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Therapeutic intervention in relapsing autoimmune demyelinating disease through induction of myelin-specific regulatory CD8 T cell responses

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ABSTRACT

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). We have shown that CNS-specific CD8 T cells (CNS-CD8) possess a disease suppressive function in MS and its animal model, experimental autoimmune encephalomyelitis (EAE). Previous studies have focused on the role of these cells predominantly in chronic models of disease, but the majority of MS patients present with a relapsing-remitting disease course. In this study, we evaluated the therapeutic role of CD8 T cells in the context of relapsing-remitting disease (RR-EAE), using SJL mice. We found that $PLP_{178-191}$- and MBP_{84-104}-CD8 ameliorated disease severity in an antigen-specific manner. In contrast, $PLP_{139-151}$-CD8 did not suppress disease. $PLP_{178-191}$-CD8 were able to reduce the number of relapses even when transferred during ongoing disease. We further ascertained that the suppressive subset of CD8 T cells was contained within the $CD25^+$ CD8 T cell compartment post-*in vitro* activation with $PLP_{178-191}$. Using *Listeria monocytogenes* (LM) encoding CNS antigens to preferentially prime suppressive CD8 T cells *in vivo*, we show that LM infection induced disease suppressive CD8 T cells that protected and treated $PLP_{178-191}$ disease. Importantly, a combination of $PLP_{178-191}$-CD8 transfer boosted by LM-$PLP_{175-194}$ infection effectively treated ongoing disease induced by a non-cognate peptide ($PLP_{139-151}$), indicating that this approach could be effective even in the context of epitope spreading. These data support a potential immunotherapeutic strategy using CD8 transfer and/or LM vaccination to boost disease regulatory CD8 T cells.

1. Introduction

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) characterized by the infiltration of immune cells, resulting in neurological dysfunction [1,2]. It is widely understood that MS pathogenesis is mediated by IL-17 and IFNγ-producing CD4 T cells [3], thus the vast majority of studies have focused on the role of these cells in driving demyelinating disease. However, studies have shown that T cells in MS lesions are predominantly of CD8 origin with evidence of oligoclonal expansion [4], indicating an important and understudied role for these cells.

Studies from our lab demonstrate that myelin-specific CD8 T cells possess a regulatory function in MS, and are protective in various types of experimental autoimmune encephalomyelitis (EAE), a murine model of MS [5–9]. A regulatory role for CD8 T cells has been implicated in other autoimmune diseases including type 1 diabetes, rheumatoid arthritis, and inflammatory bowel disease [10–14]. We have observed that unlike conventional regulatory T cell populations, these regulatory CD8 T cells lack Foxp3 expression and do not depend on anti-inflammatory cytokine production (eg. IL-4 or IL-10). Rather, they resemble cytotoxic CD8 T cells, as they depend on IFNγ, granzyme B, and perforin [9,15].

The majority of MS patients (85%) develop a relapsing-remitting disease course [16]. Interestingly, we have shown that regulatory CD8 T cells are deficient in their suppressive capacity during MS relapses [6, 17], suggesting that maintaining intact CD8 T cell regulatory function may prevent disease exacerbation. Therefore, interrogating CD8 T cell regulatory potential in a relapsing-remitting disease setting is an important step in understanding their therapeutic utility in human patients. Relapsing-remitting EAE (RR-EAE) can be induced in SJL/J mice by immunization with various myelin peptide antigens (e.g., $PLP_{139-151}$,

$PLP_{178-191}$, and MBP_{84-104} here referred to as P139, P178, and MBP respectively) [18]. In this model, relapses occur due to epitope spreading in CD4 T cell responses. Furthermore, the hierarchy of this epitope spreading can be predicted given the antigenic dominance of the CD4 T cell response, starting from the inducing epitope and followed by intramolecular and intermolecular spreading [19]. Using this model, we tested whether regulatory CD8 T cells can mediate their protective effects against relapses, a phenomenon not yet understood.

In an effort to convert our findings into an effective MS therapy, we also developed a system of endogenous priming of CNS-CD8 using *Listeria monocytogenes* (LM) engineered to express myelin epitopes. Infection with LM-containing P178 (LM-P178) produced myelin-specific CD8 T cells that were non-pathogenic and, in fact, capable of reducing CNS immune infiltration and suppressing clinical symptoms. These LM-induced regulatory CD8 T cells require perforin and IFNγ, similar to what we have observed in CD8 T cell adoptive transfer models [9]. These studies implicate the potential of using LM as a "vaccination" strategy to endogenously prime regulatory CD8 T cells. Here, we employed CD8 T cell transfer as well as LM infection to develop a therapeutic strategy that could mitigate relapsing-remitting disease. These findings further support the concept of inducing regulatory CD8 T cells for therapeutic intervention in MS.

2. Materials and methods

2.1. Mice

Female SJL/J 6-8-wk-old mice bought from The Jackson Laboratory, (Bar Harbor, ME) were housed in specific pathogen-free animal facilities and transferred to biosafety level 2 conditions for infection studies at the University of Iowa. All animal experiments were approved by the Institutional Animal Care and Use Committee Protocols.

2.2. Induction and evaluation of RR-EAE

Mice were immunized s.c. with 50 μg of PLP peptides or 150 μg MBP emulsified in 1:1 vol with complete Freund's adjuvant (CFA) distributed over two sites on the flank. All mice except those that were immunized with $PLP_{139-151}$, received 250 ng total pertussis toxin on days 0 and 2. Clinical scores were assessed daily in a blinded manner, and animals were scored using the previously defined criteria [7]: 0-normal mouse, 1-limp tail, 2-mild hind limb weakness, 3- moderate hind limb weakness/partial paralysis, 4- bilateral complete hind limb paralysis, and 5-moribund. Relapses were defined as decrease in a score ≥ 1 for at least 2 days following remission. Relapse rate was defined as the number of relapses in a group of mice divided by the number of mice in that group for each day [20].

2.3. CD8 T cell adoptive transfer

Donor mice were immunized with PLP, MBP, or OVA in CFA and administered 250 ng pertussis toxin. At day 15, spleens and inguinal lymph nodes were harvested and reactivated with rIL-2 and cognate antigen for 72hr in culture as previously described [7,9,21]. CD8 T cells were magnetically isolated using CD8α Ly-2 microbeads (Miltenyi) and $5-10 \times 10^6$ live cells were transferred i.v. into recipient mice at times indicated. For experiments isolating $CD25^+$ CD8 T cells, CD8 T cells were first magnetically sorted using a negative selection CD8 T cell isolation kit followed by a CD25 positive selection sort (Miltenyi), and $1-5 \times 10^6$ were transferred i.v. to recipient mice. The $CD25^+$ sort resulted in enrichment rather than a pure population of $CD25^+$ CD8 T cells, the purity increased from about 3% of CD8 T cells being $CD25^+$ pre-sort to 15% $CD25^+$ CD8 T cells post-sort.

2.4. Infection with recombinant myelin epitope-expressing Listeria

Attenuated ($\Delta actA/\Delta inlB$) recombinant *L. monocytogenes* expressing proteolipid protein (PLP) T cell epitopes was generated as described previously [9,22,23]. Briefly, we generated LM codon-optimized constructs containing amino acid coding sequences of the defined proteolipid protein sequences of $PLP_{139-151}$ and $PLP_{178-191}$ and the myelin basic protein (MBP) sequence of MBP_{84-104}, with three flanking amino acids on each end to encourage natural processing. Recombinant LM strains were grown and prepared for injection as previously described. Mice were injected i.v. with 10^7 or 10^8 cfu of recombinant LM in 200 μl sterile saline.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). Single comparisons of two means were analyzed by the Welch's *t*-test. For multi-parametric data, an ANOVA test was used. P values < 0.05 were considered significant.

3. Results

3.1. P178-CD8 both protect and treat in RR-EAE, whereas P139-CD8 fail to ameliorate disease

We previously demonstrated that autoreactive P178-CD8 can attenuate EAE [8,9,21]. These prior studies elucidating the regulatory role of CD8 T cells have been primarily conducted in the C57BL/6 chronic EAE model. However, the majority of MS patients present with relapsing-remitting disease course. To clarify the role of CD8 T cells in the RR-EAE model, we first asked if P178-CD8 could ameliorate relapsing disease in SJL/J mice. P178-CD8 or OVA ($OVA_{323-339}$)-CD8 controls were adoptively transferred and recipient mice were immunized with P178/CFA the following day and monitored for disease progression. In agreement with our previous findings, we found that P178-CD8 transfer resulted in significant reduction of disease severity compared to OVA-CD8 controls (Fig. 1A, top panel). Additionally, P178-CD8 recipients had a reduced relapse rate, suggesting these mice experienced fewer relapses overall compared to control recipients (Fig. 1A, bottom panel).

As mentioned above, there are three peptides classically used to induce RR-EAE for study of epitope spreading [18]. To further understand the antigen-specificity of regulatory CD8 T cells, we tested the ability of MBP- and P139-CD8 to mitigate disease. MBP-CD8 were adoptively transferred into naïve recipients prior to MBP/CFA immunization and monitored for clinical disease. Like P178-CD8, MBP-CD8 were able to mitigate disease severity and reduce the number of relapses (Fig. 1B). In agreement with our prior observations [8], mice that received P139-CD8 showed no difference in disease severity or relapse rates (Fig. 1C), suggesting that P139-CD8 lack a disease suppressing function in RR-EAE. Together, these findings suggest that CD8 T cells of various specificities differ in their capacity to regulate demyelinating disease.

3.2. Activated $CD25^+$ CD8 T cells are enriched for regulatory function

We have observed in multiple models that the suppression of EAE by CNS-CD8 is based on the induction of EAE by the same cognate antigen [5,8,15]. It makes intuitive sense that the suppressive fraction of the transferred CD8 population would be the cells that are specific for the same antigen. Thus, we hypothesized that after 3-day *in vitro* culture, the $CD25^+$ fraction would harbor the suppressive activity (as a surrogate marker for antigenic specificity [17,24,25]). To test this, we harvested cells from P178-immunzed donors, cultured them *in vitro*, then sorted for $CD25^+$ vs. $CD25^-$ CD8 T cells. These cells were transferred into naïve recipient mice, followed by active induction of EAE the following day.
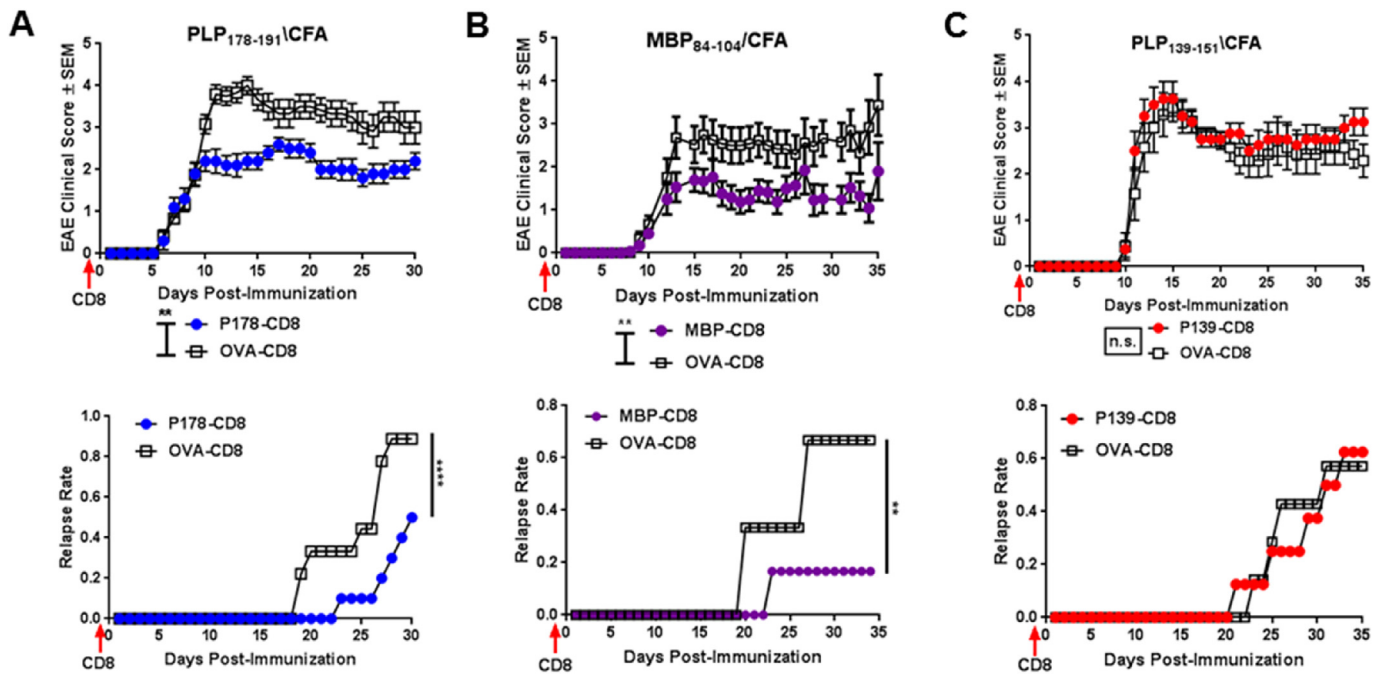
Fig. 1. CD8 T cells with various specificities differ in regulatory capacity in RR-EAE. On d-1, mice received (A) P178, (B) MBP, or (C) P139-CD8, derived from immunized donor mice, followed by 3 day *in vitro* activation. Activated OVA-CD8 were used as controls. The following day, mice were immunized with cognate antigen and CFA to induce RR-EAE and monitored for clinical disease. Mean clinical scores (top row) and relapse rates (bottom row) are shown. Data are representative of 2–3 independent experiments each with at least 5 mice per group per experiment. **p < 0.01, ****p < 0.0001, ns = not significant.

Mice that received the CD25+ CD8 T cells had significantly reduced disease in comparison to mice that received the OVA-CD8 controls or the CD25− fraction of P178-CD8 (Fig. 2A). CD25− CD8 T cells did not suppress disease, when transferred at either 1×10^6 (data not shown) or even at 5×10^6 (Fig. 2A). In contrast, 1×10^6 CD25+ CD8 T cells were sufficient to confer protection, compared to $5–10 \times 10^6$ bulk CD8 T cells used to see clinical disease suppression in Fig. 1. Similar to bulk P139-CD8, neither CD25+ P139-CD8 nor CD25− P139-CD8 were able to ameliorate disease (Fig. 2B).

3.3. P178-CD8 effectively treat ongoing RR-EAE

We next wanted to test whether P178-CD8 could treat mice during actively ongoing disease. Thus, P178-CD8 were transferred during the initial acute phase of P178-induced EAE (day 12), and mice were monitored for clinical disease. Mice that received P178-CD8 had significantly ameliorated disease (Fig. 3A) and reduced relapse rate (Fig. 3B) compared to OVA-CD8 controls. Interestingly, this disease suppression occurs rapidly after CD8 T cell transfer, suggesting CD8 T cells could be an effective treatment strategy.

3.4. LM-P178 infection ameliorates disease whereas LM-P139 infection does not

In a recent study, we demonstrated a model of endogenous priming of myelin-specific CD8 T cells using *Listeria monocytogenes* (LM) strains
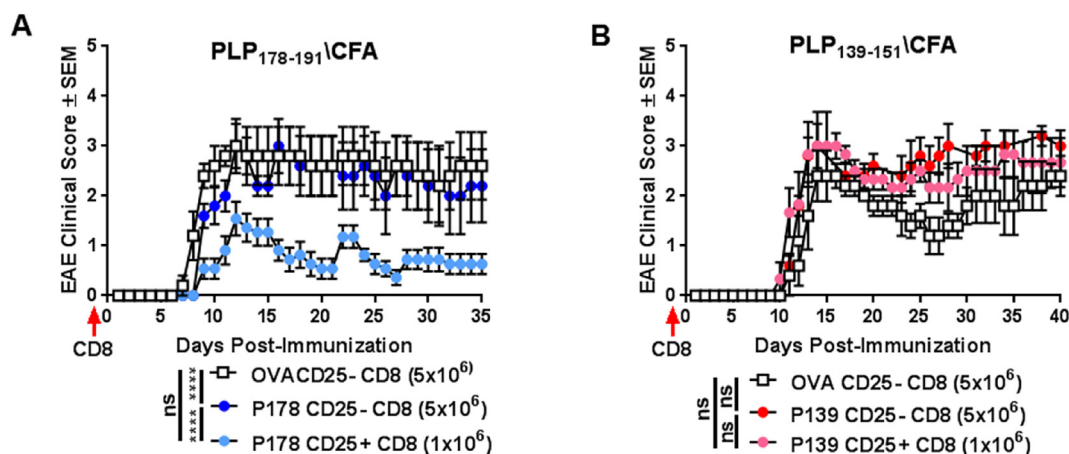
Fig. 2. Activated CD25+ CD8 T cells are enriched for disease suppressive ability. (A) CD8 T cells from P178- or OVA-immunized donor mice were activated *in vitro* for 3 days. P178 CD25+ CD8 T cells, P178 CD25− CD8 T cells or OVA-CD8 were transferred i.v. into naïve recipients on d-1, followed by immunization with P178/CFA the following day and monitoring for clinical disease. (B) CD25+ P139-CD8, CD25− P139-CD8 T cells or OVA-CD8 were transferred i.v. into naïve recipients on d-1. Recipient mice were immunized with P139/CFA the following day and monitored for clinical disease. Data are representative of 3 independent experiments each with at least 3–5 mice per group. ****p < 0.0001 and ns = not significant.
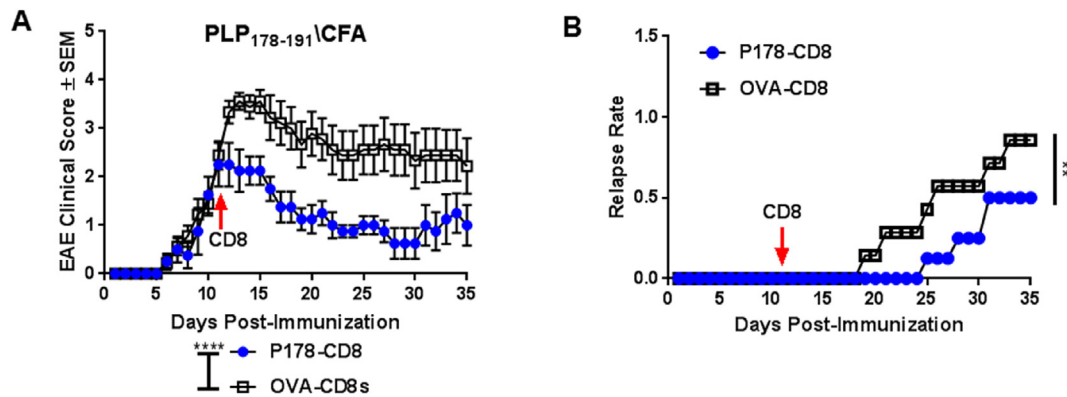
Fig. 3. P178-CD8 effectively treat RR-EAE. P178-CD8 were transferred i.v. into recipient mice on d11 after immunization with P178/CFA. OVA-CD8 were used as controls. Mean clinical scores (A) and relapse rates (B) are shown. Data are representative of 2–3 independent experiments with at least 5 mice per group. **p < 0.01 and ****p < 0.0001.

engineered to express relevant myelin peptides. Infection with LM-P178 preferentially induced a robust CD8 T cell response and these LM-primed CD8 T cells were able to reduce disease severity in chronic EAE. Similar to our experience from C57BL/6 mice [9], neither LM-P178 infection nor LM-P139 infection can induce paralytic disease in SJL mice, with or without pertussis toxin administration (data not shown). Using this system, we tested whether LM-P178 infection/vaccination would have a comparable disease suppressive effect in RR-EAE. Mice were infected on d-7 with LM-P178, immunized with P178/CFA on d0, and monitored for clinical disease. Mice that received LM-P178 had significantly ameliorated disease and reduced relapse rate compared to mice that received control LM-OVA infection (Fig. 4A). We also evaluated the efficacy of LM-P178 as a treatment for RR-EAE. Mice that were infected with LM-P178 during the acute phase of disease experienced fewer relapses than their LM-OVA infected counterparts (Fig. S1). In contrast to

LM-P178 infection, but in agreement with our CD8 T cell adoptive transfer experiments, we found that LM-P139 infection did not result in significant change in disease severity in P139-induced EAE (Fig. 4B), again suggesting that LM-induced CD8 T cell responses mimic those induced by immunization with exogenous peptides.

Next, we wanted to determine whether the LM-P178-induced CD8 population could be used to adoptively transfer protection to naïve mice and if it was also contained in the CD25$^+$ fraction, similar to immunization-derived CD8 T cells. Donor mice were infected with LM-P178 and 7 days later, splenocytes were harvested, activated *in vitro* for 72 h, sorted into CD25$^+$ and CD25$^-$ CD8 fractions, and then transferred into naïve recipient mice. Recipients were immunized with P178/CFA the following day and monitored for clinical disease. Mice that received CD25$^+$ CD8 T cells experienced significantly ameliorated disease compared to those mice that received CD25$^-$ CD8 T cells or OVA-
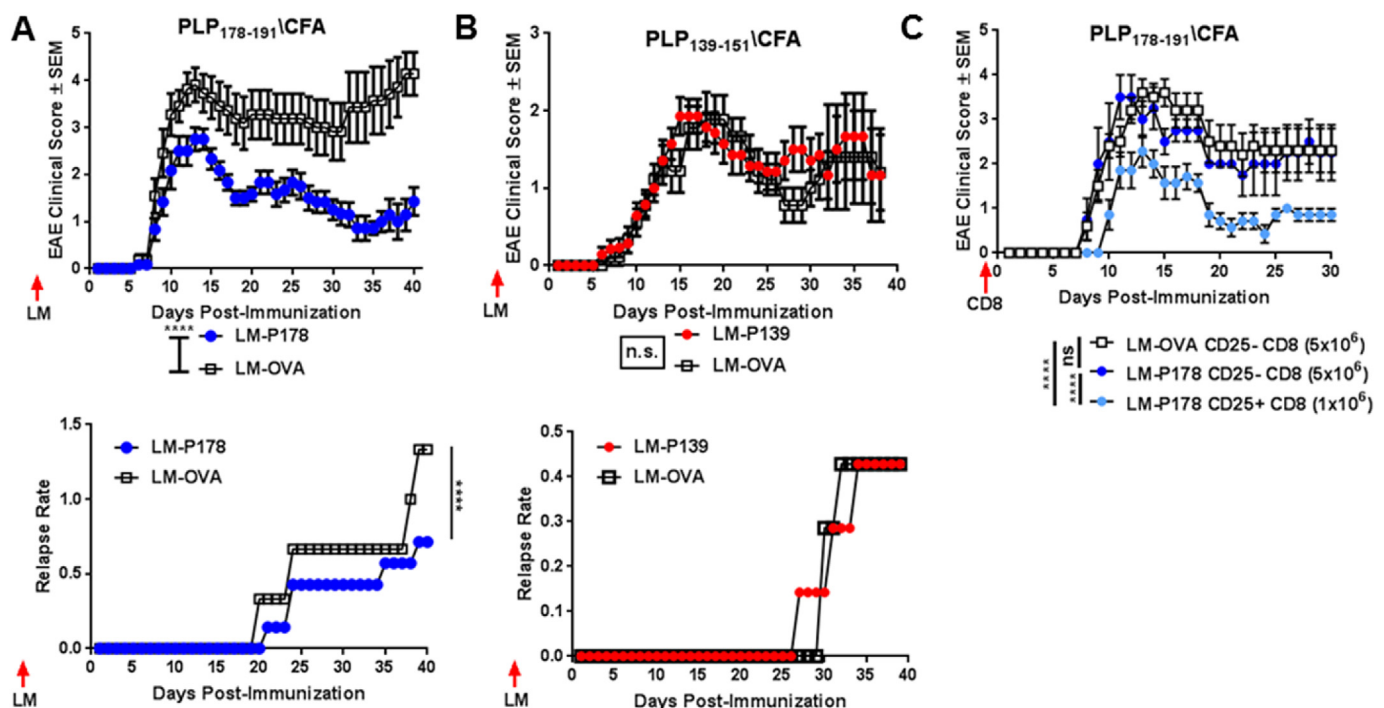
Fig. 4. LM-P178 vaccination ameliorates disease, while LM-P139 vaccination does not. Mice were infected with either (A) LM-P178 or (B) LM-P139 on d-7 and immunized with cognate antigen and CFA on d0. LM-OVA infection was used as a control. Mice were monitored for clinical disease (top panels) and relapse rates were calculated (bottom panels). (C) CD8 T cells from mice infected with LM-P178 were activated *in vitro* and sorted into CD25$^+$ and CD25$^-$ fractions and transferred i.v. into naïve recipient mice on d-1 (OVA-CD8 served as controls). Recipients were immunized with P178/CFA on d0 and monitored for clinical disease. Data are representative of 2-3 independent experiments with at least 5 mice per group per experiment. ****p < 0.0001 and ns=not significant.

CD8 (Fig. 4C). This suggests that LM priming induces a CD25$^+$ regulatory CD8 T cell population like we identified in our adoptive transfer studies.

3.5. LM-P178 infection suppresses ongoing P139-induced EAE

Epitope spreading has been well described and characterized as the primary cause of relapses in the RR-EAE model [19,26]. The pattern of this phenomenon is predictable, such that in P139-immunized mice, the acute phase of disease is mediated by P139-CD4 responses, whereas the primary relapse is predominantly mediated by P178-CD4, and the secondary relapse by MBP-CD4. Therefore, we asked whether disease relapse could be targeted using a CD8-based approach.

To test this, mice immunized with P139/CFA were treated with P178-CD8 at various stages of disease course and monitored for clinical disease. P178-CD8 were not efficient at mitigating disease when given prior to disease initiation, during acute phase of disease, or during the first remission of disease (Fig. S2). This suggested that adoptive transfer of regulatory P178-CD8 was not sufficient to prevent relapses caused by P178-CD4.

Next, we asked if LM-P178 vaccination would serve as a better strategy to prevent P178-induced relapses in P139-induced disease. Thus, mice immunized with P139/CFA were infected with LM-P178 at various stages of disease. LM-P178 infection prior to disease induction or during the acute phase of the disease did not mitigate epitope spreading effects (Fig. S3). However, infection during remission resulted in delayed amelioration of disease (Fig. 5A). Furthermore, a high dose LM-P178 infection (10^8 cfu) given during remission of P139 disease was able to

ameliorate relapses earlier (Fig. 5B). This disease suppression was not simply due to the higher dose of LM, but rather an antigen-specific phenomenon as LM-P139 given during remission at standard or high dose infection resulted in no change in P139 disease severity (Fig. S4).

Although LM-P178 infection could ameliorate epitope spreading, the duration and degree of this suppression varied between experiments. Therefore, we next tested if combining CD8 T cell transfer with and LM-priming infection would be a better therapeutic strategy. For this, *ex vivo*-derived P178-CD8 (not re-stimulated in culture) were given to P139-immunized mice at the start of remission (d19) followed by an LM-P178 boost the next day. Mice that received both P178-CD8 and LM-P178 infection had significantly reduced disease compared to mice that received OVA-CD8 and LM infection (Fig. 5C) or mice that received LM-P178 infection alone (data not shown). This supports a therapeutic strategy in which myelin-specific CD8 T cells transferred and later or subsequently boosted with an LM vaccination for treating relapsing-remitting demyelinating disease.

4. Discussion

MS is the leading cause of disability in young adults [14,16]. Of the 2.3 million people diagnosed with MS worldwide, approximately 85% experience a relapsing-remitting disease pattern [16]. Treatments for relapses are still limited to slowing disease progression [27–29]. To better understand how to stop progression of relapsing-remitting MS, we must understand the different components of the immune processes underlying its pathogenesis and regulation. The function of CD8 T cells in
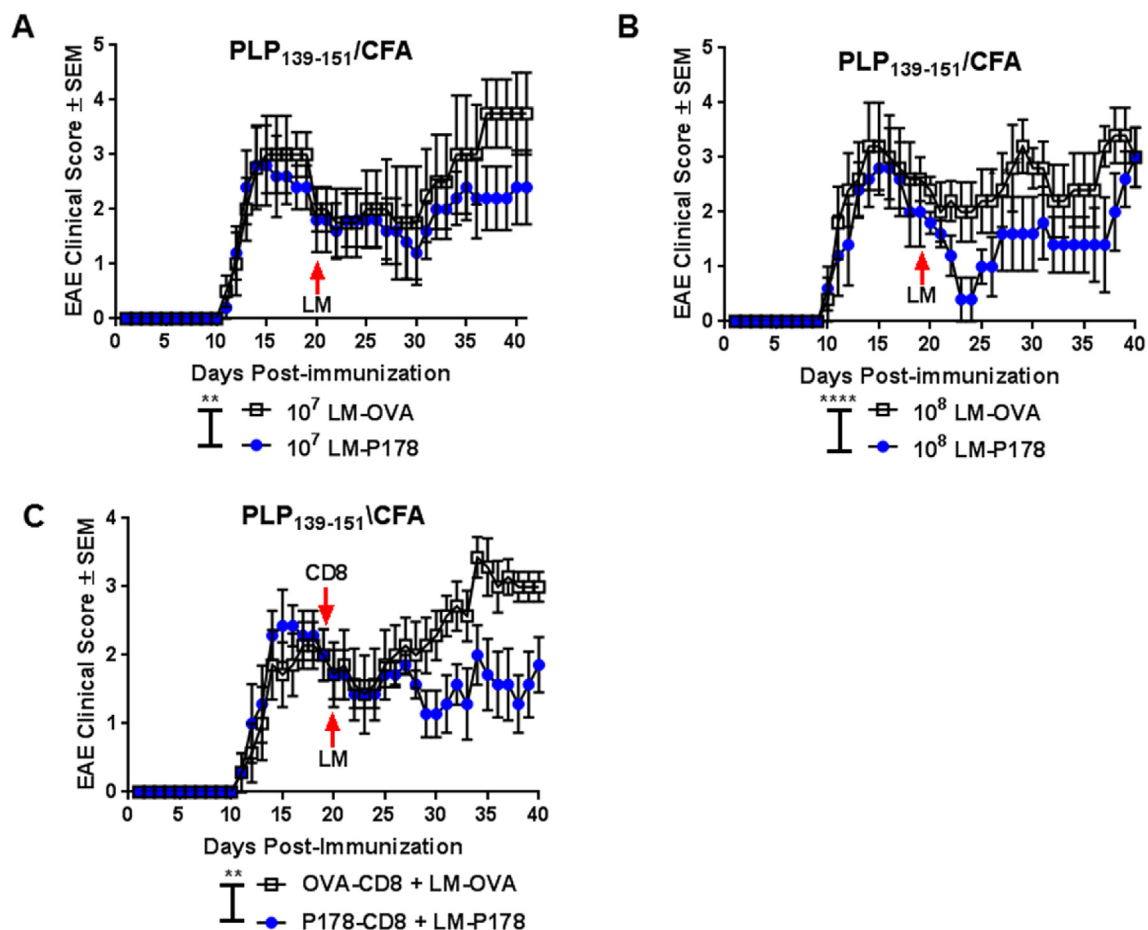
Fig. 5. A combination of CD8 T cell transfer with an LM boost is an effective therapy to suppress relapses. (A) EAE was induced with P139/CFA, followed by infection with 10^7 cfu of LM-P178 or LM-OVA (control) on d20 (disease remission). (B) EAE was induced with P139/CFA, followed by high dose infection (10^8 cfu) with LM-P178 or LM-OVA on d20. (C) Mice immunized with P139/CFA were given P178-CD8 i.v. on d19. The next day, mice were infected with 10^7 cfu of LM-P178. **p < 0.01, ****p < 0.0001, and ns = not significant.

MS is poorly understood compared to CD4 T cells, which have been implicated in the etiology of this demyelinating disease. We have now demonstrated that unlike CD4 T cells, CNS-CD8 not only fail to transfer or exacerbate demyelinating disease, but unexpectedly protect and suppress EAE [5,7–9,15,21]. This holds true for both CD8 T cells exogenously induced by peptide immunization and endogenously primed though infection with myelin-encoding LM [9]. Importantly, we have also observed this regulatory role of CD8 T cells in studies of human MS, with MS patients undergoing an acute relapse exhibiting an immunoregulatory defect in their CD8 T cell compartment [6,17,30], indicating an important role for CD8 T cells in mitigating relapse biology.

Recent work from our lab has demonstrated that the P178-CD8 are a potent suppressor of EAE in both the B6 and SJL models ([8,9,21] and confirmed here in Fig. 1A). Here we show that both P178- and MBP-CD8 significantly suppressed disease and lower relapse rate (Fig. 1). Furthermore, P178-CD8 suppress ongoing disease and subsequent relapses (Fig. 3). Interestingly, P139-CD8 appear to lack this disease-ameliorating function ([8] and confirmed in Fig. 1C). There are a few possibilities to explain this difference. In SJL mice, the DM20 isoform of PLP (which lacks the P139 region) is more abundantly expressed in the thymus than full length PLP, leading to a larger percussor frequency of P139-CD4 [31]. Additionally, P139 lies in the cytosolic region of the PLP protein, as opposed to the extracellular region of P178 [32–34]. Perhaps the antigen processing and presentation or the thymic expression of this cytosolic region does not induce a regulatory CD8 T cell response great enough to overcome the pathogenicity of CD4 T cells. While not the focus of the current studies, understanding the inherent dysfunction of P139-CD8 will be an important focus of future studies and could lead to insight into possible regulatory CD8 T cell dysfunction during MS relapses.

In previously published studies, we have shown that regulatory CD8 T cells are classically MHC Class Ia-restricted and require IFNγ and perforin, but they do not express Foxp3 nor require IL-4 or IL-10 production to mediate their disease suppressive effects [5,15]. We have also shown that the efficiency of these cells relies on *in vivo* presentation of cognate antigen [8], suggesting that it was the action of CNS-specific CD8 T cells, despite being transferred as part of bulk activated cultures. Here, we hypothesized that if this regulatory action was mediated by CNS-CD8, it would be sufficient to transfer the activated fraction of cells alone, as indicated by CD25 upregulation following *in vitro* stimulation. The CD25$^+$ P178-CD8 were not only capable of disease suppression, but showed potent suppression at far fewer numbers than their CD25$^-$ counterparts (Fig. 2A). Furthermore, we observed that LM-P178 induced CD25$^+$ CD8 T cells followed a similar pattern (Fig. 5D). These data once again suggest that these unconventional regulatory CD8 T cells are likely conventional cytotoxic/effector CD8 T cells. Rather than resembling an anti-inflammatory phenotype, these CD8 T cells are an activated (CD25$^+$) population that are using typical cytotoxic killing mechanisms (IFNγ and degranulation) to suppressive their autoreactive targets.

Several other immune cells have exhibited regulatory function in the context of autoimmune demyelinating disease, including regulatory B cells and conventional CD4$^+$CD25$^+$Foxp3$^+$ Tregs, which have been shown to ameliorate EAE [35]. It remains to be seen whether autoregulatory CNS-specific CD8 T cells interact directly or indirectly with these populations. We have previously observed that in CD8-/- mice that develop exacerbated disease (compared to wild-type mice), while there is an increase in effector Th17 and Th1 infiltration, CD4 Treg populations were unaffected, suggesting potentially non-intersecting pathways [15]. Some reports (from non-EAE studies) suggest that a large quantity of regulatory CD8 T cells is necessary to facilitate CD4 Treg function and restore disease regulation [36,37]. Other reports suggest regulatory CD8 T cells can suppress disease without inducing CD4 Tregs, but when transferred together these two cell types act synergistically to treat disease [38,39].

The IL-2RA (CD25) has long been implicated in T cell activation,

where it is upregulated in response to antigen stimulation, but mostly absent on naïve and memory cells [25,40]. In fact, a monoclonal antibody blocking the alpha subunit of the high affinity of IL-2R was developed as a treatment for relapsing-remitting MS. Daclizumab was thought to lead to the reduction of both activated effector T cells and Tregs, while enhancing NK cell responses [40,41]. It was reported that Daclizumab reduced relapses in clinical trials, but was withdrawn in 2018 due to the development of adverse events including secondary autoimmune disease against the CNS [40,41]. Although the biology underlying these adverse effects is not entirely understood, our studies suggest that the effect of daclizumab on regulatory CD8 T cells could potentiate increased survival of effector T cells recognizing "self" antigens, causing secondary autoimmunity. Our data on the efficiency of CD25$^+$ CD8 T cells in mediating disease may lead to better insight on better ways of targeting CD25 in future therapeutic ventures.

The endogenous induction of regulatory CD8 T cells using myelin antigen-expressing LM infection was described in a recent publication [9]. Here, we showed that LM-P178 infection induced disease-suppressive CD8 T cells in the context of relapsing-remitting disease (Fig. 4A). In agreement with our CD8 T cell adoptive transfer data, LM-P139 was unable to prime regulatory CD8 T cells (Fig. 4B). The safety and efficacy of live attenuated double-deficient (LADD) LM vaccines that stimulate both the innate and adaptive immune systems has been demonstrated in human clinical trials [42]. This supports the idea that *in vivo* stimulation of autoreactive CD8 T cells through genetically engineered LM may be a promising avenue for disease treatment.

We have previously shown that the regulatory CD8 T cell population exists within the terminally differentiated fraction in both healthy subjects as well as MS patients [17]. Furthermore, our studies show there is a loss of these suppressive CD8 T cells in patients undergoing a relapse [6, 17], suggesting that therapeutically enhancing or recovering their function could keep patients in remission. Developing antigen-specific strategies to induce tolerance in MS patients has been an attractive target for some time, as this approach would avoid potential adverse effects while maintaining efficiency [43,44]. Clinical trials using a mixture of known human myelin peptides alone [45] or coupled to lymphocytes using ethylene carbodiimide have shown promising results [46,47], as well as DNA vaccination to MBP [48]. Alternatively, using a nanoparticle platform to deliver a mixture of myelin-specific peptides has had promising results in EAE and is likely moving to clinical trials [49,50]. Our studies demonstrate broad antigenic reactivity of CNS-targeted CD8 T cells in MS, with different specificities in different patients [6,51]. Thus, a similar approach that covers a broad number of CNS antigens (including epitopes from MBP, PLP, MOG and others) could be used in a vaccination strategy to prime the appropriate regulatory CD8 T cells to downregulate disease in patients.

Taken together, the data presented in this study may hold implications for the immunotherapeutic potential of both adoptive CD8 T autoregulatory cell transfer and LM induction of CD8 T cells. In relapsing-remitting EAE, relapses are caused by epitope spreading patterns that have been well characterized [19]. In MS patients, it is likely that epitope spreading also occurs, though it may not be the sole driver of relapse [19]. The main focus of this study was to determine whether CD8 T cells could be used to mitigate relapses, even in the context of epitope spreading. We hypothesized that using a CD8 T cells specific to the upcoming CNS epitope driving the relapse might provide robust immunotherapeutic intervention. After evaluating multiple strategies, we determined that regulatory CD8 T cell intervention was most effective against epitope spreading during the first remission of the disease course (Fig S2, S3 and 5A). Remission is an attractive target for disease relapse therapy, as priming of the next CD4 T cell response occurs during this phase [19,26]. It is likely that CD8 T cells given during remission are able to target and suppress CD4 T cells during this priming phase, preventing further CNS damage, though we did not asses this directly. A high dose LM-P178 infection could also produce initial protection against epitope spreading, but the effects did not consistently last (Fig. 5B). The most

efficient therapeutic strategy seemed to be a combination of CD8 T cell transfer followed by an LM infection to boost the action of these cells *in vivo* (Fig. 5C). The efficacy of this strategy suggests there may be a memory component to regulatory CD8 T cells, as LM is boosting their function in an antigen-specific manner. Future studies of this potential memory pool of regulatory CD8 T cells could be applicable in halting the progression of human disease. We believe that these findings translate to a novel immunotherapeutic strategy for relapsing-remitting MS.

5. Conclusions

Our prior studies have focused on the role of myelin-specific CD8 T cells predominantly in the chronic C57BL/6 model of EAE [5,7–9,15,21]. The role of CD8 T cells is less understood in the context of relapsing-remitting disease. Here, we offer important insights into the role of both exogenously and endogenously primed regulatory CD8 T cells in relapsing-remitting demyelinating disease. We highlight the therapeutic potential of autoregulatory CD8 T cells by demonstrating that these cells in conjunction with an LM prime-boost efficiently suppress relapsing-remitting disease.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2019.100010.

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