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# Differential Effects of IL4I1 Protein on Lymphocytes From Healthy and Multiple Sclerosis Patients

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## ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease characterized by immune-mediated demyelination of the central nervous system, resulting in extensive neurological deficit and remyelination impairment. We have previously found that interleukin-four induced one (IL4I1) protein modulates CNS inflammation and enhances remyelination in mouse models of experimental demyelination. However, it remained unclear if IL4I1 regulates lymphocyte activity in MS. To assess the therapeutic potential of IL4I1 in MS, we investigated the impact of IL4I1 treatment on human lymphocytes from peripheral blood mononuclear cells (PBMCs) obtained from healthy individuals and MS patients. We found that IL4I1 increased the relative densities of Th2 and regulatory T-cells, while reducing Th17 cell density in healthy control (HC) samples. Furthermore, IL4I1-treated lymphocytes promoted CNS remyelination when grafted into demyelinated spinal cord lesions in mice. We found that baseline endogenous *IL4I1* expression was reduced in people with MS. However, unlike HCs, IL4I1 treatment had no significant effect on *IL17* or *TOB1* expression in lymphocytes derived from MS patients. These results suggest that IL4I1 skews $CD4^+$ T-cells to a regulatory state in healthy human lymphocytes, which may be essential for promoting remyelination. However, IL4I1 appears unable to exert its influence on lymphocytes in MS, indicating that impaired IL4I1-mediated activity may underlie MS pathology.

## 1 | Introduction

Multiple sclerosis is a chronic inflammatory disease, which is characterized by demyelination and neurodegeneration in the CNS [1–3]. While spontaneous repair of demyelinated lesions is successful during early stages of MS, remyelination ultimately fails in the progressive phase of the disease, leading to axonal dystrophy and irreversible neurological disability [4, 5]. The underlying mechanisms responsible for the transition from successful to failed CNS remyelination are not well understood. It has been proposed that uncontrolled inflammation plays a key role in MS pathogenesis and progression [6, 7], with autoreactive T cells driving the disease process [8]. Given the pro-inflammatory skew implicated in MS pathology, immunomodulation becomes a crucial target for promoting the natural repair processes that are deficient in progressive MS.

Interleukin-4 induced protein 1 (IL4I1), a macrophage-secreted enzyme [9–11], has been shown to modulate inflammation and enhance CNS remyelination in mice by reducing $CD4^+$ T-cell driven inflammation [11]. Additionally, administration of IL4I1 to mice with experimental autoimmune encephalomyelitis (EAE) has been found to promote motor recovery [11]. In humans, the *IL4I1* gene has been mapped to chromosomal locus 19q13.3–13.4, a region associated with autoimmune susceptibility to lupus, rheumatoid arthritis,

diabetes type 1, and MS [12, 13]. Furthermore, it has been suggested that IL4I1 maintains high expression levels of TOB1, an antiproliferative protein that primarily affects Th17 cells [14]. Notably, expression levels of TOB1 have been inversely correlated with the risk of developing MS from clinically isolated syndrome (CIS) [15]. However, it remains unknown whether IL4I1 affects immune cell activity in MS, or whether altered plasma IL4I1 levels correlate with disease severity or progression. To evaluate its potential as a therapeutic target in MS, here, we investigated the effects of IL4I1 treatment on human lymphocytes derived from PBMCs obtained from healthy individuals and MS patients.

## 2 | Methods

### 2.1 | Patient Groups

All human subject work was conducted in accordance with the Declaration of Helsinki principles, and was approved by the Georgetown University Institutional Review Board (IRB #2015–1048). All participants provided written informed consent prior to inclusion in this study, and were identified by numerical codes thereafter. Thirty-seven patients with clinically definitive MS were included in this study. Of these patients, 11 were characterized as having active relapsing–remitting MS (aRRMS), nine as having non-active relapsing–remitting disease (naRRMS), and 6 as having secondary progressive MS (SPMS). All patients were non-pregnant, DMD-naïve females aged 18–65 who were being seen by neurologists at Georgetown Multiple Sclerosis and Neuroimmunology Center (GMSNC) and were residents of the Washington D.C. metropolitan area. MS diagnosis and clinical subtype was defined according to the revised McDonald criteria (2010 revisions [16]), and Lublin and Reingold clinical course definitions (2013 revisions [17]). Specifically, aRRMS phenotype was defined as having clinically definitive RRMS with a radiological or clinical relapse within 12 months. naRRMS subtype consisted of individuals with clinically definitive RRMS who had not experienced a relapse or radiological changes within the last 12 months. Patients diagnosed with SPMS had not experienced relapses in the past 12 months and exhibited accumulation of disability, defined by a worsening score on the *Kurtzke Expanded Disability Status Scale* (EDSS) [18] that persisted for 6 months or more. Healthy control (HC) subjects were defined as having no history of neurological pathology or autoimmune disease, and satisfied the same inclusion/exclusion criteria as their patient counterparts. Commercially available HC samples (Precision Medicine) were also used in this study.

### 2.2 | Collection and Handling of Patient Samples

Patient blood was drawn and obtained from GMSNC or Georgetown University's Clinical Research Unit (GU-CRU). Three 8 mL CPT-citrate vacutainers (BD cat. 362761) were drawn per patient. PBMCs were isolated from CPT-citrate tubes by density gradient centrifugation at 1500 g, for 30 min at room temperature (RT). Supernatant was removed and pellets were resuspended in PBS and cell count was obtained to make ~$1 \times 10^6$ cells per 2 mL cryovial aliquots (ThermoFisher) and stored at −80° for 24 h before transfer to liquid nitrogen.

### 2.3 | PBMC Culture and T-Cell Activation

Peripheral blood mononuclear cells were thawed and plated following relevant sections contained the Helmholtz Zentrum Munich Clinical Cooperation Group Immune Monitoring Protocol. Cells were thawed quickly in 37°C water bath, rinsed twice, centrifuged and resuspended in 10 mL 37°C C.T.L. Test medium (ImmunoSpot cat. CTLT-005 plus 1% GlutaMAX and 1% penicillin–streptomycin). Total live cell count was performed with Trypan Blue (Sigma). Cell density was brought to $1 \times 10^6$ cells/ml in test medium, and plated as 900 μL cell suspension per well of a 12-well culture plate. For T-cell stimulation and treatment, plated cells were immediately supplemented with ImmunoCult human CD3/CD28/CD2 T cell activator tetrameric antibody complex (Stemcell Technologies cat. 10970) at 20 μL/mL, or PBS, and incubated at 37°C for 48 h, followed by addition of recombinant human IL4I1 protein (200 ng/mL) (R&D cat. # 5684-AO) or 1XPBS (sham-treatment). After 24 h incubation, cells were prepared for qRT-PCR analysis or for engrafting into athymic mouse lesions.

### 2.4 | cDNA Synthesis and qRT-PCR

Total RNA was extracted from cells with TRIzol. High integrity RNA (RIN > 7) was used to synthesize cDNA with iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad). PCR primers for human *B-ACTIN, IFNG, IL10, IL17A, IL4I1, TGFβ, TNFα*, and *TOB1* were purchased from Bio-Rad. Sybr-Green RT-PCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and analyzed by the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate and each amplicon was verified to produce a single peak in the melt curve analysis following qPCR. Results were based on raw Ct values, normalized against *B-ACTIN*, and expressed as mean ± SEM.

### 2.5 | Flow Cytometry

Flow cytometric analysis was performed on HC PBMCs after 48 h stimulation with ImmunoCult human CD3/CD28/CD2 T cell activator tetrameric antibody complex (Stemcell Technologies cat. 10970) at 20 μL/mL, or PBS (sham-stimulation) and 24 h treatment with recombinant human IL4I1 protein (R&D cat. 5684-AO) or 1XPBS. All protocols and reagents used were designed and purchased from BioLegend. Protein transport inhibitor Brefeldin A was included in the last 4 h of cell culture activation in order to block cytokine transport. Then the cells were stained with CD4, CD8, IL10, IFNγ, L17, CD56, CD14 and CD15 primary antibodies following the BioLegend intracellular cytokine staining protocol. Stained cells were analyzed at the Georgetown Lombardi Comprehensive Cancer Center Flow Cytometry & Cell Sorting Shared Resource (FCSR). CD3 antibody was not used because the cells were treated with anti-CD3/CD28/CD2 for activation. Cells that were negative for CD65/CD14/CD15

were considered as CD3+. For the T helper cell population, cells were gated on Live/Dead and CD3+; analyzed for CD4 and CD8. For CD4+ T helper cell subtypes, cells were gated on Live/Dead, CD3+, CD4+; analyzed for IFNγ, L17, and IL10. Only a single experimental replicate was performed due to constraints on sample availability and resource limitations.

### 2.6 | Mouse Demyelination and Lymphocyte Grafting

All animal experiments were performed in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines, and in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols of Georgetown University. Female athymic, "nude" mice (Foxn1$^{nu}$) were purchased from Jackson Laboratory (Bar Harbor, ME). Focal demyelination was induced by injecting 1.0% lysolecithin (Sigma) in 1XPBS into the spinal cord dorsal horn of female athymic mice at 8–10 weeks old. PBMCs from HC donors were stimulated with anti-CD3/CD28/CD2, and treated with IL4I1 or PBS. Treated cells were collected in media, centrifuged at 600 × *g* for 10 min at 4°C, and resuspended in 1XPBS at $10^5$ live cells/μl. 1 μL PBMCs suspension was injected into the lesion site, which was previously marked by charcoal, at 48 h after lysolecithin injection. Non-grafted control mice (NGC) did not undergo the grafting procedure. Mice in each of three groups (NGC, PBS-grafted and IL4I1-grafted), with $n = 3–5$ per group, were sacrificed 15 days after the lysolecithin-injection was performed.

### 2.7 | Tissue Sectioning and Immunohistochemistry (IHC)

Mice were perfusion-fixed with 4% (w/v) paraformaldehyde (PFA). Spinal cord tissues were dissected and postfixed for 45 min in 4% PFA at RT. Tissues were cryoprotected in 20% (w/v) sucrose before freezing in OCT, and cryosectioned at 12 μm on SuperFrostPlus slides (Stellar Scientific), and stored at −80°C until use. Sections were thawed for 30 min at RT and incubated in blocking solution (0.1% [v/v] Triton X-100 and 10% fetal bovine serum in TBS) for 1 h at RT. Rat anti-myelin basic protein (MBP) primary antibody (AbD Serotec) was diluted 1:400 in TBS blocking solution and applied to sections overnight at 4°C. AlexaFluor 488 secondary antibody (ThermoFisher) was used at a concentration of 1:500 and applied for 45 min at RT. FluoroMyelin dye (ThermoFisher) at was applied for 45 min at 4°C, at a concentration of 1:75 in TBS blocking solution. CC1 mouse antibody (Millipore), at a concentration of 1:100 in blocking buffer, and Olig2 rabbit (Millipore), at a concentration of 1:500 in blocking buffer, were added on tissues for 48 h at 4°C after Mouse-On-Mouse Detection (Vector Laboratories). To label nuclei, Hoechst stock (ThermoFisher) was diluted 1:20000 in TBS and applied with secondary antibodies for 45 min.

### 2.8 | Imaging and Quantification of IHC

For quantification of IHC, stained areas were manually captured under the LSM880 confocal microscope, and a minimum of 3 sections from $n = 3–5$ mice were examined. ImageJ software was used to quantify MBP intensity. Images were de-identified such that analyses were blinded to experimental conditions. Demyelinated lesions were identified as areas of high cellular density, as defined by nuclear (DAPI) staining. Fluorescence intensity in non-lesioned areas was measured on each channel in order to correct for background fluorescence. Corrected Fluorescence Intensity (CFI) was calculated as: *CFI = IntDensity of lesion – (area of lesion* x *mean fluorescence of background*) per channel. CFIs were averaged per mouse per condition and transferred to GraphPad Prism 7 (La Jolla, CA, USA) for normalization and graphic representation. For Olig2/CC1 quantifications, lesion areas were identified based on nuclei density, and Olig2/CC1 positive cells were quantified and lesion area assessed via ImageJ software.

### 2.9 | Statistics

All statistics were performed using GraphPad Prism 7 (La Jolla, CA, USA). Data is represented as mean ± SEM after identifying statistical outliers. Student's *t* tests were conducted for paired, within-group analyses; ordinary one-way ANOVA with Tukey's multivariable comparisons *post hoc* analysis (alpha = 0.05) are used for unpaired, between-group comparisons. Statistical significance is reported as ns, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

## 3 | Results

### 3.1 | IL4I1 Skews CD4$^+$ T Cell Populations From Healthy Donors Towards a Regulatory State

To examine the effect of IL4I1 on human lymphocytes, cells (PBMCs) were isolated from healthy donor blood samples and activated in culture using anti-CD3/CD28/CD2 microbeads. After 48 h of stimulation, recombinant human IL4I1 or PBS (vehicle control) were added for 24 h, followed by flow cytometry analysis (Table 1). We found that the relative percentages of CD4$^+$ and CD8$^+$ T-cells were similar between IL4I1- and PBS-treated samples (Figure 1A). However, within the CD4$^+$ T-cell populations, we found that IL4I1 treatment led to an increase in the percentage of regulatory T-cells (Tregs cells: CD4$^+$ IL10$^+$ IFNγ$^-$) by over five-fold (3.48% vs. 0.66%), and Th2 cells (CD4$^+$ IL17$^-$ IFNγ$^-$ IL10$^-$) by nearly two-fold (67.18% vs. 37.16%) compared to control (Figure 1B). Furthermore, pro-inflammatory Th17 (CD4$^+$ IL17$^+$ IFNγ$^-$) populations were reduced substantially in IL4I1-treated samples by over thirty-fold (0.96% vs. 30.80%) (Figure 1B). The results in this study were based on a single experiment. Therefore, additional experiments with larger sample sizes are necessary to confirm whether IL4I1 influences the proliferation or differentiation of CD4+ T-cell subtypes in human cells. Nevertheless, the potential immunomodulatory effect of IL4I1 on human T cells, particularly in regulating Th17 cell proliferation, has been observed previously [14, 19], and is consistent with our previous findings in mouse models of MS [11], suggesting that IL4I1 plays a role in modulating CD4+ T-cell function.

**TABLE 1** | List of antibodies used for flow cytometry.

| Target | Antibody | Product |
|---|---|---|
| GrnzB | Pacific Blue anti-human/mouse Granzyme B | BioLegend #515407 |
| L/D | Zombie Aqua Fixable Viability Kit | BioLegend #423101 |
| CD197 (R7) | Brilliant Violet 605 anti-human CD197 (CCR7) | BioLegend #353223 |
| CD45RA | Brilliant Violet 711 anti-human CD45RA | BioLegend #304137 |
| IL-10 | Alexa Fluor 488 anti-human IL-10 | BioLegend #501413 |
| IFNg | PE anti-human IFN-γ | BioLegend #506506 |
| CD4 | PerCP/Cy5.5 anti-human CD4 | BioLegend #317427 |
| IL-17A | Alexa Fluor 647 anti-human IL-17A | BioLegend #512309 |
| CD8a | Alexa Fluor 700 anti-human CD8a | BioLegend #301027 |
| CD56/CD14/CD15 | APC/Fire 750 anti-human CD3 | BioLegend #300469 |

### 3.2 | Remyelination Is Enhanced in Mouse Lesions Engrafted With IL4I1-Treated Human Lymphocytes

A recent study by El Behi and colleagues demonstrated that human lymphocytes from MS patients significantly impairs remyelination efficiency in athymic nude mice (*Foxn1*$^{nu}$) when engrafted directly into demyelinated lesions [20]. To determine if the effect of IL4I1 on healthy human lymphocytes affects CNS remyelination in vivo, we grafted IL4I1- or vehicle-treated human lymphocytes ($1 \times 10$ [5]) into focally demyelinated lesions of *Foxn1*$^{nu}$ mouse spinal cord at 2 days post lesion (dpl). Additionally, CNS lesions without cell engraftment were examined as control. To quantify the relative extent of remyelination, FluoroMyelin staining and myelin basic protein (MBP) immunostaining were performed at 15 dpl, corresponding with oligodendrocyte differentiation and early remyelination [21], and the relative fluorescence intensities within lesions were examined. We found that lesions engrafted with IL4I1-treated cells exhibited greater FluoroMyelin and MBP staining intensity compared to those engrafted with vehicle-treated cells (Figure 2A,B). Moreover, MBP staining in lesions with vehicle treated grafts were not significantly different from lesions without any cells engrafted. Quantification of staining intensity revealed that engraftment of IL4I1-treated cells significantly increased FluoroMyelin labeling in lesions by two-fold (Figure 2B). To confirm that increased myelin staining resulted from increased oligodendrocytes in lesions, co-immunostaining analysis for the oligodendrocyte lineage cell marker Olig2 and the mature oligodendrocyte marker CC1 was performed. We found that lesions that received IL4I1-treated grafts exhibited significantly greater number of Olig2$^{+}$CC1$^{+}$ oligodendrocytes compared to PBS-treated grafts, and to those without grafts (Figure 2C). Moreover, no difference in oligodendrocyte density was observed between non-grafted control lesions and lesions containing PBS-treated grafts, supporting previous finding [20]. These results suggest that lymphocyte presence alone does not affect remyelination in lesions, and that IL4I1 modulation of CD4$^{+}$ T-cells is necessary to promotes oligodendrocyte remyelination.

### 3.3 | Effect of IL4I1 on PBMCs of MS-Derived Lymphocytes

The observation that IL4I1 modulates T-cells and promotes remyelination raises the question whether endogenous IL4I1 expression or function is impaired in MS. To answer this question, we examined the expression of *IL4I1* mRNA expression in non-activated PBMCs of HC and MS patients. Baseline qRT-PCR analysis of HC-derived PBMCs confirmed that *IL4I1* is expressed at detectable levels (Figure 3A) To identify potential correlation between MS disease state and *IL4I1* levels, *IL4I1* mRNA expression in PBMCs from active relapsing remitting MS (aRRMS), non-active relapsing remitting MS (naRRMS), and SPMS were compared with HC (see Methods for MS classifications). We found that *IL4I1* expression was significantly reduced in naRRMS compared to HC, with aRRMS and SPMS trending towards reduced levels (Figure 3B). These findings suggest that *IL4I1* is reduced in some MS disease stages.

To examine the effect of IL4I1 on inflammatory cell activity in the context of MS and non-disease states, lymphocytes from HC, aRRMS, naRRMS and SPMS groups were treated with IL4I1 or PBS (control) followed by qRT-PCR analysis for *IL17* expression, since Th17 cells from healthy lymphocytes were most profoundly affected by IL4I1 treatment (Figure 1B). We found that IL4I1 treated HC lymphocytes exhibited reduced *IL17* expression relative to PBS control (Figure 4A). However, its expression was unaffected by IL4I1 in all of the MS derived lymphocytes tested (Figure 4A). This result suggests that IL4I1 is unable to modulate Th17 cells in MS despite its ability to alter Th17 cell activity in healthy patients. Since previous study suggested that IL4I1 targets *TOB1*, an anti-proliferative regulator to prevent Th17 cell expansion [14], we next examined the expression of *TOB1* in HC- and MS-derived lymphocytes after IL4I1 or PBS (control) treatment. We found that IL4I1 increased *TOB1* expression in HC lymphocytes compared to control (Figure 4B). However, IL4I1 had no effect on *TOB1* expression in MS lymphocytes in all the disease stages examined (Figure 4B). Collectively, these data suggest that IL4I1 affects *IL17* and *TOB1* expression in

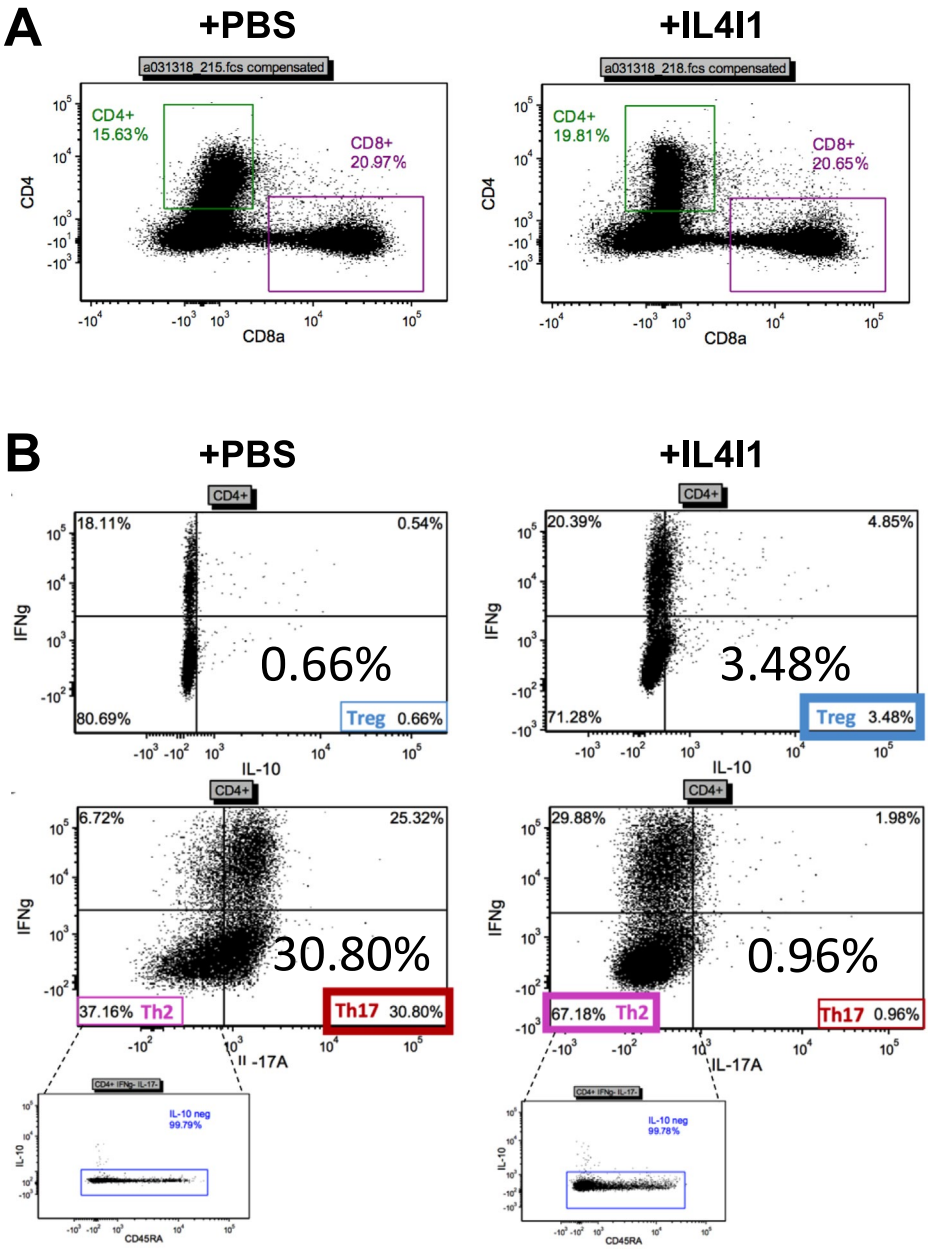


**FIGURE 1** | IL4I1 increases Treg and Th2 cell densities, and decreases Th17 cell density in PBMC-derived lymphocytes. Flow cytometry analysis of PBMC-derived lymphocytes from healthy control donor under untreated (+PBS) and treated (+IL4I1) conditions ($n = 1$ experiment per condition was performed). (A) Analysis of $CD4^+$ versus $CD8^+$ cells. (B) Percentage of regulatory T cell (Treg), type 2 helper T cell (Th2), T helper 17 cell (Th17) subtypes among $CD4^+$ T-cells based on IFNγ versus IL10 and IFNγ versus IL17 expressions.

healthy lymphocytes, but this relationship is perturbed in MS. Moreover, these results suggest that MS patients exhibit impaired IL4I1-TOB1 signaling that may lead to uncontrolled Th17 cell proliferation.

## 4 | Discussion

In this study, we have found that IL4I1 skews lymphocytes from healthy individuals towards a regulatory state by reducing Th17 cells while enriching for Tregs and Th2 cell populations. This shift in lymphocyte subtypes appears to be crucial for promoting for CNS repair. A caveat to this observation is that this study was based on a single experimental replicate, which limits the robustness of our conclusion. Although the role of IL4I1 expression in human T-cells have been described by others [14, 19, 22, 23], future experiments with larger sample sizes are necessary to validate the observed effects of IL4I1 treatment on human CD4+ T cells to confirm the reproducibility of our findings and strengthen the statistical power of the analyses.

Recent studies suggest that lymphocyte-derived factors facilitate oligodendrocyte differentiation via interactions with microglia, and that Tregs play a critical role in remyelination success in mice [20, 24]. We found that IL4I1 treated human lymphocytes significantly enhanced remyelination in vivo

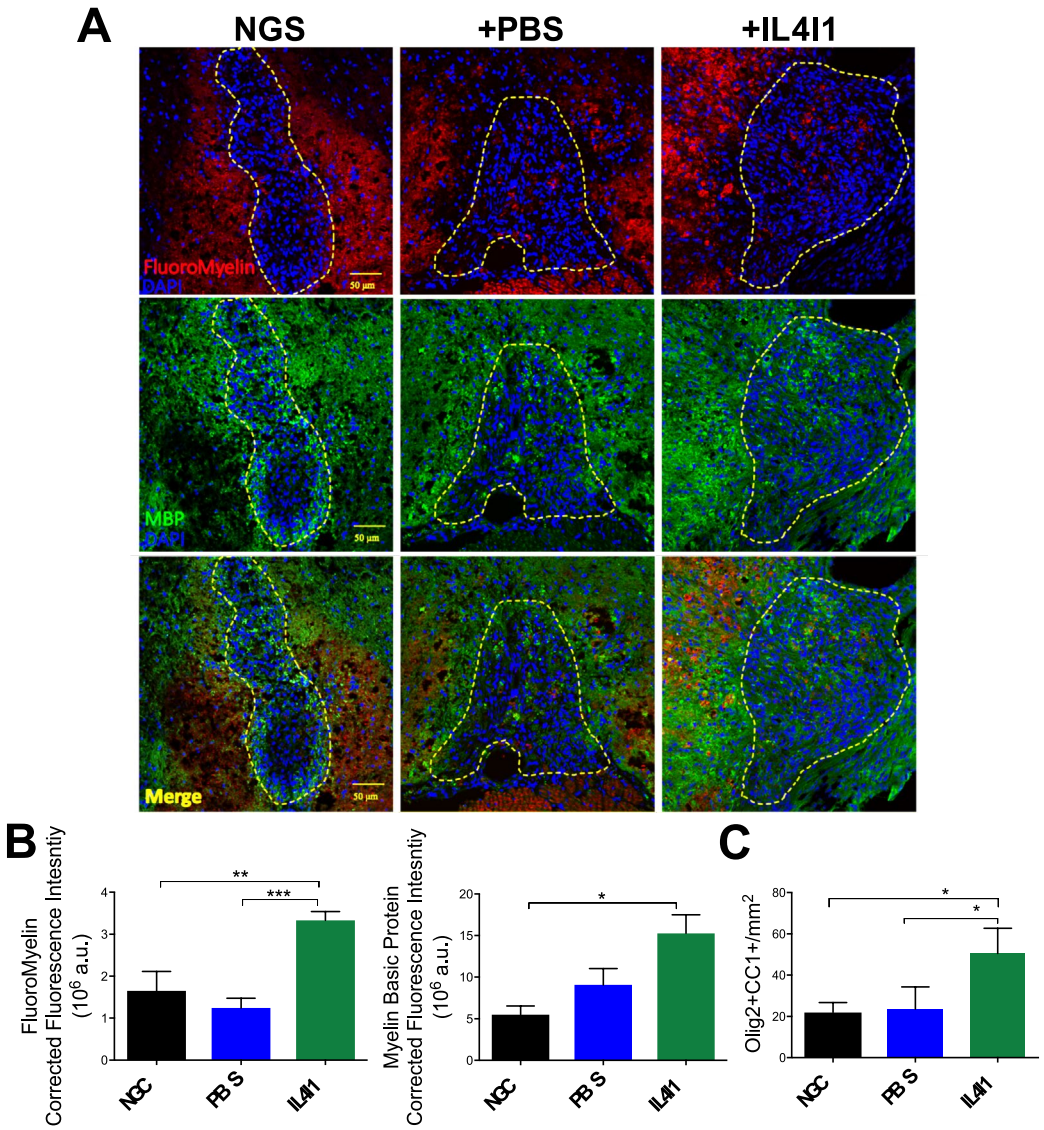


**FIGURE 2** | IL4I1-treated human lymphocyte grafts enhance remyelination in nude mouse demyelinated lesions. IHC staining and quantification of myelin within spinal cord dorsal horn lesions of nude mice at 15 days-post lesion (dpl). At 2dpl, lesions were engrafted with IL4I1-treated human lymphocytes (IL4I1; $n = 4$), sham-treated human lymphocytes (PBS; $n = 5$) or were used as non-grafted controls (NGC; $n = 3$). Representative 20× images showing (A) FluoroMyelin dye, myelin basic protein (MBP) stain, and merged staining in NGC, PBS and IL4I1 lesions. Lesioned areas are outlined in yellow. Quantification of (B) FluoroMyelin and MBP fluorescence intensities, and (C) $Olig2^{+}CC1^{+}$ cell densities within NGC, PBS-grated and IL4I1-grafted lesions. Scale bar, 50 µm. One-way ANOVA with Tukey's multiple comparisons test; Data represented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

when transplanted into lesions in nude mouse. Our result suggests that IL4I1 modulates the inflammatory microenvironment driven by T-cells, thereby promoting remyelination. Moreover, since PBMCs can be easily obtained from patients, this strategy may serve as a predictive preclinical assay to determine whether immunomodulatory drugs can improve remyelination in MS.

The mechanisms by which IL4I1-treated lymphocytes enhance remyelination remain unclear but may involve IL4I1's role as an aromatic amino acid oxidase, which modulates amino acid availability within immune cells. IL41 has been shown to promote FoxP3+ regulatory T cells differentiation through phenylalanine consumption [22]. Additionally, it is known to limit Th17 cell expansion in a cell-autonomous manner by down-regulating CD3ζ chain expression in the T-cell receptor (TCR) complex [14]. Our recent work indicates that alpha-keto acids generated by IL4I1 modulate myeloid cell activity and activate the aryl hydrocarbon receptor (AhR) pathway [25]. Alpha-keto acids, such as indole-3-pyruvic acid, are also known to activate AhR signaling in T cells, promoting an anti-inflammatory phenotype [26]. Therefore, IL4I1 may modulate T cell activity via the production of alpha-keto acids production, contributing to an immunoregulatory environment to promote remyelination.

Additionally, we found that lymphocytes derived from MS patients exhibited lower expression of *IL4I1* compared to HCs, suggesting a transcriptional dysregulation of *IL4I1* in MS. Since the *IL4I1* genomic locus is situated within a chromosomal region linked to autoimmune susceptibility [13], our

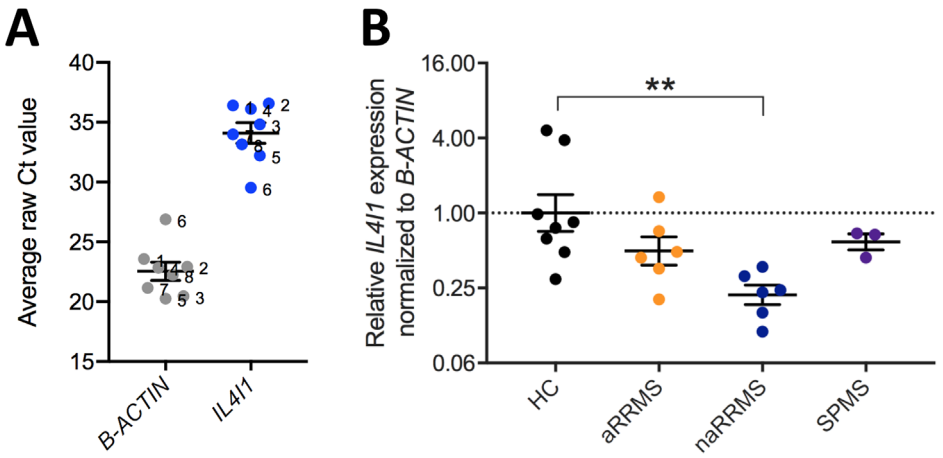


**FIGURE 3** | Baseline *IL4I1* expression is reduced in MS patient PBMCs. (A) *IL4I1* mRNA expression in unstimulated PBMCs from HC by qRT-PCR. *B-Actin* served as a loading control. qRT-PCR Ct values are shown with numbered dots representing individual donors ($n = 8$). (B) Relative *IL4I1* mRNA expression in unstimulated PBMCs from HC ($n = 8$) versus patients with MS. Female donors with aRRMS ($n = 6$), naRRMS ($n = 6$), and SPMS ($n = 3$) are shown relative to control. One-way ANOVA with Tukey's multiple comparisons test; Data represented as mean ± SEM; $^{**}p < 0.01$.

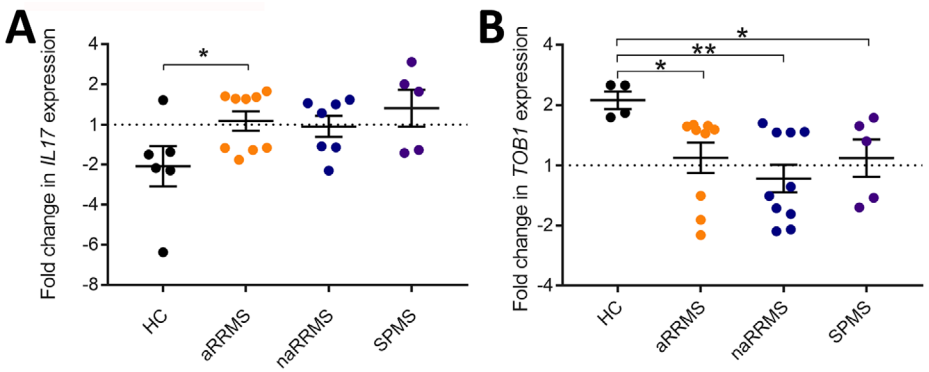


**FIGURE 4** | IL4I1 does not affect *IL17* or *TOB1* expression in MS lymphocytes. Fold change of (A) *IL17* and (B) *TOB1* in PBMC-derived lymphocytes from HC ($n = 6$), aRRMS ($n = 9$), naRRMS ($n = 6$), and SPMS ($n = 5$) after treatment with IL4I. Relative expressions were normalized to their respective PBS-treated controls. One-way ANOVA with Tukey's multiple comparisons test; Data represented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

results support the potential role for *IL4I1* dysfunction in MS. While IL4I1 treatment altered *IL17* and *TOB1* expression in healthy lymphocytes, no effect on their expression in MS-derived lymphocytes was observed, suggesting that T-cells in MS are resistant to IL4I1 modulation, which may contribute to persistent inflammation and remyelination impairment in MS lesions. However, it will be important to perform longitudinal studies that assess the stability of IL4I1's effects over time and under varying conditions, such as different stages of disease progression in MS patients. Future research to determine how IL4I1 dysregulation impacts T-cell function in MS could improve our understanding of MS pathogenesis and potentially lead the development of treatments targeting IL4I1 signaling to promote repair in MS.

## 5 | Significance Statement

We found that the amino acid oxidase, IL4I1, promotes human CD4+ T-cells towards a regulatory state and enhances CNS remyelination in mice when grafted into demyelinated lesions. However, in contrast to healthy cells, we found that MS derived lymphocytes were unresponsive to IL4I1, suggesting that impaired IL4I1-mediated activity may underlie MS pathology.

### Author Contributions

S.E.D., F.S.A., A.W. and J.K.H. initiated and designed this study. S.E.D. conducted in vitro and in vivo experiments, performed gene expression analysis, analyzed data, and drafted the manuscript for intellectual content. J.H. prepared samples for flow cytometry analysis, assisted with the in vivo experiments, and contributed to data analysis. S.E.N. and M.N.K. assisted with spinal cord sectioning and performed immunostaining. M.B. and S.E.N. assisted in perfusions and contributed to data analyses. H.C.O. assisted with in vitro experiments. K.P. assisted in the in vivo experiments. F.S.A. oversaw patient recruitment and assisted in blood collection. A.W. contributed to qPCR and PBMC analysis. S.E.D., J.H., and J.K.H. wrote the manuscript. J.K.H. oversaw the project.

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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

There are no datasets in this study.

### References

1. R. Dutta and B. D. Trapp, "Relapsing and Progressive Forms of Multiple Sclerosis: Insights From Pathology," *Current Opinion in Neurology* 27 (2014): 271–278.

2. A. Compston and A. Coles, "Multiple Sclerosis," *Lancet* 359 (2002): 1221–1231.

3. D. S. Reich, C. F. Lucchinetti, and P. A. Calabresi, "Multiple Sclerosis," *New England Journal of Medicine* 378 (2018): 169–180.

4. R. Dutta and B. D. Trapp, "Mechanisms of Neuronal Dysfunction and Degeneration in Multiple Sclerosis," *Progress in Neurobiology* 93 (2011): 1–12.

5. R. J. M. Franklin, C. ffrench-Constant, J. M. Edgar, and K. J. Smith, "Neuroprotection and Repair in Multiple Sclerosis," *Nature Reviews. Neurology* 8 (2012): 624–634.

6. D. Fitzner and M. Simons, "Chronic Progressive Multiple Sclerosis—Pathogenesis of Neurodegeneration and Therapeutic Strategies," *Current Neuropharmacology* 8 (2010): 305–315.

7. R. J. M. Franklin, "Why Does Remyelination Fail in Multiple Sclerosis?," *Nature Reviews. Neuroscience* 3 (2002): 705–714.

8. A. Compston and A. Coles, "Multiple Sclerosis," *Lancet* 372 (2008): 1502–1517.

9. Y. Yue, W. Huang, J. Liang, et al., "IL4I1 Is a Novel Regulator of M2 Macrophage Polarization That Can Inhibit T Cell Activation via L-Tryptophan and Arginine Depletion and IL-10 Production," *PLoS One* 10 (2015): e0142979.

10. A. Carbonnelle-Puscian, C. Copie-Bergman, M. Baia, et al., "The Novel Immunosuppressive Enzyme IL4I1 Is Expressed by Neoplastic Cells of Several B-Cell Lymphomas and by Tumor-Associated Macrophages," *Leukemia* 23 (2009): 952–960.

11. K. Psachoulia, K. A. Chamberlain, D. Heo, et al., "IL4I1 Augments CNS Remyelination and Axonal Protection by Modulating T Cell Driven Inflammation," *Brain: A Journal of Neurology* 139 (2016): 3121–3136.

12. K. G. Becker, R. M. Simon, J. E. Bailey-Wilson, et al., "Clustering of Non-major Histocompatibility Complex Susceptibility Candidate Loci in Human Autoimmune Diseases," *Proceedings of the National Academy of Sciences of the United States of America* 95 (1998): 9979–9984.

13. S. S. Chavan, W. Tian, K. Hsueh, D. Jawaheer, P. K. Gregersen, and C. C. Chu, "Characterization of the Human Homolog of the IL-4 Induced Gene-1 (Fig1)," *Biochimica et Biophysica Acta* 1576 (2002): 70–80.

14. V. Santarlasci, L. Maggi, A. Mazzoni, et al., "IL-4-Induced Gene 1 Maintains High Tob1 Expression That Contributes to TCR Unresponsiveness in Human T Helper 17 Cells," *European Journal of Immunology* 44 (2014): 654–661.

15. J.-C. Corvol, D. Pelletier, R. G. Henry, et al., "Abrogation of T Cell Quiescence Characterizes Patients at High Risk for Multiple Sclerosis After the Initial Neurological Event," *Proceedings of the National Academy of Sciences of the United States of America* 105 (2008): 11839–11844.

16. C. H. Polman, S. C. Reingold, B. Banwell, et al., "Diagnostic Criteria for Multiple Sclerosis: 2010 Revisions to the McDonald Criteria," *Annals of Neurology* 69 (2011): 292–302.

17. F. D. Lublin, S. C. Reingold, J. A. Cohen, et al., "Defining the Clinical Course of Multiple Sclerosis: The 2013 Revisions," *Neurology* 83 (2014): 278–286.

18. J. F. Kurtzke, "Rating Neurologic Impairment in Multiple Sclerosis: An Expanded Disability Status Scale (EDSS)," *Neurology* 33 (1983): 1444–1452.

19. V. Santarlasci, L. Maggi, M. Capone, et al., "Rarity of Human T Helper 17 Cells Is due to Retinoic Acid Orphan Receptor-Dependent Mechanisms That Limit Their Expansion," *Immunity* 36 (2012): 201–214.

20. M. El Behi, C. Sanson, C. Bachelin, et al., "Adaptive Human Immunity Drives Remyelination in a Mouse Model of Demyelination," *Brain: A Journal of Neurology* 140 (2017): 967–980.

21. N. D. Jeffery and W. F. Blakemore, "Remyelination of Mouse Spinal Cord Axons Demyelinated by Local Injection of Lysolecithin," *Journal of Neurocytology* 24 (1995): 775–781.

22. C. Cousin, A. Aubatin, S. Le Gouvello, L. Apetoh, F. Castellano, and V. Molinier-Frenkel, "The Immunosuppressive Enzyme IL4I1 Promotes FoxP3(+) Regulatory T Lymphocyte Differentiation," *European Journal of Immunology* 45 (2015): 1772–1782.

23. F. Lasoudris, C. Cousin, A. Prevost-Blondel, et al., "IL4I1: An Inhibitor of the $CD8^+$ Antitumor T-Cell Response In Vivo," *European Journal of Immunology* 41 (2011): 1629–1638.

24. Y. Dombrowski, T. O'Hagan, M. Dittmer, et al., "Regulatory T Cells Promote Myelin Regeneration in the Central Nervous System," *Nature Neuroscience* 20 (2017): 674–680.

25. J. Hu, G. S. Melchor, D. Ladakis, et al., "Myeloid Cell-Associated Aromatic Amino Acid Metabolism Facilitates CNS Myelin Regeneration," *Nature Partner Journals Regenerative Medicine* 9 (2024): 1.

26. R. Aoki, A. Aoki-Yoshida, C. Suzuki, and Y. Takayama, "Indole-3-Pyruvic Acid, an Aryl Hydrocarbon Receptor Activator, Suppresses Experimental Colitis in Mice," *Journal of Immunology (Baltimore, Md.: 1950)* 201 (2018): 3683–3693.