Circulating Myeloid-Derived Suppressor Cells Predict Differentiated Thyroid Cancer Diagnosis and Extent

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Background: Establishing the preoperative diagnosis and long-term prognosis of differentiated thyroid cancer (DTC) remain challenging in some patients. Myeloid-derived suppressor cells (MDSC) are tumor-induced cells mediating immune tolerance that are detectable in the peripheral blood of cancer patients. The authors previously developed a novel clinical assay to detect the phenotypes of two human MDSC subsets in peripheral blood, and hypothesize that higher MDSC levels measured by this assay correlate positively with both malignancy and worse patient outcomes.

Methods: A prospective observational pilot study was performed of patients undergoing thyroidectomy for a solitary thyroid nodule. The presence of a thyroid nodule >1 cm was confirmed sonographically, and fine-needle aspiration biopsy performed prior to surgery in all cases. Peripheral blood collected preoperatively was analyzed using a novel flow cytometry–based immunoassay to detect and quantify two subsets of human MDSC. Circulating MDSC levels were compared by histopathologic diagnosis, stage, and presence of persistent disease after treatment.

Results: Of 50 patients included in this study, MDSC measurement was successful in 47 (94%). One patient was found to have a concurrent cancer, leaving 46 patients for primary analysis. The cytologic diagnoses were benign in five (10.8%), atypia or follicular lesion of undetermined significance in five (10.8%), suspicious for follicular neoplasm in five (10.8%), suspicious for malignant in three (6.5%), and malignant in 28 (60.1%) of the 46 nodules. Final histopathology was benign in 11 (24%) and DTC in 35 (76%), encompassing 34 PTC cases and one follicular thyroid carcinoma. Mean percentages of CD11b⁺HLA-DR^{low}HIF1a⁺ MDSC (CD11b⁺MDSC) were 14.0 ± 6.2% and 7.9 ± 3.6% in DTC versus benign nodules, respectively ($p < 0.005$). A cutoff of 12% yielded a specificity of 0.91, a sensitivity of 0.72, and a likelihood ratio of 7.9. Mean CD11b⁺MDSC levels increased linearly with higher TNM stage ($p < 0.01$), and were 19.4 ± 5.4 in patients with persistent cancer after surgery compared with 13.2 ± 6.8 in those without evidence of disease ($p < 0.05$).

Conclusion: MDSC measurement using this flow cytometry–based assay represents a novel approach for preoperatively assessing malignancy risk and cancer extent in patients with thyroid nodules. While further validation is needed, these data suggest that MDSC assessment may serve as a useful adjunct when cytology is indeterminate, and predict tumor stage and recurrence risk in cases of thyroid cancer.

Introduction

THYROID NODULES ARE A COMMON FINDING, and though usually benign, diagnostic evaluation by ultrasound-guided fine-needle aspiration (FNA) biopsy is recommended for most nodules >1–1.5 cm because 5–15% of such nodules are malignant (1–4). Unfortunately, FNA yields an indeterminate result in 15–25% of cases, and surgical resection of the nodule is often recommended, despite most proving

benign (5). Surgery exposes patients with benign lesions to unnecessary risks, while an initial diagnostic surgery may be suboptimal for those with malignancy (6,7).

The Bethesda System for Reporting Thyroid Cytology reclassifies cytologic findings into categories that further stratify cancer risk (8), but frequently this does not provide sufficient reassurance to allow monitoring *in lieu* of diagnostic surgery (3,4). Adjuvant molecular tests have improved the preoperative diagnostic assessment of indeterminate

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nodules, but do not have ideal accuracy, require the invasive FNA procedure, and do not predict tumor burden when thyroid cancer is present (9–12).

Differentiated thyroid cancer (DTC) is the most common endocrinologic malignancy, and its incidence has increased threefold in recent decades due in part to greater detection of low-risk papillary thyroid carcinoma (PTC) (13,14). Autopsy studies confirm 20–36% of people harbor PTC during their life without clinical consequences, and there is evidence that most patients with the least aggressive PTC can be safely monitored without surgical resection (15,16). Though some PTC may have minimal clinical impact, 15–30% of treated patients suffer a recurrence, and 5–10% succumb to the disease. The determination of which thyroid cancers will follow an indolent course is incompletely understood (17), and represents an important area for improving care.

Immune dysfunction is now recognized as a fundamental component of human cancers (18). The immune system has the capacity to recognize and eliminate neoplastic cells, termed tumor immune surveillance, and cancer progression requires dysregulation of antitumor immune responses (19). An important mechanism through which cancers escape immune destruction is via recruitment or induction of suppressor immune cells, including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) (20–22). Human MDSC are a heterogeneous population of immature myeloid cells that inhibit T cell effector function through a range of mechanisms, such as arginase-1 and inducible nitric oxide synthase (23–25). While rare in healthy individuals, MDSC may accumulate in the settings of severe trauma, sepsis, or cancer (26).

It has previously been shown that many if not all cancer types induce human MDSC as a component of tumor-driven immune dysfunction (27–30). The universal induction of MDSC in cancer patients and the correlation between their accumulation and increasing tumor burden indicate that MDSC measurement may be a useful clinical tool for cancer detection and monitoring. A challenge to the clinical application of MDSC measurement is their phenotypic heterogeneity, frequently requiring functional definitions (28). To address this, the authors previously (27) identified functionally suppressive human MDSC phenotypes in the cancer setting, namely granulocytic CD11b⁺HLA-DR^{low} or monocytic CD33⁺HLA-DR^{low}, with expression of either HIF1 α ⁺ or C/EBP β ⁺. Then, a flow cytometric clinical assay was developed to detect these specific phenotypes in routine venipuncture samples in order to facilitate clinical application of MDSC measurement in patients (27).

The purpose of the present study was to assess the ability of preoperative MDSC measurement in the peripheral blood using this novel MDSC clinical assay to noninvasively predict the diagnosis of thyroid cancer, cancer stage, and recurrence risk in patients undergoing surgery for a thyroid nodule.

Methods and Materials

Study population

After approval by the Institutional Review Board of the University of Southern California (HS-11-00676), adult patients (≥ 21 years of age) undergoing surgery for a solitary thyroid nodule or recurrent DTC at the Los Angeles County-University of Southern California Medical Center (LAC-USC) or Keck Hospital of USC were prospectively enrolled from

February 2012 to December 2013. Patients were excluded if there was concurrent malignancy, recent use of nonsteroidal anti-inflammatory drugs (NSAID), tobacco smoking, chronic viral infection (e.g., hepatitis B or C, HIV, etc.), or autoimmune disease other than chronic thyroiditis. Informed consent was obtained in all cases.

Demographic, pathologic, and outcome data were prospectively collected. The cytologic and final histopathologic diagnoses were made by academic clinical pathologists specializing in thyroid evaluation using Bethesda classifications and American Joint Committee on Cancer (AJCC) Tumor-Nodal-Metastasis (TNM) staging (31), respectively. Patients were assessed for anatomic or biochemical cancer persistence at one year after initial treatment. Anatomic persistence was defined as thyroid cancer identified by ultrasound, cross-sectional imaging (typically computed tomography), or ¹³¹I imaging, with biopsy-proven disease required except in cases of ¹³¹I avidity or those demonstrating a typically appearance of pulmonary micrometastases in the appropriate clinical context. Biochemical persistence was defined as stimulated thyroglobulin (Tg) >2 ng/dL in those who received ¹³¹I ablation or unstimulated Tg >1 ng/dL with a concurrent suppressed thyrotropin level. Patients with only the presence of Tg antibody, whether persistent or incident during follow-up, were not considered to have persistent disease.

Specimen collection and processing

Prior to surgery, 20 mL of peripheral blood was obtained from subjects via routine venipuncture using heparin-sodium tubes. CD11b⁺ and CD33⁺ MDSC were isolated with other peripheral blood leukocyte (PBL) populations by differential density gradient separation (Ficoll-Paque; Sigma-Aldrich, St. Louis, MO) as previously described (27,32), and aliquots of 2×10^6 cells/mL were stored in 10% dimethyl sulfoxide media at -170°C for batched analysis.

Measurement of peripheral blood immune cells

Monoclonal antibodies against CD11b (OKM-1), CD33 (11-1), HLA-DR (L243), HIF1 α (564-16), and C/EBP β (48-17) were custom fluorescently labeled (BD Biosciences, San Jose, CA) to identify two MDSC phenotypes (27). Additionally antibodies (BD Biosciences) used were against CD14 (M Φ P9) and CD15 (HI98) for further characterization of MDSC, CD25 (MA251), CD4 (RPA-T4), CD3 (UCHT1), FoxP3 (259D/C7), CD14 (M5E2), CD19 (SJ25C1), CD56 (B159), and CD45 (HI30) for identification of other immune populations and included appropriate isotype-matched control antibodies.

For analysis, one PBL aliquot for each subject was thawed, then transferred into RPMI-1641, pelleted and washed in FACS buffer, and resuspended in 100 μL . Samples were incubated with primary antibodies against surface MDSC markers at 4°C for 30 min, and then washed twice with FACS buffer. Cells were then fixed and permeabilized (eBioscience, San Diego, CA) prior to staining with antibodies to intracellular targets at 4°C for 30 min. Samples were washed twice and resuspended for analysis using an LSRII flow cytometer (BD Biosciences). Isotype controls were prepared in parallel. Fluorescence Activated Cell Sorting (FACS) was performed by the University of Southern California Flow Cytometry Core Facility using a BD Bioscience FACSaria II. Analysis of data was performed using FlowJo software (TreeStar, Inc., Ashland, OR).

Statistical analyses

Descriptive statistics are shown as mean $\pm$ standard deviation (*SD*) or median with interquartile range, as indicated. Differences in immune cells between groups were analyzed using an unpaired Students *t*-test, after determination of normality using the D'Agostino and Pearson normality test, or Fisher's exact test, as appropriate. Receiver operating characteristic (ROC) analysis was performed to assess the diagnostic utility of immune cell measurement and the sensitivity, specificity, and likelihood ratio for the diagnosis of thyroid cancer. Evaluation by analysis of variance for trend was used to determine the presence of a linear trend in the mean immune cell values between groups based on TNM stage, MACIS (distant Metastasis, Age, Completeness of resection, local Invasion, and tumor Size) score (33), or American Thyroid Association (ATA) recurrence risk stratification (3). For all tests, $\alpha=0.05$. Statistical analysis was performed using GraphPad Prism v6.0 (La Jolla, CA), and graphs were created using GraphPad and Adobe Photoshop (San Jose, CA).

Results

Patient data

Fifty patients undergoing surgery for a solitary thyroid nodule satisfied inclusion and exclusion criteria, of whom peripheral blood cells were successfully isolated in 47 (94%). One patient was found to have a concurrent renal cell carcinoma after data acquisition, leaving 46 patients for primary analysis. The cytologic diagnoses were benign (Bethesda II) in five (10.8%), atypia of undetermined significance or follicular lesion of undetermined significance (Bethesda III) in five (10.8%), suspicious for follicular neoplasm (Bethesda VI) in five (10.8%), suspicious for malignant (Bethesda V) in three (6.5%), and malignant (Bethesda VI) in 28 (60.1%) of the 46 nodules. Final histopathology was benign in 11 (24%) and DTC in 35 (76%), encompassing 34 PTC cases and one follicular thyroid carcinoma. As shown in Table 1, the median patient age of 59.3 ± 11.9 years versus 49.3 ± 11.1 years was greater in patients with benign nodules versus DTC, respectively ($p < 0.05$). Other patient and nodule characteristics did not differ significantly between groups (Table 1).

The pathologic characteristics of malignant tumors are shown in Table 2. Mean tumor size was 2.1 ± 1.7 cm, with 21/35 (60%) measuring < 2 cm. In three cases, surgery was performed for local soft-tissue recurrences of PTC, and these were not included in the analysis of diagnostic accuracy. Of the 32 primary tumors, invasion was present in 17 (53.1%), and lymph node metastasis was present in 12 (37.5%). At presentation, 19 patients had AJCC TNM stage I–II thyroid cancer, while 16 patients had stage III–VI disease. Two patients presented with distant metastases, both in the lungs.

Preoperative levels of circulating MDSC predict the diagnosis of DTC

The preoperative levels of two MDSC subsets in the peripheral blood, characterized as $CD11b^+HLA-DR^{low}HIF1\alpha^+$ ($CD11b^+$ MDSC) or $CD33^+HLA-DR^{low}HIF1\alpha^+$ ($CD33^+$ MDSC), were evaluated by flow cytometry measurement, and expressed as the percentage of live-gated leukocytes (% $CD11b^+$ MDSC or % $CD33^+$ MDSC, respectively). Results

TABLE 1. PATIENT DEMOGRAPHIC, CYTOLOGIC, AND PATHOLOGIC DATA

	All patients (n = 46)	Benign (n = 11)	DTC (n = 35)
Sex			
Male	8 (17.4)	1 (9.1)	7 (20.0)
Female	38 (82.6)	10 (91.9)	28 (80.0)
Mean patient age, years $\pm$ SD	51.7 ± 12.0	59.3 ± 11.9	49.3 ± 11.1^a
Mean nodule size, cm		3.2 ± 1.5	2.1 ± 1.7
FNA cytology (Bethesda class)			
Benign (II)	5 (10.8)	5 (45.4)	0 (0)
AUS/FLUS (III)	5 (10.8)	2 (27.3)	3 (8.6)
SFN/FN (IV)	5 (10.8)	4 (27.3)	1 (2.8)
SUSP (V)	3 (6.5)	0 (0)	3 (8.6)
Malignant (VI)	28 (60.1)	0 (0)	28 (80.0)
Histopathology			
Benign colloid nodule		7 (63.6)	
Follicular adenoma		4 (36.4)	
Papillary thyroid carcinoma			34 (97.1)
Follicular thyroid carcinoma			1 (2.9)

^a $p < 0.05$, benign versus DTC groups.

DTC, differentiated thyroid cancer; SD, standard deviation; FNA, fine-needle aspiration; AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; SFN/FN, suspicious of follicular neoplasm/follicular neoplasm; SUSP, suspicious for malignancy.

were compared to gold-standard histopathology to assess the diagnostic utility of preoperative MDSC measurement.

As shown in Figure 1, higher mean percentage $CD11b^+$ MDSC levels were measured in patients with cancerous compared to benign thyroid nodules ($14.0 \pm 6.2\%$ compared with $7.9 \pm 3.6\%$, respectively; $p < 0.005$). Receiver operating characteristic (ROC) curve analysis demonstrated an area under the curve (AUC) of 0.80 [confidence interval (CI) 0.67–0.93]. For percentage $CD33^+$ MDSC, similar mean values of $0.8 \pm 0.9\%$ and $1.1 \pm 1.2\%$ were measured in malignant and benign cases, respectively. Mean MDSC percentages were not associated with individual pathologic features for either MDSC subtype studied (Table 2).

Further characterization of the $CD11b^+$ MDSC population by flow cytometry revealed a subset with CD15 expression and absent significant CD14 expression (Fig. 2). Isolation of this population by FACS and subsequent Wright–Giemsa staining demonstrated immature granulocytic appearing cells with prominent nucleoli and occasional band forms (Fig. 2F).

The diagnostic potential of percentage $CD11b^+$ MDSC measurement was assessed using a cutoff of 12% based on ROC analysis. Percentage $CD11b^+$ MDSC levels were $> 12\%$ in 1/11 (9%) benign cases and 21/32 (65.6%) DTC cases ($p < 0.005$), yielding a specificity of 0.91, sensitivity of 0.72, and likelihood ratio of 7.9. Of 13 patients with indeterminate Bethesda cytologies, this cutoff correctly categorized 12/13 patients.

Assessment of circulating $CD3^+CD4^+FoxP3^+$ Treg as a percentage of lymphocytes found mean Treg percentages of

TABLE 2. PATHOLOGY FINDINGS IN PATIENTS WITH DIFFERENTIATED THYROID CANCER

	<i>DTC cases</i>	<i>Mean ± SD % CD11b⁺MDSC</i>	<i>Fraction with >12% CD11b⁺MDSC (%)</i>
Recurrent DTC	3/35 (8.6)	23.2 ± 8.1	3/3 (100)
Primary DTC	32/35 (91.4)	13.9 ± 6.1	23/32 (71.9)
<i>Tumor size</i>			
<1 cm	8 (25.0)	11.0 ± 5.4	5/8 (62.5)
>1 cm	24 (75.0)	14.9 ± 6.1	19/24 (79.2)
<i>Invasion</i>			
Encapsulated	15 (46.9)	12.5 ± 6.5	9/15 (60.0)
Invasive ^a	17 (53.1)	15.2 ± 5.7	14/17 (82.4)
<i>Nodes</i>			
Nx, N0	20 (62.5)	13.6 ± 6.7	13/20 (65.0)
N1 ^b	12 (37.5)	14.6 ± 5.2	10/12 (83.3)
<i>Distant metastasis at presentation</i>			
No	30 (94.3)	14.0 ± 6.3	21/30 (70.0)
Yes ^c	2 (5.7)	13.3 ± 0.2	2/2 (100)
<i>AJCC TNM stage</i>			
I–II	19 (59.4)	12.4 ± 6.6	8/19 (50.0)
III	8 (25.0)	16.0 ± 4.6	6/8 (75.0)
IV	5 (15.6)	16.1 ± 2.1	5/5 (100)
<i>ATA risk</i>			
Low	11 (37.4)	12.6 ± 7.1	6/11 (54.5)
Int	14 (43.8)	13.1 ± 6.1	9/14 (64.3)
High	7 (21.8)	16.6 ± 3.8	7/7 (100)
<i>MACIS score</i>			
<6	15 (46.9)	10.9 ± 5.3	7/15 (46.7)
6–7	8 (25.0)	15.2 ± 7.7	6/8 (75.0)
>7	9 (28.1)	16.9 ± 3.7	9/9 (100)
<i>Response to treatment</i>			
No evidence of disease		13.2 ± 6.8	13/21 (62.0)
Persistent disease		19.4 ± 5.4	6/6 (100)

^aIncludes lymphovascular invasion, and extracapsular and/or extrathyroidal extension.

^bIncludes central neck (N1a) and/or lateral neck (N1b).

^cTwo patients with lung metastasis at presentation.

AJCC TNM, American Joint Committee on Cancer Tumor-Nodal-Metastasis staging; ATA, American Thyroid Association; MACIS, distant Metastasis, Age, Completeness of resection, local Invasion, and tumor Size.

5.7 ± 2.7% and 5.3 ± 2.8% in benign and malignant cases, respectively ($p = \text{n.s.}$). Similarly, there were no significant differences between mean levels of T cells (CD45⁺CD3⁺), B cells (CD45⁺CD19⁺), or a pan-monocyte populations (CD45⁺CD14⁺).

Preoperative levels of circulating CD11b⁺MDSC correlate with the DTC aggressiveness

Preoperative mean percentage CD11b⁺MDSC levels were compared to postoperative cancer assessment by AJCC TNM stage, MACIS score, and ATA recurrence risk stratification (Table 2). As shown in Figure 3, there was a significant linear trend in the mean percentage CD11b⁺MDSC levels with increasing TNM stage ($p < 0.01$), MACIS score ($p < 0.005$), and ATA risk category ($p < 0.01$). It is evident that thyroid cancers in patients with a percentage CD11b⁺MDSC level <12% were predominantly categorized as more indolent (TNM Stage I/II, ATA low risk, MACIS <6), whereas patients undergoing surgery for recurrent cancer, though only numbering three in this study, demonstrated among the highest percentage CD11b⁺MDSC values observed.

When patients with at least one year of follow-up after initial cancer therapy were analyzed for treatment response,

percentage CD11b⁺MDSC levels were significantly higher in subjects with persistent disease compared with those with an excellent response (negative thyroglobulin and post-therapy scan if ¹³¹I was given). In six patients with persistent disease, of whom two were stage I/II and four were stage III/IV, mean percentage CD11b⁺MDSC levels were 19.4 ± 5.4% compared with 13.2 ± 6.8% in patients without evidence of disease ($p < 0.05$; Fig. 3D). Additionally, the percentage CD11b⁺MDSC level was >12% in all patients with persistent disease after initial treatment.

Discussion

Thyroid nodules are common, and while FNA biopsy is currently the most accurate diagnostic evaluation, it is invasive and requires time, specialized equipment, and cytopathologic assessment that has suboptimal reproducibility (34). Adjunctive testing can enhance diagnostic accuracy but remains imperfect (11,35), and there continues to be a need for improved methods to determine the risk posed by a thyroid nodule preoperatively.

This study evaluates circulating MDSC levels as a diagnostic test for malignancy in thyroid nodules using a novel clinical assay. To the authors' knowledge, there are no

FIG. 1. Quantification of immune cell populations in benign nodules compared with differentiated thyroid carcinomas (DTC). Individual patient data and mean ($\pm$ standard deviation [SD]) percentage for (A) CD11b⁺HLA-DR^{low}HIF1a⁺ myeloid-derived suppressor cells (% CD11b⁺MDSC) and (B) CD33⁺HLA-DR^{low}HIF1a⁺ MDSC demonstrate significantly higher mean percentage CD11b⁺MDSC levels in patients with DTC compared with benign thyroid lesions ($13.96 \pm 6.16\%$ compared with $7.86 \pm 3.58\%$, respectively; $p < 0.005$). Receiver operator characteristic (ROC) curve analysis demonstrated an area under the curve (AUC) of 0.80 [confidence interval (CI) 0.67–0.93], but no significant difference for CD33⁺ MDSC accumulation. Applying a cutoff of 12% showed that 1/11 (9%) benign cases and 21/32 (65.6%) malignant cases had $>12\%$ CD11b⁺MDSC (Fisher's exact test, $p < 0.005$), yielding a specificity of 0.91, a sensitivity of 0.72, and a likelihood ratio of 7.9. (C) Individual patient data with mean ($\pm$ SD) percentage for the percent CD3⁺CD4⁺FoxP3⁺ T-reg cells of CD45⁺ cells in benign and malignant cases were similar and did not demonstrate diagnostic utility ($5.67 \pm 2.72\%$ versus $5.30 \pm 2.82\%$, respectively; AUC = 0.52 [CI 0.3–0.73]). Similarly, measurement of (D) CD3⁺ T cells and (E) CD14⁺ monocytes and CD19⁺ B cells did not differ significantly between benign and malignant cases. ** $p < 0.005$.

studies specifically analyzing MDSC levels to determine if a suspicious lesion is benign or malignant prior to intervention, despite numerous studies comparing cancer patients with healthy controls (28,29). In patients undergoing surgery for a solitary thyroid nodule, the mean percentage of granulocytic CD11b⁺HLA-DR^{low}HIF1a⁺ MDSC was significantly higher in the presence of malignant compared with benign nodules. These phenotypic markers are increasingly recognized as identifying a clinically relevant MDSC population (22,27,32,36,37). In this prospective evaluation, a cutoff value of $>12\%$ CD11b⁺MDSC yielded a specificity of 91% for the diagnosis of DTC. Nodules with indeterminate cytology, while not investigated separately, were correctly identified in all but one case, indicating that MDSC measurement may be useful in this situation. Interesting, one patient with a benign thyroid nodule was subsequently found to have a concurrent renal cell carcinoma. In this case, the percentage CD11b⁺MDSC value of 13.8% is consistent with the identification of malignancy, suggesting that measurement of this MDSC phenotype may be useful across cancer types.

Preoperative MDSC levels were also predictive of disease burden and prognosis in this study. Patients with a percentage

CD11b⁺MDSC level $<12\%$ (11/32; 34%) were almost exclusively those with more indolent DTC tumors (AJCC Stage I/II, ATA low risk, MACIS score <6). The sole Stage III malignancy that would have been missed using this cutoff was a 2.4-cm classical PTC with minimal capsular extension and limited level VI lymph node metastases (intermediate ATA risk, MACIS score <6), and the patient achieved an undetectable stimulated thyroglobulin level at one year postoperatively. These data suggest that patients with PTC and low preoperative MDSC levels may harbor the most clinically indolent disease. This finding has potentially important clinical implications. Since prospective observational data suggest many PTC remain clinically indolent even without resection (16), less aggressive treatment may be reasonable for low-risk disease (3), and any intervention at all may constitute superfluous therapy in some patients. The ability of MDSC measurement to predict cancer extent and prognosis preoperatively may improve the management of PTC by identifying patients optimal for conservative management.

Two previous studies of thyroid cancer did not find increased MDSC (defined as CD14⁺CD11b⁺CD33⁺ cells) in

FIG. 2. Flow cytometry assessment of human peripheral blood to identify and characterize CD11b⁺ myeloid derived suppressors cells. Representative flow cytometry dot plots of (A) live cell scatter; (B) doublet exclusion by single-cell gating, which encompassed >95% of cells; (C) isotype (left), patient with a benign thyroid nodule (middle), and patient with thyroid cancer (right) for CD11b and HLA-DR, showing gating for the CD11b⁺HLA-DR^{low} population; (D) HIF1a⁺ positivity of CD11b⁺HLA-DR^{low} cells; and (E) CD15 and CD14 evaluation for CD11b⁺HLA-DR^{low}HIF1a⁺ cells, showing a subset with CD15 expression and absent significant CD14 expression consistent with a granulocytic origin. (F) Photomicrograph (original magnification 400×) of CD11b⁺HLA-DR^{low}HIF1a⁺ cells isolated by fluorescence activated cell sorting and subsequently stained with Wright-Giemsa demonstrating immature granulocytic appearing cells with prominent nucleoli and occasional band forms.

FIG. 3. CD11b⁺HLA-DR^{low}HIF1a⁺ myeloid-derived suppressor cell levels in patients with DTC stratified by prognostic schemes and patient outcome. The levels of CD11b⁺HLA-DR^{low}HIF1a⁺ myeloid-derived suppressor cells as a percentage of live-gated leukocytes (% CD11b⁺MDSC) are shown demonstrating a significantly correlation between mean percentage CD11b⁺MDSC values and increasing disease burden when stratified by (A) American Joint Committee on Cancer (AJCC) Tumor-Nodal-Metastasis (TNM) stage, (B) distant Metastasis, Age, Completeness of resection, local Invasion, and tumor Size (MACIS) score, and (C) American Thyroid Association (ATA) risk stratification. Cancer patients with <12% CD11b⁺MDSC exclusively harbored low-risk papillary thyroid cancers (TNM Stage I/II, MACIS <6, ATA low risk). (D) Comparison of mean percentage CD11b⁺MDSC levels between patients without evidence of persistent thyroid cancer and those with persistent/recurrent disease during two years of prospective follow-up. In patients with persistent disease, percentage CD11b⁺MDSC levels were 19.42 ± 5.4% compared with 13.15 ± 6.8% in patients without evidence of persistent disease ($p < 0.05$). B, benign nodule; R, recurrent disease. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

PTC patients compared with controls, but did show accumulation in patients with anaplastic thyroid cancer (38,39). In contrast, the current study shows significantly higher circulating MDSC levels were observed in a population consisting predominantly of PTC patients. The reason for this discrepancy may be the different MDSC phenotypes evaluated between the studies.

Consistent with the present findings, many previous studies investigating similar human MDSC phenotypes in other cancer types have demonstrated a correlation between higher MDSC levels and more advanced clinical stage (30,40,41), metastatic burden (41,42), disease progression (41,42,43), and worse patient survival (28,29,44,45). The current data further confirm the role of circulating MDSC as a tool for disease detection and monitoring across cancer types. Importantly, the three-marker phenotypes employed in this study correlated with functionally suppressive MDSC induced *in vitro* (27), obviating the need to perform laborious functional evaluations, and can be evaluated on standard clinical flow cytometers, addressing two critical aspects for clinical implementation of this technique.

There are several limitations to the current study. The small sample size and high prevalence of thyroid cancers compared with benign nodules prevents relevant assessment of positive and negative predictive values, which would require a sample representative of the clinical population, and a larger study population with longer follow-up duration is necessary to evaluate overall and disease-specific survival for DTC. While the correlation of circulating MDSC with the diagnosis, extent, and prognosis of malignancy is increasingly accepted, optimal markers for clinical MDSC measurement remain unclear. Peripheral MDSC measurement for diagnostic and/or prognostic purposes has been attempted in the breast, lung, urogenital, prostate, gastrointestinal and pancreatic, head and neck, skin, and brain cancers using >20 phenotypes (28,29). The markers studied here are not meant to be inclusive of all MDSC subsets, but are felt to reflect a population with clinical utility that can be feasibly measured in patient specimens.

Several clinical and methodological factors may complicate MDSC measurement. Non-malignant conditions, such as severe trauma or sepsis, may increase circulating MDSC levels (26). In this study, however, blood samples for MDSC measurement were taken from patients without acute illnesses presenting for a scheduled surgery. Further, in the design of this study, major immunomodulatory conditions, including immunodeficiency and active infection, were excluded, thereby limiting possible confounding of MDSC levels. While the MDSC population defined by CD33 positivity was not found to have diagnostic utility in this study, recent data suggest loss of this marker during cryopreservation (32), and therefore other markers for monocytic MDSC such as CD14 may be superior for measurement of this subset clinically.

In conclusion, this proof-of-concept prospective study demonstrates the potential of MDSC measurement in the evaluation of thyroid nodules to improve preoperative diagnostic and prognostic assessment. Specifically, using a three-marker flow cytometric assay human MDSC can be quantified in readily available peripheral blood samples with accuracy sufficient to yield clinically relevant information. Additional studies are necessary to validate the diagnostic utility and cost-effectiveness of MDSC measurement for indeterminate

cytology nodules, and demonstrate that thyroid cancer management is improved when incorporating the prognostic information obtained. Such larger investigations of MDSC measurement are now in progress for thyroid cancer and other malignancies to elucidate further the clinical value of MDSC assessment.

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Author Disclosure Statement

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