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1 **Immisation strategies against prion diseases: prime-boost immunisation with a PrP**
2 **DNA vaccine containing foreign helper T-cell epitopes does not prevent mouse scrapie**

3

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11 *Running Title:* prime-boost immunisation against the prion protein

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42 **Abstract**

43 Vaccination against prion diseases constitutes a promising approach for the treatment
44 and prevention of the disease. Passive immunisation with antibodies binding to the cellular
45 prion protein (PrP^C) can protect against prion disease. However, immunotherapeutic strategies
46 with active immunisation are limited due to the immune tolerance against the self-antigen. In
47 order to develop an anti-prion vaccine, we designed a novel DNA fusion vaccine composed of
48 mouse PrP and immune stimulatory helper T-cell epitopes of the tetanus toxin that have
49 previously been reported to break tolerance to other self-antigens. This approach provoked a
50 strong PrP^C-specific humeral and cellular immune response in PrP null mice, but only low
51 antibody titres were found in vaccinated wild-type mice. Furthermore, prime-boost
52 immunisation with the DNA vaccine and recombinant PrP protein increased antibody titres in
53 PrP null mice, but failed to protect wild-type mice from mouse scrapie.

54
55 *Key words:* prion protein, DNA fusion vaccine, tetanus toxin, P30

57 **1. Introduction**

58 Prion diseases or transmissible spongiform encephalopathies (TSE) belong to a group
59 of fatal neurodegenerative diseases affecting humans and animals (Prusiner et al., 1998). The
60 causative infectious agent, designated prion, is mainly composed of PrP^{Sc}, a detergent-
61 insoluble and partially protease-resistant pathological conformer of the cellular prion protein,
62 PrP^C (Caughey and Lansbury, 2003; Prusiner, 2004). To date, no prophylactic or effective
63 treatment is available for prion diseases (Cashman and Caughey, 2004; Mallucci and
64 Collinge, 2005; Weissmann and Aguzzi, 2005). Successful vaccination studies for the
65 treatment of neurodegenerative diseases like Alzheimer's disease (Citron, 2004; Monsonego
66 and Weiner, 2003) have stimulated various immunotherapeutic approaches against prion
67 diseases (Aguzzi and Sigurdson, 2004; Buchholz et al., 2006; Cashman and Caughey, 2004).

68 Evidence for a possible therapeutic effect of PrP^C-specific antibodies in diminishing prion
69 infectivity was first reported from *in vitro* studies (Gabizon et al., 1988). Further studies have
70 shown that anti-PrP antibodies or Fab fragments and single-chain molecules derived thereof
71 have a pronounced effect in suppressing prion replication in cultured cells (Donofrio et al.,
72 2005; Enari et al., 2001; Peretz et al., 2001). Passive immunisation of mice with PrP^C-specific
73 antibodies or transgenic expression of a monoclonal anti-PrP antibody delays the
74 accumulation of prions in lymphoid organs and the onset of disease in mice (Heppner et al.,
75 2001; Sigurdsson et al., 2003; White et al., 2003). However, the efficacy of active
76 immunisations is limited due to immunological tolerance to PrP^C, which is a ubiquitously
77 expressed GPI-anchored protein on the cell surface mainly of neurons, but also on glial and
78 peripheral cells, albeit to a lesser extent (Flechsigs and Weissmann, 2004; Ford et al., 2002).
79 The lack of immunity to PrP has been attributed to T-helper tolerance, and studies have been
80 initiated to overcome tolerance by presenting PrP to the immune system with a variety of
81 adjuvants and in diverse forms, such as peptides (Magri et al., 2005; Schwarz et al., 2003),
82 protein (Koller et al., 2002; Sigurdsson et al., 2002), protein dimer (Gilch et al., 2003;
83 Polymenidou et al., 2004), retroviral particle (Nikles et al., 2005) or *Salmonella* vaccine (Goni
84 et al., 2005). In most of the initial studies, moderate levels of PrP-specific antibodies were
85 induced in wild-type mice, which were able to delay the onset of prion disease by a few days.
86 However, some studies were challenged by the observation that adjuvant by itself also had an
87 effect on the survival time of prion-infected animals (Sethi et al., 2002; Tal et al., 2003). In an
88 alternative approach, DNA PrP vaccines have been generated, in which mouse PrP has been
89 fused either to ubiquitin or the lysosomal membrane protein LIMPII to enhance antigen
90 presentation via the major histocompatibility complex (MHC) class I or class II, respectively
91 (Fernandez-Borges et al., 2006). Finally, immunisation studies in mice have been undertaken
92 with recombinant PrP molecules from other species, which are immunogenic in mice because
93 of their different amino acid compositions eliciting antibodies not only specific to the injected

94 PrP molecules but also to the corresponding mouse PrP sequences, albeit at lower levels
95 (Ishibashi et al., 2006; Kascsak et al., 1987).

96 The current study explores the efficacy of an active immunisation approach against
97 PrP by generating a novel DNA vaccine composed of mouse PrP and universally
98 immunogenic T-cell epitopes to break T-cell tolerance in wild-type mice. We hypothesised
99 that prime-boost immunisation with the designed DNA vaccine and recombinant mouse PrP
100 could induce immune responses to PrP with a therapeutic effect on mouse scrapie. Using
101 DNA fusion vaccines incorporating the antigen linked to T-cell epitopes of the tetanus toxin is
102 a well-established strategy used to break tolerance to self antigens like intracellular or surface-
103 expressed tumour antigens (Stevenson et al., 2004). Effective bypassing of immunological
104 self-tolerance in mice has been achieved against cellular proteins such as ubiquitin (Dalum et
105 al., 1997; Dalum et al., 1996), the proinflammatory cytokine TNF- α (Dalum et al., 1999),
106 cytokine IL-5, associated with allergies and asthma (Hertz et al., 2001), and the membrane
107 protein Nogo-A that is involved in neurite outgrowth and nerve regeneration (Merkler et al.,
108 2003). Immune responses to these targeted antigens were increased by multiple helper T-cell
109 epitopes of the non-toxic C-terminal fragment of the tetanus toxin (FrC) or shorter sequences
110 thereof, including P2 and P30 (Panina-Bordignon et al., 1989). In this study, we generated a
111 DNA vaccine composed of mouse PrP and the P30 sequence of the tetanus toxin. Vaccination
112 provoked a strong immune response in PrP null mice, but only low antibody titres were found
113 in a few wild-type mice. Prime-boost immunisation with the DNA vaccine and recombinant
114 PrP protein increased antibody titres in PrP null mice, but failed to protect wild-type mice
115 from mouse scrapie.

116

117 **2. Material and Methods**

118 *2.1 Animals and scrapie infection*

119 All mice were kept under specific pathogen-free conditions at the Institute of Virology
120 and Immunobiology and all experiments were carried out according to legal animal welfare
121 requirements for animal experiments. For immunisation, wild-type (C57BL/6) mice were
122 purchased from Janvier (Le Genest-St-Isle, France) and PrP null (*Prnp^{0/0}*) mice (Büeler et al.,
123 1992) were bred at the Institute. Immunised wild-type mice were challenged with a mouse-
124 adapted Rocky Mountain Laboratories (RML) prion inoculum prepared as described (Kaeser
125 et al., 2001). The newly generated stock (RML.5) was a 10% (w/v) homogenate of RML-
126 infected, terminally sick CD1 mouse brains in 0.32M sucrose with a titre of 8.6 log LD₅₀
127 units/ml as determined by endpoint titration (Reed and Muench, 1938). Mice were challenged
128 intraperitoneally (i.p.) with 0.1 ml of the diluted stock containing 3.6 log LD₅₀ units in
129 phosphate-buffered saline (PBS) with 5% bovine serum albumin (BSA). Mice were
130 monitored every second day, and scrapie was diagnosed using standard clinical signs of
131 mouse scrapie (Büeler et al., 1993).

132

133 *2.2 Vectors and immunisation*

134 For DNA immunisation, cDNA of mouse PrP was cloned under control of the CMV
135 promoter into a pCG expression plasmid (Schlereth et al., 2000) resulting in the vector pCG-
136 PrP. A synthetic linker encoding the codon-optimised sequence of the tetanus toxin (residues
137 947-967), designated P30 (Panina-Bordignon et al., 1989), was inserted between amino acid
138 223 and 224 of mouse PrP. The open reading frame of the modified mouse PrP was cloned in
139 the multiple cloning site of the pCG vector, yielding pCG-PrP-P30. For protein vaccination,
140 recombinant full-length mouse PrP (rPrP) was expressed in *E. coli* and purified as described
141 previously (Zahn et al., 1997). Mice were vaccinated (0.1mg; four times intradermally
142 followed by two subcutaneous injections) every four weeks either with pCG-PrP, pCG-PrP-
143 P30, or the empty pCG vector. For prime-boost immunisation with DNA and rPrP, mice were
144 immunised every four weeks five times with 0.1mg of either pCG, pCG-PrP or pCG-PrP-P30

145 DNA followed by two injections of 10 μ g rPrP and CpG-1668 as adjuvant. The
146 oligodeoxynucleotide CpG-1668 (5'-tcc-atg-acg-ttc-ctg-atg-ct-3') was purchased from MWG
147 (Germany, Ebersberg).

148

149 *2.3 Determination of antibody titres by ELISAs*

150 Ten days after each immunisation, antibody titres were determined in the blood of
151 immunised *Prnp*^{0/0} and wild-type mice against rPrP coated on ELISA plates (50ng/well). Sera
152 of *Prnp*^{0/0} mice were diluted 1:250 and 1:1000 for the experiments shown in Figures 1 and 3,
153 respectively. Sera of wild-type mice were analysed at a dilution of 1:40. After washing, the
154 plates were probed with a horseradish peroxidase-conjugated goat anti-mouse Ig, IgG1 or
155 IgG2a antibody (DAKOCytomation, Carpintera, CA) and developed with BD OptiEIA (BD-
156 Pharmingen, Germany, Heidelberg); the optical density was measured at 450 nm.

157

158 *2.4 Flow cytometric analysis*

159 For determination of PrP^C-specific antibody binding, mouse neuroblastoma N2a cells
160 were incubated for 2h at 4°C with sera of immunised mice, or anti-PrP antibody 6H4
161 (Prionics, Switzerland) as a positive control. After washing, cells were incubated for 1h at 4°C
162 with FITC-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany). Samples were
163 analysed on a FACS Calibur (Becton Dickinson, Heidelberg, Germany) and data analysis was
164 undertaken with Cell Quest software (Becton Dickinson).

165

166 *2.5 Histology*

167 Paraffin-embedded sections of 2-4 μ m in thickness, prepared from brains and spleens
168 of terminally sick mice, were stained with haematoxylin/eosin (HE). Immunostaining for glial
169 fibrillary acidic protein (GFAP) was performed by using rabbit antiserum against GFAP

170 (1:5000 dilution; DAKOCytomation, Carpintera, CA) followed by incubation with avidin-
171 peroxidase and staining with diaminobenzidine (Sigma, Taufkirchen, Germany).

172

173 *2.6 Proliferation assay*

174 For T-cell proliferation, spleens of mice immunised with DNA or control mice were
175 used and isolated cells of each mouse were tested in quadruplicate. Cells were plated in 96-
176 well plates, at a density of 3×10^5 cells per well. Spleen cells were re-stimulated with 2.5
177 $\mu\text{g/ml}$ of rPrP. Cells were maintained for 72h and pulsed with [^3H] thymidine (0.5 $\mu\text{Ci/well}$)
178 18h prior to analysis with a beta counter (Perkin Elmer, Rodgau-Jügesheim, Germany).

179

180 *2.7 Inhibition assay of PrP^{Sc} accumulation in cultured cells and western blot analysis*

181 Serum from immunised mice was added to the culture media of RML prion-infected
182 N2a (ScN2a) cells at a dilution of 1:40 and renewed three times in 5 days. Cell lysates (1
183 mg/ml) were treated with proteinase K (20 $\mu\text{g/ml}$) for 30min at 37°C. Treated lysates (150
184 μg) were resolved by SDS-PAGE (12%) and transferred to PVDF membrane. Polyclonal
185 rabbit antiserum SA2124 raised against rPrP (1:5000) and goat-anti-rabbit antibody
186 conjugated with horseradish peroxidase (DAKOCytomation, Carpintera, CA; 1:10.000) were
187 used. PrP signals were visualized by incubation with ECL substrate (Pierce, Germany, Bonn)
188 and exposed to Hyperfilm (Amersham Biosciences, Buckinghamshire, UK).

189

190 **3. Results**

191 *3.1 Humoral responses against PrP after DNA immunisation*

192 Prior to immunisation, newly generated DNA vaccines were tested in transient
193 transfections of HEK 293 cells to ensure that the encoded proteins were appropriately
194 expressed. Both vaccines, the non-modified wild-type PrP (pCG-PrP) and the modified PrP
195 with the P30 sequence (pCG-PrP-P30), were expressed on the cell surface, as detected by

196 flow cytometric analysis (data not shown). After immunisation with each DNA vaccine,
197 antibody titres were determined against recombinant full-length mouse PrP (rPrP) by ELISA.
198 *Prnp*^{0/0} mice developed a strong antibody response to rPrP after immunisation with pCG-PrP
199 or pCG-PrP-P30, but not with the empty vector pCG (Fig. 1A). No difference in antibody
200 titres was found between the two groups. In contrast, only weak antibody levels were
201 detectable in a few wild-type mice after more than six immunisations, but most animals had
202 no detectable anti-PrP antibodies (Fig. 1C). The signals in these wild-type mice were similar
203 to those found in mice given the empty DNA vector. In *Prnp*^{0/0} mice, both PrP vaccines
204 induced IgG1 and IgG2a isotypes at comparable levels suggesting the induction of a mixed T-
205 helper type 1 (Th1) and type 2 (Th2) response to PrP^C (Fig. 1B). Since binding to PrP^C is
206 crucial for the anti-prion effect of antibodies (Enari et al., 2001; Polymenidou et al., 2004), we
207 performed flow cytometric analysis of the serum from immunised mice on neuroblastoma
208 (N2a) cells expressing mouse PrP^C. Strong binding of sera from *Prnp*^{0/0} mice receiving pCG-
209 PrP or pCG-PrP-P30 was found (Fig. 1D). The binding intensities correlated with the anti-PrP
210 antibody titres of individual *Prnp*^{0/0} mice. In contrast, only one serum from each group of
211 wild-type mice immunised with pCG-PrP or pCG-PrP-P30 showed a weak binding to PrP^C on
212 N2a cells (Fig. 1E).

213 To determine whether the elicited anti-PrP antibodies inhibited PrP^{Sc} accumulation in
214 cultured cells, RML prion-infected N2a (ScN2a) cells were treated for five days with the
215 serum from each mouse. Sera from *Prnp*^{0/0} mice immunised with pCG-PrP or pCG-PrP-P30
216 reduced PrP^{Sc} levels on ScN2a cells to almost undetectable levels (Fig. 1F), whereas sera
217 from wild-type mice had no effect (Fig. 1G). Anti-PrP immune sera of *Prnp*^{0/0} mice also
218 reduced PrP^C levels of ScN2a cells (Fig. 1F), as previously reported (Gilch et al., 2003).
219 These data indicate that both DNA vaccines induce anti-PrP antibodies in *Prnp*^{0/0} mice, which
220 were able to inhibit PrP^{Sc} accumulation *in vitro*, yet they failed to break immunological
221 tolerance in wild-type mice.

222

223 *3.2 Cellular responses against PrP after DNA immunisation*

224 To evaluate the cellular immune response to the PrP vaccines, splenocytes were
225 harvested after six immunisations and re-stimulated with rPrP *in vitro*. In *Prnp*^{0/0} mice, both
226 PrP vaccines induced T-cell proliferation in contrast to the pCG control vector (Fig. 2A). The
227 stimulation indices (SI) were significantly higher after vaccination with pCG-PrP-P30
228 compared to the pCG-PrP vaccine ($P < 0.05$; Mann-Whitney test). Splenocytes from wild-type
229 mice did not show any PrP-specific proliferation, regardless of the PrP vaccine used (data not
230 shown). Although T-cell proliferation could be induced in *Prnp*^{0/0} mice, no differences in
231 cytokine production by CD4⁺ or CD8⁺ T cells were detectable upon re-stimulation with anti-
232 CD3/CD28 antibodies *in vitro* (data not shown). All groups of vaccinated *Prnp*^{0/0} mice,
233 including the pCG control, showed comparable percentages of T cells producing TNF- α or
234 IFN- γ .

235

236 *3.3 Humoral responses against PrP after prime-boost immunisation*

237 The ability of prime-boost immunisations with DNA and protein to enhance immune
238 responses has been demonstrated in various animal models (Ramshaw and Ramsay, 2000).
239 Therefore, we immunised *Prnp*^{0/0} mice and wild-type mice with either pCG, pCG-PrP or
240 pCG-PrP-P30 DNA followed by two injections of rPrP mixed with the synthetic
241 oligodeoxynucleotide, CpG-1668, as adjuvant. After the final immunisation, antibody titres in
242 *Prnp*^{0/0} mice receiving pCG-PrP or pCG-PrP-P30 and rPrP/CpG-1668 were more than four
243 times higher than in mice immunised with DNA alone (Fig. 3A). The antibody responses
244 were dominated by IgG1, suggesting a shift towards Th2-type responses due to the protein
245 boosts (data not shown). The PrP specificity of the IgG antibodies was confirmed by
246 demonstrating binding to PrP on N2a cells (Fig. 3B). In contrast, vaccinated wild-type mice
247 showed antibody titres against rPrP when looked at using ELISA regardless of the PrP

248 vaccine or the empty vector (Fig. 3A), but these antibodies were largely unspecific because
249 they were not able to bind PrP^C on N2a cells (data not shown). These results show that a
250 prime-boost vaccination with DNA and rPrP-enhanced antibody titres in *Prnp*^{0/0} mice, but
251 failed to induce PrP^C-specific antibodies in wild-type mice.

252

253 *3.4 Development of scrapie after prime-boost immunisation and peripheral prion challenge*

254 To determine whether prime-boost immunisation protects against infection with mouse
255 scrapie, wild-type mice were immunised five times with 0.1mg of pCG, pCG-PrP or pCG-
256 PrP-P30 followed by two injections of 10µg rPrP/CpG-1668, and subsequent intraperitoneal
257 (i.p.) challenge with 3.6 logLD₅₀ units of RML prions. All the mice developed mouse scrapie
258 with similar incubation times as found in non-immunised controls (Fig. 3C) and they all had
259 comparable levels of PrP^{Sc} in spleen and brain at the terminal stage, as determined by Western
260 blotting (data not shown). In addition, the brains of vaccinated and non-vaccinated mice
261 showed similar histopathology including microvacuolation and reactive gliosis. No overt
262 differences in the splenic microarchitecture were found between immunised and non-
263 immunised animals (Fig. 3D).

264

265 **4. Discussion**

266 In this study we have assessed the ability of a DNA fusion vaccine composed of
267 mouse PrP and three universally immunogenic T-cell epitopes (P30) to break tolerance to PrP
268 in wild-type mice. The DNA vaccine was able to elicit immune responses against
269 recombinant mouse PrP only in PrP null mice, but not in wild-type mice. Only antibodies that
270 bound to recombinant PrP but failed to recognise native PrP expressed on N2a cells were
271 found in vaccinated wild-type mice. This is consistent with studies in which modest levels of
272 anti-PrP antibodies were detectable in wild-type mice vaccinated with tandem PrP or PrP
273 displayed on viral particles by using a sensitive binding assay (Nikles et al., 2005;

274 Polymenidou et al., 2004). Limited by the lower sensitivity of our assay, we cannot exclude
275 the presence of low levels of IgM antibodies in our animals. Although the DNA vaccine could
276 induce IgM to IgG class switch in PrP null mice, PrP^C-specific IgG antibodies were only
277 induced in a few wild-type mice. Other vaccines, like retroviral particles, have been shown to
278 induce class switch in wild-type mice, but the switch to PrP^C-specific IgG antibodies was less
279 pronounced in wild-type than in PrP null mice (Nikles et al., 2005). The general potential of
280 P30 to break tolerance was demonstrated in various models (Stevenson et al., 2004). For
281 example, P30 fused to the inflammatory cytokine IL-5 bypassed B-cell tolerance and induced
282 neutralising antibodies (Hertz et al., 2001). However, in some cases the P30 sequence
283 encoding only three T-cell epitopes was not as efficient as the multiple T-cell epitopes of the
284 entire FrC fragment of the tetanus toxin (Stevenson et al., 2004), suggesting a higher potential
285 for a PrP-FrC DNA vaccine.

286 The PrP-P30 DNA vaccine also failed to mediate cellular immune responses in wild-
287 type mice. However, the vaccine induced T-cell proliferation in PrP null mice, but only of low
288 magnitude. PrP might be involved in T-cell activation (Cashman et al., 1990; Mabbott et al.,
289 1997) or may even be a component of the signalling complex (Mattei et al., 2004). Indeed,
290 PrP^C is up-regulated on T-cells upon stimulation with concavalin A, whereas the T-cell
291 response to concavalin A is significantly reduced in PrP null mice (Mabbott et al., 1997).
292 Thus, the lack of PrP^C on T-cells could lead to generally low T-cell activation levels, which
293 might explain the weak T-cell response to the PrP DNA vaccine in PrP null mice.

294 To improve the efficiency of the DNA vaccine, we performed prime-boost
295 immunisation with DNA and recombinant mouse PrP mixed with CpGs as adjuvant. Making
296 use of CpGs has recently been suggested as an adjuvant for vaccination studies in wild-type
297 mice (Rosset et al., 2004). Although our strategy increased antibody titres in PrP null mice, no
298 antibodies were detectable in wild-type mice as judged by binding to native PrP on N2a cells.
299 It might be conceivable that anti-PrP antibodies were present but that they formed complexes

300 with PrP^C, which prevented their detection. Although we have not determined whether such
301 complexes exist, it could be possible that even low levels of the complexes can protect against
302 prion infection in a similar mechanism as shown for soluble dimeric PrP fused to an Fc-
303 γ immunoglobulin (Meier et al., 2003). To address this hypothesis, wild-type mice vaccinated
304 with DNA and recombinant mouse PrP were peripherally challenged with a low dose of
305 mouse prions. However, the incubation times of vaccinated mice were similar to those found
306 in non-vaccinated mice. Moreover, PrP^{Sc} levels and histopathological changes were similar in
307 the brains of vaccinated and non-vaccinated mice at the terminal state of the disease. Since no
308 evidence for any therapeutic effect was found, it is unlikely that the PrP DNA vaccine
309 induced any biologically relevant antibody responses in our model. The efficiency of the
310 antigen presentation of our DNA vaccines in wild-type mice was not analysed, but targeting
311 them specifically to MHC class I or class II pathways might improve their potential to break
312 tolerance (Leifert et al., 2004). As recently described, DNA vaccination with a PrP fused to a
313 lysosomal protein-enhancing MHC class II antigen increased the cellular and humoral
314 response to PrP in PrP null mice. Surprisingly, vaccination with this vaccine delayed the onset
315 of the disease but not the overall survival time after contracting the disease (Fernandez-
316 Borges et al., 2006).

317 Recently, it has been reported that an attenuated *Salmonella* vaccine containing a
318 tandem PrP fused to the FrC fragment induced mucosal immunity that prolonged the survival
319 time of mice after oral infection (Goni et al., 2005). Therefore, making use of multiple T-cell
320 epitopes of the entire FrC fragment fused to PrP, in combination with administration of the
321 chimeric protein, might improve approaches to inducing systemic immunity against prion
322 diseases.

323

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329

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486 **Figure Legends:**

487

488 **Fig. 1: Antibody responses of *Prnp*^{0/0} and wild-type mice after DNA vaccinations and**
 489 **their ability to bind to native PrP and inhibit PrP^{Sc} in cultured cells.**

490 Antibody titres in *Prnp*^{0/0} (A) and wild-type (C) mice vaccinated with pCG-PrP or pCG-PrP-
 491 P30 or the empty vector (pCG) were determined ten days after the 6th immunisation by
 492 ELISA. The titre of each mouse is indicated by a symbol and the mean values of each group
 493 are given in bars. *Prnp*^{0/0} mice show a strong and comparable antibody response (dilution
 494 1:250) with both PrP vaccines (A) whereas wild-type mice (C) show low PrP antibody titres
 495 (dilution 1:40) and similar levels to the empty vector (pCG). (B) Both DNA vaccines induce
 496 comparable levels of PrP^C-specific IgG1 (filled column) and IgG2a (open column) in *Prnp*^{0/0}
 497 mice. Flow cytometric analysis shows binding of induced IgG antibodies to PrP^C on N2A
 498 cells in sera from representative *Prnp*^{0/0} mice (D) but only low induction of PrP^C-specific
 499 antibodies in individual wild-type mice (E). Prion-infected ScN2a cells were treated with
 500 serum (dilution 1:40) from vaccinated *Prnp*^{0/0} (F) or wild-type (G) mice and analysed by
 501 western blotting. PrP^{Sc}, and to some extent also PrP^C, levels on ScN2a cells are largely
 502 diminished after treatment with representative sera from *Prnp*^{0/0} (F) mice vaccinated with
 503 pCG-PrP or pCG-PrP-P30, whereas no inhibitory effect is detectable with sera from
 504 vaccinated wild-type mice (G) and from a *Prnp*^{0/0} mouse immunised only with the pCG
 505 vector. PrP^{Sc} levels of ScN2a were determined in the absence (-) or presence (+) of proteinase
 506 K treatment of the cell lysates (PK) prior to electrophoresis. Blots were probed with a
 507 polyclonal rabbit anti-PrP serum and a goat-anti-rabbit antibody conjugated with horseradish
 508 peroxidase as described in Material and Methods.

509

510 **Fig. 2: Cellular immune responses of *Prnp*^{0/0} mice after DNA vaccination.**

511 Analyses were performed seven days after the 6th immunisation with the indicated vaccine.
512 Proliferation of splenocytes was determined 48h after re-stimulation of cells with rPrP *in*
513 *vitro*. Stimulation index (SI) represents the ratio of thymidine incorporation of PrP-stimulated
514 versus non-stimulated cells. Each symbol represents the mean SI of four parallel cultures for
515 each mouse. Differences in SI between the pCG-PrP-P30 vaccine group compared to the
516 pCG-PrP group were statistically significant ($P < 0.05$; Mann-Whitney test).

517

518 **Fig. 3: Prime-boost vaccination with DNA and PrP protein induces antibody responses**
519 **in *Prnp*^{0/0} mice, but failed to protect wild-type mice from prion disease.**

520 (A) *Prnp*^{0/0} and wild-type mice receiving indicated DNA and rPrP/CpG-1668 were analysed
521 ten days after the last immunisation. Sera of *Prnp*^{0/0} and wild-type mice were diluted 1:1000
522 and 1:40 for ELISA, respectively. The titre of each mouse is indicated by a symbol and the
523 mean values of each group are given in bars. The increase in antibody titres in vaccinated
524 wild-type mice most likely reflects unspecific binding to rPrP on the ELISA plate, because
525 these sera do not bind to native PrP^C on N2A cells (data not shown). (B) Flow cytometric
526 analysis of sera from representative *Prnp*^{0/0} mice show binding of induced IgG antibodies to
527 PrP^C on N2a cells. (C) Incubation times of wild-type mice after intraperitoneal infection with
528 RML prions. No difference in survival time was found between vaccinated and unvaccinated
529 mice. Mean survival times with standard deviation and numbers of affected/inoculated mice
530 are given in parenthesis. (D) Brains of terminally ill mice, of immunised mice and of non-
531 treated controls display similar pathological changes (HE: hematoxylin-eosin staining; GFAP:
532 glial fibrillary acidic protein; scale bar: 50µm). Spleen sections of mice show no gross
533 abnormalities in the formation of the microarchitecture when compared to non-treated mice
534 (HE; scale bar: 100µm).

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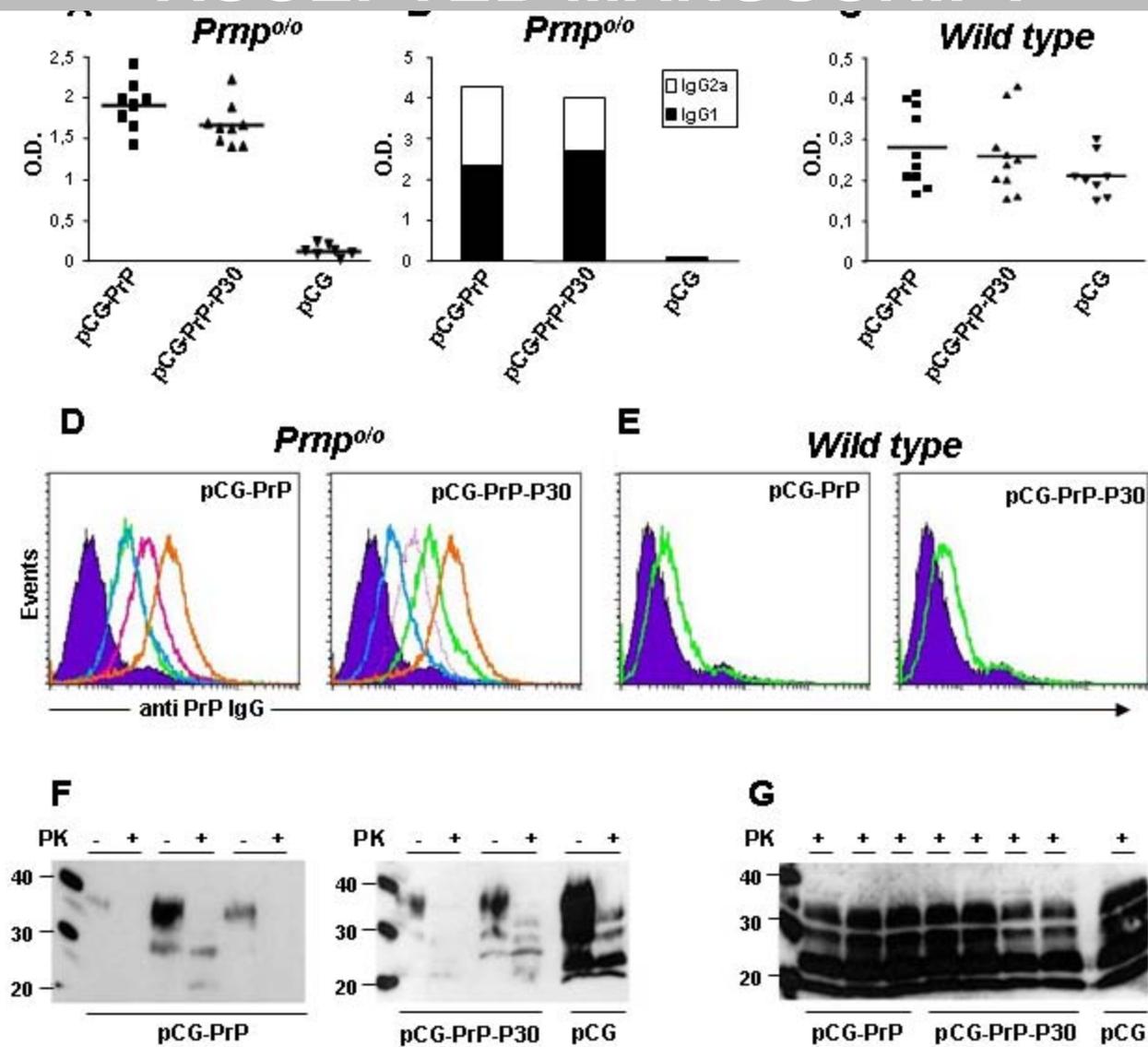


Figure 1

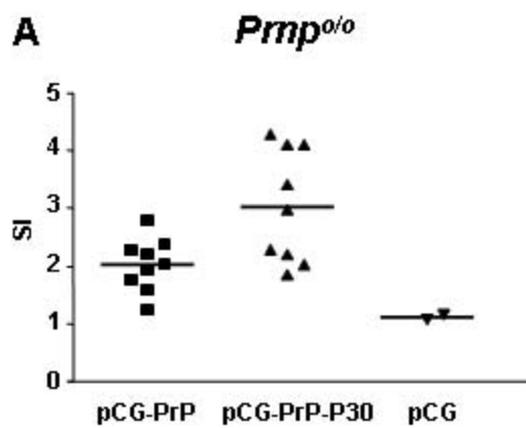


Figure 2

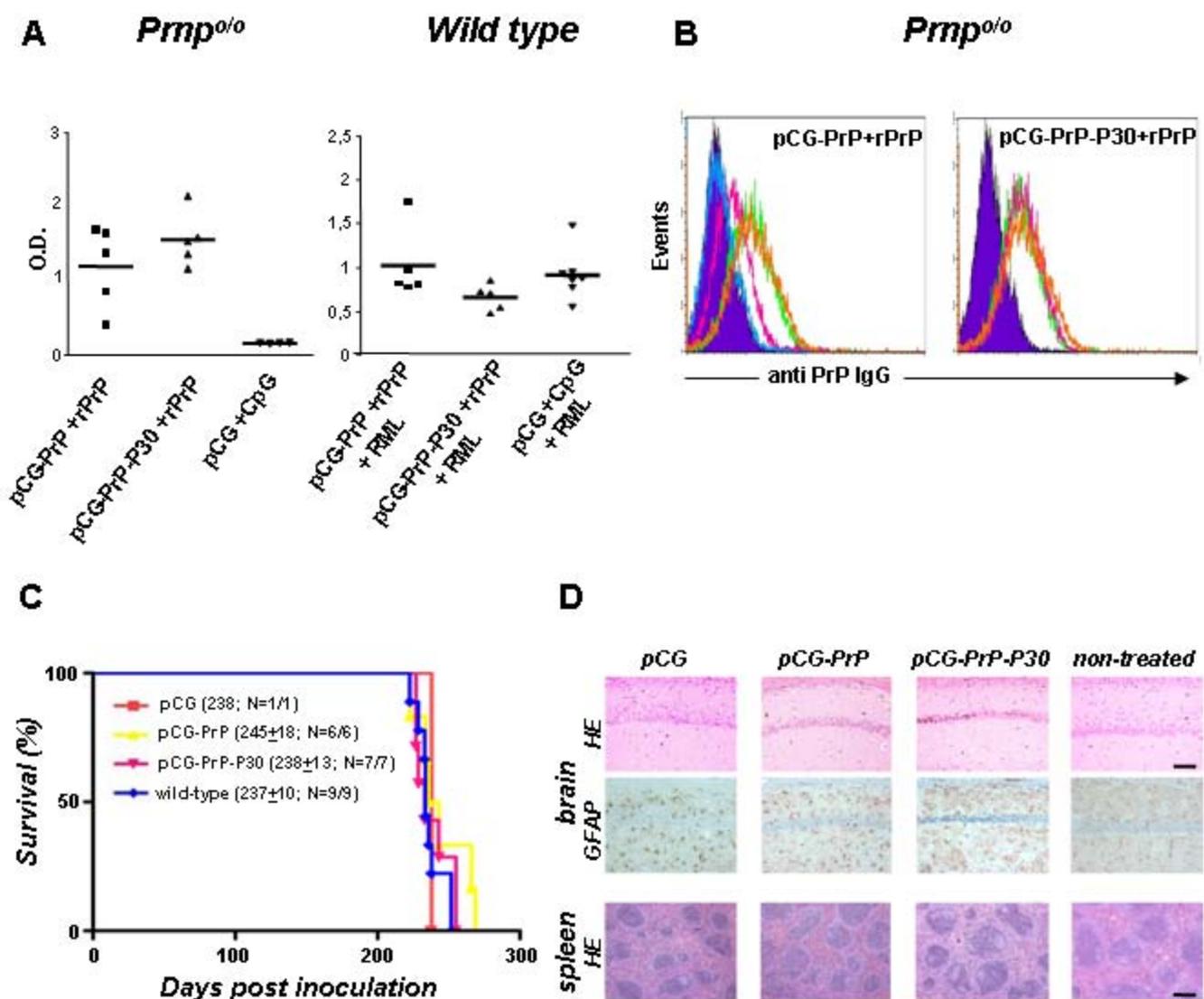


Figure 3