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14. ABSTRACT

Relapsing remitting multiple sclerosis (RRMS) is demyelinating disease that affects both men and women and is characterized by cycles of acute myelin loss, followed by remission with active myelin repair. The mechanism and the cellular source for remyelination are still in discussion, but there is evidence for the involvement of adult oligodendrocyte progenitor cells(OPCs). In this study, we hypothesized that circulating precursor cells identified by the presence of the cell surface marker CD133 may be of relevance during MS and for CNS repair processes. We measured the abundance of CD133+ and CD34+ cells in peripheral blood collected from RRMS patients and healthy controls. Our results showed that circulating CD34+ cells were not significantly affected by the disease. In contrast, CD133+ cells were significantly reduced in the RRMS patients recruited in this study. Interestingly, when CD133+ values from RRMS women were compared to RRMS men, we found that women had significantly lower values than the men ($p<0.029$). The fraction of hematopoietic CD133+ cells that were positive for the CD34+ marker was significantly elevated in RRMS patients. Our findings point to gender differences in the number of circulating progenitor cells in MS patients, and suggest that a reductions in CD133+ cells in RRMS may be exploited as a tool to diagnose RRMS.

15. SUBJECT TERMS
multiple sclerosis, neuroinflammation, stem cells, neurogenesis, hematopoiesis, T-cells.

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INTRODUCTION.

Preliminary experiments from our laboratory tested the working hypothesis that during Multiple Sclerosis (MS), the opening of the Blood-Brain-Barrier (BBB) could allow redistribution of progenitors with neural characteristics (NPs) from sites where they are born to those areas of demyelination where they may engage in myelin repair. In these preliminary experiments, we have found that peripheral blood mononuclear cells (PBMCs) isolated from a small group of patients with RRMS expressed the Olig2 gene –a transcription factor involved in the generation of oligodendrocytes-. This finding strongly indicates that mobilization of NPs with oligodendrocyte characteristics appears to occur in some patients. We speculate that NPs can be mobilized from two sources: 1) during disease, ruptures or transient openings of the BBB permit NPs to exit the nervous system and be circulated by the blood. These NPs are born or derive from endogenous NSCs residing within the nervous system; 2) during disease, the damaged nervous system signals or beacons the recruitment of peripheral stem cells to adopt a neural phenotype and thus be mobilized into the blood. In both scenarios, our application assumes that blood-borne neural precursors (BNPs) have the ability to either neuroprotect the MS nervous system and/or engage in remyelination by differentiating in myelinating oligodendrocytes once infiltrated in the nervous system of the MS patient. The goal of this application is to determine the identity of mobilized BNPs and to characterize their in vivo and in vitro potential to engage in remyelination.

BODY.

Enrollment of patients for blood donation. Forty one patients with a RRMS diagnosis according to McDonald's criteria (McDonald, Compston et al. 2001; Polman, Reingold et al. 2011) were enrolled in this study. The control population was composed of 25 healthy volunteers. Blood samples were collected from all individuals. We were able to collect second blood samples from only five MS patients. Most MS patients did not follow a six-month follow up.

Collection of blood samples. In all cases, about 20 ml of blood were collected in heparinized tubes and diluted 1:1 using 2 mM EDTA/PBS. Plasma and cells were separated by centrifugation. Plasma from each individual was aliquoted in 1 ml aliquots and kept frozen at -80C.

Mononuclear fractionation. The mononuclear fraction was obtained by centrifugation in a Ficoll density gradient. We had an average of 2.0 x 10^6 mononuclear cells per ml of control blood and 1.97 x 10^6 mononuclear cells per ml of RRMS blood. For each sample, mononuclear cells were aliquoted in 3 fractions: one aliquot of cells was frozen and reserved for protein and mRNA preparation; a second aliquot of cells was transferred to 10% FBS DMEM 15% DMSO and liquid nitrogen-stored for subsequent characterization and a third aliquot of cells were used for culture and/or isolation of stem cells and for flow cytometry analyses.

Isolation of CD133+ stem cells from RRMS blood: We have tested different culture media and supplements in order to find the best growing condition for the blood-borne neural progenitors (BNPs). These tests were performed with healthy control cells. In those experiments, we found that the basic neural stem cell medium that we normally use for growing neural stem cells as neurospheres inappropriate to promote survival and/or proliferation of cells. We reformulated a human neural stem cell medium (HNSM), which is composed by DMEM/F-12 supplemented with 20 ng/ml EGF, 10 ng/ml bFGF and 1ng/ml heparin, and 10% FBS, to decrease the survival of hematopoietic cells and promoting that of BNPs. With this medium, although colony-like structures formed after 6-8 days in culture, the cultures still degenerated during the second week of culture. For this reason, we have dedicated our efforts to enrich our cultures in stem cells.

Very immature multipotential stem cells in the blood express a cell surface marker –CD133- which is also shared by brain derived neural stem cells. Because of the potential usefulness of using this marker for enrichement of a population of BNPs, a protocol for their isolation from freshly prepared mononuclear fractions was set up and optmized in our laboratory.

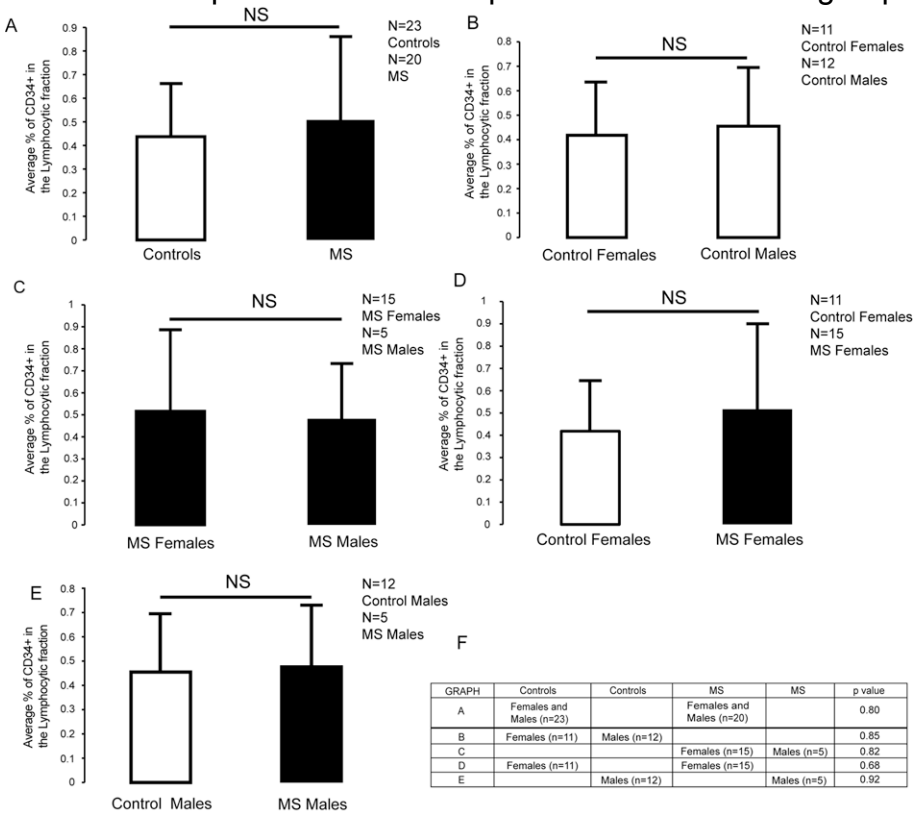
CD133+ stem cells were selected from freshly isolated mononuclear cells using the CD133 Microbead Kit (Miltenyi Biotec). The recovery yields were averaging 3,000 CD133+ cells per million of mononuclear cells. Aliquots of CD133+ cells were frozen for cell culture, and aliquots of 10,000 cells were saved for gene array analysis.

Flow cytometry analysis of CD133 and CD34 in blood. Fluorescence-Activated Cell Sorting (FACS) experiments were carried out as followed. Following the removal of platelets, PBMCs were adjusted to one million cells per ml in 0.5% BSA, 2mM EDTA, PBS, centrifuged at 3,000 g for 5 min, and resuspended in a final volume of 100 µl of human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Each tube containing one million cells were incubated for 15-30 min at 4°C with one µg of either a mouse anti-human CD133 antibody-PE (Phycoerythrin), a mouse anti-human CD34 antibody-FITC (fluorescein isothiocyanate), or both. Negative controls included incubations with IgG1-PE and IgG2a-FITC. All antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). After two washes at 300 g, cells were transferred to flowcytometry tubes and incubated with 7-amino-actinomycin D (7AAD) (BD, Franklin Lakes, NJ) to label dead cells, which were subtracted from the total cell count. Whenever possible, about 200,000 events were counted in G2 using a LSR I flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). Cells counted by FACS were expressed as percentage from the total gated lymphocytic PBMCs. The analysis of the raw data was performed with Summit Version 4.3, (Dako, Glostrup, Denmark). To calculate the ratios of CD133+CD34+ as a percentage of CD133+ cells, the sum of the percentages of IgG control cells was subtracted first from the sum of the percentages of double labeled CD133+CD34+ plus CD133+ (single labeled) cells. This number was then divided by the percentage of CD133+ (single labeled) cells. To correct for percentage values higher than 100%, the value of zero for single labeled CD133+ was used for the calculation whenever the background IgG PE was shown to be higher than or equal to the value of percentage of single labeled CD133+ (non-CD133+CD34+).

Statistics. All statistical analyses were performed using free-access non-parametric tests from Delaware University. The non-parametric Spearman correlation and Kruskal-Wallis analysis of variance tests were used due to data being not Gaussian. Significance was taken as two-tailed with a p value < 0.05.

Abundance of CD34 and CD133 cells in the circulating blood of patients with RRMS. Since our last report, we have completed the analyses for 23 blood control samples and 20 blood samples from MS patients by flow cytometry. Abundance of CD133+ and CD34+ cells was analyzed for each sample. To determine whether or not MS patients had a restricted capacity for hematopoiesis, we first quantified by flow cytometry the abundance of CD34+ stem cells, which form the majority of the hematopoietic stem cells. We compared the fractions of CD34+ cells, as percentage of PBMCs freshly collected from healthy controls (N=23), to that in MS patients (N=20). We found a slight increase in the percentages of CD34+ cells in MS patients, which averaged 0.51% ± 0.35 standard deviations, when compared to the control population, which averaged 0.43% ± 0.22 standard deviations (Figure 1A). However, this difference did not reach statistical significance ($p=0.8$). Because the patient and control groups were composed of individuals of both genders, this lack of significance could be due to a compensation of values between individuals of different genders. To rule out that possibility we compared control

women (N=11, 0.42 0.22) to control men (N=12, 0.45 ± 0.24), which did not show any statistical difference (p=0.85), (Figure 1B). Similarly, CD34 values from women (N=15, 0.52 0.37) and men (N=5, 0.48 0.25) from the MS group (Figure 1C) were not significantly different (p=0.82).. Within-gender comparisons also did not reveal any statistically significant differences in CD34+ values for the women control (N=11, 0.42 0.22) versus MS patients (N=15, 0.52 0.37; p=0.68); or for the men control (N=12, 0.45 ± 0.24) versus MS patients (N=5, 0.48 0.25; p=0.92). Taken together, these data indicate that the abundance of circulating CD34+ cells is not compromised in RRMS patients versus control groups.

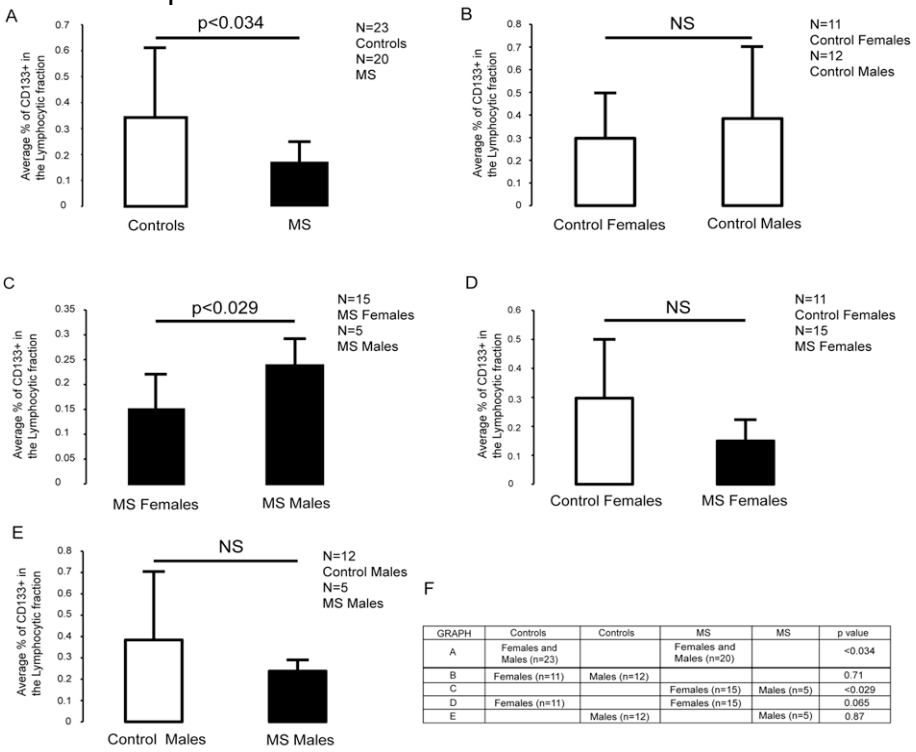


GRAPH	Controls	Controls	MS	MS	p value
A	Females and Males (n=23)		Females and Males (n=20)		0.80
B	Females (n=11)	Males (n=12)			0.85
C			Females (n=15)	Males (n=5)	0.82
D	Females (n=11)		Females (n=15)		0.68
E		Males (n=12)		Males (n=5)	0.92

Figure 1. Percentage of CD34+ hematopoietic stem cells in the lymphocytic fraction of RRMS patients or healthy individuals. Peripheral blood mononuclear cells (PBMCs) from healthy volunteers or relapsing remitting multiple sclerosis (RRMS) patients were fractionated from venous blood. About $1x10^6$ PBMCs were analyzed by FACS with a Fluorescein-conjugated anti-hCD34 antibody. All values of CD34+ cells are expressed as a percentage of the lymphocyte fraction. A. Comparison between 23 healthy volunteer individuals (Control) and 20 patients (MS). B. Comparison among control genders (11 females and 12 males). C. Comparison among MS genders (15 females and 5 males). D. Comparison between 11 control females and 15 MS females. E. Comparison between 12 control males and 5 MS males. F. Summary of the comparisons above with the p values obtained by statistical analysis using a non-parametric test (Kruskal-Wallis, two tails). No significant differences between all the comparisons were found. Error bars represent standard deviation of the mean. Open bars represent controls; black solid bars represent MS patients.

Next, we compared the abundance of CD133+ cells by flow cytometry in the same group of subjects. CD133+ cells were found significantly (P<0.034) decreased in the blood from RRMS patients (0.17 ± 0.08) when compared to controls (0.34 ± 0.27) (Figure 2A). Interestingly, a gender effect appears to influence the abundance of CD133+ cells in MS. In controls, the abundance of

CD133+ cells in women (0.30 ± 0.20) was not significantly different from that in men (0.38 ± 0.32; P=0.71) (Figure 2B). In contrast, there was a significant (P<0.029) decrease in CD133+ cells in MS women (0.15 ± 0.07) vs MS men (0.24 ± 0.05) (Figure 2C). Control women hadhigher CD133+ values than MS women (Figure 2D), a difference which almost reached statistical significance(P=0.065); while no differences were detected in CD133+ levels between control and MS men (Figure 2E). Taken together, these results indicate a clear decrease in the percentage of CD133+ cells in RRMS patients versus controls, which may be primarily driven by a more pronounced reduction in women versus men patients.



GRAPH	Controls	Controls	MS	MS	p value
A	Females and Males (n=23)		Females and Males (n=20)		<0.034
B	Females (n=11)	Males (n=12)			0.71
C			Females (n=15)	Males (n=5)	<0.029
D	Females (n=11)		Females (n=15)		0.065
E		Males (n=12)		Males (n=5)	0.87

Figure 2. Percentage of CD133+ hematopoietic stem cells in the lymphocytic fraction of RRMS patients or healthy individuals. PBMCs from healthy volunteers or relapsing remitting multiple sclerosis (RRMS) patients were fractionated from venous blood. About 1x10^6 PBMCs were incubated with a phycoerythrin-conjugated (PE-conjugated) anti-hCD133 antibody and analyzed by FACS. All values of CD133+ cells are expressed as a percentage of the lymphocyte fraction. A. Comparison between the 23 healthy volunteer individuals (Control), and 20 patients (MS) showed significant difference (p<0.034). B. Comparison between the controls (11 females and 12 males). C. Comparison between MS patients (15 females and 5 males) showed significant difference (p<0.029). D. Comparison between 11 control females and 15 MS females produce a marked trend, which did not reach significance (p=0.065). E. Comparison between 12 control males and 5 MS males. F. Summary of the comparisons above. Statistical analysis performed (Kruskal-Wallis, two tails) showed only significance between controls and MS patients and between females and males in the MS group. Error bars represent standard deviation of the mean. Open bars represent controls; black solid bars represent MS patients.

Correlation between CD133+ cells abundance and clinical variables. Because aging correlates with a decrease in repair and regeneration in the nervous system, we tested if this is a factor influencing the circulating levels of CD133+ cells. However, there was no statistically significant correlation found between the abundance of CD133+ cells and age in the healthy controls (Figure

3A) or the MS patients (Figure 3B) . To determine if the levels of CD133+ cells had a predictive value for the degree of severity in MS, a correlation analysis was performed comparing EDSS values to CD133+ abundance; however, we did not detect any statistically significant correlation between these 2 parameters (Figure 4). These results suggest that EDSS is not a good predictor of the CD133+ levels and consequently patients with low EDSS show equal variability of CD133+ cell values when compared to patients with high EDSS .

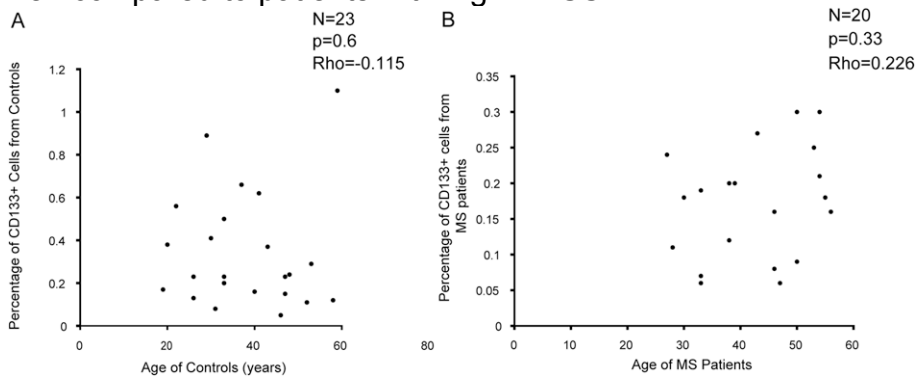


Figure 3. Correlation between age and the percentage of CD133+ hematopoietic stem cells in the lymphocytic fraction of healthy controls or RRMS patients. Clinical data obtained from the medical records was used to test if a correlation between the percentage of CD133+ in the lymphocytic population and the age of individuals could be found using the Spearman non-parametric test. A. Relationship between CD133+ cells and age of controls was not significant (n=23, rho=-0.115, p=0.6). B. Relationship between CD133+ cells and age of MS patients was not significant (n=20, rho=0.226, p=0.33).

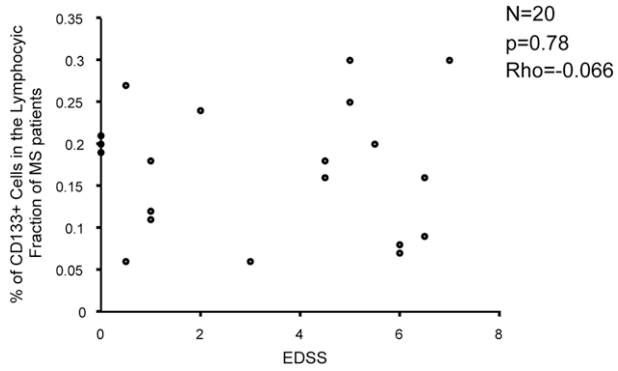


Figure 4. Correlation between Expanded Disability Status Scale (EDSS) and the percentage of CD133+ hematopoietic stem cells in the lymphocytic fraction of RRMS patients. Absence of correlation between the EDSS and the percentage of CD133+ cells from MS patients was found by Spearman test (rho= -0.066, p=0.78) indicating that EDSS is not a predictor of the percentage of CD133+ cells.

We then investigated whether the therapeutic treatment had any effect on CD133+ cell abundance (Figure 5). Despite a modest decrease of CD133+ cell in patients treated with interferon- (median 0.11), when compared to the group treated with Tysabri (median 0.175) and a composite third group encompassing patients under other drug treatments (median 0.2), no significant difference was found (Kruskal-Wallis non parametric test, two tails (p=0.15) . Likewise, there was no correlation between disease duration and the lymphocytic CD133+ fraction (Spearman p=0.72) (Figure 6A); or between disease severity as

measured by the progression index (EDSS/disease duration) and the lymphocytic CD133+ fraction (spearman=0.69) (Figure 6B).

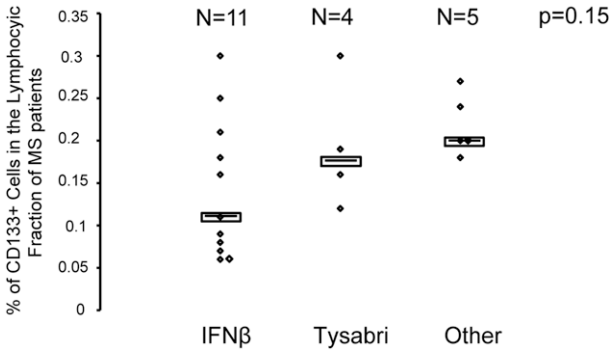


Figure 5. Treatments do not have a significant effect on the percentage of CD133+ cells of RRMS patients. Comparison between the percentages of CD133+ cells from lymphocytes of MS patients treated with interferon- (N=11, median=0.11%), Tysabri (N=4, median=0.175%), or other drugs (cyclophosphamide, N=1, glatiramer acetate, N=3, maraviroc, N=1; median=0.2%) showed no significant difference (Kruskal-Wallis, two-tailed, p=0.15).

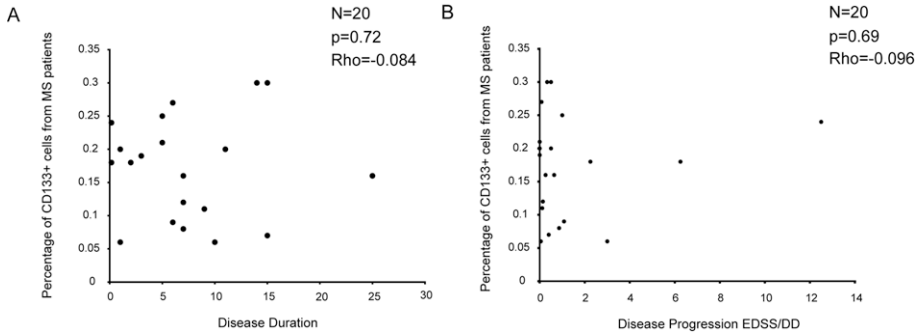


Figure 6. Correlation between disease duration or disease progression and CD133+ percentage in the lymphocytic fraction of MS patients. A. Disease duration (DD) showed no correlation to CD133+ cells by Spearman test (rho= -0.084, p=0.72; N=20 patients). B Disease progression, defined as EDSS/DD showed no correlation to the levels of CD133+ cells in the lymphocytic fraction (Spearman rho= -0.096, p=0.69; N=20 patients).

We tested the possibility that CD133+ cells may be differentiating towards a hematopoietic lineage in MS patients. A prediction of this hypothesis is that a higher percentage of cells will coexpress both CD133 and CD34 markers. Our analysis shows that the MS patients in this study had a significantly higher percentage of doubly labeled CD133+ and CD34+ cells than controls (Kruskal-Wallis, two tails $p<0.042$) (Figure 7).

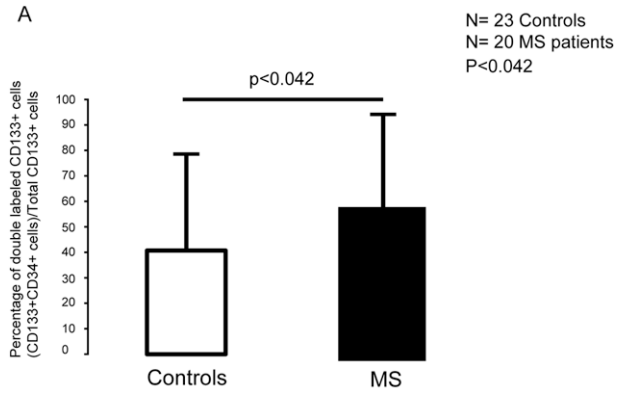


Figure 7. Comparison between the percentages of CD133+CD34+ cells and total CD133+ cells in the lymphocytic fraction of healthy controls and MS patients. CD133+CD34+ cells of all 23 healthy controls and all 20 MS patients were expressed as a percentage of their respective totals of CD133+ cells and compared in both subject populations. MS patients showed significant higher numbers of double stained CD133+CD34+ cells (56% ± 29.6%, mean ± SD, horizontal bars) than controls (40.7% ± 38%, mean ± SD, horizontal bars). Statistical analysis by Kruskal-Wallis, p<0.042, two-tailed).

Gene expression analysis. RNA was isolated from mononuclear cells from 16 MS patients and from 23 healthy volunteers. Total RNA was isolated using the RNAqueous-Micro Kit (Ambion). Twenty micrograms of fluorescence-labeled amplified RNA were prepared using the Amino Allyl MessageAmp II Kit (Ambion). We used 2 µg of each sample to hybridize -in triplicate- with full genome Human OneArray (Phalanx Biotech) according to the manufacturer's recommendations. The arrays were scanned using ScanArray Lite (Perkin Elmer). Scanned images were analyzed with ScanArray System software to obtain gene expression ratios. Statistical analysis was performed using LIMMA (Smyth,G.K. 2004. *Statistical Applications in Genetics and Molecular Biology* 3, Article 3).

To gain more accuracy and statistical from our full analysis, we compared labeled RNA from individual female or male patients to labeled RNA from pooled either female or male controls (reference RNA). We analyzed our data using the RRC facility, as above, which included scanning, normalization and statistical analysis of the data. We obtained a set of signals representing putative genes that are significantly de-regulated in all the male patients or in all the female patients. For the females, a total of 58 putative genes were significantly de-regulated. Out of these, 16 putative genes showed on average lower expression than in control females, while 42 putative genes showed higher expression than in control females. For the males, a total of 127 putative genes showed significant de-regulation. Out of these, 54 putative genes showed on average lower expression than in control males, while 73 putative genes showed higher expression than in control males.

Our microarray data produced significant difference between females and the corresponding female pool control as a group. A partial list of deregulated genes is presented in table I below, and summarized in a volcano plot in figure 8 below.

Table I. List of deregulated genes in MS female patients

Gene_symbol	Gene_description
COL28A1	Collagen alpha-1(XXVIII) chain Precursor [Source:UniProtKB/Swiss-Prot;Acc:Q2UY09]
XXbac-B135H6.15	processed transcript
ZC3H12B	Probable ribonuclease ZC3H12B (EC 3.1.-.-)(Zinc finger CCCH domain-containing protein 12B)(MCP-induced protein 2) [Source:UniProtKB/Swiss-Prot;Acc:Q5HYM0]
GNGT2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2 Precursor (G gamma-C)(G-gamma-8)(G-gamma-9) [Source:UniProtKB/Swiss-Prot;Acc:O14610]
C1orf220	Putative uncharacterized protein C1orf220 [Source:UniProtKB/Swiss-Prot;Acc:Q5T0J3]
CDK2AP2	Cyclin-dependent kinase 2-associated protein 2 (CDK2-associated protein 2)(DOC-1-related protein)(DOC-1R) [Source:UniProtKB/Swiss-Prot;Acc:O75956]
C2orf18	Transmembrane protein C2orf18 Precursor [Source:UniProtKB/Swiss-Prot;Acc:Q8N357]
BDNF	Brain-derived neurotrophic factor Precursor (BDNF)(Abrineurin) [Source:UniProtKB/Swiss-Prot;Acc:P23560]
NOTCH4,	Notch 4
HCN4	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 [Source:UniProtKB/Swiss-Prot;Acc:Q9Y3Q4]
ATP8B3	Probable phospholipid-transporting ATPase IK (EC 3.6.3.1)(ATPase class I type 8B member 3) [Source:UniProtKB/Swiss-Prot;Acc:O60423]
CALML3	Calmodulin-like protein 3 (Calmodulin-related protein NB-1)(CaM-like protein)(CLP) [Source:UniProtKB/Swiss-Prot;Acc:P27482]
ARL10	ADP-ribosylation factor-like protein 10 [Source:UniProtKB/Swiss-Prot;Acc:Q8N8L6]
TSP50	Testis-specific protease-like protein 50 Precursor (Cancer/testis antigen 20)(CT20) [Source:UniProtKB/Swiss-Prot;Acc:Q9UI38]
F2RL2	Proteinase-activated receptor 3 Precursor (PAR-3)(Thrombin receptor-like 2)(Coagulation factor II receptor-like 2) [Source:UniProtKB/Swiss-Prot;Acc:O00254]
FAM47DP,PRRG1	NA
ALAS2	5-aminolevulinate synthase, erythroid-specific, mitochondrial Precursor (EC 2.3.1.37)(5-aminolevulinic acid synthase)(Delta-aminolevulinate synthase)(Delta-ALA synthetase)(ALAS-E) [Source:UniProtKB/Swiss-Prot;Acc:P22557]
TMEM45B	Transmembrane protein 45B [Source:UniProtKB/Swiss-Prot;Acc:Q96B21]
SLC25A37	Mitoferrin-1 (Mitochondrial iron transporter 1)(Solute carrier family 25 member 37)(Mitochondrial solute carrier protein) [Source:UniProtKB/Swiss-Prot;Acc:Q9NYZ2]
TMEM159	Promethin (Transmembrane protein 159) [Source:UniProtKB/Swiss-Prot;Acc:Q96B96]
AIP	AH receptor-interacting protein (AIP)(Aryl-hydrocarbon receptor-interacting protein)(Immunophilin homolog ARA9)(HBV X-associated protein 2)(XAP-2) [Source:UniProtKB/Swiss-Prot;Acc:O00170]
MVP	Major vault protein (MVP)(Lung resistance-related protein) [Source:UniProtKB/Swiss-Prot;Acc:Q14764]
MTHFS	5-formyltetrahydrofolate cyclo-ligase (EC 6.3.3.2)(5,10-methenyl-tetrahydrofolate synthetase)(Methenyl-THF synthetase)(MTHFS) [Source:UniProtKB/Swiss-Prot;Acc:P49914]
GPR172A	Porcine endogenous retrovirus A receptor 1 Precursor (PERV-A receptor 1)(Protein GPR172A) [Source:UniProtKB/Swiss-Prot;Acc:Q9HAB3]
RNASEH2C	Ribonuclease H2 subunit C (RNase H2 subunit C)(Ribonuclease HI subunit C)(Aicardi-Goutieres syndrome 3 protein)(AGS3)(RNase H1 small subunit) [Source:UniProtKB/Swiss-Prot;Acc:Q8TDP1]
HSD17B10	3-hydroxyacyl-CoA dehydrogenase type-2 (EC 1.1.1.35)(3-hydroxyacyl-CoA dehydrogenase type II)(Type II HADH)(3-hydroxy-2-methylbutyryl-CoA dehydrogenase)(EC 1.1.1.178)(17-beta-hydroxysteroid dehydrogenase 10)(Mitochondrial ribonuclease P protein 2)(Mitochondrial RNase P protein 2)(Endoplasmic reticulum-associated amyloid beta-peptide-binding protein)(Short-chain type dehydrogenase/reductase XH98G2) [Source:UniProtKB/Swiss-Prot;Acc:Q99714]
GATAD2A	Transcriptional repressor p66-alpha (Hp66alpha)(GATA zinc finger domain-containing protein 2A) [Source:UniProtKB/Swiss-Prot;Acc:Q86YP4]
PIP5K2A	Phosphatidylinositol-5-phosphate 4-kinase type-2 alpha (EC 2.7.1.149)(Phosphatidylinositol-5-phosphate 4-kinase type II alpha)(1-phosphatidylinositol-5-phosphate 4-kinase 2-alpha)(PtdIns(5)P-4-kinase isoform 2-alpha)(PIP4KII-alpha)(Diphosphoinositide kinase 2-alpha)(PtdIns(4)P-5-kinase B isoform)(PIP5KIII)(PtdIns(4)P-5-kinase C isoform) [Source:UniProtKB/Swiss-Prot;Acc:P48426]
NFAT5	Nuclear factor of activated T-cells 5 (NF-AT5)(T-cell transcription factor NFAT5)(Tonicity-responsive enhancer-binding protein)(TonE-binding protein)(TonEBP) [Source:UniProtKB/Swiss-Prot;Acc:O94916]
RAB37	Ras-related protein Rab-37 [Source:UniProtKB/Swiss-Prot;Acc:Q96AX2]
DHX15	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (EC 3.6.1.-)(DEAH box protein 15)(ATP-dependent RNA helicase #46) [Source:UniProtKB/Swiss-Prot;Acc:O43143]
TTC38	Tetratricopeptide repeat protein 38 (TPR repeat protein 38) [Source:UniProtKB/Swiss-Prot;Acc:Q5R3I4]
SMEK1	Serine/threonine-protein phosphatase 4 regulatory subunit 3A (SMEK homolog 1) [Source:UniProtKB/Swiss-Prot;Acc:Q6IN85]
SH2D1B	SH2 domain-containing protein 1B (EWS/FLI1-activated transcript 2)(EAT-2) [Source:UniProtKB/Swiss-Prot;Acc:O14796]

PGDS	Prostaglandin-H2 D-isomerase Precursor (EC 5.3.99.2)(Lipocalin-type prostaglandin-D synthase)(Glutathione-independent PGD synthetase)(Prostaglandin-D2 synthase)(PGD2 synthase)(PGDS2)(PGDS)(Beta-trace protein)(Cerebrin-28) [Source:UniProtKB/Swiss-Prot;Acc:P41222]
TBX21	T-box transcription factor TBX21 (T-box protein 21)(Transcription factor TBLYM)(T-cell-specific T-box transcription factor T-bet) [Source:UniProtKB/Swiss-Prot;Acc:Q9UL17]
FCRL6	Fc receptor-like protein 6 Precursor (FcR-like protein 6)(FcRL6)(Fc receptor homolog 6)(FcRH6)(IFGP6) [Source:UniProtKB/Swiss-Prot;Acc:Q6DN72]
CCNT1	Cyclin-T1 (CycT1)(Cyclin-T) [Source:UniProtKB/Swiss-Prot;Acc:O60563]
ZNF833	processed transcript
CNX	calnexin
GALC	galactosylceramidase

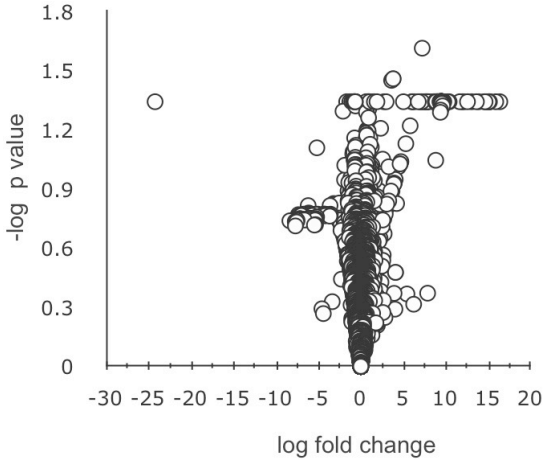


Figure 8. Schematic representation using a volcano plot, of all the genes in the female group. The plot was generated by plotting the -logarithm of the p-value (Y axis) vs. the logarithm of the fold change of the signal (X axis). p=0.05 is at y=1.3.

Validation of gene expression changes of selected genes by Quantitative Real-Time PCR

To determine whether genes in the neural or oligodendroglia lineage were also expressed and/or deregulated in MS patients, we selected a group of genes in the array that matched that condition. We normalized the expression with respect to GAPDH and compared it to the QR-PCR using the same total RNA from pool female controls and MS females. The assay produced consistent results when compared to the microarray data. A summary of some of the genes analyzed is shown in figure 9.

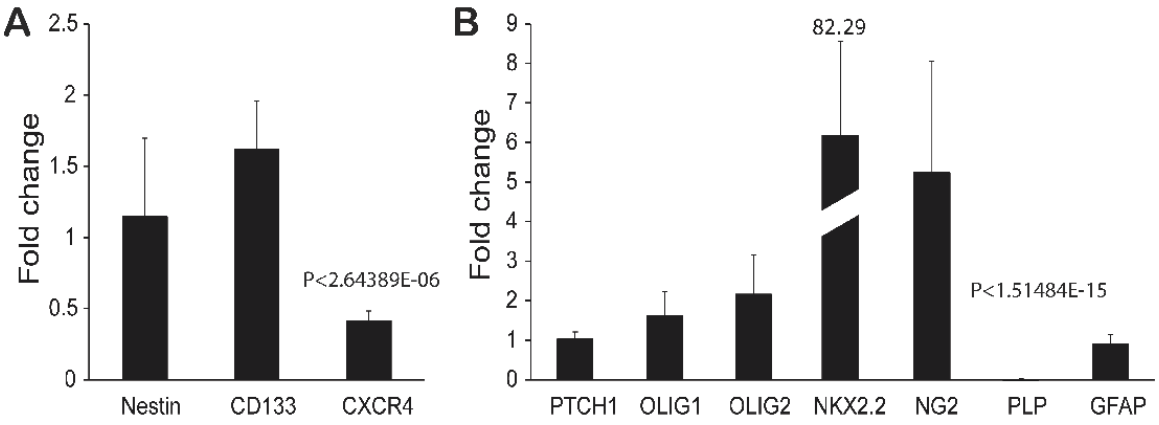


Figure 9. Peripheral blood of twelve female RRMS patients showed to express some neural genes. A, B) RNA was isolated from freshly drawn PBMCs and subjected to QRT-PCR using specific primers contained within exon sequences for the genes of interest. Samples were run in triplicates using an Applied Biosystems 7900HT-AB PCR machine. Fluorescent amplifications were measured and the fold change of gene expression over the control samples was calculated using the DCT method, with GAPDH as a housekeeping gene (GAPDH =1).

Immunoblotting analysis. PBMCs pellets have been obtained from controls and MS patients and were being processed for immunoblotting analysis of expression for neural markers as proposed. A summary of some of the genes analyzed is shown in figure 10.

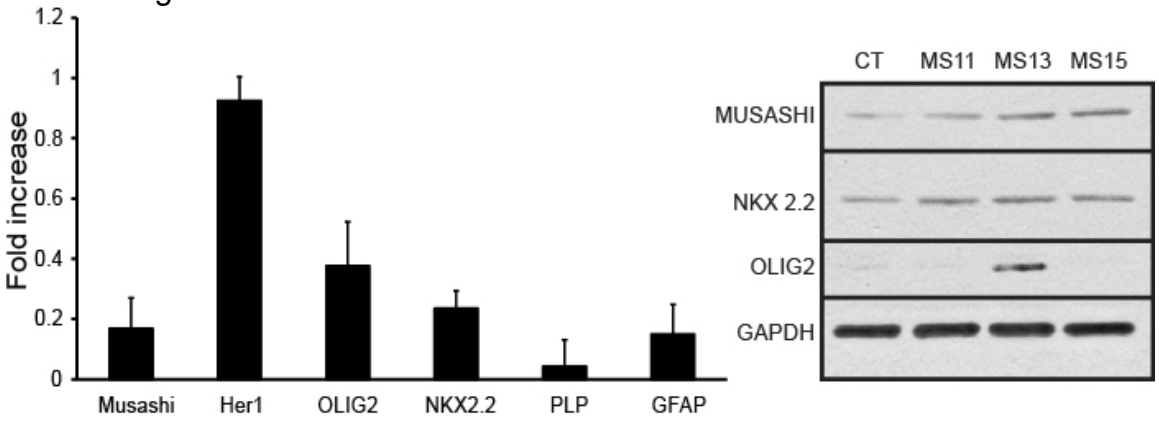


Figure 10. Immunoblot analysis of neural gene expression in MSPBMCs. PBMC cell pellets (5x106 cells) were lysed and used for SDS-PAGE/western blotting analyses. Twenty micrograms of total protein from one control and three RRMS patients were fractionated on a 4-20 % acrylamide SDS-gel and electrotransferred onto PVDF membranes. Blots were incubated with specific antibodies. Immunoreactions were developed using chemiluminiscent ECL reagents and films scanned for ImageJ analysis (NIH). Signals were normalized vs GADPH and expressed as fold increase respect to the control.

In vitro differentiation analyses. Cultures of CD133 cells were tested under various different culture conditions to study differentiation into hematopoietic and neural lineages. Hematopoietic conditions were established and experiments were performed to determine differences in differentiation potential of CD133 stem cells from MS patients.

Testing Differentiation potential of CD133 cells from MS patients versus controls. To test the differentiation potential of CD133 cells from MS patients

and controls (N=3) we compared CD133 cells isolated from PBMCS as explained above and culture them in a specially formulated media, Methocult™ (H4434 StemCell Technologies) for 15 days to allow for the differentiation of CD133 cells into myeloid, erythroid and lymphoid lines. We found no significant difference between their differentiation potential or the number of colonies. The results are summarized in Figure 11.

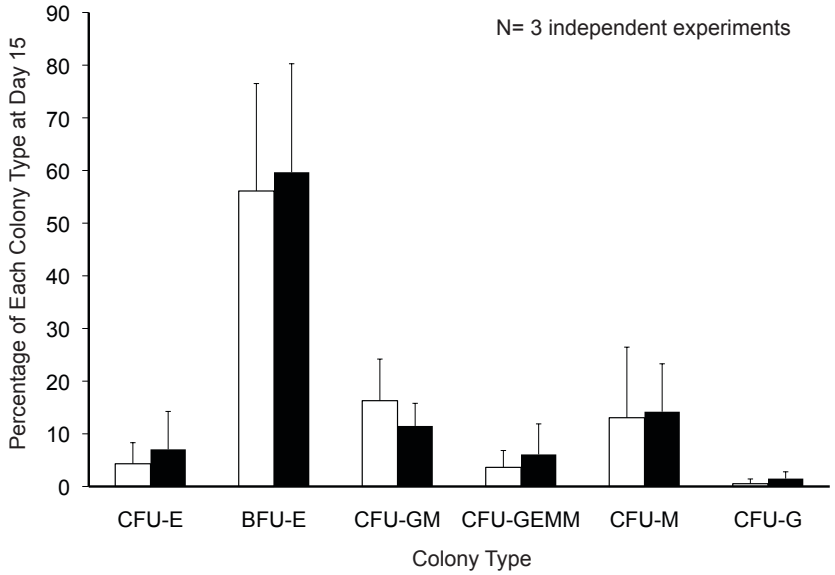


Figure 11. Colony Forming cells derived from CD133+ cells and from MS patients showed no significance in their morphology (not shown) or number of differentiated colonies. Summary of culturing 3000 CD133+ cells per 35mm plate from normal individuals or MS patients in 2 ml of human Methocult™ media (H4434 StemCell Technologies) for after 15 days.

Testing the in vitro cell proliferation potential of CD133 cells from MS patients and controls. To test the in vitro capacity of CDD133+ cells to proliferate we isolated CD133+ cells from healthy controls or MS patients as above. These cells were grown on each well of a Poly-L-Laminin (10μg/ml) coated six well plate at a density of $30x10^3$ cells per well in Cell-Gro-SCGM medium. One half of the plate was supplemented with the following growth factors: FLTK3 ligand at 150ng/ml, SCF at 150ng/ml IL-3 at 20 ng/ml to promote cell proliferation, while the other half was left as a reference control. Cells were photographed and viability was measured, then counted using the help of ImageJ counter subroutine (figure 12). After normalization with respect to the untreated reference control, percentages were used to compare statistically the percentage increase in numbers in Student paired *t*-test.

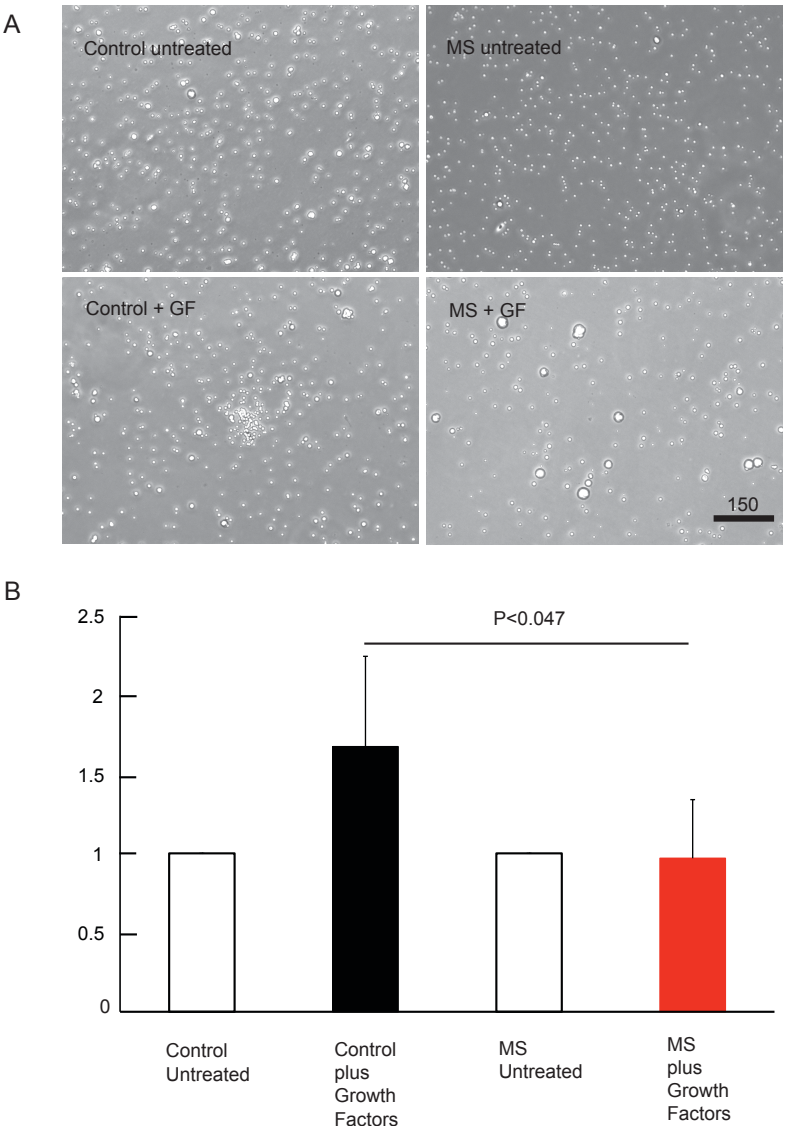


Figure 12. Cell Proliferation Assay of CD133+ cells from healthy controls and MS patients. A. Photograph of cells showing their appearance after 6 days in culture. B. Statistical representation of the analysis of three independent experiments performed in triplicate (scale bar 150μm. *t*-Test, P<0.047, bars are standard deviation.). Abbreviations, GF: growth factors, MS: multiple sclerosis patient).

Testing the response of CD133 to SDF-1 alpha. To test if CD133 from MS patients differed from their normal counterparts in their capacity to respond to SDF-1 alpha we isolated CD133+ cells from MS patients or healthy controls, labeled them with 10μM Calcein AM, and plated at concentration of $20X10^3$ per 50μl of MEM on top of a 8μm-pore size filter (NeuroProbe) that was in touch with the bottom well of a 96-well plate. One half of the six wells that were used per assay contained only MEM and was used as negative control, the other half contained SDF-1α at 10ng/ml. After two hour incubation at 37°C, 5% CO2, the fluorescence values of the bottom wells were used as an indirect measurement for the number of cells that had migrated into the bottom wells. These values were normalized to the fluorescence from the wells that did not have SDF-1α.

Results from three independent experiments performed in triplicate are shown in the graph below (Figure 13), bar graphs represent standard deviation.

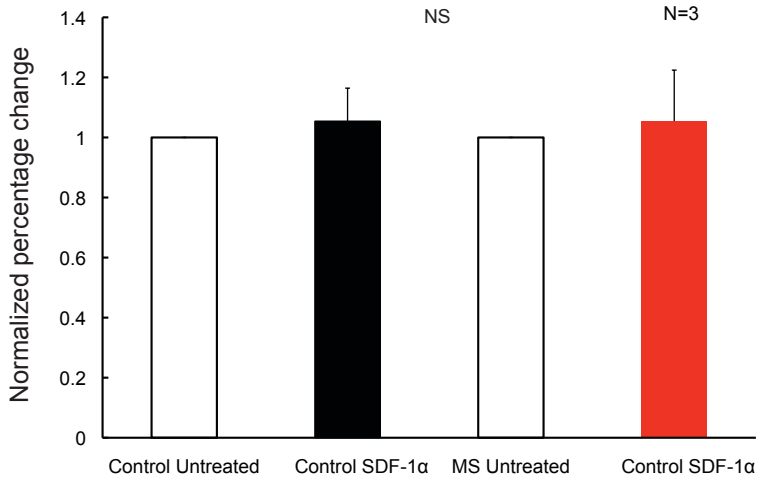


Figure 13. CD133 cells from MS patients or controls do not differ significantly in their capacity to migrate towards a source of SDF-1 alpha.

In vitro neurogenic differentiation of CD133 stem cells. Various different culture conditions were tried but none was found to induce the differentiation of CD133 into any of the neural lineages. We suspect that the neurogenic differentiation might require in vivo microenvironments that are not supported by the in vitro conditions. This is being analyzed using the mutant Shiverer as a host for transplantation of CD133 cells. Animals have been transplanted with CD133 cells marked with Cell Tracker (Invitrogen). Transplanted mice are being analyzed.

Confirmation by QRT-PCR of the microarray expression pattern of Brain-Derived Neurotrophic Factor (BDNF). BDNF is a neurotrophin which belongs to a family of neural factors involved neuronal survival, synaptic formation and neuronal differentiation. It has been shown to be de-regulated in Peripheral blood mononuclear cells of patients of RRMS. Consistent with these data, we have previously shown in our microarray analysis that women with RRMS have increased levels of mRNA encoding BDNF. We have confirmed this by quantitative RT-PCR performed with primers targeting BDNF in the same women (Figure 14). We are currently determining for changes in protein levels.

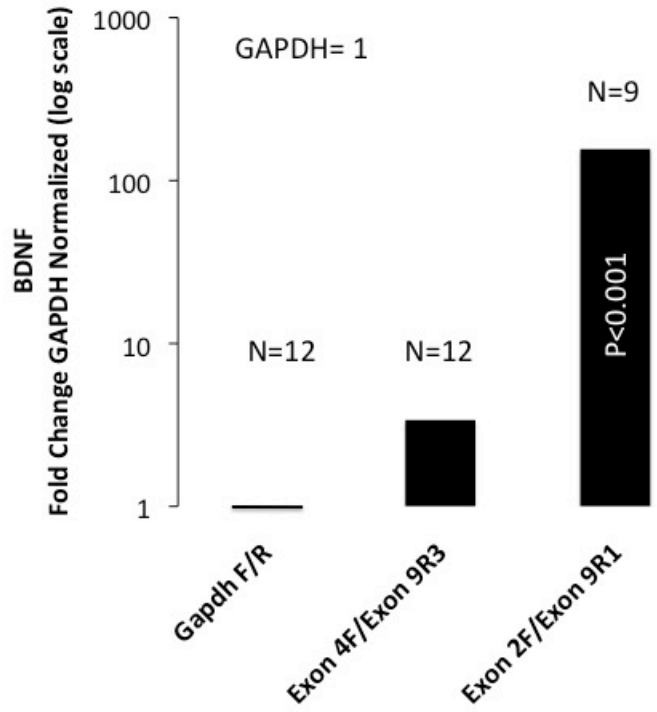


Figure 14. QRT-PCR analysis shows BDNF is increased in women with RRMS. Total RNA from 12 women or pool controls was used to detect mRNA coding for BDNF using Sybergreen. The data were normalized with respect to GAPDH and differences were calculated with the DeltaCT method.

Changes in Calnexin and Galactosylceramidase levels in PBMCs from RRMS patients. Two other interesting gens that were identified in our gene array analysis were calnexin (CNX) and Galactosylceramidase (GALC). We performed quantitative tests to measure for the abundance of mRNAs and proteins for both CNX and GALC. Quantitative real time polymerase chain reaction (qRT-PCR) analysis confirmed a significant reduction in the levels of CNX mRNA in a subset (n=7) of the RRMS mRNAs used for the wide genome analysis (Figure 15A). Furthermore, immunoblotting analysis confirmed a decrease in the abundance of the CNX proteins in total extracts of PBMCs from these RRMS patients. Figure 15B shows CNX protein levels by immunoblotting in just two different cases from the cohort of MS samples. These results confirmed that CNX expression is significantly lower than normal in PBMCs from RRMS patients. This may indicate that RRMS is compounded by a dysfunctional ER quality control apparatus, at least in PBMCs. Whether a similar defect is also present in myelin forming cells (OLs) in the brain and spinal cord of RRMS patients is presently unknown. Because CNX is one chaperone of fundamental importance for the production of correctly folded proteins, a reduction of CNX may lead to the introduction of errors during protein synthesis, transport and assembly, debilitating the mechanisms to maintain myelin during adult life.

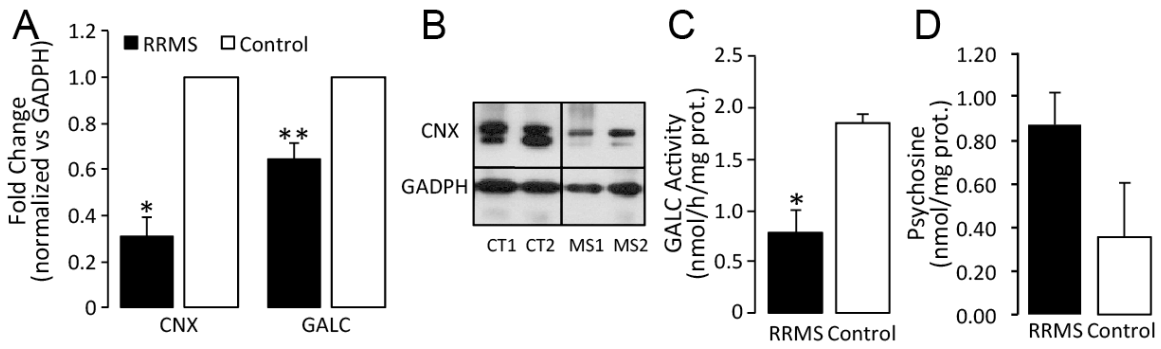


Figure 15. Quantitative analyses of CNX and GALC levels in RRMS PBMCs. A) Quantitative controls for CNX and GALC mRNA levels in RRMS PBMCs were performed using quantitative real time PCR. PBMC RNA was isolated from RRMS patients (n=7) and healthy volunteers (n=25). Real time PCR was performed using syber-green specific primers for human CNX and GALC and normalized for GAPDH. CNX and GALC levels in control PBMCs are set to =1. *p=0.0047; **p=0.00022 (Anova). B) Immunoblotting analysis for CNX in RRMS PBMCs. PBMCs from two control (CT1, CT2) and two RRMS (MS1, MS2) patients were analyzed using a specific anti-CNX antibody. GAPDH was used as loading control. C) GALC activity was measured in PBMCs isolated from RRMS donors (n=6) and healthy volunteers (n=4) by fluorometriy. *p=0.019 (Anova). D) Psychosine content was measured in lipid extracts prepared from total PBMCs (106 cell/ sample) from RRMS donors (n=3) and healthy volunteers (n=3) by liquid chromatography-mass spectrometry.

Analyses for GALC expression also confirmed our original finding in the gene array study. qRT-PCR analysis measured significant reductions of GALC mRNA in seven of the RRMS mRNAs used for the microarray analysis (Figure 15A). We measured the residual level of GALC enzymatic activity in protein extracts of RRMS PBMCs using a highly sensitive fluorometric method. Figure 14C shows quantitative data revealing a reduction of about 50% of GALC activity in RRMS PBMCs respect control values. Deficient GALC activity is known to lead to abnormal degradation of GALC substrates. To examine if RRMS PBMCs had altered levels of psychosine, we processed 3 of the RRMS PBMCs samples for lipid extraction and purification of psychosine. Psychosine levels were measured using LC-MS-MS, which provides an unparalleled level of sensitivity in the order of picomolar concentrations. Figure 15D shows LC-MS-MS quantitative data indicating increased levels of psychosine in RRMS PBMCs in comparison to control levels. These results indicate a defective catabolic pathway of the neurotoxin psychosine in PBMCs from RRMS. Because psychosine is a potent neurotoxin for OLs, accumulation of psychosine may also contribute as a compounding pathogenic factor during demyelination in RRMS.

KEY RESEARCH ACCOMPLISHMENTS

1) Enrollment of MS patients from UIC and Rush University sites was completed.
2) Successfully quantitated the abundance of CD133+ stem cells from peripheral blood from healthy and MS volunteers and anayzed their hematopoietic potential. Neural potential is still being studied in an in vivo model.
3) Gene array analyses of PBMCs from MS and healthy controls have been done and yielded very significant data. New research lines are derived from this project.

REPORTABLE OUTCOMES

1) One manuscript with the findings of CD133 stem cells in blood is in consideration for publication.
2) A second manuscript on gene array data is prepared and being corrected for final submission.
3) Two symposium presentations of results were done at the last annual meeting of the American Society for Neurochemistry, Baltimore, march 2012 and at the annual meeting of the Society for Neuroscience, Washington 2011.
4) A repository of cells and plasma from RRMS patients is available in our laboratory.
5) Part of this work supported a funding request to the National Multiple Sclerosis Society. This request was approved and funded for one year on July 2012.
6) Part of this work supported a funding request to DoD. This application passed the first level of review and is pending scientific review.

CONCLUSION

Our experiments have focussed determining the levels of circulating CD133+ stem cells in the blood of RMMS patients. CD133 stem cells are significally at low levels in the blood of MS patients. The significance of this is still unclear, but it may relate to the changes in abundance of stem cells during the disease process or rapid turn over or rapid differentiation in specific cell lineages. In vitro and in vivo experiments are undergoing to characterize the properties of these cells and to determine their potential to generate remyelinating oligodendrocytes. In addition, we have ideintified several genes which are deregulated in MS. Two of these, calnexin and galactosylceramidase, are of interest because they are associated with myelin conditions. We are evaluating their relevance in MS in two funding applications. In respect to the timeline for the third year of this study, we have worked on proposed experiments and have optimized the majority of experimental conditions. We have requested a no cost extension to complete final experiments and publication of relevant data. This request was approved by DoD.

REFERENCES

N/A

APPENDICES

N/A

SUPPORTING DATA

N/A