

# UC Irvine

## UC Irvine Electronic Theses and Dissertations

### Title

Regulatory T cell Mediated Repair Following Neural Stem Cell Transplantation in Murine Models of Multiple Sclerosis

### Permalink

<https://escholarship.org/uc/item/1p901358>

### Author

McIntyre, Laura

### Publication Date

2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,  
IRVINE

Regulatory T cell Mediated Repair Following Neural Stem Cell Transplantation  
in Murine Models of Multiple Sclerosis

DISSERTATION

Submitted in partial satisfaction of the requirements  
For the degree of

DOCTOR OF PHILOSOPHY

in Molecular Biology and Biochemistry

by

Laura Lynn McIntyre

Dissertation Committee:  
Professor Craig M. Walsh, Chair  
Professor Mathew Blurton- Jones  
Professor Matthew A. Inlay

2018



## **DEDICATION**

To my favorite lab, Brody.



## TABLE OF CONTENTS

|                                                                                                                              | Page  |
|------------------------------------------------------------------------------------------------------------------------------|-------|
| LIST OF FIGURES                                                                                                              | iv    |
| LIST OF TABLES                                                                                                               | vi    |
| LIST OF ABBREVIATIONS                                                                                                        | vii   |
| ACKNOWLEDGEMENTS                                                                                                             | xi    |
| CURRICULUM VITAE                                                                                                             | xiii  |
| ABSTRACT OF THE DISSERTATION                                                                                                 | xviii |
| CHAPTER 1: Promoting remyelination through cell replacement therapies in a model of viral- induced neurodegenerative disease | 1     |
| CHAPTER 2: 'Ex-Treg' derived neural antigen-specific regulatory T cells promote remyelination                                | 36    |
| CHAPTER 3: Visualizing regulatory T cell and neural stem cell dynamics within the murine spinal cord                         | 106   |
| CHAPTER 4: Regulatory T cells: Promising Immunotherapies for Autoimmunity and Tissue Repair                                  | 127   |
| CHAPTER 5: Conclusion                                                                                                        | 154   |

## LIST OF FIGURES

|                    |                                                                                                                                       | Page |
|--------------------|---------------------------------------------------------------------------------------------------------------------------------------|------|
| <b>Chapter One</b> |                                                                                                                                       |      |
| Figure 1.1         | Axonal damage in JHMV-infected mice is reversed following NPC engraftment.                                                            | 20   |
| Figure 1.2         | JHMV replicates in glial cells derived from mouse NPCs                                                                                | 21   |
| Figure 1.3         | Intraspinal transplantation of iPSC-derived NPCs into JHMV-infected mice.                                                             | 22   |
| Figure 1.4         | Intraspinal transplantation of hNPCs into JHMV-infected mice.                                                                         | 23   |
| <b>Chapter Two</b> |                                                                                                                                       |      |
| Figure 2.0         | 'Ex-Treg' derived neural antigen-specific regulatory T cells promote remyelination- graphic abstract                                  | 38   |
| Figure 2.1         | Transplantation of embryonic stem cell derived human neural stem cells promotes remyelination and Tregs in the EAE mouse spinal cord. | 66   |
| Figure 2.2         | Tregs accumulate at the site of hNSC injection in the EAE spinal cord                                                                 | 68   |
| Figure 2.3         | hNSCs expand Tregs <i>in vitro</i>                                                                                                    | 69   |
| Figure 2.4.        | hNSCs display unique CDR3 $\beta$ sequences                                                                                           | 71   |
| Figure 2.5         | Tregs from hNSC cultures are CNS antigen specific                                                                                     | 73   |
| Figure 2.6         | hNSC expanded Tregs are derived from the 'exTreg' pool                                                                                | 75   |
| Figure 2.7         | hNSC expanded Tregs display unique gene expression patterns                                                                           | 77   |
| Figure S2.1        | Mouse neural stem cells engraft, migrate and remyelinated in an EAE mouse model of MS with no effect upon CNS immune environment      | 79   |
| Figure S2.2        | EB-derived human neural stem cells are multipotent and express neural lineage markers                                                 | 81   |

|              |                                                                                                                                |    |
|--------------|--------------------------------------------------------------------------------------------------------------------------------|----|
| Figure S2.3  | hNSC, but not HDFs promote remyelination                                                                                       | 82 |
| Figure S2.4  | Immune cell populations are unaffected following hNSC transplant                                                               | 84 |
| Figure S2.5  | PC61.5 treatment of mice depleted Tregs without affecting Tconv cell population or EAE clinical score                          | 86 |
| Figure S2.6  | hNSCs expand Tregs <i>in vitro</i> and display greater expression of CD25 and FoxP3                                            | 88 |
| Figure S2.7  | HDFs and mNSCs do not expand Tregs <i>in vitro</i>                                                                             | 90 |
| Figure S2.8  | hNSC-Tregs recognize neurofilament antigens                                                                                    | 92 |
| Figure S2.9  | Neural antigen-specific T cells upregulate FoxP3 upon antigen exposure <i>in vivo</i>                                          | 94 |
| Figure S2.10 | Following expansion in lymphopenic hosts neural antigen-specific T cells upregulate FoxP3 upon antigen exposure <i>in vivo</i> | 96 |

### Chapter Three

|            |                                                                                             |     |
|------------|---------------------------------------------------------------------------------------------|-----|
| Figure 3.1 | Mapping of spinal cord homing Tregs in EAE following hNSC Transplant.                       | 120 |
| Figure 3.2 | hNSC Transplantation does not alter Treg motility behavior within the spinal cord.          | 121 |
| Figure 3.3 | hNSC expanded Tregs interact with endogenous PLP <sup>+</sup> cells within the spinal cord. | 123 |

### Chapter Four

|            |                                        |     |
|------------|----------------------------------------|-----|
| Figure 4.1 | Therapies involving regulatory T cells | 142 |
|------------|----------------------------------------|-----|

## LIST OF TABLES

|           |                                                                           | Page |
|-----------|---------------------------------------------------------------------------|------|
|           | <b>Chapter One</b>                                                        |      |
| Table 1.1 | Overview of mouse and human stem cell engraftment into JHMV-infected mice | 25   |

## LIST OF ABBREVIATIONS

|                       |                                                  |
|-----------------------|--------------------------------------------------|
| 2P                    | two-photon                                       |
| ACT                   | adoptive cellular therapy                        |
| ADGRG1                | adhesion G-protein coupled receptor G1 precursor |
| APC                   | antigen presenting cell                          |
| BBB                   | blood brain barrier                              |
| CAR                   | chimeric antigen receptor                        |
| CCL                   | chemokine ligand                                 |
| Ccr                   | chemokine receptor                               |
| CD                    | cluster of differentiation                       |
| cLN                   | cervical lymph node                              |
| CNS                   | central nervous system                           |
| CVB3                  | Coxsackievirus B3                                |
| Dkk3                  | Dickkopf-3                                       |
| DMT                   | disease-modifying therapy                        |
| EAE                   | experimental autoimmune encephalomyelitis        |
| ESC                   | embryonic stem cell                              |
| FoxP3                 | forkhead box 3                                   |
| FoxP3 <sup>EGFP</sup> | FoxP3-enhanced green fluorescent protein         |
| FTY720                | Fingolimod                                       |
| GALT                  | gut associated lymphoid tissue                   |
| GFP                   | green fluorescent protein                        |
| GVHD                  | graft-versus-host-disease                        |

|           |                                                 |
|-----------|-------------------------------------------------|
| Gzmb      | granzyme B                                      |
| HA        | hemagglutinin                                   |
| HDF       | human Dermal Fibroblast                         |
| HLA       | human leukocyte antigen                         |
| hNSC      | human neural stem cell                          |
| hNSC-T    | hNSC-transplanted                               |
| hNSC-Treg | hNSC expanded Treg                              |
| HSCT      | hematopoietic stem cell transfer                |
| HSV-1     | herpes simplex virus type 1                     |
| IFN       | interferon                                      |
| IL        | interleukin                                     |
| IPEX      | immunodysregulation polyendocrinopathy X-linked |
| iPSC      | induced pluripotent stem cell                   |
| iTreg     | induced Treg                                    |
| JHM       | John Howard Muller                              |
| JHMV      | JHM strain of mouse hepatitis virus             |
| mAb       | monoclonal antibody                             |
| MBP       | myelin basic protein                            |
| MFI       | mean fluorescent intensity                      |
| MHC       | major histocompatibility complex                |
| MOG       | myelin oligodendrocyte glycoprotein             |
| MS        | Multiple Sclerosis                              |
| mTORC1    | mammalian target of rapamycin 1                 |

|                |                                              |
|----------------|----------------------------------------------|
| NK             | natural killer                               |
| NKT            | natural killer T cell                        |
| NOD            | non-obese diabetic                           |
| NPC            | neural progenitor or precursor cell          |
| NPLC           | neural precursor like cell                   |
| NSC            | neural stem cell                             |
| NT             | non-transplanted                             |
| OPC            | oligodendrocyte progenitor or precursor cell |
| OVA            | Ovalbumin                                    |
| p.i.           | post-immunization                            |
| p.t.           | post-transplant                              |
| PBS            | phosphate buffered saline                    |
| PD-1           | programmed cell death 1                      |
| PD-L1          | programmed cell death ligand 1               |
| PLP            | proteolipid protein                          |
| PMD            | Pelizaeus-Merbacher disease                  |
| PPAR- $\gamma$ | proliferator-activated receptor gamma        |
| pTregs         | peripheral Tregs                             |
| R2D2           | <i>RAG2</i> <sup>-/-</sup> 2D2 <sup>+</sup>  |
| RA             | rheumatoid arthritis                         |
| RAE            | retinoic acid early precursor transcript     |
| RAG            | recombinase activating gene                  |
| RRMS           | relapse-remitting forms of MS                |

|             |                                 |
|-------------|---------------------------------|
| S1P         | sphingosine 1 phosphate         |
| SC          | spinal Cord                     |
| SCI         | spinal cord injury              |
| SLE         | systemic lupus erythematosus    |
| T1DM        | type 1 diabetes                 |
| T9          | thoracic vertebrae 9            |
| TCR         | T cell receptor                 |
| TCR $\beta$ | T cell receptor beta            |
| TdT         | TdTomato                        |
| Tconv       | conventional T cell             |
| Teff        | effector T cell                 |
| TG2         | transglutaminase-2              |
| TGF $\beta$ | transforming growth factor beta |
| Treg        | regulatory T cells              |
| tTregs      | thymus Tregs                    |
| YFP         | yellow fluorescent protein      |



## ACKNOWLEDGEMENTS

I would like to thank my thesis mentor Dr. Craig Walsh for his encouragement and for allowing me the freedom to develop into an independent researcher. I would also like to thank my thesis committee Dr. Mathew Blurton-Jones and Dr. Matthew Inlay for their encouragement and wisdom. In addition, I would like to thank my advancement committee Dr. Melissa Lodoen and Dr. Jennifer Prescher for their support. Thank you to my lab mates in the Walsh Lab; Julio Ayala Angulo, Lu Chen, Kody Dang, Kelley Fracchia, Scott Greilach, Herlinda Guzman, Edna Hingo, Jimmy Huynh, Justin Lengfeld, Jackie Mitchell, Louis Parker, Quan Pham, Warren Plaisted, Freddy Rezk, Solmaz Sadari, Jorge Silva, Estelle Vu, Brian Wi, Jason Weinger, and Angel Zavala and all of my collaborators; Mathew Blurton-Jones, Morgan Coburn, Hayk Davtyan, Jonny Hasselman, Amanda McQuade, Jessica Sanchez-Ramirez, Michael Cahalan, Othy Shivashankar (Shiva), Kai Kessenbrock, Quy Nguyen, Hamad Saleh O Alsheteiwi, Matthew Inlay, Yasamine Ghorbanian, Alborz Karimzadeh, Ankita Shukla, Erika Varady, Tom Lane, Collen Worne, Stephen Schoenberger, Joey Lee, Albert Zlotnik, Jovani Catalan-Dibene, Jenna Kastenschmidt and Daniel Haensel for their generous encouragement and support. I would like to extend my gratitude to the staff at the UCI Sue and Bill Gross Stem Cell Research Center, Jennifer Atwood at the UCI Institute for Immunology, Opal Allen and Brian Dailey at the University of Utah Genomics core, Melanie Oakes at the UCI Genomics Core, Chad Caraway and Ryan Bohannon at the UCI ADRC, and Jon Neumann and Shimako Kawauchi at the UCI TMF for technical support and use of core facilities. This work was supported by the California Institute for Regenerative Medicine (TR05603 to CMW), the National Multiple Sclerosis Society (CA1058 to CMW), and the

National Institutes of Health (1T32NS082174 to LLM; 5R01NS092042 to TEL;  
5R01AI121945 to MDC and CMW; 1RF1AG055524 to MBJ and CMW).

# CURRICULUM VITAE

## Laura McIntyre

29 Hummingbird Lane Aliso Viejo, CA 92656

Phone: (619) 665-9998

Email: [mcintyrl@uci.edu](mailto:mcintyrl@uci.edu)

[www.linkedin.com/in/mcintyrl](http://www.linkedin.com/in/mcintyrl)

## Education

### University of California Irvine, Irvine, CA

2013- 2018

Doctoral Student

Laboratory of Craig Walsh PhD.

Clinical Co-Mentor Ardith Courtney D.O.

Department of Molecular Biology and Biochemistry

GPA: 3.96

Coursework: Human Embryonic Stem Cell Techniques Course, Induced Pluripotent Stem Cells Techniques Course, Integrative Immunology, Nucleic Acids Structure and Function, Public Speaking, Regulation of Gene Expression, Topics in Stem Cell Ethics, Translational Neuroscience, Cancer Immunology Journal Club, Immunology Journal Club

### San Diego State University, San Diego, CA

2009- 2013

Bachelor of Science: Microbiology

GPA: 3.33

Major GPA: 3.66

Coursework: Biochemistry (Cell and Molecular Biology) I, II, and III, Ecology and the Environment, Genetics and Evolution, Immunology, Medical Microbiology, Microbial Genetics and Physiology, Microbiology, Neurobiology, Organic Chemistry I and II, Virology.

## Grants, Honors, and Awards

NIH/ NINDS Pre-Doctoral Training Grant

2015- 2017

NIH Award# NS082174

University of California Irvine

American Association of Immunologists

2015, 2016, 2017

Young Investigator Award

## Research Experience

### The Scripps Research Institute

Laboratory of Charles D. Surh, PhD.

Research Intern

La Jolla, CA

2009- 2012

### San Diego State University, Cell and Molecular Biology

Laboratory of Ralph Feuer PhD.

San Diego, CA

## Publications

**McIntyre, L.L.**, Greilach, S.A., Hasselmann, J., Othy, S., Ngyuen, Q., Wi, B., Sears-Kraxberger, I., Silva, J., Ayala-Angulo, J., Vu, E., Pham, Q., Dang, K., Rezk, F., Steward, O., kessenbrock, K., Loring, J.F., Cahalan, M.D., Blurton-Jones, M., Lane, T.E., Walsh, C.M. (2018) 'Ex-Treg' derived neural antigen-specific regulatory T cells promote remyelination. *Cell*. (submitted)

Riazifar, M., Mohammadi, M.R., Pone, E.J., **McIntyre, L.L.**, Yeri, A., Lasser, C., Segaliny, A., Shelke, G., Hutchins, E., Hamamoto, A., Calle, E., Crescitelli, R., Liao, W., Pham, V., Yin, Y., Walsh, C.M., Van Keuren-Jensen, K., Lotvall, J., Zhao, W. (2018) Stem Cell-derived Exosomes as Nanotherapeutics for Autoimmune and Neurodegenerative Disorders. *ACS Nano*. (in review)

Shukla, A.K., **McIntyre, L.L.**, Marsh, S.E., Schneider, C.A., Hoover, E.M., Walsh, C.M., Lodoen, M.B., Blurton-Jones, M., Inlay, M.A. (2018) CD11a expression distinguishes infiltrating myeloid cells from plaque-associated microglia in Alzheimer's Disease. *Glia*. (accepted)

Catalan, J., **McIntyre, L.L.**, Zlotnik, A. (2018) Interlukin-30 to Interleukin-40. *J Interferon Cytokine Res*. 38(10): 423-439

**McIntyre, L.L.**, Mangale, V., Walsh, C.M., Loring, J.F., and Lane, T.E. (2018) Promoting remyelination through cell replacement therapies in a model of viral-induced neurodegenerative disease. *Dev Dynamics*. doi: 10.1002/dvdy.24658

Sin, J., **McIntyre, L.L.**, Stotland, A., Feuer, R., Gottlieb, R.A. (2017) Coxsackievirus B escapes the infected cell in ejected mitophagosomes. *J. Virol*. 91(24). Pii: e01247-17

Robinson, S.M., Tsueng, G., Sin, J., Mangale, V., Rahawi, S., **McIntyre, L.L.**, Williams, W., Kha, N., Cruz, C., Sayen, M.R., Hilton, B.J., Segall, A.M., Wolkowicz, R., Cornell, C.T., Whitton, J.L., Gottlieb, R.A., Feuer, R. (2014) Coxsackievirus B Exits the Host Cell in Shed Microvesicles Displaying Autophagosomal Markers. *PLoS Pathog*. 10(4): e1004045

## Presentations

**La Jolla Immunology Conference**  
Salk Institute, San Diego, CA  
Poster Presentation

2018

**Keystone Symposia Conference New Frontiers in Neuroinflammation**  
Keystone, Colorado

2018

Poster Presentation

**American Association of Immunologists Annual Meeting** 2018

Austin, Texas

Oral and Poster Presentation, AAI Trainee Award

**UCI Associated Graduate Students Symposium** 2018

University of California, Irvine

Poster Presentation, People's Choice Award

**UCI Molecular Biology and Biochemistry Retreat** 2018

UCLA Lake Arrowhead Conference Center

Oral and Poster Presentation

**UCI Immunology Fair** 2017

University of California, Irvine

Poster Presentation, Best Poster Presentation Award

**La Jolla Immunology Conference** 2017

Salk Institute, San Diego, CA

Poster Presentation

**Immunology LA** 2017

Skirball Cultural Center

Oral Presentation

**American Association of Immunologists Annual Meeting** 2017

Washington D.C.

Poster Presentation, AAI Trainee Poster Award

**UCI Molecular Biology and Biochemistry Retreat** 2017

UCLA Lake Arrowhead Conference Center

Poster Presentation

**Herbert Gavin Eye Institute Bench to Bedside Symposium** 2017

Beckman Center, Irvine, CA

Poster Presentation

**UCI Immunology Fair** 2016

University of California, Irvine

Oral Presentation, Best Oral Presentation Award

**UCI Stem Cell Awareness Day** 2016

University of California, Irvine

Poster Presentation, Best Poster Award

|                                                                                                                                                            |      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| <b>La Jolla Immunology Conference</b><br>Salk Institute, San Diego, CA<br>Poster Presentation, AAI Young Investigator Award                                | 2016 |
| <b>So Cal Flow Summit</b><br>Beckman Center, Irvine, CA<br>Poster Presentation                                                                             | 2016 |
| <b>UCI Immunology Fair</b><br>University of California, Irvine<br>Poster Presentation                                                                      | 2015 |
| <b>UCI Stem Cell Awareness Day</b><br>University of California, Irvine<br>Poster Presentation                                                              | 2015 |
| <b>La Jolla Immunology Conference</b><br>Salk Institute, San Diego, CA<br>Poster Presentation, AAI Young Investigator Award                                | 2015 |
| <b>Graduate Student Symposium</b><br>San Diego State University                                                                                            | 2013 |
| <b>Student Research Symposium</b><br>San Diego State University<br>Poster Presentation, Undergraduate Research Excellence Award                            | 2013 |
| <b>CSU Program for Education Research in Biotechnology</b><br>Anaheim, California<br>Poster Presentation, Nagel Undergraduate Research Award               | 2013 |
| <b>Cardiovascular Regeneration International Symposium</b><br>San Diego State University Integrated Regenerative Research Institute<br>Poster Presentation | 2012 |
| <b>MBRS Undergraduate Student Research Symposium</b><br>San Diego State University<br>Oral Presentation                                                    | 2012 |

## **Skills**

Laboratory skills: Stem cell and iPSC culture (CIRM training course certified), tissue culture, flow cytometry, fluorescence microscopy, tissue culture, stem cell culture, ELISA, qPCR, murine husbandry, bacterial culture, familiar with cloning, and electron microscopy.

Computer skills: MacOS, Windows operating systems (XP and Vista), MS Office, PRISM, BD FACS DIVA, FlowJo (flow cytometry data analysis software), Image J.

### **Teaching Experience**

|                                            |            |
|--------------------------------------------|------------|
| Advanced Immunology Lab Teaching Assistant | 2015, 2016 |
| General Microbiology Teaching Assistant    | 2015, 2017 |
| Molecular Biology Lab Teaching Assistant   | 2017       |

### **Academic Recognitions**

|                                              |      |
|----------------------------------------------|------|
| UCI AGS People's Choice Award                | 2018 |
| AAI Trainee Award                            | 2018 |
| UCI Immunology Fair Best Poster Presentation | 2017 |
| AAI Trainee Poster Award                     | 2017 |
| UCI Immunology Fair Best Oral Presentation   | 2016 |
| UCI Stem Cell Awareness Day Best Poster      | 2016 |
| AAI Young Investigator Award                 | 2016 |
| AAI Young Investigator Award                 | 2015 |

### **Campus Involvement and Professional Memberships**

|                                                      |               |
|------------------------------------------------------|---------------|
| UCI AGS Symposium                                    | 2018          |
| UCI Grad Slam Finals                                 | 2018          |
| UCI AGS Symposium                                    | 2017          |
| UCI Grad Slam                                        | 2017          |
| UCI Graduate Professional Success BIOMED Program     | 2014- 2018    |
| Undergraduate Research Mentor                        | 2014- 2018    |
| Excellence in Mentoring                              | 2014          |
| Teaching Assistant Professional Development Training | 2014          |
| Sigma Delta Epsilon Graduate Women in Science        | 2013- 2018    |
| AAI Member                                           | 2013- Present |

### **Volunteer Experience**

|                                                      |                  |
|------------------------------------------------------|------------------|
| UCI Multiple Sclerosis Walk                          | 2015, 2016, 2017 |
| UCI Stem Cell Awareness Day Tours                    | 2015, 2016, 2017 |
| Irvine Unified School District Ask-A-Scientist Night | 2014             |
| UCI Stem Cell Awareness Day Tours                    | 2014             |

## **ABSTRACT OF THE DISSERTATION**

Regulatory T cell Mediated Repair Following Neural Stem Cell Transplantation  
in Murine Models of Multiple Sclerosis

By

Laura Lynn McIntyre

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Craig Michael Walsh, Chair

Multiple Sclerosis (MS) is a chronic, autoimmune disease of the central nervous system (CNS), for which there is no cure. Deficits in neurological function occur as a result of widespread demyelination and axonal loss caused by infiltrating immune cells, mainly lymphocytes (T cells) and myeloid cells. Regulatory T cells (Tregs), characterized by expression of CD4 and FoxP3, are a specialized subset of T cells that are essential for the maintenance of T cell homeostasis and prevention of autoimmunity. Thus, dysregulation of Tregs has been linked to MS disease pathogenesis. Current therapies for MS are only focused on immunosuppression and fail to address the need for sustained remyelination and axonal protection. Transplantation of neural stem cells (NSCs) or neural precursor/ progenitor cells (NPCs) is a promising therapeutic strategy to treat conditions and diseases affecting white matter integrity, such as MS. NSCs and NPCs are a mixed population of self-renewing stem cells and cells that are poised to differentiate into the three types of neural cells found in the CNS; neurons, astrocytes, and myelin-producing oligodendrocytes. Early studies employing animal models of demyelination



have demonstrated that surgical implantation of NSCs into the CNS reduces neuroinflammation and enhances remyelination. However, the mechanism by which these cells promote tissue repair remains poorly understood.

The focus of this dissertation is to elucidate the mechanisms by which NSCs promote remyelination following transplantation in murine models of MS. Viral infection has long been considered a potential triggering mechanism involved in immune-mediated demyelination, therefore viral models of demyelination are relevant for modeling human demyelinating diseases, such as MS. Intracranial inoculation of susceptible mice with the neurotropic JHM variant of mouse hepatitis virus (JHMV) leads to immune-mediated demyelination and axonopathy. We have demonstrated that intraspinal injection of syngeneic mouse NSCs into the CNS of JHMV-infected mice results in remyelination associated with axonal sparing due to cell replacement. In addition, JHMV-infected mice that received transplants of xenogeneic human NPCs also displayed sustained remyelination and decreased neuroinflammation, which was associated with an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Tregs. Importantly, recovery was not a result of cell replacement, hNPCs underwent xenograft rejection, rather due to immune modulation (**Chapter 1**). Chapter 1 is a re-print of a review article published in *Developmental Dynamics* that summarizes our recent findings transplanting mNPCs and hNPCs into the spinal cord of JHMV infected mice, and serves as an introduction to the dissertation. Immunization of mice with myelin oligodendrocyte glycoprotein is the prototypic murine model of MS, experimental autoimmune encephalomyelitis (EAE), which is characterized by ascending immune-mediated inflammation and demyelination of axonal tracks, as well as neuronal death resulting in motor deficits. In support of our previous findings, engraftment of

syngeneic mNSCs facilitated remyelination in EAE mice with no effect upon the immune system. Transplantation of xenogeneic hNSCs into EAE mice resulted in dampened neuroinflammation and remyelination due to expansion of neural antigen-specific Tregs. Ablation of Tregs abrogated remyelination. Additionally, hNSCs promoted expansion of neural antigen-specific Tregs (hNSC-Tregs) *in vitro*, from the 'exTreg' pool, a population of thymically derived Tregs that lost FoxP3 expression in the periphery. hNSCs reinvigorated FoxP3 expression in 'exTregs' following co-culture with hNSCs, supporting the hypothesis that self-peptide/MHC is important for maintenance of FoxP3 expression and Treg function. hNSC-Tregs display a unique gene expression signature, upregulating molecules known to be involved in suppression of neuroinflammation, Dickkopf 3, and Transglutaminase 2 which facilitates oligodendrocyte progenitor cell differentiation to mature, myelin producing oligodendrocytes (**Chapter 2**). Utilizing two-photon microscopy of spinal cord explants, we discovered that Tregs localize to the sites of hNSC transplantation and interact with cells, most likely oligodendrocytes, that produce a component of the myelin sheath, proteolipid protein (**Chapter 3**). These findings support the concept that Tregs not only function in suppressing inflammatory responses, but also support their role as potentiators of tissue repair. Therefore, there has been an increasing interest utilizing Tregs as immunotherapies to treat autoimmune diseases. Therapies to promote expansion of Tregs and adoptive T cell therapy are currently under investigation in clinical trials for autoimmune diseases including, graft versus host disease and type I diabetes myelitis, although the ability of Tregs to modulate tissue repair is not well studied in the clinic (**Chapter 4**). Thus, hNSC expanded Tregs may provide new therapeutic strategies for treatment of autoimmune, neurodegenerative diseases, such as MS.

## CHAPTER ONE

### **Promoting remyelination through cell transplantation therapies in a model of viral-induced neurodegenerative disease**

Laura L. McIntyre\*, Vrushali Mangale\*, Craig M. Walsh, Jeanne F. Loring and Thomas E. Lane

\*These authors contributed equally to the manuscript.

## **Summary**

Multiple sclerosis (MS) is a central nervous system (CNS) disease characterized by chronic neuroinflammation, demyelination, and axonal damage. Infiltration of activated lymphocytes and myeloid cells are thought to be primarily responsible for white matter damage and axonopathy. A number of FDA-approved therapies exist that impede activated lymphocytes from entering the CNS thereby limiting new lesion formation in patients with relapse-remitting forms of MS (RRMS). However, a significant challenge within the field of MS research is to develop effective and sustained therapies that allow for axonal protection and remyelination. In recent years, there has been increasing evidence that some kinds of stem cells and their derivatives seem to be able to mute neuroinflammation as well as promote remyelination and axonal integrity. Intracranial infection of mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in immune-mediated demyelination and axonopathy, making this an excellent model to interrogate the therapeutic potential of stem cell derivatives in evoking remyelination. This review provides a succinct overview of our recent findings using intraspinal injection of mouse CNS neural progenitor cells and human neural precursors into JHMV-infected mice. JHMV-infected mice receiving these cells display extensive remyelination associated with axonal sparing. In addition, we discuss possible mechanisms associated with sustained clinical recovery.

**Keywords:** demyelination, virus, remyelination, neural precursor cells, multiple sclerosis

## **Introduction**

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS) characterized by extensive myelin destruction (Steinman, 1996). While the cause of MS is unknown, disease onset has been attributed to multiple factors including the genetic background of the individual as well as environmental influences (Oksenberg et al., 1993; Poser, 1994). Histologic characterization of lesions reveals the presence of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as macrophages, which are thought to act in concert with reactive microglia to release a milieu of pro-inflammatory factors that lead to oligodendrocyte dysregulation (Lassmann et al., 2007; Traugott et al., 1983). Multifocal demyelinating lesions eventually lead to various clinical symptoms such as impaired motor skills, cognitive decline, behavioral deficits and vision loss (Lassmann et al., 2007; Neumann et al., 2002; Prineas and Graham, 1981). Disease-modifying therapies (DMTs) for MS focus on reducing T lymphocyte infiltration into the CNS in an attempt to prevent formation of new lesions. With the exception of Ocrelizumab (anti-CD20) (Frampton, 2017), which was recently approved for progressive MS, all FDA approved DMTs are indicated for relapsing-remitting form of MS (Weinshenker et al., 1989).

Remyelination failure in MS patients is complex and the result of a variety of factors that culminate in the inability of oligodendrocyte precursor cells (OPCs) to mature into myelin-producing oligodendrocytes. Endogenous OPCs are spread throughout the CNS and appear in high density within some subacute lesions during early stages of MS (Chang et al., 2000). Remyelination following OPC maturation leads to the formation of shadow plaques, in which patches of remyelinated white matter are composed of disproportionately thin myelin sheaths surrounding axons (Chang et al., 2000; Halfpenny

et al., 2002; Lassmann, 1983; Lucchinetti et al., 1999; Prineas et al., 1989; Roy et al., 1999; Schlesinger, 1909). Therefore, understanding mechanisms associated with impaired OPC differentiation and triggering maturation of these cells into mature myelin-producing oligodendrocytes has potential for profound clinical relevance. With this in mind, one critically important aspect related to OPC-mediated remyelination is that myelin-debris needs to be cleared by phagocytic cells, including neutrophils (Lindborg et al., 2017), inflammatory macrophages (Healy et al., 2017; Karamita et al., 2017) and resident microglia (Karamita et al., 2017; Kucharova and Stallcup, 2017; Zhu et al., 2016). The ability to efficiently phagocytize myelin is dependent upon age in mice; macrophages from older mice have impaired ability to engulf myelin compared to macrophages derived from younger mice. Elegant studies by Franklin and colleagues (Ruckh et al., 2012) employed heterochronic parabiosis to assess recovery in old mice that had undergone experimentally induced demyelination. When conjoined to younger mice, the old mice showed increased remyelination; this effect was attributed to increased clearance of myelin debris in older animals by macrophages provided from younger animals. A recent study identified a potential mechanism associated with diminished phagocytic activity by aged macrophages. Cantuti-Castelvetri et al. (Cantuti-Castelvetri et al., 2018) demonstrated via transmission electron microscopy that lipids are rapidly released in response to a demyelinating injury, and this can mute OPC differentiation and remyelination. In contrast to older macrophages, young macrophages were able to efficiently engulf and process myelin lipids. Old macrophages were deficient in lipid processing, which led to formation of cholesterol crystals, phagolysosomal rupture and stimulated inflammasomes that ultimately led to an inability to resolve inflammation.

One therapeutic option to treat progressive MS would be to replenish or rejuvenate the pool of endogenous OPCs that show limited remyelination potential in the later stages of disease. Several groups have employed high-throughput screening of small molecule compounds to identify potential drugs that enhance OPC maturation, with the goal of promoting remyelination in pre-clinical animal models of MS (Deshmukh et al., 2013; Mei et al., 2014; Mei et al., 2016b). Using this approach, Lairson and colleagues (Deshmukh et al., 2013) demonstrated that benztropine, an anti-muscarinic receptor compound, increased OPC maturation and remyelination in mice with experimental autoimmune encephalomyelitis (EAE), the prototypic model of MS (Deshmukh et al., 2013). More recently, clemastine, another anti-muscarinic receptor compound, was also shown to enhance OPC maturation in EAE (Mei et al., 2016a). These results are consistent with the observation in EAE mice that ablation of the M1 muscarinic receptor in oligodendroglia resulted in accelerated remyelination, diminished axonal loss and improved clinical outcome, arguing that clemastine may be functioning by binding to this specific receptor (Mei et al., 2016a).

Cellular replacement therapies for human neurologic diseases have also emerged as a clinically relevant area of research. NPCs possess the ability to develop into neurons, astrocytes, and oligodendrocytes (Gage, 2000). Additionally, quiescent adult NPCs have been shown to proliferate, differentiate and migrate into response to acute CNS damage in spinal cord injury, inflammatory demyelination and stroke (Picard-Riera et al., 2002; Yagita et al., 2001; Zhang et al., 2004). In animal models of chronic spinal cord injury, NPCs have been reported to differentiate and promote locomotor recovery (Salazar et al., 2010). Transplantation of NPCs improved cognition in a murine model of Alzheimer's

disease (AD) by increasing brain derived neurotrophic factor (BDNF) (Ager et al., 2015; Blurton-Jones et al., 2009). Engraftment of NPCs into murine and primate models of Huntington's disease (HD) restore motor skills through differentiation into mature striatal neurons (Dunnett et al., 2000; Kendall et al., 1998; Palfi et al., 1998; Reidling et al., 2018).

It has also been reported that peripheral administration of hNPCs in a non-human primate experimental autoimmune encephalomyelitis (EAE) model reduces disease severity through immune regulation (Pluchino et al., 2009). A small clinical study reported that transplantation of human fetal-derived NPCs into the frontal lobes of children with Pelizaeus-Merzbacher disease (PMD), a rare hypo-myelination disorder in children, resulted in measurable gains in motor and/or cognition associated with remyelination (Gupta et al., 2012).

**JHMV infection as a model of neuroinflammation and demyelination.** Intracranial inoculation of C56BL/6 mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV) results in widespread dissemination of virus throughout the brain and spinal cord (Bergmann et al., 2006; Glass et al., 2004; Hosking and Lane, 2009). Oligodendrocytes, astrocytes and microglia are susceptible to infection while neurons are spared (Fleming et al., 1986). Type I interferons have essential roles for protecting the host against JHMV infection, as mice deficient in the IFN- $\alpha/\beta$  receptor show elevated viral load within the CNS and higher mortality, and exogenous treatment of mice with type I interferon limits dissemination of virus (Ireland et al., 2008; Minagawa et al., 1987; Smith et al., 1987). Virus-specific CD4<sup>+</sup> T cells function as support cells for CD8<sup>+</sup> T cells, promoting CD8<sup>+</sup> T cell expansion in the periphery and enhancing survival and cytolytic targeting of infected



cells within the CNS (Phares et al., 2012; Zhou et al., 2005). In addition, CD4<sup>+</sup> T cells can control viral spread through their release of IFN- $\gamma$ , which serves dual roles by inhibiting viral replication within oligodendrocytes and also inducing upregulation of MHC class II expression on microglia (Bergmann et al., 2003; Gonzalez et al., 2006; Parra et al., 1999b; Phares et al., 2012; Ramakrishna et al., 2004). Depletion of CD4<sup>+</sup> T cells alters CD8<sup>+</sup> T cell-mediated control of viral replication within the CNS, mainly a result of reduced IFN- $\gamma$  expression and elevated CD8<sup>+</sup> T cell apoptosis (Phares et al., 2012). Virus-specific CD8<sup>+</sup> T cells are the primary cytolytic effector cell within the CNS during JHMV infection and their peak accumulation coincides with viral clearance from glia (Lin et al., 1997; Parra et al., 1999b; Ramakrishna et al., 2004). A recent study by Perlman and colleagues (Wheeler et al., 2018) employed an inhibitor of colony-stimulating factor 1 receptor (CSF1R) that depletes microglia to demonstrate that microglia were required during the early days after infection to limit JHMV replication within the CNS and protect against clinical disease and death. Moreover, depletion of microglia resulted in impaired T cell responses, leading to elevated viral titers within the CNS. These results reveal nonredundant, critical roles for microglia in the early innate and virus-specific T cell responses and for subsequent host protection from viral encephalitis.

Mice that survive acute JHMV infection progress into the immune-mediated chronic demyelinating phase of the disease, with clinical symptoms manifesting as ataxia and partial-to-complete hind limb paralysis that peaks 2-3 weeks post-infection. Histologic analysis of spinal cords from mice undergoing JHMV-induced demyelination shows that oligodendrocyte dysfunction and loss of myelin integrity within white matter tracts is not due to widespread apoptosis or necrosis of mature oligodendrocytes, but instead is

closely associated with the presence of both inflammatory leukocytes and presentation of viral antigen via MHC-I and MHC-II (Redwine et al., 2001; Stohlman and Hinton, 2001; Wu and Perlman, 1999). Moreover, a paucity of infectious viral particles within the CNS during chronic disease suggests that productive infection of new glial cells does not amplify demyelination. More likely, viral RNA quasispecies present within the CNS of persistently infected mice promote chronic inflammation and demyelination (Adami et al., 1995; Fleming et al., 1995; Rowe et al., 1997). Luxol fast blue staining of spinal cord sections during persistent JHMV-infection reveals lesion formation primarily within the lateral funiculus and posterior funiculus (Wang et al., 1992). Additionally, there have been reports that axonal degeneration within the white matter tracts of spinal cords of JHMV-infected mice, as assessed by SMI-32 or Bielschowsky's *silver* impregnation stain, occurred at the same time as demyelination, while axon damage is argued to precede oligodendrocyte dysregulation in MS (Dandekar et al., 2001; Das Sarma et al., 2009).

Several studies have reported that T cells and macrophages are the main inducers of demyelination during chronic JHMV infection, rather than viral-induced lysis of oligodendrocytes. This idea stems from results showing that JHMV-infection of *RAG1*<sup>-/-</sup> immunodeficient mice (lacking functional T and B lymphocytes) results in limited demyelination while there is extensive viral replication within oligodendrocytes. (Pewe and Perlman, 2002; Wu and Perlman, 1999) Moreover, adoptive transfer of JHMV-sensitized splenocytes from wild type mice into JHMV-infected *RAG1*<sup>-/-</sup> mice results in demyelination. Subsequent studies indicate that both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets are capable of contributing to demyelination following JHMV infection (Pewe and Perlman, 2002) (Lane et al., 2000). Other factors, such as epitope spreading and autoreactive T

cells against host neuroantigens, are not thought to contribute to demyelination in these animals. Together, this evidence suggests that demyelination is multifaceted and numerous factors could contribute to pathology.

**Effects of mouse neural precursor engraftment in JHMV-infected mice.** As a first approach toward understanding the effects of transplanting NPCs, early studies used a syngeneic transplant protocol, in which H-2<sup>b</sup> haplotype-matched mouse striatal NPCs from post-natal day 1 (P1) C56BL/6 mice were transplanted intraspinally into the T8 region of C57BL/6 recipient mice undergoing JHMV-induced demyelination (Totoiu et al., 2004). Initial results demonstrated that transplanted NPCs readily proliferated and migrated up to 12mm both rostral and caudal from the transplant site and preferentially differentiated into oligodendrocyte-lineage cells (Totoiu et al., 2004). Quantification of remyelinated axons resulted in up ~70% of axons remyelinated compared to 10% for non-transplanted controls, suggesting that NPCs can survive within the inflammatory niche and functionally incorporate throughout demyelinated white matter tracts following differentiation into mature oligodendrocytes (Totoiu et al., 2004). Additional studies by Carbajal et al. (Carbajal et al., 2010) demonstrating that transplanted mouse GFP-NPCs were shown to selectively colonize demyelinated white matter regions within the ventral and lateral funiculus regions of the spinal cord. Positional migration of NPCs was mediated, in part, by responding to the CXC chemokine ligand CXCL12 via the receptor CXCR4 expressed by engrafted NPCs (Carbajal et al., 2010). NPC transplantation did not alter the accumulation of T cells or macrophages within the CNS nor proinflammatory chemokine and cytokine gene expression, suggesting that the enhanced remyelination

and recovery following transplantation was not a result of NPC bystander effects attenuating the inflammatory response (Hardison et al., 2006).

As an additional step to better understand the therapeutic potential of engraftment of NPCs in promoting clinical and histologic recovery, we have transplanted MHC-mismatched mouse NPCs into JHMV-infected mice with established demyelination to determine whether allogeneic NPCs are recognized as foreign and rejected via immunological mechanisms. Transplantation of allogeneic NPCs is clinically relevant, because transplantation of human neural stem cells into PMD patients required administration of immunosuppressive drugs to limit potential rejection (Gupta et al., 2012). Similarly, transplantation of hESC-OPCs into individuals with spinal cord injuries also was performed in conjunction with administration of immunosuppressive drugs. Studies by Palmer and colleagues (Chen et al., 2011; Phillips et al., 2013) have shown an important role for components of the innate immune response including NK cells in recognizing and rejecting MHC-mismatched NPCs following transplantation into the brains of mice. Similarly, we have demonstrated that engraftment of allogeneic NPCs into spinal cords of JHMV-infected mice results in rejection mediated, in part, by both T lymphocytes as well as NK cells (Weinger et al., 2014; Weinger et al., 2012). NPCs respond to both IFN- $\gamma$  as well as viral infection; they react by expressing MHC class I and II that allows for T lymphocyte recognition, and retinoic acid early precursor transcript (RAE)-1 that enables NK cell recognition (Plaisted et al., 2014; Weinger et al., 2014; Weinger et al., 2012). Collectively, these findings highlight that NPCs are recognized by cellular components of both the innate and adaptive immune system, indicating that administration of

immunosuppressive drugs must be considered in order to promote long-term survival and function.

We have recently employed two-photon microscopy to assess intercellular interactions of transplanted mouse NPCs *ex vivo* (Greenberg et al., 2014a). JHMV-infected Thy1-YFP mice, which express yellow fluorescent protein (YFP) from medium-to-large caliber axons within the spinal cord, received subventricular zone-derived NPCs that express GFP following their differentiation into oligodendrocytes (PLP-GFP). Several important observations were derived from this study, including the finding that JHMV-infected Thy1-YFP mice displayed extensive axonal damage earlier than expected during JHMV-induced disease, suggesting that appearance of axonopathy precedes robust immune-mediated demyelination. This argues that axonal damage may be important in contributing to white matter damage and myelin loss. It is not yet clear whether viral infection of neurons and/or transport of viral proteins along axons is important in this process (Das Sarma et al., 2009). In addition, 2-photon imaging showed that engrafted NPCs interacted with damaged axons and this resulted in improved axonal integrity and remyelination as determined by YFP expression (**Figure 1.1 A-D**) (Greenberg et al., 2014a; Kerschensteiner et al., 2005).

We have also examined the effect of S1P receptor antagonism on the biology of mouse NPCs following transplantation into JHMV-infected mice. Earlier studies from our laboratory showed that treatment of JHMV-infected mice with FTY720 (fingolimod), the first oral drug approved by the FDA for treatment of patients with the relapsing-remitting form of MS, mutes effective anti-viral immune responses by affecting migration and accumulation of virus-specific T cells within the CNS during acute viral-induced

encephalomyelitis (Blanc et al., 2014). FTY720 treatment reduced the severity of neuroinflammation-mediated demyelination by restricting the access of disease-causing lymphocytes into the CNS, but this did not result in viral recrudescence. As a result of this work, we were interested if the therapeutic benefit of mouse NPC transplantation into JHMV-infected mice would be augmented if FTY720 was also administered, since previously published studies showed a beneficial effect of FTY720 in combination with benztropine in reducing clinical disease and increasing remyelination in the mouse EAE model of MS (Deshmukh et al., 2013). We found that cultured NPCs expressed transcripts for S1P receptors S1P1, S1P2, S1P3, S1P4, and S1P5. Administration of FTY720 to JHMV-infected mice resulted in enhanced migration and increased proliferation of transplanted NPCs following spinal cord engraftment. FTY720 treatment did not improve clinical disease, diminish neuroinflammation or the severity of demyelination and did not increase remyelination (Blanc et al., 2015).

Glial-committed neural precursor cells have been previously suggested as a potential treatment for autoimmune demyelinating diseases such as MS, as they are sources for generation of mature remyelinating oligodendrocytes (Ben-Hur et al., 1998; Brustle et al., 1999). Glial progenitors derived from NPCs can remyelinate axons following transplantation into regions of experimentally induced demyelination (Keirstead et al., 1999). Transplantation of these cells into rodent autoimmune models of demyelination resulted in improved clinical outcomes as a result of migration of cells into the inflamed white matter tracts (Ben-Hur et al., 2003). Glial precursor cells have been suggested to act either as modulators of the immune system or by replacement of the damaged or lost endogenous neural precursors in animal models of MS (Aharonowiz et al., 2008; Pluchino

et al., 2009; Pluchino et al., 2003). Most of these studies used models of demyelination caused by injury or infiltration of myelin-reactive T cells to demonstrate the effect of implanting myelin-competent NPCs in promoting remyelination. But viral infections have also been considered as potential triggers of MS in genetically susceptible individuals (Giovannoni et al., 2006), and a clinically relevant question is whether glial-committed stem cells can ameliorate demyelination caused by persistent neurotropic viruses. To address this question, we have shown that engraftment of glial-committed progenitors in JHMV infected mice with established neurological disease resulted in remyelination and axonal sparing (Totoiu et al., 2004). This result raises another relevant question, whether glial cells derived from NPCs are susceptible to viral infection. There are several known neurotropic viruses that have been shown to infect and replicate in NPCs and cells derived from NPCs. For example, a neonatal neurotropic virus called Coxsackievirus B3 (CVB3) persists in the CNS and preferentially infects proliferating neural stem cells and infiltrating myeloid cells (Tabor-Godwin et al., 2010). CVB3 persists within the murine neurogenic region and infects neural stem cells, causing cell death, decrease in brain size, and eventually developmental defects (Ruller et al., 2012). This suggests that persistent viral infections in the CNS can have long-term neurological sequelae (Ruller et al., 2012). Borna disease virus, a human pathogen associated with behavioral disorders, is capable of severely impairing neurogenesis by infecting human neural progenitors (Brnic et al., 2012). Another human neurotropic virus, herpes simplex virus type 1 (HSV-1) that causes herpes simplex encephalitis, was shown to infect and deplete mouse NPCs in the subventricular zone, causing a loss of neuroblasts (Chucair-Elliott et al., 2014). Furthermore, NPCs are depleted by viral-induced lysis due to their susceptibility to

infection by Enterovirus 71 (Huang et al., 2014). In addition, human ESC-derived oligodendrocyte progenitors are highly susceptible to infection by JC virus, the causative pathogen of progressive multifocal leukoencephalopathy (Schaumburg et al., 2008). We have shown that glial cells derived from murine NPCs are susceptible to JHMV infection and these cells can actively replicate JHMV, as evidenced by increasing viral titers and extensive distribution of viral antigen throughout the infected monolayer (**Figures 1.2A and B**) (Whitman et al., 2009). IFN- $\gamma$  plays an important role in controlling JHMV infection of persistently infected mice (Parra et al., 1999a). Treatment of JHMV-infected cells with IFN- $\gamma$  led to inhibition of viral replication in a dose-dependent manner (Whitman et al., 2009). IFN- $\gamma$  treatment also limited the cytopathic effects of JHMV infection, demonstrating the importance of this cytokine in host defense following JHMV infection (Whitman et al., 2009). JHMV is capable of infecting and replicating in primary OPC cultures, indicating that these cells are susceptible to infection *in vivo*. Remyelination is relatively slow in JHMV-infected mice, yet OPCs can be found in the vicinity of on-going demyelination. Overall, these findings suggest that susceptibility of NPCs and their derivatives to viral infection should be considered in plans to use these cells for cell replacement therapy for neurological disorders. Immunosuppression used to prevent rejection of allogeneic cells may cause reemergence of persistent neurotropic viruses. These reactivated viruses could infect and diminish the transplanted cells, impeding therapeutic benefits. Problems associated with immunosuppression could be mitigated by using patient-specific induced pluripotent stem cells (iPSCs) to produce immune-matched cells for transplantation. Interestingly, we recently learned that mouse iPSC-derived NPCs expressed low levels of the JHMV receptor- CEACAM1a, which made them



resistant to infection and viral induced cell death *in vitro* (Mangale et al., 2017). This suggests that iPSC-derived cells may be a good option for cell replacement therapy, because they would avoid both rejection and viral-mediated cell death. An overview of our results with transplantation of mouse NPCs into JHMV-infected mice is provided in **Table 1.1**.

**Effects of transplantation of human pluripotent stem cell-derived cells in virally-induced models of neuroinflammation and demyelination.** The long-term goal of studying MS model mice is to guide the development of effective treatments for the human disease. In our early work, we saw very limited clinical recovery after transplantation of pre-differentiated human OPCs in mice undergoing JHMV-induced demyelination (Hatch et al., 2009). Engrafted cells were rejected within 2 weeks after transplantation, even in the presence of immunosuppressive drugs targeting activated T lymphocytes. There was only a slight increase in remyelination near the transplant site compared to mice receiving a saline control (Hatch et al., 2009). This in contrast to earlier studies using human embryonic stem cell (hESC)-derived early stage OPCs in a model of spinal cord injury in rat, in which enhanced remyelination and improved motor function were observed following transplantation (Keirstead et al., 2005). Less mature human neural lineage cells have previously been shown to exert neuroprotective effects in mouse and non-human primate models of EAE, suggesting that they possess broader functionality *in vivo* (Aharonowiz et al., 2008; Pluchino et al., 2009)

When we transplanted NPCs derived from human induced pluripotent stem cells (iPSCs) into the spinal cords of JHMV-infected mice, the cells were rejected, but

there was focal remyelination at the site of transplantation (**Figures 1.3A and B**) (Plaisted et al., 2016). There was also reduced recruitment of CD4<sup>+</sup> T cells into the CNS, and a transient increase in CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs was observed (**Figures 1.3C and D**). Importantly, ablation of Tregs via PC61.5 treatment abrogated histopathological recovery. These findings support an immunomodulatory role for Tregs, where they may suppress neuroinflammation or promote tissue repair mechanisms. The cells used for this study were generated by an embryoid-body-based technique; they were characterized by gene expression analysis and found to be positive for the transcription factor *PAX6*, a classical marker of CNS neural precursor cells. However, the results differed when we transplanted a population of *PAX6*-negative hPSC-derived cells that we referred to as “neural precursor-like cells” (NPLCs) into JHMV-infected mice. The NPLC transplantation resulted in clinical and histological improvement out to 6 months post-transplant, despite the rejection of transplanted cells within 8 days (**Figures 1.4A and B**) (Chen et al., 2014a). Strikingly, while the transplanted cells did not migrate from the site of implantation, the remyelinated axons were distributed both rostrally and caudally, rather than localized to the region of cell delivery (**Figures 1.4C and D**). The remyelination was not likely to be the result of acute inflammatory-mediated rejection, as the spinal cords had reduced infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells compared to controls, and the total number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) within the spinal cords was elevated (**Figure 1.4D**) (Chen et al., 2014a). Depletion of Tregs in NPLC-transplanted mice via anti-CD25 (PC61.5) treatment abolished the therapeutic benefits, highlighting the likely importance of Tregs in this more extensive recovery (**Figure 1.4E**). The *PAX6*-negative NPLCs were not classic neural precursor cells; they were produced by a method

that enhanced the differentiation of peripheral neural lineage cells rather than CNS neural lineage derivatives. The differences were confirmed by gene expression studies, which showed that the NPLCs had an expression profile that considerably differed from the CNS-NPCs as well as ineffective fibroblasts and undifferentiated hESCs and iPSCs (Plaisted et al., 2016). The gene expression signature gave clues to the characteristics that may underlie the disease-modifying activity of NPLCs; for example, these cells produced higher levels of TGF- $\beta$ 2 than NPCs, fibroblasts, and undifferentiated hESC cells that did not elicit clinical recovery (Chen et al., 2014a). Previous work has shown that this anti-inflammatory cytokine promotes FoxP3 expression in the peripheral Treg compartment, influencing the frequency and suppressive activity of Tregs (Marie et al., 2005). Tregs have been shown to have an important role during both acute and chronic JHMV-infection (Anghelina et al., 2009). IL-10-expressing virus-specific Tregs dampen proliferation of virus-specific effector CD4<sup>+</sup> T cells, and depletion of Tregs increases mortality, suggesting that during acute JHMV infection, Tregs limit immunopathological disease without negatively impacting viral clearance. In addition, studies from Trandem et al. (Trandem et al., 2010) have shown that adoptive transfer of Tregs into JHMV-infected mice attenuates clinical disease severity by dampening neuroinflammation and subsequent demyelination. An overview of our results with transplantation of human progenitor cells into JHMV-infected mice is provided in **Table 1.1**.

### **Concluding Remarks**

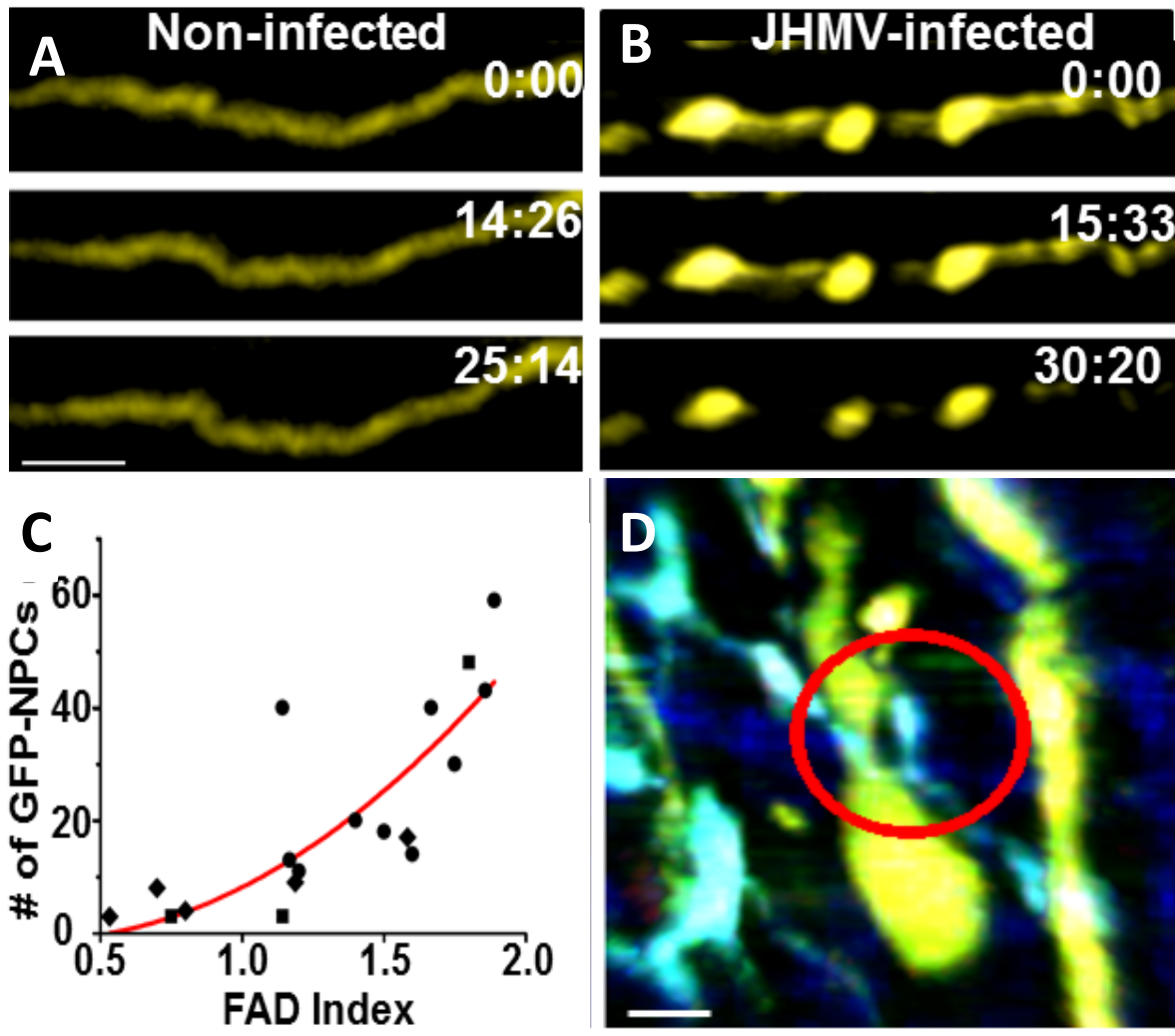
Research using a mouse model of virally-induced demyelination has provided support for the potential of cell transplantation therapy for human disease. Experiments indicate that

transplantation of certain types of cells can promote sustained recovery both through promoting remyelination and limiting ongoing demyelination by muting neuroinflammation. These reports also highlight the importance of comparing differing cell types transplanted to the same model of human disease. In designing cell therapies for human disease, it is important to standardize criteria for defining cell types to be used for transplantation. Our analysis of gene expression profiles of a variety of human precursors and stem cells revealed that they are very diverse; for example, while pluripotent stem cells were very similar to each other, cells that had been designated as neural stem cells were clustered into multiple subgroups (Muller et al., 2008). Similarly, mesenchymal stem cells are very divergent in their behavior and capabilities depending on fundamental factors, including organ or tissue of origin, age of donor, preparation methods, degree and means of expansion, and assays used to assess their differentiation capabilities (Robey, 2017).

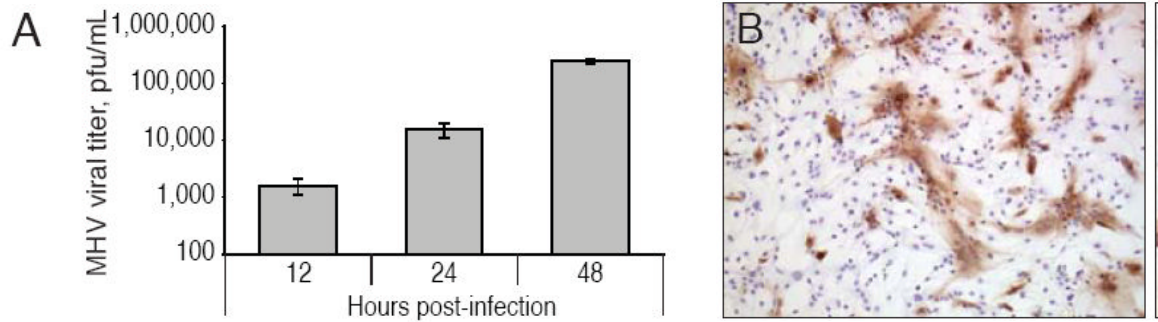
The mechanisms by which different transplanted cells elicit clinical improvements appear to be different, but the experimental evidence converges on common themes. The transplanted cells all appear to mute the effects of inflammatory immune cells and involve signaling by Tregs, which are anti-inflammatory. Some of the cell types either function as OPCs or to stimulate remyelination by endogenous OPCs. In order for cell therapies to advance to clinical relevance, the properties of each cell type should be examined by multiple methods to determine what characteristics are responsible for clinical recovery in mouse models of demyelinating disease. This approach could lead to identification of the best cell type for transplantation therapy, or

perhaps more promising, identification of the key ameliorative factors that can be translated into therapy without the need for cells.

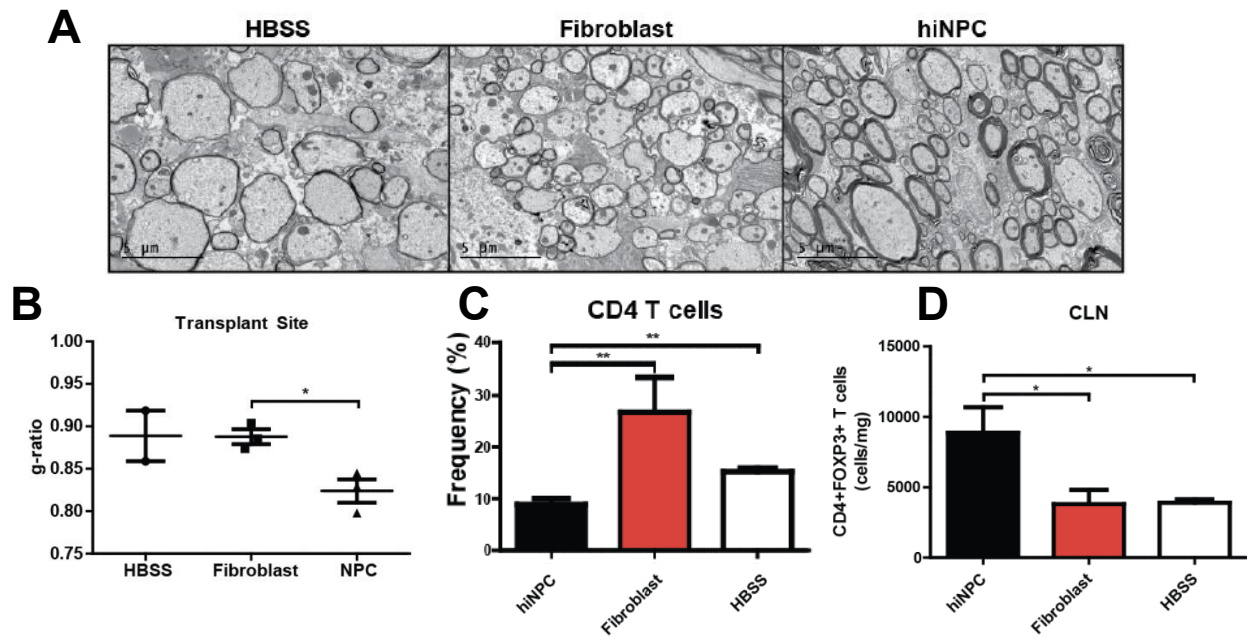
**Figure 1.1. Axonal damage in JHMV-infected mice is reversed following NPC engraftment. (A)** Time-lapse images (times marked in mins) depicting absence of focal axonal degeneration (FAD) in a non-infected Thy1-YFP spinal cord. **(B)** Time-lapse images showing progression of FAD in a Thy1-YFP spinal cord 7 days following JHMV infection; scale bar = 20  $\mu$ m. **(C)** GFP-NPC localization correlates with the FAD severity of lesions in the JHMV infected Thy1-YFP spinal cord 8 days post-transfer. Number of transferred GFP-NPCs found in lesions is plotted vs. FAD severity of the lesions for each  $10^{-5}$   $\text{cm}^3$  imaging volume. **(D)** Time-lapse images showing GFP-NPCs initiating intercellular interactions with “Stage 1 FAD” axons in the JHMV infected Thy1-YFP spinal cord 8 days post-transfer. Circle indicates a GFP-NPC actively extending a process toward the axon; scale bars = 10  $\mu$ m. Figures derived from Greenberg et al., 2014.



**Figure 1.2. JHMV replicates in glial cells derived from mouse NPCs.** (A) Differentiated progenitor cultures were infected with JHMV (multiplicity of infection = 0.1) and viral titers in supernatants determined at 12, 24, and 48 h post- infection (p.i.) by plaque assay. (B) Immunocytochemical staining for viral antigen at 24 h p.i. revealed wide-spread distribution of virus throughout the cell culture (100× magnification). Figures derived from Whitman et al., 2008.

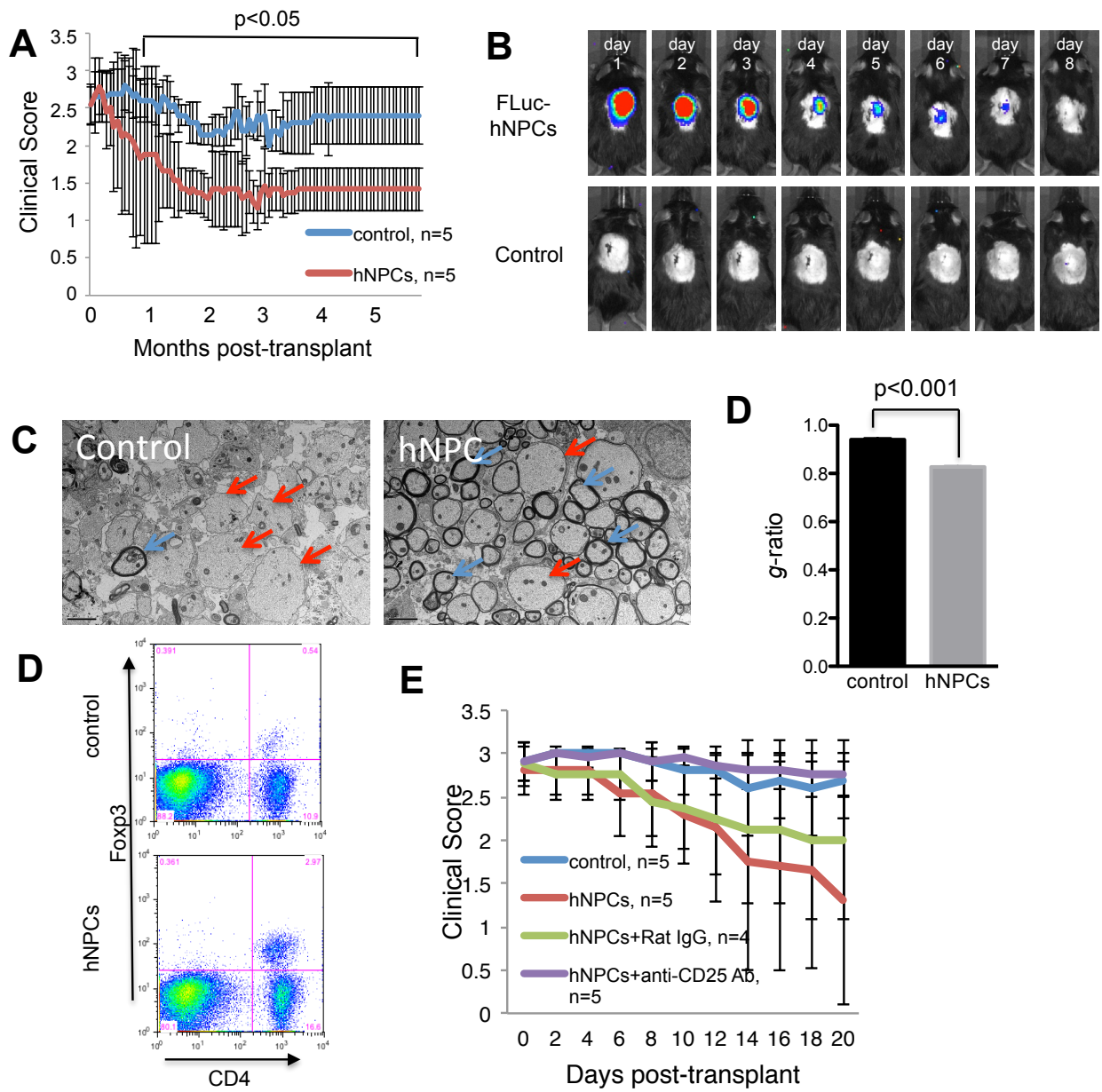


**Figure 1.3. Intraspinal transplantation of iPSC-derived NPCs into JHMV-infected mice.** (A) Focal remyelination in animals transplanted with hiNPCs. Representative electron micrographs of coronal spinal cord sections from HBSS, fibroblast, and hiNPC injected mice. (B) Analysis of the ratio of the axon diameter vs. total fiber diameter (g-ratio) confirmed enhanced remyelination. (C) Quantification of the percent of CD4+ T cells demonstrated a significant ( $p < 0.05$ ) decrease in the cLNs of hiNPC transplanted mice compared to controls at 5 days post-transplant (p.t.) (D) Quantification of the number of CD4+FoxP3+ Tregs demonstrated a significant ( $p < 0.05$ ) increase in the CLNs of hiNPC transplanted mice compared to controls at 5 days p.t. Figures derived from Plaisted et al., 2016.





**Figure 1.4. Intraspinal transplantation of hNPCs into JHMV-infected mice. (A)** Improved ( $p < 0.05$ ) clinical recovery in hNPC-transplanted JHMV-infected mice was sustained out to 168 days post-transplantation (p.t.) when compared to infected mice treated with vehicle alone. **(B)** Daily IVIS<sup>®</sup> imaging of luciferase-labeled hNPCs revealed that following intraspinal transplantation, cells are reduced to below the level of detection by day 8 post-transplantation; representative mice are shown. IVIS<sup>®</sup> imaging was performed on vehicle-transplanted mice as a control. **(C)** Quantification of Treg numbers in spinal cords of mice indicated a significant ( $p < 0.05$ ) increase in numbers of Tregs in hNPC-transplanted mice versus controls between 8-10 days post-transplantation. Data are representative of three independent experiments with a minimum of 3 mice per group; data are presented as average  $\pm$ SEM. Mann-Whitney t-tests were used to determine the p values. **(D)** hNPC-transplanted mice receiving anti-CD25 antibody (purple line) did not display recovery in motor skills as compared to either hNPC-treated mice (red line), hNPC-treated mice receiving isotype-matched control antibody (green line), or vehicle control mice (blue line). Figures derived from Chen et al., 2014.



**Table 1.1: Overview of mouse and human stem cell engraftment into JHMV-infected mice**

| Cell Type    | Antigenicity | Cell Survival & Migration | Clinical Improvement | Spinal Cord Demyelination | Spinal Cord Remyelination   | Immuno-modulation | Reference                                                                                    |
|--------------|--------------|---------------------------|----------------------|---------------------------|-----------------------------|-------------------|----------------------------------------------------------------------------------------------|
| <b>Mouse</b> | NPCs         | Yes                       | Yes                  | Yes                       | Yes                         | No                | Totoiu et al., 2004<br>Carbajal et al., 2010<br>Greenberg et al., 2014<br>Blanc et al., 2015 |
|              | NPCs         | No                        | No                   | Yes                       | Not Determined              | No                | Weinger et al., 2012<br>Weinger et al., 2014                                                 |
| <b>Human</b> | ESC-OPCs     | No                        | No                   | Yes                       | Focal at site of transplant | Not Determined    | Hatch et al., 2009                                                                           |
|              | ESC-NCLCs    | No                        | Yes                  | Reduced                   | Yes                         | Yes               | Chen et al., 2014                                                                            |
|              | iPSC-NPCs    | No                        | No                   | Reduced                   | Focal at site of transplant | Yes               | Plaisted et al., 2016                                                                        |

NPCs: neural progenitor cells

ESC-OPCs: Embryonic stem cell-derived oligodendrocyte progenitor cells

ESC-NCLCs: Embryonic stem cell-derived neural crest like cells

iPSC-NPCs: inducible pluripotent cell-derived neural progenitor cells

## **Acknowledgements**

This work was supported by National Institutes of Health (NIH) grants R01 NS041249 and R01 NS074987 (TEL) as well as National Multiple Sclerosis Society Collaborative Research Center grant RG 4925 (TEL) and funding from the Ray & Tye Noorda Foundation (TEL); NIH grant R01 AI121945 (CMW); California Institute for Regenerative Medicine (CIRM) grants RM1-01717 and CL1-00502 (JFL) and TR3-05603 (CMW and JFL); and training grant NIH 5T32NS082174 (LLM).

## References

- Adami, C., Pooley, J., Glomb, J., Stecker, E., Fazal, F., Fleming, J.O., and Baker, S.C. (1995). Evolution of mouse hepatitis virus (MHV) during chronic infection: quasispecies nature of the persisting MHV RNA. *Virology* *209*, 337-346.
- Ager, R.R., Davis, J.L., Agazaryan, A., Benavente, F., Poon, W.W., LaFerla, F.M., and Blurton-Jones, M. (2015). Human neural stem cells improve cognition and promote synaptic growth in two complementary transgenic models of Alzheimer's disease and neuronal loss. *Hippocampus* *25*, 813-826.
- Aharonowiz, M., Einstein, O., Fainstein, N., Lassmann, H., Reubinoff, B., and Ben-Hur, T. (2008). Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis. *PLoS One* *3*, e3145.
- Anghelina, D., Zhao, J., Trandem, K., and Perlman, S. (2009). Role of regulatory T cells in coronavirus-induced acute encephalitis. *Virology* *385*, 358-367.
- Ben-Hur, T., Einstein, O., Mizrachi-Kol, R., Ben-Menachem, O., Reinhartz, E., Karussis, D., and Abramsky, O. (2003). Transplanted multipotential neural precursor cells migrate into the inflamed white matter in response to experimental autoimmune encephalomyelitis. *Glia* *41*, 73-80.
- Ben-Hur, T., Rogister, B., Murray, K., Rougon, G., and Dubois-Dalcq, M. (1998). Growth and fate of PSA-NCAM+ precursors of the postnatal brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* *18*, 5777-5788.
- Bergmann, C.C., Lane, T.E., and Stohlman, S.A. (2006). Coronavirus infection of the central nervous system: host-virus stand-off. *Nature reviews Microbiology* *4*, 121-132.
- Bergmann, C.C., Parra, B., Hinton, D.R., Chandran, R., Morrison, M., and Stohlman, S.A. (2003). Perforin-mediated effector function within the central nervous system requires IFN-gamma-mediated MHC up-regulation. *J Immunol* *170*, 3204-3213.
- Blanc, C.A., Grist, J.J., Rosen, H., Sears-Kraxberger, I., Steward, O., and Lane, T.E. (2015). Sphingosine-1-phosphate receptor antagonism enhances proliferation and migration of engrafted neural progenitor cells in a model of viral-induced demyelination. *Am J Pathol* *185*, 2819-2832.
- Blanc, C.A., Rosen, H., and Lane, T.E. (2014). FTY720 (fingolimod) modulates the severity of viral-induced encephalomyelitis and demyelination. *J Neuroinflammation* *11*, 138.
- Blurton-Jones, M., Kitazawa, M., Martinez-Coria, H., Castello, N.A., Muller, F.J., Loring, J.F., Yamasaki, T.R., Poon, W.W., Green, K.N., and LaFerla, F.M. (2009). Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci U S A* *106*, 13594-13599.

- Brnic, D., Stevanovic, V., Cochet, M., Agier, C., Richardson, J., Montero-Menei, C.N., Milhavet, O., Eloit, M., and Couplier, M. (2012). Borna disease virus infects human neural progenitor cells and impairs neurogenesis. *J Virol* 86, 2512-2522.
- Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285, 754-756.
- Cantuti-Castelvetri, L., Fitzner, D., Bosch-Queralt, M., Weil, M.T., Su, M., Sen, P., Ruhwedel, T., Mitkovski, M., Trendelenburg, G., Lutjohann, D., *et al.* (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. *Science* 359, 684-688.
- Carbajal, K.S., Schaumburg, C., Strieter, R., Kane, J., and Lane, T.E. (2010). Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 107, 11068-11073.
- Chang, A., Nishiyama, A., Peterson, J., Prineas, J., and Trapp, B.D. (2000). NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20, 6404-6412.
- Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, Craig M., Sears-Kraxberger, I., Steward, O., Macklin, Wendy B., Loring, Jeanne F., *et al.* (2014). Human Neural Precursor Cells Promote Neurologic Recovery in a Viral Model of Multiple Sclerosis. *Stem Cell Reports*.
- Chen, Z., Phillips, L.K., Gould, E., Campisi, J., Lee, S.W., Ormerod, B.K., Zwierzchoniewska, M., Martinez, O.M., and Palmer, T.D. (2011). MHC mismatch inhibits neurogenesis and neuron maturation in stem cell allografts. *PLoS One* 6, e14787.
- Chucair-Elliott, A.J., Conrady, C., Zheng, M., Kroll, C.M., Lane, T.E., and Carr, D.J. (2014). Microglia-induced IL-6 protects against neuronal loss following HSV-1 infection of neural progenitor cells. *Glia* 62, 1418-1434.
- Dandekar, A.A., Wu, G.F., Pewe, L., and Perlman, S. (2001). Axonal damage is T cell mediated and occurs concomitantly with demyelination in mice infected with a neurotropic coronavirus. *J Virol* 75, 6115-6120.
- Das Sarma, J., Kenyon, L.C., Hingley, S.T., and Shindler, K.S. (2009). Mechanisms of primary axonal damage in a viral model of multiple sclerosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 10272-10280.
- Deshmukh, V.A., Tardif, V., Lyssiotis, C.A., Green, C.C., Kerman, B., Kim, H.J., Padmanabhan, K., Swoboda, J.G., Ahmad, I., Kondo, T., *et al.* (2013). A regenerative approach to the treatment of multiple sclerosis. *Nature* 502, 327-332.

- Dunnett, S.B., Nathwani, F., and Bjorklund, A. (2000). The integration and function of striatal grafts. *Prog Brain Res* 127, 345-380.
- Fleming, J.O., Adami, C., Pooley, J., Glomb, J., Stecker, E., Fazal, F., and Baker, S.C. (1995). Mutations associated with viral sequences isolated from mice persistently infected with MHV-JHM. *Advances in experimental medicine and biology* 380, 591-595.
- Fleming, J.O., Trousdale, M.D., Elzaatari, F.A.K., Stohlman, S.A., and Weiner, L.P. (1986). PATHOGENICITY OF ANTIGENIC VARIANTS OF MURINE CORONAVIRUS JHM SELECTED WITH MONOCLONAL-ANTIBODIES. *J Virol* 58, 869-875.
- Frampton, J.E. (2017). Ocrelizumab: First Global Approval. *Drugs* 77, 1035-1041.
- Gage, F.H. (2000). Mammalian neural stem cells. *Science* 287, 1433-1438.
- Giovannoni, G., Cutter, G.R., Lunemann, J., Martin, R., Munz, C., Sriram, S., Steiner, I., Hammerschlag, M.R., and Gaydos, C.A. (2006). Infectious causes of multiple sclerosis. *Lancet Neurol* 5, 887-894.
- Glass, W.G., Hickey, M.J., Hardison, J.L., Liu, M.T., Manning, J.E., and Lane, T.E. (2004). Antibody targeting of the CC chemokine ligand 5 results in diminished leukocyte infiltration into the central nervous system and reduced neurologic disease in a viral model of multiple sclerosis. *J Immunol* 172, 4018-4025.
- Gonzalez, J.M., Bergmann, C.C., Ramakrishna, C., Hinton, D.R., Atkinson, R., Hoskin, J., Macklin, W.B., and Stohlman, S.A. (2006). Inhibition of interferon-gamma signaling in oligodendroglia delays coronavirus clearance without altering demyelination. *Am J Pathol* 168, 796-804.
- Greenberg, M.L., Weinger, J.G., Matheu, M.P., Carbajal, K.S., Parker, I., Macklin, W.B., Lane, T.E., and Cahalan, M.D. (2014). Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A*.
- Gupta, N., Henry, R.G., Strober, J., Kang, S.M., Lim, D.A., Bucci, M., Caverzasi, E., Gaetano, L., Mandelli, M.L., Ryan, T., *et al.* (2012). Neural stem cell engraftment and myelination in the human brain. *Science translational medicine* 4, 155ra137.
- Halfpenny, C., Benn, T., and Scolding, N. (2002). Cell transplantation, myelin repair, and multiple sclerosis. *Lancet Neurol* 1, 31-40.
- Hardison, J.L., Nistor, G., Gonzalez, R., Keirstead, H.S., and Lane, T.E. (2006). Transplantation of glial-committed progenitor cells into a viral model of multiple sclerosis induces remyelination in the absence of an attenuated inflammatory response. *Exp Neurol* 197, 420-429.

- Hatch, M.N., Schaumburg, C.S., Lane, T.E., and Keirstead, H.S. (2009). Endogenous remyelination is induced by transplant rejection in a viral model of multiple sclerosis. *J Neuroimmunol* 212, 74-81.
- Healy, L.M., Jang, J.H., Won, S.Y., Lin, Y.H., Touil, H., Aljarallah, S., Bar-Or, A., and Antel, J.P. (2017). MerTK-mediated regulation of myelin phagocytosis by macrophages generated from patients with MS. *Neurol Neuroimmunol Neuroinflamm* 4, e402.
- Hosking, M.P., and Lane, T.E. (2009). The Biology of Persistent Infection: Inflammation and Demyelination following Murine Coronavirus Infection of the Central Nervous System. *Curr Immunol Rev* 5, 267-276.
- Huang, H.I., Lin, J.Y., Chen, H.H., Yeh, S.B., Kuo, R.L., Weng, K.F., and Shih, S.R. (2014). Enterovirus 71 infects brain-derived neural progenitor cells. *Virology* 468-470, 592-600.
- Ireland, D.D., Stohlman, S.A., Hinton, D.R., Atkinson, R., and Bergmann, C.C. (2008). Type I interferons are essential in controlling neurotropic coronavirus infection irrespective of functional CD8 T cells. *J Virol* 82, 300-310.
- Karamita, M., Barnum, C., Mobius, W., Tansey, M.G., Szymkowski, D.E., Lassmann, H., and Probert, L. (2017). Therapeutic inhibition of soluble brain TNF promotes remyelination by increasing myelin phagocytosis by microglia. *JCI Insight* 2.
- Keirstead, H.S., Ben-Hur, T., Rogister, B., O'Leary, M.T., Dubois-Dalcq, M., and Blakemore, W.F. (1999). Polysialylated neural cell adhesion molecule-positive CNS precursors generate both oligodendrocytes and Schwann cells to remyelinate the CNS after transplantation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19, 7529-7536.
- Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., and Steward, O. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25, 4694-4705.
- Kendall, A.L., Rayment, F.D., Torres, E.M., Baker, H.F., Ridley, R.M., and Dunnett, S.B. (1998). Functional integration of striatal allografts in a primate model of Huntington's disease. *Nat Med* 4, 727-729.
- Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., and Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med* 11, 572-577.
- Kucharova, K., and Stallcup, W.B. (2017). Distinct NG2 proteoglycan-dependent roles of resident microglia and bone marrow-derived macrophages during myelin damage and repair. *PLoS One* 12, e0187530.



Lane, T.E., Liu, M.T., Chen, B.P., Asensio, V.C., Samawi, R.M., Paoletti, A.D., Campbell, I.L., Kunkel, S.L., Fox, H.S., and Buchmeier, M.J. (2000). A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J Virol* 74, 1415-1424.

Lassmann, H. (1983). Comparative neuropathology of chronic experimental allergic encephalomyelitis and multiple sclerosis. *Schriftenreihe Neurologie* 25, 1-135.

Lassmann, H., Bruck, W., and Lucchinetti, C.F. (2007). The immunopathology of multiple sclerosis: an overview. *Brain pathology* 17, 210-218.

Lin, M.T., Stohlman, S.A., and Hinton, D.R. (1997). Mouse hepatitis virus is cleared from the central nervous systems of mice lacking perforin-mediated cytolysis. *J Virol* 71, 383-391.

Lindborg, J.A., Mack, M., and Zigmond, R.E. (2017). Neutrophils Are Critical for Myelin Removal in a Peripheral Nerve Injury Model of Wallerian Degeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 37, 10258-10277.

Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (1999). A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. *Brain : a journal of neurology* 122 ( Pt 12), 2279-2295.

Mangale, V., Marro, B.S., Plaisted, W.C., Walsh, C.M., and Lane, T.E. (2017). Neural precursor cells derived from induced pluripotent stem cells exhibit reduced susceptibility to infection with a neurotropic coronavirus. *Virology* 511, 49-55.

Marie, J.C., Letterio, J.J., Gavin, M., and Rudensky, A.Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *The Journal of experimental medicine* 201, 1061-1067.

Mei, F., Fancy, S.P.J., Shen, Y.A., Niu, J., Zhao, C., Presley, B., Miao, E., Lee, S., Mayoral, S.R., Redmond, S.A., *et al.* (2014). Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. *Nat Med* 20, 954-960.

Mei, F., Lehmann-Horn, K., Shen, Y.A., Rankin, K.A., Stebbins, K.J., Lorrain, D.S., Pekarek, K., S, A.S., Xiao, L., Teuscher, C., *et al.* (2016a). Accelerated remyelination during inflammatory demyelination prevents axonal loss and improves functional recovery. *Elife* 5.

Mei, F., Mayoral, S.R., Nobuta, H., Wang, F., Desponts, C., Lorrain, D.S., Xiao, L., Green, A.J., Rowitch, D., Whistler, J., *et al.* (2016b). Identification of the Kappa-Opioid Receptor as a Therapeutic Target for Oligodendrocyte Remyelination. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36, 7925-7935.

Minagawa, H., Takenaka, A., Mohri, S., and Mori, R. (1987). Protective effect of recombinant murine interferon beta against mouse hepatitis virus infection. *Antiviral research* 8, 85-95.

Muller, F.J., Laurent, L.C., Kostka, D., Ulitsky, I., Williams, R., Lu, C., Park, I.H., Rao, M.S., Shamir, R., Schwartz, P.H., *et al.* (2008). Regulatory networks define phenotypic classes of human stem cell lines. *Nature* 455, 401-405.

Neumann, H., Medana, I.M., Bauer, J., and Lassmann, H. (2002). Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci* 25, 313-319.

Oksenberg, J.R., Begovich, A.B., Erlich, H.A., and Steinman, L. (1993). Genetic factors in multiple sclerosis. *Jama* 270, 2362-2369.

Palfi, S., Conde, F., Riche, D., Brouillet, E., Dautry, C., Mittoux, V., Chibois, A., Peschanski, M., and Hantraye, P. (1998). Fetal striatal allografts reverse cognitive deficits in a primate model of Huntington disease. *Nat Med* 4, 963-966.

Parra, B., Hinton, D.R., Marten, N.W., Bergmann, C.C., Lin, M.T., Yang, C.S., and Stohlman, S.A. (1999a). IFN-gamma is required for viral clearance from central nervous system oligodendroglia. *J Immunol* 162, 1641-1647.

Parra, B., Hinton, D.R., Marten, N.W., Bergmann, C.C., Lin, M.T., Yang, C.S., and Stohlman, S.A. (1999b). IFN-gamma is required for viral clearance from central nervous system oligodendroglial. *J Immunol* 162, 1641-1647.

Pewe, L., and Perlman, S. (2002). Cutting edge: CD8 T cell-mediated demyelination is IFN-gamma dependent in mice infected with a neurotropic coronavirus. *J Immunol* 168, 1547-1551.

Phares, T.W., Stohlman, S.A., Hwang, M., Min, B., Hinton, D.R., and Bergmann, C.C. (2012). CD4 T cells promote CD8 T cell immunity at the priming and effector site during viral encephalitis. *J Virol* 86, 2416-2427.

Phillips, L.K., Gould, E.A., Babu, H., Krams, S.M., Palmer, T.D., and Martinez, O.M. (2013). Natural killer cell-activating receptor NKG2D mediates innate immune targeting of allogeneic neural progenitor cell grafts. *Stem cells* 31, 1829-1839.

Picard-Riera, N., Decker, L., Delarasse, C., Goude, K., Nait-Oumesmar, B., Liblau, R., Pham-Dinh, D., and Baron-Van Evercooren, A. (2002). Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. *Proc Natl Acad Sci U S A* 99, 13211-13216.

Plaisted, W.C., Weinger, J.G., Walsh, C.M., and Lane, T.E. (2014). T cell mediated suppression of neurotropic coronavirus replication in neural precursor cells. *Virology* 449, 235-243.

Plaisted, W.C., Zavala, A., Hingco, E., Tran, H., Coleman, R., Lane, T.E., Loring, J.F., and Walsh, C.M. (2016). Remyelination Is Correlated with Regulatory T Cell Induction Following Human Embryoid Body-Derived Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis. *PLoS One* 11, e0157620.

Pluchino, S., Gritti, A., Blezer, E., Amadio, S., Brambilla, E., Borsellino, G., Cossetti, C., Del Carro, U., Comi, G., Hart, B., *et al.* (2009). Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. *Ann Neurol* 66, 343-354.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., *et al.* (2003). Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422, 688-694.

Poser, C.M. (1994). The epidemiology of multiple sclerosis: a general overview. *Ann Neurol* 36 *Suppl* 2, S180-193.

Prineas, J.W., and Graham, J.S. (1981). Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* 10, 149-158.

Prineas, J.W., Kwon, E.E., Goldenberg, P.Z., Ilyas, A.A., Quarles, R.H., Benjamins, J.A., and Sprinkle, T.J. (1989). Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Laboratory investigation; a journal of technical methods and pathology* 61, 489-503.

Ramakrishna, C., Stohlman, S.A., Atkinson, R.A., Hinton, D.R., and Bergmann, C.C. (2004). Differential regulation of primary and secondary CD8(+) T cells in the central nervous system. *J Immunol* 173, 6265-6273.

Redwine, J.M., Buchmeier, M.J., and Evans, C.F. (2001). In vivo expression of major histocompatibility complex molecules on oligodendrocytes and neurons during viral infection. *Am J Pathol* 159, 1219-1224.

Reidling, J.C., Relano-Gines, A., Holley, S.M., Ochaba, J., Moore, C., Fury, B., Lau, A., Tran, A.H., Yeung, S., Salamati, D., *et al.* (2018). Human Neural Stem Cell Transplantation Rescues Functional Deficits in R6/2 and Q140 Huntington's Disease Mice. *Stem Cell Reports* 10, 58-72.

Robey, P.G. (2017). "Mesenchymal stem cells": fact or fiction, and implications in their therapeutic use *F1000Research* 524.

Rowe, C.L., Baker, S.C., Nathan, M.J., and Fleming, J.O. (1997). Evolution of mouse hepatitis virus: detection and characterization of spike deletion variants during persistent infection. *J Virol* 71, 2959-2969.

Roy, N.S., Wang, S., Harrison-Restelli, C., Benraiss, A., Fraser, R.A., Gravel, M., Braun, P.E., and Goldman, S.A. (1999). Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19, 9986-9995.

Ruckh, J.M., Zhao, J.W., Shadrach, J.L., van Wijngaarden, P., Rao, T.N., Wagers, A.J., and Franklin, R.J. (2012). Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* 10, 96-103.

Ruller, C.M., Tabor-Godwin, J.M., Van Deren, D.A., Jr., Robinson, S.M., Maciejewski, S., Gluhm, S., Gilbert, P.E., An, N., Gude, N.A., Sussman, M.A., *et al.* (2012). Neural stem cell depletion and CNS developmental defects after enteroviral infection. *Am J Pathol* 180, 1107-1120.

Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., and Anderson, A.J. (2010). Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. *PLoS One* 5, e12272.

Schaumburg, C., O'Hara, B.A., Lane, T.E., and Atwood, W.J. (2008). Human embryonic stem cell-derived oligodendrocyte progenitor cells express the serotonin receptor and are susceptible to JC virus infection. *J Virol* 82, 8896-8899.

Schlesinger, H. (1909). Zur Frage der akuten multiplen Sklerose und der encephalomyelitis disseminata im Kindesalter. *Arb Neurol Inst [Wien]* 17, 410-432.

Smith, A.L., Barthold, S.W., and Beck, D.S. (1987). Intranasally administered alpha/beta interferon prevents extension of mouse hepatitis virus, strain JHM, into the brains of BALB/cByJ mice. *Antiviral research* 8, 239-245.

Steinman, L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* 85, 299-302.

Stohlman, S.A., and Hinton, D.R. (2001). Viral induced demyelination. *Brain pathology* 11, 92-106.

Tabor-Godwin, J.M., Ruller, C.M., Bagalso, N., An, N., Pagarigan, R.R., Harkins, S., Gilbert, P.E., Kiesses, W.B., Gude, N.A., Cornell, C.T., *et al.* (2010). A novel population of myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 8676-8691.

Totoiu, M.O., Nistor, G.I., Lane, T.E., and Keirstead, H.S. (2004). Remyelination, axonal sparing, and locomotor recovery following transplantation of glial-committed progenitor cells into the MHV model of multiple sclerosis. *Exp Neurol* 187, 254-265.

Trandem, K., Anghelina, D., Zhao, J., and Perlman, S. (2010). Regulatory T cells inhibit T cell proliferation and decrease demyelination in mice chronically infected with a coronavirus. *J Immunol* 184, 4391-4400.

Traugott, U., Reinherz, E.L., and Raine, C.S. (1983). Multiple sclerosis: distribution of T cell subsets within active chronic lesions. *Science* 219, 308-310.

Wang, F.I., Hinton, D.R., Gilmore, W., Trousdale, M.D., and Fleming, J.O. (1992). Sequential infection of glial cells by the murine hepatitis virus JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Laboratory investigation; a journal of technical methods and pathology* 66, 744-754.

Weinger, J.G., Plaisted, W.C., Maciejewski, S.M., Lanier, L.L., Walsh, C.M., and Lane, T.E. (2014). Activating Receptor NKG2D Targets RAE-1-Expressing Allogeneic Neural Precursor Cells in a Viral Model of Multiple Sclerosis Stem cells.

Weinger, J.G., Weist, B.M., Plaisted, W.C., Klaus, S.M., Walsh, C.M., and Lane, T.E. (2012). MHC mismatch results in neural progenitor cell rejection following spinal cord transplantation in a model of viral-induced demyelination. *Stem cells* 30, 2584-2595.

Weinshenker, B.G., Bass, B., Rice, G.P., Noseworthy, J., Carriere, W., Baskerville, J., and Ebers, G.C. (1989). The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. *Brain : a journal of neurology* 112 ( Pt 1), 133-146.

Wheeler, D.L., Sariol, A., Meyerholz, D.K., and Perlman, S. (2018). Microglia are required for protection against lethal coronavirus encephalitis in mice. *J Clin Invest* 128, 931-943.

Whitman, L., Zhou, H., Perlman, S., and Lane, T.E. (2009). IFN-gamma-mediated suppression of coronavirus replication in glial-committed progenitor cells. *Virology* 384, 209-215.

Wu, G.F., and Perlman, S. (1999). Macrophage infiltration, but not apoptosis, is correlated with immune-mediated demyelination following murine infection with a neurotropic coronavirus. *J Virol* 73, 8771-8780.

Yagita, Y., Kitagawa, K., Ohtsuki, T., Takasawa, K., Miyata, T., Okano, H., Hori, M., and Matsumoto, M. (2001). Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke* 32, 1890-1896.

Zhang, R., Zhang, Z., Wang, L., Wang, Y., Gousev, A., Zhang, L., Ho, K.L., Morshead, C., and Chopp, M. (2004). Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. *J Cereb Blood Flow Metab* 24, 441-448.

Zhou, J., Hinton, D.R., Stohlman, S.A., Liu, C.P., Zhong, L., and Marten, N.W. (2005). Maintenance of CD8+ T cells during acute viral infection of the central nervous system requires CD4+ T cells but not interleukin-2. *Viral Immunol* 18, 162-169.

Zhu, K., Sun, J., Kang, Z., Zou, Z., Wu, G., and Wang, J. (2016). Electroacupuncture Promotes Remyelination after Cuprizone Treatment by Enhancing Myelin Debris Clearance. *Front Neurosci* 10, 613.

## CHAPTER TWO

### **'Ex-Treg' derived neural antigen-specific regulatory T cells promote remyelination**

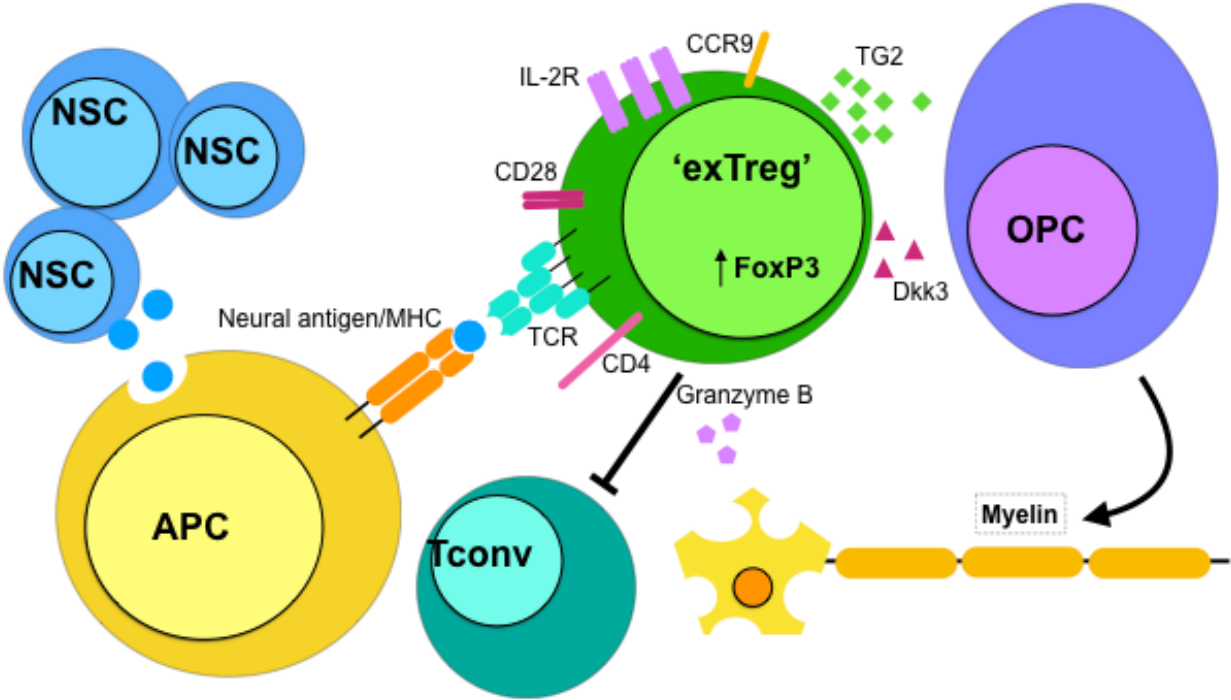
Laura L. McIntyre, Scott A. Greilach, Jonathan Hasselmann, Shivashankar Othy, Quy H. Nguyen, Brian Wi, Ilse Sears-Kraxberger, Jorge Silva, Julio Ayala-Angulo, Estelle Vu, Quan Pham, Kody Dang, Fady Rezk, Oswald Steward, Kai Kessenbrock, Michael D. Cahalan, Mathew Blurton-Jones, Thomas E. Lane and Craig M. Walsh

## Summary

Regulatory T cells (Tregs) are crucial for maintenance of homeostasis and self-tolerance. Recently, Tregs have also been implicated as potentiators of tissue repair. We report that human neural stem cells (hNSCs) promote expansion of neural antigen-specific Tregs that activate endogenous repair pathways to promote remyelination in a murine model of Multiple Sclerosis. We observed remyelination, decreased neuroinflammation and an increase in central nervous system- resident CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in experimental autoimmune encephalomyelitis mice receiving an intraspinal transplant of hNSCs. Ablation of Tregs abrogated histopathological improvement. hNSCs promoted expansion of neural antigen-specific Tregs *in vitro*. hNSC expanded Tregs (hNSC-Tregs) are expanded from the 'exTreg' pool. Intriguingly, hNSC-Tregs display a unique gene expression signature, upregulating Transglutaminase 2 which facilitates oligodendrocyte maturation. These findings support the concept that Tregs not only function in reducing neuroinflammation, but also influence CNS tissue repair. Therefore, neural-antigen specific Tregs represent a promising cellular therapy treatment of neurodegenerative diseases.

**Keywords:** Regulatory T cells, Multiple Sclerosis, Neural Stem Cells, Neuroinflammation, Remyelination

Figure 2.0. 'Ex-Treg' derived neural antigen-specific regulatory T cells promote remyelination- graphical abstract





## **Introduction**

Regulatory T cells (Tregs), characterized by expression of CD4 and FoxP3, are essential for prevention of autoimmunity and maintenance of T cell homeostasis (Josefowicz et al., 2012; Sakaguchi et al., 1995). While recent studies have also implicated Tregs in non-immunomodulatory roles, such as, promotion of wound healing in the skin and muscle repair (Burzyn et al., 2013; Nosbaum et al., 2016). Multiple Sclerosis (MS) is a chronic, autoimmune disease of the central nervous system (CNS), for which there is no cure, which owes its deficits in neurological function to widespread demyelination and axonal loss caused by infiltrating immune cells (Steinman, 1996). While the etiology of MS remains unknown, a breakdown in central tolerance leads to autoreactive T cell infiltration into the CNS. Previous studies have shown that dysregulation of Tregs has been linked to MS disease pathogenesis (Haas et al., 2007; Venken et al., 2008); and patients with MS typically report a decrease in their numbers of Treg. Conversely, it has been reported that MS patients have normal numbers of Tregs, but these cells are defective resulting in tissue damage (Venken et al., 2008). Regardless of disease ontogeny, current therapies are only focused on immunosuppression and are directed at the most common clinical form of MS, Relapsing-Remitting MS (RRMS), with efficacy in only 30% of RRMS patients (Lassmann et al., 1997), possibly due to the fact that these therapies fail to promote remyelination or repair damage already done to the CNS.

Early studies employing animal models of demyelination support that transplantation of neural stem cells (NSCs) may offer a promising therapeutic strategy to treat conditions and diseases affecting white matter integrity. Indeed, multiple studies have demonstrated that surgical implantation of NSCs into the CNS, mutes

neuroinflammation and enhance remyelination (Ben-Hur et al., 2013; Greenberg et al., 2014b; Mozafari et al., 2015; Pluchino et al., 2009; Salazar et al., 2010). However, much of the work evaluating NSC transplantation has been conducted using grafts from syngeneic donors or in immune suppressed subjects. From a clinical perspective, syngeneic NSC transplants are not a feasible option for patients with MS because NSCs may have a genetic defect; in addition, MHC matched cells are difficult to obtain, donor transplants are likely to be allogeneic, and life-long systemic immune suppression increases the risk of infection for the patient. Therefore, it is important to consider the potential for transplanted cells to elicit an immune response. The mechanism(s) through which NSCs affect the pathophysiology of experimental autoimmune encephalomyelitis (EAE), a mouse model of immune mediated demyelination have not been defined.

Here we report that human NSCs (hNSCs) expand neural antigen-specific Tregs that activate endogenous repair pathways and promote remyelination in EAE, the prototypic murine model of MS. EAE mice receiving intraspinal transplants of hNSCs displayed remyelination that correlated with dampened neuroinflammation, which was paralleled by an increase in central nervous system (CNS)-resident CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. Furthermore, hNSCs promoted Treg expansion in co-cultures *in vitro* and these Tregs were found to respond to neural self-antigens, including neurofilament. Additionally, a majority of the observed Treg expansion appeared to have originated from a population of Tregs that lost FoxP3 expression, termed 'exTregs' (Rubtsov et al., 2010; Zhou et al., 2009). Our findings support the hypothesis that continuous exposure to self-antigen is important for the maintenance of FoxP3 in CNS-antigen targeted Tregs, and that these

Tregs play a vital role in promoting tissue regeneration within the CNS during autoimmune neuroinflammation.

## **Methods**

### Derivation and maintenance of mNSCs and hNSCs

mNSCs Enhanced Green Fluorescent Protein expressing mouse Neural Stem Cells (mNSCs), derived from C57BL/6 mice were cultured as previously described (Lu et al., 2002). mNSCs were maintained in mNSC medium (DMEM/F12 w/ GlutaMax, 250µg/mL Amphotericin B, 100U/mL penicillin, 100µg/mL streptomycin, and 1X N2; all from Thermo Fisher) supplemented with 20µg/mL EGF (Thermo Fisher). 0.05% Trypsin (Thermo Fisher) was used to split cells when the cell density reached 80-90% confluence.

hNSCs WA09 hESCs were adapted to feeder-free conditions and maintained in Essential 8 medium (Thermo Fisher) on Geltrex™- coated plates (Thermo Fisher). hESCs were differentiated into EB-NSCs according to established methods (Chambers et al., 2009). Briefly, feeder-free hESCs were dissociated using Accutase (Thermo Fisher), transferred to ultra-low adherence 6-well plates, and cultured for 5 days in human ESC medium (DMEM/F12+ GlutaMAX, 20% Knockout Serum Replacement, 1X non-essential amino acids, and 0.1M 2-mercaptoethanol; all from Thermo Fisher) supplemented with 500ng/mL recombinant Noggin (R&D Systems) and 10µM SB431542 (Torcris). On the fifth days of culture, hESCs formed embryoid body-like structures (EBs) and 20-50 EBs were transferred to each well of a Geltrex™-coated 6-well dish. Increasing amounts of N2 medium (DMEM/F12+ GlutaMAX and 1X N2 Supplement; Thermo Fisher) supplemented with 500ng/mL Noggin and 10µM SB431542 were added every other day for 14 days.

Resulting columnar rosette structured were collected using Accutase and stained with anti-human CD184, anti-human CD24, anti-human CD44, anti-human, CD271 according to manufacturer recommendations using the BD Stemflow Human Neural Cell Sorting Kit. Cells were sorted on a FACS Aria Fusion (BD) and sorted hNSCs were maintained in hNSC maintenance medium (DMEM/F12+ GlutaMAX, 0.5X N2, 0.5X B27 without vitamin A, 20ng/mL bFGF; all from Thermo Fisher) on Geltrex™-coated dishes using Accutase to split cells when the cell density reached 80-90% confluence.

hNSCs were characterized by flow cytometry analysis. hNSCs were collected using Accutase and stained with mouse anti-human Nestin (1:200; BD), mouse anti-human Sox1 (1:200, BD), and mouse anti-human Sox2 (1:200; BD). For immunofluorescence microscopy differentiated NSCs were seeded on Geltrex-coated chamber slides, fixed with 4% paraformaldehyde, and stained with mouse anti-beta-III-tubulin (1:500; Abcam), rabbit anti-GFAP (1:200; Thermo Fisher), or rabbit anti-NG2 (1:200; Chemicon) before addition of respective secondary antibodies (goat anti-rabbit AlexaFluor™ 488, goat anti-rabbit AlexaFluor™ 568; Thermo Fisher). Slides were coverslipped and mounted using VectaShield, Hard Set Mounting Medium with DAPI (Vector Labs), and all images were captured using a Nikon Eclipse Ti inverted microscope.

### EAE mouse model of MS and Transplantation

Animal care and EAE immunization *All experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee.* Age-matched (8 weeks) male C57BL/6 mice (H-2b, Charles River) FoxP3<sup>EGFP</sup> or R2D2 mice were immunized by subcutaneous injections with 100µl of emulsion containing 100µg of myelin

oligodendrocyte glycoprotein MOG<sub>35-55</sub> (MEVGWYRSPFSRVVHLYRNGK-COOH; Pierce) in PBS, with complete Freund's adjuvant (CFA) containing 200µg *Mycobacterium tuberculosis* H37Ra (DIFCO Laboratories). The day of NSC transplant is designated as day 0. Mice received intraperitoneal (I.P.) injections with 400ng of *Bordetella pertussis* toxin (List Biological Laboratories) on day -21 and day -19 prior to transplant. Clinical evaluation was performed double-blind and based on the following scoring system; 0, asymptomatic; 0.5, ruffled fur; 1, flaccid tail; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund. Mice were sacrificed via inhalation of a lethal dose of isoflurane and cardiac perfusion with PBS was performed at defined time points post-transplant for tissue harvesting and analysis. Age and sex-matched (4-8 week old) C57BL/6 FoxP3<sup>EGFP</sup> mice were obtained from Jackson Laboratories (B6.Cg-Foxp3<sup>tm2(EGFP)Tch</sup>/J, Stock No: 006772; Jackson Laboratories) RAG2<sup>-/-</sup>2D2<sup>+</sup> mice were generated by crossing RAG2<sup>-/-</sup> (B10;B6- Rag2<sup>tm1Fwa</sup>; Taconic) x 2D2 (C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J, Stock No: 006912; Jackson Laboratories) RAG1<sup>-/-</sup>OT-II<sup>+</sup> mice were a kind gift from Stephen Schoenberger at the La Jolla Institute for Allergy and Immunology. 'exTreg' reporter mice were generated by breeding female FoxP3<sup>YFP/Cre+/-</sup> (B6.129 (Cg)-FoxP3<sup>tm4(YFPicre)Ayr</sup>/J, Stock No: 016959; Jackson Laboratories) x loxP-TdTomato Ai14<sup>+/+</sup> (B6.Cg-Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hze</sup>/J, Stock No: 007914; Jackson Laboratories)

Transplantation of NSCs Mice immunized with MOG were injected with 2.5 x10<sup>5</sup> mNSCs, hNSCs in 2.5µl of PBS or PBS alone as a control, at thoracic vertebrae 9 (T9) on day 21 post-immunization (p.i) (Day 0) as previously described (Carbajal et al., 2011).

*In vivo* Treg ablation EAE mice were injected I.P. with 150µg of purified, monoclonal anti-mouse CD25 (rat anti-mouse CD25, clone PC61.5 (Huss et al., 2016) (Biolegend) or control rat immunoglobulin G (Sigma) at day -2, day 0 and day 2 p.t., as previously described (Chen et al., 2014b) Efficiency of anti-CD25 treatment was determined by collection of peripheral blood from the peri-orbital sinus of anesthetized animals, followed by quantification of circulating Tregs by flow cytometry analysis.

#### Flow Cytometric Analysis of Tissues and *in vitro* co-cultures

Flow Cytometric Analysis of Tissues Cervical lymph nodes, spleens, or spinal cords were dissected into single- suspension, depleted of red blood cells using tris-acetic-acid-chloride (TAC) as previously described (Lane et al., 2000). Cells were filtered, washed, and counted before being blocked and stained with anti-mouse CD16/32 (1:100), B220-BV510, CD3- BV421, CD4-PE or BV785, CD8- FITC or PE/Cy7 or APC/Cy7, CD11b-Alexa 700, CD25-PE or APC, CD44- PB, CD45- PE/Cy7, CD62L- PE/Cy7, FoxP3-Alexa 488, Gr-1- BV605, Helios- FITC, Neuropillin-1- APC, NK1.1- APC, Propidium iodine, Zombie Aqua. For intracellular cytokine analysis, cells were fixed and permeabilized according to manufacturer specifications using the eBioscience FoxP3/ Transcription Factor Staining Buffer Set (Thermo Fisher). All antibodies were purchased from BD Biosciences, Biolegend, or eBiosciences. Single-stain samples and FMO controls were used to establish PMT voltages, gating, and compensation parameters. Cells were processed using an LSR II flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

*In vitro* splenocyte and NSC co-cultures Spleens dissected from naïve age-matched (4-8-week-old) C57/BL6, FoxP3<sup>EGFP</sup>, RAG1<sup>-/-</sup>OT-II<sup>+</sup>, or R2D2 mice were isolated into single-suspension, depleted of red blood cells. Splenocytes were stained with proliferation dye CFSE or ef670 (Thermo Fisher). Splenocytes were combined with NSCs at defined proportions (1.5x 10<sup>5</sup> cells per well) in round bottom 96-well plates and incubated at 37°C, 5% CO<sub>2</sub> for 4 days in 200µl final volume, of complete RPMI medium (RPMI-1640, 10% FBS (Atlanta Biologicals) 1X non-essential amino acids, 100U/mL penicillin, 100µg/mL streptomycin, 1mM sodium pyruvate, 55µM 2-mercaptoethanol; all from Thermo Fisher). Splenocytes were activated with plate bound anti-Armenian hamster IgG (30µg/mL; Vector or Jackson Immuno), Armenian hamster, anti-mouse CD3 (1µg/mL, 2C-11, Biolegend) and soluble anti-mouse CD28 (1µg/mL, Biolegend or Tonbo) were indicated. Addition of recombinant human IL-2 (100U/mL) and recombinant human TGFβ (10ng/mL) were added to cultures were indicated. After 4 days, cells were filtered, and washed before being blocked and stained with anti-mouse CD16/32 (1:100), CD4- PE/Cy7 or APC/Cy7 or PB, CD8- PE/Cy7 or APC, CD25- PE or APC, FoxP3-Alexa 647, PI. For intracellular cytokine analysis, cells were fixed and permeabilized according to manufacturer specifications using the eBioscience FoxP3/ Transcription Factor Staining Buffer Set (Thermo Fisher). All antibodies were purchased from BD Biosciences, Biolegend, or eBiosciences. For experiments where cells were sorted, cells were first filtered, washed and subjected to negative T cell isolation using magnetic separation and then sorted using FACS. Cells were counted and resuspended at [1x10<sup>8</sup>/mL] in T cell isolation buffer (1x PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Thermo Fisher) containing 2%FBS (Atlanta Biologicals) and 1mM EDTA (Thermo Fisher)) and 50ul/ mL Normal Rat Serum

(StemCell Technologies) with 10 $\mu$ l/mL anti-mouse B220 biotin and 10 $\mu$ l/mL anti-mouse CD11b biotin (Biolegend) for 10 min. at room temperature (RT). 50 $\mu$ l/mL Mojosort Streptavidin Nanobeads (Biolegend) were added and incubated for 2.5 min. at RT. Addition of T cell isolation buffer was added up to 5mL and samples were placed in EasySep magnet (StemCell Tech) for 2.5 min. at RT, supernants were collected. Cells were sorted into RPMI complete medium or Trizol (Thermo Fisher) when indicated. Single-stain samples and FMO controls were used to establish PMT voltages, gating, and compensation parameters. Cells were processed using an LSR II or LSRFortessa X-20 flow cytometer or sorted using a FACSAria Fusion (BD) and analyzed using FlowJo software (Tree Star). For quantification analysis, cell counts were normalized to a starting population of 150,000 splenocytes.

### Microscopy

Immunohistochemistry and analysis of histopathology Spinal cords were dissected, fixed overnight in 4% paraformaldehyde, and soaked in 30% sucrose for 7 days before being embedded in OCT compound. Frozen tissues were serially sectioned and stained with Luxol Fast Blue (LFB) and counterstained with hematoxylin and eosin (H&E) to assess the severity of demyelination. The total area of the white matter was quantified and compared to the area of demyelinated regions using ImageJ software (National Institutes of Health (NIH)). All demyelination measurements were conducted double blind. Images were obtained on a Nikon TE inverted microscope and processed using ImageJ software (NIH).



Transmission Electron Microscopy and G-ratio Analysis Mice were sacrificed via inhalation of a lethal dose of isoflurane and cardiac perfusion was performed using 1M cacodylate buffer containing 2% paraformaldehyde and 2% glutaraldehyde. Spinal cords were dissected and embedded in Epon epoxy resin before being ultra-serially sectioned, stained with uranyl acetate-lead citrate and analyzed as previously described (Liu et al., 2001). G-ratios were determined by measuring axon diameter and comparing it to the total fiber diameter (axon diameter/total fiber diameter) using ImageJ software (NIH). Measurements were performed double blind by two investigators at more than 200 axons were measured per experimental group.

2-Photon Imaging For 2-photon imaging, cell trace yellow (CTY) labeled hNSCs were transplanted into spinal cords of Foxp3<sup>EGFP</sup> mice undergoing EAE at thoracic vertebrae 9 (T9) on Day 21 and spinal cords were carefully dissected 3 days after p.t, fixed in 4% paraformaldehyde washed in PBS and imaged in PBS at room temperature. Two photon imaging was performed using a custom-built 2-photon system based on an Olympus BX51 upright microscope as previously described (Matheu et al., 2015), fitted with a Nikon 25x (NA= 1.1) water-immersion objective, and equipped with 3 PMTs and excitation generated by a tunable Chameleon femtosecond laser (Coherent) set to 890nm for optimal excitation of GFP and CTY. 495 nm and 560 nm dichroic filters were arranged in series to separate blue, green and red signals. Several 3D image stacks of x=500 µm, y=250 µm, and z=500 µm (XYZ voxel size 1 µm x 1 µm x 5 µm) were acquired using image acquisition software Slidebook (Intelligent Imaging Innovations) and motorized Z-Decks stage (Prior Scientific). 3D Image blocks were stitched in Slidebook and Imaris

version 9.2.1 (Bitplane) was used for rendering, final image size 3 mm X 12 mm X 0.5 mm.

### Genomic Analysis

RNA isolation, sequencing and analysis Cells were pelleted and 500ul of Trizol LS (Thermo Fisher) was added to each sample and stored at -80°C until RNA extraction. RNA was extracted from cells by adding 140ul of TET (10mM Tris 8.0/0.01mM EDTA/0.05% Tween20) and then 140ul chloroform: Isolamyl alcohol 24:1 (Sigma). Samples were then centrifuged at 15,000xg for 10 min. at 4°C. The aqueous phase was collected and added to 1.5ul of glycol blue (Thermo Fisher) and 10% volume 3M sodium acetate and 1 volume isopropanol. Samples were mixed by inverting and stored at -20°C overnight. Samples were then spun at 15,000xg for 30 min. at 4°C. Supernatants were removed from pellets, and 500ul of 75% ethanol was added to the pellet. Samples were then spun at 15,000xg for 30 min. at 4°C, and supernatants were removed from pellets. RNA pellets were resuspended in 30ul of H<sub>2</sub>O and used immediately or aliquoted and stored at -80°C. RNA concentrations were quantified using the Qubit Fluorometer (Thermo Fisher) and RNA quality of each sample was determined using the 2100 Bioanalyzer (Agilent Technologies) to obtain an RNA Integrity Number (RIN). Sequencing libraries were prepared from samples with acceptable RINs (9.0-10.0) using Illumina TruSeq Stranded mRNA Library prep according to manufacturer's instructions (Illumina). Libraries were then sequenced as a 150bp, paired-end run on a NovaSeq2 (Illumina).

FASTQ files were preprocessed using BBDuk (Bushnell, 2018) to trim Illumina adapters (ktrim=r, mink=8, k=23, hdist=1) followed by filtering of ribosomal RNA and PhiX reads as well as quality trimming to remove regions falling below a PHRED score of 28 (k=31, hdist=1, qtrim=rl, trimq=28, minlen=45). FASTQC (Andrews, 2014) analysis was then performed to verify the quality of the sequencing files and all files were determined to be of sufficient quality for downstream processing. Reads were then pseudoaligned to the mus musculus GRCm38.p6 transcriptome (Schneider et al., 2017; Zerbino et al., 2018) (Ensembl release version 94) using Kallisto (Bray et al., 2016) and transcripts were summarized to the gene level via tximport (Soneson et al., 2015). A batch effect was evident by PCA analysis so differential gene expression analysis was performed using DESeq2 (Love et al., 2014) while modeling the batch effect in the experiment design (design=~Batch + Cell Type).

Data was normalized and converted to a  $\log_2$  scale for visualization using DESeq2's (Love et al., 2014) varianceStabilizingTransformation() (VST) followed by batch correction using the removeBatchEffect() command from limma (Ritchie et al., 2015) Heatmaps were generated using the R "Pheatmap" package (Kolde, 2018) and scaled by row via mean centering. Volcano and bar plots were generated using the "ggplot2" package (Wickham, 2016).

TCR Repertoire Analysis iTregs or hNSC-Tregs were FACS purified and sorted directly into Trizol LS. RNA from samples was isolated, 100ng of RNA was sent to iRepertoire for CDR3 $\beta$  sequencing and analysis.

qPCR SuperscriptIII reverse transcriptase kit (Thermo Fisher) was used to generate cDNAs from RNA collected from samples. qRT-PCR was performed on the cDNAs using

TaqMan gene expression master mix (Thermo Fisher) to quantify transcript levels using TaqMan expression probes FoxP3, MOG and neurofilament (Thermo Fisher) in an ABI ViiA7 thermocycler. Human brain tissues samples were obtained from the UCI ADRC and used as controls for MOG and neurofilament expression. Data was analyzed using comparative Ct method (Schmittgen and Livak, 2008).

### Statistical Analyses

Data were analyzed using Prism software (GraphPad) or the R programming language. Comparisons were performed using a two-tailed T test and two-way analysis of variance, where indicated. Clinical scores were analyzed using two-tailed T test with a Mann-Whitney post-test. For all statistical models and tests described above, the significance is displayed as follows: NS  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## **Results**

### **ES-derived hNSCs promote remyelination and regulatory T cell expansion in the EAE mouse spinal cord**

In support of previous findings, engraftment of syngeneic mouse NSCs promotes remyelination in EAE with no effect upon the immune system (**Supplemental Figure 2.1**) (Carbajal et al., 2010; Carbajal et al., 2011; Greenberg et al., 2014b). To evaluate the potential of xenogeneic hNSCs to promote remyelination in EAE, we transplanted  $2.5 \times 10^5$  human embryonic stem cell (ESC)-derived NSCs, human dermal fibroblasts (HDFs) (as a xenogeneic control), or administered phosphate buffered saline (PBS; as a vehicle control) into the spinal cord of EAE immunized mice 21 days post immunization (p.i.), a

time point at which mice display demyelinating lesions (**Figure 2.1A**) (Constantinescu et al., 2011; McCarthy et al., 2012). hESCs were differentiated into NSCs using dual SMAD inhibition as previously described (**Supplemental Figure 2.2A**) (Chambers et al., 2009). In brief, differentiated cells were enriched for NSCs by FACS sorting for CD184<sup>+</sup>CD24<sup>+</sup>CD271<sup>-</sup>CD44<sup>-</sup> cells (**Supplemental Figure 2.2B**) (Plaisted et al., 2016; Yuan et al., 2011). As shown in Supplemental Figures 2.2C and 2.2D, sorted cells strongly expressed NESTIN (81.6%±2.91), a known marker of stem cells, and stained double positive for the NSC markers SOX1 and SOX2 (92.7%±0.61) (**Supplemental Figures 2.2C and 2.D**). Upon growth factor withdrawal, these cells differentiated and expressed markers associated with the three neural lineages:  $\beta$ III-tubulin (neurons), GFAP (astrocytes) and NG2 (oligodendrocytes) (**Supplemental Figure 2.2E**).

Consistent with previous studies from our group, hNSCs underwent xenograft rejection and are undetectable 8 days post-transplant (p.t.) (Chen et al., 2014b; Plaisted et al., 2016). However, luxol fast blue (LFB) staining at 21 days p.t. revealed that EAE mice transplanted with hNSCs have less demyelinated white matter compared to PBS treated controls near the transplant site (**Figures 2.1B-D**). Estimating the number of CD4<sup>+</sup> T cells 7 days p.t. revealed a decrease in neuroinflammation in mice receiving hNSCs compared to PBS controls. Remyelination was associated with a decrease in CD4<sup>+</sup> T cells and an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in the spinal cord (SC) and cervical lymph node (cLN) of hNSC transplanted mice 7 days p.t. (**Figure 2.1E**). To confirm that hNSC-induced remyelination is not a result of mere rejection of xenogeneic cells, we used human dermal fibroblasts (HDFs) as a xenogeneic control. EAE mice transplanted with HDFs did not result in noticeable differences in myelin (**Supplemental Figures 2.3A and**

**2.3B**), indicating that the mechanism of hNSC-mediated immune response is indeed mediated by hNSCs and not due to a xeno-specific immune response. Notably, clinical scores of mice receiving either hNSCs or HDFs were unaltered (**Supplemental Figure 2.3C**). These results are consistent with our previous findings in JHM strain of mouse hepatitis virus (JHMV)- infected animals receiving hNSCs (Plaisted et al., 2016). The decrease in CD4<sup>+</sup> T cells and increase in Tregs appeared to be transient, and corresponds to the time period at which hNSCs are rejected. Accordingly, we did not detect a difference in these populations 21 days p.t. (**Supplemental Figure 2.3D**) nor did we detect a difference in frequency in other immune cell populations (CD45<sup>lo</sup> CD11b<sup>+</sup> Microglia, CD45<sup>hi</sup> CD11b<sup>+</sup> Macrophages, CD45<sup>+</sup>, CD11b<sup>+</sup>Gr-1<sup>+</sup> Neutrophils, CD11b<sup>+</sup>B220<sup>-</sup> cDCs, CD11b<sup>+</sup>B220<sup>+</sup> pDCs, B220<sup>+</sup> B cells, NK1.1<sup>+</sup> NK cells, CD3<sup>+</sup>NK1.1<sup>+</sup> NK T cells, or CD3<sup>+</sup> T cells) day 7 p.t. (**Supplemental Figure 2.4A and 2.4B**). Altogether, transplantation of hNSCs into the spinal cord during EAE increases the local number of Tregs, while decreasing the overall CD4<sup>+</sup> T cells, resulting in site specific remyelination. Sustained remyelination up to several days after rejection of hNSCs suggests that SC-Tregs are recruited and expanded to the site of transplantation are promoting endogenous repair mechanisms.

### **Tregs are necessary for remyelination**

To determine whether Tregs were necessary for remyelination in hNSC mice, EAE mice were depleted of Tregs using an anti-CD25 antibody (PC61.5) (Huss et al., 2016; Setiady et al., 2010). EAE mice received no antibody treatment, PC61.5 or an IgG isotype control, and were then transplanted with hNSCs or PBS 21 days post immunization

**(Supplemental Figure 2.5A).** Ablation of Tregs abrogated remyelination along with PC61.5 treated mice displaying a decreased frequency and number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in peripheral blood 3 and 9 days p.t. **(Supplemental Figure 2.5B and 2.5C)** with no effect upon the frequency and number of CD4<sup>+</sup> T cells **(Supplemental Figure 2.5D and 2.5E)** or clinical outcome **(Supplemental Figure 2.5F)**. We compared the ratio of the inner axonal diameter to that of total myelinated fiber (g-ratio) between EAE mice transplanted with hNSCs, hNSCs+ PC61.5, hNSCs+ IgG control, or PBS control mice. Lower g-ratios indicate more remyelination (Liu et al., 2001). Mice transplanted with hNSCs displayed extensive signs of remyelination at the transplant site **(Figure 2.1F)**. G-ratio analyses confirmed a significant increase ( $p < 0.001$ ) in myelin at the transplant site; hNSC transplanted mice had significantly lower calculated g-ratios ( $0.7414 \pm 0.0063$ ) compared to those transplanted with hNSCs but depleted of Tregs (hNSCs+PC61.5) ( $0.7808 \pm 0.0057$ ) **(Figure 2.1G; Supplemental Figure 2.3E)**. These results confirm that Tregs are necessary for remyelination; depletion of Tregs abrogates histopathological improvement.

### **Tregs localize to the site of hNSC injection in EAE Spinal cord**

To investigate whether Tregs interacted with transplanted hNSCs *in vivo*, we utilized two-photon (2P) microscopy of spinal cord explants to reveal cellular interactions. We transplanted cell trace yellow (CTY) labeled hNSCs or PBS into FoxP3<sup>EGFP</sup> EAE immunized mice 21 days p.i. Five days p.t., spinal cord explants were imaged by 2P microscopy (Greenberg et al., 2014b; Weinger et al., 2015). Montage images revealed that FoxP3<sup>EGFP+</sup> Tregs localize to the ventral lateral spinal cord at the site of hNSC

injection (**Figures 2.2A and 2.2B**). These images demonstrate that Tregs interact with hNSCs *in vivo* to promote repair.

### **hNSCs expand regulatory T cells *in vitro***

To determine if hNSCs directly mediated the observed increase in Tregs, we developed an *in vitro* co-culture system utilizing naïve splenocytes from wild-type B6 or FoxP3<sup>EGFP</sup> reporter mice and hNSCs (**Figure 2.3A**). Employing this approach, we detected a significant dose-dependent increase in the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was observed in cultures containing hNSCs (**Figure 2.3B**). In the CNS, microglia secrete copious amounts of TGF $\beta$  and IL-2 (Gehrmann et al., 1995), factors that are essential for Treg generation and function (Horwitz et al., 2008; Tischner et al., 2012). To better mimic physiologic conditions of antigen presentation in the CNS, TGF- $\beta$  and IL-2 were added to co-cultures of naïve splenocytes stimulated with  $\alpha$ -CD3 and  $\alpha$ -CD28 and hNSCs. Cultures with hNSCs yielded two-fold higher levels of Tregs (hNSC-Tregs) compared to induced Treg (iTreg) controls (**Figures 2.3C and 2.3D; Supplemental Figure 2.6A-C**). hNSC-Treg generation was consistent among various ratios of splenocytes to hNSCs (**Figure 2.3E**), and specific to hNSCs; co-cultures with syngeneic mouse mNSCs or HDFs did not yield Tregs (**Supplemental Figures 2.3A-D**). Additionally, Tregs from co-cultures with hNSCs displayed 1.9-fold and 1.2-fold higher expression of FoxP3 and CD25, respectively, as measured by mean fluorescent intensity (MFI), compared to iTregs (**Supplemental Figures 2.6D and 2.6E**). Higher expression of FoxP3 in hNSC-Tregs was confirmed by qPCR, where Tregs expressed 14.2 ( $\pm$ 2.6) fold higher expression of FoxP3 compared to iTreg controls (**Supplemental Figure 2.6F**). This suggests that



hNSC-cocultured Tregs have a more activated, functional phenotype (Chauhan et al., 2009) .

To determine whether viable hNSCs were necessary to obtain more Tregs, co-cultures were performed with hNSCs cultured under standard conditions or cultured overnight in PBS (PBS-killed hNSCs). Both live or PBS-killed hNSCs were able to induce more Tregs in co-cultures (**Figure 2.3F; Supplemental Figure 2.6G**). Accordingly, PBS-killed mNSCs and HDFs failed to increase the frequencies or numbers of Tregs (**Supplemental Figures 2.7E-H**) confirming hNSC specific response. Altogether, co-culture of live or dead hNSCs with splenocytes lead to significant Treg expansion suggesting involvement of hNSC specific antigens.

### **Antigen specificity of hNSC-Tregs**

To understand the antigen specificity of hNSC-Tregs we compared iTreg TCR $\beta$  sequences to that of hNSC-Tregs using high-throughput TCR $\beta$  repertoire analyses. These analyses identified 7,427 unique CDR3 $\beta$  sequences for iTregs and hNSC-Tregs. To determine the clonal dominance in each population, we used the clonality index (inverse of Shannon's entropy) (Stewart et al., 1997), with 0 indicating that each clone only occurs once and 1 being a monoclonal population. While both populations of Tregs were polyclonal, with a diversity index of less than 0.2, only 57 TCR $\beta$  sequences were common to both Treg populations while 5,549 TCR $\beta$  sequences were unique to hNSC-Tregs (**Figure 2.4A**). Additionally, using the diversity index where 0 is a monoclonal population and 50 is a diverse population, hNSC-Tregs had a lower diversity score (5.3-9) compared to iTregs (7.5-15.5). Although both populations of Tregs were diverse, clonal

dominance can be estimated by the contribution of the top 10 most abundant clones. Many of the top 10 hNSC-Treg clones were distinct compared to the iTreg repertoire. In addition, hNSC-Treg clones displayed similar peptide sequences (**Figure 2.4B**). When comparing CDR3 $\beta$  sequences of iTregs and hNSC-Tregs to HDF-induced Tregs, the latter were the least diverse (diversity index=3) with only 1,127 unique clones (**Supplemental Figure 2.8A**). These results suggest that although there is high diversity within the TCR sequence of the hNSC-Treg group, many of these clones bear a striking similarity to one another and may recognize different epitopes of the same antigen.

Next, we sought to determine whether antigen presentation was necessary for hNSC-Treg generation. In activating conditions, antigen presenting cells (APCs) upregulate CD80 and MHC-II. APCs cultured in the presence of hNSCs expressed greater levels of CD80 and MHC-II than conditions without hNSCs, suggesting that hNSCs enhance antigen presentation on APCs (**Supplemental Figure 2.8B**). We utilized splenocytes from T cell receptor (TCR) transgenic *RAG1*<sup>-/-</sup>OT-II<sup>+</sup> mice, which express a T cell receptor repertoire restricted to ovalbumin (OVA), and thus do not recognize any hNSC antigens presented by APCs in the co-cultures (Barnden et al., 1998). Co-culture of hNSCs with polyclonal B6 splenocytes resulted in a dose-dependent increase in frequency and number of Tregs (**Figure 2.5A and 2.5B**), however co-culture of hNSCs with *RAG1*<sup>-/-</sup>OT-II<sup>+</sup> splenocytes did not show an increase in Tregs with increasing numbers of hNSCs (**Figure 2.5C and 2.5D**). Thus, when T cells cannot recognize hNSC antigens, they are unable to expand as hNSC numbers increase. This observation indicates that antigen presentation and Treg TCR specificity play a critical role in hNSC mediated Treg induction.

To determine if hNSC-Tregs recognize neural self-antigens, we generated TCR transgenic *RAG2<sup>-/-</sup>2D2<sup>+</sup>* (R2D2) mice, which have a TCR repertoire restricted to myelin oligodendrocyte glycoprotein (MOG) and neurofilament, respectively (Bettelli et al., 2003; Lucca et al., 2014). Co-cultures of naïve R2D2 splenocytes with hNSCs resulted in a dose-dependent expansion of Tregs (**Figure 2.5E and 2.5F**). Mouse and human MOG peptide sequences are 89% homologous, and neurofilament medium shares 97% homology. Gene expression analyses revealed that hNSCs express neurofilament medium (**Supplemental Figure 2.8C and 2.8D**). This observation suggests that hNSC-Tregs recognize the antigen neurofilament. Together these data support that hNSC-Tregs possess a unique TCR repertoire that recognize neural antigens including neurofilament.

### **hNSCs expand Tregs from the ‘exTreg’ pool**

Foxp3<sup>+</sup> Tregs can be derived in the thymus (tTregs), or in the periphery (pTregs) through the conversion of conventional T cells. To determine whether hNSC-Tregs are derived from the thymus or the periphery, we co-cultured FACS-purified FoxP3<sup>EGFP</sup>- naïve splenocytes from FoxP3<sup>EGFP</sup> reporter mice with hNSCs. Similar increases in the frequency and number of hNSC-Tregs were observed regardless of the presence of FoxP3<sup>+</sup> cells in the starting splenocyte population (**Figure 2.6A and 2.6B**). Intriguingly, hNSC-Tregs from both co-cultures expressed Helios and Neuropilin-1, markers of thymically derived Tregs (data not shown) (Singh et al., 2015; Thornton et al., 2010). A population of thymically derived Tregs possessing unstable FoxP3 expression, termed ‘exTregs’, has been described in the periphery (Rubtsov et al., 2010; Zhou et al., 2009). Therefore, we sought to determine whether hNSCs were inducing the conversion of

conventional T cells to Tregs or, alternatively, reinvigorating cells that had downregulated FoxP3 expression ('exTregs'). To address this question, we used 'exTreg' reporter mice; FoxP3<sup>YFP/Cre</sup> mice that express a knocked-in yellow fluorescent protein/iCre-recombinase fusion protein under the control of the Treg specific FoxP3 promoter (Rubtsov et al., 2008) and combined them with Ai14 Cre reporters (Madisen et al., 2010) that have a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein (tdTomato). Resulting 'exTreg' reporter mice (loxP-tdTomato (TdT) x FoxP3<sup>YFP/Cre</sup>) have FoxP3<sup>+</sup>Tregs which express YFP<sup>+</sup> and TdT<sup>+</sup> concomitantly, while cells that lose FoxP3 expression are permanently marked TdT<sup>+</sup>, but lose YFP expression. Thus, using these mice, we FACS purified Treg populations containing all Tregs (YFP<sup>+</sup>TdT<sup>+</sup>; orange gate), populations containing 'exTregs' that previously expressed FoxP3 (YFP<sup>-</sup>TdT<sup>+</sup>; red gate), or populations of T cells that never expressed FoxP3 (YFP<sup>-</sup>TdT<sup>-</sup>; grey gate) (**Figure 2.6C**). We cultured each of the splenocyte populations containing each of the purified Tregs with hNSCs for 4 days, then using flow cytometry calculated the frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3/YFP<sup>+</sup>Tdt<sup>+</sup> cells. Co-cultures of splenocytes containing all Tregs (YFP<sup>+</sup>TdT<sup>+</sup>) and hNSCs resulted in a 2-fold increase in the frequency of Tregs (**Figure 2.6D**), consistent with our previous results (**Figure 2.3E**; **Supplemental Figure 2.6C**). Moreover, co-cultures of splenocytes without cells currently expressing FoxP3 (YFP<sup>-</sup>TdT<sup>+</sup>) significantly increased the frequency of Tregs (**Figure 2.6E**). However, in co-cultures lacking CD4<sup>+</sup> T cells that currently or formerly expressed FoxP3<sup>+</sup> (YFP<sup>-</sup>TdT<sup>-</sup>; grey), there was no increase in Tregs (**Figure 2.6F**). These data suggest that hNSC-Tregs are expanded from the thymically derived 'exTreg' pool.

'ExTregs' display memory phenotype markers (Zhou et al., 2009). Self-peptide/MHC contact is essential for the survival of naïve T cells and co-stimulation with IL-7 promotes conversion of conventional T cells to memory phenotype (Sprent and Surh, 2011). Additionally, *in vivo* conversion of naïve CD4<sup>+</sup> T cells has been reported following low dose antigen exposure (Kretschmer et al., 2005; Kretschmer et al., 2006) and also following adoptive transfer into lymphopenic hosts (Haribhai et al., 2009). These reports, along with our own results, lead us to hypothesize that exposure to self-antigen may be vital for the maintenance of FoxP3 expression in tTregs as well; notably, R2D2 mice lacked FoxP3<sup>+</sup> cells in the periphery under homeostatic conditions (**Supplemental Figure 2.9A**). To determine if exposure to self-antigen expanded CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in R2D2 mice, we immunized R2D2 mice with MOG peptide. Examination of cLN and SC tissues 10 days p.i. revealed an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells within MOG immunized mice compared to sham immunized mice (**Supplemental Figures 2.9B-E**). Albeit a small fraction of CD4<sup>+</sup> cells, most CD25<sup>+</sup>FoxP3<sup>+</sup> cells expressed Helios (**Supplemental Figure 2.9C**), suggesting that these cells were thymically derived. Transfer of naïve CD4<sup>+</sup> T cells into lymphopenic *RAG*-deficient hosts induces their conversion into CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs (Haribhai et al., 2009). We tested this model by transferring purified naïve R2D2 CD4<sup>+</sup> T cells into *RAG2 $\gamma$ C<sup>-/-</sup>* lymphopenic hosts, which were subsequently immunized with MOG or a sham control. Five weeks post-transfer and immunization, we observed an expansion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in the cervical lymph nodes and brain of immunized mice, but not sham-immunized controls (**Supplemental Figure 2.10A**). Tregs in the brain were CD62L<sup>-</sup>CD44<sup>+</sup>, indicative of an effector memory phenotype (**Supplemental Figure 2.10B**). These data support the

conclusion that self-peptide/MHC contact is essential for the maintenance of FoxP3 expression in Tregs, similar to survival of conventional T cells.

### **hNSC-Tregs have a distinct gene expression signature**

Multiple subtypes of *ex vivo* Tregs have been defined genomically (Feuerer et al., 2010). To more precisely define the hNSC-Treg gene expression signature, we performed RNAseq analyses. We aimed to determine whether hNSC-Tregs possess a unique gene expression profile distinct from iTregs isolated from *in vitro* co-cultures. Comparing iTregs versus hNSC-Tregs from three independent co-cultures, we identified a unique gene expression signature of hNSC-Tregs (**Figures 2.7A and 2.7B**). While the majority of transcripts were similar (**Figure 2.7C**), we found 56 upregulated and 26 downregulated genes in hNSC-Tregs versus iTregs (**Figure 2.7D**). For example, hNSC-Tregs displayed an induction of Chemokine Receptor 9 (*Ccr9*) and Granzyme B (*Gzmb*) and downregulation of *Sox4*, a profile previously attributed to *ex vivo* Tregs from gut associated lymphoid tissues (GALT) (**Figure 2.7E**) (Hill et al., 2007). Significantly, transglutaminase-2 (*Tgm2*), an adhesion G-protein coupled receptor G1 precursor (ADGRG1) oligodendrocyte progenitor (OPC) ligand (Giera et al., 2018; Gundemir et al., 2012), and Dickkopf-3 (*Dkk3*), a modulator of T cell responses (Meister et al., 2015) were found to be upregulated in hNSC-Tregs. Both Treg populations expressed FoxP3<sup>+</sup> “signature genes”; *Ctla4*, *Foxp3*, *Ikzf2*, *Il10*, *Il2ra*, *Itgae*, and *Tgfb1* (**Figure 2.7F**) (Fontenot et al., 2005b; Hill et al., 2007; Huehn et al., 2004). Taken together, these data indicate that hNSC-Tregs possess a unique gene expression signature that contributes to their function *in vivo*.

## Discussion

Tregs are critical for controlling inflammation and prevention of autoimmunity (Sakaguchi et al., 2006). Deficiencies in FoxP3 result in systemic autoimmunity in Scurfy mutant mice and human patients with immunodysregulation polyendocrinopathy and enteropathy X-linked (IPEX) syndrome (Ziegler, 2006). Impairments in peripheral Treg function have also been reported in patients with MS, however the role of Tregs in the CNS is not well understood (Kumar et al., 2006; Vigiotta et al., 2004). The CNS has long been considered 'immune privileged', although this concept has recently been challenged (Carson et al., 2006). Kipnis et al reported that activated microglia recruit Tregs into the CNS via production of CCL22 in neurodegenerative diseases (Kipnis et al., 2004). Indeed, both neurons and astrocytes have been shown to induce Tregs from conventional T cells in the CNS (Liu et al., 2006; Trajkovic et al., 2004). Additionally, these Tregs have been shown to prevent disease progression in EAE through suppression of inflammation (Liu et al., 2006). However, the mechanism by which Tregs facilitate tissue repair within the CNS is not well understood.

In this study, we provide evidence of a role for interactions of NSCs with Tregs further implicating Tregs not only as suppressors of inflammatory T cell responses, but, importantly, also as modulators of tissue repair during EAE. Mice receiving hNSC transplants displayed significant remyelination at the transplant site, although we did not detect a significant clinical response. The lack of clinical response is likely due to local Treg recruitment to the site of transplant, whereas other areas of the spinal cord remain damaged. Future studies will focus on methods to expand Treg recruitment and repair throughout the spinal cord. Moreover, our data support the hypothesis that, under

homeostatic conditions, tissue-restricted antigen-specific Tregs lose FoxP3 expression and convert into 'exTregs.' Subsequent to tissue damage, normally sequestered self-antigens are presented by APCs to expand Tregs that are capable of promoting tissue repair and regeneration.

While little is known about the maintenance of Treg homeostasis, here we report that self-peptide/MHC is essential for the maintenance of FoxP3 expression in regulatory T cells. Our data strongly supports the hypothesis that neural antigen-specific Tregs expand from the 'exTreg' pool. Co-culture of FACS-sorted naïve CD4<sup>+</sup> FoxP3<sup>-</sup> splenocytes with hNSCs yielded increased frequencies and numbers of Tregs. However, these cultures contained T cells that previously expressed FoxP3, cells that have been termed 'exTregs'. Exposure to hNSCs reinvigorated FoxP3 expression in 'exTregs', but not conventional naïve CD4<sup>+</sup> T cells, indicating that hNSCs do not promote conversion of conventional T cells to Tregs. These data suggest that access to self-antigens plays an important role in the recruitment of 'exTregs' and re-expression of FoxP3. Supporting this, R2D2 mice lack Tregs in the periphery, but CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs emerge following MOG self-antigen exposure *in vivo*. Purified naïve CD4<sup>+</sup> R2D2 T cells also convert to CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in lymphopenic hosts following immunization. These Tregs display a memory phenotype, which has recently been implicated in regulating immune contributions to tissue repair (Rosenblum et al., 2016).

This model of 'exTreg' FoxP3 re-expression is further supported by the work of Caton and colleagues (Jordan et al., 2001) who reported that TCR transgenic TS1 mice, which possess T cells specific for the major I-Ed determinant (S1) or influenza hemagglutinin (HA) do not develop Tregs. However, when these mice were crossed with



HA transgenic (HA28) mice, Tregs were detected following HA exposure, indicating that Treg development is dependent on the presence of their specific antigen. In addition, studies performed using the TCR transgenic *RAG*<sup>-/-</sup> DO11.10, a TCR-transgenic mouse where all T cells express a TCR specific for a peptide of Ovalbumin (OVA) peptide revealed a failure in Treg development (Itoh et al., 1999). When these mice were crossed with mice expressing OVA in the thymus and pancreas, Tregs successfully emerged (Walker et al., 2003). We hypothesize that HA specific Tregs may be expanded from the 'exTreg' pool upon exposure to HA antigen whereas OVA-specific Tregs maintain FoxP3 expression due to continual antigen exposure, resulting in tonic TCR signaling. It is well established that TCR signaling regulates positive T cell selection and homeostasis in conventional T cells (Gaud et al., 2018). While it is known that Tregs respond to low abundance but high affinity antigens (Li and Rudensky, 2016), the importance of tonic signals released through self-peptide/MHC contact has not been implicated in Treg function until now.

Our work also highlights the ability of Tregs to act as potentiators of tissue repair by promoting remyelination in the CNS. We observed an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in the CNS and cervical lymph nodes of MOG immunized animals transplanted with hNSCs, but not in saline controls or animals that received HDFs. Utilizing 2P microscopy we observed Tregs accumulating at the site of hNSC transplant within the CNS. An increased frequency of Tregs was associated with a decrease in CD4<sup>+</sup> T cells. G-ratio analyses revealed that mice receiving hNSC transplants had significantly more myelin compared to controls. Importantly, depletion of Tregs abrogated histopathological improvement by preventing remyelination. We note that Tregs responding to hNSC

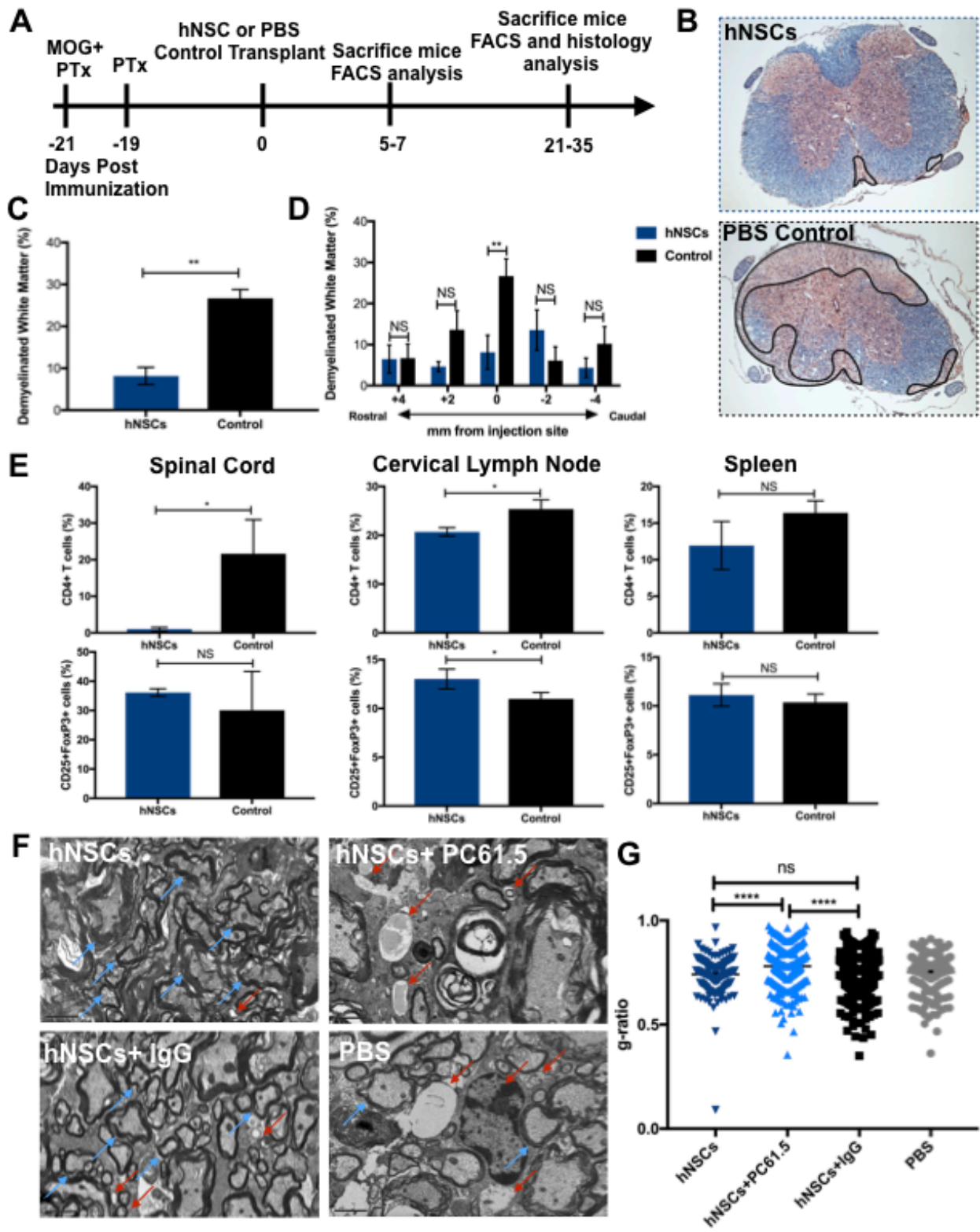
antigens *in vitro* displayed a unique gene expression signature, upregulating *Ccr9* and *Gzmb* and downregulating *Sox4*, a profile previously attributed to *ex vivo* Tregs from the GALT (Hill et al., 2007). This unique gene transcriptional profile is important for maintaining T cell homeostasis, but may also be exploited as a therapeutic strategy for recruitment of Tregs to the CNS. The CCR9 ligand, CCL25 facilitates recruitment of *Ccr9*<sup>+</sup> cells. Osmotic infusion of CCL25 into the CNS could therefore recruit additional Tregs to the CNS to promote repair (Loomis et al., 1987; Svensson and Agace, 2006).

Gene expression analyses also revealed that hNSC-Tregs secrete molecules known to modulate neuroinflammation and promote repair in the CNS. Dickkopf-3 (encoded by *Dkk3*), a known suppressor of inflammatory T cell responses was significantly upregulated in hNSC-Tregs. *Dkk3* encodes a secreted protein that shows highest expression in 'immune-privileged' organs, such as the CNS (Nakamura et al., 2007; Niehrs, 2006). Moreover, genetic deletion or antibody neutralization of *Dkk3* exacerbates EAE symptoms (Vogelpoel et al., 2015). *Dkk3* is also a positive regulator of Wnt signaling, important for promoting neurogenesis and maturation of oligodendrocyte progenitors (OPCs) (Munji et al., 2011; Rosso and Inestrosa, 2013). Transglutaminase-2 (TG2), encoded by *Tgm2*, a Ca<sup>2+</sup>-dependent protein cross-linking enzyme, was also found to be highly upregulated in hNSC-Tregs. TG2 acts on adhesion G-protein coupled receptor G1 precursor (ADGRG1) on oligodendrocyte progenitors leading to oligodendrocyte maturation and increased remyelination (Giera et al., 2018), and TG2 knock out mice treated with cuprizone (chemically-induced demyelination) exhibited impaired remyelination (Torkildsen et al., 2008; Van Strien et al., 2011). Induction of TG2 expression via pharmacological agents promotes OPC maturation (Giera et al., 2018),

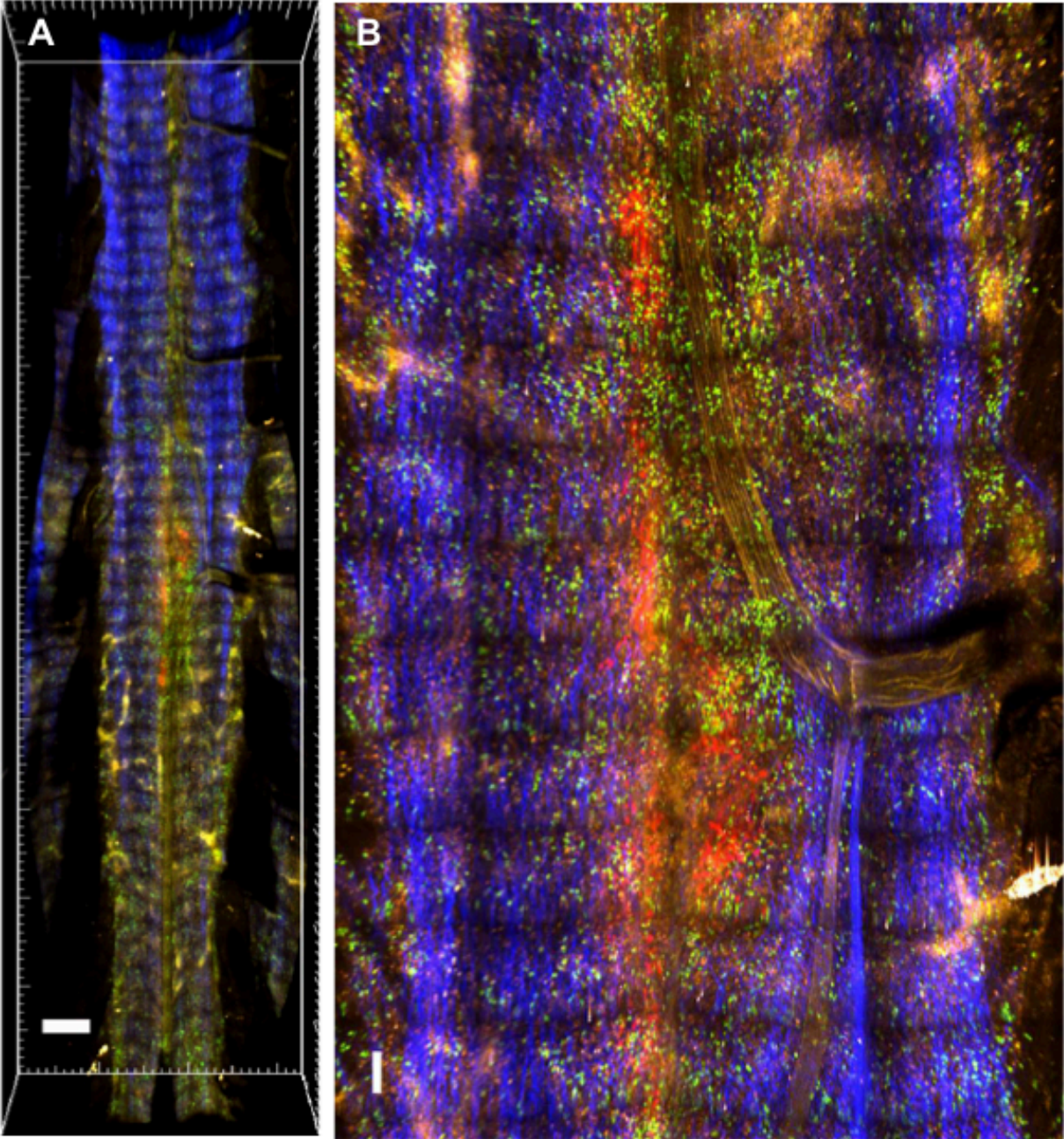
and pharmacological and biological modulators of ADGRG1 are currently under development (Salzman et al., 2016; Stoveken et al., 2016). Both strategies predict novel therapeutic avenues for promoting remyelination.

Administration of neural antigen-specific Tregs may potentially be exploited as a therapeutic strategy to suppress inflammation and promote remyelination in MS, as *ex vivo* expanded Tregs have been shown to suppress autoimmune diabetes (Putnam et al., 2009; Tang et al., 2004). Given the aforementioned attributes of hNSC-elicited Tregs, *ex vivo* expansion of MS patient-derived, neural antigen-specific Tregs represents a promising cellular therapy to promote remyelination in MS. Taken together, our work highlights a potential novel therapeutic approach utilizing neural antigen-specific Tregs or molecular compounds secreted by Tregs for the treatment of autoimmune or neuroinflammatory diseases.

**Figure 2.1. Transplantation of embryonic stem cell derived human neural stem cells promotes remyelination and Tregs in the EAE mouse spinal cord. (A)** Timeline of EAE induction (MOG immunization and Pertussis Toxin (PTx) administration), hNSC transplant and analysis. **(B)** Representative brightfield images of coronal spinal cord sections stained with luxol fast blue (LFB) and counterstained with hematoxylin and eosin (H&E), areas of demyelinated white matter outlined in black. **(C)** Quantification of demyelination in the ventral white matter of hNSC (n=8) and control PBS (n=8) transplanted mice revealed significantly ( $p \leq 0.01$ ) reduced demyelination at the injection side in the spinal cords of hNSC transplanted mice, 28 days p.t. **(D)** Quantification of demyelination in areas rostral and caudal to the site of injected revealed that reduced demyelination was not sustained throughout the spinal cord. **(E)** Quantification of the frequency of CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD8<sup>+</sup> T cells 7 days p.t. (n=3 each for saline and hNSCs) demonstrated a significant ( $p < 0.05$ ) decrease in the percentage of CD4<sup>+</sup> T cells within the spinal cord (top left) and draining cervical lymph node (top middle). A trend toward an increase in frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was observed in the spinal cord (lower left) and a significant ( $p < 0.05$ ) increase in frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was observed in the draining cervical lymph node (lower middle) of hNSC transplanted mice. A difference in CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells was not observed in the spleen. For **(C-E)** Data represents three independent experiments. **(F)** Representative electron micrographs of coronal spinal cord sections from hNSC, hNSC+PC61.5, hNSC+IgG, and control PBS injected mice. Demyelinated axons indicated by red arrows. Remyelinated axons indicated by blue arrows. **(G)** Quantification of ratio of inner axon diameter versus total myelinated fiber (g-ratio) G-ratio of hNSCs ( $0.7414 \pm 0.0063$ ), hNSCs+PC61.5 ( $0.7808 \pm 0.0057$ ), hNSCs+IgG ( $0.748 \pm 0.0049$ ), and PBS ( $0.7512 \pm 0.0054$ ). More than 200 axons were measured per group. All data is presented as average  $\pm$  SEM and analyzed by either using one-way ANOVA followed by Tukey's multiple comparison test (**C, D and G**) or an unpaired, two-tailed T-Test (**E**).



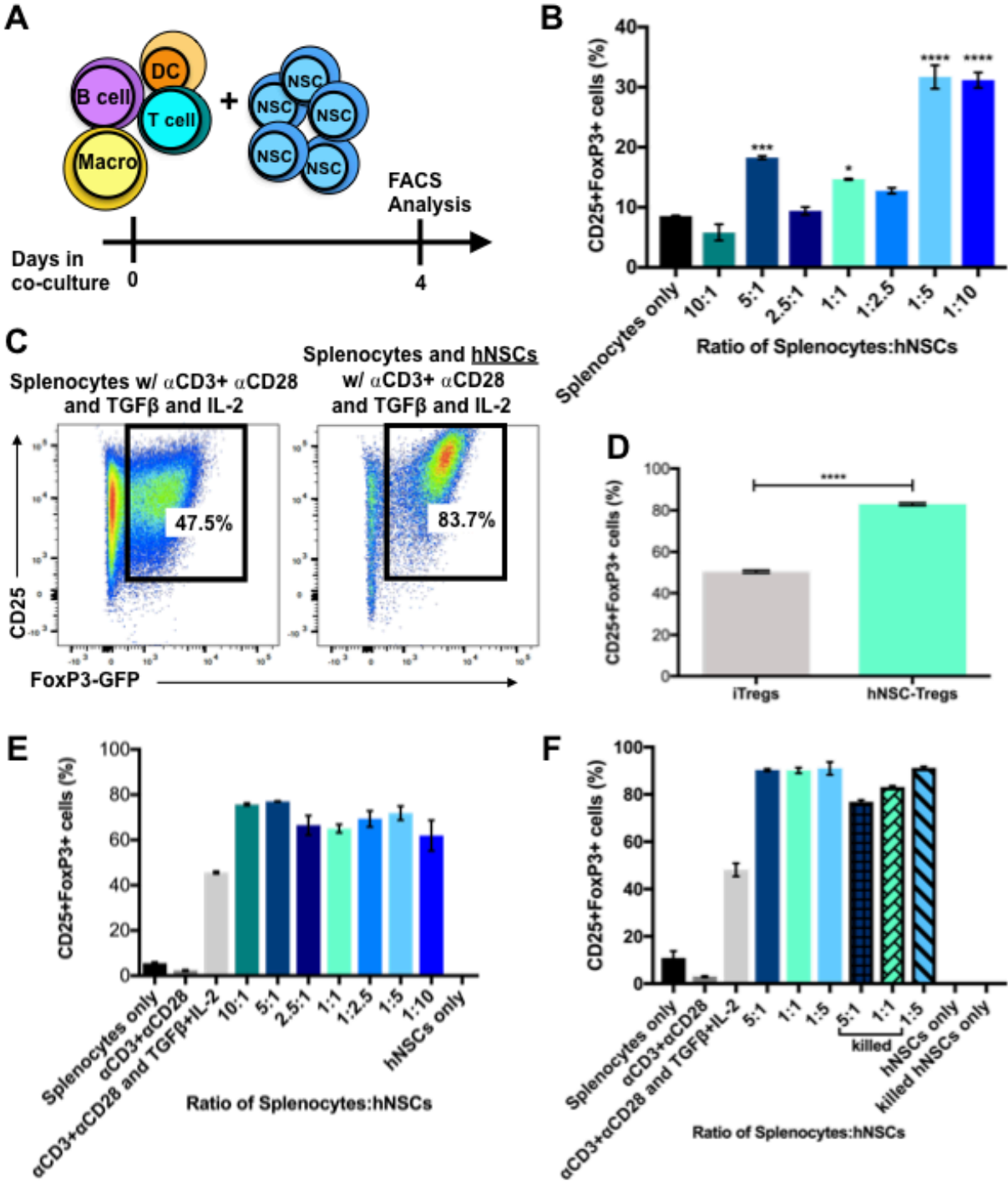
**Figure 2.2. Tregs accumulate at the site of hNSC injection in the EAE Spinal cord.** Spinal cord montage (A) and zoomed in image (B) showing distribution of FoxP3<sup>EGFP+</sup> Tregs (green), transplanted CTY-hNSC (red), second-harmonic signal from collagen (blue) in the ventral side of the spinal cord, 3 days p.t. Images are maximal intensity projections through the z-axis of x-y-z stacks (Image size A = 3 mm X 12 mm X 0.5 mm), scale bar in A = 500  $\mu$ m, scale bar in B = 100  $\mu$ m.



Tregs Collagen hNSCs Autofluorescence

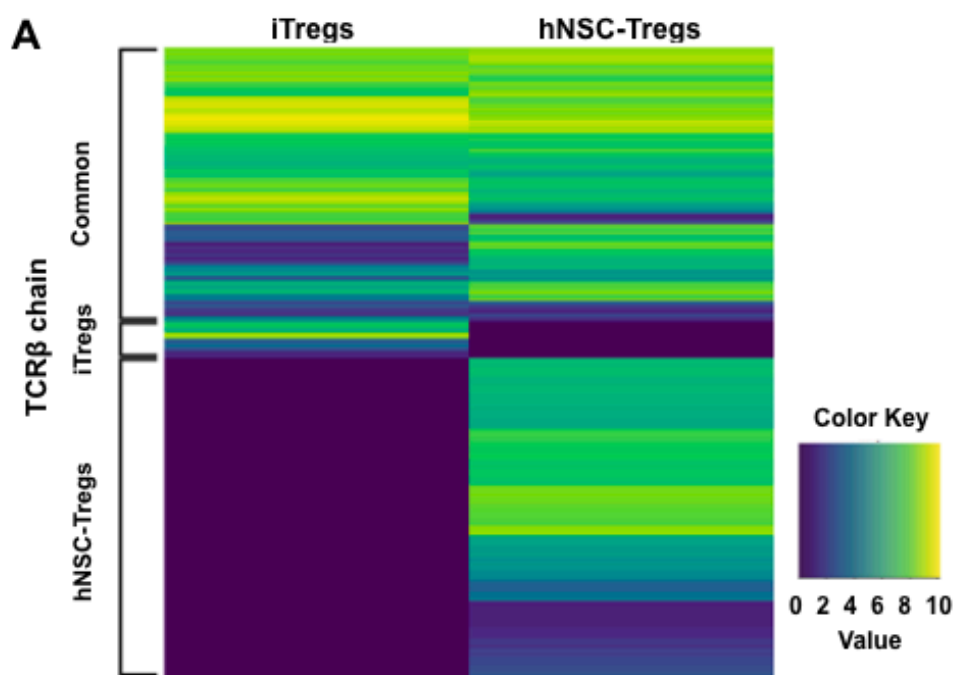


**Figure 2.3. hNSCs expand Tregs *in vitro*.** **(A)** Timeline of splenocyte and hNSC co-culture. **(B)** Frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> expressing CD4<sup>+</sup> T cells is significantly increased in the presence of hNSCs at a naïve splenocyte to T cell ratio of (5:1, 1:1, 1:5, and 1:10; p<0.01, p<0.05, p<0.0001, p<0.0001, respectively) compared to naïve splenocytes only control. **(C)** Representative FACS plots of CD25<sup>+</sup>FoxP3<sup>+</sup> Treg (gated on live, CD4<sup>+</sup> cells) from naïve splenocytes cultured with 1µg/mL α-CD3, 1µg/mL α-CD28, 10ng/mL TGFβ and 100U/mL IL-2 in the absence (left) or presence of hNSCs (right) for 4 days. The frequency **(D)** and number **(Supplemental Figure 6C)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was significantly (p<0.0001) increased in the presence of hNSCs. **(E)** Naïve splenocyte cultures were mixed with hNSCs at varying ratios of splenocytes to hNSCs; a significant (P<0.0001) increase in the percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was observed at all ratios with hNSCs. Co-cultures of naïve splenocytes and live or killed hNSCs revealed that killed hNSCs expanded frequency **(F)** and number **(Supplemental Figure 6G)** of Tregs similar to live hNSCs. All data is presented as average ± SEM, representative of three independent experiments, and analyzed by either one-way ANOVA with a Tukey's multiple comparisons test **(B, E and F)** or an unpaired, two-tailed T-test **(D)**.

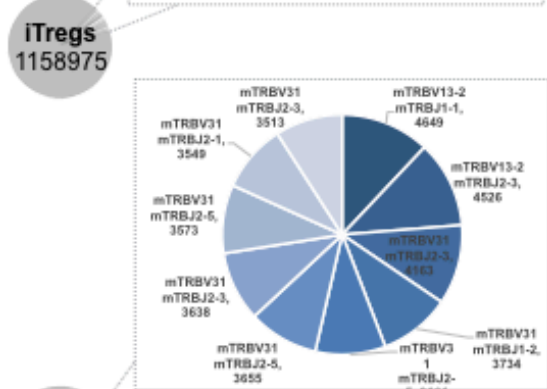
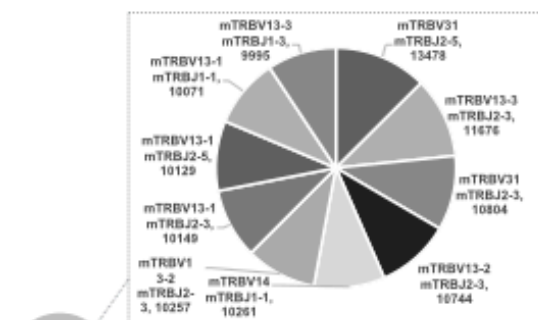




**Figure 2.4. hNSC-Tregs display unique CDR3 $\beta$  sequences.** (A) TCR repertoire analysis of TCR $\beta$  chains from sorted CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs from *in vitro* cultures in the absence (iTregs) or presence of hNSCs (hNSC-Tregs) revealed a diverse TCR repertoire, with expanded clones unique to hNSC-Tregs. (B) The top 10 TCR $\beta$  chain clones from iTregs are different than hNSC-Tregs. Data is representative of two independent experiments.



**B** Top 10 TCR clones



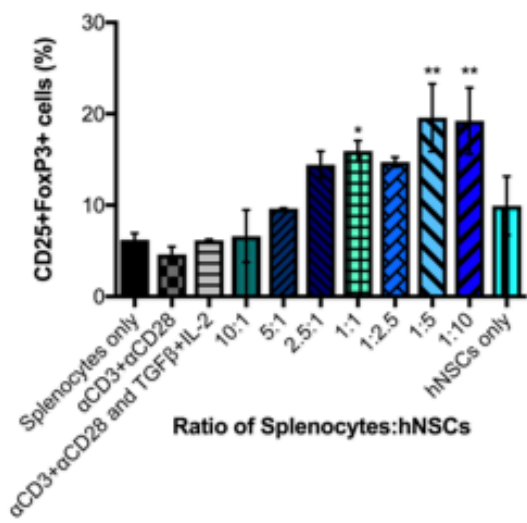
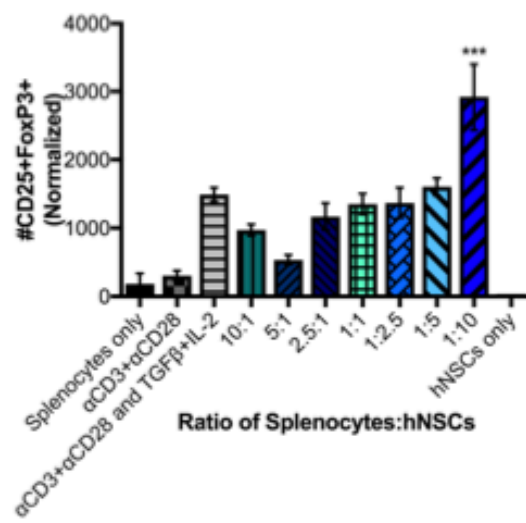
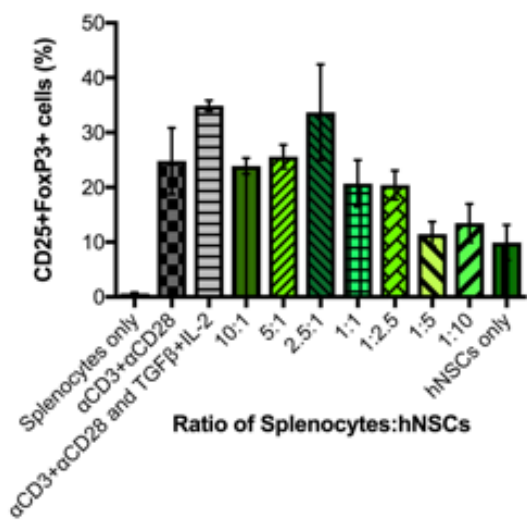
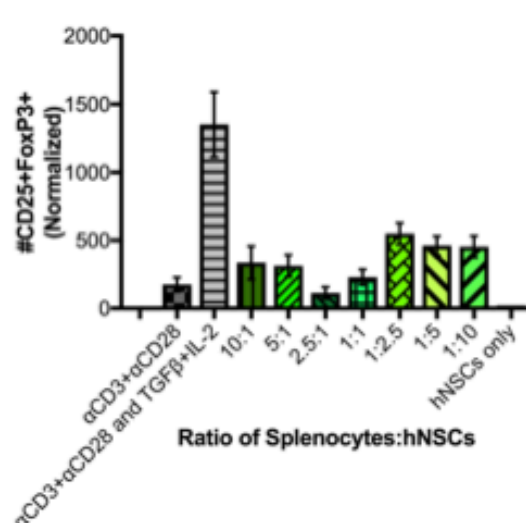
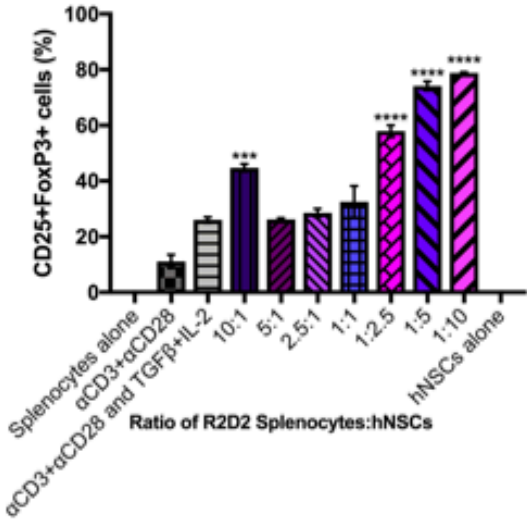
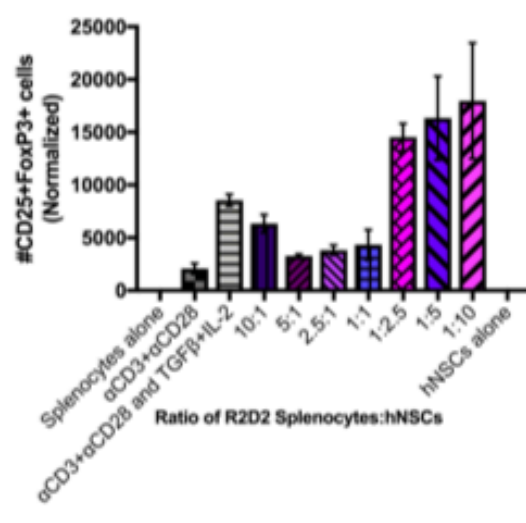
**iTregs**

| Vβ        | Jβ       | CDR3β (peptide) | Count |
|-----------|----------|-----------------|-------|
| mTRBV13-2 | mTRBJ1-1 | ASGAANTEVF      | 4649  |
| mTRBV13-2 | mTRBJ2-3 | ASGDASAETLY     | 4526  |
| mTRBV31   | mTRBJ2-3 | AWSLSAETLY      | 4163  |
| mTRBV31   | mTRBJ1-2 | AWSHRGRNSDYT    | 3734  |
| mTRBV31   | mTRBJ2-5 | AWSPGLNQDTQY    | 3661  |
| mTRBV31   | mTRBJ2-5 | AWSLGNFQDTQY    | 3655  |
| mTRBV31   | mTRBJ2-3 | AWGLGASAETLY    | 3638  |
| mTRBV31   | mTRBJ2-5 | AWSRGGDTQY      | 3573  |
| mTRBV31   | mTRBJ2-1 | AWSQDNYAEQF     | 3549  |
| mTRBV31   | mTRBJ2-3 | AWSKTGTETLY     | 3513  |

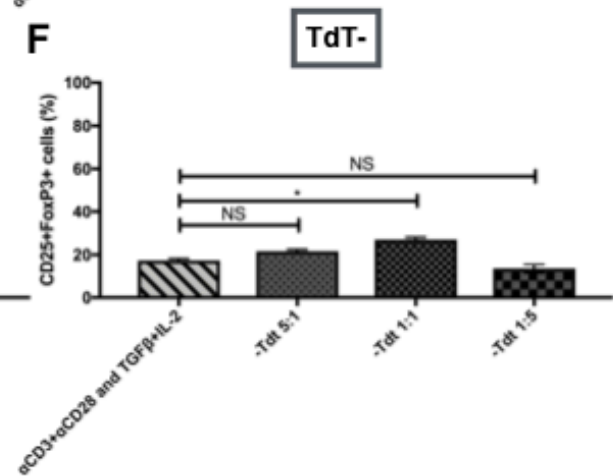
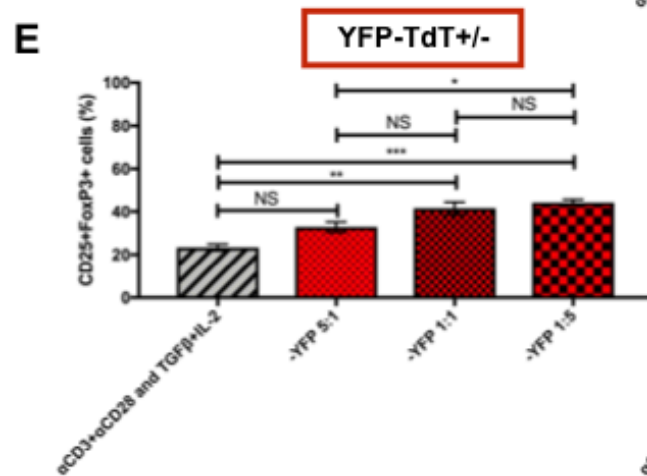
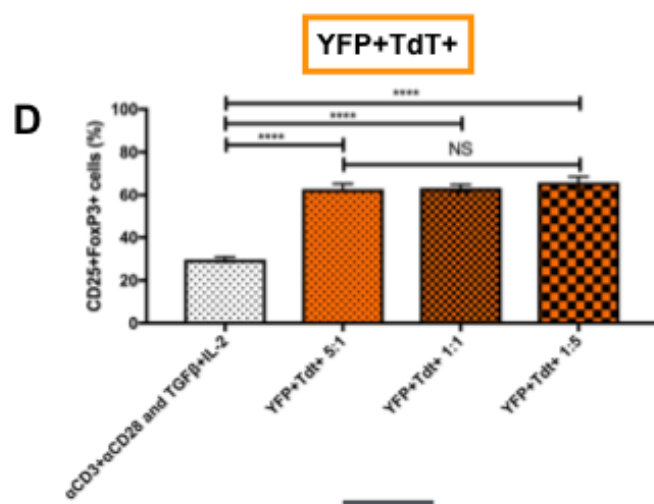
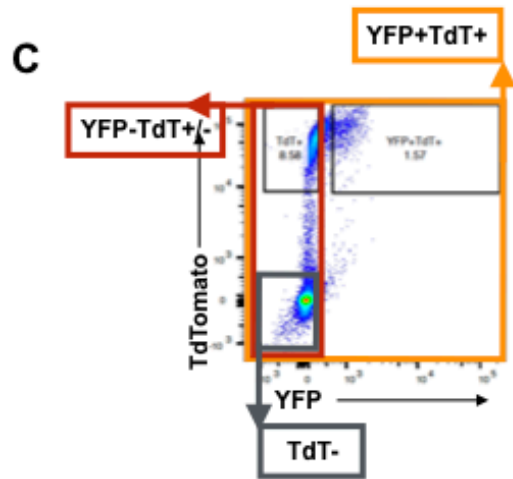
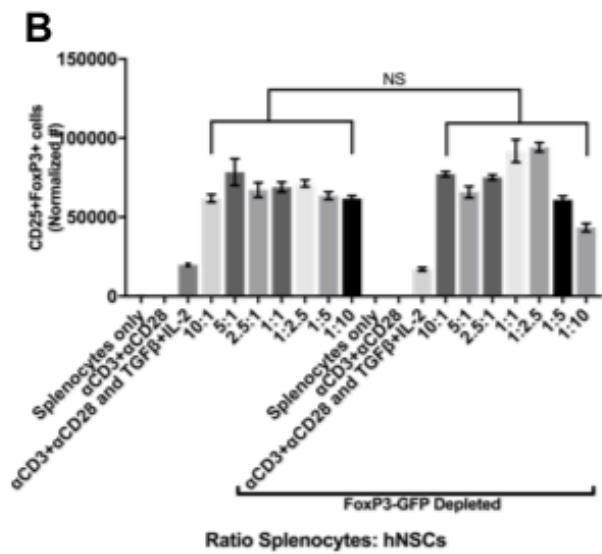
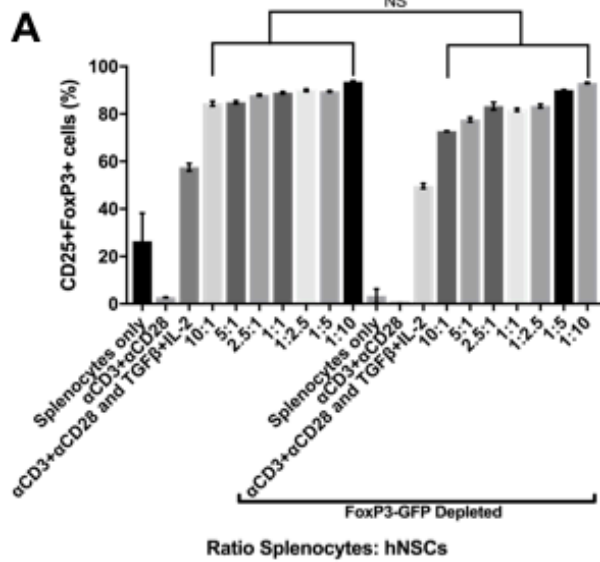
**hNSC-Tregs**

| Vβ        | Jβ       | CDR3β (peptide) | Count |
|-----------|----------|-----------------|-------|
| mTRBV31   | mTRBJ2-5 | AWSPWVNQDTQY    | 13478 |
| mTRBV13-3 | mTRBJ2-3 | ASNDWGSSAETLY   | 11676 |
| mTRBV31   | mTRBJ2-3 | AWSLGLGGAETLY   | 10804 |
| mTRBV13-2 | mTRBJ2-3 | ASGDGAETLY      | 10744 |
| mTRBV14   | mTRBJ1-1 | ASSRGNTEVF      | 10261 |
| mTRBV13-2 | mTRBJ2-3 | ASELGGGAETLY    | 10257 |
| mTRBV13-1 | mTRBJ2-3 | ASSGGAETLY      | 10149 |
| mTRBV13-1 | mTRBJ2-5 | ASSDGAQDTQY     | 10129 |
| mTRBV13-1 | mTRBJ1-1 | ASSGTGFYTEVF    | 10071 |
| mTRBV13-3 | mTRBJ1-3 | ASRGDNSGNTLY    | 9995  |

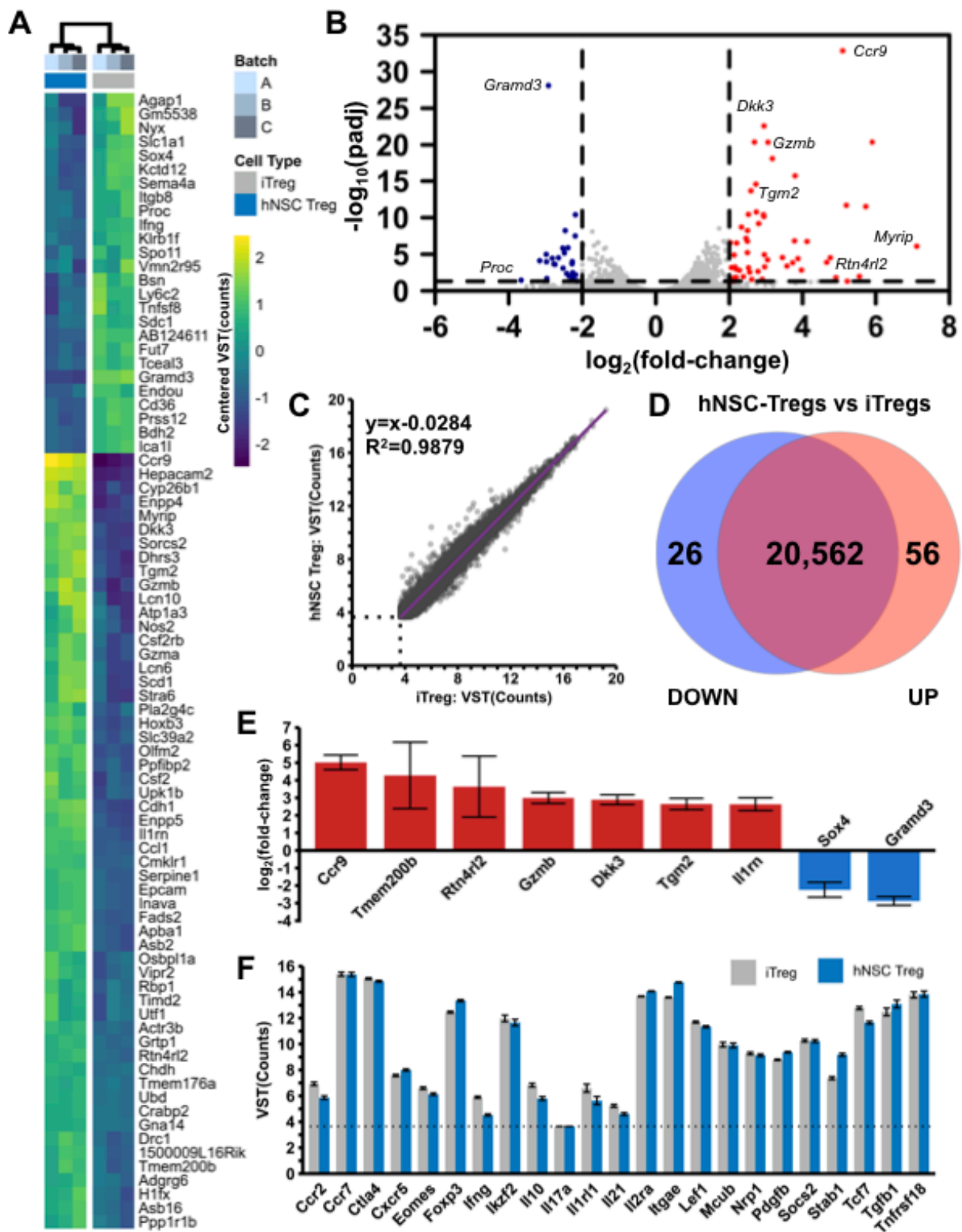
**Figure 2.5. Tregs from hNSC cultures are CNS antigen specific.** Naïve splenocytes were cultured at different ratios of splenocytes to hNSCs and activated in the presence of 1ug/mL  $\alpha$ -CD3, 1ug/mL  $\alpha$ -CD28, 10ng/mL TGF $\beta$  and 100U/mL IL-2. Co-cultures with polyclonal B6 splenocytes revealed a significant increase in the frequency at ratios of splenocytes to hNSCs (1:1, 1:5, and 1:10; p<0.05, p<0.01, p<0.01, respectively) **(A)** and number at ratios (1:10, p<0.001) **(B)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs after 4 days in culture. Co-cultures of naïve splenocytes from *RAG1*<sup>-/-</sup> OT-II<sup>+</sup> mice with a TCR repertoire specific for ovalbumin and hNSCs did not reveal a difference in frequency **(C)** or number **(D)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs compared to controls without hNSCs. Co-cultures of naïve splenocytes from *RAG2*<sup>-/-</sup>2D2<sup>+</sup> (R2D2) with a TCR repertoire specific for myelin oligodendrocyte glycoprotein (MOG) and neurofilament revealed a significant increase in frequency at ratios of splenocytes and hNSCs (10:1, 1:2.5, 1:5, and 1:10; p<0.01, p<0.001, p<0.001, p<0.001, respectively) **(E)** and number **(F)** of CD25<sup>+</sup>FoxP3<sup>+</sup> cells. For **(A-D)** data are representative of two independent experiments (n=3). For **(E)** and **(F)** data are representative of three independent experiments (n=3), data is presented as average +/- SEM. All data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test.

**A****B6 splenocytes****B****C****RAG1<sup>-/-</sup>OT-II<sup>+</sup> splenocytes****D****E****RAG2<sup>-/-</sup>2D2<sup>+</sup> splenocytes****F**

**Figure 2.6. hNSC expanded Tregs are derived from the ‘exTreg pool’.** Co-cultures of naïve splenocytes with and without FoxP3<sup>EGFP+</sup> cells and hNSCs were performed. No significant difference was observed in the frequency **(A)** or number **(B)** of CD25<sup>+</sup>FoxP3<sup>+</sup> cells after 4 days in cultures containing natural Tregs or depleted of Tregs at the start. **(C)** Sample FACS gating strategy for sorting T cell populations from FoxP3<sup>YFP/Cre</sup> x loxP-TdTomato (‘exTreg’ reporter) mice. T cell populations included naïve splenocytes with tTregs (YFP<sup>+</sup>Tdt<sup>+</sup>; orange), sort for splenocytes without FoxP3<sup>+</sup> cells (YFP-Tdt<sup>+/-</sup>; red), and sort for splenocytes without FoxP3<sup>+</sup> cells or cells previously expressing FoxP3 (‘exTregs’) (YFP-Tdt; grey). **(D)** Co-cultures of naïve splenocytes with hNSCs confirmed a significant increase in frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> cells at ratios of splenocytes to hNSCs at ratios (5:1, 1:1 and 1:5; p<0.0001) compared to cultures without hNSCs. **(E)** Co-cultures of splenocytes without nTregs (FoxP3/YFP<sup>+</sup>Tdt<sup>+</sup>) and hNSCs displayed a significant increase in the frequency of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs at ratios of splenocytes to hNSCs (1:1 and 1:5; p<0.01 and p<0.001 respectively). **(F)** Co-cultures of splenocytes without nTregs (FoxP3/YFP<sup>+</sup>Tdt<sup>+</sup>) and ‘exTregs’ (Tdt<sup>+</sup>) did not display an expansion of CD25<sup>+</sup>FoxP3<sup>+</sup> cells compared to controls without hNSCs. Data is representative of three and two independent experiments, respectively n=3. Data is presented as average ± SEM. All data were analyzed using a one-way ANOVA followed by a Tukey’s multiple comparison test.

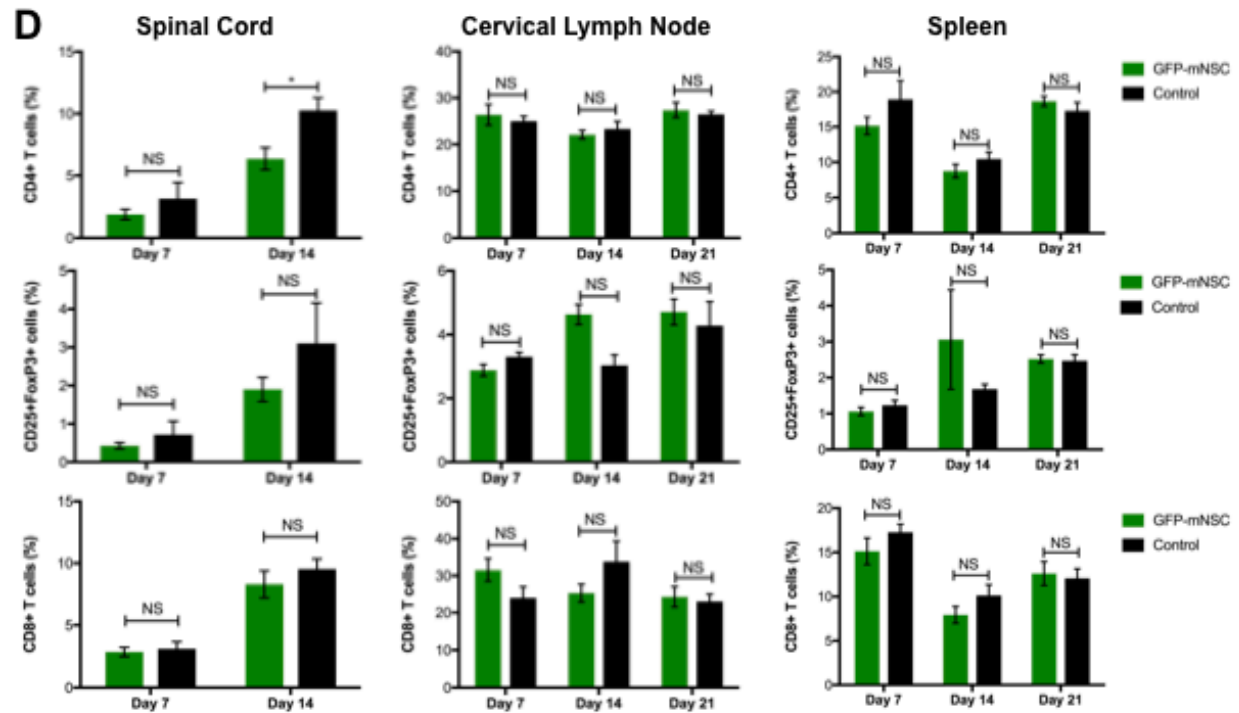
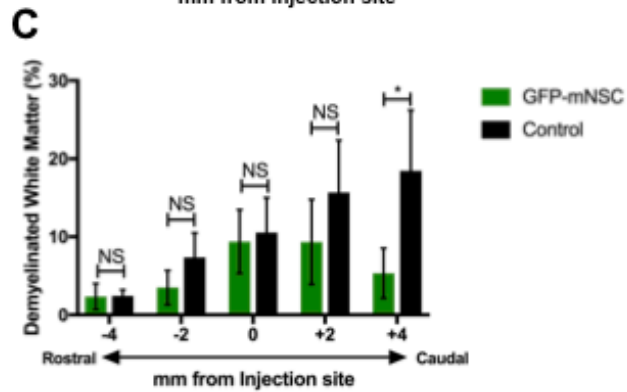
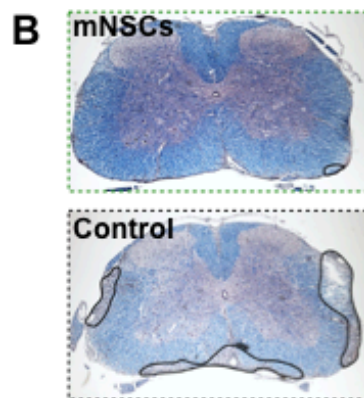
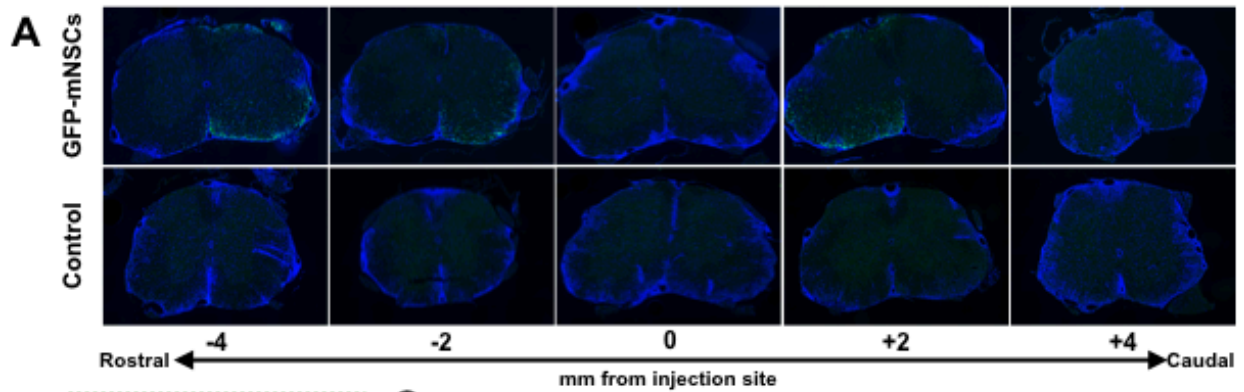


**Figure 2.7. hNSC expanded Tregs display unique gene expression patterns.** CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs FACS purified from co-cultures with hNSCs or iTregs were subjected to gene-expression analyses using RNA-Seq. **(A)** Heat map comparing gene expression of hNSC-Tregs versus iTregs. Data represent differentially expressed genes (FDR= 0.05, LFC=±2). **(B)** Volcano plots displaying genes that are either significantly upregulated (red) or downregulated (blue) as an FDR cutoff of 0.05 and an LC cutoff of ±2. Labeled genes are related to Treg signature. **(C)** Regression analysis comparing iTregs versus hNSC-Tregs. **(D)** Venn diagram depicting differentially expressed genes from iTregs versus hNSC-Tregs that are upregulated (red) or downregulated (blue). **(E)** Bar graphs comparing genes of interest found in gene expression analysis comparing iTregs versus hNSC-Tregs. **(F)** Bar graphs comparing Treg signature genes expressed in iTregs and hNSC-Tregs. The dotted line represents genes with no expression following variance stabilization between groups.

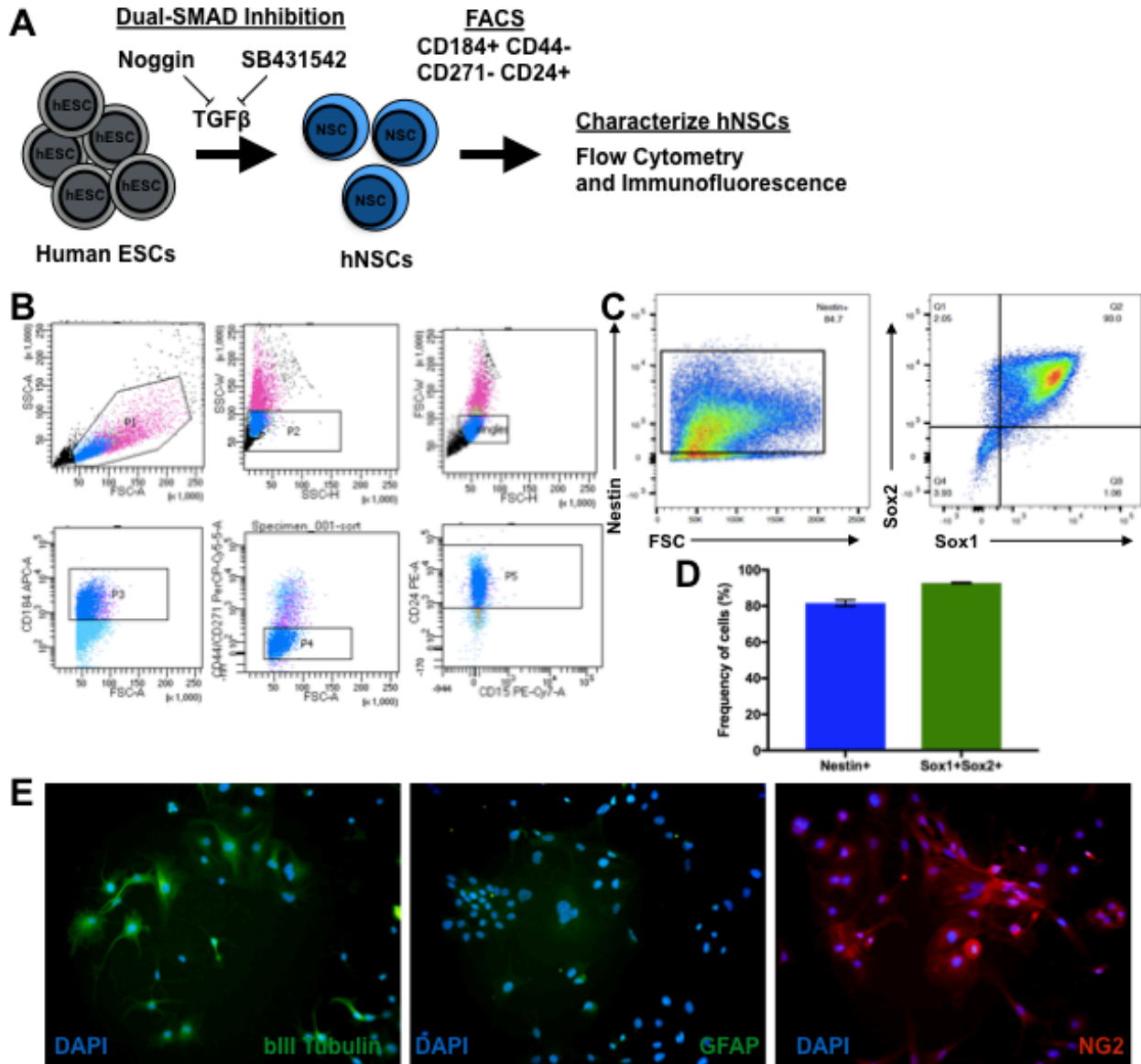




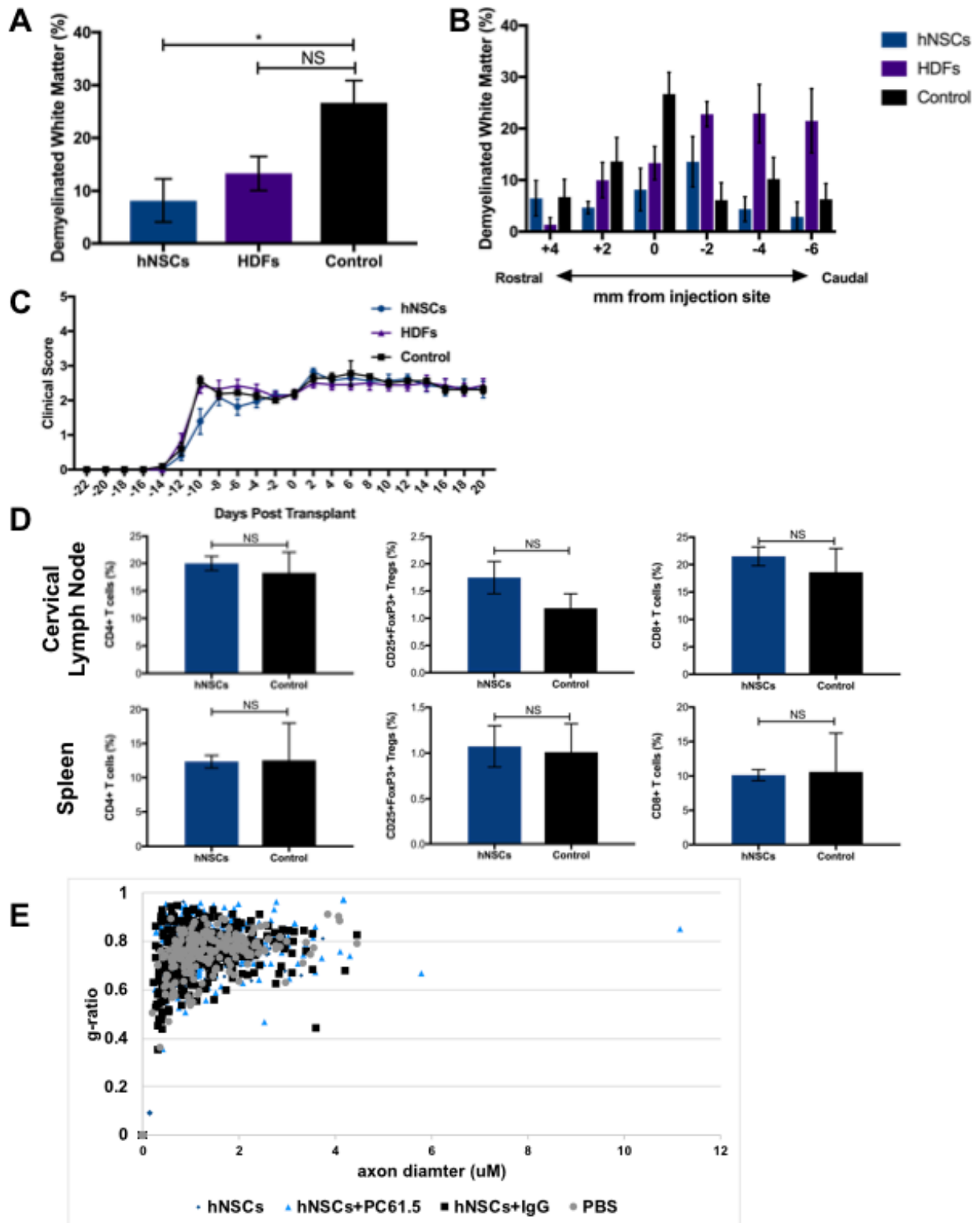
**Supplemental Figure 2.1. mouse neural stem cells engraft, migrate and remyelinated in an EAE mouse model of MS with no effect upon CNS immune environment.** **(A)** Representative serial sections of spinal cord rostral and caudal to the site of implantation (T9) show that GFP<sup>+</sup>mNSCs survive 28 days post-transplant and migrate towards areas of demyelination compared to PBS treated controls. **(B)** Representative brightfield images of coronal spinal cord sections stained with luxol fast blue (LFB) and counterstained with hematoxylin and eosin (H&E), areas of demyelinated white matter outlined in black. **(C)** Quantification of demyelination in the ventral white matter of GFP<sup>+</sup>mNSC and control PBS transplanted mice revealed significantly ( $p < 0.05$ ) reduced demyelination at sections caudal to the injection site. **(D)** Quantification of the frequency of adaptive immune cell populations CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD8<sup>+</sup> T cell subsets within the spinal cord, draining cervical lymph node, and spleen of GFP<sup>+</sup>mNSC or control PBS injected mice revealed no significant difference in cell populations at day 7 p.t. Data represents two independent experiments. For **(C)**, day 28 p.t. demyelinated white matter analysis GFP<sup>+</sup>mNSCs (n=12) and control PBS (n=13). Data is presented as average  $\pm$  SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. For **(D)**, day 7 p.t. analysis GFP<sup>+</sup>mNSCs (n=5) and control PBS (n=5). Data is presented as average  $\pm$  SEM and analyzed using a two-way ANOVA with a Sidak's multiple comparisons.



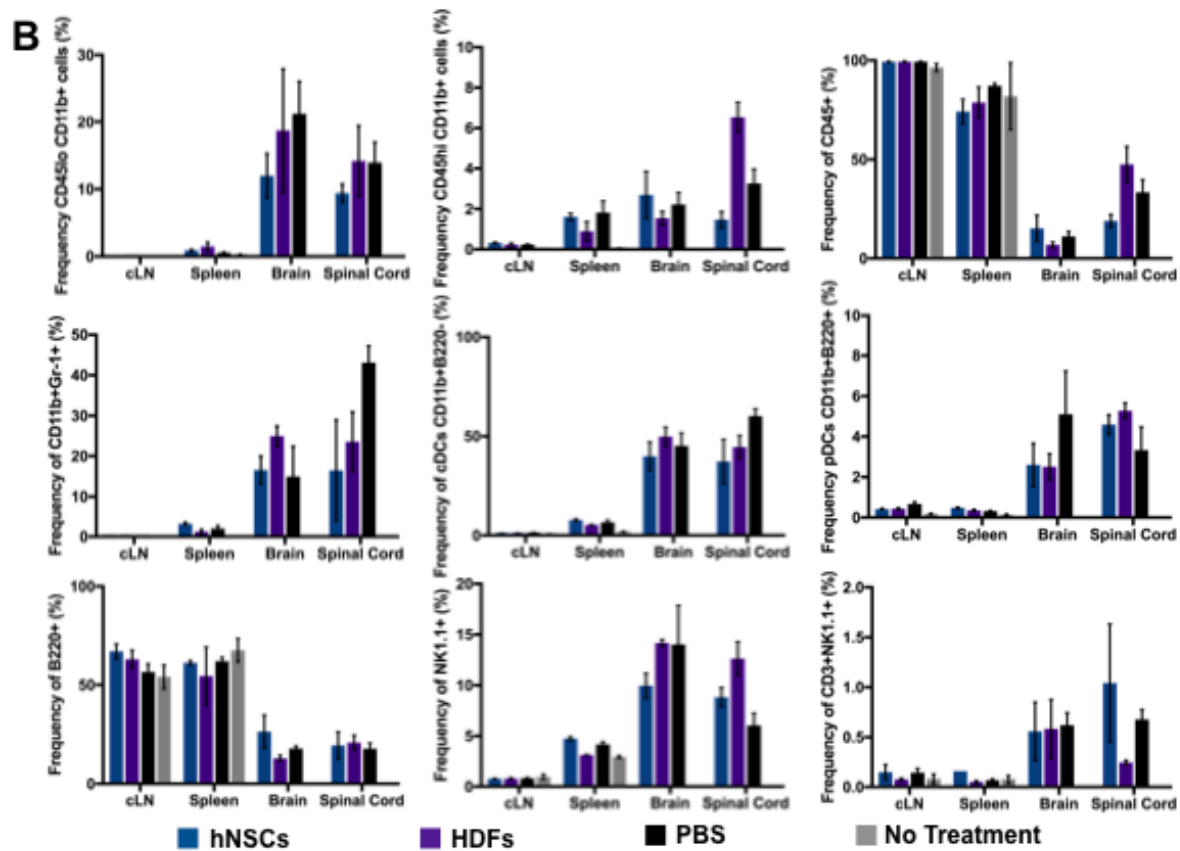
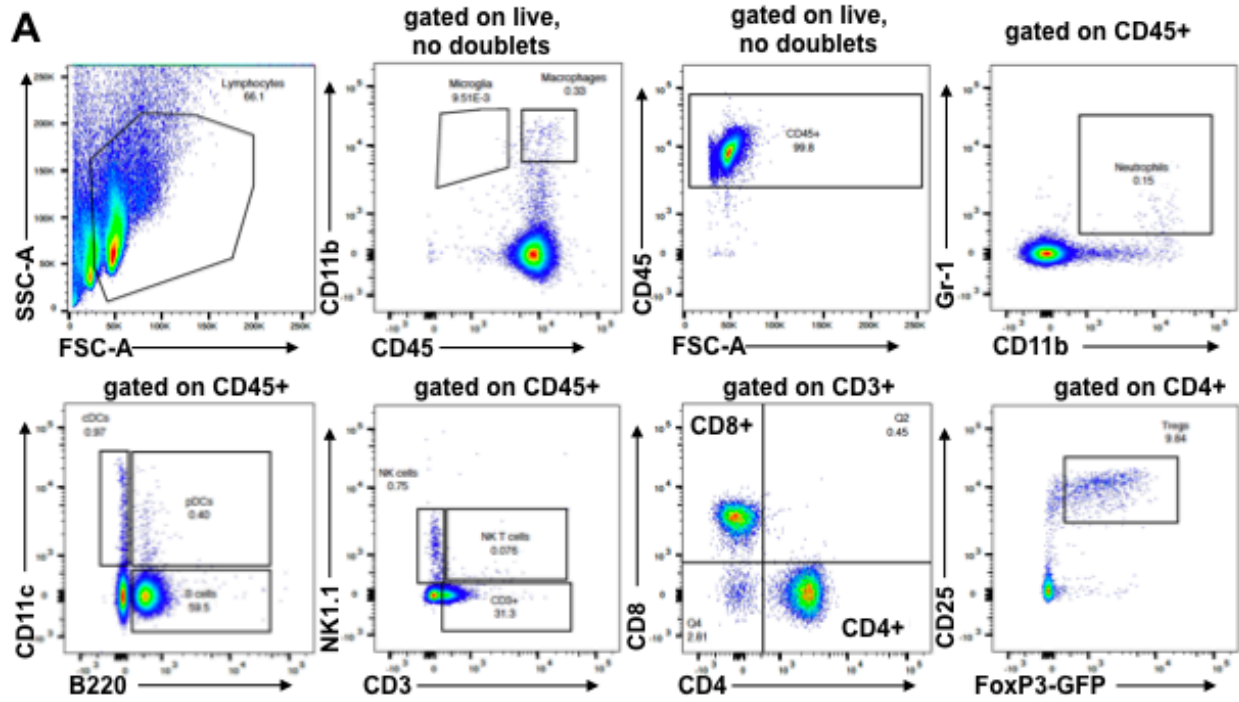
**Supplemental Figure 2.2. EB-derived human neural stem cells are multipotent and express neural lineage markers. (A)** Diagram depicting derivation and differentiation of hNSCs. **(B)** Representative FACS sort plots for CD184<sup>+</sup>CD44<sup>-</sup>CD271<sup>-</sup>CD24<sup>+</sup> hNSCs **(C)** FACS plots of sorted hNSCs for expression of NSC markers Nestin, Sox1 and Sox2. **(D)** Frequency of hNSCs expressing NSC makers Nestin (81.6%±2.91) and Sox1 and Sox2 (92.7%±2.91). Data is presented as average ± SEM. **(E)** Microscopy of differentiated hNSCs expressed markers of neurons ( $\beta$ III-tubulin; left), astrocytes (GFAP; middle) and oligodendrocytes (NG2; right). Magnification =20x.



**Supplemental Figure 2.3. hNSC, but not HDFs promote remyelination. (A)** Quantification of demyelination in the ventral white matter of hNSC, HDF and control PBS transplanted mice revealed significantly ( $p < 0.01$ ) reduced demyelination at the injection side in the spinal cords of hNSC but not HDF transplanted mice, 28 days p.t. **(B)** Quantification of demyelination in areas rostral and caudal to the site of injected revealed that reduced demyelination was not sustained throughout the spinal cord. For **(A and B)** Data represents two independent experiments. Data is presented as average  $\pm$  SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test **(C)** Graph of clinical scores of mice injected intraspinally with hNSCs (blue), HDFs (purple), or control PBS (black) at defined timepoints post-transplant. Clinical evaluation was performed double-blind and based on the following scoring system; 0, asymptomatic; 0.5, ruffled fur; 1, flaccid tail; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund. Data represents two independent experiments and is presented as average  $\pm$  SEM. No significant difference in locomotor function were observed. Data was analyzed using a two-way ANOVA. **(D)** Quantification of the frequency of adaptive immune cell populations  $CD4^+$ ,  $CD4^+CD25^+FoxP3^+$  and  $CD8^+$  T cell subsets within the spinal cord, draining cervical lymph node, and spleen of hNSC ( $n=3$ ) or control PBS ( $n=3$ ) injected mice revealed no significant difference in cell populations at day 28 p.t. Data represents two independent experiments. **(E)** Scatter plot displaying g-ratio of individual axons as a function of axonal diameter.

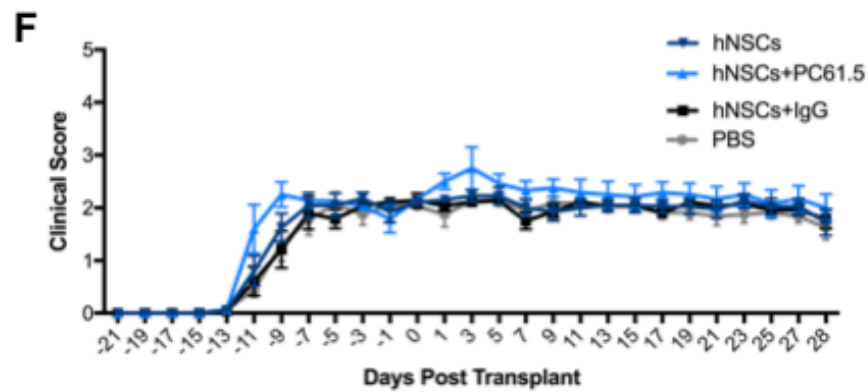
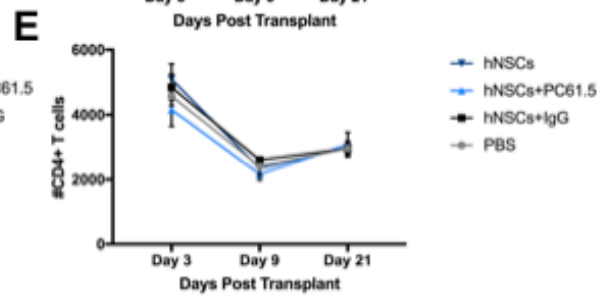
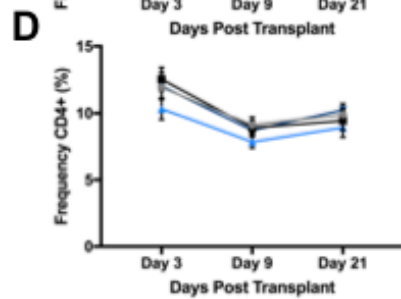
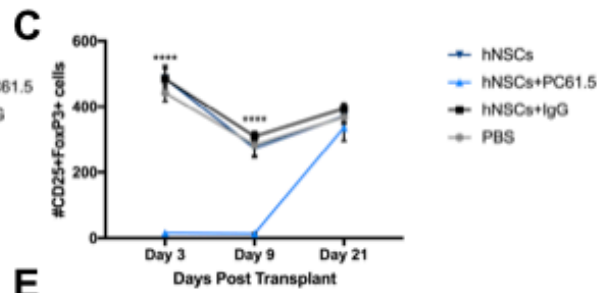
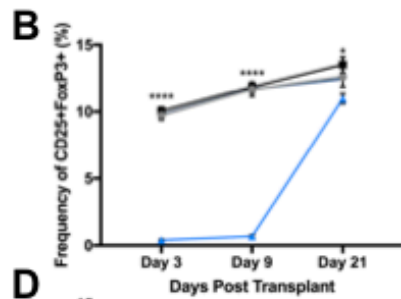
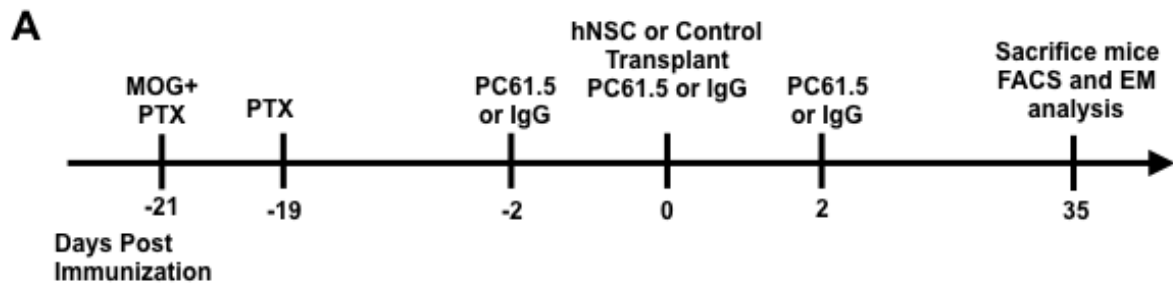


**Supplemental Figure 2.4. Immune cell populations are unaffected following hNSC Transplant.** (A) Representative FACS plots showing gating strategy for immune cells. (B) Quantification of the frequency and number of immune cell populations in cervical lymph nodes, spleen, spinal cord and brain of hNSC (n=6), HDF (n=6), PBS (n=6), or non-transplanted (n=6) control mice 7 days p.t. No difference in immune cell populations (CD45<sup>lo</sup> CD11b<sup>+</sup> Microglia, CD45<sup>hi</sup> CD11b<sup>+</sup> Macrophages, CD45<sup>+</sup>, CD11b<sup>+</sup>Gr-1<sup>+</sup> Neutrophils, CD11b<sup>+</sup>B220<sup>-</sup> cDCs, CD11b<sup>+</sup>B220<sup>+</sup> pDCs, B220<sup>+</sup> B cells, NK1.1<sup>+</sup> NK cells, CD3<sup>+</sup>NK1.1<sup>+</sup> NK T cells, or CD3<sup>+</sup> T cells) were observed. Data represents two independent experiments. Data is presented as average  $\pm$  SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test.

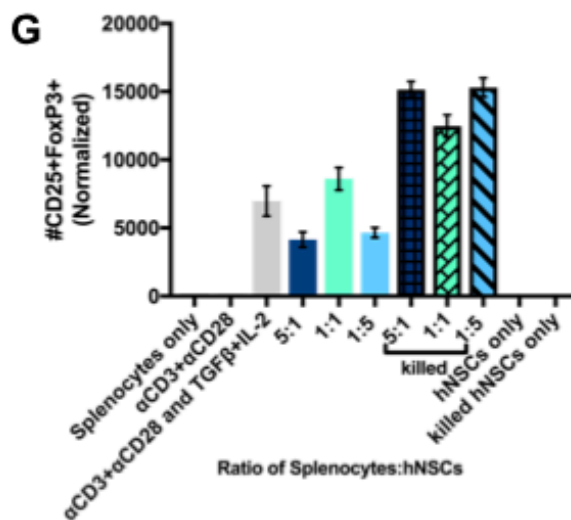
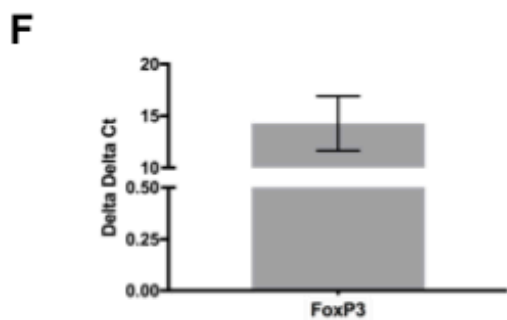
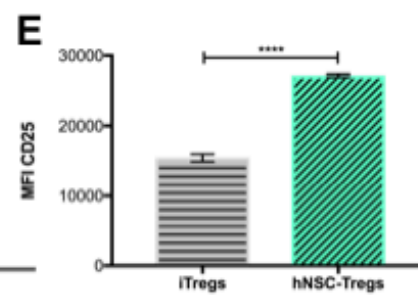
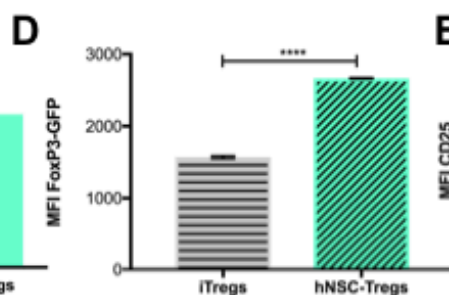
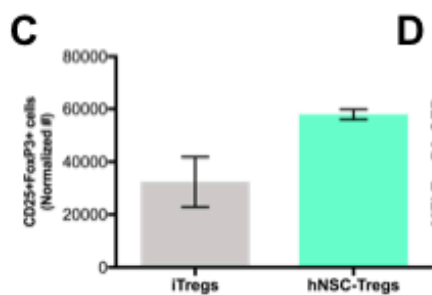
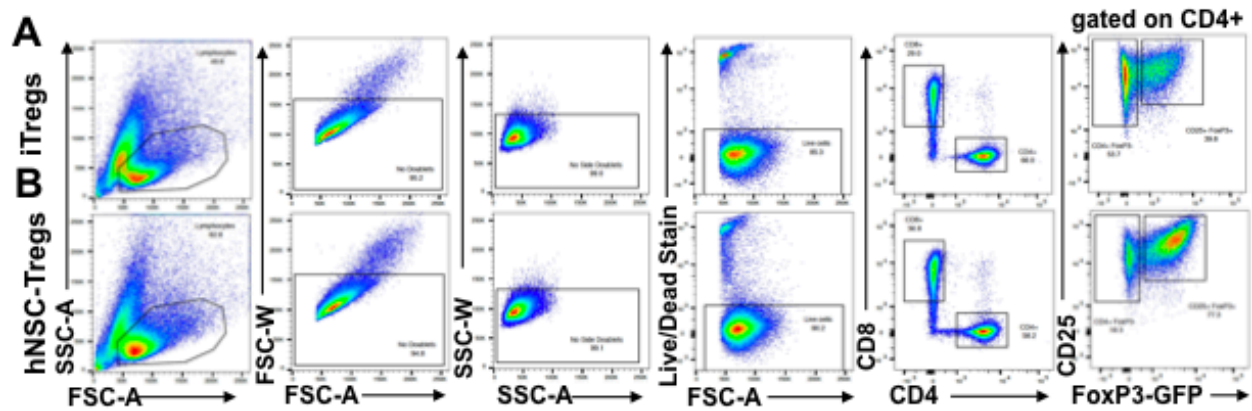


**Supplemental Figure 2.5. PC61.5 treatment of mice depleted Tregs without affecting Tconv cell population or EAE clinical score. (A)** Timeline of experiment. EAE mice received intraspinal transplant of hNSCs and intraperitoneal (i.p.) PBS, hNSCs and anti-CD25 (clone PC61.5) i.p., hNSCs and IgG i.p., or PBS as a control. Anti-CD25 antibody selectively depletes Tregs, IgG was given as an isotype control. EAE mice were administered anti-CD25 and IgG antibodies -2, 0, and 2 days p.t. of hNSCs. Quantification of frequency **(B)** and number **(C)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in peripheral blood 3, 9, and 21 days p.t. Quantification of frequency **(D)** and number **(E)** of CD4<sup>+</sup> Tconv cells in peripheral blood 3, 9, and 21 days p.t. **(F)** Graph of clinical scores of mice injected intraspinally with hNSCs (dark blue), hNSCs+PC61.5 (light blue), hNSCs+IgG (black), or PBS (grey) at defined timepoints post-transplant. PC61.5 treatment does not impact clinical score. Data is presented as average  $\pm$  SEM and analyzed using two-way ANOVA followed by Tukey's multiple comparison test (\* $p=0.01$ ; \*\*\*\* $p<0.0001$ ).



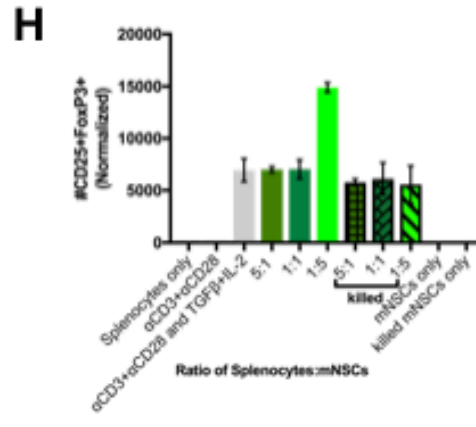
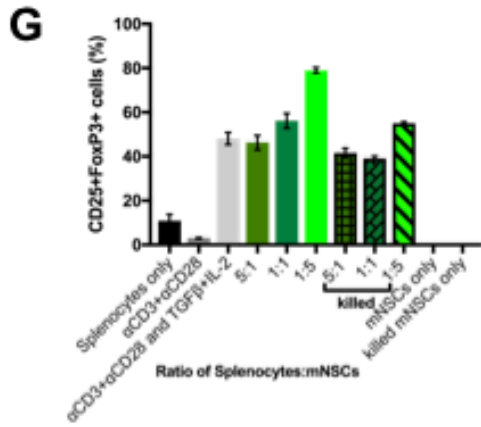
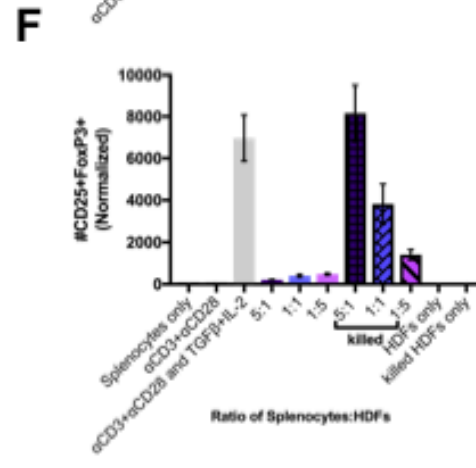
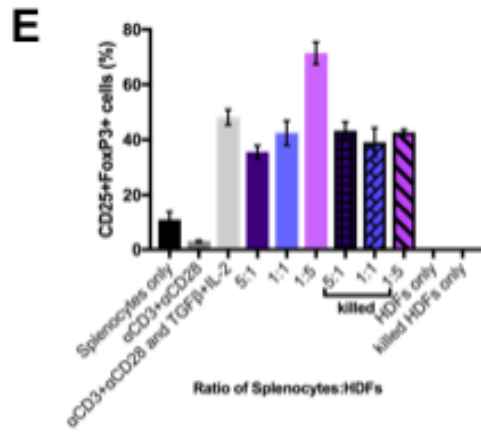
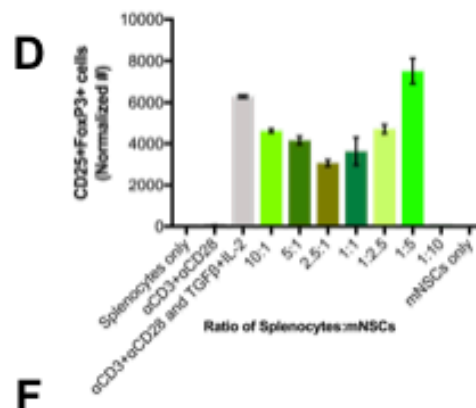
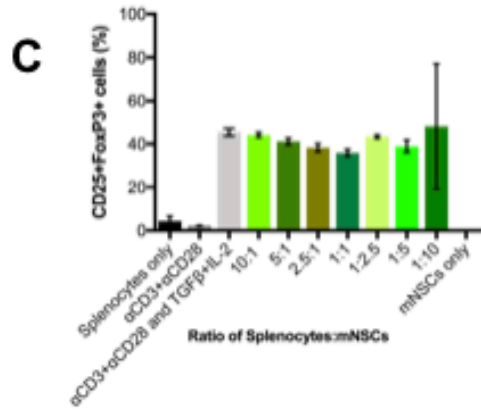
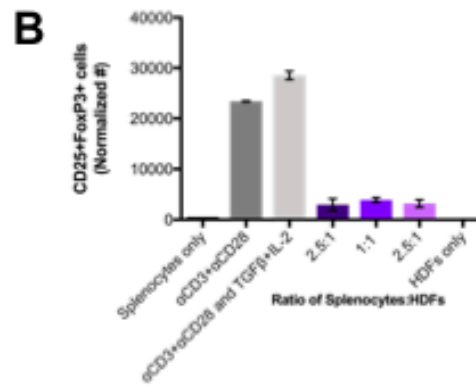
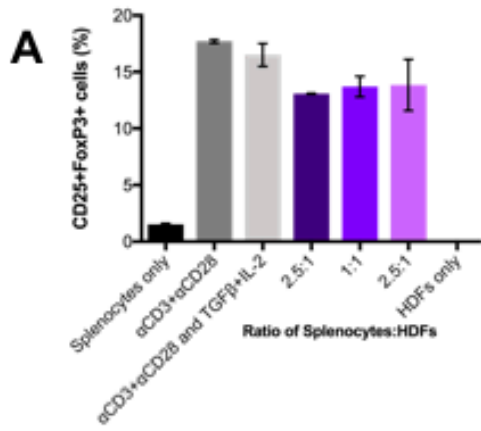


**Supplemental Figure 2.6. hNSCs expand Tregs *in vitro* and display greater expression of CD25 and FoxP3.** Naïve splenocytes were cultured at different ratios of splenocytes to hNSCs, HDFs, or GFP<sup>+</sup>mNSCs and activated in the presence of 1ug/mL  $\alpha$ -CD3, 1ug/mL  $\alpha$ -CD28, 10ng/mL TGF $\beta$  and 100U/mL IL-2. Representative FACS gating strategy for sorting iTregs **(A)** and hNSC-Tregs **(B)** from *in vitro* co-cultures. The frequency **(Fig 2D)** and number **(C)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs was significantly ( $p < 0.0001$ ) increased in the presence of hNSCs. A significant ( $p < 0.0001$ ) increase in the mean fluorescent intensity (MFI) of FoxP3 **(D)** and CD25 **(E)** of the CD4<sup>+</sup> cells in the presence of hNSCs was also observed. Data represents three independent experiments. data was analyzed using an unpaired, two-tailed T test and is presented as average  $\pm$  SEM. **(F)** qPCR analysis of sorted Tregs from co-cultures revealed that hNSC-Tregs express 15-fold higher levels of FoxP3. Co-cultures of naïve splenocytes and live or killed hNSCs revealed that killed hNSCs expanded percentage (Fig 2F) and number **(G)** of Tregs similar to live hNSCs.

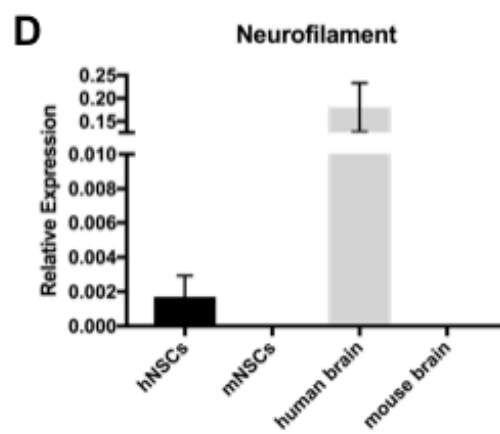
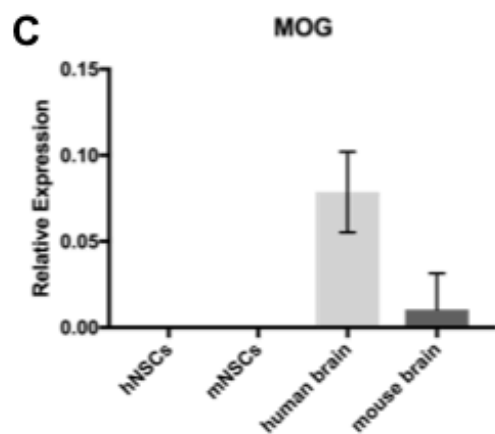
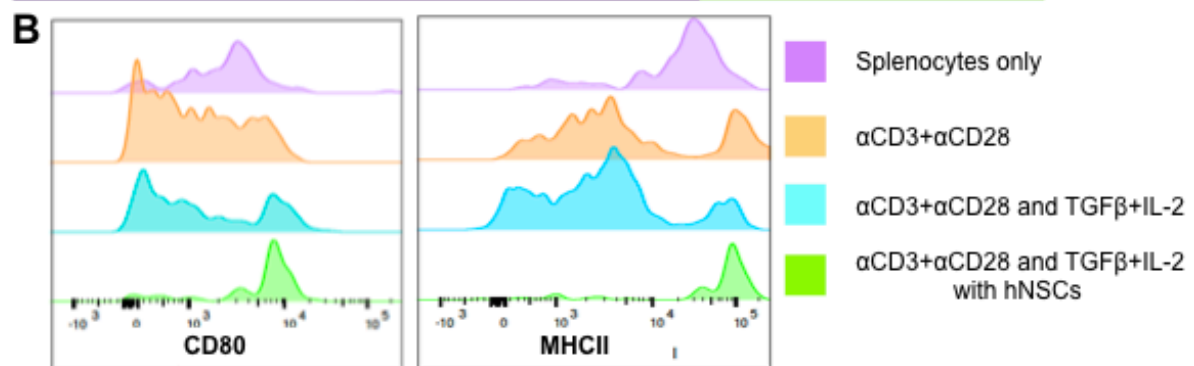
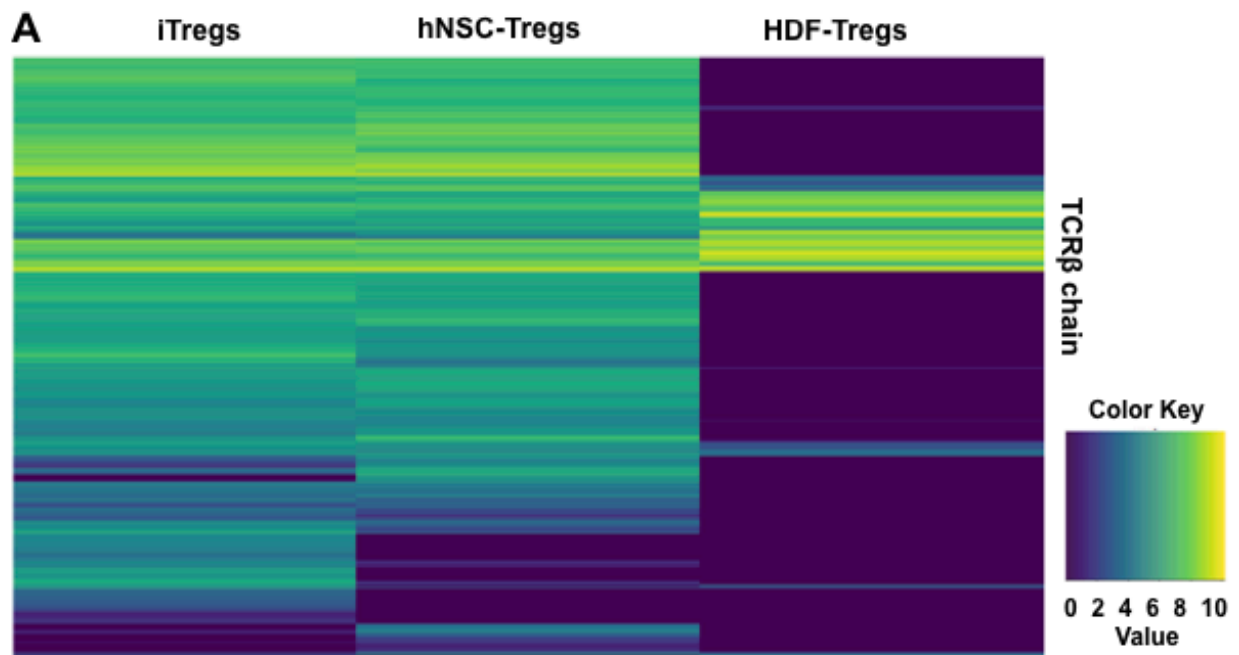


**Supplemental Figure 2.7. HDFs and mNSCs do not expand Tregs *in vitro*.**

Co-cultures with polyclonal B6 FoxP3<sup>EGFP</sup> splenocytes and HDFs resulted in no significant difference in the frequency **(A)** or number **(B)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs at ratios of splenocytes to HDFs (2.5:1, 1:1, or 2.5:1, respectively). Co-cultures with polyclonal B6 FoxP3<sup>EGFP</sup> splenocytes and mNSCs resulted in no significant difference in the frequency **(C)** or number **(D)** of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs at ratios of splenocytes to HDFs (2.5:1, 1:1, or 2.5:1, respectively). For **(A-D)** data are representative of two independent experiments n=3. Co-cultures of naïve B6 FoxP3<sup>EGFP</sup> splenocytes and live or killed HDFs or GFP-mNSCs revealed that killed have a similar, non-statistically significant, percentage **(E and F, respectively)** and number **(G and H, respectively)** of Tregs similar to live cells. For **(E-H)** Data is representative of three independent experiments, data is presented as average  $\pm$  SEM and analyzed using a one-way ANOVA with a Tukey's multiple comparisons test and is presented as average  $\pm$  SEM.



**Supplemental Figure 2.8. hNSC-Tregs recognize neurofilament antigens. (A)** TCR repertoire analysis of TCR  $\beta$  chains from sorted CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs from *in vitro* cultures of splenocytes under Treg skewing conditions 1 $\mu$ g/mL  $\alpha$ -CD3, 1 $\mu$ g/mL  $\alpha$ -CD28, 10ng/mL TGF $\beta$  and 100U/mL IL-2 (iTregs; left), with addition of hNSCs (hNSC-Tregs; middle), or with addition of HDFs (HDFs-Tregs; right) revealed a diverse TCR repertoire, with expanded clones unique to hNSC-Tregs. HDF-Tregs displayed a limited TCR repertoire. **(B)** FACS plots displaying higher levels of CD80 and MHCII expression on B220<sup>+</sup> cells from *in vitro* co-cultures splenocytes and hNSCs (green) compared to controls; splenocytes only (red), splenocytes activated in the presence of 1 $\mu$ g/mL  $\alpha$ -CD3, 1 $\mu$ g/mL  $\alpha$ -CD28 (blue), or splenocytes activated in the presence of Treg skewing conditions 1 $\mu$ g/mL  $\alpha$ -CD3, 1 $\mu$ g/mL  $\alpha$ -CD28, 10ng/mL TGF $\beta$  and 100U/mL IL-2 (orange). **(C)** Relative expression ( $2^{\Delta\Delta Ct}$ ) of myelin oligodendrocyte glycoprotein (MOG). qPCR analysis revealed that hNSCs and mNSCs do not express MOG. **(D)** Relative expression ( $2^{\Delta\Delta Ct}$ ) of neurofilament. qPCR analysis revealed that hNSCs, but not mNSCs express neurofilament.

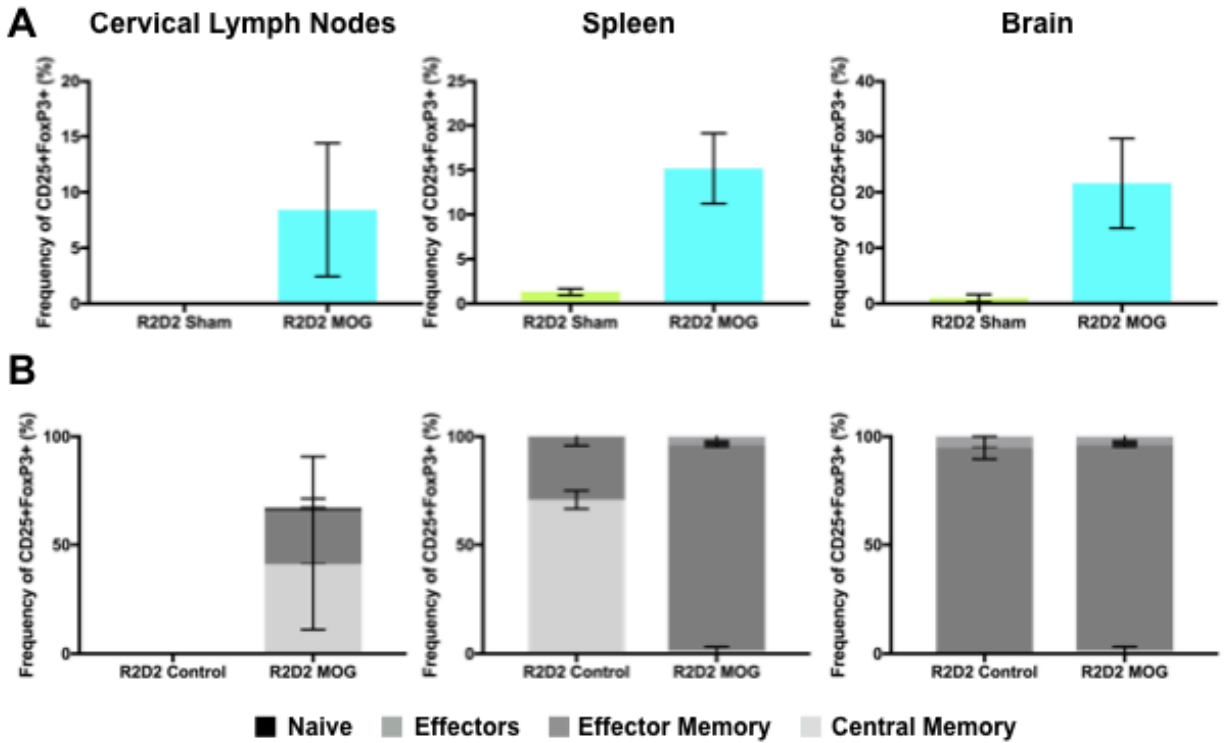


**Supplemental Figure 2.9. Neural antigen-specific T cells upregulate FoxP3 upon antigen exposure *in vivo*.** (A) Representative flow cytometry analysis of  $RAG2\gamma C^{-/-}2D2^{+}$  TCR transgenic splenic T cells, showing R2D2 mice lack  $CD4^{+}CD25^{+}FoxP3^{+}$  Tregs under homeostatic conditions in the periphery. (B) Representative FACS plots from  $CD4^{+}$  T cells isolated from cervical lymph nodes, spleen, and spinal cords of R2D2 mice sham immunized (R2D2 control; green) and MOG immunized counterparts (R2D2 MOG; blue). (C) Histograms of Helios expression in  $CD4^{+}CD25^{+}FoxP3^{+}$  T cells; FMO control for Helios- FITC in grey. Bar plots quantifying percentage of Helios expressing  $CD4^{+}CD25^{+}FoxP3^{+}$  T cells. Quantification of frequency (D) and absolute number (normalized) (E) of  $CD4^{+}CD25^{+}FoxP3^{+}$  T cells.





**Supplemental Figure 2.10. Following expansion in lymphopenic hosts neural antigen-specific T cells upregulate FoxP3 upon antigen exposure *in vivo*.** (A) Quantification of frequency of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells isolated from cervical lymph nodes (left), spleen (middle), or brain (right) from *RAG2 $\gamma$ c<sup>-/-</sup>* mice receiving adoptive transfer of R2D2 T cells, 35 days p.t. (B) Quantification of naïve (CD62L<sup>+</sup>CD44<sup>-</sup>), effector (CD62L<sup>-</sup>CD44<sup>int</sup>), effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>), or central memory (CD62L<sup>+</sup>CD44<sup>+</sup>) CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells isolated from cervical lymph nodes (left), spleen (middle), or brain (right) from *RAG2 $\gamma$ c<sup>-/-</sup>* mice receiving adoptive transfer of R2D2 T cells, 10 days p.t.



## **Acknowledgements**

We would like to thank the staff at the UCI Sue and Bill Gross Stem Cell Research Center, Jennifer Atwood at the UCI Institute for Immunology, Opal Allen and Brian Dailey at the University of Utah Genomics core, Melanie Oakes at the UCI Genomics Core, Chad Caraway and Ryan Bohannon at the UCI ADRC, and Jon Neumann and Shimako Kawauchi at the UCI TMF for technical support and use of core facilities. We would also like to thank Warren, Plaisted, Edna Hingco, Angel Zavala, Louis Parker, Jackie Mitchell and Collene Worne for technical support. We thank Stephen Schoenberger and Joey Lee for the generous contribution of *RAG1<sup>-/-</sup>OT-II<sup>+</sup>* mice. This work was supported by the California Institute for Regenerative Medicine (TR05603 to CMW), the National Multiple Sclerosis Society (CA1058 to CMW), and the National Institutes of Health (1T32NS082174 to LLM; 5R01NS092042 to TEL; 5R01AI121945 to MDC and CMW; 1RF1AG055524 to MBJ and CMW).

## **Author Contributions**

Conceptualization, L.L.M., S.A.G., J.H., S.O., Q.H.N., K.K, O.S., M.C., T.E.L. and C.M.W.; Methodology, L.L.M.; Software, J.H. and Q.N.; Investigation, L.L.M., S.A.G., J.H., S.O., Q.H.N., B.W., I.S., J.S., J.A., E.V., Q.P., K.D, and F.R.; Resources, S.O., M.D.C., and T.E.L., Resources, I.S., O.S., K.K., and M.B.J.; Writing- Original Draft, L.L.M.; Writing- Reviewing & Editing, L.L.M., S.A.G., J.H., S.O., M.D.C., T.E.L. and C.M.W; Funding Acquisition L.L.M., M.D.C., T.E.L., and C.M.W.

## References

Andrews, S. (2014). FastQC: a quality control tool for high throughput sequence data. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.

Barnden, M.J., Allison, J., Heath, W.R., and Carbone, F.R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76, 34-40.

Ben-Hur, T., Fainstein, N., and Nishri, Y. (2013). Cell-based reparative therapies for multiple sclerosis. *Curr Neurol Neurosci Rep* 13, 397.

Bettelli, E., Pagany, M., Weiner, H.L., Linington, C., Sobel, R.A., and Kuchroo, V.K. (2003). Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197, 1073-1081.

Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* 34, 525-527.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A.J., Benoist, C., *et al.* (2013). A special population of regulatory T cells potentiates muscle repair. *Cell* 155, 1282-1295.

Bushnell, B. (2018). BBDuk. Available online at: [sourceforge.net/projects/bbmap](https://sourceforge.net/projects/bbmap).

Carbajal, K.S., Schaumburg, C., Strieter, R., Kane, J., and Lane, T.E. (2010). Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 107, 11068-11073.

Carbajal, K.S., Weinger, J.G., Whitman, L.M., Schaumburg, C.S., and Lane, T.E. (2011). Surgical transplantation of mouse neural stem cells into the spinal cords of mice infected with neurotropic mouse hepatitis virus. *J Vis Exp*, e2834.

Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., and Ploix, C.C. (2006). CNS immune privilege: hiding in plain sight. *Immunol Rev* 213, 48-65.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27, 275-280.

Chauhan, S.K., Saban, D.R., Lee, H.K., and Dana, R. (2009). Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. *J Immunol* 182, 148-153.

Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, C.M., Sears-Kraxberger, I., Steward, O., Macklin, W.B., Loring, J.F., *et al.* (2014). Human neural precursor cells

promote neurologic recovery in a viral model of multiple sclerosis. *Stem Cell Reports* 2, 825-837.

Constantinescu, C.S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* 164, 1079-1106.

Feuerer, M., Hill, J.A., Kretschmer, K., von Boehmer, H., Mathis, D., and Benoist, C. (2010). Genomic definition of multiple *ex vivo* regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A* 107, 5919-5924.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005). Regulatory T cell lineage specification by the forkhead transcription factor *foxp3*. *Immunity* 22, 329-341.

Gaud, G., Lesourne, R., and Love, P.E. (2018). Regulatory mechanisms in T cell receptor signalling. *Nat Rev Immunol* 18, 485-497.

Gehrmann, J., Matsumoto, Y., and Kreutzberg, G.W. (1995). Microglia: intrinsic immuneffector cell of the brain. *Brain Res Brain Res Rev* 20, 269-287.

Giera, S., Luo, R., Ying, Y., Ackerman, S.D., Jeong, S.J., Stoveken, H.M., Folts, C.J., Welsh, C.A., Tall, G.G., Stevens, B., *et al.* (2018). Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells. *Elife* 7.

Greenberg, M.L., Weinger, J.G., Matheu, M.P., Carbajal, K.S., Parker, I., Macklin, W.B., Lane, T.E., and Cahalan, M.D. (2014). Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 111, E2349-2355.

Gundemir, S., Colak, G., Tucholski, J., and Johnson, G.V. (2012). Transglutaminase 2: a molecular Swiss army knife. *Biochim Biophys Acta* 1823, 406-419.

Haas, J., Fritzsching, B., Trubswetter, P., Korporal, M., Milkova, L., Fritz, B., Vobis, D., Krammer, P.H., Suri-Payer, E., and Wildemann, B. (2007). Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol* 179, 1322-1330.

Haribhai, D., Lin, W., Edwards, B., Ziegelbauer, J., Salzman, N.H., Carlson, M.R., Li, S.H., Simpson, P.M., Chatila, T.A., and Williams, C.B. (2009). A central role for induced regulatory T cells in tolerance induction in experimental colitis. *J Immunol* 182, 3461-3468.

Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). *Foxp3* transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27, 786-800.

Horwitz, D.A., Zheng, S.G., Wang, J., and Gray, J.D. (2008). Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. *Eur J Immunol* 38, 912-915.

Huehn, J., Siegmund, K., Lehmann, J.C., Siewert, C., Haubold, U., Feuerer, M., Debes, G.F., Lauber, J., Frey, O., Przybylski, G.K., *et al.* (2004). Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med* 199, 303-313.

Huss, D.J., Pellerin, A.F., Collette, B.P., Kannan, A.K., Peng, L., Datta, A., Wipke, B.T., and Fontenot, J.D. (2016). Anti-CD25 monoclonal antibody Fc variants differentially impact regulatory T cells and immune homeostasis. *Immunology* 148, 276-286.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162, 5317-5326.

Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Hohenbeck, A.E., Lerman, M.A., Naji, A., and Caton, A.J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2, 301-306.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564.

Kipnis, J., Avidan, H., Caspi, R.R., and Schwartz, M. (2004). Dual effect of CD4+CD25+ regulatory T cells in neurodegeneration: a dialogue with microglia. *Proc Natl Acad Sci U S A* 101 Suppl 2, 14663-14669.

Kolde, R. (2018). Pheatmap: pretty heatmaps. R package version 1.0.10. Available online at: <https://CRAN.R-project.org/package=pheatmap>.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M.C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6, 1219-1227.

Kretschmer, K., Heng, T.S., and von Boehmer, H. (2006). De novo production of antigen-specific suppressor cells in vivo. *Nat Protoc* 1, 653-661.

Kumar, M., Putzki, N., Limmroth, V., Remus, R., Lindemann, M., Knop, D., Mueller, N., Hardt, C., Kreuzfelder, E., and Grosse-Wilde, H. (2006). CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *J Neuroimmunol* 180, 178-184.

Lane, T.E., Liu, M.T., Chen, B.P., Asensio, V.C., Samawi, R.M., Paoletti, A.D., Campbell, I.L., Kunkel, S.L., Fox, H.S., and Buchmeier, M.J. (2000). A central role for

CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J Virol* 74, 1415-1424.

Lassmann, H., Bruck, W., Lucchinetti, C., and Rodriguez, M. (1997). Remyelination in multiple sclerosis. *Mult Scler* 3, 133-136.

Li, M.O., and Rudensky, A.Y. (2016). T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat Rev Immunol* 16, 220-233.

Liu, M.T., Keirstead, H.S., and Lane, T.E. (2001). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol* 167, 4091-4097.

Liu, Y., Teige, I., Birnir, B., and Issazadeh-Navikas, S. (2006). Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. *Nat Med* 12, 518-525.

Loomis, C.W., Milne, B., and Cervencko, F.W. (1987). Determination of cross tolerance in rat spinal cord using intrathecal infusion via sequential mini-osmotic pumps. *Pharmacol Biochem Behav* 26, 131-139.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550.

Lu, B., Kwan, T., Kurimoto, Y., Shatos, M., Lund, R.D., and Young, M.J. (2002). Transplantation of EGF-responsive neurospheres from GFP transgenic mice into the eyes of rd mice. *Brain Res* 943, 292-300.

Lucca, L.E., Desbois, S., Ramadan, A., Ben-Nun, A., Eisenstein, M., Carrie, N., Guery, J.C., Sette, A., Nguyen, P., Geiger, T.L., *et al.* (2014). Bispecificity for myelin and neuronal self-antigens is a common feature of CD4 T cells in C57BL/6 mice. *J Immunol* 193, 3267-3277.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., *et al.* (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* 13, 133-140.

Matheu, M.P., Othy, S., Greenberg, M.L., Dong, T.X., Schuijs, M., Deswarte, K., Hammad, H., Lambrecht, B.N., Parker, I., and Cahalan, M.D. (2015). Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. *Nat Commun* 6, 6219.

McCarthy, D.P., Richards, M.H., and Miller, S.D. (2012). Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Biol* 900, 381-401.

Meister, M., Papatriantafyllou, M., Nordstrom, V., Kumar, V., Ludwig, J., Lui, K.O., Boyd, A.S., Popovic, Z.V., Fleming, T.H., Moldenhauer, G., *et al.* (2015). Dickkopf-3, a tissue-derived modulator of local T-cell responses. *Front Immunol* 6, 78.

Mozafari, S., Laterza, C., Roussel, D., Bachelin, C., Marteyn, A., Deboux, C., Martino, G., and Baron-Van Evercooren, A. (2015). Skin-derived neural precursors competitively generate functional myelin in adult demyelinated mice. *J Clin Invest* 125, 3642-3656.

Munji, R.N., Choe, Y., Li, G., Siegenthaler, J.A., and Pleasure, S.J. (2011). Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors. *J Neurosci* 31, 1676-1687.

Nakamura, R.E., Hunter, D.D., Yi, H., Brunken, W.J., and Hackam, A.S. (2007). Identification of two novel activities of the Wnt signaling regulator Dickkopf 3 and characterization of its expression in the mouse retina. *BMC Cell Biol* 8, 52.

Niehhs, C. (2006). Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 25, 7469-7481.

Nosbaum, A., Prevel, N., Truong, H.A., Mehta, P., Ettinger, M., Scharschmidt, T.C., Ali, N.H., Pauli, M.L., Abbas, A.K., and Rosenblum, M.D. (2016). Cutting Edge: Regulatory T Cells Facilitate Cutaneous Wound Healing. *J Immunol* 196, 2010-2014.

Plaisted, W.C., Zavala, A., Hingco, E., Tran, H., Coleman, R., Lane, T.E., Loring, J.F., and Walsh, C.M. (2016). Remyelination Is Correlated with Regulatory T Cell Induction Following Human Embryoid Body-Derived Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis. *PLoS One* 11, e0157620.

Pluchino, S., Gritti, A., Blezer, E., Amadio, S., Brambilla, E., Borsellino, G., Cossetti, C., Del Carro, U., Comi, G., t Hart, B., *et al.* (2009). Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. *Ann Neurol* 66, 343-354.

Putnam, A.L., Brusko, T.M., Lee, M.R., Liu, W., Szot, G.L., Ghosh, T., Atkinson, M.A., and Bluestone, J.A. (2009). Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58, 652-662.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47.

Rosenblum, M.D., Way, S.S., and Abbas, A.K. (2016). Regulatory T cell memory. *Nat Rev Immunol* 16, 90-101.

Rosso, S.B., and Inestrosa, N.C. (2013). WNT signaling in neuronal maturation and synaptogenesis. *Front Cell Neurosci* 7, 103.



Rubtsov, Y.P., Niec, R.E., Josefowicz, S., Li, L., Darce, J., Mathis, D., Benoist, C., and Rudensky, A.Y. (2010). Stability of the regulatory T cell lineage in vivo. *Science* 329, 1667-1671.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr., *et al.* (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28, 546-558.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006). Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212, 8-27.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164.

Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., and Anderson, A.J. (2010). Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. *PLoS One* 5, e12272.

Salzman, G.S., Ackerman, S.D., Ding, C., Koide, A., Leon, K., Luo, R., Stoveken, H.M., Fernandez, C.G., Tall, G.G., Piao, X., *et al.* (2016). Structural Basis for Regulation of GPR56/ADGRG1 by Its Alternatively Spliced Extracellular Domains. *Neuron* 91, 1292-1304.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3, 1101-1108.

Schneider, V.A., Graves-Lindsay, T., Howe, K., Bouk, N., Chen, H.C., Kitts, P.A., Murphy, T.D., Pruitt, K.D., Thibaud-Nissen, F., Albracht, D., *et al.* (2017). Evaluation of GRCh38 and de novo haploid genome assemblies demonstrates the enduring quality of the reference assembly. *Genome Res* 27, 849-864.

Setiady, Y.Y., Coccia, J.A., and Park, P.U. (2010). In vivo depletion of CD4<sup>+</sup>FOXP3<sup>+</sup> Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcγRIII<sup>+</sup> phagocytes. *Eur J Immunol* 40, 780-786.

Singh, K., Hjort, M., Thorvaldson, L., and Sandler, S. (2015). Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naive mice. *Sci Rep* 5, 7767.

Soneson, C., Love, M.I., and Robinson, M.D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res* 4, 1521.

Sprent, J., and Surh, C.D. (2011). Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. *Nat Immunol* 12, 478-484.

- Steinman, L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* 85, 299-302.
- Stewart, J.J., Lee, C.Y., Ibrahim, S., Watts, P., Shlomchik, M., Weigert, M., and Litwin, S. (1997). A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol Immunol* 34, 1067-1082.
- Stoveken, H.M., Bahr, L.L., Anders, M.W., Wojtovich, A.P., Smrcka, A.V., and Tall, G.G. (2016). Dihydromunduletone Is a Small-Molecule Selective Adhesion G Protein-Coupled Receptor Antagonist. *Mol Pharmacol* 90, 214-224.
- Svensson, M., and Agace, W.W. (2006). Role of CCL25/CCR9 in immune homeostasis and disease. *Expert Rev Clin Immunol* 2, 759-773.
- Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199, 1455-1465.
- Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and Shevach, E.M. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184, 3433-3441.
- Tischner, D., Wiegers, G.J., Fiegl, H., Drach, M., and Villunger, A. (2012). Mutual antagonism of TGF-beta and Interleukin-2 in cell survival and lineage commitment of induced regulatory T cells. *Cell Death Differ* 19, 1277-1287.
- Torkildsen, O., Brunborg, L.A., Myhr, K.M., and Bo, L. (2008). The cuprizone model for demyelination. *Acta Neurol Scand Suppl* 188, 72-76.
- Trajkovic, V., Vuckovic, O., Stosic-Grujicic, S., Miljkovic, D., Popadic, D., Markovic, M., Bumbasirevic, V., Backovic, A., Cvetkovic, I., Harhaji, L., *et al.* (2004). Astrocyte-induced regulatory T cells mitigate CNS autoimmunity. *Glia* 47, 168-179.
- Van Strien, M.E., Baron, W., Bakker, E.N., Bauer, J., Bol, J.G., Breve, J.J., Binnekade, R., Van Der Laarse, W.J., Drukarch, B., and Van Dam, A.M. (2011). Tissue transglutaminase activity is involved in the differentiation of oligodendrocyte precursor cells into myelin-forming oligodendrocytes during CNS remyelination. *Glia* 59, 1622-1634.
- Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J.L., and Stinissen, P. (2008). Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* 180, 6411-6420.
- Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 199, 971-979.

- Vogelpoel, L.T., Baeten, D.L., de Jong, E.C., and den Dunnen, J. (2015). Control of cytokine production by human fc gamma receptors: implications for pathogen defense and autoimmunity. *Front Immunol* 6, 79.
- Walker, L.S., Chodos, A., Eggena, M., Doods, H., and Abbas, A.K. (2003). Antigen-dependent proliferation of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in vivo. *J Exp Med* 198, 249-258.
- Weinger, J.G., Greenberg, M.L., Matheu, M.P., Parker, I., Walsh, C.M., Lane, T.E., and Cahalan, M.D. (2015). Two-photon imaging of cellular dynamics in the mouse spinal cord. *J Vis Exp*.
- Wickham, H. (2016) *ggplot2: Elegant graphics for data analysis*. Springer-Verlag.
- Yuan, S.H., Martin, J., Elia, J., Flippin, J., Paramban, R.I., Hefferan, M.P., Vidal, J.G., Mu, Y., Killian, R.L., Israel, M.A., *et al.* (2011). Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. *PLoS One* 6, e17540.
- Zerbino, D.R., Achuthan, P., Akanni, W., Amode, M.R., Barrell, D., Bhai, J., Billis, K., Cummins, C., Gall, A., Giron, C.G., *et al.* (2018). Ensembl 2018. *Nucleic Acids Res* 46, D754-D761.
- Zhou, X., Bailey-Bucktrout, S.L., Jeker, L.T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W., and Bluestone, J.A. (2009). Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* 10, 1000-1007.
- Ziegler, S.F. (2006). FOXP3: of mice and men. *Annu Rev Immunol* 24, 209-226.

## CHAPTER THREE

### **Visualizing regulatory T cell and neural stem cell dynamics within the murine spinal cord**

Laura L. McIntyre\*, Shivashankar Othy\*, Michael D. Cahalan and Craig M. Walsh

\*These authors contributed equally to the manuscript.

## **Abstract**

Multiple Sclerosis (MS) is a debilitating, autoimmune disease of the central nervous system for which there is no cure. Defects in neurological function are a result of demyelination and axonal loss caused by infiltrating immune cells. Transplantation of neural stem cells (NSCs) are a promising therapeutic strategy to treat MS. Previously, we have shown that transplantation of human NSCs leads to an increase in regulatory T cells (Tregs) in the spinal cord during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Tregs are able to modulate inflammatory immune cells responses. Interactions between transplanted hNSCs and their impact upon endogenous Tregs and myelin producing cells, oligodendrocytes remains limited. Utilizing two-photon microscopy of spinal cord explains we characterized the *in-situ* behavior of endogenous Tregs and proteolipid protein (PLP<sup>+</sup>) cells in non-transplanted or hNSC- transplanted mice during EAE. Tregs motility dynamics within the spinal cord were not altered following hNSC-transplant, however Tregs localized to sites of hNSC transplant and interacted with PLP<sup>+</sup> cells. Our data provide insight into cellular interactions following hNSC-transplant in EAE, and support a role for Tregs in immune modulation and potentiators of tissue repair.

## **Introduction**

Multiple Sclerosis (MS) is a chronic, inflammatory, autoimmune disease that affects the central nervous system (CNS). Defective neurological function is a result of demyelination and axonal loss caused by infiltrating immune cells (Steinman, 1996). MS is the leading cause of non-traumatic disability in young adults; affecting approximately 400,000 people in the United States and more than 2.3 million people worldwide (McFarlin and McFarland, 1982). During progression of disease, a breakdown in central tolerance occurs, allowing autoreactive T cells to disrupt the blood brain barrier (BBB) and gain access to the CNS where they attack the myelin sheath surrounding the axons of neurons, as well as myelin producing cells, oligodendrocytes. Regulatory T cells that are important for maintaining immunological tolerance are dysfunctional in patients with MS permeating disease progression (Josefowicz et al., 2012; Sakaguchi et al., 1995). This autoimmune attack ultimately results in neuronal loss and a defect in neurological function. Patients with MS experience perturbations in sensation, motor, autonomic, visual and cognitive symptoms.

Currently, there are no effective therapies for progressive forms of MS. Approved disease-modifying therapies (DMTs) all limit immune cell infiltration into the CNS thereby inhibiting ongoing demyelination. These treatments are approved for use in patients with the mildest form of MS, Relapsing-Remitting MS (RRMS), but do not provide long-term relief for progressive forms of MS, where spontaneous remyelination is not sustained. Consequently, there is an unmet clinical need for a method to promote remyelination while limiting immune cell infiltration into the CNS. Ultimately, remyelination and repair of

damaged axons requires oligodendrocytes derived from neural stem and progenitor cells (NSC or NPCs).

Transplantation of neural stem cells (NSCs) is a promising therapeutic strategy for treating MS (Martino et al., 2010). NSCs are comprised of a mixed population of self-renewing cells and cells poised to differentiate into the 3 cell types found in the CNS; astrocytes, oligodendrocytes, and neurons. NSCs and their derivatives have the potential to replace the cells that are damaged during MS, and they can also promote regeneration by facilitating endogenous repair pathways (Ben-Hur et al., 2013). Transplantation of NSCs into mouse models of severe dysmyelination were shown to preferentially differentiate into oligodendrocytes and promote myelination (Mozafari et al., 2015). Additionally, human NSCs have been shown to differentiate and promote locomotor recovery in animal models of chronic spinal cord injury, and show promising results in phase I/II clinical trials (Salazar et al., 2010). Furthermore, peripheral administration of hNPCs in a non-human primate experimental autoimmune encephalomyelitis (EAE) model reduces disease severity through immune regulation (Pluchino et al., 2009).

Utilizing a viral mouse model demyelination, our lab has previously shown recovery when JHMV-infected mice received an intra-spinal transplant of syngeneic mNSCs, human embryonic stem cell (hESC) or induced pluripotent stem cell (iPSC) derived NPCs. Transplantation of mNSCs into JHMV-infected mice results in migration, proliferation, and differentiation of transplanted cells, suggesting recovery is due to cell replacement (Carbajal et al., 2010; Carbajal et al., 2011). In animals receiving hNPCs, recovery was not a result of cell replacement by NPCs because transplanted cells underwent rapid xenograft rejection (Chen et al., 2014b; Plaisted et al., 2016). Recovery was associated

with remyelination, a decrease in neuroinflammation, and an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells (Tregs). Using another model of immune mediated demyelination, EAE, we recently reported similar findings as in the viral model (McIntyre et al., 2018, submitted). EAE mice transplanted with mNSCs displayed remyelination with little effect upon the immune system. Mice receiving hNSC transplants displayed remyelination and decreased neuroinflammation, and an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. We found that Tregs are essential for promoting remyelination through their interactions with endogenous myelin producing cells, oligodendrocytes. However, the interactions between Tregs and oligodendrocytes is not well understood.

Two-photon microscopy enables real-time visualization of cellular interactions within intact organs (Germain et al., 2012). Previously our lab has developed a method to visualize NPCs and CNS resident cells within the intact spinal cord *ex vivo* (Weinger et al., 2015). Using 2P, we observed that mNSCs engrafted into JHMV-infected spinal cords and preferentially accumulated within areas of axonal damage and initiated remyelination (Greenberg et al., 2014b). Interactions between transplanted hNSCs and endogenous CNS and immune cells have never been visualized in real time. Here we report that following hNSC transplantation in to EAE immunized mice, Tregs home to the site of hNSC injection and interact with endogenous cells expressing proteolipid protein (PLP), which is expressed in oligodendrocytes. These data indicate that hNSCs facilitate endogenous repair through Treg and oligodendrocyte interactions.



## Methods

Animal care and EAE Immunization All experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee. C57BL/6 B6 FoxP3<sup>EGFP</sup> mice were obtained from Jackson Laboratories (B6.Cg-Foxp3<sup>tm2(EGFP)Tch</sup>/J, Stock No: 006772) (Lin et al., 2007). FoxP3<sup>EGFP</sup> mice were crossed with Ai-14 mice LSL-TdT<sup>+</sup> (B6.Cg-Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hze</sup>/J, Stock No: 007914) mice to generate FoxP3-eGFP<sup>+</sup> LSL-TdT<sup>+</sup>. Resulting F1 homozygote females (FoxP3-eGFP<sup>+/+</sup>LSL-TdT<sup>+/+</sup>) were bred to PLP/creER (B6.Cg-tg(Plp1-cre/ERT)3Pop/J, Stock No: 005975) males to generate FoxP3-GFP<sup>+</sup>PLP-TdT<sup>+</sup> mice, and >8 week old males were immunized with 100 $\mu$ l of emulsion containing 100 $\mu$ g of myelin oligodendrocyte glycoprotein MOG<sub>35-55</sub> (MEVGWYRSPFSRVVHLYRNGK-COOH; Pierce) in PBS, with complete Freund's adjuvant (CFA) containing 200 $\mu$ g *Mycobacterium tuberculosis* H37Ra (DIFCO Laboratories) subcutaneously. Mice received intraperitoneal injections of 400 $\eta$ g of Bordetella pertussis toxin (List Biological Laboratories) on day 0 and 2 p.i. Clinical evaluation was performed double-blind and based on the following scoring system; 0, asymptomatic; 0.5, ruffled fur; 1, flaccid tail; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund. Mice were sacrificed via inhalation of a lethal dose of isoflurane and cardiac perfusion with PBS was performed at 26 days p.t. for tissue harvesting and analysis.

hNSC cell culture hNSCs were generated from WA09 hESCs as previously described according to established methods (Chambers et al., 2009; Yuan et al., 2011). hNSCs were cultured on Geltrex<sup>TM</sup>- coated 6- well tissue culture treated plates (Thermo Fisher)

in hNSC maintenance medium (DMEM/F12+ GlutaMAX, 0.5X N2, 0.5X B27 without vitamin A, 20ng/mL bFGF; all from Thermo Fisher). Cells were split using Accutase (ThermoFisher) when cell density reached 80-90%.

Transplantation of hNSCs Mice immunized with MOG were injected with  $2.5 \times 10^5$  hNSCs in 2.5 $\mu$ l of PBS (hNSC-transplanted) or received no transplant (non-transplanted), at thoracic vertebrae 9 (T9) on day 21 p.i. as previously described (Carbajal et al., 2011).

Ex vivo spinal cord preparation, Two-photon Imaging and Analysis Two photon imaging was performed using a custom-built 2-photon system based on an Olympus BX51 upright microscope as previously described (Matheu et al., 2015), fitted with a water-immersion objective, and equipped with 3 PMTs and excitation generated by a tunable Chameleon femtosecond laser (Coherent). Explanted spinal cords were imaged with a laser set to 920nm for optimal excitation of GFP and TdT. 495 nm and 560 nm dichroic filters were arranged in series to separate blue, green and red signals.

*For montage images*, spinal cords were carefully dissected 5 days p.t., 26 days p.i., fixed in 4% paraformaldehyde washed in PBS and imaged in PBS at room temperature. Several 3D image stacks of  $x=1000 \mu\text{m}$ ,  $y=700 \mu\text{m}$ , and  $z=500 \mu\text{m}$  (XYZ voxel size  $1 \mu\text{m} \times 1 \mu\text{m} \times 5 \mu\text{m}$ ) were acquired using 10x objective (Zeiss, NA=0.5 WD=3.7mm) water dipping objective, image acquisition software Slidebook (Intelligent Imaging Innovations) and motorized Z-Decks stage (Prior Scientific). 3D Image blocks were stitched in Slidebook and Imaris version 9.2.1 (Bitplane) was used for rendering, final image size 3.5 mm X 14 mm X 0.75 mm.

*For live cell imaging*, spinal cords were isolated from thoracic vertebra 4 to lumbar vertebra 2 and embedded in a 5% agarose gel as previously described (Greenberg et al., 2014b). Spinal cords were oriented with the ventral side facing the dipping objective, and superfused with warmed, oxygenated medium. 3D image stacks of  $x=350\ \mu\text{m}$ ,  $y=350\ \mu\text{m}$ , and  $z=52\ \mu\text{m}$  (XYZ voxel size  $.68\ \mu\text{m} \times .68\ \mu\text{m} \times 4\ \mu\text{m}$ ) were sequentially acquired at 11 second intervals using 25x objective (Nikon, NA=1.1 WD=2mm) water dipping objective.

Cell motility data were processed and analyzed using Imaris software. A combination of manual and automatic tracking was used to generate highly accurate cell tracks. The x,y,z coordinates of the tracks were used to calculate mean track velocity, instantaneous speed, meandering index, and to plot tracks as described previously (Kerschensteiner et al., 2005; Matheu et al., 2015).

Statistical Analyses Data were analyzed using Prism software (GraphPad). Comparisons were performed using a Student's *t* test or with a Mann-Whitney U test, where indicated. For all statistical models and tests described above, the significance is displayed as follows: NS  $p>0.05$ , \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

## **Results**

### **Regulatory T cells accumulate at the site of hNSC transplant**

Previously, we observed an increase in  $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$  Tregs within the spinal cord of EAE mice receiving an intraspinal transplant of hNSCs (McIntyre et al., 2018, submitted). To visualize the distribution of Tregs within the cord of hNSC transplanted animals, we utilized two-photon (2P) microscopy to image spinal cord explants

(Greenberg et al., 2014b). We utilized FoxP3-eGFP<sup>+</sup>PLP-TdT<sup>+</sup> mice visualize endogenous Tregs and oligodendrocytes. FoxP3-eGFP mice contain a bicistronic FoxP3-enhanced green fluorescent protein (FoxP3-eGFP) that reliably express GFP under the control of the Treg specific FoxP3 promoter (Haribhai et al., 2007). FoxP3-eGFP PLP-TdT mice express the fluorescent reporter TdTomato (Tdt) under the control of the myelin proteolipid promoter (PLP-Cre) (Doerflinger et al., 2003). Use of these mice allowed us to visualize endogenously labeled Tregs (green) and oligodendrocytes expressing PLP (red). MOG immunized FoxP3-eGFP<sup>+</sup>PLP-TdT<sup>+</sup> mice were transplanted with hNSCs or received no transplant at thoracic vertebra 9 (T9) 21 days post immunization (p.i), a timepoint at which chronic demyelination is established (**Figure 3.1A**) (Constantinescu et al., 2011; McCarthy et al., 2012). Five days post-transplant (p.t.) spinal cord explants were imaged using a 2P microscope (Weinger et al., 2015). Montage images of the spinal cord from non-transplanted (**Figures 3.1B and 3.1C**) and hNSC-transplanted (**Figures 3.1D and 3.1E**) mice revealed that there is an abundance of FoxP3-eGFP<sup>+</sup> Tregs within the EAE spinal cord. FoxP3-eGFP<sup>+</sup> Tregs were distributed throughout the ventral spinal cord, amassing at sites of damage in both hNSC-transplanted and non-transplanted animals. In animals receiving hNSCs, FoxP3-GFP<sup>+</sup> Tregs were localized to the ventral lateral spinal cord at the site of hNSC injection (**Figure 3.1E**).

## **hNSC Transplantation does not alter Treg motility behavior within the spinal cord of EAE mice**

To observe Treg behavior following hNSC transplant, FoxP3-eGFP<sup>+</sup>PLP-TdT<sup>+</sup> MOG immunized mice were transplanted with hNSCs 21 days p.i. and Tregs were monitored *ex vivo* under 2P excitation 5 days p.t. (26 days p.i). Live imaging at the site of hNSC injection (T9) FoxP3-eGFP<sup>+</sup> Tregs in non-transplanted mice (**Figure 3.2A**) displayed similar motility compared to those in mice that received hNSCs (**Figure 3.2B**). To analyze migratory behavior of Tregs we overlaid T cell tracks from >50 individual cells on an x-y plane. Under homeostatic conditions, Tregs display a 'random walk' pattern of motility which shows no progressive displacement over time (Miller et al., 2003; Miller et al., 2002). Whereas, during a chemotaxis response Tregs display directional movement. Tregs in hNSC-transplanted and non-transplanted mice displayed 'random walk' motility, as indicated by random tracks (**Figure 3.2C**). We also did not observe a difference in the meandering index (total displacement/path length of a cell track) of Tregs whether or not mice were transplanted with hNSCs, suggesting Tregs were not responding to a chemotaxis gradient to hNSCs (**Figure 3.2D**). To further understand whether Tregs were responding to molecules secreted by hNSCs we performed an *in vitro* co-culture of naïve splenocytes with hNSCs in contact, or separated by a transwell. Transwells allow soluble factors to pass through a 0.4µM permeable membrane, but prevents direct contact of cells (Fujii et al., 1989). As previously reported, *in vitro* co-cultures of naïve splenocytes with hNSCs resulted in a two-fold increase of Tregs (McIntyre et al., 2018, submitted). However, separation of splenocytes and hNSCs by a transwell, prevented an expansion

of Tregs (data not shown). These data support their hypothesis that the expansion of Tregs is contact, and antigen dependent, rather than mediated by chemotaxis.

Additionally, time-lapse images of intraspinal Tregs revealed no significant difference in their basal motility characteristics. *In vivo*, Tregs exhibit a stop-and-go pattern of motility, rather 'lunging' forward at high velocity and then balling up. Tregs have been reported to obtain maximum velocities exceeding  $25\mu\text{m}\cdot\text{min}^{-1}$ , with average velocities of  $10.2\text{-}11.5\mu\text{m}\cdot\text{min}^{-1}$  (Miller et al., 2003). Tregs in mice that were non-transplanted hNSC-transplanted displayed similar frequencies of cells with similar instantaneous velocities (**Figure 3.2E**). Additionally, mean velocities of Tregs in mice that received no transplant ( $12.24\pm 0.6743\ \mu\text{m}\cdot\text{min}^{-1}$ ) were similar to those that received hNSCs ( $11.13\pm 0.5908\ \mu\text{m}\cdot\text{min}^{-1}$ ) (**Figure 3.2F**), consistent with previously reported average velocities of T cells in lymph nodes. Together, these data indicate that transplantation of hNSCs does not influence Treg motility.

### **hNSC expanded Tregs preferentially colocalize with cells expressing proteolipid protein within the spinal cord**

Although transplantation of hNSCs did not directly influence directionality or velocity of Tregs, distribution of Tregs within the spinal cord is altered in hNSC-transplanted animals. We hypothesize that Tregs secrete molecules to support maturation of oligodendrocyte progenitors (OPCs) to promote endogenous remyelination. To determine if Tregs interacted with oligodendrocytes we mapped the distance between FoxP3-eGFP<sup>+</sup> Tregs with respect to PLP-TdT<sup>+</sup> cells. Distance measurements revealed that Tregs preferentially colocalized with PLP<sup>+</sup> cells in hNSC-transplanted mice, but not non-

transplanted mice (**Figure 3.3A**). In addition, montage images of the ventral spinal cord revealed that hNSC-transplanted mice had increased numbers of Tregs compared to non-transplanted mice (**Figure 3.3B**). These microscopy results are consistent with our previous reports of increased frequency and numbers of Tregs in the spinal cords detected by flow cytometry (McIntyre et al., 2018, submitted). Upon further analysis, we discovered that hNSC-transplanted animals also displayed a greater proportion of Tregs within a radius of 30 $\mu$ m from PLP<sup>+</sup> cells compared to non-transplanted controls (**Figure 3.3B**), suggesting that Tregs are directly interacting with OPCs *in vitro*. These data support the hypothesis that hNSCs expand antigen-specific Tregs that facilitate oligodendrocyte maturation to promote remyelination within the EAE mouse spinal cord.

## **Discussion**

Transplantation of neural stem and progenitor cells are a promising therapeutic strategy to treat neurological disorders, such as MS. Studies have mainly focused on elucidating mechanisms of cellular replacement, while little attention is given to the impact transplanted cells exert on the endogenous cells. The majority of the work evaluating NSC transplantation has been conducted using genetically identical donors or immune suppressed subjects. For human patients, NSC transplants are likely to be genetically mismatched (allogeneic). Therefore, when evaluating NSC transplantation as a therapeutic for MS, the potential for engrafted cells to elicit an immune response or modulate the immune response, as well as the consequences of engraftment in an inflammatory environment must be evaluated.

Previously, our lab reported remyelination and decreased neuroinflammation in mice receiving an intra-spinal transplant of syngeneic NSCs, which replaced damaged cells (Carbajal et al., 2010). Alternatively, mice receiving transplants of xenogeneic human NSCs displayed remyelination, a decrease in neuroinflammation, and an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. Recovery was not a result of direct cell replacement as hNSCs underwent xenograft rejection, suggesting endogenous cells mediated remyelination (Plaisted et al., 2016). In addition, we discovered FoxP3-eGFP<sup>+</sup> localized to the site of hNSC injection.

Here, our results confirm that FoxP3-eGFP<sup>+</sup> home to the site of hNSC injection and interact with PLP<sup>+</sup> cells. Tregs have been shown to promote maturation of OPCs (Dombrowski et al., 2017). However, the behavior of these Tregs within the CNS has not been visualized. In this study, we established a model system to observe Treg motility behavior and Treg- oligodendrocyte cellular interactions within the intact spinal cord utilizing an *ex vivo* imaging platform. Tregs are distributed throughout the spinal cord and accumulate in areas of damage in EAE. hNSC-transplanted mice displayed an accumulation of Tregs around the site of injection (T9). Although hNSC transplant did not influence Treg motility behavior, Tregs were found to be in close proximity to PLP<sup>+</sup> cells in hNSC-transplanted mice compared to non-transplanted controls. These data suggest Tregs are interacting with endogenous PLP<sup>+</sup> cells to promote remyelination.

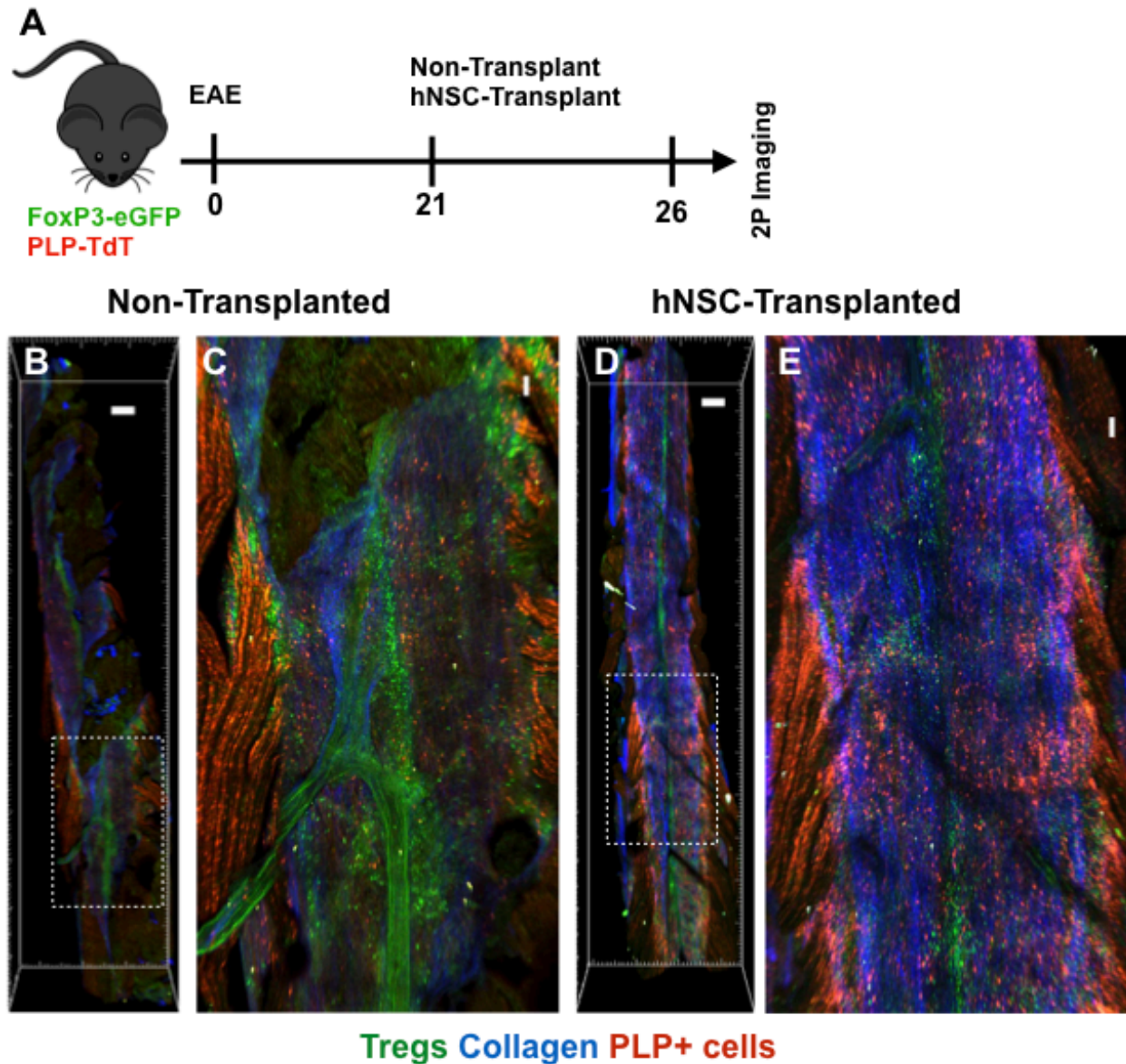
To further elucidate the mechanisms governing Treg mediated repair within the CNS, it would be useful to visualize axonal remyelination directly. To address the relationship between Treg and oligodendrocyte interactions, and ensuing remyelination, future studies utilizing Thy1-YFP+PLP-Tdt+ FoxP3-GFP+ mice where endogenous axons



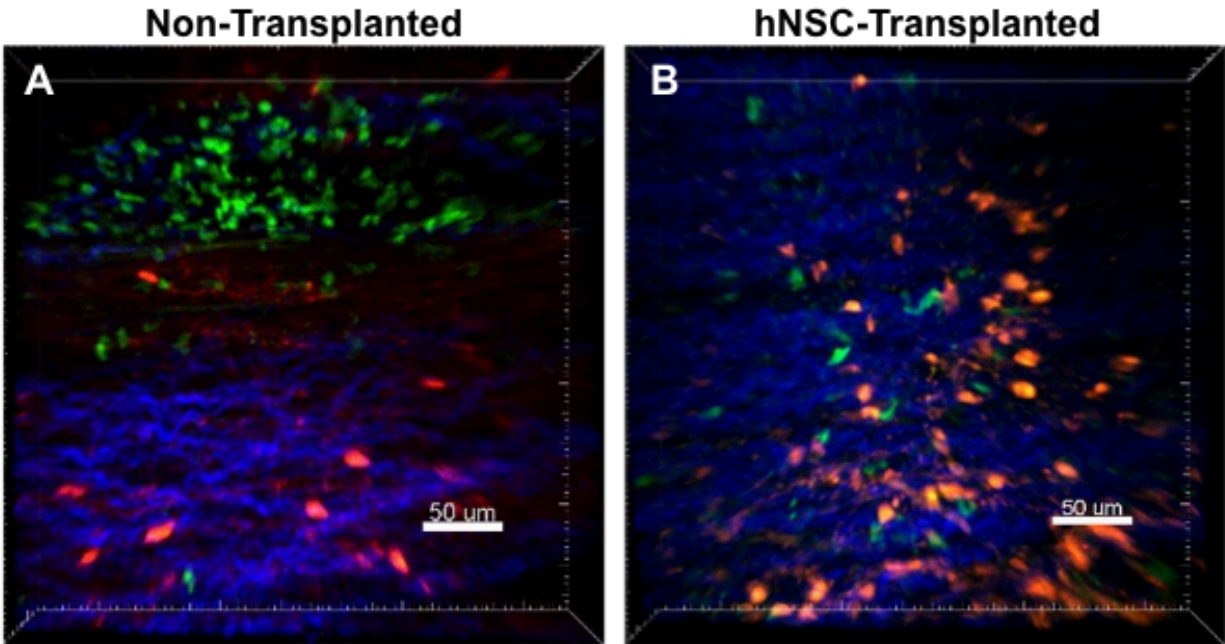
of neurons are labeled with yellow fluorescent protein (YFP) under the control of the Thy1 promoter (Feng et al., 2000; Kerschensteiner et al., 2005) could be utilized to reveal real-time cellular interactions in EAE mice following hNSC transplant.

We have previously shown that transplanted mNSCs and hNSCs preferentially colonize areas of demyelination (McIntyre et al., 2018, submitted). However, the motility of hNSCs was not previously examined. Here, our results support the hypothesis that Tregs respond to hNSC antigens and interact with endogenous myelin producing cells to promote remyelination. In addition, 2P imaging of Tregs and PLP+ cells allow for visualization of cellular interactions in EAE mice transplanted with hNSCs.

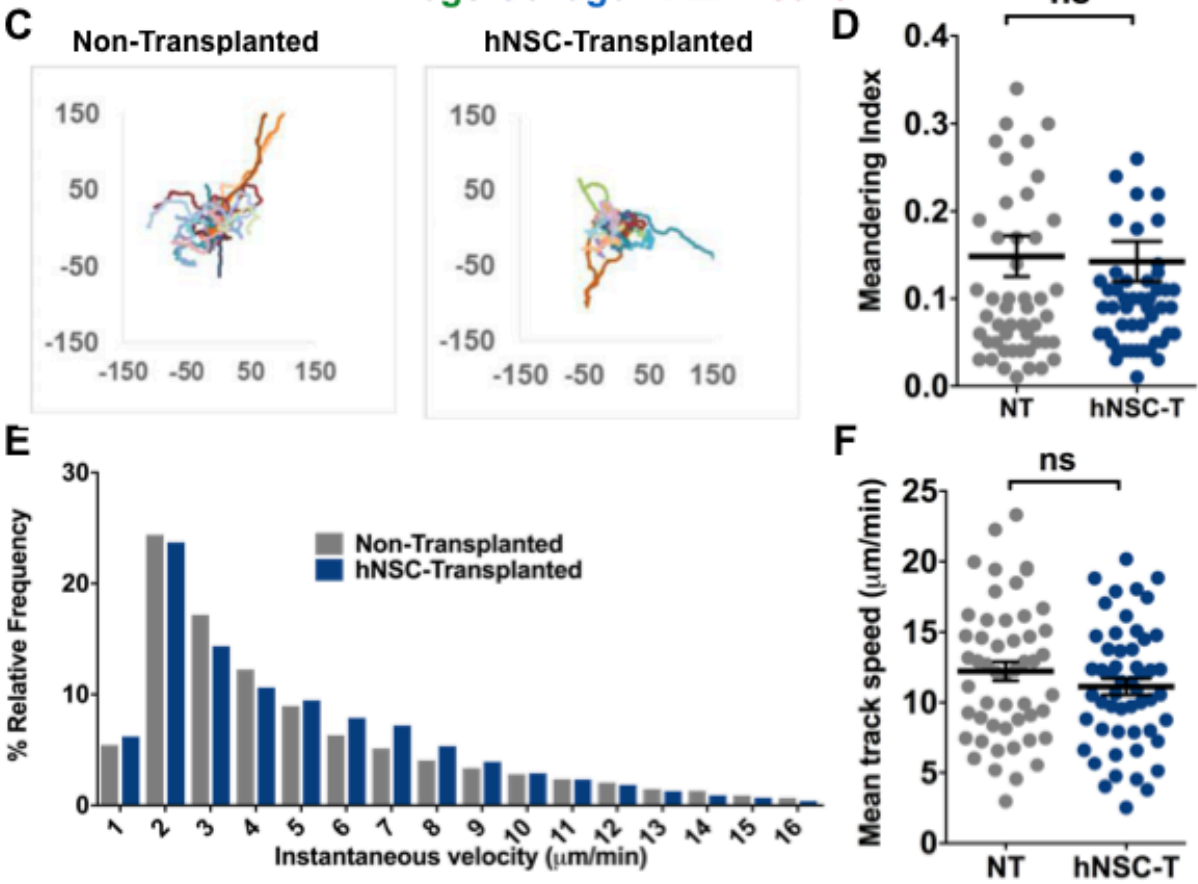
**Figure 3.1. Mapping of spinal cord homing Tregs in EAE following hNSC Transplant.** (A) Timeline of EAE induction, hNSC transplant and 2P Imaging. Analysis of FoxP3-eGFP<sup>+</sup> PLP-TdT<sup>+</sup> mice. Spinal cord montage from EAE mice non-transplanted (B) and zoomed in image (C) compared to hNSC-transplanted (D) and zoomed in image (E). Images showing the distribution of FoxP3<sup>+</sup> Tregs (green) and proteolipid protein (PLP)<sup>+</sup> cells (red), second harmonic signal from collagen (blue) in the ventral side of the spinal cord 5 days p.t. Images are maximum intensity projections through z-axis. Scale bar in A and C= 500  $\mu$ m, scale bar in B and D =100  $\mu$ m.



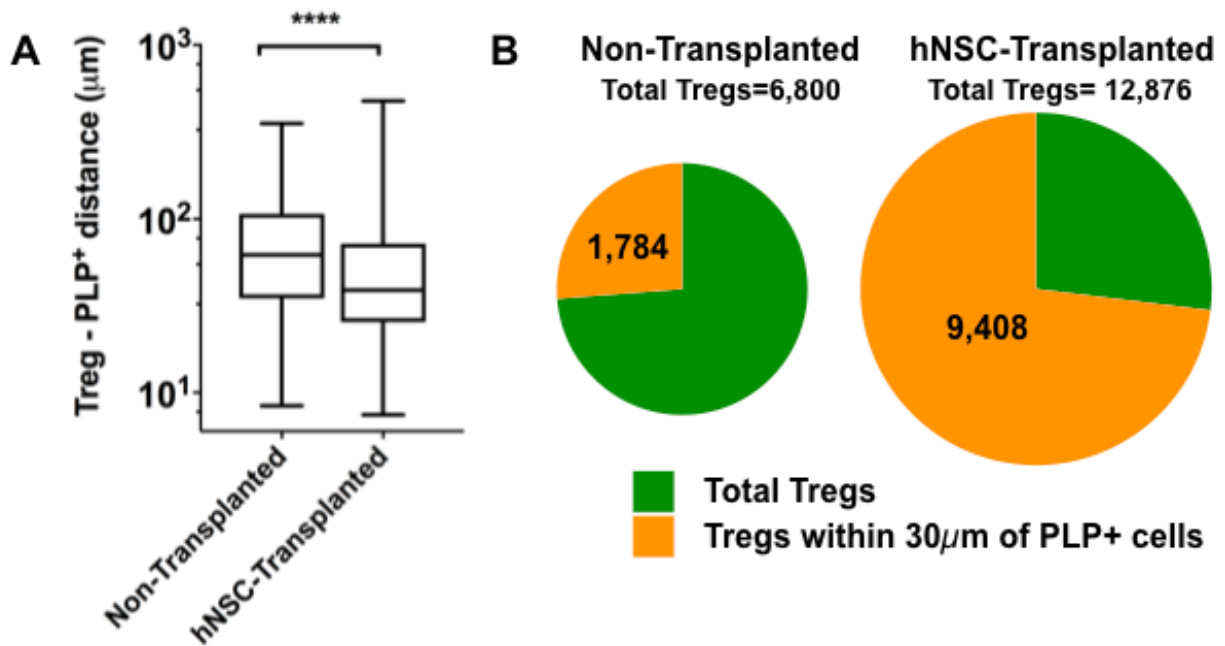
**Figure 3.2. hNSC Transplantation does not alter Treg motility behavior within the spinal cord.** Static images from 2P videos of spinal cord explants from non-transplanted **(A)** or hNSC-transplanted **(B)** EAE mice. Images showing the distribution of FoxP3+ Tregs (green) and proteolipid protein (PLP)+ cells (red), second harmonic signal from collagen (blue) in the ventral side of the spinal cord at the site of transplant (T9) 5 days p.t. **(C)** Displacement plots of Treg movement in non-transplanted (left) and hNSC-transplanted EAE mice(right) show no difference in Treg directional movement. **(D)** Meandering index (total displacement/path length of a cell track) of non-transplanted (NT) or hNSC-transplanted (hNSC-T) mice. **(E)** Frequency of instantaneous Treg velocities with the spinal cord of non-transplanted (NT) or hNSC-transplanted (hNSC-T) EAE mice. Each circle represents measurements from individual cell tracks; black bars indicate overall mean values and P values are marked in the figures. Mean track speed **(F)** of FoxP3-GFP+ Tregs within the spinal cord of non-transplanted (NT) and hNSC-transplanted (hNSC-T) EAE mice revealed no significant difference. Data is presented as average  $\pm$  SEM and analyzed using a t-test with a Mann-Whitney posttest.



Tregs Collagen PLP+ cells



**Figure 3.3. hNSC expanded Tregs interact with endogenous PLP+ cells within the spinal cord. (A)** Box and whisker plots quantifying distance between FoxP3-GFP+ Tregs and PLP+ cells in non-transplanted and hNSC-transplanted mice. hNSC-transplanted mice have significantly more Tregs in closer proximity to PLP+ cells ( $p < 0.0001$ ). Data was analyzed using a t-test with a Mann-Whitney posttest. **(B)** hNSC-transplanted mice have greater total number of Tregs  $n = 12876$  (green) and Tregs within  $30 \mu\text{m}$  of PLP+ cells  $n = 9408$  (orange) compared to non-transplanted controls  $n = 6800$ ,  $n = 1784$ ; respectively.



## References

- Ben-Hur, T., Fainstein, N., and Nishri, Y. (2013). Cell-based reparative therapies for multiple sclerosis. *Curr Neurol Neurosci Rep* 13, 397.
- Carbajal, K.S., Schaumburg, C., Strieter, R., Kane, J., and Lane, T.E. (2010). Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 107, 11068-11073.
- Carbajal, K.S., Weinger, J.G., Whitman, L.M., Schaumburg, C.S., and Lane, T.E. (2011). Surgical transplantation of mouse neural stem cells into the spinal cords of mice infected with neurotropic mouse hepatitis virus. *J Vis Exp*, e2834.
- Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27, 275-280.
- Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, C.M., Sears-Kraxberger, I., Steward, O., Macklin, W.B., Loring, J.F., *et al.* (2014). Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis. *Stem Cell Reports* 2, 825-837.
- Constantinescu, C.S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* 164, 1079-1106.
- Doerflinger, N.H., Macklin, W.B., and Popko, B. (2003). Inducible site-specific recombination in myelinating cells. *Genesis* 35, 63-72.
- Dombrowski, Y., O'Hagan, T., Dittmer, M., Penalva, R., Mayoral, S.R., Bankhead, P., Fleville, S., Eleftheriadis, G., Zhao, C., Naughton, M., *et al.* (2017). Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci* 20, 674-680.
- Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28, 41-51.
- Fujii, T., Sugita, N., Kobayashi, Y., Saito, K., Iijima, H., Matsunaga, K., Ando, T., Oguchi, Y., Morita, I., Yoshikumi, C., *et al.* (1989). Treatment with Krestin combined with mitomycin C, and effect on immune response. *Oncology* 46, 49-53.
- Germain, R.N., Robey, E.A., and Cahalan, M.D. (2012). A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 336, 1676-1681.
- Greenberg, M.L., Weinger, J.G., Matheu, M.P., Carbajal, K.S., Parker, I., Macklin, W.B., Lane, T.E., and Cahalan, M.D. (2014). Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 111, E2349-2355.

Haribhai, D., Lin, W., Relland, L.M., Truong, N., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* 178, 2961-2972.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564.

Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., and Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med* 11, 572-577.

Lin, W., Haribhai, D., Relland, L.M., Truong, N., Carlson, M.R., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8, 359-368.

Martino, G., Franklin, R.J., Baron Van Evercooren, A., Kerr, D.A., and Stem Cells in Multiple Sclerosis Consensus, G. (2010). Stem cell transplantation in multiple sclerosis: current status and future prospects. *Nat Rev Neurol* 6, 247-255.

Matheu, M.P., Othy, S., Greenberg, M.L., Dong, T.X., Schuijs, M., Deswarte, K., Hammad, H., Lambrecht, B.N., Parker, I., and Cahalan, M.D. (2015). Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. *Nat Commun* 6, 6219.

McCarthy, D.P., Richards, M.H., and Miller, S.D. (2012). Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Biol* 900, 381-401.

McFarlin, D.E., and McFarland, H.F. (1982). Multiple sclerosis (second of two parts). *N Engl J Med* 307, 1246-1251.

Miller, M.J., Wei, S.H., Cahalan, M.D., and Parker, I. (2003). Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy. *Proc Natl Acad Sci U S A* 100, 2604-2609.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 296, 1869-1873.

Mozafari, S., Laterza, C., Roussel, D., Bachelin, C., Marteyn, A., Deboux, C., Martino, G., and Baron-Van Evercooren, A. (2015). Skin-derived neural precursors competitively generate functional myelin in adult demyelinated mice. *J Clin Invest* 125, 3642-3656.

Plaisted, W.C., Zavala, A., Hingco, E., Tran, H., Coleman, R., Lane, T.E., Loring, J.F., and Walsh, C.M. (2016). Remyelination Is Correlated with Regulatory T Cell Induction Following Human Embryoid Body-Derived Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis. *PLoS One* 11, e0157620.

Pluchino, S., Gritti, A., Blezer, E., Amadio, S., Brambilla, E., Borsellino, G., Cossetti, C., Del Carro, U., Comi, G., Hart, B., *et al.* (2009). Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. *Ann Neurol* 66, 343-354.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164.

Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., and Anderson, A.J. (2010). Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. *PLoS One* 5, e12272.

Steinman, L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* 85, 299-302.

Weinger, J.G., Greenberg, M.L., Matheu, M.P., Parker, I., Walsh, C.M., Lane, T.E., and Cahalan, M.D. (2015). Two-photon imaging of cellular dynamics in the mouse spinal cord. *J Vis Exp*.

Yuan, S.H., Martin, J., Elia, J., Flippin, J., Paramban, R.I., Hefferan, M.P., Vidal, J.G., Mu, Y., Killian, R.L., Israel, M.A., *et al.* (2011). Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. *PLoS One* 6, e17540.



## **CHAPTER FOUR**

### **Regulatory T cells: Promising Immunotherapies for Autoimmunity and Tissue Repair**

**Abstract**

Regulatory T cells (Tregs) are essential for maintenance of T cell homeostasis and preventing autoimmunity. Defective Treg function has been reported in numerous autoimmune diseases including insulin-dependent type 1 diabetes, multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis. Immunotherapies using Tregs aim to suppress unwanted inflammatory responses and restore self-tolerance. Therapies to expand Tregs and cell-based strategies are being studied in pre-clinical animal models and are currently underway in clinical trials. These therapies offer tremendous hope to patients afflicted with autoimmune diseases, however, future studies are necessary to improve survival and efficacy of Tregs.

**Keywords:** Regulatory T cells, immunotherapy, autoimmunity, cellular therapy, immune tolerance

## **Introduction**

The immune system is tasked with the complicated role of protecting the host from foreign pathogens, such as viruses and bacteria, meanwhile maintaining tolerance to self-tissues. Inadequate immune responses result in life-threatening infections and tumor growth, whereas over-exuberant immune responses result in autoimmunity. Maintaining this delicate balance between pro- and anti-inflammatory immune responses is challenging, but there are a number of check points in the thymus and periphery that govern that govern this process (Bluestone et al., 2015a). Regulatory T cells (Tregs) are crucial for maintaining peripheral self-tolerance and preventing autoimmunity (Josefowicz et al., 2012). Autoimmune diseases, including insulin-dependent type 1 diabetes myelitis (T1DM), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) all occur as a result of break down in immune self-tolerance. These diseases are estimated to afflict between 3-5% of individuals, with prevalence of up to 12.5% of individuals in developed countries (Cooper et al., 2009). Although, patients with autoimmune diseases possess Tregs (Venken et al., 2008; Viglietta et al., 2004), these cells are enigmatically dysfunctional in their suppressive capabilities (Ehrenstein et al., 2004; Venken et al., 2008; Viglietta et al., 2004). Patients with autoimmunity often experience life-long, debilitating symptoms due to both disease and treatment side-effects. Immune therapies aim to restore the balance between pro- and anti-inflammatory responses in autoimmunity, a topic addressed here.

## **Mechanisms of Regulatory T cell Function**

Tregs are a specialized subset of T lymphocytes that are responsible for modulating inflammatory responses, preventing autoimmunity, and promoting tissue repair through a variety of mechanisms (Plitas and Rudensky, 2016). Tregs are characterized by their expression of CD4<sup>+</sup> and the master transcriptional regulator, Forkhead Box 3 (FoxP3) (Sakaguchi et al., 1995). A mutation in FoxP3, identified in Scurfy mutant mice, results in a fatal lymphoproliferative disorder as a result of defective T cell tolerance (Brunkow et al., 2001). In humans, patients with mutations in FoxP3 develop immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome which presents as multi-organ autoimmunity (Ziegler, 2006). Additionally, Tregs constitutively express high levels of CD25 (IL-2R $\alpha$ ) (Sakaguchi et al., 1995), and are therefore reliant on IL-2 for survival and FoxP3<sup>+</sup> expression/ effector function (Barron et al., 2010; Fontenot et al., 2005a).

Thymus-derived Tregs (tTreg) are responsible for maintenance of homeostasis and tissue repair (Abbas et al., 2013), whereas those arising from naïve conventional T cells in the periphery (pTregs) are important for controlling local inflammatory responses (Yadav et al., 2013). Although these populations comprise two distinct subsets, they possess many of the same effector functions. Tregs exert their suppressive capabilities via contact-independent and contact dependent mechanisms. Tregs can directly suppress effector T cells (Teff) via programmed cell death (PD-1)- PD-1Ligand (PD-L1) coupling and CD39-CD73 receptor engagement resulting in apoptotic death of effector cells (Bopp et al., 2007; Ohta and Sitkovsky, 2014). Tregs also secrete cytotoxic molecules perforin and granzyme to induce apoptosis of neutrophils (Alberu et al., 2012). Since Tregs express high levels of CD25, they are capable of sequestering IL-2

necessary for Teff, resulting in granzyme and perforin mediated death. In addition, Tregs prevent co-stimulation of effectors via antigen presenting cells (APCs) by regulating CD80/86 expression on APCs through CTLA-4 or by competing for CD28 binding (Paust et al., 2004; Qureshi et al., 2011; Wing et al., 2008). Tregs secrete immunosuppressive cytokines including TGF $\beta$ , IL-10 and IL-35 (Fox et al., 1989).

While Tregs have traditionally been implicated in suppressing inflammatory T cell responses, an emerging role for Tregs as potentiators of tissue repair has recently been highlighted (Li et al., 2018). The involvement of innate immune cells, neutrophils and monocytes, is well characterized in tissue repair (Laurent et al., 2017). Neutrophils are the first cells recruited to the sites of tissue damage, where they phagocytose debris and promote inflammation essential for later phases of tissue repair (Silverstein and Rabadan, 2012). Monocytes and macrophages are then recruited to sites of tissue damage, where pro-inflammatory macrophages (M1) (Wynn and Barron, 2010) act as phagocytes, and then alternatively activated reparative macrophages (M2) aid in healing (Nahrendorf et al., 2007). Recently, other immune cells including  $\gamma\delta$  T cells (Jameson et al., 2002), innate lymphoid cells (ILCs) (Artis and Spits, 2015; Klose and Artis, 2016) and Tregs (Arpaia et al., 2015) have also be implicated in tissue healing. Tregs promote tissue repair indirectly by influencing immune responses, such as recruiting neutrophils (Carbone et al., 2013; D'Alessio et al., 2009; Weirather et al., 2014), influencing macrophage M1 or M2 polarization (Aurora et al., 2014; Lavine et al., 2014), and suppressing conventional T cells (Chen et al., 2014b; Weirather et al., 2014). In addition, Tregs facilitate tissue regeneration directly by facilitating progenitor cell activity (Ali et al., 2017; Castiglioni et al., 2015; Dombrowski et al., 2017).

Tissue-Tregs found in the visceral adipose tissue (VAT) are one of the first examples of Tregs assisting in the tissue repair process. VAT Tregs express peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), which regulates adipocyte differentiation and glucose metabolism (Feuerer et al., 2009; Tontonoz and Spiegelman, 2008). Tregs have also been shown to promote osteoblast differentiation necessary for bone regeneration (Glowacki et al., 2013). Tregs are also important for establishing immunological tolerance with commensal microbes in the skin (Scharschmidt et al., 2015), and ablation of Tregs in the skin delays wound healing (Nosbaum et al., 2016). Moreover, Tregs interact with progenitor cells, is in the skin, where Tregs localize to hair follicles where they express the Notch ligand *Jag1*, which is required for cycling of hair follicle stem cells (Ali and Rosenblum, 2017).

Lung epithelial cell proliferation is strongly correlated with Treg abundance, suggesting Tregs play a reparative role following lung injury (Arpaia et al., 2015; Mock et al., 2014). Scarring of cardiac tissue following myocardial infarction leads to loss of cardiomyocytes and ultimately cardiac failure (Ertl and Frantz, 2005). Mice lacking CD4<sup>+</sup> cells have increased cardiac inflammation, impaired wound healing, and decreased survival (Hofmann et al., 2012), possibly due to a lack of Treg-included repair. Indeed, Tregs have been shown to attenuate cardiac injury via expression of CD39 *in vitro* (Xia et al., 2015) and via modulation of macrophage polarization *in vivo* (Weirather et al., 2014). Perhaps, the most well characterized role for Tregs in tissue repair has been shown in the skeletal muscle (Burzyn et al., 2013). In the mdx mouse model of Duchenne muscular dystrophy, Tregs accumulate in the injured muscle and produce the growth factor Amphiregulin which promotes muscle satellite cells to facilitate muscle repair

(Burzyn et al., 2013). Tregs are also essential for remyelination and repair within the central nervous system. In a mouse model of spinal cord injury (SCI), Tregs participate in neuronal survival (Raposo et al., 2014; Walsh et al., 2014). Following ischemic stroke, Tregs accumulate in the brain and their depletion exacerbates brain damage (Liesz et al., 2009; Wang et al., 2018). Although, the benefits of Tregs in the CNS following SCI or ischemic stroke remain controversial, they are likely dependent upon spatial and temporal factors (Kleinschnitz et al., 2013; Walsh et al., 2014). Mice deficient in CD4<sup>+</sup> or CD8<sup>+</sup> T cells display impaired remyelination following chemical demyelination (Bieber et al., 2003). In addition, Treg deficient mice also display impaired remyelination (Gadani et al., 2015). We and others have shown that Tregs promote remyelination by promoting oligodendrocyte progenitor cell maturation (Chen et al., 2014b; Dombrowski et al., 2017; Plaisted et al., 2016). The ability of Tregs to facilitate tissue repair may be impaired in patients with autoimmune diseases, like MS and RA, where disability is a direct result of irreversible tissue damage.

### **Current regulatory T cell-based therapies**

Tregs are a tantalizing therapy to suppress autoimmune and alloimmune responses because of their ability to suppress unwanted inflammatory immune responses. There are currently two types of Treg based therapies; those that promote expansion of Tregs *in vivo* and cellular based Treg therapies (**Figure 4.1**). Here we discuss both types of therapies in pre-clinical and clinical trials for human disease.

## **Current regulatory T cell-based therapies that promote Treg expansion and function**

### Rapamycin

Rapamycin is a metabolite known to enhance Treg expansion and stabilize FoxP3 expression (Battaglia et al., 2006b; Battaglia et al., 2005; Zhang et al., 2013). First discovered in a soil sample from Easter Island (Rapa Nui), rapamycin is an antifungal metabolite produced by *Streptomyces hygroscopicus* (Li et al., 2014). Rapamycin promotes Treg expansion by interacting with the mammalian target of rapamycin complex 1 (mTORC1) inhibiting P13K/AKT signaling (Ohkura et al., 2011; Powell and Delgoffe, 2010). Non-obese diabetic (NOD) mice, a model of insulin-dependent type 1 diabetes (T1DM), treated with rapamycin combined with IL-10 prevented disease (Battaglia et al., 2006a). Moreover, patients with T1DM treated with rapamycin displayed expansion of Tregs with enhanced suppressive capabilities (Battaglia et al., 2006b; Monti et al., 2008).

### Interleukin-2

Interleukin-2 (IL-2) is essential for Treg development and homeostasis (Chinen et al., 2016), and has been shown to expand Tregs *in vitro* and *in vivo*, as well as enhance their suppressive capacity (Scheffold et al., 2005). Tregs express high levels of the trimeric IL-2R $\alpha\beta\gamma_c$ , thus low-dose IL-2 preferentially activates Tregs (Yu et al., 2015). Administration of low-dose IL-2 to NOD mice resulted in increased Tregs and the prevention or reversal of diabetes (Grinberg-Bleyer et al., 2010; Tang et al., 2008). In patients with T1DM, low-dose IL-2 was found to be well tolerated and expanded Tregs (Hartemann et al., 2013; Klatzmann and Abbas, 2015). Administration of low-dose IL-2 to experimental



autoimmune encephalomyelitis (EAE) mice, a mouse model of MS, resulted in prevention of autoimmunity mediated by Tregs, although this treatment was unable to attenuate disease when administered after disease onset (Rouse et al., 2013). Low-dose IL-2 has also demonstrated efficacy in clinical trials for chronic graft-versus-host-disease (GVHD) (Matsuoka et al., 2013) and hepatitis C virus-induced vasculitis (Saadoun et al., 2011). However, IL-2 is not only involved in expansion of Tregs since NK cells and CD8<sup>+</sup> T cells express the dimeric IL-2R $\beta\gamma_c$  (Yu et al., 2015). Therefore, dosing and administration should be investigated carefully to avoid induction of these proinflammatory cell types. Celgene, Roche, Amgen, and other groups are developing new cytokine-antibody conjugates called IL-2 mutein Fc fusion proteins that preferentially increase Tregs rather than Teff, and IL-2 therapies are currently being tested in clinical trials for ankylosing spondylitis, autoimmune hepatitis, Behcet's disease, Crohn's disease, psoriasis, RA, sclerosing cholangitis, SLE, Takayasu's disease, Wegener's granulomatosis, and ulcerative colitis (ClinicalTrials.gov).

#### Other therapies that enhance Tregs

CD3 monoclonal antibodies (mAb) that bind to the T cell receptor (TCR) complex, have been used to deplete effector T cells, and intriguingly, these mAbs were reported to cause a transient, systemic rise in the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs in mice (Belghith et al., 2003; Penaranda et al., 2011). Although this increase has not been seen in humans, Ablamunits *et al.* reported an increase in regulatory CD8<sup>+</sup> T cells (Ablamunits et al., 2010). Treatment of NOD and EAE mice with CD3 mAbs resulted in prevention of disease, with NOD mice even displaying a reversal of disease when anti-CD3 mAbs were administered

at the time of disease onset (Chatenoud et al., 1997; Kuhn et al., 2011). Administration of teplizumab, an anti-CD3 mAb, increases T cell homing to the gut and in increase in FoxP3<sup>+</sup> T cells in mice and humans (Esplugues et al., 2011; Waldron-Lynch et al., 2012). In addition, administration of CD3 mAbs has also been shown to suppress autoimmunity via enhancement of gut Tregs in animal models of encephalitis, collagen-induced arthritis and SLE (Hu et al., 2013; Ishikawa et al., 2007; Ochi et al., 2006; Wu et al., 2009; Wu et al., 2010; Wu et al., 2008).

There has also been considerable interest in other molecules that promote Treg generation and prevent the activation of Teffs. Alefacept is a LFA3 fusion molecule that binds CD2 on Teff cells, thereby preventing co-stimulatory activation signals. Clinical trials in patients with psoriasis and T1DM revealed increased numbers of Tregs, similar to those involving anti-CD3 mAb treatment (Ellis et al., 2001; Krueger and Ellis, 2003; Lebwohl et al., 2003). Tolerizing nanoparticle technology has also been developed to expand Tregs in animal models of relapsing-remitting MS (Park et al., 2011; Steenblock and Fahmy, 2008). In this case, APCs uptake nanoparticles containing self-antigen and present self-peptides bound to MHC, and along with rapamycin, these APCs promote the induction of Tregs.

## **Current cellular based regulatory T cell therapies**

### Ex vivo expansion of Tregs

Adoptive cellular therapies (ACTs) utilizing Tregs to promote self-tolerance and control unwanted inflammatory immune responses are currently being tested and developed for autoimmune diseases including GVHD, T1DM, and many others, as reviewed in

(Gliwinski et al., 2017). Early studies using pre-clinical animal models demonstrated that Treg transfer in combination with hematopoietic stem cell transfer (HSCT) protected mice from GVHD (Edinger et al., 2003). However, the low number of tTregs in peripheral blood (only 1-2% of total peripheral blood, 5-10% of CD4<sup>+</sup> cells in humans proved to be problematic when attempting to translate these results to human clinical trials, likely due to the large number of Tregs necessary for transfer (Sakaguchi et al., 2008; Tang and Lee, 2012). To circumvent this issue, researchers have developed methods for *ex vivo* expansion of polyclonal Tregs (Masteller et al., 2006).

Cell surface expression of CD4 and CD25 has allowed researchers to isolate and expand Tregs *ex vivo*. The Bluestone group has recently reported methods to expand Tregs up to 2000-fold for clinical use using a 14-day expansion protocol with anti-CD3, anti-CD28, and IL-2 (Tang and Lee, 2012). Indeed, *ex vivo* expanded Tregs adoptively transferred into NOD mice traffic to the pancreas where they suppress Teff cells and preserve islet function (Green et al., 2003; Tang et al., 2004). In phase I clinical trials for patients with T1DM, infusion of autologous, polyclonal *ex vivo* expanded Tregs were found to be well-tolerated, with a reported improvement in glycogen-stimulated C-peptide responses, a measure of pancreatic beta cell function (Bluestone et al., 2015b; Leighton et al., 2017; Marek-Trzonkowska et al., 2014). Notably, adoptively transferred Tregs were observed after more than one year post infusion (Busch et al., 2007). Furthermore, adoptive transfer of Tregs has been shown to prevent EAE in animal models of MS (Kohm et al., 2002). Similar results were reported in animals with multiorgan autoimmunity (Ono et al., 2006). TCR transgenic mice possessing a TCR specific for myelin basic protein (MBP) displayed reduced clinical symptoms when administered polyclonal Tregs (Hori et

al., 2002). Disease symptoms were further reduced when animals received auto-antigen specific Tregs (Hori et al., 2002). Similar results were reported in NOD mice receiving polyclonal or auto-antigen specific Tregs (Masteller et al., 2005; Tang et al., 2004), supporting their hypothesis that autoantigen specific Tregs confer a greater benefit than polyclonal Tregs.

### Engineered antigen-specific Tregs

Tregs engineered to express a TCR with predetermined antigen-specificity are currently in development. Antigen-specific Tregs have been engineered via gene transfer using viral vectors encoding specific TCRs (TCR-Tregs) or chimeric antigen receptors (CAR). Strauss and colleagues demonstrated that TCR-Tregs expressing a TCR that recognizes ovalbumin suppressed Teff inflammatory responses in a murine model of autoimmune inflammatory arthritis (Wright et al., 2009). Additional studies have shown that TCR-Tregs suppress Teff responses in animal models of hemophilia and T1DM (Curran et al., 2012; Kim et al., 2015).

As an alternative to TCR gene transfer, chimeric antigen receptor-Tregs (CAR-Tregs) have been developed. CAR-Tregs are an engineered TCR alternative, consisting of an antigen binding domain of a monoclonal antibody linked via an extracellular hinge to a transmembrane region and intracellular signaling motifs for T cell activation (Zhang et al., 2018). Since Treg expansion using APCs is inefficient, CAR-Tregs offer an MHC-independent method of generating antigen-specific Tregs, and are therefore applicable to patients irrespective of MHC haplotype (Boardman et al., 2016). In 2017, the first two CAR-T cell therapies were approved for the treatment of CD19<sup>+</sup> B cell lymphomas, and

other CAR-T cell therapies are now being widely tested in many clinical trials to treat other types of cancer (Quintas-Cardama, 2018), although CAR-Tregs have yet to be evaluated in patients. In pre-clinical animal models of human autoimmune diseases, CAR-Tregs were reported to traffic to the gut, suppress inflammation in an antigen-specific manner independent of MHC, and maintain their Treg effector phenotype in a murine model of colitis (Blat et al., 2014; Elinav et al., 2009). CAR-Tregs have also been shown to be suppressive in murine models of hemophilia (Yoon et al., 2017), GVHD (MacDonald et al., 2016), skin allograft rejection (Trauner et al., 2017), EAE (Fransson et al., 2012) and allergic asthma (Hombach et al., 2009).

#### **Future Directions: what lies ahead for regulatory T cell therapies**

Tregs are a promising therapeutic strategy to treat patients with autoimmune disease, and there are currently 818 trials involving Tregs (ClinicalTrials.gov). Therapies using Tregs are attractive for treating autoimmune disorders because of their intrinsic ability to suppress inflammatory responses and promote self-tolerance. Tregs are capable of exerting their suppressive function through a variety of mechanisms via contact-independent and contact-dependent modalities exerting effects on a wide range of innate immune cells as well as T<sub>H</sub>17 cells (Josefowicz et al., 2012; Steinman et al., 2012). Recently, Tregs have also been implicated in promoting tissue repair through suppression of inflammation, and more directly through interactions with tissue-resident progenitor cells involved in regeneration (Li et al., 2018).

Methods have been developed and validated in clinical trials to promote expansion of Tregs (**Figure 4.1**), including administration of rapamycin, IL-2 and antibody-mediated

therapies. These strategies have been effective in pre-clinical animal models and clinical trials for the treatment of T1DM and GVHD, although these therapies do have significant side-effects. Rapamycin induces whole body immunosuppression, which renders the host susceptible to otherwise innocuous viral and bacterial infections. IL-2 expands Treg populations, but also NK cells and CD8<sup>+</sup> Teff cells. Therefore, these therapies have considerable drawbacks that must be overcome.

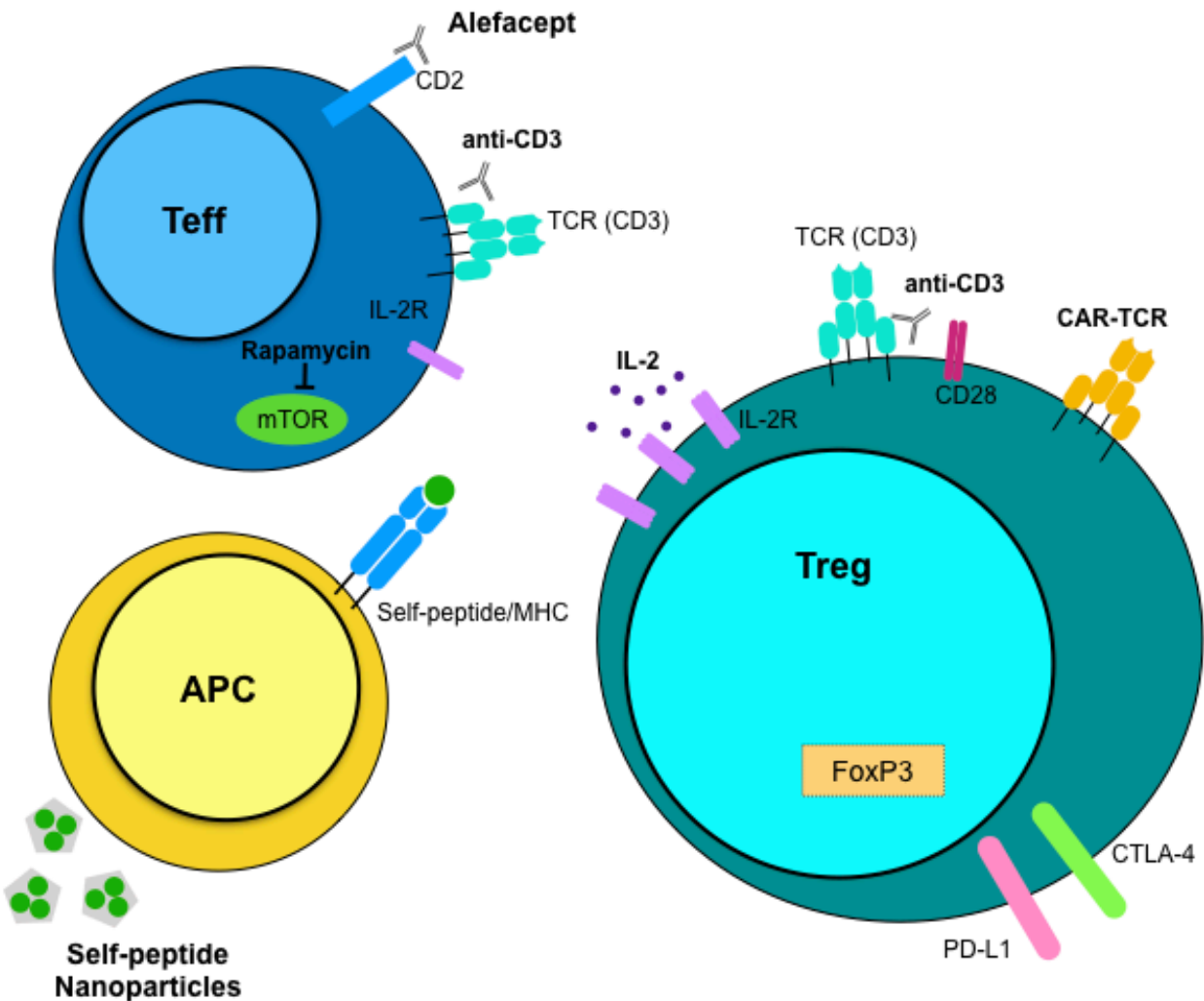
Treg cellular therapy has also been developed for treatment of autoimmune diseases (**Figure 4.1**). ACT has been most well-studied and employed in animal models and patients with T1DM (Bluestone et al., 2015b). A significant problem with ACT is the difficulty in obtaining the large numbers of Tregs needed for effective therapies. Methods for *ex vivo* expansion of Tregs have augmented this caveat, although expanded Tregs are polyclonal, whereas monoclonal Tregs are known to possess greater therapeutic efficacy. While promising in many animals of autoimmunity including T1DM and EAE, adoptive T cell therapy was found to be ineffective in animal models of collagen induced arthritis (Zhou et al., 2010) and SLE (Bagavant and Tung, 2005). CAR-Tregs provide an alternative to *ex vivo* expansion as they can be generated against specific self-antigens. However, CAR-Tregs have not yet been tested in patients.

The biology of Tregs is not completely understood, and therefore the stability of FoxP3 expression and suppressive function is a concern with Treg therapy. T cells are intrinsically plastic, as they must respond to foreign pathogens but maintain tolerance to self-antigens (Sakaguchi et al., 2013). Bluestone and colleagues reported that Tregs are unstable in inflammatory tissue microenvironments, losing FoxP3 expression under these

conditions (McClymont et al., 2011). Given this important caveat, the stability of Tregs and their suppressive capacity following ACT warrants further investigation.

As described, Tregs are currently being therapeutically evaluated in a number of autoimmune diseases for their suppressive capabilities. However, their tissue repair functions have not yet been evaluated in the clinic. Recently, we and others have highlighted a role for Tregs in directly promoting repair in the bone, CNS, cardiac tissue, lung, skeletal muscle and skin, tissues often targeted in autoimmunity (Li et al., 2018). Based upon these studies, Tregs are likely to be beneficial in tissue repair and therefore, should be considered for regenerative as well as anti-inflammatory therapies. Immunotherapies employing Tregs for the treatment of autoimmune and alloimmune responses aim to restore disrupted immune homeostasis. These therapies are currently being evaluated in clinical trials and in pre-clinical models of human disease. Future studies are likely to build upon these initial trials to improve the stability, suppressive function, survival, and tissue repair capacities of Tregs.

**Figure 4.1. Therapies involving regulatory T cells.** Therapies promote Tregs; Rapamycin, IL-2, anti-CD3 monoclonal antibodies, Alefacept, and nanoparticles have been used in clinical trials of autoimmune diseases. In addition, adoptive cell transfer of Tregs has been conducted using autologous and donor Tregs that are polyclonal or self-antigen specific. Tregs can be expanded ex vivo using anti-CD3, anti-CD28, and IL-2. Chimeric antigen receptor (CAR) Tregs are also being developed which possess an engineered, antigen-specific TCR that is MHC independent.





## References

- Abbas, A.K., Benoist, C., Bluestone, J.A., Campbell, D.J., Ghosh, S., Hori, S., Jiang, S., Kuchroo, V.K., Mathis, D., Roncarolo, M.G., *et al.* (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* *14*, 307-308.
- Ablamunits, V., Bisikirska, B., and Herold, K.C. (2010). Acquisition of regulatory function by human CD8(+) T cells treated with anti-CD3 antibody requires TNF. *Eur J Immunol* *40*, 2891-2901.
- Alberu, J., Vargas-Rojas, M.I., Morales-Buenrostro, L.E., Crispin, J.C., Rodriguez-Romo, R., Uribe-Uribe, N.O., Carrasco, G., Gomez-Martin, D., and Alcocer-Varela, J. (2012). De Novo Donor-Specific HLA Antibody Development and Peripheral CD4(+)CD25(high) Cells in Kidney Transplant Recipients: A Place for Interaction? *J Transplant* *2012*, 302539.
- Ali, N., and Rosenblum, M.D. (2017). Regulatory T cells in skin. *Immunology* *152*, 372-381.
- Ali, N., Zirak, B., Rodriguez, R.S., Pauli, M.L., Truong, H.A., Lai, K., Ahn, R., Corbin, K., Lowe, M.M., Scharschmidt, T.C., *et al.* (2017). Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell* *169*, 1119-1129 e1111.
- Arpaia, N., Green, J.A., Molledo, B., Arvey, A., Hemmers, S., Yuan, S., Treuting, P.M., and Rudensky, A.Y. (2015). A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* *162*, 1078-1089.
- Artis, D., and Spits, H. (2015). The biology of innate lymphoid cells. *Nature* *517*, 293-301.
- Aurora, A.B., Porrello, E.R., Tan, W., Mahmoud, A.I., Hill, J.A., Bassel-Duby, R., Sadek, H.A., and Olson, E.N. (2014). Macrophages are required for neonatal heart regeneration. *J Clin Invest* *124*, 1382-1392.
- Bagavant, H., and Tung, K.S. (2005). Failure of CD25+ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis. *J Immunol* *175*, 944-950.
- Barron, L., Doms, H., Hoyer, K.K., Kuswanto, W., Hofmann, J., O'Gorman, W.E., and Abbas, A.K. (2010). Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory T cells. *J Immunol* *185*, 6426-6430.
- Battaglia, M., Stabilini, A., Draghici, E., Migliavacca, B., Gregori, S., Bonifacio, E., and Roncarolo, M.G. (2006a). Induction of tolerance in type 1 diabetes via both CD4+CD25+ T regulatory cells and T regulatory type 1 cells. *Diabetes* *55*, 1571-1580.
- Battaglia, M., Stabilini, A., Migliavacca, B., Horejs-Hoeck, J., Kaupper, T., and Roncarolo, M.G. (2006b). Rapamycin promotes expansion of functional

CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 177, 8338-8347.

Battaglia, M., Stabilini, A., and Roncarolo, M.G. (2005). Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105, 4743-4748.

Belghith, M., Bluestone, J.A., Barriot, S., Megret, J., Bach, J.F., and Chatenoud, L. (2003). TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 9, 1202-1208.

Bieber, A.J., Kerr, S., and Rodriguez, M. (2003). Efficient central nervous system remyelination requires T cells. *Ann Neurol* 53, 680-684.

Blat, D., Zigmond, E., Alteber, Z., Waks, T., and Eshhar, Z. (2014). Suppression of murine colitis and its associated cancer by carcinoembryonic antigen-specific regulatory T cells. *Mol Ther* 22, 1018-1028.

Bluestone, J.A., Bour-Jordan, H., Cheng, M., and Anderson, M. (2015a). T cells in the control of organ-specific autoimmunity. *J Clin Invest* 125, 2250-2260.

Bluestone, J.A., Buckner, J.H., Fitch, M., Gitelman, S.E., Gupta, S., Hellerstein, M.K., Herold, K.C., Lares, A., Lee, M.R., Li, K., *et al.* (2015b). Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med* 7, 315ra189.

Boardman, D., Maher, J., Lechler, R., Smyth, L., and Lombardi, G. (2016). Antigen-specificity using chimeric antigen receptors: the future of regulatory T-cell therapy? *Biochem Soc Trans* 44, 342-348.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., *et al.* (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204, 1303-1310.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paepfer, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27, 68-73.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A.J., Benoist, C., *et al.* (2013). A special population of regulatory T cells potentiates muscle repair. *Cell* 155, 1282-1295.

Busch, R., Neese, R.A., Awada, M., Hayes, G.M., and Hellerstein, M.K. (2007). Measurement of cell proliferation by heavy water labeling. *Nat Protoc* 2, 3045-3057.

Carbone, F., Nencioni, A., Mach, F., Vuilleumier, N., and Montecucco, F. (2013). Pathophysiological role of neutrophils in acute myocardial infarction. *Thromb Haemost* 110, 501-514.

Castiglioni, A., Corna, G., Rigamonti, E., Basso, V., Vezzoli, M., Monno, A., Almada, A.E., Mondino, A., Wagers, A.J., Manfredi, A.A., *et al.* (2015). FOXP3+ T Cells Recruited to Sites of Sterile Skeletal Muscle Injury Regulate the Fate of Satellite Cells and Guide Effective Tissue Regeneration. *PLoS One* 10, e0128094.

Chatenoud, L., Primo, J., and Bach, J.F. (1997). CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol* 158, 2947-2954.

Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, C.M., Sears-Kraxberger, I., Steward, O., Macklin, W.B., Loring, J.F., *et al.* (2014). Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis. *Stem Cell Reports* 2, 825-837.

Chinen, T., Kannan, A.K., Levine, A.G., Fan, X., Klein, U., Zheng, Y., Gasteiger, G., Feng, Y., Fontenot, J.D., and Rudensky, A.Y. (2016). An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* 17, 1322-1333.

Cooper, G.S., Bynum, M.L., and Somers, E.C. (2009). Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun* 33, 197-207.

Curran, K.J., Pegram, H.J., and Brentjens, R.J. (2012). Chimeric antigen receptors for T cell immunotherapy: current understanding and future directions. *J Gene Med* 14, 405-415.

D'Alessio, F.R., Tsushima, K., Aggarwal, N.R., West, E.E., Willett, M.H., Britos, M.F., Pipeling, M.R., Brower, R.G., Tuder, R.M., McDyer, J.F., *et al.* (2009). CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *J Clin Invest* 119, 2898-2913.

Dombrowski, Y., O'Hagan, T., Dittmer, M., Penalva, R., Mayoral, S.R., Bankhead, P., Fleville, S., Eleftheriadis, G., Zhao, C., Naughton, M., *et al.* (2017). Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci* 20, 674-680.

Edinger, M., Hoffmann, P., Ermann, J., Drago, K., Fathman, C.G., Strober, S., and Negrin, R.S. (2003). CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 9, 1144-1150.

Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., and Mauri, C. (2004). Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med* 200, 277-285.

Elinav, E., Adam, N., Waks, T., and Eshhar, Z. (2009). Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor. *Gastroenterology* 136, 1721-1731.

Ellis, C.N., Krueger, G.G., and Alefacept Clinical Study, G. (2001). Treatment of chronic plaque psoriasis by selective targeting of memory effector T lymphocytes. *N Engl J Med* 345, 248-255.

Ertl, G., and Frantz, S. (2005). Healing after myocardial infarction. *Cardiovasc Res* 66, 22-32.

Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T., Wan, Y.Y., O'Connor, W., Jr., Rongvaux, A., Van Rooijen, N., Haberman, A.M., *et al.* (2011). Control of TH17 cells occurs in the small intestine. *Nature* 475, 514-518.

Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S., *et al.* (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* 15, 930-939.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6, 1142-1151.

Fox, R.I., Saito, I., Chan, E.K., Josephs, S., Salahuddin, S.Z., Ahlashi, D.V., Staal, F.W., Gallo, R., Pei-Ping, H., and Le, C.S. (1989). Viral genomes in lymphomas of patients with Sjogren's syndrome. *J Autoimmun* 2, 449-455.

Fransson, M., Piras, E., Burman, J., Nilsson, B., Essand, M., Lu, B., Harris, R.A., Magnusson, P.U., Brittebo, E., and Loskog, A.S. (2012). CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* 9, 112.

Gadani, S.P., Walsh, J.T., Smirnov, I., Zheng, J., and Kipnis, J. (2015). The glia-derived alarmin IL-33 orchestrates the immune response and promotes recovery following CNS injury. *Neuron* 85, 703-709.

Gliwinski, M., Iwaszkiewicz-Grzes, D., and Trzonkowski, P. (2017). Cell-Based Therapies with T Regulatory Cells. *BioDrugs* 31, 335-347.

Glowacki, A.J., Yoshizawa, S., Jhunjunwala, S., Vieira, A.E., Garlet, G.P., Sfeir, C., and Little, S.R. (2013). Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes. *Proc Natl Acad Sci U S A* 110, 18525-18530.

Green, E.A., Gorelik, L., McGregor, C.M., Tran, E.H., and Flavell, R.A. (2003). CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells control anti-islet CD8<sup>+</sup> T cells through TGF- $\beta$ -TGF- $\beta$  receptor interactions in type 1 diabetes. *Proc Natl Acad Sci U S A* 100, 10878-10883.

Grinberg-Bleyer, Y., Baeyens, A., You, S., Elhage, R., Fourcade, G., Gregoire, S., Cagnard, N., Carpentier, W., Tang, Q., Bluestone, J., *et al.* (2010). IL-2 reverses

established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* 207, 1871-1878.

Hartemann, A., Bensimon, G., Payan, C.A., Jacqueminet, S., Bourron, O., Nicolas, N., Fonfrede, M., Rosenzweig, M., Bernard, C., and Klatzmann, D. (2013). Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol* 1, 295-305.

Hofmann, U., Beyersdorf, N., Weirather, J., Podolskaya, A., Bauersachs, J., Ertl, G., Kerkau, T., and Frantz, S. (2012). Activation of CD4+ T lymphocytes improves wound healing and survival after experimental myocardial infarction in mice. *Circulation* 125, 1652-1663.

Hombach, A.A., Kofler, D., Rappl, G., and Abken, H. (2009). Redirecting human CD4+CD25+ regulatory T cells from the peripheral blood with pre-defined target specificity. *Gene Ther* 16, 1088-1096.

Hori, S., Haury, M., Coutinho, A., and Demengeot, J. (2002). Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A* 99, 8213-8218.

Hu, C., Ding, H., Zhang, X., Wong, F.S., and Wen, L. (2013). Combination treatment with anti-CD20 and oral anti-CD3 prevents and reverses autoimmune diabetes. *Diabetes* 62, 2849-2858.

Ishikawa, H., Ochi, H., Chen, M.L., Frenkel, D., Maron, R., and Weiner, H.L. (2007). Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody. *Diabetes* 56, 2103-2109.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. *Science* 296, 747-749.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564.

Kim, Y.C., Zhang, A.H., Su, Y., Rieder, S.A., Rossi, R.J., Ettinger, R.A., Pratt, K.P., Shevach, E.M., and Scott, D.W. (2015). Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T- and B-cell responses. *Blood* 125, 1107-1115.

Klatzmann, D., and Abbas, A.K. (2015). The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol* 15, 283-294.

Kleinschnitz, C., Kraft, P., Dreykluft, A., Hagedorn, I., Gobel, K., Schuhmann, M.K., Langhauser, F., Helluy, X., Schwarz, T., Bittner, S., *et al.* (2013). Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood* 121, 679-691.

- Klose, C.S., and Artis, D. (2016). Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* 17, 765-774.
- Kohm, A.P., Carpentier, P.A., Anger, H.A., and Miller, S.D. (2002). Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169, 4712-4716.
- Krueger, G.G., and Ellis, C.N. (2003). Alefacept therapy produces remission for patients with chronic plaque psoriasis. *Br J Dermatol* 148, 784-788.
- Kuhn, C., You, S., Valette, F., Hale, G., van Endert, P., Bach, J.F., Waldmann, H., and Chatenoud, L. (2011). Human CD3 transgenic mice: preclinical testing of antibodies promoting immune tolerance. *Sci Transl Med* 3, 68ra10.
- Laurent, P., Jolivel, V., Manicki, P., Chiu, L., Contin-Bordes, C., Truchetet, M.E., and Pradeu, T. (2017). Immune-Mediated Repair: A Matter of Plasticity. *Front Immunol* 8, 454.
- Lavine, K.J., Epelman, S., Uchida, K., Weber, K.J., Nichols, C.G., Schilling, J.D., Ornitz, D.M., Randolph, G.J., and Mann, D.L. (2014). Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proc Natl Acad Sci U S A* 111, 16029-16034.
- Lebwohl, M., Christophers, E., Langley, R., Ortonne, J.P., Roberts, J., Griffiths, C.E., and Alefacept Clinical Study, G. (2003). An international, randomized, double-blind, placebo-controlled phase 3 trial of intramuscular alefacept in patients with chronic plaque psoriasis. *Arch Dermatol* 139, 719-727.
- Leighton, E., Sainsbury, C.A., and Jones, G.C. (2017). A Practical Review of C-Peptide Testing in Diabetes. *Diabetes Ther* 8, 475-487.
- Li, J., Kim, S.G., and Blenis, J. (2014). Rapamycin: one drug, many effects. *Cell Metab* 19, 373-379.
- Li, J., Tan, J., Martino, M.M., and Lui, K.O. (2018). Regulatory T-Cells: Potential Regulator of Tissue Repair and Regeneration. *Front Immunol* 9, 585.
- Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., Giese, T., and Veltkamp, R. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 15, 192-199.
- MacDonald, K.G., Hoeppli, R.E., Huang, Q., Gillies, J., Luciani, D.S., Orban, P.C., Broady, R., and Levings, M.K. (2016). Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest* 126, 1413-1424.
- Marek-Trzonkowska, N., Mysliwiec, M., Dobyszuk, A., Grabowska, M., Derkowska, I., Juscinska, J., Owczuk, R., Szadkowska, A., Witkowski, P., Mlynarski, W., *et al.* (2014).

Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clin Immunol* 153, 23-30.

Masteller, E.L., Tang, Q., and Bluestone, J.A. (2006). Antigen-specific regulatory T cells--ex vivo expansion and therapeutic potential. *Semin Immunol* 18, 103-110.

Masteller, E.L., Warner, M.R., Tang, Q., Tarbell, K.V., McDevitt, H., and Bluestone, J.A. (2005). Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. *J Immunol* 175, 3053-3059.

Matsuoka, K., Koreth, J., Kim, H.T., Bascug, G., McDonough, S., Kawano, Y., Murase, K., Cutler, C., Ho, V.T., Alyea, E.P., *et al.* (2013). Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med* 5, 179ra143.

McClymont, S.A., Putnam, A.L., Lee, M.R., Esensten, J.H., Liu, W., Hulme, M.A., Hoffmuller, U., Baron, U., Olek, S., Bluestone, J.A., *et al.* (2011). Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J Immunol* 186, 3918-3926.

Mock, J.R., Garibaldi, B.T., Aggarwal, N.R., Jenkins, J., Limjunyawong, N., Singer, B.D., Chau, E., Rabold, R., Files, D.C., Sidhaye, V., *et al.* (2014). Foxp3+ regulatory T cells promote lung epithelial proliferation. *Mucosal Immunol* 7, 1440-1451.

Monti, P., Scirpoli, M., Maffi, P., Piemonti, L., Secchi, A., Bonifacio, E., Roncarolo, M.G., and Battaglia, M. (2008). Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+CD25+FOXP3+ regulatory T-cells. *Diabetes* 57, 2341-2347.

Nahrendorf, M., Swirski, F.K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.L., Libby, P., Weissleder, R., and Pittet, M.J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 204, 3037-3047.

Nosbaum, A., Prevel, N., Truong, H.A., Mehta, P., Ettinger, M., Scharschmidt, T.C., Ali, N.H., Pauli, M.L., Abbas, A.K., and Rosenblum, M.D. (2016). Cutting Edge: Regulatory T Cells Facilitate Cutaneous Wound Healing. *J Immunol* 196, 2010-2014.

Ochi, H., Abraham, M., Ishikawa, H., Frenkel, D., Yang, K., Basso, A.S., Wu, H., Chen, M.L., Gandhi, R., Miller, A., *et al.* (2006). Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat Med* 12, 627-635.

Ohkura, N., Hamaguchi, M., and Sakaguchi, S. (2011). FOXP3+ regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends Pharmacol Sci* 32, 158-166.

Ohta, A., and Sitkovsky, M. (2014). Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol* 5, 304.

Ono, M., Shimizu, J., Miyachi, Y., and Sakaguchi, S. (2006). Control of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25+ and CD25- regulatory T cells. *J Immunol* 176, 4748-4756.

Park, J., Gao, W., Whiston, R., Strom, T.B., Metcalfe, S., and Fahmy, T.M. (2011). Modulation of CD4+ T lymphocyte lineage outcomes with targeted, nanoparticle-mediated cytokine delivery. *Mol Pharm* 8, 143-152.

Paust, S., Lu, L., McCarty, N., and Cantor, H. (2004). Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A* 101, 10398-10403.

Penaranda, C., Tang, Q., and Bluestone, J.A. (2011). Anti-CD3 therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells. *J Immunol* 187, 2015-2022.

Plaisted, W.C., Zavala, A., Hingco, E., Tran, H., Coleman, R., Lane, T.E., Loring, J.F., and Walsh, C.M. (2016). Remyelination Is Correlated with Regulatory T Cell Induction Following Human Embryoid Body-Derived Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis. *PLoS One* 11, e0157620.

Plitas, G., and Rudensky, A.Y. (2016). Regulatory T Cells: Differentiation and Function. *Cancer Immunol Res* 4, 721-725.

Powell, J.D., and Delgoffe, G.M. (2010). The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 33, 301-311.

Quintas-Cardama, A. (2018). CD19 directed CAR T cell therapy in diffuse large B-cell lymphoma. *Oncotarget* 9, 29843-29844.

Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., *et al.* (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332, 600-603.

Raposo, C., Graubardt, N., Cohen, M., Eitan, C., London, A., Berkutzki, T., and Schwartz, M. (2014). CNS repair requires both effector and regulatory T cells with distinct temporal and spatial profiles. *J Neurosci* 34, 10141-10155.

Rouse, M., Nagarkatti, M., and Nagarkatti, P.S. (2013). The role of IL-2 in the activation and expansion of regulatory T-cells and the development of experimental autoimmune encephalomyelitis. *Immunobiology* 218, 674-682.

Saadoun, D., Rosenzweig, M., Joly, F., Six, A., Carrat, F., Thibault, V., Sene, D., Cacoub, P., and Klatzmann, D. (2011). Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 365, 2067-2077.



Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164.

Sakaguchi, S., Vignali, D.A., Rudensky, A.Y., Niec, R.E., and Waldmann, H. (2013). The plasticity and stability of regulatory T cells. *Nat Rev Immunol* 13, 461-467.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell* 133, 775-787.

Scharschmidt, T.C., Vasquez, K.S., Truong, H.A., Gearty, S.V., Pauli, M.L., Nosbaum, A., Gratz, I.K., Otto, M., Moon, J.J., Liese, J., *et al.* (2015). A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity* 43, 1011-1021.

Scheffold, A., Huhn, J., and Hofer, T. (2005). Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango. *Eur J Immunol* 35, 1336-1341.

Silverstein, S.C., and Rabadan, R. (2012). How many neutrophils are enough (redux, redux)? *J Clin Invest* 122, 2776-2779.

Steenblock, E.R., and Fahmy, T.M. (2008). A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. *Mol Ther* 16, 765-772.

Steinman, L., Merrill, J.T., McInnes, I.B., and Peakman, M. (2012). Optimization of current and future therapy for autoimmune diseases. *Nat Med* 18, 59-65.

Tang, Q., Adams, J.Y., Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., Piccirillo, C.A., Salomon, B.L., and Bluestone, J.A. (2008). Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28, 687-697.

Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199, 1455-1465.

Tang, Q., and Lee, K. (2012). Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transplant* 17, 349-354.

Tontonoz, P., and Spiegelman, B.M. (2008). Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* 77, 289-312.

Trauner, M., Fuchs, C.D., Halilbasic, E., and Paumgartner, G. (2017). New therapeutic concepts in bile acid transport and signaling for management of cholestasis. *Hepatology* 65, 1393-1404.

Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J.L., and Stinissen, P. (2008). Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* 180, 6411-6420.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 199, 971-979.

Waldron-Lynch, F., Henegariu, O., Deng, S., Preston-Hurlburt, P., Tooley, J., Flavell, R., and Herold, K.C. (2012). Teplizumab induces human gut-tropic regulatory cells in humanized mice and patients. *Sci Transl Med* 4, 118ra112.

Walsh, J.T., Zheng, J., Smirnov, I., Lorenz, U., Tung, K., and Kipnis, J. (2014). Regulatory T cells in central nervous system injury: a double-edged sword. *J Immunol* 193, 5013-5022.

Wang, X., Xuan, W., Zhu, Z.Y., Li, Y., Zhu, H., Zhu, L., Fu, D.Y., Yang, L.Q., Li, P.Y., and Yu, W.F. (2018). The evolving role of neuro-immune interaction in brain repair after cerebral ischemic stroke. *CNS Neurosci Ther* 24, 1100-1114.

Weirather, J., Hofmann, U.D., Beyersdorf, N., Ramos, G.C., Vogel, B., Frey, A., Ertl, G., Kerkau, T., and Frantz, S. (2014). Foxp3+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation. *Circ Res* 115, 55-67.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322, 271-275.

Wright, G.P., Notley, C.A., Xue, S.A., Bendle, G.M., Holler, A., Schumacher, T.N., Ehrenstein, M.R., and Stauss, H.J. (2009). Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. *Proc Natl Acad Sci U S A* 106, 19078-19083.

Wu, H.Y., Center, E.M., Tsokos, G.C., and Weiner, H.L. (2009). Suppression of murine SLE by oral anti-CD3: inducible CD4+CD25-LAP+ regulatory T cells control the expansion of IL-17+ follicular helper T cells. *Lupus* 18, 586-596.

Wu, H.Y., Maron, R., Tukpah, A.M., and Weiner, H.L. (2010). Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP+ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. *J Immunol* 185, 3401-3407.

Wu, H.Y., Quintana, F.J., and Weiner, H.L. (2008). Nasal anti-CD3 antibody ameliorates lupus by inducing an IL-10-secreting CD4+ CD25- LAP+ regulatory T cell and is associated with down-regulation of IL-17+ CD4+ ICOS+ CXCR5+ follicular helper T cells. *J Immunol* 181, 6038-6050.

Wynn, T.A., and Barron, L. (2010). Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 30, 245-257.

Xia, N., Jiao, J., Tang, T.T., Lv, B.J., Lu, Y.Z., Wang, K.J., Zhu, Z.F., Mao, X.B., Nie, S.F., Wang, Q., *et al.* (2015). Activated regulatory T-cells attenuate myocardial ischaemia/reperfusion injury through a CD39-dependent mechanism. *Clin Sci (Lond)* 128, 679-693.

Yadav, M., Stephan, S., and Bluestone, J.A. (2013). Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol* 4, 232.

Yoon, J., Schmidt, A., Zhang, A.H., Konigs, C., Kim, Y.C., and Scott, D.W. (2017). FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII. *Blood* 129, 238-245.

Yu, A., Snowwhite, I., Vendrame, F., Rosenzweig, M., Klatzmann, D., Pugliese, A., and Malek, T.R. (2015). Selective IL-2 responsiveness of regulatory T cells through multiple intrinsic mechanisms supports the use of low-dose IL-2 therapy in type 1 diabetes. *Diabetes* 64, 2172-2183.

Zhang, P., Tey, S.K., Koyama, M., Kuns, R.D., Olver, S.D., Lineburg, K.E., Lor, M., Teal, B.E., Raffelt, N.C., Raju, J., *et al.* (2013). Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. *J Immunol* 191, 5291-5303.

Zhang, Q., Lu, W., Liang, C.L., Chen, Y., Liu, H., Qiu, F., and Dai, Z. (2018). Chimeric Antigen Receptor (CAR) Treg: A Promising Approach to Inducing Immunological Tolerance. *Front Immunol* 9, 2359.

Zhou, X., Kong, N., Wang, J., Fan, H., Zou, H., Horwitz, D., Brand, D., Liu, Z., and Zheng, S.G. (2010). Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. *J Immunol* 185, 2675-2679.

Ziegler, S.F. (2006). FOXP3: of mice and men. *Annu Rev Immunol* 24, 209-226.

## **CHAPTER FIVE**

### **Conclusion**

## **Summary and Significance**

Multiple Sclerosis (MS) is a chronic, autoimmune disease of the central nervous system (CNS) due to infiltrating immune cells resulting in demyelination and axonal loss (Steinman, 1996). Inflammatory lymphocytes, mainly effector T cells (Teff), and myeloid cells are responsible for ensuing damage, whereas regulatory T cells (Tregs) are responsible for maintenance of immune cell homeostasis and preventing autoimmunity (Josefowicz et al., 2012). Previous studies have shown that dysregulation of Tregs has been linked to MS disease pathogenesis (Haas et al., 2007; Venken et al., 2008) and patients with MS have reduced numbers of Tregs, whereas Venken et al. reported that Tregs have less potent suppressor functions (Venken et al., 2008). Current disease modifying therapies focus on immunosuppression, limiting cell entry into the CNS thereby inhibiting ongoing demyelination. These treatments are only available for the mildest form of MS, Relapsing-Remitting MS (RRMS), with efficacy in only 30% of RRMS patients (Lassmann et al., 1997), and do not provide long-term relief for progressive forms of MS. Consequently, there is an unmet clinical need for therapies that sustain remyelination and promote axonal repair once the damage has already occurred.

Transplantation of neural stem cells (NSCs) may be a viable therapeutic strategy for MS. Indeed, studies have proven that surgical implantation of NSCs into the CNS of animal models of demyelination have been shown to suppress neuroinflammation and enhance remyelination (Ben-Hur et al., 2013; Greenberg et al., 2014b; Mozafari et al., 2015; Pluchino et al., 2009). While early animal studies are promising, the majority of work evaluating NSC transplantation has been conducted using genetically identical, syngeneic donors or immune suppressed subjects. In the clinic, grafts are likely to be

genetically mismatched (allogenic) because samples from MHC matched donors are difficult to obtain and autologous therapies are not feasible for MS because the etiology is not entirely understood, therefore autologous cells could possess genetic defects. Additionally, life-long immune suppression renders the host susceptible to viral and bacterial infections. Therefore, when evaluating NSC transplantation as a therapeutic, it is important to consider the potential for cells to elicit an immune response.

The goal of this dissertation is to elucidate the mechanisms by which NSCs promote remyelination following transplantation in murine models of MS. This knowledge has the potential to improve therapeutic approaches for modulating immune responses and promoting sustained remyelination in patients with MS. To accomplish this goal, we evaluated the impact of NSCs following transplantation into two murine models of immune mediated demyelination; a viral model, using the neuro-adapted JHM strain of murine hepatitis virus (JHMV) and experimental autoimmune encephalomyelitis (EAE). Intracranial inoculation of susceptible mice with JHMV leads to an acute encephalomyelitis and immune-mediated demyelination followed by axonal loss (Lane and Hosking, 2010). Previous, our group reported that intraspinal transplantation of syngeneic mouse NSCs into JHMV-infected mice resulted in engraftment, differentiation of NSCs into oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes that is associated with increased axonal remyelination (Carbajal et al., 2010). Transplanted NSCs were found accumulate within areas of axonal damage where they were directly responsible for wrapping myelin around damaged axons (Greenberg et al., 2014b). JHMV- infected mice transplanted with human embryonic stem cell (hESC)- or human induced pluripotent stem cell (iPSC)- derived neural precursor cells (NPCs) displayed

remyelination and reduced neuroinflammation that was associated with an increase of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs (Chen et al., 2014b; Plaisted et al., 2016) (**reviewed in Chapter 1**).

Building upon these findings, we found that hNSCs expand neural antigen-specific Tregs from the 'exTreg' pool to facilitate remyelination following hNSC transplant in EAE (**Chapter 2**). In the most commonly used animal model for MS, EAE, immunization of mice with myelin oligodendrocyte glycoprotein (MOG) results in demyelinating lesions caused by infiltrating T cells, B cells, and macrophages (McCarthy et al., 2012). Consistent with previous findings in JHMV-infected mice, EAE mice receiving intraspinal transplants of syngeneic mNSCs displayed less demyelination compared to controls, with no effect upon immune cell populations. These results suggest repair is due to cell replacement, rather than immune modulation. Transplantation of hESC-derived hNSCs into the spinal cord of EAE mice resulted in dampened neuroinflammation and enhanced remyelination that was correlated with an increase in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. Tregs accumulated near the site of hNSC transplantation, and importantly, ablation of Tregs inhibited remyelination. hNSCs expanded neural antigen-specific Tregs *in vitro*. Tregs were found to respond to the neural antigen, neurofilament, expressed by hNSCs, Neural antigen-specific Tregs were found to be expanded from a population of thymus derived Tregs that lost FoxP3 expression in the periphery, termed 'exTregs' (Rubtsov et al., 2008; Zhou et al., 2009). Intriguingly, co-culture of 'exTreg' containing splenocytes with hNSCs reinvigorated FoxP3 expression. While, it is well established that self-peptide/ MHC is essential for homeostasis of conventional T cells (Sprent and Surh, 2011), the factors which influence Treg homeostasis remain elusive. We present the novel concept that self-

peptide/MHC is important for the homeostasis of Tregs and maintenance of FoxP3 expression. Supporting this, we generated a T cell receptor (TCR) transgenic mouse which only recognizes MOG and neurofilament antigens ( $RAG2^{-/-}2D2^{+}$  also known as R2D2). These mice are deficient in peripheral Tregs, but upon MOG exposure *in vivo*  $CD4^{+}CD25^{+}FoxP3^{+}$  Tregs emerge. Together these data strongly support a role for tonic TCR signaling through self-peptide/MHC contact in Treg homeostasis.

In addition, our work also highlights a role for Tregs as not only suppressors of inflammation, but also a novel role for Tregs in CNS remyelination in the context of EAE. hNSC- Tregs possessed a unique gene transcriptional profile compared to induced (iTregs), upregulating genes associated with maintaining T cell homeostasis, immunosuppression in the CNS and maturation of OPCs. hNSC-Tregs significantly upregulated Dickkopf-3, (*Dkk3*) and Transglutaminase-2 (TG2) which are known to influence differentiation of OPCs to mature, myelin producing oligodendrocytes (Giera et al., 2018; Munji et al., 2011; Rosso and Inestrosa, 2013). Induction of TG2 via pharmacological agents are currently under development for other neurological disorders (Salzman et al., 2016; Stoveken et al., 2016) and could also be used to treat MS. Future studies utilizing conditional knock out of TG2 in Tregs would be useful in confirming the role of Treg secreted TG2 in remyelination.

Interactions between transplanted hNSCs and endogenous CNS and immune cells have never been visualized in real time, until now. Utilizing two-photon microscopy we were able to visualize real-time cellular interactions following hNSC-transplant (**Chapter 3**). Tregs in mice receiving hNSC-transplants accumulated at the site of transplant, but did not display altered motility dynamics (mean track speed, velocity or



meandering index). However, Tregs interacted with cells, most likely oligodendrocytes, that produced proteolipid protein (PLP), a component of the myelin sheath. These data support the hypothesis that Tregs interact with endogenous OPCs that remyelinate in the CNS of EAE mice.

Clinical trials involving Tregs are currently underway to suppress autoimmune and alloimmune responses in human diseases including graft-versus-host-disease and insulin-dependent type 1 diabetes. Tregs have the ability to suppress inflammatory responses, prevent autoimmunity, and have recently been implicated as modulators of tissue repair, therefore making Tregs an attractive immunotherapy for autoimmune diseases, such as MS. Current Treg based therapies attempt to promote Treg expansion in hosts, or use adoptive cellular therapy (ACT) to induce immune tolerance, but these therapies may also be useful in tissue regeneration (**Chapter 4**). Furthermore, self-antigen specific Tregs have superior efficacy in pre-clinical models of Treg ACT compared to polyclonal Tregs (Tang and Bluestone, 2013; Tang et al., 2012). Therefore, hNSC-Treg ACT may be a therapeutic approach to promote sustained remyelination in patients with MS, although, stability and function of hNSC-Tregs warrants further investigation.

NSC transplantation expands neural antigen-specific Tregs from the 'exTreg' pool that promote endogenous repair pathways in EAE. These findings support a crucial role for recognition of self-antigen/MHC in Treg homeostasis and maintenance of FoxP3, which is important for suppressive and tissue repair programs. A better understanding of the mechanisms by which neural antigen-specific Tregs mediate endogenous remyelination following NSC transplantation will give us new insight to develop novel therapies, such as hNSC-Treg ACT, and treat neurodegenerative disease, such as MS.

## References

- Abbas, A.K., Benoist, C., Bluestone, J.A., Campbell, D.J., Ghosh, S., Hori, S., Jiang, S., Kuchroo, V.K., Mathis, D., Roncarolo, M.G., *et al.* (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* 14, 307-308.
- Ablamunits, V., Bisikirska, B., and Herold, K.C. (2010). Acquisition of regulatory function by human CD8(+) T cells treated with anti-CD3 antibody requires TNF. *Eur J Immunol* 40, 2891-2901.
- Adami, C., Pooley, J., Glomb, J., Stecker, E., Fazal, F., Fleming, J.O., and Baker, S.C. (1995). Evolution of mouse hepatitis virus (MHV) during chronic infection: quasispecies nature of the persisting MHV RNA. *Virology* 209, 337-346.
- Ager, R.R., Davis, J.L., Agazaryan, A., Benavente, F., Poon, W.W., LaFerla, F.M., and Blurton-Jones, M. (2015). Human neural stem cells improve cognition and promote synaptic growth in two complementary transgenic models of Alzheimer's disease and neuronal loss. *Hippocampus* 25, 813-826.
- Aharonowiz, M., Einstein, O., Fainstein, N., Lassmann, H., Reubinoff, B., and Ben-Hur, T. (2008). Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis. *PLoS One* 3, e3145.
- Alberu, J., Vargas-Rojas, M.I., Morales-Buenrostro, L.E., Crispin, J.C., Rodriguez-Romo, R., Uribe-Urbe, N.O., Carrasco, G., Gomez-Martin, D., and Alcocer-Varela, J. (2012). De Novo Donor-Specific HLA Antibody Development and Peripheral CD4(+)CD25(high) Cells in Kidney Transplant Recipients: A Place for Interaction? *J Transplant* 2012, 302539.
- Ali, N., and Rosenblum, M.D. (2017). Regulatory T cells in skin. *Immunology* 152, 372-381.
- Ali, N., Zirak, B., Rodriguez, R.S., Pauli, M.L., Truong, H.A., Lai, K., Ahn, R., Corbin, K., Lowe, M.M., Scharschmidt, T.C., *et al.* (2017). Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell* 169, 1119-1129 e1111.
- Anghelina, D., Zhao, J., Trandem, K., and Perlman, S. (2009). Role of regulatory T cells in coronavirus-induced acute encephalitis. *Virology* 385, 358-367.
- Arpaia, N., Green, J.A., Molledo, B., Arvey, A., Hemmers, S., Yuan, S., Treuting, P.M., and Rudensky, A.Y. (2015). A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* 162, 1078-1089.
- Artis, D., and Spits, H. (2015). The biology of innate lymphoid cells. *Nature* 517, 293-301.

- Aurora, A.B., Porrello, E.R., Tan, W., Mahmoud, A.I., Hill, J.A., Bassel-Duby, R., Sadek, H.A., and Olson, E.N. (2014). Macrophages are required for neonatal heart regeneration. *J Clin Invest* 124, 1382-1392.
- Bagavant, H., and Tung, K.S. (2005). Failure of CD25+ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis. *J Immunol* 175, 944-950.
- Barnden, M.J., Allison, J., Heath, W.R., and Carbone, F.R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76, 34-40.
- Barron, L., Doms, H., Hoyer, K.K., Kuswanto, W., Hofmann, J., O'Gorman, W.E., and Abbas, A.K. (2010). Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory T cells. *J Immunol* 185, 6426-6430.
- Battaglia, M., Stabilini, A., Draghici, E., Migliavacca, B., Gregori, S., Bonifacio, E., and Roncarolo, M.G. (2006a). Induction of tolerance in type 1 diabetes via both CD4+CD25+ T regulatory cells and T regulatory type 1 cells. *Diabetes* 55, 1571-1580.
- Battaglia, M., Stabilini, A., Migliavacca, B., Horejs-Hoeck, J., Kaupper, T., and Roncarolo, M.G. (2006b). Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 177, 8338-8347.
- Battaglia, M., Stabilini, A., and Roncarolo, M.G. (2005). Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105, 4743-4748.
- Belghith, M., Bluestone, J.A., Barriot, S., Megret, J., Bach, J.F., and Chatenoud, L. (2003). TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 9, 1202-1208.
- Ben-Hur, T., Einstein, O., Mizrachi-Kol, R., Ben-Menachem, O., Reinhartz, E., Karussis, D., and Abramsky, O. (2003). Transplanted multipotential neural precursor cells migrate into the inflamed white matter in response to experimental autoimmune encephalomyelitis. *Glia* 41, 73-80.
- Ben-Hur, T., Fainstein, N., and Nishri, Y. (2013). Cell-based reparative therapies for multiple sclerosis. *Curr Neurol Neurosci Rep* 13, 397.
- Ben-Hur, T., Rogister, B., Murray, K., Rougon, G., and Dubois-Dalcq, M. (1998). Growth and fate of PSA-NCAM+ precursors of the postnatal brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18, 5777-5788.
- Bergmann, C.C., Lane, T.E., and Stohlman, S.A. (2006). Coronavirus infection of the central nervous system: host-virus stand-off. *Nature reviews Microbiology* 4, 121-132.

Bergmann, C.C., Parra, B., Hinton, D.R., Chandran, R., Morrison, M., and Stohlman, S.A. (2003). Perforin-mediated effector function within the central nervous system requires IFN-gamma-mediated MHC up-regulation. *J Immunol* 170, 3204-3213.

Bettelli, E., Pagany, M., Weiner, H.L., Linington, C., Sobel, R.A., and Kuchroo, V.K. (2003). Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197, 1073-1081.

Bieber, A.J., Kerr, S., and Rodriguez, M. (2003). Efficient central nervous system remyelination requires T cells. *Ann Neurol* 53, 680-684.

Blanc, C.A., Grist, J.J., Rosen, H., Sears-Kraxberger, I., Steward, O., and Lane, T.E. (2015). Sphingosine-1-phosphate receptor antagonism enhances proliferation and migration of engrafted neural progenitor cells in a model of viral-induced demyelination. *Am J Pathol* 185, 2819-2832.

Blanc, C.A., Rosen, H., and Lane, T.E. (2014). FTY720 (fingolimod) modulates the severity of viral-induced encephalomyelitis and demyelination. *J Neuroinflammation* 11, 138.

Blat, D., Zigmond, E., Alteber, Z., Waks, T., and Eshhar, Z. (2014). Suppression of murine colitis and its associated cancer by carcinoembryonic antigen-specific regulatory T cells. *Mol Ther* 22, 1018-1028.

Bluestone, J.A., Bour-Jordan, H., Cheng, M., and Anderson, M. (2015a). T cells in the control of organ-specific autoimmunity. *J Clin Invest* 125, 2250-2260.

Bluestone, J.A., Buckner, J.H., Fitch, M., Gitelman, S.E., Gupta, S., Hellerstein, M.K., Herold, K.C., Lares, A., Lee, M.R., Li, K., *et al.* (2015b). Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med* 7, 315ra189.

Blurton-Jones, M., Kitazawa, M., Martinez-Coria, H., Castello, N.A., Muller, F.J., Loring, J.F., Yamasaki, T.R., Poon, W.W., Green, K.N., and LaFerla, F.M. (2009). Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci U S A* 106, 13594-13599.

Boardman, D., Maher, J., Lechler, R., Smyth, L., and Lombardi, G. (2016). Antigen-specificity using chimeric antigen receptors: the future of regulatory T-cell therapy? *Biochem Soc Trans* 44, 342-348.

Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., *et al.* (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204, 1303-1310.

Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* 34, 525-527.

Brnic, D., Stevanovic, V., Cochet, M., Agier, C., Richardson, J., Montero-Menei, C.N., Milhavet, O., Eloit, M., and Couplier, M. (2012). Borna disease virus infects human neural progenitor cells and impairs neurogenesis. *J Virol* 86, 2512-2522.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27, 68-73.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285, 754-756.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A.J., Benoist, C., *et al.* (2013). A special population of regulatory T cells potentiates muscle repair. *Cell* 155, 1282-1295.

Busch, R., Neese, R.A., Awada, M., Hayes, G.M., and Hellerstein, M.K. (2007). Measurement of cell proliferation by heavy water labeling. *Nat Protoc* 2, 3045-3057.

Cantuti-Castelvetri, L., Fitzner, D., Bosch-Queralt, M., Weil, M.T., Su, M., Sen, P., Ruhwedel, T., Mitkovski, M., Trendelenburg, G., Lutjohann, D., *et al.* (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. *Science* 359, 684-688.

Carbajal, K.S., Schaumburg, C., Strieter, R., Kane, J., and Lane, T.E. (2010). Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 107, 11068-11073.

Carbajal, K.S., Weinger, J.G., Whitman, L.M., Schaumburg, C.S., and Lane, T.E. (2011). Surgical transplantation of mouse neural stem cells into the spinal cords of mice infected with neurotropic mouse hepatitis virus. *J Vis Exp*, e2834.

Carbone, F., Nencioni, A., Mach, F., Vuilleumier, N., and Montecucco, F. (2013). Pathophysiological role of neutrophils in acute myocardial infarction. *Thromb Haemost* 110, 501-514.

Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., and Ploix, C.C. (2006). CNS immune privilege: hiding in plain sight. *Immunol Rev* 213, 48-65.

Castiglioni, A., Corna, G., Rigamonti, E., Basso, V., Vezzoli, M., Monno, A., Almada, A.E., Mondino, A., Wagers, A.J., Manfredi, A.A., *et al.* (2015). FOXP3+ T Cells Recruited to Sites of Sterile Skeletal Muscle Injury Regulate the Fate of Satellite Cells and Guide Effective Tissue Regeneration. *PLoS One* 10, e0128094.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27, 275-280.

- Chang, A., Nishiyama, A., Peterson, J., Prineas, J., and Trapp, B.D. (2000). NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20, 6404-6412.
- Chatenoud, L., Primo, J., and Bach, J.F. (1997). CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol* 158, 2947-2954.
- Chauhan, S.K., Saban, D.R., Lee, H.K., and Dana, R. (2009). Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. *J Immunol* 182, 148-153.
- Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, Craig M., Sears-Kraxberger, I., Steward, O., Macklin, Wendy B., Loring, Jeanne F., *et al.* (2014a). Human Neural Precursor Cells Promote Neurologic Recovery in a Viral Model of Multiple Sclerosis. *Stem Cell Reports*.
- Chen, L., Coleman, R., Leang, R., Tran, H., Kopf, A., Walsh, C.M., Sears-Kraxberger, I., Steward, O., Macklin, W.B., Loring, J.F., *et al.* (2014b). Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis. *Stem Cell Reports* 2, 825-837.
- Chen, Z., Phillips, L.K., Gould, E., Campisi, J., Lee, S.W., Ormerod, B.K., Zwierzchoniewska, M., Martinez, O.M., and Palmer, T.D. (2011). MHC mismatch inhibits neurogenesis and neuron maturation in stem cell allografts. *PLoS One* 6, e14787.
- Chinen, T., Kannan, A.K., Levine, A.G., Fan, X., Klein, U., Zheng, Y., Gasteiger, G., Feng, Y., Fontenot, J.D., and Rudensky, A.Y. (2016). An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* 17, 1322-1333.
- Chucair-Elliott, A.J., Conrady, C., Zheng, M., Kroll, C.M., Lane, T.E., and Carr, D.J. (2014). Microglia-induced IL-6 protects against neuronal loss following HSV-1 infection of neural progenitor cells. *Glia* 62, 1418-1434.
- Constantinescu, C.S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* 164, 1079-1106.
- Cooper, G.S., Bynum, M.L., and Somers, E.C. (2009). Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun* 33, 197-207.
- Curran, K.J., Pegram, H.J., and Brentjens, R.J. (2012). Chimeric antigen receptors for T cell immunotherapy: current understanding and future directions. *J Gene Med* 14, 405-415.

- D'Alessio, F.R., Tsushima, K., Aggarwal, N.R., West, E.E., Willett, M.H., Britos, M.F., Pipeling, M.R., Brower, R.G., Tuder, R.M., McDyer, J.F., *et al.* (2009). CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *J Clin Invest* 119, 2898-2913.
- Dandekar, A.A., Wu, G.F., Pewe, L., and Perlman, S. (2001). Axonal damage is T cell mediated and occurs concomitantly with demyelination in mice infected with a neurotropic coronavirus. *J Virol* 75, 6115-6120.
- Das Sarma, J., Kenyon, L.C., Hingley, S.T., and Shindler, K.S. (2009). Mechanisms of primary axonal damage in a viral model of multiple sclerosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 10272-10280.
- Deshmukh, V.A., Tardif, V., Lyssiotis, C.A., Green, C.C., Kerman, B., Kim, H.J., Padmanabhan, K., Swoboda, J.G., Ahmad, I., Kondo, T., *et al.* (2013). A regenerative approach to the treatment of multiple sclerosis. *Nature* 502, 327-332.
- Doerflinger, N.H., Macklin, W.B., and Popko, B. (2003). Inducible site-specific recombination in myelinating cells. *Genesis* 35, 63-72.
- Dombrowski, Y., O'Hagan, T., Dittmer, M., Penalva, R., Mayoral, S.R., Bankhead, P., Fleville, S., Eleftheriadis, G., Zhao, C., Naughton, M., *et al.* (2017). Regulatory T cells promote myelin regeneration in the central nervous system. *Nat Neurosci* 20, 674-680.
- Dunnett, S.B., Nathwani, F., and Bjorklund, A. (2000). The integration and function of striatal grafts. *Prog Brain Res* 127, 345-380.
- Edinger, M., Hoffmann, P., Ermann, J., Drago, K., Fathman, C.G., Strober, S., and Negrin, R.S. (2003). CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 9, 1144-1150.
- Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., and Mauri, C. (2004). Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med* 200, 277-285.
- Elinav, E., Adam, N., Waks, T., and Eshhar, Z. (2009). Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor. *Gastroenterology* 136, 1721-1731.
- Ellis, C.N., Krueger, G.G., and Alefacept Clinical Study, G. (2001). Treatment of chronic plaque psoriasis by selective targeting of memory effector T lymphocytes. *N Engl J Med* 345, 248-255.
- Ertl, G., and Frantz, S. (2005). Healing after myocardial infarction. *Cardiovasc Res* 66, 22-32.

Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T., Wan, Y.Y., O'Connor, W., Jr., Rongvaux, A., Van Rooijen, N., Haberman, A.M., *et al.* (2011). Control of TH17 cells occurs in the small intestine. *Nature* 475, 514-518.

Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28, 41-51.

Feuerer, M., Herrero, L., Ciolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S., *et al.* (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* 15, 930-939.

Feuerer, M., Hill, J.A., Kretschmer, K., von Boehmer, H., Mathis, D., and Benoist, C. (2010). Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A* 107, 5919-5924.

Fleming, J.O., Adami, C., Pooley, J., Glomb, J., Stecker, E., Fazal, F., and Baker, S.C. (1995). Mutations associated with viral sequences isolated from mice persistently infected with MHV-JHM. *Advances in experimental medicine and biology* 380, 591-595.

Fleming, J.O., Trousdale, M.D., Elzaatari, F.A.K., Stohlman, S.A., and Weiner, L.P. (1986). PATHOGENICITY OF ANTIGENIC VARIANTS OF MURINE CORONAVIRUS JHM SELECTED WITH MONOCLONAL-ANTIBODIES. *J Virol* 58, 869-875.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005a). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6, 1142-1151.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005b). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329-341.

Fox, R.I., Saito, I., Chan, E.K., Josephs, S., Salahuddin, S.Z., Ahlashi, D.V., Staal, F.W., Gallo, R., Pei-Ping, H., and Le, C.S. (1989). Viral genomes in lymphomas of patients with Sjogren's syndrome. *J Autoimmun* 2, 449-455.

Frampton, J.E. (2017). Ocrelizumab: First Global Approval. *Drugs* 77, 1035-1041.

Fransson, M., Piras, E., Burman, J., Nilsson, B., Essand, M., Lu, B., Harris, R.A., Magnusson, P.U., Brittebo, E., and Loskog, A.S. (2012). CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* 9, 112.

Fujii, T., Sugita, N., Kobayashi, Y., Saito, K., Iijima, H., Matsunaga, K., Ando, T., Oguchi, Y., Morita, I., Yoshikumi, C., *et al.* (1989). Treatment with Krestin combined with mitomycin C, and effect on immune response. *Oncology* 46, 49-53.



Gadani, S.P., Walsh, J.T., Smirnov, I., Zheng, J., and Kipnis, J. (2015). The glia-derived alarmin IL-33 orchestrates the immune response and promotes recovery following CNS injury. *Neuron* 85, 703-709.

Gage, F.H. (2000). Mammalian neural stem cells. *Science* 287, 1433-1438.

Gaud, G., Lesourne, R., and Love, P.E. (2018). Regulatory mechanisms in T cell receptor signalling. *Nat Rev Immunol* 18, 485-497.

Gehrmann, J., Matsumoto, Y., and Kreutzberg, G.W. (1995). Microglia: intrinsic immuneffector cell of the brain. *Brain Res Brain Res Rev* 20, 269-287.

Germain, R.N., Robey, E.A., and Cahalan, M.D. (2012). A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 336, 1676-1681.

Giera, S., Luo, R., Ying, Y., Ackerman, S.D., Jeong, S.J., Stoveken, H.M., Folts, C.J., Welsh, C.A., Tall, G.G., Stevens, B., *et al.* (2018). Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells. *Elife* 7.

Giovannoni, G., Cutter, G.R., Lunemann, J., Martin, R., Munz, C., Sriram, S., Steiner, I., Hammerschlag, M.R., and Gaydos, C.A. (2006). Infectious causes of multiple sclerosis. *Lancet Neurol* 5, 887-894.

Glass, W.G., Hickey, M.J., Hardison, J.L., Liu, M.T., Manning, J.E., and Lane, T.E. (2004). Antibody targeting of the CC chemokine ligand 5 results in diminished leukocyte infiltration into the central nervous system and reduced neurologic disease in a viral model of multiple sclerosis. *J Immunol* 172, 4018-4025.

Gliwinski, M., Iwaszkiewicz-Grzes, D., and Trzonkowski, P. (2017). Cell-Based Therapies with T Regulatory Cells. *BioDrugs* 31, 335-347.

Glowacki, A.J., Yoshizawa, S., Jhunjunwala, S., Vieira, A.E., Garlet, G.P., Sfeir, C., and Little, S.R. (2013). Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes. *Proc Natl Acad Sci U S A* 110, 18525-18530.

Gonzalez, J.M., Bergmann, C.C., Ramakrishna, C., Hinton, D.R., Atkinson, R., Hoskin, J., Macklin, W.B., and Stohlman, S.A. (2006). Inhibition of interferon-gamma signaling in oligodendroglia delays coronavirus clearance without altering demyelination. *Am J Pathol* 168, 796-804.

Green, E.A., Gorelik, L., McGregor, C.M., Tran, E.H., and Flavell, R.A. (2003). CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells control anti-islet CD8<sup>+</sup> T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc Natl Acad Sci U S A* 100, 10878-10883.

Greenberg, M.L., Weinger, J.G., Matheu, M.P., Carbajal, K.S., Parker, I., Macklin, W.B., Lane, T.E., and Cahalan, M.D. (2014a). Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A*.

Greenberg, M.L., Weinger, J.G., Matheu, M.P., Carbajal, K.S., Parker, I., Macklin, W.B., Lane, T.E., and Cahalan, M.D. (2014b). Two-photon imaging of remyelination of spinal cord axons by engrafted neural precursor cells in a viral model of multiple sclerosis. *Proc Natl Acad Sci U S A* 111, E2349-2355.

Grinberg-Bleyer, Y., Baeyens, A., You, S., Elhage, R., Fourcade, G., Gregoire, S., Cagnard, N., Carpentier, W., Tang, Q., Bluestone, J., *et al.* (2010). IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* 207, 1871-1878.

Gundemir, S., Colak, G., Tucholski, J., and Johnson, G.V. (2012). Transglutaminase 2: a molecular Swiss army knife. *Biochim Biophys Acta* 1823, 406-419.

Gupta, N., Henry, R.G., Strober, J., Kang, S.M., Lim, D.A., Bucci, M., Caverzasi, E., Gaetano, L., Mandelli, M.L., Ryan, T., *et al.* (2012). Neural stem cell engraftment and myelination in the human brain. *Science translational medicine* 4, 155ra137.

Haas, J., Fritzsching, B., Trubswetter, P., Korporal, M., Milkova, L., Fritz, B., Vobis, D., Krammer, P.H., Suri-Payer, E., and Wildemann, B. (2007). Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol* 179, 1322-1330.

Halfpenny, C., Benn, T., and Scolding, N. (2002). Cell transplantation, myelin repair, and multiple sclerosis. *Lancet Neurol* 1, 31-40.

Hardison, J.L., Nistor, G., Gonzalez, R., Keirstead, H.S., and Lane, T.E. (2006). Transplantation of glial-committed progenitor cells into a viral model of multiple sclerosis induces remyelination in the absence of an attenuated inflammatory response. *Exp Neurol* 197, 420-429.

Haribhai, D., Lin, W., Edwards, B., Ziegelbauer, J., Salzman, N.H., Carlson, M.R., Li, S.H., Simpson, P.M., Chatila, T.A., and Williams, C.B. (2009). A central role for induced regulatory T cells in tolerance induction in experimental colitis. *J Immunol* 182, 3461-3468.

Haribhai, D., Lin, W., Relland, L.M., Truong, N., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* 178, 2961-2972.

Hartemann, A., Bensimon, G., Payan, C.A., Jacqueminet, S., Bourron, O., Nicolas, N., Fonfrede, M., Rosenzweig, M., Bernard, C., and Klatzmann, D. (2013). Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol* 1, 295-305.

Hatch, M.N., Schaumburg, C.S., Lane, T.E., and Keirstead, H.S. (2009). Endogenous remyelination is induced by transplant rejection in a viral model of multiple sclerosis. *J Neuroimmunol* 212, 74-81.

Healy, L.M., Jang, J.H., Won, S.Y., Lin, Y.H., Touil, H., Aljarallah, S., Bar-Or, A., and Antel, J.P. (2017). MerTK-mediated regulation of myelin phagocytosis by macrophages generated from patients with MS. *Neurol Neuroimmunol Neuroinflamm* 4, e402.

Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27, 786-800.

Hofmann, U., Beyersdorf, N., Weirather, J., Podolskaya, A., Bauersachs, J., Ertl, G., Kerkau, T., and Frantz, S. (2012). Activation of CD4<sup>+</sup> T lymphocytes improves wound healing and survival after experimental myocardial infarction in mice. *Circulation* 125, 1652-1663.

Hombach, A.A., Kofler, D., Rappl, G., and Abken, H. (2009). Redirecting human CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells from the peripheral blood with pre-defined target specificity. *Gene Ther* 16, 1088-1096.

Hori, S., Haury, M., Coutinho, A., and Demengeot, J. (2002). Specificity requirements for selection and effector functions of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A* 99, 8213-8218.

Horwitz, D.A., Zheng, S.G., Wang, J., and Gray, J.D. (2008). Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3<sup>+</sup>CD4<sup>+</sup> Treg. *Eur J Immunol* 38, 912-915.

Hosking, M.P., and Lane, T.E. (2009). The Biology of Persistent Infection: Inflammation and Demyelination following Murine Coronavirus Infection of the Central Nervous System. *Curr Immunol Rev* 5, 267-276.

Hu, C., Ding, H., Zhang, X., Wong, F.S., and Wen, L. (2013). Combination treatment with anti-CD20 and oral anti-CD3 prevents and reverses autoimmune diabetes. *Diabetes* 62, 2849-2858.

Huang, H.I., Lin, J.Y., Chen, H.H., Yeh, S.B., Kuo, R.L., Weng, K.F., and Shih, S.R. (2014). Enterovirus 71 infects brain-derived neural progenitor cells. *Virology* 468-470, 592-600.

Huehn, J., Siegmund, K., Lehmann, J.C., Siewert, C., Haubold, U., Feuerer, M., Debes, G.F., Lauber, J., Frey, O., Przybylski, G.K., *et al.* (2004). Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4<sup>+</sup> regulatory T cells. *J Exp Med* 199, 303-313.

- Huss, D.J., Pellerin, A.F., Collette, B.P., Kannan, A.K., Peng, L., Datta, A., Wipke, B.T., and Fontenot, J.D. (2016). Anti-CD25 monoclonal antibody Fc variants differentially impact regulatory T cells and immune homeostasis. *Immunology* 148, 276-286.
- Ireland, D.D., Stohlman, S.A., Hinton, D.R., Atkinson, R., and Bergmann, C.C. (2008). Type I interferons are essential in controlling neurotropic coronavirus infection irrespective of functional CD8 T cells. *J Virol* 82, 300-310.
- Ishikawa, H., Ochi, H., Chen, M.L., Frenkel, D., Maron, R., and Weiner, H.L. (2007). Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody. *Diabetes* 56, 2103-2109.
- Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162, 5317-5326.
- Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. *Science* 296, 747-749.
- Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Holenbeck, A.E., Lerman, M.A., Najj, A., and Caton, A.J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2, 301-306.
- Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564.
- Karamita, M., Barnum, C., Mobius, W., Tansey, M.G., Szymkowski, D.E., Lassmann, H., and Probert, L. (2017). Therapeutic inhibition of soluble brain TNF promotes remyelination by increasing myelin phagocytosis by microglia. *JCI Insight* 2.
- Keirstead, H.S., Ben-Hur, T., Rogister, B., O'Leary, M.T., Dubois-Dalcq, M., and Blakemore, W.F. (1999). Polysialylated neural cell adhesion molecule-positive CNS precursors generate both oligodendrocytes and Schwann cells to remyelinate the CNS after transplantation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19, 7529-7536.
- Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., and Steward, O. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25, 4694-4705.
- Kendall, A.L., Rayment, F.D., Torres, E.M., Baker, H.F., Ridley, R.M., and Dunnett, S.B. (1998). Functional integration of striatal allografts in a primate model of Huntington's disease. *Nat Med* 4, 727-729.

Kerschensteiner, M., Schwab, M.E., Lichtman, J.W., and Misgeld, T. (2005). In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med* 11, 572-577.

Kim, Y.C., Zhang, A.H., Su, Y., Rieder, S.A., Rossi, R.J., Ettinger, R.A., Pratt, K.P., Shevach, E.M., and Scott, D.W. (2015). Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T- and B-cell responses. *Blood* 125, 1107-1115.

Kipnis, J., Avidan, H., Caspi, R.R., and Schwartz, M. (2004). Dual effect of CD4+CD25+ regulatory T cells in neurodegeneration: a dialogue with microglia. *Proc Natl Acad Sci U S A* 101 Suppl 2, 14663-14669.

Klatzmann, D., and Abbas, A.K. (2015). The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol* 15, 283-294.

Kleinschnitz, C., Kraft, P., Dreykluft, A., Hagedorn, I., Gobel, K., Schuhmann, M.K., Langhauser, F., Helluy, X., Schwarz, T., Bittner, S., *et al.* (2013). Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood* 121, 679-691.

Klose, C.S., and Artis, D. (2016). Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* 17, 765-774.

Kohm, A.P., Carpentier, P.A., Anger, H.A., and Miller, S.D. (2002). Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169, 4712-4716.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M.C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6, 1219-1227.

Kretschmer, K., Heng, T.S., and von Boehmer, H. (2006). De novo production of antigen-specific suppressor cells in vivo. *Nat Protoc* 1, 653-661.

Krueger, G.G., and Ellis, C.N. (2003). Alefacept therapy produces remission for patients with chronic plaque psoriasis. *Br J Dermatol* 148, 784-788.

Kucharova, K., and Stallcup, W.B. (2017). Distinct NG2 proteoglycan-dependent roles of resident microglia and bone marrow-derived macrophages during myelin damage and repair. *PLoS One* 12, e0187530.

Kuhn, C., You, S., Valette, F., Hale, G., van Endert, P., Bach, J.F., Waldmann, H., and Chatenoud, L. (2011). Human CD3 transgenic mice: preclinical testing of antibodies promoting immune tolerance. *Sci Transl Med* 3, 68ra10.

Kumar, M., Putzki, N., Limmroth, V., Remus, R., Lindemann, M., Knop, D., Mueller, N., Hardt, C., Kreuzfelder, E., and Grosse-Wilde, H. (2006). CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *J Neuroimmunol* 180, 178-184.

Lane, T.E., and Hosking, M.P. (2010). The pathogenesis of murine coronavirus infection of the central nervous system. *Crit Rev Immunol* 30, 119-130.

Lane, T.E., Liu, M.T., Chen, B.P., Asensio, V.C., Samawi, R.M., Paoletti, A.D., Campbell, I.L., Kunkel, S.L., Fox, H.S., and Buchmeier, M.J. (2000). A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J Virol* 74, 1415-1424.

Lassmann, H. (1983). Comparative neuropathology of chronic experimental allergic encephalomyelitis and multiple sclerosis. *Schriftenreihe Neurologie* 25, 1-135.

Lassmann, H., Bruck, W., Lucchinetti, C., and Rodriguez, M. (1997). Remyelination in multiple sclerosis. *Mult Scler* 3, 133-136.

Lassmann, H., Bruck, W., and Lucchinetti, C.F. (2007). The immunopathology of multiple sclerosis: an overview. *Brain pathology* 17, 210-218.

Laurent, P., Jolivel, V., Manicki, P., Chiu, L., Contin-Bordes, C., Truchetet, M.E., and Pradeu, T. (2017). Immune-Mediated Repair: A Matter of Plasticity. *Front Immunol* 8, 454.

Lavine, K.J., Epelman, S., Uchida, K., Weber, K.J., Nichols, C.G., Schilling, J.D., Ornitz, D.M., Randolph, G.J., and Mann, D.L. (2014). Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proc Natl Acad Sci U S A* 111, 16029-16034.

Lebwohl, M., Christophers, E., Langley, R., Ortonne, J.P., Roberts, J., Griffiths, C.E., and Alefacept Clinical Study, G. (2003). An international, randomized, double-blind, placebo-controlled phase 3 trial of intramuscular alefacept in patients with chronic plaque psoriasis. *Arch Dermatol* 139, 719-727.

Leighton, E., Sainsbury, C.A., and Jones, G.C. (2017). A Practical Review of C-Peptide Testing in Diabetes. *Diabetes Ther* 8, 475-487.

Li, J., Kim, S.G., and Blenis, J. (2014). Rapamycin: one drug, many effects. *Cell Metab* 19, 373-379.

Li, J., Tan, J., Martino, M.M., and Lui, K.O. (2018). Regulatory T-Cells: Potential Regulator of Tissue Repair and Regeneration. *Front Immunol* 9, 585.

Li, M.O., and Rudensky, A.Y. (2016). T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat Rev Immunol* 16, 220-233.

- Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., Giese, T., and Veltkamp, R. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* 15, 192-199.
- Lin, M.T., Stohlman, S.A., and Hinton, D.R. (1997). Mouse hepatitis virus is cleared from the central nervous systems of mice lacking perforin-mediated cytolysis. *J Virol* 71, 383-391.
- Lin, W., Haribhai, D., Relland, L.M., Truong, N., Carlson, M.R., Williams, C.B., and Chatila, T.A. (2007). Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8, 359-368.
- Lindborg, J.A., Mack, M., and Zigmond, R.E. (2017). Neutrophils Are Critical for Myelin Removal in a Peripheral Nerve Injury Model of Wallerian Degeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 37, 10258-10277.
- Liu, M.T., Keirstead, H.S., and Lane, T.E. (2001). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol* 167, 4091-4097.
- Liu, Y., Teige, I., Birnir, B., and Issazadeh-Navikas, S. (2006). Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. *Nat Med* 12, 518-525.
- Loomis, C.W., Milne, B., and Cervenko, F.W. (1987). Determination of cross tolerance in rat spinal cord using intrathecal infusion via sequential mini-osmotic pumps. *Pharmacol Biochem Behav* 26, 131-139.
- Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550.
- Lu, B., Kwan, T., Kurimoto, Y., Shatos, M., Lund, R.D., and Young, M.J. (2002). Transplantation of EGF-responsive neurospheres from GFP transgenic mice into the eyes of rd mice. *Brain Res* 943, 292-300.
- Lucca, L.E., Desbois, S., Ramadan, A., Ben-Nun, A., Eisenstein, M., Carrie, N., Guery, J.C., Sette, A., Nguyen, P., Geiger, T.L., *et al.* (2014). Bispecificity for myelin and neuronal self-antigens is a common feature of CD4 T cells in C57BL/6 mice. *J Immunol* 193, 3267-3277.
- Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (1999). A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. *Brain : a journal of neurology* 122 ( Pt 12), 2279-2295.
- MacDonald, K.G., Hoeppli, R.E., Huang, Q., Gillies, J., Luciani, D.S., Orban, P.C., Broady, R., and Levings, M.K. (2016). Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest* 126, 1413-1424.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., *et al.* (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* *13*, 133-140.

Mangale, V., Marro, B.S., Plaisted, W.C., Walsh, C.M., and Lane, T.E. (2017). Neural precursor cells derived from induced pluripotent stem cells exhibit reduced susceptibility to infection with a neurotropic coronavirus. *Virology* *511*, 49-55.

Marek-Trzonkowska, N., Mysliwiec, M., Dobyszuk, A., Grabowska, M., Derkowska, I., Juscinska, J., Owczuk, R., Szadkowska, A., Witkowski, P., Mlynarski, W., *et al.* (2014). Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. *Clin Immunol* *153*, 23-30.

Marie, J.C., Letterio, J.J., Gavin, M., and Rudensky, A.Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *The Journal of experimental medicine* *201*, 1061-1067.

Martino, G., Franklin, R.J., Baron Van Evercooren, A., Kerr, D.A., and Stem Cells in Multiple Sclerosis Consensus, G. (2010). Stem cell transplantation in multiple sclerosis: current status and future prospects. *Nat Rev Neurol* *6*, 247-255.

Masteller, E.L., Tang, Q., and Bluestone, J.A. (2006). Antigen-specific regulatory T cells--ex vivo expansion and therapeutic potential. *Semin Immunol* *18*, 103-110.

Masteller, E.L., Warner, M.R., Tang, Q., Tarbell, K.V., McDevitt, H., and Bluestone, J.A. (2005). Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. *J Immunol* *175*, 3053-3059.

Matheu, M.P., Othy, S., Greenberg, M.L., Dong, T.X., Schuijs, M., Deswarte, K., Hammad, H., Lambrecht, B.N., Parker, I., and Cahalan, M.D. (2015). Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. *Nat Commun* *6*, 6219.

Matsuoka, K., Koreth, J., Kim, H.T., Bascug, G., McDonough, S., Kawano, Y., Murase, K., Cutler, C., Ho, V.T., Alyea, E.P., *et al.* (2013). Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med* *5*, 179ra143.

McCarthy, D.P., Richards, M.H., and Miller, S.D. (2012). Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Biol* *900*, 381-401.

McClymont, S.A., Putnam, A.L., Lee, M.R., Esensten, J.H., Liu, W., Hulme, M.A., Hoffmuller, U., Baron, U., Olek, S., Bluestone, J.A., *et al.* (2011). Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J Immunol* *186*, 3918-3926.



McFarlin, D.E., and McFarland, H.F. (1982). Multiple sclerosis (second of two parts). *N Engl J Med* 307, 1246-1251.

Mei, F., Fancy, S.P.J., Shen, Y.A., Niu, J., Zhao, C., Presley, B., Miao, E., Lee, S., Mayoral, S.R., Redmond, S.A., *et al.* (2014). Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. *Nat Med* 20, 954-960.

Mei, F., Lehmann-Horn, K., Shen, Y.A., Rankin, K.A., Stebbins, K.J., Lorrain, D.S., Pekarek, K., S, A.S., Xiao, L., Teuscher, C., *et al.* (2016a). Accelerated remyelination during inflammatory demyelination prevents axonal loss and improves functional recovery. *Elife* 5.

Mei, F., Mayoral, S.R., Nobuta, H., Wang, F., Despons, C., Lorrain, D.S., Xiao, L., Green, A.J., Rowitch, D., Whistler, J., *et al.* (2016b). Identification of the Kappa-Opioid Receptor as a Therapeutic Target for Oligodendrocyte Remyelination. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36, 7925-7935.

Meister, M., Papatriantafyllou, M., Nordstrom, V., Kumar, V., Ludwig, J., Lui, K.O., Boyd, A.S., Popovic, Z.V., Fleming, T.H., Moldenhauer, G., *et al.* (2015). Dickkopf-3, a tissue-derived modulator of local T-cell responses. *Front Immunol* 6, 78.

Miller, M.J., Wei, S.H., Cahalan, M.D., and Parker, I. (2003). Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy. *Proc Natl Acad Sci U S A* 100, 2604-2609.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 296, 1869-1873.

Minagawa, H., Takenaka, A., Mohri, S., and Mori, R. (1987). Protective effect of recombinant murine interferon beta against mouse hepatitis virus infection. *Antiviral research* 8, 85-95.

Mock, J.R., Garibaldi, B.T., Aggarwal, N.R., Jenkins, J., Limjunyawong, N., Singer, B.D., Chau, E., Rabold, R., Files, D.C., Sidhaye, V., *et al.* (2014). Foxp3+ regulatory T cells promote lung epithelial proliferation. *Mucosal Immunol* 7, 1440-1451.

Monti, P., Scirpoli, M., Maffi, P., Piemonti, L., Secchi, A., Bonifacio, E., Roncarolo, M.G., and Battaglia, M. (2008). Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+CD25+FOXP3+ regulatory T-cells. *Diabetes* 57, 2341-2347.

Mozafari, S., Laterza, C., Roussel, D., Bachelin, C., Marteyn, A., Deboux, C., Martino, G., and Baron-Van Evercooren, A. (2015). Skin-derived neural precursors competitively generate functional myelin in adult demyelinated mice. *J Clin Invest* 125, 3642-3656.

Muller, F.J., Laurent, L.C., Kostka, D., Ulitsky, I., Williams, R., Lu, C., Park, I.H., Rao, M.S., Shamir, R., Schwartz, P.H., *et al.* (2008). Regulatory networks define phenotypic classes of human stem cell lines. *Nature* 455, 401-405.

- Munji, R.N., Choe, Y., Li, G., Siegenthaler, J.A., and Pleasure, S.J. (2011). Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors. *J Neurosci* 31, 1676-1687.
- Nahrendorf, M., Swirski, F.K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.L., Libby, P., Weissleder, R., and Pittet, M.J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 204, 3037-3047.
- Nakamura, R.E., Hunter, D.D., Yi, H., Brunken, W.J., and Hackam, A.S. (2007). Identification of two novel activities of the Wnt signaling regulator Dickkopf 3 and characterization of its expression in the mouse retina. *BMC Cell Biol* 8, 52.
- Neumann, H., Medana, I.M., Bauer, J., and Lassmann, H. (2002). Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci* 25, 313-319.
- Niehrs, C. (2006). Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 25, 7469-7481.
- Nosbaum, A., Prevel, N., Truong, H.A., Mehta, P., Ettinger, M., Scharschmidt, T.C., Ali, N.H., Pauli, M.L., Abbas, A.K., and Rosenblum, M.D. (2016). Cutting Edge: Regulatory T Cells Facilitate Cutaneous Wound Healing. *J Immunol* 196, 2010-2014.
- Ochi, H., Abraham, M., Ishikawa, H., Frenkel, D., Yang, K., Basso, A.S., Wu, H., Chen, M.L., Gandhi, R., Miller, A., *et al.* (2006). Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4<sup>+</sup> CD25<sup>-</sup> LAP<sup>+</sup> T cells. *Nat Med* 12, 627-635.
- Ohkura, N., Hamaguchi, M., and Sakaguchi, S. (2011). FOXP3<sup>+</sup> regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends Pharmacol Sci* 32, 158-166.
- Ohta, A., and Sitkovsky, M. (2014). Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol* 5, 304.
- Oksenberg, J.R., Begovich, A.B., Erlich, H.A., and Steinman, L. (1993). Genetic factors in multiple sclerosis. *Jama* 270, 2362-2369.
- Ono, M., Shimizu, J., Miyachi, Y., and Sakaguchi, S. (2006). Control of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25<sup>+</sup> and CD25<sup>-</sup> regulatory T cells. *J Immunol* 176, 4748-4756.
- Palfi, S., Conde, F., Riche, D., Brouillet, E., Dautry, C., Mittoux, V., Chibois, A., Peschanski, M., and Hantraye, P. (1998). Fetal striatal allografts reverse cognitive deficits in a primate model of Huntington disease. *Nat Med* 4, 963-966.

- Park, J., Gao, W., Whiston, R., Strom, T.B., Metcalfe, S., and Fahmy, T.M. (2011). Modulation of CD4+ T lymphocyte lineage outcomes with targeted, nanoparticle-mediated cytokine delivery. *Mol Pharm* 8, 143-152.
- Parra, B., Hinton, D.R., Marten, N.W., Bergmann, C.C., Lin, M.T., Yang, C.S., and Stohlman, S.A. (1999a). IFN-gamma is required for viral clearance from central nervous system oligodendroglia. *J Immunol* 162, 1641-1647.
- Parra, B., Hinton, D.R., Marten, N.W., Bergmann, C.C., Lin, M.T., Yang, C.S., and Stohlman, S.A. (1999b). IFN-gamma is required for viral clearance from central nervous system oligodendroglial. *J Immunol* 162, 1641-1647.
- Paust, S., Lu, L., McCarty, N., and Cantor, H. (2004). Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A* 101, 10398-10403.
- Penaranda, C., Tang, Q., and Bluestone, J.A. (2011). Anti-CD3 therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells. *J Immunol* 187, 2015-2022.
- Pewe, L., and Perlman, S. (2002). Cutting edge: CD8 T cell-mediated demyelination is IFN-gamma dependent in mice infected with a neurotropic coronavirus. *J Immunol* 168, 1547-1551.
- Phares, T.W., Stohlman, S.A., Hwang, M., Min, B., Hinton, D.R., and Bergmann, C.C. (2012). CD4 T cells promote CD8 T cell immunity at the priming and effector site during viral encephalitis. *J Virol* 86, 2416-2427.
- Phillips, L.K., Gould, E.A., Babu, H., Krams, S.M., Palmer, T.D., and Martinez, O.M. (2013). Natural killer cell-activating receptor NKG2D mediates innate immune targeting of allogeneic neural progenitor cell grafts. *Stem cells* 31, 1829-1839.
- Picard-Riera, N., Decker, L., Delarasse, C., Goude, K., Nait-Oumesmar, B., Liblau, R., Pham-Dinh, D., and Baron-Van Evercooren, A. (2002). Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. *Proc Natl Acad Sci U S A* 99, 13211-13216.
- Plaisted, W.C., Weinger, J.G., Walsh, C.M., and Lane, T.E. (2014). T cell mediated suppression of neurotropic coronavirus replication in neural precursor cells. *Virology* 449, 235-243.
- Plaisted, W.C., Zavala, A., Hingco, E., Tran, H., Coleman, R., Lane, T.E., Loring, J.F., and Walsh, C.M. (2016). Remyelination Is Correlated with Regulatory T Cell Induction Following Human Embryoid Body-Derived Neural Precursor Cell Transplantation in a Viral Model of Multiple Sclerosis. *PLoS One* 11, e0157620.
- Plitas, G., and Rudensky, A.Y. (2016). Regulatory T Cells: Differentiation and Function. *Cancer Immunol Res* 4, 721-725.

Pluchino, S., Gritti, A., Blezer, E., Amadio, S., Brambilla, E., Borsellino, G., Cossetti, C., Del Carro, U., Comi, G., Hart, B., *et al.* (2009). Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. *Ann Neurol* 66, 343-354.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., *et al.* (2003). Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422, 688-694.

Poser, C.M. (1994). The epidemiology of multiple sclerosis: a general overview. *Ann Neurol* 36 *Suppl* 2, S180-193.

Powell, J.D., and Delgoffe, G.M. (2010). The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 33, 301-311.

Prineas, J.W., and Graham, J.S. (1981). Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* 10, 149-158.

Prineas, J.W., Kwon, E.E., Goldenberg, P.Z., Ilyas, A.A., Quarles, R.H., Benjamins, J.A., and Sprinkle, T.J. (1989). Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Laboratory investigation; a journal of technical methods and pathology* 61, 489-503.

Putnam, A.L., Brusko, T.M., Lee, M.R., Liu, W., Szot, G.L., Ghosh, T., Atkinson, M.A., and Bluestone, J.A. (2009). Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 58, 652-662.

Quintas-Cardama, A. (2018). CD19 directed CAR T cell therapy in diffuse large B-cell lymphoma. *Oncotarget* 9, 29843-29844.

Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., *et al.* (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332, 600-603.

Ramakrishna, C., Stohlman, S.A., Atkinson, R.A., Hinton, D.R., and Bergmann, C.C. (2004). Differential regulation of primary and secondary CD8(+) T cells in the central nervous system. *J Immunol* 173, 6265-6273.

Raposo, C., Graubardt, N., Cohen, M., Eitan, C., London, A., Berkutzki, T., and Schwartz, M. (2014). CNS repair requires both effector and regulatory T cells with distinct temporal and spatial profiles. *J Neurosci* 34, 10141-10155.

Redwine, J.M., Buchmeier, M.J., and Evans, C.F. (2001). In vivo expression of major histocompatibility complex molecules on oligodendrocytes and neurons during viral infection. *Am J Pathol* 159, 1219-1224.

Reidling, J.C., Relano-Gines, A., Holley, S.M., Ochaba, J., Moore, C., Fury, B., Lau, A., Tran, A.H., Yeung, S., Salamati, D., *et al.* (2018). Human Neural Stem Cell Transplantation Rescues Functional Deficits in R6/2 and Q140 Huntington's Disease Mice. *Stem Cell Reports* 10, 58-72.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47.

Robey, P.G. (2017). "Mesenchymal stem cells": fact or fiction, and implications in their therapeutic use *F1000Research* 524.

Rosenblum, M.D., Way, S.S., and Abbas, A.K. (2016). Regulatory T cell memory. *Nat Rev Immunol* 16, 90-101.

Rosso, S.B., and Inestrosa, N.C. (2013). WNT signaling in neuronal maturation and synaptogenesis. *Front Cell Neurosci* 7, 103.

Rouse, M., Nagarkatti, M., and Nagarkatti, P.S. (2013). The role of IL-2 in the activation and expansion of regulatory T-cells and the development of experimental autoimmune encephalomyelitis. *Immunobiology* 218, 674-682.

Rowe, C.L., Baker, S.C., Nathan, M.J., and Fleming, J.O. (1997). Evolution of mouse hepatitis virus: detection and characterization of spike deletion variants during persistent infection. *J Virol* 71, 2959-2969.

Roy, N.S., Wang, S., Harrison-Restelli, C., Benraiss, A., Fraser, R.A., Gravel, M., Braun, P.E., and Goldman, S.A. (1999). Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19, 9986-9995.

Rubtsov, Y.P., Niec, R.E., Josefowicz, S., Li, L., Darce, J., Mathis, D., Benoist, C., and Rudensky, A.Y. (2010). Stability of the regulatory T cell lineage in vivo. *Science* 329, 1667-1671.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr., *et al.* (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28, 546-558.

Ruckh, J.M., Zhao, J.W., Shadrach, J.L., van Wijngaarden, P., Rao, T.N., Wagers, A.J., and Franklin, R.J. (2012). Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* 10, 96-103.

Ruller, C.M., Tabor-Godwin, J.M., Van Deren, D.A., Jr., Robinson, S.M., Maciejewski, S., Gluhm, S., Gilbert, P.E., An, N., Gude, N.A., Sussman, M.A., *et al.* (2012). Neural stem cell depletion and CNS developmental defects after enteroviral infection. *Am J Pathol* 180, 1107-1120.

- Saadoun, D., Rosenzweig, M., Joly, F., Six, A., Carrat, F., Thibault, V., Sene, D., Cacoub, P., and Klatzmann, D. (2011). Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 365, 2067-2077.
- Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006). Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212, 8-27.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-1164.
- Sakaguchi, S., Vignali, D.A., Rudensky, A.Y., Niec, R.E., and Waldmann, H. (2013). The plasticity and stability of regulatory T cells. *Nat Rev Immunol* 13, 461-467.
- Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell* 133, 775-787.
- Salazar, D.L., Uchida, N., Hamers, F.P., Cummings, B.J., and Anderson, A.J. (2010). Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. *PLoS One* 5, e12272.
- Salzman, G.S., Ackerman, S.D., Ding, C., Koide, A., Leon, K., Luo, R., Stoveken, H.M., Fernandez, C.G., Tall, G.G., Piao, X., *et al.* (2016). Structural Basis for Regulation of GPR56/ADGRG1 by Its Alternatively Spliced Extracellular Domains. *Neuron* 91, 1292-1304.
- Scharschmidt, T.C., Vasquez, K.S., Truong, H.A., Gearty, S.V., Pauli, M.L., Nosbaum, A., Gratz, I.K., Otto, M., Moon, J.J., Liese, J., *et al.* (2015). A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity* 43, 1011-1021.
- Schaumburg, C., O'Hara, B.A., Lane, T.E., and Atwood, W.J. (2008). Human embryonic stem cell-derived oligodendrocyte progenitor cells express the serotonin receptor and are susceptible to JC virus infection. *J Virol* 82, 8896-8899.
- Scheffold, A., Huhn, J., and Hofer, T. (2005). Regulation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell activity: it takes (IL-)two to tango. *Eur J Immunol* 35, 1336-1341.
- Schlesinger, H. (1909). Zur Frage der akuten multiplen Sklerose und der encephalomyelitis disseminata im Kindesalter. *Arb Neurol Inst [Wien]* 17, 410-432.
- Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3, 1101-1108.
- Schneider, V.A., Graves-Lindsay, T., Howe, K., Bouk, N., Chen, H.C., Kitts, P.A., Murphy, T.D., Pruitt, K.D., Thibaud-Nissen, F., Albracht, D., *et al.* (2017). Evaluation of

GRCh38 and de novo haploid genome assemblies demonstrates the enduring quality of the reference assembly. *Genome Res* 27, 849-864.

Setiady, Y.Y., Coccia, J.A., and Park, P.U. (2010). In vivo depletion of CD4+FOXP3+ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcγRIII+ phagocytes. *Eur J Immunol* 40, 780-786.

Silverstein, S.C., and Rabadan, R. (2012). How many neutrophils are enough (redux, redux)? *J Clin Invest* 122, 2776-2779.

Singh, K., Hjort, M., Thorvaldson, L., and Sandler, S. (2015). Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naive mice. *Sci Rep* 5, 7767.

Smith, A.L., Barthold, S.W., and Beck, D.S. (1987). Intranasally administered alpha/beta interferon prevents extension of mouse hepatitis virus, strain JHM, into the brains of BALB/cByJ mice. *Antiviral research* 8, 239-245.

Soneson, C., Love, M.I., and Robinson, M.D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Res* 4, 1521.

Sprent, J., and Surh, C.D. (2011). Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. *Nat Immunol* 12, 478-484.

Steenblock, E.R., and Fahmy, T.M. (2008). A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. *Mol Ther* 16, 765-772.

Steinman, L. (1996). Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* 85, 299-302.

Steinman, L., Merrill, J.T., McInnes, I.B., and Peakman, M. (2012). Optimization of current and future therapy for autoimmune diseases. *Nat Med* 18, 59-65.

Stewart, J.J., Lee, C.Y., Ibrahim, S., Watts, P., Shlomchik, M., Weigert, M., and Litwin, S. (1997). A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol Immunol* 34, 1067-1082.

Stohlman, S.A., and Hinton, D.R. (2001). Viral induced demyelination. *Brain pathology* 11, 92-106.

Stoveken, H.M., Bahr, L.L., Anders, M.W., Wojtovich, A.P., Smrcka, A.V., and Tall, G.G. (2016). Dihydromunduletone Is a Small-Molecule Selective Adhesion G Protein-Coupled Receptor Antagonist. *Mol Pharmacol* 90, 214-224.

Svensson, M., and Agace, W.W. (2006). Role of CCL25/CCR9 in immune homeostasis and disease. *Expert Rev Clin Immunol* 2, 759-773.

Tabor-Godwin, J.M., Ruller, C.M., Bagalso, N., An, N., Pagarigan, R.R., Harkins, S., Gilbert, P.E., Kiosses, W.B., Gude, N.A., Cornell, C.T., *et al.* (2010). A novel population of myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 8676-8691.

Tang, Q., Adams, J.Y., Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., Piccirillo, C.A., Salomon, B.L., and Bluestone, J.A. (2008). Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28, 687-697.

Tang, Q., and Bluestone, J.A. (2013). Regulatory T-cell therapy in transplantation: moving to the clinic. *Cold Spring Harb Perspect Med* 3.

Tang, Q., Bluestone, J.A., and Kang, S.M. (2012). CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. *J Mol Cell Biol* 4, 11-21.

Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., and Bluestone, J.A. (2004). In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199, 1455-1465.

Tang, Q., and Lee, K. (2012). Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transplant* 17, 349-354.

Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and Shevach, E.M. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184, 3433-3441.

Tischner, D., Wieggers, G.J., Fiegl, H., Drach, M., and Villunger, A. (2012). Mutual antagonism of TGF-beta and Interleukin-2 in cell survival and lineage commitment of induced regulatory T cells. *Cell Death Differ* 19, 1277-1287.

Tontonoz, P., and Spiegelman, B.M. (2008). Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* 77, 289-312.

Torkildsen, O., Brunborg, L.A., Myhr, K.M., and Bo, L. (2008). The cuprizone model for demyelination. *Acta Neurol Scand Suppl* 188, 72-76.

Totoiu, M.O., Nistor, G.I., Lane, T.E., and Keirstead, H.S. (2004). Remyelination, axonal sparing, and locomotor recovery following transplantation of glial-committed progenitor cells into the MHV model of multiple sclerosis. *Exp Neurol* 187, 254-265.

Trajkovic, V., Vuckovic, O., Stosic-Grujicic, S., Miljkovic, D., Popadic, D., Markovic, M., Bumbasirevic, V., Backovic, A., Cvetkovic, I., Harhaji, L., *et al.* (2004). Astrocyte-induced regulatory T cells mitigate CNS autoimmunity. *Glia* 47, 168-179.



- Trandem, K., Anghelina, D., Zhao, J., and Perlman, S. (2010). Regulatory T cells inhibit T cell proliferation and decrease demyelination in mice chronically infected with a coronavirus. *J Immunol* *184*, 4391-4400.
- Traugott, U., Reinherz, E.L., and Raine, C.S. (1983). Multiple sclerosis: distribution of T cell subsets within active chronic lesions. *Science* *219*, 308-310.
- Trauner, M., Fuchs, C.D., Halilbasic, E., and Paumgartner, G. (2017). New therapeutic concepts in bile acid transport and signaling for management of cholestasis. *Hepatology* *65*, 1393-1404.
- Van Strien, M.E., Baron, W., Bakker, E.N., Bauer, J., Bol, J.G., Breve, J.J., Binnekade, R., Van Der Laarse, W.J., Drukarch, B., and Van Dam, A.M. (2011). Tissue transglutaminase activity is involved in the differentiation of oligodendrocyte precursor cells into myelin-forming oligodendrocytes during CNS remyelination. *Glia* *59*, 1622-1634.
- Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J.L., and Stinissen, P. (2008). Natural naive CD4+CD25+CD127<sup>low</sup> regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* *180*, 6411-6420.
- Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* *199*, 971-979.
- Vogelpoel, L.T., Baeten, D.L., de Jong, E.C., and den Dunnen, J. (2015). Control of cytokine production by human fc gamma receptors: implications for pathogen defense and autoimmunity. *Front Immunol* *6*, 79.
- Waldron-Lynch, F., Henegariu, O., Deng, S., Preston-Hurlburt, P., Tooley, J., Flavell, R., and Herold, K.C. (2012). Teplizumab induces human gut-tropic regulatory cells in humanized mice and patients. *Sci Transl Med* *4*, 118ra112.
- Walker, L.S., Chodos, A., Eggena, M., Dooms, H., and Abbas, A.K. (2003). Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* *198*, 249-258.
- Walsh, J.T., Zheng, J., Smirnov, I., Lorenz, U., Tung, K., and Kipnis, J. (2014). Regulatory T cells in central nervous system injury: a double-edged sword. *J Immunol* *193*, 5013-5022.
- Wang, F.I., Hinton, D.R., Gilmore, W., Trousdale, M.D., and Fleming, J.O. (1992). Sequential infection of glial cells by the murine hepatitis virus JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Laboratory investigation; a journal of technical methods and pathology* *66*, 744-754.

Wang, X., Xuan, W., Zhu, Z.Y., Li, Y., Zhu, H., Zhu, L., Fu, D.Y., Yang, L.Q., Li, P.Y., and Yu, W.F. (2018). The evolving role of neuro-immune interaction in brain repair after cerebral ischemic stroke. *CNS Neurosci Ther* 24, 1100-1114.

Weinger, J.G., Greenberg, M.L., Matheu, M.P., Parker, I., Walsh, C.M., Lane, T.E., and Cahalan, M.D. (2015). Two-photon imaging of cellular dynamics in the mouse spinal cord. *J Vis Exp*.

Weinger, J.G., Plaisted, W.C., Maciejewski, S.M., Lanier, L.L., Walsh, C.M., and Lane, T.E. (2014). Activating Receptor NKG2D Targets RAE-1-Expressing Allogeneic Neural Precursor Cells in a Viral Model of Multiple Sclerosis Stem cells.

Weinger, J.G., Weist, B.M., Plaisted, W.C., Klaus, S.M., Walsh, C.M., and Lane, T.E. (2012). MHC mismatch results in neural progenitor cell rejection following spinal cord transplantation in a model of viral-induced demyelination. *Stem cells* 30, 2584-2595.

Weinshenker, B.G., Bass, B., Rice, G.P., Noseworthy, J., Carriere, W., Baskerville, J., and Ebers, G.C. (1989). The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. *Brain : a journal of neurology* 112 ( Pt 1), 133-146.

Weirather, J., Hofmann, U.D., Beyersdorf, N., Ramos, G.C., Vogel, B., Frey, A., Ertl, G., Kerkau, T., and Frantz, S. (2014). Foxp3+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation. *Circ Res* 115, 55-67.

Wheeler, D.L., Sariol, A., Meyerholz, D.K., and Perlman, S. (2018). Microglia are required for protection against lethal coronavirus encephalitis in mice. *J Clin Invest* 128, 931-943.

Whitman, L., Zhou, H., Perlman, S., and Lane, T.E. (2009). IFN-gamma-mediated suppression of coronavirus replication in glial-committed progenitor cells. *Virology* 384, 209-215.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322, 271-275.

Wright, G.P., Notley, C.A., Xue, S.A., Bendle, G.M., Holler, A., Schumacher, T.N., Ehrenstein, M.R., and Stauss, H.J. (2009). Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. *Proc Natl Acad Sci U S A* 106, 19078-19083.

Wu, G.F., and Perlman, S. (1999). Macrophage infiltration, but not apoptosis, is correlated with immune-mediated demyelination following murine infection with a neurotropic coronavirus. *J Virol* 73, 8771-8780.

Wu, H.Y., Center, E.M., Tsokos, G.C., and Weiner, H.L. (2009). Suppression of murine SLE by oral anti-CD3: inducible CD4+CD25-LAP+ regulatory T cells control the expansion of IL-17+ follicular helper T cells. *Lupus* 18, 586-596.

Wu, H.Y., Maron, R., Tukpah, A.M., and Weiner, H.L. (2010). Mucosal anti-CD3 monoclonal antibody attenuates collagen-induced arthritis that is associated with induction of LAP+ regulatory T cells and is enhanced by administration of an emulsome-based Th2-skewing adjuvant. *J Immunol* 185, 3401-3407.

Wu, H.Y., Quintana, F.J., and Weiner, H.L. (2008). Nasal anti-CD3 antibody ameliorates lupus by inducing an IL-10-secreting CD4+ CD25- LAP+ regulatory T cell and is associated with down-regulation of IL-17+ CD4+ ICOS+ CXCR5+ follicular helper T cells. *J Immunol* 181, 6038-6050.

Wynn, T.A., and Barron, L. (2010). Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 30, 245-257.

Xia, N., Jiao, J., Tang, T.T., Lv, B.J., Lu, Y.Z., Wang, K.J., Zhu, Z.F., Mao, X.B., Nie, S.F., Wang, Q., *et al.* (2015). Activated regulatory T-cells attenuate myocardial ischaemia/reperfusion injury through a CD39-dependent mechanism. *Clin Sci (Lond)* 128, 679-693.

Yadav, M., Stephan, S., and Bluestone, J.A. (2013). Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol* 4, 232.

Yagita, Y., Kitagawa, K., Ohtsuki, T., Takasawa, K., Miyata, T., Okano, H., Hori, M., and Matsumoto, M. (2001). Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke* 32, 1890-1896.

Yoon, J., Schmidt, A., Zhang, A.H., Konigs, C., Kim, Y.C., and Scott, D.W. (2017). FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII. *Blood* 129, 238-245.

Yu, A., Snowwhite, I., Vendrame, F., Rosenzweig, M., Klatzmann, D., Pugliese, A., and Malek, T.R. (2015). Selective IL-2 responsiveness of regulatory T cells through multiple intrinsic mechanisms supports the use of low-dose IL-2 therapy in type 1 diabetes. *Diabetes* 64, 2172-2183.

Yuan, S.H., Martin, J., Elia, J., Flippin, J., Paramban, R.I., Hefferan, M.P., Vidal, J.G., Mu, Y., Killian, R.L., Israel, M.A., *et al.* (2011). Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. *PLoS One* 6, e17540.

Zerbino, D.R., Achuthan, P., Akanni, W., Amode, M.R., Barrell, D., Bhai, J., Billis, K., Cummins, C., Gall, A., Giron, C.G., *et al.* (2018). Ensembl 2018. *Nucleic Acids Res* 46, D754-D761.

Zhang, P., Tey, S.K., Koyama, M., Kuns, R.D., Olver, S.D., Lineburg, K.E., Lor, M., Teal, B.E., Raffelt, N.C., Raju, J., *et al.* (2013). Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. *J Immunol* *191*, 5291-5303.

Zhang, Q., Lu, W., Liang, C.L., Chen, Y., Liu, H., Qiu, F., and Dai, Z. (2018). Chimeric Antigen Receptor (CAR) Treg: A Promising Approach to Inducing Immunological Tolerance. *Front Immunol* *9*, 2359.

Zhang, R., Zhang, Z., Wang, L., Wang, Y., Gousev, A., Zhang, L., Ho, K.L., Morshead, C., and Chopp, M. (2004). Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. *J Cereb Blood Flow Metab* *24*, 441-448.

Zhou, J., Hinton, D.R., Stohlman, S.A., Liu, C.P., Zhong, L., and Marten, N.W. (2005). Maintenance of CD8+ T cells during acute viral infection of the central nervous system requires CD4+ T cells but not interleukin-2. *Viral Immunol* *18*, 162-169.

Zhou, X., Bailey-Bucktrout, S.L., Jeker, L.T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W., and Bluestone, J.A. (2009). Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* *10*, 1000-1007.

Zhou, X., Kong, N., Wang, J., Fan, H., Zou, H., Horwitz, D., Brand, D., Liu, Z., and Zheng, S.G. (2010). Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. *J Immunol* *185*, 2675-2679.

Zhu, K., Sun, J., Kang, Z., Zou, Z., Wu, G., and Wang, J. (2016). Electroacupuncture Promotes Remyelination after Cuprizone Treatment by Enhancing Myelin Debris Clearance. *Front Neurosci* *10*, 613.

Ziegler, S.F. (2006). FOXP3: of mice and men. *Annu Rev Immunol* *24*, 209-226.