Nuclear export inhibitors avert progression in preclinical models of inflammatory demyelination

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Axonal damage has been associated with aberrant protein trafficking. We examined a newly characterized class of compounds that target nucleo-cytoplasmic shuttling by binding to the catalytic groove of the nuclear export protein XPO1 (also known as CRM1, chromosome region maintenance protein 1). Oral administration of reversible CRM1 inhibitors in preclinical murine models of demyelination significantly attenuated disease progression, even when started after the onset of paralysis. Clinical efficacy was associated with decreased proliferation of immune cells, characterized by nuclear accumulation of cell cycle inhibitors, and preservation of cytoskeletal integrity even in demyelinated axons. Neuroprotection was not limited to models of demyelination, but was also observed in another mouse model of axonal damage (that is, kainic acid injection) and detected in cultured neurons after knockdown of Xpo1, the gene encoding CRM1. A proteomic screen for target molecules revealed that CRM1 inhibitors in neurons prevented nuclear export of molecules associated with axonal damage while retaining transcription factors modulating neuroprotection.

Focal axonal damage is a pathological hallmark of neurodegenerative disorders such as Alzheimer’s disease, amyotrophic lateral sclerosis and multiple sclerosis¹⁄². It is characterized by cytoskeletal disruption and impaired axonal transport, resulting in localized axonal swellings¹³. Dysregulated nucleo-cytoplasmic shuttling is one of the mechanisms hypothesized to contribute to the pathogenesis of this localized damage in several neurological disorders⁴⁄⁶. For this reason, we tested whether targeting nuclear transport could be considered a venue of therapeutic intervention with potentially broad implications for neurodegenerative diseases. We previously identified exportin 1 (XPO1, CRM1)-mediated nuclear export as being necessary for focal axonal damage in multiple sclerosis (MS)⁴. CRM1 is a member of the nuclear export family of proteins that recognizes cargoes containing leucine-rich nuclear export sequences² and transports them from the nucleus to the cytoplasm⁸–¹⁰. Neurotoxicity has been associated with the accumulation in the axoplasm of molecules interfering with axonal cytoskeleton such as TAU¹¹ and RNA transport such as those present in the cytoplasm at the lesion border, gray matter, the presence of phagocytes containing MBP-positive myelin degradation products in the cytoplasm at the lesion border, and the presence of numerous foamy phagocytes expressing CD68 (Fig. 1a). In the adjacent demyelinated gray matter, numerous NeuN⁺ neurons were present, some of them with peri-somatic neurofilament immunopositivity, which was suggestive of neuronal damage.

We also examined their beneficial therapeutic role in two preclinical models of axonal damage in the context of autoimmune demyelination and in a model of neurotoxicity following kainic acid injection. Finally, using a proteomic approach, we identified nuclear retention of protein targets that have been implicated in the pathogenesis of other neurological disorders, thereby suggesting the CRM1 inhibitors as potential candidates for the development of therapeutic strategies broadly targeting neuroprotection.

RESULTS
CRM1 protein levels are upregulated in human MS brains
Previous data from our lab revealed aberrant localization of nuclear protein in axons characterized by focal axonal damage in the brains of individuals with MS⁴. An important regulator of nucleo/cytoplasmic shuttling is CRM1 (refs. 8–10). We therefore sought to define the pattern of expression of CRM1 by immunohistochemistry of leukocortical MS brain lesions. Actively demyelinating lesions were defined on the basis of the reduced immunoreactivity to antibodies specific for myelin basic protein (MBP) in both the white and gray matter, the presence of phagocytes containing MBP-positive myelin degradation products in the cytoplasm at the lesion border, and the presence of numerous foamy phagocytes expressing CD68 (Fig. 1a). In the adjacent demyelinated gray matter, numerous NeuN⁺ neurons were present, some of them with peri-somatic neurofilament immunopositivity, which was suggestive of neuronal damage.
Figure 1 Expression of CRM1 in MS gray and white matter lesion areas. All pictures are derived from the same leukocortical MS lesion. (a) Immunohistochemical staining of a leukocortical MS lesion, which was characterized by staining for MBP for myelin, NeuN for neurons, and CD68 for monocytes and microglia. The dotted line indicates the border between gray and white matter. Note the loss of myelin and the presence of myelin-laden phagocytes at the edges of the lesion (inset, scale bar represents 25 µm). CD68+ cells were detected in gray and white matter, with higher numbers being present in the white matter part of the lesion. (b) Left, perivascular infiltrate in the white matter part of the lesion. Arrow indicates the numerous CD68+ cells in the infiltrate as well as in the parenchyma. Damaged neurons were identified by somatic staining for neurofilament. (c) The fluorescent images show at higher magnification the large inflammatory infiltrate located at the border between white and gray matter indicated by an arrow in b. Numerous CD45+ leukocytes (green) expressed CRM1 (red). Nuclei were counterstained with DAPI (blue). Scale bar represents 100 µm. (d–e) Low- and high-magnification confocal images of lesional areas of MS gray matter and normal-appearing gray matter stained for NeuN (green) to identify neurons and CRM1 (red). Scale bars represent 40 µm (d) and 15 µm (e). (f) Western blot analysis of protein extracts of post-mortem human gray matter tissue prepared from non-neurological controls (n = 5) and MS patients (n = 8), which were probed with antibodies specific for CRM1. ACTIN was used as a loading control. Blots were cropped and full-length images are presented in Supplementary Figure 11.

Characterization of inhibitors of CRM1

Of several molecules targeting the catalytic groove of CRM1, KPT-276 and KPT-350 were two inhibitors, structurally defined by a difluoroazetidinepropenonenyl group, that substantially differed from other published inhibitors, including leptomycin B9,20. Both KPT-276 and KPT-350 shared a trifluoromethyl phenyl triazole scaffold (Figure 2a), with previously reported compounds KPT-185 (ref. 21) and KPT-251 (ref. 22) (Supplementary Fig. 1a–c). In vitro, KPT-276 was capable of disrupting Ran-dependent CRM1 binding to the nuclear export sequence (NES) of the protein kinase A inhibitor protein (PKI; Fig. 2b), a bona fide NES-containing CRM1 target. The 1.8-Å resolution crystal structure of KPT-276 bound to CRM1 revealed covalent conjugation of

Figure 2 KPTs selectively and covalently bind CRM1 and inhibit binding to NES with pharmacokinetic properties that favor blood brain barrier permeability. (a) Chemical structure of KPT-276 and KPT-350. (b) Inhibition of CRM1-NES binding by KPT-276 resulting from direct blockade of the NES binding groove. Pull-down assay of ~ 15 µg of 10 µM H2CRM1 binding to either immobilized GST or GST-PKINES in the presence of RanGTP and either buffer or 100 µM KPT-276. (c) The 1.8-Å resolution crystal structure of KPT-276 bound to CRM1 showing KPT-276 binding in the NES-binding groove. (d) Magnified view of KPT-276 bound to CRM1 showing interactions between the inhibitor and CRM1 with the composite omit map of the inhibitor shown as a green mesh. (e) Pharmacological properties of KPT-276 and KPT-350, including molecular weight (MW), calculated logarithm of partition coefficient (clogP), topological polar surface area (tPSA) and molecular formula. (f) Pharmacokinetic properties of orally gavaged KPT-276 and KPT-350 in Sprague-Dawley rats.
the inhibitor to the reactive cysteine residue in the NES-binding groove of CRM1 and a binding mode similar to that of KPT-185 and KPT-251 (Cys359 in a modified yeast CRM1; Fig. 2c,d and Supplementary Table 2). KPT-276 had a molecular weight of 426.27 g mol⁻¹, a partition coefficient of 4.44 and a topological polar surface area of 48.27 (Fig. 2e). KPT-350, a related and more potent CRM1 inhibitor, was characterized by a molecular weight of 449.35 g mol⁻¹, a partition coefficient of 3.48 and a topological polar surface area of 86.16. The two inhibitors were designed for oral administration and have the ability to cross the blood-brain barrier with different partition coefficients between blood plasma and the parenchyma, as determined by pharmacokinetic measurements in rats (Fig. 2f). When we tested them against an extensive panel of 150 different kinases and phosphatases, we found no binding (data not shown), further supporting a direct effect of the newly synthesized inhibitors on CRM1, rather than on off-targets. Crystal structures of CRM1 bound to KPT-276 or to previously reported inhibitors (KPT-185 and KPT-251) further showed the specificity of the difluoroazetidinepropenonenyl group for the catalytic binding pocket of the CRM1 protein (Supplementary Fig. 1d–i). To begin characterizing the potential of antagonizing CRM1 function for treating demyelinating disorders, we first characterized Xpo1 expression levels in multiple cell types in the CNS and in immune cells of the periphery (Supplementary Fig. 2a,b), which revealed a ubiquitous expression. Another important feature of CRM1 inhibitors was the low cytotoxicity in post-mitotic cells. Evaluation of survival using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) mitochondrial reductase activity assay in cultured neurons derived from the spinal cord or cortex, or in mature oligodendrocytes, astrocytes or splenocytes did not reveal any toxicity at a concentration range between 0.1 and 1,000 nM (Supplementary Fig. 2c–h). The only exception was proliferating oligodendrocyte progenitor cells, which were sensitive to high dose of the compounds, far above the therapeutic range.

**CRM1 inhibitors decrease the severity of EAE**

To test the translational value of the newly synthesized CRM inhibitors, we first used a widely accepted preclinical model of demyelination, experimental autoimmune encephalomyelitis (EAE), which shares many pathological hallmarks of MS, including immune cell activation and CNS infiltration, demyelination and axonal damage23. To ascertain the potential for therapeutic application of the CRM1 inhibitors, we designed a double-blind experiment in which treatment started after mice developed hindlimb paralysis (EAE clinical score of 2.5), which on average occurred 16 d after immunization (Fig. 3a and Supplementary Video 1). Mice were gavaged every other day with either vehicle or the CRM1 inhibitors KPT-276 (75 mg per kg of body weight) or KPT-350 (7.5 mg per kg) at doses that were consistent with their in vitro binding affinity and well below the maximum tolerated dose defined in toxicology studies (data not shown). No overt signs of toxicity were detected in the different treatment groups, as we did not detect increased mortality or adverse effect on weight (Supplementary Fig. 3a) or body condition (Supplementary Fig. 3b) in the treated mice compared with controls. The therapeutic efficacy of CRM1 inhibitors was demonstrated by their ability to decrease clinical progression, in treated mice compared with vehicle-treated controls. Although the vehicle-treated mice progressed to full quadriplegia (Supplementary Video 2), the motor signs of mice treated with KPT-276 (Supplementary Video 3) and KPT-350 (Supplementary Video 4) substantially improved over time, as reflected by the decrease in overall cumulative disease score in the KPT-276 (60 ± 4.72%) and KPT-350 (75 ± 4.32%) groups (Fig. 3b). When toluidine blue sections were analyzed at drug start time, which corresponded to a clinical score of 2.5 in the EAE model, we observed limited areas of myelin
damage, as measured by Fluoromyelin (Supplementary Fig. 3c,d), localized to areas with immune cell infiltrates (Fig. 3c). Treatment of mice with the CRM1 inhibitors KPT-276 and KPT-350 largely preserved both myelinated and demyelinated axons in the lumbo-sacral area of the spinal cord (Fig. 3d), and this was in sharp contrast with vehicle-treated controls, whose spinal cords were characterized by large areas of axonal destruction, as clearly detected by a three-dimensional serial electron microscopic analysis (Fig. 3d).

To further determine whether the presence of a higher number of myelinated fibers in mice treated with the CRM1 inhibitors could

Figure 4 Therapeutic treatment of EAE mice with KPT reduces inflammatory burden in the spinal cord and impairs CD4+ T cell proliferative capacity, but does not affect their ability to produce cytokines. (a) Histopathological characterization of inflammatory infiltrates, as detected in hematoxylin- and eosin-stained coronal sections from lumbar spinal cord of mice at treatment start time (day 16), and end (day 28); dotted lines delineate inflammatory lesions (scale bar represents 1 mm). (b) Graphs representing the number of infiltrates in a, n = 3 mice per condition, 5 sections per mouse from 2 independent experiments. (c) Representative FACS plots of CD4+, CD8+ B220+ or CD11b+ cells in the spinal cord of mice treated with therapeutic vehicle, KPT-276 or KPT-350. Numbers in FACS plots indicate the percentage in the live cell gate. (d) Quantification of the number of cells positive for CD45low among splenic CD4+ T cells from therapeutic treated vehicle and KPT-350 mice and quantification. Numbers in FACS plots indicate the percentage in the CD4+ gate (e, n = 3 mice per group, representative of two biological replicate). (f) Relative transcript levels of cytokines in the spleen of mice treated with either vehicle, KPT-276 or KPT-350, measured by quantitative PCR (qPCR) and normalized to 18S rRNA (n = 8 per condition, from two independent experiments). (g) Relative transcript levels of cytokines in spinal cords of mice treated with either vehicle, KPT-276 or KPT-350, measured by quantitative PCR (qPCR) and normalized to 18S rRNA (n = 8 per condition, from two independent experiments). (h) Quantification by flow cytometry of splenic populations in vehicle-, KPT-276- or KPT-350-treated mice. Monocytes were identified as CD11c+ CD45high and monocytes as CD11b+ CD45high (n = 3–4 mice per condition). (i) Relative transcript levels of cytokines in the spleen of mice treated with either vehicle, KPT-276 or KPT-350, measured by qPCR and normalized to 18S rRNA (n = 3 per condition). (j) Representative example of IFNγ and IL17a intracellular staining among splenic CD4+ T cells from therapeutic treated vehicle and KPT-350 mice and quantification. Numbers in FACS plots indicate the percentage in the CD4+ gate (n = 4 mice per group, representative of two biological replicate). (k) Quantification of CD4+ T cell proliferation in cells treated with either vehicle or KPT-350 and stimulated with antibodies to CD3 and CD28 in the presence or absence of IL-2 for 48 h (–IL-2, P = 0.001; +IL2, P = 0.0001). (l) Nuclear accumulation of the cell cycle inhibitor p27Kip1 (red) in CD4+ splenocytes acutely isolated from vehicle- and KPT-350–treated EAE animals, cells were counterstained with DAPI (blue). Scale bars represent 3 μm. All graphs represent mean ± s.e.m. Statistical differences in b, d and f were determined using ANOVA with Dunnett’s correction (*P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle-treated animals). Statistical differences in j and k were determined using independent t tests with Bonferroni correction (P < 0.001).
be the result of enhanced regenerative processes induced in oligodendrocyte lineage cells, we performed immunohistochemistry with antibodies specific for NG2 to label progenitors and CC1 to label mature oligodendrocytes. We then quantified the number of progenitors and mature oligodendrocytes outside the lesion areas in KPT-276- and in KPT-350–treated mice compared with controls and did not detect any change in cell numbers (Fig. 3e) or myelin gene transcripts (Supplementary Fig. 4a), which was consistent with the similar number of remyelinated axons counted in semithin spinal cord sections from mice treated with CRM1 inhibitors compared with vehicle controls (Fig. 3f). To further evaluate cell-specific direct effects of CRM1 inhibitors on oligodendrocyte lineage cells, we treated cultured oligodendrocytes with KPT-276 or KPT-350 and assessed proliferation (Supplementary Fig. 4d) and differentiation (Supplementary Fig. 4b,c) using immunocytochemistry, which did not reveal any difference between treatment and control groups. These data suggest that the effect of CRM1 inhibitors in EAE could not be attributed to a direct effect on oligodendrocyte lineage cells.

**CRM1 inhibitors reduce the inflammatory burden of the CNS**

To define the mechanisms underlying the positive effect of the compounds in preventing clinical progression in EAE, we stained the lumbosacral cord with hematoxylin and eosin. The start point of treatment was characterized by the presence of several inflammatory infiltrates in the spinal cord (Fig. 4a). Analysis after 12 d of treatment revealed fewer inflammatory lesions in the lumbosacral spinal cord of mice treated with the CRM1 inhibitors compared with controls (Fig. 4b). Fluorescence-activated cell sorting (FACS) analysis of immune cells from the spinal cord at the end of treatment (Fig. 4c) confirmed a reduction in the numbers of CD45+ leukocytes (P < 0.05 for KPT-276, P < 0.01 for KPT-350), B220+ B cells (P < 0.05), and CD4+ and CD8+ T cells (P < 0.05 for KPT-276,
Figure 6  KPT inhibitors prevent locomotion decline and partially restore motor function in a localized EAE mouse model. (a) Schematic diagram of focal EAE model used to assess locomotor activity using the Basso Motor Scale (BMS). Locomotion of focal EAE mice were assessed daily using BMS; arrow indicates treatment window and asterisks indicate dosing regimen (n = 3 mice for drug start, n = 11 for vehicle, n = 12 for KPT-350), from three independent experiments. (b) Scatter plots depicting quantification of cumulative BMS score from a (**P = 0.0127, independent t test). (c) Representative coronal spinal cord sections from focal EAE mice were stained with FluoroMyelin (green). Scale bar represents 200 µm. (d) Quantification of FluoroMyelin intensity from focal EAE animals. (e) Representative confocal images of axonal damage were assessed in coronal sections from focal EAE mice by staining with SMI-32 (green) and NFH (red). Scale bar represents 200 µm. (f) Quantification of SMI-32 intensity from e. (g) IBA1+ (green) stained sections from EAE spinal cord; sections were counterstained with DAPI (blue). Scale bar represents 200 µm. (h) Quantification of IBA1+ microglia from confocal sections in g, n = 3 animals per treatment group. Bar graphs represent mean ± s.e.m. Statistical differences were determined in f by one-way ANOVA with Tukey’s correction (**P < 0.01); d and h by one-way ANOVA with Dunnett’s correction (***P < 0.001 versus drug start).

**P < 0.01 for KPT-350) in treated mice compared with vehicle-treated controls (Fig. 4d). This number was also lower than that at treatment start (Fig. 4d). Microglial cells were also reduced in treated mice compared with controls (Fig. 4d-f). The reduced inflammatory burden in the CNS resulted in decreased cytokine messenger RNA levels (Il6, Tgfb1, Il10, Il17a, Tnf, Ifng Fig. 4g). Treatment with CRM1 inhibitors also affected the peripheral immune system (Fig. 4h), as shown by the overall decrease of total splenocytes (Supplementary

Figure 7  Treatment of cultured neurons with KPT-350 or knockdown of CRM1 prevents the induction of focal axonal damage and prevents damage in models independent of inflammation. (a) Representative low- and high-magnification images of rat hippocampal neurons treated with glutamate (50 µM) and TNFα (200 ng mL⁻¹) for 4 h and stained with neurofilament NFH (red) to identify neuronal processes and SMI-32 (green) to detect damaged neurites with localized swellings. Scale bars represent 25 µm (left) and 5 µm (right). (b) Quantification of focal axonal damage expressed as percentage of neuronal processes with beading as shown in a, n = 10 neurites per field, 10 fields per experiment, three independent biological replicates. (c) Representative confocal images of neurons infected with either control or Xpo1 knockdown lentiviral particles. Xpo1 knockdown was confirmed by staining for CRM1 (blue). Focal axonal damage was evaluated by staining neuronal cultures with neurofilament NFH (red) to detect neuronal processes and SMI-32 (green) to detect damaged neurites in neuronal cultures treated kainic acid. Scale bars represent 25 µm (left) and 5 µm (right). (d) Representative still images of mitochondria from videos of cultured neurons stained with the live MitoTracker Green FM dye. Scale bar represents 25 µm. (e) Quantification of mitochondrial velocity from d, n ≥ 10 mitochondria per condition in three independent biological replicates. (f) Quantification of mitochondrial length from d, n = 150 mitochondria per condition, from three independent experiments. Bar graphs represent mean pixel intensity ± s.e.m. Statistical significance was determined in b using one-way ANOVA with Tukey’s test (***P < 0.001 versus Glut + TNFα); in e and f using one-way ANOVA with Dunnett’s correction (**P < 0.05, ***P < 0.001 versus control).
Figure 8 Proteomic screen of neurons treated with KPT-350 led to the identification of target molecules explaining the neuroprotective effect. (a) Schematic diagram of the methodology used to determine the mechanism of neuroprotection induced by KPT molecules. The Venn diagrams represents the cytoplasmic molecules identified from the proteomics analysis of neurons treated with vehicle, with glutamate (Glut) + TNFα or KPT-350 + glutamate + TNFα. The table lists identified protein targets that contain predicted NESs that were not enriched in the cytoplasmic compartment following treatment with glutamate + TNFα + KPT-350. The predicted NES was determined using NetNES 1.1 Server. (b) Schematic diagram of neuronal culture system used to validate proteomics targets. Validation of TAU and NRF2 as targets in cultured KPT-350 treated neurons. Western blot analysis of cytosolic (c) and nuclear (n) protein extracts from cultured cortical neurons treated with glutamate + TNFα or KPT-350 + glutamate + TNFα. Antibodies for MEK1/2 and HISTONE H3 were used as internal controls for cytosolic and nuclear proteins, respectively (n = 3 samples per condition, two independent experiments). Blots were cropped and full length images are presented in Supplementary Figure 12. (c) Quantification of the results in nuclear extracts from the experiments described in b (P = 0.0341 for TAU and P = 0.0213 for NRF2, independent t tests with Bonferroni correction, versus vehicle). (d) Schematic diagram of system used to validate nuclear retained targets in EAE mice. Validation of the KPT-350 targets in spinal cord extracts from treated mice. Western blots of cytosolic (c) and nuclear (n) protein extracts from the spinal cords of mice harvested after treatment with vehicle or KPT-350. Blots were probed with antibodies specific for TAU, FUS and NRF2. MEK1/2 and HISTONE H3 were used both as loading controls and to verify correct cytosolic and nuclear fractionation, respectively (n = 3 samples per condition, two independent experiments). Blots were cropped and full length images are presented in Supplementary Figure 13. (e) Quantification of western blots of nuclear extracts as described in d (P = 0.0413 for TAU and P = 0.0257 for NRF2, independent t tests with Bonferroni correction, versus vehicle). (f) Representative confocal images of EAE lumbar spinal cords from mice at treatment onset (drug start) or treated for 12 d with vehicle or KPT-350. Sections were stained with antibodies for NRF2 (green) and NeuN (red). Scale bar represents 20 µm, n = 3 fields per animal in 12 mice from two independent experiments. (g) Quantification of the number of NeuN+ NRF2+ cells per field; n = 10 fields per animal, n = 6 mice per group in two independent experiments. (h) Transcript levels for Nfe2I2 and the NRF2 target genes Nqo1 and Gclc (n = 8 RNA samples per condition from two independent experiments. **P = 0.0010 for Nqo1 and ***P = 0.0021 for Gclc). Bar graphs represent mean ± s.e.m. Statistical differences in g and h were determined using independent t tests, with Bonferroni correction (**P < 0.01 versus vehicle).

Fig. 5a,b) and of B, T cells, monocytes, neutrophils and dendritic cell populations (Fig. 4h and Supplementary Fig. 5c). However, treatment with CRM1 inhibitors did not impair the ability of splenocytes to express cytokines (Fig. 4i), as shown by the ability of splenocytes acutely isolated from treated mice to make IFNγ and IL17a (Fig. 4j). Thus, immune cells remained functional after CRM1 inhibition, and the overall decrease of distinct cell populations in the spleen and spinal cord was the consequence of decreased proliferation (Fig. 4k), associated with a fourfold increase (P = 0.0003, data not shown) in nuclear accumulation of cell cycle inhibitory molecules (for example, p27Kip1; Fig. 4l), and possibly by the induction of G1/S cell cycle arrest24. Thus, the immunomodulatory effect of the CRM1 inhibitors was mediated by an effect on cell proliferation rather than a toxic effect on immune cells.

To further demonstrate the immunomodulatory role of the CRM1 inhibitors, we tested whether prophylactic treatment of EAE mice at the time of immunization would prevent disease onset. Consistent with the predicted outcome, both disease onset and severity (Supplementary Fig. 6a,b) were decreased in treated mice compared with controls. No effect was observed on mouse weight (Supplementary Fig. 6c) or body condition (Supplementary Fig. 6d), and immunophenotypic profiling of the spinal cord of treated mice revealed a reduction of B220+ B cells, and CD4+ and CD8+ T cells without altered microglia or monocyte numbers (Supplementary Fig. 6e). In addition, we detected an effect of treatment on the total number of splenocytes (Supplementary Fig. 6f) and reductions in splenic cell populations (Supplementary Fig. 6g). Together, these data suggest that CRM1 inhibitors have a non-toxic reversible effect on immune cell number in the periphery. To confirm the lack of cytotoxicity, we performed a washout experiment of CRM1 inhibitors in naive mice and detected a recovery of the number of splenocytes (Supplementary Fig. 7a,b), thereby supporting the reversibility of CRM1 inhibition and the safety of treatment with the compounds.

CRM1 inhibitor treatment preserves axonal integrity
In addition to the immunomodulatory role of CRM1 inhibitors, we asked whether the block of clinical progression after treatment could also be attributed to a direct neuroprotective effect. At the treatment start point, when mice exhibited hindlimb paralysis, detectable, but modest, axonal damage could be detected in association

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with inflammatory infiltrates (Fig. 5a,b). After 12 d, however, spinal cord sections from vehicle-treated control mice were characterized by extensive axonal damage, whereas sections from mice treated with CRM1 inhibitors displayed the same levels of damage as at the start time point (Fig. 5a,b). Although massive areas of neuronal loss and severely damaged axons with extensive cytoskeletal disruption and localized enlargements were detected in vehicle-treated spinal cords (Fig. 5c), the cytoskeletal integrity and axonal diameter were well preserved in mice treated with CRM1 inhibitors, despite myelin loss or myelin damage (Fig. 5c–f). Quantification of these findings revealed fewer axons with Wallerian degeneration (Fig. 5d) and an overall preservation of axons per unit area in the lumbosacral spinal cord of treated mice (Fig. 5e). These morphological data were also supported by higher transcript levels of neurofilament heavy chain (Neftl) in treated mice compared with controls (Supplementary Fig. 8a). Together, these results show a robust effect of KPT-350 treatment in preserving axonal integrity regardless of the presence or absence of myelin, thereby suggesting a direct neuroprotective effect of CRM1 inhibitor treatment. We therefore asked whether CRM1 inhibitors could also be protective in other models of direct axonal damage, independent of immune cell activation. We selected a model of axonal damage consequent to excitotoxicity and injected mice with kainic acid, a non–degradable analog of glutamate that induces excitotoxic death of pyramidal neurons in the CA3 region of the hippocampus. Subcutaneous administration of kainic acid (2 mg per kg) was associated with the detection of axonal damage in the CA3 hippocampal region of control mice, but not in mice that were treated with CRM1 inhibitors (Fig. 5g,h). To further ascertain that the protective effect of CRM1 inhibitor treatment resulted from a direct action in neuronal cells rather than a potential modulation of microglial numbers, we also stained sections for microglial markers that revealed a similar number of cells (Fig. 5i,j). Direct neuroprotection was then assessed in hippocampal slice cultures, which preserve the cytoarchitecture of the hippocampus. Treatment of slice cultures with kainic acid (5 µM) for 18 h resulted in extensive axonal damage in the CA3 region that was clearly detected by SMI-32 immunoreactivity (Supplementary Fig. 9a). The same experiment conducted in the presence of CRM1 inhibitors, however, resulted in axonal protection and reduced SMI-32 immunoreactivity (Supplementary Fig. 9b). Thus, CRM1 inhibitors act as broad neuroprotective agents and prevent axonal damage induced by excitotoxicity.

To more carefully evaluate the effect of CRM1 inhibitors on mouse locomotion, we adopted a model of localized inflammatory demyelination targeting the dorsal column of the sciatic spinal cord and monitored hind paw position and coordination in mice using the Basso Motor Scale (BMS). Briefly, mice were sensitized to MOG and localized lesions in the dorsal columns were obtained by local injection of TNFα and IFNγ in the sciatic spinal cord by adapting published protocols. This procedure induced a steady decline in motor function that could be quantified. To test the effectiveness of CRM1 inhibition in this model, mice were administered either vehicle or KPT-350 (7.5 mg per kg) when they displayed a BMS score of 5, which was characterized by lack of coordination of the hindlimbs and external rotation of the hind paws. Daily monitoring in an open field platform was performed in a double-blinded fashion. Although vehicle-treated mice progressed to almost full disability, KPT-350–treated mice stopped the progression of the motor dysfunction and partially recovered motor movement (Fig. 6a), as indicated by statistically significant differences in the cumulative BMS score (P = 0.0127; Fig. 6b). The improved motor function was not associated with any detectable changes in the level of FluoroMyelin staining (Fig. 6c,d), but with clear differences in the number of damaged axons (Fig. 6e,f) and microglial numbers (Fig. 6g,h). Thus, also in a localized model of localized axonal damage, CRM1 inhibitors were effective in preventing clinical disease progression and retaining motor function.

**Viral knockdown of Xpo1 protects from axonal damage**

To further evaluate the direct neuroprotective effect of CRM1 inhibition, we also used cultured neurons treated with cytokine and excitatory amino acids to induce focal axonal damage, as revealed by the presence of localized swellings co-stained with neurofilament H and the antibody SMI-32, which recognizes damaged axons (Fig. 7a). A statistically significant reduction in the number of damaged neuronal processes was detected in KPT-350–treated cultures (x = 79 ± 1.09%, n = 60, P < 0.001) compared with controls (Fig. 7b). Viral knockdown of Xpo1 (Fig. 7c) was obtained using lentiviral vectors, which reduced the relative nuclear CRM1 intensity to 36 ± 3% of the control value (P = 0.0034, data not shown). The SMI-32 immunoreactivity in cultures with reduced CRM1 levels was also reduced to 38 ± 16% (P = 0.0231, data not shown) of that of neuronal cultures exposed to the excitotoxin. These results suggest that reduction of CRM1 protects neurons from kainic acid–induced excitotoxic damage (Fig. 7c). The similarity of the neuroprotective effect of pharmacological inhibition and viral knockdown of Xpo1 in neurons further supports a direct neuroprotective role of CRM1 inhibition.

Additional support for the protective effect of CRM1 inhibitor treatment on axonal damage was the evidence of rescued mitochondrial motility in models of neurotoxicity, as shown by live microscopy of fluorescently labeled mitochondria using MitoTracker Green FM dye (Fig. 7d). Treatment of neurons with glutamate and TNFα significantly reduced mitochondrial speed from 7.2 ± 1.6 to 3.0 ± 0.6 µm s−1 (P < 0.05; Fig. 7e), an effect that was also associated with decreased mitochondrial length (Fig. 7f), and both events were blocked by treatment with the CRM1 inhibitor KPT-350 (P > 0.05 versus control). Because altered mitochondrial dynamics is often associated with impaired function, we also measured the oxygen consumption rate using the Seahorse Bioanalyzer in neurons treated with glutamate and TNFα in the presence or absence of CRM1 inhibitors. Glutamate and TNFα treatment induced a reduction of neuronal mitochondria spare respiratory capacity (x = 24 ± 4.14%, n = 12 cultures, P < 0.05 versus control), which was not detected in neurons treated with the CRM1 inhibitors (Supplementary Fig. 10a,b). Given that decreased mitochondrial function is hallmark of axonal damage in MS, these data further support the importance of CRM1 inhibitors as therapeutic agents preventing clinical progression.

**Molecular targets of CRM1 inhibition in vitro and in vivo**

To define the molecular mechanism underlying the neuroprotective effect of KPT350 in cultured neurons, we used mass spectrometry to analyze molecules with cytosolic accumulation in neurons treated with glutamate and TNFα in the absence of the KPT compound, but not in its presence (Fig. 8a). We reasoned that these molecules must be targets of CRM1 nuclear export in pathological conditions and that they also contained nuclear export signals. Among the identified molecules that we detected were TAU and amyloid precursor protein, which have been associated with Alzheimer’s disease, FUS and TDP-43, which are associated with axonal damage in amyotrophic lateral sclerosis (ALS), and NRF2, a transcription factor modulating the expression of genes involved in the response to oxidative stress in several pathologies, including MS. To further validate the subcellular localization of these molecules in our experimental system, we performed
western blots of cytosolic and nuclear fractions prepared from cultured neurons exposed to glutamate and TNFα with and without KPT-350 (Fig. 8b). Immunoblot analysis confirmed the nuclear retention of TAU and NRF2 in protected neurons (Fig. 8c)17,18.

Similar results were also detected in vivo, in the spinal cords of vehicle and KPT-350–treated mice (Fig. 8d). These experiments further supported the nuclear retention of TAU and NRF2 in neurons in the spinal cord of KPT-treated mice (Fig. 8e) compared with controls, a finding which was also confirmed by immunohistochemistry (Fig. 8f).

The number of NeuN+ neurons with nuclear NRF2 was significantly increased in KPT-350-treated spinal cords compared with controls (P < 0.01 versus vehicle; Fig. 8g). The transcript levels of NRF2 target genes such as Nqo1 (nicotinamide adenine dinucleotide phosphate dehydrogenase (quinone 1), encoding a protein involved in detoxification of cytotoxic quinones, and Gdc (glutamate-cysteine ligase catalytic subunit), encoding an enzyme involved in the biosynthesis of glutathione, were also increased in the KPT-treated mice (Fig. 8h), suggesting an overall enhancement of the antioxidant response. Combined, these data suggest that CRM1 inhibitors halt clinical disease progression in preclinical models of demyelination by a dual mechanism that targets both immune cell function and neuroprotection.

DISCUSSION

Aberrant nuclear export has been reported in a large number of pathologies associated with axonal damage, including traumatic brain injury29, Alzheimer’s disease30, ALS31,32 and MS4. We previously proposed that cyttoplasmic localization of molecules that, in physiological conditions, are localized in the nucleus results in aberrant function and axonal damage in MS33. However, nucleo-cyttoplasmic shuttling is impaired in several neurodegenerative diseases characterized by axonal damage. For instance, in Alzheimer’s disease, the nuclear export of cell cycle inhibitors in neurons has been implicated in aberrant cell cycle re-entry (reviewed in ref. 34). Export of TAU, a microtubule binding protein, has been associated with axonal damage when it accumulates in the cytoplasm and exerts a neuroprotective function when localized in the nucleus35,36. In MS brains and in models of axonal damage associated with exposure to cytokines and excitotoxins, nuclear export of HDAC1 into the axoplasm has been shown to interfere with mitochondrial movement, by sequestration of motor proteins4. Nuclear localization of the transcription factor NRF2 has been shown to be important for transcription of genes involved in anti-oxidant defense and its activation is neuroprotective. Nuclear export of NRF2 in cerebellar neurons has been associated with impaired response to oxidative stress15–18. Taken together, these studies suggest that neurodegeneration and axonal damage are correlated with mis-localization of nuclear molecules to the cytoplasm, whereas protection is favored by their retention in the nuclear compartment, which leads to the hypothesis that targeting nuclear export could be effective for reducing axonal damage.

Nuclear export is a highly regulated cellular function mediated by two large families of proteins: the importins (16 family members), which are involved in the transport from the cytosol to the nucleus of cells, and the exportins (6 family members), which are responsible for the selective export of molecules from the nucleus to the cytosol37,38. The exportin family includes several members with distinct recognition domains and substrate specificity. Among the family members, CRM1, which recognizes leucine-rich motifs in proteins, has been associated with the occurrence of axonal damage4,29. This molecule is upregulated in models of traumatic brain injury and we found that it was upregulated in neurons in MS lesions, which is consistent with aberrant nuclear export detected in these conditions. Furthermore, CRM1-dependent transport has been shown to modulate subcellular localization of NRF2 (ref. 18). We also found that NRF2 and TAU were retained in the nucleus in neurons as neuroprotective mechanisms associated with CRM1 inhibition. We examined the pre-clinical efficacy of a previously unknown class of pharmacological compounds targeting CRM1 in three distinct animal models: MOG-induced EAE, localized inflammatory demyelination of dorsal columns in the spinal cord and a mouse model of excitotoxicity induced by kainate injection.

Neuroprotection resulting from CRM1–mediated inhibition of axonal damage was associated with a dual targeting of immune and neuronal cells. In neurons, inhibition of CRM1–mediated nuclear export resulted in improved mitochondrial transport and function possibly resulting from nuclear retention of ‘pro-noxious’ molecules (for example, TAU), and consequent preservation of cytoskeletal integrity. Inhibition of nuclear export also favored accumulation of ‘protective’ molecule such as transcription factors involved in modulation of the anti-oxidant response (for example, NRF2), thereby increasing the protective response of neurons to oxidative stress. In immune cells, CRM1 inhibitors decreased proliferation of immune cells by favoring nuclear accumulation of molecules involved in cell cycle arrest and thereby decreasing the inflammatory burden31,32. Treatment was not cytotoxic on immune cells, as their number was rapidly restored following washout of the drug and immune cells retained the ability to produce cytokines. Thus, inhibition of CRM1–mediated nuclear export favored neuroprotection by acting both on neuroinflammation and on axonal transport disruption and neuronal defense against oxidative stress.

Several studies have focused on potential causes of axonal damage in MS that are caused by mechanisms independent of peripheral immune cell infiltration and the subsequent cytotoxicity that is characteristic of inflammatory demyelination39. These other signals, which are also shared with several neurodegenerative diseases, include resident microglia activation40, energetic failure of neurons resulting from mitochondrial functional impairment41, and mitochondrial fragmentation/movement defects42 and the accumulation in the cytoplasm of nuclear proteins. Our data indicate that KPT compounds provide neuroprotection by targeting multiple causes of axonal damage. The KPT inhibitors protected axons by directly targeting neuronal CRM1 and indirectly by reducing inflammation.

Many of the current available therapies for MS target either peripheral immune cell function42 or their ability to cross into the brain parenchyma; however, these compounds have not been able to stop the progression of axonal damage despite immunomodulatory intervention43. Advancing other therapies that target also other components of MS pathophysiology has proven to be more challenging. For example, targeting microglial activation has been shown to have both detrimental and beneficial roles in axonal physiology44. Remyelination strategies have been recently proposed as providing neuroprotective effects on myelinated axons. Our study provides a new approach that is both immunomodulatory and neuroprotective. The eventual combination with remyelination therapies and/or symptomatic therapies may prove useful to address the current multiple therapeutic needs of multiple sclerosis patients. CRM1 inhibitors are also orally bioavailable and cross the blood brain barrier, and additional safety and efficacy studies will be needed to test its utility for patients who require long-term therapy options.

METHODS

Methods and any associated references are available in the online version of the paper.
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AUTHOR CONTRIBUTIONS
P.C., S.S., J.D.H. and D.M. were responsible for overall analysis and study design. In vivo and in vitro experiments were performed by J.D.H., O.H., B.d.l.H., O.G.V. and G.A.M. Q.S., H.Y.F.J. and Y.M.C. crystallized CRM1 bound to SIN1 and performed gel shift assays. S.A. and T.K. performed the human brain immunohistochemistry experiments. G.J.K. helped analyze and interpret the three-dimensional electron microscopy results. Immunology experiments were conceived and interpreted by O.H. and K.A. Pharmacokinetic experiments and characterization of drug properties were performed by D.M. and S.S. The paper was written by J.D.H. and P.C.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.
ONLINE METHODS

Animals. All animal experiments were approved by the Icahn School of Medicine at Mount Sinai Animal Care Committee. All mice were obtained from Jackson Laboratories, and rats from Charles River Laboratories. Mice were housed at a maximum of five animals per cage. Rats were housed as a single pregnant female per cage. All animals were exposed to a normal 12-24 h light-dark cycle, and provided ad libitum with food and water.

Reagents and supplies. All reagents and supplies were from Fisher Scientific or Sigma-Aldrich. Karyopharm Therapeutics supplied the in vitro and in vivo formulations of KPT-276, and KPT-350, and the vehicle control compounds, Pluronic F-68 and PVP K-29/32.

Structural determination of KPT-276 bound CRML1. Crystal structure determination is as previously described20–22,45. Briefly, 56CRML1 (Thr539 mutated to cysteine to allow inhibitor binding; residues 377–413 and 1,059–1,084 removed to improve crystal quality), human Ran and S. cerevisiae RanBP1 (residues 1–61 removed to improve crystal quality) were expressed and purified separately and then assembled to form a ternary complex. The CRML1–Ran–RanBP1 complex was mixed with KPT-276 at 1:5 ratio and further purified by size exclusion chromatography followed by crystallization and structure determination. Figures showing the structures were prepared using PyMol (DeLano Scientific) and the omit electron density map was generated using CCP4 (ref. 46).

In vitro GST pull-down inhibition assay. 10 µM of purified recombinant 56CRML1 was incubated with or without 100 µM KPT-276 in buffer containing 10 mM Tris pH 7.5, 100 mM sodium chloride, and 5 mM magnesium acetate, in a total volume of 100 µl. ~15 µg of GST or recombinant GST–NES (NES is residues 33–50 of the protein kinase-A inhibitor (PKI)) immobilized on glutathione sepharose beads, and molar excess of RanGTP were added to the CRML1 proteins and rotated for 30 min at 4 °C. The glutathione sepharose beads were then washed extensively with 20 mM HEPES pH 7.5, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EDTA, and 15% glycerol. Bound proteins were separated by SDS-PAGE gel and stained with Coomassie dye.

Pharmacokinetic (PK) measurements. KPT-276 (2 mg per kg) and KPT-350 (3 mg per kg) was administered via oral gavage in male Sprague-Dawley rats, with a mean body weight of approximately 210–230 g. At nine designated time points (1, 5, 15, 30 min and 1, 2, 4, 7 and 24 h) post-gavage, the animals were killed by CO2 inhalation and the brain tissue was harvested. The whole brain was collected, rinsed with cold saline, dried on filtre paper, weighed and then snap frozen by placing into dry ice. All samples were stored at –80 °C until further analysis. The PK parameters were determined by a non-compartmental module of WinNonlin Professional 6.2.

EAE. EAE was performed as previously described47. Briefly, 8-week-old female C57BL/6J mice (Jackson Laboratories) were immunized with 300 µg of murine myelin oligodendrocyte glycoprotein, peptide sequence 35–55 (MOG35–55) in complete Freund’s adjuvant (10 ml of incomplete Freund’s adjuvant containing 500 µg of Mycobacterium tuberculosis). Mice were injected with 200 µl of a solution consisting of equal amounts of 1.5 mg ml–1 MOG35–55 peptide, 2.5 mg ml–1 Mycobacterium tuberculosis and 50% Incomplete Freund’s Adjuvant. On day 8 and day 2, the mice are injected intraperitoneally with 500 ng pertussis toxin (from Bordetella pertussis) in 200 µl phosphate-buffered saline (PBS). The MOG35–55/CFA injection was repeated on the opposite hind limb after seven days to boost the immunological reaction. Mice were monitored daily for clinical score based on a five-point scale, where 0 represents no disability a score of 1 corresponds to a flaccid tail, 2 is weak hind legs, 3 is hindlimb paralysis, 4 is quadriplegia and a score of 5 is death. Mice were also assessed for body condition (score BC1 - BC5, where BC3 represents a healthy body coat and weight; BC5 represents an obese mouse; BC1 is an anorexic mouse), and weighed daily. At the onset of disability, the mice were provided ad libitum with water–moistened food that was supplemented with flavored gelatin. In addition, Nutritional was provided as a high calorie supplement in order to prevent weight loss. Any mice that did not develop clinical symptoms (score 0) were excluded before the start of treatment. All other mice were assigned to treatment groups in a random manner, and scored and drug-treated in double-blinded fashion. Any mice that did not develop symptoms of EAE were not used in the treatment groups. All treatment groups were only revealed at the end of the experiments to ensure experimenter bias was not introduced. All measurements were taken in the vivarium during the light cycle.

Localized EAE to dorsal column of the spinal cord and mouse locomotion testing. Focal EAE was performed according to a modified version of previously described protocols27,28. 8-week-old female C57BL/6J mice (Jackson Laboratories) were sensitized with a single dose of 300 µg of murine myelin oligodendrocyte glycoprotein emulsified in complete Freund’s adjuvant (as described above). 7 d later, mice were anesthetized with ketamine and xylazine and a hemi-laminectomy was performed at thoracic level T5, and the spinal cord was stereotactically injected into the dorsal column of the spinal cord over a three minute period with a 1 µl cytotax mixture containing 250 ng recombinant mouse TNFα 250 (R&D Systems), and 150 U of recombinant mouse interferon-γ (R&D Systems). Mice were monitored daily for motor symptoms. Motor function was evaluated by hind-limb locomotor performance using the open-field Basso Mouse Scale (BMS)29. The scale consists of a nonlinear scoring scale ranging from 0 (complete paralysis) to 9 (normal mobility), with each score represents a distinct motor functional state. Focal EAE mice were randomly assigned to treatment groups (either vehicle or KPT-350) starting at a BMS score of 5. Locomotor activity was assessed daily in an open field for 4 min and each mouse was assessed by two individual investigators that were blinded to the treatment groups. The treatment groups were only revealed at the completion of the experiment.

Organotypic slice cultures. Hippocampal slice cultures were prepared using the interface culture method48 adapted for 5–7-d-old C57BL6J male mouse pups. Briefly, hippocampi were dissected under sterile conditions and sliced transversely at 400 µm with a McIlwain tissue chopper. Slices were plated on porous (0.4 µm) insert membranes (Millipore) and maintained at 37 °C in a 5% CO2 incubator. Culture medium was changed every 2–3 d and experiments were performed after at least 2 weeks in culture. For inducing excitotoxicity, slices were first treated with 10 nM KPT-350 for 1 h, followed by 5 µM kainic acid for 18 h.

Kainic acid induced neurotoxicity in vivo. In vivo kainic acid experiments were performed in 12-month-old male C57/BL6J mice. Mice were given KPT-350 (7.5 mg per kg) for 1 d before the subcutaneous injection of 2 mg per kg kainic acid which was used to induce neuronal damage in the CA3 region of the hippocampus. Mice were closely monitored for symptoms of seizure. After 1 h, all mice were intraperitoneally injected with 40 mg per kg of sodium pentobarbital to temporarily anesthetize the animals. After 18 h mice were perfused with 4% paraformaldehyde and the brains were post-fixed for 1 d in paraformaldehyde, followed by soaking in 30% sucrose and embedding in OCT. Hippocampal sections were stained to assess neuronal damage.

Drug preparation, dosing and administration. The vehicle solution was comprised of 0.6% Pluronic F-68 (wt/vol) and 0.6% PVP K-29/32 (wt/vol) in water. The final concentration of drug given to mice was 75 mg per kg of KPT-276 and 7.5 mg per kg of KPT-350. The drugs and vehicle were dosed three times per week (Monday, Wednesday and Friday) via oral gavage. For therapeutic EAE, treatment was started at day 16, at the onset of hindlimb paralysis (EAE clinical score of 2.5), and animals were dosed for a 12-d time window, and harvested on day 28. For prophylactic EAE, drug administration was started at the same time as immunization with MOG35–55 and mice were treated for a total of 19 d.

Primary neuronal and oligodendrocyte cultures. Primary neuronal cultures were prepared as previously described35,49. Briefly, pregnant Sprague-Dawley rats, or pregnant C57/BL6J mice at gestational day 16 were killed by carbon dioxide asphyxiation. Cortices and hippocampi from embryos were removed, dissociated into a single cell suspension using 0.05% trypsin and mechanical disruption, and plated in Neurobasal media supplemented with B27 supplement (Invitrogen), glutamine and penicillin/streptomycin. For immunocytochemistry, hippocampal neurons were plated at a density of 1.3 × 104 cells per ml on poly-d-lysine–coated...
MTT toxicity assays. Cells were plated in 48-well plates at a density of 25,000 cells cm⁻². For total splenocytes and GM-CSF induced, bone marrow–derived dendritic cells, 10⁶ cells were plated in 96-well plates. Both cortical and spinal cord neurons were treated with 0.1 to 1,000 nM KPT-276 or KPT-350 for 24 h and then MTT reagents were added for 4 h. The media was aspirated and the formazan crystals were solubilized in DMSO, and the solute was measured spectrophotometrically at 540 nm with background subtraction at 655 nm. Data were normalized as percent of control.

Protein extraction, cytotoxic and nuclear fractionations, proteomics, SDS-PAGE and western blotting. Human post-mortem cortical gray matter from MS patients and controls was provided by the United Kingdom Brain Bank. A small piece gray matter was micro-dissected on a cold steel block under a stereomicroscope to ensure the sample was free of white matter or meningeal material. Tissue was lysed with RIPA lysis buffer in a Qiagen Tissue lysis buffer, and centrifuged at 15,000 g to obtain a post-nuclear supernatant for western blot analysis. For cytotoxic and nuclear fractionation of cultured cells and mouse spinal cord tissue, a method employing a hypotonic lysis buffer, followed by NP40 detergent isolation was used (ActiveMotif). Proteomics was performed on cytotoxic fractions of cortical neurons, and on EAE spinal cords. Proteomics was performed on a Thermo Q-Exactive LC-MS/MS instrument. The data were filtered to include only the 1,573 gene products that had six or more spectra when considering the combined three replicates. Protein amounts were determined using the Bradford protein assay, and samples (25 μl per lane) were resolved by SDS-PAGE on pre-cast BioRad gels and transferred to PVDF membranes. Membranes were blocked in 5% (wt/vol) skim milk powder in TBS-tween (TBST) and incubated overnight with primary antibodies (CRM1, BD Transduction Labs, cat no: 611832, 1:1,000; ACTIN clone AC-15, Sigma, cat no: A5541, 1:5,000; MEK1/2, Sigma, cat no: M5795, 1:10,000; HISTONE H3, Abcam, cat no: AB1791, 1:5,000; FUS, Abcam, cat no: EP1207Y, 1:250; total TAU, clone 86E/C11, Millipore, cat no: 05-803, 1:1,000; NRF2, Santa Cruz, cat no: sc-722, 1:100) diluted in 5% bovine serum albumin in TBST. The next day, membranes were washed with TBST and incubated with secondary antibody diluted 1:2,500 (goat anti-mouse-HRP, or goat-anti-rabbit-HRP, JacksonImmunoResearch) for 1 h. Blots were incubated with Amersham ECL reagent, and imaged and quantified on a BioRad Molecular Imager ChemiDoc with Image Lab XRS+ Software.

RNA extraction and qPCR. An equal size weight (10 mg) piece of spleen or lumbar-sacral region of spinal cord region from therapeutically treated EAE mice harvested at day 28 was dissected and flash frozen on dry ice for RNA extraction. RNA was extracted using Trizol (Invitrogen, #15596-018) and purified with the RNeasy Micro kit (Qiagen, #74004) following the manufacturer’s protocol. The total RNA amount was quantified on nanodrop, and 1 μg of total RNA was reverse transcribed with qScript cDNA Supermix (Quanta, #9504) and qRT-PCR was performed using Perfecta Sybr Fast Mix Rox 1250 (Quanta, #Q1014-278) (primers are listed in Supplementary Table 3). After normalization to 18S rRNA, the average values for each transcript was calculated based on the values obtained in all the samples included for each experimental condition. Transcripts levels were normalized based on calculated primer efficiencies.

Immunocytchemistry, immunohistochemistry and quantitative confocal microscopy. Following experimental treatments, cells were washed once with 1× PBS and fixed with 4% (wt/vol) paraformaldehyde. Cells were permeabilized with blocking buffer (0.1 M phosphate buffer (PB), 10% (vol/vol) normal goat serum (Vector Laboratories) and 0.5% (vol/vol) Triton X-100) and incubated with primary antibodies (NFH, Millipore, cat no: MAB5448, 1:400; SMI-32, Millipore, cat no: NE1023, 1:5,000; OLI2, G2) and imaged (See below). The nuclei were counterstained with DAPI (0.1 μg ml⁻¹) for 5 min before mounting. For immunohistochemistry, mice were anesthetized and perfused with 4% (wt/vol) paraformaldehyde. Spinal cords were removed, post-fixed, and cryo-preserved in 30% (wt/vol) sucrose, embedded in OCT and sectioned longitudinally (8 μm). Spinal cord sections were permeabilized with blocking buffer (0.1 M phosphate buffer (PB), 10% (vol/vol) normal goat serum (Vector Laboratories)

Viral knockdowns of Xpo1. Mouse hippocampal neurons (10 DIV) were infected with lentiviral particles (multiplicity of infection = 5) containing shRNA for knockdown of Xpo1 (pLKO.1-CMV-GFP, cat no: TRCN0000046624, 5'-TTG-TGA-CTT-TGC-CGG-CTT-TGA-3') or TurboGFP control (pLKO.1-CMV-GFP Empty Vector Control) in the presence of 4 μg ml⁻¹ of hexamethrinle bromide (Sigma). After two, infected cells were identified by GFP fluorescence on a live imaging microscope, and then cells were treated with 5 μM kainic acid for 2 h. Following kainic acid treatment, cells were fixed with 4% paraformaldehyde, and stained with antibodies for CRM1 (1:200, Santa Cruz, cat no: sc-5595), NFH (1:400, Millipore, cat no: MAB5448), SMI-32 (1:5,000, Millipore, cat no: NE1023). Axonal damage was assessed by quantifying the intensity of SMI-32 immunoreactivity and expressing it as a ratio to the expression of NFH.
and 0.5% (vol/vol) Triton X-100) and incubated with stains (FluoroMyelin, Invitrogen 1:300) or primary antibodies (CC1/APC, Calbiochem, cat no: 8080, 1:200; MBP, Millipore, cat no: MAB386, 1:400; NG2, Millipore, cat no: 24447, 1:250; IBA1, Wako, cat no: 019-17941) overnight and then washed 3 x 5 min with PB and incubated with the primary antibody overnight, and incubated with corresponding secondary antibodies, followed by counterstaining with DAPI (as above).

For human tissue lesional and non-lesional areas of paraformaldehyde fixed, paraffin embedded human brain biopsies were sectioned and antigen retrieval was performed using a microwave or a steamer and sodium citrate buffer, pH 6.0. Slides were incubated overnight with antibodies for anti-MBP (Dako, cat no: 1: 1,000), anti-neurofilament (Dako, 1:2,000), anti-CD45 (Dako, 1:800), anti-CRM1 (Santa Cruz, cat no: sc-5595, 1:100), anti-NeuN (Chemicon, cat no: MAB377, 1:100). To identify blood-derived monocytes, microglia and their activated derivatives, we used the antibody KiM1P (1: 5,000, kindly provided by H.-J. Radzun, University of Göttingen). This antibody recognizes the antigen CD68 and has been especially designed to label all CD68-positive cells in paraffin embedded human tissue samples including monocytes, ramified microglia and foamy phagocytes. Secondary antibodies were biotinylated anti-rabbit (Vector Laboratories, 1:400) or anti-mouse (GE Health care, 1:400) antibodies followed by ExtrAvidin-Peroxidase (1: 100, Sigma). DAB was used as a chromogen and sections were counterstained using hematoxylon. For double staining, sections were incubated with Cy3 (Jackson Immunoresearch, 1:200) or Alexa488 (Jackson Immunoresearch, 1:200) conjugated antibodies and counterstained with DAPI (Invitrogen, 1:5,000). All images were taken on a Zeiss LSM-710 confocal microscope with Zen software or an Olympus fluorescent microscope. Axonal damage was assessed by either assessing neuritic beading, which was defined as neurites exhibiting a non-smooth morphology, characterized by a pattern of successive swellings/enlargements, and/or severed neurites. A second measure of axonal damage was obtained by expressing the ratio of SMI-32 to NFH immunoreactivity, which was quantified using ImageJ.

Hematoxylin and eosin staining. EAE mice were perfused at day 28 and spinal cords were removed, soaked in 30% sucrose solution in PBS (wt/vol), embedded in OCT and frozen on dry ice. 10-µm-thick frozen sections were cut on a cryostat, and washed twice in xylene, with subsequent washes in 100%, and 95% ethanol for 3 min each. Following this, the slides are washed in water, and placed in hematoxylon solution for three minutes. The slides are then washed with water, and acidified alcohol (95% alcohol + 5% glacial acetic acid), followed by washing in 5% ammonium hydroxide. The slides are incubated in eosin solution, followed by dehydration again in ethanol (100% and 95%), with a final wash in xylene before mounting with a coverslip.

Serial electron microscopy. For serially-reconstructed electron microscopy, animals (n = 3 per group) were rapidly perfused with 4% paraformaldehyde, 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2. Spinal columns were removed and spinal cords were processed in L-4.5 region with embedded in Epon plastic. Semithin sections (1 mm) were stained for myelin with 1% toluidine blue/1% sodium borate. Following identification of an appropriate region for electron microscopy analysis, tissue was embedded and cut into semi-thin sections for three-dimensional electron microscopy (Renovo Inc, Cleveland, OH). For ultrastructural imaging, a SigmaVP microscope from Zeiss was used with a 3 view door from Gatan. A low kilovolt backscatter detector (Gatan) was used to detect the signal. For cytoskeletal analysis, 200 axons were analyzed, and scored based on the percent microtubules either intact, or with varying degrees of disruption.

Proliferation of CD4+ T cells. CD4+ cells were negatively selected from total splenocytes by use of a cocktail of antibody-coated magnetic beads from Miltenyi Biotech (CD91a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, and Ter-119), according to manufacturer’s instructions. CD4+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies Inc.) and cultured at 10^5 cells per well for 48 h in anti-CD3 (BD) and anti-CD28 (BD) coated microplates (5 µg ml^-1) with or without mouse rIL2 (BD) (10 ng/ml). Cells were cultured in RPMI-1640 supplemented with Glutamax, 10% FCS, 0.02 mM 2-mercaptoethanol, and antibiotics. CFSE dilution analyzed by flow cytometry was used to determine the percentage of proliferating CD4+.

FACS analysis. Mice were killed using CO2 and the spleen and spinal cord were immediately removed and placed into cold PBS. The tissue was dissociated with a 40-µm filter and centrifuged to obtain a single cell suspension, APC-conjugated anti-CD45R (B220; RA3-6B2), anti–IFN-γ (XMG1.2), and anti–MHCI (AF6-120), PE-conjugated, anti–IL-17 (TC11-18H10) were from BD. EFluor450-conjugated anti-CD11b (M1/70), CD8α (53-6.1), APC-conjugated anti-CD115 (AFS98), PE-Cy7–conjugated anti-CD11c (N418), anti-CD4 (GK1.5), Alexa fluoro 700-conjugated CD45 (50-F11), PerCP/Cy5.5-conjugated CD3ε (145-2C11) were from eBioscience. For intracellular cytokine staining, lymphocytes were stimulated in vitro with leukocyte activation cocktail (BD) according to the manufacturer's instructions. Surface staining was performed before permeabilization using an intracellular staining kit (eBioscience). For staining of spinal cord cells, spinal columns were flushed with cold PBS, and the cord was mashed through a cell strainer. Mononuclear cells were separated by density gradient centrifugation on Percoll (GE Healthcare). Forward scatter and side scatter were used to gate cells excluding debris and cell aggregates and 7-AAD (BioLegend) was used to gate out dead cells. Samples were analyzed using a flow cytometer (LSRFortessa; BD) and the resulting data were analyzed using Flowjo software (Tree Star).

Relative spleen cell population calculations. The populations analyzed by FACS in the spleen (B220+ cells, CD4+ cells, CD8+ cells, monocytes, neutrophils and dendritic cells) represent approximately 90% of spleen cellularity when erythrocytes are excluded. In order to represent relative spleen cell populations in pie graph format, the sum of these leucocyte numbers was considered to be 100% of total cells.

NES prediction. NES predictions were performed using NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES/). Mouse protein accession numbers were accessed through the National Library of Medicine, and the primary amino acid sequence was queried for containing leucine-rich sequences indicative of an NES^10.

Sample size, data analysis and statistics. All data were analyzed using GraphPad Prism 5.0 software. All data represents means ± s.e.m. All statistical tests were two-tailed, and performed using parametric statistics (independent t tests with Bonferroni correction, one-way ANOVA with Dunnett's correction, or linear regression analysis) when data were normally distributed (tested using GraphPad), and the assumptions underlying the given tests were met. Non-parametric equivalent statistical tests (for example, Mann-Whitney U tests, or Kruskal-Wallis tests with Dunn's correction) were used when data were not normally distributed, or when variances were largely different between groups. No statistical methods were used to pre-determine sample sizes, but our samples sizes are similar or higher than those employed in the field. Human sample sizes were limited, and thus we used the number of samples we had access to. All data were considered significant at P < 0.05.

A Supplementary Methods Checklist is available.

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