Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg

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Key words

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Abbreviations

MBP; myelin basic protein, PLP; proteolipid protein

Abstract

The potential use of $CD4^+Foxp3^+$ Treg as a cellular therapy for autoimmune disease is of great interest. For clinical translation the key objective is to reverse established disease. Here we demonstrate that myelin basic protein (MBP)-reactive $CD4^+CD25^+$ Treg from TCR Tg mice, but not polyclonal (non-MBP-reactive) Treg, can transfer efficient protection against MBP-induced EAE when used either directly from donor mice, or after in vitro expansion. MBP-reactive Treg transfer also showed some ability to improve recovery from EAE initiated by T cells recognizing a distinct CNS autoantigen, proteolipid protein. Importantly, we also demonstrate for the first time in the context of EAE that in-vitro expanded naturally-occurring MBP-reactive Treg can prevent disease relapse when given after the onset of clinical EAE. Our study also contains data pertaining to the most effective Treg sub-population in vivo ($CD4^+CD25^+$CD62Lhi) and shows that their potent suppressive effects reflect stable expression of Foxp3, although not CD25 or CD62L. Our data provide proof of principle that Treg-based therapies can cure CNS autoimmune disease, highlight the challenges for clinical translation and open new avenues for assessing how changing immune function via Treg activity might impact on neurodegeneration and repair.

Introduction

Degeneracy in recognition of foreign and self Ag by the adaptive immune system provides the risk of autoimmune disease in every individual. Although negative selection in the thymus can purge the immune repertoire of many overtly self-reactive T cells, these processes are incomplete [1]. Complex peripheral mechanisms, therefore, reinforce immune tolerance to self, and include the deletion and functional inactivation of some self-reactive cells, in addition to an essential role for active immune regulation [2]. $CD4^+$ Treg expressing the Foxp3 transcription factor are now recognized to be of primary importance in the regulation of potentially autoaggressive immune responses [3]. These Treg arise naturally during thymic selection and the expression of Foxp3 is essential both for their development and for their function [3-5]. Humans and mice with inactive Foxp3 genes develop severe multi-organ inflammatory diseases, together with autoantibody production.[6-11] Moreover, in a variety of rodent models, experimental enhancements of Treg numbers and/or function have been shown to ameliorate disease [12]. The IL-2 receptor α chain, CD25, remains at present the most useful cell-surface marker for the enrichment of CD4+Foxp3+ Treg, despite the fact that not all Foxp3+ Treg are CD25+, and CD25 is also expressed on activated non-Treg [13, 14].

The function of Treg cells in MS and its primary laboratory model, EAE, has been the focus of considerable attention [15, 16]. MS studies have reported that, although no obvious numerical deficit is apparent, the Treg isolated from the peripheral blood of patients have functional impairments as assessed with in vitro assays of suppression [17-19]. The conclusion that stems from these studies is that if we can enhance Treg function, this might have some therapeutic benefit in re-establishing an equilibrium between Treg and T effector cells in MS. Studies in EAE provide support for this notion. Substantially increasing Treg numbers through adoptive transfer of CD4+CD25+ cells prior to immunization with myelin autoantigens can provide some degree of protection [20, 21]. The opposite approach of compromising the Treg repertoire through the in vivo use of depleting anti-CD25 antibodies renders mice more vulnerable to disease upon immunization [14, 22]. In our own work we have focused on the natural development of the Treg response, specifically within the CNS during EAE. Using a naturally resolving model, we found a striking accumulation of Treg within the inflamed CNS, which appears to drive the progression to resolution (Treg-depleted mice did not recover) [22]. These CNS Treg are actively triggered by the inflammation within the CNS which drives their rapid proliferation [23]. Importantly, we found that transfer of low numbers of Treg recovered from the CNS of sick mice could provide protection in recipient mice; a protection that was not found using Treg derived from the peripheral lymphoid organs of the same EAE donors [22]. This most likely reflects two facets, that the Treg extracted from the inflamed CNS were particularly activated and that they were particularly enriched for recognition of the relevant myelin autoantigen [23, 24].

There is now strong interest in the possibility of using a patient's own Treg as a cellular therapy in autoimmune disease [25-27]. Because of the low frequencies of Treg within the human peripheral blood $CD4^+$ T cell population, such an approach would most likely require an

in vitro expansion phase to produce sufficient Treg numbers. In mice, this can now be achieved by the culture of purified Treg subsets with either beads coated with anti-CD3 and anti-CD28 (to provide TCR ligation and costimulation, respectively) or activated dendritic cells (DC), in the presence of high doses of IL-2 [28, 29]. Here we used EAE to test whether such an approach is valid for the treatment of autoimmune disease of the CNS. By making use of T cells from mice expressing a transgenic TCR recognizing myelin basic protein (MBP), we show that only Treg recognizing an EAE-relevant Ag can suppress EAE. Moreover these MBP-reactive Treg could effectively control ongoing disease, preventing chronic impairment. These data provide proof of principle for this approach in CNS autoimmune disease and also give insight into the best prospects for the translation of Treg-based therapies.

Results

CD62Lhi Tg4 Treg give better protection against EAE than their CD62Llo counterparts.

In order to test the ability of MBP(Ac1-9)-responsive Treg to control EAE we made use of the Tg4 TCR transgenic mouse. Polyclonal (non-MBP-reactive) Treg populations were sourced from syngeneic B10.PL mice. Sorted CD4+CD25+ cells from unimmunised mice routinely showed >95% purity for Foxp3+ cells (Figure 1A). These sorted populations could however be further divided based on their expression of CD62L (Figure 1A). CD62L (L-selectin) is a key homing marker allowing access to the lymph nodes across high endothelial venules and is lost upon TCR activation. Thus the CD62Lhi and CD62Llo fractions should reflect naive and Ag-experienced populations, respectively. In confirmation of other studies [30], sorting of CD4+CD25+ Tg4 cells based on CD62L showed that both the CD62Lhi and the CD62Llo fractions could function as suppressors in vitro (Figure 1B). Treg are suppressive only if activated with their cognate Ag, although once activated they can suppress other responder T cells with different Ag-reactivity [31-33]. Using CD4+CD25- Tg4 cells as responders, we found that sub-sets of Tg4 CD4+CD25+ T cells could suppress equally well, regardless of their CD62L expression, in cultures stimulated with either anti-CD3 or the Ac1-9 peptide (Figure 1B; left panels). However, when CD4+CD25+ T cells isolated from B10.PL mice (i.e., not MBP-reactive) were tested, both fractions could suppress Tg4 responders stimulated with anti-CD3, but only CD62Llo CD25+ T cells could suppress Ac1-9-stimulated cultures (Figure 1B; right panels). These CD62Llo Treg had presumably been activated prior to isolation and so would not require further TCR stimulation in order to function in culture. In contrast, the B10.PL $CD25^{+}$CD62Lhi population would not have been activated in vivo and so would require in vitro TCR ligation in order to suppress; this would be provided by stimulation with anti-CD3 but not Ac1-9.

From their pre-activated phenotype, the CD62Llo population might be predicted to be the most effective at inhibiting EAE. However, in other disease models, such as Type 1 diabetes [30] and graft-versus-host disease [34, 35], CD25+CD62Llo cells were found to lack in vivo suppressive activity. To verify these observations in the context of EAE we transferred $1x10^{5}$ CD4+CD25+ Tg4 cells, sorted according to CD62L expression, into naïve B10.PL mice prior to immunization with Ac1-9. This EAE model shows a chronic disease course. The CD25+CD62Lhi Tg4 population gave almost complete protection throughout the disease course. In contrast, although the CD25+CD62Llo population gave reasonable protection from initial EAE onset, disease appeared to break through at later time-points (Figure 1C,D).

CD62Lhi Treg maintain Foxp3 expression, but lose CD25 in response to Ag in vivo

In order to address whether differences in the stability of their Treg phenotype (ie. Foxp3 expression), or in their expansion potential in draining lymphoid organs, could underlie the contrasting in vivo potency of CD62Lhi vs CD62Llo CD25+ cells, we transferred traceable Treg populations from Tg4xCD45.1 mice into B10.PL (CD45.2) mice one day prior to immunization.

We also transferred CD4+CD25- Tg4 cells as controls. At the dose available for transfer ($2x10^5$ per host mouse), the frequency of donor cells could not be accurately distinguished above background at early time points after immunization (day 1, data not shown), preventing identification of any early differences in the relative survival and/or trafficking of the different subsets to lymphoid organs. However, on day 6 after immunization, all the different donor cell subsets had expanded and were readily identified in both the draining lymph nodes and spleen (Figure 2). As would be expected, CD4+CD25- effector cells had expanded and populated the host in the highest numbers. The chimerism seen with CD25+CD62Lhi cells was around 4-fold lower than CD25- cells, but ~3-fold higher than that seen with CD25+CD62Llo cells (Figure 2A; upper panels). Most strikingly, although the transferred CD25+CD62Lhi cells maintained Foxp3 expression at ~80%, this had dropped to only 20-40% in the transferred CD25+CD62Llo population (Figure 2A; lower panels). Thus the net effect was that mice that had received CD25+CD62Lhi cells had 6-fold (spleen) to 11-fold (lymph node) higher numbers of MBP-reactive Foxp3+ donor cells than those mice receiving CD25+CD62lo cells. It seems reasonable to conclude that these differences would impact significantly on the T effector / Treg balance, ultimately accounting for the differences in protection afforded by the two sub-sets of CD4+CD25+ cells. As an aside, it is worth noting that the CD25+CD62Lhi donor cells themselves had reduced expression of CD62L by day 6 (a predictable sign of their in vivo activation in response to Ac1-9 immunization) (Figure 2B; left panel). However, we did not expect to find that these cells had also lost CD25 expression (Figure 2B; right panel). This novel finding means that CD25 may well be dynamically expressed in vivo making it a less attractive marker for Treg isolation. Moreover, it may mean that Foxp3+CD25+ and Foxp3+CD25- populations are not separate Treg lineages.

Loss of Foxp3 in CD62Llo Treg is not due to outgrowth of contaminating Foxp3- cells

The restrictions on cell numbers in the transfer experiments described above made it difficult to precisely follow the fate of individual cell populations over time. For this purpose we utilized an in vitro expansion culture using beads coated with anti-CD3 and anti-CD28 in the presence of high doses of IL-2. The Foxp3 purity of both subsets after cell sorting of Tg4 cells was consistently high (Figure 3A). Although the fold expansion was essentially the same for each population over a seven day period (Figure 3B; left panel), the CD25+CD62Lhi population had maintained Foxp3 expression above 80%, whereas this had dropped to below 40% in their CD62Llo counterparts (Figure 3A and 3B; right panel), precisely mirroring the difference in Foxp3 expression seen in vivo (Figure 2). Cell cycle analysis by BrdU incorporation revealed that, in both populations, Foxp3+ cells had divided in culture by day 5, but over half of the BrdU+ CD62Llo cells were now Foxp3-, whereas Foxp3 was retained in the CD62Lhi population (Figure 3C). In order to rule out possible differing levels of Foxp3- cell contamination in the starting cultures, we isolated CD25+Foxp3+ cells from Foxp3-GFP reporter C57BL/6 mice. When CD62Llo and CD62Lhi populations were submitted to in vitro culture we again saw the selective loss of Foxp3 in the CD62Llo population, in this case from day 6 to day 9 (Figure 3E),

even though these CD62Llo cells had a higher starting purity of Foxp3 than the CD62Lhi cells (Figure 3D).

Only MBP-reactive Treg can protect against MBP-induced EAE

Thus far we had established the superior regulatory potency of CD25+CD62Lhi Tg4 cells over their CD62Llo counterparts and that this reflected more stable Foxp3 expression following stimulation both in vivo and in vitro. Therefore focusing on CD4+CD25+CD62Lhi cells as our starting population, we next addressed the requirement for Ag recognition by the transferred Treg population. We had found that the in vitro culture gave significant expansion of Treg with stable Foxp3 expression (Figure 3). Furthermore, the anti-CD3 coated beads provide TCR stimulation for Treg irrespective of their Ag-reactivity, and we had already shown that recently activated (CD62Llo) polyclonal CD25+ cells could suppress naïve CD25- Tg4 T cell activation in vitro (Figure 1B). We therefore expanded CD25+CD62Lhi cells from Tg4 or B10.PL donors and found that each were equally effective at suppressing naïve Tg4 cells stimulated with Ac1-9 (i.e., when the B10.PL Treg did not receive further TCR stimulation in the assay) (Figure 4A). Since others have reported that transfer of very large numbers of "naïve" polyclonal CD4+CD25+ cells can have some suppressive effects in EAE [20, 21], we reasoned that our expanded polyclonal B10.PL Treg might also have good suppressive potential in the disease. In fact, we found that transfer of $1x10^5$ expanded Tg4 Treg to B10.PL mice one day prior to immunization gave complete protection against EAE. In contrast, transfer of $2x10^5$ expanded B10.PL Treg gave no protection whatsoever (Figure 4B). From these data we can conclude that to provide effective protection against CNS autoimmune disease, we need to use Treg that recognize a CNS autoantigen.

MBP-reactive Treg can reduce EAE induced with a different myelin autoantigen

To further test the potential for these MBP-reactive Treg to protect against EAE, we switched to using B10.PLxSJL F_1 mice as hosts. This allows immunization with myelin Ag other than Ac1-9, notably the PLP(139-151) peptide. Furthermore, there is greater scope in these mice for diversification of the immune response through "epitope spreading" than in B10.PL mice. We transferred expanded Tg4 CD25+CD62Lhi cells prior to immunization either with Ac1-9 or PLP(139-151) alone, or with a mixture of the two peptides. As predicted from our observations in B10.PL mice, Tg4 Treg also provided robust protection from the chronic-relapsing Ac1-9-induced EAE in B10.PLXSJL mice from the outset (Figure 5A). Chronic-relapsing disease was also seen after immunization with the MBP and PLP peptides in combination. In this case the presence of transferred Tg4 Treg had a lesser influence on the first phase of disease, although the recovery from primary disease was enhanced and the severity of disease relapses was significantly reduced (Figure 5B). As yet it is not clear which CNS autoantigen(s) is/are recognized by those cells that drive different phases of disease after immunization with the Ac1-9/139-151 mix. Nevertheless, these data suggested that transfer of Treg recognizing a single CNS autoantigen could have an impact on a more complex disease model. To test this

we provided Tg4 Treg prior to immunization with PLP(139-151) alone. In such a scenario the Tg4 Treg would not have substantial amounts of MBP to act as a TCR trigger until inflammation was underway in the CNS, releasing MBP in greater amounts and/or in a more immunologically relevant form (associated with MHC class II on activated APC), potentially within the inflamed CNS itself. As seen when immunizing with PLP(139-151) and Ac1-9 in combination (Figure 5B), the presence of Tg4 Treg did not substantially influence the acute phase of disease, but again there was a significant improvement in recovery, resulting in a dampening of the chronic phase (Figure 5C). These data suggest that a process in which MBP-reactive Treg access the CNS after inflammation is established by PLP-reactive autoaggressive T cells, and can have a beneficial influence on later stages of the disease, (or that MBP presented in lymph nodes after the establishment of disease in the CNS results in their ability to be activated and affect later events).

MBP-reactive Treg can cure chronic/relapsing EAE.

The above model is pertinent because the clinical need would be to reduce ongoing disease. To test this we used either B10.PL (Figure 6A) or B10.PLxSJL (Figure 6B) hosts that had been immunized with Ac1-9. A single administration of in vitro-expanded Tg4 Treg was given towards the end of the first phase of disease. In each host, this was sufficient to significantly reduce the severity of disease relapse. This was most notable in the B10.PLxSJL recipients, which were essentially disease-free by the end of the experiment. Transfer of expanded polyclonal Treg did not have any effect on ongoing disease (data not shown).

Discussion

The powerful suppressive effects of Foxp3$^+$ Treg in various rodent models of immune pathology have sparked the ambition to translate their effects to the clinic as treatments for autoimmune diseases [25-27]. In one approach, the patient's existing Treg would be purified (most likely from peripheral blood), selectively expanded in vitro, and re-implanted to boost Treg numbers. Our data show that such an approach can be remarkably potent in either preventative or curative regimens in CNS autoimmune disease. They also provide important information relating to how best to purify populations with the most potent in vivo suppressive capacity, the stability of Foxp3 expression on Treg subsets in vitro and in vivo, and the issue of whether Treg need to recognise disease relevant Ag in order to be protective.

The greater in vivo potency we find with CD62Lhi Treg is in broad agreement with data from other studies using autoimmune diabetes [29],[30] and graft-versus-host disease [34, 35]. In contrast, both CD62L$^+$ and CD62L$^-$ CD25$^+$ cells were reported to prevent gastritis and colitis in lymphopenic mice [36], perhaps reflecting a greater opportunity for CD62Llo Treg to home to, and expand in, the appropriate location in those models. Previous studies have suggested that the different in vivo functional capacity of CD62Lhi and CD62Llo CD4$^+$CD25$^+$ T cells might be attributed to a differential ability to migrate to secondary lymphoid organs, as supported by observed differences in the expression of chemokine receptors by these subsets [30].

A key observation from our study that is likely to be the dominant factor in determining in vivo potency is the loss of Foxp3 expression on CD25$^+$CD62Llo cells during their expansion, both in vitro and in vivo. In vitro, this may have reflected an absence of TGF-β, which can stabilize Foxp3 expression [37]. In that study, Foxp3 expression could only be induced by TGFβ in CD4+CD25- cells with an Ag-experienced (CD45RBlo) but not naïve (CD45RBhi) phenotype [38]. Taken together with our data, this suggests that a subset of Ag-experienced (CD45RBlo/CD62Llo) CD4+ cells exists in which Foxp3 expression (and regulatory activity) is labile and perhaps dependent on the continued presence of TGF-β. The CD25+CD62Lhi cells, on the other hand, appear to represent a more stable subset of Foxp3+ Treg. Such a loss of Foxp3 expression by CD25$^+$CD62Llo cells after in vitro culture was not observed in another study in which cells were expanded with DC rather than beads [29], suggesting that DC-derived factors may have contributed to the maintenance of Foxp3 stability in those cells [29].

The other key issue addressed here was that of whether the Treg used needed to recognize a CNS-relevant autoantigen. Clinical translation would of course be easier if polyclonal Treg were suppressive, obviating the challenge of defining a CNS autoantigen that Treg from MS patients can recognize. However, our data clearly show that only Treg that recognize MBP could function in our EAE models. These findings contrast with reports that supplementation of the host's existing Treg with very high numbers of naïve polyclonal CD4+CD25+ Treg can somewhat reduce the severity of disease elicited by subsequent immunization [20, 21]. They do, however, confirm other findings that polyclonal Treg failed to provide consistent protection in either EAE [39], or autoimmune diabetes [28, 40]. Moreover,

those reports in which polyclonal Treg had some effect could never show what is essentially complete protection seen in our experiments using Tg4 Treg. In a spontaneous model of EAE (MBP-reactive TCR transgenic mice on a RAG-/- background), early administration of CD4+CD25+ cells either from RAG-sufficient TCR transgenic (MBP-reactive), or from non-transgenic donors could protect against the development of disease [41], although donor CD25+ cells from the RAG-sufficient TCR transgenic mice were only effective if they expressed the clonotypic MBP-reactive TCR, presumably due to the restricted repertoire diversity in the remaining non-clonotypic cells. The reason polyclonal Treg work in that spontaneous disease setting is most likely because the activation status of dendritic cells is low (i.e. the mice were not immunized). In this context, multiphoton analysis of the MBP-reactive and polyclonal Treg interactions with DC in lymph nodes showed that both were able to condition DC to render them less able to provide full antigenic stimulation to other cells [42]. We believe that when autoantigen is presented under strong pro-inflammatory conditions, such as those provided by immunization with the autoantigen in CFA, there is a need for Treg that can recognize the autoantigen.

The precise targets of pathogenic T cells within the CNS remain unknown (and indeed these may vary over time in the same patient). Thus the approach most likely to succeed would involve an element of "bystander suppression", whereby a regulatory population recognizing one Ag can counteract the aggressive actions of an effector T cell population recognizing another Ag. Although Tg4 Treg had a negligible effect on the early stages of primary disease induced with PLP(139-151), there was a small but significantly improved recovery from primary disease, resulting in a lessening of the chronic phase. These findings are similar to those in which PLP-reactive CD25+ TCR transgenic Treg induced by exposure to PLP-Ig could suppress EAE induced by peptides from either MBP or MOG [43]. However, in that study, such bystander effects required the pre-activation of the Treg cells in vitro prior to transfer.

The mode of action of transferred Treg remains to be fully elucidated. The possibility that Treg work within the inflamed CNS is supported by our previous observations of dramatic increases in the numbers of highly activated Treg within the CNS during MOG-induced EAE and that this process is essential for the spontaneous recovery seen in that model [22]. A contrasting possibility, that Treg may endow other immune cells with suppressive function in the periphery, comes from a study indicating "infectious tolerance" in host lymph node cells that was not ablated following the removal of the transferred Treg population [44]. Other Treg transfer studies have suggested a key role of IL-10 in their protective effects [21, 43, 44]. Indeed, we first identified the Treg that promote the natural recovery from EAE by looking for IL-10-producing cells in the CNS [22]. The precise mechanism(s) at play in our model will require further detailed investigation.

The imperative for clinical translation is to cure rather than prevent disease. In this regard, the data shown in Figure 6B are particularly compelling, with the treated group eventually becoming disease-free. So far relatively few reports suggest that transfer of

naturally occurring Treg can provide such effective treatment against autoimmune/inflammatory disease, rather than prevention at the initiation stage [28, 29, 39, 45, 46]. The only other study pertaining to the treatment of EAE with Treg used 'redirected' Treg from genetically modified mice expressing chimeric receptors in which TCR signalling was initiated by cognate interaction of pathogenic MBP-reactive T cells with MBP peptide complexed with MHC on the surface of the Treg (which may act in a different manner compared with the naturally occurring Treg used in this study) [39].

This study provides proof of principle that naturally occurring Treg-based therapy can be curative in a complex chronic-relapsing model of CNS autoimmune disease and outlines key features for their most effective use. What are the ramifications for clinical translation? Our novel finding that CD25+CD62Llo Treg cells lose Foxp3 expression both in vivo and during in vitro culture mean that in vitro analyses of Foxp3 stability will most likely be informative of potential fate in vivo. The most important point of this study is the need to use Treg that recognize a CNS relevant autoantigen. Together with our previous observations on the essential role of Treg within the inflamed CNS, this raises questions over the extent to which assays using polyclonal (usually anti-CD3) stimulation of Treg from patients' peripheral blood can tell us about the level of relevant immune regulation during disease and therapy. More importantly, the logical extension of our observations is that any Treg therapy would need to target CNS-relevant Treg. Currently the need to specifically expand CNS-reactive Treg in vitro is a substantial obstacle, because we do not know what Ag are recognized by Treg. Studies in mouse models of diabetes have succeeded in expanding protective Treg from polyclonal populations by substituting a pancreatic peptide-MHC complex for the anti-CD3 usually attached to the expansion beads (i.e. these beads would only provide stimulation for those Treg bearing pancreas-reactive TCR) [47]. Translation of such bespoke approaches, perhaps on an individual patient basis, would be a substantial undertaking. A further complication is that, although they maintained Foxp3, a large proportion of CD62Lhi Tg4 Treg lost CD25 expression after in vivo encounter with Ag. If this process proves to be the case in humans also, isolation of CD25hi cells might not provide the most relevant starting Treg population for expansion. The alternative of expanding the appropriate Treg population with Ag in vivo rather than in vitro might be more achievable.

Materials and Methods

Mice and peptides

B10.PL (H-2^u), B10.PLxSJL (H-2^{uxs}), Tg4 mice, expressing a transgenic MBP(Ac1-9)-reactive TCR [48] and Tg4.CD45.1 mice (backcrossed > 6 generations onto the Tg4 background) were bred and maintained in specific-pathogen free conditions at the University of Edinburgh. The MBP(Ac1-9) peptide (Ac-ASQKRPSQR) and the proteolipid protein (PLP) 139-151 peptide (HSLGKWLGHPDKF) were synthesised by Advanced Biotechnology Centre, (Imperial College, London). Foxp3-GFP reporter mice [49] on the C57BL/6 background were kindly provided by Dr A. Rudensky, Seattle. Animal studies received University of Edinburgh ethical approval and were performed in accordance with UK legislation.

Antibodies and flow cytometry

Cells were stained with appropriately titrated antibodies for 20 min on ice and washed with PBS+2%FCS. Foxp3 staining was performed using a Foxp3 Staining Set (eBioscience, Insight Biotechnology, UK) according to the manufacturer's instructions. Data were acquired on a LSR cytometer (BD Biosciences) and analysed using FlowJo software (Treestar, CA, USA). BrdU staining was performed on cells expanded in the presence of 10μM BrdU for 20-24 hours before harvest (Sigma Aldrich) using a protocol described previously [23].

Treg purification

$CD4^+$ T cells were enriched from spleen and lymph nodes (cervical, mesenteric, brachial, axillary, inguinal and iliac) by negative selection involving incubation on ice with the following mixture of antibodies (purified from hybridomas): RAB632 (anti-B220), 53-6.72 (anti-CD8), M1/70 (anti-Mac1), and M5/114.15.2 (anti-MHC class II), followed by washing and incubation with M450 Sheep-anti-RatIgG Dynabeads (Dynal, UK). Cells were then fractionated according to CD25 expression using anti-CD25-PE (Clone 7D4-Miltenyi Biotec), followed by incubation with anti-PE microbeads (Miltenyi Biotec) and separation on columns according to the manufacturers' instructions. Highly pure $CD4^+CD25^+$ cells fractionated according to CD62L expression were obtained after further staining of $CD25^+$-enriched cells with CD62L-FITC and CD4-PerCP and cell sorting on a FACS ARIA flow cytometer (BD Biosciences). Foxp3 expression of sorted cell populations was tested by fixation and staining of cells with Foxp3-APC (eBioscience).

In vitro suppression assay

Cells were cultured in triplicate in U-bottomed 96-well plates in T cell medium (RPMI (Gibco, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated FCS (Sigma, Poole, UK), 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 50μM 2-mercaptoethanol (all from Gibco)). Responder $CD4^+CD25^-$ Tg4 T cells were added at $2.5x10^4$ cells per well, with or without the indicated ratio of the relevant Treg population, and were stimulated with anti-CD3 mAb (5μg/ml) or Ac1-9 peptide (1μM) in the presence of $5x10^4$

irradiated (30 Gy) B10.PL splenocytes as APC. 0.5μCi ^{3}[H]-thymidine (Amersham Biosciences, UK) was added for the last 18h of a 90h culture period. Data shown are the mean±SD of triplicate wells.

Induction of EAE

In B10.PL mice, EAE was induced by subcutaneous immunization with 200μg of Ac1-9 peptide emulsified in CFA (Sigma) and injected as 50μl/leg in each hind leg. Pertussis toxin (200ng, Health Protection Agency, UK) was administered intraperitoneally on the same day and two days later. In B10.PLxSJL mice, EAE was induced in the same manner by subcutaneous immunization with 50μg of Ac1-9 peptide, 200μg of PLP 139-151 peptide or a combination of both 50μg Ac1-9 and 200μg PLP(139-151). Mice were monitored daily for clinical signs of EAE using the following scores: 0; healthy, 1; limp tail, 2; impaired gait/righting reflex, 3; partial hind limb paralysis, 4; total hind limb paralysis, 5; total hind limb paralysis plus front leg weakness, 6; moribund or dead. Comparison of disease incidence was performed by the Fisher's exact test, and overall disease burdens (clinical scores) were compared using the Mann-Whitney U test for data collected over the entire course of the experiment.

In vitro expansion of Treg cells

Highly pure FACS-sorted $CD4^{+}CD25^{+}$ T cell populations were expanded in 96-well U-bottomed plates at a starting concentration of 10^{5} cells per well in the presence of mouse CD3/CD28 T cell expander Dynabeads (Dynal Biotech, Invitrogen) at a 4:1 bead:cell ratio in T cell medium supplemented with 1000U/ml recombinant IL-2. Cells were maintained at a concentration of 0.5-1 $x10^{6}$ cells/ml.

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Conflict of interest

The authors have no competing financial or commercial interests to declare.

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Figure legends

Figure 1.

$CD4^+CD25^+CD62Lhi$ Tg4 cells provide better protection against EAE than $CD4^+CD25^+CD62Llo$ Tg4 cells, although both populations are suppressive in vitro.

(A) Representative flow cytometric analysis of CD62L and Foxp3 expression on gated $CD4^+CD25^+$ cells from pooled spleen and lymph nodes of Tg4 mice. (B) CD62Lhi and CD62Llo $CD4^+CD25^+$ cells from Tg4 mice (left) and syngeneic B10.PL mice (right) were tested for their ability to suppress the proliferation of $CD4^+CD25^-$ Tg4 cells in vitro in response to polyclonal stimulation with anti-CD3 (5μg/ml), or to Ac1-9 peptide stimulation (1μg/ml) in the presence of irradiated B10.PL splenocytes. Results are representative of at least 2 experiments. (C) 10^5 CD62Lhi or CD62Llo $CD4^+CD25^+$ cells isolated from Tg4 mice were injected intravenously into B10.PL mice one day before EAE induction with 200μg Ac1-9/CFA. Groups that received either $CD25^+$CD62Lhi cells or $CD25^+$CD62Llo cells had a significant reduction in overall disease burden compared to control mice ($p<0.0001$, one-tailed Mann-Whitney U test). The protection afforded by CD62Lhi Treg was significantly greater than CD62Llo Treg ($p<0.0001$, two-tailed Mann-Whitney U test). Data shown (means +/- SEM) are from one of three experiments giving consistent results, with 5-6 mice per group in each experiment. (D) Disease incidence was plotted from mice treated as in Figure 1C. Data were pooled from 3 experiments and p values were calculated using a one-tailed Fisher's exact test.

Figure 2.

Expansion and phenotypic stability of transferred Tg4 cell subsets on day 6 after priming with Ac1-9/CFA.

(A) $2x10^5$ of the indicated T cell population isolated from Tg4.$CD45.1^+$mice were transferred i.v. into B10.PL recipients a day before immunization with Ac1-9/CFA. Six days after immunization, cell suspensions from draining lymph nodes and spleens were assessed by FACS for percentage, absolute number and Foxp3 expression of transferred donor cells ($V\beta8^+CD45.1^+$). Each data point represents gated $CD4^+$ cells from an individual mouse, and the experiment shown is representative of 2 separate experiments. (B) CD25 and CD62L phenotype of gated $CD4^+Foxp3^+$ donor ($CD45.1^+V\beta8^+$) cells from mice injected with $2x10^5$ $CD25^+$CD62Lhi Tg4 cells. Analyses were performed 6 days after immunization with Ac1-9/CFA. Each data point represents an individual mouse and data are pooled from 2 experiments.

Figure 3.

Proliferation and Foxp3 stability of sorted CD62Lhi and CD62Llo $CD4^+CD25^+$ cells during short-term culture in vitro.

(A-C) Sorted CD62Lhi and CD62Llo $CD4^+CD25^+$ cells isolated from Tg4 mice were cultured with anti-CD3 and anti-CD28-coated Dynabeads and 1000U/ml IL-2. (A) Representative FACS plots of Foxp3 expression immediately after sorting (day 0) and after 7 days of culture. Initial Foxp3

expression was 97.3±2.1 % (*n*=6) of cells in the $CD25^+CD62Lhi$ population and 92.9±0.8 % (*n*=6) of $CD25^+CD62Llo$ cells. (B) Cells were harvested at the indicated time points, viability counts (trypan blue exclusion) provided the fold expansion of cells and Foxp3 expression was measured by intracellular FACS analysis. (C) Proliferation of cells was determined by flow cytometric analysis of BrdU incorporation (following a 20-24h pulse with 10μM BrdU in the culture medium) on day 5 of culture. (D,E) CD62Lhi and CD62Llo subsets of CD4+Foxp3GFP+CD25+ cells were sorted from Foxp3-GFP C57BL/6 mice and cultured with anti-CD3 and anti-CD28-coated Dynabeads and 1000U/ml IL-2. (D) Foxp3 versus CD62L at the start of culture. (E) Fold-expansion (left) and % Foxp3+ cells (right) during in vitro expansion.

Figure 4.

MBP(Ac1-9)-reactive, but not polyclonal, Treg protect mice against MBP(Ac1-9)-induced EAE, although both are suppressive in vitro.

(A) Expanded $CD4^+CD25^+CD62Lhi$ cells from Tg4 or B10.PL mice were added in increasing ratios to a fixed number of naive Tg4 $CD4^+CD25$- cells and stimulated with anti-CD3 (5μg/ml) or Ac1-9 peptide (1μg/ml) in the presence of irradiated B10.PL splenocytes and proliferation was measured. (B) $CD4^+CD25^+CD62Lhi$ Treg from Tg4 ($1x10^5$) or B10.PL ($2x10^5$) mice were expanded in vitro for 7 days and injected intravenously into B10.PL recipients immediately prior to EAE induction with 200μg of Ac1-9/CFA (five mice per group). Foxp3 expression on both subsets was equivalent on the day of transfer. Data (means +/- SEM) are from one of two experiments giving consistent results.

Figure 5.

Effects of MBP(Ac1-9)-reactive Treg on EAE induced with MBP(Ac1-9) and/or PLP(139-151).

B10.PLxSJL mice received 4-$5x10^5$ expanded $CD25^+CD62Lhi$ Tg4 Treg or PBS alone intravenously prior to EAE induction using either (A) 50μg Ac1-9, (B) a mixture of 50μg Ac1-9 and 200μg PLP(139-151), or (C) 200μg PLP(139-151), emulsified in CFA. Administration of Tg4 Treg provided significant protection against EAE induced with either regime ($p<0.0001$ in each case, one-tailed Mann-Whitney U test). A,B) Data (means +/- SEM) are from one of three experiments giving consistent results (5 mice per group in each experiment). C) Data are pooled from five experiments (a total of 26 mice for the PBS group and 27 mice for the Tg4 Treg group).

Figure 6.

Expanded MBP(Ac1-9)-reactive Treg given after primary disease reduce the severity of disease relapse.

(A) B10.PL mice received $3x10^5$ expanded Tg4 $CD25^+CD62Lhi$ Treg or PBS alone intravenously on day 18 (indicated by arrow, 4 mice per group) after EAE induction with 200μg Ac1-9

peptide/CFA. (B) B10.PLxSJL mice received 10^6 expanded Tg4 $CD25^+$CD62Lhi Treg or PBS alone intravenously on day 18 (indicated by arrow, 8 mice per group) after EAE induction with 50μg Ac1-9 peptide/CFA. Data shown (means +/- SEM) are each from one of two experiments for each mouse strain giving consistent results. Treatment of either mouse strain with Treg significantly decreased the cumulative disease score when analyzing every time-point throughout the experiment (i.e. including days before cell transfer) ($p<0.0001$, one-tailed Mann–Whitney U test).

Figure 1

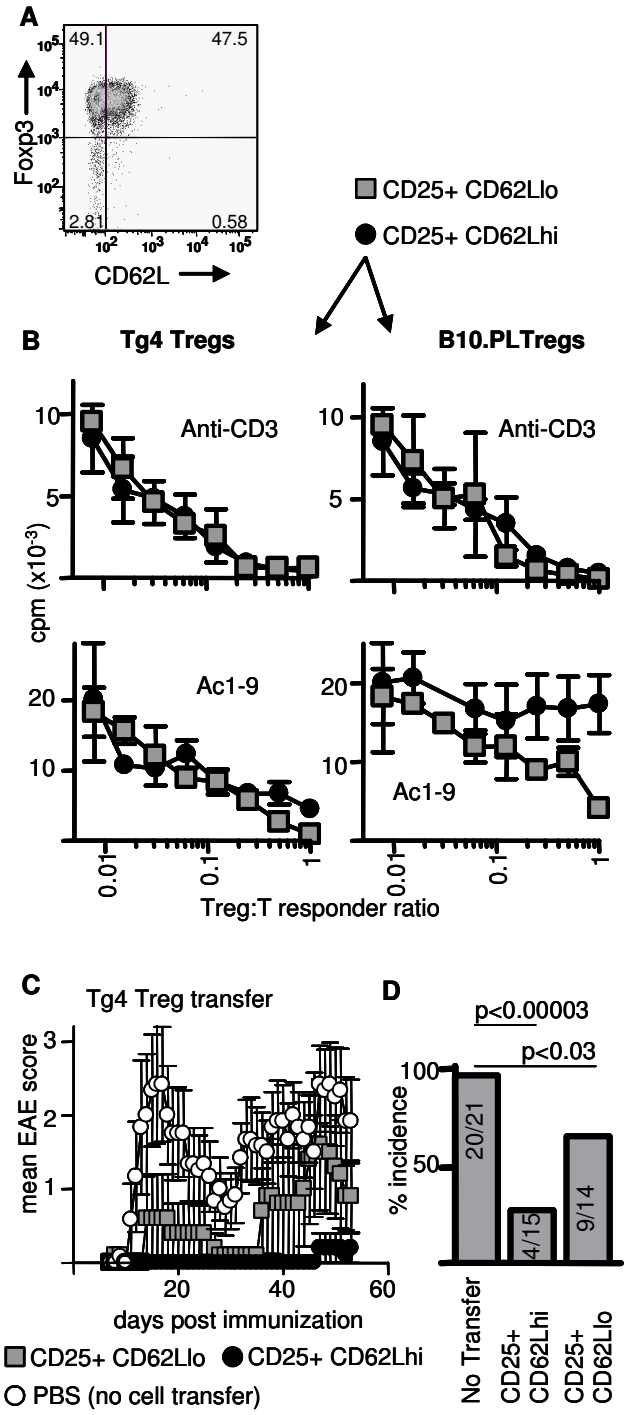

A
49.1
47.5
2.81
0.58
Foxp3
CD62L
CD25+ CD62Llo
CD25+ CD62Lhi
B
Tg4 Tregs
B10.PLTregs
Anti-CD3
Ac1-9
cpm (x10-3)
Treg:T responder ratio
C
Tg4 Treg transfer
mean EAE score
days post immunization
CD25+ CD62Llo
CD25+ CD62Lhi
PBS (no cell transfer)
D
p<0.00003
p<0.03
% incidence
20/21
4/15
9/14
No Transfer
CD25+ CD62Lhi
CD25+ CD62Llo

Figure 2

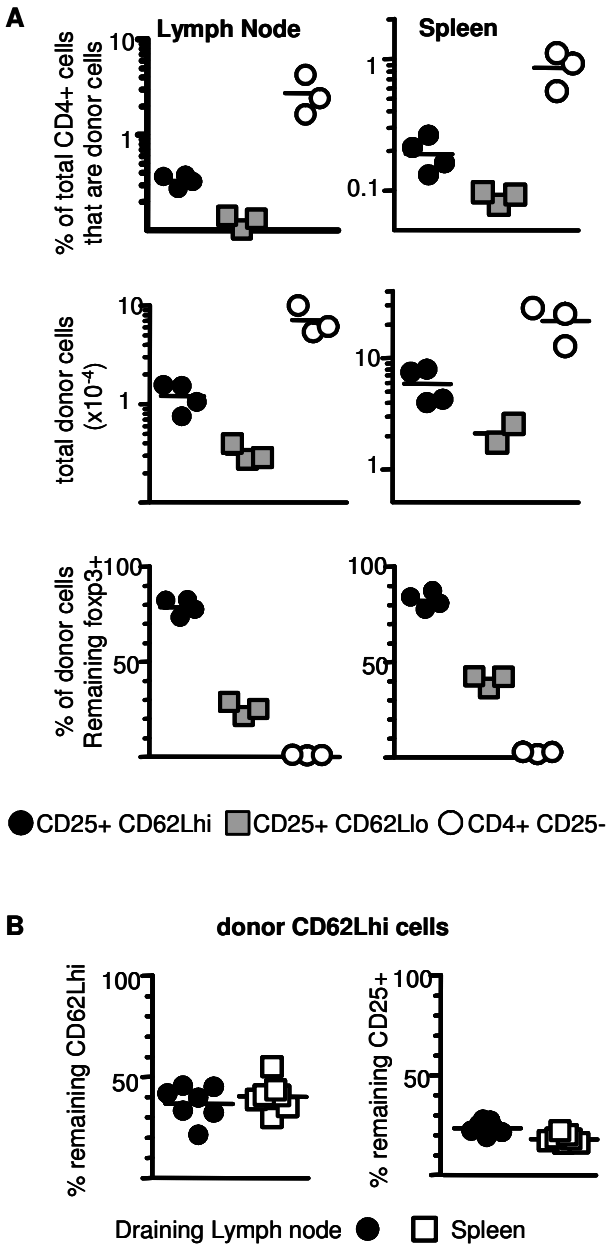

A
Lymph Node
Spleen
% of total CD4+ cells that are donor cells
10
1
1
0.1
total donor cells (x10⁻⁴)
10
1
10
1
% of donor cells Remaining foxp3+
100
50
100
50
CD25+ CD62Lhi
CD25+ CD62Llo
CD4+ CD25-
B
donor CD62Lhi cells
% remaining CD62Lhi
100
50
% remaining CD25+
100
50
Draining Lymph node
Spleen

Figure 3

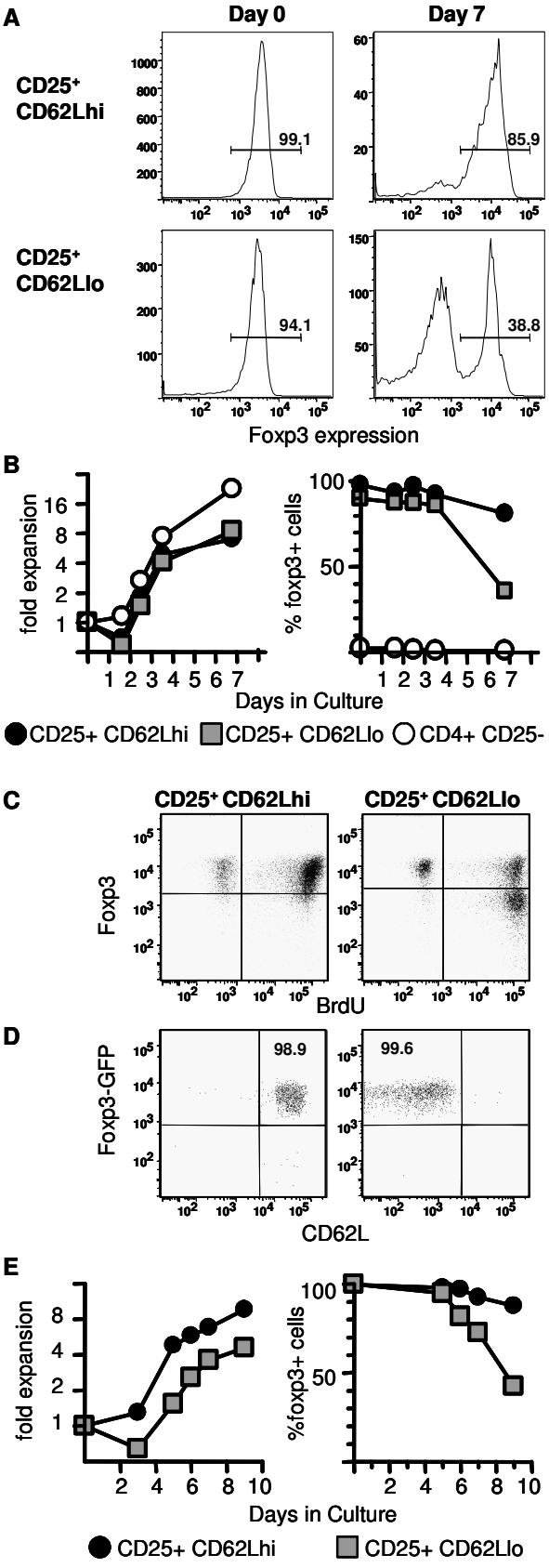

A
Day 0
Day 7
CD25+ CD62Lhi
CD25+ CD62Llo
99.1
85.9
94.1
38.8
Foxp3 expression
B
fold expansion
% foxp3+ cells
Days in Culture
CD25+ CD62Lhi
CD25+ CD62Llo
CD4+ CD25-
C
CD25+ CD62Lhi
CD25+ CD62Llo
Foxp3
BrdU
D
Foxp3-GFP
98.9
99.6
CD62L
E
fold expansion
%foxp3+ cells
Days in Culture
CD25+ CD62Lhi
CD25+ CD62Llo

Figure 4

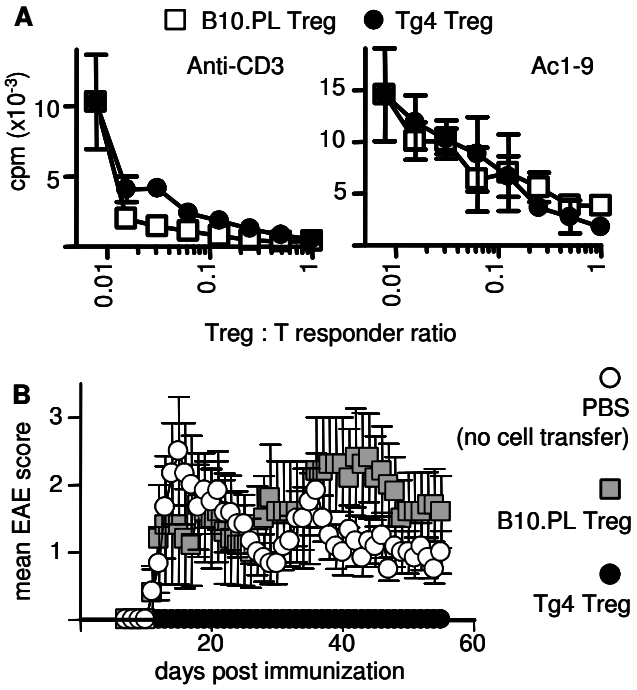

A
B10.PL Treg
Tg4 Treg
Anti-CD3
Ac1-9
cpm (x10-3)
10
5
15
10
5
0.01
0.1
1
0.01
0.1
1
Treg : T responder ratio
B
mean EAE score
3
2
1
20
40
60
days post immunization
PBS
(no cell transfer)
B10.PL Treg
Tg4 Treg

Figure 5

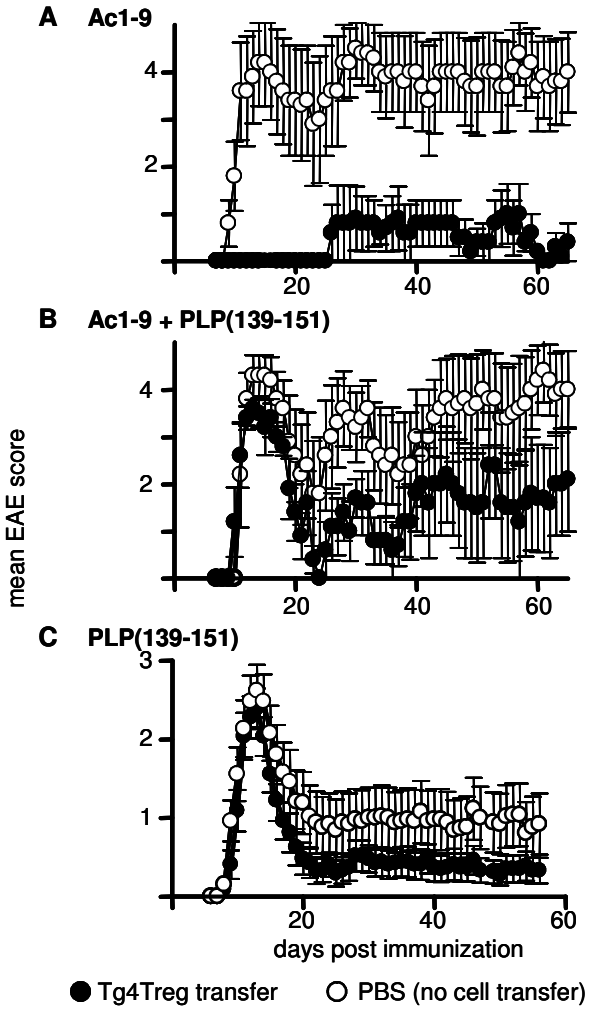
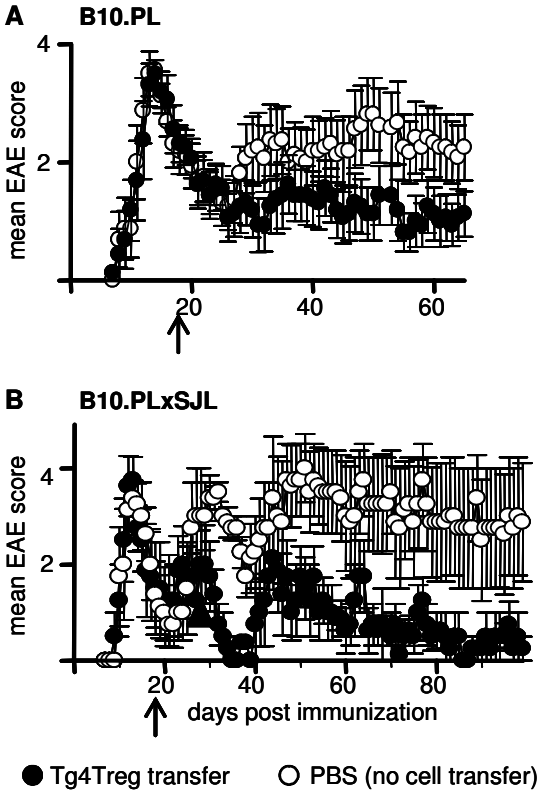

A Ac1-9
B Ac1-9 + PLP(139-151)
C PLP(139-151)
mean EAE score
days post immunization
Tg4Treg transfer
PBS (no cell transfer)

Figure 6


A
B10.PL
mean EAE score
4
2
20
40
60
B
B10.PLxSJL
mean EAE score
4
2
20
40
60
80
days post immunization
Tg4Treg transfer
PBS (no cell transfer)