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Monocyte function in patients with Myelodysplastic Syndrome

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Abstract

Myelodysplastic syndrome (MDS) is a malignant hematopoietic stem cell disorder that frequently evolves into acute myeloid leukemia (AML). Patients with MDS are prone to infectious complications, in part due to the presence of severe neutropenia and/or neutrophil dysfunction. However, not all patients with neutropenia become infected, suggesting that other immune cells may compensate in these patients. Monocytes are also integral to immunologic defense; however, much less is known about monocyte function in patients with MDS. In the current study, we monitor the composition of peripheral blood monocytes and several aspects of monocyte function in MDS patients, including HLA-DR expression, LPS-induced inflammatory cytokine production, and phagocytosis. We find that monocytes from MDS patients exhibit relatively normal innate immune functions compared to monocytes from healthy control subjects. We also find that HLA-DR expression is moderately increased in monocytes from MDS patients. These results suggest that monocytes could compensate for other immune deficits in MDS patients to help fight infection. We also find that the range of immune functions in monocytes from MDS patients correlates with several key clinical parameters, including blast cell count, monocyte count, and Revised International Prognostic Scoring System score, suggesting that disease severity impacts monocyte function in MDS patients.

Keywords

MDS; PBMC; lipopolysaccharide; phagocytosis; HLA-DR

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INTRODUCTION

Myelodysplastic syndrome (MDS) is a heterogeneous malignant hematopoietic stem cell disorder characterized by arrested differentiation, dysplastic blood cell production, and evolution to acute myeloid leukemia [1–4]. There are believed to be as many as 15,000 new diagnoses of MDS each year in the United States; MDS disproportionately impacts the elderly, with 36/100,000 diagnosed annually in the 80 years and older group [5]. MDS has a poor prognosis, with a 3-year survival rate of only 35% [6]. Infection or infectious complications are one of the most frequent causes of death in MDS patients [7–14]. Neutropenia and neutrophil dysfunction are common features of this disease [15–24]; however, many patients with significant neutropenia do not become infected [9, 11–13, 15, 19, 24–28]. One possibility is that other immune cells may compensate, at least in part, for neutrophil dysfunction in these patients. Like neutrophils, monocytes are critical for combating infection [29], and their importance may be even greater in the setting of neutropenia [30–32]. Monocytes from patients with chronic lymphocytic leukemia (CLL) exhibit significant immune dysfunction including an altered monocyte profile and a diminished inflammatory response induced by lipopolysaccharide (LPS) [33–35]. However, the function (or dysfunction) of monocytes in MDS patients remains unclear; the few prior studies concerning this topic have suggested that these immune cells may retain significant function [21, 36–38].

To clarify this issue, we have now analyzed monocyte function from the peripheral blood of 43 patients with MDS (27 untreated and 16 treated) and 14 healthy control subjects. Unlike neutrophils in MDS patients or monocytes in CLL patients, peripheral blood monocytes from patients with MDS retained significant immune function, perhaps helping explain the residual ability to fight infection in some MDS patients with neutropenia.

MATERIALS AND METHODS

Subject enrollment and sample collection

These studies were approved by the University of Colorado and National Jewish Health Institutional Review Boards. All subjects, or an appropriate proxy, gave written informed consent. Peripheral blood from MDS patients was collected as part of an IRB-approved tissue acquisition protocol at the University of Colorado. Peripheral blood from healthy subjects was obtained from two sources: an IRB-approved tissue collection protocol at National Jewish Health (Denver, Colorado) and Astarte Biologics (Bothell, WA). Blood samples were processed via mononuclear cell enrichment by Ficoll gradient processing and were subsequently cryopreserved in liquid nitrogen.

MDS patient clinical data

Clinical parameters for MDS patients were collected through retrospective chart review and included date of diagnosis, World Health Organization (WHO) subtype [39], revised International Prognostic Scoring System (IPSS-R) scores [40], treatment history, absolute peripheral blood monocyte count, and for patients diagnosed after 2014, a next-generation targeted resequencing panel analyzing the status of 54 genes (Supplemental Table 1). Next

generation sequencing of DNA from bone marrow aspirate using the TruSight myeloid sequencing panel with 568 amplicons interrogating 54 genes was performed with genomic DNA isolated using the Promega Maxwell® RSC Blood DNA Kit (Promega). 100 micrograms of DNA were processed using the TruSight Myeloid Sequencing Panel (Illumina), and the normalized libraries were pooled and loaded onto a MiSeq for sequencing using the 600-cycle MiSeq Reagent Kit v3 to generate 2×250 bp reads. Results of the targeted resequencing are listed in Supplemental Table 2.

Flow cytometry analysis

Immediately after thawing frozen PBMCs, an aliquot of cells was fixed and stained with the following antisera: anti-CD14-FITC (BD Biosciences), anti-CD16-PerCP (Invitrogen), anti-HLA-DR-PE (ebioscience), anti-CD20-PacBlue (Biolegend), and anti-CD15-PacBlue (Biolegend). Isotype-matched antisera were used as negative controls. Samples were analyzed using a BD LSR II flow cytometer running BD FACSDIVA™ acquisition software. Data were analyzed using FlowJo. The “non-lymphocyte” gate excludes small lymphocytes as well as dead cells and debris from the thaw. CD15 and CD20 were used to exclude neutrophils and B cells. All samples (healthy volunteers and MDS patients) were collected and treated similarly using identical freezing, thawing, and staining procedures. Fidelity and consistency of analysis were maintained via the batch processing function in FlowJo using exactly the same gates for all sample analyses.

Isolation of monocytes

Monocytes were isolated using a two-step purification protocol. First, frozen PBMCs were thawed in X-Vivo 10 Hematopoietic Cell Medium (Lonza), and monocytes were enriched using a Human Pan Monocyte Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. Enriched monocytes were counted using a hemocytometer and were further purified by plate adherence to tissue culture plastic in X-Vivo 10 medium supplemented with 3% heat inactivated platelet-poor plasma (HIPPP) and 1% Penicillin/Streptomycin (Fisher). HIPPP was produced by pooling platelet-poor plasma from 7 healthy donors, was isolated via Percoll separation and centrifugation, and was provided by Silvia Caceres and Ken Malcolm. Monocytes were plated at 100,000 cells per well in 96-well format. After one hour, the few non-adherent cells were removed by aspiration and adherent monocytes were used as outlined below.

Assaying monocyte response to LPS

Adherent monocytes (100,000 cells per well in 96-well format) were exposed to 20 ng/ml LPS (List Biological Labs) in X-Vivo 10 medium supplemented with 3% HIPPP and 1% Pen/Strep for four hours. After four hours, supernatants were collected and cytokine protein production (TNFα and IL-6) was monitored by ELISA (R&D Biosystems). RNA for qPCR analysis was prepared by lysing the adherent cells in RLT buffer (Qiagen) and using the Qiagen RNAeasy kit for RNA purification. qPCR was performed using the Qiagen Quantitect SYBR-Green RT-PCR kit and an ABI 7900 Thermocycler. Data were normalized relative to βactin using the ddCt method. Primer sequences used for qPCR are listed in Supplemental Table 3.

Assaying monocyte phagocytosis

The ability of adherent monocytes to phagocytose FITC-labeled *E. coli* was monitored using a BioTek Synergy HT plate reader and the Vybrant Phagocytosis assay Kit (Molecular Probes) according to the manufacturer's instructions.

Statistical Methods and limitations

To allow for comparison between experiments, the ELISA, qPCR, and phagocytosis data were all assayed relative to a common untreated control sample; these data were in turn normalized so that the healthy volunteers averaged to 1. The range of cytokine values in the ELISA data for healthy donors was 890 ± 510 pg/ml (avg $\pm$ stdev) for IL-6 and 540 ± 480 pg/ml (avg $\pm$ stdev) for TNF α . The qPCR data is all relative quantitation (ddCt method), and phagocytosis is assayed in arbitrary fluorescence units. All data were analyzed using Graphpad Prism 5. Unpaired *t*-tests were used to test for statistically significant differences in the flow cytometry data. Statistical analyses comparing data from MDS patients and healthy volunteers were performed using one-way Anova. To explore the association between monocyte function and clinical factors in MDS patients, single variable linear regression was used to test for association between these clinical factors and monocyte function. Statistical significance was considered $P < 0.05$.

One limitation to this study is that we do not have access to unfrozen monocytes for analysis. We did observe some loss of viability of frozen cells; however, cell recovery did not correlate with the monocyte population distribution, HLA-DR levels, or LPS-induced cytokine production, suggesting that the use of frozen samples does not account for the comparisons between healthy subjects and MDS patients.

RESULTS AND DISCUSSION

Monocytes from patients with chronic lymphocytic leukemia (CLL) exhibit significant immune dysfunction including an altered monocyte profile and a diminished inflammatory response induced by lipopolysaccharide (LPS) [33–35]. To determine if monocytes from MDS patients exhibit similar dysfunction, we analyzed monocyte function from the peripheral blood of 43 patients with MDS (27 untreated and 16 treated) and 14 healthy control subjects (Table 1).

To determine if the monocyte composition in peripheral blood was altered in MDS patients, we used flow cytometry to analyze CD14 and CD16 expression on peripheral blood mononuclear cells (PBMCs) from untreated MDS patients and age-matched healthy blood donors. We found that the frequency of classical (CD14⁺ CD16[−]), intermediate (CD14⁺ CD16⁺), and non-classical (CD14[−] CD16⁺) monocytes was not altered in untreated MDS patients compared to healthy donors (Figure 1A–E). This is quite different from monocytes in CLL patients, where the non-classical monocyte population is significantly increased [33, 34]. We did observe a significant increase in HLA-DR expression in the classical and intermediate monocyte subsets in the untreated MDS patients (Figure 1F–H); this finding also differs from monocytes isolated from CLL patients, which exhibit moderately decreased HLA-DR expression [33].

Monocytes have multiple key functions in the immune system, including the secretion of cytokines in response to immune challenge and phagocytosis of bacteria. We monitored these functions in monocytes isolated from MDS patients to determine if they exhibit immune defects. Monocytes from healthy blood donors, untreated MDS patients, or treated MDS patients were first enriched from PBMCs using a Human Pan Monocyte Isolation Kit (Miltenyi Biotech) and then further purified by plate adherence to tissue culture plastic. After one hour, the few remaining non-adherent cells were removed, and the percentage of monocytes in the enriched cell population was then checked by flow cytometry.

The production of pro- and anti-inflammatory cytokines induced by lipopolysaccharide (LPS, 20 ng/ml for 4 hours) was measured at the protein level by ELISA and at the mRNA level by qPCR. TNF α or IL-6 protein production induced by LPS was not significantly altered in monocytes from MDS patients, regardless of treatment status (Figure 2A,B). Likewise, TNF α or IL-6 mRNA levels did not differ in MDS patients compared to healthy control subjects, regardless of treatment status (Figure 2C,D). The level of mRNA for the anti-inflammatory cytokine IL-10 also was similar among these three groups (Figure 2E).

We also monitored uptake of fluorescently labeled bacteria. Phagocytosis of fluorescent *E. coli* particles was similar in monocytes isolated from untreated MDS patients, treated MDS patients, and healthy control subjects (Figure 2F).

Thus, our data indicate that there was little or no difference in some basic monocyte functions (LPS-induced inflammatory cytokine production and phagocytosis) in either treated or untreated MDS patients compared to healthy subjects of a similar age. This is a distinct finding from monocytes from CLL patients, which exhibit significant immune deficits [33], and also contrasts with the strong immune dysfunction present in neutrophils from MDS patients [15, 17]. This may help explain why some MDS patients with neutropenia or neutrophilic dysfunction do not have significant clinical complications from infection.

We also tested if clinical parameters in MDS patients affect monocyte function, focusing on untreated MDS patients to remove variability due to differences in treatment strategies. Clinical parameters for MDS patients were collected through retrospective chart review and included date of diagnosis, World Health Organization subtype [39], revised International Prognostic Scoring System (IPSS-R) scores [40], treatment history, absolute peripheral blood monocyte count, and number of mutations present.

LPS-induced IL-6 and TNF α protein production were both strongly positively associated with absolute peripheral blood monocyte count (Figure 3A,C), suggesting that patients producing higher levels of monocytes in their blood also produce monocytes that are more responsive to LPS. While not statistically significant, we also noted a trend towards an inverse correlation between bone marrow blast percentage and LPS-induced IL-6 and TNF α protein production (Figure 3B,D). These inverse associations between cytokine production and either marrow blasts or peripheral blood monocyte counts were consistent with our observation that marrow blast percentage and peripheral blood monocyte counts were inversely associated in untreated MDS patients (Figure 3E). In contrast to the association

between cytokine production observed with monocyte count and the trend towards an association with blast percentage, IL-6 or TNF α protein levels were not associated with either IPSS-R score or with the number of mutations present (Supplemental Table 4).

The association between IL-6 and peripheral blood monocyte count also extended to the mRNA level, as IL-6 mRNA levels were strongly positively associated with peripheral blood monocyte count (Figure 3F) and trended towards an inverse association with marrow blast cell percentage (Figure 3G). Consistent with the protein data, IL-6 mRNA was not associated with IPSS-R score or mutation count (Supplemental Table 4). Interestingly, TNF α mRNA was not associated with any of these four clinical parameters (Supplemental Table 4). In contrast, IL-10 mRNA levels were positively associated with IPSS-R score (Figure 3H) but not the other parameters (Supplemental Table 4). We also found that phagocytic ability of monocytes isolated from untreated MDS patients did not significantly associate with any of the four clinical factors tested (Supplemental Table 4). These associations indicate that MDS clinical parameters may affect monocyte function in these patients.

In conclusion, we find that monocyte composition, monocyte LPS response, and monocyte phagocytic ability are relatively unchanged in peripheral blood from patients with MDS. A prior study [37] indicated that monocytes from MDS patients exhibit an increased response to CD40 activation. Coupled with the increased MHC Class II production observed in the current study, this may suggest that the enhanced ability to present antigen and activate T cells could compensate, at least in part, for decreased neutrophil function present in these patients. Such a possibility should be examined in a larger study that examines several of these immune cell populations simultaneously. We also note that we have only analyzed a subset of monocyte functions, and it is possible that other monocyte functions may be altered in these patients. However, overall we find that, surprisingly, monocytes from MDS patients in the peripheral blood do not have gross immune deficits, and thus may contribute to fighting infection in these patients. Future large prospective clinical studies correlating monocyte number or function with infectious complications should be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

AML	acute myeloid leukemia
CLL	chronic lymphocytic leukemia
HLA-DR	Human Leukocyte Antigen – antigen D Related

IPSS-R	Revised International Prognostic Scoring System
LPS	lipopolysaccharide
MDS	Myelodysplastic Syndrome
MFI	mean fluorescence intensity
PBMC	Peripheral blood mononuclear cell
WHO	World Health Organization

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Summary

Monocytes from patients with Myelodysplastic Syndrome exhibit relatively normal immune function and could contribute to infection control in these patients.

Figure 1. Normal composition of monocyte subsets in MDS patients

Immediately after thawing frozen PBMCs from either healthy volunteers or from untreated patients with MDS, an aliquot of cells was fixed and subsequently stained for flow cytometry analysis. An identical gating strategy using FlowJo batch processing was used to analyze monocyte subsets in all samples. Small lymphocytes and dead cells were omitted from the initial cell gate (“non-lymphocytes”); then cells with high CD15 or CD20 were removed using a dump channel strategy; and finally CD14 and CD16 levels were assayed. Representative examples of this gating strategy are displayed for a healthy volunteer (**A**) and an untreated MDS patient (**B**). Panels **C–E** display the frequency of monocyte subsets.

Panels **F–H** display HLA-DR levels (MFI) normalized relative to control in each of the three monocyte subsets (average of control defined as 1). Those comparisons that were significantly different have P values listed; all other comparisons were not significant.

Figure 2. Monocytes from MDS patients do not exhibit significant immune defects

Monocytes from either healthy volunteers, untreated MDS patients, or treated MDS patients were exposed to 20 ng/ml LPS for four hours and production of the indicated cytokine proteins (A,B) or mRNAs (C–E) was monitored. (F) Monocytes were incubated for two hours with fluorescently labeled *E. coli* particles and phagocytosis of these fluorescent particles was monitored using the Vybrant Phagocytosis assay Kit (Molecular Probes). All values normalized so that healthy volunteer data averages to 1. None of the monitored parameters were statistically different from each other as determined by one-way Anova.

Figure 3. Association of clinical factors with monocyte function in patients with MDS

The graphs display the indicated cytokine proteins (A–D) or cytokine mRNAs (F–H) produced by monocytes (from untreated MDS patients) challenged with 20 ng/ml LPS for four hours compared to the indicated clinical factors in these patients (absolute peripheral blood monocyte count, marrow blast cell percentage, or IPSS-R score). Panel E depicts a comparison of absolute peripheral blood monocyte count and marrow blast cell percentage in these same patients. Solid lines depict the predicted variables from single variable linear regression. The p values from these analyses also are indicated. Further statistical information relevant to this figure is presented in Supplemental Table 4.

Table 1

Patient Demographics

	Control	MDS untreated	MDS treated
N	14	27	16
Age ^a	60.9 ± 6.5	65.8 ± 12.1	68.8 ± 8.5
Gender			
Female	8	12	7
Male	6	15	9
Race/ethnicity			
White	14	24	15
Black	0	0	1
Hispanic	0	2	0
Asian	0	1	0
Treatment			
Azacitidine	n/a	n/a	9
Decitabine	n/a	n/a	2
Lenalidomide	n/a	n/a	2
Multiple	n/a	n/a	3
Selective Disease Features			
High grade MDS ^b	n/a	2	4
Treatment Related MDS	n/a	3	1
del5q MDS	n/a	1	0
IPSS-R Risk Categories			
Low (≤ 3)	n/a	13	3
Intermediate (>3–4.5)	n/a	4	7
High (>4.5)	n/a	10	6

^a Age displayed as mean ± stdev^b Blasts account for 5–19% of marrow cells and/or 3–19% of blood cells