred in the absence of PrP. Taken together, these results confirm the specificity of the PrP detection in capsule and trabeculae of the mouse spleen.

Besides the strong PrP signal in the splenic capsule and trabeculae, specific immunostaining was also found in the FDC network of the germinal centers (Fig. 1A). This FDC-associated staining was of variable intensity in various germinal centers within the same spleen, and relatively weak compared with the signal in the capsule and trabeculae. In addition, a nonspecific staining was observed widely distributed in the splenic stroma. This staining was produced by the use of secondary Abs alone (Fig. 1, G and H), being particularly prominent when the anti-mouse isotype was used (Fig. 1H). Thus, this stromal labeling was considered to reflect a background staining of areas rich in lymphocytes. Support for this rendition comes from staining experiments of *Rag-1<sup>-/-</sup>* spleens (Fig. 1, J–L) as described below.

The prominence of immunostaining in trabeculocapsular regions compared with other splenic areas does not necessarily reflect a higher abundance of PrP. Superior accessibility of the PrP Ag could also cause a stronger signal. To address and to rule out this possibility, a quantitative analysis of the PrP abundance was performed by Western blot. The method was initially tested using total spleen homogenates from mice without PrP (*Prnp<sup>0/0</sup>*), wt controls, or a PrP-overexpressing line (*Prnp<sup>Tga20</sup>*) (Fig. 2A). Notably, the resulting PrP bands appeared somewhat blurred, as compared with PrP-specific Western blot data from other studies. This might be explained, at least in part, by the low abundance of the normal PrP in splenic tissue of uninfected animals. Indeed, there are only a few reports detailing normal splenic PrP expression (19, 20), and these show either a barely visible signal or similar results to those shown in this study (Fig. 2A). The signals detected in this study appeared in the correct m.w. range for PrP and were absent using

probes of PrP-deficient mice which confirmed the PrP specificity of the detection. Furthermore, the bands did not smear toward small molecular sizes, which excluded significant occurrence of



**FIGURE 2.** Immunoblots exhibit a PrP-specific signal which is stronger in the capsular region than in the core portion of the spleen. Homogenates of splenic samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Signals obtained after immunostaining of PrP and the house-keeping protein(s) actin (and Rab4) were analyzed by densitometry. The PrP-related values were corrected for the sample loading variations reflected by the actin (and Rab4) values and expressed as relative densitometry values. A, Analysis of spleen samples from *Prnp<sup>0/0</sup>*, PrP-overexpressing *Prnp<sup>Tga20</sup>*, and normal C57BL/6 mice is shown. B, The capsular and the core fraction from the same C57BL/6 spleen are compared.

partial PrP degradation. We feel that these results confirm the usefulness of the Western blot data for quantitative PrP analysis.

Consequently, two tissue fractions were prepared from normal mouse spleen, one consisting of the capsular region and the other of the noncapsular core region. Because the separation of the capsule from adjacent tissue occurred by simple peeling, the obtained fractions after separation were not pure. Rather, the capsular fraction was contaminated with small portions of the stroma, whereas the core fraction still contained the capsule-related trabeculae. Nevertheless, the results revealed a large difference in PrP expression typical for the distinct fractions (Fig. 2B). By normalized densitometry, PrP expression in the capsular fraction was three times higher than in the core fraction. Considering the aforementioned technical difficulties, the concentration of PrP on the splenic capsule (and the related trabeculae) may well be even more pronounced than observed by Western blot analysis. The two splenic fractions were further analyzed by real-time RT-PCR for PrP-specific RNA content. A 3-fold PrP-specific RNA abundance was observed in the capsular fraction compared with the core fraction (Table I), reflecting the difference seen at the protein level. This suggests that the trabeculocapsular PrP has not accumulated from other tissue sites, but is expressed in situ by capsular and trabecular cells.

The relatively high abundance of trabeculocapsular PrP prompted the question of whether it might play a role in splenic propagation of the prion pathogen. To fully address this question it would be necessary to use a model in which the trabeculocapsular PrP was selectively depleted. To our knowledge, such a model is currently not available. Therefore, we addressed the question indirectly by analyzing the spleens of Rag-1<sup>-/-</sup> mice for trabeculocapsular PrP expression. Rag-1<sup>-/-</sup> mice do not harbor mature lymphocytes or develop germinal centers in the secondary lymphoid organs (21). Intriguingly, in a previous study the prion pathogen was found not to replicate in spleens of Rag-1-deficient mice (22). Our immunohistochemical staining of Rag-1<sup>-/-</sup> spleens showed that PrP is abundantly and exclusively expressed in the capsule and trabeculae (Fig. 1, J–L). This result suggests that abundant trabeculocapsular PrP expression is not sufficient for splenic prion propagation. Furthermore, the absence of background staining in the stroma of Rag-1<sup>-/-</sup> spleens indicates that, as seen in wt spleens, nonspecific background staining is associated with lymphocytes.

Table I. PrP-specific RNA in mouse spleen and fibroblast samples<sup>a</sup>

RNA source	$\Delta C_T(\text{PrP-GAPDH})$	Rel. RNA amount ( $\pm$ SD)
<i>Prnp</i> <sup>+/+</sup> , naive	4.4, 5.4, 5.1, 5.2, 5.0, 5.1	1.00 $\pm$ 0.26
<i>Prnp</i> <sup>+/+</sup> , IC	5.2, 5.1, 5.1, 4.9	0.95 $\pm$ 0.08
<i>Prnp</i> <sup>+/+</sup> , VSV	5.0, 5.4, 4.9, 4.5, 4.9	1.06 $\pm$ 0.23
<i>Prnp</i> <sup>+/+</sup> , naive, capsular	3.6, 3.6, 4.0	2.42 $\pm$ 0.37
<i>Prnp</i> <sup>+/+</sup> , naive, core	5.4, 5.2	0.81 $\pm$ 0.08
<i>Prnp</i> <sup>0/0</sup> , naive	>33	0.00
<i>Prnp</i> <sup>Tga20</sup> , naive	6.1, 6.3, 6.1, 5.8	0.48 $\pm$ 0.07
<i>Prnp</i> <sup>Tga20</sup> , naive	1.8	9.17
3T3 cells, subconfluent	6.0	0.50
3T3 cells, confluent	2.2	6.95

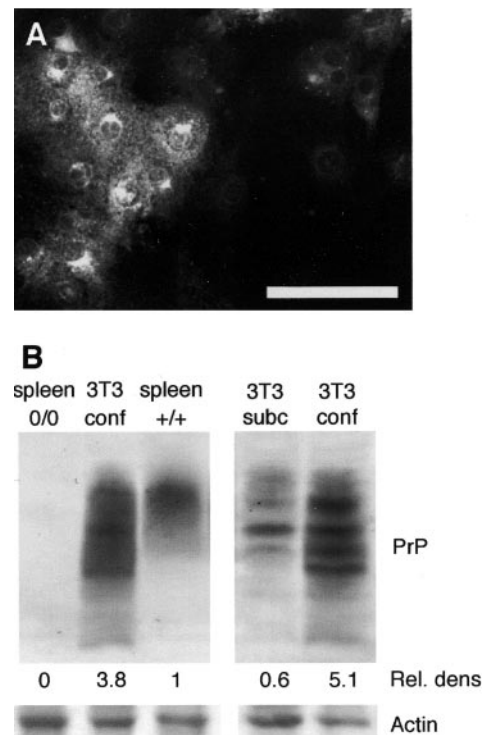
<sup>a</sup> Total RNA from spleens of mice with two (*Prnp*<sup>+/+</sup>), no (*Prnp*<sup>0/0</sup>), one (*Prnp*<sup>+/-</sup>), or multiple (*Prnp*<sup>Tga20</sup>) copies of the PrP-related gene, or from 3T3 mouse fibroblasts was analyzed by real-time RT-PCR with primer-probe sets specific for GAPDH- and PrP-related RNA species. Resulting  $C_T$  were used to calculate the relative amount of PrP-related RNA.

### Cultivated fibroblasts grown to confluency abundantly express PrP

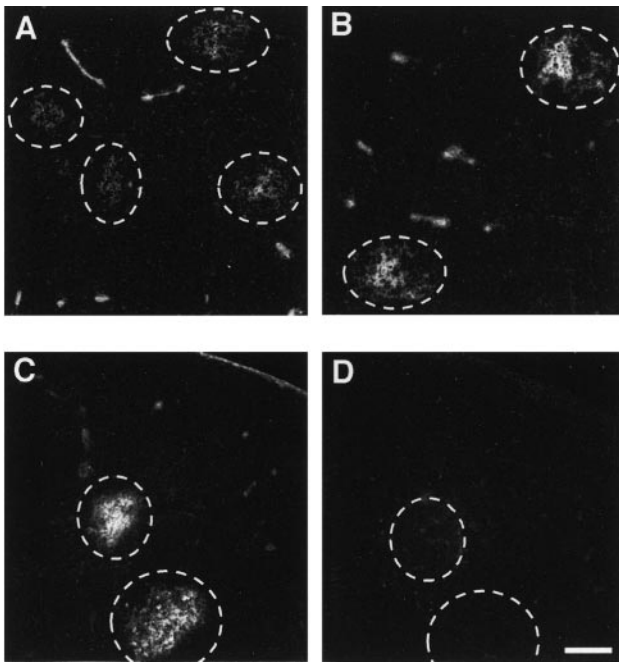
Fibroblasts constitute the predominant cell type in the capsule and trabeculae of the mouse spleen (23). Hence, these cells are likely candidates for the high expression of trabeculocapsular PrP. Therefore, we examined whether an analogous PrP expression in cultivated Swiss 3T3 mouse fibroblasts could be observed. Immunocytochemistry (Fig. 3A) and Western blot analysis (Fig. 3B) revealed a strong signal for PrP. Interestingly, the level of PrP expression was heavily up-regulated when cultivated cells had grown to confluency. The increased expression of PrP in confluent vs subconfluent fibroblasts was also reflected by a higher abundance of PrP-related RNA (Table I).

### PrP abundance in the FDC network is strongly increased after treatment with ICs or infection with VSV

We next examined the effect of immune stimuli on PrP expression. For this purpose, mice were injected i.v. with a single bolus of IC or live VSV. Both IC treatment and VSV infection resulted in strongly increased signals for immunohistochemically detected PrP in the FDC network of germinal centers; in contrast, the capsular and trabecular signals were not affected (Fig. 4, A–C). Unspecific staining by the rabbit serum directed against PrP following immune stimulus was excluded through control staining with PrP-unrelated, LCMV-specific rabbit serum on a consecutive spleen section. The LCMV staining revealed no signal, in either the FDC network or the capsule (Fig. 4D). PrP-specific staining of an immune-stimulated *Prnp*<sup>0/0</sup> spleen also yielded no signal in the FDC network (see Fig. 7E). These two controls argue against unspecific



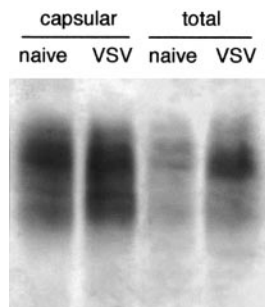
**FIGURE 3.** PrP is strongly expressed by confluent 3T3 mouse fibroblasts. *A*, Immunocytochemistry on 3T3 cell cultures produced strong PrP-specific fluorescence in areas of confluent cell cultures. The staining was most prominent intracellularly in perinuclear Golgi-like compartments. Cells of subconfluent areas were only weakly stained. *B*, Immunoblots of samples from spleens and 3T3 fibroblasts revealed PrP-specific signals that differed considerably between subconfluent and confluent cell cultures. Bar = 100  $\mu$ m.



**FIGURE 4.** Immune stimulation specifically induces an increased PrP signal in the FDC network of germinal centers. Cryostat sections of non-fixed spleens from a naive (A), an IC-treated (B), and a VSV-infected wt mouse (C and D) were stained with rabbit sera specific for PrP (A–C) and LCMV (D), respectively. In the capsular and trabecular structures, the PrP staining seemed equivalent in all spleens. In contrast, the PrP staining in the germinal centers (circled) was much stronger after immune stimulation. The LCMV-specific rabbit serum did not produce any staining. Bar = 100  $\mu$ m.

capturing of Abs used for immunohistochemistry by immune-stimulated FDCs. The stimulatory effect of IC treatment or VSV infection on germinal center PrP occurred by day 4 following challenge and was very pronounced at days 8 and 12. More detailed studies on the kinetics are currently being performed. All data presented were obtained at day 8.

The immunohistochemical findings of elevated PrP in germinal centers were confirmed by Western blot analysis which revealed a clear increase of PrP abundance in total spleen homogenates following immune stimulation. By solely investigating the capsular fractions of control and stimulated spleens, no significant difference could be observed (Fig. 5). The latter result is in accordance with the immunohistochemical data and suggests that the trabeculocapsular structures do not contribute to the increase of total splenic PrP. This is further supported by experiments with spleens



**FIGURE 5.** PrP is significantly elevated in the total spleen but not in the capsular subfraction. The spleens of a naive and a VSV-infected mouse were each prepared to yield a capsular homogenate as well as a homogenate representing the total spleen. PrP abundance in the homogenates was revealed by immunoblotting.

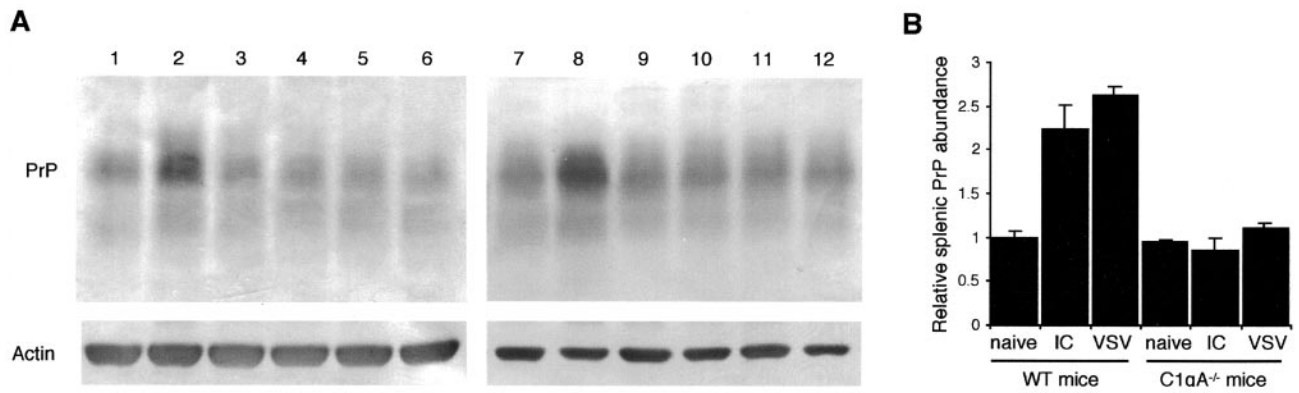
from Rag-1<sup>-/-</sup> mice, which exhibit PrP exclusively in the capsule and trabeculae. Following IC treatment of Rag-1<sup>-/-</sup> mice, no alterations of splenic PrP were observed by immunohistochemical or Western blot analysis (data not shown). A summary of the densitometric quantifications of Western blot analyses is provided in Fig. 6B. These data demonstrate the up-regulation (be 2- to 2.5-fold) of total splenic PrP upon immune stimulation by IC or VSV.

We next attempted to estimate the magnitude of PrP up-regulation in the FDC network of germinal centers because PrP induction appeared to be restricted to these areas. For this purpose the most evident approach might be a Western blot analysis of FDCs isolated from control and immune stimulated spleens. Thielen et al. (17) had previously reported a protocol for the enrichment of FDC clusters which preserved PrP expressed on the cell surface. However, we cannot rule out the possibility that the enzymatic procedure per se modifies the functional state of the isolated FDCs. To avoid artificial effects on the PrP expression level following isolation of FDCs, we instead decided to quantitate expression by immunohistochemical detection methods using spleen sections of control and IC-treated mice. Micrographs representing comparable splenic areas were analyzed by densitometry, yielding relative values for PrP abundance in cross sections of trabeculocapsular and germinal center structures (Table II). Assuming that the trabeculocapsular PrP signal was not altered, as indicated by Western blot analysis of capsular fractions, the average PrP signal in the germinal center was increased nearly 6-fold in treated spleen as compared with controls. Calculations of the total spleen PrP signal, which was mainly constituted by the trabeculocapsular and the germinal center PrP signals, suggested a global increase of splenic PrP by a factor of  $\sim$ 2-fold following IC treatment. This value for the global PrP increase was comparable to the results obtained by Western blot analysis of total spleen homogenates.

In our analyses of splenic capsular and core fractions of naive mice and of cultivated fibroblasts, increased PrP expression correlated with higher specific RNA abundance, as measured by real-time PCR. In fact, this method was found to reliably detect differences in the PrP-specific RNA content with a lower limit of sensitivity of 2-fold or less (Table I). Surprisingly, PrP-specific RNA in spleen samples of treated mice did not reveal significant differences compared with untreated controls. This data suggests that the up-regulation of PrP in treated mice occurred posttranscriptionally.

#### *Up-regulation of PrP is abolished in the C1q-deficient mice*

The complement component C1q has been shown to facilitate peripheral prion pathogenesis (9, 10). Based on the assumption that elevated expression levels of splenic PrP would be expected to enhance susceptibility to prions, we speculated that C1q may play a role in the up-regulation of PrP in response to immune stimulation. To test this hypothesis, transgenic mice lacking C1q (C57BL/6 C1qA<sup>-/-</sup> mice) (24) were given an i.v. injection of IC or VSV. In contrast to control mice, C1q-deficient mice did not respond to the immune stimuli with an increase of splenic PrP, as demonstrated by Western Blot analysis (Fig. 6) and immunohistochemistry (Fig. 7, A–D). Notably, immunohistochemistry revealed that PrP staining of FDC networks was not completely absent in C1qA<sup>-/-</sup> spleens. Rather the PrP expression seemed to be constitutively low. Spleen sections were costained for the mouse CR1 (Fig. 7, E and F), which is considered to be a marker of functional FDCs (25). The staining did not reveal any abnormalities in the constitution of the FDC network in germinal centers due to C1q deficiency. This finding is in agreement with previous reports (9, 26), demonstrating the presence of a network of cells within the germinal center of C1q-deficient mice that stains positively for the FDC marker FDC-M1.



**FIGURE 6.** Deficiency in C1q prevents PrP up-regulation upon IC treatment or VSV infection. *A*, Splenic PrP abundance was detected by Western blot analysis of spleen homogenates. The gels were loaded with samples from mice of the following groups: naive wt (lanes 1 and 7), IC-treated wt (lane 2), naive C1qA<sup>-/-</sup> (lanes 3, 4, and 9), IC-treated C1qA<sup>-/-</sup> (lanes 5 and 6), VSV-infected wt (lane 8), and VSV-infected C1qA<sup>-/-</sup> (lanes 10, 11, and 12). The graph in *B* integrates all the numeric data from the immune stimulation experiments and densitometric Western blot analysis of splenic PrP abundance. Results are represented as means ± SD.

Altogether, our data indicate that the up-regulation of PrP may be dependent on C1q. Moreover, this suggests that C1q could be involved in the spread of prions from the periphery to the CNS through its role in splenic PrP expression.

## Discussion

In the mouse spleen, the prion protein has been found to be associated with FDCs in both uninfected and scrapie-challenged animals (3). PrP expression by FDCs appears to be critical for splenic prion replication (8), but a normal function of PrP expression by FDCs has remained elusive. In the present report, we show that the PrP abundance in the FDC network is heavily increased following immune stimulation through i.v. administration of either preformed Ag-IgG complexes or live VSV. In mice deficient of complement component C1q, a similar up-regulation of splenic PrP could not be provoked, indicating that C1q is required. These results strongly suggest that PrP plays a role in the acute engagement of FDCs in humoral immune responses. The function of FDCs has been defined as the presentation of intact Ag and costimulatory signals to B cells, as a requirement for germinal center formation and hence for the maturation and maintenance of secondary immune responses (27). It can be speculated that the localization of immune-complexed Ag to the splenic follicles triggers activation of FDCs toward their tasks in the germinal center reaction. Up-regulation of PrP may be a consequence of FDC activation, and paucity of PrP up-regulation in the absence of C1q could be ex-

plained by the fact that IC trapping is impaired in germinal centers of C1qA<sup>-/-</sup> mice (26). In the case of VSV-induced PrP up-regulation, C1q might bind directly to VSV or to virus complexed by IgM (28). Of course, one could envisage alternative models that link immune stimuli to PrP up-regulation in the splenic FDC network and to C1q function. Additional experiments with diverse immune stimulators and with mice deficient of particular components of the immune system are expected to provide a more factual view of the connections.

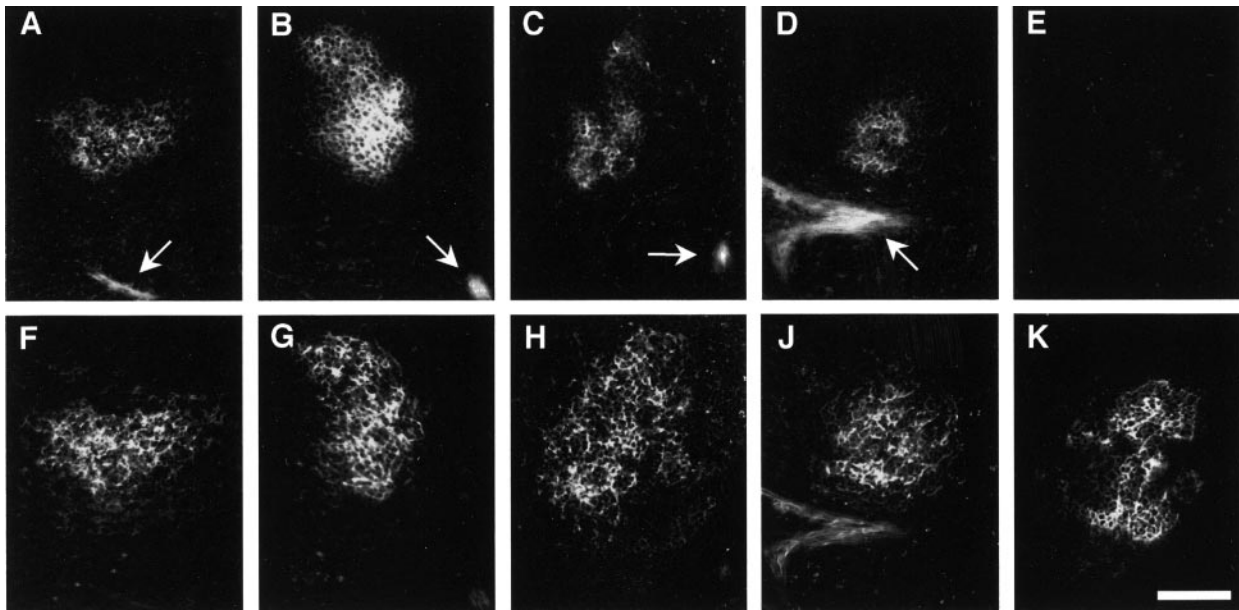
The immunologically induced up-regulation of the PrP in the FDC network is not only interesting with regard to disclosing a putative function of PrP, but it also concerns prion pathology. The level of PrP expression is rate-limiting for TSE development (29). This may apply in particular to PrP expressing cells known to be critical for prion pathogenesis like splenic FDCs. We hypothesize that an increased abundance of PrP in the FDC network following an opportune immune stimulus would enhance the susceptibility toward peripheral prion infection. The appropriate experiment to test this hypothesis needs to be done. Intriguingly, treatment of mice with the immune-stimulatory mitogens PHA or LPS has been previously found to render mice more susceptible to scrapie (30). The regulation of splenic PrP might also explain how complement facilitates early prion pathogenesis. It has previously been proposed that complement components opsonize the infectious agent and thereby mediate its localization and retention in FDC networks (9, 10). In light of our new data, we propose that complement may instead, or in addition, mediate the up-regulation of splenic PrP, resulting in increased susceptibility. The immune stimulus for PrP up-regulation may come from the prion itself or a prion-independent mechanism.

A remarkable feature of splenic PrP up-regulation is its apparent independence of transcription, because the level of PrP-related RNA was not detectably altered. This is in contrast to the differences in PrP expression observed in splenic capsular and core regions of naive mice, and between subconfluent and confluent 3T3 fibroblasts, which are reflected by corresponding differences in RNA abundance. The increase of FDC-associated PrP in IC-treated or VSV-infected mice independent of transcription raises the question of whether FDCs express any PrP at all. To date, it has not been proven formally (e.g., by *in situ* hybridization) that FDC-associated PrP is synthesized by the FDCs themselves. Hence, it might be considered that the up-regulation of PrP actually reflects a strongly increased capacity of FDCs to capture extrinsic PrP after

Table II. Quantitative analysis of PrP-specific immunofluorescence on spleen cryosections<sup>a</sup>

	Naive	IC	IC/Naive
T (Pixel count × corrector)	7208 n	6553 i	0.91 i/n
G (Pixel count × corrector)	2184 n	11695 i	5.35 i/n
G/T	0.30	1.79	5.89
(G + T)/T	1.30	2.79	2.14

<sup>a</sup> PrP-specific immunofluorescence was analyzed on spleen sections of a naive and an IC-treated mouse, respectively. In digital micrographs covering morphologically equivalent areas of 7.2 mm<sup>2</sup> (16.1 Megapix), pixels of fluorescence signals were counted and typed either T, when associated with trabeculae or capsule, or G, when located in a germinal center. To account for methodical differences in the recording of the pixel counts between the samples, correctors (correction factors) n (naive) and i (IC) were formally invented. If  $T_{IC} = T_{naive}$ , as is suggested by immunofluorescence and Western blot, then  $(G/T)_{IC}/(G/T)_{naive} = G_{IC}/G_{naive}$  follows. If, in addition,  $G + T = \text{total}$ , i.e., signals associated with neither G nor T are only marginal, then  $((G + T)/T)_{IC}/((G + T)/T)_{naive} = \text{total}_{IC}/\text{total}_{naive}$  follows.



**FIGURE 7.** Immunologically induced PrP up-regulation in FDC networks is impeded in  $C1qA^{-/-}$  mice. Cryostat sections from spleens of naive wt (A and F), VSV-infected wt (B and G), naive  $C1qA^{-/-}$  (C and H), VSV-infected  $C1qA^{-/-}$  (D and J) and VSV-infected  $Prnp^{0/0}$  mice (E and K) were immuno-double-stained for PrP (A–E) and CR1 (F–K). The staining of CR1 within the germinal center area suggests the presence of functional FDC networks in all spleen samples. VSV infection of wt mice led to an increased PrP signal in the FDC network. A likewise increase was not provoked in  $C1qA^{-/-}$  mice. In immune-stimulated spleens deficient of PrP, no PrP staining was produced. Arrows indicate PrP-positive profiles of trabeculae. Bar = 100  $\mu\text{m}$ .

immune activation. To reconcile this hypothesis with results from experiments using chimeric mice (obtained by grafting bone marrow from PrP-deficient knockout mice into PrP-expressing mice and vice versa; Ref. 8), the putative donors of extrinsic PrP cannot be PrP-expressing lymphocytes or myeloid cells. The chimera study implies instead that PrP is produced by radiation-resistant long-lived nondividing cells. Therefore, the authors of this study suggest that FDCs themselves produce PrP (8). Consequently, our results indicate that the PrP level on FDCs is regulated posttranscriptionally. The concept of posttranscriptional regulation of PrP expression is well-supported by recent reports. In the rat brain, the distribution and activity of PrP-related RNA on polysomes is suggestive of translational regulation (31). Furthermore, in the mouse *Prnp* gene upstream AUGs have been identified that are capable of modulating PrP translation in vitro (32). Alternatively to translational regulation, posttranslational mechanisms that control the cellular PrP turnover have been proposed to explain the marked disparity between prion protein and mRNA level in different neurons of the mouse brain (33). Notably, in primary splenic cell cultures, the turnover was found to be very fast, with a half-live of PrP in splenocytes of only 1.5–2 h (19). Thus, subtle modulation of PrP stability might rapidly change PrP abundance.

In addition to the up-regulation of PrP in the FDC network, we have reported another novel observation regarding PrP expression in the mouse spleen: in naive mice, PrP was not most abundant in the germinal center region, but in the splenic capsule and trabeculae. This trabeculocapsular PrP expression appeared to be constitutive and was not subject to the regulation observed in the germinal centers. The high proportion of trabeculocapsular PrP has been critical in our study to estimate the magnitude of PrP up-regulation in the germinal centers. Whether it is also significant for peripheral prion pathogenesis cannot be definitely answered at this point, however, two findings clearly argue against this possibility. First, an accumulation of the pathological PrP, which is a hallmark and marker of prion diseases, has never been described in the

capsule or trabeculae. One may argue that the pathological PrP was overlooked in the capsule and trabeculae, as was the normal PrP. Yet, the relative ease of immunohistochemical detection of the accumulating pathological form makes its ignorance in the abundant capsular and trabecular structures unlikely. Second, spleens of mice deficient in Rag-1 do not propagate the prion agent (22), however, we report that their capsule and trabeculae abundantly harbor PrP. Thus, trabeculocapsular PrP does not appear to be sufficient for splenic prion propagation.

Although a function for the trabeculocapsular PrP expression is yet to be defined, we hypothesize that the cells expressing PrP may be fibroblasts, as fibroblasts were characterized as the predominant cell type in the splenic capsule and trabeculae (23). In addition, we show that cultivated 3T3 mouse fibroblasts are capable of abundant PrP expression. Interestingly, the expression was massively higher in confluent than in subconfluent cells, both at the RNA and protein level. This finding is reminiscent of PrP up-regulation in primary cultures of human fibroblasts, induced by migration inhibitory factor-related protein 8 (34). In this study, the authors proposed that the up-regulation of PrP may be relevant to cell growth arrest and differentiation.

In summary, our data provides two new interesting features of PrP expression in the mouse spleen. We first described a constitutively high PrP expression level in the splenic capsule and trabeculae. We next observed a variable PrP expression level in the FDC network of germinal centers, which was strongly increased following immune stimulation of mice with ICs or live VSV. These observations may be critical steps in the search of physiological functions of PrP, as well as in the determination of its exact role in peripheral prion pathogenesis. In this respect, the second observation may be especially valuable, as it contributes to an already extensive web of immunological and pathological information, facilitating the correct reading of results from ongoing and succeeding experiments.

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