Prevention of Relapses in Mice with Ongoing EAE via Combined Allogeneic Bone Marrow and Neural Stem Cell Transplantation

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Abstract

Multilineage mixed hematopoietic chimerism induces transplantation tolerance to allogeneic solid organ transplants in both animal models and humans. Yet we still have an imperfect understanding of the underlying mechanism and potential applications. I hypothesized that mixed chimerism could restore self-tolerance in the context of experimental autoimmune encephalomyelitis (EAE). To test this tolerizing strategy I designed a model whereby mixed chimerism could be induced in mice with ongoing EAE and which was amenable to downstream perturbations. I divided my investigation into two main studies; (1) an examination of induction of mixed chimerism with a subsequent neural stem cell transplant (Chapter 2), and (2) an examination of extracellular vesicles (EVs) replacing bone marrow in the aforementioned model (Chapter 3).

In the first study, I found that mixed chimerism alone was insufficient to prevent, and could only delay, further EAE relapses. However, the addition of a donor-matched neural stem cell transplant could induce self-tolerance. Specifically, I found that lymphocytes from mice receiving this treatment were not responsive to restimulation with the relevant self-antigen *in vitro*. However, the mice themselves still possessed memory CD4 T cells, which could

demonstrate a memory response upon reimmunization. In this manner I showed that deletion of autoreactive T cells alone cannot be responsible for the restoration of self-tolerance and it likely relies upon a regulatory effect.

In the second study, I examined whether EVs derived from allogeneic bone marrow could replace bone marrow, in order to delay relapses in mice with ongoing EAE. I found that these EVs could in fact delay relapses in a manner consistent with results obtained using bone marrow. Additionally, while this effect was found to be dose-independent above a certain threshold, the effect could be prolonged by repeated injections at lower doses.

Examining tolerance in this model has enabled the discovery of a novel mechanism of regulation by mixed chimerism as well as described a logical pathway by which these results could be translated into the clinic. This extension of our scientific understanding should advance further explorations of the mechanism of mixed chimerism and its application to human autoimmunity.

Table of Contents

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Glossary

APC – Antigen presenting cell
ATG – Anti-thymocyte globulin
BMC – Bone marrow cell
BMT – Bone marrow transplantation
C3H – Mouse strain (H-2^k)
C57BL/6 – Mouse strain (H-2^b)
CFA - Complete Freund's adjuvant
CNS – Central nervous system
EAE – Experimental autoimmune encephalomyelitis
EV – Extracellular vesicle
GVHD – Graft versus host disease
H&E – Hematoxylin and Eosin
LFB – Luxol fast blue
IFA – Incomplete Freund's adjuvant
IV - Intravenous
mAb – Monoclonal antibody
MHC – Major histocompatibility complex
MOG – Myelin oligodendrocyte glycoprotein
MS – Multiple sclerosis
NOD – Non-obese diabetic, mouse strain (H-2^{g7})
NOR – Non-obese diabetes resistant, mouse strain (H-2^{g7})
NSC – Neural stem cell
OCB – Oligoclonal band
OVA - Ovalbumin
PLP – Proteolipid protein
SJL – Swiss Jim-Lambert, mouse strain (H-2^s)
T1D – Type-1 diabetes
TBI – Total body irradiation
Treg – $CD4^+Foxp3^+$ Regulatory T cell

For my brother Ahsha, who always made me finish my chores

1

Introduction

"How does immune tolerance function here?" is a question I have asked to time and again during my time as a PhD student. Within this dissertation, I discuss the application of that question in the context of the restoration of self-tolerance in EAE, using mixed chimerism and concurrent NSC transplantation. I also discuss some additional studies undertaken as a direct consequence of the results from that project.

In this introductory chapter, I provide the background to understand the state of research in the field, and the unanswered questions that existed when I first undertook this project. First, I discuss MS, the problem I hope to address, and EAE, the murine model of T cell-mediated demyelination I have used in these experimental studies. Next, I introduce mixed chimerism, the methodology by which I endeavored to generate tolerance. While the two areas of study in many ways seem far apart, I believe that mixed chimerism may be an ideal tool to examine questions about T cell tolerance in EAE.

Chapter Two consists primarily of a first author article submitted for publication and represents the primary work generated during my PhD. I also introduce NSCs as a potential therapeutic in MS and as a tool used in the application of mixed chimerism to EAE. In Chapter Three, I summarize the studies arising as a direct consequence of my work in which we attempt to understand how the bone marrow (or a certain aspect of it) modulates the immune system when mixed chimerism is applied to EAE. I will also discuss EVs

and describe their use as a tool that may one day be able to replace bone marrow in the induction of tolerance in autoimmunity.

Finally, in the fourth chapter, I offer concluding remarks, and a discussion of future directions for this research. Although the practice of science has many ups and downs, I have sincerely enjoyed pursuing this project and what I have learned along this journey. I hope that you enjoy reading it as well.

1.1 Multiple sclerosis

MS is a progressive neurodegenerative autoimmune disease (Hans Lassmann, 2018; Popescu & Lucchinetti, 2012) and is one of the leading causes of non-traumatic disability in the United States (Howard, Trevick, & Younger, 2016; Kutzelnigg & Lassmann, 2014; Hans Lassmann, 2018; Winsen, Polman, Dijkstra, Tilders, & Uitdehaag, 2010). Although MS is a highly heterogeneous disease with multiple described phenotypes, the primary pathologic feature is damage to the myelin sheath surrounding neuronal axons within the CNS (Hans Lassmann, 2018; Popescu & Lucchinetti, 2012) and eventual axonal damage (Kutzelnigg & Lassmann, 2014; Hans Lassmann, 2018; Popescu & Lucchinetti, 2012). This damage presents as focal lesions, known as plaques, in both the gray and white matter of the brain and spinal cord (Hans Lassmann, 2018; Popescu & Lucchinetti, 2012). Clinically, MS most commonly presents in a relapsing-remitting form where patients have discrete episodes of disability with accumulation of disability over time. This is the phenotype of EAE that I will discuss almost exclusively in this document.

1.2 An evolving understanding of MS

The first published evidence of MS is attributed to Carswell and Cruveilhier, who published anatomical drawings of neuropathology in the 1830s (Carswell, 1838; Cruveilhier, 1835). Carswell, who was working at University College in London at the time, published *Pathological Anatomy: Illustrations of the Elementary Forms of Disease* (Carswell, 1838) wherein drawings from autopsied patients showed characteristic MS lesions in the CNS. Concurrently, Cruveilhier published a multi-volume work entitled *Anatomie Pathologique du Corps Humain* (Cruveilhier, 1835) which also contained drawings clearly describing prototypical MS lesions. Although no clear consensus has emerged as to which anatomist first described MS pathology, these drawings were the first pieces of evidence in developing a clear definition of the disease as a whole.

Following these descriptions of gross MS pathology, the publication of Rindfleisch's *Histologisches Detail zur grauen Degeneration von Gehirn und Rückenmark* (Rindfleisch, 1863) added significant pathological detail by offering microscopic analysis of autopsied brains with MS pathology for the first time. Notably, Rindflesich described perivascular inflammation as a microscopic hallmark of the lesions, consistent with our understanding of the pathology today. Up to this point, however, while multiple and varied pathologic and clinical accounts existed, no one had successfully integrated the available evidence into a coherent description of the disease.

It was not until 1868, 30 years after the work of Carswell and Cruveilhier, that Charcot, who is considered the "father" of neurology (Kumar, Aslinia, Yale,

& Mazza, 2011), gave the first unifying description of the disease that we now call MS during his lectures at La Salpêtrière hospital (Charcot, 1879). His description of "la sclérose en plaques" profoundly impacted the then nascent field of neurology. By the end of the 19th century many prominent physicians across the Western world recognized that MS was in fact a specific disease (Charcot, 1879; Kumar et al., 2011; Pearce, 2005).

1.3 MS as an autoimmune disease

1933 marked the first year that evidence of MS as an autoimmune disease was published. At The Rockefeller Institute Rivers published a paper that clearly demonstrated that injections of a "vaccine virus" in Rhesus Macaques would not cause an MS-like disease, while brain extracts from rabbits were capable of inducing an "acute disseminated encephalomyelitis" with features similar to MS (Rivers, Sprunt, & Berry, 1933). This paper was followed up by two more that demonstrated myelin itself might play a role in the induction of disease in primates (Rivers & Schwentke, 1935; Schwentker & Rivers, 1934). These studies were the beginnings of the EAE model of demyelinating disease which would prove integral for the development of many MS therapeutics.

It was not until after World War II however, that Kabat reproduced Rivers' EAE model using Rhesus Macaque brain tissue in Rhesus Macaques and demonstrated that self-tissue could induce the disease as well (Kabat, Wolf, & Bezer, 1947). However, a better understanding of the immune system and the

components therein would be required to more fully elucidate the various actors in MS.

In the latter part of the 20th century progress in both technology and our understanding of the immune system allowed for the identification of the various immune cells within the MS brain. This in turn led to the hypothesis that MS might have an autoimmune etiology (Allegretta, Nicklas, Sriram, & Albertini, 1990; Iivanainen, 1981; Prineas & Wright, 1978; Weiner L. & Schocket L., 1979). It was also during this period that genetic studies identified MHC II alleles as the largest genetic risk-factors, which led to the concept of MS as a $CD4^+$ T cell mediated disease. This hypothesis was further reinforced by more rigorous genome-wide association studies, which confirmed MHC II alleles as playing a role in MS risk (Fogdell, Hillert, Sachs, & Olerup, 1995; Jersild et al., 1973).

The above studies, along with advances using the EAE model, have led to the hypothesis that MS is a T cell mediated autoimmune disease wherein the immune system either loses some aspect of self-regulation or is activated aberrantly (Baecher-Allan, Kaskow, & Weiner, 2018; Howard et al., 2016; Hans Lassmann, 2018; Popescu & Lucchinetti, 2012; Wallin et al., 2019). However, there is no consensus on this point.

An alternative hypothesis has been proposed that neurodegeneration is the primary mechanism with immune activation as a secondary response, which is supported by some pathology studies, as well as work in primary progressive MS (Barnett & Prineas, 2004; Winsen et al., 2010). Regardless which hypothesis

is true, it is clear that MS is accompanied by a loss of immune self-tolerance, which will need to be addressed by any eventual curative treatment.

Whatever their etiology, MS lesions show evidence of immune involvement. The plaques are highly heterogenous in shape, size, and distribution. Although lesion distribution is prejudiced toward periventricular white matter, the cerebellum, subpial spinal cord, and other areas within the CNS can also be affected (Eden et al., 2019; Haider et al., 2016; Hans Lassmann, 2018; Popescu & Lucchinetti, 2012). The lesions are often perivenous. Thus, venous density correlates with lesion location (although this is not exclusively the case) (Grabner et al., 2011; Haacke, Garbern, Miao, Habib, & Liu, 2010).

Lesions may be broadly categorized as active or inactive, although more detailed classification systems have been proposed (Kuhlmann et al., 2017; Hans Lassmann, 2018). Active lesions are those currently undergoing demyelination. They contain a large number of phagocytic cells, which have morphology consistent with activated microglia/macrophages (Hu & Lucchinetti, 2009; H Lassmann, 2011; Hans Lassmann, 2018; G. F. Wu & Alvarez, 2011). The macrophages contain myelin fragments (G. F. Wu & Alvarez, 2011), consistent with recent demyelination. Active lesions also have large numbers of infiltrating lymphocytes. While these are primarily $CD8^+$ T cells, $CD20^+$ B cells and $CD4^+$ T cells are also present.

Conversely, inactive lesions, often the most abundant type, do not show the same phagocyte density. Instead, they are relatively hypocellular, although reactive astrocytosis is present and a glial scar replaces the damaged

oligodendrocytes. Lymphocytic infiltrates are still present, but significantly fewer in number (Filippi et al., 2018; Hans Lassmann, 2018; G. F. Wu & Alvarez, 2011).

1.4 Immune cells in MS

Although it is believed that MS involves a loss of self-tolerance, the exact role of the various immune cells is still being studied. Traditionally, $CD4^+$ T cells have been considered the primary driver of inflammation in MS (Filippi et al., 2018), although recently a more nuanced understanding has emerged that recognizes the involvement of B cells, $CD4^+$ and $CD8^+$ T cells, Tregs, and peripheral myeloid cells in MS immune pathology (Baecher-Allan et al., 2018; Danikowski, Jayaraman, & Prabhakar, 2017; Filippi et al., 2018; Jelcic et al., 2018; Kutzelnigg & Lassmann, 2014; Legroux & Arbour, 2015).

$CD4^+$ T cells are believed to become activated in MS via an unknown mechanism, then traffic to the CNS. Once there they may participate in demyelination (Legroux & Arbour, 2015; Petermann & Korn, 2011), although the precise mechanism is unknown. It is known that a variety of inflammatory molecules are secreted within MS lesions, and while some have been identified, their role is still poorly understood. It is believed that IL-17-expressing $CD4^+$ T cells play a role in pathology, an observation supported by work in EAE models (Jäger, Dardalhon, Sobel, Kuchroo, & Bettelli, 2009; Lee et al., 2012; Petermann & Korn, 2011). Cytokines such as IFNγ, TNFα, IL-12, IL-6, IL-1β, and GM-CSF have also all been implicated, although their contribution to pathology is not well characterized (Kaskow & Baecher-Allan, 2018; Kroenke & Segal, 2011; Panitch,

Haley, Hirsch, & Johnson, 1987; Petermann & Korn, 2011). $CD8^+$ T cells are the single most abundant lymphocyte in MS lesions and are likely involved in the cytokine milieu encountered therein (Elong Ngono et al., 2012; Kaskow & Baecher-Allan, 2018; Kuhlmann et al., 2017; G. F. Wu & Alvarez, 2011).

There is also a compelling body of evidence for alterations in immune system regulation in MS patients. For example, dysfunctional Tregs have been observed in MS patients in multiple studies. It was found by Dominguez-Villar et al. that, while the overall number of Tregs is the same in both MS patients and healthy controls, a greater proportion of Tregs in MS patients produce IFNγ and show reduced suppressive activity, a finding that has been reproduced by multiple studies. Other studies have found that the MS treatment, Copaxone (glatiramer acetate), can restore Treg suppressive activity, lending further credence to the hypothesis that MS pathology involves a breakdown in regulatory tolerance (Dominguez-Villar, Baecher-Allan, & Hafler, 2011; Frisullo et al., 2009; Haas et al., 2005; Putheti, Soderstrom, Link, & Huang, 2003; Viglietta, Baecher-Allan, Weiner, & Hafler, 2004). The body of evidence showing a decline of Treg function in MS patients has also spurred research into replacing Tregs or restoring Treg activity as a means of treating the disease (Long & Buckner, 2011; Stephens, Malpass, & Anderton, 2009)

B cells are present in MS lesions (Hans Lassmann, 2018; Prineas & Wright, 1978). In fact, the identification of OCBs in CSF samples from MS patients led to interest in B cell related therapies, based upon the hypothesis that autoantibodies were an important facet of immune pathology (Dobson,

Ramagopalan, Davis, & Giovannoni, 2013; Iivanainen, 1981; Rahmanzadeh, Weber, Brück, Navardi, & Sahraian, 2018). The most compelling evidence for their involvement comes from the success of αCD20 mAbs in the treatment of relapsing-remitting MS (Baecher-Allan et al., 2018; Rahmanzadeh et al., 2018). It should be noted that B cell therapy does not have an association with changes to OCBs in treated patients, which implies a mechanism of action beyond the hypothesized reduction in autoantibodies.

Despite the compelling evidence for lymphocyte involvement in MS, and the relative success of various immune modulating treatments (Gasperini, Ruggieri, & Tortorella, 2014; Rahmanzadeh et al., 2018; Schmied, Duda, Krieger, Trollmo, & Hafler, 2003), there is no consensus view as to the antigen or antigens driving the immune response. Various myelin antigens have been proposed (Elong Ngono et al., 2012; Mirshafiey & Kianiaslani, 2013), but evidence remains scant. Although antigen-specific induction of tolerance has been successful in EAE (Turley & Miller, 2010), where the relevant self-antigen is known, the translation of these strategies to the clinic is hindered by this unanswered question.

In summary, while many advances have been made in our understanding of MS, and the restoration of self-tolerance appears to be a worthwhile goal, unanswered questions obstruct our ability to effectively develop curative treatments for the disease. Any strategy must account for the fact that we do not know the relevant self-antigens, if a discrete set of self-antigens even exists in such a highly heterogeneous disease. Therefore, a reasonable tolerance

induction strategy would be one that does not require *a priori* knowledge of self-antigens. Additionally, while it may eventually be plausible to easily deplete the memory T cells involved in the autoimmune assault, currently such a goal would be exceedingly difficult in humans and a successful tolerance induction strategy must deal with for these autoreactive memory T cells (Mohty, 2007; Sykes & Sachs, 1988a).

1.5 EAE as a tool for studying MS

Animal models of disease remain immensely valuable in our understanding of various pathologies, as well as in the development of therapeutics/therapeutic strategies. EAE, although often referred to as a model of MS, might be more accurately called a model of T cell mediated demyelination. Regardless, its study has led to the development of multiple therapeutics (Terry, Ifergan, & Miller, 2014).

Although there are many possible ways to induce EAE, I have focused my work on the $PLP_{139-151}$ peptide model of EAE wherein SJL mice are immunized subcutaneously with peptide emulsified in CFA (Terry et al., 2014). This model is ideal for answering questions about the induction of tolerance in chronic disease because the mice develop a predictable relapsing remitting disease course. In addition, while monophasic models of EAE may involve specificity against a single antigen, EAE in the SJL mouse has been described to involve epitope spreading, where a response against other antigens is observed during relapses (McRae, Vanderlugt, Dal Canto, & Miller, 1995). Thus, in the chronic

disease state, memory T cells specific for multiple antigens are present and, similar to MS, any strategy to restore self-tolerance must account for this fact.

1.6 Mixed Chimerism in transplantation

Multilineage mixed hematopoietic chimerism is a biological state wherein the lymphohematopoietic system of an organism is composed of a mixture of cells from different MHC backgrounds. This state is most often achieved using allogeneic BMT (Zuber & Sykes, 2017). An organism may be deemed a mixed chimera when both donor and recipient cells are identifiable using an assay such as flow cytometry (usually when they comprise 1-99% of the population with the most common population assayed being peripheral blood leukocytes) (Sachs, Kawai, & Sykes, 2014). Mixed chimerism induces tolerance of allogeneic solid organ transplants in animal models and humans without risk of GVHD (Sachs et al., 2014). BMT has also been used to treat autoimmunity (Nash et al., 2015).

The original report describing mixed chimerism in mice relied upon lethal TBI followed by a bone marrow transplant of a mix of donor and recipient bone marrow cells (Ildstad & Sachs, 1984; Sykes & Sachs, 1988a, 1988b). To avoid GVHD and lymphocyte mediated rejection the donor and recipient bone marrow were both T cell-depleted. Mixed chimeras were then able to accept donor-matched skin grafts even across a complete MHC mismatch (Ildstad & Sachs, 1984). Although this method successfully induced tolerance of an allograft without GVHD (which would be unacceptable in clinical applications), the

conditioning still needed to be significantly less toxic to be clinically suitable (Sachs et al., 2014).

Our understanding of the mechanism by which mixed chimerism can induce allograft tolerance was significantly expanded by the discovery that employing T cell-depleting mAbs and thymic irradiation to deplete the host of peripheral and thymic T cells respectively could also induce mixed chimerism after BMT (Sharabi & Sachs, 1989). This enabled more specific studies of how central and peripheral tolerance are modified in mixed chimeras. For example, the finding that APCs derived from both donor and recipient bone marrow could populate the thymus described a key mechanism in the induction of tolerance by mixed chimerism (Khan, Tomita, & Sykes, 1996; Tomita, Khan, & Sykes, 1994; Wekerle et al., 1998).

In parallel, the discovery and characterization of costimulatory pathways, in particular CD40 – CD40L, significantly enhanced our understanding of T cell activation in the immune response (Armitage et al., 1992; Lederman et al., 1992; Noelle et al., 1992). Shortly after T cells become activated (24-48 hours), they begin to upregulate CD40L. Although this molecule doesn't directly activate T cells it has a variety of functions regarding immune activation (Elgueta et al., 2009). Primarily it enables the main function of CD4 T cells, which is to help macrophages and B cells. T cell CD40L binds CD40 expressed on APCs and this interaction helps activate APCs, particularly DCs and B cells, causing them to upregulate B7 molecules and increase cytokine secretion, leading to a positive feedback loop of T cell activation (Elgueta et al., 2009). In fact, the application of

costimulatory blockade using αCD40L mAbs is such a powerful inhibitor of immune activation that the use of these antibodies abrogated the need for CD4+ T cell depleting mAbs and thymic irradiation in the induction of mixed chimerism (Wekerle et al., 2000, 1998).

Mechanistically, in the context of transplant, anti-donor cells are deleted both peripherally (Domenig et al., 2005; Fehr et al., 2008; Kurtz et al., 2004; Wekerle et al., 1998) and intrathymically (Tomita et al., 1994; Wekerle et al., 1998). Additional work showed that thymectomy prior to induction of mixed chimerism resulted in a failure of tolerance induction and suggested that intrathymic deletion of alloreactive T cell clones is the primary mechanism in the maintenance of tolerance (Khan et al., 1996). Concurrently, although a variety of studies found no evidence of a regulatory mechanism for $CD4^+$ T cell tolerance in this model (Ito, Kurtz, Shaffer, & Sykes, 2001; Kurtz et al., 2004; Takeuchi et al., 2004), there is some evidence that while the tolerance of bone marrow may rely on deletion, Tregs could play a role in tolerance of the subsequent solid organ transplant (Shinoda et al., 2014).

1.7 Mixed chimerism in autoimmunity

Just as the induction of transplant tolerance is a therapeutic goal within the field of transplantation, the restoration of self-tolerance is a goal in the treatment of autoimmunity. For this reason, it is reasonable to hypothesize that mixed chimerism may be applicable in autoimmunity due to its ability to induce tissue-specific tolerance.

The first studies examining the use of mixed chimerism in autoimmunity were conducted in the NOD mouse model of T1D. A series of studies in the late 1990s evaluated the original mixed chimerism protocols (lethal TBI + BMT of combined donor and recipient BMCs) in the NOD model (H. Li et al., 1996; Mathieu, Casteels, Bouillon, & Waer, 1997). Interestingly, while it was possible to induce stable mixed chimerism, the initial protocols required more conditioning to induce mixed chimerism successfully in autoimmune prone mice (i.e. NOD) than in non-autoimmune prone strains (H. Li et al., 1996). It was found that the induction of allogeneic mixed chimerism prevented the occurrence of diabetes in NOD mice. In the context of the intense T cell depletion of these protocols, it was found that the thymus was unnecessary for this protective effect, and that adoptive transfer of NOD splenocytes from these mice alone would be significantly less effective at initiating disease (Mathieu et al., 1997). Although regulation and deletion were not specifically examined in these early studies, researchers concluded that some durable tolerance effect was occurring.

Subsequent studies, which employed mixed chimerism models using costimulatory blockade in the NOD model, demonstrated that the induction of chimerism in this context was still capable of preventing the onset of disease (Beilhack, Landa, Masek, & Shizuru, 2005; Zhang et al., 2007). According to Zhang et al. (2007), mixed chimerism could cure early stage diabetes in these mice, even if it was ineffective in the late stage of the disease. Finally, many studies suggested that mixed chimerism was effective as a method of inducing tolerance to transplanted allogeneic islets, or controlling the autoimmune attack

such that recipient type islets could be transplanted without subsequent destruction (Nikolic et al., 2010; Nikolic, Takeuchi, Leykin, & Fudaba, 2004; Wang et al., 2014; Zhang et al., 2010).

One consistent observation across multiple studies was that MHC-mismatched chimerism is required in the NOD model. Both for prevention of disease or tolerance of islet transplants (Beilhack et al., 2005; Nikolic et al., 2010; J. Racine et al., 2011; Wang et al., 2014). For example, Wang et al. (2014) found that in the NOD model, MHC-matched congenic bone marrow from C56BL/6 (H-2K^{d}, I-A^{g7}) mice, which share the same MHC and background genes as the NOD mouse, was ineffective in conferring immune tolerance to transplanted islets, while MHC-mismatched C57BL/6 (H-2K^{b}, I-A^{b}) BMCs succeeded in inducing immune tolerance.

Mixed chimerism has also been applied to other models of autoimmunity. In a mouse model of lupus, not only did researchers find that mixed chimerism can reverse autoimmunity, but that the addition of a histone deacetylase inhibitor allows for the induction of chimerism without radiation or costimulatory blockade (N. Li et al., 2008; Smith-Berdan, Gille, Weissman, & Christensen, 2007). More recent work in the EAE model has shown that by using a T cell depleting (Chung et al., 2007; Mariotti et al., 2011) model of mixed chimerism (pentostatin + cyclophosphamide + ATG), relapses could be prevented in ongoing EAE (L. Wu et al., 2015). The finding that thymectomy prevented the induction of tolerance in this model implies a strong role for thymic deletion of

autoreactive T cell clones. Similar to the NOD model, MHC-mismatched chimerism was also required for this effect.

Mechanistically, Racine et al. (2011), observed that donor APC expression of MHC II was required for the tolerizing effect of mixed chimerism in the NOD model, although the reason for this requirement remains unclear. While there is evidence for deletion of autoreactive lymphocytes (J. J. Racine, Wang, Zhang, & Zeng, 2014; L. Wu et al., 2015), there is also evidence for a possible regulatory role. Wang et al. (2014) found that autoreactive memory T cells persist after the induction of mixed chimerism in the NOD model. Wu et al. (2015) found increases in the number of $CD4^+$ $Foxp3^+$ T cells in the spleen and lymph nodes following the induction of chimerism in EAE. Further evidence is found in Nikolic et al. (2010), where it was shown that adoptive transfer of T cells from mixed chimera NOD mice would induce disease if donor T cells were depleted, whereas non-depleted T cells would not. Taken together, these data imply that while deletion may be the primary mechanism for allograft tolerance, regulation may also play a primary role in mixed chimerism induced tolerance in autoimmunity.

There are many unanswered questions in the application of mixed chimerism to autoimmunity. It seems that greater success in terms of tolerance induction is achieved in the context of strong T cell depletion. However, in the context of human MS T cell depletion may be more difficult, as memory T cells are resistant to common T cell depleting therapeutics (Bouvy et al., 2016; Mohty, 2007; L. Wu et al., 2015). Any tolerance induction strategy that will successfully

reach the clinic will have to deal with the problem of memory T cells and any model to ask questions about it will have to do so as well.

1.8 References

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2

Prevention of relapses in mice with ongoing EAE via combined allogeneic bone marrow and neural stem cell transplantation

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2.1 Abstract

Previous work has shown a potential for induction of self-tolerance in EAE by allogeneic mixed hematopoietic chimerism. Here we show that allogeneic mixed chimerism alone is insufficient to induce durable self-tolerance. However, the addition of donor matched NSCs to the allogeneic mixed chimerism protocol completely prevents relapses in mice with ongoing EAE.

We induced EAE with $PLP_{139-151}$ in 6-8 week old female SJL mice. Upon entering the first disease remission we induced mixed chimerism using $CD8^+$ T-cell depletion, 300 cGy total-body irradiation, αCD40L antibodies, and $50x10^6$ BMCs from C57BL/6 (allogeneic), or SJL (syngeneic) donors. Following BMT the two groups were given a C57BL/6, SJL, or sham NSC transplant.

Only mice that received allogeneic BMT and allogeneic NSCs remained relapse free for the duration of the study. All other groups relapsed within the 100 day post-BMT monitoring period. Allogeneic bone marrow recipients had detectable chimerism and remained tolerant to donor antigens, despite EAE relapses. Splenocytes from mixed chimeras transplanted with donor matched NSCs were unresponsive to PLP peptide restimulation, in contrast to mixed chimeras that received a sham treatment. Reimmunization of these mice with PLP peptide was able to break tolerance and demonstrated that autoreactive memory $CD4^+$ T cells were still present and capable of responding to stimulation.

These data indicate that inducing mixed chimerism combined with donor-matched NSC transplantation can completely prevent relapses in chronic, established EAE by preventing the reactivation of autoreactive memory $CD4^+$ T

cells. Allogeneic mixed chimerism is necessary but not sufficient to induce self-tolerance while the addition of donor-matched NSCs maintains self-tolerance for at least 100 days. These results may provide new insights into tolerance induction and memory T cell activation in autoimmunity.

2.2 Introduction

MS is a progressive autoimmune disease characterized by CNS inflammation, demyelination, and eventual axonal degradation (Hemmer, Archelos, & Hartung, 2002; Hu & Lucchinetti, 2009; Petermann & Korn, 2011). The most common form of MS presents in a relapsing-remitting pattern with periods of relatively little disease activity punctuated by relapses with a progressive increase in disability (Baecher-Allan, Kaskow, & Weiner, 2018). $CD4^+$ T cell dysregulation is considered to be the driving force behind the etiology and pathogenesis of MS (Baecher-Allan et al., 2018; Popescu & Lucchinetti, 2012), although $CD8^+$ T cells and B cells appear to also play a role (Lassmann, 2018; Rahmanzadeh, Weber, Brück, Navardi, & Sahraian, 2018).

EAE induced via immunization of mice with certain myelin proteins delivered in an inflammatory environment is a classical mouse model for T cell-mediated demyelination similar to MS (Terry, Ifergan, & Miller, 2014). A number of immune tolerance induction strategies have been shown to prevent EAE onset via deletion and/or suppression of some autoreactive $CD4^+$ T cells specific for the immunizing autoantigen (Miller, Turley, & Podojil, 2007a). However, such strategies have been difficult to translate to humans. It is therefore critical to design new strategies capable of restoring T cell tolerance of in MS patients.

Tolerance of allogeneic organ transplants has been achieved in rodents and patients via mixed hematopoietic chimerism. In this setting, allogeneic BMT under non-myeloablative conditioning resulted in coexistence of the host and the donor immune systems and achieved allograft tolerance with minimal risks of

GVHD. It is firmly established that stable MC in mice leads to continuous presentation of allogeneic MHC molecules in the host's thymus and subsequent deletion of developing alloreactive T cells (Zuber & Sykes, 2017). On the other hand, the contribution of peripheral T cell regulation, if any, in chimerism-induced allograft tolerance using αCD40L costimulatory blockade is still unclear (Kurtz, Shaffer, et al., 2004; Kurtz, Wekerle, & Sykes, 2004).

Based on similar principles, genetic resistance to autoimmunity transferred via BMT has been considered as a potential therapy for autoimmune diseases. Supporting this view, several reports showed that BMT could prevent the occurrence of type 1 diabetes in NOD mice (Ikehara et al., 1985; LaFace & Peck, 1989; Naji, Silvers, Bellgrau, & Barker, 1981; Takao et al., 1995). Likewise, mixed chimerism prevented diabetes and reversed isletitis in myeloablated prediabetic mice (H. Li et al., 1996; Mathieu, Casteels, Bouillon, & Waer, 1997). Since allogeneic BMT achieves tolerance of alloantigens, it was conceivable that this strategy could be used in combination with inoculation of pancreatic islets from the same donor and cure diabetes. Indeed, costimulatory blockade plus sublethal TBI achieved mixed chimerism with tolerance of donor skin allografts and lack of autoimmunity recurrence upon transplantation of donor islets in diabetic NOD mice (H. Li et al., 1996).

In another study, tolerance of allogeneic islet grafts from male NOR donors could be induced in female NOD recipients by simultaneous islet and bone marrow transplantation under fludarabine phosphate-based nonmyeloablative conditioning therapy(Sherer & Shoenfeld, 1998). At the same time, studies from

M. Sykes' laboratory showed that MC induced via non-myeloablative conditioning involving a low dose of TBI, recipient peripheral T cell depletion, and αCD154 mAb treatment allowed successful islet transplantation and reversal of destructive autoimmunity and disease in NOD mice with ongoing diabetes (Nikolic, Takeuchi, Leykin, & Fudaba, 2004). In a subsequent study from the same group, it was reported that partial depletion of NOD $CD4^+$ T cells was needed to achieve reversal of isletitis via mixed chimerism, but was not required for induction of tolerance to alloantigens (Nikolic, Onoe, Takeuchi, Khalpey, et al., 2010). Finally, similar hematopoietic chimerism procedures have been shown to prevent or ameliorate other autoimmune diseases including systemic lupus erythematosus and EAE in mice (N. Li et al., 2008; L. Wu et al., 2015).

Cellular therapy has a great deal of promise in the treatment of MS. Although multiple cell types are being evaluated, one population of particular interest in MS are neural stem cells. NSCs are multipotent stem cells which are found in the CNS, especially in the ependyma of the subventricular zone, and are able to differentiate into both glia and neurons (Azari, Rahman, Sharififar, & Reynolds, 2010). NSCs have been examined in EAE, where they have been shown to treat disease by an as yet unknown mechanism. Despite this promise, issues such as route of administration, the source of cells to be used in therapies, and the immune response to an allogeneic transplant remain obstacles to their clinical use.

NSCs have a variety of beneficial effects within the CNS of animals with ongoing EAE. Syngeneic NSC administration has been shown to induce recovery

in ongoing, chronic EAE (Bai, Hecker, Kerstetter, & Miller, 2013; Einstein et al., 2007; Pluchino et al., 2003). The exact mechanism of NSC-mediated repair in EAE is poorly understood. Although it is known that NSCs can differentiate into oligodendrocytes, and may be able to mediate repair directly (Ben-Hur et al., 2003; Einstein et al., 2006), there is evidence that they may not actually enact repair themselves and may instead enable endogenous repair mechanisms in the host (Bai et al., 2013; S. Wu et al., 2013).

Although NSCs have a great deal of potential as a cellular therapy, there exist a number of obstacles to their use in a clinical setting. As of now, there is no source of syngeneic NSCs suitable for transplant in humans and therefore allogeneic transplants must be considered. For this reason, the primary barrier to NSC transplantation, as with other transplants is immune rejection (Tullis, Spears, & Kirk, 2014).

Originally, it was hypothesized that allogeneic NSC transplantation into the CNS would not necessarily result in immune rejection due to the immune-privileged nature of the CNS and also low/absent expression of MHC molecules on the NSC surface (Hori et al., 2003; L. Li et al., 2004). It has since become clear, however, that in inflammatory environments (such as MS) NSCs can be rejected (Johansson, Price, & Modo, 2008; Ubiali et al., 2007; Weinger et al., 2012), and for this reason the immune consequences of allogeneic transplantation must be dealt with for NSCs to be a viable therapeutic option. Although immunosuppression has been effective at preventing NSC rejection in a model of spinal cord injury (Rosenzweig et al., 2018), lifelong

immunosuppression is less appealing in the context of MS and therefore a tolerance induction strategy would be necessary.

Another consideration in the use of NSCs is the route of administration. Initial studies in this field used the intravenous route (Bai et al., 2013; Pluchino et al., 2005). Recent data suggest that NSCs transplanted in this way do not specifically traffic to the CNS, and may be found in peripheral lymph nodes, where they are broadly immunosuppressive (Reekmans et al., 2011; Yang et al., 2009). Although surgical transplantation directly into the CNS is an option, and perhaps the preferred route for spinal cord injury (Rosenzweig et al., 2018), another non-invasive option is intranasal delivery. By this route NSCs may be easily transplanted and trafficking to the CNS is efficient and highly specific (Danielyan et al., 2009; S. Wu et al., 2013).

While the aforementioned studies demonstrate that hematopoietic chimerism can prevent or halt the progression of autoimmunity, it is unlikely to restore physiological functions once organs and tissues have been already destroyed. To address this problem, we tested whether a combination of mixed chimerism and NSC transplantation could achieve both immune tolerance and remyelination of damaged neural tissues, respectively, in mice with ongoing EAE. We found that while mixed chimerism alone only delayed relapses in mice with ongoing EAE, its combination with NSC transplantation cured the disease. These findings may have important implications for the design of new therapies in MS.

2.3 Materials and Methods

Mice: C57BL/6J, C3H/J, and SJL/J mice were obtained from the Jackson laboratory (Bar Harbor, ME). 6-8 week old female mice were used throughout the study. All animal care and handling was performed according to institutional guidelines.

Reagents: Antibodies: PerCP/Cy5.5 αCD4 (BioLegend 100433), Brilliant Violet 510 αCD8α (BioLegend 100751), FITC αCD19 (BioLegend 115505), APC αCD11b (BioLegend 101211), APC αCD11c (BioLegend 117309), Brilliant Violet 421 αCD45.2 (BioLegend 109831), PE/Cy7 αCD335 (BioLegend 137617).

EAE Reagents: Incomplete Freund's Adjuvant (Sigma Aldrich F5506), Heat-killed Mycobacterium Tuberculosis H37 (Difco 231141).$PLP_{139-151}$ (HCLGKWLGHPDKF), MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK), MOG_{92-106} (DEGGYTCFFRDHSYQ), and $PLP_{178-191}$ (NTWTTCQSIAFPSK) were provided by Dr. Ashok Khatri (Massachusetts General Hospital, Charlestown, MA).

Mixed Chimerism Reagents: αCD8α (Clone 2.43, BioXCell BE0061), αCD40L (Clone MR1, BioXCell, BE0017-1), Shepherd Cesium Irradiator.

Experimental Autoimmune Encephalomyelitis: CFA was prepared at a 5mg/ml concentration of heat-killed Mycobacterium Tuberculosis in IFA mixed

very well. CFA was used fresh or within 1-2 days for all experiments. Emulsions were prepared by combining equal amounts of CFA and $PLP_{139-151}$ dissolved in PBS at 150 μg/ml using two 3ml syringes and a three-way stopcock system. Female 6-8 week old SJL/J mice were given a 100μl subcutaneous injection of emulsion (comprising of 75μg $PLP_{139-151}$) ventrally on the right side of the chest situated near the axial lymph nodes using a 1ml syringe with a 22 gauge needle (to ensure smooth flow of the emulsion). Five days after immunization daily monitoring for EAE symptoms began. Symptoms would routinely begin 10-14 days post-immunization. Mice were assessed for symptoms using a 5-point scale as follows: 1 – Complete tail paralysis, 2 – Score 1 plus hind-limb weakness as assessed by gait disturbance or inability to right after flipping, 3 – Score 1 plus complete hind-limb paralysis, 4 – Score 3 plus complete forelimb paralysis, 5 – Death. Half-point scores were awarded in cases of incomplete paralysis (e.g. a score of 0.5 for partial tail paralysis). Remission was defined as two consecutive days of decreased score following the peak acute-phase score (Miller & Karpus, 2007). Relapse was defined as an increase in score after the nadir of remission combined with two days of score 1 or above. Reimmunization was performed in a manner similar to the initial immunization with the following modifications: IFA was used in place of CFA and the immunization was given ventrally on the left side of the chest situated near the axial lymph nodes. All EAE experiments were performed blinded, where the researcher scoring the mice was unaware of their treatment status. Mice were considered self-tolerant if they were relapse-free for at least 100 days following mixed chimerism induction.

Mixed Chimerism: On the first day of remission as measured by population average (defined as Day -1 mixed chimerism induction) within the experiment, mice were given 500μg of αCD8α intraperitoneally. On day 0 bone marrow cells were isolated from 6-8 week old female donor mice as previously described (Amend, Valkenburg, & Pienta, 2016). Recipient mice were treated with 300 rads total-body irradiation, 1mg αCD40L IP, and 50 x 10^6 bone marrow cells IV. Beginning at day 28 post-chimerism induction and at regular intervals thereafter, peripheral blood mononuclear cells were obtained via cheek bleeding and examined via flow cytometry for $CD45.2^+$ (donor) cells. Mice were considered chimeric if they had detectable donor cells above background.

Neural Stem Cell Isolation and Transplantation: Neural stem cells were isolated as previously described (Azari et al., 2010). Briefly, the subventricular zone was microdissected from 4-6 week old mice and subjected to digestion with trypsin. Following this the cells were cultured in Neurocult basal medium with Neurocult proliferation supplement, recombinant human epidermal growth factor, recombinant human β–fibroblastic growth factor, and heparin. The cells were cultured and the medium regularly refreshed until neurospheres of sufficient size formed, at which point the cells were passaged by trypsin digestion, washing, counting, and resuspension in fresh medium at $1x10^6$ cells/ml. At passage 4, neurospheres were cryopreserved by the addition of 8% DMSO to the culture medium, freezing, and storage in liquid nitrogen. Prior to transplantation cells were thawed, washed, and cultured until neurospheres of

sufficient size were visible in the medium. On the day of transplant, neurospheres were disassociated by trypsin digestion, counted, and resuspended in PBS. Concurrently, mice were treated with 100U of hyaluronidase in PBS in each nostril. 30 minutes post-enzymatic treatment, the mice were given $1x10^6$ NSCs, split into $5x10^5$ NSCs in each nostril. Sham treated mice received hyaluronidase treatment and subsequently PBS in each nostril.

***In Vitro* Cell Restimulation:** Following euthanasia by CO_2 asphyxiation, spleens were isolated and a single cell suspension was obtained by passage through a 70 micron mesh into PBS. Red blood cells were lysed using ACK Lysis Buffer, and cells were washed and resuspended in cDMEM. $2.0x10^5$ splenocytes were culture in the presence of $OVA_{323-339}$, $PLP_{139-151}$, or MOG_{35-55} at 20μg/ml. Alternatively, cells would be cultured with equivalent numbers of irradiated B6, SJL, or C3H cells. αCD3/αCD28 Dynabeads were used a positive control. Cells were cultured for 5 days at which point they were stained for analysis by flow cytometry.

Flow Cytometry: Samples were acquired using a BD FACSVerse flow cytometer and analyzed using FlowJo V10. Cells to be acquired were first washed in PBS to remove protein which would interfere with viability staining. Cells were resuspended in [livedeadstain] according to the manufacturers instructions and incubated for 20 minutes. Following that cells were washed with 2% FBS in PBS (hereafter referred to as FACS buffer). Cells were then stained for cell-surface

molecules using pre-determined antibody titrations in FACS buffer for 30 minutes. For experiments not requiring intracellular staining the cells were washed twice in FACS buffer, resuspended in 200µl of FACS buffer, and acquired immediately. For cells requiring intracellular staining following the two washes the cells were resuspended in BD Cytofix for 10 minutes, the cells were then washed in BD Cytoperm twice and resuspended in BD Cytoperm with the appropriate antibody for 45 minutes. The cells were then washed twice and resuspended in FACS buffer before acquisition.

2.4 Results

2.4.1 Stable mixed hematopoietic chimerism can be induced in mice with ongoing EAE

Mixed hematopoietic chimerism in which the donor and recipient immune systems coexist is regularly achieved in naïve mice via non-myeloablative conditioning and bone marrow transplantation (Sachs, Kawai, & Sykes, 2014). In this study, we first investigated whether stable mixed chimerism could be achieved in the context of inflammation in mice with ongoing EAE.

First, we evaluated various doses of PLP peptide 139-151 ($PLP_{139-151}$) emulsified in CFA for their ability to induce EAE in SJL ($H\text{-}2^s$, CD45.1) mice. As shown in supplemental Figure 1, 50μg $PLP_{139-151}$ resulted in an acute EAE attack slightly over two weeks after immunization with subsequent remission followed by relapses consistent with prior reports (Terry et al., 2014). Increasing the dose of peptide (100μg) or adding pertussis toxin resulted in either no or milder EAE symptoms (Figure S1). A dose of 75μg $PLP_{139-151}$ was chosen for further EAE experiments in this study.

Next, SJL mice entering the first disease remission were conditioned with a non-myeloablative regimen consisting of 500μg intraperitoneal αCD8 mAb on day -1, 3 Gy TBI, 1mg intraperitoneally of αCD40L mAb, and IV injection of $50x10^6$ BMCs isolated from a fully allogeneic C57BL/6 mouse (B6, $H\text{-}2^b$, CD45.2) on day 0 (Figure 2.1a and 2.1b).

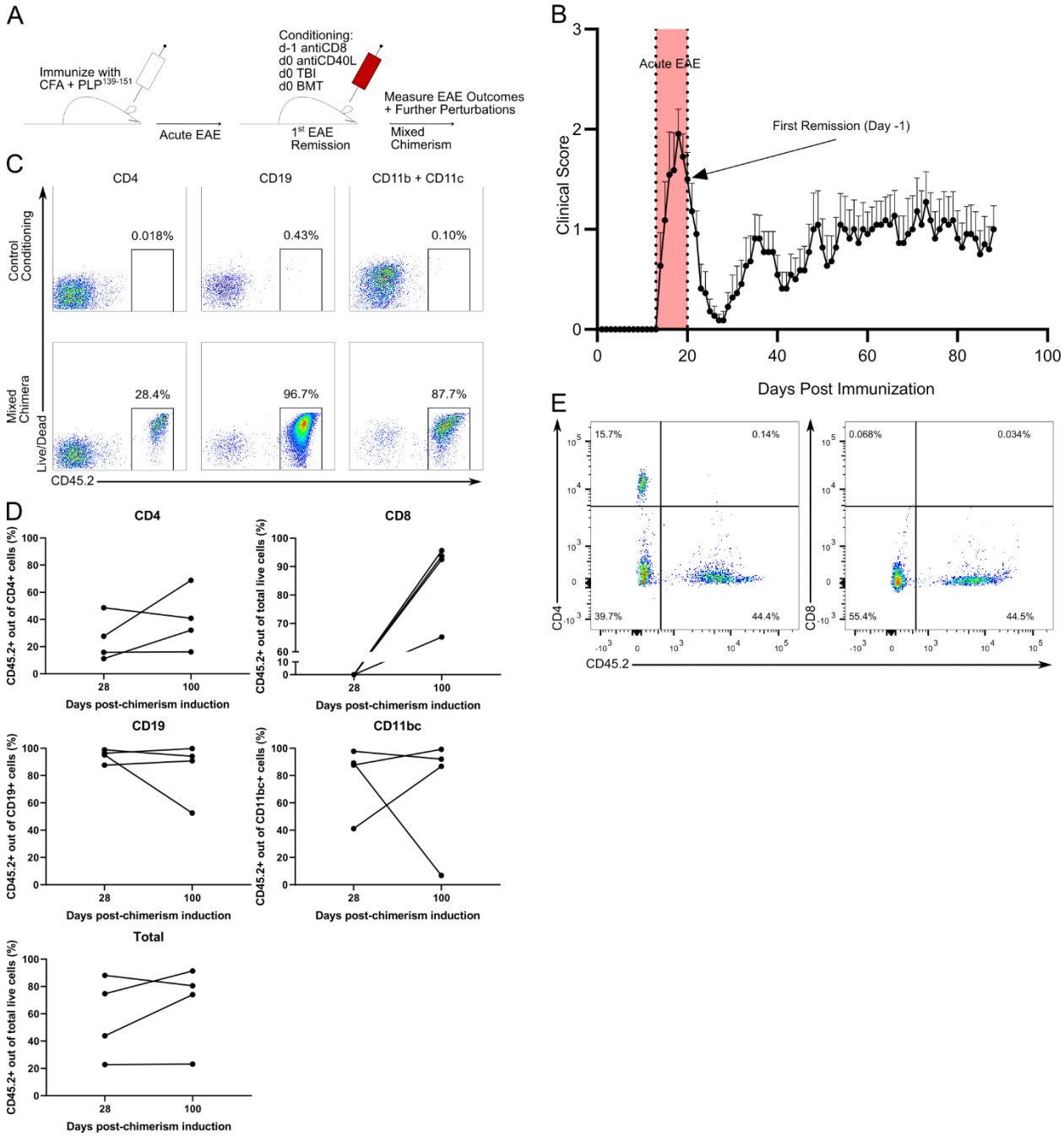


Figure 2.1: Induction of mixed chimerism in EAE. A. Cartoon of experimental model. **B**. Sample EAE graph depicting typical course of EAE and points of intervention. The red bar indicates the first acute EAE attack, with the left boundary indicating onset and the right indicating the first day of remission. **C**. Representative plot of chimerism values in a mixed chimera and a mouse that received control conditioning from day 28 post-chimerism induction. Peripheral blood mononuclear cells were analyzed by flow cytometry using the following gating strategy: Singlets (FSC-H vs FSC-A), live/dead discrimination, the indicated leukocyte subset (CD4, CD8, CD19, or CD11b + CD11c), and CD45.2 discrimination. **D**. XY plot of chimerism

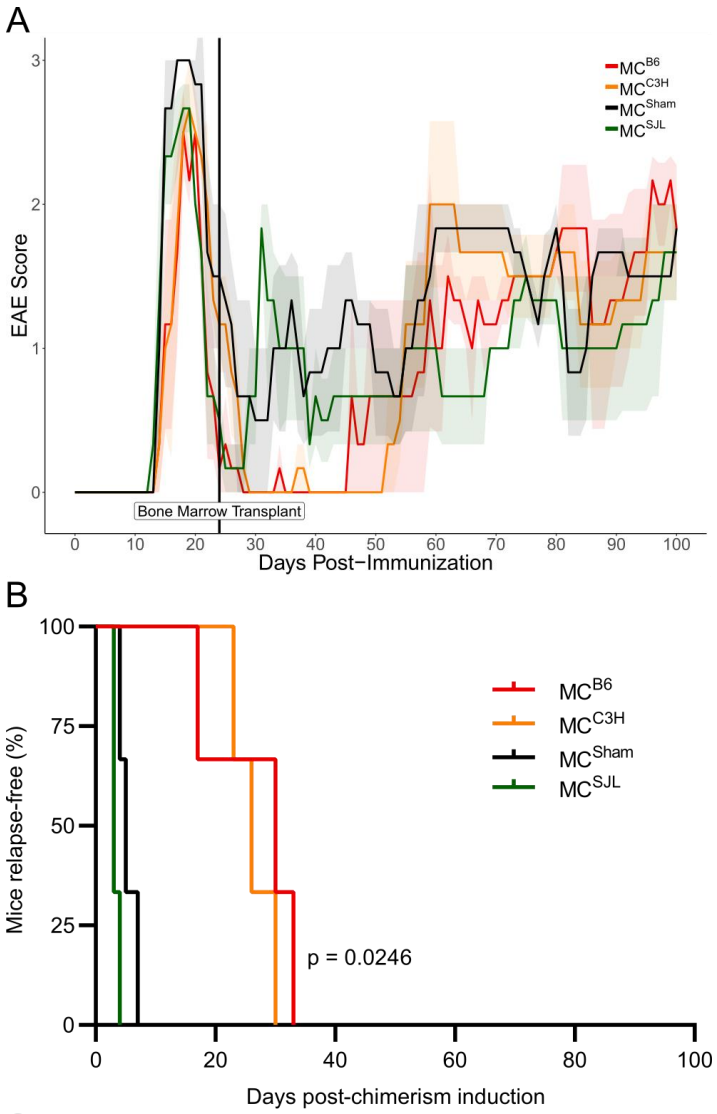
Figure 2.1 (continued) values at days 28 and 100 post-chimerism induction in a single experiment. Values are presented as percentage of CD45.2 positive cells within the marked leukocyte subset. (N=4) **E**. Representative plot of donor and recipient proportions of CD4 and CD8 positive cells at day 14 post-chimerism induction. Gating strategy as in B. Results are representative of multiple experiments.

The level of donor hematopoietic chimerism among peripheral blood leukocytes was then monitored via CD45.2 expression at regular intervals for 100 days using flow cytometry. As shown in Figure 2.1c and Figure S2, on day 28 post-chimerism induction, sustained mixed chimerism including B cells, macrophages, and dendritic cells was observed in all mice. 100 days post-chimerism induction, mice remained chimeric (Figure 2.1d). On the other hand, 14 days after transplantation, the $CD8^+$ compartment was entirely depleted while the recipient $CD4^+$ compartment remained intact (Figure 2.1e).

2.4.2 Mixed hematopoietic mixed chimerism delays but does not prevent disease relapses

Next, we evaluated the effects of mixed chimerism on disease relapses. SJL ($H\text{-}2^s$) mice were conditioned during the first remission phase and injected with syngeneic BM cells (referred to as MC^{SJL}), fully allogeneic BM cells from a B6 ($H\text{-}2^b$) (MC^{B6}), or a C3H ($H\text{-}2^k$) (MC^{C3H}) donor. Other mice received saline instead of BMCs (MC^{sham}). As shown in Figure 2.2a-c, MC^{Sham} mice experienced significant disease burden almost immediately post-transplant. Alternatively, allogeneic BMT significantly delayed the onset of disease relapses ($p=0.0246$). Nevertheless, all MC^{C3H} and MC^{B6} mice ultimately experienced relapses of equivalent severity by day 50. Finally, SJL mice that received syngeneic SJL BMCs (BM^{SJL}) developed

relapses similar to that observed in control animals (Figure 2.2a-c). Therefore, allogeneic, but not syngeneic, BMT delays the onset of relapses in EAE mice but fails to cure the disease.



C

# of mice	BM Donor	Days relapse-free (mean)	95% CI
18	B6	38.78	29.41-48.15
3	C3H	26.33	17.61-35.06
9	SJL	12.11	3.565-20.66
12	Sham	8.889	6.214-11.56

Figure 2.2: Mixed chimerism delays, but does not prevent, EAE relapses. Mice were immunized with 75µg $PLP_{139-151}$ emulsified in CFA. Upon EAE remission mice were given conditioning and bone marrow transplant as detailed in materials and methods. **A**. Mice were given $50x10^6$ B6 bone marrow cells (MC^{B6}, N=9), $50x10^6$ C3H bone marrow cells (MC^{C3H}, N=3), $50x10^6$ SJL bone marrow cells (MC^{SJL}, N=9), or sham transplant (MC^{Sham}, N=9). The vertical line so labeled indicates the day of bone marrow transplant. **B**. Survival analysis of mice from A. Following bone marrow transplant, mice were monitored for disease relapse as described in materials and methods. Kaplan-Meier analysis was performed to identify differences in time until relapse. Results are representative of multiple experiments. **C**. Descriptive statistics of mice that underwent the experimental protocol by bone marrow donor.

2.4.3 Mixed hematopoietic mixed chimerism combined with neural stem cell transplantation prevents disease relapses

NSCs have been shown to differentiate into various cells of the CNS and thereby promote myelin repair in a mouse model of MS (Einstein et al., 2006; Pluchino et al., 2003, 2005; S. Wu et al., 2013). This prompted us to test whether combining hematopoietic chimerism with NSC transplantation could both restore T cell tolerance to PLP autoantigen peptide and ensure remyelination of damaged nerves, respectively, in mice with ongoing EAE. NSCs from the brain of B6 and SJL mice were prepared as previously described (Azari et al., 2010).

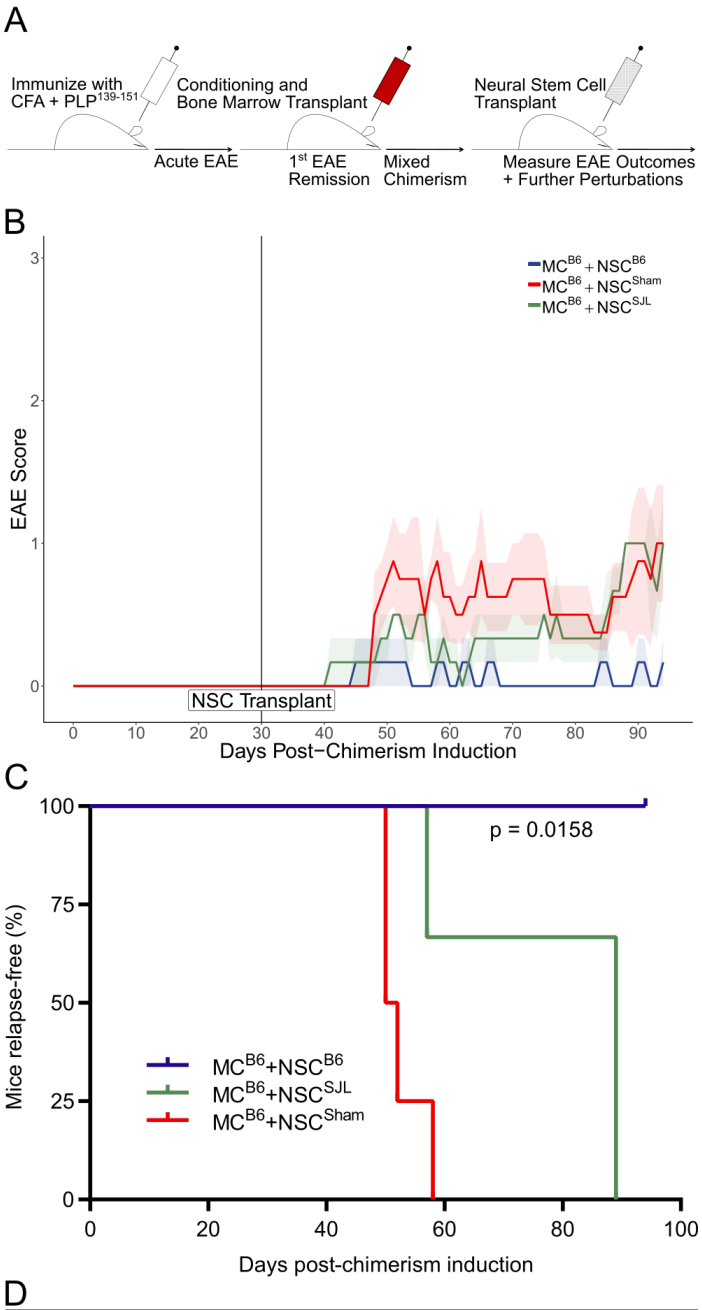
Next, SJL mice with ongoing EAE were treated with our mixed chimerism procedure (B6 BMT) during the first remission phase and one month later received 10^6 allogeneic (NSC^{B6}) or syngeneic (NSC^{SJL}) NSCs given intranasally, as described elsewhere (S. Wu et al., 2013) (Figure 2.3a). Control mice received saline instead of NSCs (NSC^{sham}). The onset of EAE relapses was monitored for 100 days post-chimerism induction. In addition, at the time of sacrifice, mouse brains and spinal cords were harvested, fixed, and embedded in paraffin for histological examination.

As shown in Figure 2.3b-d, SJL chimeric mice that received B6 NSCs showed virtually no signs of relapses while chimeras injected with saline developed relapses between 30-50 days post-allogeneic BMT ($p=0.0158$). Surprisingly, SJL chimeras that received recipient type i.e. syngeneic SJL NSCs

developed relapses in a fashion similar to that observed in control NSC^{sham} mice although with a slight delay and reduced severity (Figure 2.3b-d).

Figure 2.3: Donor-matched NSC transplant completely prevents relapses in mixed chimeras. A. Cartoon of experimental model including a neural stem cell transplant following chimerism induction. **B.** Mice were immunized and chimerism was induced upon entering remission as before. 30 days post-chimerism induction mice were given an NSC transplant. Mice received 1 x 10^6 B6 NSCs (NSC^{B6}, N=3), 1 x 10^6 SJL NSCs (NSC^{SJL}, N=3), or sham treatment (NSC^{Sham}, N=4). **C.** Survival analysis of mice from B. Following NSC transplant, mice were monitored for disease relapse as described in materials and methods. Kaplan-Meier analysis was performed to identify differences in time until relapse. Results are representative of multiple experiments. **D**. Descriptive statistics of mice that underwent the experimental protocol by bone marrow donor.

Figure 2.3(continued)



D

# of mice	NSC Donor	Days relapse-free (mean)	95% CI
19*	B6	no relapses observed	-
9	SJL	57.22	40-94-73.5
14	Sham	50.36	45.24-55.47
*7 out of 19 mice were reimmunized at day 60 post-chimerism induction			

2.4.4 Mixed chimerism and combined NSC transplantation result in a concomitant reduction in CNS inflammation and lymphocytic infiltration

Histological examination of spinal cord and brain revealed that both the $MC^{B6}+NSC^{Sham}$ and $MC^{B6}+NSC^{SJL}$ groups had significant inflammation visible within the spinal cord. In particular inflammation was noted within the meninges and perivascular lymphocytic infiltrates were observed (Figure 2.4a). Neither meningeal inflammation nor perivascular lymphocytic infiltrates were observed within the $MC^{B6}+NSC^{B6}$ group (Figure 2.4a). These infiltrates contained many $CD3^+$ cells, (Figure 2.4b). In contrast, within the $MC^{B6}+NSC^{B6}$ group, $CD3^+$ cells were not detected within the spinal cord (Figure 2.4b). In addition, luxol fast blue staining showed areas of spinal cord demyelination in $MC^{B6}+NSC^{Sham}$ and $MC^{B6}+NSC^{SJL}$ groups consistent with active EAE, inflammation, and an ongoing demyelinating disease (Figure 2.4c). Altogether, these observations correlated well with the disease phenotype of the mice at this time point, as the $MC^{B6}+NSC^{Sham}$ and $MC^{B6}+NSC^{SJL}$ groups experienced relapses and had ongoing EAE while the $MC^{B6}+NSC^{B6}$ group was symptom free. Taken together, these results show that allogeneic but not syngeneic NSCs prevented disease relapses in mixed chimeric mice.

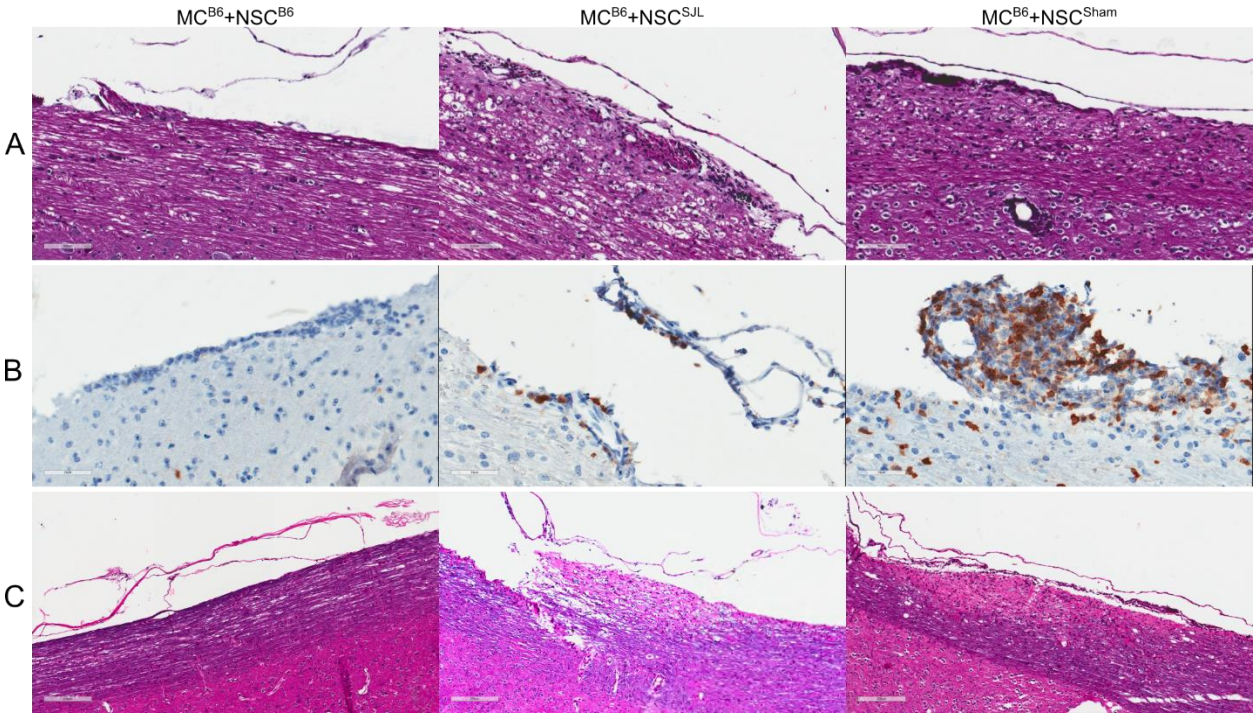


Figure 2.4: Histology of spinal cords in MCB6 mice receiving B6 NSCs, SJL NSCs, or sham treatment. Spinal cords from mice that received the indicated treatment were prepared 100 days post-chimerism induction as described in Materials and Methods. **A.** Hematoxylin and Eosin (H&E) staining. **B.** immunohistochemistry with αCD3. **C.** Luxol fast blue staining with H&E counterstaining. Results are representative of multiple experiments.

2.4.5 Mechanisms of disease prevention

Splenocytes from mice which had experienced relapses were collected at day 100 post-BMT and tested for the presence of donor hematopoietic cells using flow cytometry. As shown in Figure 2.5a, MC^{B6}+NSC^{Sham} mice that had relapsed maintained chimerism. Furthermore, there was no correlation between the level of chimerism and the severity of relapses in mixed chimeras (Figure S3). Therefore, the onset of EAE relapses in mice, which had received allogeneic BM cells, was not due to a loss of chimerism.

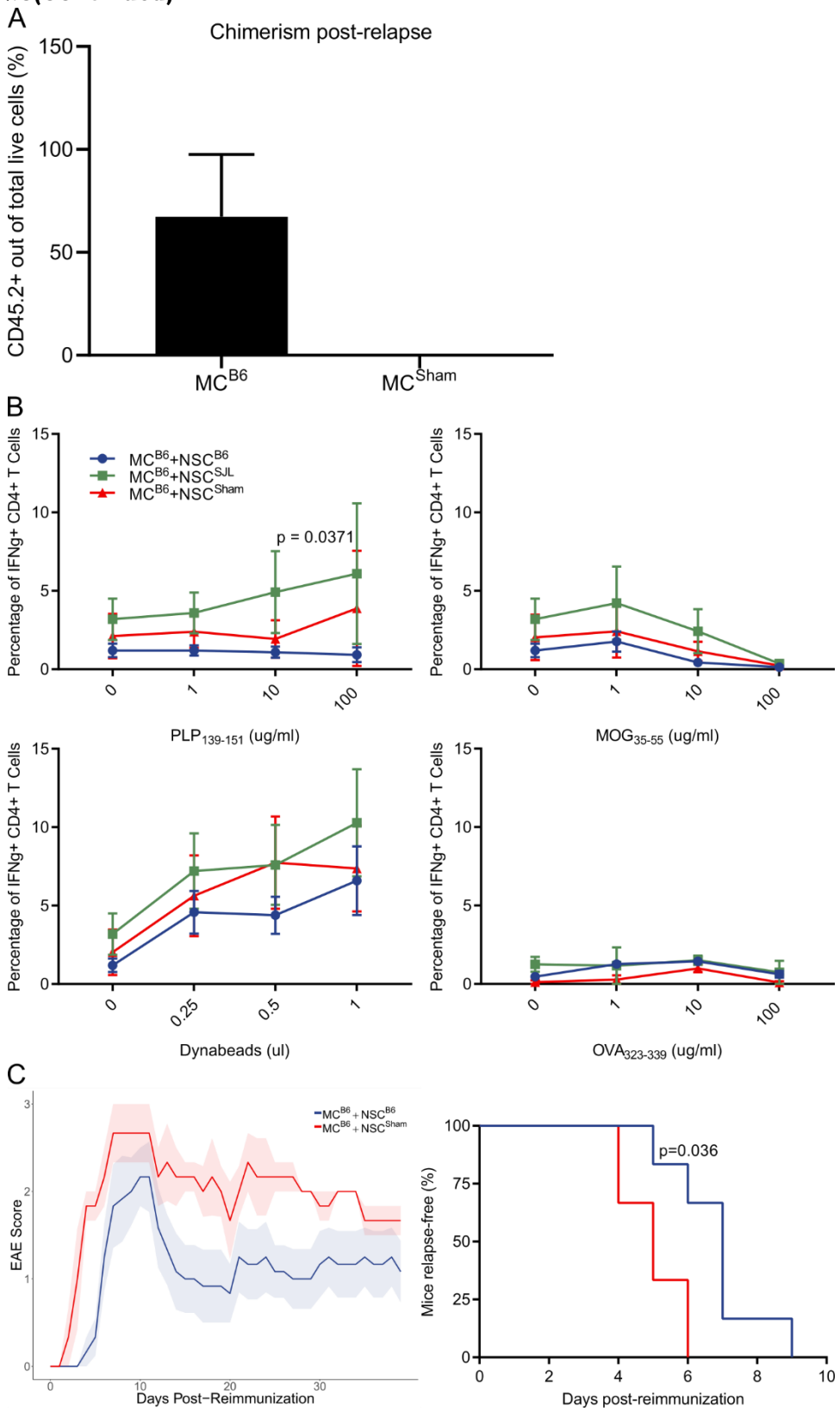
Next, spleen T cells of mixed chimeric SJL mice, which had received either B6 BM cells alone or B6 BM cells along with syngeneic or allogeneic NSCs were tested for their response against B6 allogeneic cells (alloimmunity) or myelin antigens (autoimmunity). Spleen cells were harvested 100 days after chimerism induction and re-stimulated in vitro for 5 days with irradiated allogeneic B6 cells to measure the direct alloresponse (mixed lymphocyte reaction) or with myelin autoantigen peptides, $PLP_{139-151}$ and MOG_{35-55} to assess autoimmunity. To assess inflammatory T cell responses, the frequencies of IFNγ-producing $CD4^+$ cells were measured via flow cytometry after intra-cellular cytokine staining. Control antigens included syngeneic SJL stimulators and irrelevant $OVA_{323-339}$ peptides for allo- and auto-immunity, respectively. T cells from all mixed chimeras, including those treated with NSCs uniformly produced inflammatory cytokine IFNγ in response to stimulation with αCD3/αCD28 mAb-coated beads (Figure 2.5b and S4). On the other hand, T cells from mice which received BMT alone or BMT + syngeneic NSCs produced IFNγ after $PLP_{139-151}$ peptide

stimulation. In contrast, mixed chimeras that had received allogeneic B6 NSCs showed no response to this peptide (Figure 2.5b). There was no significant response to stimulation with MOG_{35-55} or $OVA_{323-339}$ peptides, an encephalitogenic peptide and control class II peptide the mice had never been immunized against.

Finally, SJL mixed chimeras, which had received either B6 NSCs or saline were reimmunized at day 90 post-BMT with $PLP_{139-151}$ peptide emulsified in incomplete Freund's adjuvant. As shown in Figure 2.5c, MC^{B6}+NSC^{B6} mice relapsed more slowly than MC^{B6}+NSC^{Sham} control mice and experienced less severe disease ($p=0.036$). All mice, however, relapsed between 4-9 days after reimmunization. Altogether, these results showed that administration of mixed chimeras with donor-matched allogeneic B6 NSCs resulted in suppression of $CD4^+$ T cell inflammatory responses to PLP autoantigen peptide and some disease protection upon reimmunization with PLP peptide.

Figure 2.5: Mechanistic Studies into the role of tolerance. A. Flow cytometry of splenocytes from MC^{B6}+NSC^{Sham} mice 100 days post-chimerism induction. Graph of CD45.2+ cells as a percentage of live cells. All mice had relapsed by this timepoint. **B.** Splenocytes from mice 100 days post-chimerism induction were cultured in the presence of the indicated peptides or αCD3/αCD28 Dynabeads for 5 days then analyzed by flow cytometry. Values are percentage of IFNγ+ cells out of total CD4+ T cells with the following gating strategy: singlets, live cells, lymphocytes, CD4+. **C.** 30 days post NSC transplant (60 days post-chimerism induction) non-relapsed MC^{B6}+NSC^{B6} (N=6) and MC^{B6}+NSC^{Sham} (N=3) mice were restimulated with 75μg $PLP_{139-151}$ emulsified in IFA. EAE Score graph is presented as mean + SEM. Results are representative of multiple experiments.

Figure 2.5(Continued)



2.5 Discussion

Multiple lines of evidence from both human and animal studies indicate a loss of self-regulation of lymphocytes as the driving force behind MS pathology (Haas et al., 2005; Huan et al., 2005; Koutrolos, Berer, Kawakami, Wekerle, & Krishnamoorthy, 2014; Saunders et al., 2012; Venken et al., 2008; Viglietta, Baecher-Allan, Weiner, & Hafler, 2004). Therapeutic strategies with the potential to restore self-tolerance therefore hold great promise for the treatment of MS.

Currently, the application of tolerance strategies in MS is complicated because the majority of tolerance induction strategies which have shown promise in animal models (Miller, Turley, & Podojil, 2007b; Peron et al., 2010; Pires, Marques, Sousa, Cerqueira, & Pinto, 2016; Turley & Miller, 2010) or are being attempted in humans (Lutterotti et al., 2013; Pires et al., 2016) rely on knowledge of the self-antigen involved, but this antigen is unknown in MS (Elong Ngono et al., 2012; Hemmer et al., 2002). Additionally, while inducing immune tolerance in mouse models has been accomplished in a variety of ways, the majority of these strategies have been difficult to translate to large animals/humans. Thus, any tolerance induction strategy in MS must both translate successfully from small animal models and also be able to induce self-tolerance in the absence of knowledge of the pathogenic self-antigen.

Mixed hematopoietic chimerism is a method of tolerance induction which has been used successfully to induce tolerance to alloantigens in large animals and humans (Kawai, 2013; Sachs et al., 2014). Since mixed chimerism can induce tissue-specific, rather than antigen-specific, tolerance and has been used

successfully in humans it represents an ideal potential therapeutic strategy in MS.

Mixed chimerism has previously been applied in the NOD mouse model of diabetes (Nikolic, Onoe, Takeuchi, & Khalpey, 2010; Nikolic et al., 2004), where it was shown to effectively halt autoimmune attack and also was able to induce tolerance to transplanted allogeneic islets (Nikolic, Onoe, Takeuchi, Khalpey, et al., 2010; Nikolic et al., 2004). Additionally, mixed chimerism has been used in EAE along with a T cell depleting conditioning regimen where it was able to induce long-term protection from relapses (L. Wu et al., 2015). However, whether T cell depletion (which is not 100% effective in humans (Mohty, 2007)) is necessary for mixed chimerism induced tolerance remains an open question. Furthermore, whether MC acts on autoimmune disease through a deletional, regulatory, or mixed pathway is still unknown.

$CD4^+$ T cell depletion is a highly effective treatment in EAE but $CD4^+$ T cell depletion in humans is both difficult to accomplish and hazardous for the patient, making strategies that don't rely on $CD4^+$ depletion more valuable. We have developed a model using a non-$CD4^+$ T cell depleting regimen whereby mixed chimerism may be induced in mice with ongoing EAE. Our chimerism induction strategy allowed us to both preserve the $CD4^+$ compartment and to minimize immunosuppression.

We hypothesized that the first remission after acute EAE would be the most ideal point for chimerism induction for the following reasons: 1) The autoimmune disease has at that point developed the capability of a secondary

response (McRae, Vanderlugt, Dal Canto, & Miller, 1995; Terry et al., 2014; Vanderlugt & Miller, 2002) which allows us to examine tolerance induction in the context of ongoing disease; 2) the first day of remission provides a clear intervention point based upon disease progression; and 3) we speculated that tolerance induction should be more achievable in the absence of significant disease activity.

Based upon work in the other autoimmune models, we hypothesized that mixed chimerism using a non-T cell depleting conditioning regimen would have a positive effect upon ongoing EAE. While there was a disease-free period following mixed chimerism induction, without any further intervention mice universally suffered a relapse. This relapse did follow a significant delay relative to mice with failed chimerism or control conditioning alone. At no point in any experiment were mice observed to lose chimerism. We concluded from this result that while mixed chimerism with a strong T cell depleting conditioning regimen has previously been shown to induce self-tolerance (L. Wu et al., 2015), mixed chimerism alone is insufficient to achieve self-tolerance when autoreactive T cells are not depleted.

One possible explanation for the eventual relapse could be that while mixed chimerism helps induce self-tolerance, maintenance of tolerance may occur via another mechanism. When MC^{B6} mice eventually relapsed, there was no observed difference in the severity of the disease relative to MC^{sham}. While the exact mechanism behind relapses in $PLP_{139-151}$ peptide-induced EAE is unknown (McRae et al., 1995; Terry et al., 2014), the results from Figure 2.2 support the hypothesis that mixed chimerism succeeds in inducing but not maintaining

tolerance in these animals. Regardless it is possible to conclude that the autoreactive cells in these animals remain as potent as in the control conditions, and capable of inducing autoimmunity.

Since we saw an increase in relapse-free survival using allogeneic (B6) bone marrow donors but not syngeneic (SJL) bone marrow donors, it was of interest to determine whether this effect was specific to B6 or could be observed with any allogeneic bone marrow. The other allogeneic donor examined (C3H) in Figure 2.2 produced an equivalent delay in relapse, suggesting that the delay in relapse was likely due to the allogeneic nature of the transplant rather than to the specific donor background.

Although we confirmed that successful induction of mixed chimerism had an ameliorating effect on ongoing EAE, that effect was more limited than had previously been reported (L. Wu et al., 2015). To address this problem, we tested whether a combination of mixed chimerism and NSC transplantation could result in an increased regenerative capacity within the CNS.

Previous studies have reported transplantation of syngeneic NSCs into the CNS (Ben-Hur et al., 2003; Einstein et al., 2007). In our case, we sought to utilize NSCs from the same allogeneic donor strain that was used for induction of mixed chimerism. In addition to providing a tissue to which mixed chimerism would be expected to tolerize the host, this choice is more relevant to eventual clinical applications, since it would be difficult, if not impossible, to produce and utilize syngeneic NSCs in humans.

Based upon previous data in the NOD model using islet transplants (Nikolic, Onoe, Takeuchi, & Khalpey, 2010; Nikolic et al., 2004), we decided to give the NSC transplant thirty days post-chimerism induction. We hypothesized that this delay would give time for induction of allo-tolerance and also be outside any windows of acute inflammation or immune modulation due to the mixed chimerism treatment. During the period of the study, which was continued for 100 days following bone marrow transplant, there was no evidence of EAE activity in the MC^{B6}+NSC^{B6} group while the MC^{B6}+NSC^{Sham} group experienced relapses. This led us to conclude that a donor-matched NSC transplant following induction of mixed chimerism restores self-tolerance in ongoing EAE. One possible interpretation would be that the NSC transplant results in maintenance of tolerance which did not occur during mixed chimerism alone, although more studies would be required to confirm this hypothesis.

To determine whether any NSC could provide the necessary stimulus to increase relapse-free survival or whether the NSC needed to be syngeneic to the source of bone marrow used to produce mixed chimerism, we repeated this experiment using host-type NSC. We hypothesize the lack of effect from syngeneic NSCs is because the relevant donor antigens need to be expressed within the tissue of interest (in this case the CNS). To further study the basis of these results, we performed histological examinations of the CNS of MC^{B6}+NSC^{B6}, MC^{B6}+NSC^{Sham}, and MC^{B6}+NSC^{SJL} mice to assay for lymphocytic infiltrates

When performing the histological examinations, we wanted to ensure any EAE phenotype observed was chronic, and that any non-relapsing mice were truly long-term tolerant. For this reason we performed histological analyses at day 100 post-chimerism induction. Despite the clear tolerant phenotype in the $MC^{B6}+NSC^{B6}$ group, we were not able to find any GFP^{+} NSCs cells in the CNS of these animals (mirroring the results of the $MC^{B6}+NSC^{Sham}$ and $MC^{B6}+NSC^{SJL}$ groups, where no GFP signal was seen either) (Data not shown). Although this does not preclude the cells being present, we did conclude from these results that the NSC transplant was not directly responsible for repair/remyelination within the CNS of these animals.

We were unable to determine whether the normal myelination in the $MC^{B6}+NSC^{B6}$ group was due to protection from demyelination, or efficient remyelination. Additionally, the healthy tissue observed in the LFB and H&E histological assays supported our hypothesis that the $MC^{B6}+NSC^{B6}$ mice were self-tolerant, especially considering the significant demyelination, meningeal inflammation, and perivascular lymphocytic infiltrates observed in the $MC^{B6}+NSC^{Sham}$ and $MC^{B6}+NSC^{SJL}$ groups.

Based on these results, we concluded that a donor-matched NSC transplant was sufficient to restore self-tolerance in mixed chimeric mice, but the mechanism remained unclear. If the $MC^{B6}+NSC^{Sham}$ group had lost mixed chimerism, this could have explained the difference induced by NSC^{B6}. However, no changes in mixed chimerism occurred in relapsed mice (Figure 2.5a), leading to the conclusion that while it is possible that mixed chimerism may induce

tolerance, mixed chimerism alone cannot be responsible for the maintenance of tolerance.

Since no change in mixed chimerism status presaged relapse, we conclude that autoreactive cells are either regulated by another mechanism or deleted in the MC^{B6}+NSC^{B6} mice. If the cells were being deleted, we should not have found autoreactive cells in these mice. If the cells were being regulated, it could be either in terms of halting/preventing activation or preventing their entering the CNS. Since mixed chimeras spontaneously relapse, we know that at least prior to the NSC transplant, autoreactive cells are present and capable of responding to stimulation. The lack of *in vitro* response to stimulation with $PLP_{139\text{-}151}$ (see Figures 2.5b and 2.5c) did not allow us to rule out either regulation or deletion as a possible mechanism, since either could result in the blunted response observed.

Since an *in vitro* response to stimulation may not provide a full picture of the immune system in these mice, we also performed a reimmunization. In naïve mice receiving their first immunization with peptide and CFA, symptoms appear in 10-14 days (Terry et al., 2014). The response observed in the MC^{B6}+NSC^{B6} mice, which were immunized with PLP peptide, was clearly consistent with a memory T cell response, leading us to conclude that autoreactive cells are present in the MC^{B6}+NSC^{B6} mice and are capable of responding to stimulation, but either fail to be stimulated or are suppressed.

Taken together, the response to reimmunization conclusively rules out deletion as the primary mechanism by which self-tolerance is restored in this

model and provides evidence for T cell regulation as the driving force behind the maintenance of tolerance in this model. Future research will focus on elucidating the mechanisms by which regulation occurs, specifically what requirements for donor and/or recipient Tregs exist for the maintenance of tolerance in MC^{B6}+NSC^{B6} mice.

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3

Allogeneic extracellular vesicles abrogate the need for bone marrow to delay EAE relapses using a mixed chimerism conditioning protocol

This chapter is adapted from a manuscript being prepared for submission: William Orent, Natacha Carnel, Bruno Gonzalez-Nolasco, David H. Sachs, and Gilles Benichou. (2019) Allogeneic extracellular vesicles abrogate the need for bone marrow to delay EAE relapses using a mixed chimerism protocol. Manuscript being prepared for submission.

3.1 Abstract

Previously, we demonstrated that induction of allogeneic mixed chimerism using a $CD4^+$ T cell-sparing conditioning regimen could delay, but not prevent, relapses in mice with ongoing EAE (Manuscript submitted for publication, Chapter 2). Here we show that allogeneic, bone marrow-derived EVs abrogate the need for bone marrow in our mixed chimerism protocol. Additionally, repeated injection of these vesicles reduces overall disease burden.

We induced EAE with $PLP_{139-151}$ in 6-8 week old female SJL mice. Upon entering the first disease remission we conditioned the recipients using $CD8^+$ T cell depletion, 300 cGy total-body irradiation, and αCD40L antibodies. Mice then received $1x10^9$ C57BL/6 (allogeneic) bone marrow derived EVs, $50x10^6$ bone marrow cells from C57BL/6 donors, or a sham injection via the IV route.

As seen previously, conditioning plus bone marrow delayed but did not prevent relapses. Mice that received a single dose of $1x10^9$ allogeneic EVs also showed a significant delay in relapses compared to conditioning alone. In a dose-response experiment, an injection of a lower dose ($1x10^6$ EVs) following conditioning resulted in relapses significantly quicker than $1x10^9$ EVs, while a higher dose ($1x10^{11}$ EVs) had a similar delay in relapse to $1x10^9$ EVs, but higher overall disease burden.

In a separate experiment, if mice received $1x10^8$ EVs daily for 8 days following the initial $1x10^9$ EV treatment, they had significantly less overall disease than mice that received the single dose of $1x10^9$ EVs. The same effect as

the daily treatment was not observed if mice received an additional $1x10^9$ EVs one week after the original treatment.

These data indicate that both EVs and BMCs, after conditioning, can delay relapses in mice with ongoing EAE, suggesting, but not proving, that both BMCs and EVs may act through the same mechanism. These results may provide new insights into the restoration of tolerance in autoimmunity and could have clinical implications.

3.2 Introduction

MS is a progressive, neurodegenerative autoimmune disease (Lassmann, 2018). The defining characteristic of MS is CNS demyelination, presenting as focal plaques (Hemmer, Archelos, & Hartung, 2002; Hu & Lucchinetti, 2009; Petermann & Korn, 2011). As T cell dysregulation and a loss of self-tolerance are hypothesized to be primary drivers of MS pathology (Baecher-Allan, Kaskow, & Weiner, 2018; Lassmann, 2018; Popescu & Lucchinetti, 2012; Rahmanzadeh, Weber, Brück, Navardi, & Sahraian, 2018), the restoration of self-tolerance is a therapeutic goal in the treatment MS.

Allogeneic BMT combined with non-myeloablative conditioning induces a state referred to as mixed chimerism (Sachs, Kawai, & Sykes, 2014; Zuber & Sykes, 2017). Mixed chimerism, in turn, induces tolerance of allogeneic organ transplants in both animal models and in humans (Ildstad & Sachs, 1984; Kawai et al., 2014; Sachs et al., 2014). Mechanistically, mixed chimerism results in ongoing presentation of donor MHC molecules in the recipient thymus which leads to deletion of alloreactive thymocytes (Zuber & Sykes, 2017).

Work from our lab (manuscript submitted for publication) and others (Wu et al., 2015), has shown that the induction of mixed chimerism augments the immune response and is capable of delaying relapses in ongoing EAE, an animal model of T cell mediated demyelination useful in the study of MS (Terry, Ifergan, & Miller, 2014). Although a large body of research exists on the mechanisms by which mixed chimerism functions in tissue transplantation (Sachs et al., 2014; Zuber & Sykes, 2017), comparatively little is known about its mechanism in

autoimmunity, especially EAE. As such, how inducing tolerance inhibits autoreactive T cell reactivation remains a major unanswered question in the field.

EV is an umbrella term for any of a number of vesicular bodies released from cells (including microvesicles, exosomes, apoptotic bodies, and others), and are used by a wide variety of cell types in their normal physiological functions (Théry, Ostrowski, & Segura, 2009). They are released into the extracellular environment and typically act as a mechanism for intercellular communication. EVs may express MHC and other surface molecules and have a cargo capacity for proteins, mRNAs, miRNAs, or other molecules (Raposo & Stoorvogel, 2013; Théry et al., 2009).

It has also been shown recently that EVs play a role in the immune response, particularly in the development of alloresponses against transplanted tissue (Gonzalez-Nolasco, Wang, Prunevieille, & Benichou, 2018; Marino et al., 2016; Théry et al., 2009). Work in our lab and others has shown that after tissue transplantation, EVs carry donor MHC molecules into recipient lymphoid organs (Marino et al., 2016; Smyth, Lechler, & Lombardi, 2017). Once there, recipient APCs may acquire these vesicles and then present intact donor MHC to T cells. Tracking experiments have revealed that, even in a syngeneic setting, EVs have a very short half-life after injection, on the scale of minutes to hours (Takahashi et al., 2013).

Considering that stable mixed chimerism results in presentation of donor MHC in the host thymus (Zuber & Sykes, 2017), and that allogeneic EVs are

capable of carrying donor MHC into lymphoid organs for presentation by APCs (Marino et al., 2016; Smyth et al., 2017), a reasonable hypothesis is that allogeneic EVs may be used instead of bone marrow, along with conditioning, in the induction of tolerance. The hypothesis that the presentation of donor MHC is a necessary part of the mechanism of tolerance induction is central to this premise. If the mixed chimerism-mediated treatment effect in autoimmunity works by a similar mechanism, then EVs along with non-myeloablative conditioning should be an effective treatment in autoimmunity as well.

Here we have used allogeneic EVs along with non-myeloablative conditioning to treat mice with ongoing EAE. We found that EVs may be effectively used in place of bone marrow to reduce disease burden and delay the time until relapse relative to a sham transplant. Moreover, we found that EV treatment is dose-dependent, and multiple doses over time confer superior durability of the treatment as measured by overall disease burden. These findings may have important implications for advancing our understanding of the mechanisms of tolerance in, and designing novel therapies for the treatment of MS.

3.3 Materials and Methods

Mice: C57BL/6J, and SJL/J mice were obtained from the Jackson laboratory (Bar Harbor, ME). 6-8 week old female mice were used throughout the study. All animal care and handling was performed according to institutional guidelines.

Reagents: Antibodies: PerCP/Cy5.5 αCD4 (BioLegend 100433), Brilliant Violet 510 αCD8α (BioLegend 100751), FITC αCD19 (BioLegend 115505), APC αCD11b (BioLegend 101211), APC αCD11c (BioLegend 117309), Brilliant Violet 421 αCD45.2 (BioLegend 109831), PE/Cy7 αCD335 (BioLegend 137617).
EAE Reagents: Incomplete Freund's Adjuvant (Sigma Aldrich F5506), Heat-killed Mycobacterium Tuberculosis H37 (Difco 231141).
$PLP_{139-151}$ (HCLGKWLGHPDKF) was provided by Dr. Ashok Khatri (Massachusetts General Hospital, Charlestown, MA).

Mixed Chimerism Reagents: αCD8α (Clone 2.43, BioXCell BE0061), αCD40L (Clone MR1, BioXCell, BE0017-1), Shepherd Cesium Irradiator.

Experimental Autoimmune Encephalomyelitis: CFA was prepared at a 5mg/ml concentration of heat-killed Mycobacterium Tuberculosis in IFA mixed very well. CFA was used fresh or within 1-2 days for all experiments. Emulsions were prepared by combining equal amounts of CFA and $PLP_{139-151}$ dissolved in PBS at 150 μg/ml using two 3ml syringes and a three-way stopcock system.

Female 6-8 week old SJL/J mice were given a 100µl subcutaneous injection of emulsion (comprising of 75µg $PLP_{139-151}$) ventrally on the right side of the chest situated near the axial lymph nodes using a 1ml syringe with a 22 gauge needle (to ensure smooth flow of the emulsion). Five days after immunization daily monitoring for EAE symptoms began. Symptoms would routinely begin 10-14 days post-immunization. Mice were assessed for symptoms using a 5-point scale as follows: 1 – Complete tail paralysis, 2 – Score 1 plus hind-limb weakness as assessed by gait disturbance or inability to right after flipping, 3 – Score 1 plus complete hind-limb paralysis, 4 – Score 3 plus complete forelimb paralysis, 5 – Death. Half-point scores were awarded in cases of incomplete paralysis (e.g. a score of 0.5 for partial tail paralysis). Remission was defined as two consecutive days of decreased score following the peak acute-phase score (Miller & Karpus, 2007). Relapse was defined as an increase in score after the nadir of remission combined with two days of score 1 or above. Reimmunization was performed in a manner similar to the initial immunization with the following modifications: IFA was used in place of CFA and the immunization was given ventrally on the left side of the chest situated near the axial lymph nodes. All EAE experiments were performed blinded, where the researcher scoring the mice was unaware of their treatment status.

Extracellular Vesicle Isolation and Characterization: Bone marrow cells were isolated from 6-8 week old female donor mice as previously described (Amend, Valkenburg, & Pienta, 2016), and 50 million were cultured in RPMI 1640 medium

supplemented with 10% exosome-depleted FBS overnight at 37C and 5% CO_2. Extracellular vesicles were isolated from the cell culture supernatant using sequential ultracentrifugation with the following steps. The initial culture medium was subjected to a 300g spin for 10 minutes to remove cells and any large clumps/debris. Following this, the supernatant was spun at 16,000g for 30 minutes to remove any remaining cell debris and large extracellular vesicles (greater than 200nm in diameter). The remaining small extracellular vesicle fraction (around 100nm) was pelleted by centrifuging at 100,000g for 60 minutes. Finally, the extracellular vesicle pellet was washed with twice-filtered PBS to remove contaminating protein aggregates and was spun at 100,000g for an additional 60 minutes to pellet the purified extracellular vesicles. The samples were then resuspended in 500 microliters of PBS which had been filtered twice using a 0.2 micrometer filter and frozen prior to quantification. To quantify the number of extracellular vesicles a small sample was diluted 10-fold in twice-filtered PBS. This sample was then measured on a Nanosight LM10 equipped with a 405nm laser. The instrument was calibrated using 100nm standard beads. Vesicles were kindly provided by Dr. Bruno Gonzalez-Nolasco.

Mixed Chimerism and Extracellular Vesicle Treatment: On the first day of remission as measured by population average (defined as Day -1 mixed chimerism induction) within the experiment, mice were given 500μg of αCD8α intraperitoneally. On day 0 bone marrow cells were isolated from 6-8 week old female donor mice as previously described (Amend et al., 2016). Recipient mice

were treated with 300 cGy TBI, and 1mg αCD40L IP. Mice were then given either $50x10^6$ bone marrow cells, extracellular vesicles (at the amount described in a given experiment), or a sham injection IV. Beginning at day 28 post-chimerism induction and at regular intervals thereafter, peripheral blood mononuclear cells were obtained via cheek bleeding and examined via flow cytometry for CD45.2+ (donor) cells. Mice were considered chimeric if they had detectable donor cells above background.

3.4 Results

3.4.1 Allogeneic EVs isolated from bone marrow can delay relapses in ongoing EAE

Previous work in our lab has shown that donor-type allogeneic EVs are detectable in recipient lymph nodes as early as 1.5 days after skin grafting and these EVs are capable of "cross-dressing" recipient cells in the thymus (Marino et al., 2016). Based upon this phenomenon and the ability of these EVs to initiate an alloresponse (Marino et al., 2016), we hypothesized it may be possible to develop a model wherein EVs may be used in conjunction with conditioning, as a treatment for ongoing EAE, in a manner similar to mixed chimerism.

To test this, we employed a model similar to that shown in Figure 2.1a (in the previous chapter). SJL mice with ongoing EAE were treated with the conditioning regimen at the first remission (Figure 3.1a), and concurrently given a transplant of $1x10^9$ allogeneic (C57BL/6J) EVs IV. Control groups of mice were given $50x10^6$ B6 BMCs or sham treatment (Control Conditioning) IV.

We compared the overall disease burden for each group in the post-transplant period (0-30 days post-transplant) by calculating the area under the curve of the EAE score and using a one-way ANOVA with Tukey's multiple comparison test. We chose a 30-day period in order to ensure sufficient time had passed for relapses to occur while still specific enough to see differences in the various groups (since we hypothesized all mice will eventually relapse, on a long enough timeline the overall disease burden will revert to the mean). As shown in Figure 3.1b-c, in the 30-day post-transplant period, both the mixed chimerism

and B6 EV grousp had significantly less overall disease burden compared to the control conditioning group.

Previously, we showed that mixed chimeras in the post-transplant period had decreased disease burden due to a delay in relapses (Figure 2.2b). In the current study, we examined if the same was true in EV-treated mice by performing survival analysis of the time to relapse after transplant. We found that mixed chimeras and EV-treated mice both had a significant delay in time to relapse after transplant compared to sham-treated mice (Figure 3.1d).

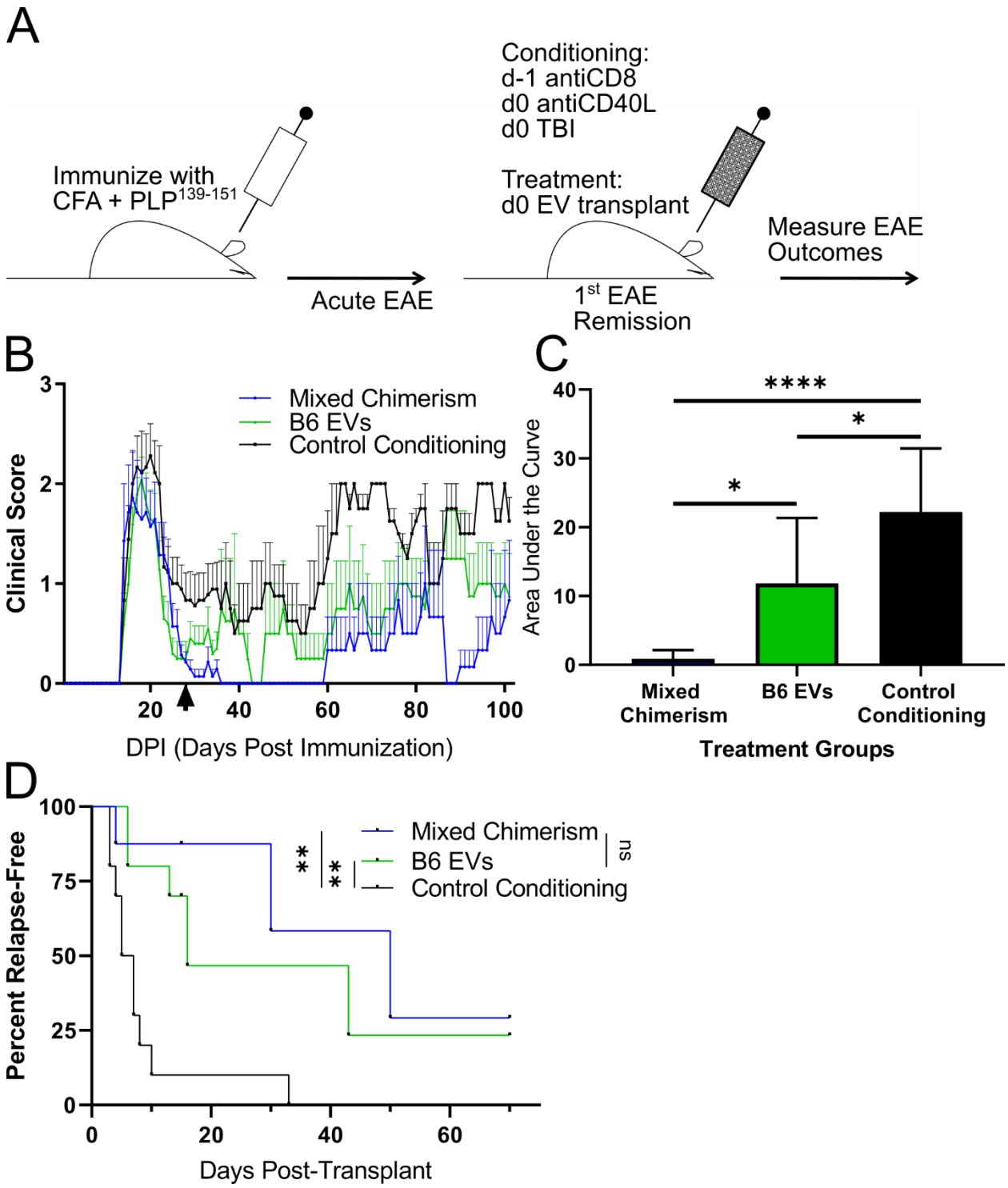

Figure 3.1 Allogeneic EVs with conditioning delay relapses in ongoing EAE. A. Cartoon of experimental model. **B.** Mice were immunized with 75μg $PLP_{139-151}$ in CFA, Upon EAE remission (black arrow) mice were given conditioning and then $1x10^9$ B6 EVs (B6 EVs, n=10), 50 x 10^6 B6 bone marrow cells (Mixed Chimerism, n=7), or sham treatment (Control Conditioning, n=9). **C.** Area under the curve of the EAE score for the 30 days post-transplant in each group was calculated. One-way ANOVA with post-hoc Tukey's multiple comparison test was performed. **D.** Survival analysis of the time to relapse post-transplant in each group. Kaplan-meier analysis for

Figure 3.1 (continued): multiple comparisons with post-hoc analysis was performed. Results are representative of multiple experiments.

3.4.2 EV-induced delay in relapses is dose-responsive

One possible explanation for the effect of EV treatment observed in Figure 3.1 could be the presence of a molecular mediator common to EVs and BMCs (e.g. a protein or other macromolecule). If a molecular mediator is responsible, it might be expected that the treatment response follows a dose-effect curve. To test this hypothesis, we repeated the experiment outlined in Figure 3.1a with three groups receiving increasing amounts of allogeneic EVs concurrent with conditioning: $1x10^6$, $1x10^9$ (the same dose as in the previous experiment), and $1x10^{11}$.

Using the same analysis as in Figure 3.1, we found that in the 30-day post-transplant window, mice treated with the $1x10^9$ EV dose had significantly less overall disease burden as measured by area under of the curve of the EAE score in the post-transplant period than the low ($1x10^6$) or high ($1x10^{11}$) EV dose (Figure 3.2a-b). While the $1x10^9$ dose had a lower overall disease burden than either of the other doses, both the $1x10^9$ and $1x10^{11}$ dose had a delay in relapse relative to the $1x10^6$ dose (Figure 3.2c).

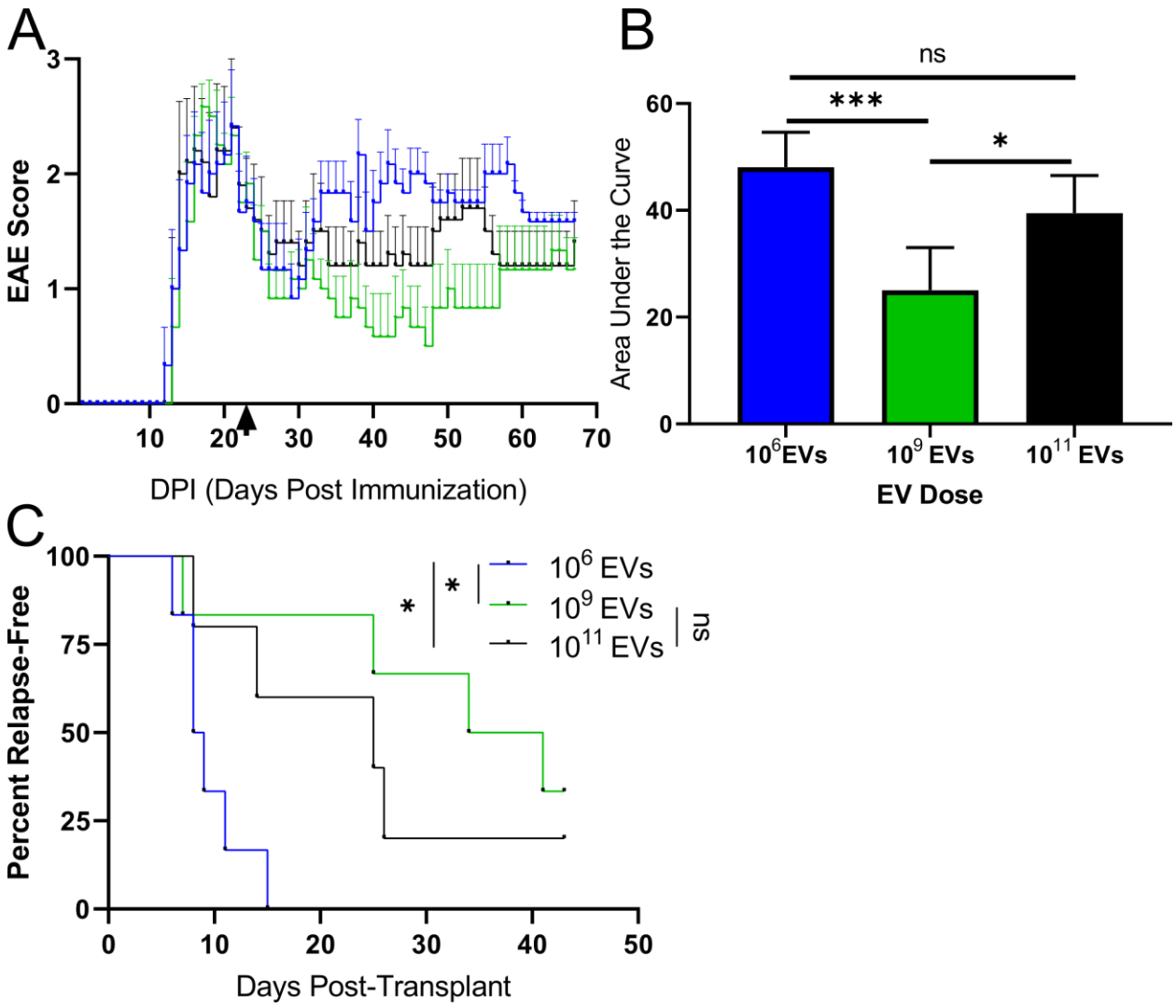

Figure 3.2 Allogeneic EV dose response curve in EAE. A. Mice were immunized with 75μg $PLP_{139-151}$ in CFA, Upon EAE remission (black arrow) mice were given conditioning and then $1x10^6$ B6 EVs (10^6 EVs, n=6), $1x10^9$ B6 EVs (10^9, n=6), or $1x10^{11}$ B6 EVs (10^{11}, n=5). **B.** Area under the curve of the EAE score for the 30 days post-transplant in each group was calculated. One-way ANOVA with post-hoc Tukey's multiple comparison test was performed. **C.** Survival analysis of the time to relapse post-transplant in each group. Kaplan-meier analysis for multiple comparisons with post-hoc analysis was performed. Results are representative of multiple experiments.

3.4.3 Repeated EV injections lowered overall disease burden relative to a single injection

Although a higher ($1x10^{11}$) EV dose on day 0 of treatment did not significantly improve outcome relative to the $1x10^{9}$ dose, we hypothesized that due to the short half-life of EVs in the circulation (Takahashi et al., 2013), repeated doses over time might be able to prolong the treatment effect. To answer this question, we modified the model from Figure 3.1a to include four treatment groups as shown in Figure 3.4a. The first group, Daily, received a loading dose of $1x10^{9}$ allogeneic EVs on day 0, then $1x10^{8}$ allogeneic EVs every day thereafter for 8 days. The second group, Weekly, received $1x10^{9}$ allogeneic EVs on day 0, and then $1x10^{9}$ allogeneic EVs on day 8. The third group, $1x10^{9}$ EVs, received $1x10^{9}$ allogeneic EVs on the initial day 0 and then no other treatment. Finally, the fourth group, Sham Tx, received sham treatment.

As before, we looked at the area under the curve of the EAE score in the 30 days post-transplant and compared the four treatment groups with one-way ANOVA followed by Tukey's multiple comparison test. We found that the Daily treatment group had significantly less post-transplant overall disease burden than the Sham Tx group or the $1x10^{9}$ EV group, but not the Weekly treatment group (Figure 3.3b-c).

We also analyzed the time until relapse post-transplant. The Daily and $1x10^{9}$ EV groups both had a significant delay in the time until relapse compared to the Sham Tx group, while the Weekly group did not significantly differ from

Sham Tx. The Daily, Weekly, and $1x10^9$ groups had no significant differences amongst each other (Figure 3.3d).

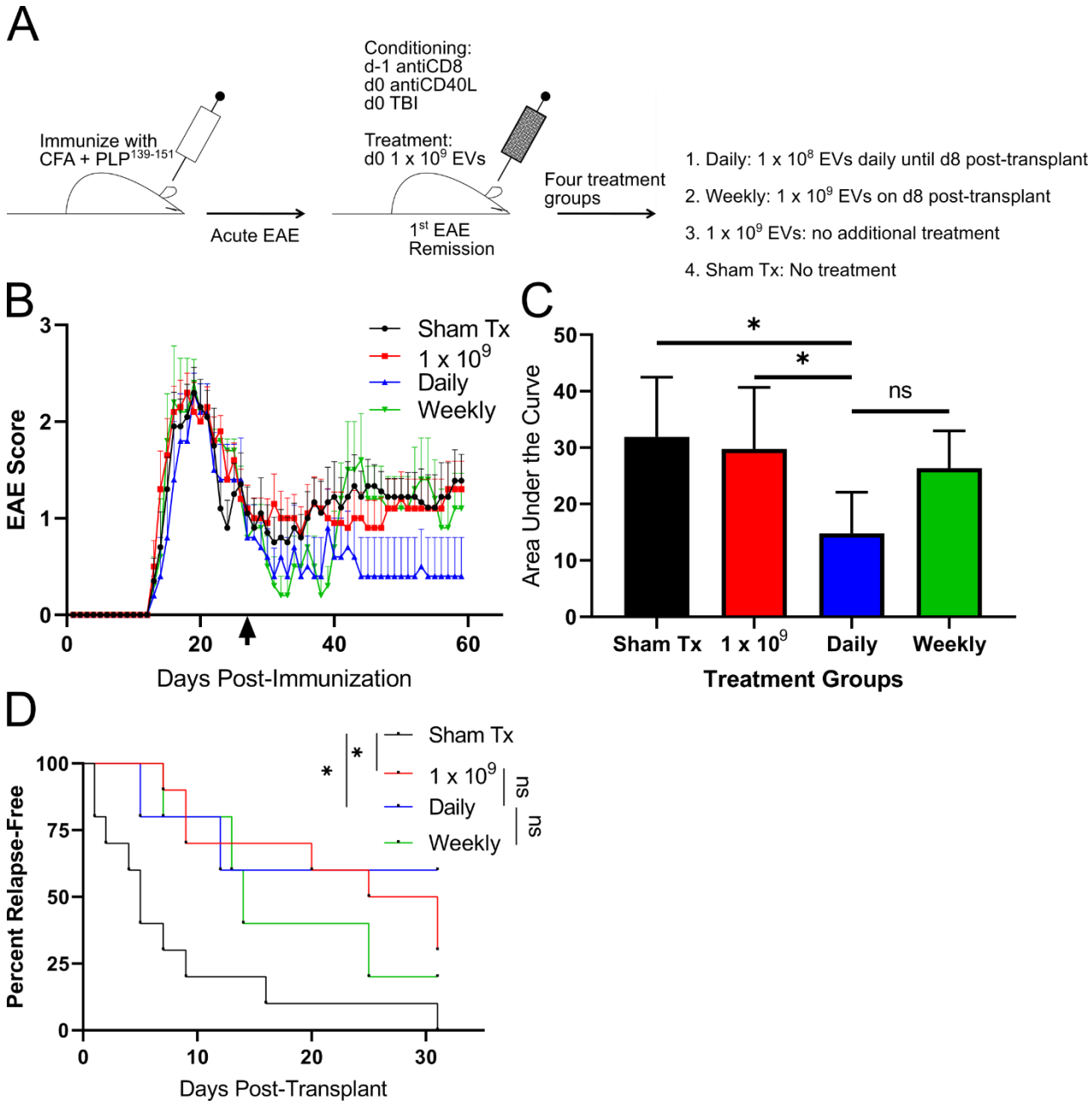

Figure 3.3 Repeated allogeneic EV treatment is superior to one-time treatment A. Cartoon of updated model. Mice were immunized as before, and upon entering remission were conditioned and divided into one of four treatment groups as shown: 1. Daily (n=5), 2. Weekly (n=5), 3. 1x10^9 EVs (n=10), 4. Sham Tx (n=10). All groups except Sham Tx received d0 1x10^9 allogeneic EVs **B.** EAE score graph. Black arrow indicates d0 of treatment. **C.** Area under the curve of the EAE score for the 30 days post-transplant in each group was calculated. One-way ANOVA with post-hoc Tukey's multiple comparison test was performed. **D.** Survival analysis of the time to relapse post-transplant in each group. Kaplan-meier analysis for multiple comparisons with post-hoc analysis was performed.

3.5 Discussion

Previously, we showed that allogeneic mixed chimerism can delay relapses in EAE using a non-T cell-depleting, costimulatory blockade conditioning regimen (manuscript submitted for publication). Although relapses could be prevented entirely by the addition of NSCs, many questions remain regarding why mixed chimerism can impact ongoing autoimmune processes.

In transplantation, mixed chimerism may induce tolerance to a solid organ transplant of the same MHC haplotype as the bone marrow donor (Ildstad & Sachs, 1984; Sachs et al., 2014). Following conditioning, donor and recipient APCs repopulate the thymus and delete thymocytes reactive for the donor or the recipient (Khan, Tomita, & Sykes, 1996; Tomita, Khan, & Sykes, 1994; Wekerle et al., 1998). Anti-donor cells are also deleted peripherally (Fehr et al., 2008; Kurtz et al., 2004; Wekerle et al., 1998). The fact that tolerance to a tissue transplant of the same MHC haplotype as the bone marrow results from this process is logical. The bone marrow "takes care of its own" so to speak. In contrast, the mechanism by which allogeneic BMT can temporarily halt autoreactive cells which are not necessarily allospecific does not lend itself quite so easily to logical deduction. Thus, a significant unanswered question in the field is what aspect of the bone marrow (and its subsequent engraftment in the recipient) enables the delay of relapses in EAE.

EVs have been of great interest in the field of transplant tolerance because of their observed ability to traffic to lymphoid organs, cross-dress recipient APCs, and generate alloreactive T cell responses subsequent to transplant (Marino et

al., 2016). Work in our lab by Dr. Gonzalez-Nolasco et al. has also shown that EVs, along with conditioning similar to that used in the induction of mixed chimerism, can significantly delay rejection of skin grafts (Unpublished data). Based upon these observations I hypothesized that conditioning along with an EV transplant would delay relapses in EAE similar to that observed with mixed chimerism. If so, I believed that developing this model would provide an ideal tool for asking mechanistic questions about the induction of tolerance in EAE.

We began with a simple modification of the model used in our previous studies (Figure 3.1a), by replacing allogeneic bone marrow with allogeneic bone-marrow-derived EVs. We decided to use $1x10^9$ bone marrow-derived EVs as opposed to another cellular EV source based upon the hypothesis they would have protein expression signatures more similar to that of BMCs and thus would better enable downstream experiments to identify a molecular basis for any observed effect. We chose the quantity based of EVs ($1x10^9$) upon the amount of EVs necessary to see an effect in skin graft rejection (Gonzales-Nolasco et al., Unpublished data).

When we compared the overall disease burden following transplant and the time until relapse, we found that the EV transplant did have a similar effect to that achieved with bone marrow. Like mixed chimeras, EV-treated mice displayed an immediate and significant drop in overall disease burden. EV-treated mice would also eventually relapse, similar to mixed chimeras. This led us to conclude that allogeneic EVs along with conditioning delay relapses in EAE with outcomes similar to mixed chimerism.

Interestingly, while successful mixed chimeras generally have very low disease burden in the immediate post-transplant period, this effect was not as pronounced in EV treated mice. One possible explanation for this is that, while successful bone marrow engraftment in mixed chimeras can be easily measured, there are no analogous measures for successful "engraftment" of EVs. It may be the case that a subset of EV-treated mice has greater disease burden because EVs have not successfully initiated whatever event is required to delay relapse.

We hypothesized the EV-mediated treatment effect could be the result of donor EVs cross-dressing recipient cells (likely APCs) and then delaying reactivation of autoreactive memory T cells through a mechanism mediated by a molecule that is likely present in both BMCs (or a cell they differentiate into) and EVs. We further hypothesized this would be MHC based on Racine et al.'s (2011) results showing donor MHC II is required for the induction of tolerance by mixed chimerism. If there is a molecular mediator of the observed treatment effect from EVs, it would be a reasonable hypothesis that this effect is dose-dependent.

To explore this hypothesis, we designed an experiment to measure a dose-response curve to EV treatment. We tested three separate doses: $1x10^6$, $1x10^9$, and $1x10^{11}$ EVs. These doses were chosen with the goal of testing a range over multiple orders of magnitude. Although the $1x10^9$ EV dose was arguably the most effective, the $1x10^{11}$ dose induced an equivalent delay in time to relapse (Figure 3.2c). We concluded the $1x10^6$ dose was clearly less effective and, based on these results, it appears the treatment effect is dose-responsive up to some amount above $1x10^6$ EVs. Based upon results with the highest ($1x10^{11}$) dose, we

concluded there was no further benefit for increasing the dose beyond $1x10^9$. Since the effect appears dose responsive to a certain point, this could imply there is either a certain threshold for activation of a binary effect, or that the maximum achievable effect is reached by, at most, $1x10^9$ EVs, although these results do not prove either of those hypotheses conclusively.

Because the half-life of EVs in the periphery after injection is very short (Takahashi et al., 2013), if the treatment effect was dose dependent, repeated injections might allow for a prolongation of that effect. To test this hypothesis, we modified our EV treatment model to include repeated treatment as described in Figure 3.3a.

Interestingly, the experiment demonstrated that the daily dose was the most effective. Not only did the daily treatment regimen delay relapses but it resulted in a significant and prolonged suppression of disease burden. Although the weekly dose was also effective in delaying relapses, it did not result in a statistically significant drop in overall disease burden as measured by the area under the curve. It is likely the weekly group (which received a higher overall dose) were subject to a longer period without circulating EVs, in contrast to the daily treatment. This data supports the hypothesis that the effect is dose-dependent and relies on the continued presence of donor EVs.

Taken together, the above data show that allogeneic EVs may be used to delay relapses in ongoing EAE when combined with conditioning. The effect is dose-dependent up to a certain threshold at which point higher dose confers no additional benefit. Although it is unclear why there is no additional benefit to the

higher dose, if the EV-mediated treatment effect relies upon the previously identified phenomenon of MHC transfer from EVs to recipient cells (Marino et al., 2016), it is possible that there is saturation of the recipient cell population responsible for the effect and thus the maximum treatment effect is reached by the 10^9 treatment. Finally, daily treatments result in lower overall disease burden. This study provides support for the hypothesis that allogeneic EVs may replace bone marrow in the treatment of ongoing in EAE, and also that a specific molecular mediator present upon both EVs and BMCs (or cells differentiated from them), is responsible for the delay in relapses.

Future work will focus on conclusively determining whether specific molecules present on EVs are responsible (particularly MHC) for the treatment effect, and whether EV treatment combined with NSC transplantation is capable of inducing tolerance similar to that induced by mixed chimerism and NSC transplantation.

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4

Conclusions and future directions

4.1 General conclusion

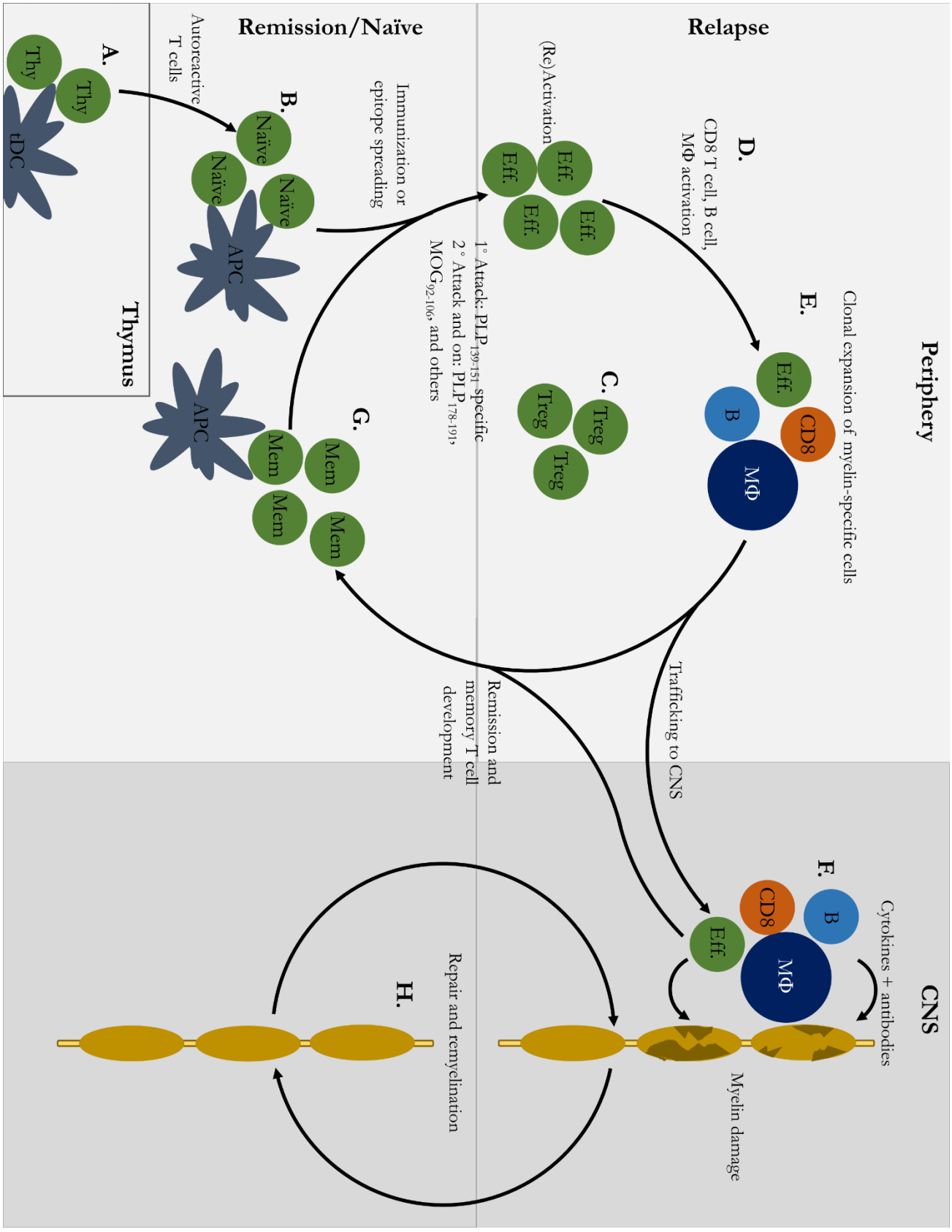
In the results presented here, we have tested hypotheses about how tolerance may be restored in the context of a chronic autoimmune disease and in the presence of autoreactive T cells. The concept of what exactly constitutes tolerance is not precisely defined within the field of immunology and deserves a moment of discussion. For the purposes of this work, we define tolerance as the absence of a detectable immune response for 100 days without (i) the use of immunosuppression and, (ii) without immune activation that would not normally be encountered (for example, reimmunization, which we are defining as breaking tolerance). Although it would certainly be possible to define tolerance differently, this is the definition we use in our descriptions of the state of the mice in our studies to have a common framework for discussion.

In Chapter 2, we developed a model for studying how mixed chimerism may be applied to ongoing EAE and developed a treatment wherein the delay in relapses afforded by mixed chimerism may be extended indefinitely when combined with a donor-matched NSC transplant. In Chapter 3, we studied the use of allogeneic, bone marrow-derived EVs, and how EVs may replace bone marrow in the treatment of ongoing EAE within our model. Below, I summarize our results, then discuss some of the implications and future directions. I also include models of CD4 T cell involvement in EAE and potential mechanisms by which mixed chimerism, mixed chimerism and NSC transplantation, and EV treatment may delay or present relapses, which are presented in Figures 4.1 and 4.2.

First, we developed a model for the induction of tolerance in EAE using mixed chimerism and concurrent NSC transplantation. We then tested various hypotheses regarding the nature of that tolerance. We showed that mixed chimerism can be successfully induced in ongoing EAE using a costimulatory blockade protocol that preserves the memory $CD4^+$ T cell compartment In addition, we showed that allogeneic mixed chimerism delays relapses in ongoing EAE, while syngeneic BMT with conditioning does not. The addition of a donor-matched NSC transplant to this protocol resulted in long term prevention of relapses. We also found that in mixed chimeras receiving a donor-matched NSC transplant, no CNS infiltrates were observed, while mixed chimeras showed meningeal inflammation, $CD3^+$ T cell infiltration, and demyelination. The mixed chimerism and NSC-treated mice did not respond to restimulation with the autoantigen $PLP_{139-151}$, while other groups did. Finally, the mixed chimerism and NSC treated mice were less sensitive to reimmunization. We further examined whether allogeneic EVs may replace bone marrow in our model and found that EVs successfully delay relapses in ongoing EAE. Finally, we found that the EV treatment effect was dose-dependent up to a certain threshold, and that repeated treatment over time increased the effect in terms of overall disease burden.

Figure 4.1: CD4 T cells in EAE:
A. Autoreactive, myelin-specific CD4 T cells mature in the thymus. (Thy = CD4 Thymocyte, tDC = thymic dendritic cell)
B. Autoreactive CD4 T cells are activated by immunization with their cognate antigen. In future relapses, myelin-specific memory CD4 T cells and additional naïve myelin-specific CD4 T cells are activated, as evidence by intra- and intermolecular epitope spreading (McRae, Vanderlugt, Dal Canto, & Miller, 1995; Vanderlugt & Miller, 2002). (Naïve = naïve myelin-specific CD4 T cell)
C. Endogenous Tregs fail to suppress the autoreactive immune activation. (Treg = CD4 Foxp3+ Treg)
D. Activated CD4 T cells go on to promote the activation and expansion of myelin-specific CD8 T cells and B cells, and further activate APC effector functions. (Eff = Activated CD4 Effector T Cell, B = B cell, CD8 = CD8 T cell, MF = Macrophage)
E. Activated lymphocytes undergo clonal expansion.
F. Immune cells traffic to the CNS, where they induce CNS inflammation and demyelination.
G. Following the acute attack (or relapse), immune activity remits and quiescent memory T cells remain. (Mem = Memory CD4 T cell)
H. Endogenous repair mechanisms attempt to repair and remyelinate any damage incurred during the relapse.

Figure 4.1:(Continued)



4.2 Future directions for mixed chimerism and concurrent NSC transplantation

MS is a disease of unanswered questions. We do not understand the etiology, neither do we fully understand the pathology. Regardless of whether the MS lesion is initiated by a neurodegenerative process or an autoimmune one, it is clear that there is a loss of self-tolerance at some point in the disease process. It is also highly likely that memory $CD4^+$ T cells play some role in the disease. Thus, any tolerance induction strategy must address this problem of immunologic memory towards the self in order to successfully treat the disease. Figure 4.1 summarizes the role of CD4 T cells in EAE. As seen in Figure 4.1g, during remission memory, CD4 T cells are present and, as we showed through reimmunization in Chapter 2, capable of causing disease in mice, even those treated with mixed chimerism and concurrent donor-matched NSC transplantation.

In the two studies using mixed chimerism to treat ongoing EAE, one study (from our laboratory) found that mixed chimerism can only delay relapses, while the other (Wu et al., 2015) found that relapses can be prevented entirely. I speculate that the major differences lie in the conditioning. In our study, we used costimulatory blockade, which leaves the $CD4^+$ T cell compartment intact (and capable of having a memory response after reimmunization), while Wu et al. (2015) used a strong T cell-depleting regimen.

There are a variety of potential mechanisms by which mixed chimerism and NSC transplantation could be inducing tolerance, as diagrammed in Figure

4.2 below. One of the mechanisms of tolerance in mixed chimerism is renewed thymic selection of T cells (Zuber & Sykes, 2017). It would be logical then that, in mixed chimeras, new autoreactive T cells are successfully deleted or otherwise handled by thymic selection, and do not make it to the periphery (Figure 4.2a). However, in the costimulatory blockade protocol, it is possible the memory $CD4^+$ T cells that are already in the periphery eventually become activated (by some unknown relapse mechanism, likely involving epitope spreading) and cause a relapse (Figure 4.2b-g). If these mature memory T cells are removed in the conditioning stage, that would prevent later relapses.

The results from our model seem to imply that mixed chimerism alone does not actually induce tolerance, but this result is masked if the T cells are depleted. In contrast, when we combine mixed chimerism with the neural stem cell transplant, however, it does appear to induce tolerance, so far as we can measure it.

Future work should endeavor to understand the mechanism by which tolerance is induced by the addition of the NSC transplant. While we have shown that deletion cannot be the *sole* mechanism, this does not exclude it from playing a role. In fact, after the induction of chimerism, it is still likely that there is a renewed thymic selection that is better able to delete autoreactive thymocytes (Figure 4.2a). However, since deletion cannot be the whole answer, it is very likely that regulation (in particular, Tregs) are involved in the mechanism of tolerance in our mixed chimerism and NSC model.

We have found both direct and indirect evidence for Treg involvement in mixed chimerism and NSC transplant-induced tolerance in ongoing EAE. The *in vitro* restimulation experiments in Figure 2.5b show that splenocytes from treated mice are unresponsive to stimulation in contrast to controls, implying the cells fail to activate. At the same time, the reimmunization experiments in Figure 2.5c show the cells are not deleted, are present, and can respond to stimulation under the right conditions. Additionally, Figure S5 shows that when splenocytes from various groups are examined 100 days post chimerism induction, recipient-type Tregs are elevated in mixed chimeras that received a donor-matched NSC transplant, but not in other groups. Taken together, it seems very likely that Tregs are somehow involved in the induction of tolerance in this model.

To answer the mechanistic questions posed above, I propose four possible avenues of research for further focus, which may be able to help unlock the secrets of this model:

First, it is possible that NSCs are primarily a source of antigen that is presented in some tolerogenic context to the immune system (Figure 4.2f, h). For instance, myelin peptides could be presented by donor MHC, and by linked suppression, the recipient immune system could be tolerized. Repeating the basic experiment (as modeled in Figure 2.3a) and using NSCs where myelin peptides have been knocked out would be one approach. Additionally, performing the experiment and transplanting cells without the potential to differentiate into oligodendrocytes, but with a similar ability to traffic to the CNS

(for instance, another glial precursor), would also provide insight into this question. Finally, more direct application of antigen may also be worth pursuing. For example, transduction of donor cells to express PLP and their transplant into the CNS. These strategies would allow us to examine whether various antigens are required for the restoration of tolerance in this model, the kinetics of these requirements, and how they might be modified to enable the clinical application of this approach.

The second possible avenue of inquiry is based on the hypothesis that the NSCs themselves somehow interact with the recipient immune system to induce tolerance. This could be through a soluble mediator (such as TGFβ), or through a cell-cell interaction within the CNS (Figure 4.2e, f). An initial approach would be to examine differences in soluble molecules in the CNS between mixed chimeras and mixed chimeras that received an NSC transplant. Mass spectrometry could be invaluable in identifying differences between these two groups. More sensitive labeling and tracking studies of transplanted NSCs would also allow us to monitor their interactions within the CNS, and examine whether they upregulate any particular molecules involved in immune regulation. TGFβ knockout NSCs could also be tested to look at requirements for cytokine expression by NSCs.

The third avenue regards the role of Tregs in tolerance. In order to understand whether Tregs (of donor or recipient origin) are responsible for the maintenance of tolerance in our model, direct perturbations will be necessary. There are two primary models by which this could be accomplished.

The first model relies upon the Foxp3^{DTR} mouse, where Foxp3+ Tregs may be depleted specifically by application of diphtheria toxin (Kim, Rasmussen, & Rudensky, 2007). By breeding this allele onto the SJL strain, it would be possible to use Foxp3^{DTR} bone marrow as the donor, transplant wild-type B6 bone marrow into an SJL Foxp3^{DTR} recipient, or have both donor and recipient with the Foxp3^{DTR} allele. In this manner, it would be possible to deplete donor, recipient, or both donor and recipient Tregs after the induction of tolerance to determine what the exact requirements for Tregs are.

Adoptive transfer is the second model by which Treg requirements could be examined. Following induction of tolerance, splenocytes could be sorted to remove Tregs from the donor, recipient, or any other population of interest (this would be aided greatly by using a Foxp3 reporter mouse). Although this model may require more manipulation than the Foxp3^{DTR} model, it also lends itself well to adding back in populations of interest. Thus, Tregs from tolerant mice could be transferred along with non-tolerant splenocytes to see if they are capable of inducing tolerance on their own.

The third avenue would be a novel or otherwise unanticipated mechanism of tolerance. Such a discovery would likely rely upon experimental decisions when examining the first two avenues of inquiry that ensure negative results allow researchers to ask more informed questions.

Figure 4.2: Potential Mechanisms of Mixed Chimerism and NSC transplantation in EAE

A. After the thymus is repopulated with donor DCs (red), thymic selection may eliminate new recipient myelin-specific T cells from entering the periphery. Transplanted EVs (red) may transfer MHC (or other molecules) to recipient thymic cells and influence thymic selection.

B. Donor-type APCs (red) may present EAE-relevant antigens without costimulation, preventing the activation of naïve cells. Transplanted EVs may transfer MHC to recipient APCs, which may then present EAE-relevant antigens as well.

C. Donor Tregs (red) may suppress activation of naïve or memory myelin-specific T cells. Transplanted EVs may induce suppressive responses in recipient Tregs.

D. In mixed chimerism alone or EV treatment, cells eventually escape any suppression and follow the pathway of activation to cause a relapse.

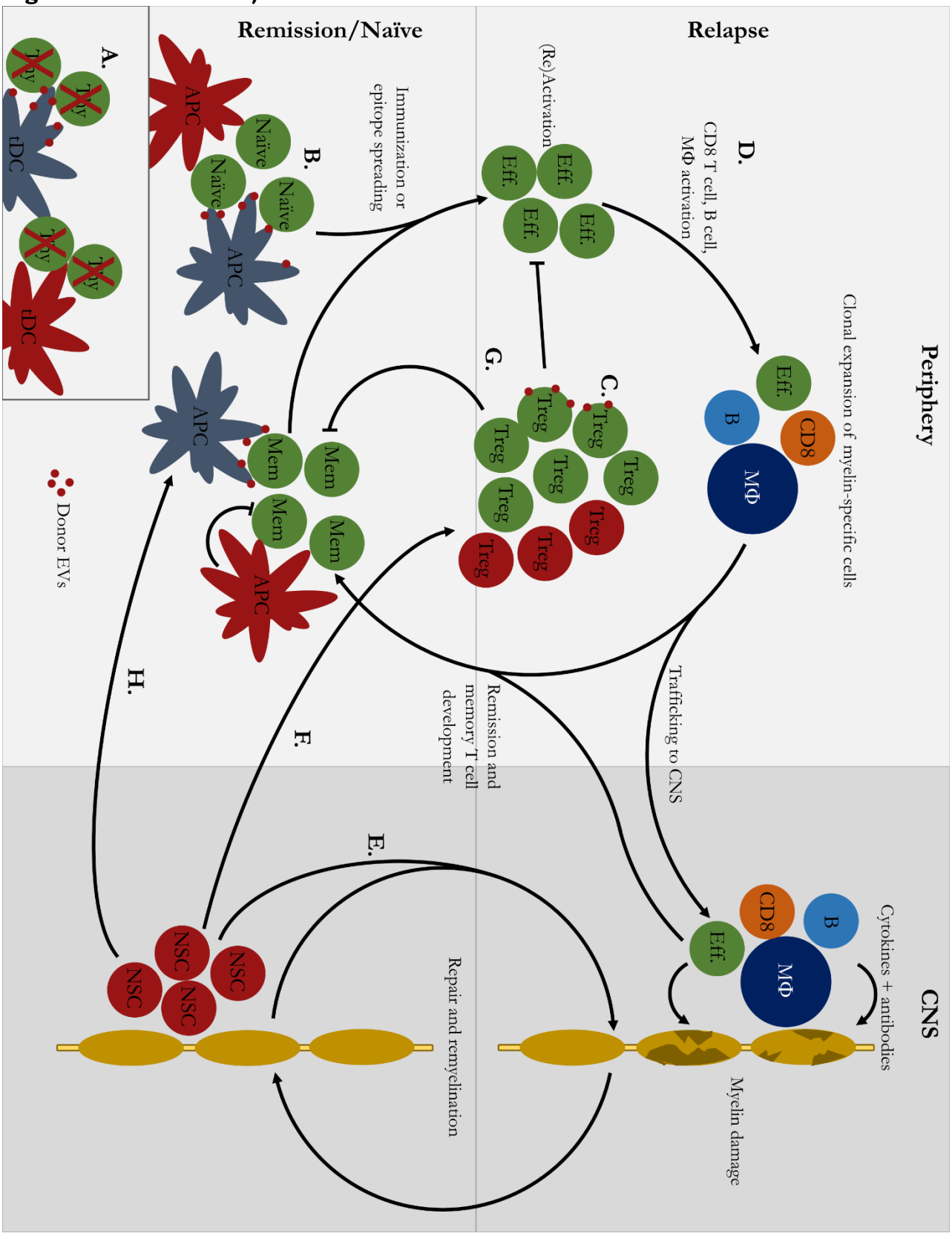
E. In mixed chimeras with concurrent NSC transplantation, donor NSCs (red) mediate improved CNS recovery by recipient-type cells.

F. NSCs influence Tregs, possibly directly (by presenting myelin antigens in the context of donor-type MHC) or indirectly (through APCs), resulting in an expansion of recipient-type peripheral Tregs.

G. Expanded recipient-type Tregs (as well as donor-type Tregs) could more effectively suppress memory T cell reactivation and naïve T cell recruitment, preventing relapses.

H. NSCs interacting with APCs, directly (in the CNS) or indirectly (via antigen draining from the CNS to the periphery), could serve a tolerogenic function, thus resulting in those APCs not activating peripheral T cells.

Figure 4.2: Continued)



4.3 Future directions for the use of EVs to delay relapses in EAE

Mechanistically, it seems likely that EVs recapitulate some necessary aspect of the bone marrow to prevent EAE relapses. If so, based upon results showing that MHC II is required for mixed chimerism to effectively treat diabetes in the NOD model (Racine et al., 2011), MHC II seems a likely candidate for mediating this treatment effect. A repeat experiment using MHC II knockout EVs and MHC II overexpressing EVs could shed further light on the mechanisms of the mixed chimerism effect.

Although MHC II is a likely candidate molecular mediator, it is certainly not the only one; more non-specific treatments of the EVs prior to injection could help answer this question. For example, a proteinase or protein denaturing treatment could be tested to see if it reverses the treatment effect. In this case, such treatments could be used to rule in or rule out various molecules within the EVs as the one(s) responsible for the treatment effect.

Finally, where EVs are required and what cells they are acting upon is also unknown. APCs within the thymus appear to be likely candidates (Figure 4.2a), although other lymphoid organs may also be involved. It is also possible that EVs could be interacting with Tregs, either activating them to suppress relapses or altering the Tregs' specificity to allow them to respond to aberrant immune activation (Figure 4.2c). Repeating the earlier experiments with a more targeted injection of EVs would be invaluable to answering this question. Additionally, examining the various lymphoid organs of recipients via microscopy at multiple time points after transplant to directly observe EV-cell interactions would be

critical in understanding how the EV transplant functions in this context. The use of fluorescent EVs to enable better tracking and measurement in the recipient would also greatly enable these studies.

In conclusion, it appears that mixed chimerism, or EV treatment plus conditioning, are effective strategies for modulating the immune response in EAE. The addition of the NSC transplant allows for a researcher to shift the model in-between autoimmunity and tolerance. This model offers a method by which tolerance and mechanisms of tolerance induction may be thoroughly investigated. Further, these studies are part of a growing research effort to apply such strategies to autoimmunity that will hopefully offer pathways toward a more complete understanding of immune tolerance, as well as ideas for developing clinically applicable therapeutics for the treatment of autoimmunity.

4.4 Concluding remarks

To offer a final grace note to this dissertation, I would like to mention that the practice of science, and the process of getting a PhD in particular, has been a series of experiences that were at times so exciting I thought I might change the world, and at others so frustrating I wondered why I was even doing it.

I began this project very excited to apply my advisor's method of tolerance induction to autoimmunity. At the time (in 2013), I felt confident that mixed chimerism would induce tolerance in EAE, so imagine my surprise when the mice relapsed. Conversely, I anticipated the NSC transplant would offer an

improvement in disease burden at best, and I am still a little in awe of how it completely changed the disease course in the experimental animals.

Towards the end of this experience, I have realized how important it is to design experiments properly, do everything in your power to minimize bias, and follow the data where it leads. To this day, I am excited about possible future applications of this work and its potential to answer fundamental questions about tolerance. I am grateful to have had the opportunity to make a small contribution to our knowledge in this field in pursuit of this PhD and look forward to seeing where the research goes next.

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Appendix

Supplemental figures

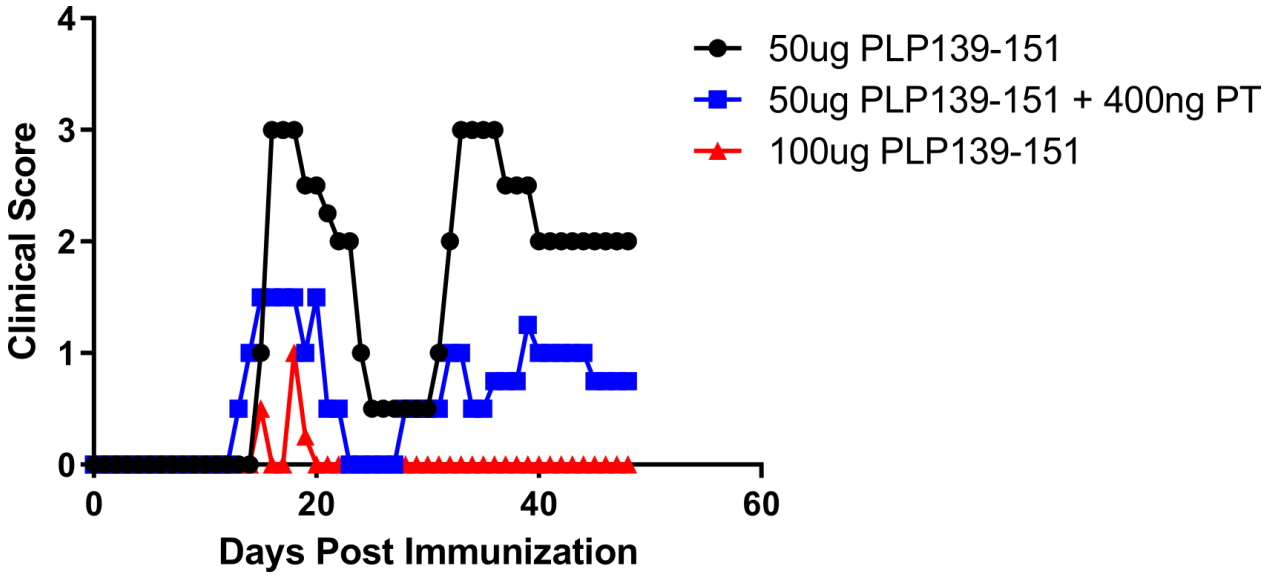

Figure S1: EAE is successfully induced in SJL mice. EAE was induced in 6-8 week old female SJL mice as described in the materials and methods with the indicated amounts of $PLP_{139-151}$ and with or without the addition of pertussis toxin. N=3 in each group.

A

Frequency of Parent	Total CD45.1	Total CD45.2	CD4.1	CD4.2	CD8.1	CD8.2	CD11bc.1	CD11bc.2	CD19.1	CD19.2	CD335.1	CD335.2
Control Conditioning 1	93.7	0.021	99.9	0	100	0	99.9	0	100	0	97.8	0
Control Conditioning 2	93.2	0.43	99.4	0	75	0	88	11.7	99.9	0.011	99	0.1
Control Conditioning 3	83.1	0.025	99.7	0	100	0	99.6	0	100	0	93.3	0
Control Conditioning 4	89.8	0.019	99.4	0	0	0	100	0	100	0	97.3	0
Mixed Chimera 1	78.9	13.7	98.7	0.038	50	50	38.7	59	91.9	7.04	91.2	3.85
Mixed Chimera 2	69.5	16.6	85.4	0.19	68.7	2	10.7	78.9	93.7	1.37	66.7	5.18
Mixed Chimera 3	65.4	16.9	87.2	0.42	6.67	26.7	18.6	69.1	76.2	14.3	66.6	7.96
Failed Chimera 1	99.2	0.015	100	0	93.3	0	100	0	100	0	99.7	0
SJL	83.6	0.013	100	0	99.5	0	99.5	0.11	100	0	99.7	0
B6	0	98	0	100	0	100	0	100	0	99.9	0	99.9
Counts per 10,000 cells	Total CD45.1	Total CD45.2	CD4.1	CD4.2	CD8.1	CD8.2	CD11bc.1	CD11bc.2	CD19.1	CD19.2	CD335.1	CD335.2
Control Conditioning 1	9370	2	3103	0	2	0	252	0	5390	0	246	0
Control Conditioning 2	9322	43	1057	0	2	0	207	27	7537	1	388	0
Control Conditioning 3	8309	2	3397	0	15	0	227	0	3956	0	209	0
Control Conditioning 4	8976	2	756	0	0	0	359	0	7308	0	363	0
Mixed Chimera 1	7890	1370	1816	1	1	1	473	722	4971	381	367	15
Mixed Chimera 2	6950	1662	2290	5	150	4	131	966	3398	50	159	12
Mixed Chimera 3	6538	1686	2355	11	0	2	170	629	3045	573	173	21
Failed Chimera 1	9918	1	5455	0	4	0	211	0	3461	0	195	0
SJL	8356	1	2726	0	1172	0	220	0	3570	0	324	0
B6	0	9800	0	1890	0	1290	0	311	0	5445	0	366

B

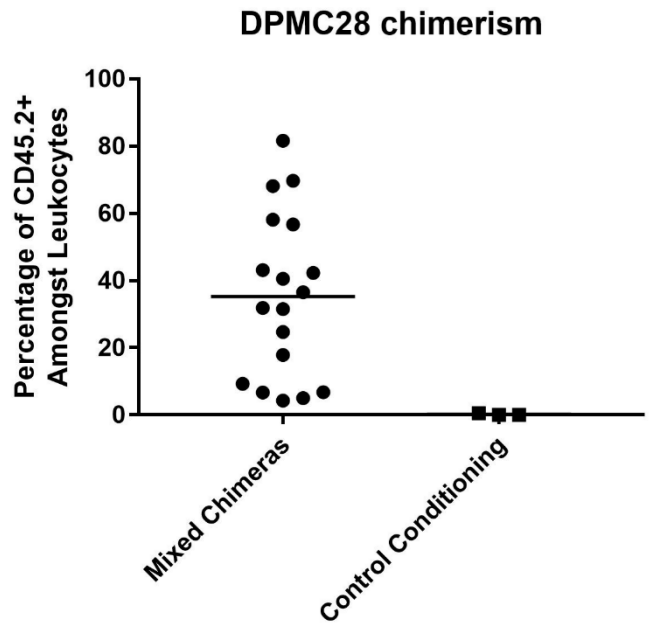

Figure S2: Mixed chimerism is successfully induced in EAE. On day 28 post-chimerism induction, peripheral blood mononuclear cells were obtained via cheek bleed from mice and examined by flow cytometry for the presence of donor-type (CD45.2+) cells. **A.** Table of values presented as frequency of parent population and absolute count. Columns with the suffix ".2"

Figure S2:(Continued)refer to CD45.2+ cells, while columns with the suffix ".1" refer to CD45.1+ cells of the given prefix. **B.** Total chimerism values among mice over multiple experiments at day 28 post chimerism induction compared to control conditioning.

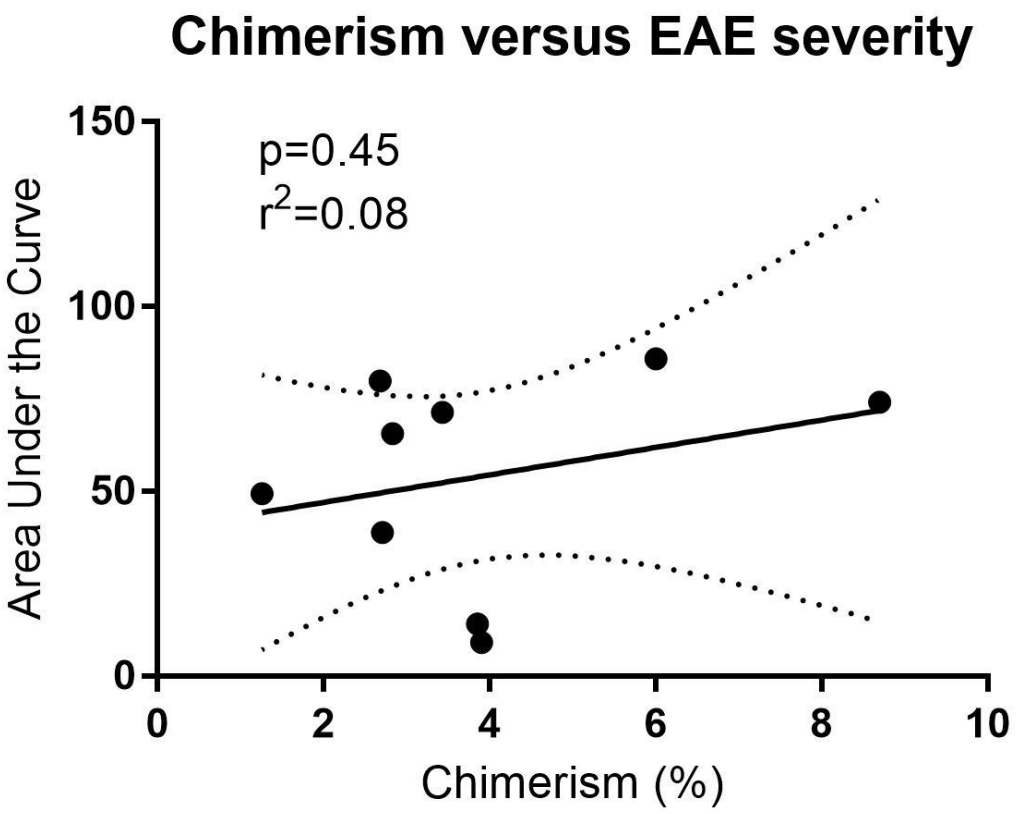

Figure S3: No correlation between chimerism and disease severity. Chimerism was induced as described in the materials and methods in 6-8 week old SJL mice with ongoing EAE. Successful chimeras were kept for 100 days post-chimerism induction. Chimerism values day 100 post chimerism induction in total splenocytes were compared to the EAE score area under the curve for the period after chimerism induction and a regression analysis was performed.

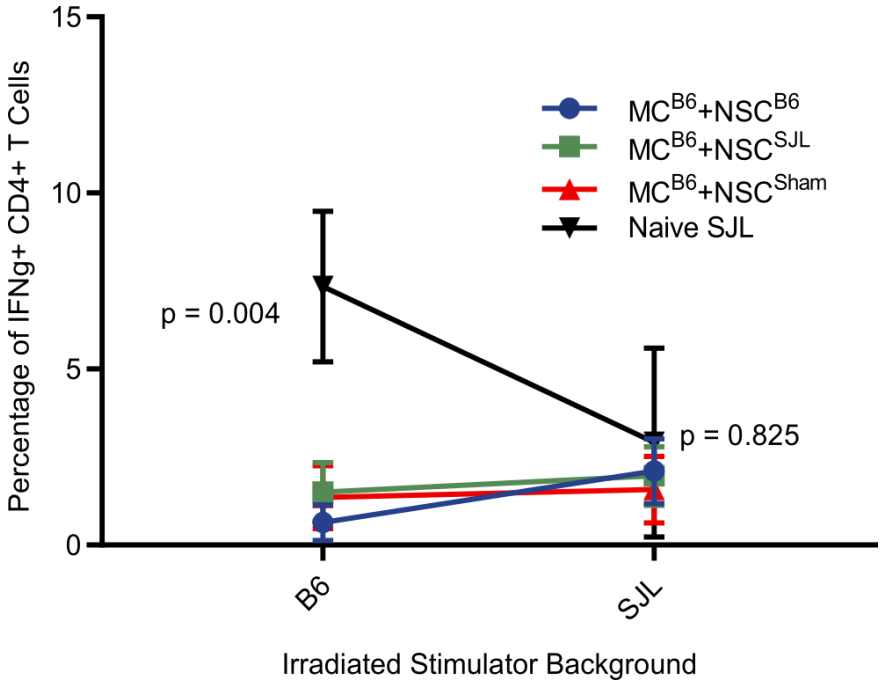

Figure S4: Restimulation of splenocytes with allogeneic and syngeneic stimulator cells. $2x10^5$ splenocytes were cultured in the presence of equivalent numbers of irradiated stimulator cells of the indicated background for 5 days and then stained for intracellular IFNγ expression. Results are shown as Mean + SD. One-way ANOVA was performed on each group (B6 and SJL respectively).

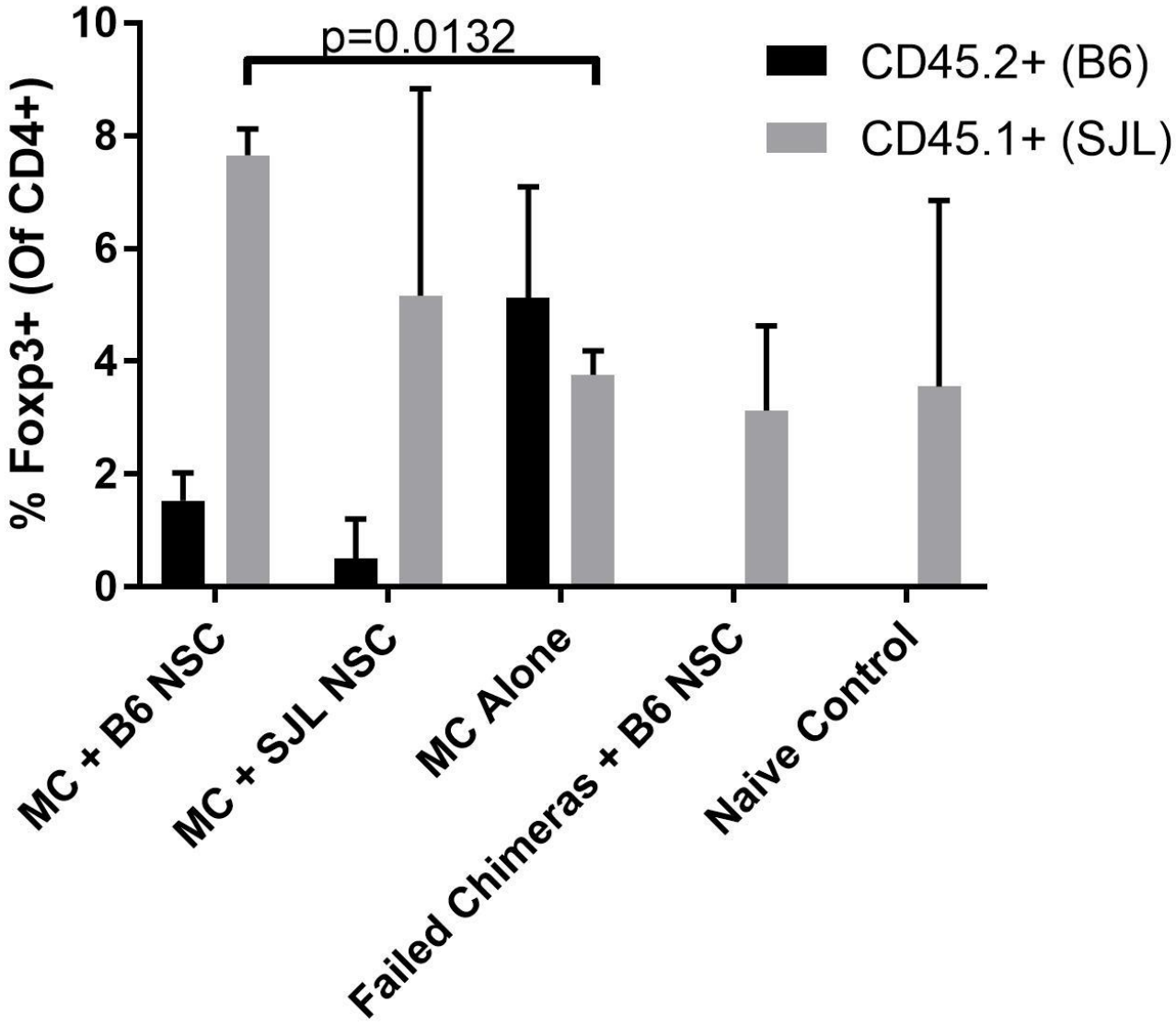

Figure S5: Proportion of Tregs in various treatment groups. On day 100 post-chimerism induction, splenocytes were obtained from mice in the listed treatment groups. Cells were examined by flow cytometry for the presence of Foxp3+ Tregs. Gating strategy as follows: Singlets > live cells > lymphocytes > CD4+ > CD45.2 or CD45.1 (donor or recipient respectively) > Foxp3+.

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