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Characterization of myeloid-derived suppressor cells in the peripheral blood and bone marrow of patients with chronic idiopathic neutropenia

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Chronic idiopathic neutropenia (CIN) is characterized by the persistent and unexplained reduction of peripheral blood (PB) absolute neutrophil counts (ANCs).[1,2] The pathogenesis of CIN has been associated with increased apoptosis of the granulocytic progenitor cells due to an inflammatory bone marrow (BM) microenvironment consisting of activated T lymphocytes, proinflammatory monocytes, and proapoptotic cytokines.[3–5] The myeloid-derived suppressor cells (MDSCs) are immature myeloid cells, deviating from the standard differentiation pathway during emergency myelopoiesis, that display immunomodulatory properties mainly by suppressing T-cell responses. They are recognized by the immunophenotype $CD11b^+CD33^+HLA-DR^{-/low}$ and further characterized as $CD14^+$ (monocytic, M-MDSCs) and $CD15^+$ (polymorphonuclear, PMN-MDSCs) subpopulations.[6–13]

In the present study, we explore, for the first time, the possible involvement of MDSCs in the pathophysiology of CIN by investigating their number, functional characteristics, and transcriptome profile in a group of patients ($n = 102$) and age- and sex-matched healthy controls ($n = 77$). The patients fulfilled the previously described diagnostic criteria for CIN (File S1).[2,14,15] Sixteen patients had clonal hematopoiesis identified by next-generation sequencing analysis of 40 recurrently mutated myeloid genes.[15] The clinical and laboratory data of the patients are presented in Supporting Information S1: Tables 1 and 2. The study was approved by the Institutional Review Board of the

University Hospital of Heraklion and informed consent was obtained from all subjects.

MDSC subsets were quantitated and sorted by flow cytometry in the PB mononuclear cell (PBMC) and BM mononuclear cell (BMMC) fractions according to the recommended protocol.[6] The gating strategies for MDSC quantification and sorting are presented in Figure 1A,B respectively. The methodology of the T-cell suppression assay to evaluate the function of MDSCs was performed according to the recommended standards (File S1).[6,18,19] In brief, the suppression of normal T cells was demonstrated in a heterologous system including co-culture of immunomagnetically sorted carboxy-fluorescein succinimidyl ester (CFSE)-stained T cells with PMN-MDSCs or M-MDSCs (Figure 1C) and an autologous system including cultures of CFSE-stained PBMCs versus CD33-immunomagnetically depleted PBMCs (Figure 1D). To identify the biochemical and molecular parameters associated with MDSC characterization,[6] we performed transcriptional profiling of MDSCs from patients ($n = 6$) and healthy controls ($n = 5$) using RNA sequencing (File S1 and Supporting Information S1: Table 3). The data were analyzed using the GraphPad Prism Statistical software with the nonparametric Mann–Whitney U test, the Wilcoxon signed ranks test for paired samples, and the Kruskal–Wallis test for comparison between multiple groups. The Spearman test was used for the identification of correlations between

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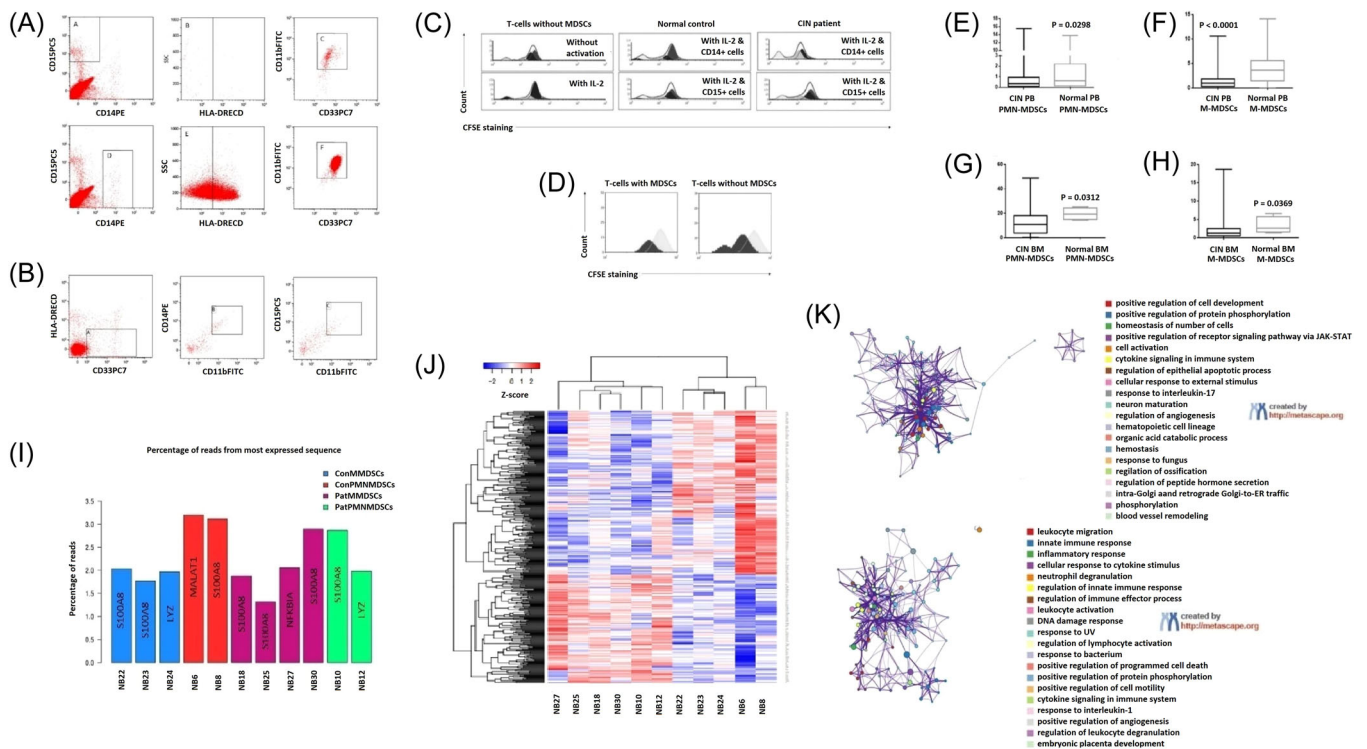

FIGURE 1 Flow cytometric analysis of MDSC subsets, quantitative analysis of PB and BM MDSC subsets in CIN patients and healthy subjects, T-cell suppression assay for testing the MDSC suppressive capacity, and distinct pattern of gene expression between CIN patients and normal controls. (A) The graphs show the gating strategy for the identification of the MDSC populations. We first gated the live cells based on the Forward Scatter (FSC) and Side Scatter (SSC) properties (not shown). For the quantification of the polymorphonuclear $CD11b^+/CD33^+/HLA-DR^{-/low}/CD15^+$ MDSCs (PMN-MDSCs) (upper graphs), analysis was initially performed in the $CD15^+$ cells gated on the live cells (Gate A), followed by analysis in the $HLA-DR^{-/low}$ cells gated on the $CD15^+$ cells (Gate B) and finally in the $CD11b^+/CD33^+$ positive cells gated on the $CD15^+HLA-DR^{-/low}$ cells (Gate C). For the quantification of the monocytic $CD11b^+/CD33^+/HLA-DR^{-/low}/CD14^+$ MDSCs (M-MDSCs) (lower graphs), we performed the same analysis following the initial gating in the $CD14^+$ cells gated on the live cells. Using this back-gating strategy, results were expressed as a proportion (%) of $CD11b^+/CD33^+/HLA-DR^{-/low}/CD15^+$ and $CD11b^+/CD33^+/HLA-DR^{-/low}/CD14^+$ MDSCs in the live PBMCs. The same gating strategy was followed for the quantification of PMN-MDSCs and M-MDSCs in the BMMC fraction. (B) The graphs show the gating strategy for the sorting of the PMN-MDSC and M-MDSC populations from the PBMC fraction. We initially gated the live cells based on the FSC and SSC properties (not shown). Gating was then performed in the $CD33^+/HLA-DR^{-/low}$ cell compartment gated on the live cells (left graph), followed by gating and sorting of the $CD11b^+/CD15^+$ cells (middle graph) and $CD11b^+/CD14^+$ cells (right graph) gated on the previous cell compartment. (C) Characteristic pictures that show CFSE staining of sorted T cells on day 3 of culture. The graph on the left depicts normal T-cell proliferation without the presence of MDSCs, the middle graph shows the decreased T-cell proliferation in the presence of MDSCs from a representative healthy control, and the graph on the right the decreased T-cell proliferation in the presence of MDSCs from a representative CIN patient. (D) Characteristic pictures that show CFSE staining of $CD5^+$ PBMCs from a healthy individual on Day 3 of culture. The graph on the left depicts the T-cell proliferation in PBMC cultures containing MDSCs, while the graph on the right shows the T-cell proliferation in PBMC cultures depleted of MDSCs (i.e., following depletion of $CD33^+$ cells). The box plots in graphs (E) and (F) show the median (horizontal line) and the 25% and 75% percentiles of the distribution of the proportions of PMN-MDSCs (graph E) and M-MDSCs (graph F) detected by flow cytometry in the PBMC fraction of CIN patients (n = 102) and healthy controls (n = 77). The respective box plots in graphs (G) and (H) depict the proportions of PMN-MDSCs and M-MDSCs in the BMMC fraction of patients (n = 37) and healthy controls (n = 8). The whiskers indicate the minimum and maximum values of the respective distributions. Comparisons have been performed using the nonparametric Wilcoxon signed ranks test for paired samples, the nonparametric Mann–Whitney U test for nonpaired data, and the statistically significant p values are shown. (I) Percentage of "reads" associated with the gene expression sequence having the highest number for each sample. (J) Pivot cluster heatmap of our data that shows the differences in the gene expression between patients and controls. In the heatmap, each row in the y-axis represents one gene and each column in the x-axis represents one case. NB22, NB23, and NB24 represent M-MDSCs from healthy controls, NB6 and NB8 represent PMN-MDSCs from healthy controls, NB18, NB25, NB27, and NB30 represent M-MDSCs from CIN patients, while NB10 and NB12 represent PMN-MDSCs from CIN patients. (K) Networks of enriched terms from the upregulated (upper part) and downregulated (lower part) genes in CIN MDSCs colored by cluster, where nodes that share the same cluster are typically close to each other. The network was visualized using Cytoscape (cytoscape.org)[16] through Metascape (metascape.org).[17] ANC, absolute neutrophil count; BM, bone marrow; BMMC, BM mononuclear cells; CFSE, carboxy-fluorescein succinimidyl ester; CIN, chronic idiopathic neutropenia; Con, control; MDSC, myeloid derived suppressor cells; M-MDSC, monocytic MDSC; Pat, patient; PB, peripheral blood; PBMC, PB mononuclear cells; PMN-MDSC, polymorphonuclear MDSC.

different parameters. Data are presented as mean value ± 1 standard deviation.

We found that CIN patients displayed significantly lower proportions of MDSCs in both the PBMC (n = 102) and BMMC (n = 37) fractions compared to healthy controls (n = 77 and n = 8, respectively) (Table 1). The proportion of PB total MDSCs (T-MDSCs: PMN-MDSCs plus M-MDSCs) was statistically significantly lower in patients compared

to controls ($p < 0.0001$). This decrease was due to the lower proportion of both PMN-MDSC and M-MDSC subsets in patients compared to controls ($p = 0.0298$ and $p < 0.0001$, respectively) (Table 1 and Figure 1E,F). The proportions of T-MDSCs and their subsets, that is, the PMN-MDSCs and M-MDSCs, were correlated with the ANCs in the total group of subjects studied ($r = 0.399$, $p < 0.0001$; $r = 0.1757$, $p = 0.0219$; $r = 0.4115$, $p < 0.0001$, respectively) (Supporting Information

TABLE 1 Proportion of PBMC and BMMC MDSC subsets in the total group and subgroups of CIN patients and age- and sex-matched healthy controls.

PBMC	CIN patients (n = 102)			Healthy controls (n = 77)
	ANC <1.0 × 10⁹/L (n = 25)	ANC ≥ 1.0 ×10⁹/L (n = 77)	Total CIN group (n = 102)	
T-MDSCs (%)				
Mean ± 1SD	2.571% ± 2.651%	2.343% ± 2.620%	2.399% ± 2.616%	5.584% ± 3.898%
Median (range)	1.769% (0.211–11.24)	1.446% (0.012–16.15)	1.497% (0.012–16.15)	5.486% (0.013–17.07)
p Value*	0.0002	<0.0001	<0.0001	
p Value**	0.6313			
PMN-MDSCs				
(%) Mean ± 1SD	0.743% ± 1.399%	1.097% ± 2.322%	1.010% ± 2.132%	1.689% ± 2.490%
Median (range)	0.332% (0.021–7.155)	0.369% (0.006–15.55)	0.341% (0.006–15.55)	0.608% (0.003–13.83)
p Value*	0.1368	0.0454	0.0298	
p Value**	0.9072			
M-MDSCs (%)				
Mean ± 1SD	1.828% ± 2.384%	1.246% ± 1.274%	1.389% ± 1.623%	3.895% ± 2.915%
Median (range)	0.986% (0.005–10.59)	0.947% (0.003–6.149)	0.963% (0.003–10.59)	3.674% (0.010–14.10)
p Value*	0.0002	<0.0001	<0.0001	
p Value**	0.4162			
BMMC	**CIN patients (n = 37)**			**Healthy controls (n = 8)**
T-MDSCs (%)				
Mean ± 1SD	15.45% ± 11.03%			22.77% ± 5.59%
Median (range)	12.93% (0.51–49.75)			23.80% (15.42–28.06)
p Value*	0.0312			
PMN-MDSCs (%)				
Mean ± 1SD	13.03% ± 11.27%			19.49% ± 4.46%
Median (range)	11.00% (0.05–48.84)			19.35% (14.11–25.16)
p Value*	0.0312			
M-MDSCs (%)				
Mean ± 1SD	2.42% ± 3.66%			3.28% ± 2.17%
Median (range)	1.28% (0.00–18.69)			2.59% (1.31–6.64)
p Value*	0.0369			

Note: Comparisons between each CIN group and healthy individuals as well as between CIN patient subgroups (i.e., between patients with varying degrees of neutropenia) have been performed with the Mann–Whitney U test.

Abbreviations: BMMC, bone marrow mononuclear cells; CIN, chronic idiopathic neutropenia; MDSC, myeloid-derived suppressor cells; M-MDSCs, monocytic MDSCs; PBMC, peripheral blood mononuclear cells; PMN-MDSCs, polymorphonuclear MDSCs; SD, standard deviation; T-MDSCs, total MDSCs (PMN-MDSCs plus M-MDSCs).

*Comparison with the healthy control group.

**Comparison between CIN patients with mild (ANC < 1.0 × 10⁹/L) and more severe (ANC ≥ 1.0 × 10⁹/L) neutropenia.

S1: Figures 1A–C). However, when a subset analysis was performed in the group of patients only, no correlation was identified between MDSC subsets and ANCs, suggesting that apparently more factors are involved in the development of neutropenia in CIN. In favor of this assumption, the proportions of T-MDSCs, PMN-MDSCs, and M-MDSCs were not statistically significantly different between patients with mild (ANC ≥ 1.0 × 10⁹/L) and more severe (ANC < 1.0 × 10⁹/L) neutropenia (Table 1). Both patient groups, that is, patients with mild (n = 77) and more severe (n = 25) neutropenia, displayed significantly lower T-MDSC proportions compared to healthy individuals ($p < 0.0001$ and $p = 0.0002$, respectively), mainly due to the lower proportions of M-MDSCs ($p < 0.0001$ and $p = 0.0002$, respectively). PMN-MDSC proportions were also lower in both patient groups compared to healthy individuals, but the decrease was more profound in the group with mild neutropenia

($p = 0.045$), apparently due to the higher number of patients in this group (Table 1). As expected, given that M-MDSCs are part of the monocytes, the proportions of M-MDSCs were correlated with the absolute monocyte counts in both the total study population ($r = 0.4197$, $p < 0.0001$) and the CIN group ($r = 0.3566$, $p = 0.0002$). Finally, a subset analysis was performed in MDSC subsets between patients with clonal and non-clonal hematopoiesis (Supporting Information S1: Table 4). No statistically significant difference was identified between these patient groups, suggesting that the MDSC numbers are not associated with the presence of clonal disease in CIN. The proportion of T-MDSCs in the BMMC fraction was significantly lower in patients compared to controls ($p = 0.0312$) (Table 1). The subset analysis showed that both PMN-MDSC and M-MDSC proportions were significantly lower in the patients compared to controls ($p = 0.0312$ and $p = 0.0369$, respectively)

(Table 1 and Figure 1G,H). These data indicate defective BM production of PMN-MDSCs and M-MDSCs in CIN patients compared to healthy individuals.

CIN patient MDSCs displayed normal T-cell suppressive activity. In the total set of experiments evaluating the T-cell suppressive activity of patient MDSCs, the undivided cells in the cultures were significantly increased in the presence of MDSCs ($1.58\% \pm 4.61\%$, median 0.02%, range 0%–20.31%) compared to those with absence of MDSCs ($0.34\% \pm 1.51\%$, median 0.00%, range 0%–7.09%) ($p = 0.001$) (Supporting Information S1: Figure 2A). There were no statistically significant differences in the proportion of undivided cells between PMN-MDSCs ($0.14\% \pm 0.20\%$, median 0.02%, range 0%–0.47%) and M-MDSCs ($0.48\% \pm 0.75\%$, median 0.03%, range 0%–1.74%, $p = 0.5$) or between healthy ($0.39\% \pm 0.69\%$, median 0.00%, range 0%–1.96%) and CIN cultures ($1.83\% \pm 5.48\%$, median 0.06%, range 0%–19.99%, $p = 0.4821$) (Supporting Information S1: Figure 2B,C).

The transcriptomic analysis of MDSCs from CIN patients showed an altered profile compared to healthy individuals. A significant number of mapped reads (1202 for PMN-MDSCs and 239 for M-MDSCs) were differentially expressed (log2fold change (FC) $\geq |2|$ and $p < 0.05$) between patients and controls. In patient PMN-MDSCs and M-MDSCs, 524 and 162 mapped reads, respectively, were upregulated, whereas 678 and 77 mapped reads, respectively, were downregulated. All differentially expressed mapped reads are given in Supporting Information S1: Tables 5 and 6. Patient and control MDSCs expressed genes typically associated with their immunomodulatory properties. Specifically, the genes of S100 calcium-binding protein A8/A9 (S100A8/A9) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which are typically associated with MDSC immunomodulatory properties, were among the most highly expressed sequences in both patients and controls (Figure 1I). However, alternative genes associated with MDSC expansion, phenotype, and functionality were downregulated (CEBPB, TGFBR2, VEGFB, IL4R), while others, such as IRF8, which is typically expressed at a low level in normal MDSCs, were upregulated in patient MDSCs. Taken together, the transcriptomic analysis[17] revealed that MDSCs from CIN patients, compared to those from healthy controls, display an altered immunomodulatory profile associated with upregulation of pathways related to inflammatory responses (e.g., IL6, IL1B, CCR2, CD86) and T-cell activation/responses (e.g., IL6, CD28) and downregulation of genes related to cellular viability and responses to damage (e.g., POLB, RPA4, ERCC4) and phagocytic functions (e.g., BLTP1, ABCA1, NCF4) (Figure 1J,K and Supporting Information S1: Figure 3).

Overall, we have shown that CIN patients have significantly lower proportions of MDSCs in the PBMC and BMMC fractions compared to healthy controls. MDSCs of CIN patients sufficiently suppress T cells ex vivo but display altered transcriptome properties implying intrinsic functional defects, beyond the T-cell suppression capacity, in vivo. Interestingly, low numbers of MDSCs and abnormal transcriptome profiles associated with intrinsic defects of MDSCs such as downregulation of genes related to responses to inflammatory cytokines and upregulation of genes related to DNA damage and apoptosis have been also found in patients with aplastic anemia and these defects have been associated with the immunopathogenesis of the disease.[20] The downregulation of genes related to MDSC expansion (e.g., CEBPB), along with the contracted BM myeloid progenitor cell compartment,[3] may have a role in the defective MDSC production in CIN patients. A higher apoptotic rate of MDSCs might be an additional mechanism for the decreased MDSC numbers in CIN as was indicated by the higher transcriptome expression of genes related to apoptosis (e.g., DAPK2) and lower expression of genes related to cell viability and repair after stress (e.g., POLB, RPA4, ERCC4), compared to healthy individuals. The low numbers and altered properties of MDSCs probably result in inadequate suppression of the aberrant inflammatory processes known to underlie CIN, contributing therefore to the increased levels of circulating inflammatory cytokines and chemokines. Our results depict for the first time the alterations of MDSCs in CIN and trigger more mechanistic studies in the future to further explore the exact role of these cells in the disease.

AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST STATEMENT

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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