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Environmental Factors, Genetics, and Epigenetics in MS Susceptibility and Clinical Course

Investigation of probiotics in multiple sclerosis

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Abstract: None of the disease-modifying therapies (DMTs) currently being used for the management of multiple sclerosis (MS) are 100% effective. In addition, side effects associated with the use of these DMTs have limited the practice of combination therapy. Hence, there is a need for safe immunomodulatory agents to fine-tune the management of MS. The gut microbiome plays an important role in autoimmunity, and several studies have reported alterations in the gut microbiome of MS patients. Studies in animal model of MS have identified members of the gut commensal microflora that exacerbate or ameliorate neuroinflammation. Probiotics represent an oral, non-toxic immunomodulatory agent that could be used in combination with current MS therapy. We designed a pilot study to investigate the effect of VSL3 on the gut microbiome and peripheral immune system function in healthy controls and MS patients. VSL3 administration was associated with increased abundance of many taxa with enriched taxa predominated by *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species. At the immune level, VSL3 administration induced an anti-inflammatory peripheral immune response characterized by decreased frequency of intermediate monocytes ($CD14^{high}CD16^{low}$), decreased mean fluorescence intensity (MFI) of CD80 on classical monocytes as well as decreased human leukocyte antigen–antigen D related (HLA-DR) MFI on dendritic cells.

Keywords: Probiotic, gut microbiome, MS, peripheral immune response

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Introduction

The gut microbiome has been implicated in several autoimmune disorders including inflammatory bowel disease, rheumatoid arthritis, and MS.[6–9]Studies in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, have shown that perturbation of the composition of the gut microbiota affects mice susceptibility to develop EAE.[10,11] Others have shown that colonization of mice with *Bacteroides fragilis* ameliorates EAE and that colonization of mice with segmented filamentous bacteria (SFB), a Th17 inducer, exacerbates EAE.9,10 Tryptophan-derived Aryl hydrocarbon receptor (AHR) ligands produced by intestinal commensals can reach the central nervous system (CNS) to modulate astrocyte function and suppress inflammation and neurodegeneration.[14] A recent study showed that the human gut–derived commensal *Prevotella* suppresses EAE in a human leukocyte antigen (HLA) class II transgenic mouse model.[15] We have reported alteration in the gut microbiome of MS patients.[6] Several other groups have also reported dysbiosis in the gut microbiome of MS subjects.[8,9] Recently, it was shown that high frequency of intestinal TH17 cells correlates with alterations in gut microbiota composition as well as increased disease activity in MS.[16] These findings suggest that manipulation of the gut microbiome by the use of probiotics, for example, could potentially be beneficial to MS patients. Probiotic VSL3 is a cocktail of eight bacteria that has a good safety profile. Probiotic VSL3 has been shown to induce interleukin (IL)-10^{+} and IL-10-dependent transforming growth factor beta (TGF-β)-bearing regulatory cells in the gut of a mouse model of colitis.[17] Furthermore, VSL3 has been shown to promote an anti-inflammatory response in the gut of a mouse model of peanut allergy and diabetes.[18,19] A recent study conducted in antiretroviral therapy (ART)-treated HIV-1-positive patients reports an association between VSL3 administration and decreased T cell activation in the peripheral blood and in gut-associated lymphoid tissue.[20] VSL3 immunomodulatory properties extend to the CNS. For example, VSL3 has been shown to promote neuroprotection in a mouse model of traumatic spinal cord injury.[21] Another study

reported that VSL3 administration was associated with decreased microglial cell activation as well as decreased CNS monocytes infiltration leading to improve sickness behavior in a mouse model of liver inflammation.[22] In humans, VSL3 has been shown to be beneficial to patients with pouchitis, ulcerative colitis, and diabetes.[23–25] Very little is known about the effect of VSL3 on peripheral immune function in humans. Hence, the goal of this study is to investigate the effect of probiotic VSL3 on peripheral immune function in healthy controls and MS subjects.

Material and methods

Subjects

Relapsing-remitting multiple sclerosis (MS) subjects on glatiramer acetate ($N = 7$) or untreated ($N = 2$) and healthy controls ($N = 13$) were orally administered VSL3 twice daily (total 3600 billion CFU/day) for 2 months. The MS and control cohorts had comparable demographic characteristics except that the MS cohort was older (35 ± 14 vs 50 ± 10, $p = 0.01$) and had higher body mass index (BMI; 25.8 ± 4.1 vs 31.1 ± 5.6, $p = 0.03$). The subjects enrolled in this study were not on a dietary restriction. VSL3 is a probiotic mixture containing 3×10^{11}/g of viable lyophilized bacteria including four strains of *Lactobacillus* (*Lactobacillus paracasei* DSM 24734, *Lactobacillus plantarum* DSM 24730, *Lactobacillus acidophilus* DSM 24735, and *Lactobacillus delbruckeii* subspecies *bulgaricus* DSM 24734), three strains of *Bifidobacterium* (*Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, and *Bifidobacterium breve* DSM 24732), and one strain of *Streptococcus* (*Streptococcus thermophilus* DSM 24731) currently sold in the United States under the brand name Visbiome and in Europe under the brand name Vivomixx. None of the MS patients had an active relapse at the time of study enrollment. Stool specimens were collected prior to, at discontinuation of therapy, and 3 months thereafter. Frozen peripheral blood mononuclear cells (PBMCs) were used for immune cells profiling. Stool samples were used for 16S profiling by Illumina MiSeq. This study was approved by the Partners Human Research Committee and all participants signed an informed consent before any data collection or study procedure.

Sample collection. Stool samples were obtained from patients by providing them with stool containers. Subjects collected samples produced at anytime of day with no specific dietary restrictions. Collection containers were then placed in boxes with ice packs for immediate shipment to our laboratory via overnight delivery at a maintained temperature of 0°C. On receipt of samples, they were frozen at −80°C until DNA extraction.

16S rRNA microbial community profiling. Fecal DNA was isolated (MoBio PowerLyzer PowerSoil Kit) and the V4 region of the bacterial 16S rRNA gene was amplified using barcoded forward primers (515f, 926R) from the Earth Microbiome Project[1] and http://www.earthmicrobiome.org/protocols-and-standards/16s/. Samples were sequenced by paired end 250 base pair reads at the Harvard Medical School Biopolymer Facility using the MiSeq platform (Illumina). Sequence quality was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The median Phred quality scores were above Q30 for the first 230 basepairs and above Q20 for the entire sequence for forward read; however, the median Phred Q scores for read 2 dropped below Q20 within the first 50 bases of the reverse read. Thus, we utilized only the forward read of 250 bases, corresponding to the V4 region of the microbial 16S rRNA. Downstream analysis was performed in QIIME (Qualitative Insights into Microbial Ecology).[2] Sequences were demultiplexed and quality filtered in which reads are truncated if two consecutive bases fall below a quality score of Q20 (1% error), and reads that are <75% of full length are discarded.[1] Operational taxonomic units (OTUs) were picked using the open reference method Sumaclust (http://metabarcoding.org/sumatra) and SortMeRNA.[3] Taxonomy is picked against the Greengenes 13.8 release database (http://greengenes.secondgenome.com) using a 97% similarity threshold. Alpha diversity was calculated with the phylogenetic diversity_whole_tree, Shannon diversity, and richness metrics, and beta diversity was calculated using weighted and unweighted UniFrac Distances.

Fluorescence-activated cell sorting analysis. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ). The PBMCs were stored in liquid nitrogen until use and samples from the same individuals were processed and analyzed together to minimize batch-to-batch variability and to allow for comparisons of mean fluorescence intensities (MFIs). All antibodies used for surface staining were obtained from Biolegend. The viability dyes, violet fluorescent reactive dye (VVD) and Efluor 506, were obtained from Life Technologies and BD Biosciences respectively. All antibodies were titrated to obtain optimal signal-to-noise ratio. Samples were acquired using a BD LSR II flow cytometer (BD Bioscience) and analyzed using flowjow software.

Statistical analyses. Statistical differences for alpha and beta diversities were calculated with PRISM 7.0

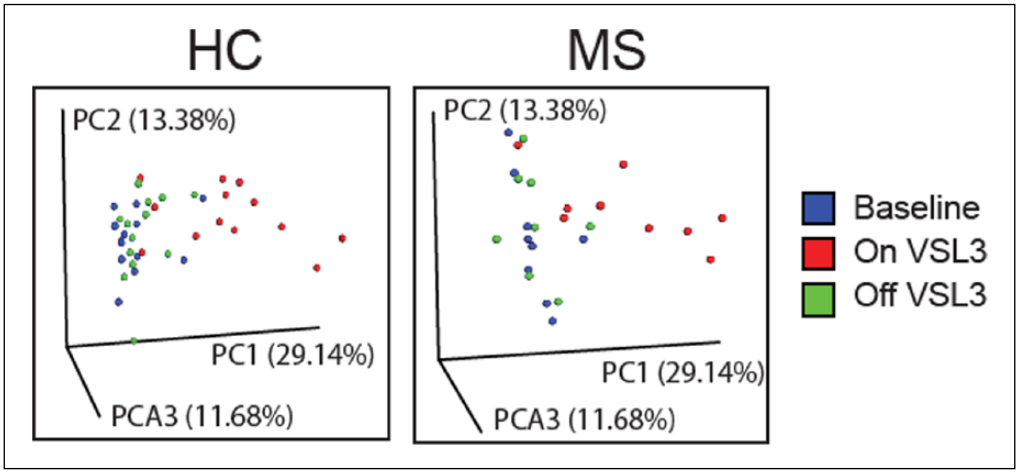

Figure 1. Probiotic VSL3 effect on microbial community structure. Principal coordinate analysis of weighted Unifrac distances colored according to time points in healthy controls (HC) and multiple sclerosis patients (MS).

software (GraphPad) using paired one-way analysis of variance (ANOVA) with the Tukey post-test to assess significance at the 0.05 level and to correct for multiple comparisons. Differences in beta-diversity visualized on the PCoA plots were calculated with the ADONIS test at 999 permutations[4] using the R vegan package.[5] Change over time in fluorescence-activated cell sorting (FACS) analyses was assessed using the Wilcoxon signed rank test. For this analysis, each pair of time points was compared, and the two groups were treated separately.

Product tolerability and safety

All study participants were greater than 90% compliant with probiotic supplementation. No serious adverse reactions were reported by the patients or identified by the clinicians. None of the patients experienced a relapse during or after probiotic supplementation.

Results

Administration of probiotic VSL3 is associated with changes in the structure and composition of the gut microbiome in healthy controls and MS subjects

Microbial DNA was extracted from fecal samples and 16S rRNA gene sequencing was performed on Illumina MiSeq platforms using primers targeting the V4 variable region. To assess overall differences in microbial community structure in MS patients and controls, we calculated measures of alpha and beta diversities. Alpha diversity is a measurement of the ecological diversity within a given sample (i.e. types of microbial species in a microbiota sample), whereas beta diversity compares the bacterial composition between samples (i.e. whether the microbial species are similar between microbiota samples). Shannon index, an alpha diversity measurement of both richness (number of species present) and evenness (whether there are predominant and rare species or equal representation of all species), was measured at multiple sequencing depths using rarefaction curves. We found that administration of VSL3 was associated with decreased alpha diversity in healthy controls. No change in alpha diversity was observed in MS subjects following VSL3 administration.

To determine whether overall microbial community structure was different among the three time points, we calculated differences in beta diversity using the weighted UniFrac metric. Statistical analysis of the resulting matrices using the ADONIS test for clustering revealed that following VSL3 administration, there was a change in overall microbial community structure in controls (p=0.048, Figure 1). We saw a similar trend in MS patients (p=0.10, Figure 1). The overall microbial community structure shifted back to baseline level following discontinuation of VSL3 in controls and MS patients (p=0.58, p=0.54, respectively; Figure 1). We next investigated whether the relative abundance of specific bacteria strains differed following administration and discontinuation of VSL3 in both controls and MS patients.

VSL3 administration was associated with an increase in the relative abundance of several species including *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species in both controls and MS patients. The relative abundance of these bacteria strains returned to baseline following discontinuation of VSL3. Of note, the reduced alpha diversity observed in controls most

likely results from the fact that probiotic administration was associated with an increase in the relative abundance of specific bacteria strains, thereby reducing the evenness within the community and likely also competitively reducing the number of other bacteria species in controls. We do see a similar trend in our MS population, thus the failure to see a statistically significant change in alpha diversity in MS patients may just be a power issue. Alternatively, VSL3 may have a more competitive action in controls compared to MS patients and limits colonization of other microbiota.

VSL3 effect on peripheral immune function

Blood samples from all subjects were collected at baseline, 2 months after initiation of VSL3, and at 3 months post discontinuation of VSL3. PBMCs were isolated from these blood samples at all three time points and subsequently used for immune cell profiling by flow cytometry. Given prior reports of increased latency-associated peptide (LAP) and IL-10$^+$ T regulatory cells (T regs) in the gut following VSL3 administration, we conducted experiments to investigate the effect of probiotic VSL3 on peripheral T regulatory cells. No significant change in the relative frequencies of CD4$^+$CD127^{low}CD25^{high},[26] CD4$^+$IL-10$^+$ or CD39$^+$CD127^{low}CD25^{high} T regs were observed following administration of VSL3. Discontinuation of VSL3 was associated with decreased relative frequency of IL-10$^+$ T regs (2.88% vs 1.63%, $p = 0.0269$) as well as decreased relative frequency of CD39$^+$CD127^{low}CD25^{high} T regs (71.1% vs 66.9%, $p = 0.011$) in controls. We also observed a trend toward decreased frequency of LAP$^+$ T regs in controls (1.00% vs 0.61%, p=0.065). Subsequently, we evaluated the frequencies of CD4$^+$ and CD8$^+$ T cells. We found a trend toward increase in frequency of effector memory CD8 T cells following probiotic supplementation in MS patients (23.6% vs 30.4%, $p = 0.080$). The relative frequencies of Th1 and Th17 cells were trending down following VSL3 administration in both controls and MS patients. We also observed that the relative frequency of IL-10$^+$ T regs was trending up following VSL3 administration in MS patients.

We did not observe a change in the frequencies of naive CD4 or naive CD8 T cells, central memory CD4 T cells, effector memory CD4 T cells, or central memory CD8 T cells following VSL3 supplementation in our cohort.

Next, we performed immune cell profiling of monocytes from healthy control and MS patients at the three time points and found that administration of VSL3 was associated with decreased frequency of intermediate monocytes (CD14^{high}CD16^{low}) in MS patients (9.07% vs 7.58%, $p = 0.039$). In controls, we observed a trend toward decreased frequency of inflammatory monocytes (CD14^{low}CD16^{high}) following VSL3 supplementation (6.15% vs 4.68%, p=0.068). Discontinuation of VSL3 was associated with increased frequency of inflammatory monocytes (CD14^{low}CD16^{high}) in healthy controls (4.68% vs 7.08%, $p = 0.033$). In healthy controls, we also observed decreased MFI of costimulatory marker CD80 on classical monocytes (88 vs 80.6, p=0.048) following administration of VSL3. In MS patients, we observed decreased MFI of human leukocyte antigen–antigen D related (HLA-DR) on myeloid-derived dendritic cells (CD45$^+$LIN$^-$ CD11c$^+$) following administration of VSL3 (1890 vs 1510, $p = 0.0156$). Of note, we did not observe a change in the frequencies of B cells, natural killer (NK) cells, and myeloid or plasmacytoid dendritic cells following administration of VSL3 in our cohort.

Conclusion

Our studies showed that administration of probiotic VSL3 was associated with increase in the relative abundance of several species with a predominant enrichment of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species in both controls and MS patients. At the immune level, VSL3 effect was predominantly seen on monocytes and dendritic cells. VSL3 administration induced an anti-inflammatory peripheral innate immune response characterized by decreased frequency of intermediate monocytes (CD14^{high}CD16^{low}) in MS subjects as well as decreased MFI of costimulatory marker CD80 on classical monocytes in controls. In controls, we also found a trend toward decreased frequency of inflammatory monocytes following VSL3 supplementation. Administration of VSL3 was also associated with decreased MFI of HLA-DR on myeloid-derived dendritic cells (CD45$^+$LIN$^-$CD11c$^+$) in MS subjects. On the other hand, discontinuation of VSL3 induced a pro-inflammatory immune response characterized by increased frequency of inflammatory monocytes (CD14^{low}CD16^{high}) in controls. We also observed decreased frequency of IL-10$^+$ T regs in controls as well as a trend toward decreased frequency of LAP$^+$ T regs following discontinuation of VSL3. Taken together, these findings suggest that the use of probiotic VSL3 can induce changes in the gut microbiota composition that are associated with an anti-inflammatory peripheral innate immune response in controls and MS patients. These immunomodulatory effects did not persist after discontinuation of VSL3. Additional studies

are needed to validate these findings in a larger MS cohort and to determine if VSL3-induced anti-inflammatory peripheral immune response is associated with improved disease outcome in MS patients.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Stephanie K Tankou has received honorarium from Teva Pharmaceuticals during the conduct of the study.
Dr. Stankiewicz reports grants from Teva Neuroscience during the conduct of the study.
Dr. Howard L Weiner reports grant from Teva Neuroscience during the conduct of the study.
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