Mouse vaccination with dendritic cells loaded with prion protein peptides overcomes tolerance and delays scrapie

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Prion diseases are presumed to be caused by the accumulation in the brain of a pathological protein called prion protein (PrP) scrapie which results from the transconformation of cellular PrP, a ubiquitous glycoprotein expressed in all mammals. Since all isoforms of PrP are perceived as self by the host immune system, a major problem in designing efficient immunoprophylaxis or immunotherapy is to overcome tolerance. The present study was aimed at investigating whether bone-marrow-derived dendritic cells (DCs) loaded with peptides previously shown to be immunogenic in PrP-deficient mice, can overcome tolerance in PrP-proficient wild-type mice and protect them against scrapie. Results show that, in such mice, peptide-loaded DCs elicit both lymphokine release by T cells and antibody secretion against native cellular PrP. Repeated recalls with peptide-loaded DCs reduces the attack rate of 139A scrapie inoculated intraperitoneally and retards disease duration by 40 days. Most interestingly, survival time in individual mice appears to be correlated with the level of circulating antibody against native cellular PrP.

INTRODUCTION

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders which progress slowly, induce severe neurological loss and are transmissible. The agent is most presumably the scrapie prion protein (PrP) or PrPSc, a misfolded protein, rich in beta-sheets, resulting from the transconformation of cellular PrP (PrPc). PrPc is a ubiquitous, membrane-anchored protein, which has been remarkably conserved through evolution, but whose function in health is still enigmatic. The presence of PrPSc in tissues of diseased individuals confirms the diagnosis TSE (Prusiner *et al.*, 1998; Wisniewski *et al.*, 1998).

Prions do not spontaneously evoke antibody (Ab) or T-cell responses in diseased subjects, most likely because PrPSc, like PrPc, is not perceived as foreign or 'dangerous' (Matzinger, 1998) by the host immune system. In recent years, however, following encouraging immunotherapy results in Alzheimer's disease patients (Schenk *et al.*, 1999), several teams have attempted to passively or actively generate adaptive immune responses against prions in

wild-type (wt) mice and have shown that passive Ab treatment (Sigurdsson *et al.*, 2003; White *et al.*, 2003), constitutive transgenic secretion of anti-PrP IgM (Heppner *et al.*, 2001) or active immunization using whole PrP (Sigurdsson *et al.*, 2002), PrP peptides (Schwarz *et al.*, 2003), PrP fragments (Bade *et al.*, 2006) or DNA constructs encoding *Prnp* gene sequences (Fernandez-Borges *et al.*, 2006) had beneficial effects on disease evolution.

In order to identify major histocompatibility (MHC) class II-restricted T-cell epitopes in C57BL6 (B6)–H-2^b mice, we previously screened a bank of overlapping peptides of mouse PrP and identified two main epitopes. One is contained in peptide PrP$_{97-128}$, the other is shared by peptides PrP$_{143-172}$ and PrP$_{158-187}$. Immunization of mice made genetically deficient for PrP (*Prnp$^{-/-}$*) with PrP$_{97-128}$ induces moderate T-cell responses but good Ab secretion, including Abs to native, cell-bound PrPc, whereas PrP$_{158-187}$ elicits strong cellular responses but few Abs to the native protein (Gregoire *et al.*, 2004). When injected into wt mice, the same peptides trigger no response unless they are administered together with a potent adjuvant such as CpG-ODN (Rosset *et al.*, 2004). Furthermore, the Abs generated under such conditions bind to plastic-immobilized recombinant PrP, but not to cell-surface native PrPc, suggesting that B cells which recognize native PrPc with good affinity are deleted during ontogeny (Gregoire *et al.*, 2005;

Polymenidou *et al.*, 2004). Given the current assumption that immune protection against TSEs is primarily effected by Ab specific for cell-bound PrP, a major objective is to develop vaccines which will generate Abs of such specificity in wt mice. Nikles *et al.* (2005) have obtained promising results by using retroviral particles which display the C-terminal 111 aa of PrP (Nikles *et al.*, 2005). We propose here an alternative strategy based on vaccination with syngeneic dendritic cells (DCs) loaded with the previously identified immunogenic PrP peptides. Here, we show firstly that pulsed DCs can successfully elicit T- and B-cell-responses against PrPc in wt mice and secondly, that repeated DC recalls delay scrapie progression in peripherally infected mice.

RESULTS

### PrP-expressing DCs elicit anti-PrP immune responses in Prnp$^{-/-}$ mice

Before probing DCs in wt mice, we verified that they could raise anti-PrP T- and B-cell-responses in non-tolerant *Prnp*$^{-/-}$ mice. Four female mice were immunized twice intraperitoneally (i.p.) at 15 day intervals with 1×10^6 DCs differentiated from wt bone-marrow (BM) B6 precursors and thus expressing PrPc (PrP$^+$ DCs). Control mice received PrP$^-$ DCs differentiated from *Prnp*$^{-/-}$ BM donors. Spleen T cells and sera were collected 10 days after the second challenge. As shown in Fig. 1(a), T cells from all four mice injected with PrP$^+$ DCs secreted gamma

interferon (IFN-γ) upon *in vitro* challenge with antigen-presenting cells (APCs) + PrP$_{158-187}$. In mice that had received PrP$^-$ DCs or PBS, secretion was at background levels. The number of spots increased with the *in vitro* concentration of peptide, confirming that cytokine release depended on specific antigen recognition (Fig. 1b).

Next, we looked for Abs against cell-bound PrPc. A typical overlay (Fig. 1c) shows EL4 cells with secondary Ab only, cells incubated with a normal mouse serum plus secondary Ab and cells incubated with a serum from a DC-vaccinated mouse plus secondary Ab. We checked that the Abs present in serum were also staining PrP$^+$ splenocytes from wt mice and not PrP$^-$ splenocytes from *Prnp*$^{-/-}$ mice (data not shown). As Fig. 1(d) shows, the sera from the four *Prnp*$^{-/-}$ mice immunized with PrP$^+$ DCs produced mean fluorescent intensity (MFI) values well above those of sera from mice injected with PrP$^-$ DCs or PBS. Differences were significant between the three groups by Kruskal–Wallis test ($P<0.05$) and by Dunn's two by two between PrP$^+$ DCs and PBS ($P<0.01$).

Overcoming tolerance in wt mice requires peptide-loaded DCs

To elicit responses in wt mice, we injected either unloaded PrP$^+$ DCs or PrP$^+$ DCs loaded with PrP$_{98-127}$ or PrP$_{158-187}$. Viable DCs (1×10^6) were administered at 15 day intervals and spleen T cells and sera were collected 10 days after the last challenge. Fig. 2(a) shows that only mice injected with DCs loaded with PrP$_{158-187}$ mounted a substantial IFN-γ
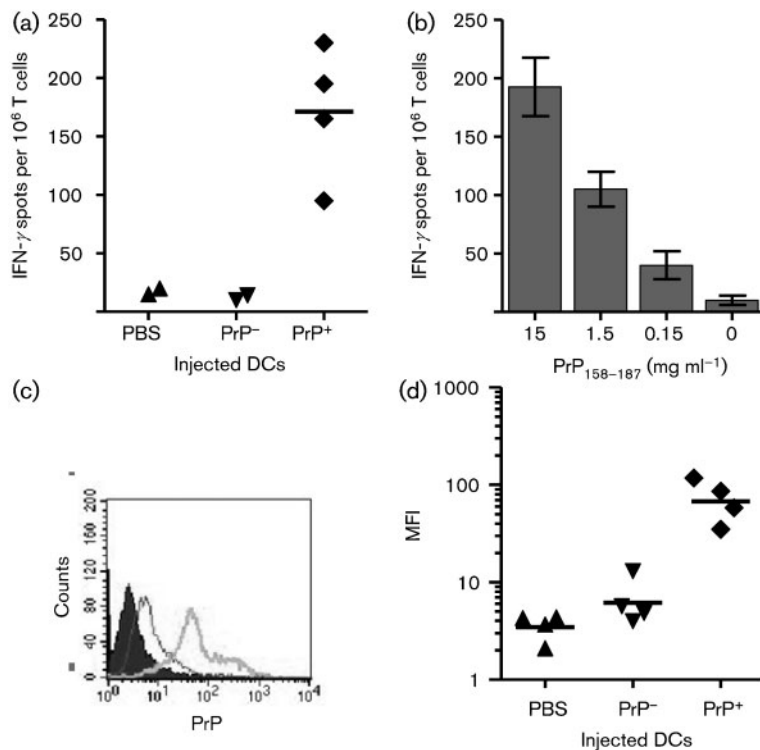
Fig. 1. PrP$^+$ DCs elicit T- and B-cell responses in non-tolerant *Prnp*$^{-/-}$ mice. Mice were injected i.p. twice at a 15 day interval with 1×10^6 unloaded DCs differentiated from wt PrP$^+$ or *Prnp*$^{-/-}$ BM. A third group received PBS. (a) Each point represents the frequency of IFN-γ spots per 1×10^6 T cells from individual mice probed with 15 µg ml^{-1} PrP$_{158-187}$. The short horizontal line shows the median. (b) Spot frequency increases with the concentration of peptide PrP$_{158-187}$ present in the ELISPOT assay. Each column shows the mean number of spots ± SD of triplicate cultures. (c) Typical overlay of activated EL4 cells incubated with the secondary Ab only (shaded), incubated with a normal mouse serum and secondary Ab (thin black line) or with an immune serum (1:20 dilution) and secondary (thick grey line). (d) MFI values (shown at 1:20 serum dilution) of mice immunized with PrP$^+$ DCs, PrP$^-$ DCs or PBS. Short horizontal lines show the geometric means.
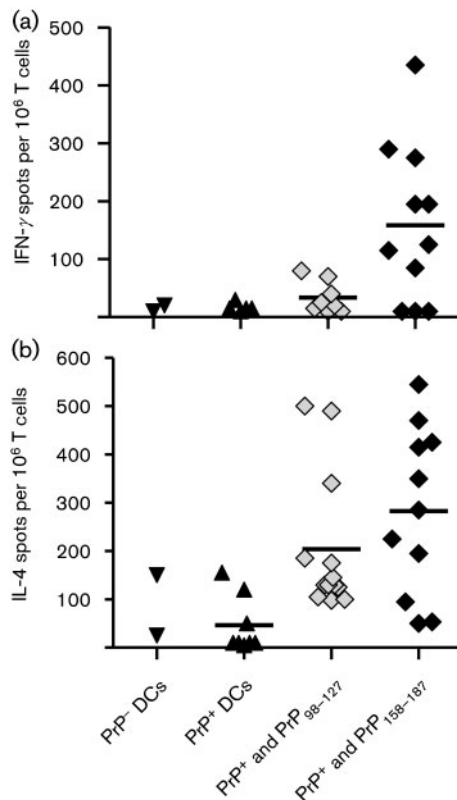
Fig. 2. Lymphokine secretion by T cells in wt mice immunized with loaded or unloaded PrP$^+$ DCs. (a) Frequencies of IFN-γ-secreting T cells in response to challenge with the same peptide with which they were loaded (15 μg ml^{-1}). T cells immunized with unloaded DCs were challenged *in vitro* with PrP$_{158-187}$. Medians (short horizontal lines) between groups were significantly different by Kruskal–Wallis test ($P<0.001$). Dunn's two by two comparison showed a significant difference ($P<0.01$) between DCs loaded with PrP$_{158-187}$ and DCs loaded with PrP$_{98-127}$ or unloaded DCs. (b) Frequencies of IL-4-secreting T cells probed as described in (a). Medians between groups were statistically different by Kruskal–Wallis ($P<0.001$). Dunn's two by two comparison showed a significant difference between PrP$_{158-187}$-loaded DCs and unloaded DCs ($P<0.01$) and between PrP$_{98-127}$-loaded DCs and unloaded DCs ($P<0.05$), but not between the two peptide-loaded groups.

response, with a median spot frequency around 200. In contrast, few spots were generated in mice injected with DCs plus PrP$_{98-127}$ and even less were generated in mice that had received unloaded PrP$^+$ DCs. Contrasting with their poor IFN-γ responses, mice immunized with DCs plus PrP$_{98-127}$ mounted good interleukin (IL)-4 responses (Fig. 2b), the differences were not significant by the Mann–Whitney test, from mice that had received DCs loaded with PrP$_{158-187}$. Mice that had been injected with unloaded DCs remained unresponsive. In summary, T cells of wt B6 mice responded only to peptide-loaded DCs. Loading with PrP$_{158-187}$ elicits a double response of IFN-γ and IL-4, while loading with PrP$_{98-127}$ induces mostly IL-4 secretion.

Next, we assayed the sera from DC-immunized wt mice for the presence of Abs against native PrP^c. Consistent with what had been observed for lymphokine secretion, Ab responses were essentially seen in wt mice that had been injected with peptide-loaded DCs. Both PrP$_{158-187}$ and PrP$_{98-127}$ generated similar response patterns with large variations between individual mice, indicating that tolerance had not been equally overcome. Differences between the four vaccination regimens were significant by Kruskal–Wallis test ($P=0.018$). Dunn two by two indicated that there was no significant difference between the two groups immunized with the two respective peptides. Characterization of anti-PrP Ab subclasses revealed that both IgG1 and IgG2a were secreted, thereby confirming the involvement of T-cell help in the anti-PrP response (Fig. 3b). We checked that the response was really targeted at PrP by testing sera on recombinant PrP in an ELISA assay (Fig. 3c, d).

DCs must express constitutive PrPc, in addition to loaded peptides

Next, we asked whether T and B cells cooperating for the Ab response recognized different antigenic determinants from the loaded peptides, or whether B cells were directly activated by the PrP^c constitutively expressed on the DC cell surface. First, we compared responses generated by peptide-loaded DCs which did or did not express constitutive PrP^c on their surface. Fig. 4(a) shows that PrP$^-$ DCs were significantly less efficient at eliciting Ab than PrP$^+$ DCs, irrespective of the loading peptide.

Epitope mapping further confirmed that B cells recognize antigenic motives of PrP^c on the DC surface, absent from the loading peptide. In addition, sera displayed recurrent epitope patterns, irrespective of whether they had been raised with PrP$_{98-127}$-loaded or PrP$_{158-187}$-loaded DCs (Fig. 4b). In all cases, PrP$_{23-52}$, which has no overlap with the two loading peptides, was best recognized, followed by PrP$_{83-112}$, PrP$_{98-127}$ and PrP$_{143-172}$. Of note, PrP$_{158-187}$ was not recognized by Abs.

Vaccination with peptide-loaded DCs confers protection

Having shown that peptide-loaded DCs raised immune responses to PrP in tolerant wt mice, we attempted to use DCs as a vaccine to confer protection against scrapie. We chose PrP$_{98-127}$ on the basis of several observations, indicating that this peptide is more Th2-oriented (Fig. 2b). Ten wt mice receiving a total of seven injections of PrP$_{98-127}$-loaded DCs and 12 controls receiving PBS were infected i.p. with a lethal dose of 139A scrapie, 7 days after the second DC injection, i.e. 21 days after the beginning of the protocol. These mice were allowed to develop scrapie to its terminal phase. In addition, two PBS controls and four vaccinated mice were culled at 75 days post-inoculation (p.i.), after five DC injections, to evaluate the effect of DC vaccination on prion lymphoinvasion.
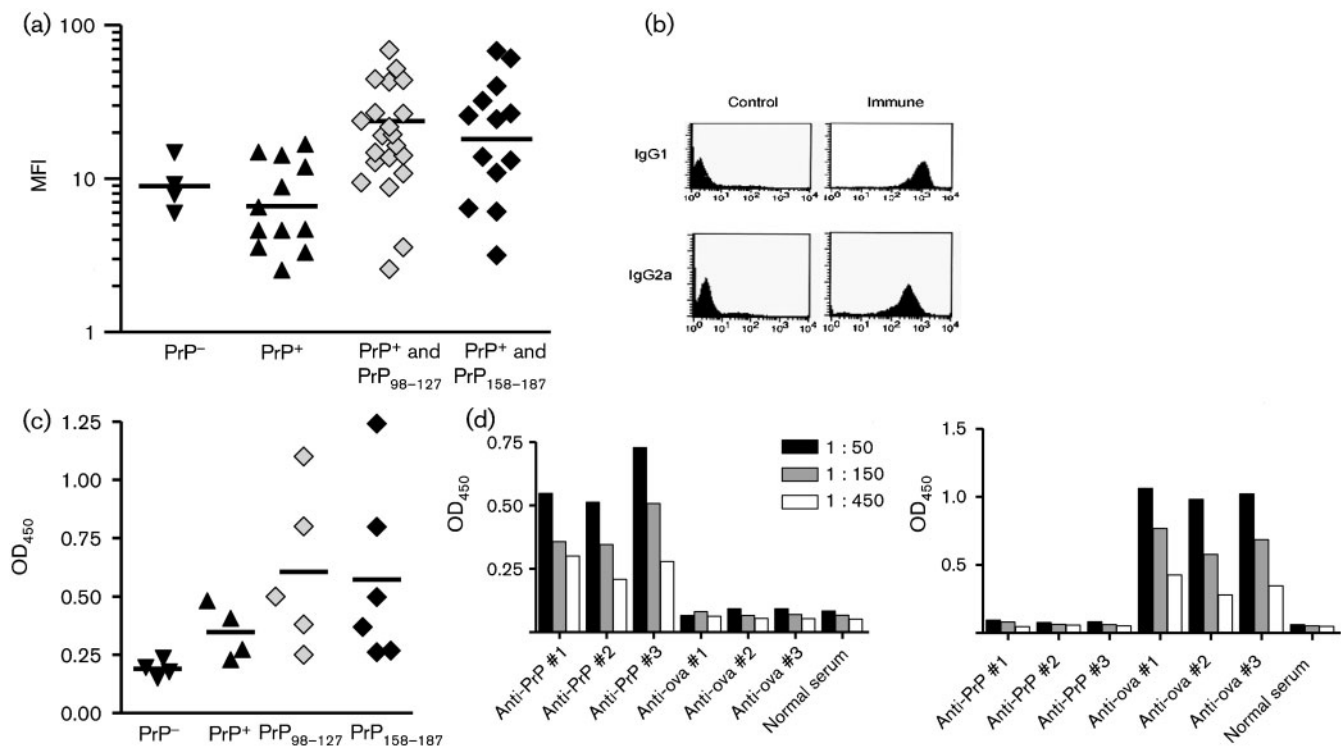
Fig. 3. MFI values produced by Abs against native PrPc in wt mice immunized with loaded or unloaded DCs. (a) Compilation of three independent fluorescence experiments on activated EL4 cells. Sera are shown at a 1 : 20 dilution. Samples were collected from mice immunized at least two or three times at 15 day intervals with DCs. Short horizontal lines represent the geometric means. (b) Representative histograms of EL4 cells stained with normal mouse serum (control) or with an immune serum from a mouse injected twice with PrP_{98-127}-loaded DCs (immune) and revealed with anti-IgG1 or anti-IgG2a Abs. Sera were diluted at 1 : 20. (c) ELISA against coated recombinant murine PrP. As in (a), the best Ab responses against recombinant PrP, measured by ELISA, were again obtained with DCs loaded with peptide. ANOVA followed by a Dunn's two by two test reached significance between either one of the peptide-loaded groups versus PrP-negative DCs, but not between unloaded PrP^+ DCs and PrP^- controls. (d) Assessment of the specificity of the Ab for recombinant PrP. As shown by a cross-ELISA, sera raised by DC vaccination recognized plastic-bound PrP, but not ovalbumin, while anti-ovalbumin sera recognized only their specific antigen.

PrPSc was examined directly in mesenteric lymph nodes by immunohistochemistry and measured quantitatively with an anti-PrPSc ELISA in the spleen. PrPSc had accumulated less in mesenteric lymph nodes of DC-vaccinated mice compared with controls (Fig. 5a) and quantitative assessment of PK-resistant PrPSc by ELISA clearly showed that vaccinated mice had also accumulated significantly less PrPSc in their spleen at 75 days p.i. (Fig. 5b). In light of these results, DC vaccination seems to slow down lymphoinvasion.

We also examined the effect of DC vaccination on mouse survival. The individual disease courses are shown in Table 1. Two of the ten DC-vaccinated mice remained totally free of disease after more than 1 year's observation. The eight other mice became sick, with a median incubation period of 191 days p.i. versus 167 days p.i. in controls ($P<0.0005$ by Mann–Whitney test; Fig. 5c). Neurological symptoms also developed more slowly (59

versus 44.5 days p.i., $P<0.005$) resulting in an extension of the median total disease duration to 254 versus 214 days p.i. ($P<0.0005$).

Although vaccinated and control mice which succumbed to the disease displayed typical neurological signs of 139A scrapie, we looked for the presence of PrPSc in brains, asking whether the amount of the pathological protein was reduced in brains of protected mice. Fig. 5(d) shows the typical three band electrophoretic profile of PK-digested PrPSc. All five lanes including one PBS control sample and four vaccinated samples, loaded at 3 mg total brain protein, gave a comparable signal. Thus, at the terminal stage, protected and control mice contain similar amounts of pathological protein. No difference was seen between vaccinated mice #3 and #4, which survived longer and produced higher MFI values, and vaccinated mice #1 and #2.
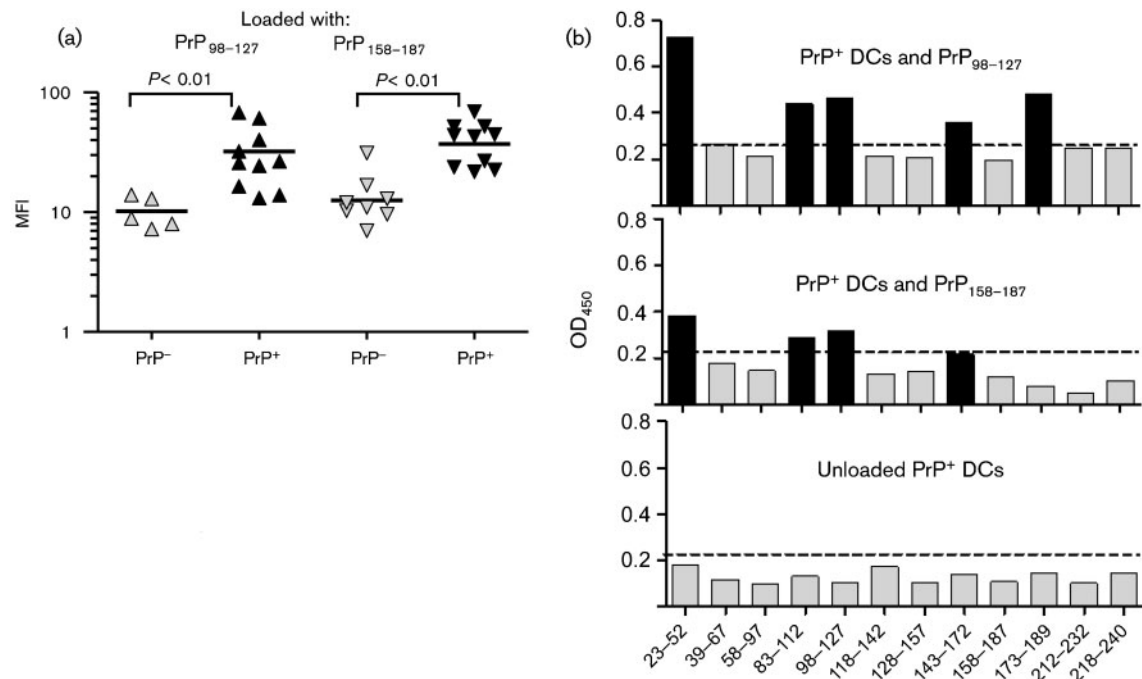
Fig. 4. Requirement for constitutive PrPc expression on loaded DCs. (a) MFIs of sera from wt mice immunized with PrP$_{98-127}$-loaded (△ or ▲) or PrP$_{158-187}$-loaded (▽ or ▼) PrP$^-$ DCs (open) or PrP$^+$ DCs (filled). Sera are shown at a dilution of 1:20. (b) Epitope mapping of polyclonal sera generated with loaded DCs. Sera were assayed by ELISA as described in Methods. Each serum is representative of two others tested under the same conditions. Peptides are positioned in their natural order from N- to C-terminus as shown on the x-axis. Black columns are the peptides recognized in common by the two sets of immune sera.

Table 1. Clinical course in individual DC-vaccinated versus control mice

Mouse no.	Vaccinated			Mouse no.	Controls		
	Onset (days p.i.)	Total duration (days p.i.)	Clinical phase (days)		Onset (days p.i.)	Total duration (days p.i.)	Clinical phase (days)
1	175	235	60	11	164	212	48
2	191	238	47	12	170	214	44
3	191	245	54	13	164	209	45
4	191	249	58	14	164	200	36
5	191	254	63	15	164	215	51
6	191	254	63	16	170	216	46
7	200	255	55	17	170	214	44
8	200	269	69	18	164	208	44
9	>365	>365	No signs	19	164	222	58
10	>365	>365	No signs	20	170	205	35
				21	170	222	52
				22	170	214	44
Median day	191*	254*	59†		167	214	44.5

*$P<0.0005$ by Mann–Whitney test between vaccinated and control mice.
†$P<0.005$ by Mann–Whitney test between vaccinated and control mice.
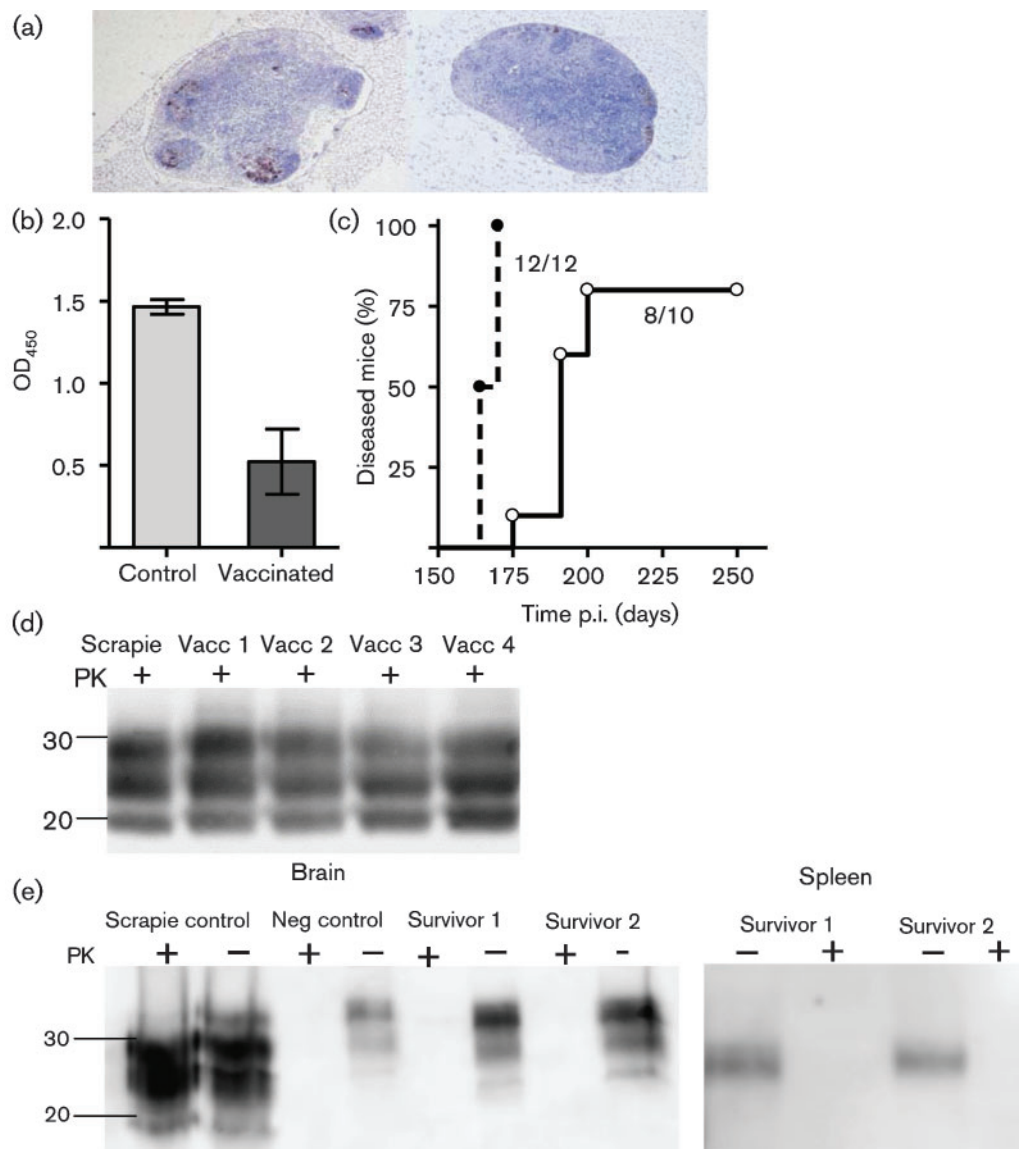
Fig. 5. (a) Immunohistochemical detection of PK-resistant PrPSc on mesenteric LN sections of a vaccinated (left) and a control (right) mouse (×40). (b) PK-resistant anti-PrPSc measured by ELISA in spleen lysates of vaccinated ($n=2$) and control ($n=4$) mice. Data shown are the mean±SEM OD_{450} values. (c) Onset of disease in vaccinated (○) versus control (●) mice. (d) Western blot detection of PrPSc in brains of terminally sick mice. The five lanes show PK-digested brain samples: there is one PBS control and four DC-vaccinated mice with a total disease duration of 235, 238, 245 and 249 days p.i., respectively, compared with 214 days p.i. for the PBS control. A precipitate extract corresponding to 3 mg brain was loaded in each well; blots were exposed for 3 min. (e) No PrPSc was detected by Western blot in the brains and spleens of the two DC-vaccinated survivors (survivor 1 and 2). Infected control is a brain extract of a mouse from the control group which was infected and died from scrapie; normal brain is from a healthy B6 mouse. A precipitate extract equivalent to 10 mg tissue, digested (+) or not (−) was loaded in each well and exposure was for 1 min.

We also looked for the presence of PrPSc in the brains and spleens of the two vaccinated mice that did not develop clinical scrapie. Western blots were run with an equivalent of 10 mg protein. Still, PK-digested lanes were totally free of PrPSc and remained negative even at long exposures (Fig. 5e).

Protection is correlated with Ab response and not disorganized germinal centre (GC) micro-architecture

In order to assess a correlation between survival and Ab secretion with maximal confidence, we increased the sensitivity of the EL4 assay, as described in Methods.

MFI values detected under such conditions ranged from 25 to 300 arbitrary units, at a serum dilution of 1 : 50. Plotting individual serum values at 45 days p.i. against individual disease duration gave a positive linear correlation (Fig. 6a), with an r^2 value of 0.7584 and significance of $P<0.005$. Most interestingly, the serum MFI values of the two mice still free of symptoms at 400 days p.i. (therefore not shown in Fig. 6a) were remarkably high, compared with the MFIs of the other eight mice in the group (Fig. 6b), again underlining the importance of Abs in disease protection.

In order to rule out the possibility that multiple injections of loaded DCs might have disorganized GC micro-architecture, resulting in disease retardation, we examined in detail lymph nodes (LNs) from mice after five injections of 1×10^6 peptide-loaded DCs. Microphotographs of a typical LN reveal definite PrPSc deposits in the GC area (Fig. 7a). H&E staining of serial sections confirms the presence of normally developed GC (Fig. 7b). A GC seen at higher magnification (Fig. 7c) reveals the typical presence of tingible body macrophages and of cells in mitosis. A follicular DC (FDC) with its two pale nuclei and tightly associated lymphocytes with chromatin-dense nuclei is shown in Fig. 7d. In view of these data, characteristic of many other data, it seems unlikely that repeated DC injections cause GC disorganization resulting in delayed scrapie evolution.

DISCUSSION

The aim of the present study was to find out whether DCs loaded with immunogenic, class II-restricted peptides of PrP can break self tolerance, induce T- and B-cell-responses in PrP-expressing wt mice and thus be used as a vaccine against TSEs. DCs have been recognized for a long time as the most effective inducers of T-cell responses and have been used in numerous protocols of cancer immunotherapy. Most groups have focused on the activation of cytotoxic CD8$^+$ T cells as these can lyse MHC class I-positive tumour cells. The induction of

cytotoxic and IFN-γ-secreting CD8$^+$ T cells through DC vaccination was achieved in mice and humans by various techniques (Nair et al., 2000; Nestle et al., 1998; Yu et al., 2004). More recently, some investigators have developed DC protocols to generate responses against MHC class II-restricted tumour peptides. In human patients, Fay et al. (2006) have observed IFN-γ secretion by CD4$^+$ T cells in response to repeated injections of DCs loaded with melanoma peptide. Our protocol of immunization was inspired by that study and others which involve monthly injections of DCs directed against weakly immunogenic tumours (Palucka et al., 2006; Wierecky et al., 2006). Recent mouse studies (Charalambous et al., 2006) suggest that targeting a xenogeneic tumour antigen to maturing DCs via DEC-205 receptors is a very effective way to induce CD4$^+$ immunity to this antigen. Although DCs have also been shown to stimulate B cells, fewer studies aimed at generating humoral responses have been reported so far. Most of these attempts deal with antigens expressed on infectious agents and are intended to be developed as anti-infectious vaccines (Boscardin et al., 2006; Gruber et al., 2007; Lu et al., 2003). Still, there is increasing evidence of in vivo physiological interactions between DCs and B cells which allows the generation of Abs. Experiments published some years ago showed that DCs had an important role in initiating and regulating Ab synthesis by acquiring and keeping protein antigens in a non-degraded form long enough to allow DC migration to secondary lymphoid organs and deliver antigen to naive, recirculating B cells (Wykes et al., 1998). A novel non-degradative pathway of antigen presentation to B cells was also recently described, involving internalization of antigen-IgG immune complexes by DCs via their inhibitory FcγRIIB receptor (Bergtold et al., 2005). Finally, it was recently shown by vital microscopy that, after exiting high endothelial venules and before entering into lymph node follicles, B cells survey locally concentrated DCs. Direct engagement of the B-cell receptor on the DC-associated antigen leads to B-cell activation, migration arrest, antigen acquisition and extra follicular accumulation (Qi et al., 2006).
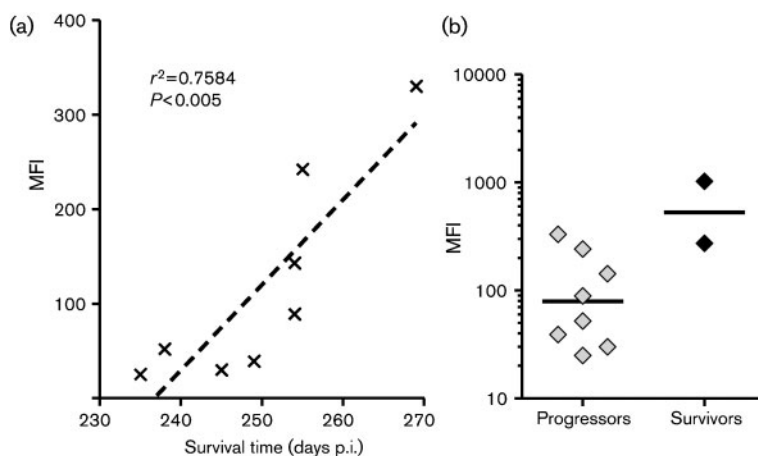
Fig. 6. (a) Correlation analysis between disease survival and MFI values of sera collected at 45 days p.i. in the eight mice that died from scrapie. Ab visualization was achieved in two steps as described in Methods. Sera were tested at several dilutions (1 : 50 is shown). (b) Scatter plot of MFI values of the eight DC-vaccinated mice that progressed toward terminal scrapie compared with the two DC-vaccinated mice that survived over 400 days p.i.
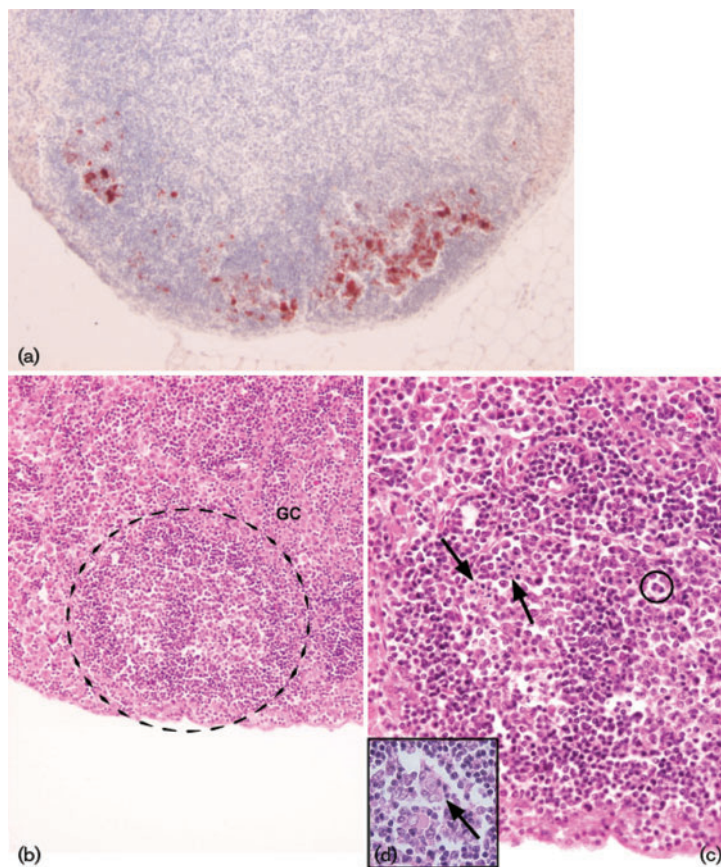
Fig. 7. Photographs of a LN at 75 days p.i., i.e. 1 week after the fifth consecutive injection of loaded DCs. (a) Immunohistochemistry reveals the presence of PrPSc decorating GCs (×100 magnification). (b) Serial sections stained with HE showing normally developed GC (×200) (lower left). (c) GC at higher magnification (×400) displays two tingible body macrophages (arrows) and one cell in mitosis (circled). (d) A typical FDC with two chromatin light nuclei and chromatin-dense tightly associated lymphocytes (oil immersion, ×1000).

In the present study, the injection of PrP$^+$ DCs into *Prnp*$^{-/-}$ mice efficiently induced the generation of T cells producing cytokines and of B cells secreting Ab directed against cell-bound native PrPc. This occurred without peptide loading or addition of free PrP or peptides, suggesting that PrP$^+$ DCs do naturally process MHC class II-restricted peptides in sufficient amounts to activate CD4$^+$ T cells and display enough PrPc conformational epitopes for activating B cells. Presentation to T and B cells was most probably effected by the injected DCs themselves, as no other cell in the recipient expressed PrPc. Still, the possibility remains that recipient DCs engulf or trogocytose elements of donor DCs and present them in turn to T and B cells, as already reported in other models of DC immunization (Herrera *et al.*, 2004).

Injecting PrP$^+$ DCs into wt recipients under the same conditions was clearly insufficient to either elicit cytokine release by CD4$^+$ T cells (Fig. 2) or anti-PrPc Abs by B cells (Fig. 3). PrP$^+$ DCs had to be loaded with PrP$_{98-127}$ or PrP$_{158-187}$ in order to elicit cytokines and Abs. Consistent with previous studies in *Prnp*$^{-/-}$ mice (Gregoire *et al.*, 2004), loading DCs with PrP$_{158-187}$ gave a potent T cell stimulus, and triggered both IFN-γ and IL-4. Loading with PrP$_{98-127}$ elicited a more discrete response almost exclusively centred on IL-4. Most importantly, immunizing with PrP$_{158-187}$- or PrP$_{98-127}$-loaded DCs resulted in Ab

production which steadily increased with the number of injections (data not shown). Mice injected with PBS or PrP$^-$ DCs gave MFIs ranging between 7 and 15 arbitrary units, which corresponds to background values given by normal mouse sera. PrP$_{158-187}$ and PrP$_{98-127}$ were equally potent at eliciting Ab responses (Fig. 3a). The fact that responses were dispersed and globally lower than those of the *Prnp*$^{-/-}$ MFIs in Fig. 1(d) suggests that B- and T-cell precursors are limiting, due to immune tolerance, and that the repertoire which escapes tolerance in wt mice is more difficult to activate. The fact that Abs to cell-surface PrPc are nevertheless generated in wt mice proves that helper T- and B-cell repertoires directed against PrPc epitopes are not totally purged as previously suggested (Gregoire *et al.*, 2005; Polymenidou *et al.*, 2004).

Lastly, we tested the possibility that peptide-loaded DCs might be used as vaccines to prevent prion diseases. We chose PrP$_{98-127}$ because it elicits a more exclusively Th2-oriented response, thus avoiding putative Th1 complications. Iterative injections of PrP$_{98-127}$-loaded DCs resulted in a 20 % reduction of the attack rate. Two of 10 vaccinated mice never became sick. At the time of sacrifice (400 days p.i.) these mice looked healthy and Western blots of proteins from their brains and spleens showed no traces of PrPSc (Fig. 5e). The eight vaccinated mice that finally succumbed to scrapie, had a delayed onset and a slightly

slower clinical course. The fact that, at 75 days p.i., mesenteric lymph nodes and spleens of DC-vaccinated mice contain less PrPSc than a similar control organ suggests that the delayed onset is probably related to a delayed lymphoinvasion. A correlation was also established between disease duration and Ab MFI values (Fig. 6a; correlation coefficient r^2=0.7464 and P-value=0.0057), strongly indicating that Abs to native PrPc are critical to convey anti-scrapie protection. However, the possibility that T-cell responses concomitantly elicited by peptide-loaded DCs may have also contributed to protection cannot be excluded. This issue is currently under investigation in our laboratory by adoptive cell transfer experiments. Given that no correlation was found between prolonged survival in DC-vaccinated mice and the amount of PrPSc deposits in the brain (Fig. 5), it can be concluded that at the terminal stage, prion expansion is the same whatever the prior course of the disease may have been. The fact that surviving mice had absolutely no PrPSc in their spleens and high Ab titres suggests that DC vaccination can in some cases completely block PrPSc replication/accumulation in lymphoid organs, rather than simply hamper neuroinvasion.

We could exclude the possibility that scrapie retardation in vaccinated mice resulted from a GC architecture made chaotic by multiple injections of loaded DCs (Brown *et al.*, 1999; Heikenwalder *et al.*, 2004; Mabbott *et al.*, 2000; Montrasio *et al.*, 2000). LN sections performed after mice had received five DC administrations show a normal GC architecture with abundant PrPSc deposits, a typical presence of tingible body macrophages, cells in mitosis and FDCs.

Compared with other protocols of active therapy against TSE, DC vaccination appears to be among the most efficient. Active vaccination with recombinant PrP in complete Freund's adjuvant was tested by Sigurdsson *et al.* (2002). It delayed onset after i.p. infection and extended survival by 16 days compared with controls. Active immunization with PrP$_{98-127}$ or PrP$_{158-187}$ in the presence of CpG induced T cells and Ab production but prolonged mouse survival by only 20 days (Sacquin *et al.*, 2008). More encouraging results have been obtained by Goni *et al.* (2008) following oral immunization with *Salmonella typhimurium* encoding one or two copies of mouse *Prnp* in their genome. The attack rate was reduced down to zero in mice immunized six times prior to challenge and selected for high IgA production. The advantage of using DCs as a vaccine support was also demonstrated in a recent study performed by a member of our group (Rosset *et al.*, 2009). There, the main principle relied on the transduction of DCs from wt mice with a recombinant adenovirus coding for human PrP (hPrP), followed by administration into wt mice subsequently infected with a scrapie agent. Clinical disease was significantly delayed in the groups which received DCs expressing mouse PrPc and transduced with adenovirus (Ad) encoding hPrPc or transduced with a control Ad. However, the best protection was obtained after vaccination with transgenic DCs expressing hPrP and transduced by a control Ad.

In the study by Rosset *et al.* (2009), both PrP-specific cytotoxic T cells and Ab responses were analysed and circulating Abs against native mouse PrPc were significantly increased; however, the exact immune mechanisms involved were not precisely identified. Interestingly, Abs against native mouse PrP were far less efficiently generated in mice directly immunized with recombinant adenoviruses encoding hPrP, thereby underlining the advantage of targeting antigen to DCs.

The present study, in contrast with that of Rosset *et al.* (2009), used exclusively non-transduced DCs administered immediately after differentiation/maturation and loaded with well-defined peptides. Special attention was given to understanding the role of each immunogenic constituent, i.e. the PrPc expressed on DCs and the loaded peptides. Results show that in order to break tolerance in PrP$^+$ mice, DCs must not only be loaded with peptide but also express constitutive PrPc (Fig. 4a). This result suggests that T and B cells recognize determinants of different origin on DCs. T cells bind to class II-restricted epitopes which result from the processing of the loading peptides, whereas B cells are probably activated by conformational PrPc epitopes displayed at the surface of the DC. Because we have reported in a previous study that PrPc is involved in the formation of the T/DC synapse and its absence or its masking by Ab hampers T/DC interactions (Ballerini *et al.*, 2006), we cannot totally rule out the possibility that the reduced immunogenicity of PrP$^-$ DCs may also result from a weaker activation of helper T cells. Still, an additional proof that B cells are activated by determinants independent of the loading peptides was provided by epitope mapping of Abs. The pattern of peptide recognition was remarkably recurrent among sera, regardless of whether DCs had been loaded with PrP$_{98-127}$ or PrP$_{158-187}$. Interestingly, dominantly recognized epitopes were concentrated in the flexible N-terminal domain of the protein which is likely to be the most exposed (Donne *et al.*, 1997). Whereas PrP$_{158-187}$ is a dominantly recognized T-cell peptide, there was no Ab binding to it, confirming the independence between Ab fine specificities and T-cell epitopes.

From the perspective of applying DC vaccination to human patients, DCs cultured *in vitro* can be used with no further manipulation and by simple loading with synthetic, pure and well-defined immunogenic peptides. Virally transduced DCs also afford good protection and have the advantage of not requiring the identification of peptides restricted to a given MHC context. On the other hand, they involve a supplementary step of recombinant adenovirus production and transduction and may therefore be less safe or easy to use than DCs expanded *in vitro* from a patient and simply loaded with synthetic peptides before reinjection. In any case, introducing DC vaccine protocols in the field of neurodegenerative diseases opens promising

perspectives. The ongoing work produced in cancer therapy will be most useful for developing safe and efficient DC vaccines against TSE and, more generally, against neurodegenerative conditions.

METHODS

Mice. $Prnp^{-/-}$ mice were from the original $129 \times$ B6 Zurich nucleus (Bueler *et al.*, 1992) (with kind permission from C. Weissmann, Scripps Institute, Florida). The null mutation was subsequently back-crossed in our facility onto B6 mice. $Prnp^{-/-}$ mice used throughout this study were homozygous offspring derived from back-cross 12. B6 wt mice are fully histocompatible with $Prnp^{-/-}$ mice, as assessed by reciprocal skin grafting. All mice were raised and maintained under strictly monitored specific-pathogen-free conditions. Experimentation was conducted in accordance with the French legislation and European Community recommendations.

Peptides. PrP_{97-128} and $PrP_{158-187}$ are two immunogenic peptides recognized by $CD4^+$ T cells in a class II, I-A^b context. They were previously identified out of a bank of 13 peptides, mostly 30-mers, with a 15 aa overlap on each side (Gregoire *et al.*, 2004). The bank was synthesized by NeoMPS (Strasbourg, France) with a minimum of 80 % purity. Both PrP_{98-127} and $PrP_{158-187}$ are soluble in water without addition of DMSO, at a 10× concentration of 150 µg ml^{-1} and are LPS-free.

DC generation. BM-derived DCs (BMDCs) were differentiated from femur and tibia BM of 8- to 10-week-old wt or $Prnp^{-/-}$ female mice (Weigel *et al.*, 2002). Cells were cultured in RPMI 1640 (Gibco Invitrogen) supplemented with 10 % FCS, penicillin/streptomycin (1000 IU ml^{-1}/10 mg ml^{-1}), 2 mM glutamine and 0.05 mM 2-mercaptoethanol. Granulocyte-macrophage colony-stimulating factor (PeproTech) was added at 200 U ml^{-1} from the onset and renewed at days 3 and 6. On day 7, DCs were recovered, adjusted to 1×10^6 cells ml^{-1} and matured overnight with LPS at 2 µg ml^{-1} (Sigma). They were collected the following day and pulsed for 4 h, at a concentration of 2×10^6 cells ml^{-1}, with 10 µM peptide PrP_{98-127} or $PrP_{158-187}$ and then washed three times in PBS. All batches of DCs were monitored, by flow cytometry, for high CD11c, MHC class II, CD80 and CD86 expression (data not shown), using a FACSCalibur and CellQuest Pro software (Becton Dickinson).

DC injections. DCs were prepared as described above and injected i.p. in PBS at a concentration of 1×10^6 per mouse. Mice periodically treated against scrapie received a total of seven DC injections; the first three were at 15 day intervals and the remaining four were at monthly intervals. Seven days after the second DC injection (d21), mice were infected i.p. with a 139A inoculum of 2×10^4 LD$_{50}$.

ELISPOT assay. IFN-γ- and IL-4-producing T cells were enumerated by ELISPOT as described previously (Ballerini *et al.*, 2006). T cells (2×10^5 per well) enriched by negative magnetic selection and spleen DCs (1.5×10^4 per well) positively selected with a CD11c magnetic kit (Miltenyi Biotec) were plated together onto nitrocellulose-based microplates (Millipore). Abs against IFN-γ were clone R4-6A2 for capture and XMG1.2 for revelation (PharMingen). For IL-4, we used the Ab from the IL-4 ELISPOT kit (R&D). PrP_{98-127} or $PrP_{158-187}$ were added at 15, 1.5, 0.15 and 0 µg ml^{-1} and plates were incubated for 24 h for IFN-γ or 48 h for IL-4. Spots were counted using an automatic ELISPOT plate counter (AID). Results are expressed as the average number of spots per 1×10^6 T cells. Because unloaded DCs are sufficient to elicit non-specific stimulation of T cells from DC-vaccinated mice (Palucka *et al.*, 2005), results are presented as: (the number of spots generated in the presence of DCs+

peptide)−(background number of spots generated with unloaded DCs).

Solid phase ELISA. ELISA against a bank of plastic-immobilized peptides was performed as described previously (Gregoire *et al.*, 2005). Sera were distributed as duplicates at a 1 : 50 dilution and left for 2 h at room temperature on antigen-coated polypropylene microtitre plates (Maxisorb). Bound Ab was revealed with goat anti-total mouse Ig or goat anti-mouse IgM, IgG1 or IgG2a, coupled to peroxidase (Roche). The chromogenic reaction was generated in the presence of H_2O_2 with phenylenediamine in 0.05 M citrate buffer (Sigma) and was stopped with 25 µl 2 N sulfuric acid. SAF84, a monoclonal Ab directed against mouse PrP, served as a positive standard.

Indirect immunofluorescence on EL4 cells for anti-native PrPc Abs. Ab binding to cell-surface native PrPc was detected by immunofluorescence on the EL4 T cell line as described previously (Gregoire *et al.*, 2004). Since EL4 cells express relatively low levels of membrane PrPc under spontaneous conditions, they were stably transfected with the *Prnp* gene and were activated before each test in order to optimize the sensitivity of Ab detection. EL4 cells collected 24 h after their last passage, were incubated overnight with anti-CD3 Ab (clone 2C11), plated at 10 µg ml^{-1}. Dilutions of sera at 1 : 20, 1 : 50 and 1 : 100 were incubated for 20 min with 1×10^5 activated EL4 cells. Bound Ab was revealed with the following secondary antibodies: either mark1, a rat anti-mouse κ-chain monoclonal Ab coupled to PE (Cormont *et al.*, 1986), or polyclonal rat anti-mouse IgG1 or anti-IgG2a coupled to FITC or PE (Becton Dickinson). Every assay includes normal B6 mouse sera as background controls and anti-PrPc SAF-84 monoclonal Ab as the upper limit. Cell-surface fluorescence is expressed as the geometric MFI, measured by flow cytometry on a FacsCalibur with CellQuest-Pro software. In the assays aimed at correlating MFIs with survival (specified in Results and in legend to Fig. 6), the MFI signal was reinforced by a two step visualization: firstly with a biotinylated rat anti-mouse IgG and secondly with streptavidin–allophycocyanin (all from Becton Dickinson).

Scrapie inoculation. The 139A strain of scrapie has been serially passaged by intracerebral injection into B6 mice. An infectious stock (10 % w/v in PBS) was prepared out of a pool of brains from terminally ill mice. Mice were infected i.p. with 0.1 ml homogenate at 0.5 % representing 2×10^4 LD$_{50}$ units, 7 days after the second DC immunization, therefore 21 days after the start of the vaccination process. They were scored as clinically sick after three consecutive observations of reduced activity in the cage and poor gait control when crossing a set of regularly spaced bars, as described previously (Aucouturier *et al.*, 2001).

Anti-PrPSc ELISA. The ELISA was performed with the TeSeE Sheep/ Goat ELISA kit (Bio-Rad) according to the manufacturer's recommendations, with minor modifications. Spleens were resuspended in 10 % (w/v) lysis buffer. ELISA was performed on 100 µl spleen lysate.

Western blots. Brain or spleen samples were prepared at 10 % (w/v) in PBS plus protease inhibitors (Complete, Roche Diagnostic). Homogenization was performed with the Fast Prep method applied for 40 s. One aliquot was digested at 37 °C for 15 min with proteinase K (PK) (Roche, Meylan, France) at 10 µg ml^{-1}, the other was left intact. Aliquots equivalent to 3 mg or 10 mg of original tissue, as specified in the legend to Fig. 5d, e and Results, were separated on 12 % SDS-PAGE. Samples were subsequently transferred to nitro-cellulose membranes and revealed with mAb SAF-84 at 1 : 5000, followed by incubation with a goat anti-mouse Ig coupled to peroxidase and chemiluminescent substrate. Signal was captured with a Fujifilm LAS3000 camera for 1 min. Longer exposures (not shown)

were done to demonstrate complete PK-digestion of PrPc and the absence of any trace of PrPSc in protected mice

Histology. GC micro-architecture was analysed on fixed 5 μm serial sections, stained with haematoxylin and eosin, and examined at various magnifications. Photographs were taken with an Olympus BX51 microscope equipped with an Olympus DP50 camera.

PrPSc was detected by immunohistochemistry on fixed 5 μm contiguous sections as described previously (Levavasseur *et al.*, 2007). Sections were treated with 98 % formic acid, heated for 10 min at 121 °C in 10 mM citrate buffer pH 6.1 and digested with 10 μg ml^{-1} PK. PrP resistant to proteinase K digestion (PrPres) was revealed for 60 min with mAb SAF84, followed by 30 min with the peroxidase-conjugated EnVision reagent (K4000, Dako). Slides were counterstained with Mayer's haematoxylin.

Statistics. Analyses were performed with GraphPad software and values were considered significantly different for $P<0.05$. When more than two groups of data were compared, the Kruskal–Wallis multivariate analysis was used, followed by a Dunn's post-test. A non-parametric Mann–Whitney test was used for comparing two sets of data. Correlation coefficient r^2 and statistical significance was established by linear regression.

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