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ORIGINAL ARTICLE

CD161$^+$ NKT Cell Proportion as a Predictive Biomarker for Bortezomib Treatment Response in Newly Diagnosed Multiple Myeloma Patients

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SUMMARY

Background: **Multiple myeloma (MM) remains incurable, with drug resistance being a key clinical challenge. Impaired natural killer T (NKT) cell function may contribute to MM immune escape, while the significance of the inhibitory receptor CD161 expression on NKT cells is unclear. This study investigated the association between the peripheral blood CD3$^+$CD56$^+$CD161$^+$ NKT cell proportion and response to bortezomib plus dexamethasone therapy in newly diagnosed MM (NDMM) patients.**

Methods: **Seventy-two NDMM patients receiving bortezomib plus dexamethasone and 37 healthy controls (HCs) were enrolled. Flow cytometry assessed the peripheral blood CD3$^+$CD56$^+$CD161$^+$ cell proportion before and after treatment. Treatment response was evaluated according to IMWG criteria (responders: ≥ partial response [PR]; non-responders: ≤ stable disease [SD]). Receiver operating characteristic (ROC) curve analysis evaluated predictive value. Correlation with clinical parameters (ISS stage, LDH, β_2-MG, etc.) was analyzed.**

Results: **The baseline CD3$^+$CD56$^+$CD161$^+$ proportion was significantly lower in NDMM patients than in HCs (2.25% vs. 4.20%, $p < 0.05$). After treatment, it increased to 3.10% ($p < 0.05$). Responders had a significantly higher baseline proportion than non-responders (3.40% vs. 1.60%, $p < 0.0001$). ROC analysis showed the baseline proportion predicted treatment response with an AUC of 0.789 (95% CI: 0.675 - 0.903). At the optimal cutoff of 1.85%, sensitivity was 87.9% and specificity was 71.8%. Patients with low proportions (< 1.85%) had a higher frequency of ISS stage III ($p < 0.05$) and significantly elevated LDH and β_2-MG levels (both $p < 0.05$).**

Conclusions: **Low expression of peripheral blood CD3$^+$CD56$^+$CD161$^+$ NKT cells is associated with increased tumor burden and bortezomib resistance in NDMM, suggesting its potential as a predictive biomarker for treatment response.**

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INTRODUCTION

Multiple Myeloma (MM) is a clonal plasma cell malignancy that remains incurable [1]. Although novel agents like proteasome inhibitors (e.g., bortezomib) combined with dexamethasone have significantly improved patient survival, drug resistance persists as a major clinical challenge [2]. Natural killer (NK) cells, as central effectors of

innate immunity, can eliminate malignant plasma cells and modulate adaptive immune responses [3]. However, in MM patients, NK cell function is significantly impaired, characterized by insufficient activation and reduced cytotoxicity [4], directly contributing to the immune system's inability to effectively recognize and eliminate tumor cells. Notably, the immune system harbors cell populations exhibiting characteristics of both T cells and NK cells (e.g., some NKT cells and γδ T cells). These cells participate in innate immune responses and may influence the MM immune microenvironment [5]. CD161 is a C-type lectin receptor expressed on most NK cells ($CD56^+CD3^-$). It inhibits NK cell cytotoxicity through interaction with its ligand, lectin-like transcript 1 (LLT1/CLEC2D) [6]. Beyond NK cells, CD161 is also expressed on subsets of peripheral blood T cells, including NKT cells ($CD56^+CD3^+$) [7].

Based on this background, this study focused on CD161 expression on NKT cells and its clinical significance. We investigated changes in the proportion of $CD161^+$ NKT cells in peripheral blood before and after four cycles of bortezomib plus dexamethasone chemotherapy in NDMM patients, evaluated its correlation with treatment response, and determined its prognostic predictive value.

MATERIALS AND METHODS

Study subjects

Seventy-two NDMM patients initially treated with bortezomib plus dexamethasone between January 2022 and December 2024 at our hospital were enrolled. The cohort comprised 40 males and 32 females, with a mean age of 69.03 ± 7.05 years. ISS stages were: I (n = 18), II (n = 24), III (n = 30). Thirty-seven age- and sex-matched healthy controls (HCs) without any signs of disease or infection were also included (21 males, 16 females; mean age 67.03 ± 5.62 years). Treatment response for the 72 NDMM patients was assessed after initial therapy according to IMWG criteria [8]. Patients achieving partial response (PR) or better were classified as responders. Patients achieving stable disease (SD), progressive disease (PD), or failing to achieve PR were classified as non-responders. Consequently, 33 patients were responders and 39 were non-responders.

Data collection

Patient clinical data, including gender, age, clinical stage, and bone marrow plasma cell percentage, were collected. Clinical staging used the International Staging System (ISS) established by the International Myeloma Working Group (IMWG). Baseline laboratory parameters were recorded: serum creatinine (SCr), hemoglobin (Hb), calcium (Ca), albumin (ALB), lactate dehydrogenase (LDH), β_2-microglobulin (β_2-MG), C-reactive protein (CRP), and platelet count (PLT).

Mononuclear cell preparation

Before starting chemotherapy and after completing four cycles, 2 mL of fresh peripheral blood was collected from each patient into EDTA vacuum tubes and processed within 60 minutes. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Solarbio, China) via density gradient centrifugation.

Flow cytometry analysis

Surface phenotypes of freshly isolated PBMCs were identified using the following monoclonal antibody cocktail: 20 μL CD45-PerCP, 20 μL CD4-FITC, 20 μL CD161-PE, 10 μL CD8-APC, 10 μL CD56-PE-Cy7, 10 μL CD3-APC-H7 (all antibodies from BD Biosciences, San Jose, CA, USA). The antibody mixture was incubated with cells at room temperature (20 - 25°C) in the dark for 30 minutes. After incubation, cells were washed twice with phosphate-buffered saline (PBS). Immunophenotyping was performed using an 8-color multiparameter flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA, USA). Analysis involved sequential gating: lymphocytes were selected first, followed by the $CD3^+CD56^+$ cell population, and finally, CD161 expression was analyzed within this gated population.

Statistical analysis

The Shapiro-Wilk test assessed data normality. Normally distributed continuous variables are presented as mean ± standard deviation (SD) and compared using independent samples *t*-tests. Non-normally distributed continuous variables are presented as median (interquartile range, IQR) and compared using the Mann-Whitney U test. Categorical variables are presented as frequency (%) and compared using the chi-squared test (χ^2 test). Receiver operating characteristic (ROC) curve analysis evaluated the predictive performance of the $CD3^+CD56^+CD161^+$ proportion, calculating the area under the curve (AUC) and the optimal diagnostic cutoff value. All statistical analyses were two-sided, and $p < 0.05$ was considered statistically significant.

RESULTS

Proportion of $CD3^+CD56^+CD161^+$ cells in NDMM patients before and after treatment

Before treatment, the proportion of $CD161^+$ cells within the $CD3^+CD56^+$ population in NDMM patients was 2.25% (IQR: 1.23, 3.73), significantly lower than the 4.20% (IQR: 1.40, 5.90) observed in HCs ($p < 0.05$). After completing four cycles of chemotherapy, the proportion in NDMM patients increased significantly to 3.10% (IQR: 1.60, 5.13) ($p < 0.05$ vs. baseline) and was no longer significantly different from HCs ($p > 0.05$). Notably, responders had a significantly higher baseline $CD3^+CD56^+CD161^+$ proportion (3.40%, IQR: 2.25, 4.40) compared to non-responders (1.60%, IQR: 0.90, 2.40) ($p < 0.0001$) (Figure 1).

Predictive value of baseline $CD3^+CD56^+CD161^+$ proportion for initial treatment response in NDMM

ROC curve analysis was performed to assess the predictive value of the baseline $CD3^+CD56^+CD161^+$ proportion for

Table 1. Comparison of clinical characteristics between patients with different CD3+CD56+CD161+ Levels.

Clinical characteristic	Low level (n = 28)	High level (n = 44)	t/χ^2 value	p-value
Gender				
Male	15 (53.57%)	24 (54.55%)	0.007	0.936
Female	13 (46.43%)	20 (45.45%)		
Age (mean ± SD, years)	67.84 ± 7.81	70.89 ± 5.25	1.821	0.073
ISS Stage				
I + II	11 (39.29%)	31 (70.45%)	5.617	0.018
III	17 (60.71%)	13 (29.55%)		
Bone marrow plasma cells (%)				
< 30	13 (46.43%)	20 (45.45%)	0.018	0.893
≥ 30	15 (53.57%)	24 (54.55%)		
SCr				
< 177 μmol/L	16 (57.13%)	21 (47.73%)	0.289	0.591
≥ 177 μmol/L	12 (42.86%)	23 (52.27%)		
Hb				
< 110 g/L	18 (64.29%)	19 (43.18%)	2.264	0.132
≥ 110 g/L	10 (35.71%)	25 (56.82%)		
ALB				
< 35 g/L	19 (67.86%)	24 (45.45%)	2.216	0.106
≥ 35 g/L	9 (32.14%)	20 (54.55%)		
Ca				
< 2.75 mmol/L	23 (82.14%)	30 (68.18%)	1.073	0.300
≥ 2.75 mmol/L	5 (17.86%)	14 (31.82%)		
LDH				
< 245 U/L	6 (21.43%)	24 (54.55%)	6.419	0.011
≥ 245 U/L	22 (78.57%)	20 (45.45%)		
β2-MG				
< 3.5 mg/L	4 (14.29%)	19 (43.18%)	5.310	0.021
≥ 3.5mg/L	24 (85.71%)	25 (56.82%)		
CRP				
< 10 mg/L	8 (28.57%)	17 (38.64%)	0.385	0.535
≥ 10 mg/L	20 (71.43%)	27 (61.36%)		
PLT (x 10⁹/L)				
< 74	7 (25.00%) *	15 (34.09%)	0.307	0.580
≥ 74	21 (75.00%)	29 (65.91%)		

* SCr Serum Creatinine, Hb Hemoglobin, ALB Albumin, Ca Calcium, LDH Lactate Dehydrogenase, β2-MG β2-Microglobulin, CRP C-Reactive Protein, PLT Platelet Count. Statistically significant p-values are in underlined line.
* Discrepancy in total % noted in original table; value adjusted to add up to 100% based on n = 28.

treatment response and determine its optimal cutoff value. The analysis revealed an AUC of 0.789 (95% CI: 0.675 - 0.903, p < 0.0001). At the optimal cutoff value of 1.85%, sensitivity was 87.90% and specificity was 71.80% (Figure 2).

Comparison of clinical characteristics between patients with different CD3+CD56+CD161+ levels

Based on the optimal baseline CD3+CD56+CD161+ proportion cutoff (1.85%), the 72 NDMM patients were divided into a high-level group (≥ 1.85%, n = 44) and a low-level group (< 1.85%, n = 28). Compared to the high-level group, the low-level group had a significantly higher pro-

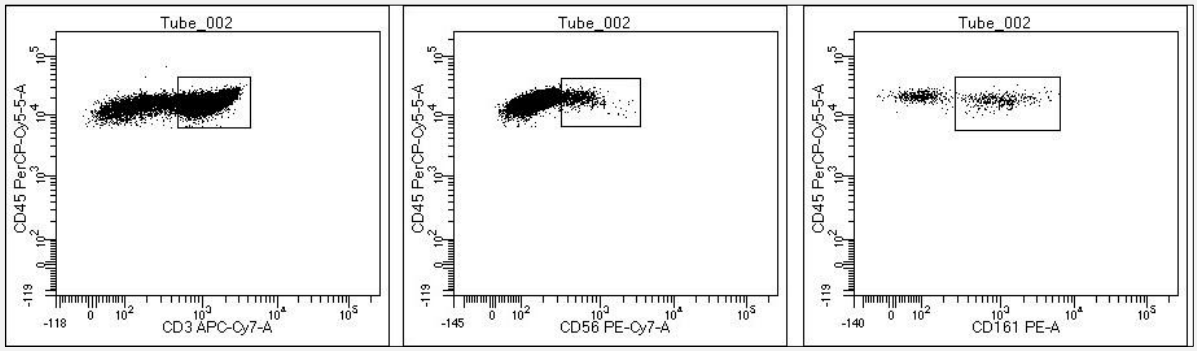

Figure 1. Representative flow cytometry analysis dot plots.

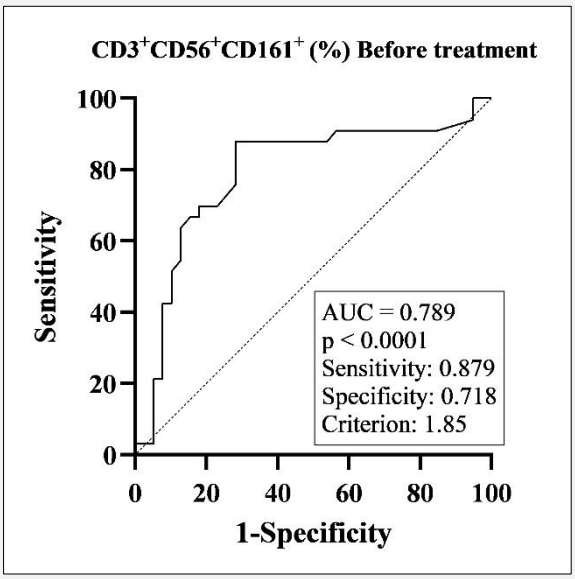

Figure 2. ROC curve of $CD3^+CD56^+CD161^+$ percentage for predicting initial treatment efficacy in NDMM patients.

portion of patients with ISS stage III ($p < 0.05$) and significantly higher serum LDH and β_2-MG levels ($p < 0.05$). No significant differences were found between the groups regarding gender, age, bone marrow plasma cell percentage, or other clinical parameters ($p > 0.05$) (Table 1).

DISCUSSION

As the second most common hematologic malignancy in adults, the incurability of MM remains a significant clinical challenge [9]. Although advances in novel agents and treatment strategies have markedly extended patient survival [10], in-depth exploration of prognostic factors is crucial for optimizing individualized therapy.

Substantial evidence indicates that MM progression is closely linked to systemic immune dysfunction. Beyond

humoral immune suppression, key effector cells of the innate and adaptive immune systems - such as NK cells, NKT cells, and γδ T cells, which possess the ability to directly kill malignant plasma cells - exhibit significant numerical and functional abnormalities [11]. Notably, the CD161 receptor plays an important role in this immunoregulation: this inhibitory receptor is highly expressed on NK cells (> 90%) and subsets of T cells (including NKT cells) [12]. It transmits inhibitory signals via its intracellular ITIM (immunoreceptor tyrosine-based inhibitory motif) domain, negatively regulating immune cell activity [13]. However, in some diseases, the frequency of peripheral blood $CD161^+$ immune cells (including NK and NKT cell subsets) is significantly reduced, and CD161 expression on T cells (including NKT cells) is markedly downregulated [14-17].

Building on this background, our study focused on the clinical value of $CD3^+CD56^+CD161^+$ NKT cells. While the regulatory mechanisms of NK cells on malignant plasma cells are relatively well-defined, research on this specific cellular phenotype as a prognostic marker in MM remains scarce. Furthermore, patients resistant to proteasome inhibitor-based regimens like bortezomib and dexamethasone have an extremely poor prognosis (median overall survival only 6 - 12 months) [18,19], highlighting the urgent need for biomarkers capable of early prediction of treatment response.

Our study found that the baseline $CD3^+CD56^+CD161^+$ proportion was significantly lower in NDMM patients compared to HCs. Within the NDMM cohort, non-responders had a significantly lower baseline proportion than responders. These statistically significant differences suggest that the baseline level of this marker may help predict response to bortezomib plus dexamethasone therapy. Our observation of reduced baseline peripheral blood $CD3^+CD56^+CD161^+$ NKT cells aligns with some reports linking this finding to disease progression, often attributed to decreased levels of cytokines required to maintain this cell subset [20]. Notably, classical studies suggest that in murine models of systemic lupus erythematosus (SLE), reduced numbers of invariant NKT (iNKT) cells (Type I NKT cells) correlate with disease activity and can be restored after treatment [21,22]. The reduced $CD161^+$ NKT cell proportion observed in our study may partially reflect a decrease in iNKT cell numbers.

ROC curve analysis confirmed that the baseline $CD3^+CD56^+CD161^+$ proportion has good predictive value for treatment response (AUC = 0.789). At the optimal cutoff (1.85%), sensitivity and specificity were 87.90% and 71.80%, respectively. Compared to traditional indicators like β_2-MG, serum free light chains, or M-protein, the $CD3^+CD56^+CD161^+$ NKT cell proportion, as an immunological marker, may be less influenced by factors such as nutritional status or renal function. We analyzed the relationship between baseline $CD3^+CD56^+CD161^+$ level (grouped by the cutoff) and clinicopathological features. Results showed that the low-level group had a significantly higher proportion of patients with ISS stage III, indicating that patients with low baseline expression of this marker often present with more advanced disease. β_2-MG level is a key indicator reflecting MM tumor burden, indicative of tumor proliferation and invasiveness, playing a central role in ISS staging, and serving as an independent prognostic risk factor [23]. LDH also reflects MM disease status and is closely associated with tumor burden [24]. Our study further found that patients in the low-level group had significantly higher serum LDH and β_2-MG levels than those in the high-level group, supporting an inverse correlation between baseline $CD3^+CD56^+CD161^+$ proportion and MM tumor burden.

Future studies should involve multi-center collaboration, enrolling larger cohorts encompassing different MM subtypes and disease stages, and include long-term follow-up to observe the predictive value of this marker for overall survival and progression-free survival, thereby more comprehensively validating our findings.

CONCLUSION

This study enhances our understanding of the $CD3^+CD56^+CD161^+$ level as a predictive biomarker for treatment response, potentially providing a basis for optimizing individualized treatment strategies and improving patient outcomes. However, these findings are based on the bortezomib plus dexamethasone regimen; further research is needed to validate their applicability to other treatment regimens (e.g., those containing immunomodulatory drugs or monoclonal antibodies).

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The authors have no acknowledgments to declare.

Data Availability Statement:
The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Statement:
This study was approved by the Ethics Committee of the First Affiliated Hospital of Hebei North University in September 2022 (Approval Number: K2022922). Informed consent was obtained from all participants in writing prior to sample collection.

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Declaration of Interest:
The authors declare no potential conflicts of interest.

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