Prions are infectious proteins responsible for a group of fatal neurodegenerative diseases called TSEs (transmissible spongiform encephalopathies) or prion diseases. In mammals, prions reproduce themselves by recruiting the normal cellular protein PrP\(^\text{sc}\) and inducing its conversion into the disease-causing isoform denominated PrP\(^\text{Sc}\). Recently, anti-prion antibodies have been shown to permanently cure prion-infected cells. However, the inability of full-length antibodies and proteins to cross the BBB (blood-brain barrier) hampers their use in the therapy of TSEs in vivo. Alternatively, brain delivery of prion-specific scFv (single-chain variable fragment) by AAV (adeno-associated virus) transfer delays the onset of the disease in infected mice, although protection is not complete. We investigated the anti-prion effects of a recombinant anti-PrP (D18) scFv by direct addition to scrapie-infected cell cultures or by infection with both lentivirus and AAV-transducing vectors. We show that recombinant anti-PrP scFv is able to reduce proteinase K-resistant PrP content in infected cells. In addition, we demonstrate that lentiviruses are more efficient than AAV in gene transfer of the anti-PrP scFv gene and in reducing PrP\(^\text{sc}\) content in infected neuronal cell lines. Finally, we have used a bioinformatic approach to construct a structural model of the D18scFv-PrP\(^\text{Sc}\) complex. Interestingly, according to the docking results, Arg\(_{151}\) (Arg\(_{151}\) from prion protein) is the key residue for the interactions with D18scFv, anchoring the PrP\(^\text{Sc}\) to the cavity of the antibody. Taken together, these results indicate that combined passive and active immunotherapy targeting PrP might be promising strategies for therapeutic intervention in prion diseases.

Key words: adeno-associated virus, immunotherapy, lentivirus, cellular prion protein (PrP\(^\text{C}\)), scrapie prion protein (PrP\(^\text{Sc}\)), single-chain variable fragment (scFv).

INTRODUCTION

TSEs (transmissible spongiform encephalopathies) or prion diseases are fatal neurodegenerative disorders that affect humans and animals. The mechanisms involved in their pathogenesis are not completely understood, but they are associated with the accumulation in the brain of a misfolded form, PrP\(^\text{Sc}\), of the endogenously expressed PrP\(^\text{C}\) [cellular PrP (prion protein)] [1]. Industrial cannibalism has been responsible for one of the most infamous TSE epidemics, BSE (bovine spongiform encephalopathy), paralleled with an increasing number of human cases with vCJD (variant form of Creutzfeldt-Jakob Disease) from BSE-contaminated beef products [2].

Direct interaction between the pathogenic PrP\(^\text{Sc}\) template and the endogenous PrP\(^\text{C}\) substrate and the following conformational conversion of PrP\(^\text{C}\) into PrP\(^\text{Sc}\) are proposed to drive the formation of nascent infectious prions [3]. Various lines of evidence indicate that the process of conversion is the key element in pathogenesis. Knockout mice (Prnp\(^{-/-}\)) are resistant to prion diseases and do not propagate infectivity [4], and brain tissue homogenates from Prnp\(^{+/+}\) cows are resistant to prion propagation in vitro [5]. Moreover, Prnp\(^{+/+}\) brain tissue surrounding prion-infected Prnp\(^{-/-}\) neurografts does not develop neuropathological changes typical of prion diseases [6].

Over the past several years, many anti-prion compounds have been identified in vitro models of prion replication, such as polysulphated polyanionic compounds, polyanime, tetrypyrroles, polynene antibiotics, peptides, tetracyclic and tricyclic compounds. These prion antagonists can be targeted towards the selective binding of PrP\(^\text{C}\) or PrP\(^\text{Sc}\) and/or to the process of conversion (reviewed in [7–9]). However, most of these molecules were found to be toxic or ineffective in animal models of prion diseases (reviewed in [7–9]).

Interestingly, by taking advantage of the ‘prion-resistant’ polymorphisms Q171R and E219K that naturally exist in sheep and humans, respectively, a therapeutic approach based on the use of lentiviral vectors carrying mouse Prnp mutated gene has recently been evaluated [10]. The expression of these PrP variants, acting as dominant-negative, inhibits prion replication in permanently prion-infected cells and in animals [10]. In addition, suppression of PrP expression by lentiviral vectors carrying mouse Prnp mutated gene has recently been evaluated [10]. The expression of these PrP variants, acting as dominant-negative, inhibits prion replication in permanently prion-infected cells and in animals [10]. In addition, suppression of PrP expression by lentiviral vector-mediated RNAi (RNA interference) prolongs survival of scrapie-infected mice [11].

Alternatively, anti-PrP antibodies have been shown to have an anti-prion effect in cellular and animal models [12–15]. Early indications of the potential of antibody therapy for prion disease came from in vitro studies showing a reduction in infectivity of prions after incubation with an anti-PrP antibody [16]. Immunization with PrP peptides reduced PrP\(^\text{Sc}\) in a peripheral model of mouse scrapie [17]. Different publications report that antibodies directed against the middle portion of PrP (residues 91–110 and 132–156) can cure scrapie-infected cells in culture and reduce PK (proteinase K)-resistant PrP in spleens of infected mice (reviewed in [12,13,18]). In cell systems, anti-prion antibodies were able to purge the cells entirely of PrP\(^\text{Sc}\) [12], and their potency...
correlated with their ability to recognize the total population of PrPSc molecules on the cell surface. Despite these encouraging results, development of effective immunotherapy presents several problems in both active and passive approaches. An important obstacle in the development of efficacious regimens for active immunization is host tolerance to endogenous PrPSc, which limited the therapeutic efficacy of this immunization approach [15,19]. In comparison, passive immunization suffers from the intrinsic problem of poor antibody diffusion from vessels into tissues, especially in the nervous tissue: administration of monoclonal antibodies has been shown to prevent the pathogenesis only when applied simultaneously, or shortly after, peripheral prion infection [18]. Moreover, production of large amounts of monoclonal antibodies for therapy is technically challenging and expensive.

In 2001, Perez et al. [12] analysed the ability of some recombinant antibody antigen-binding fragments, Fabs, to inhibit prion propagation in a cultured mouse neuroblastoma cell line infected with PrPSc, ScN2a. The most effective Fab, D18, was found to abolish prion replication and to clear pre-existing PrPSc, eliminating 50% of PrPSc from the cells within approx. 24 h. The activity of D18 was attributed to its ability to specifically recognize the total population of PrPSc molecules on the cell surface. In PrPSc, the D18 epitope spans residues 132–156 and incorporates helix A. This sequence lies within the region of the protein thought to bind PrPSc, an essential step for prion propagation: therefore, it can be argued that D18 operates mechanistically by directly blocking or modifying interaction of PrPSc with PrPSc [12]. Compared with full-length antibodies, Fabs are smaller molecules and their monovalent nature may be beneficial, since intracerebral injection of some anti-PrP IgG antibodies seemed to provoke neuronal apoptosis [20]. However, their effectiveness in prion disease therapy in vivo has not yet been evaluated. The major problem in using anti-prion molecules in vivo is that peptide and protein therapeutics are generally excluded from transport to the brain, owing to the negligible permeability of these drugs to the brain capillary endothelial wall, which makes up the BBB (blood-brain barrier) in vivo. Fab fragments did not penetrate through the BBB, unless they are hydrophobized by adding stearyl chloride [21] or cationized [22]. One promising solution to this problem is offered by the use of scFv (single-chain variable fragments). ScFvs are monovalent mini-antibodies, constituted by a single fusion polypeptide comprising the variable region of the light and heavy chain (\( \nu_L, \nu_H \)); they maintain antigen specificity and can be engineered for intracellular expression or secretion. So far, several groups have investigated the use of scFv in prion-infected cell culture systems. One group showed the paracrine inhibition of prion replication by RD-4 cells expressing and secreting scFv(6H4) on co-cultured ScN2a cells [23]. Another interesting study showed the retention of PrPSc in the ER (endoplasmic reticulum) of HEK (human embryonic kidney)-293 and PC12 cells after the expression of a scFv from antibody 8H4 and 8F9, containing the ER retention sequence KDEL [24]. Starting from these results, a subsequent set of in vivo analyses was carried out showing that mice intracerebrally injected with KDEL-8H4-NGF-differentiated PC12 cells infected with scrapie did not develop scrapie clinical signs or show any brain damage [25]. Moreover, scFvs directed against the LRP/LR (37/67 kDa laminin receptor) have been recently used as therapeutic approach in prion diseases. Delivery of these scFv both by passive immunotransfer [26] and by AAV (adeno-associated virus)-mediated gene transfer [27] resulted in significant reduction of the peripheral PrPSc propagation. Therefore, these results support the use of scFv as a therapeutic approach.

Additional support to the potential therapeutic value of intracerebral antibody production has recently been given by two studies showing that brain delivery of either prion-specific scFv by AAV transfer [28] or a soluble prion antagonist (PrP-Fc) by lentivirus transfer [29] delays the onset of the disease in infected mice, although protection in these studies was not complete. Because infectious prion replication occurs peripherally within the lymphoreticular system organs, the incomplete protection given by gene transfer of anti-prion molecules in the brain may be consequent to the fact that the peripheral pool of PrPSc continues to replicate and then migrate to the CNS (central nervous system) by neuroinvasion [28].

In order to develop a system that could have therapeutic and not only prophylactic utility, in this study we combined active and passive immunotherapeutic approaches. First, we have produced a monovalent version of the anti-prion D18Fab, D18scFv, and we have tested it in prion-infected cells. The big improvement of our strategy compared with the previous gene transferring is that our molecule might have effects both peripherically and into the brain. We show here that this recombinant anti-prion scFv is able to reduce PK-resistant PrP accumulation in different infected cell models of prion diseases. Furthermore, in order to provide a localized supply of D18scFv (that could obviate the needs of repetitive injection into the brain), we evaluated and compared the in vitro efficiency of two virus-mediated gene transfer systems. We produced two viral vectors, one based on recombinant AAV serotype 2 (AAV2) and the other on HIV-1, carrying the D18scFv gene, and showed that the latter is more efficient in reducing prion levels in cultured infected cells in vivo. These results provide additional support to use small antibody fragments for active and passive immunotherapy in prion diseases and encourage the in vivo evaluation of an anti-prion scFv-based treatment for the therapy of prion disorders.

**EXPERIMENTAL**

**Vector production and protein expression and purification**

The D18scFv sequence was assembled by fusion PCR of the \( \nu_L \) and \( \nu_H \) of the D18Fab by inserting a 20 AA (amino acid) linker. The resulting sequence was cloned into a pET22b(+) vector (Novagen) digested by BamHI and NotI in order to maintain the pelB leader in the N-terminal and the 6-His tag in the C-terminal part. XL1-blue (Strategene) and BL21(DE3) Escherichia coli (Strategene) were transformed both to amplify and to express the D18scFv respectively.

For protein expression, transformed BL21(DE3) were scooped into LB (Luria–Bertani) broth (with 100 \( \mu \)g/ml ampicillin) and grown overnight at 37°C. The overnight culture was diluted 1:50 and grown at 37°C up to \( D_{600} = 0.6 \) before induction with 0.5 mM IPTG (isopropyl \( \beta \)-D-thiogalactoside) for 3 h at 37°C or overnight at 30°C.

Bacteria aliquots (1 ml) were centrifuged and analysed for total protein expression by SDS/PAGE on 12% acrylamide gels and either Coomassie Blue stained or Western blotted, using 1:50 HisProbe-HRP (where HRP is horseradish peroxidase) (Pierce Biotechnology).

To test the recovery of D18scFv from periplasm, bacteria were submitted to osmotic shock with 5 mM MgSO4 at 4°C in order to allow the release of periplasmic proteins in the aqueous phase. For cytosolic fraction preparation, the bacteria pellet was resuspended in BugBuster Master Mix (Novagen) by pipetting and vortexing and then incubated on a shaking platform for 10–20 min at room temperature (25°C), pelleted, resuspended in BugBuster Master Mix and shaken again twice. Aliquots of pellets and supernatants were analysed by SDS/PAGE followed by either Coomassie Blue staining or Western blot, in order to
analyse insoluble and soluble cytosolic fractions. The last pellet was resuspended three times in 1:10 diluted BugBuster Master Mix (2.5 ml/g of the original weight) and centrifuged at 4°C twice at 5000 g and finally at 16000 g. Since the protein was found to be insoluble, it was purified by IMAC (immobilized metal-ion-affinity chromatography) in denaturing conditions and then refolded by fast dilution. Briefly, the pellet was resuspended in 10 ml (for a 1 litre culture) of denaturing buffer A (6 M guanidium/HCl, 0.1 M sodium phosphate buffer and 0.01 M Tris/HCl, pH 8.4) and incubated with of 1 × Ni-NTA (Ni2+ -nitrilotriacetate) His-bound resin (Novagen) (1 ml/4 ml of denatured protein) by gently shaking for 3–4 h at room temperature. After incubation, protein-bound resin was washed twice with buffer B (8 M urea, 0.1 M sodium phosphate buffer and 0.01 M Tris/HCl, pH 6.3) and then the D18scFv was eluted with 4 × 1 ml of buffer C (8 M urea, 0.1 M sodium phosphate buffer and 0.01 M Tris/HCl, pH 5.9), followed by 4 × 1 ml of buffer D (8 M urea, 0.1 M sodium phosphate buffer and 0.01 M Tris/HCl, pH 4.5). Sample aliquots were taken at each step for analysis by SDS/PAGE followed by either Coomassie Blue staining or Western blot using 1:50 were taken at each step for analysis by SDS/PAGE followed by either Coomassie Blue staining or Western blot using 1:50

Activity assays

ELISA

D18scFv was tested for binding to recombinant PrP-Fc. Plates were coated with 20 μg/ml of goat α-human Fc in 0.1 M NaHCO3, pH 8.6, overnight at room temperature, washed three times with TBST (20 mM Tris, 137 mM NaCl and 0.05 % Tween 20, pH 7.5) and then incubated with PBS with 0.25 % BSA and 0.05 % Tween 20 for 1 h at room temperature. PrP-Fc was incubated (containing 1–10 μg/ml of protein) [30] was pre-incubated with several amounts of D18scFv (0.05 up to 10 μg/well) for 15 min at room temperature, and this solution was then incubated with the goat α-human Fc present in the wells for 2 h at room temperature. After washing with TBST and incubation with 1:50 HisProbe-HRP for 1 h at room temperature, plates were developed with TMB (3,3′,5,5′-tetramethylbenzidine) solution (Pierce) for 15 min and 2 M H2SO4 and absorbance was measured at 450 nm. Several concentrations of D18Fab were tested as controls.

Western blot competition assay

Aliquots of 5 μl 10% brain homogenate from FVB mice were loaded on to 12.5 % acrylamide gels and subjected to SDS/PAGE and Western blot. Membranes were saturated with 5 % (w/v) non-fat dried skimmed milk in TBST and incubated for 1 h with several concentration of D18scFv (0.05 to 2.5 μg/ml) together with D18Fab by utilizing Fab/scFv ratios of 1:1, 1:10, 1:50 and 1:100, and then with a goat anti-human IgG-F(ab′)-HRP.

AAV2 production

The D18scFv fragment, including the C-terminal 6-His tag and the N-terminal secretion signal, was cloned into the AAV backbone of the plasmid pAAV-MCS (Stratagene) to obtain pAAV-D18scFv.

Infectious AAV2 vector particles were generated by the AVU (AAV Vector Unit) at ICGEB Trieste in HEK-293 cells, using dual plasmid co-transfection procedure with pDG as packaging helper plasmid [provided by J. A. Kleinschmidt, DKFZ (Deutsches Krebsforschungszentrum), Germany], as previously described [31]. AAV-D18scFv vector stocks were purified by caesium chloride centrifugation and titration of viral particles was performed by real-time PCR quantification of the number of viral genomes, as described previously [31]; the viral preparations used in this work had titres between 1 × 1011 and 1 × 1012 vgp (viral genome particles) per ml.

Lentivirus production

D18scFv cDNA was cloned into the lentiviral vector pRRL.sin. cPPT.hCMV.GFP.Wpre in place of GFP (green fluorescent protein) by digestion with AgeI and SalI. The signal peptide from PrP sequence was inserted at the N-terminal (BclI/AgeI). Vector stocks were generated by co-transfection of 293T cells with pCMV-ΔR8.91,13 pMD.G,13 and pRRL.sin.cPPT.hCMV, D18scFv vectors. HEK-293T-conditioned media was filtered and ultracentrifuged to concentrate the vector. Particle content was measured by HIV-1 p24 antigen immunocapture. HIV-1 p24 concentration was approx. 70 ng/ml (corresponding to 100 000 infectious units per ng of p24).

Cell cultures

Sc2N2 cells and mouse GnRH (gonadotropin-releasing hormone) neuronal cells (ScGT1-1) were maintained in DMEM (Dulbecco’s modified Eagle’s media), with 10 % FBS (fetal bovine serum) in a humidified 37°C incubator with 5 % CO2.
Figure 2  Expression and purification of the D18scFv

(A) Expression of the D18scFv in E. coli. BL21 (DE3) Rosetta-gami E. coli strain was transformed and induced by using 0.5 mM IPTG (+) at 30 or 37 ºC. Note that a higher expression of the molecule was found at 37 ºC. (B) Cytoplasmic extraction of the D18scFv. D18scFv was recovered from periplasmic (P) or both soluble (S) and insoluble (I) cytosolic preparations. Equivalent aliquots of the different obtained fractions were analysed by Coomassie Blue staining (top panel) and Western blot (WB) by using an anti-6-His probe (bottom panel). Note that the larger amount of D18scFv was recovered in the insoluble cytosolic fraction. (C) Purification of insoluble D18scFv. Insoluble D18scFv was purified by IMAC after denaturation using 6 M guanidium/HCl. After elution, eight different fractions were collected and analysed by Coomassie staining (top panel) and Western blot by using an anti-6-His probe (bottom panel). Note that the scFv shows a high degree of homogeneity and is consistent in all the analysed fractions. (D) Solubility of the D18scFv after ultracentrifugation. Purified D18scFv refolded at different pH values (6.5, 7.5 and 8.5) was ultracentrifuged at 100 000 g (+) and analysed by Coomassie Blue staining. Note that D18scFv is more soluble at pH 8.5. Arrows indicate D18scFv.

Studies of D18scFv inhibition

Varying concentrations (0.1 up to 5 μg/ml) of D18scFv were added to ScGT1-1 cells and incubated for 1 week. The cells were fed three times per week with replacement media containing the appropriate amount of scFv. Cells were collected by washing three times with calcium- and magnesium-free PBS and resuspended in 500 μl of lysis buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 0.5 % sodium deoxycholate and 0.5 % Nonidet P-40). Cell nuclei were removed from the lysate by centrifugation at 2000 g for 2 min, and the protein concentration of the supernatant was measured by Bradford assay (Pierce) and subjected to PK resistance assay.

PK resistance assay

ScN2a and ScGT1 cell lysates (500 μg) were treated with 20 μg/ml of PK (total protein:enzyme ratio = 50:1) for 30 min at 37 ºC. Proteolytic digestion was terminated by the addition of PMSF to a final concentration of 2 mM. PK-resistant material was pelleted by centrifugation at 18 000 g for 1 h and then resuspended in reducing SDS sample buffer, boiled for 5 min, cleared by centrifugation and resolved by SDS/12 % PAGE. Samples were transferred on to nitrocellulose membrane and blocked with 5 % (w/v) non-fat dried skimmed milk in TBST. PrP was detected using SAF61 antibody. Blots were developed with ECL® (enhanced chemiluminescence) reagent (Amersham) and exposed to ECL Hypermax film (Amersham).

Studies of D18scFv-virus inhibition

Different titres of lentivirus [10 x 10⁴ to 10 x 10⁶ TU (titration units)] and rAAV2 (10 x 10¹⁰ to 10 x 10¹² TU) carrying D18scFv gene were used to infect both ScGT1-1 and ScN2a cells. For AAV2 infection, cells were pre-treated with 1 mM idrossiurea for approx. 16 h. One week post-infection, cells were collected, resuspended in 500 μl of lysis buffer and subjected to PK resistance assay as described below.
ScFv in prion disease therapy

Figure 3  Analysis of activity of the D18scFv

(A) D18scFv binding to recombinant PrP-Fc. ELISA assays were performed to test the binding to recombinant PrP-Fc of D18scFv (left graph) in comparison with FabD18 (right graph). Note that no significant difference was found for the different pH refolding conditions and that D18scFv maintains the same affinity for PrP as the original D18Fab. (B) D18scFv binding to PrPc present in mouse brain homogenate. Western blot competition assay between the D18scFv and the D18Fab was set up as described in the Experimental section. Different concentrations of D18scFv were tested in competing D18Fab detection of PrPc. Note that the reduction of PrP signal by utilizing a Fab/scFv ratio of 1:50 and 1:100 clearly showed the ability of the scFv to specifically interact with PrP to compete with the Fab for its binding.

Bioinformatic models

The sequence of the D18scFv [32] was compared with the sequences of known 3D (three-dimensional) structures collected in Protein Data Bank [33] in order to find templates for homology modelling. The anti-PrP scFv fragment (PDB code: 2HH0) [34] was identified and further used as the template for the modelling. 3D models were built using MODELLER 6v2 [35]. Amino acids of a signal sequence and poly-glycine parts of the sequences were neglected in the modelling. The structure of the D18scFv model was then used to dock the PrP (PDB code: 1HJM) [36] by means of the HADDOCK 2.0 program [37,38]. Residues determined to be important for protein–protein recognition between PrP and D18scFv were reported in the literature [32]. For the purpose of docking, they were filtered according to the solvent accessibility with the NACCESS program. All His residues were deprotonated. Cluster analysis was then performed with programs available in HADDOCK 2.0 [37,38].

RESULTS AND DISCUSSION

It has been recently shown that anti-prion scFvs could be delivered to the CNS of mice by AAV and are able to delay the onset of the disease when used in prophylaxis, but not when used after prion exposure [28]. Contribution to the incomplete protection could derive from down-regulation of the scFv expression by PrPSc neuroinvasion. In addition, because infectious prions replicate peripherally within the lymphoreticular system organs, the incomplete protection may be consequent to the fact that the peripheral pool of PrPSc continues to replicate and migrate to the CNS by neuroinvasion. These observations make inefficient anti-prion treatments based exclusively on active immunotherapy. Since it has been shown that small proteins can be rapidly delivered and spread in the brain after intracerebral injection or after nasal administration [39,40], we have evaluated, in cell culture, an alternative approach to treat TSEs based on passive immunotherapy.

To this aim, we have produced a smaller version of the D18Fab, the D18scFv in which the variable regions of the light and heavy chains of Fab were fused together by PCR, by inserting a 20 AA linker and adding a 6-His tag in the C-terminal part (Figure 1). The sequence corresponding to the scFv (Figure 1A) was cloned in a bacterial expression system (Figure 1B) and its expression was induced by using 0.5 mM IPTG at 30 or 37 °C. A higher expression of the molecule was found at 37 °C, as shown in Figure 2(A). To test the recovery of D18scFv from periplasm or cytosol, bacteria were resuspended in lysis buffer, and after shaking, centrifugation was performed to separate the soluble from an insoluble protein fraction (Figure 2B). Equivalent aliquots of different fractions were analysed by Coomassie Blue staining and Western blot (Figure 2B). Since the larger amount of D18scFv was recovered in the insoluble cytosolic fraction, it was purified by IMAC in denaturing conditions. In all recovered fractions, D18scFv was shown to be highly homogeneous and in considerable amounts (Figure 2C) and therefore refolded by fast-dilution. In order to test for functional refolding after fast dilution at different pH values (6.5, 7.5 and 8.5), D18scFv was analysed for its solubility by ultracentrifugation at 100000 g (Figure 2D). We found that D18scFv is more soluble at pH 8.5, suggesting that, at this pH, it has lower tendency to precipitate and aggregate and therefore its conformation is more stable.

In order to test directly for interaction and anti-prion activity, D18scFv was evaluated for its binding to PrP. Specifically, we tested the ability of this molecule to bind recombinant PrP-Fc...
[30] in an ELISA assay, and no significant difference was found for the different pH refolding conditions (Figure 3A, right graph). Promisingly, D18scFv was found to maintain the same affinity for PrP of the original D18Fab used as control (Figure 3A, left graph). We also tested the ability of the scFv to bind PrPc present in brain homogenate from FVB mice. We set up a competition assay between the D18scFv and the D18Fab, which could be detected in Western blot by an anti-human F(ab′)2-HRP (Figure 3B). The reduction of PrP signal by utilizing a Fab/scFv ratio of 1:50 and 1:100 clearly showed the ability of the scFv to specifically interact with PrP and to compete with the Fab for its binding. Moreover, we did not find any difference among the scFvs refolded at different pH values (results not shown).

Owing to the interest in how D18scFv interacts with the PrPc, we have used bioinformatic approaches to construct a structural model of the D18scFv–PrPc complex. This kind of approach is commonly used as a first attempt to identify 3D structures of the proteins and protein–protein interactions in the absence of detailed experimental data. Firstly, the 3D structure of D18scFv was modelled based on anti-PrP scFv fragment (PDB code: 2HH0) [34]. This fragment was crystallized in a complex with a small peptide. Owing to the presence of a small peptide, the CDR3 (complementarity determining region 3) is further apart than in non-complexed antibody structures. Therefore, this structure was taken as a template for the model of the D18scFv since it allows an easy approach of the PrPc to the cavity of the antibody in the docking procedure (Figure 4A). Then a D18scFv–PrPc complex was built by docking of PrPc (PDB code: 1HJM) [36] to the model of D18scFv. According to the docking results, ArgPrPc151 (Arg151 from prion protein) is the key residue for the interactions with D18scFv. It anchors the PrPc to the cavity of the antibody, forming H-bonds with TyrVH32 (Tyr32 in heavy variable chain), AsnVH33, AspVH35 and TyrVL210 (Tyr210 in light variable chain), along with van der Waals interactions with TrpVL205 (Figure 4B). A series of other H-bonds and hydrophobic interactions contribute further to the D18scFv–PrPc complex stability.

Owing to the lack of available mutational data, it is difficult to evaluate our models accurately. However, the modelled structure of the D18scFv–PrPc complex might provide an insight into the relevant interactions between the two proteins and give new suggestions for mutational studies.

The next step was to test D18scFv for its effect on scrapie replication in scrapie-infected cell lines (Figure 5). After incubation of ScGT1 cells with different concentrations (0.1, 0.5, 1 and 5 μg/ml) of scFv for 1 week, scFv was found to reduce PK-resistant PrP levels in a concentration-dependent manner, similarly to what was found for the Fab in ScN2a cells [12]. In particular, we found 20 ± 4%, 50 ± 7%, 80 ± 3% and 95 ± 1% PK-resistant PrP reduction by using respectively 0.1, 0.5, 1 and 5 μg/ml D18scFv. We have used R2Fab (10 μg/ml), that was shown to be ineffective in reducing PK-resistant PrP in ScN2a cells [12], and D18Fab (1 and 5 μg/ml) was used as positive control. Interestingly, the same concentration of Fab and scFv (compare lanes with 5 μg/ml) is required to achieve the same

Figure 4 Model of interaction between D18scFv and PrP
(A) Complex between D18scFv (silver) and PrP (blue). Epitope residues of PrP (yellow) interact with the six CDR loops (red, CDR3; green, CDR1,2) of the antibody. (B) Arg151 anchors the PrP to the cavity of the antibody through the H-bond and van der Waals interactions with Tyr32, Asn33, Asp35, Trp205 and Tyr210.

Figure 5 Effect of D18scFv in curing scrapie-infected GT1 cells
ScGT1 cells were incubated for 1 week with different concentrations (0.1, 0.5, 1 and 5 μg/ml) of scFv refolded at different pH values (pH 6.5, 7.5 and 8.5). As controls, R2 (10 μg/ml) and D18Fab (1 and 5 μg/ml) were used. PrPSc present in cell lysates was digested with PK as described in the Experimental section and detected by Western blot using the SAF61 antibody. Note that scFv reduces PK-resistant PrP level by 20 ± 4%, 50 ± 7%, 80 ± 3% and 95 ± 1% using 0.1, 0.5, 1 and 5 μg/ml D18scFv respectively. Blots of PK-resistant assays were quantified by ImageJ (n = 3) and the Student’s t test was used to calculate significance of data (P < 0.05). No difference was found among the scFvs refolded at the different pH values.
ScFv in prion disease therapy

Figure 6 Production and cellular test of lentivirus and AAV2 containing the D18scFv

(A) Cloning of the D18scFv in pTTLsin.PPT.hPGK.GFPpre and in pAAV-MCS. For lentiviral vector production (left panel), the gene encoding for D18scFv (Figure 1A) was cloned in defective lentiviral vector (pTTLsin.PPT.hPGK.GFPpre) between Agel and SalI restriction enzyme sites. The signal peptide of the PrP sequence was cloned upstream between BclI and Agel. For AAV vector production (right panel), the gene encoding for D18scFv was cloned in pAAV-MCS between the HindIII and BglII restriction enzyme sites. For secretion, the signal peptide of the immunoglobulin sequence was added between HindIII and ApalI. (B) Expression of D18scFv after transduction of ScGT1 and ScN2a cells. Lentivirus (10 × 10^4 TU) and 10 × 10^10 TU of rAAV2 were used to transduce both ScGT1 and ScN2a cells. One week post-infection, D18scFv expression was tested by Western blot by using an anti-6-His tag antibody. (C) Effect of the lentivirus and rAAV2 carrying the D18scFv in curing scrapie-infected neuronal cell lines. Titres of 10 × 10^4–10 × 10^6 TU of the lentivirus (left panels) and 10 × 10^10–10 × 10^15 TU of the rAAV2 (right panels) carrying the D18scFv were used to infect both ScGT1 and ScN2a cells. One week post-infection, PrP^Sc present in cell lysates was digested with PK as described in the Experimental section and detected by Western blot by using SAF61 antibody. Blots of PK-resistant assays were quantified by ImageJ (n = 3) and the Student's t test was used to calculate significance of data (P < 0.05). By using increasing titre of lentivirus, levels of PK-resistant PrP were reduced by 7 ± 2%, 75 ± 3% and 80 ± 7% in ScGT1 and 76 ± 2%, 80 ± 7% and 90 ± 3% in ScN2a cells. By using increasing titre of AAV2, levels of PK-resistant PrP were reduced by 2 ± 3%, 5 ± 3% and 5 ± 4% in ScGT1 and 4 ± 2%, 4 ± 7% and 20 ± 5% in ScN2a cells. Note that levels of PK-resistant PrP were significantly reduced in both cell systems by using the highest lentiviral titre tested (10 × 10^6 TU).

extent of reduction in PrP^Sc level. However, since the scFv is approximately half the molecular mass compared with the Fab, D18scFv is 2-fold less effective than Fab with respect to the reduction of the PrP^Sc level in ScGT1 cells. After 1 week of treatment, PK-resistant PrP was reduced to undetectable levels but was not completely eliminated from the cells because it reappeared after 1 additional week of growth in the absence of the scFv (results not shown). However, after 2 or 3 weeks of scFv treatment, PK-resistant PrP remained undetectable for up to 3 weeks of culture without adding any scFv (results not shown), as it has been shown for the original Fab [12]. Also in this case we found no difference among the scFv refolded at the different pH levels (Figure 5). These results indicate that the small monovalent antibody fragment might be a very useful tool in therapy because...
it is able to interfere with prion metabolism (either reducing conversion or promoting degradation) in infected cells. The big improvement of our strategy compared with the previous gene transfer-based approaches [28] is that our molecule might have effects both peripherally and into the brain, both of them sites of prion replication.

*In vivo*, the putative anti-prion effects of the D18scFv expressed as recombinant protein might be enhanced by combining passive and active immunotherapeutic approaches. Recently, different gene transfer approaches in anti-prion prophylaxis of infected animals have been successfully used by using either lentivirus carrying a soluble prion antagonist [29] or AAV transducing either anti-LRP/LR [27] or anti-PrP specific scFvs [28]. Therefore, in order to have a continuous supply of our recombinant scFv to be eventually used in immunotherapy in animals and to test the effectiveness of these two viral systems, we cloned the gene encoding for D18scFv (Figure 1A) either in a defective lentiviral vector (pTTLsin.PPT.hPGK.GFPpre) (Figure 6A, left panel) or in an AAV vector (pAAV-MCS) (Figure 6A, right panel).

The lentivirus vector was transfected in association with a three-plasmid expression system in HEK-293T cells in order to produce a functional lentivirus. A lentivirus containing a GFP reporter gene was used to test the efficiency of viral transduction. At 48 h post-transduction, ScGT1 cells were analysed by immunofluorescence and we found that 35 ± 5% of ScGT1 cells were GFP positive (results not shown). Lentivirus carrying the D18scFv was titred and tested for its effectiveness in reducing PK-resistant PrP content in infected cell cultures. Titres of 10 × 10^6 up to 10 × 10^8 TU were used to transduce both ScGT1 and ScN2a cells. One week post-transduction D18scFv was detected (Figure 6B) and the levels of PK-resistant PrP were reduced by 80 ± 7% and 90 ± 3% respectively in ScGT1 and ScN2a cells by using the highest viral titre tested (10 × 10^6 TU) (Figure 6C). Interestingly, the effect on PK-resistant PrP content after lentivirus transduction was more pronounced in ScN2a cells, where even a lower titre of 10 × 10^5 TU was effective (Figure 6C). Alternatively, we also produced a recombinant AAV2 (see Experimental) expressing D18scFv and subsequently packaged into virus stocks for CNS delivery (see the Experimental section). Titres of 10 × 10^3 to 10 × 10^5 TU were used to transduce both ScGT1 and ScN2a cells and transgene expression (Figure 6B), and PK-resistant PrP levels (Figure 6C) were checked 1 week post-transduction. Interestingly, only the highest titre tested (10 × 10^5 TU) was able to reduce the PK-resistant PrP level by 20 ± 5% in ScN2a cells (Figure 6C). The same result is achieved after longer period of viral transduction (up to 3 weeks) (results not shown). Also in this case we used an AVV2-containing GFP to evaluate the efficiency of viral transduction. At 48 h post-transduction, 15 ± 8% of ScGT1 cells were GFP positive (results not shown). These results indicate that, at least in cell culture models, lentivirus transduces D18scFv in a functional form able to prevent or interfere with PrP^\text{\textsuperscript{sc}} conversion and seems to represent a better delivery system compared to AAV. In comparison, due to the well-known high tropism of recombinant AAVs for brain neurons *in vivo*, recently supported by its efficacy in different clinical trials for neurodegenerative disorders (see, among others, [41]), AAV-scFv remains a valuable potential tool for the *in vivo* treatment of prion disease. How these viruses act in reducing PK-resistant PrP levels still has to be elucidated. One possibility could be that anti-PrP scFvs directly block the conversion reaction. Alternatively, PK-resistant PrP reduction could be an indirect effect of down-regulation of PrP^\text{\textsuperscript{sc}} expression. Nevertheless, the eventual regulation of PrP^\text{\textsuperscript{sc}} expression is specifically due to the scFv, because viruses expressing other constructs (GFP–PrP) do not reduce PK-resistant PrP content in scGT1 cells (results not shown).

In conclusion, we validated here the use of D18scFv as a potential anti-prion immunotherapeutic both as purified protein and after viral transduction in cultured neuronal cells. The availability of our lentiviral and AAV2 vectors expressing the same therapeutic molecule will now allow us to directly compare the efficacy of the two viral vectors in sick animals *in vivo*.

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