Molecular Therapy
Methods & Clinical Development
Original Article

Induction of antigen-specific tolerance by hepatic AAV immunotherapy regardless of T cell epitope usage or mouse strain background

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***In vivo* induction of antigen (Ag)-specific regulatory T cells (Treg) is considered the holy grail of therapeutic strategies for restoring tolerance in autoimmunity. Unfortunately, in the autoimmune disease multiple sclerosis, an effective and durable therapy targeting the diverse repertoire of emerging Ags without compromising the patient's natural immunity has remained elusive. To address this deficiency, we have developed an Ag-specific adeno-associated virus (AAV) immunotherapy that will restore tolerance in a Treg-dependent manner. Using multiple strains of mice with different genetic and immunological backgrounds, we demonstrate that a liver directed AAV vector expressing a single transgene can prevent experimental autoimmune encephalomyelitis from developing and effectively mitigate pre-existing or established disease that was induced by one or more auto-reactive myelin oligodendrocyte glycoprotein-derived peptides. Overall, the results suggests that AAV can efficiently restore Ag-specific immune tolerance to an immunogenic protein that is neither restricted by the major histocompatibility complex haplotype, nor by the specific antigenic epitope(s) presented. These findings may pave the way for developing a comprehensive Ag-specific immunotherapy that does not require prior knowledge of the specific immunogenic epitopes and that may prove to be universally applicable to all MS patients, and adaptable for other autoimmune diseases.**

INTRODUCTION

Induction of antigen (Ag)-specific tolerance is a promising therapeutic strategy to restore homeostasis in an autoimmune disease like multiple sclerosis (MS). Unfortunately, the available immunotherapies only affect the immune system in a non-Ag-specific way.[1] Such an approach could selectively dampen the pathogenic autoimmune response without affecting the physiological functions of the immune system.[2,3] Ideally, an Ag-specific immunotherapy could provide long-term therapeutic protection without the need for continuous generalized systemic immune suppression. Unfortunately, developing an Ag-specific therapy, especially for MS, has remained elusive. Autoimmune diseases are complex and are often driven by multiple autoantigens. Although T and B cell responses against numerous myelin and neuronal Ags have been identified in MS patients, none have shown to be a specific driver because of patient-to-patient epitope variability.[4]

MS is a protracted, organ specific-inflammatory, autoimmune disease of the central nervous system (CNS) in which lymphocytes cross the blood-brain barrier and cause localized CNS inflammation, demyelination, and axonal damage.[1] Various studies have documented a failure of central and peripheral regulatory mechanisms (particularly regulatory T cells [Tregs]) to maintain self-tolerance and control autoreactive lymphocytes, which is thought to be key in the development and pathogenesis of MS.[5–10] In the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), both Treg depletion and Treg adoptive transfer experiments have demonstrated that Tregs do indeed have a critical role in controlling disease development and severity.[11] Currently, there are no approved MS therapies designed to specifically remove Ag-specific auto-reactive immune cells and induce sustained *in vivo* restoration of tolerance.[12]

Historically, having specific knowledge of the target Ag(s) or epitopes responsible for disease has been a pre-requisite for developing an Ag-specific therapy. However, identifying the relevant and emerging autoantigens has proven difficult.[13] The complexity of disease initiation and progression in MS makes determining the specific Ags challenging, especially since multiple antigenic epitopes can exist in within a single neuroprotein, and there are several neuroproteins involved.

Adding to the difficulty is the process of epitope spreading, which is known to occur in both EAE and MS patients.[14] Epitope spreading was first described by Lehmann et al.[14] in 1992 as the shifting or spreading of an immune response to epitopes distinct from, and

Received 22 September 2022; accepted 22 December 2022;
https://doi.org/10.1016/j.omtm.2022.12.011.

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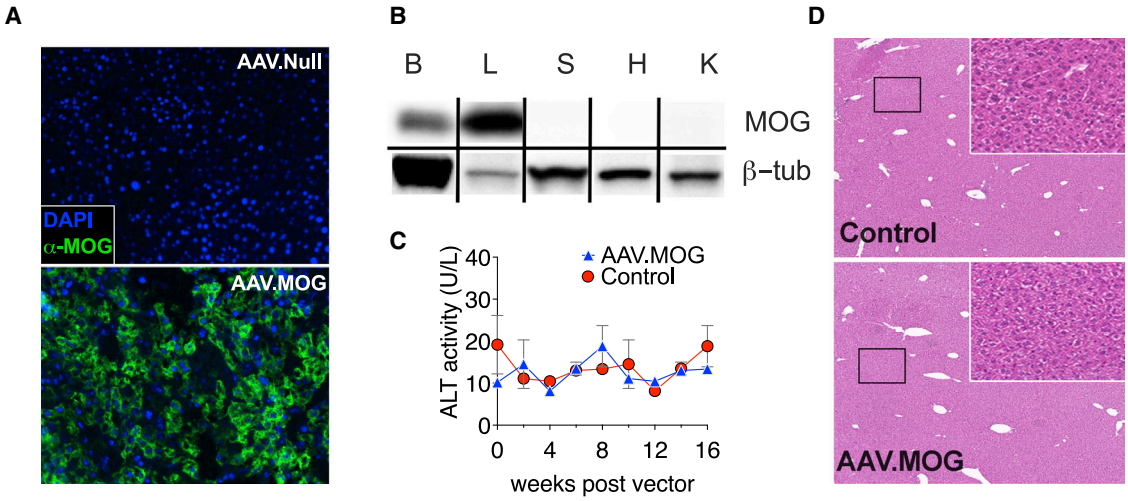


Figure 1. Liver expression of AAV.MOG

Female C57BL/6 mice were injected with 1 × 10^{11}vg AAV.MOG or control vector. (A) Representative immunofluorescent image of frozen liver tissue stained with α-MOG antibody (green) and DAPI stained nuclei (blue) to indicate total cells (100×). (B) Western blot analysis demonstrating transgene expression of MOG in the liver, but not in other organs such as heart (H), spleen (S), or kidney (K) (α-MOG and α-β-tubulin). Tissues lanes were run on the same gel, but were noncontiguous. (C) No significant difference in the ALT levels detected in the plasma of treated mice compared with controls over the indicated time (shown as mean ± SEM, n = 5–7). (D) Hematoxylin and eosin-stained liver tissue showing a lack of overt inflammation or liver pathology.

non-cross reactive with, the original disease causing epitope.[14,15] In other words, as autoimmunity develops and tissue damage increases, priming to additional self-antigens can occur.

Although all the specific Ag(s) responsible for T cell activation in MS remain unknown, MS and EAE share many of the principle encephalitogenic proteins.[11] As such, studies using EAE mice have provided significant insight into the various myelin-directed responses in the CNS.[16,17] Similar to MS, EAE is an autoimmune disease that is mediated by myelin reactive $CD4^+$ T helper (Th) 1 cells and interleukin-17-producing (Th17) cells. These auto-reactive lymphocytes infiltrate the CNS and contribute to demyelination/neurodegeneration.

To address the diversity of epitopes, we developed a novel Ag-specific adeno-associated virus (AAV) gene immunotherapy that induces Ag-specific Tregs, restores tolerance, and is capable of preventing and reversing EAE.[18] While informative, the initial reporting was inadequate in demonstrating the full therapeutic scope because EAE was exclusively induced in C57BL/6 (B6) mice using the single immunodominant myelin oligodendrocyte glycoprotein epitope ($MOG_{35–55}$).

In the present study, we aimed to demonstrate that our AAV.MOG vector is dynamically responsive in mitigating disease induced with multiple MOG epitopes, not just the well known prototypic epitope previous used. Since the vector was engineered to deliver the full nucleotide coding sequence of MOG, this would enable transduced cells to have the intrinsic capacity to synthesize the protein material necessary to process and present all possible MOG epitopes by the major histocompatibility complex (MHC). As designed, the vector is not limited to expressing predetermined epitopes.

To demonstrate the dynamic therapeutic potential of this single AAV.MOG immunotherapy vector to induce Treg-dependent immune tolerance against a variety possible immunogenic epitope(s) of MOG, we used $MOG_{35–55}$, $MOG_{79–96}$, $MOG_{92–106}$, $MOG_{119–132}$, and $MOG_{1–125}$ peptides, singularly and in combination, to induce EAE in multiple strains of genetically diverse mice (e.g., B6 [$I\text{-}A^b$], SJL [$I\text{-}A^s$], DBA1 [$I\text{-}A^q$], and [C57BL/6xSJL]F1 [$I\text{-}A^{b/s}$]). The results show that AAV.MOG immunotherapy provides long-lasting protection when given prophylactically or during pre-existing active disease, regardless of the encephalitogenic epitope used or the genetic background of the mice.

RESULTS

Liver restricted AAV.MOG transgene expression without liver inflammation

To leverage the therapeutic potential of the liver to induce immune tolerance during an autoimmune disease, we designed an AAV vector that would transduce liver cells and express the full-length neuroprotein, MOG, under the control of a liver-specific promoter.[18] To validate that MOG protein was expressed in liver, 1 × 10^{11} vector genomes (vg) (a dose previously shown to transduce >38% of mouse hepatocytes)[19] of AAV.MOG or AAV.Null (a promoterless control vector) was intravenously injected into B6 mice. Transgene expression was evaluated 2 weeks later via immunofluorescent microscopy (Figure 1A). The fluorescent staining pattern observed suggests MOG expression is membrane bound. To confirm that AAV.MOG resulted in tissue-specific transgene expression with no off-target expression, protein expression was evaluated in liver, spleen, heart, and kidney tissue via western blot analysis (Figure 1B).

Table 1. EAE scores

Score	Clinical presentation
0.0	No clinical signs
0.5	Partial paralysis/limp tail
1.0	Paralyzed tail
1.5	Impaired coordination/balance
2.0	Hindlimb paresis
2.5	One hindlimb paralyzed
3.0	Complete hindlimb paralysis (paraplegia)
3.5	Hindlimb paralysis and unable to right self
4.0	Hindlimb paralysis and forelimb paresis
5.0	Moribund/dead

Mice euthanized or found deceased were recorded as 5 for the remainder of the time.

Elevation in plasma alanine transaminase (ALT) levels is routinely used to screen for liver inflammation and cell-mediated immunity directed against AAV-transduced cells. Elevated ALT levels often correlate with an anti-capsid immune response, resulting in transgene loss.[20] Although AAV hepatic gene therapy in mice does not usually result in elevated ALT levels, we wanted to assess possible liver damage considering the expressed transgene is a non-secreted, membrane bound neuroprotein. Plasma samples obtained before AAV injection (naive) and every 2 weeks thereafter were analyzed for ALT activity. Over a total of 16 weeks, there was no significant difference in the ALT levels detected in the plasma of treated mice compared with controls (Figure 1C). Additionally, histological evaluation of serial cut liver sections from both the treated and control groups showed no overt qualitative differences between the two groups (Figure 1D).

AAV.MOG prevents disease to secondary immunogenic peptides in I-A^b mice

An *in vivo* immunotherapy that restores Ag-specific tolerance needs to be dynamically capable of responding to dominate and secondary encephalitogenic epitopes. Historically, the majority of pre-clinical studies have focused on the prototypic MOG_{35-55} epitope, located within the extracellular domain, and ignored other identified encephalitogenic MOG epitopes.[18,21,22] We previously demonstrated a single dose of AAV.MOG vector could prevent the onset of EAE induced by the immunodominant MOG_{35-55} epitope.[18] However, MS and EAE are complex diseases where multiple MOG epitopes have been identified in disease progression.[17] One such epitope in B6 (I-A^b) mice is $MOG_{119-132}$, a sequestered transmembrane sequence that induces EAE with similar severity as MOG_{35-55}.[21]

To determine whether our AAV.MOG immunotherapy can respond to such cryptic epitopes, we evaluated its ability to prophylactically protect mice against $MOG_{119-132}$ induced EAE. B6 (I-A^b) mice were tolerized with AAV.MOG or control vector 2 weeks before EAE was induced with $MOG_{119-132}$ peptide emulsified in complete Freund's adjuvant (CFA). Mice were monitored daily for clinical signs of disease according to a standard 5-point scale (Table 1). Like disease induced with the prototypic MOG_{35-55} epitope, the control mice began showing neurological deficits approximately 9 days after EAE induction characterized by ascending para lysis. Clinically presenting with hindlimb paralysis (paraplegia), disease peaked with a mean EAE score of 3.2 ± 0.2 (mean ± standard error of the mean [SEM]) (Figure 2A). Mice were monitored for 40 days, over which time control mice maintained severe disease (EAE score $\geq$2.5), in stark contrast with AAV.MOG-treated mice that showed no signs of disease throughout the experimental period.

The presence of inflammation and large confluent demyelinating lesions in the CNS is a hallmark of MS pathology and in B6 mice occurs primarily in the spinal cord.[23,24] Therefore, we qualitatively evaluated the level of cellular infiltration and demyelination between AAV.MOG-treated mice and controls using serial cut tissue sections from similar regions of the spinal cord (Figure 2B). AAV.Null mice exhibited numerous localized foci of cellular infiltrates in the white matter of the spinal cord (hematoxylin and eosin stain). These areas corresponded with extensive demyelination (Luxol fast blue staining) seen on adjacent sections. In contrast, there were little to no signs of localized lymphocyte infiltration or demyelination seen within the spinal cords of AAV.MOG-treated mice. This suggests that our vector immunotherapy protects against CNS inflammation and subsequent axonal damage.

AAV.MOG establishes long-term protection

Like chronic MS, MOG-induced EAE is a disease in which the inflammatory response results in the continuous exposure of immunogenic epitopes and degradation of the myelin sheath. Considering this, we questioned if the long-term suppressive effects of our treatment are simply due to a resolution of CFA-induced inflammation. Ideally, an immunotherapy should be robust enough to remain effective, even in the presence of multiple or continuous insults. To test this, we first assessed the long-term stability and robustness of the AAV immunotherapy to prevent $MOG_{119-132}$-induced disease. EAE was induced with $MOG_{119-132}$ in B6 mice that were tolerized with AAV.MOG or AAV.Null 2 weeks earlier. Consistent with the previous results, treated mice failed to develop clinical signs of disease, whereas the control mice developed persistent (125 days) and significant neurological deficits beginning approximately on day 9 (Figure 2C). On day 125 mice, were re-challenged with Ag emulsion (Figure 2C, dotted line). Rapidly after the re-challenge, disease severity in control mice (AAV.Null) increased to the point mice began exhibiting quadriplegia and 80% succumbed to the disease (Figure 2D). In sharp contrast, 100% of the AAV.MOG-treated mice continued to remain symptom free until termination of the experiment on day 145.

AAV.MOG vector reverses pre-existing disease

Having demonstrated that prophylactic AAV.MOG administration effectively prevents EAE induced by both primary (MOG_{35-55}) and secondary ($MOG_{119-132}$) immunogenic epitopes, we addressed the more clinically relevant question whether AAV immunotherapy can effectively reduce or reverse pre-established EAE (Figure 2E). To test this, before administering AAV.MOG, we first induced EAE in B6 mice using the alternate $MOG_{119-132}$ peptide. As mice reached

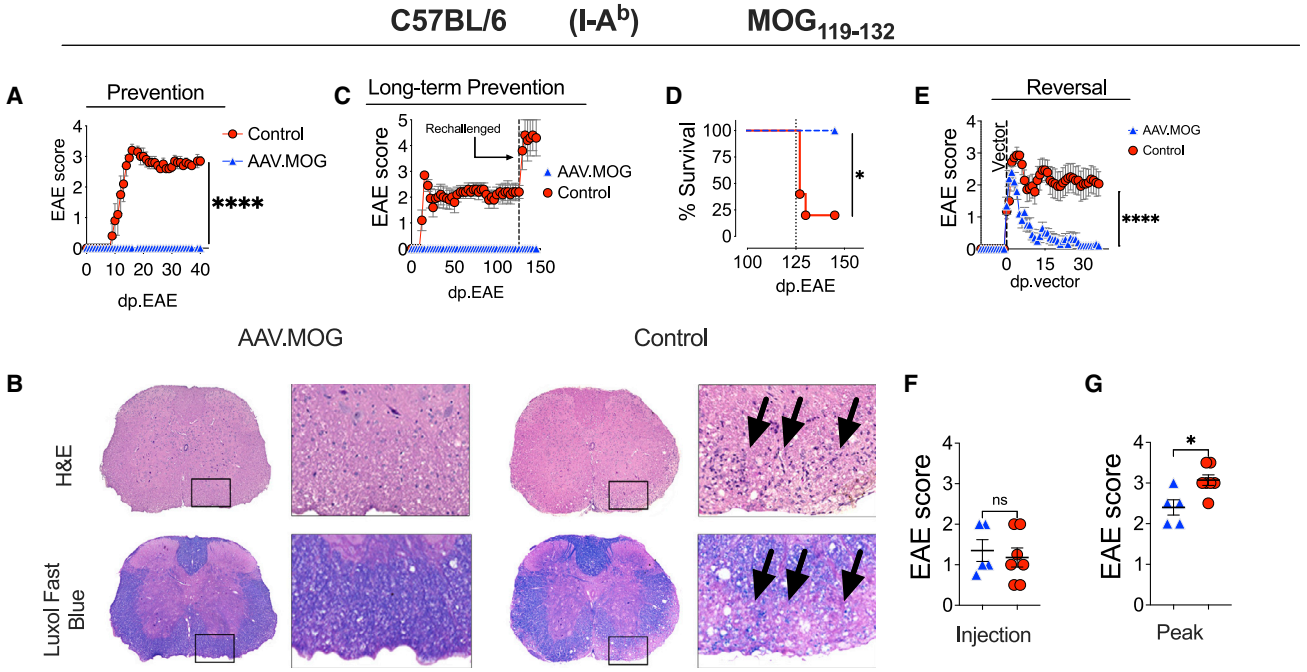


Figure 2. AAV.MOG prevents and reverses MOG$_{119-132}$ induced EAE in C57BL/6 (I-A^b) mice EAE

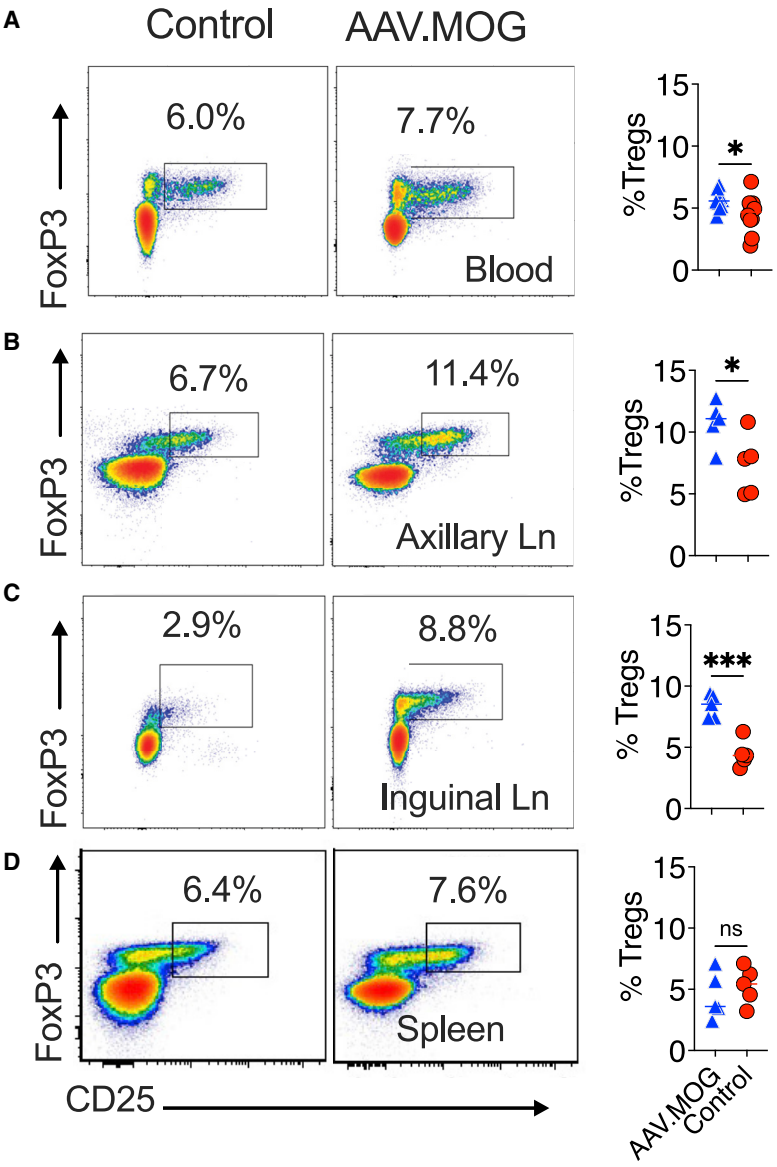
(A) Prevention of EAE disease. Female C57BL/6 mice were intravenously injected via the tail vein with 10^{11} vg/mouse of either AAV.MOG or control vector. Two weeks later, EAE was induced with MOG$_{119-132}$. The mean EAE score of mice is shown as mean ± SEM, n = 5/group). Data are representative of at least two repeat experiments. (B) Representative histological images of spinal cord demonstrating multiple foci of inflammation in the white matter of control mice (hematoxylin and eosin stain) and a serial cut section of spinal cord from the same mouse showing multifocal demyelination associated with the areas of inflammation (Luxol fast blue). AAV.MOG treated mice showed little to no inflammation or demyelination. (C) Long-term Prevention: mice were tolerized with AAV.MOG or control 2-weeks before being immunized with MOG$_{119-132}$ to induce EAE. On day 125 post EAE (dashed line), all mice were re-challenged with MOG$_{119-132}$/CFA. Disease severity shown as MCS ±SEM (n = 5/group). (D) Survival curve of long-term mice before and after re-challenge (p = 0.0285; log rank [Mantel-Cox] test). (E) Reversal of EAE disease. EAE was induced with MOG$_{119-132}$ in age-matched female C57BL/6 mice (9–10 weeks old) and allowed to progress. (F) As mice reached an EAE score of 1.0 or greater (shown as mean ± SEM; n = 5–7/group), individual mice received 10^{12} vg/mouse of either AAV.MOG or control vector via the tail vein. Treated mice quickly recovered to near baseline, whereas disease was maintained in control mice. (p < 0.001; the Mann-Whiney test was used to determine differences). (G) Peak scores of each group (p = 0.0328).

a target score of approximately 1.0 (complete tail paralysis), they were divided in an alternating fashion into two cohorts and administered either AAV.MOG or AAV.Null. Both cohorts of animals received vector at statistically similar scores of 1.4 ± 0.3 (AAV.MOG) and 1.2 ± 0.2 (AAV.Null) (Figure 2F). EAE continued to develop after vector treatment in both groups, although the AAV.MOG-treated mice developed slightly less severe disease, peaking at 2.4 ± 0.2, as compared to the AAV.Null control group peaking at 2.9 ± 0.1 (Figure 2G). By day 12, the AAV.MOG-treated mice had nearly recovered and by day 30 were essentially disease free, having an average score of 0.1 ± 0.1. In contrast, significant neurological deficits persisted in the control mice for the remainder of the experiment (average score of 2.1 ± 0.4) (Figure 2E). These combined results clearly demonstrate that AAV.MOG can also reverse established EAE that is induced with a cryptic autoantigen through a mechanism that decreases inflammation and demyelination of the CNS.

AAV.MOG induces Tregs

Using AAV hepatic gene therapy, we have shown that Ag-specific CD4$^+$CD25$^+$FoxP3$^+$ Tregs are induced *in vivo* and play a key role in restoring tolerance.[18,25,26] However, it remains unclear if our treatment results in an increase in Treg frequencies within the peripheral lymphatics. Using freshly isolated peripheral blood mononuclear cells from MOG$_{119-132}$-induced EAE, we confirmed a small but significant increase in the relative frequencies of Tregs 2 weeks after AAV.MOG injection (Figure 3A). Additional analysis of the Treg frequency at 40 days after EAE revealed a significant increase within the draining axillary (Figure 3B) and inguinal (Figure 3C) lymph nodes in treated mice as compared with controls, although no statistical difference was seen in the spleen (Figure 3D).

To further demonstrate the therapeutic effectiveness of AAV.MOG is dependent on the induction of FoxP3$^+$Tregs, we used transgenic reporter mice (DTR-Foxp3^{gfp+}) that express the human diphtheria toxin (DT) receptor under the control of the FoxP3$^+$ promoter. In this system, the administration of DT results in the rapid and specific depletion of FoxP3$^+$ Tregs, regardless of Ag specificity.[27] In this experiment, FoxP3$^+$Tregs were depleted at the time of vector administration once mice developed early stage tail paralysis and 2 days later. Unlike the AAV.MOG mice, EAE disease continued to progress



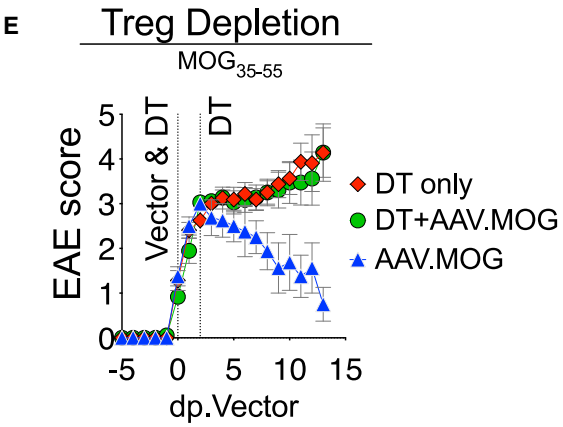
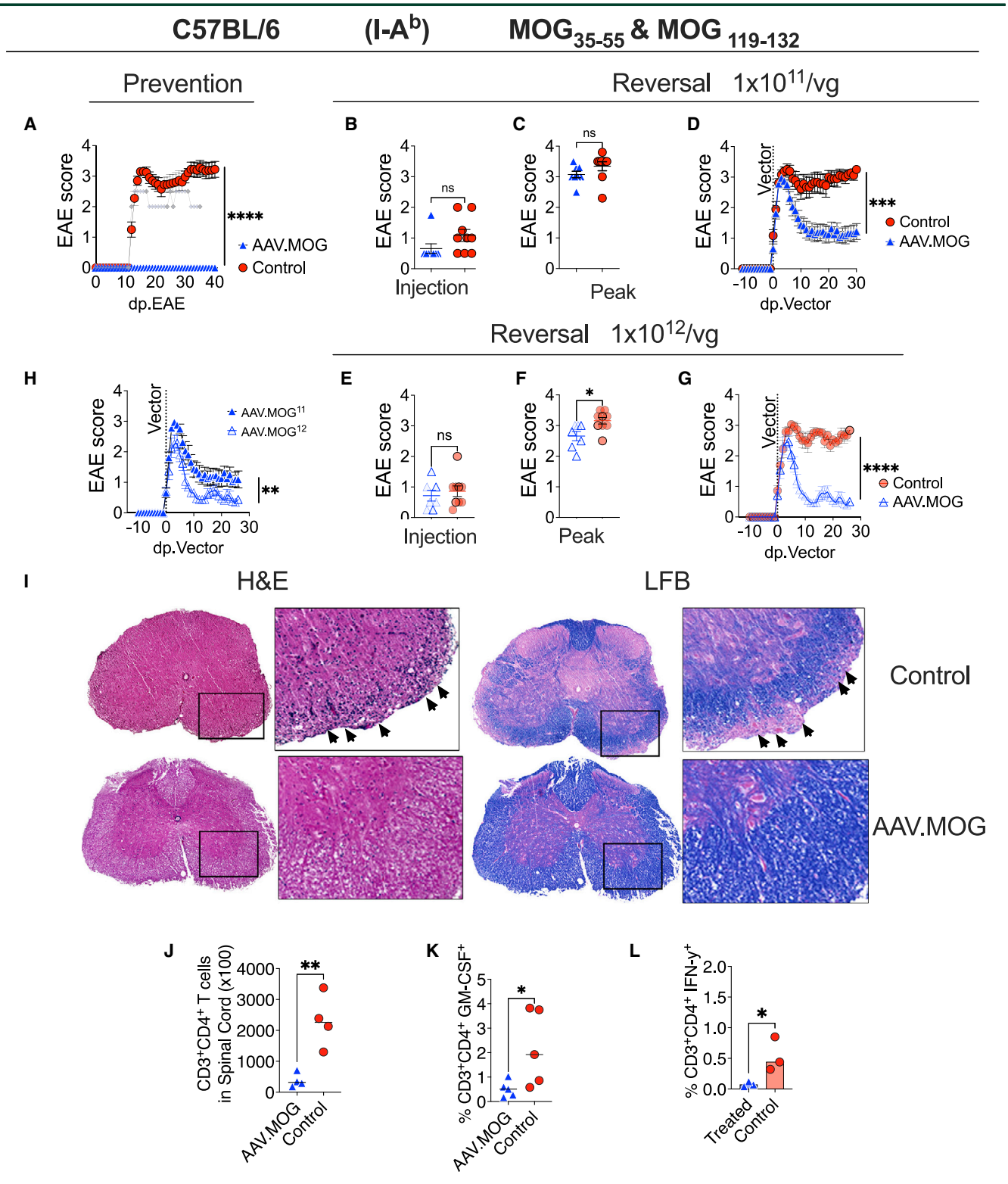


Figure 3. AAV.MOG is Treg Dependent

Female C57BL/6 mice were intravenously injected via the tail vein with 10^{11} vg/mouse of either AAV.MOG or control vector. Two weeks later, EAE was induced with $MOG_{119–132}$. (A) The frequency of $CD4^+CD25^+FOXP3^+$ Tregs (mean ± SEM) present in blood 2 weeks after vector administration (n = 10/group; unpaired two-tailed t test; p = 0.0459). (B) Frequency of Tregs (mean ± SEM, n = 5/group; unpaired two-tailed t test) isolated from the axially lymph node (p = 0.03), (C) inguinal lymph node (p < 0.001), and (D) spleen. (E) To demonstrate that induced Tregs are responsible for protection EAE was induced with $MOG_{35–55}$. At first sign of disease (EAE score of approximately 1), vector was administered and Tregs were systemically depleted concomitantly, and 3 days later using DT (the Mann-Whiney test was used to determine differences).



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in those treated with DT (Figure 3E). Cumulatively, these results strengthen the hypothesis that AAV.MOG immunotherapy restores tolerance and prevents early disease progression through a Treg-mediated mechanism.

AAV.MOG is effective at preventing and reversing autoimmune disease induced with multiple MOG epitopes simultaneously

For an immunotherapy to be maximally effective against MS, it must be effective against the simultaneous presentation of multiple encephalitogenic epitopes. While we have demonstrated that AAV.MOG treatment is effective against various epitopes individually, the fact is that the concurrent presentation of several epitopes occurs in EAE and MS patients, especially as disease progresses. Considering this point, we postulated that the natural processing of the full-length transgene protein delivered by our AAV vector would result in generation of the Ag-specific Tregs clones needed to suppress the various epitope-specific effector T cells used to induce in EAE.

To assess whether AAV.MOG is effective at attenuating EAE that has been induced by multiple immunogenic epitopes derived from MOG protein, B6 mice were tolerized with AAV.MOG or AAV.Null 2 weeks before EAE was induced with a mixture of both $MOG_{35–55}$ and $MOG_{119–132}$ Ags in equal concentration emulsified in CFA. Much like previous observations, between days 10 and 14 control mice developed severe clinical signs of disease that peaked with an EAE score of 3.3 ± 0.2 and persisted until termination of the experiment (Figure 4A). In contrast, 90% of animals in the treated group never developed EAE (single non-responder graphed independently).

Next, we evaluated the ability of AAV immunotherapy to reverse pre-existing disease performed above using the same mixture Ags (Figures 4B–4D). As expected, both groups developed severe neurological deficits peaking with similar EAE scores (Figure 4C). However, the AAV.MOG group quickly responded to treatment and ended with a significantly lower level of disability (1.2 ± 0.3) compared with the control group (3.3 ± 0.1) (Figure 4D). To see if disease reduction could be further improved, we repeated the experiment using a higher vector dose (10^{12}vg) (Figures 4E–4G). The results did indeed reveal a significant dose-dependent recovery (Figure 4H).

Pathological studies of spinal cords revealed that control animals contained multiple foci of cellular infiltration that corresponded with areas of severe demyelination on serial cut sections (Figure 4I). In striking contrast, the spinal cords from treated animals showed little to no signs of inflammation or demyelination. The difference in $CD3^+CD4^+$ T cell infiltrates was confirmed by flow cytometric analysis of cells isolated by a density gradient from individual spinal cord tissue (Figure 4J).

We also probed the isolated cells for specific cytokine expression by intracellular staining to begin elucidating potential mechanisms that may be involved. Cytokines are small, signaling proteins responsible for immune modulation and migration. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an inflammatory cytokine that plays an important role in a host of autoimmune diseases.[28,29] It has been demonstrated that GM-$CSF^{-/-}$ mice are resistant to EAE, display decreased Ag-specific proliferation of splenocytes, and fail to sustain immune cell infiltrates in the CNS.[30] In patients suffering from MS, GM-CSF has been shown to be up-regulated in cerebrospinal fluid, along with increased frequencies of GM-CSF producing T memory cells.[31-33] Interferon-gamma (IFN-γ) is a pleiotropic cytokine historically considered the hallmark Th1 cytokine responsible for driving inflammation in MS.[34] Increased IFN-γ levels are associated with exacerbation of disease severity and development of demyelinating lesions.[35-38] This is likely due to the fact that IFN-γ production results in increased C-C motif chemokine ligand 2 production and is thus responsible for the recruitment of monocytes and macrophages to the CNS.[39-41] This is corroborated by the fact that animals lacking IFN-γ producing T cells fail to develop EAE.[42,43] To determine if the lack of cellular infiltrates may be due, in part, to AAV.MOG modulating cytokine levels within the CNS, we performed intracellular cytokine staining for GM-CSF and IFN-γ in T cells harvested at peak of disease from the spinal cord of treated and control B6 mice induced with $MOG_{35–55}$. AAV.MOG-treated animals showed significantly reduced levels of GM-CSF (Figure 4K) and IFN-γ-producing T cells (Figure 4L) in the CNS as compared with controls.

AAV.MOG protects mice with different genetic backgrounds

Disease heterogeneity is a hallmark characteristic of MS and likely stems from the complex interplay of a patient's genetic background and environmental factors.[44] The human leukocyte antigen (HLA)

Figure 4. AAV.MOG prevention and reversal of disease induced with multiple immunogenic epitopes

(A) Prevention of EAE disease. Age-matched female C57BL/6 (I-A^b) mice (9–10 weeks old) were intravenously injected via the tail vein with 10^{11} vg/mouse of either AAV.MOG or control vector and 2 weeks later EAE was induced with a mixture of $MOG_{35–55}$ and $MOG_{119–132}$ in equal concentrations. Mean EAE score of mice is shown as mean ± SEM (n = 10/group). Reversal of EAE disease. EAE was induced before vector administration with a mixture of $MOG_{35–55}$ and $MOG_{119–132}$ in equal concentrations. At first signs of disease (shown as mean ± SEM; n = 8–9/group) individual mice received (B) 10^{11} or (E) 10^{12} vg/mouse of either AAV.MOG or control vector via the tail vein. (C and F) Peak scores of each group during onset (<10 days). (D and G) Treated mice had a significant reduction in score compared to control mice ($p < 0.001$ and $p < 0.0001$, respectively; the Mann-Whiney test was used to determine differences). (H) Comparison of 10^{11} and 10^{12} vector doses ($p = 0.0094$; the Mann-Whiney test was used to determine differences). (I) Representative histological images of spinal cord demonstrating multiple foci of inflammation in the white matter of control mice (hematoxylin and eosin stain [H&E]) and a serial cut section of spinal cord from the same mouse showing multifocal demyelination associated with the areas of inflammation (Luxol fast blue [LFB]). AAV.MOG-treated mice showed little to no inflammation or demyelination (bottom row). (J) Number of $CD4^+CD3^+$ T cells (mean ± SD) isolated from individual spinal cords at endpoint approximately 30 days of prevention study (n = 4/group; unpaired two-tailed t test; $p = 0.0049$). (K) Percentage of $CD4^+CD3^+GM\text{-}CSF^+$ T cells isolated from the spinal cords (mean ± SD; n = 5/group; $p = 0.0456$, unpaired two-tailed t test). (L) Percentage of $CD4^+CD3^+INF\text{-}\gamma^+$ T cells isolated from the spinal cords at peak of disease (mean ± SD, n = 3/group; $p = 0.0470$; unpaired two-tailed t test).

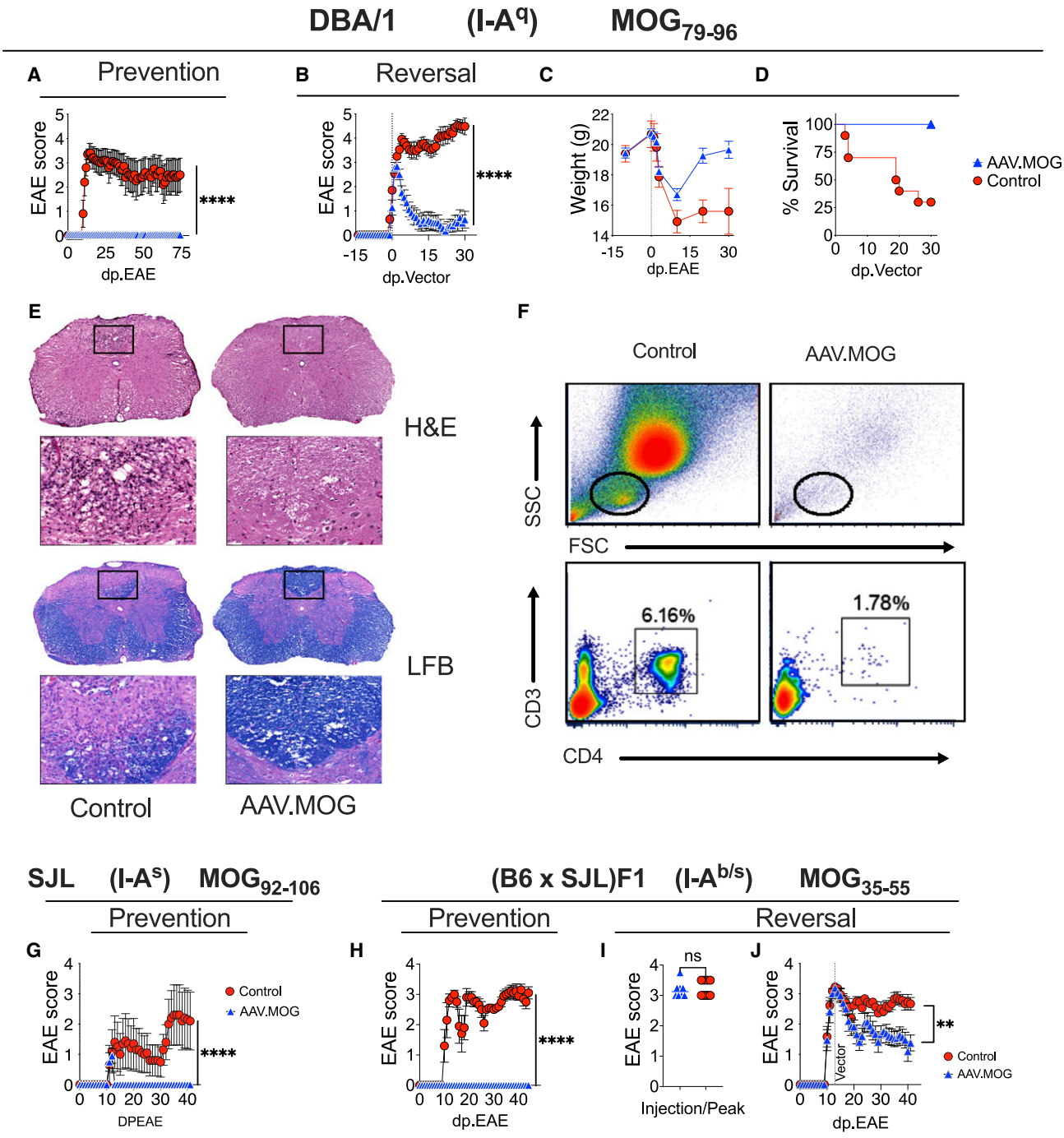

Figure 5. Efficacy of AAV.MOG immunotherapy in various strains of genetically diverse mice

DBA/1 with MHC haplotype I-A^q. (A) To demonstrate prevention of disease, age-matched female mice were intravenously injected via the tail vein with 10^{12} vg/mouse of either AAV.MOG or control vector 2 weeks before EAE was induced with MOG$_{79-96}$. The mean EAE score of mice is shown as mean ± SEM (n = 5/group; $p < 0.0001$; the Mann-Whiney test was used to compare differences). (B) For reversal of disease, EAE was induced before vector administration. As mice developed disease with a score of approximately 1–2, individual mice received 10^{12} vg/mouse of AAV.MOG or control vector via the tail vein. Treated mice rapidly responded, returning to near baseline, compared with the control mice (mean ± SEM; n = 9–10/group; $p < 0.0001$; the Mann-Whiney test was used to compare differences). (C) Comparison of weight loss between treated and control mice (g ± SEM). (D) Kaplan-Meier analysis showing 70% of the control mice failed to survive, compared with 100% of the treated mice. (E) Representative histological images of spinal cord showing an area of inflammation in the white matter of control mice (hematoxylin and eosin stain [H&E]) and a serial cut section of spinal cord from the same mouse showing demyelination in the corresponding area associated with the inflammation (Luxol fast blue [LFB]). AAV.MOG-treated mice showed little to no

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system is an important part of the immune system and is responsible for encoding the MHC. The inherent variability allows individuals to present and recognize different epitopes as immunogenic. This natural variability is a difficult obstacle for Ag-specific therapies to address. Even if the epitopes for one MS patient are known, a different patient, with a distinct HLA background, might respond to an entirely different set of epitopes. Therefore, an Ag-specific immunotherapy needs to be universally effective, regardless of the genetic background of the patient.

Within EAE, it is known that both the genetic background and the specific disease-inducing epitope influence disease severity.[14,44] While our studies clearly show that in B6 (I-A^b) animals AAV gene immunotherapy is highly protective in response to multiple MOG epitopes when given singularly or simultaneously, it is yet to be determined if it would be effective in mice that are genotypically different.

To resolve this concern, we evaluated the vector efficacy in other EAE-susceptible strains of mice that varied in their genetic background, including their MHC haplotype. Unlike B6 mice, the immunodominant MOG epitope in DBA/1 mice is $MOG_{79–96}$ which is presented by I-A^q. Thus, we tolerized DBA/1 mice with AAV.MOG or AAV.Null 2 weeks before EAE was induced with $MOG_{79–96}$. By day 10, mice in the control group began displaying signs of ascending paralysis that progressed to severe neurological deficits (Figure 5A), whereas Treated mice remained disease free throughout the 75-day experiment. We then evaluated the ability of AAV.MOG to reverse active disease and the results again demonstrated that our immunotherapy can significantly ameliorate disease (Figures 5B and 5C). Notably, the severity of EAE in the control group resulted in 70% of the animals succumbing to disease. (Figure 5D).

Pathological analysis of spinal cords was performed to determine if our treatment modulated cellular infiltration and demyelination in I-A^q mice. Unlike treated mice, multiple foci of cellular infiltrates were found in control animals (Figure 5E). These areas typically corresponded with areas of demyelination in serially cut sections. The paucity of infiltrates in treated mice was confirmed by flow cytometric analysis which showed a significantly higher percentage of $CD3^+CD4^+$ cells isolated from the spinal cords of control animals (Figure 5F).

To further test the range of genetic and epitope diversity, we evaluated the ability of the vector to prevent EAE induced by the immunogenic epitope $MOG_{92–106}$ in SJL (I-A^s) mice. After resolving an initial mild tail paralysis between days 11 and 12, all treated mice remained symptom free compared with the control mice, which developed severe neurological deficits (Figure 5G).

As a final demonstration of the versatility of the vector to establish tolerance in complex genetically diverse mice, we used the offspring from a B6 (I-A^b) female crossed with an SJL (I-A^s) male. The F1 generation is heterozygous for both alleles at all loci (I-$A^{b/s}$ mixed haplotype). Consistent with the previous examples, prophylactic AAV.MOG immunotherapy completely prevented clinical symptoms (Figure 5H). Translationally important, when vector was administered therapeutically at peak of disease on day 13 after EAE (Figure 5I), disease was attenuated significantly in treated mice compared with controls (Figure 5J). Overall, this series of experiments further supports the hypothesis that AAV.MOG immunotherapy is effective not only against multiple immunogenic epitopes of MOG, but also across genetically diverse strains of mice.

DISCUSSION

MS is a chronic neurodegenerative disease where CNS inflammation and demyelination are mediated by an unregulated immune response to multiple encephalitogenic autoantigens.[45] The ideal therapy for MS would be an Ag-specific therapy that would suppress only the destructive autoimmune responses, while leaving the immune system intact. It would provide patients with long-term protection without the need for extended systemic immune suppression.[46] In the search for such a therapy, various approaches for inducing Ag-specific Tregs have been investigated (well-reviewed in Serra et al.[3]). Traditionally, prior identification of the immunogenic epitopes to be targeted has been considered a prerequisite for developing Ag-specific therapies.[47] Despite some experimental success in animal models, the development of Ag-specific therapies for MS has been hindered by the complexity of identifying all the possible epitopes. For example, within a single myelin protein such as MOG, there may be multiple immunoreactive epitopes. In fact, it has been shown that T cells from MS patients proliferated strongly to $MOG_{35–55}$, $MOG_{119–130}$, and $MOG_{186–200}$ epitopes.[22] In another report, Forsthuber et al.[14] showed that $MOG_{97–108}$ was the dominant epitope in patients of the HLA-DRB1*0401 background. This type of epitope diversity is not limited to just MOG. Both myelin basic protein and proteolipid protein have been shown to contain multiple antigenic epitopes.[48]

Another major obstacle hindering the development of Ag-specific treatments for MS is intramolecular or intermolecular epitope spreading of T cell responses. Epitope spreading is when an immune response develops to an unpredicted, cryptic, or secondary epitope(s) substantially different from the original pathogenic epitope.[14] Ordinarily, peripheral T cell responses to dominant myelin epitopes develop first during initial disease. However, as demyelination and tissue damage progress, the autoreactive T cell repertoire may increase to newly emerging, encephalitogenic myelin epitopes. This new reactivity is

inflammation or demyelination (bottom, right). (F) Representative flow cytometry demonstrating the sparsity of inflammatory cells able to be isolated and the lack of $CD3^+CD4^+$ T cells infiltrating the spinal cords of treated mice compared to control mice. SJL mice with MHC haplotype I-A^s. (G) Mice received 10^{12} vg/mouse of AAV.MOG or control vector via the tail vein 3 weeks before EAE was induced with $MOG_{92–106}$ (mean ± SEM; n = 5/group; $p < 0.0001$; the Mann-Whiney test was used to compare differences). F1 generation of B6xSJL mice with mixed MHC haplotype I-$A^{b/s}$ using $MOG_{35–55}$ epitope for (H) prevention (mean ± SEM; n = 5/group; $p < 0.0001$; the Mann-Whiney test was used to compare differences) and (I and J) reversal of disease, as before (mean ± SEM; n = 9–10/group; $p = 0.0011$; the Mann-Whiney test was used to compare differences) mice received 10^{11} vg/mouse of AAV.MOG or control vector via the tail vein.

initiated in part by *in vivo* phagocytic processing of myelin debris and subsequent presentation of *de novo* myelin epitopes by CNS-resident antigen-presenting cells (APCs).[49] As a result, trying to accurately predict or define all the target epitopes may be futile.

To overcome these and other challenges, various Ag-specific strategies have been clinically tested with mixed results.[13] One promising clinical trial from Juryńczyk et al.[50] used transdermal patches to deliver a cocktail of three myelin epitopes, which significantly decreased myelin-specific T cell proliferation in patients as compared with placebo controls. The decrease in proliferation was, in part, attributed to induction of regulatory cells to multiple Ags. While somewhat successful, this method still relied on prior knowledge of target epitopes and will arguably remain ineffective against cryptic epitopes or those resulting from epitope spreading.

In our initial report, we detailed the development of a liver-directed AAV gene therapy for an autoimmune disorder.[14] We showed that AAV.MOG gene immunotherapy induced $MOG_{35–55}$ specific Tregs and was capable of preventing and reversing EAE induced by the immunodominant $MOG_{35–55}$ epitope.[18] Although the results were remarkable, the study was limited because EAE was only induced by a single MOG epitope and is not representative of the complex of epitope diversity seen in MS. To correct this experimental weakness, we re-evaluated our AAV.MOG immunotherapy in more complex EAE models driven by an increased repertoire of autoreactive T cells clones targeting multiple MOG epitopes in various strains of genetically diverse mice.

Unlike some other technologies, prior knowledge of specific immunogenic epitopes is not required for our AAV immunotherapy. By engineering the vector to encode the full-length MOG protein, it conceivably contains all possible epitopes. This approach exploits the liver's naturally effective mechanism for the induction and maintenance of peripheral tolerance and circumvents the need to pre-identify specific epitopes.

In sum, we clearly show that a single treatment of AAV.MOG can prevent and significantly decrease or reverse EAE induced by multiple immunogenic epitopes of MOG, regardless of the genetic background of the mice.[18,51,52] Given the ability to protect against all epitopes tested further suggests that our AAV.MOG immunotherapy will be effective against epitope spreading. Importantly, this discovery represents a significant advancement in Ag-specific therapies and may be Ehrlich's "magic bullet" for MS[53] and the larger autoimmune and gene therapy fields.

MATERIALS AND METHODS

Animals and immunization

Female inbred C57BL/6, SJL/J, (C57BL/6xSJL/J)F1, and DBA/1J mice were purchased from The Jackson Laboratory. B6.129(Cg) $Foxp3^{tm3(DTR/GFP)Ayr}$/J ($FOXP3^{DTR}$) breeder pairs were purchased from The Jackson Laboratory and were bred in house at the University of Florida. Animals were approximately 8–10 weeks old at the time of disease induction. EAE was induced by 2 subcutaneous injections each containing 100 μg peptide ($MOG_{35–55}$, $MOG_{119–132}$, prevention of $MOG_{79–96}$, $MOG_{92–106}$, and $MOG_{1–125}$) or 50 μg peptide ($MOG_{119–132}$/$MOG_{35–55}$ for a mixed total of 100 μg, and reversal of $MOG_{79–96}$) emulsified in CFA (Sigma Aldrich) supplemented with 9 mg/mL *Mycobacterium tuberculosis* (Difco Laboratories). Pertussis Toxin (200 ng or 500 ng for $MOG_{92–106}$ induction only, List Biological Laboratories, Inc., was injected intraperitoneally 2 and 24 h after immunization with CFA. All peptides were based on mouse proteins.

Clinical scoring of disease

Animals were monitored daily and the mean clinical score was recorded according to a 5-point scale: 0, no clinical signs of disease; 1.0, complete tail paralysis; 2.0, loss of coordinated movement or dragging of one hind foot; 2.5, dragging of both hind feet or complete paralysis in one hindlimb but other hindlimb showing a lesser form of paralysis; 3.0, complete hindlimb paralysis or both hind limbs capable of moving but not forward of the hip; 3.5, complete hindlimb paralysis and unable to right oneself when placed on side or hindquarters flat like a pancake; 4.0, complete hindlimb paralysis and partial forelimb paralysis; and 5.0, moribund. Mice were euthanized at any score higher than a 4.0 or when animals remained a 4.0 for 48 h (Table 1).

Vector production

A recombinant scAAV vector expressing full length MOG under a liver-specific promoter (AAV.MOG) or control vector expressing lacZ without a promoter (AAV.Null) was produced by the method of transfection using polyethyleneimine maximum molecular weight 40,000 (PEI) (Polysciences, Inc.) as a transfection reagent into human embryonic kidney (HEK293) cells, below passage 50. Two plasmid DNAs—recombinant construct flanked by the AAV inverted terminal repeats, pAAV apolipoprotein E/human alpha-1 antitrypsin, and a helper plasmid for AAV8 serotype (pDG8) mixed in equimolar amount—totaling 30 μg per 15-cm plate—were added to each plate containing 80%–90% confluent cells. Media was changed 3 h after transfection and supplemented with 5% fetal bovine serum. Both media and cells were collected on day 3 after transfection.

Cells were resuspended in 20 mM Tris/HCl (pH 8.5)/15 mM NaCl lysis buffer, 10 mL per 1–2 × 10^8 cells. The virus was precipitated from media with 1/4 collected volume of 40% polyethylene glycol 8,000/2.5 M NaCl solution stored overnight at 4°C then pelleted and resuspended in 10-mL lactated Ringers' solution. Cells were lysed by one-time freeze/thaw cycle and three 1-minute rounds of sonication on ice. The virus recovered from both media and cells was incubated at 37°C with 2.2 μL Turbonuclease from Serratia marcescens (Sigma Aldrich) and saturated MgCl per 10mL of volume for 30 min. Virus recovered only from media was incubated for an additional 30 min with 0.5 mL 10% NA-deoxycholate per 10 mL volume. Clarified lysates ran on a step iodixanol density gradient and dialyzed/concentrated on Amicon Ultra centrifugal filters with 1× PBS/0.35 M NaCl/5% sorbitol final vector solution. The titer of each preparation was estimated using a ddPCR-evergreen assay, as previously described.[54]

Vector administration

Prophylactic

Animals were injected with 10^{11} or 10^{12} vg of either AAV.MOG or AAV.Null via the tail vein 2 weeks before EAE induction.

Therapeutic

EAE was induced first and at a predetermined EAE score; mice were treated with 10^{11} or 10^{12} vg of either AAV.MOG or AAV.Null via the tail vein.

Treg depletion by DT administration

To deplete Tregs in DTR-Foxp3^{gfp+} mice, mice received two intraperitoneal injections of DT 50 μg/kg at the time of vector administration and 2 days later. The animals were bred and housed under specific pathogen-free conditions at the University of Florida and treated under approved protocols of the Institutional Animal Care and Use Committee.

Alanine aminotransferase activity assay

Hepatocellular injury was assessed using plasma samples in duplicate at 1:2 dilution with an Alanine Aminotransferase Activity Assay kit according to the manufacturer's protocol (Sigma-Aldrich).

Intracellular staining

Animals were euthanized at peak of disease (approximately 15 days after disease induction, MCS approximately 3.5) and perfused with PBS. Spinal cords and spleens were harvested and processed into single cell suspensions. We coated 12-well plates with 2×10^6 APCs/well from spleen in 10% complete RPMI. Lymphocytes were isolated from spinal cords via a Percoll gradient and added to APC cultures. Positive control wells contained 10 ng/mL PMA and 500 ng/mL ionomycin; negative control wells contained APCs and lymphocytes only in complete media, and sample wells contained APCs and lymphocytes in the presence of 10 μg/mL MOG_{35-55}. Cells were allowed to rest at 37°C and 5% CO_2 for 4 h, then Brefeldin A was added (1×). Cells were incubated in the presence of Brefeldin A (Invitrogen, Carlsbad, CA) for 12 h. Cells were then harvested, extracellularly stained, intracellularly stained, and analyzed via flow cytometry (below).

Flow cytometry

Peripheral blood, spleens, and lymph nodes were harvested from mice and processed into single cell suspensions. Suspensions were stained with antibodies: CD3 (145-2C11), CD4 (RMF4-5), CD25 (PC61), CD62L (MEL14), CTLA-4 (UC10-4B9), CD44 (IM7), PD-1 (29F.1A12), Nrp-1 (3E12) (Biolegend, San Diego, CA), CD39 (24DMS1), Foxp3 (FJK-16s) GM-CSF (MP1-22E9), or IFN-γ (XMG1.2). Intracellular staining, and red blood cell lysis, were performed using the FOXP3 staining kit (Invitrogen). Samples were analyzed on a Fortessa flow cytometer (BD Biosciences). Analyses were performed using FCS Express 6 (De Novo Software).

Histology

Histological analyses were performed on formalin-fixed, paraffin-embedded CNS and liver tissues. Serial sections (10 μm) were used. Sections were stained with Hematoxylin and Eosin to identify gross pathology and cellular infiltrates and Luxol Fast Blue staining was used to identify demyelination. Liver sections were stained with only hematoxylin and eosin. Standard staining procedures as previously described were used for both stains.[18]

Immunofluorescence

Immunofluorescent analyses were performed on frozen liver tissues embedded in Tissue-Tek Optimal Cutting Temperature compound. Serial liver sections were cut at 10 μm using a cryostat. Tissue sections were methanol fixed for 20 min in −20°C methanol and placed in blocking solution containing 1% donkey serum for 1 h. Mouse Anti-MOG antibody (AF2439; R&D Systems) was applied to tissue sections at 1 μg/mL and incubated overnight at 4°C. Sections were washed with PBS three times and incubated with 10 μg/mL of Alexa Fluor 488 donkey anti-goat IgG (A11055; Invitrogen) for 1 h at room temperature in the dark. Slides were washed as described previously and coverslips were placed containing ProLong Diamond Antifade Mountant with DAPI (P36962; Invitrogen). Slides were analyzed and photographed on the Keyence BZ-X800.

Western blot

Total protein was extracted from liver, spleen, kidney, and heart tissues using TPER Tissue Extraction Reagent (Thermo Fisher Scientific) and quantified via a bicinchoninic acid protein assay (Thermo Fisher Scientific) per assay protocol. Samples were run on a 4%–20% MiniProtean TGX gels (BioRad) and then transferred to a polyvinylidene fluoride membrane via standard protocols. Membranes were blocked with 1% fat free dried milk in 1× Tris-buffered saline with Tween (TBS) and then incubated with anti-MOG or anti-β actin antibody in 1% fat-free milk for 1 h at room temperature. Membranes were washed with TBS and incubated with horseradish peroxidase-conjugated secondary antibodies. Signal was detected with ECL 2 western blot substrate (Thermo Fisher Scientific).

Statistical analyses

All EAE scores are reported as mean ± standard error of the mean. Prism v9 statistical software (GraphPad) was used to perform statistical analyses identified in the Figure legends.

Study approval

All studies involving animals were carried out in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.12.011.

ACKNOWLEDGMENTS

Funding for this work was provided by NIAID R01 AI128074 and the UF Foundation: Gene Immunotherapy Fund F025797.

AUTHOR CONTRIBUTIONS

G.D.K., C.D.G., A.S.A., K.G.S., I.C., M.R., D.Mi., D.Ma., and B.P. maintained and evaluated mice, processed tissues, performed assays, and collected data. B.E.H., C.D.G., and G.D.K. designed and conducted experiments and analyzed data. G.D.K. and B.E.H. wrote the manuscript; it was edited by C.D.G.

DECLARATION OF INTERESTS

B.E.H. hold patents related to AAV gene therapy. All other authors declare no competing financial interest.

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