HHS Public Access

Author manuscript

*J Immunol*. Author manuscript; available in PMC 2024 June 01.

Published in final edited form as:

*J Immunol*. 2023 June 01; 210(11): 1677–1686. doi:10.4049/jimmunol.2200798.

# Presentation of Human Neural Stem Cell Antigens Drives Regulatory T cell induction

**Scott A. Greilach**[1,**], **Laura L. McIntyre**[1,**], **Quy H. Nguyen**[2], **Jorge Silva**[1], **Kai Kessenbrock**[2], **Thomas E. Lane**[3], **Craig M. Walsh**[1,*]

[1]Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA, 92697

[2]Department of Biological Chemistry, University of California, Irvine, Irvine, CA, 92697

[3]Department of Neurobiology and Behavior, University of California, Irvine, Irvine, CA, 92697

## Abstract

Transplantation of human Neural Stem Cells (hNSCs) is a promising regenerative therapy to promote remyelination in patients with Multiple sclerosis (MS). Transplantation of hNSCs has been shown to increase the number of CD4+CD25+Foxp3+ T regulatory cells (Tregs) in the spinal cords of murine models of MS, which is correlated with a strong localized remyelination response. However, the mechanisms by which hNSC transplantation leads to an increase in Tregs in the CNS remains unclear. We report that hNSCs drive the conversion of T conventional cells (Tconv) into Tregs in vitro. Conversion of Tconv cells is antigen-driven and fails to occur in the absence of TCR stimulation by cognate antigenic self-peptides. Furthermore, CNS antigens are sufficient to drive this conversion in the absence of hNSCs in vitro and in vivo. Importantly, only antigens presented in the thymus during T cell selection drive this Treg response. This work provides new insights into the mechanisms by which hNSC antigens drive the conversion of Tconv cells into Tregs and may provide key insight needed for development of MS therapies.

### Keywords

Multiple sclerosis; experimental autoimmune encephalomyelitis; regulatory T cells; stem cells; neural stem cells; immunological tolerance

## Introduction

Multiple sclerosis (MS) is a debilitating autoimmune disease caused by autoreactive T cells that results in progressive damage to the central nervous system (CNS)(1). Current

*Correspondence: cwalsh@uci.edu.

**Authors contributed equally to this work

Author Contributions

**S.A.G.**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing- Original Draft, Writing- Reviewing & Editing, Visualization, Funding acquisition **L.L.M.**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing- Original Draft, Writing- Reviewing & Editing, Visualization, Funding acquisition; **Q.H.N.**: Conceptualization, Software, Investigation, Visualization; **J.S.**: Investigation; **K.K.**: Conceptualization, Resources; **T.E.L.**: Conceptualization, Resources, Writing- Reviewing & Editing, Funding acquisition; **C.M.W.**: Conceptualization, Writing- Reviewing & Editing, Supervision, Funding acquisition.

FDA-approved therapies for the relapsing-remitting form of MS (RRMS) function by suppressing autoreactive immune cells or by limiting access to the CNS, but because they do not promote either substantial remyelination or preservation of axons, these treatments do not provide long-term relief and are not effective for progressive forms of MS that lack spontaneous remyelination. An important unmet clinical need for MS patients is an effective method for promoting remyelination that can ameliorate clinical symptoms associated with demyelination and restore motor function while limiting immune cell infiltration into the CNS (2, 3).

Neural Stem Cell (NSC) transplantation has long been considered a potential therapeutic approach to repairing and replacing damaged cells in the CNS(4, 5). In murine models of MS, syngeneic mouse NSC (mNSC) transplants into the spinal cord have been shown to effectively engraft, differentiate, and repair and replace damaged neurons and oligodendrocytes(6-10). Due to the autoimmune nature of these models, it is however, difficult to assess the capacity of human NSCs (hNSCs) to repair damage in the context of a xenogeneic transplant because the murine immune system causes transplanted hNSCs to be rapidly rejected (6, 11). Interestingly, despite this rejection, hNSC transplantation leads to a reduction in the number of activated inflammatory CD4+ T conventional cells (Tconv) in the CNS and periphery, as well as a large, localized increase in CD4+CD25+Foxp3+ T regulatory cells (Tregs) at the site of hNSC transplantation in the spinal cord(6, 11-14). Of note, this Treg influx also results in localized remyelination, and repair in the CNS, and depletion of such Tregs abrogates the repair induced by hNSC transplantation (6, 11). Therefore, it is critical to understand how hNSC transplantation drives this localized increase in Tregs to gain insight into this mechanism and potentially employ this process as a future therapeutic modality.

Tregs are the primary suppressive cells of the adaptive immune system and function to maintain immune homeostasis. They are characterized by their expression of the master transcription factor fork head box P3 (Foxp3) and the high affinity IL-2 receptor alpha chain, CD25 (15, 16). Tregs are known for their capacity to actively suppress immune responses against self-antigens and their absence results in massive systemic autoimmunity (17, 18). Current models of autoimmunity hypothesize that Treg disfunction is one of the root causes of autoimmune disease, including MS and Type I diabetes (19-22). Recent work has also shown that Tregs are capable of directly interacting with a variety of distinct cell types and tissues to instigate repair as well as serving homeostatic functions (13, 16, 23-31).

This knowledge paired with our findings of a localized Treg response post-hNSC transplantation has led us to explore the possibility that these Tregs are responsible for both the suppression of Tconv cells in the CNS as wells as their ability to promote tissue repair within the CNS in the form of remyelination(6, 11, 12). This study investigated the mechanisms by which hNSCs generate a Treg response. Our data provides evidence that hNSCs indirectly promote Treg induction through increasing the availability of CNS specific antigens, resulting in the generation of peripheral Tregs (pTregs) from the Tconv population.

## Materials and Methods

### Mice

All experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee. C57BL/6 mice (Strain: 027) were obtained from Charles River Laboratories. Foxp3-GFP+ mice (B6.Cg-Foxp3tm2Tch/J, Stock No: 006772) were obtained from The Jackson Laboratory(58, 59). RAG2−/−2D2+ mice were generated by breeding RAG2−/− (B6.129S6-Rag2tm1FwaN12; Taconic Model #RAGN12)(60) and 2D2 T cell receptor transgenic (TCR-tg) mice recognizing Myelin Oligodendrocyte Glycoprotein (MOG) 35-55 (C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J; The Jackson Laboratory Stock No: 006912)(40). RAG2−/−OT-II+ TCR-tg mice recognizing ovalbumin (OVA) 323-339 were obtained from Taconic (B6. 129S6-Rag2tm1FwaTg(TcreTcrb) 425Cbn, Model #11490)(60, 61). Splenocytes from RAG1−/−OT-II+ TCR-tg mice recognizing OVA 323-339 were kindly provided by Stephen Schoenberger at the La Jolla Institute for Allergy and Immunology.

RAG2−/−2D2+ mice were immunized by subcutaneous injections with 100ul of emulsion containing 100ug of MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK-COOH; Pierce), Neurofilament Medium (NFM) 18-30 (TETRSSFSRVSGS; GenScript) or OVA 323-339 (ISQAVHAAHAEINEAGR; Neta Scientific) in Phosphate Buffered Saline (PBS), with Complete Freund's Adjuvant (CFA) containing 200ug Mycobacterium tuberculosis H37Ra (DIFCO Laboratories). Mice received intraperitoneal (I.P.) injections with 200ng of Bordetella pertussis toxin (List Biological Laboratories) day 0 and day 2 post-immunization (p.i.). Mice were sacrificed via inhalation of a lethal dose of isoflurane and cardiac perfusion with PBS was performed at defined time points p.i. for tissue harvesting and analysis.

### Flow cytometry

Cervical lymph nodes, spleens, brains or spinal cords were dissociated into single-cell suspension, depleted of red blood cells using tris-acetic-acid-chloride (TAC) as previously described(62). Cells from brain and spinal cord samples were further purified using a 23% percoll gradient(63). Cells were filtered, washed and counted before being stained with Zombie Violet™ or Zombie Aqua™ Fixable Viability Kit (Biolegend), blocked with CD16/32 (2.4G2) and stained with surface antibodies against CD3 (145-2C11), CD4 (RM4-5), CD25 (PC61.5). Intracellular staining for FoxP3 (FJK-16S) was performed 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 a BD™ LSR II or BD LSRFortessa™ flow cytometer and analyzed using FlowJo software (Tree Star).

### Cell lines

hESCs were derived from WA09 human embryonic stem cells and differentiated into EB-NSCs and sorted according to established methods as previously described (64, 65). 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.

## In vitro assays

**Peptide cultures.—**Spleens dissected from naïve, age-matched (6-8-week-old) RAG2−/−OT-II+ or RAG2−/−2D2+ mice were isolated into single-cell suspension. 1.5 x$10^5$ splenocytes per well were cultured in round bottom 96-well plates and incubated at 37°C, 5% CO2 for 4 days in 200ul final volume of complete RPMI medium (RPMI-1640, 10% FBS (Atlanta Biologicals) 1X non-essential amino acids, 100U/mL penicillin, 100ug/mL streptomycin, 1mM sodium pyruvate, 55uM 2-mercaptoethanol; all from Thermo Fisher). Splenocytes were cultured alone; activated with plate bound anti-Armenian hamster IgG (30ug/mL; Vector or Jackson Immuno), Armenian hamster, anti-mouse CD3 (1ug/mL, 2C-11, Biolegend) and soluble anti-mouse CD28 (1ug/mL, Biolegend or Tonbo); with 20ug/mL MOG 35-55 peptide (Pierce); with 20ug/mL NFM 18-30 peptide (GenScript) with 20ug/mL OVA peptide (Neta Scientific); or with 3ug/mL high molecular weight Poly (I:C) (Invivogen) (39). After 4 days, cells were filtered and washed before being stained with Zombie Aqua™ Fixable Viability Kit (Biolegend), blocked with CD16/32 (2.4G2) and surface antibodies against CD4 (RM4-5) and CD25 (PC61.5). Intracellular staining for FoxP3 (FJK-16S) was performed 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 a BD™ LSR II or BD LSRFortessa™ flow cytometer and analyzed using FlowJo software (Tree Star).

**Splenocyte and hNSC co-cultures.—**Spleens dissected from naïve, age-matched (6-8-week-old) C57/BL6, FoxP3-GFP+, RAG1−/−OT-II+ or RAG2−/−2D2+ mice were isolated into single- suspension, depleted of red blood cells, as previously described(62). Splenocytes were combined with hNSCs at defined proportions (Supplemental Figure 4) with 1.5x $10^5$ cells total per well in round bottom 96-well plates and incubated at 37°C, 5% CO2 for 4 days in 200ul final volume of complete RPMI medium. Splenocytes were activated with plate bound anti-Armenian hamster IgG (30ug/mL; Vector or Jackson Immuno), Armenian hamster, anti-mouse CD3 (1ug/mL, 2C-11, Biolegend) and soluble anti-mouse CD28 (1ug/mL, Biolegend or Tonbo). Addition of recombinant human IL-2 (100U/mL) and recombinant human TGFβ (7ng/mL) were added to cultures where indicated. After 4 days, cells were filtered and washed before being stained for flow cytometry analysis as previously mentioned. For quantification, cell counts were normalized to a starting population of 150,000 splenocytes.

For iTreg and hNSC Treg sorts, splenocytes from FoxP3-GFP+ mice were cultured with or without hNSCs as described above. After 4 days, cells were filtered, washed and subjected to negative T cell isolation using magnetic separation and then sorted using FACS. Cells were counted and resuspended at [1x10^8/mL] in T cell isolation buffer (1x PBS without Ca2+ and Mg2+ containing 2%FBS and 1mM EDTA) and 50ul/ mL Normal Rat Serum (StemCell Technologies) with 10ul/mL anti-mouse B220 biotin and 10ul/mL anti-mouse CD11b biotin (Biolegend) for 10 min. at room temperature (RT). 50ul/mL Mojosort™ Strepavidin 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, supernatants were collected. Cells were stained with Propidium Iodine Viability Dye, blocked with CD16/32 (2.4G2) and surface antibodies against CD4 (RM4-5) and CD25 (PC61.5). All antibodies were from Biolegend or BD Biosciences. Cells were sorted using a BD FACSAria II™ into Trizol LS (Thermo Fisher) and stored at −80°C until RNA extraction. For co-cultures utilizing depleted splenocytes, splenocytes from Foxp3-GFP+ were sorted using a BD FACSAria II™, then cultured with hNSCs.

### TCR repertoire analysis

iTregs or hNSC Tregs were FACS purified as described above. 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 H2O 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). 100ng of RNA with acceptable RINs (9.0-10.0) was sent to iRepertoire for CDR3ε sequencing and analysis.

**qPCR—**SuperscriptIII reverse transcriptase kit (Thermo Fisher) was used to generate cDNA 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 *Mog* and *Nfm* (Thermo Fisher) in an ABI ViiA7 thermocycler. Human brain tissues samples were obtained from the UCI ADRC and used as controls for *Mog* and *Nfm* expression. Data was analyzed using comparative Ct method (66).

### Statistical analysis

Data were analyzed using Prism software (GraphPad). Comparisons were performed using a two-tailed T test and two-way analysis of variance, where indicated. For all statistical models and tests described above, the significance is displayed as follows; NS is not significant, *p<0.05, **p<0.01, ***p<0.001,****p<0.0001.

## Results

### Human Neural Stem Cells expand Tregs *in vitro*

We have previously reported that transplantation of human neural stem cells (hNSCs) into the spinal cords of mice with immune-mediated demyelination results in an increase in CD4+CD25+Foxp3+ Tregs (11, 12). To investigate how hNSCs contributed to the observed increase in Tregs *in vivo*, we developed an in vitro co-culture system in which naïve mouse splenocytes are mixed with different ratios of H9 human embryonic stem cell-derived

hNSCs (Figure 1A, Supplemental Figure 1A,B). Co-culture of splenocytes with hNSCs resulted in a small but significant increase in frequency of Tregs at 1:5 a ratio compared to splenocytes alone (Supplemental Figure 1C). In subsequent experiments, naïve splenocytes were cultured in conditions that support T cell activation and survival, with splenocytes stimulated with αCD3 and αCD28, as well as TGF-β and IL-2 in the presence or absence of hNSCs. TGF-β and IL-2 were added to co-cultures to better mimic physiologic conditions of antigen presentation in the CNS, as microglia secrete TGFβ and IL-2 (32), factors that are essential for Treg generation and function (33, 34). Splenocytes cultured with hNSCs yielded two-fold higher proportions of Tregs (hNSC-Tregs) when compared to induced Treg (iTreg) controls generated by stimulation under Treg skewing conditions alone (Figures 1B-D). Additionally, Tregs cultured with hNSCs displayed 1.9-fold and 1.2-fold higher expression of Foxp3 (Figure 1E) and CD25 (Figure 1F), respectively, as measured by mean fluorescent intensity (MFI) when compared to iTregs. hNSC Treg generation was consistent among various ratios of C57BL/6 splenocytes to hNSCs (Supplemental Figure 1C,D) and specific to hNSCs; co-cultures with xenogeneic human dermal fibroblasts(Supplemental Figure 1E,F) did not yield an expanded fraction of CD25+Foxp3+ Tregs. These data suggest that hNSCs directly contribute to the expansion of Tregs *in vitro*.

## hNSC-Tregs are expanded from the conventional T cell pool

Foxp3+ Tregs can be derived from the thymus (tTregs) during thymic selection or in the periphery (pTregs) through the conversion of Tconv cells (T conv) into Tregs [31, 32]. To determine whether hNSCs expanded Tregs from the tTreg pool or by conversion of Tconv cells into pTregs, unsorted splenocytes and splenocytes depleted of Foxp3-GFP+ cells from Foxp3-GFP reporter mice were co-cultured with hNSCs (Figure 2A). An increase in the frequency (Figure 2B) and number (Figure 2C) of CD25+Foxp3+ hNSC-Tregs were observed regardless of the presence of Foxp3+ cells in the starting splenocyte population, demonstrating that hNSCs are actively involved in converting Tconv cells into CD25+Foxp3+ Tregs.

## hNSC-Tregs have a unique TCR repertoire

To further investigate the differences between hNSC-Tregs and iTregs, we compared their T cell receptor (TCR) β sequences using high-throughput TCRβ repertoire analyses. These analyses identified 7,427 unique CDR3ɐ sequences for iTregs and hNSC Tregs. To determine the clonal dominance in each population, we used the clonality index (inverse of Shannon's entropy)(35), 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 TCRβ sequences were common to both Treg populations while 5,549 TCRβ sequences were unique to hNSC-Tregs (Figure 3A). 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 when compared to the iTreg repertoire. In addition, hNSC-Treg clones displayed similar peptide sequences (Supplemental Figure 2A,B). Vβ13 was one of the most predominant clones in the iTreg sample, which encodes the protein Vβ8.1 and has been implicated in

conferring protection in a viral murine model of demyelination (36, 37). Vβ31, encoding for the protein Vβ14, was one of the most predominant clones in hNSC Tregs. Vβ14 is known to recognize part of myelin oligodendrocyte glycoprotein (MOG), but it is not the predominant myelin reactive clone Vβ11(38). The gene Vβ16 encodes for Vβ11 and was absent in the top 10 clones of both iTregs and hNSC-Tregs. Furthermore, when comparing CDR3ε 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 (data not shown). 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. Taken together, these TCR repertoire studies provide evidence that hNSCs elicit a unique TCR repertoire within the expanded Treg population in these co-cultures, supporting the concept that the response requires antigen presentation.

## hNSC Antigens Drive Treg Increases

To determine if the mechanism through which hNSCs promote Treg expansion occurs via passive antigen expression or via active expression of factors such as cytokines that promote Treg differentiation, we tested the ability of killed hNSCs to elicit Treg expansion. Since hNSCs do not survive longer than 8 days post-transplantation(12), we hypothesized that hNSCs were not required to be viable for the observed increase in Tregs, and that their antigens alone may be sufficient for the observed increase in Tregs. To test this hypothesis, we characterized co-cultures of splenocytes mixed with hNSCs cultured under normal conditions (live) or hNSCs cultured overnight in PBS to induce cell death (PBS-killed). Both live and PBS-killed hNSCs generated more Tregs in co-cultures compared to iTreg controls (Figures 3B, C). Although there was not a detectible difference in the frequency of CD25+Foxp3+ cells with live or PBS-killed hNSCs compared to one another (Figure 3B), there was an increase in number of CD25+Foxp3+ cells in conditions with PBS-killed hNSCs compared to live cells (Figure 3C). These data suggest that direct T cell-hNSC interactions or hNSC secreted cytokines are not responsible for the observed increase in Tregs. The fact that hNSCs did not need to be alive to expand Tregs led us to investigate whether or not TCR antigenic recognition is responsible for this Treg expansion.

To confirm that hNSC antigens drive the observed increases in hNSC-Tregs, we characterized co-cultures employing splenocytes from two different TCR transgenic mouse lines that bear restricted, monoclonal TCR repertoires. We used RAG2−/−2D2+ mice which have T cells that recognize myelin oligodendrocyte glycoprotein (MOG) and neurofilament medium (NFM) self-antigens and RAG1−/−OT-II+ mice with T cells that recognize the non-self-antigen chicken ovalbumin (OVA). If hNSCs antigens are necessary for Treg expansion, we would anticipate an increase in Tregs in RAG2−/−2D2+ splenocyte cultures, but not in RAG1−/−OT-II+ splenocyte cultures since hNSCs express NFM (Supplemental Figure 2 C,D). Supporting the hypothesis that hNSC antigen recognition drives an increase in Tregs, 4-day co-cultures of RAG2−/−2D2+ splenocytes and hNSCs had a significant, dose-dependent expansion of CD25+Foxp3+Tregs (Figures 4A, B). In contrast, co-cultures of RAG1−/−OT-II+ splenocytes and hNSCs did not result in an increase in CD25+Foxp3+ Tregs (Figures 4C, D). Importantly, RAG2−/−2D2+ mice lack Tregs in the periphery, suggesting that exposure to hNSC antigens promotes the conversion of naïve Tconv

cells into CD25+Foxp3+ Tregs, similar to the results seen when hNSC co-cultures were performed with splenocytes depleted of tTregs.

### Self-antigen recognition is sufficient to drive Treg Expansion

It is clear that TCR engagement with presented self-antigen is critical in promoting an increase in hNSC-Tregs, however it remained unclear if peptide presentation is sufficient to upregulate Foxp3 expression in the absence of hNSCs. To examine this further, splenocytes from RAG2−/−2D2+ and RAG2−/−OT-II+ mice were cultured for 4 days ex vivo in the presence of various stimuli: alone, with αCD3 + αCD28, MOG peptide, NFM peptide, OVA peptide, or Poly (I:C), the latter serving as a non-specific inducer of inflammation. T cells from RAG2−/−2D2+ splenocytes cultured in the presence of αCD3 + αCD28, MOG and NFM all became activated and upregulated CD25, although to a lesser extent than in the MOG and NFM conditions (Figure 5A). Strikingly, only RAG2−/−2D2+ splenocytes cultured with MOG or NFM upregulated Foxp3 (1.47% ± 0.34 and 3.43% ± 0.57, respectively) (Figures 5A, C, D). Addition of TGFβ and IL-2 to MOG and NFM cultures greatly enhanced the generation of CD25+Foxp3+ Tregs (26.26% ± 5.10 and 30.3% ± 1.70, respectively), even when compared to αCD3 + αCD28 iTreg controls (6.97% ± 1.89) (Supplemental Figure 3A, C, D). While all conditions containing MOG and NFM resulted in the generation of CD25+Foxp3+ RAG2−/−2D2+ cells, there was a greater frequency of CD25+Foxp3+ cells under conditions containing NFM compared to MOG. Of note, RAG2−/−2D2+ splenocytes cultured in the presence of Poly (I:C) did not upregulate Foxp3, suggesting that Foxp3 expression was not due to bystander inflammation(39), but instead was mediated via TCR antigenic stimulation. Thus, exposure to self-pepitde:MHC (pMHC) is sufficient to upregulate Foxp3 *in vitro*. RAG2−/−OT-II+ splenocytes failed to develop CD25+Foxp3+ Tregs under any of these stimulation conditions, although cells did upregulate CD25+ when cultured in the presence of αCD3 + αCD28 and OVA, suggesting that these CD4+ cells are capable of recognizing peptide and becoming activated, but ultimately do not differentiate into Tregs (Figure 5B, E, F). RAG2−/−OT-II+ splenocytes were capable of generating CD25+Foxp3+ induced Tregs (iTregs) when TGFβ and IL-2 were added to αCD3 + αCD28 and OVA cultures (34.53% ± 7.12 and 3.05% ± 0.82, respectively) (Supplemental Figure 3B,E, F).

### Self-pMHC exposure upregulates FoxP3 *in vivo*

Next, we sought to address whether self-pMHC exposure was sufficient to upregulate Foxp3 expression in RAG2−/−2D2+ T cells *in vivo*. Immunization of RAG2−/−2D2+ mice with MOG peptide has been used as a model for autoimmune mediated demyelination, mimicking certain features of MS(40). However, previous studies have not examined Foxp3 expression following immunization, possibly because these mice do not possess CD25+Foxp3+ cells under homeostatic conditions. To investigate this further, we immunized RAG2−/−2D2+ mice with their cognate self-peptides $MOG_{35-55}$ or $NFM_{15-35}$, or $OVA_{323-339}$ as an irrelevant antigen control. Mice immunized with MOG and NFM peptides developed Foxp3+ Tregs within the brain and spinal cord (Figure 6A) 10 days post-immunization (p.i.), displaying an increase in both frequency (Figure 6B) and number (Figure 6C) of CD25+Foxp3+ Tregs as compared to OVA immunized controls. We also observed a small population of CD25+Foxp3+ cells in the cervical lymph nodes of MOG

immunized mice, but not within the spleen under any immunization conditions (Figure 6A), indicating that CD25+Foxp3+ Tregs are found primarily in tissues were cognate antigen is expressed. Taken together, these data suggest that exposure to self-pMHC is sufficient to upregulate Foxp3 *in vivo*.

## Discussion

In this study, we demonstrate that hNSCs drive the antigen-specific conversion of Tconv cells into a CD25+Foxp3+ T regulatory phenotype in vitro. Co-culturing of splenocytes (containing peripheral T cells and antigen presenting cells) with hNSCs resulted in a doubling in both the percentage and number of Tregs. This doubling occurred when thymic Tregs (tTregs) were excluded from culture, suggesting that the observed increase in Tregs is the result of Tconv conversion as opposed to expansion of tTregs (Figure 2). Furthermore, Treg conversion is unlikely a result of direct signaling or secretion of inductive factors by hNSCs, as Treg conversion occurred in the absence of viable hNSCs (Figure 3A,B). Additionally, hNSCs failed to drive this observed increase in the absence of TCR signaling by cognate antigen, as was seen in hNSC RAG1−/−OTII+ co-cultures (Figure 4A,C), but did occur when antigen was presented as seen in the hNSC RAG2−/−2D2+ co-cultures (Figure 4B,D). Further, in the absence of hNSCs, cognate self-antigen was shown to be sufficient to drive Treg conversion when RAG2−/−2D2+ splenocytes were cultured in the presence of their cognate self-peptide MOG and NFM. These conversions failed to occur with polyclonal stimulation of the TCR via αCD3 and αCD28 and were not the result of bystander inflammation since Poly (I:C) failed to induce a similar response (Figure 5). Of significant note, RAG−/−OTII+ T cells failed to generate a Treg response when stimulated with their cognate antigen OVA. These results strongly support the hypothesis that Treg conversion of naïve Tconv in the periphery is driven through recognition of self-antigens that are presented in the thymus during thymic selection. These findings were also confirmed *in vivo* where RAG2−/−2D2+ mice immunized with MOG and NFM generated strong Treg responses despite lacking a pool of tTregs in the periphery (Figure 6). This response was not the result of general inflammation since OVA immunization of RAG2−/−2D2+ mice failed to illicit a Treg response. While these findings may seem narrow in their impact, they have broader immunological implications and impacts on therapies.

The activation of cross-reactive TCRs is one of many hypotheses to account for the genesis of autoimmunity, however the importance of cross-reactive TCRs in tolerance is not well understood (41). The cross reactivity of the 2D2 TCR to MOG and NFM is well understood and has been thoroughly examined through the lens of EAE (42-44). Despite their nearly identical peptide sequence, immunization with NFM fails to generate a sufficient MOG-reactive T cell response to induce EAE (44). Further, knock out of NFM, but not MOG, results in fulminant EAE, suggesting that NFM may play a tolerizing role in the thymus or periphery (44). Additionally, previous work has demonstrated that NFM does not prophylactically tolerize against MOG-mediated EAE, but suppresses disease progression when administered at the peak of disease. These findings suggest that NFM may not tolerize against MOG reactivity in the peripheral lymph nodes, but instead broadly suppresses MOG reactive T cells in the CNS during disease progression (45). However, these studies to do not examine the role of Tregs in their findings. The present study demonstrates that NFM

peptide is capable of generating a pTreg response, which may add to our understanding of previous findings in the literature. Critically, we also recognize the limitations of the TCRs and peptides we examined in our efforts to understand how hNSC antigens drive pTreg generation and acknowledge that there are likely additional antigenic hNSC self-peptides that drive this conversion in wild type mice. Additionally, tolerization by presentation of apoptotic hNSCs may further contribute to the strength of the Treg response *in vivo* and *in vitro*.

The prevailing opinion in Treg biology has been that tTregs are generated following agonist selection against self-antigens in the thymus and, conversely, pTregs are induced in the periphery by TCR reactivity to non-self-antigens such as those produced by commensals on the skin and in the gut. However, his paradigm has begun to shift as more research has demonstrated that non-self-antigens are presented by peripheral dendritic cells (DCs) that traffic into the thymus (46, 47). Moreover, it has been demonstrated that pTreg generation in the periphery is dependent upon epigenetic modifications that occur earlier during thymic development (48, 49). Interestingly, although these studies revealed that pTregs were generated in the periphery based on these epigenetic changes, the influence of self- versus non-self-antigen recognition was not examined. Our findings suggest that pTregs are generated in response to self-antigens from the CNS within the periphery, since an increase in Tregs was also observed in the CNS draining cervical lymph nodes. Of note, our *in vitro* data suggest that peripheral presentation and recognition of thymically presented cognate self-antigen is sufficient to induce expression of Foxp3 in Tconv cells(50, 51). This finding may lend further refinement of work presented by others suggesting that presentation of antigen via tolerizing DCs is sufficient to induce Tregs, since RAG2−/−OTII+ mice failed to generate Tregs when presented with OVA, a non-self-antigen that is not expressed in the thymus of these mice(52). Instead, it may be the case that naïve Tconv cells are poised to convert into pTreg when they are presented with self-antigens that they have previously encountered during thymic selection. While the local inflammatory environment may contribute to Treg induction, inflammation is not sufficient to generate pTregs, since Poly (I:C) failed to generate a Treg response. Additionally, this response requires antigenic recognition since polyclonal stimulation by αCD3 and αCD28 failed to induce Tregs. Evidence that Treg induction is based on specific signaling that may be imparted in the thymus. These findings shine new light on our understanding of auto-reactivity in T cells leaving the thymus. Instead of being overtly autoreactive, T cells with affinity to self may offer a mechanism of tolerance under circumstances in which antigens fail to generate Tregs in the thymus or rarely presented antigens become available during tissue damage.

Stem cell transplantation remains a promising therapeutic approach to repair the damage caused by the recurrent inflammation within the CNS that occurs in MS. While cell replacement therapy remains a primary goal for these treatments, there has been a shift in our understanding of how NSC transplantation drives repair in the CNS, particularly with evidence that NSCs induce repair through the secretion of neurotropic factors(53-55). These novel approaches have the advantage of eliminating the problems associated with stem cell transplantation. Adding to this promise, our findings that hNSC antigens drive Treg generation is particularly exciting. Previous studies in our laboratory demonstrated that Tregs promote strong remyelination responses in mouse models of MS following hNSC

transplantation (6, 13) and while the mechanism by which Tregs induce remyelination remains unknown, these findings are part of a growing literature that demonstrates a reparative role for Tregs outside of their immune suppressive activities (13, 27, 28, 30, 56). This makes the findings in this study particularly exciting as it may in fact be possible to simply expose patients to boluses of CNS antigens to both reduce autoimmune infiltration as wells as inducing repair in the CNS by generating pTregs. There are currently a number of clinical trials underway examining the potential of vaccination against myelin epitopes as a method for inducing tolerance against these antigens. While these trials show promise, they target induction of peripheral tolerance through antigen presentation in the periphery as opposed to presentation within the CNS. More research is required to examine the importance of local versus peripheral presentation to determine if peripheral immunization is capable of inducing Treg infiltration into the CNS.

This study provides new insight into the generation of peripheral Tregs and reveals novel interactions between transplanted hNSCs and the adaptive immune system. In contrast to studies that suggest a direct role for hNSCs in suppression of autoimmune responses(14, 57), we have found that presentation of self-antigens derived from hNSCs drives tolerance through the induction of pTregs. This study further emphasizes the importance of understanding how the immune system interacts with transplanted cells in models of stem cell transplantation in the CNS and highlights the importance of studying Tregs not only for their suppressive capacity but their capacity to generate repair responses in the CNS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This work was supported by grantsT32 NS082174 (to LLM and SAG), NIH R01 AI121945 (to CMW), CIRM TR3-05693 (to CMW and TEL) and National Multiple Sclerosis Society CA 1058-A-8 (to CMW and TEL).

We would like to thank the animal care staff at UCI, Vanessa Scarfone and Pauline Nguyen at the Sue and Bill Gross Stem Cell Research Center, Jennifer Atwood at the UCI Institute for Immunology, the UCI ADRC for human brain tissue samples and Melanie Oakes at the UCI Genomics Core. We thank Stephen Schoenberger and Joey Lee for the generous contribution of RAG1−/−OT-II+ mice.

## References

1. Steinman L 1996. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Cell 85: 299–302. [PubMed: 8616884]
2. Hauser SL, and Cree BAC. 2020. Treatment of Multiple Sclerosis: A Review. Am J Med 133: 1380–1390 e1382. [PubMed: 32682869]
3. Chari DM 2007. Remyelination in multiple sclerosis. Int Rev Neurobiol 79: 589–620. [PubMed: 17531860]
4. Gage FH 2000. Mammalian neural stem cells. Science 287: 1433–1438. [PubMed: 10688783]
5. Ben-Hur T, Fainstein N, and Nishri Y. 2013. Cell-based reparative therapies for multiple sclerosis. Curr Neurol Neurosci Rep 13: 397. [PubMed: 24078453]
6. McIntyre LL, Greilach SA, Othy S, Sears-Kraxberger I, Wi B, Ayala-Angulo J, Vu E, Pham Q, Silva J, Dang K, Rezk F, Steward O, Cahalan MD, Lane TE, and Walsh CM. 2020. Regulatory T cells promote remyelination in the murine experimental autoimmune encephalomyelitis model

of multiple sclerosis following human neural stem cell transplant. Neurobiol Dis 140: 104868. [PubMed: 32276110]

7. Totoiu MO, Nistor GI, Lane TE, and Keirstead HS. 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. [PubMed: 15144852]
8. Hardison JL, Nistor G, Gonzalez R, Keirstead HS, and Lane TE. 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. [PubMed: 16297915]
9. Whitman LM, Blanc CA, Schaumburg CS, Rowitch DH, and Lane TE. 2012. Olig1 function is required for remyelination potential of transplanted neural progenitor cells in a model of viral-induced demyelination. Exp Neurol 235: 380–387. [PubMed: 22449475]
10. Greenberg ML, Weinger JG, Matheu MP, Carbajal KS, Parker I, Macklin WB, Lane TE, and Cahalan MD. 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. [PubMed: 24843159]
11. Chen L, Coleman R, Leang R, Tran H, Kopf A, Walsh CM, Sears-Kraxberger I, Steward O, Macklin WB, Loring JF, and Lane TE. 2014. Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis. Stem Cell Reports 2: 825–837. [PubMed: 24936469]
12. Plaisted WC, Zavala A, Hingco E, Tran H, Coleman R, Lane TE, Loring JF, and Walsh CM. 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. [PubMed: 27310015]
13. Dombrowski Y, O'Hagan T, Dittmer M, Penalva R, Mayoral SR, Bankhead P, Fleville S, Eleftheriadis G, Zhao C, Naughton M, Hassan R, Moffat J, Falconer J, Boyd A, Hamilton P, Allen IV, Kissenpfennig A, Moynagh PN, Evergren E, Perbal B, Williams AC, Ingram RJ, Chan JR, Franklin RJM, and Fitzgerald DC. 2017. Regulatory T cells promote myelin regeneration in the central nervous system. Nat Neurosci 20: 674–680. [PubMed: 28288125]
14. Nazmi A, Mohamed Arif I, Dutta K, Kundu K, and Basu A. 2014. Neural stem/progenitor cells induce conversion of encephalitogenic T cells into CD4+-CD25+- FOXP3+ regulatory T cells. Viral Immunol 27: 48–59. [PubMed: 24605788]
15. 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. [PubMed: 7636184]
16. Josefowicz SZ, Lu LF, and Rudensky AY. 2012. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol 30: 531–564. [PubMed: 22224781]
17. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, and Ramsdell F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 27: 68–73. [PubMed: 11138001]
18. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, and Ochs HD. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 27: 20–21. [PubMed: 11137993]
19. Bluestone JA, Tang Q, and Sedwick CE. 2008. T regulatory cells in autoimmune diabetes: past challenges, future prospects. J Clin Immunol 28: 677–684. [PubMed: 18716861]
20. Haas J, Fritzsching B, Trubswetter P, Korporal M, Milkova L, Fritz B, Vobis D, Krammer PH, 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. [PubMed: 17617625]
21. Venken K, Hellings N, Broekmans T, Hensen K, Rummens JL, 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. [PubMed: 18424765]

22. Wan YY, and Flavell RA. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 445: 766–770. [PubMed: 17220876]

23. Li J, Tan J, Martino MM, and Lui KO. 2018. Regulatory T-Cells: Potential Regulator of Tissue Repair and Regeneration. Front Immunol 9: 585. [PubMed: 29662491]

24. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, and Mathis D. 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. [PubMed: 19633656]

25. Tontonoz P, and Spiegelman BM. 2008. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem 77: 289–312. [PubMed: 18518822]

26. Glowacki AJ, Yoshizawa S, Jhunjhunwala S, Vieira AE, Garlet GP, Sfeir C, and Little SR. 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. [PubMed: 24167272]

27. Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, Ahn R, Corbin K, Lowe MM, Scharschmidt TC, Taravati K, Tan MR, Ricardo-Gonzalez RR, Nosbaum A, Bertolini M, Liao W, Nestle FO, Paus R, Cotsarelis G, Abbas AK, and Rosenblum MD. 2017. Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. Cell 169: 1119–1129 e1111. [PubMed: 28552347]

28. Arpaia N, Green JA, Moltedo B, Arvey A, Hemmers S, Yuan S, Treuting PM, and Rudensky AY. 2015. A Distinct Function of Regulatory T Cells in Tissue Protection. Cell 162: 1078–1089. [PubMed: 26317471]

29. Mock JR, Garibaldi BT, Aggarwal NR, Jenkins J, Limjunyawong N, Singer BD, Chau E, Rabold R, Files DC, Sidhaye V, Mitzner W, Wagner EM, King LS, and D'Alessio FR. 2014. Foxp3+ regulatory T cells promote lung epithelial proliferation. Mucosal Immunol 7: 1440–1451. [PubMed: 24850425]

30. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, Sefik E, Tan TG, Wagers AJ, Benoist C, and Mathis D. 2013. A special population of regulatory T cells potentiates muscle repair. Cell 155: 1282–1295. [PubMed: 24315098]

31. Ito M, Komai K, Mise-Omata S, Iizuka-Koga M, Noguchi Y, Kondo T, Sakai R, Matsuo K, Nakayama T, Yoshie O, Nakatsukasa H, Chikuma S, Shichita T, and Yoshimura A. 2019. Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery. Nature 565: 246–250. [PubMed: 30602786]

32. Gehrmann J, Matsumoto Y, and Kreutzberg GW. 1995. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev 20: 269–287. [PubMed: 7550361]

33. Horwitz DA, Zheng SG, Wang J, and Gray JD. 2008. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. Eur J Immunol 38: 912–915. [PubMed: 18395858]

34. Tischner D, Wiegers GJ, 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. [PubMed: 22322859]

35. Stewart JJ, Lee CY, 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. [PubMed: 9519765]

36. Drescher KM, Johnston SL, Hogancamp W, Nabozny GH, David CS, Rimm IJ, Wettstein PJ, and Rodriguez M. 2000. V(beta)8(+) T cells protect from demyelinating disease in a viral model of multiple sclerosis. Int Immunol 12: 271–280. [PubMed: 10700462]

37. Wilson RK, Lai E, Concannon P, Barth RK, and Hood LE. 1988. Structure, organization and polymorphism of murine and human T-cell receptor alpha and beta chain gene families. Immunol Rev 101: 149–172. [PubMed: 2965094]

38. Mendel Kerlero de Rosbo N, and Ben-Nun A. 1996. Delineation of the minimal encephalitogenic epitope within the immunodominant region of myelin oligodendrocyte glycoprotein: diverse V beta gene usage by T cells recognizing the core epitope encephalitogenic for T cell receptor V beta b and T cell receptor V beta a H-2b mice. Eur J Immunol 26: 2470–2479. [PubMed: 8898962]

39. Thompson LJ, Lai JF, Valladao AC, Thelen TD, Urry ZL, and Ziegler SF. 2016. Conditioning of naive CD4(+) T cells for enhanced peripheral Foxp3 induction by nonspecific bystander inflammation. Nat Immunol 17: 297–303. [PubMed: 26752376]
40. Bettelli E, Pagany M, Weiner HL, Linington C, Sobel RA, and Kuchroo VK. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. J Exp Med 197: 1073–1081. [PubMed: 12732654]
41. Wucherpfennig KW, and Strominger JL. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell 80: 695–705. [PubMed: 7534214]
42. Krishnamoorthy G, Saxena A, Mars LT, Domingues HS, Mentele R, Ben-Nun A, Lassmann H, Dornmair K, Kurschus FC, Liblau RS, and Wekerle H. 2009. Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis. Nat Med 15: 626–632. [PubMed: 19483694]
43. Lucca LE, Desbois S, Ramadan A, Ben-Nun A, Eisenstein M, Carrie N, Guery JC, Sette A, Nguyen P, Geiger TL, Mars LT, and Liblau RS. 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. [PubMed: 25135834]
44. Blanchfield L, Sabatino JJ Jr., Lawrence L, and Evavold BD. 2017. NFM Cross-Reactivity to MOG Does Not Expand a Critical Threshold Level of High-Affinity T Cells Necessary for Onset of Demyelinating Disease. J Immunol 199: 2680–2691. [PubMed: 28887429]
45. Ramadan A, Lucca LE, Carrie N, Desbois S, Axisa PP, Hayder M, Bauer J, Liblau RS, and Mars LT. 2016. In situ expansion of T cells that recognize distinct self-antigens sustains autoimmunity in the CNS. Brain 139: 1433–1446. [PubMed: 27000832]
46. Ennamorati M, Vasudevan C, Clerkin K, Halvorsen S, Verma S, Ibrahim S, Prosper S, Porter C, Yeliseyev V, Kim M, Gardecki J, Sassi S, Tearney G, Cherayil BJ, Bry L, Seed B, and Jain N. 2020. Intestinal microbes influence development of thymic lymphocytes in early life. Proc Natl Acad Sci U S A 117: 2570–2578. [PubMed: 31964813]
47. Breed ER, Lee ST, and Hogquist KA. 2018. Directing T cell fate: How thymic antigen presenting cells coordinate thymocyte selection. Semin Cell Dev Biol 84: 2–10. [PubMed: 28800929]
48. Owen DL, Mahmud SA, Sjaastad LE, Williams JB, Spanier JA, Simeonov DR, Ruscher R, Huang W, Proekt I, Miller CN, Hekim C, Jeschke JC, Aggarwal P, Broeckel U, LaRue RS, Henzler CM, Alegre ML, Anderson MS, August A, Marson A, Zheng Y, Williams CB, and Farrar MA. 2019. Thymic regulatory T cells arise via two distinct developmental programs. Nat Immunol 20: 195–205. [PubMed: 30643267]
49. Dikiy S, Li J, Bai L, Jiang M, Janke L, Zong X, Hao X, Hoyos B, Wang ZM, Xu B, Fan Y, Rudensky AY, and Feng Y. 2021. A distal Foxp3 enhancer enables interleukin-2 dependent thymic Treg cell lineage commitment for robust immune tolerance. Immunity 54: 931–946 e911. [PubMed: 33838102]
50. Kuczma MP, Szurek EA, Cebula A, Ngo VL, Pietrzak M, Kraj P, Denning TL, and Ignatowicz L. 2021. Self and microbiota-derived epitopes induce CD4(+) T cell anergy and conversion into CD4(+)Foxp3(+) regulatory cells. Mucosal Immunol 14: 443–454. [PubMed: 33139845]
51. Kalekar LA, Schmiel SE, Nandiwada SL, Lam WY, Barsness LO, Zhang N, Stritesky GL, Malhotra D, Pauken KE, Linehan JL, O'Sullivan MG, Fife BT, Hogquist KA, Jenkins MK, and Mueller DL. 2016. CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors. Nat Immunol 17: 304–314. [PubMed: 26829766]
52. Maldonado RA, and von Andrian UH. 2010. How tolerogenic dendritic cells induce regulatory T cells. Adv Immunol 108: 111–165. [PubMed: 21056730]
53. Pluchino S, Gritti A, Blezer E, Amadio S, Brambilla E, Borsellino G, Cossetti C, Del Carro U, Comi G, t Hart B, Vescovi A, and Martino G. 2009. Human neural stem cells ameliorate autoimmune encephalomyelitis in non-human primates. Ann Neurol 66: 343–354. [PubMed: 19798728]
54. Muller FJ, Snyder EY, and Loring JF. 2006. Gene therapy: can neural stem cells deliver? Nat Rev Neurosci 7: 75–84. [PubMed: 16371952]

55. Neirinckx V, Coste C, Rogister B, and Wislet-Gendebien S. 2013. Concise review: adult mesenchymal stem cells, adult neural crest stem cells, and therapy of neurological pathologies: a state of play. Stem Cells Transl Med 2: 284–296. [PubMed: 23486833]

56. Laurent P, Jolivel V, Manicki P, Chiu L, Contin-Bordes C, Truchetet ME, and Pradeu T. 2017. Immune-Mediated Repair: A Matter of Plasticity. Front Immunol 8: 454. [PubMed: 28484454]

57. Arad T, Fainstein N, Goldfarb S, Lachish M, Ganz T, and Ben-Hur T. 2021. CD200 -dependent and -independent immune-modulatory functions of neural stem cells. Stem Cell Res 56: 102559. [PubMed: 34634761]

58. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA, and Ziegler SF. 2013. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol 14: 307–308. [PubMed: 23507634]

59. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, and Chatila TA. 2007. Regulatory T cell development in the absence of functional Foxp3. Nat Immunol 8: 359–368. [PubMed: 17273171]

60. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, and et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68: 855–867. [PubMed: 1547487]

61. Barnden MJ, Allison J, Heath WR, and Carbone FR. 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. [PubMed: 9553774]

62. Lane TE, Liu MT, Chen BP, Asensio VC, Samawi RM, Paoletti AD, Campbell IL, Kunkel SL, Fox HS, and Buchmeier MJ. 2000. A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. J Virol 74: 1415–1424. [PubMed: 10627552]

63. Juan WS, Lin HW, Chen YH, Chen HY, Hung YC, Tai SH, Huang SY, Chen TY, and Lee EJ. 2012. Optimal Percoll concentration facilitates flow cytometric analysis for annexin V/propidium iodine-stained ischemic brain tissues. Cytometry A 81: 400–408. [PubMed: 22311734]

64. Chambers SM, Fasano CA, Papapetrou EP, 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. [PubMed: 19252484]

65. Yuan SH, Martin J, Elia J, Flippin J, Paramban RI, Hefferan MP, Vidal JG, Mu Y, Killian RL, Israel MA, Emre N, Marsala S, Marsala M, Gage FH, Goldstein LS, and Carson CT. 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. [PubMed: 21407814]

66. Schmittgen TD, and Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108 [PubMed: 18546601]

**Key Points**

- Human neural stem cells (hNSCs) induce CD25+ Foxp3+ Tregs from conventional T cells.
- This conversion to Tregs is driven by hNSC antigens and not secreted factors.
- hNSC antigens require thymic presentation for peripheral Treg conversion.

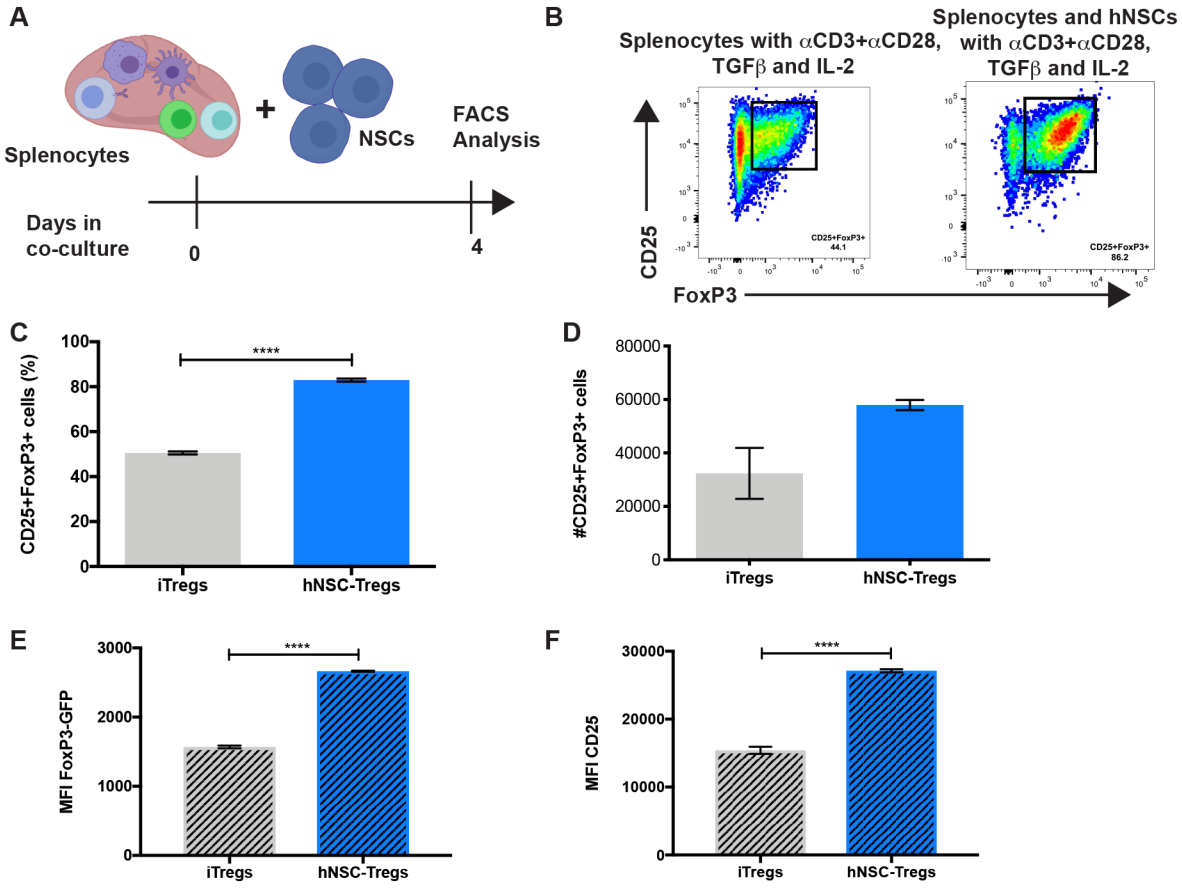


**Figure 1. hNSCs expand antigen specific Tregs.**

Splenocytes were isolated from 6-8-week-old C57BL/6 wild type mice and cultured for 4 days ex vivo with 1μg/mL αCD3+ 1μg/mL αCD28+ 7ng/mL TGFβ+ 100U/mL IL-2 in the presence or absence of hNSCs at various ratios of splenocytes to hNSCs (A). Representative dot plots of CD25+Foxp3+ Tregs gated sequentially on lymphocytes then excluding forward and side scatter doublets, then gated on live, CD4+ cells after 4 days in culture with Treg inducing (iTregs) conditions with αCD3+ αCD28, TGFβ and IL-2 (left) and with hNSCs (right) (B). Gating strategy used to determine CD25+Foxp3+ cells can be found in Supplemental Figure 1. Bar plots displaying the frequency (%) and number, respectively, of CD4+CD25+Foxp3+ T cells from cultures of C57BL/6 splenocytes (C and D). A significant (****p<0.0001) increase in the mean fluorescent intensity (MFI) of Foxp3 (E) and CD25 (F) of the CD4+ cells in the presence of hNSCs was also observed. Experimental groups consisted of 3 samples; experiments were independently replicated 3 times. Data is presented as average ± SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test comparing each condition with hNSCs to iTregs control. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001, NS is not significant.

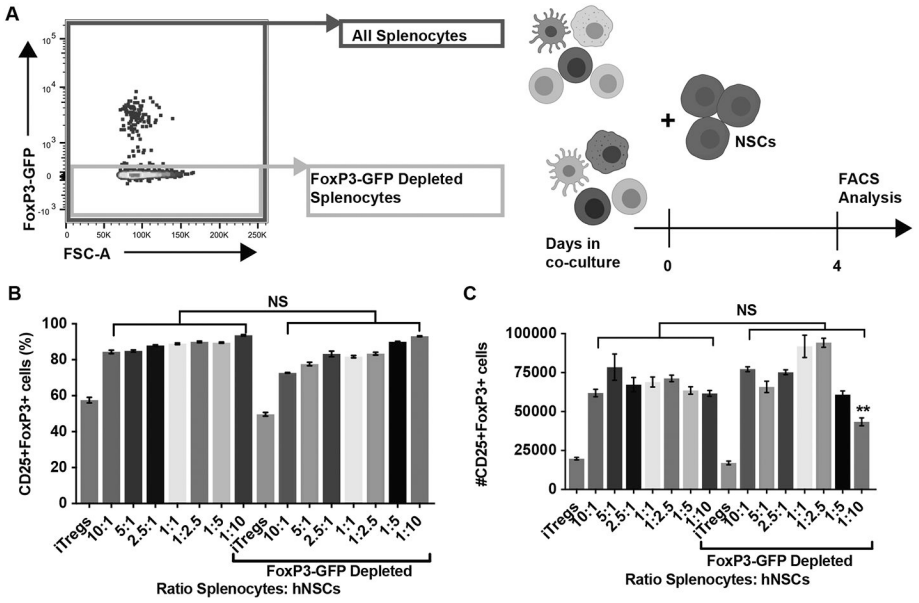


**Figure 2. hNSCs induce Tregs from T conventional cells.**

Splenocytes isolated from 6-8-week-old Foxp3-GFP+ mice were isolated and subjected to FACS of all splenocytes (blue) or splenocytes depleted of Foxp3-GFP+ cells (grey) and cultured for 4 days ex vivo with 1μg/mL αCD3+ 1μg/mL αCD28+ 7ng/mL TGFβ+ 100U/mL IL-2 in the presence or absence of hNSCs at various ratios of splenocytes to hNSCs (A). No significant difference was observed in the frequency (B) or number (C) of CD25+Foxp3+ cells after 4 days in cultures containing starting splenocyte populations of natural Tregs (all splenocytes) or depleted of Foxp3-GFP+ Tregs. Experimental groups consisted of 3 samples; experiments were replicated 3 times. Data is presented as average ± SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test comparing each ratio of all splenocytes condition to the same ratio of Foxp3-GFP depleted condition (ie. 10:1 all splenocytes to hNSCs (blue) compared against 10:1 Foxp3-GFP depleted splenocytes to hNSCs (grey)). A significant difference between iTreg and all ratios of all splenocytes to hNSCs and all ratios of Foxp3-GFP depleted splenocytes to hNSCs was observed consistent with previous results. **p< 0.0059, ****p<0.0001, NS is not significant.

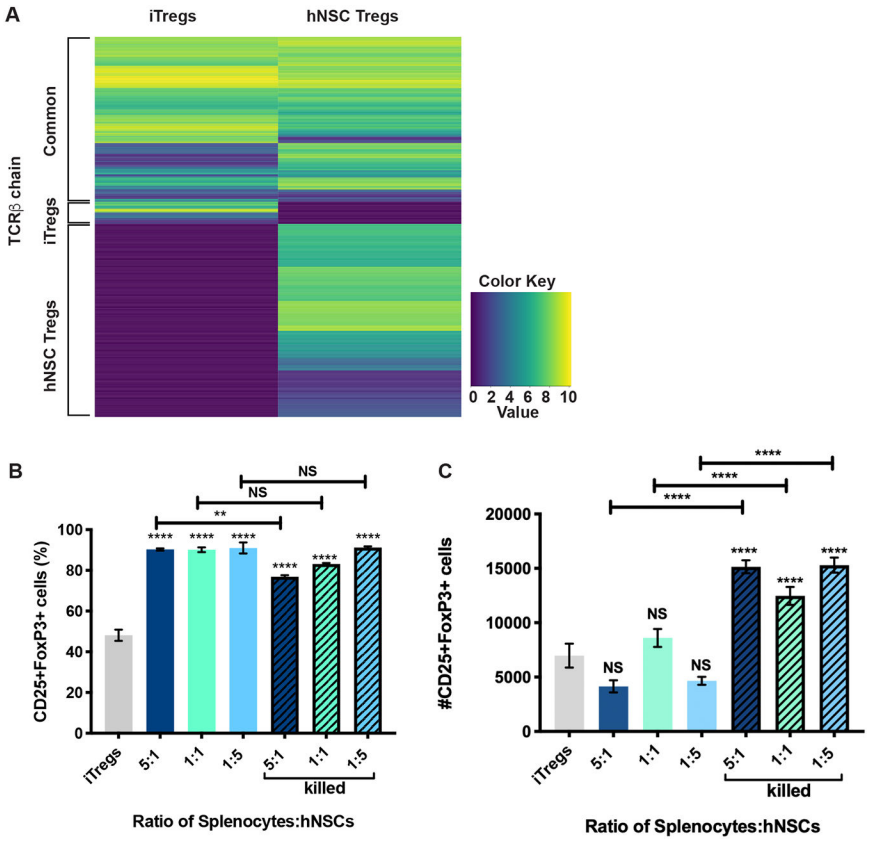


**Figure 3. hNSC Tregs display unique CDR3e sequences, and killed hNSCs expand CD25+FoxP3+ cells.**

(A) TCR repertoire analysis of TCRβ chains from sorted CD4+CD25+Foxp3+ 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. The top 10 TCRβ chain clones from iTregs are different than hNSC Tregs (Supplemental Figure 2). Data is representative of two independent experiments. (B and C) Splenocytes were isolated from 6-8-week-old Foxp3-GFP+ mice and cultured for 4 days ex vivo with 1μg/mL αCD3+ 1μg/mL αCD28+ 7ng/mL TGFβ+ 100U/mL IL-2 in the presence or absence of viable or killed hNSCs, at various ratios of splenocytes to cells. Cells were killed by culture in PBS for 24 hours prior to co-culture, inducing apoptotic cell death, confirmed by flow cytometry (data not shown). Bar plots displaying the frequency (%) and number, respectively, of CD25+Foxp3+ cells from cultures of C57BL/6 Foxp3-GFP+ splenocytes and hNSCs (B and C). Experimental groups consisted of 3 samples and were repeated 3 times. Data is presented as average ± SEM and analyzed using a one-way ANOVA with a Tukey's multiple comparisons test comparing all groups to each other. NS is not significant, $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

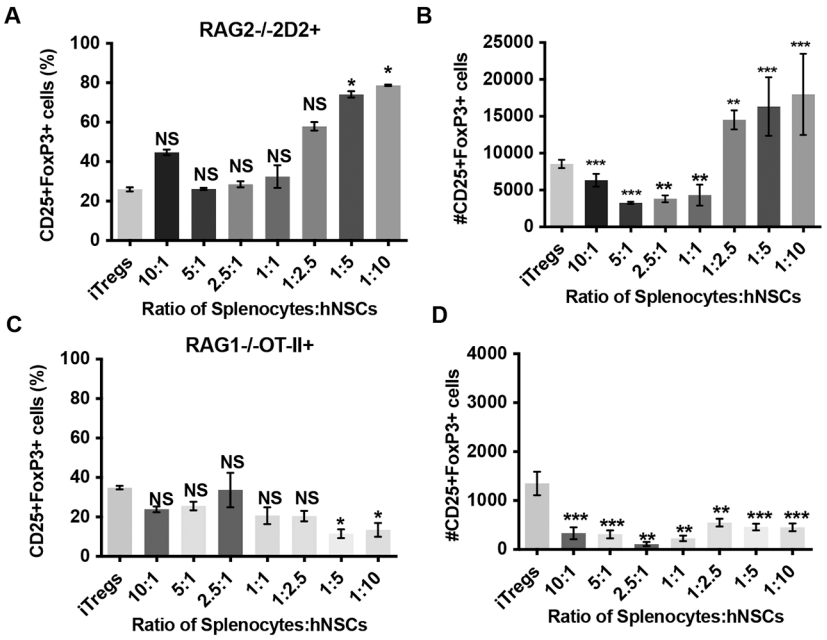


**Figure 4. hNSCs expand antigen specific Tregs.**

Splenocytes were isolated from 6-8-week-old RAG2−/−2D2+ TCR-tg and RAG1−/−OT-II+ TCR-tg mice and cultured for 4 days ex vivo with 1μg/mL αCD3+ 1μg/mL αCD28+ 7ng/mL TGFβ+ 100U/mL IL-2 in the presence or absence of hNSCs at various ratios of splenocytes to hNSCs. Bar plots displaying the frequency (%) and number, respectively, of CD25+Foxp3+ cells from cultures RAG2−/−2D2+ splenocytes (A and B) and RAG1−/−OT-II+ splenocytes (C and D) with hNSCs. Co-cultures of RAG2−/−2D2+ splenocytes with hNSCs revealed a significant increase in the frequency of CD25+Foxp3+ cells at the following ratios of splenocytes to hNSCs compared to iTregs control; 10:1, 1:2.5, 1:5, 1:10. Co-cultures of RAG1−/−OT-II+ splenocytes with hNSCs did not show a significant increase in the frequency of CD25+Foxp3+ cells at all ratios of splenocytes to hNSCs compared to iTregs control. Experimental groups consisted of 3 samples; experiments were independently replicated 3 times. Data is presented as average ± SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test comparing each condition with hNSCs to iTregs control. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001, NS is not significant.

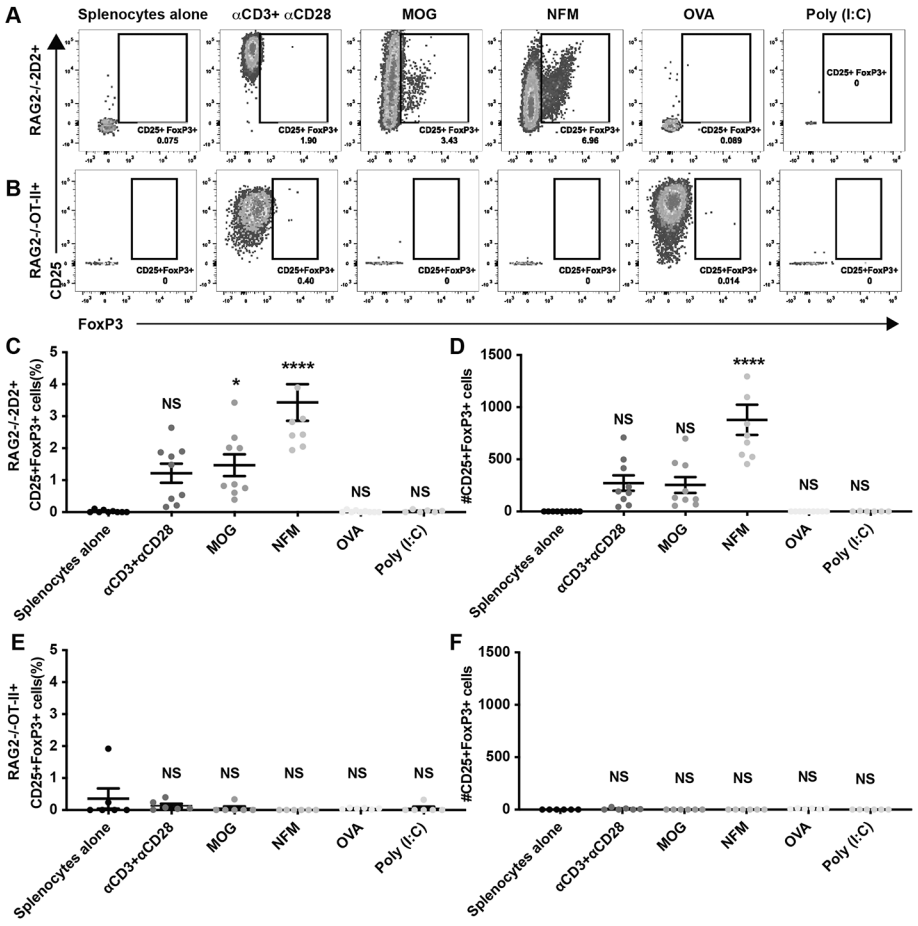


**Figure 5. RAG2−/−2D2+ splenocytes upregulate Foxp3 following cognate peptide exposure in vitro.**

Splenocytes were isolated from 5-8-week-old RAG2−/−2D2+ TCR-tg and RAG2−/−OT-II+TCR-tg mice and cultured for 4 days ex vivo in the presence of various stimulus. Representative dot plots gated on lymphocytes, excluding forward and side scatter doublets, live, CD4+ display frequency (%) of CD25+Foxp3+ cells from RAG2−/−2D2+ TCR-tg mice (A) and RAG2−/−OT-II+ TCR-tg (B) cultured in the following conditions, from left to right; splenocytes alone, with 1μg/mL αCD3+1μg/mL αCD28, 20μg/mL MOG peptide, 20μg/mL NFM peptide, 20μg/mL OVA peptide, or 3μg/mL Poly (I:C). Bar graphs display frequency (%) (C) and number (D) of CD4+CD25+Foxp3+ cells from RAG2−/−2D2+ TCR-tg splenocytes. Bar graphs display frequency (%) (E) and number (F) of CD4+CD25+Foxp3+ cells from RAG2−/−OT-II+ TCR-tg splenocytes. Experimental groups consisted of 3 samples; experiments were replicated 2 times using RAG2−/−OT-II+ splenocytes and 3 times using RAG2−/−2D2+ splenocytes. Data is presented as average ± SEM and analyzed using one-way ANOVA followed by Tukey's multiple comparison test comparing each stimulation condition to splenocytes alone control. *p=0.0213, ****p<0.0001, NS is not significant.

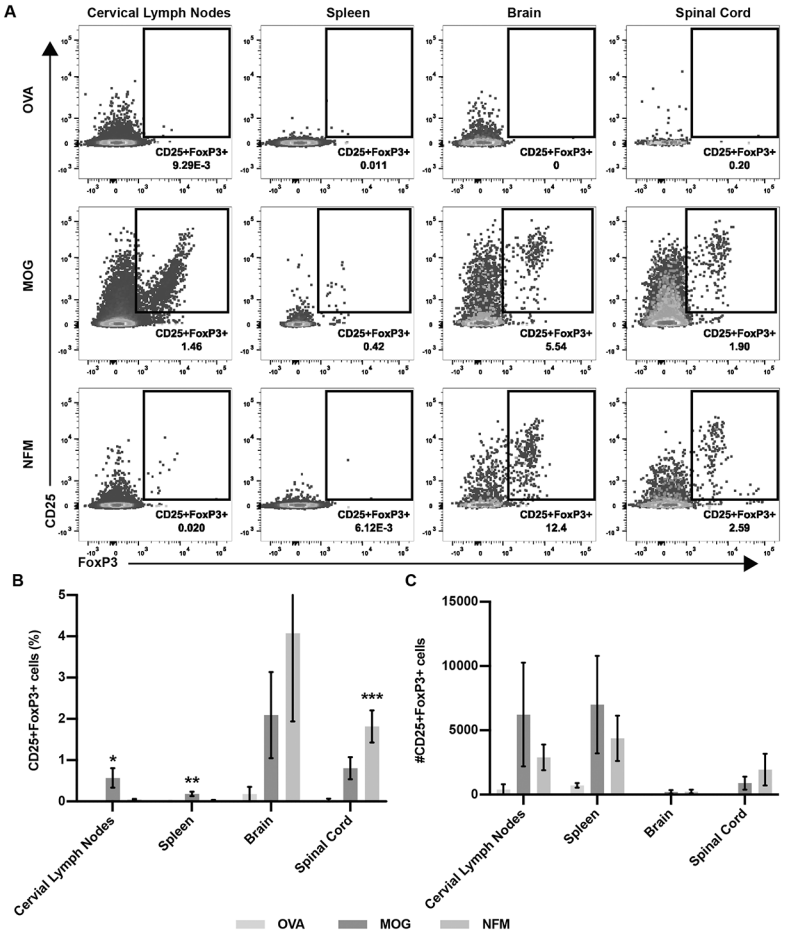


**Figure 6. RAG2−/−2D2+ T cells upregulate Foxp3 following cognate peptide exposure in vivo.**

Immunization of 8-week-old RAG2−/−2D2+ TCR-tg mice with OVA 323-329 peptide (n=6; green), MOG 35-55 peptide (n=7; purple) or NFM 18-30 peptide (n=5; blue) was performed. Ten days post immunization mice were sacrificed; cervical lymph nodes, spleen, brain and spinal cord were harvested and analyzed using flow cytometry. Panels show representative dot plots of CD25+Foxp3+ Tregs gated on lymphocytes, excluding forward and side scatter doublets, live, CD4+ cells (A). Bar graphs show frequency (%) (B) and total number (C) of CD25+Foxp3+ Tregs present in each tissue. Experimental groups consisted of 5-7 mice. Data is presented as average ± SEM and analyzed using a one-way ANOVA with multiple comparisons, comparing OVA control to MOG or NFM. *p=0.0381, **p=0.0059, ***p=0.0007, NS is not significant.