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No proinflammatory signature in CD34+ hematopoietic progenitor cells in multiple sclerosis patients

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Abstract
Autologous hematopoietic stem cell transplantation (aHSCT) has been used as a therapeutic approach in multiple sclerosis (MS). However, it is still unclear if the immune system that emerges from autologous CD34+ hematopoietic progenitor cells (HPC) of MS patients is pre-conditioned to re-develop the proinflammatory phenotype. The objective of this article is to compare the whole genome gene and microRNA expression signature in CD34+ HPC of MS patients and healthy donors (HD). CD34+ HPC were isolated from peripheral blood of eight MS patients and five HD and analyzed by whole genome gene expression and microRNA expression microarray. Among the differentially expressed genes (DEGs) only TNNT1 reached statistical significance (logFC=3.1, p<0.01). The microRNA expression was not significantly different between MS patients and HD. We did not find significant alterations of gene expression or microRNA profiles in CD34+ HPCs of MS patients. Our results support the use of aHSCT for treatment of MS.

Keywords
multiple sclerosis, hematopoietic stem cell transplantation, gene expression

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Introduction
Intense immunosuppression followed by autologous hematopoietic stem cell transplantation (aHSCT) is a potential treatment for patients suffering from aggressive multiple sclerosis (MS).1 aHSCT is able to induce a long-lasting remission of inflammatory disease activity, which can persist years beyond complete immune reconstitution. The rationale for aHSCT in MS is based on the concept that lympho-/myeloablative conditioning eliminates pathogenic autoreactive immune cells and facilitates the regeneration of a new and tolerant immune system from CD34+ hematopoietic progenitor cells (HPC). In fact, thorough analysis of the T cell repertoire in the regenerating immune system after aHSCT in MS supports that a new and antigen-naïve T cell repertoire develops from the HPC compartment via thymic regeneration.2 To date, it remains unresolved whether autoimmunity in MS is merely a consequence of loss of peripheral immune tolerance or whether it results from immune dysregulation, which is already predetermined in HPC. To

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approach this key point we compared the global gene- and miRNA expression profiles of CD34+ and CD34- cells collected from MS patients and healthy donors (HD).

**Patients and methods**

**Patients and controls**

MS patients (n=8) with relapsing–remitting (RRMS) or secondary-progressive (SPMS) disease (mean disease duration 10 years, range 6–16 years) were treated with aHSCT at the University of Hamburg, Germany (four female SPMS) and the Haematology Unit, Careggi Hospital of Florence, Italy (two male RRMS and two female SPMS). All patients had previously received immunomodulatory and/or immunosuppressive therapy. Control HPC samples were obtained from five age-matched HD (three female). All patients provided written informed consent and all study protocols were in accordance with the Declaration of Helsinki and approved by Institutional Review Boards at each centre.

**Mobilization and collection of CD34+ cells**

Before collecting HPC from peripheral blood by leukopheresis, the Hamburg MS cohort and the five HD were mobilized with subcutaneous injection of granulocyte colony-stimulating factor (G-CSF) analogue (2x5µg/kg/day) for 5–8 days. The Florence cohort was mobilized with intravenous cyclophosphamide (Cy, 4g/m²) and G-CSF (5µg/kg/day) until cell harvest by leukocytapheresis. Cell collections were frozen in liquid nitrogen according to standard procedures. All samples were thawed and processed at one centre by a standardized protocol and CD34+ HPC purified by magnetic bead separation using the autoMACS system (Miltenyi). The control samples consisted of the remaining CD34- negative cell fraction after magnetic bead separation, i.e. a population of peripheral blood mononuclear cells. Purity and viability of CD34+ cells were analyzed by FACS and revealed a mean of 84.8% (range 73.5 – 89.7%) viable CD34+ cells. There was no difference in the purity or viability of cells between MS patients and HD (see supplemental methods).

**Microarray analysis**

Whole genome gene expression was analyzed with the Human 4x44K Design Array (Agilent-Technologies). Differentially expressed genes (DEGs) of interest were confirmed by quantitative rtPCR. miRNA profiling was performed with the Human miRNA Array V2.0 (Agilent-Technologies). The microarray data were generated conforming to the MIAME guidelines and are deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27694).

**Statistics and bioinformatics**

Standard microarray analysis methods were used for processing intensity data and normalization (see supplemental methods). Individual genes were considered differentially expressed above a fold-change of 1.7 (logFC>0.7). P-values were corrected for multiple testing. miRNA data were analyzed in an analogous way.

**Results**

**Gene expression analysis**

Principal component analysis (PCA) confirmed separation between CD34+ and CD34- samples and showed a clear clustering of CD34+ cells according to the mobilization regimen.
Accordingly, we found 2801 DEGs in CD34+ (adj. \( p \)-value\( \leq 0.05 \)) and 9440 DEGs in CD34- (adj. \( p \)-value\( \leq 0.05 \)) cells comparing MS patients mobilized with G-CSF only or Cy/G-CSF, respectively.

Comparing DEGs in CD34+ HPC of MS patients and HD, both mobilized with G-CSF only, we found 297 DEGs (logFC>0.7), but the TNNT1 gene was the only DEG with statistical significance after Benjamini-Hochberg correction (logFC=3.1, adj. \( p \)<0.01; Table 1).

Comparing CD34- cells between MS and HD we found 167 DEGs (logFC>0.7), however none reached statistical significance.

### miRNA expression analysis

miRNA expression was analyzed in samples obtained from MS patients mobilized with G-CSF only and HD mobilized with G-CSF only. None of the miRNA showed statistically significant differential expression levels comparing MS patients and HD mobilized with G-CSF only.

### Discussion

The immunologic rationale for aHSCT as treatment for autoimmune diseases like MS is being discussed intensively.

| Table 1. Ten most up- and down-regulated genes in CD34+ HPCs comparing MS patients and healthy donors. |
|---|---|---|---|---|
| **Gene symbol** | **Description** | **logFC** | **\( p \)-value\(^1\)** | **Function** |
| **Upregulated** |  |  |  |  |
| TNNT1 | Troponin T type 1 (skeletal, slow) | 3.197 | 0.0000001\(^2\) | Subunit of troponin, striated muscle contraction |
| FOXE1 | Forkhead box E1 (thyroid transcription factor 2) | 2.269 | 0.02534 | Thyroid transcription factor |
| HLA-DQB1 | Major histocompatibility complex class II. DQ beta | 1.922 | 0.00560 | HLA class II beta chain, expressed in antigen presenting cells |
| SOX17 | SRY (sex determining region Y)-box 17 | 1.869 | 0.03167 | Transcription factor, determination of the cell fate |
| GPR141 | G protein-coupled receptor 141 | 1.780 | 0.11199 | Rhodopsin family of G protein-coupled receptors |
| NEUROG1 | Neurogenin 1 | 1.771 | 0.05177 | Promotes neurogenesis, inhibits astrocyte differentiation |
| LOC647121 | Embigin homolog (mouse) pseudogene | 1.696 | 0.02507 | Not known |
| TNXB | Tenascin XB | 1.587 | 0.06001 | Extracellular matrix glycoprotein, anti-adhesive effect |
| DKFZP434I0714 | Hypothetical protein DKFZP434I0714 | 1.574 | 0.04861 | Not known |
| ZSCAN10 | Zinc finger and SCAN domain containing 10 | 1.568 | 0.07729 | Transcription factor, metal ion binding |
| **Downregulated** |  |  |  |  |
| DDX3Y | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3 | -2.454 | 0.14760 | Putative RNA helicases |
| PRSS21 | Protease.serine. 21 (testisin) | -2.209 | 0.00003 | Cell-surface anchored serine protease |
| CTSG | Cathepsin G | -1.983 | 0.04575 | Member of the peptidase S1 protein family, neutrophil granulocytes |
| MPO | Myeloperoxidase | -1.909 | 0.00095 | Neutrophil azurophilic granules, microbicidal activity |
| FAM78A | Family with sequence similarity 78.member A | -1.844 | 0.06178 | Not known |
| EIF1AY | Eukaryotic translation initiation factor 1A | -1.695 | 0.09977 | Eukaryotic translation initiation factor |
| JARID1D | Jumonji. AT rich interactive domain 1D | -1.692 | 0.06539 | Protein containing zinc finger domains |
| CYorf15A | Chromosome Y open reading frame 15A | -1.580 | 0.09665 | Not known |
| ELA2 | Elastase 2.neutrophil | -1.516 | 0.00429 | Serine protease, neutrophil granulocytes |
| DNTT | Deoxynucleotidyltransferase. terminal | -1.428 | 0.00428 | Template-independent DNA polymerase |

HPCs: hematopoietic progenitor cells; logFC: log fold change; \(^1\): \( p \)-value (not adjusted); \(^2\): only gene which maintained statistical significance after correction (Benjamini-Hochberg).
among basic and clinical immunologists in recent years.2,7,8 A key issue has been the question whether replacement of the autoreactive immune system by autologous HPC is able to stop the autoimmune process for long or forever, or alternatively whether the autoaggressive immunity will rebound after hematologic reconstitution. If the latter occurred it would indicate that the autoimmune process is pre-programmed in HPCs of genetically predisposed individuals rather than evolving at the stage of mature T cells and in the peripheral immune system. In this study we approached this question by comparing the gene expression profile of CD34+ HPCs collected from MS patients before autologous transplantation with CD34+ HPCs or from HDs. To the best of our knowledge, this is the first study to analyze the gene expression profile of CD34+ HPC in an autoimmune disease.

The results of this study support the view that HPC of MS patients are not pre-conditioned towards autoimmunity. We did not find significant alteration in the gene expression profile of CD34+ HPC in MS. Only one DEG (TNN1) maintained statistical significance after correction for multiple comparisons (Table 1). TNN1 encodes a subunit of troponins involved in contraction of slow skeletal muscle. Of note, the TNN1 gene is expressed on chromosome 19q13, which carries predisposing loci for several autoimmune diseases, but with conflicting results in MS.9–11 A recent genome-wide association study did not find SNPs (single nucleotide polymorphism) associated with MS in the TNN1 gene.12

Comparison of miRNA expression profiles of CD34+ HPC between MS and HD did not reveal statistically significant differences, thereby corroborating our DEG results lacking substantial alterations in CD34+ cells in MS. There were no statistically significant DEGs in CD34- cells comparing MS and HD. The interpretation of our results must consider that the mobilization regimen with G-CSF provides a strong stimulus to the peripheral immune compartments and the stem cell niche and might thereby overshadow more subtle differences in the gene expression pattern of CD34- and CD34+ cells. Currently, experts recommend that the mobilization regimen for HSCT in MS should include G-CSF and cyclophosphamide, which precludes any comparison with HD. Since the mobilization regimen clearly influenced gene expression and HD are always mobilized by G-CSF only, our patient cohort provided a unique opportunity to directly compare the gene and miRNA expression profile of highly purified CD34+ cells from MS patients with HD. Consistent with our results, it has been shown that both the gene and miRNA expression differ depending on the stem-cell source and the mobilization regimen used.13–15 Studies analyzing gene and miRNA expression in hematopoiesis or hematological malignancies mainly used HPC obtained by bone marrow aspiration or from in-vitro cultured cells, precluding a direct comparison with our results. A caveat in the interpretation of our study is the small number of samples, which leaves the possibility of a false negative result.

In summary, we did not find significant alterations of gene expression or miRNA profiles in CD34+ HPCs of MS patients. Thus, we provide evidence that the immune deviation seen in the peripheral immune system in MS patients is probably not at the CD34+ precursor cell stage. One must consider that the immune changes seen in MS may represent a secondary response to a primary CNS pathology. Nevertheless, we feel that the lack of significant alterations of gene expression or miRNA profiles in CD34+ HPCs of MS patients supports the use of autologous HPC for HSCT in MS.

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Conflict of interest statement
All authors declare no conflict of interests.

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