# VITAMIN D INHIBITS EXPRESSION OF PROTEIN ARGININE DEIMINASE 2 AND 4 IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS MODEL OF MULTIPLE SCLEROSIS

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Travis William McCain

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Vitamin D Inhibits Expression of Protein Arginine Deiminase 2 and 4 in Experimental Autoimmune Encephalomyelitis Model of Multiple Sclerosis

Multiple sclerosis (MS) is a disabling disease that afflicts an estimated two million people worldwide. The disease is characterized by degradation of the myelin sheath that insulates neurons of the central nervous system manifesting as a heterogeneous collection of symptoms. Two enzymes, protein arginine deaminases type 2 and 4 (PAD2 and PAD4) have been implicated to play an etiologic role in demyelination and neurodegeneration by catalyzing a post-translational modification of arginine peptide residues to citrulline. The pathogenesis of MS is poorly understood, though vitamin D deficiency is a well-associated risk factor for developing the disorder. Using the experimental autoimmune encephalomyelitis (EAE) model of MS we demonstrate vitamin D treatment to attenuate over-expression of PAD 2 and 4 in the brain and spine during EAE. In addition, we identify two molecules produced by peripheral immune cells, $IFN_{\gamma}$ and IL-6, as candidate signaling molecules that induce PAD expression in the brain. We demonstrate vitamin D treatment to inhibit $IFN_{\gamma}$ mediated up regulation of PAD2 and PAD4 both directly within the brain and by modulating PAD-inducing cytokine production by infiltrating immune cells. These results provide neuroprotective rational for the supplementation of vitamin D in MS patients. More importantly, these results imply an epigenetic link between vitamin D deficiency and the pathogenesis of MS that merits further investigation.

Patricia J. Gallagher, Ph.D.

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LIST OF ABBREVIATIONS

| Abbreviation | Description | Page |
|---|---|---|
| MS | Multiple sclerosis | 1 |
| EAE | Experimental autoimmune encephalomyelitis | 1 |
| CNS | Central nervous system | 1 |
| Th1 | T helper cell type 1 | 2 |
| Th17 | T helper cell type 17 | 2 |
| PAD | Protein (or peptidyl) arginine diminase | 2 |
| TNFα | Tumor necrosis factor alpha | 2 |
| MBP | Myelin basic protein | 3 |
| NAWM | Normal appearing white matter | 3 |
| iNOS | Inducible nitric oxide synthase | 4 |
| MOG | Myelin oligodendrocyte glycoprotein | 5 |
| VDR | Vitamin D receptor | 5 |
| $IFN_{\gamma}$ | Interferon gamma | 7 |
| IL-12 | Interleukin 12 | 7 |
| Con A | Concanavalin A | 8 |
| IL-6 | Interleukin 6 | 8 |
| IL-17 | Interleukin 17 | 8 |
| i.p. | Intraperiotoneal injection | 9 |
| IL-8 | Interleukin 8 | 36 |

# INTRODUCTION

## Multiple Sclerosis, a Heterogeneous Disorder

Multiple sclerosis (MS) is a disabling disorder that afflicts over 2 million people worldwide (Anderson et al., 1992). The heterogeneous disorder is characterized by degradation of the myelin sheath and axonal loss in neurons of the central nervous system (CNS) resulting in a collection of physical disabilities and cognitive impairment. The diverse pathophysiology of MS is classified into four clinical immunopatterns involving various degrees of inflammation, gliosis and neurodegeneration (Popescu and Pirko, 2013). The disorder is studied in several animal models that collectively reflect the heterogeneity of the disorder (Moscarello et al., 2013). Classically, MS is considered an autoimmune disease modeled by experimental autoimmune encephalomyelitis (EAE) in which peripheral immune cells cross the blood brain barrier and react with the myelin sheath under direction of CNS-reactive Th1/Th17 cells resulting in neuroinflammation (Robinson et al., 2014). However, in several animal models of MS including the neurotoxic cuprizone model (Star et al., 2012), the transgenic ND4 mouse model (Mastronardi et al., 1993), and the viral Theiler's urine encephalomyelitis model (Fujinami, 2011) neurodegenerative stress precedes peripheral immune cell invasion of the CNS. Diffuse neurodegeneration apart from plaques is well observed in MS brain (Haider et al., 2014). These results suggest degenerative mechanisms to work in concert with or to even potentiate demylination by the adaptive immune response of invading peripheral immune cells.

**Protein Arginine Deaminases Expression in Brain and Peripheral Immune Cells**

One molecular target at the intersection of auto-inflammation by peripheral immune cells and inflammation-independent oligodendrocyte degeneration is the protein arginine deiminase (PAD) family of enzymes. Over-expression and up-regulation of PAD activity are associated both with autoimmune diseases, such as Lupus (Nissinen et al., 2003; Knight et al., 2013) and rheumatoid arthritis (Jones et al., 2009; Nissinen et al., 2003), as well as neurodegenerative diseases including Alzheimer's (Ishigami et al., 2005; (Whiteley, 2014), Parkinson's (Nicholas, 2011) and prions disease (Jang et al., 2013). Of the five enzymes that compose the family, only PAD2 and PAD4 enzymes are expressed both in the CNS and peripheral immune cells (Vossenaar et al., 2003). PAD 2 and PAD4 catalyze an irreversible and calcium-dependent post-translational deimination of peptidyl arginine residues to citrulline resulting in a loss of cationical charge and mass at the converted residue. PAD4 contains a nuclear localization sequence and has been demonstrated to citrullinate histone H3 under stimulation of TNFα where it is implicated to play a role in oligodendrocyte apoptosis (Mastronardi et al., 2006). Both enzymes localize to the intracellular space between oligodendrocytes and neurons (Wood et al., 2008), with PAD2 contributing to the majority of citrullination (Beers et al., 2013; Jin et al., 2013).

**PAD Activity During MS**

Expression of both PAD2 and PAD4, total PAD activity and the amount of citrullinated protein are all up regulated in the brain during MS (Wood et al., 2008) where the amount of citrullinated myelin basic protein (MBP) correlates with clinical severity. An average of 18% of MBP extracted from control adult normal appearing white matter (NAWM) has been demonstrated to be citrullinated at the C-8 moiety as compared to 45% of MBP in NAWM during chronic MS (Moscarello et al., 1994). During Marburg's Disease, or acute MS, the proportion of citrullinated MBP increases to 90% (Wood et al., 1996). Hypercitrullination in the CNS occurs in several animal models of MS including murine experimental autoimmune encephalomyelitis (Nicholas et al., 2005; Raijmakers et al., 2005) as well as models that are not Th1 cell mediated. PAD2 over-expression precedes demyelination and protein citrullination by one month in ND4 mice that express 70 copies of the cDNA for the myelin proteolipid DM20, implying citrullination to play an etiologic role in the biochemistry of demylenation (Moscarello et al., 2002). PAD2 over-expression alone is sufficient to cause spontaneous demyelination at six months of age in mice transfected with 30 copies of PAD2 cDNA under the MBP promoter (Musse et al., 2008). Finally, irreversible inhibition of the PAD family enzymes with 2-chloroacetamidine attenuates or entirely resolves clinical symptoms in four independent animal models of MS (Moscarello et al., 2013). Together, these results indicate an important role for PAD enzyme activity across a variety of demyelination processes.

**PADs in Auto-Inflammation and Oligodendrocyte Apoptosis**

Citrullination of MBP and other myelin sheath proteins by PAD2 has potential to cause neurodegeneration independent of infiltrating immune cells as well as trigger enhanced auto-inflammation against the myelin sheath by infiltrating immune cells. Moscarello has demonstrated less-cationically charged MBP to loose its compact tertiary structure and cause poor lipid bilayer packing by several techniques including X-ray diffraction (Brady et al., 1981), electron spin resonance (Natl et al., 1982), circular dichroism (Epand and Wheeler, 1974) and NMR (Deber et al., 1986). Citrullinated MBP dissociates more readily from the lipid bilayer than other modified forms of the protein. In vitro recapitulation of this dissociation in mixed microglia/oligodendrocyte culture results in iNOS induction in microglia causing apoptosis of oligodendrocytes via mitochondrial pathway (Shanshiashvili et al., 2012). These observations demonstrate citrullinated MBP to play an etiopathic role in demyelination independent of Th1 cell mediated inflammation.

Citrullinated MBP also represents a novel epitope to infiltrating leukocytes and increases the repertoire of auto-reactive T cells under the Th1 model of MS. T cell polarization by citrullinated MBP occurs preferentially in MS patients as compared to controls suggesting the increased myelin citrullination during MS to exacerbate Th1 cell mediated inflammation by infiltrating leukocytes in the MS brain (Deraos et al., 2008; Tranquill et al., 2000). This exacerbation of the Th1 response by the peptidyl citrulline epitope in myelin sheath proteins can be recapitulated in the EAE model of MS. Mice with EAE that are injected with T cells activated by citrullinated myelin oligodendrocyte

glycoprotein (MOG) have been shown to develop exaggerated pathology (Carrillo-vico et al. 2014). How PADs become over-expressed in order to contribute to oligodendrocyte degeneration and Th1 mediated inflammation during MS is poorly understood.

**PADs and Vitamin D**

The etiology of MS is unknown, but one's risk for developing the disorder is thought to involve a genetic predisposition triggered by a number of interacting environmental risk factors including smoking, viral infection and vitamin D status that contribute to one's risk for developing MS from conception into adulthood (Pierrot-deseilligny and Souberbielle, 2013). Maternal parent of origin effects, birth month effects and differences in occurrence of the disorder between monozygous twins further imply epigenetic aetiologic mechanisms that can be imprinted in utero and are highly stable, such as DNA methlyation patterns. The CpG island of the PAD2 promoter has been demonstrated to be hypomethlyated in NAWM of MS brain (Mastronardi et al., 2007). We speculate that hypomethlyation of the PAD2 promoter region mediated by an environmental risk factor early in life might predispose an individual to PAD2 over-expression and demyelination later in life. A vitamin D receptor binding site is located within an exon of PAD3 clustered by PAD2 and PAD4 on chromosome one. The genes have previously been demonstrated responsive to the vitamin D receptor (VDR)-1,25(OH)2D3 complex in keratinocytes (Lu et al., 2005). Chip analysis has revealed the VDR-1,25(OH)2D3 complex to repress expression of certain genes by mediating

hypermethylation of target gene promoter regions (Fu et al., 2013), providing a plausible mechanism for vitamin D to directly repress PAD expression.

**Vitamin D and MS/EAE**

The association between vitamin D status and risk of developing MS is well characterized (Pierrot-deseilligny and Souberbielle 2013; (Simon, Munger and Ascherio, 2012) and first suggested quite some time ago (Goldberg, 1974). The prevalence of the disorder is stratified geographically with the highest prevalence far north and south of the equator and lowest prevalence near the equator (Freedman, Dosemeci, and Alavanja, 2000). Since de novo vitamin D synthesis from 7-dehydrocholesterol requires exposure of the skin to UV light, the latitude-dependent distribution of MS demonstrates increased risk for developing MS in populations geographically predisposed to be vitamin D deficient. Variants of vitamin D related genes have been investigated with suggestive results. Most notably, rare variants of the vitamin D activating enzyme *CYP27B1*, associated with decreased 25(OH)D activation, to 1,25(OH)2D3 carry an increased risk for developing MS suggesting a causal role for vitamin D deficiency in MS pathogenesis (Ramagopalan et al., 2011). Individuals deficient in circulating 25(OH)D have a greater risk for developing MS (Munger et al., 2014) and in patients newly diagnosed, vitamin D status predicts the extent of future disability (Smolders et al., 2008) and risk of new T2 lesions (Mowry et al., 2012). Vitamin D supplementation has been shown to increase long-term memory during MS (Koven et

al., 2013). Most MS patients supplement with vitamin D and a large clinical trial on high dose vitamin D on disease progression is currently underway.

Immunomodulatory effects of vitamin D have been well characterized. We have previously demonstrated treatment with the active form of vitamin D 1,25(OH)2D3 to ameliorate clinical symptoms in the chronic EAE model of MS by regulating Th1 differentiation through the $IFN_{\gamma}$/IL-12 axis (Bright et al., 2006), but its direct neuroprotective effects are less well understood with no previous study relating vitamin D to PAD expression in the brain during MS.

**Hypothesis**

We hypothesized that Vitamin D attenuates chronic EAE by modulating expression of PAD 2 and 4.

**Brief Results**

Using the chronic EAE model of MS, we demonstrate vitamin D treatment to repress PAD2 and 4 expression in the brain and identify the $IFN_{\gamma}$/IL-12 axis of infiltrating peripheral immune cells as an inducer of PAD expression. Our results demonstrate vitamin D to attenuate peripheral immune cell mediated induction of PADs in the brain. In addition, we show vitamin D to directly inhibit PAD expression in the brain implying a causal mechanism for vitamin D deficiency to preempt increased PAD expression during EAE.

METHODS & MATERIALS

## Animals and cells

The C57BL/6 mice were purchased from Harlan Laboratory (Indianapolis, IN, USA) and maintained in the animal care facility at the Indiana University Health Methodist Research Institute. All animal use protocols were approved by IACUC institutional review. Mixed brain cell and spleen cell cultures were prepared from C57BL/6 mice in DMEM and RPMI medium, respectively, and co-cultured in DMEM. T Cell cultures were prepared from spleens of C57BL/6 mice in RPMI medium (2 X $10^6$ cells/ml) and stimulated with 5 µg/ml of Concanavalin A (Con A.) (Pharmacia Biotech, Uppsla, Sweden) for three days. RAW cells obtained from ATCC (Manassas, VA, USA) were cultured in DMEM. All cultures were supplemented with 10% heat-inactivaed FBS (Gibco BRL, Rockville, MD, USA) at 37 °C and 5% $CO_2$.

## Reagents

The peptide [MEVGWYRSPFSRVVHLYRNGK] corresponding to mouse MOGp35-55 (>96.81% pure) was purchased from Genemed Synthesis (San Antonio, TX, USA). 1,25(OH)2D3, LPS and ATP were obtained from Sigma Aldrich (St. Louis, MO, USA). WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) used in proliferation assays was purchased from Roche Diagnostics (Indianapolis, IN, USA). Recombinant mouse $IFN_{\gamma}$, IL-17 and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). The High Capacity cDNA Reverse Transcription kit was

purchased from Applied Biosystem (Foster City, CA, USA). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) master mix and primers for PADI2 (PAD2), PADI4 (PAD4) and 18S were purchased from Qiagen (Valencia, CA, USA). The rabbit anti-citrulline antibody and goat anti-rabbit Alexa Fluor antibody were purchased from EMD Millipore (Billerica, MA, USA) and Invitrogen (Eugene, OR, USA), respectively. Immobilion-FL membranes were purchased from EMD Millipore (Billerica, MA, USA). The SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL, USA).

**Induction and Treatment of EAE**

EAE was induced in six 6- to 8-week-old female mice by subcutaneous immunization with 100 µg MOGp35-55 peptide in 150 µl emulsion of Complete Freund's Adjuvant (Difco, Detroit, MI) at the lower dorsum on days 0 and 7. In addition, the mice were injected intraperiotoneally (i.p.) with 100 ng pertussis toxins in 100 µl phosphate-buffered saline (PBS) on days 0 and 2. The mice comprising the treatment group received (i.p.) 100 ng 1,25(OH)2D3 in 25 µl DMSO every other day while the mice of the EAE control group received (i.p.) 25µl DMSO alone and age-matched naïve females were used as controls.

**qRT-PCR**

The effect of 1,25(OH)2D3 on PAD2 and PAD4 mRNA was determined by qRT-PCR. To characterize the in vivo response of 1,25(OH)2D3 on PAD expression, naïve and

EAE-induced mice of each treatment group were sacrificed on day 14 to isolate brain, spinal cord and spleen tissues. To determine the spleen ex vivo response in PAD expression to neural antigen stimulation, portions of each isolated spleen were pooled within treatment groups and cultured in 12-well plates at 2 X $10^6$ cells/ml with RPMI in the presence or absence of 10 µg/ml MOGp35-55 antigen. Cells and culture supernatants were collected after 72 hr. Next, the ex vivo response of PAD expression in the brain by immune cell secreted cytokines was characterized by culturing mixed brain cells from naïve mice in DMEM medium containing 10% culture supernatants collected from 72 hr spleen cultures of naïve, EAE and 1,25(OH)2D3-treated mice sacrificed at day 14. The in vitro brain expression of PADs in response to secreted cytokines in the presence or absence of 1,25(OH)2D3 was characterized by 48 hr treatment of mixed cell brain cultures of naïve mice with 100 nM 1,25(OH)2D3 in the presence or absence of 10% supernatant from day 14 spleen cell culture supernatants of EAE mice.

Finally, mixed brain cell cultures of naïve mice were treated with 100 nM 1,25(OH)2D3 in the presence of either 2 ng/ml IL-17, 4 ng/ml $IFN_{\gamma}$, or 2.5 ng/ml IL-6 for 48 hr. IL-17 and $IFN_{\gamma}$ treated mixed cell brain cultures were stimulated with 1 µg/ml LPS and 1 mM ATP at hour 2 and 42, respectively. Total RNA from all tissues and cells was extracted and quantified using TRIzol reagent and Quibit RNA assay kit, respectively, following manufacturer's instructions (Invitrogen, Madison, WI, USA). Extracted RNA was reverse transcribed to cDNA using TaqMan reverse transcription kit in the 2720 Thermocycler and then amplified in the ABI 7900 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Qiagen master mix with probe and

primers. PAD 2 and 4 gene expression (normalized to 18S) was calculated using the delta delta Ct study software (Applied Biosystems, Foster City, CA, USA) and presented as relative quantity compared to control.

**Immuno Dot Blot**

The effect of 1,25(OH)2D3 on citrullination in the spleen was measured using immuno dot blot. Protein was extracted into Laemmli sample buffer from spleens isolated from naïve or EAE-induced mice treated (i.p.) every other day with either 100 ng of 1,25(OH)2D3 in 25µl DMSO or DMSO alone. Samples were pooled between groups and loaded in duplicate (5 µg/600 µl sample buffer/well) into the Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA) fitted with a PVDF membrane. Blots were rinsed three times for 20 min each in TBST, blocked in TBST containing 10% milk for 1hr and then incubated over night at 4 °C in TBST containing 5% milk with either rabbit anti-citrulline antibody (1:500) or mouse anti-ß actin antibody (1:1000). Membranes were then rinsed three times and treated with peroxidase-conjugated anti-IgG antibodies in TBST containing 5% milk (1:5000) for 2 hr. Blots were developed using SuperSignal enhanced chemiluminescene detection system and imaged using the Alpha Innotech Light Cabinet (San Leandro, CA, USA) to determine average density of each dot. The relative citrullination of each sample was expressed as citrulline absorbance normalized to the ß-actin absorbance.

**Proliferation Assays**

The effect of neural antigen induced stimulation and 1,25(OH)2D3 treatment on immune cell proliferation was measured using WST-1 assay. First, the ex vivo proliferative effect of neural antigen stimulation on whole-spleen cell culture was characterized from spleens isolated from naïve and EAE mice. Spleen cells harvested at day 14 from naïve, EAE and 1,25(OH)2D3 treated EAE mice were cultured in RPMI medium for 96 hr in 96-well tissue culture plates ($2X10^5$/200µl/well) with 0, 2.5, 5 or 10 µg/ml MOGp35-55. Next, the in vitro effect of 1,25(OH)2D3 on spleen cell proliferation was determined by culturing spleen cells isolated from EAE mice with 10 µg/ml MOGp35-55 in the same manner as above with either 0, 5, 10, 25, 50, 100 or 250 nM 1,25(OH)2D3. Splenocytes were isolated from spleens of naïve animals and cultured in RPMI medium for 96 hr in 96-well plates ($2X10^5$/200µl/well) in the presence of either 0, 1, 2.5 or 5 µg/ml of Con A. The in vitro effect of 1,25(OH)2D3 on cell proliferation was determined by culturing splenocytes with 5 µg/ml Con A and either 10, 25, 50 or 100 nM 1,25(OH)2D3. RAW cells were cultured in DMEM medium for 96 hr in 96-well plates ($10^4$/200µl/well) in the presence of either 0, .5 or 1 µg/ml LPS. The in vitro effect of 1,25(OH)2D3 on RAW cell proliferation was determined by culturing RAW cells with 1 µg/ml LPS and 10, 25 or 100 nM 1,25(OH)2D3. In all proliferation assays, 10 µl/well of WST-1 reagent was added at hour 90 before reading the OD at 450 nm during hour 96 using a titer-plate reader (Alpha Diagnostics, San Antonio, TX, USA). All absorbance values are reported as means less the absorbance of culture medium.

**Statistical Analysis**

The values presented are mean +/- standard error and analyzed using one-way ANOVA analysis of variance (and linear correlation in the case of proliferation assays) using Prism 6.0 software (GraphPad, San Diego, CA, USA). Significance of results are expressed in the figures as */#, **/## and ***/### for p values < .05, .01 and .001, respectively.

## RESULTS

### Vitamin D Inhibits PAD2 AND PAD4 Expression in Brain and Spine During Chronic EAE

Earlier studies have shown that induction of EAE increases expression and activity of PAD2 in the brain during EAE (Raijmakers et al., 2006) and that treatment with 1,25(OH)2D3 alleviates clinical scores of disease progression (Bright et al., 2006a). To understand the effects of vitamin D on PAD expression during chronic EAE we compared the relative gene expression of PAD2 (Fig. 1A,B,C) and PAD4 (Fig. 1D,E,F) in the brain, spine and spleen between naive and EAE-induced C57BL/6 mice. Expression of both PAD2 and PAD4 are increased in the brain and spine of EAE animals as compared to naïve. The most robust induction occurred in the brain where PAD2 and PAD4 increased 5.2-fold (Fig. 1A, $p<0.05$, n=3) and 12.2-fold (Fig. 1D, $p<0.001$, n=3), respectively. However, expression of each gene decreased in the spleen during EAE (Fig. 1C,F, p=not significant, n=3).

Next, we compared the expression of PAD2 and PAD4 between chronic EAE animals injected (i.p.) every other day from day 0-30 with either 100 ng 1,25(OH)2D3 dissolved in DMSO or DMSO alone. Treatment with 1,25(OH)2D3 decreased expression of both PADs during chronic EAE in brain (Fig. 1A,D) and spine (Fig. 1B,E) to the level expressed by naive animals. In the brain, 1,25(OH)2D3 treatment reduced PAD2 and PAD4 expression 5.6-fold (Fig. 1A, $p<0.05$, n=3) and 10.4-fold (Fig. 1D, $p<0.001$, n=3), respectively. In the spleen, 1,25(OH)2D3 treatment repression of each gene was not significant (Fig. 1C,F) suggesting that vitamin D has little effect on PAD repression in

immune cells. These results demonstrate that PAD expression induced during chronic EAE is most relevant to the brain and that this induction can be prevented by treatment with 1,25(OH)2D3.

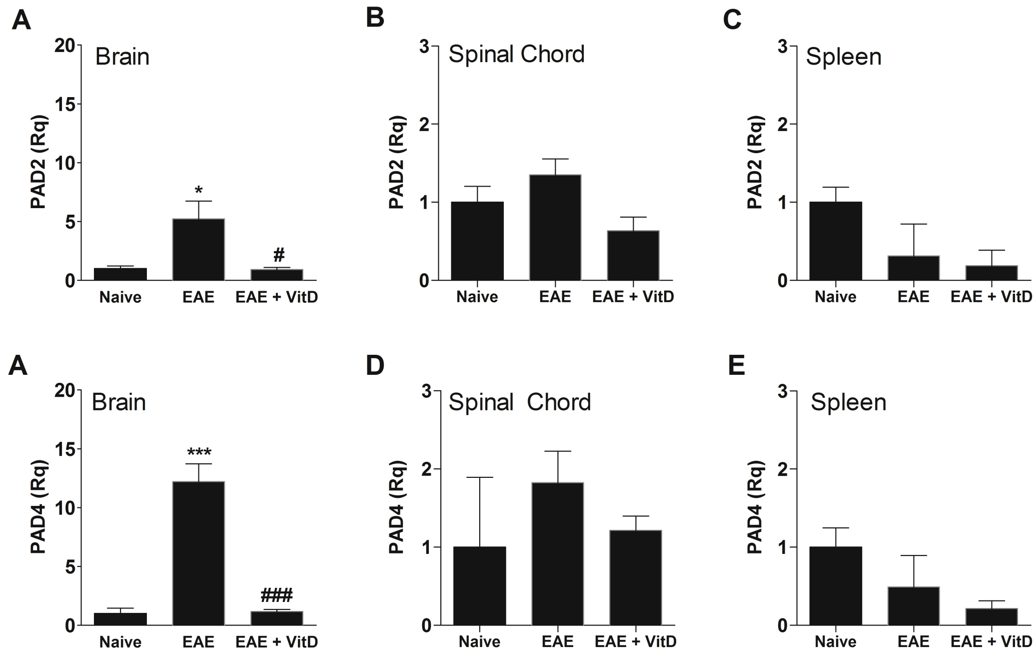


**Fig. 1. Expression of PADs in brain, spine and spleen during EAE**

(A-C) PAD2 expression at day 14 in brain (A, $p<0.05$), spinal cord (B), and spleen (C) tissue homogenates of naïve, EAE and 1,25(OH)2D3 treated EAE mice. (D-F) PAD4 expression at day 14 in brain (D, $p<0.001$), spinal cord (E) and spleen (F) tissue homogenates of naïve, EAE and 1,25(OH)2D3 treated EAE mice. In all assays, n=3.

**Vitamin D Inhibits Citrullination in the Spleen During Chronic EAE**

We examined the citrullination status of peripheral immune cells during EAE by immuno dot blot analysis of spleen protein extracts pooled between groups of animals. As shown (Fig. 2A) and quantified (Fig. 2B), more citrullinated peptides were detected in spleens of chronic EAE animals as compared to naïve. This induced citrullination status observed during chronic EAE is decreased 33% by treatment with 100 ng of 1,25(OH)2D3 every other day for 30 days. These results were replicated in a separate trial. The results demonstrate that citrullination in peripheral immune cells increases during chronic EAE suggesting that the increase in citrullination status observed previously in brain tissue during chronic EAE could be due in part to the contribution of citrulline by immune cells infiltrating the CNS.

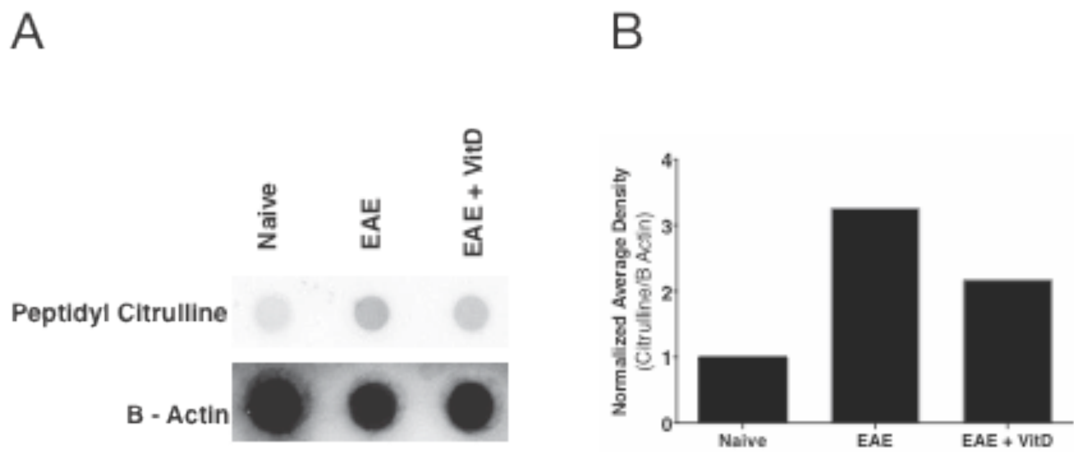


**Fig. 2. Citrulline status of spleen decreased following vitamin D treatment**
(A) Immunoslot blot of protein extracted from pooled (n=2) spleen tissue samples of naïve, EAE and 1,25(OH)2D3 treated EAE mice sacrificed at day 14. Separate membranes were probed for peptidyl citrulline and β actin. (B) Histogram of citrulline expression normalized to β actin showing an increase in peptidyl citrulline in the spleen during EAE that was attenuated by 1,25(OH)2D3 treatment. These results are representative of two immunoslot blot experiments.

**MOG Stimulation Does Not Directly Affect PAD Gene Expression in Splenocytes**

To understand whether the increase in PAD expression observed in the brain was result of neural antigen induced PAD expression of invading peripheral immune cells, we incubated splenocytes isolated from naïve, chronic EAE and chronic EAE-1,25(OH)2D3-treated animals with 10 µg/ml of MOGp35-55 peptide for 48 hr. The relative difference in PAD2 and PAD4 gene expression between splenocytes of naïve, chronic EAE and 1,25(OH)2D3 observed in vivo (Fig. 1C,F) are conserved during the 48 hr ex vivo incubation. Incubation with MOG does not cause a significant change in the expression of either PAD2 (Fig. 3A,B,C) or PAD4 (Fig. 3D,E,F) in splenocytes of any group. These results demonstrate that neural antigen stimulation does not directly affect PAD gene expression in peripheral immune cells.

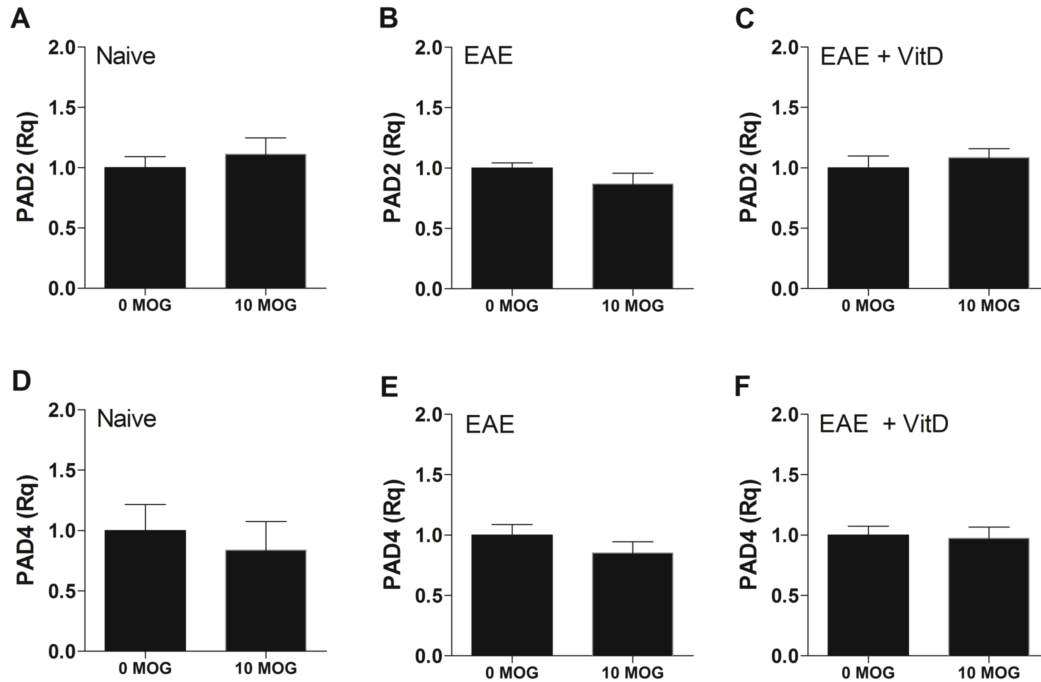


**Fig. 3. Expression of PADs in splenocytes following MOG stimulation**
(A-C) PAD2 expression in splenocytes isolated and pooled from naïve (A), EAE (B) and 1,25(OH)2D3 treated EAE mice (C) following 72 hr stimulation with 0 or 10 µg/ml of the MOGp35-55 antigen. (D-F) PAD4 expression in splenocytes isolated and pooled from naïve (D), EAE (E) and 1,25(OH)2D3 treated EAE mice (F) following 72 hr stimulation with 0 or 10 µg/ml of the MOGp35-55 antigen. In all assays, n=3.

**Vitamin D Does Not Affect Splenocyte Viability in EAE**

To confirm that the splenocytes isolated from each group were auto-reactive to the MOGp35-55 peptide of the ex vivo experiments, we assayed the viability of each group following incubation with the peptide. Incubation with increasing concentration of MOGp35-55 from 0 to 10 μg/ml exerted no observable effect on splenocyte viability isolated from naïve animals (Fig. 3A), but increased viability in a dose-dependent manner of both chronic EAE (Fig 3B, $p<0.0001$, n=3) and 1,25(OH)2D3-treated chronic EAE animals (Fig 3C, $p<0.001$, n=3) indicating that only EAE spleens contained a sufficient neural antigen reactive T cells reactive population. In vivo 1,25(OH)2D3 treatment did not exert an effect on splenocyte viability following ex vivo neural antigen stimulation. In vitro treatment of spleen cells isolated from EAE mice with increasing dose of vitamin D slightly decreased cell viability in a dose-dependent manner (Fig 3D).

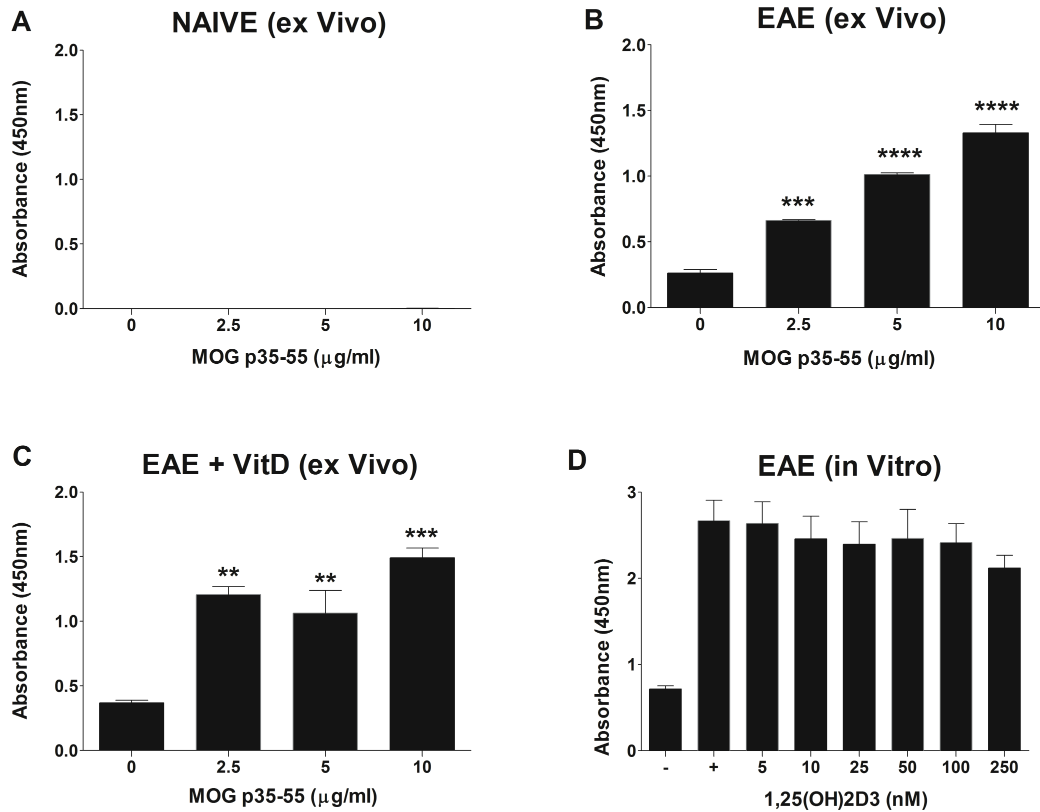


**Fig. 4. Ex vivo and in vitro splenocyte viability**
(A-C) WST-1 assay of splenocytes isolated and pooled from naïve mice (A), EAE mice (B, $p<0.0001$) and 1,25(OH)2D3 treated EAE mice (C, $p<0.001$) were stimulated with 0, 2.5, 5, or 10 µg/ml of the MOGp35-55 antigen for 96hr. (D) WST-1 assay of splenocytes isolated and pooled from EAE mice stimulated with 0 (-) or 10 (+) µg/ml of the MOGp35-55 antigen and treated with either 5, 10, 25, 50, 100 or 250 nM 1,25(OH)2D3 for 96hr (p=not significant). In all assays, n=3.

**Vitamin D Inhibits Viability of Macrophages and T Cells In Vitro**

To study the effect of vitamin D on viability of specific peripheral immune cell types, we performed a WST-1 assay of T cells and RAW cells in the presence of increasing doses of 1,25(OH)2D3 in vitro. T cell viability is increased up to 2.76-fold during 96 hr incubation in a dose dependent manner by lectin Con-A from 0 to 5ng/ml (Fig. 5A, $p<0.05$, n=3). Co-treatment of T cells with 2.5 ng/mL Con-A and an increasing dose of 1,25(OH)2D3 from 10 to 100 nM drastically decreased T cell viability to undetectable levels at 50 and 100 nM (Fig. 5B, $p<0.05$, n=3). RAW cell stimulation with increasing dose of Lipopolysaccharide (LPS) from 0 to 1 µg/ml slightly increased RAW cell viability up to 33% (Fig. 5C, $p<0.01$, n=3). Incubation of RAW cells with 1µg/mL LPS with an increasing dose of 1,25(OH)2D3 from 10 to 100 nM decreased RAW cell proliferation by up to 34% (Fig. 5D, $p<0.05$, n=3).

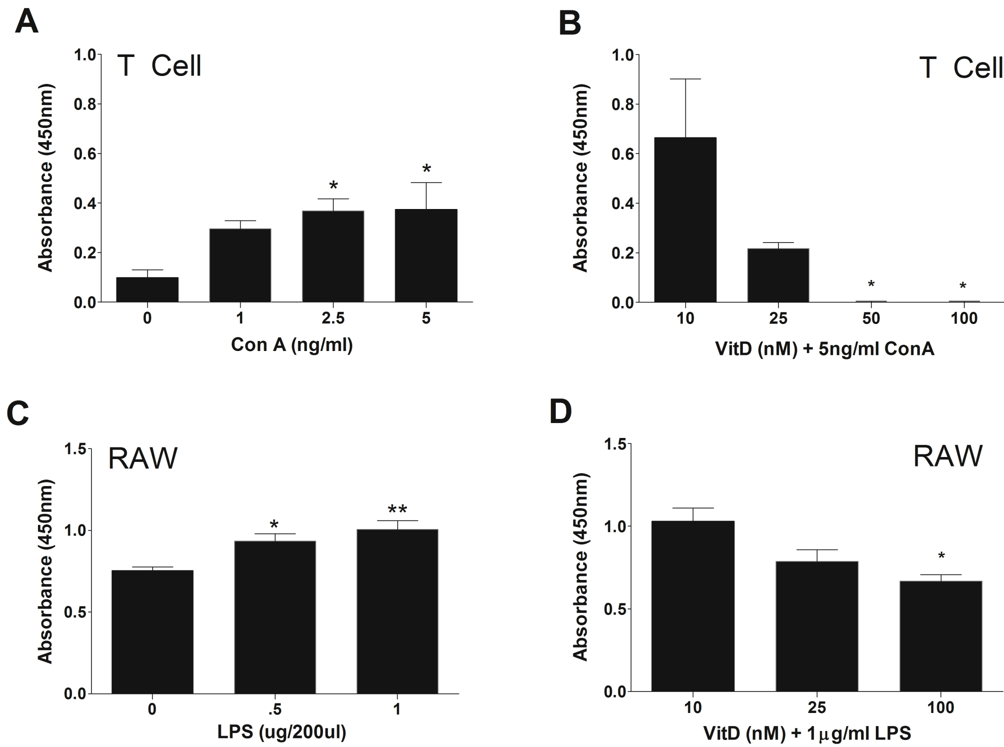


**Fig. 5. T cell and RAW cell proliferation decreased by vitamin D**

(A) WST-1 assay of T cells treated with 0, 1, 2.5 or 5 ng/ml Con A for 96hr ($p<0.05$). (B) WST-1 assay of T cells treated with 5 ng/ml Con A and either 10, 25, 50 or 100 nM 1,25(OH)2D3 for 96 hr ($p<0.05$). (C) RAW macrophage cells stimulated with 0, .5 or 1 µg/ml LPS for 96hr ($p<0.01$). (D) WST-1 assay of RAW macrophage cells stimulated with 1 µg/ml LPS in the presence of either 10, 25 or 100 nM 1,25(OH)2D3 for 96hr ($p<0.05$). In all assays, n=3.

**Vitamin D Inhibits PAD-Inducing Cytokine Production From Infiltrating Immune Cells**

To understand the role of cytokines released by infiltrating peripheral immune cells on the expression of PADs in brain tissue, we examined how supernatants collected from spleen cell cultures affected expression of the two genes within the brain in vitro. Naïve mixed cell brain cultures were incubated in media containing 10% supernatant collected at hour 72 from ex vivo spleen cell cultures of either naïve, chronic EAE or 1,25(OH)2D3-treated chronic EAE mice. Supernatants from chronic EAE spleen were able to increase the expression of PAD2 and PAD4 in the naïve brain by 3.4-fold (Fig. 6A,B, $p<0.01$, $n=3$) and 1.7-fold (Fig 6C,D, p=not significant, $n=3$) respectively, compared to supernatants collected from naïve spleen. The supernatant from splenocyte cultures of 1,25(OH)2D3-treated chronic EAE animals were less potent at inducing expression of PADs, increasing PAD2 by 2-fold (Fig. 6A) and PAD4 by 1.4-fold (Fig. 6C). These fold changes are 41% and 23% less than the fold changes induced by supernatants from spleen cultures of chronic EAE animals not treated with 1,25(OH)2D3. Addition of 100 nM 1,25(OH)2D3 to the mixed brain cell culture containing chronic EAE spleen supernatant reduced PAD2 expression by 13% (Fig. 6B) and PAD4 expression 48% (Fig. 6D).

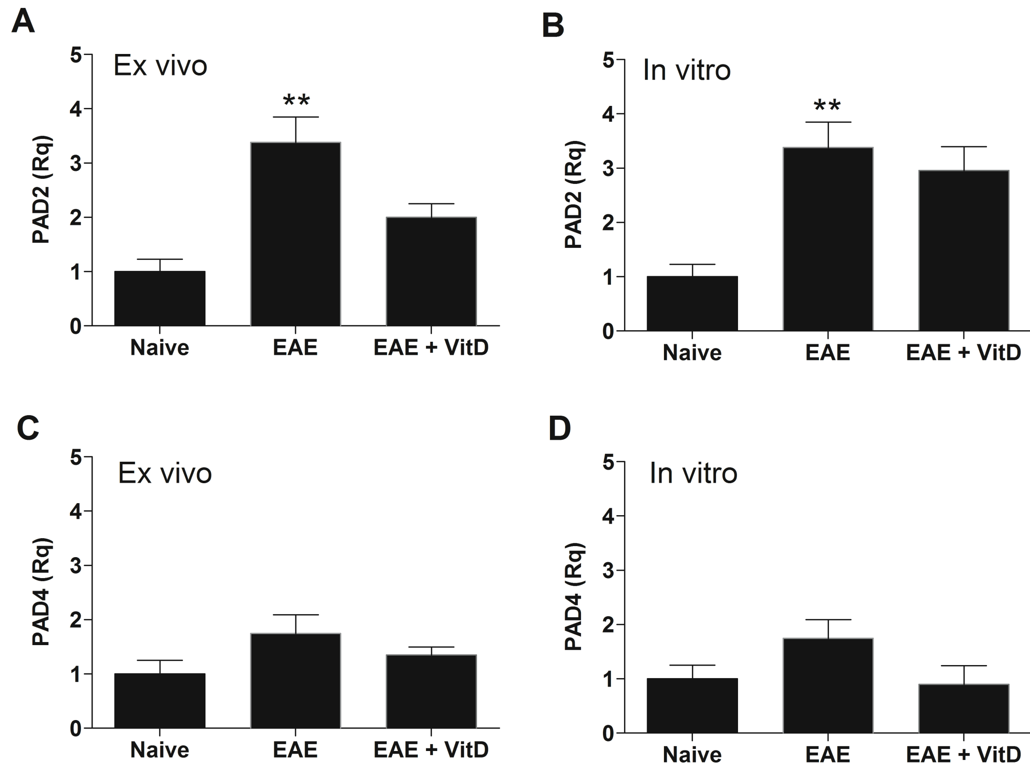


**Fig. 6. Splenocyte induced PAD expression in mixed brain cell culture**
Supernatants were collected after 72 hr from ex vivo stimulated splenocyte cultures of naïve, EAE and 1,25(OH)2D3 treated EAE mice to determine the effect of cytokines on the expression of PAD2 (A,B) and PAD4 (C,D) in mixed brain cell culture. Expression of PAD2 (A, $p<0.01$) and PAD4 (C) in mixed brain cell cultured for 48hr with 10% supernatants from splenocyte cultures of either naïve, EAE or 1,25(OH)2D3 treated EAE mice. Expression of PAD2 (B, $p<0.01$) and PAD4 (D) in naïve mixed brain cell cultured for 48 hr with either 10% supernatant from naïve splenocyte culture, 10% supernatant from EAE splenocyte culture or 10% supernatant from EAE splenocyte culture and 100 nM 1,25(OH)2D3 added directly to the mixed brain cell culture. In all assays, n=3.

**IFN$_\gamma$ Induces PAD Expression in the Naïve Brain**

To assay which cytokines might be responsible for the increased expression of PADs observed in the brain during culture with splenocyte culture supernatants, we treated mixed cell brain cultures with IL-6, IL-17 and IFN$_\gamma$ to measure expression of PAD2 (Fig. 7A,B,C) and PAD4 (Fig. 7D,E,F). Mixed brain cell cultures were stimulated with 1 µg/ml LPS and 1 mM ATP for 46 and 6 hr during 48 hr treatment with IL-17 or IFN$_\gamma$. Treatment of stimulated cells with 2 ng/ml IL-17 had an insignificant effect on PAD2 (Fig. 7A) and PAD4 (Fig. 7D) gene expression with and without co-treatment of 100 nM 1,25(OH)2D3. In contrast, treatment of stimulated cells with 4 ng/ml IFN$_\gamma$ induced PAD2 expression 2.2-fold (Fig. 7B, $p<0.01$, n=3) and PAD4 expression 1.6-fold (Fig. 7E, $p<0.01$, n=3) compared to stimulated control. Co-treatment of IFN$_\gamma$ with 100 nM 1,25(OH)2D3 decreased PAD2 expression 72% (Fig. 7B, $p<0.01$, n=3) and decreased PAD4 expression 52% (Fig. 7E, $p<0.05$, n=3), repressing expression of each gene below that of the stimulated control.

48 hr treatment of naïve mixed brain cell culture with 2.5 ng/ml recombinant mouse IL-6 resulted in a 1.2-fold and 2.0-fold increase in the expression of PAD2 (Fig. 7C, p=not significant, n=3) and PAD4 (Fig. 7F, $p<0.01$, n=3), respectively. However, this induction was not affected significantly during co-treatment with 100 nM 1,25(OH)2D3.

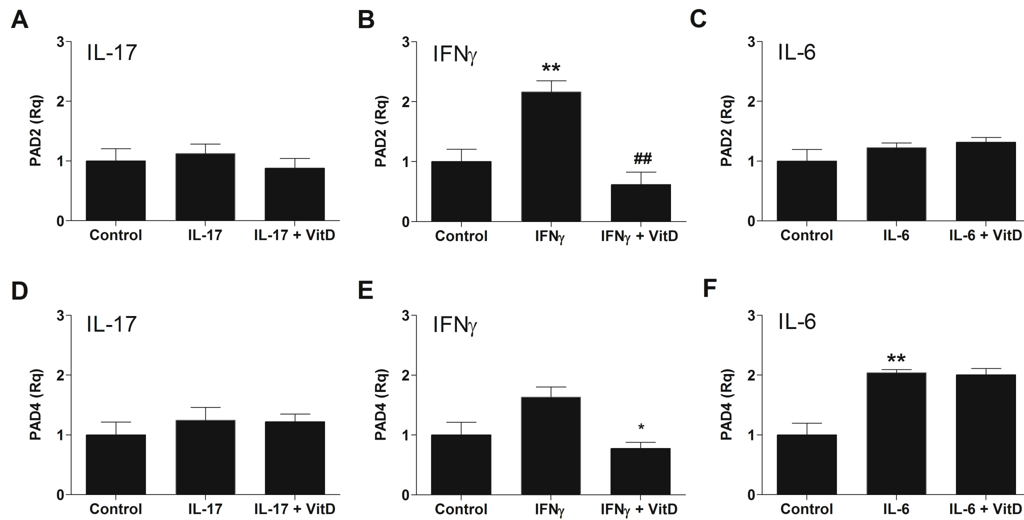


**Fig. 7. PAD expression in mixed brain cell culture in presence of IL-17 , IFN$_\gamma$ and IL-6**
Expression of PAD2 (A-C) and PAD4 (D-F) in 48 hr naïve mixed brain cell cultures. (A & D) Treatment with 1 µg/ml LPS and 1 mM ATP at hour 2 and 42, respectively, with 0 or 2 ng/ml IL-17 and 0 or 100 nM 1,25(OH)2D3 (p=not significant). (B & E) Treatment with 1 µg/ml LPS and 1 mM ATP at hour 2 and 42, respectively, with 0 or 4 ng/ml IFN$_\gamma$ and 0 or 100 nM 1,25(OH)2D3. Induction of PAD2 (B, p<0.01) and PAD4 (E, p=not significant) by IFN$_\gamma$. Significant inhibition of IFN$_\gamma$ induction by 1,25(OH)2D3 in PAD2 expression (B, p<0.01) and PAD4 expression (E, p<0.05). (C & F) Treatment with 0 or 2.5 ng/ml IL-6 and 0 or 100 nM 1,25(OH)2D3. Significant induction of PAD4 expression by IL-6 (F, p=<0.01). In all assays, n=3.

**1,25(OH)2D3 Directly Inhibits PAD2 and PAD4 Expression in the Brain**

To understand the epigenetic role of vitamin D on the expression of PAD2 and PAD4 in the brain, we examined the direct role of the 1,25(OH)2D3 on the expression of the two genes in the naïve mixed brain cell culture. 48 hr treatment of mixed brain cell culture with 100nM 1,25(OH)2D3 reduces expression of PAD2 expression by 57% (Fig. 8A, $p<0.01$, $n=4$) and PAD4 by 47% (Fig. 8B, $p<0.05$,$n=4$).

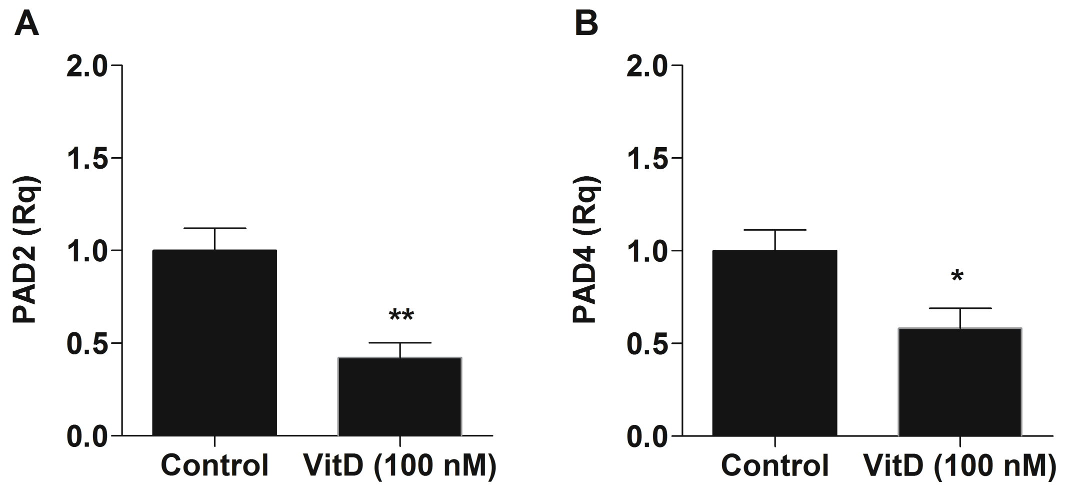


**Fig. 8. Vitamin D inhibits PAD expression in mixed brain cell culture**

(A) Expression of PAD2 in 48 hr mixed brain cell culture treated with 0 or 100 nM 1,25(OH)2D3 ($p<0.01$, $n=4$). (B) Expression of PAD4 in 48 hr mixed brain cell culture treated with 0 or 100 nM 1,25(OH)2D3 ($p<0.05$, $n=4$).

DISCUSSION

## Relevance of Findings

MS is a disorder heterogeneous in clinical presentation, pathological mechanisms and etiology. In this study we have demonstrated a link between a known risk factor for developing the disease and a biochemical pathway at the intersection of myelin destabilization and auto-inflammation. Here we show vitamin D to inhibit expression of PAD2 AND PAD4 in the CNS during EAE. A consensus on the role of hypercitrullinated proteins in the pathophysiology of MS has yet to be reached, but must lie somewhere on a continuum between irrelevant and causative. In favor of the former, previous work has demonstrated citrullinated MOG antigen to be insufficient for disease induction during EAE (Carrillo-vico et al., 2014) and knock down of PAD2, the major contributor of citrullination, does not prevent EAE induction (Raijmakers et al., 2006). However, over-expression of PAD2 is sufficient to cause hypercitrullination and spontaneous demyelination in transgenic mice (Musse et al., 2008). No single model reflects the heterogeneity of MS, but inhibition of PAD activity has recently been demonstrated to attenuate symptoms across four independent animal models of MS, including both EAE models as well as two models that do not begin with immune cell infiltration of the CNS (Moscarello et al., 2013). These results imply PAD activity to be conserved across various demyelinating processes involving both biochemical perturbations of the myelin sheath and auto-inflammatory mechanisms.

Here we have demonstrated robust induction of PAD2 and PAD4 in the CNS during EAE as is observed in MS. Previous studies have demonstrated PAD activity and citrullination in the brain to increase along with PAD mRNA levels indicating PAD gene expression to reflect citrullination status of the brain (Musse et al., 2008). To our knowledge, this is the first study to show vitamin D to inhibit expression of PAD2 and PAD4 in the CNS during EAE. This finding greatly expands the role of vitamin D in the pathogenesis of EAE/MS outside of its role as an immunomodulator. Future studies need to be performed to confirm that the decrease in PAD expression during vitamin D treatment corresponds to a reduction in the citrullination status as the process of deimination is regulated at multiple levels outside of mRNA expression assessed here (Ve, Serre, and Simon, 2010).

**Multiple Inhibitory Roles for Vitamin D in Expression of PAD 2 and 4**

Both immune and neural cells express PAD2 and PAD4, so the gene induction observed in brain and spine tissue during EAE could be result of PAD expression within immune cells infiltrating the CNS. In mice, expression of PAD2 is two-fold higher in the brain than spleen, but bone marrow derived mast cells express PAD2 about twenty-fold greater than brain tissue (Arandjelovic et al., 2014). Mast cells localize to active lesions and regions of perivascular infiltration in the brain during MS (Toms, Weiner, and Johnson, 1990), indicating that the increase in PAD expression we observed in the brain could be due in part to PAD mRNA produced by mast cells. Relevant to the Th1 model of MS, macrophages and monocytes have been demonstrated to increase expression of

PAD2 and PAD4 during contact and cytokine stimulation by activated T cells (Ferrari-lacraz et al., 2012) as would be expected in areas of inflamed neural tissue in MS.

Our viability assays demonstrate vitamin D to inhibit T cell and macrophage viability in vitro but the effect of vitamin D on whole spleen cell culture was much reduced. These results suggest neural antigen stimulated T cells to be resistant to the viability modulation by vitamin D or that other splenocytes contribute to T cell and macrophage viability during vitamin D treatment in the whole spleen culture. Previously, we have shown 1,25(OH)2D3 in vitro treatment to profoundly inhibit splenocyte proliferation when measured using thymidine uptake (Bright et al., 2006). The seemingly conflicting results from these two methods indicate vitamin D to play a role modulating splenocyte replication but not viability during 96 hr treatment with vitamin D.

These results in conjunction with the observed reduction in brain tissue PAD expression following vitamin D treatment suggest a few possible mechanistic roles for vitamin D in the pathogenesis of MS (Fig. 9); vitamin D decreases PAD expression in the brain by limiting the number of infiltrating T cell and macrophages; vitamin D attenuates peripheral immune cell production of PAD inducing cytokine production on the neural tissue; and vitamin D attenuates neural tissue PAD expression in response to infiltrating cells. Vitamin D's direct inhibition of PAD expression in infiltrating immune cells is less likely since the compound induces PAD expression in RAW macrophages (results not shown) and it is not expressed in appreciable amounts by T cells.

Interestingly, PAD2 is expressed fifty fold higher in smooth muscle as compared to whole brain tissue homogenate (Arandjelovic et al., 2014). There is little agreement

about what neural cell types contribute most to the increase in PAD expression observed during MS with oligodendrocytes and astrocytes implicated to play a role (Vossenaar et al., 2003). The potential for neural vasculature to contribute to PAD expansion is especially intriguing given heavy citrullination status of MBP in active lesions often located in perivascular regions. Future studies are required to investigate the relevance of vascular smooth muscle contribution to PAD expression in the brain during EAE.

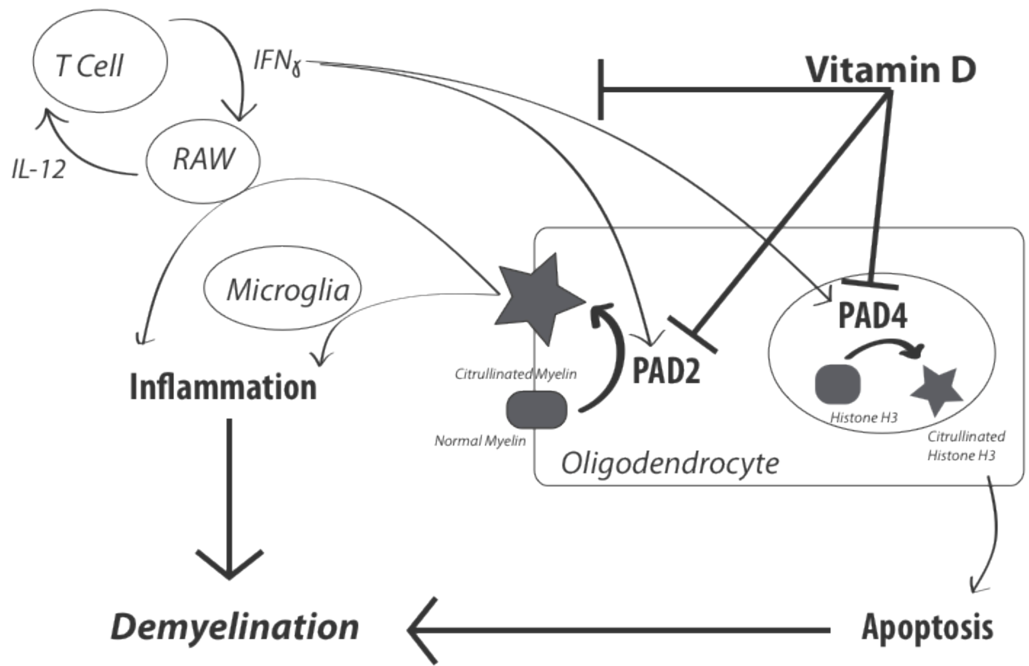


**Fig. 9. Multiple roles of vitamin D inhibiting PAD expression in the brain**
A summary of vitamin D mediated inhibition of PAD expression in the brain. Vitamin D - inhibits proliferation of T and RAW cells. Vitamin D inhibits production of PAD2 and PAD4 inducing cytokines, notably $IFN_{\gamma}$. Vitamin D directly inhibits expression of PAD2 and PAD4 in the brain, alone and during peripheral immune cell cytokine stimulation. Citrullinated MBP, primarily mediated by PAD2, increases inflammation by both local glial cells and infiltrating peripheral immune cells. Citrullinated H3 is associated with oligodendrocyte apoptosis. Both inflammation and oligodendrocyte apoptosis result in demyelination.

The expression of PAD in mixed brain cell culture in response to splenocyte supernatants demonstrate immune cell secreted cytokines to induce PAD expression in the brain. These results support the latter two mechanistic roles for vitamin D mediated inhibition of PAD expression introduced above; showing vitamin D to reduce the ability of splenocytes to induce PAD expression in the brain and vitamin D to inhibit PAD induction in the brain in response to secreted cytokines. Mixed brain cell cultures incubated with splenocyte supernatants demonstrate neural antigen induced splenocytes of EAE mice to release a cytokine profile more capable of inducing PAD expression in the naïve brain as compared to supernatant from splenocytes isolated from naïve or vitamin D treated EAE mice. PAD expression by neural tissue in response to peripheral immune cell cytokine profiles could represent a negative feedback loop that becomes over-activated during MS pathology. Previous studies have demonstrated citrullination to alter immunomodulatory functions of several cytokines including TNFα (Moelants et al., 2013) and IL-8 (Proost et al., 2008). Continued stimulation of PAD expression by neural tissue in response to infiltrated immune cells in combination with the low specificity of PAD2 and PAD4 might represent a pathway by which citrullination of myelin proteins accumulates during MS.

**Implications of $IFN_{\gamma}$ and IL-6 As Inducers of PAD Expression**

Our results show both IL-6 and $IFN_{\gamma}$ to be inducers of PAD expression. IL-6 is involved in both the autoimmune response by invading peripheral immune cells and acts as an important mediator of neural inflammation independent of infiltration.

Previous studies have shown IL-6 production by dendritic cells important to T cell priming during EAE induction (Leech et al. 2014). Venous insufficiency has been implicated in MS and IL-6 blockade attenuates brain inflammation during hypoxia (SH Yang et al. 2013). IL-6 is known to signal gene induction through the SP1 transcriptional factor (Gene et al., 2008) and PAD2 expression has been shown to be modulated by four SP1 binding sites (Serre et al., 2005), which also lie in the vicinity of PAD4 on chromosome one. Our results demonstrate IL-6 to induce PAD4 but not PAD2. Interestingly, vitamin D had no inhibitory effect on this induction. PAD4 contains a nuclear localization sequence where as PAD2 does not. The citrullination of histone H3 by PAD4 following TNFα stimulation is associated with oligodendrocyte apoptosis (Mastronardi et al., 2006). Our results outline a possible mechanistic role for IL-6 to potentiate neurodegeneration via induction of PAD4.

Here we show $IFN_{\gamma}$ to increase expression of both PAD2 and PAD4, identifying a novel role for the $IFN_{\gamma}$ / IL-12 axis in the pathogenesis of MS. Previously, we have demonstrated vitamin D to attenuate $IFN_{\gamma}$ production by Th1 cells (Bright et al., 2006). Here we also show vitamin D to inhibit PAD induction in the brain in response to $IFN_{\gamma}$. Since citrullination of myelin sheath proteins auto-reacts with a broader repertoire of T cells, these results imply that in the absence of vitamin D, T cell mediated induction of PAD expression in the brain might predispose the myelin sheath to further auto-aggression and a cycle of inflammation.

**Concluding Remarks and Future Directions**

Some consider MS to be a collection of disorders that begins with oligodendrocyte apoptosis before invasion of the CNS by the peripheral immune cells (Barnett and Prineas 2006). There are several transgenic models that demonstrate biochemical perturbations of the myelin sheath to play an etiologic role in MS. Increased PAD expression and hypercitrullination precede symptoms in the ND4 model, which involves destabilization of the myelin sheath by over expression of the DM20 proteolipid (F G Mastronardi et al., 1993). And PAD2 over-expression in the CNS is sufficient to cause spontaneous demyelination (Musse et al. 2008). The role of protein hypercitrullination across demyelinating models is mounting. Here we have shown vitamin D to inhibit immune cell induction of PADs in the brain and demonstrated a direct role for vitamin D to attenuate endogenous PAD expression in the brain without invading immune cells.

Regardless as to whether hypercitrullination is a causative factor, or simply potentiates demyelination across various mechanisms, the relevance of vitamin D deficiency to predispose enhanced PAD expression in the CNS needs investigating. Specifically, does vitamin D modulate the methylation status of the PAD2 promoter and if so, can this imprinting be modulated in utero and early life? Vitamin D deficiency from the time of conception to about twenty years old increases one's risk for developing MS (Pierrot-deseilligny and Souberbielle 2013). This time period is associated with the vast majority of neurodevelopment and a reduction in the citrullination of MBP. However MS adults have a citrullination status similar to that of early childhood (Wood et al., 1996).

Perhaps vitamin D deficiency during this critical period allows for hypomethlyation of the PAD2 promoter predisposing the CNS to become hypercitrullinated from an innumerable collection of environmental insults later in life.

TRANSLATIONAL IMPACT

## Clinical issue

Multiple sclerosis (MS) is a disabling disorder that afflicts an estimated 2.3 million people. The etiology of the disorder is poorly understood, but vitamin D deficiency appears to increase risk of developing MS. The disorder involves demyelination of the central nervous system (CNS) associated with peripheral immune cells infiltrating the blood brain barrier and contributing to demyelination processes. In addition, various biochemical perturbations to the myelin sheath including posttranslational modifications and over expression of various proteolipids have been demonstrated to trigger myelination apart from infiltrating immune cells. The diverse pathophysiology of MS is represented in a variety of animal models that collectively reflect the heterogeneity of disease mechanisms and clinical symptoms observed across MS. Recently, the inhibition of protein arginine deiminase (PAD) family enzyme has been shown to attenuate or entirely avoid disease progression in four independent animal models of multiple sclerosis demonstrating the relevance of PAD activity across various demyelination processes. PAD enzymes carry out a post-translational modification of arginine peptide residues to citrulline resulting in a loss of mass and positive charge to each residue modified. PAD2 and PAD4 are over-expressed during MS, but the mechanisms underlying why are largely unexplored.

## Results

Using the experimental autoimmune encephalomyelitis (EAE) model of MS, we demonstrate vitamin D to inhibit expression of PAD2 and PAD4 in CNS tissue during the disorder. We demonstrate PADs induction in the brain by stimulation with peripheral immune cell cytokine profile, but no change in PAD expression during neural antigen stimulation of splenocytes. Specifically, we identify $IFN_{\gamma}$ released by infiltrating T cells to be an inducer of each gene and outline multiple modes by which vitamin D inhibits the ability of invading immune cells to induce PAD expression in the brain. In addition, we show vitamin D to exert a direct role inhibiting PAD expression within neural tissue.

## Implications and Further Directions

Our results imply that the vitamin D deficiency risk factor associated with MS might predispose the CNS to over express PADs and potentiate demyelination spontaneously, by environmental insult or during immune cell infiltration. Future investigations should determine if vitamin D is responsible for modulating the methylation status of the PAD2 promoter region, which has previously been demonstrated to be hypomethylated in MS patients. Identifying a direct, mechanistic link between a well-characterized environmental risk factor and biochemical process involved in MS disease progression will enable a better understanding of the pathogenesis of MS and other complex epigenetic disorders.

# APPENDIX: ADDITIONAL METHODS

Within this appendix are additional methods I learned throughout the year in the Dr. Bright's laboratory. Some descriptions are expanded notes upon methods used in the experiments of the thesis, while others include methods that never panned out. Each involved troubleshooting, lending a variety of null results. The protocols and selected results for some methods are included here as reference for future trials. A list of the figures in this appendix are included below.


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## Cell Culture and Techniques

### *Neural Stem Cell Culture*

Harvest brain from newborn mouse pup and homogenate without shearing by pressing the brain between two frosted glass slides. Culture cells in neural basal medium (NBM) containing 10% FBS, 20 µl B27 per ml NBM, and 1 ng/ml each of EGF and FGF for about one week until neural spheres proliferate. Add additional medium during initial culture if medium changes color from pink to yellow. Harvest cells into a conical tube and allow neural spheres to settle to base of tube for 5 min. Remove media and other mixed cells in supernatant. Do not centrifuge neural stem cell spheres.

### *EOC20 Cell Culture*

Microglia cells cultured in DMEM with heat-inactivated 10% FBS and 10% LADMAC culture soup at high cell concentrations. EOC20 culture takes weeks to become confluent as compared to RAW macrophage culture.

### *DB29 Cell Culture*

Immortalized human oligodendrocyte cell line cultured in DMEM with heat-inactivated 10% FBS. Grow relatively quickly with diffuse projections. Must harvest by trypsinizing cells from culture flask.

*Awaking & Freezing Cell Lines*

Awaken cells as quickly as possible. Freeze cells slowly. To awaken: thaw cells in the water bath quickly, suspend in PBS, spin down at 1500 rpm for 5 min, remove supernatant and resuspend in fresh media. To freeze: collect cells and spin down in vials, remove culture media from pellet, resuspend pellet in freezing media (70% culture media, 10% DMSO, 20% FBJ), place vials into Nalgene freezing container and then move to liquid nitrogen the following morning.

*Harvesting Cells with Trypsin*

Remove media. Gently rinse cells with PBS. Add Trypsin solution for 5 min while in 37 °C incubator. Check to see that all cells are detached using the light microscope. Add media to neutralize the trypsin. Take a sample to count the cells if necessary. Spin down cells at 1500 rpm for 5 min. Re-suspend cell pellet in fresh media.

## Gene Detection Techniques

*RNA Extraction*

Spin down cells 1500 rpm for 5 min and remove medium. Rinse pellet with PBS and spin down 2000 rpm for 5 min. Discard PBS and add 500 µl Trizol reagent. Vortex for 15 sec or sonicate over ice if neural stem cells. Centrifuge for 15 min at 12,000 rcf. Add 200 µl chloroform and vortex for 15 sec, then incubate at room temperature for 3 min. Centrifuge for 15 min at 12,000 rcf. Transfer clear aqueous layer only into fresh epindorf tubes containing 500 µl of 2-propanol. Incubate at room temperature for 10 min. Centrifuge for 10 min at 12,000 rcf. Discard supernatant and add 500 µl of 75% ethanol to each and vortex. Centrifuge for 5 min at 7,500 rcf. Discard supernatant, invert and let dry briefly. Add 40 µl of DPEC and then quantify before storing at -80 °C.

*Reverse Transcription Notes*

When using the High Capacity Reverse Transcription kit (Applied Biosystems) use 4 µl 10x Reverse Transcriptase Buffer, 1.6 µl DNTPs, 4 µl random primers, 2 µl reverse transcriptase per 20 µl total of sample to be reverse transcribed. RNA should be loaded ideally 100-500 ng per sample, though can be much lower if all are matched. Volume of RNA should range 2-4 µl per sample, allowing the remaining volume to be made up to 20 µl with RNA-ase free water.

*PCR Notes*

The volume of the reaction chamber well can usually be much lower than suggested by Applied Biosystems if run for 60 cycles instead of the default 40. Saravanan's protocol includes 10.5 µl of PCR master mix and 2 µl of cDNA, for a total of ~13 µl per reaction well. Make master mix by adding 6.25 µl Quiagen sybgr master mix, .5 µl primer and 3.75 µl RNA-ase free water. Add 10.5 µl PCR master mix per well and then 2 µl of cDNA.

## Protein Detection Techniques

### *Protein Extraction*

Create lysis solution using 11 ml 1x RIPA lysis buffer, 1 ml .1% SDS, 12.5 µl each of leupoprotein and apoprotein 1000x, and 125 µl PMSF 100x. Add 20 µl of lysis solution to cell pellet and lyse on sonicator. Rock at 4 °C for 1 hr. Centrifuge at 10,000 rpm for 10 min. Retain supernatant, which contains the soluble protein, and discard the pellet. Load 10 µl each of samples and standards onto a 96 well plate. Mix BCA Protein Assay solution (Thermo) 5ml A to 100 µl B, then add 200 µl of solution to each sample and dark incubate at room temperature for 30 min. Read and compare sample absorbance to standard curve.

### *Western Blot*

Cast 5ml mini resolving gel using 1.6 ml water, 2.0 ml 30% acrylamide mix, 1.3 ml 1.5 M Tris (pH 8.8), 50 µl 10% SDS, 50 µl 10% ammonium persulfate, and 2 µl TEMED. Allow to set before casting stacking gel. Cast 2ml mini stacking gel using 1.4 ml water, .33 ml 30% acrylamide mix, .13 ml 1.5 M Tris (pH 8.8), 10 µl 10% SDS, 10 µl 10% ammonium persulfate, and 1 µl TEMED. Add comb and allow to set. Add equal µg of protein between samples into loading buffer, boil for 10 min, spin down and load into gel. Run gel at 100 mV.

Rinse a membrane in methanol for 3 min. Place three filter papers into transfer apparatus and wet with transfer buffer, place membrane atop filter papers, remove gel and place on membrane, then place two filter papers atop gel. Wet the entire stack with

transfer buffer, removing any air bubbles by rolling a glass pipette over the stack. Dry the area around the stack and then close apparatus. Run at 100 mA for 90 min.

Remove membrane and block in 5% milk TBST for 1 hr at room temperature. Add primary antibody in 2.5% milk TBST overnight on rocker at 4 C. The following morning, remove from refrigerator and rock at room temperature for 1 hr. Rinse in PBST 3x at 20 min each. Incubate with biotin conjugated secondary antibody in 2.5% milk TBST for 1 hr at room temperature. Rinse in PBST 3x at 20 min each. Add 2ml of each part of ECL solution (Supersignal West Pico, Thermo) and rock at room temperature for 5 min and then image as described in slot blot. Examples of trials are included (Fig. A1,2,3).

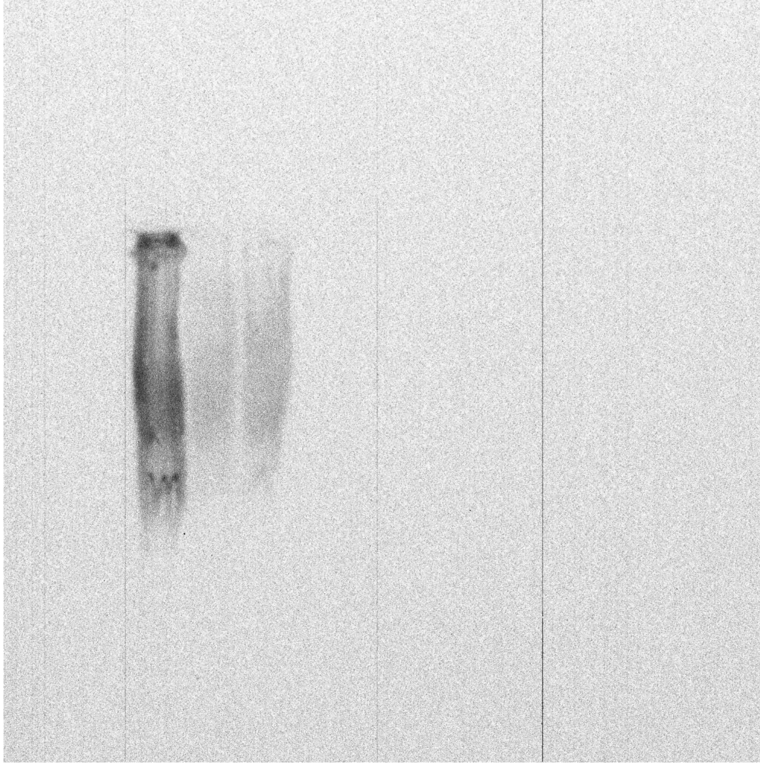

**Fig. A1. Example of peptidyl citrulline western blot**

Western blot of brain protein extracts probed for peptidyl citrulline (1:500). The antibody binds any proteins expressing citrulline resulting in smearing. The far left brain sample was contaminated with blood resulting in a much darker band.

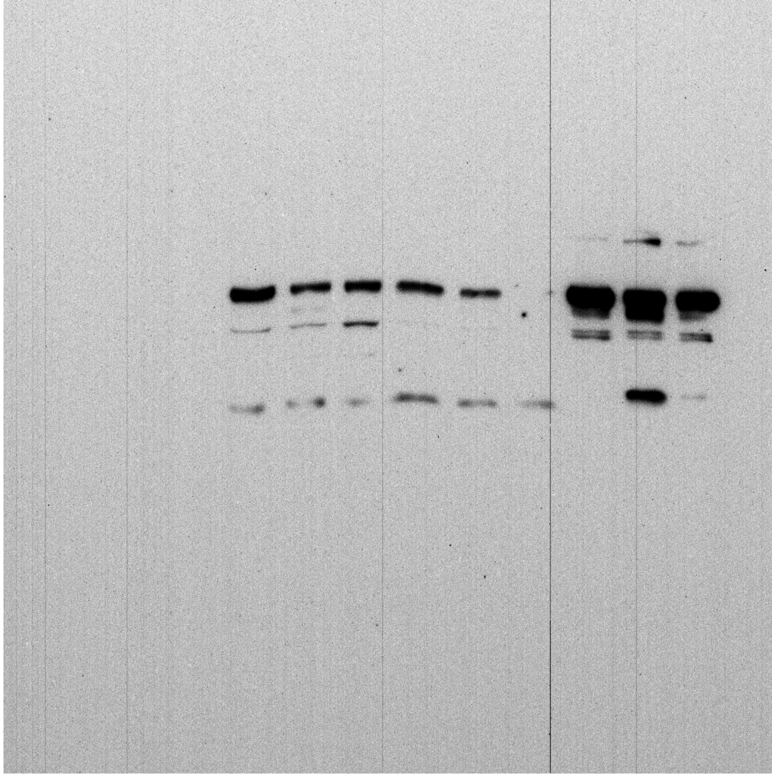

**Fig. A2. Example of beta actin and MOG western blot**
Western blot of brain, spine and spleen protein extracts run in triplicate from left to right probed for beta actin and MOG.

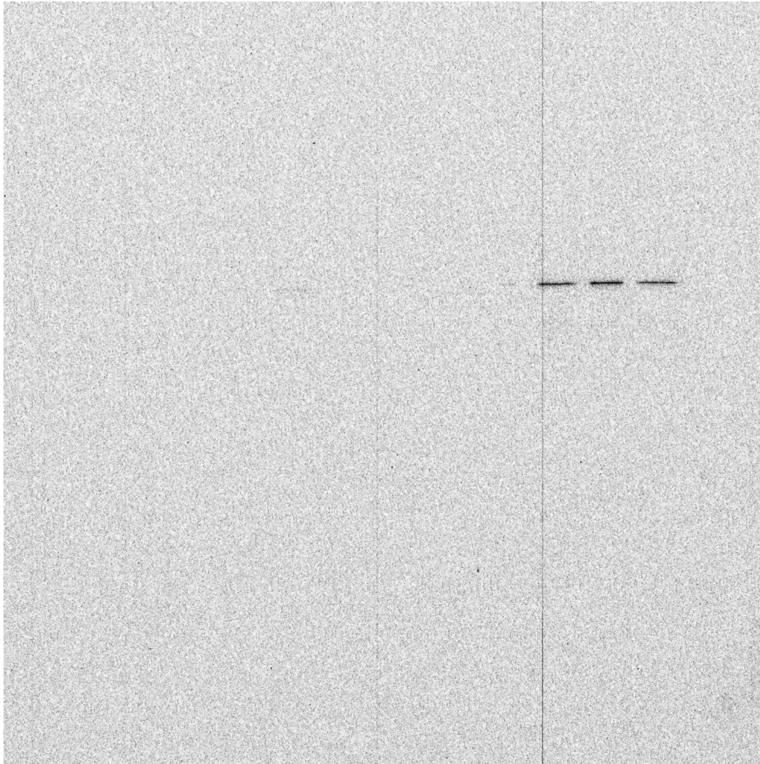

**Fig. A3. Example of beta actin western blot**
Western blot of brain, spine and spleen protein extracts run in triplicate from left to right probed for beta actin. Beta actin was only detectable in the spleens on this trial.

*Cytokine ELISA*

The effect of neural antigen stimulation and 1,25(OH)2D3 on $IFN_{\gamma}$ and IL-12 secretion in whole-spleen cell culture was determined by ELISA. Spleen cell cultures were created from pooled spleen tissue samples isolated at day 14 from naïve and EAE-induced mice treated (i.p.) every other day with either 100 ng of 1,25(OH)2D3 in 25 µl DMSO or 25 µl DMSO alone. To study the ex vivo response to neural antigen stimulation, spleen cells were cultured ($1X10^6$/ml) in 12-well plates containing RPMI with 0 or 10 µg/ml MOGp35-55 antigen. The in vitro response to 1,25(OH)2D3 was determined by treating spleen cell cultures isolated from chronic EAE animals with 0, 5, 10, 25, 50, 100 or 250 nM 1,25(OH)2D3. Supernatants from all spleen cell cultures were harvested at 72 hr to measure the concentration of $IFN_{\gamma}$ and IL-12 by ELISA. In short, 96-well ELISA plates were coated with capture antibodies by incubating 50 µl/well of either anti-$IFN_{\gamma}$ or anti-IL-12 in .06M carbonate buffer (pH 9.6) overnight at 4°C. Excess antibodies were washed with three rinses of PBST (PBS containing .05% Tween) and excess protein binding sites blocked with 3% BSA in PBS for 1 hr. Supernatant and standard samples were plated in triplicate and incubated at 4 °C overnight. Plates were rinsed with PBST three times and then incubated with biotin-conjugated secondary antibodies for 2 hr at room temperature. Excess antibodies were rinsed with three washes of PBST before incubating plates with avidin-alkaline phosphatase (1:5000) for 45 min at room temperature. Plates were again rinsed and then developed over 2 hr by incubation with 1 mg/ml *p*-nitrophenyl phosphate. Absorbance was read at 405 nm and

the concentration of $IFN_{\gamma}$ and IL-12 in each sample calculated from the standard curve.

Examples of standard curves are provided (Fig. A4,5).

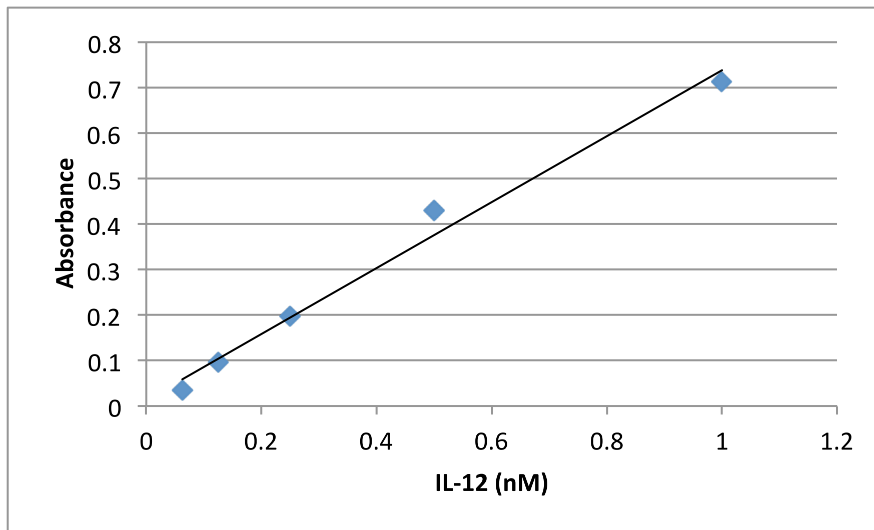


**Fig. A4. Example of IL-12 Alkaline Phosphatase ELISA**
Standard curve of an Avadine-Alkaline Phosphatase ELISA with IL-12 capture antibody incubated with IL-12 from .0625 to 1 nM. In this trial, the $IFN_{\gamma}$ standard did not develop.

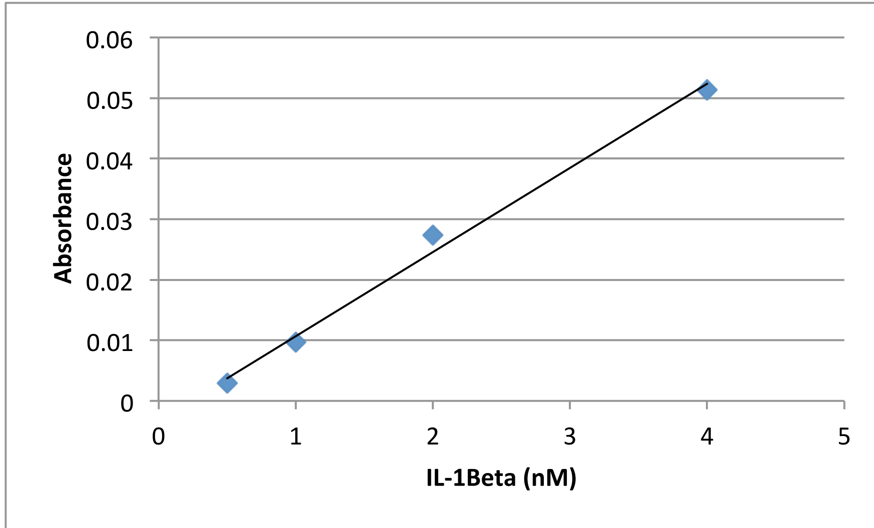


**Fig. A5. Example of IL-1B Alkaline Phosphatase ELISA**

Standard curve of an Avadine-Alkaline Phosphatase ELISA with IL-1B capture antibody incubated with IL-1B from .5 to 4 nM.

*Immunohistochemistry*

Mixed brain cells were plated in an poly-D-lysein coated 8-well chamber slide (BD Biosciences, Bedford, MA, USA) with 200µl of DMEM medium. Experimental groups were incubated for 48 hr with either 100nM 1,25(OH)2D3, 4 ng/mL $IFN_{\gamma}$, or 100 nM 1,25(OH)2D3 and 4 ng/mL $IFN_{\gamma}$. One group was stimulated for 48 and 1 hr with 1 µg/ml LPS and 100 mM ATP, respectively. Cells were incubated with fixative and peremabilization solution at 4 °C for 15 min and then rinsed twice with cold PBS before blocking with 1% BSA in PBS at 4 °C for 1 hr. The slide was rinsed one time with cold PBS before staining with primary anti-peptidyl citrulline and MOG antibodies (both 1:500) in 1% BSA at 4 °C. Following overnight incubation with primary antibodies, the plate was rinsed three times with cold PBS and then stained with secondary antibodies (both 1:1000) in 1% BSA at 4 °C for 2 hr. Cells were then rinsed three times with cold PBS before addition of Vectashield mounting medium. Fluorescence of goat anti-rabbit, donkey anti-mouse and DAPI were attained separately with green, red and blue filters, respectively, using a Leitz DMRB microscope (Lecia Microsystems, Buffalo Grove, IL, USA) outfitted with a RT Color camera (SPOT Imaging Solutions, Sterling Heights, MI, USA) and then merged using SPOT software.

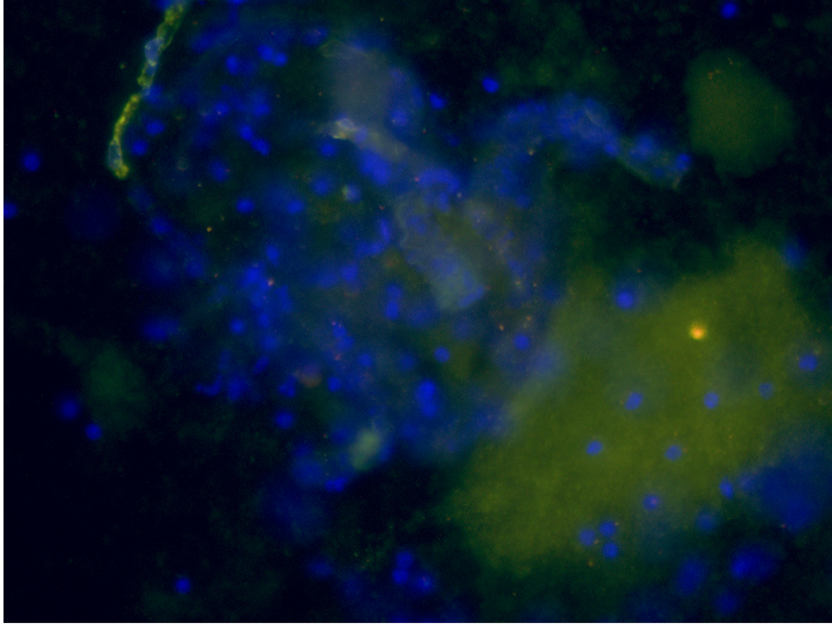

**Fig. A6. Example of triple staining of mixed brain cell culture**
Image of nuclei (blue), MOG(red), citrulline (green) and areas of co-staining (yellow).

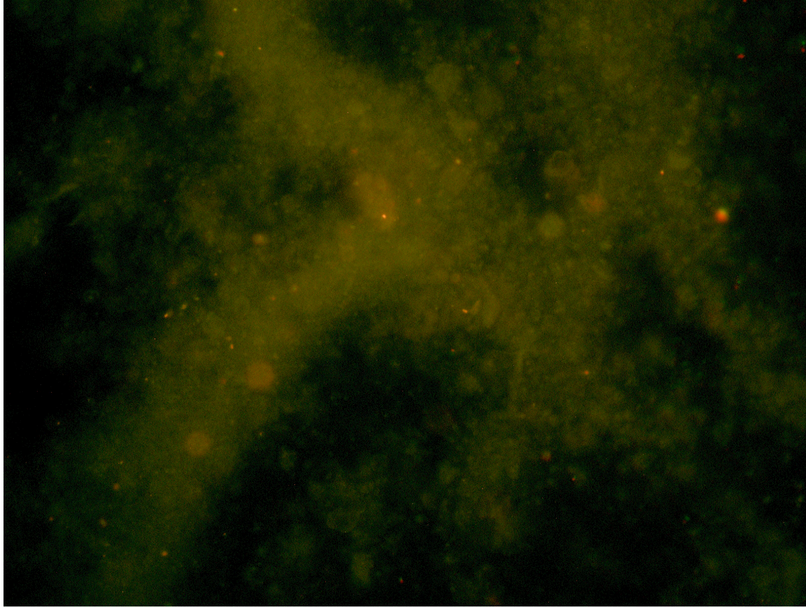

**Fig. A7. Example of double staining of mixed brain cell culture**
Image of MOG (red), citrulline (green) and areas of co-staining (yellow) reveal co-localization of anti peptidyl citrulline and anti MOG antibodies.

*Flow Cytometery*

DB29 cells were plated (200,000/well) in a 6 well chamber slide with 200 µl of DMEM medium. Experimental groups were incubated for 48 hr with either 100 nM 1,25(OH)2D3, 4 ng/mL $IFN_{\gamma}$, or 100 nM 1,25(OH)2D3 and 4 ng/mL $IFN_{\gamma}$. One set of groups was stimulated for 48 and 1 hr with 1 µg/ml LPS and 100 mM ATP, respectively. Cells were trypsinized and harvested then incubated overnight in 250 µl fixative solution at 4 °C. The following morning cells were rinsed by adding 500 µl permebilization solution to each sample and spun down before incubation with rabbit anti-peptidyl citrulline antibody (1:500) and mouse anti-MOG antibody (1:500) in permebilization solution for 2 hr at room temperature. Following incubation, cells were rinsed 2x with 1% BSA in PBS before incubation with corresponding secondary antibodies (1:1000) in permebilization solution for 1 hr at room temperature. Cells were then rinsed twice in 1% BSA in PBS before analysis.

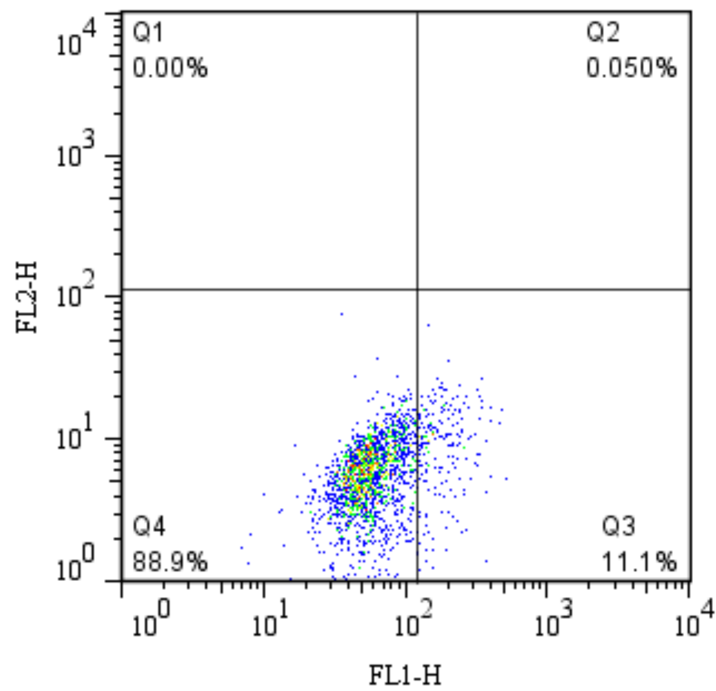


**Fig. A8. Example of flow cytometry analysis of DB29 immortalized oligodendrocytes**
Analysis of citrulline (FL1-H) vs. MOG (FL2-H) positive DB29 cells. Cell number in a few experimental samples was too low to draw conclusive remarks.

## Other Techniques

### *Nitric Oxide Assay*

Nitric oxide can be assayed using the Griess Reagent (Sigma). The amount of nitric oxide reflects the activity of iNOS and thus inflammation. The test is performed by plating 50 µl of Griess Reagent and 50 µl of standard or sample onto a 96 well plate and reading absorbance at 450 and 630 nm once a colorimetric gradation appears.

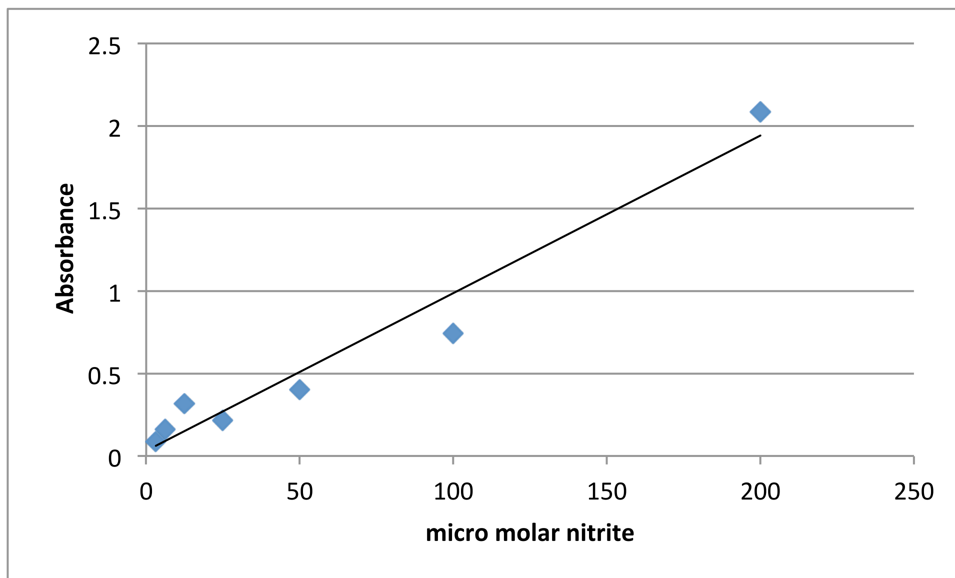


**Fig. A9. Example of nitric oxide standard curve**
Example of standard curve generated from nitrite samples ranging 3.12 to 200 μM.

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CURRICULIUM VITAE

Travis William McCain

## EDUCATION

8/13 – 9/14 **Indiana University** | Indianapolis, IN
M.S. Candidate in Cellular Physiology. September 2014. Coursework in: biochemistry, physiology, neuroscience laboratory techniques, research ethics and biostatistics.

6/13 – 8/13 **Boston College** | Boston, MA
Coursework in: organic chemistry I & II lecture and laboratory.

9/09 – 6/13 **Stanford University** | Stanford, CA
B.S. in Engineering, Product Design. Program GPA: 3.7/4.0
Coursework in: human-centered design, mechanical engineering, biotechnology, cognitive science and economics.

6/12 – 8/12 **Peking University** | Beijing, China & Liujiazhen, China
Studied cross-cultural design in the resource-poor setting of a rural Chinese village through a team service project.

1/12 – 3/12 **Stanford Bing in Washington** | Washington, DC
Studied U.S. health care policy and behavior change design through coursework and an independent-study project.

## ACADEMIC PROJECTS

10/13 – 7/14 **M.S. Graduate Thesis in Cellular and Integrative Physiology**
Trained in neuroscience research techniques while investigating a link between vitamin D and the expression of two genes involved in autoimmune and neurodegeneration during multiple sclerosis pathogenesis.

1/13 – 3/13 **Should We Use the Robot?** | MS&E 292: Health Policy Modeling
Reviewed published case studies to perform a cost-effectiveness analysis of robot-assisted vs. open pediatric pyleoplasty. Sensitivity analysis revealed scenarios under which robot assistance is and is not an incrementally affordable alternative.

3/12 – 6/12 **William Morris Craft in Surgery** | ME120: Philosophy of Design
Examined the erosion of proprioception in the evolution of least-invasive surgical tools and how this loss might affect surgeon skill acquisition and fulfillment. Analyzed primary texts, interviewed surgeons and built an interaction to demonstrate surgical situations where enhanced visual acuity cannot accommodate for a loss in haptic perception.

## EXPERIENCE

6/14–*Present* **Complex Care Coordinator, Stanford Children's Health** | Stanford,CA
My responsibilities include developing management practices to ideate, implement and evaluate new models of care delivery that help transition the hospital from fee-for-service to value-based reimbursement arrangements. I will be learning lean management principles and fostering collaboration between the hospital and Stanford Design School.

9/11 – 8/13 **Product Design Consultant, Blue Ocean Ventures** | Miami, FL
Prototyped and communicated new products and services using a user-centered design process. Coordinated development of client's online services with web developer. Iterated mechanical system design and developed reagents for water-testing kiosk. Coached client in Stanford's iterative design process and co-authored three pending patents.

1/12 – 3/12 **Intern, The World Bank Group**| Washington, DC
Quickly became familiar with the field of behavior change design while managing multiple projects including; meta-analysis of prior Bank practices, organizing case study findings for presentation, assisting outreach to potential partners of the practice and grant writing. Worked closely under supervisor to iterate deliverables and incorporate feedback.

1/12 – 3/12 **Research Assistant, Center for Design Research**| Stanford, CA
Assisted doctoral student with organization and presentation of dissertation. Developed an understanding for team behaviors and design outcomes elicited through interaction with low and high fidelity prototypes.

9/07 – 9/11 **President, Midwestern Aquatics Inc.** | Lizton, IN
Researched, designed, and developed a new material for growing coral. Secured a contract to supply an aquaculture facility with over 1/3 million parts. Negotiated with the client to develop, finance and implement specialized manufacturing equipment and operations to complete the order on time and within budget.

## PUBLICATIONS

*In Progress* Travis W. McCain, Saravanan Kanakasabi, John J. Bright. A review of epigenetic mechanisms in multiple sclerosis pathogenesis.

8/14 Travis W. McCain, Saravanan Kanakasabi, John J. Bright. In Submission 2014. Vitamin D modulates PAD2/4 expression in EAE model of multiple sclerosis. *Biology Open*.

## PRESENTATIONS & CONFERENCES ATTENDED

9/14 Stanford Medicine X. Stanford, CA.

7/14 Thesis oral defense. IU Health MRI Seminar. Indianapolis, IN.

7/14 Indiana University Health Research Day. Indianapolis, IN.

2/12 National Health Policy Conference. Washington, D.C.

9/11 Poster presentation on Radical Breaks and Incremental Improvements in Team Design Projects. Mechanical Engineering Summer Undergraduate Research Institute. Stanford, CA.

## PATENTS

8/13 Philip William Root, Travis William McCain. 61,869,917 Self-Service Kiosk for Water Analysis. 61,869,946 Cuvette Storage and Dispensing System. 61,869,940 Reagent Dispensing System.

## TEACHING

9/12 – 12/12 **Teaching Assistant, U.S. Health Care Policy** | Stanford, CA

Facilitated weekly discussion meetings with 15+ students to review course content. Created curriculum materials, led review sessions, tutored students individually and graded examinations.

8/11 – 9/11 **Teaching Assistant, Design Thinking & Art of Innovation** | Stanford, CA

Served as both a teaching and residential assistant to introduce students from a variety of academic backgrounds to Stanford's Design Thinking methodologies. Managed social outings and engaged team-based learning.

## CREATIVE WORKS

6/13 "Grip Clip: Pencil Hero." Injection molded part. Manufacturing & Finance. Campaign funded on Kickstarter.

11/12 "Icebergs." Sculpture. Steel, acrylic, plaster, wax and water.

9/12 "Preserves." Sculpture. Steel, jello, glass, plaid fabric.

10/13 "Homeless Humanity." Portraiture & Writing. Displayed at Cantor Museum and featured in Stanford Arts Review.

10/11 "Care and Disease in Milne Bay, Papua New Guinea A Photo Essay." Displayed at Wallenberg Hall, Stanford.

## SERVICE

1/14 – 7/14 **Clinic Volunteer, Gennesaret Free Clinic** | Indianapolis, IN

Opened and closed clinic. Greeted patients and managed health records during visits.

5/14 – 6/14 **Facilitator, Hugh O'Brian Youth Leadership (HOBY)** | Indianapolis, IN
Facilitated discussion in a group of young high school students to develop leadership confidence and skills.

6/11 – 3/13 **Steering Committee Chair, Pacific Free Clinic** | San Jose, CA
Connected underserved patients with specialty care providers after their clinic visit. Tracked and reported effectiveness of referral process to propose improvements. Directed scheduling and training of 30 volunteers.

6/12 – 8/12 **Team Member, Cross-Cultural Design for Service** | Liujiazhen, China
Volunteered in rural China with a team of 4 peers from Shi Lanka, China and America. Through interview, observation and prototype testing our team created and implemented a program that uses art lessons as a medium for mentorship.

7/11 – 8/11 **Clinic Volunteer, PNG Medical Project** | Alotau, Papua New Guinea
Quickly learned to communicate with patients and present history and physical findings to physicians. Researched and delivered a contextualized lecture on renal physiology and disorder. Developed a passion for photo essay while shadowing PNG's second woman surgeon.

## ADDITIONAL INFORMATION

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| Lab Skills | Techniques: EAE model of MS, rt-qPCR, proliferation assay, FACs, western blot, immunoslot blot, ELISA, proliferation assay, immunoflourscence, dissection. Cell culture: neural stem, glial, macrophage, T cell & mixed brain cell. |
| Design Skills | User observation & interview, need finding, concept generation, rapid prototype generation & testing, facilitating brainstorms, project planning, cost modeling, sketching, CAD, technical writing, and Adobe Creative Suite. |
| Honors | Sam Walton Community Scholar. Awarded two Stanford Arts Grants for photo essay works. Finished in the top 2% at the largest half marathon in the U.S. |