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RESEARCH ARTICLE

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Bone marrow free immune checkpoints as a potential biomarker for differential diagnosis of acquired bone marrow failures

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Abstract

Objective: Clinically, to make a definite diagnosis of aplastic anemia (AA), idiopathic cytopenia of undetermined significance (ICUS) or myelodysplastic syndrome (MDS), they should be distinguished from each other. AA and ICUS have some incidence to transform into MDS. Immunosuppressive therapy (IST) is effective in AA and partial ICUS patients, while other ICUSs are more likely to progress to MDS without response to IST. To date, we neither found a technical method that could easily identify AA from hypoproliferative MDS, nor a simple parameter that could indicate ICUS with a response to IST. Here, we detected the concentration of free immune checkpoints in bone marrow supernatant of AA, ICUS, and MDS patients, analyzed the differences of immune status among these three diseases, to try to find a way to predict the response to IST in ICUSs.

Methods: Seventy-four novel patients were enrolled with newly diagnosed acquired bone marrow failure (including 29 AA patients, 11 ICUS patients, and 34 MDS patients), bone marrow supernatants were collected. Luminex liquid suspension array technology was used to measure the concentrations of 17 immune checkpoints to analyze the differences of immune status among these three diseases.

Results: The levels of 17 free immune checkpoints were elevated in MDS and showed a strong correlation with each other, followed by ICUS, and with the weakest in AA. By drawing the ROC curve, we found eight immune checkpoints, including sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-2, could easily distinguish AA from hypoproliferative MDS. ICUSs with lower concentrations of these eight free immune checkpoints predicted a better IST response.

Conclusion: In conclusion, we found that there were notable differences in the immune status of AA, ICUS, and MDS. The concentrations of sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-2 could be used to identify AA

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and hypoproliferative MDS patients, as well as to distinguish ICUS patients who could benefit from IST.

KEYWORDS
aplastic anemia, bone marrow, idiopathic cytopenia of undetermined significance, immune checkpoints, myelodysplastic syndrome

1 | INTRODUCTION

Hematological diseases are complex and diverse, especially bone marrow failure, such as aplastic anemia (AA), hypoproliferative myelodysplastic syndrome (hMDS), and idiopathic cytopenia of undetermined significance (ICUS). The boundaries of these diseases are obscure and often overlap with diagnostic indicators, posing a great challenge to clinical doctors. In terms of pathogenesis, the onset of AA is caused by cytotoxic T cell-mediated destruction of hematopoietic cells, whereas MDS is known as bone marrow failure secondary to clonal hematopoietic cells due to somatic mutation. However, these two mechanisms may exist simultaneously in these two diseases.[1] ICUS could be caused by immune mechanisms or clonal hematopoiesis although the etiology has not been clarified. Accordingly, there are some connections and differences between AA, ICUS, and MDS.

To distinguish AA from MDS, we often detect cytomorphology, flow cytometry, chromosome, FISH and NGS. However, it is arduous to distinguish AA from hMDS, as evidenced by the following conditions: First, it is hard to observe pathological hematopoietic features by cytomorphology and insignificant abnormalities in cell differentiation by flow cytometry due to few nucleated cells in bone marrow failure diseases. Second, in terms of chromosome or fluorescence in situ hybridization(FISH), if there is no enough karyotype or cell for detection, the examinations turns to be clinically useless. The next-generation sequencing (NGS) technology are currently widely used in clinical. However, clonal hematopoiesis was widely spread in normal population, even though some mutations which is high related to myeloid malignancies were found in BMF patients, we could not only use NGS to confirm diagnosis to be AA or hMDS. Therefore, a more sensitive and easier method is needed to solve this problem. For ICUS, there are two different fates, one with good response to IST and good prognosis, while another with a high tendency transforming to myeloid malignancy and poor prognosis. However, the current examination assays such as FISH, chromosome, and NGS are only served to predict the transformation of ICUS to MDS, but fail to predict the response to IST. It is very important to find a biomarker that can directly observe the differences among AA, ICUS, and MDS patients, cause correct diagnosis is the basis and guarantee of effective treatment.

Immune checkpoints (ICs) were involved in the pathogenesis of various tumors and autoimmune diseases. Meanwhile, based on targeting immune checkpoint proteins or their ligands on cells, the therapeutic effect also has been improved. Previous studies have shown that different ICs' molecules are widely expressed in different immune cells, with different expression levels, always collaborating with each other, such as ICOS and CD28, CTLA-4, and PD-1(see[2]). Some ICs have a dual function, such as TIM-3, binding with different ligands could display stimulation or inhibition.[3–5] Thus, ICs constitute a complex network of immune regulation in the immune microenvironment.

Recently, it is found that ICs exist not only on the cell surface but also in the plasma or local tissue microenvironment. The latter serves as a huge buffer pool of the systemic immune system, which brings great "flexibility" to the system immunity. We believed that any disruption of immune homeostasis will inevitably leave "traces" of regulation in this buffer pool. In this study, we attempted to distinguish the benign and malignant of acquired bone marrow failures by detecting the concentration of free immune checkpoints in the supernatant of bone marrow from patients with bone marrow failures, also try to provide a new method to identify the ICUS patients who may have response to immunosuppressive therapy (IST).

2 | MATERIALS AND METHODS

2.1 | Patients

In our study, we selected 74 patients with acquired bone marrow failure (male: female = 0.95:1, median age 57 years) who consulted with clinicians at the Department of Hematology, General Hospital of Tianjin Medical University from October 2020 to February 2021, including 29 patients with aplastic anemia (AA), 11 with idiopathic cytopenia of undetermined significance (ICUS), and 34 with myelodysplastic syndromes (MDS), all of whom were newly diagnosed (see Table 1). All patients underwent bone marrow aspiration, bone marrow pathology, chromosome karyotype, histochemical analysis, and other related examinations. Infection, solid tumors, and other hematological diseases were excluded. Our experiments were performed under the Declaration of Helsinki after obtaining written informed consent and approved by the Medical Ethics Committee of Tianjin Medical University General Hospital.

2.2 | Detection of immune checkpoints

5 ml of bone marrow from AA, ICUS, MDS patients, and healthy donors was collected in centrifuge tubes containing EDTA anticoagulant. Plasma was collected by centrifugation, subpackaged into 1.5-ml

TABLE 1 Basic characteristics of 74 patients with newly diagnosed: AA, ICUS and MDS

Characteristic	AA ($n = 29$)	ICUS ($n = 11$)	MDS ($n = 34$)
Age (years)			
Median (Range)	53 (17–76)	48 (13–75)	64 (25–87)
Gender (male/female)	20/16	7/4	18/16
Disease status N (Rate)	AA type AA:14 (48.30%) SAA:11 (37.90%) AA-PNH:4 (13.80%)		MDS staging(IPSS-R) Very low-risk: 4 (11.80%) Low-Risk: 9 (26.50%) Medium-Risk: 10 (29.40%) High-risk/Very high-risk: 11 (32.30%)
Laboratory parameters			
Median (25%–75%)			
WBC (×109/L)	2.26 (1.84–4.02)	2.66 (1.80–4.19)	2.26 (1.46–3.68)
RBC (×1012/L)	2.19 (1.62–95.50)	2.61 (2.20–3.10)	1.89 (1.52–2.67)
HGB (g/L)	74.00 (56.00–95.50)	82.00 (67.00–98.00)	65.00 (57.75–76.50)
PLT (×109/L)	26.00 (8.50–36.50)	43.00 (37.00–141.00)	46.00 (21.50–152.00)
Ret (×109/L)	27.90 (12.10–55.50)	61.30 (22.60–88.20)	34.50 (17.78–48.78)

Abbreviations: AA, aplastic anemia; ICUS, idiopathic cytopenia of undetermined significance; MDS, hypoplasia myelodysplastic syndrome.

EP tubes, and stored in a refrigerator at −80°C for subsequent testing. A Luminex 200 system (Luminex Corporation) chip platform and Human Immuno-Oncology Checkpoint Protein Panel 1 (HCKP1-11 K-PX17) detection kit were used to detect the relative concentrations of 17 free immune checkpoints in bone marrow plasma of AA, ICUS, and MDS patients, including CTLA-4, CD80/B7-1, CD86/B7-2, BTLA, HVEM, PD-1, PD-L1, PD-L2, TIM-3, TLR-2, LAG-3, CD28, ICOS, CD27, CD40, GITR, and GITRL. All operations were performed following the recommendations of the kit operating manual. After the samples and standards were detected by Luminex 200 detector, the fluorescence obtained was automatically calculated and optimized by software. According to the fluorescence detection value (FI) obtained from the standard substance, the standard curve was fitted by using the multiparameter mode to obtain the Standard Curve and its equation, and the concentration unit was pg/ml. In the standard curve fitting, the software automatically corrects some deviation points and fits the effective points. Substitute the original fluorescence of each sample into the standard curve formula to calculate the sample concentration, which can be used for comparison between samples. The marker * indicates the signal value is extremely low or high, lower than the minimum standard concentration, or higher than the maximum standard concentration, but the corresponding concentration can be calculated by the multiparameter standard curve. OOR< or OOR>: The indicator concentration is too low or too high, below or above the detection range of the standard curve.

2.3 | Statistical analysis

When the Kolmogorov–Smirnov test was used to demonstrate the normality of the distributions, independent t-tests were used to compare means of analyte concentrations; otherwise, nonparametric tests were used. For non-normally distributed data, the Mann–Whitney U test was used to compare two groups of continuous variables and the Kruskal–Wallis one-way analysis of variance was used to compare three groups. The data were log-transformed to correct for non-normality in the cluster heatmap. Correlated data were tested using Spearman's rank correlation tests. Quantitative data were presented as means ± SDs and medians and were considered significant when $p < 0.05$. R statistical software for statistical analysis.

3 | RESULTS

3.1 | The concentration of free ICs in AA, ICUS, and MDS patients

As shown in the heatmap, the concentrations of all 17 of the above-mentioned free ICs in MDS patients appeared significantly higher than in AA (see Table 2, Figure 1. Box plots are available in Figure S1), suggesting that these ICs were differentially-expressed in the two disease states, thus fully illustrating that there are significant differences between AA and MDS in immune status within the bone marrow microenvironment. Furthermore, we found that the mentioned 17 ICs may participate in the pathogenesis of all of these three diseases, with the weakest correlation in AA (Figure 2A), followed by ICUS (Figure 2B), and with the strongest in MDS (Figure 2C), further suggesting that in terms of immune status, the inherency of AA and MDS is different.

3.2 | Eight immune checkpoints could be used to distinguish AA from MDS

Next, with AA as the control group and MDS as the case group, we further analyzed the diagnostic value of the immune checkpoints by ROC curves. Of these, eight free immune checkpoints were

TABLE 2 Concentrations of free immune checkpoints in bone marrow serum (median concentration (25%–75%) in pg/ml)

	AA	ICUS	MDS
BTLA	111.28 (51.78–316.85)	222.17 (178.68–285.92)	409.54 (147.29–961.14)
CD27	1150.94 (431.74–1909.81)	840.00 (567.51–1387.64)	2130.89 (1125.81–3069.42)
CD28	2090.63 (1891.69–3176.98)	3438.72 (1558.84–5935.96)	3553.13 (2354.53–6169.42)
CD40	565.86 (445.51–747.94)	790.50 (523.71–935.32)	1044.87 (694.01–1702.80)
CD80/B7-1	73.24 (38.87–129.38)	78.61 (57.07–137.70)	156.49 (44.80–384.24)
CD86/B7-2	54.14 (ORR <–146.03)	87.81 (54.14–495.47)	413.73 (121.22–1134.60)
CTLA-4	14.98 (11.56–22.24)	17.71 (15.89–24.04)	36.16 (24.03–69.41)
GITR	[a]ORR < (ORR <–73.77)	71.22 (5.18–166.51)	142.67 (110.24–283.48)
GITRL	114.53 (21.93–268.89)	260.10 (107.03–398.43)	409.32 (115.36–851.08)
HVEM	1805.09 (1497.58–2381.27)	2291.62 (1848.79–3215.55)	3691.27 (2322.19–6499.81)
ICOS	469.68 (190.94–1026.15)	638.87 (479.23–1035.14)	1517.63 (293.57–3326.40)
LAG-3	22861.75 (9580.07–46537.53)	112338.10 (74507.53–209826.82)	74816.84 (19499.17–200950.06)
PD-1	269.52 (177.44–463.35)	443.81 (278.26–690.82)	902.55 (465.70–2012.94)
PD-L1	62.96 (43.09–99.22)	99.20 (61.06–241.43)	201.59 (81.30–443.06)
PD-L2	16269.81 (13988.28–17752.72)	17337.81 (13027.05–22002.73)	21061.77 (16088.48–25242.42)
TIM-3	3463.66 (2825.01–4312.24)	4092.27 (3213.69–5803.64)	8131.17 (5600.97–14637.17)
TLR-2	861.56 (508.66–1346.31)	1498.67 (702.91–2343.43)	2738.66 (1105.57–587.13)

[a]ORR < indicates that the concentration is too low, below the standard detection range.

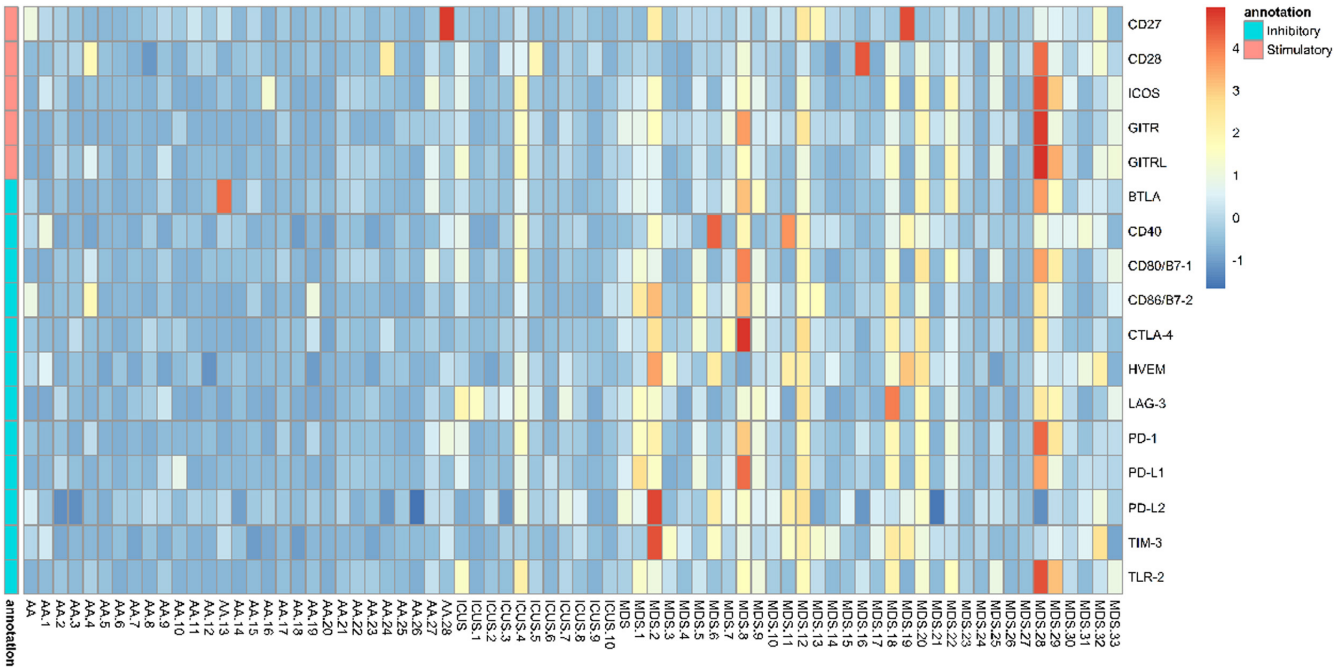

FIGURE 1 The concentrations of all 17 free-immune checkpoints in MDS patients appeared significantly higher than in AA.

significantly upregulated in MDS, with area under the curve (AUC) close to or over 8, namely sCD40 (AUC: 0.833, cutoff: 962.765), sCD86/B7-2 (AUC: 0.793, cutoff: 204.165), sCTLA-4 (AUC: 0.831, cutoff: 23.570), sGITR (AUC:0.924, cutoff: 107.5), sHVEM (AUC:0.836, cutoff: 2560.520), sPD-1 (AUC:0.824, cutoff: 467.695), sTIM-3 (AUC:0.886, cutoff: 4738.980), sTLR-2 (AUC:0.836, cutoff: 2008.360) (Figure 3A–H, the remaining immune checkpoints AUC/CUTOFF values are listed in Figure S2).

3.3 | The concentration of free ICs may predict IST response of ICUS patients

With current knowledge, ICUS was defined as a disease that may not be clearly classified as either benign or malignant bone marrow failure disease or even be recognized as a pathological status in between. Part of the ICUSs could respond to IST, but others with poor response to IST and a high rate of MDS transformation. Free ICs

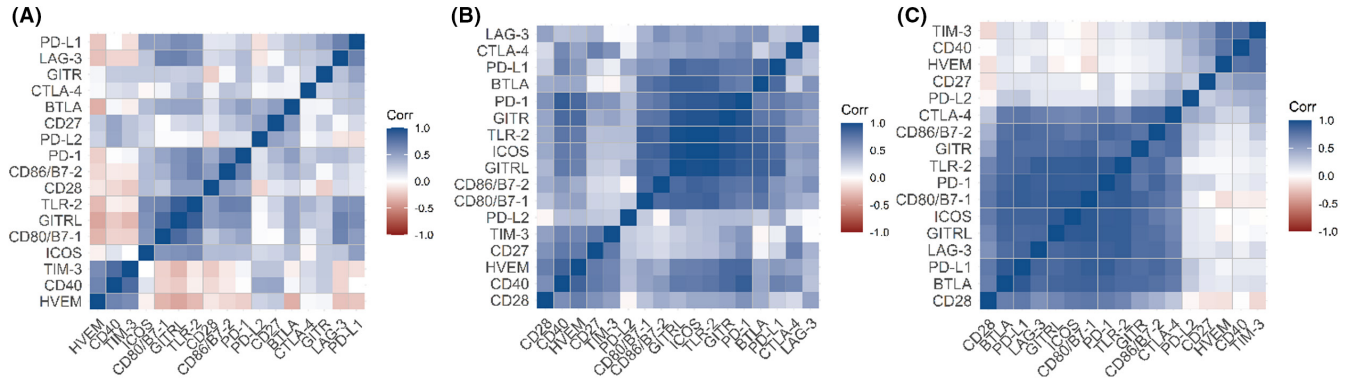

FIGURE 2 Similarity matrices are showing correlations of free immune checkpoints in AA (A), ICUS (B), and MDS (C). Heatmap was generated by spearman rank correlation using average linkage for hierarchical clustering.

testing in the supernatants of bone marrow revealed a high diversity in ICUS patients. The patients with a relatively low level of ICs always showed AA features and benefit from IST.

In this study, all 11 ICUS patients received IST including cyclosporine and glucocorticoids, along with supplementation of hemopoietic therapy including EPO, G-CSF, and TPO or TPO-RA. We found that six of 11 patients responded well with better peripheral blood recovery (Table S1, cases 6–11), while the other five patients showed poor or even lack of response to IST treatment (Table S1, cases, 1–5). To determine whether the different responses of ICUS patients to IST are related to ICs, we compared the eight ICs that we used to distinguish AA from MDS in IST response and no-response groups. We found that among the above six ICUS patients with good response to IST, two patients had six ICs below the cutoff values, and four patients had all eight ICs below each cutoff values, whereas as to the rest of five patients resistant to IST had at least four ICs above the cutoff value. The levels of the above eight immune checkpoints of ICUS patients in treatment-responsive and nonresponsive groups are listed in Figure 4A–H. Our data suggest that patients with lower levels of ICs in bone marrow are more inclined to AA by disease characteristics, and therefore may benefit from IST. On the other hand, patients with higher levels of ICs are potentially prone to MDS, IST should be evaluated and long-term follow-up of clonal hematopoiesis or MDS/AML is necessary.

4 | DISCUSSION

The discovery of ICs is a historic breakthrough in the study of neoplastic and autoimmune diseases. ICs inhibitors, as the target of cancer immunotherapy, have significantly improved the survival rate of patients with various tumors, such as lung cancer, renal cell carcinoma, gynecological tumors, and so on. However, most studies are based on the expression of their receptors on the surface of tumor cells and ICs on immune cells in the tumor microenvironment, while few studies are on the role of free immune checkpoints in plasma or other body fluids. Moreover, most studies focused on the immune target therapy of tumors, those applying ICs to the differential diagnosis of diseases are rare.

Myelodysplastic syndrome is a myeloid malignant disease of the hematological system. Previous studies have found that PD-1/PD-L1, CTLA-4, TIM-3, and other immune checkpoints were abnormally expressed on the hematopoietic stem cells and immune cells of MDS. Mechanism studies also found that the mentioned pathways were involved in the pathogenesis of MDS, and the blockade of immune checkpoints and demethylation drugs (HMA) have synergistic effects showing high response in patients with HR-MDS, CMML, or AML. In AA studies, CTLA-4 expression was down-regulated on Tregs cells in untreated AA patients compared with healthy volunteers and the expression of CTLA-4 gradually recovered after IST treatment.[6,7] TIM-3 expression was reduced on $CD4^+$ T cells as well as on NK cells and CD56dim NK subsets,[8,9] it is also restored after IST. The level of TIM-3 correlated with the severity of pancytopenia in SAA[8] indicating it participated in the onset and progression of AA. Above evidence shows that ICs were involved in both AA and MDS.

In this study, we demonstrated the differences in the concentration of free ICs in the plasma of patients with AA, ICUS, and MDS. To be specific, AA is in an immune hyperfunction status with a low level of cellular and plasm free ICs. However, MDS has an elevated level of both cellular and plasma free ICs, suggesting that it was in an immunosuppressive status. By plotting ROC curves, we calculated the cutoff values of each immune checkpoint in AA and MDS, and found that eight immune checkpoints (sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-2) showed the greatest differences between AA and MDS, which could play a more important role in the differential diagnosis of these two diseases.

Patients with ICUS characterized by one or more lineages of cytopenia do not meet the minimum diagnostic criteria for MDS, and other hematological or nonhematological diseases should also be excluded. The process of ICUS is variable and unpredictable, some of which are related to autoimmune abnormalities, others may transform into MDS or AML, and even into lymphoproliferative diseases or mast cell tumors in a few years. Different disease diagnosis determines the choice of treatment options. If the progression of ICUS could be predicted at a relatively early stage, detours will be avoided.

In this study, it was found that compared with AA or MDS, the levels of ICs in ICUS were high dispersion. Some patients tended to

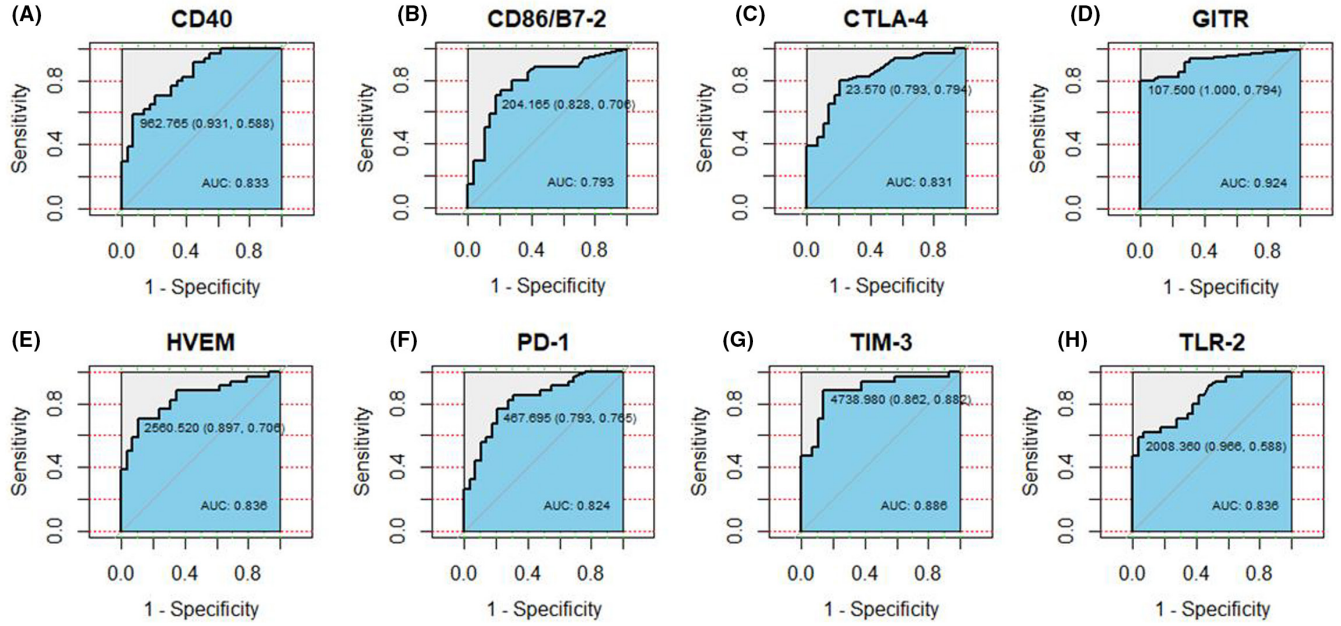

FIGURE 3 ROC curves of sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-3. These eight free immune checkpoints may distinguish AA from MDS, with area under the curve (AUC) close to or over 8. (AA as controls, MDS as case group).

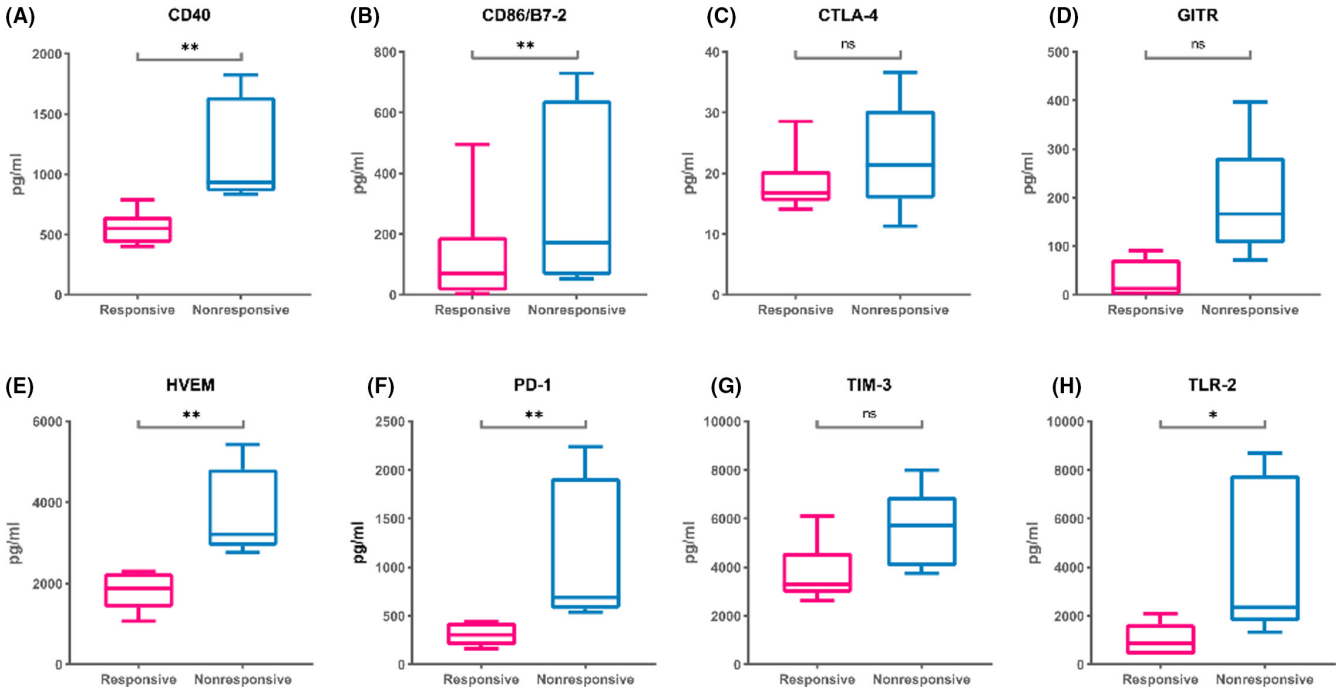

FIGURE 4 The levels of sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-3 of ICUS patients in treatment-responsive and nonresponsive groups. $^{*}p < 0.05$, $^{**}p < 0.01$, ns, no significance.

AA, with a relatively low expression level of ICs; and some tended to MDS, with a relatively high expression. Accordingly, the same treatment regimen would inevitably lead to differences in efficacy. According to the clinical efficacy of IST, we separate ICUS patients into two groups. We found that the patients with good responses to IST have a lower concentration of ICs like AA. On the contrary, the patients with poor response have a relatively higher level of ICs similar to MDS. Therefore, free ICs levels also could be used to predict the efficacy of IST in ICUS.

Similar to our results, it has been reported that CD40 and CD86, compared with normal individuals, exhibit elevated levels in hematological malignancies such as AML and MDS and correlated with lower survival rates, both of which may serve as prognostic markers and regulators of anti-tumor response.[10,11] In our experiment, we

selected more immune checkpoints and discriped immune status of AA or MDS from more dimensional; later, we do hope to perform subgroup analysis of MDS according to cytogenetic and risk stratification, as well as correlation with prognosis.

However, the flaw of our study is obvious: First, the number of patients is limited, especially ICUS group, as the low incidence of ICUS we only collected 11 patients during this period, we will expand the sample size to further consolidate our experimental results; secondly, the study lacks in-depth research on the pathogenesis of selected immune checkpoints in AA, ICUS, and MDS, which will be our future main research direction.

5 | CONCLUSION

In this article, we detected the concentrations of 17 soluble immune checkpoints in the bone marrow supernant and found that there were significant differences in immune status between AA and MDS. Among them, sCD40, sCD86/B7-2, sCTLA-4, sGITR, sHVEM, sPD-1, sTIM-3, and sTLR-2 were the most effective immune checkpoints to differentiate AA from MDS. There is heterogeneity in ICUS, and the above eight immune checkpoints can predict the efficacy of ICUS to IST. In conclusion, the bone marrow soluble immune checkpoint is expected to become a new indicator to differentiate acquired bone marrow failures such as AA, ICUS, and MDS.

AUTHOR CONTRIBUTIONS

Mengtong Zang contributed to draft the article, and collect the samples and clinical data with Qiulin Chen as well as Ningyuan Ran; Nianbin Li took responsibility for data analysis. Ting Wang put forward the design of the work and revised the draft. Zonghong Shao and Rong Fu approved the version to be published after the final revision.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this study.

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 online in the Supporting Information section at the end of this article.

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