Chapter 7

Immunobiology of Prion Diseases

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Abstract

Prion diseases are invariably lethal neurodegenerative diseases, associated with the structural conversion of the cellular isoform of the prion protein to its pathological, disease-associated isoform. The cellular isoform of the prion protein is highly conserved and virtually ubiquitously expressed; nevertheless, its physiological role remains unclear. Mounting evidence suggests its involvement in the regulation and function of the immune system. At the same time, the immune system is heavily involved in the pathogenesis of the diseases, playing a major role in the peripheral replication of the infectious agent and spread toward the central nervous system. On the other hand, immunotherapies are among the most promising means of intervention. This chapter deals with these fascinating and sometimes contrasting aspects of prion biology, with an emphasis on the immunization protocols developed for prophylaxis and treatment of prion diseases.

Keywords: prion, immunobiology, active immunization, passive immunization, DNA vaccines, mucosal vaccination

1. Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are invariably lethal neurodegenerative diseases afflicting a wide variety of species, including humans [1]. The common pathogen to all TSEs is termed prion and is believed to consist solely or primarily of the disease-associated isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}). PrP^{C} is a highly conserved, GPI-anchored sialoglycoprotein encoded by the single-copy *Prnp* gene. *Prnp* is virtually ubiquitously expressed, with its expression peaking in the neuronal tissue, whereas high *Prnp* expression levels have been reported in many cells of the immune system. PrP^{Sc} is believed to propagate by inducing the conformational conversion of PrP^{C} molecules into new

PrP^{Sc} copies. The exact mechanism governing this conversion is still under dispute, but it is widely accepted that it does not involve modifications of the primary structure of the protein.

The physiological and pathological PrP isoforms display distinct conformations. The N-terminal region of PrP^{C} is highly unstructured, as opposed to the globular C-terminal region, which contains predominantly a-helices and only a minor region encompassing two-stranded β sheet [2]. Infrared spectroscopy and circular dichroism data indicate clear differences in the secondary structure of PrP^{C} and PrP^{Sc}, in which equal amounts of a-helices and β sheets can be found [3]. This conformational difference is believed to be at the basis of the biochemical differences observed between the two isoforms, namely, the partial proteinase K resistance, the reduced solubility, and the fibril-producing potential displayed by PrP^{Sc} [4]. To date, the only known difference at the chemical level is associated with the oxidation level of the methionine residues, which was found to be elevated in PrP^{Sc} compared to PrP^{C} [5].

The physiological role of the prion protein remains obscure. Its high level of conservation among species would indicate that PrP^{C} is of crucial importance to the organism; however, $PrP^{-/-}$ mice are viable, developmentally and behaviorally normal, and do not display a prominent phenotype except for the complete protection against prion diseases [6, 7]. PrP^{C} has since been implicated in a variety of cellular functions, including cell proliferation, differentiation and survival, protection against oxidative stress, and synaptic function (reviewed in [8, 9]). Further evidence suggests it may play a role in the immune system. In line with this, it has been recently reported that $PrP^{-/-}$ mice display lower numbers of CD4 T cells and lymphoid tissue inducer (LTi) cells as well as impaired splenic T zone structures [10]. Moreover, immune responses have been reported during prion diseases progression, suggesting the involvement of the immune system in disease pathogenesis, and immune-based approaches have yielded some of the most promising results toward protection and/or treatment of spongiform encephalopathies. In this chapter these exciting aspects of prion biology will be discussed.

2. PrP and the immune system

2.1. PrP^{C} expression patterns in cells of the immune system

Even though PrP^{C} is predominantly expressed in the central and peripheral nervous system [11, 12], elevated protein expression levels have also been reported in many cells of the immune system. In long-term hematopoietic stem cells (HSCs), PrP^{C} expression levels are raised and PrP^{C} has been suggested as a marker for these cells [13]. PrP^{C} expression is retained throughout maturation either toward the myeloid [14] or the lymphoid lineage [15, 16]. Interestingly, along the granulocyte maturation lineage, PrP^{C} expression is downregulated [17].

Among cells of the lymphoid lineage, T cells, monocytes, and natural killer (NK) cells express higher PrP^{C} levels compared to B lymphocytes [18]. PrP^{C} expression levels are regulated and can vary greatly across different T-cell subtypes: $CD8^{+}$ cells display higher expression levels than $CD4^{+}$ cells, and between $CD4^{+}$ cells, $CD25^{+}$ expresses 4.5-fold higher *Prnp* levels than $CD25^{-}$ cells [19], while $CD45RO^{+}$ memory T lymphocytes express higher PrP^{C} levels

compared to naïve CD45RA$^+$ T lymphocytes [16]. It would thus appear that PrP^C expression levels in cells of the immune system are dynamic, indicating that PrP^C may play a role in the immune system.

2.2. PrP^C function in the immune system

Despite evidence that PrP^C may be associated with the function of the immune system, its role remains unclear. PrP$^{-/-}$ mice do not display gross overt effects, at least under normal conditions. However, evidence indicates that when PrP$^{-/-}$ mice are subject to immunological stress their phenotype may deviate from normal. To test whether PrP^C may act as a regulator of cellular immunity, the effect PrP^C deficiency may have on the course of experimental autoimmune encephalomyelitis (EAE) was assessed [20]. EAE is an inflammatory demyelinating disease of the central nervous system (CNS), triggered by the injection of brain extracts, proteins of the CNS such as the myelin basic protein and the myelin oligodendrocyte glycoprotein (MOG) or peptides from these proteins to experimental animals, usually mice and rats. EAE is widely used as an animal model for multiple sclerosis and acute disseminated encephalomyelitis but is also considered the prototype for T-cell–mediated autoimmune disease in general [21]. It was found that PrP$^{-/-}$ mice displayed a more aggressive disease onset and no clinical improvement during the chronic phase of the disease. These clinical findings were in agreement with the increased cytokine gene expression in MOG-primed PrP$^{-/-}$ cells and indicate that PrP^C could be involved in the attenuation of T-cell-dependent neuroinflammation.

Similar results were obtained when *Prnp* expression was silenced via treatment with small interfering ribonucleic acid (siRNA) targeting *Prnp*. In this case, siRNA administration led to effective *Prnp* silencing in the lymphoid tissue, but not the central nervous system. In agreement to the results obtained with PrP$^{-/-}$ mice, siRNA-mediated *Prnp* silencing led to marked worsening of EAE [22]. In a series of elegant experiments, it was shown that the central nervous system autoimmune disease was modulated at all stages of the disease and that PrP^C regulates activation of T lymphocytes mediated by the T-cell receptor (TCR), differentiation, and survival, thus identifying PrP^C as a regulator of cellular immunological homeostasis. The proposed immunomodulatory properties when considered in conjunction with (i) the protein's expression patterns, which overlap with immune-privileged organs and (ii) the observation that only minor phenotypes can be associated with PrP$^{-/-}$ mice under physiological conditions, but rather striking ones under stress and particularly under inflammation in immune-privileged organs, has led to the hypothesis that PrP^C may be involved in immune quiescence, protecting immune-privileged organs, such as the brain [23].

3. Prion disease pathogenesis and the immune system

The central event in the pathogenesis of all forms of transmissible spongiform encephalopathies is the conversion of PrP^C to the more thermodynamically stable PrP^{Sc} by PrP^{Sc} via a mechanism which remains at large obscure [24]. Regrettably, the actual conversion mechanism is not the only missing piece of the prion disease pathogenesis puzzle, and not much is

known on how the infectious agent enters the host or how it is transported from the periphery to the central nervous system. A series of experiments using animal models of TSEs have provided interesting data on pathogenesis.

Parenteral—usually intracranial or intraperitoneal—administration of the pathogen to hamsters or mice is among the most widely used animal TSE models. Such models are particularly useful, since most of the naturally acquired TSE cases both in humans and animals are contracted via peripheral—through the alimentary tract—exposure to the pathogen [25]. While these models provide a wealth of information regarding pathogenesis, it later became evident that different mechanisms are involved in the pathogenesis of prion infection following the intraperitoneal or the oral route of infection [26], and other factors such as the pathogen strain and the host species and/or strain can also have a major impact on the mechanisms involved [27]. For example, in a recent study in sheep with different *Prnp* polymorphisms, which confer different levels of resistance to prion infection, it was observed that following intracranial administration of the pathogen, sheep with a "resistant" genetic polymorphism did not accumulate the pathogen in lymphoid tissues [28]. Even more strikingly, it has recently been reported that the role of the immune system might be limited in case of genetic prion disease. In a murine model of late onset genetic Creutzfeldt-Jakob disease, PrP^{Sc} has not been detected in the lymph nodes or the spleens of the transgenic mice at all ages and stages of disease, indicating that in this case conversion of PrP^{C} to PrP^{Sc} occurs predominantly or even exclusively within the CNS [29].

Prion pathogenesis can be divided into phases, some of which may take place in parallel: (i) peripheral prion exposure and uptake, (ii) peripheral pathogen replication, (iii) migration through the peripheral nervous system to the CNS, and (iv) centrifugal spread from the CNS back to the periphery [25, 27]. Despite PrP^{Sc} can be detected in various sites following peripheral exposure, especially in the lymphatic system, signs of pathology, including neurodegeneration, spongiosis, and gliosis are only found within the CNS. It is important to stress that as the means available evolve, our understanding of the phenomena taking place also improves. For instance, detection of PrP^{Sc} in the brains of some peripherally challenged hamsters as early as 4 and 9 days following challenge was recently reported [30].

M cells, which are epithelial cells specialized for transepithelial transport found in the follicle-associated epithelia of the small and large intestines, tonsils and adenoids [31], were shown capable of transcytosing the TSE infectious agent *in vivo* [32]. In addition to M cells, other epithelial cells may be involved in the uptake of the pathogen in a ferritin-mediated mechanism [33]. The pathogen is first detected in gut-associated lymphoid tissue (GALT), including Peyer's patches and mesenteric lymph nodes [26]. Evidence from in vitro studies indicates that the GALT in the small rather than the large intestine plays a major role in PrP^{Sc} accumulation and eventually neuroinvasion [34].

It is not yet clear how the pathogen is transported from the entry site to the lymphoid tissue. It has been hypothesized that following pathogen uptake by M cells, the infectious agent can be transported to the M cells' intraepithelial pocket, where it can be processed by macrophages, B- and T- lymphocytes residing within this pocket or the dendritic cells, macrophages, and lymphocytes situated immediately beneath the intraepithelial pocket [35]. Of these cells, macrophages and dendritic cells appear the most plausible candidates for effective transport of

the pathogen. In line with this assumption, PrP accumulations were detected in various types of macrophages following TSE infection [36–38]. However, the role macrophages undertake remains obscure, as *in vivo* experiments have shown that macrophages may also be involved in clearing the pathogen [39, 40]. It seems that the role of the macrophages following prion infection depends on the infectious dose and the agent strain [41]. Macrophages may also be important for the delivery of the infectious agent to the neural cells, and in this case, cell death may play an important role. In recent *in vitro* studies, it was determined that coculture of killed, PrP^{Sc}-infected macrophages with N2a-3 neuroblastoma cells accelerated PrP^{Sc} transmission to the neuronal cells [42]. Dendritic cells on the other hand can be ideally located to transport the pathogen following uptake by the M cells, and some of them have already been shown to be able to transport PrP^{Sc} without degrading it [43, 44].

B lymphocytes were initially identified as the cells involved in replication of the TSE infectious agent [45], but this hypothesis was later revised, and the role of B lymphocytes in prion pathogenesis was associated with the regulated maturation of follicular dendritic cells (FDCs) [46]. Initial experiments with splenectomized or thymectomized mice indicated the dispensable role of T lymphocytes in the replication of the agent [47], whereas fractionation [48] and irradiation [49] experiments indicated that replication of the pathogen depends on radioresistant cells, localized within the stromal compartment of the spleen. FDCs fulfill all these criteria, and their crucial role for replication of the pathogen was confirmed in a series of experiments, in which depletion of mature FDCs led to prolongation of the incubation period of the disease [46, 50–53]. FDCs are of stromal origin, reside in the primary B lymphocytes follicles and germinal centers of lymphoid tissues, and are non-phagocytic and non-migratory. As a result of their large surface area and longevity, FDCs are capable of trapping and retaining antigen in its native state for months to years. FDCs retain antigens in the form of immune complexes, consisting of antigen-complement components and/or antibody and trap these complexes either through complement receptors CR1 and CR2 or through FcRIIb and FcεRII antibody receptors [35]. In agreement with the role FDCs undertake in prion pathogenesis and the involvement of complement components and receptors in antigen trapping by FDCs, it was found that the absence of complement components (C1q, C2, C3, and factor B) and cellular complement receptor can have an adverse effect on the accumulation of PrP^{Sc} in the spleen [54, 55]. However, the inability to completely inhibit disease progression via depletion of mature FDCs [46, 51], in addition to observations confirming that propagation of prion diseases is possible even in the absence of mature FDCs [41, 56–58], indicates that possibly a different cell type—most probably MOMA-1-positive macrophages [41]—is responsible for replication of the pathogen. These differences in the cell types required for pathogenesis were attributed to the dose and agent strain [41].

Peripheral replication of the pathogen precedes neuroinvasion, during which the pathogen is transported within the CNS. Both the enteric and autonomic nervous systems are believed to participate in the transport of the infectious agent [36, 59, 60]. The exact mechanism governing transport of the pathogen to the CNS remains unidentified, and has been reported to be both PrP^{C}-dependent [61, 62] and independent [63]. Interestingly, it was reported that the transfer speed of intraperitoneally administered prions relies to the distance between FDCs and splenic nerve endings [64, 65].

The immune system is greatly implicated in the peripheral pathogenesis of prion diseases but fails to provide protection. Until recently, no response against the prion pathogen has been described, and this was linked to tolerance effects due to widespread expression of the physiological isoform of the prion protein throughout the body, which prevents the host from mounting a humoral or cellular immune response against PrP^{Sc} following infection [66]. On the contrary, $PrP^{-/-}$ mice mount a robust immune response against PrP, indicating the immunogenicity of the protein. Lately it was shown that TSE infection can have adverse effects on the maturation cycle of FDCs, causing an abnormality in immune function [67]. Given the crucial role the immune system plays in the peripheral pathogenesis of prion diseases, it could be argued that it promotes rather than protects against prion pathogenesis. In agreement with this, increased susceptibility to intraperitoneal challenge with TSE agents following repetitive immunization was recently reported [68].

4. Harnessing the immune system against prion diseases

Since the immune system plays an ambivalent role in prion disease pathogenesis, the question emerged whether suppressing the immune system would be the most appropriate approach [69]. Targeting the FDCs was already proven a viable approach, providing partial protection in an animal model of prion diseases and minimizing the infectivity of the peripheral tissue of the afflicted animals [46, 50–52]. Disruption of the FDCs also appears to be the protective mechanism against TSEs following repetitive CpG administration [70]. CpG had previously been administered as a stimulator of innate immunity and was shown effective at providing partial protection in an animal model of TSEs [71]. In this case CpG was administered to stimulate the macrophages and enhance phagocytosis of the pathogen. Indeed, repetitive administration provided partial protection against TSEs [71], but as it was later shown, this protection was due to disruption of the lymphoid follicles rather than stimulation of the macrophages [70]. Interestingly, disruption of the FDCs has also been observed following immunization of wild-type mice with recombinant murine PrP aggregates and is at least in part responsible for the observed partial protection when the immunized mice were challenged with a murine strain of TSEs [72].

The first indications that the immune system might prove effective against prion diseases stemmed from *in vitro* experiments, where it was shown that treatment of TSE-infected cell cultures with monoclonal anti-PrP antibodies could effectively inhibit PrP^{Sc} replication and on some occasions clear infectivity [73–75]. Proof of principle that immunization against prion diseases can be effective against prion diseases was provided later using transgenic mice, capable of producing anti-PrP antibodies. These mice, in contrast to wild-type controls, failed to succumb to disease following challenge with a mouse-adapted scrapie strain [76]. Similarly, passive immunization of wild-type mice, by administration of anti-PrP antibodies was found to provide protection against prion diseases [77].

From this initial series of experiments, valuable conclusions emerged, most importantly, that immunization is an efficient means of therapy rather than protection, against prion diseases. Moreover, the safety of these procedures was confirmed, since immunization against a self-

antigen could always give rise to autoimmunity. Given the identical primary structure PrP^{C} and PrP^{Sc} share, adverse reactions stemming from the reaction of the anti-PrP antibodies with PrP^{C} could be expected. Autoimmunity was not induced by these immunization approaches, and furthermore the "dispensable" role of the prion protein for the appearance of a physiological phenotype was already known from studies on $PrP^{-/-}$ animals [6], as well as from transgenic animals with conditional depletion of the prion protein [78] and provided an extra layer of security. However, other findings raised some concerns over the safety of administration of anti-PrP antibodies, since it was found that intracerebral administration of anti-PrP monoclonal antibodies can give rise to cross-linking of PrP molecules on adjacent neurons and eventually cell death, triggered possibly by the initiation of death signaling [79]. These effects are clearly not associated with autoimmunity, but rather with impaired cell signaling.

4.1. Passive immunization approaches

The first indications that passive immunization could prove useful at protecting against prion diseases emerged from studies in which mice genetically modified to produce an anti-PrP monoclonal antibody (6H4μ) were fully protected against prion diseases [76]. In a more classical approach, monoclonal anti-PrP antibodies (ICSM18 and ICSM35) were administered intraperitoneally to wild-type mice briefly after intraperitoneal inoculation with the pathogen or when the first clinical signs appeared. When the antibodies were administered after the inoculation, animals receiving the antibodies survived approximately 300 days more than control mice, and the accumulation of infectivity in the peripheral tissue was markedly reduced [77]. Intraperitoneal administration of a different antibody (6D11) immediately after intraperitoneal administration of the pathogen also proved its protective efficacy, since mice receiving the antibody survived longer by approximately 36.9% compared to control mice. In a recent study, a pharmacokinetic and pharmacodynamic analysis following intraperitoneal administration of various anti-PrP antibodies was carried out. The ability of an antibody to form long-lasting complexes with PrP^{C} was found to positively correlate with its efficacy in delaying peripheral accumulation of PrP^{Sc} and, in agreement with this, intraperitoneal administration of the monoclonal antibody BAR216 led to a statistically significant prolongation of survival of the mice [80].

The therapeutic efficacy of intracerebral administration of anti-PrP monoclonal antibodies was evaluated in two recent studies. In the first one, monoclonal antibody 4H11 ($F(ab')_2$ and IgG) was intraventricularly administered to transgenic mice overexpressing PrP using osmotic pumps from d85 to d100 following intraperitoneal challenge with a mouse-adapted bovine spongiform encephalopathy (BSE) strain. The mice were not protected by this regimen, and they succumbed to disease concomitantly with the control mice. Furthermore, mice treated with the antibodies developed neuronal cell death, associated with administration of the antibodies. In addition to previously reported results [79], linking cell death to PrP cross-linking events, in this study, emerged that PrP cross-linking is not the only mechanism mediating cell death; "coating" the whole cell surface PrP with antibodies or antibodies fragments could induce other toxic signals [81]. In the second study, intraventricular administration of antibodies 106, 110, 31C6, and 44B1 to wild-type mice was not linked with neuronal cell death; however, only a minor prolongation of survival and in one of the two tested animal models

was achieved following administration of the monoclonal antibodies [82]. Differences in the epitopes recognized by the antibodies used in these two studies as well as the use of PrP over-expressing versus wild-type mice could account for the different results obtained regarding neuronal cell death. Of note, neuronal cell death has been challenged in another, more recent study, and it would be safe to assume that toxic effects are associated with the epitope and the dosage of the antibodies used [83].

A completely different passive immunization approach was used in two other studies; based on the discovery of the non-integrin 37/67 kDa lamin receptor (LRP/LR) as an interaction partner for both isoforms of PrP [84–86], polyclonal anti-LRP/LR [87] or single-chain Fv anti-LRP/LR antibodies [88] were intraperitoneally administered to wild-type mice as protective means in a mouse model of prion diseases. On both occasions, peripheral PrP^{Sc} accumulation was reduced; however, partial protection was only achieved with the polyclonal antibodies. This difference in the efficacy was attributed to differences in the pharmacokinetics and dosage regimen; polyclonal antibodies have a half-life of approximately 14 days in the blood, whereas the single-chain antibodies have a half-life of only 12 h. Moreover, the polyclonal antibodies were administered for 12 weeks, starting 1 week before administration of the pathogen, whereas the single-chain antibodies for 8 weeks. Passive immunization approaches are summarized in **Table 1**.

4.2. Active immunization approaches

Although passive immunization does protect against prion diseases, it provides a narrow window for intervention, i.e., antibodies must be administered shortly after exposure to the pathogen. In this regard, active immunization against the prion protein, which provides protection against the diseases similarly to a conventional vaccine, could prove a much more useful approach. Nevertheless, the prion protein-associated tolerance effects which prevent the immune system from mounting an immune response against the prion protein hinder development of such approaches [66].

Despite the tolerance effects, initiation of a humoral immune response against the prion protein was achieved, albeit with mediocre results in terms of protection against the disease. In the first reports, wild-type mice were immunized with recombinant murine prion protein mixed with complete Freund's adjuvant (CFA) and challenged with a mouse-adapted scrapie strain either concomitantly with the immunization (rescue treatment) or following its completion (prophylactic treatment). Although the mice developed antibodies against the prion protein, only mice of the prophylactic treatment group were partially protected against the pathogen; mice of this group succumbed to disease with a delay of approximately 16d compared to control mice [89].

4.2.1. Peptide-based active immunization

Numerous strategies were implemented to overcome the tolerance effects and promote generation of anti-prion antibodies. The most obvious approach was to use prion peptides properly modified to enhance the antigenicity of the protein (summarized in **Table 2**). Following this rationale, wild-type animals were immunized with prion protein peptides [90–93], PrP

Antibody name	Antibody type and target	Epitope	Administration protocol	*In vitro* assay	*In vivo* assay	Reference
ICSM18, ICSM35	Monoclonal, PrP	ICSM18: 143–153aa ICSM35: 93–105aa	Intraperitoneal administration twice weekly starting 7 or 30 days after administration of the pathogen or at onset of the clinical symptoms	NP	Prolonged survival interval in a mouse model of prion disease when the antibodies were administered prior to the appearance of clinical symptoms	[77]
6D11	Monoclonal, PrP	97–110aa	One intravenous administration immediately after administration of the pathogen followed by consecutive intraperitoneal administrations (twice per week for 4 or 8 weeks)	Prevention of infection and clearance of infection in already prion-infected cell lines	Prolongation of incubation period in a mouse model of prion disease	[115]
BAR236	Monoclonal, PrP	Linear epitope unidentified	Intraperitoneal (3 weekly administrations, starting 1 week after administration of the pathogen)	NP	Prolongation of survival interval in a mouse model of prion disease	[80]
4H11	Monoclonal or $F(ab')_2$ fragments, PrP	Epitope within octarepeat region (59–89aa)	Intraventricular (osmotic pump delivering antibody for 16 days starting 85 days after administration of the pathogen)	Inhibition of PrP^{Sc} propagation in an already prion-infected cell line. Recognition of PrP on the cell surface by FACS	Intraventricular administration of the antibody did not prolong survival interval in a mouse model of prion disease	[81]
106, 110, 31C6, 44B1	Monoclonal, anti-PrP	106: 88–90aa 110: 83–89aa 31C6: 143–149aa 44B1: discontinuous epitope within aa 155–231 aa	Intraventricular (osmotic pump delivering antibody for 14 days starting 60, 90, or 120 days after administration of the pathogen)	NP	Small (8%) prolongation of survival interval in a mouse model of prion diseases, even when administration of antibodies commences after appearance of first symptoms (120 days after administration of the pathogen)	[82]
pAb W3	Polyclonal anti-LRP/LR	Undefined	Intraperitoneal (12 weekly administrations starting 1 week before administration of the pathogen)	NP	Prolongation of survival interval, but not of incubation period in a mouse model of prion disease	[87]
S18	scFV, LRP	272–280aa	Intraperitoneal (8 weekly administrations starting 1 day before administration of the pathogen)	S18 prevents interaction of the recombinant human PrP with recombinant human LRP	Reduction of splenic PrP^{Sc}, but no prolongation of survival interval in a mouse model of prion disease	[88]

Antibody name	Antibody type and target	Epitope	Administration protocol	*In vitro* assay	*In vivo* assay	Reference
W226	Monoclonal, scFV	Undefined	Intraperitoneal administration twice weekly starting 2 or 28 days after administration of the pathogen or at onset of the clinical symptoms	Clearance of PrP^{Sc} in ScN2a cells	Minor delay of incubation time in immunized versus control mice	[116]
EB8, DC2, DE10, EF2	Monoclonal	EB8: 26–34aa; DC2: 35–46aa; DE10: 44–52aa and EF2: 47–52aa	NP	Clearance of PrP^{Sc} in ScGT2 cells	NP	[117]

Table 1. Summary of studies based on passive immunization against prion diseases.

dimers [94–96], or PrP aggregates [72]. In addition to homologous prion protein immunization [89], which provided proof of principle that active immunization can have a protective role against prion diseases, immunization with heterologous prion peptides also provided rather encouraging results [97]. In an attempt to enhance the immunogenicity of the prion peptides, various adjuvants, including Freund's adjuvant, Montanide IMS-1313, TiterMax, CpG, anti-OX40 antibodies—antibodies against the signaling molecule CD134, which recently has been shown to break T cell tolerance—and keyhole limpet hemocyanin, were used [95, 98], as well as different vaccine formulations, including encapsulation of the CpG-antigen complex in polylactide-coglycolide microspheres [96]. Interestingly, an early report indicates that immunization with complete Freund's adjuvant alone can provide partial protection in a mouse model of prion diseases through an unidentified mechanism [99]. Based on the extremely strong adjuvant effect exerted by heat-shock proteins, PrP molecules chemically cross-linked [100] or fused [72] to recombinant bacterial heat-shock proteins were also used to immunize wild-type mice and lead to the production of antibodies that recognized recombinant PrP.

Despite the widely accepted notion that PrP^{Sc} is not immunogenic and that the immune system does not provide protection against PrP^{Sc} in wild-type animals, when highly purified proteinase K-resistant PrP^{Sc}, originating from murine brains afflicted with an animal model of prion diseases was coadministered with CpG [101] or administered immobilized on Dynabeads coated with antibodies against PrP [102] a humoral immune response, which providing partial protection in animal model of prion diseases was elicited.

Although the protective role of the aforementioned, peptide-based approaches was not investigated on all occasions, it became evident that using various approaches the self-tolerance effects can be overcome and immune reactions against the prion protein can be obtained. However, it appears that protection against TSEs is restricted to antibodies capable of recognizing the native cell-surface PrP^{C} [95]. This requirement was met by antibodies known to provide protection against TSEs, e.g., ICSM18 [77] and 6H4 [76], whereas other antibodies

capable of recognizing recombinant PrP but unable to provide protection against TSEs also failed to recognize native PrP^C [72, 95].

4.2.2. DNA vaccines

In addition to peptide-based vaccines, DNA vaccines were also used to promote immune responses against the prion protein. In this case, nucleic acid encoding for the prion protein is administered to animals, wherein the nucleic acid is translated to the corresponding protein and an immune response is initiated. The first attempt at raising anti-PrP antibodies using DNA vaccines was only successful in $PrP^{-/-}$ mice, whereas the same approach failed to give rise to anti-PrP antibodies in wild-type mice [103]. Induction of anti-PrP antibodies using DNA vaccines in wild-type mice was triggered when the mice were immunized with a DNA construct coding for the murine prion protein fused to the lysosomal targeting signal from lysosomal integral membrane protein type II (LIMPII). Immunization with this construct leads to a remarkable delay on the onset of disease symptoms, which was not followed by a similar prolongation of survival interval. This discrepancy in the obtained results was attributed to immunopathology mediated by PrP-specific antibodies induced by the DNA vaccine used and constitutes the first report of adverse effects following active prion immunization [104].

In a different approach, DNA vaccines were used to prime wild-type mice, followed by peptide immunizations to further boost immune responses. Although this approach was successful when $PrP^{-/-}$ mice were immunized, very low antibody titers and only marginal protection were achieved when tested on wild-type mice [105]. In a recent report, wild-type mice were immunized with cDNA coding for human PrP^C fused to a T-cell stimulatory peptide. These mice developed a strong humoral immune response against the native protein, and although a bioassay was not carried out, the produced antibodies were capable of recognizing the native conformation of murine PrP^C, which—as already mentioned—constitutes a strong indicator of protective efficiency against prion diseases [106]. Studies based on DNA vaccines are summarized in **Table 3**.

4.2.3. Immunization with PrP-displaying viral constructs

A different approach to overcome the tolerance effects and stimulate the production of anti-PrP antibodies in wild-type mice is the expression of the prion protein on the surface of viral particles (summarized in **Table 4**). Virus-like particles (VLPs) are much better B lymphocytes immunogens than monovalent proteins and would be expected to trigger a stronger humoral immune response by passing tolerance.

In a first attempt, retroviral particles displaying the C-terminal portion of murine PrP were used to immunize wild-type mice. These mice developed anti-PrP antibodies, capable of recognizing the native form of PrP^C, thus displaying strong therapeutic potential [107]. A similar approach was used to insert the 9-amino-acid-, prion-pathogenesis associated-peptide pertaining to the murine/rat prion protein into the L1 major capsid protein of bovine papillomavirus type 1. These VLPs were used to immunize both wild-type rats and rabbits. The anti-sera

Antigen	Animals immunized	Humoral response	T-cell responses	*In vitro* assays	*In vivo* assays	Reference
Various murine PrP peptides	Wild-type mice	+	NP	NP	Reduction of proteinase K-resistant prion protein in a scrapie-infected tumor transplant	[93]
Recombinant murine PrP chemically cross-linked to bacterial heat-shock proteins	Wild-type mice	+	NP	NP	NP	[100]
Recombinant murine PrP	Wild-type mice	+	NP	NP	Prolongation of survival interval in a mouse model of prion disease	[89]
Recombinant murine PrP dimer	Wild-type mice, rabbits	+	NP	Polyclonal sera produced reduced PrP^{Sc} synthesis in prion-infected cell lines	NP	[94]
Recombinant murine prion peptide 105–125 linked to keyhole limpet hemocyanin and recombinant murine prion 90–230	Wild-type mice	+	NP	NP	Prolongation of survival interval in a mouse model of prion disease	[92]
Mouse prion peptides 31–50 and 211–230	Wild-type mice	NP	NP	NP	Prolongation of survival interval in a mouse model of prion disease, even when only the adjuvant Complete Freund's Adjuvant (CFA) is administered	[99]
Various murine prion peptides and adjuvants	Wild-type mice	+	ND	FACS to detect binding of the produced antibodies on native PrP	Statistically insignificant prolongation of survival time in a mouse model of prion disease	[95]
Murine prion peptides 39–67, 98–127, 143–172, and 158–187 with CFA or CpG	Wild-type mice	+	+	NP	NP	[98]

Antigen	Animals immunized	Humoral response	T-cell responses	*In vitro* assays	*In vivo* assays	Reference
Hamster prion peptides 105–128, 119–146, and 142–179	Wild-type hamsters	+	NP	NP	Prolongation of survival interval in a hamster model of prion diseases	[91]
Recombinant murine, ovine, and bovine prion protein	Wild-type mice	Detected following immunization with ovine and bovine recombinant PrP	NP	NP	Prolongation of survival interval in a mouse model of prion diseases following immunization with the bovine-recombinant protein	[97]
Recombinant murine PrP dimer and CpG encapsulated in polylactide-coglycolide microspheres	Wild-type mice	+	+	NP	NP	[96]
Murine scrapie-associated fibrils and CpG	Transgenic and wild-type mice	+	NP	NP	Prolongation of the survival interval of the wild-type mice in a mouse model of prion disease when CpG was used	[101]
Murine scrapie-associated fibrils immobilized on Dynabeads	Wild-type mice	+	NP	NP	Prolongation of survival interval in a mouse model of prion diseases with the bovine-recombinant protein	[102]
Cervid prion peptide sequences 168–182 and 145–164	Deer	+	NP	NP	Delay of incubation time in immunized versus control mice	[118]
Prion disease-derived brain material	Camelid	+	NP	Permanent abrogation of prion replication in a prion-permissive cell line	NP	[119]
rPrP aggregates, solubilized rPrP, DnaK-fused PrP	Mouse	+	NP	FACS to detect binding of the produced antibodies on native PrP	Statistically significant prolongation of survival time in a mouse model of prion disease	[72]

Table 2. Summary of studies on peptide-based active immunization against prion diseases.

Vaccine	Immunized animals	Humoral response	T-cell responses	*In vitro* assays	*In vivo* assays	Reference
DNA vaccine encoding either murine PrP or murine PrP fused to ubiquitin or to a lysosomal targeting signal	Wild-type mice	+	+	NP	Prolongation of asymptomatic period and accumulation of disease associated PrP, but not of survival interval. Death of the immunized mice was attributed to neurodegeneration associated with production of anti-PrP antbodies	[104]
DNA vaccine encoding murine PrP linked to helper T-cell epitopes Combination of DNA and peptide immunization	$PrP^{-/-}$ and wild-type mice	Achieved in $PrP^{-/-}$ mice, very low titer in wild-type mice	Detected in $PrP^{-/-}$ mice but not wild-type mice	FACS to detect binding of the produced antibodies on native PrP positive with $PrP^{-/-}$ mice sera, negative with wild-type mice sera. $PrP^{-/-}$ mice sera reduced PrP^{Sc} levels in prion-infected cell lines	Not effective	[105]
DNA vaccine encoding human PrP fused or not to a tetanus toxin stimulatory T-cell epitope and PrP protein boost	Wild-type mice	+	NP	FACS to detect binding of the produced antibodies on native PrP	NP	[106]
DNA vaccine encoding human PrP fused to ubiquitin, lysosomal integral membrane protein type II lysosome-targeting signal or an ER-targeting signal in conjunction with PrP vaccination	Wild-type mice	+	+	NP	NP	[120]

Table 3. Summary of studies on DNA vaccination against prion diseases.

Vaccine	Immunized animals	Humoral response	T-cell responses	*In vitro* assay	*In vivo* assay	Reference
Murine PrP or C-terminal murine PrP expressed on recombinant retroviral virus-like particles	$PrP^{-/-}$ and wild-type mice	+	NP	FACS to detect binding of the produced antibodies on native PrP	NP	[107]
Murine/rat prion 9/mer inserted into the L1 major capsid protein of bovine papillomavirus type 1	Wild-type rabbits and rats	+	NP	FACS to detect binding of the produced antibodies on native PrP, immunoprecipitation Rabbit immune sera inhibited de novo synthesis of PrP^{Sc} in prion-infected cells	NP	[108]
Priming with adenovirus 5 expressing the human PrP gene followed by boosting with the human PrP plasmid	$PrP^{-/-}$ and wild-type mice	+	+	FACS to detect binding of the produced antibodies on native PrP	Marginal prolongation of survival interval of the immunized mice	[109]

Table 4. Studies on active immunization approaches, using PrP-displaying virus constructs.

collected from both immunized species recognized native PrP^{Sc}, and importantly immune serum from the immunized rabbit prevented synthesis of PrP^{Sc} in scrapie-infected cell lines [108]. In a more recent approach, dendritic cells transduced with adenoviruses encoding the human prion protein were used to immunize wild-type mice. These mice developed antibodies against the murine prion protein as well, which provided partial protection against TSEs, as shown by the reduction in splenic PrP^{Sc} accumulation and prolongation of survival interval in a murine model of TSEs [109].

4.2.4. Mucosal immunization

To date, the only active immunization strategy providing complete protection against prion diseases is mucosal immunization. To trigger mucosal immunization, either transgenic, live-attenuated *Salmonella typhimurium* or cholera toxin is used. Both the attenuated *S. typhimurium* and the cholera toxin induce the production of IgA, which is the main immunoglobulin found in mucous secretions and is particularly abundant in the secretions of the gastrointestinal tract.

To induce mucosal immunization, a live-attenuated *S. typhimurium* vaccine strain engineered to express one [110] or two copies [110–112] of mouse [110, 112] or deer [111] PrP was administered orally to mice and deer, respectively. In a different approach, a murine PrP fragment was coadministered with cholera toxin either orally or intranasally [113]. The immunized animals were then orally challenged with a murine model of TSEs or chronic wasting disease (CWD)—infected brain homogenate to evaluate the protective potential of the immunization. Although both approaches promoted the generation of anti-PrP IgA, protection afforded by the

Vaccine	Immunized animals	Humoral response	T-cell responses	*In vitro* assay	*In vivo* assay	Reference
Orally administered *S. typhimurium* LVR01 expressing one or two copies of mouse PrP	Wild-type mice	+	NP	NP	Significant prolongation of survival interval in a mouse model of prion disease	[110, 112]
Intranasally, intragastrically, or intraperitoneally administered murine PrP90–231 and cholera toxin	Wild-type mice	+	NP	NP	Marginal prolongation of survival interval in a mouse model of prion disease following intranasal administration	[113]
Orally administered *S. typhimurium* LVR01 expressing two copies of elk PrP	White-tailed deers	+	NP	NP	Significant prolongation of survival interval in an elk model of prion disease. One immunized animal remained asymptomatic	[111]

Table 5. Mucosal vaccination approaches.

immunized animals varied greatly. Animals immunized with the attenuated PrP-expressing *Salmonella* survived significantly longer than control animals, and most importantly some of them were completely protected, remaining disease-free [110–112]. On the other hand, the cholera toxin was used to induce production of IgA, mice were only partially protected against oral exposure to the infectious agent, and modest prolongation of the survival interval was observed, without any mice remaining symptoms-free [113]. Studies dealing with mucosal immunization are summarized in **Table 5**.

Although mucosal immunization is only effective following oral exposure, it is important to remember that the gut is the major route of entry for prion diseases such as CWD in white-tailed deer, BSE in cattle, and variant Creutzfeldt-Jakob disease and kuru in humans. Furthermore, mucosal vaccination can be properly designed to induce a primarily humoral immune response and is unlikely to produce a significant immune response within the brain, thus minimizing the risk of appearance of adverse reactions [112].

5. Future perspectives

Despite fervent research and some very encouraging results, many facets of the involvement of the immune system in prion pathogenesis remain obscure, and a powerful immunoprotective tool has yet to emerge. Passive immunization with anti-prion antibodies and mucosal immunization were the only two approaches to provide satisfactory results but have a series of limitations associated with the narrow window of intervention and the route of infection. However, immune-based therapeutics both in their more classical immunization-based form or more modern, immunomodulatory form [114] hold great promise for prion diseases and other protein-misfolding diseases.

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