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Monoclonal full-length antibody against TAR DNA-binding protein 43 reduces related proteinopathy in neurons

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Conflict of interests
Authors declare that no conflict of interest exists. Jpj and SP are owners of a patent US 15/532,909 titled “TDP43-binding polypeptides useful for the treatment of neurodegenerative diseases”. JPJ is chief scientific officer of Imstar Therapeutics.

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Key words

Amyotrophic lateral sclerosis; full-length antibody; TDP43; in vivo delivery; immunotherapy.
Abstract

Amyotrophic Lateral Sclerosis (ALS) and FrontoTemporal Lobar Degeneration (FTLD), two incurable neurodegenerative disorders, share the same pathological hallmark named TDP43 (TAR DNA binding protein 43) proteinopathy. This event is characterized by a consistent cytoplasmic mislocalization and aggregation of the protein TDP43 which loses its physiological properties leading neurons to death. Antibody-based approaches are now emerging interventions in the field of neurodegenerative disorders. Here we tested the target specificity, in vivo distribution and therapeutic efficacy of a monoclonal full-length antibody, named E6, in TDP43 related conditions. We observed that the antibody recognizes specifically the cytoplasmic fraction of TDP43. We demonstrated its ability in targeting large neurons in the spinal cord of mice and in reducing TDP43 mislocalization and NF-κB activation. We also recognized the proteasome as well as the lysosome machineries as possible mechanisms used by the antibody to reduce TDP43 proteinopathy.

To our knowledge this is the first report showing the therapeutic efficacy and feasibility of a full-length antibody against TDP43 in reducing TDP43 proteinopathy in spinal neurons of an ALS/FTLD mouse model.
**Introduction**

TAR DNA binding protein 43 (also known as TDP43) is a DNA/RNA binding protein predominantly localized in the nucleus of cells (1). Mislocalization and accumulation of hyperphosphorylated, fragmented and ubiquitinated forms of this protein in the cytoplasm of neurons are known as TDP43 proteinopathy, a pathological hallmark of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (2, 3). ALS and FTLD are midlife onset neurodegenerative disorders, with ALS showing muscle-related symptoms, since the main cells undergoing neurodegeneration are motor neurons (4, 5), and FTLD characterized by changes in the behaviour, personality and/or language, since the cortical prefrontal and temporal neurons are affected (6). Because of TDP43 proteinopathy, ALS and FTLD are now recognized as a disease continuum (7), but same TDP43 alterations can be observed also in other disorders such as Alzheimer’s disease (AD), Parkinson’s disease (PD), and vascular dementia (1, 8, 9).

Despite different efforts in studying TDP43 during the pathological events, it remains still unclear why and how TDP43 mislocalizes and accumulates in the cytoplasm. However, once it starts to accumulate in the cytoplasm, it undoubtedly acquires cytotoxic properties (10). For this reason, therapeutic approaches aimed to reduce cytoplasmic TDP43 accumulation are now being considered (11, 12). In recent years, there has been increasing interest on the use of monoclonal antibodies as a treatment for neurodegenerative disorders (13), with the aim of targeting misfolded intra- or extracellular proteins, such as amyloid beta peptide, tau, or alpha-synuclein (reviewed in (14)). In the field of ALS, immunotherapies against SOD1 protein, involved in familial forms of the disease, have been developed and tested on cellular and mouse models with encouraging results (15–21).

Recently we demonstrated the ability of a single chain antibody against the RRM1 domain of TDP43 to target and reduce cytoplasmic mislocalization/aggregation of TDP43 and to improve motor and cognitive performances in ALS/FTLD mouse models (22). Having proven the therapeutic efficacy of the antigen binding domain, here we investigated the target recognition, in vivo distribution and
therapeutic potential of the full-length antibody, named E6, against the same RRM1 domain of TDP43.
Results

**E6 full-length antibody binds specifically to cytoplasmic TDP43**

We already knew that the E6 monoclonal antibody recognized TDP43 in nuclear cell lysates by western blot (22). Here we compared the binding property of the E6 mouse IgG2A anti-RRM1 TDP43 antibody with a commercial mouse anti-human TDP43 N-Term (Abnova). As demonstrated by dot blot analysis (Fig. 1A) and ELISA (Fig. 1B), the monoclonal anti-RRM1 antibody specifically binds human TDP43 without cross-reactivity to BSA. Interestingly, we observed that in both assays E6 antibody showed lower binding affinity for TDP43 than the commercial antibody. A mouse monoclonal IgG2A antibody generated against the G1 glycoprotein of La Crosse Virus (clone 807.33) was used as a control and it did not show any reactivity against TDP43.

To better understand the features of E6 antibody target recognition, we investigated the specificity of binding to TDP43 by immunofluorescence microscopy (Fig. 1C-D). To avoid antigen binding competition effects and secondary antibody aspecific signals, we decided to localize TDP43 with a commercial polyclonal anti C-Term TDP43 (Proteintech) and to label the E6 anti-RRM1 TDP43 or the control mouse IgG2A with a fluorescent dye (Alexa 488). We first performed experiments on human HEK293 cells (Fig. 1C and Supplementary Fig. 1A) in condition where the endogenous TDP43 is mainly localized in the nucleus, or in presence of human TDP43 over-expression which provokes an aberrant mislocalization of TDP43 protein in the cytoplasm. It is noteworthy that the E6 anti-RRM1 antibody detected primarily the cytoplasmic form of TDP43 in contrast with the commercial antibody which detected mainly nuclear TDP43 with only a faint signal of cytoplasmic TDP43. No immunoreactivity was observed with the fluorescein-labeled control antibody 807.33. On the same cells, we performed western blot analyses (Supplementary Fig. 1B) and we observed that in total extract of cells the E6 antibody showed to mainly recognize the 35KDa protein which is one of the fragmented and pathological form of the protein found in the cytoplasm (23).
We then tested the anti-RRM1-TDP43 E6 antibody on tissues from 10 months old mice over-expressing human mutant TDP43A315T, a mouse model that shows histopathological and phenotypical features of ALS/FTLD pathology such as TDP43 proteinopathy (24) (Fig.1D and Supplementary Fig.1C). The E6 antibody, but not the control antibody, detected the cytoplasmic mislocalized TDP43 in both cortical and spinal neurons, two regions of the central nervous system affected by the disease. E6 was also able to detect spot-like staining in the cytoplasm of cells, presumably TDP43 aggregates. Interestingly, as observed in Hek293 cells with endogenous expression of TDP43, E6 antibody was able to detect the physiological cytoplasmic TDP43 present in cells also in motor cortical neurons and large neurons of the spinal cord in non-transgenic mice (Supplementary Fig. 1D).

We also evaluated the binding specificity of E6 antibody with human brain and spinal cord samples (Fig.2). Prefrontal cortex of control and a TDP43 related FTLD patient samples were probed with unlabelled E6 and control antibody 807.33. Mislocalization of TDP43 was previously confirmed on tissues by immunohistochemistry for TDP43 (Supplementary Fig.1E). E6 recognized cytoplasmic TDP43 in both control and FTLD patient tissues confirming its specificity for the cytosolic form of TDP43 under physiological and pathological conditions. Moreover, in FTLD tissue sections, the cytoplasmic TDP43 aggregates were detected with both E6 and the commercial anti-TDP43 C-Term antibody (Fig. 2A). Finally, we performed immunofluorescence staining in spinal cord samples of an ALS patient (Fig.2B) where we demonstrated the peculiar co-localization of E6 with only the cytoplasmic TDP43 in large neurons of the ventral horns. Also in this pathological tissue it was evident how E6 could stain for both diffuse and aggregated forms of TDP43.

E6 full-length antibody is internalized in cells

One of the major problems in working with full-length antibodies is their low capacity to cross the cellular membrane and target intracellular proteins. The ability of E6 antibody to penetrate in living cells was investigated by treating mouse neuroblastoma cells (Neuro2A) with E6 or control antibody.
We exposed cells to 10 μg/ml of antibodies and verified the presence of the antibody intracellularly by western blot at different time points (Fig. 3A). The E6 antibody penetrated cells and internalization was detected after 3h of treatment. The amount of internalized antibodies increased with time. We also confirmed the ability of the antibody to penetrate the cells and localize mainly in the cytoplasm by immunofluorescence on cells treated for 24h with labelled E6 or control Ab (Fig. 3B and Supplementary Fig.2A). Internalization of E6 or control antibodies did not induce cellular death (Supplementary Fig. 2B). We finally verified the internalization of the antibody in N2A cells transfected for 24h with human mNLS-TDP43 which, by having a mutation inside the nuclear localization signal, allows a significant cytoplasmic distribution of the TDP43 protein (25). The E6 monoclonal antibody, differently from control, was detectable inside the cell soon after 30 min from treatment and its presence increased with the time of exposure (Supplementary Fig. 2C).

**E6 antibody reduces cytoplasmic TDP43 and NF-KB activation in cultured cells**

We previously demonstrated that E6 antibody is able to disrupt the interaction between human recombinant TDP43 and p65, the main subunit of NF-KB (22). Moreover, the single chain antibody derived from E6 had demonstrated the ability to reduce NF-KB activation in microglial cells both in vitro and in vivo. We therefore evaluated the ability of E6 antibody to reduce NF-KB activation in a well established cellular model, the BV2-p65-luc microglial cells (26) challenged with LPS. We treated cells with 2.5 μg/ml of antibody and then activated them for 4h with 500 ng/ml of LPS, for a total of 6h treatment with the antibody (Supplementary Fig. 3A). After treatment, we confirmed the presence of the antibodies inside microglial cells (Supplementary Fig. 3B) and we observed that the E6 anti-TDP43 antibody reduced by about 35% the activation of NF-KB in microglial cells after LPS challenge (Fig. 3C).

The previously studied E6-derived single chain antibody was found to reduce the amount of cytoplasmic TDP43 by enhancing ubiquitination of the protein toward the proteasome and autophagic degradative pathways (22). The Fc domain of the E6 full-length antibody may confer an advantage
in boosting degradation of protein target. It has indeed been demonstrated that internalized antibodies can bind to the Fc intracellular receptor TRIM21 which drives the antibody-target complex to the proteasomal degradation (27–29). We therefore examined if E6 can mediate TDP43 degradation by the TRIM21-dependent mechanism in N2A cells transfected with GFP-mNLS-TDP43 and treated with 10 µg/ml of E6 antibody (Supplementary Fig. 3C). Indeed, E6 antibody was able to reduce the amount of cytoplasmic TDP43 compared to the control antibody (Fig. 3D, E) by about 33%. Interestingly, by inhibiting the proteasome by MG-132 treatment, we noticed TDP43 degradation impairments only in presence of E6, suggesting the involvement of this cellular component in the E6-mediated TDP43 degradation.

We also evaluated cytoplasmic TDP43 degradation in N2A GFP-mNLS-TDP43 expressing cells co-transfected with mCherry-TRIM21 (Supplementary Fig. 3D). By overexpressing TRIM21 we observed a 59% significant reduction of TDP43 only in cells treated with E6 antibody (Fig. 3F, G), confirming the involvement of TRIM21 in the degradation of TDP43 when E6 antibody is present. Finally, by analysing the E6 localization in treated cells at higher magnification we noticed both a diffuse and a spot-like staining of the penetrating antibody (Supplementary Fig. 1E). We therefore determined whether an antibody could be internalized also in degradative vesicles (30). We found that both E6 and control antibodies, after 24h treatment, localized in late endosomes (Fig. 3H and Supplementary Fig. 3F) and lysosomes (Fig. 3I and Supplementary Fig. 3G), supporting the idea that a small portion of an antibody inside the cell, and potentially its antigen, could be lately degraded also through the lysosomal pathway.

**Tissue and cellular distribution of E6 antibody after intranasal, intraperitoneal, intracerebroventricular and intrathecal injections**

ALS/FTLD is a pathology that affects neurons in both the brain, in particular the frontal and temporal cortex, and the spinal cord. With a therapeutic perspective, we investigated various ways to deliver the E6 antibody in TDP43A315T transgenic mice with the aim of targeting neurons in the cortex and...
anterior horn of the spinal cord. Here, the most used ways of delivery of an antibody (31) have been
tested considering the less invasive techniques, i.e. intranasal (Fig. 4) and intraperitoneal (Fig. 5), but
also the most efficient in targeting the CNS, i.e. intracerebroventricular (Fig. 6) and intrathecal (Fig.
7). Single injections were performed, and tissues were analysed at different time points to evaluate
distribution and durability of antibody.

We firstly evaluated intranasal (IN) delivery by treating mice with a total of 100μg of E6 antibody or
equal volume of PBS and then sacrificed them after 24h, 48h and 72h (Supplementary Fig. 4A).
Western blot was performed on total lysates from olfactory bulb, hippocampus, frontal and posterior
cortex, cerebellum, brainstem and cervical, thoracic and lumbar spinal cord (Fig. 4A). A 50 kDa band
was visible in mice treated with E6 antibody soon after 24h although the higher intensity was
observed after 48h with a general decrease after 72h. The antibody band was clearly detectable in the
olfactory bulb more than other areas although a more intense band, compared to PBS-treated, was
detectable also in the hippocampus, cortex, brainstem and interestingly in the cervical region of spinal
cord. We confirmed the wide distribution of the antibody at 48h after intranasal injection also by
immunofluorescence (Fig. 4B), although a deeper analysis of cellular localization showed that the Ab
was mainly trapped in blood vessels structures than penetrating the tissues and localizing into cells
(Supplementary Fig. 4B).

Intraperitoneal (IP) delivery was assessed by treating mice with a total of 500μg of E6 antibody or
control antibody or equal volume of PBS then sacrificed them after 2 days, 3 days and 4 days
(Supplementary Fig. 5A). We confirmed the presence of the antibodies in the plasma of treated mice
(Fig. 5A). We also found that the antibodies could penetrate circulating peripheral blood mononuclear
cell (PBMC) (Fig. 5B) which could allow the diffusion of the antibodies also in tissues. When we
checked for brain penetration, we were able to detect the antibodies (both E6 and CTR) only after 3
days from injection as a strong and diffuse signal at the level of the third ventricle and a faint and
occasional penetration into some neurons (Fig. 5C and Supplementary Fig. 5B).
Intracerebroventricular (ICV) delivery was performed by injecting mice unilaterally into the lateral ventricle with a total of 20μg of E6 antibody or control antibody or equal volume of PBS then sacrificed them after 2h, 4h, 6h or 24 and 48h (Supplementary Fig. 6A). We confirmed the presence on the Abs in the CSF of treated mice (Fig. 6A). We then performed immunofluorescence on brain tissues of treated mice (Fig. 6B and Supplementary Fig. 6B) observing the presence of E6 and CTR Abs in the ipsilateral region of the brain in the early time points after the injection. The antibodies were clearly visible both in the cortex and in the hippocampus of treated mice as a diffuse signal. By analysing later time points (24h and 48h) we observed a neuronal uptake and internalization after 24h from injection in both regions analysed. The fluorescence signal was detectable in both ipsilateral and contralateral regions. At 48h there was a loss of fluorescence signal.

We finally investigated the intrathecal (IT) delivery, with the aim of targeting motor neurons, by injecting a total of 25 μg of E6 antibody or equal volume of PBS and sacrificing mice after 24h, 48h, 72h or 6 days (Supplementary Fig. 7A). We confirmed the presence of the antibody by western blot in the lumbar spinal cord of mice (Fig. 7A) observing a clear signal even after 72h form injection and a weaker presence also after 6 days. At 72h, immunofluorescence microscopy revealed the E6 antibody to be internalized into spinal motor neuronal-like cells (Fig. 7B).

E6 antibody can spread thorough the spinal cord after repeated intrathecal injections

As a proof of principle, we decided to assess the in vivo potential of the full-length anti-RRM1-TDP43 antibody to rescue ALS/FTLD pathological events in the TDP43A315T mouse model. As these mice exhibit motor neuron disease with aging (24), we decided to carry on the therapeutic approach with the intrathecal delivery. Due to the small volumes that can be injected in the limited intrathecal space and the possibility to deliver a fresh preparation of antibody every short period, repeated intrathecal injections rather than a continuous diffusion was preferred. By keeping the 72h as the reference time, we injected 9 months old TDP43A315T mice with PBS or 25 μg of E6 antibody
or control antibody (n=10 per group) twice a week for 5 weeks allowing mice to receive 10 injections and a total of 250 μg (Supplementary Fig. 7B).

We evaluated the diffusion of the antibodies after 5 weeks of repeated injections. The entire central nervous system was analysed by immunofluorescence for the presence of mouse IgG2A (Fig. 7C and Supplementary Fig. 7C). Interestingly, we found that both E6 and control antibodies were able to penetrate the CNS tissues from the intrathecal space and to diffuse from the sacral portion throughout all the length of the spinal cord, targeting cells both in the lumbar, thoracic and cervical portions. Although a weak signal was also detectable in the brainstem nuclei (data not shown), no signal was detected in the cerebellum and the brain of the mice.

A careful analysis by immunofluorescence revealed that E6 antibody was mainly uptaken by neurons and microglial cells, whereas no mIgG2A signal was observed within astrocytes or oligodendrocytes (Fig. 7D and Supplementary Fig. 7D).

Repeated injections of E6 antibody reduced cytoplasmic TDP43 and nuclear NF-KB in motor neurons

The therapeutic effect of the repeated intrathecal delivery of the E6 antibody was evaluated in TDP43A315T mice IT injected for 5 weeks (Supplementary Fig. 7B). No adverse effect, neurological symptom, weight loss or premature death occurred in mice throughout the course of the study, strengthening the absence of toxicity of E6 antibody previously observed in vitro.

A detailed analysis at tissue and cellular level was then performed on treated mice. We firstly evaluated the cytoplasmic levels of TDP43 in lumbar spinal cord by western blot (Supplementary Fig 8A, B) observing a slight but not significant reduction of cytoplasmic TDP43 in E6-treated mice. Since we observed a neuronal specific uptake of the antibody, the analysis of TDP43 mislocalization was then performed by immunofluorescence focusing in particular on large neurons present in the ventral horns of the lumbar spinal cord (Fig. 8A and Supplementary Fig. 8C). We analysed four mice per group (Supplementary Fig. 8D) with an average of 300 large neurons (area >250 μm²) counted.
per experimental group (Figure 8B). In these cells we quantified the nuclear to cytoplasmic ratio of TDP43 staining observing a significant reduction of cytoplasmic TDP43 mediated by E6 antibody. No motor neuron toxicity was observed in treated mice as the number of Chat+ve cells (area >250 μm²) per section remained unaltered in the three different experimental groups (Supplementary Fig. 2E, F).

Another detrimental effect of TDP43 proteinopathy considered in this study is the hyperactivation of NF-KB. We previously demonstrated that the E6 antibody was able to reduce NF-KB in vitro, therefore we decided to verify this ability also in vivo after repeated intrathecal injections.

We firstly analysed large neurons of the lumbar spinal cord ventral horns (Figure 8C and Supplementary Fig.9A) as it has been demonstrated that NF-KB activation in neuronal cells contributes to cell sensitivity to toxic pathways (32). We analysed four mice per group (Supplementary Fig. 9B) with an average or 200 large neurons (area >250 μm²) counted per experimental group (Fig. 8D). In these cells we measured the nuclear signal of acetylated p65, a known form of activated p65 (33, 34), finding a significant reduction of nuclear p65 mediated by E6 antibody treatment. Interestingly, we observed a correlation between the presence of E6 antibody and the reduced nuclear levels of p65 in neurons (Supplementary Fig. 9C).

We then analysed the effect of E6 antibody on NF-KB modulation in glial cells by quantifying microglial (CD11b staining) and astrocytes (GFAP staining) reactivity in lumbar spinal cord of treated mice. Although the antibody was able to localize also in microglial cells and to reduce microglial NF-KB activity in vitro, we did not observe any reduction of microgliosis after E6 treatment (Fig. 8E, F). On the contrary, the treatment with the E6 antibody induced a general microglial activation. No significant changes were observed in terms of astrogliosis (Supplementary Fig. 9D, E).
Discussion

Here we report for the first time a mouse monoclonal full-length antibody (named E6) generated against the RRM1 domain of TDP43 that specifically recognized cytoplasmic TDP43 species in cellular systems, in mouse models and in human samples. Moreover, we show that repeated intrathecal injections of E6 antibody resulted in large neuron penetration of the antibody and mitigation of cytoplasmic TDP43 mislocalization. Finally, evidence is presented that E6 antibody-mediated TDP43 degradation may involve the TRIM21/proteasome and lysosome degradation pathways.

Over the past decades, increasing efforts have been put in the use of therapeutic antibodies for neurodegenerative disorders such as AD, or PD (14). In the field of ALS, immunotherapy has been firstly developed against the protein SOD1 with encouraging results (15–21). Other proteins have also been the target of immunotherapy like NogoA (35, 36), IL10 (37) and RAN proteins for C9ORF72 repeats expansions (29, 38). Recently, we demonstrated that intracellular TDP43 is a therapeutic target for antibody-based approaches. Therein we showed that viral delivery of a single chain antibody against the RRM1 domain of TDP43 was able to recognize its target and to reduce the levels of cytoplasmic TDP43 accumulation together with improvement of cognitive and motor functions in two mouse models of ALS/FTLD (22).

Despite these promising results, for human clinical trial the administration of full-length antibodies would likely be safer and easier to implement than AAV-mediated transfer of single chain antibodies without expression control. In case of adverse effects, the direct administration of a monoclonal antibody allows modulation of treatment. Moreover, the presence of the constant fragment (Fc) in the full-length antibody gives therapeutic advantages related to cellular uptake in neurons expressing Fc receptors (39) and target degradation (30, 40). So, we examined the ability of the E6 full-length antibody to mitigate TDP43 proteinopathy in ALS/FTLD mouse model.
From previous studies we knew that this antibody was able to recognize TDP43 in nuclear lysates of murine cells as commercial antibodies do. Here we confirmed the ability of E6 antibody to bind to TDP43 but we also described a lower affinity for the target compared to commercial mouse anti-human TDP43 N-Term (Abnova), commonly used for diagnosis or research purposes (Fig. 1B). TDP43 can freely shuttle between nucleus and cytoplasm and in both compartments it has important biological functions (41). When it consistently mislocalizes in the cytoplasm of cells and aggregates, TDP43 acquires pathological features mainly losing the physiological ability to move between nucleus and cytoplasm and to freely bind proteins or RNA (42). Here we show that in cellular systems, animal models and human tissues the E6 monoclonal antibody recognizes mainly the cytoplasmic TDP43. Interestingly, by western blot on total cell lysates the E6 antibody showed a preference for the 35KDa form of the protein (Supplementary Fig. 1B) which is known to be one of the pathological and cytoplasmic fraction of the protein (23). This preference for the cytoplasmic protein was clearly visible in pathological conditions where TDP43 was overexpressed and mislocalized in culture cells (Fig.1C) or in cortical and large neurons of the lumbar spinal cord of mutant TDP43 overexpressing mice (Fig. 1D). In contrast to E6 antibody, commercial antibodies against TDP43 detect predominantly the nuclear TDP43 species. It is noteworthy that the E6 monoclonal antibody recognized cytoplasmic TDP43 also in human cortical neurons of control and ALS/FTLD patients (Fig. 2A) and in large neurons of the spinal cord ventral horns (Fig. 2B). The E6 immunostaining of punctate structures in the cytoplasm of pathological neurons must reflect the ability of the antibody to detect aggregated TDP43. Interestingly, in physiological conditions like untransfected Hek cells (Fig. 1C), non-transgenic mice (Supplementary Fig. 1D) or healthy individuals (Fig. 2A), where the C-Term antibody shows mainly a nuclear TDP43 signal, E6 can recognize the physiological cytoplasmic localized protein. The lack of E6 immunostaining for TDP43 in the nucleus may reflect the masking of the epitope in the RRM1 domain which interacts in this compartment with nucleic acids (43). The specificity of E6 antibody for the cytoplasmic TDP43 makes it a unique antibody with therapeutic potential to target TDP43 pathology.
We demonstrated that the E6 monoclonal antibody was also able to penetrate neurons and microglial cells both in vitro and in vivo (Fig. 3 and 7D) and to mediate a therapeutic effect once inside cells. It has been previously described that antibodies against Tau are primarily taken up by neurons via clathrin-dependent FcG receptor endocytosis (30, 39). Similarly, we observed a quick uptake of the E6 monoclonal antibody by cultured neuronal and microglial cells which appeared higher for E6 monoclonal antibody than for control. As suggested also in the case of Tau antibodies, the presence of the pathological target inside the cell may influence antibody internalization and/or retention in neurons (39). Once inside neurons, both in vitro and in vivo, the E6 antibody showed no toxicity. On the contrary, we observed a reduction in levels of cytoplasmic TDP43 (Fig. 3E and 8A, B). The reduction of the pathological form of TDP43 can be explained by two known mechanisms of action of antibodies, both of them dependent on the Fc domain of an antibody, i.e. TRIM21-dependent proteasome degradation and the lysosome system. It has been demonstrated that once inside the cells and bound to its target via the antigen binding fragment (Fab), an antibody can be recognized by TRIM21, an intracellular Fc receptor (44). TRIM21 is a E3 ubiquitin ligase which can mediate the coupling of the complex antibody/antigen to the proteosomal machinery inducing a fast degradation of endogenous cytoplasmic proteins recognized by antibodies (27, 28). The TRIM21-dependent degradation mechanism has been previously demonstrated with antibodies against Tau (40) and very recently also for antibodies targeting RAN proteins (29). In this study we demonstrated that cytoplasmic TDP43 can be degraded by the E6 monoclonal antibody and that this event was TRIM21-dependent, since inhibition of proteasome (Fig. 3E), the final step in this mechanism, or overexpression of TRIM21 (Fig. 3G) altered TDP43 degradation only in presence of the E6 monoclonal antibody. Interestingly, both the E6-derived single chain antibody previously tested (22) and the E6 full-length form can mediate a reduction of the cytoplasmic TDP43 in large neurons of about 30% compared to control antibodies. The two forms of the E6 antibody, that share the same antigen binding domain with strong specificity for the pathological protein, demonstrated to overlap in the final effect of degrading TDP43 although their mechanisms of action are different. The single
chain antibody indeed acts as a flag on TDP43 for cells that enhance the ubiquitination of the protein for the proteasome and the autophagic pathways. Instead, the full-length antibody, which possess the Fc domain, once inside the cell is recognized by a strong and effective machinery of degradation represented by TRIM21 and the proteasome. Moreover, as it happens for anti-Tau antibodies, reported to induce Tau degradation also by the endosome/autophagosome/lysosome system (30), here we demonstrated that a small portion of E6 co-localized with both late endosomes (Fig. 3H) and lysosomes (Fig. 3I), combining to the demonstrated TRIM21-mediated pathway also the possibility that the cytoplasmic TDP43 might be additionally degraded by E6 antibody via this pathway.

Another beneficial effect of E6 antibody was the reduction of NF-KB activation in large neurons of the lumbar spinal cord (Fig. 8C, D). This phenomenon may be due to either a reduction of the cytoplasmic TDP43 or to the antibody block of the TDP43/p65 interaction (22). As demonstrated previously (32), a reduced NF-KB activation in neuronal cells protects them from detrimental toxic stimuli.

Macrophages and microglial cells express FcG receptors as neurons (45, 46) supporting the presence of E6 monoclonal antibody in circulating PBMC and microglial cells both in vitro and in vivo (Supplementary Fig. 3B and Figures 5B, 7D). In vitro, we were able to demonstrate a reduction of NF-KB activation mediated by the antibody (Fig. 3C). In contrast to the in vitro results, we registered an activated phenotype for microglial cells after E6 antibody treatment in mice (Fig. 8E, F). This might reflect a protective phagocytic phenotype, which can help in neuronal TDP43 clearance (47) and also facilitate the uptake of the antibody, or an immunogenic effect of the antibody on microglial cells induced by the binding between the Fc fragment and the Fc receptors present in these cells (48, 49). On this regard, the previously studied E6-derived single chain antibody, that was lacking the Fc portion, did not induce any immunogenicity but instead reduced NF-KB activity, confirming the ability of the antigen binding fragment in blocking the TDP43/p65 interaction and in reducing inflammation. The discrepancy between the results obtained in vitro and in vivo with the full-length E6 antibody might also be due to the amount of antibody delivered and the duration of the treatment.
(2.5 μg/ml for 6h in cells and a total of 250 μg over a period of 5 weeks for mice). We might speculate that a longer treatment with a lower amount of antibody delivered or less frequent injections could result in a reduced microgliosis also in vivo. This, together with the efficiency of the E6 antibody in mediating the degradation of the cytoplasmic TDP43, might eventually result in a stronger therapeutic effect in ALS/FTLD mouse models.

Finally, few considerations should be given for the anti-TDP43 antibody delivery methods described in this study. Here we showed for the first time a comprehensive description of CNS distribution in TDP43 mutant mice of a mouse IgG2A antibody against TDP43 after systemic or local administration commonly used for antibody delivery (31). We first analysed two main non-invasive delivery methods, i.e. the intraperitoneal (IP) and the intranasal (IN) ways. IP injection (Fig. 5) unfortunately yielded only a small and weak distribution of the antibody in the brain, mainly limited to the ventricular area. Although not suitable for CNS delivery, we demonstrated a sustained and long-lasting presence of the antibody in the blood stream and in blood cells, which might be therapeutically useful in case of TDP43 induced peripheral pathological events like, for example, gastrointestinal problems described in mutant TDP43 overexpressing mice (50–52). On the same side, the internalized E6 antibody in peripheral blood cells might target and modulate TDP43 mislocalization (53) and the decreased survival ability of PBMC documented for ALS patients (54, 55) or could potentially help the antibody to reach the CNS through infiltrating lymphocytes (56, 57).

Interesting results were obtained for IN delivery (Fig. 4). Here we confirmed that IN administration of an antibody yielded a strong and wide distribution throughout the entire brain (58), which was maintained even after three days from delivery. We also demonstrated that IN allowed the antibody to reach the brainstem and the cervical tract of the spinal cord. Unfortunately, as already reported (58), the single IN administration failed to permit a cellular penetration of the antibody which remained mainly localised in blood vessels. Perhaps repeated IN administrations may lead to a better cellular localization but this hypothesis merits further investigations.
We also analysed two main ways of delivery into the CSF, i.e. the intracerebroventricular (ICV, Fig. 6) and the intrathecal (IT, Fig. 7) ways which, although more invasive then IP and IN, have the great advantage of bypassing the major obstacle for macromolecules delivery, the blood-brain barrier (BBB) (13). At 2 hours after ICV injection, we were able to detect the antibody spread from the lateral ventricle to cortical and hippocampal regions of the ipsilateral hemisphere. With time, the antibody disseminated to the contralateral hemisphere. At 24 hours after single injection there was evidence of massive penetration of antibodies into cells. At 48h, the signal was then reduced in tissue but still present in the CSF.

Efficient results in term of distribution and cellular penetration were finally obtained with IT delivery. We demonstrated the presence of the antibody in the CSF up to 3 days after a single intrathecal delivery which yielded an appreciable penetration in large neurons of the lumbar spinal cord. We demonstrated that a treatment with repeated IT injections for 5 weeks was safe and feasible for transgenic old mice expressing mutant TDP43 and with evidence of target recognition (Fig. 7C, D).

Indeed, by exploiting the CSF flux, the antibody diffused throughout the entire length of the spinal cord till the cervical region and penetrated in ALS pathological cells, i.e. motor neurons. The fact that neurons possess the Fc receptors and a higher level of the pathological target makes of this cellular population a good target for the antibody that, once internalized, mediates the therapeutic effect previously discussed. It might be interesting in future to administer the antibody through intrathecal cervical injection. This technique, used for example for the administration on analgesic compounds in pain treatment, might allow our antibody to achieve a better cervical spinal cord and brain diffusion.

In conclusion, we demonstrated for the first time that a full-length monoclonal antibody against TDP43-RRM1 domain, named E6, i) recognized specifically cytoplasmic TDP43 in cellular, animal and human samples, ii) mediated cytoplasmic TDP43 degradation in large neurons of the spinal cord, likely through TRIM21 and lysosome-dependent mechanisms, and iii) reduced NF-KB activation.
Moreover, we described and compared four ways of antibody delivery to the CNS that highlighted iv) the therapeutic validity of repeated intrathecal injections for ALS in targeting large neurons throughout the entire length of the spinal cord ventral horns, v) the capability of continuous ICV delivery to target cortical and hippocampal neurons, and vi) the potential of a wide brain distribution after IN delivery. These results demonstrated the feasibility of a full-length antibody-based therapeutic intervention against cytoplasmic TDP43 in context of ALS/FTLD.
Material and Methods

Methods related to cell cultures transfection and test, ELISA Array, immunohistochemistry and immunofluorescence, protein extractions, dot blot and western blot can be found in Supplementary material.

Aim and experimental design of the study

In this study we aimed to i) test the target specificity ii) the in vivo distribution and iii) the therapeutic efficacy of the E6 full-length monoclonal antibody. We analysed the antibody specificity in TDP43 overexpressing neuronal cells, animal tissues and ALS/FTLD human samples. The in vivo distribution was analysed at different time points in TDP43A315T mice after single IP, IN, ICV, IT injections. The therapeutic efficacy was analysed in microglial and neuronal cell lines, and in TDP43A315T mice that received repeated intrathecal injections twice a week for five weeks.

Experiments implementation and data collection were blind performed for all animal studies (operators were kept blind when administering treatment or analysing tissues). Data collection was blind performed for in vitro experiments (operators were kept blind on treatments). To evaluate the therapeutic efficacy in mice, treatment randomization was applied, although gender balance was taken in consideration among groups. No data were excluded from analyses.

Raw experimental data, supporting the findings of this study, are available from the corresponding author upon reasonable request.

Anti-TDP43 and control full-length monoclonal antibodies

Both anti-TDP43-RRM1 domain (E6) monoclonal antibody (mIgG2A), produced as previously described (22), and the control (807.33) mouse monoclonal antibody (mIgG2A) anti-G1 protein of La Crosse virus (ATCC CRL-2290) were purchased from Medimabs (Montreal, CA) after purification and lyophilisation of the antibody from media of hybridoma cells. For direct fluorescence detection, E6 and 807.33 antibodies were conjugated with Alexa fluorochrome (807.33-488 and E6-
using an Alexa Fluor 488 Antibody Labelling Kit (Thermofisher) following manufacturer’s instructions and 6h dialysis against PBS to remove unbound dye and sodium bicarbonate.

**Patients**

Control samples were obtained from a 70 year old man with no history of neurodegenerative disorder who died from multiple organ failure (Identifier 18N00032). Experimental samples were obtained from a 60 year old man affected with TDP43 related frontotemporal lobar degeneration (Identifier 15N00204) and from a 54 year old man affected with sporadic ALS (Identifier 20N00264).

**Mice treatments and tissues collection**

*TDP43A315T* mice (24) were identified by PCR on DNA form ear biopsies and constantly maintained, after more than 20 backcrosses, on a C57/BL6 strain (Charles River) as colony at the CERVO Brain Research Centre animal facility under standard conditions. Antibody distribution analyses were performed on female *TDP43A315T* mice (n=31, average age=10 months), whereas the antibody therapeutic efficacy was assessed in 9 months old *TDP43A315T* mice (n=30 in total, n=7 males and n=3 females per group). Non-transgenic (*NTg*) mice (n=3) from the same background and age (littermates) of *TDP43A315T* mice were used for immunofluorescence experiments.

For intraperitoneal administrations, mice were injected with 500µL of 0.9% saline or 1 mg/mL purified monoclonal antibody. For any other antibody delivery, mice were anesthetized with 2% isoflurane during all the procedures, and post-treated with subcutaneous buprenorphine (0.05 mg/kg) to prevent postoperative pain, except for intranasal delivery. For intranasal administration, anesthetized mice were placed on their back, and droplets of 0.9% saline or 1 mg/mL purified monoclonal antibody solution were dropped into the mouse nostrils using a pipette. Fifty microliters of solution per mouse (25µL per nostril) were administered. For intracerebroventricular delivery, mice were placed in a stereotaxic apparatus (David Kopf Instruments) and the right ventricle was then reached with a 33-gauge stainless steel cannula (Plastics One) that was connected to a 25µl Hamilton syringe with an intramedic polyethylene tubing (PE-50; Clay Adams). 0.9% saline or 2
mg/mL purified monoclonal antibody solution were administered in a maximum volume of 4μl over a total of 4 min by means of a microinjection pump (model A-99; Razel Scientific Instruments). Intrathecal injections were performed as previously described (18). Briefly, a total of 20μL volume of PBS or 1.25 mg/mL purified monoclonal antibody solution was slowly injected into the dura (L4-L5 intervertebral space) using a sterile Hamilton syringe. The presence of a reflex contraction of the hind legs or the tail was considered indicative of a successful administration. The syringe was removed 1 minute after the end of the injection to minimize CSF and solution leakage. Tissues were collected after deep anaesthesia with 10μl/g pentobarbital 12mg/kg. For protein analyses, mice were perfused intracardially with cold 0.1M phosphate buffer. Tissues were rapidly collected, frozen on dry ice, and stored at -80°C until used for protein extraction. For histological analyses, mice were first perfused intracardially with 0.1M cold phosphate buffer, and subsequently perfused with cold 4% paraformaldehyde (PFA). Tissues were then collected, post-fixed for 24h in 4% PFA and cryoprotected in sucrose 30%.

CSF was collected as previously described (59) from the cisterna magna of mice, centrifuged at 13500 x g for 5 min at 4°C and stored at -80°C until analysis.

Plasma and PBMC from mice were obtained as previously described (60, 61) with few modifications. Briefly, mice were deeply anesthetized and blood was sampled by intracardiac drawing and transferred into EDTA containing vials (Sarstedt). Blood was then diluted 1:1 with PBS and stratified on one volume of Ficol Plaque Premium 1.084 density (GE Healthcare). After centrifugation at 400 x g for 40 min plasma was collected and stored at -80°C until use for analysis, whereas the buffy coat, containing PBMC, was transferred into a new tube, resuspended with DPBS (Gibco) and centrifuged first at 500 x g for 15 min and after at 400 x g for 10 min. Pellet containing PBMC was stored at -80°C until use for analysis.

**Image acquisition and analysis**
Slides with stained human frontal cortex samples were placed on the stage of an inverted motorized microscope NIKON ECLIPSE Ti-E (Nikon Instruments Europe) equipped with a CFI SR APO TIRF 100X ON1.49 objective, a Perfect Focus System, and a Total Internal Reflection Fluorescence (TIRF) LAs2 module (Roper Scientific). Acquisition of images was obtained using Metamorph 7.7 software (Molecular Devices). Image sequences were acquired with a single-photon sensitive camera Evolve 128TM EMCCD 512 x 512 imaging array, 16 x 16 μm pixels (Photometrics).

For low magnification fluorescence imaging, slides with stained cells or animal tissues were placed on the stage of a DM5000 B wide-field microscope (Leica Microsystems) connected to a CTR5000 station and a Mi-150 Fiber Optic Illumination System (Dolan-Jenner) and equipped with 10X/0.30 Ph1 HC PL Fluotar, 20X/0.50 Ph2 HC PL Fluotar and 40X/0.75 Ph2 HCX PL Fluotar objectives. Images were acquired using a Digital FireWire Monochrome Camera (DFC350 FX) controlled by Leica Application Suite software version 4.8.0. For high magnification fluorescence Imaging, slides with animal tissues were placed on the stage of a Axio Imager Z1 wide-field microscope (Zeiss) equipped with 40X EC-plan Neofluar 40x/0.75 Ph2 M27, 63X Plan Apochromat 63x/1.40 Oil DIC M27, and 100X EC-plan Neofluar 100x/1.30 Oil Ph3 M2 oil immersion objectives. Images were acquired using an AxioCam MRm camera controlled by AxioVision software (Carl Zeiss) version 4.8.1.0.

ImageJ software was used for images reconstruction and morphometric analysis. To assess the number of motor neurons in ventral horns, neurons stained with anti-ChaT antibody were counted on pictures acquired at low magnification (20X). The nucleus/cytoplasmic ratio of TDP43 was determined for each cell at high magnification (63X) as described before (22, 62). To quantify NF-KB activation in neurons, the mean intensity of acetylated NF-KB signal was measured in the nucleus and corrected to the mean cytoplasmic signal of the same cell (considered as basal signal). TDP43 mislocalization and nuclear acetylated p65 were analysed only in large (area > 250 μm²) motor neuron-shaped cells of the ventral horns of the spinal cord with visible nucleoli. The analysis was done by an experienced neuropathologist. For astrogliosis and microgliosis assessments, an
automated intensity detection threshold (IJ_IsoData) was applied to images acquired at medium
magnification (40X), and elements higher than 30 µm were selected and analysed for area distribution
(22).

Statistics
Statistical analyses were performed using PRISM software version 5.0 for Windows (GraphPad).
Unpaired two-tailed T-Test or One-way ANOVA followed by post hoc test for multiple comparisons
corrections were performed according to the experimental design. Normal distribution and the
homoscedasticity of data was verified using Shapiro–Wilk’s test. Differences were considered
significant when p-value<0.05. No power analysis was performed before animal treatment but sample
size is consistent with other studies in the field (63).

Study approval
Post-mortem cryopreserved brain samples were obtained from the Neurodegenerative Diseases Brain
Bank of Angers University Hospital (national identifier BB-0033-00038, Regional Ethics Committee
declaration number DC-2011-146).
The Animal Care Ethics Committee of Université Laval approved all in vivo experimental protocols
used in this study. Experiments were carried out in accordance with the Guide to the Care and Use of
Experimental Animals of the Canadian Council on Animal Care.

Author contributions
SP designed the study, performed Elisa, immunofluorescence and western blot experiments in mice
and all manipulation of culture cells, analysed the data, performed statistical analyses and data
interpretation, prepared figures and wrote the manuscript. PC contributed in study design and tissues
analysis of repeated injected mice, performed immunofluorescence experiments in cells, mice and
human tissues, contributed in preparing figures and writing the manuscript. GS performed all the
injections in mice. LR helped with western blots experiments in mice and cells. PJC helped in collecting tissues for Ab distribution analysis and in purifying PBMC. KD performed preliminary experiments on full-length Ab. CB performed PCR for mice genotyping. JPJ supervised the study and revised the manuscript. All authors agreed to be personally accountable for contributions and ensured that questions related to accuracy and integrity of any part of the work are appropriately investigates, resolved and documented by literature. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful to the donors and their families. SP would like to thank Mélisse Iyera Nkurunziza and Chloé Grenon for helping with western blots experiments in cells during their training as summer students. This work was funded by the Canadian Institutes of Health Research and the ALS Society of Canada. JPJ holds a Canada Research Chair in Neurodegeneration. SP received the 2019 Marlene Reimer Brainstar of the year award from CIHR-CAN (ICT-171454).
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Figure Legends

Figure 1

**Figure 1.** Anti-RRM1 full-length antibody E6 recognizes cytoplasmic localized TDP43 in cells and TDP43 A315T mice tissues. (A) Different concentrations of human recombinant TDP43 or BSA were loaded on a dot blot membrane and probed with 0.5 μg/ml of control antibody, E6 antibody or a commercial mouse anti-human TDP43 N-Term antibody (Abnova). Ponceau was used as loading reference. (B) ELISA assay measuring the interaction of 0.4 μg/ml control antibody, E6 or a commercial anti-TDP43 N-Term antibody (Abnova) or PBS on different concentration of TDP43. Data are mean±SEM, n=3 wells per conditions (dots) and are expressed as ratio of signal (Abs, absorbance) obtained on TDP43 versus BSA. Two-way Anova (interaction F_{6,24} =167.4 P<0.001, concentration F_{2,24} =256.5 P<0.001, antibody F_{3,24} =3694 P<0.001), * P<0.05 or *** P<0.001 versus PBS, # P<0.05 or ### P<0.001 versus control antibody by Tukey’s multiple comparison test. (C) Representative immunofluorescence with anti-TDP43 C-Term (Proteintech, red), control- or E6-488 conjugated (green) on fixed HEK293 cells in standard conditions or after overexpression of hTDP43-WT, scale bar=5 μm. Merge with nuclei (Hoechst, blue) is shown in Supplementary Figure 1A. (D)
Representative immunofluorescence with anti-TDP43 C-Term (Proteintech, red), control- or E6-488 conjugated antibodies (green) and merge with nuclei (Hoechst, blue) on motor cortex or lumbar spinal cord of 10 months old TDP43A315T mice, scale bar=50 µm and scale bar=10 µm in the enlarged (5X) pictures. Spot-like staining is marked by arrow heads. Merge with nuclei in non-enlarged pictures is shown in Supplementary Figure 1C. Immunofluorescence was performed in more than three replicates for panel C and in three mice for D. Control antibody (clone 807.33).
Figure 2

Figure 2. Anti-RRM1 full-length antibody E6 recognizes cytoplasmic localized TDP43 in cortical and spinal cord tissues of ALS/FTD patients. (A) Representative immunofluorescence with anti-TDP43 C-Term (Proteintech, red), unlabelled control or E6 antibodies (green) and merge with nuclei (Hoechst, blue) on prefrontal cortex of a control non-neurodegenerative patient and a FTLD patient, scale bar=5 µm. Lipofuscin non-specific spots were visible in all fluorescence channels and are marked as arrow heads. (B) Representative immunofluorescence with anti-TDP43 C-Term (Proteintech, red), unlabelled E6 antibody (green) and merge with nuclei (Hoechst, blue) on spinal cord ventral horns of an ALS patient, scale bar=10 µm.
Figure 3. E6 full-length antibody is internalized in culture cells and reduces NF-KB activation and cytoplasmic TDP43 in vitro. (A) Representative western blot showing heavy light chain (50 kDa) form control- or E6 antibodies in total lysates of neuronal N2A cells after CHX (cycloheximide) and 10 μg/ml E6 or control antibody treatments. Total transferred proteins (TTP) were considered as loading reference. Experiment was replicated more than three times. (B) Representative fluorescence microscopy images showing control- or E6-488 conjugated antibodies (green) internalized in the cytoplasm of neuronal N2A cells after 24h treatment. Nuclei are labelled with Hoechst (blue), scale bar=20 µm. Images were acquired with automatic light intensity regulation. Single channel for antibody-conjugated signal is shown in Supplementary figure 2A. (C) NF-KB activation was evaluated in BV2-p65-luc cells by measuring the luciferase activity (RLU, relative luminescence units) after treatment. Data are mean±SEM, n=5-6 replicates from 3 independent experiments (dots) and are expressed as fold of PBS treated, unpaired t-test analysis. (D) Representative western blot and quantification (E) of the overexpressed protein (70 kDa) in the purified cytoplasmic fraction of treated cells, total transferred proteins (TTP) were considered as loading reference. Data are mean±SEM, n=4 independent experiments (dots) and are expressed as fold of cells treated with PBS,
CHX and DMSO. Two-way Anova (interaction F(1,2)=4.496 P=0.0555, treatment F(1,2)=1.032 P=0.3298, Ab treatment F(1,2)=0.0346 P=0.8554). *, p<0.05 by unpaired t-test analysis. (F)

Representative western blot and quantification (G) of the overexpressed protein (70 kDa) in the purified cytoplasmic fraction of treated cells, total transferred proteins (TTP) were considered as loading reference. Data are mean±SEM, n=3 independent experiments (dots) and are expressed as fold of cells treated with PBS and CHX. Two-way Anova (interaction F(1,8)=0.0163 P=0.9014, treatment F(1,8)=4.015 P=0.0800, Ab treatment F(1,8)=4.821 P=0.0594). *, p<0.05 by unpaired t-test analysis. (H,I) Representative merged channels of fluorescence microscopy images showing the co-localization of 488-conjugated antibodies (green) with Tsg101 (red) late endosomal marker (H) or lysotracker (red) (I) in neuronal N2A cells treated 24h with 10 µg/ml of antibodies, scale bar=20 µm. Images were acquired with automatic light intensity regulation. Single channels for the antibodies, Tsg101 or lysotracker signals are shown in Supplementary figure 3F-G. Experiments showed in panels H and I were repeated at least three times. Control antibody (clone 807.33).
Figure 4. Tissue and cellular distribution after intranasal (IN) delivery. (A) Western blot for mIgG2A and actin on total lysate from different brain and spinal cord regions of mice treated intranasally with E6 Ab or equal volume of PBS. (B) E6 Ab distribution in sagittal sections of brain. Mouse PBS treated was sacrificed after 24h whereas E6 Ab treated mouse after 48h. Pictures represent merged signal from mIgG2A (green) and nuclei (Hoechst, blue). 2.5X enlargement is shown. One mouse per condition was used but results were confirmed in multiple sections from the areas of interest.
Figure 5. Tissue and cellular distribution after intraperitoneal (IP) delivery. Western blot for mIgG2A in plasma (A) and PBMC (B) total lysate from mice injected intraperitoneally with E6, CTR Ab or equal volume of PBS. Ponceau was used as loading control. (C) Ab distribution after 3 days from injection in coronal sections of cortex (C), hippocampus (H) and third ventricle (T.V.). Signal represents mIgG2A (green). Scale bar =50 μm. One mouse per condition was used but results were confirmed in multiple sections from the areas of interest. Control antibody (clone 807.33).
Figure 6. Tissue and cellular distribution after intracerebroventricular (ICV) delivery. (A) Western blot for mlG2A in CSF from mice injected intracerebroventricularly with E6, CTR Ab or equal volume of PBS. Ponceau was used as loading control. (B) E6 Ab distribution in coronal sections of cortex (C) and hippocampus (H). Ipsilateral and contralateral sections have been both considered. Signal represents mlG2A (green). Scale bar =50 μm. One mouse per condition was used but results were confirmed in multiple sections from the areas of interest. Control antibody (clone 807.33).
Figure 7

Figure 7. Tissue and cellular distribution after intrathecal (IT) delivery. (A) Western blot for mlgG2A in total lysate from lumbar spinal cord of mice injected intrathecally with E6 or equal volume of PBS. Actin was used as loading control. (B) E6 Ab distribution in coronal sections of lumbar spinal cord. Tissues were analysed from mice after 24h from PBS treatment and 48h after E6 Ab treatment. Signal represents mlgG2A (green). Scale bar=50 µm. (C) Representative images of E6 antibody (mlgG2A staining, green merged with Hoechst, blue) distribution in the lumbar, thoracic and cervical regions of the spinal cord (S.C.) and brain (hippocampus and motor cortex) after 5 weeks of repeated intrathecal injections. Scale bar=50µm. The analysed areas are highlighted in the schematic representation of the central nervous system. Representative pictures for control antibody are shown in Supplementary figure 7C. (D) Representative channels merged images of immunofluorescence for E6 antibody (mlgG2A staining, green), markers for neurons (NeuN, red), microglial cells (CD11B, red), astrocytes (GFAP, red) and oligodendrocytes (OligoSP, red) and nuclei (Hoechst, blue) in ventral horns of lumbar spinal cord after 5 weeks of repeated injections. Harrow heads show co-localization. Scale bar=20µm. Single channels pictures are shown in Supplementary figure 7D. Results in panels B, C and D were performed in three mice per conditions and confirmed in multiple sections from the areas of interest. Control antibody (clone 807.33).
Figure 8. Treatment with E6 antibody reduces cytoplasmic TDP43 and nuclear p65 in lumbar motor neurons. (A) Representative high magnification colorimetric heat map images of TDP43 immunofluorescence. Single channels images are shown in Supplementary figure 8C. Scale bar=10 μm. Harrow heads show cytoplasmic TDP43. (B) Graph represents quantification of nuclear to cytoplasmic integrated density of TDP43 signal in single large neurons counted in ventral horns of mice lumbar spinal cord. Data are mean±SEM, number of counted neurons (dots) from four independent mice (numbered 1-4) is shown in the graph, One-way Anova F11,934=36.98 P<0.001, *** P<0.0001 by Tukey’s multiple comparison test. (C) Representative high magnification colorimetric heat map images of p65 immunofluorescence. Single channels images are shown in Supplementary figure 9A. Scale bar=10 μm. (D) Graph represents quantification of nuclear integrated density of p65 signal in single neurons counted in anterior horns lumbar spinal cord. Data are mean±SEM, number of counted neurons (dots) from four independent mice (numbered 1-4) is shown in the graph, One-way Anova F11,667=36.39 P=0.0011, *** P<0.001 by Tukey’s multiple comparison test. (E) Representative images of immunofluorescence for microglial cells (CD11b, red) merged with nuclei (Hoechst, blue) performed on lumbar spinal cord of treated mice. Scale bar=20 μm. (F) Graphs represent quantification of percentage of area covered by CD11b signal (area fraction). Data are mean±SEM, (n=4 independent mice, dots). Area Fraction One-way Anova
F2,9 = 6.906, P = 0.0151, *P < 0.05 by Tukey’s multiple comparison test. Control antibody (clone 807.33).