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DARS expression in *BCR/ABL1*-negative myeloproliferative neoplasms and its association with the immune microenvironment

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DARS*, encoding for aspartyl-tRNA synthetase, is implicated in the pathogenesis of various cancers, including renal cell carcinoma, glioblastoma, colon cancer, and gastric cancer. Its role in *BCR/ABL1*-negative myeloproliferative neoplasms (MPNs), however, remains unexplored. This study aimed to elucidate the expression of *DARS* in patients with MPNs (PV 23, ET 19, PMF 16) through immunohistochemical analysis and to examine the profiles of circulating immune cells and cytokines using flow cytometry. Our findings indicate a significant overexpression of *DARS* in all MPNs subtypes at the protein level compared to controls ($P < 0.05$). Notably, elevated *DARS* expression was linked to splenomegaly in MPNs patients. The expression of *DARS* showed a negative correlation with CD4+ T cells (R = – 0.451, $P = 0.0004$) and CD4+ T/CD8+ T cell ratio (R = – 0.3758, $P = 0.0040$), as well as with CD68+ tumor-associated macrophages (R = 0.4037, $P = 0.0017$). Conversely, it was positively correlated with *IL-2* (R = 0.5419, $P < 0.001$), *IL-5* (R = 0.3161, $P = 0.0166$), *IL-6* (R = 0.2992, $P = 0.0238$), and *IFN-γ* (R = 0.3873, $P = 0.0029$). These findings underscore a significant association between *DARS* expression in MPNs patients and specific clinical characteristics, as well as immune cell composition. Further investigation into the interplay between *DARS* and the immune microenvironment in MPNs could shed light on the underlying mechanisms of MPNs pathogenesis and immune dysregulation.

Keywords Myeloproliferative neoplasms, Immune microenvironment, Immune metabolism, *DARS*

The *BCR/ABL1*-negative myeloproliferative neoplasms (MPNs) are a group of clonal diseases that affect the hematopoietic stem and progenitor cells (HSPC) and are characterized by the abnormal proliferation of one or more myeloid cell lines[1]. Essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) are common subtypes of MPNs. They all are acquired stem cell neoplasms that arise due to somatic "driver" mutations, including the *JAK2V617F*, *CALR*, and *MPL* mutations. Additional mutations are often determinants for myelofibrotic and leukemic transformation[2]. All these conditions, especially PMF, may progress toward a fibrotic involution of BM, frequently accompanied by cytopenia and splenomegaly and shorter overall survival (OS)[3]. To improve the quality of life and survival of MPNs patients, the current clinical focus is on reducing the thrombotic risk, relieving the symptom burden, improving the prognosis, and reducing transfusion requirements[4]. Conventional interventions and surgical treatments include phlebotomy, radiotherapy, splenectomy, and allogeneic hematopoietic stem cell transplantation[5]. However, these treatments are prone to complications such as hypovolemia, hypocalcemia, bleeding, and infection[6,7]. Allogeneic hematopoietic stem cell transplantation has the potential to cure MPNs but is associated with a high risk of developing graft-versus-host disease (GVHD) and postoperative mortality[8]. Current conventional drugs for the treatment of MPNs include hydroxyurea, interferon alpha, androgens, alkylating agents, immunomodulators, and anticoagulants[9]. However,

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these drugs have the disadvantages of short-term efficacy and the potential to develop resistance or intolerance to them. Therefore, it is of great significance to explore the pathological mechanisms of MPNs and find new therapeutic approaches to improve the outcomes of patients with MPNs.

Genetic and epigenetic changes associated with cancers are often coupled with reprogramming cellular metabolism, which has been recognized as one of the hallmarks of cancers[10]. Doubtless, metabolic reprogramming in tumor cells is also present in MPNs. altered metabolic pathways are present in patients with MPNs[11]. There are also significant differences between serum metabolites in patients with MPNs and those in normal subjects[12]. Mechanistic studies suggest that altered metabolism in hematopoietic cells underlies the pathogenesis of *JAK2* mutation-driven MPNs[13]. The metabolism can affect the differentiation and function of immune cells, Immune metabolism refers to the interaction between metabolism and immune response, which has been proven to be related to immune activation in many diseases[14]. The development of MPNs as a tumor of innate immune cell progenitors is inextricably linked to the expansion of cytokines, monocytes/macrophages, and myeloid-derived suppressor cells in the bone marrow immune microenvironment as well as alterations in the function of T cells, dendritic cells, and NK cells[15]. In MPNs, the pathogenic contribution of immunity has been investigated almost exclusively by analyzing the biological and prognostic significance of serum cytokines and chemokines[16]. Fewer studies have examined the relationship between genes associated with altered tumor metabolism and abnormalities in MPNs immunity.

DARS is a member of the amino acid metabolism-associated aminoacyl-tRNA synthetases (*ARSs*) family. It encodes an aspartyl-tRNA synthetase, which is mainly expressed in the cell membrane and cytoplasm and is involved in protein and amino acid metabolic pathways[17]. It has been reported that altered or dysregulated function of the *DARS*-encoded protein is closely associated with the development of tumors such as renal cell carcinoma[18], glioblastoma[19], colon cancer, and gastric cancer[20], Aberrantly expressed *DARS* (aspartyl-tRNA synthetase) promotes tumor development by maintaining tumor proliferative signals, dysregulating cellular energy, and promoting tumor inflammation, metastasis, and angiogenesis[20,21]. The protein encoded by *DARS* also promotes gastrointestinal tumor progression by regulating immune cells such as CD8+ T cells, CD4+ T cells, macrophages, and related signaling pathways[22].

To the best of our knowledge, no study has reported the expression of *DARS* in MPNs and its relationship with immune cells. This study aimed to analyze the expression level of *DARS* in MPNs and its impact on clinical features and determine the relationship between *DARS* expression and immune cells and cytokines, thereby providing insights into novel biomarkers and therapeutic targets for MPNs.

Methods

Patient samples

To select study subjects, we re-evaluated bone marrow slides and reviewed clinical and laboratory data of patients with MPNs between January 2022 and January 2024 at the Second Hospital of Lanzhou University, Gansu, China. We studied a total of 58 patients affected by myeloproliferative neoplasm according to the 2016 World Health Organization classification and diagnostic criteria for MPNs[23]. A bone marrow biopsy was available for all triple-negative patients. In this study, "myelofibrosis" was defined as patients with MPNs whose bone marrow biopsies showed abnormal reactive deposition of bone marrow stromal network and collagen fibers [myelofibrosis (MF) ≥ 1]. Notably, PMF can be divided into pre-fibrotic (pre-PMF) and dominant fibrosis (dominant PMF) subtypes. For pre-PMF cases, MF ≤ 1 is required, so PMF patients may also have bone marrow without fibrosis[23]. As a control group, we analyzed 12 patients with benign hematological disorders (BHD) (1 with iron deficiency anemia, 6 with erythrocytosis, and 5 with thrombocytosis)[24]. Excluded from participation were pregnant women, subjects with acute infections, known autoimmune disorders, or concomitant solid tumors. The use of bone marrow specimens and clinical data was approved by the Ethics Committee of the Second Hospital of Lanzhou University (Approval No.2023A-188), and written informed consent was obtained from all participants (informed consent was obtained from parents/guardians of minors). All procedures involving human participants performed in this study followed the institutional and/or national research committee's ethical standards and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Immunohistochemistry

Tissues were fixed with 3% neutral formaldehyde fixative at room temperature for > 24 h, decalcified, routinely processed for paraffin embedding, and then cut into 3 μm thick sections for staining. The slides were then baked at 65 °C for 2 h to melt the paraffin wax. The slides were soaked using xylene and gradient ethanol (anhydrous ethanol, 95% ethanol, 85% ethanol) for 5 min each; the purpose of this step was to make the paraffin shedding complete. Thermal repair of antigens was performed using pH 8.0, 0.5 mM EDTA buffer for 15 min. Slides were incubated with peroxidase-blocking, non-specific staining blockers according to the instructions of the Immunohistochemistry SP kit ((cat.no.KIT-9710, Fujian Mai xin). Slides were incubated with rabbit anti-*DARS* antibody (dilution, 1:800; cat.no.bs-14197R; BIOSS), and rabbit anti-CD68 antibody (kit-0026, Fujian Mai xin) overnight at 4 °C. After primary antibody incubation, slides were treated sequentially with biotin-labeled goat anti-rabbit IgG polymer, and streptavidin antibiotic protein-peroxidase according to the immunohistochemistry kit instructions. DAB staining solution was configured according to the instructions of the DAB kit (cat. no.DAB-0031, Fujian Mai xin), and the slides were stained. Finally, the slides were re-stained with hematoxylin and sealed with neutral gum.

DARS and *CD68* expression were independently reviewed and scored by two clinical pathologists specializing in hematology. Different cases were re-examined until a consensus was reached. *DARS* and *CD68* expression were semi-quantitatively assessed using the IRS score, derived from the intensity score multiplied by the percentage of positive cells score. The intensity of immunostaining was scored on a scale of 0–3: negative, 0; weak,

1; moderate, 2; and strong, 3. The percentage of positive cells was scored on a 0–4 scale: less than 1%, 0; 1–10%, 1; 11–50%, 2; 50–80%, 3; 81–100%, 4[25,26].

Immunoassay of lymphocyte subsets

We used BD's Canto II flow cytometer to assess the percentage of common lymphocyte subsets (total T-cells, CD4+ T-cells, CD8+ T-cells, Treg cells, NK cells, B-cells) in peripheral blood using flow cytometry. The patient's fasting venous blood was drawn in the early morning using an EDTA-K2 anticoagulation tube for 2 ml. 100 µl of the peripheral blood specimen was taken and incubated with 5 µl of the conjugated antibody as follows: (i) an unlabeled tube for negative cells acquisition; (ii) a tube with anti-CD3 FITC, anti-CD45 PerCP, anti-CD19APC, and anti-CD16 + CD56 PE antibodies; (iii) a tube with anti-CD3 FITC, anti-CD45 PerCP, anti-CD8 PE and anti-CD4 APC antibodies; (iv) a tube with anti-CD4 FITC, anti-CD25APC, anti-CD127 PE antibodies. After the addition of the antibody, the above experimental tubes were shaken and mixed well, and incubated for 15 min at room temperature protected from light. When the incubation of the above experimental tubes was completed, 1 ml of erythrocyte lysate was added to the tubes respectively, and the tubes were shaken and mixed well, and then incubated again for 15 min protected from light. When the incubation was completed again, the tubes were centrifuged at 500*g* for 5 min, the supernatant was removed, and 500 µl of PBS was added to each tube to re-suspend the cells for testing. Supplementary Table 1 shows the antibody characteristics used for immunophenotyping.

Serum cytokine expression level assay

A comprehensive analysis of plasma cytokines was conducted using the 12 cytokines combined kit (immunofluorescence assay) from Jiangxi Saiye Biotechnology Co, measuring the levels of *IL-1β*, *IL-2*, *IL-4*, *IL-5*, *IL-6*, *IL-8*, *IL-10*, *IL-12p70*, *IL-17A*, *IFN-γ*, *TNF-α*, and *IFN-α*. The specific procedures were as follows: (1) 2 ml of fasting venous blood was drawn from the patients using EDTA-K2 anticoagulant tubes, followed by the extraction of plasma through centrifugation at 1000*g* for 10 min; (2) 2 ml of the sample dilution was transferred into a centrifuge tube with the calibrator, allowed to stand at room temperature for 15 min, gently mixed, and designated as the highest concentration; (3) 11 experimental sampling tubes labeled with dilution ratios of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048 were prepared, with 300 µl of sample dilution added to each tube; (4) Transfer 300 µl of liquid from the calibrator tube with the highest concentration into the 1:2 tube, followed by mixing. Subsequently, transfer 300 µl of liquid from the 1:2 tube into the 1:4 tube, aspirate, and mix, continuing this process until reaching the 1:2048 tube; (5) Determine the total number of experimental samples required, denoted as n [n = number of samples + 11 calibrators + 1 negative control (sample dilution)]. Calculate the necessary amount of microsphere mixture based on the number of samples (25 µl/sample); (6) Take the required mixture of captured microspheres, centrifuge at 200*g* for 5 min, discard the supernatant, add the same volume of microspheres buffer as the supernatant, vortex thoroughly mix, and incubate in the dark for 15–30 min; (7) 25 µl of the incubated and mixed capture microsphere mixture was added to each experimental tube; (8) 25 µl of gradient-diluted calibrator was added to the calibrator tube; (9) Add 25 µl of fluorescence detection reagent into each tube, incubate for 2.5–3 h, add 1 ml PBS solution, and centrifuge at 200 g for 5 min to remove the supernatant; (10) According to the requirements of the flow cytometer (FACS Canto II BD), add 100 µl PBS solution to each tube, and place it away from light, waiting for detection.

Statistical analysis

SPSS 23 and GraphPad Prism (version 10.0; GraphPad Software, Inc.) software were used for statistical analysis, we used the chi-square test and independent samples *t*-test to analyze the clinical characteristics, gene mutation status, common laboratory indicators, and tumor symptom burden of MPNs patients, $P < 0.05$ was considered a statistically significant difference. Then correlation analysis was used to explore the association between *DARS* and the immune microenvironment, and $P < 0.05$, the difference was considered statistically significant. Patients' characteristics were summarized as numbers (percentage) for qualitative variables, and with mean ± standard deviation, or median—[Inter-Quartile Range (IQR)], as appropriate, for continuous variables.

Results

Key demographics, hematology, biochemistry, and gene mutations in MPNs patients

To determine the relationship between the expression level of *DARS* and important clinical parameters in patients with MPNs, 58 cases of MPNs (19 PV, 23 ET, and 16 PMF) who underwent bone marrow biopsy were selected as study subjects, and 12 patients with benign hematological disorders were used as controls. Table 1 shows the clinical, hematological, and immunological parameters of the patients with MPNs participating in the study. These results are consistent with those reported in previous MPNs-related studies[27,28].

The expression of *DARS* in patients with MPNs

To determine *DARS* expression in MPNs patients, we examined *DARS* expression using immunohistochemical staining of bone marrow biopsy specimens from 58 MPNs patients and 12 benign disease control patients. The IRS scoring system was used to semi-quantify *DARS* immunoreactivity (Fig. 1A,B). The results showed that *DARS* expression levels were significantly higher in patients with PV, ET, and PMF compared to controls ($P < 0.001$) (Fig. 1C). Undoubtedly, the expression of DARS in MPNs as a whole was significantly higher than that in the control group ($P < 0.001$) (Fig. 1D). We also compared the expression of *DARS* in control and MPNs patients as a whole, and there is no doubt the overall expression level of *DARS* was significantly higher in MPNs patients than in controls ($P < 0.001$). To better understand the clinical characteristics of patients with different *DARS* expressions, we wanted to categorize MPN patients into high and low-expression groups based on the IRS score of *DARS* expression. The cut-off score for normal *DARS* expression was set at 6 based on the finding

Parameter	ET (23)	PV (19)	MF (16)	*P*
Sex (male/female), n (%)	13/10 (56.5/43.5)	13/6 (68.4/31.6)	6/10 (37.5/62.5)	0.184
Age, year	48.6 ± 15.2	55.2 ± 8.7	61.9 ± 15.5	0.014
HGB, g/l	140.4 ± 24.5	192.5 ± 22.3	90.6 ± 32.7	0.000
PLT, ×10 g/l	824.5 ± 366.9	423.1 ± 220.4	268.9 ± 354.1	0.000
WBC, ×10 g/l	8.9 ± 52.4	13.9 ± 7.0	9.5 ± 14.6	0.155
LDH, U/l	308.1 ± 194.1	343.2 ± 140.9	503.1 ± 340.8	0.034
Gene mutation, n (%)	23	19	16	0.274
JAK2	12 (52.2)	17 (89.5)	11 (68.8)	
CALR	6 (26.1)	1 (5.3)	3 (18.8)	
MPL	1 (4.3)	0 (0)	0 (0)	
Triple-negative	4 (17.4)	1 (5.3)	2 (12.5)	
DARS (IRS score)	7.09 ± 2.15	5.79 ± 1.93	7.06 ± 2.46	0.12
CD68 (IRS score)	4.74 ± 1.42	5.05 ± 1.78	4.81 ± 1.87	0.83
CD4+ T (%)	42.16 ± 9.52	43.90 ± 7.66	35.98 ± 8.71	0.03
CD4+ T/CD8+ T	1.74 ± 0.87	1.72 ± 0.65	1.06 ± 0.51	0.01
IL2 (pg/ml)	0.73 ± 1.01	0.53 ± 0.78	2.3 ± 4.8	0.11
IL5 (pg/ml)	0.52 ± 0.56	0.37 ± 0.45	0.72 ± 0.63	0.14
IL6 (pg/ml)	5.33 ± 6.92	5.90 ± 6.41	9.72 ± 7.55	0.16
IFN-γ (pg/ml)	0.99 ± 1.87	1.55 ± 3.46	3.44 ± 6.52	0.19

Table 1. Clinical baseline data sheets and immune-related indicators in MPN patients. *CALR* calreticulin, *ET* essential thrombocythemia, *JAK2* Janus kinase 2, *LDH* lactate dehydrogenase, *MPL* MPL protooncogene, thrombopoietin receptor, *PLT* platelet, *PMF* primary myelofibrosis, *PV* polycythemia vera, *WBC* white blood cell. The numerical results (IRS scores for *DARS* expression) are represented as mean ± standard deviation.

that no patient in the BHD group showed a score less than 6. A *DARS* score less than 6 was considered as low *DARS* expression[29].

The relationship between *DARS* expression and clinical characteristics of MPNs patients

JAK2 mutation is the most common mutation among the three subtypes of MPNs[28]. Therefore, the present study analyzed the relationship between clinical characteristics and *DARS* expression in patients with three subtypes of MPNs and patients with *JAK2*-mutated MPNs. We categorized MPNs patients into low and high *DARS* expression groups based on their IRS score of *DARS* expression. A comparison of clinical manifestations and laboratory characteristics of patients in the high and low-expression groups is shown in Tables 2, 3. In patients with ET, PMF, and *JAK2* mutations, high expression of *DARS* was associated with a greater occurrence of splenomegaly ($P < 0.05$), and this difference was also observed in patients with PV, although not statistically significant. In the ET group, high expression of *DARS* was associated with higher lactate dehydrogenase ($P = 0.01$); among patients with *JAK2* mutations, those with high expression of *DARS* were more likely to have MPN-related symptoms and elevated hemoglobin (HGB) ($P < 0.05$), a difference that was not observed in the PV and ET groups alone and PMF.

The relationship between DARS expression and immune cells

Immunometabolism is associated with the activation of a wide range of immune cells[30]. *DARS* encoding aspartyl-tRNA synthetase can influence immune metabolism by regulating aspartate synthesis[31,32]. It is of interest whether the expression of *DARS* in MPNs is associated with immune cells. We used flow cytometry to detect the frequency of common T-cells, B-cells, and NK-cells in peripheral blood, and the immunohistochemical marker CD68 to represent tumor-associated macrophages. We classified *DARS* into two groups of high and low expression according to the IRS score and compared the differences in immune cells between the two groups of patients. The results showed that the percentage of CD4+ T cells, the IRS score of CD68, and the CD4+ T/CD8+ T ratio were different between the two groups, and the differences were statistically significant ($P < 0.05$) (Fig. 2A–I). The proportion of T cells, Treg cells, CD8+ T cells, B cells, and NK cells in immune cells did not differ between groups with high and low DARS expression (Supplementary Fig. 1A–K). Therefore, we further explored the correlation between *DARS* expression and frequency of CD4+ T cells, CD68+ tumor-associated macrophages, and the ratio of CD4+ T/CD8+ T cells. Correlation analysis showed that *DARS* expression was negatively correlated with the frequency of CD4+ T cells ($R = -0.451$, $P = 0.0004$), CD4+ T/CD8+ T cell ratio ($R = -0.3758$, $P = 0.0040$) and positively correlated with CD68+ tumor-associated macrophages ($R = 0.4037$, $P = 0.0017$) (Fig. 2J–L).

The relationship between *DARS* expression and cytokines

As key mediators of immune responses, cytokines are involved in the regulation of the immune system and play a role in tumor progression and prognosis. After clarifying that *DARS* is involved in the composition of immune cells in the microenvironment, we further explored the relationship between cytokines and *DARS* expression. Considering that MPN has been recognized as an inflammatory disease—a paradigm for the relationship between

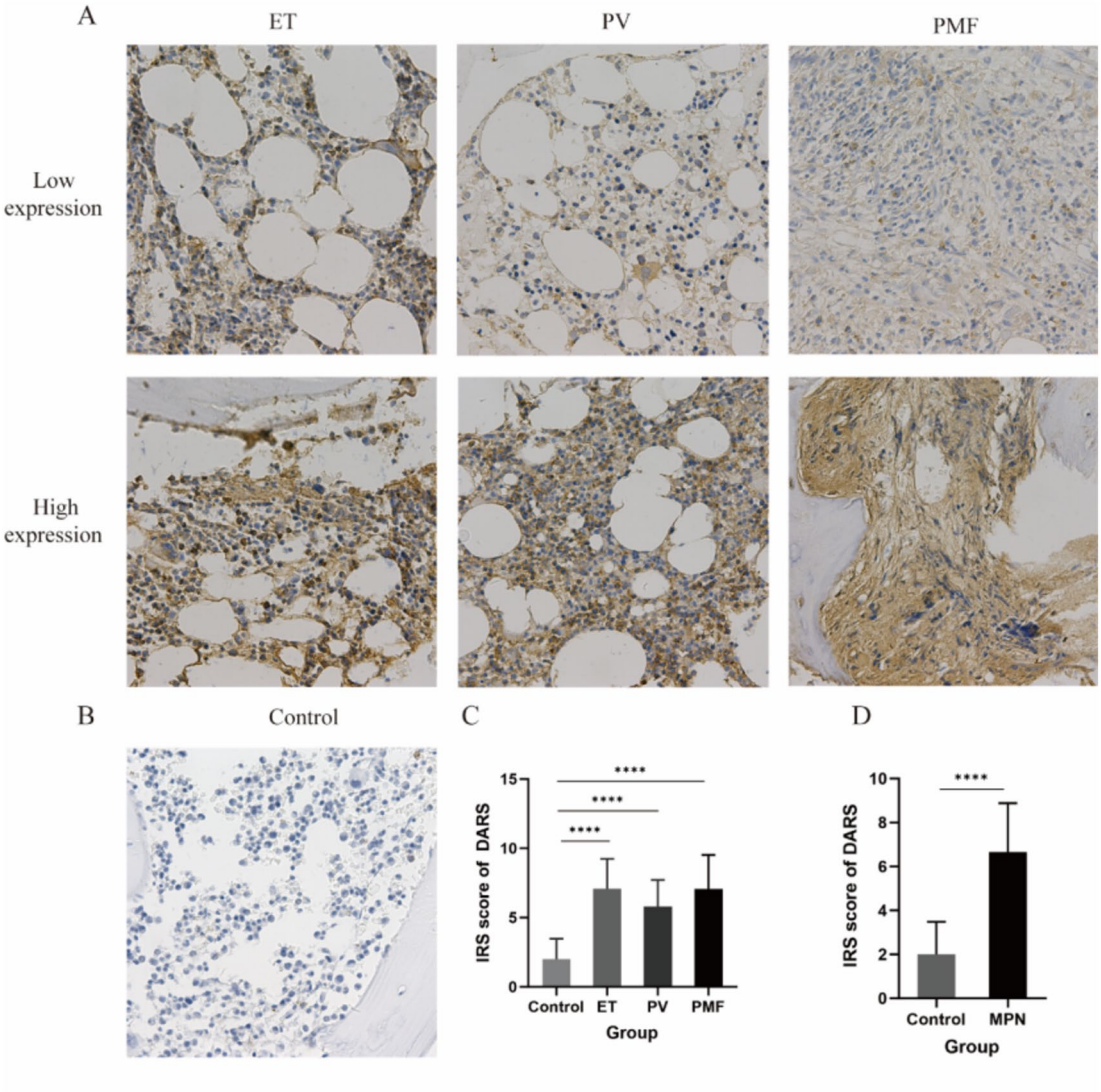

Figure 1. Representative immunohistochemical results of all myeloproliferative neoplasms groups. (**A**) Immunohistochemical analysis of *DARS* expression using paraffin-embedded bone marrow biopsy specimens in the order of ET (left panel), PV (middle panel) and PMF (right panel). (**B**) Immunohistochemical analysis of *DARS* in the control group; (**C**) IRS scores of the *DARS* expression in the PV group, the ET group, the PMF group, and the control group IRS scores; (**D**) IRS scores of *DARS* expression in all MPNs patients and controls; ***$P < 0.01$. *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *PV* true erythrocytosis. The numerical results (IRS scores for *DARS* expression)are represented as mean ± standard deviation.

chronic inflammation and tumorigenesis. We therefore focus on common inflammation-associated cytokines. We classified *DARS* into high and low-expression groups according to IRS scores and showed that there were differences in the expression of pro-inflammatory cytokines *IL-2*, *IL-5*, *IL-6*, and *IFN-γ* between the high and low-expression groups ($P < 0.05$, Fig. 3A–D). There were no differences in *IFN-α*, *IL1β*, *IL4*, *IL8*, *IL10*, *IL17A*, *TNF-α*, and *IL12p70* expression between the two groups (see Supplementary Fig. 1L–S). We further explored the correlation of *DARS* expression with the expression of cytokines *IL-2*, *IL-5*, *IL-6*, and *IFN-γ*. The results showed that the expression of *DARS* was positively correlated with the expression of *IL-2* ($R = 0.5419$, $P < 0.001$), *IL-5* ($R = 0.3161$, $P = 0.0166$), *IL-6* ($R = 0.2992$, $P = 0.0238$) and *IFN-γ* ($R = 0.3873$, $P = 0.0029$) (Fig. 3E–H).

Discussion

The oncogenic role of the *DARS* oncogene has been demonstrated in a variety of tumors including renal clear cell carcinoma[24], lung adenocarcinoma, and gastric cancer[20]. To the best of our knowledge, this study is the first to report that *DARS* is upregulated at the protein level in MPNs bone marrow cells. The results of the present study also revealed a relationship between the expression level of *DARS* and the disease load in MPNs patients. MPNs patients with high DARS expression are more likely to have splenomegaly and elevated lactate dehydrogenase, which are more readily observed in ET patients. *DARS* encodes an aspartyl tRNA synthetase that affects the correct translation of aspartic acid[20]. Increased aspartic acid in tumor cells promotes tumor cell proliferation,

Variables	DARS expression								
	ET			PMF			PV		
	High	Low	P	High	Low	P	High	Low	P
Age, years	48.00 ± 17.02	48.93 ± 14.84	0.893	61.29 ± 12.15	63.44 ± 18.35	0.888	57.00 ± 9.42	54.67 ± 8.75	0.676
Sex (male/female)	4/4 (50/50)	9/6 (60/40)	0.685	2/5 (58.3/41.7)	4/5 (44.4/55.6)	0.063	3/1 (75/25)	10/5 (66.67/33.33)	1
WBC, $\times 10^9$/l	9.45 ± 2.63	8.55 ± 2.33	0.428	13.89 ± 22.00	6.03 ± 2.28	0.301	13.13 ± 5.82	14.08 ± 7.41	0.816
HGB, g/l	140.75 ± 20.90	140.20 ± 26.90	0.960	78.86 ± 30.53	99.78 ± 33.08	0.216	193.75 ± 33.39	192.13 ± 20.06	0.902
PLT, $\times 10^9$/l	844.25 ± 464.60	813.93 ± 321.39	0.855	243.29 ± 249.82	288.89 ± 432.79	0.808	358.25 ± 258.83	440.4 ± 215.81	0.523
LDH, U/l	445.25 ± 278.71	235.00 ± 62.49	0.010	657.14 ± 424.62	383.33 ± 213.71	0.113	360.50 ± 184.52	338.60 ± 134.67	0.791
MPN-related symptoms	4 (50)	3 (20)	0.182	5 (71.4)	3 (33.3)	0.315	3 (75)	5 (33.3)	0.262
Hemorrhage at diagnosis, n (%)	1 (12.5)	0 (0)	0.348	1 (14.3)	1 (11.1)	1	0 (0)	0 (0)	–
Thrombosis at diagnosis, n (%)	1 (12.5)	0 (0)	0.348	5 (71.4)	4 (44.4)	0.358	1 (25)	2 (13.3)	0.530
Splenomegaly, n (%)	6 (75.0)	2 (13.3)	0.006	7 (100)	4 (55.6)	0.034	4 (100)	7 (46.7)	0.103

Table 2. Comparison of clinical data between high and low DARS expression groups in MPN patients.

Variables	DARS expression		
	Jak2+ mutation		
	High	Low	P
Age, years	55.62 ± 14.35	57.74 ± 13.17	0.645
Sex (male/female)	6/7 (46.2/53.8)	16/11 (59.3/40.7)	0.509
WBC, $\times 10^9$/l	14.04 ± 15.56	10.59 ± 6.19	0.319
HGB, g/l	125.69 ± 49.42	160.44 ± 45.03	0.033
PLT, $\times 10^9$/l	582.31 ± 502.84	425.07 ± 258.29	0.196
LDH, U/l	377.31 ± 194.10	322.41 ± 157.99	0.345
MPN-related symptoms	10 (76.9)	8 (29.6)	0.007
Hemorrhage at diagnosis, n (%)	1 (7.7)	1 (3.7)	1
Thrombosis at diagnosis, n (%)	5 (38.5)	6 (22.2)	0.451
Splenomegaly, n (%)	13 (100)	9 (33.3)	0.000

Table 3. Comparison of clinical data between high and low DARS expression groups in MPN patients of JAK2+ mutation. *CALR* calreticulin, *ET* essential thrombocythemia, *JAK2* Janus kinase 2, *LDH* lactate dehydrogenase, *MPL* MPL proto-oncogene, thrombopoietin receptor; MPN, myeloproliferative neoplasm; PLT, platelet, *PMF* primary myelofibrosis, *PV* polycythemia vera, *WBC* white blood cell, *HGB* hemoglobin. The numerical results (IRS scores for *DARS* expression) are represented as mean ± standard deviation.

metastasis, and chemoresistance through activation of the mTORC1 pathway, in addition to regulating metabolic reprogramming of tumor cells[33,34]. These results, combined with studies related to the *DARS* oncogene[35], suggest that *DARS* may be a marker for malignant clones of MPNs myeloid cells.

Chronic inflammation characterizes the immune system in MPNs, and both the cytokine environment and the immune system are dysregulated in MPNs patients[36]. Lymphocyte subsets can reflect the immune function of the body[37]. Studies have shown that lymphocyte subsets are impaired in patients with MPNs and that B, T, and NK cell lineages are involved in MPNs malignant clones[36]. To understand the changes in each lymphocyte subset in MPNs patients, we analyzed the proportions of lymphocyte subsets in MPNs patients using flow cytometry. We consider that amino acids support immune cell function through multiple mechanisms such as redox homeostasis and epigenetic modifications. Aspartic acid has an important role in promoting nucleotide synthesis and driving translational processes in immune cells[31]. As a gene that influences the coding of aspartic acid, we are interested in the role of *DARS* in the MPNs immune microenvironment. We therefore also analyzed the relationship between *DARS* expression and lymphocyte subsets. We were surprised to find that *DARS* expression was negatively correlated with the frequency of CD4+ T cells, the ratio of CD4+ T/CD8+ T. In other words, patients with high expression of *DARS* have a depletion of circulating CD4+ T cells. CD4+ T cells can induce immune cells in vivo and participate in the activation of B lymphocytes, macrophages, and cytotoxic CD8+ T cells, while CD8+ T cells have limited antitumor effects in the absence of CD4+ T cells[38,39]. Decreased

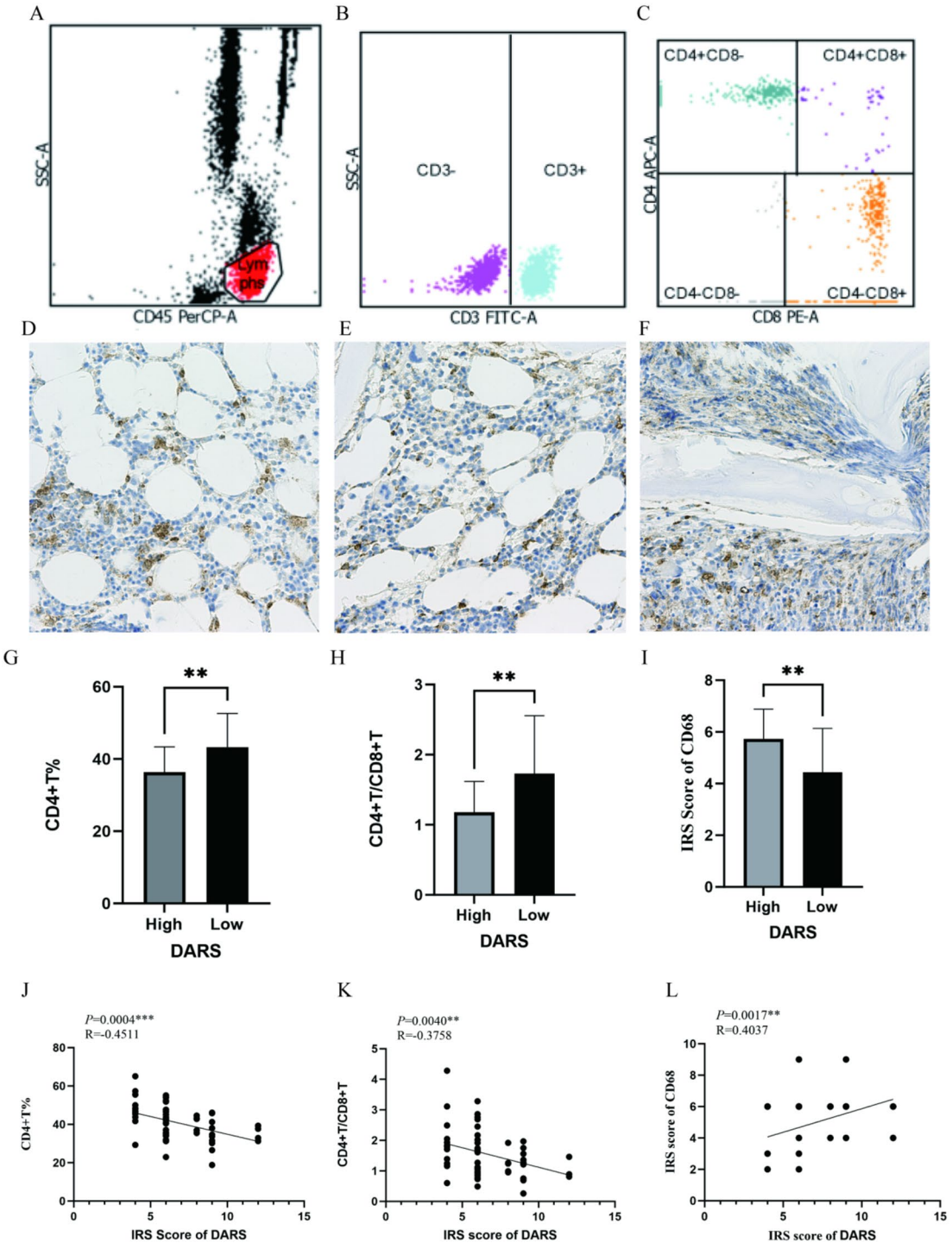

Figure 2. Relationship between *DARS* expression and circulating immune cells. (**A**–**C**) Percentage of CD45 (**A**), CD3 (**B**), and CD4/CD8 (**C**) Cells in Circulating Cells of MPN Patients detected by Flow Cytometry; (**D**–**F**) CD68 expression of ET (**D**), PV (**E**), PMF (**F**) in patients with myeloproliferative neoplasms; (**G**,**H**) Based on the IRS score of *DARS* expression, MPNs patients were classified into the *DARS* high and low expression groups, and the differences in CD4+ T percentage (**G**) and CD4+ T/CD8+ T ratio (**H**) between the two groups were analyzed; (**I**) Semi-quantification of *CD68* expression according to IRS score and comparison of *CD68* differences between *DARS* high and low expression groups; (**J**–**L**) Based on the IRS score of *DARS* expression, and analyzed the correlation between the *DARS* scores and CD4+ T percentage (**J**), CD4+ T/CD8+ T ratio (**K**); (**L**) Correlation between *DARS* scores and CD68 scores was analyzed based on IRS scores for *DARS* and *CD68* immunoreactivity. The numerical results (IRS scores for *CD68* expression, percentages of CD4+ T cells, CD4+/CD8+ ratios) are represented as mean ± standard deviation.

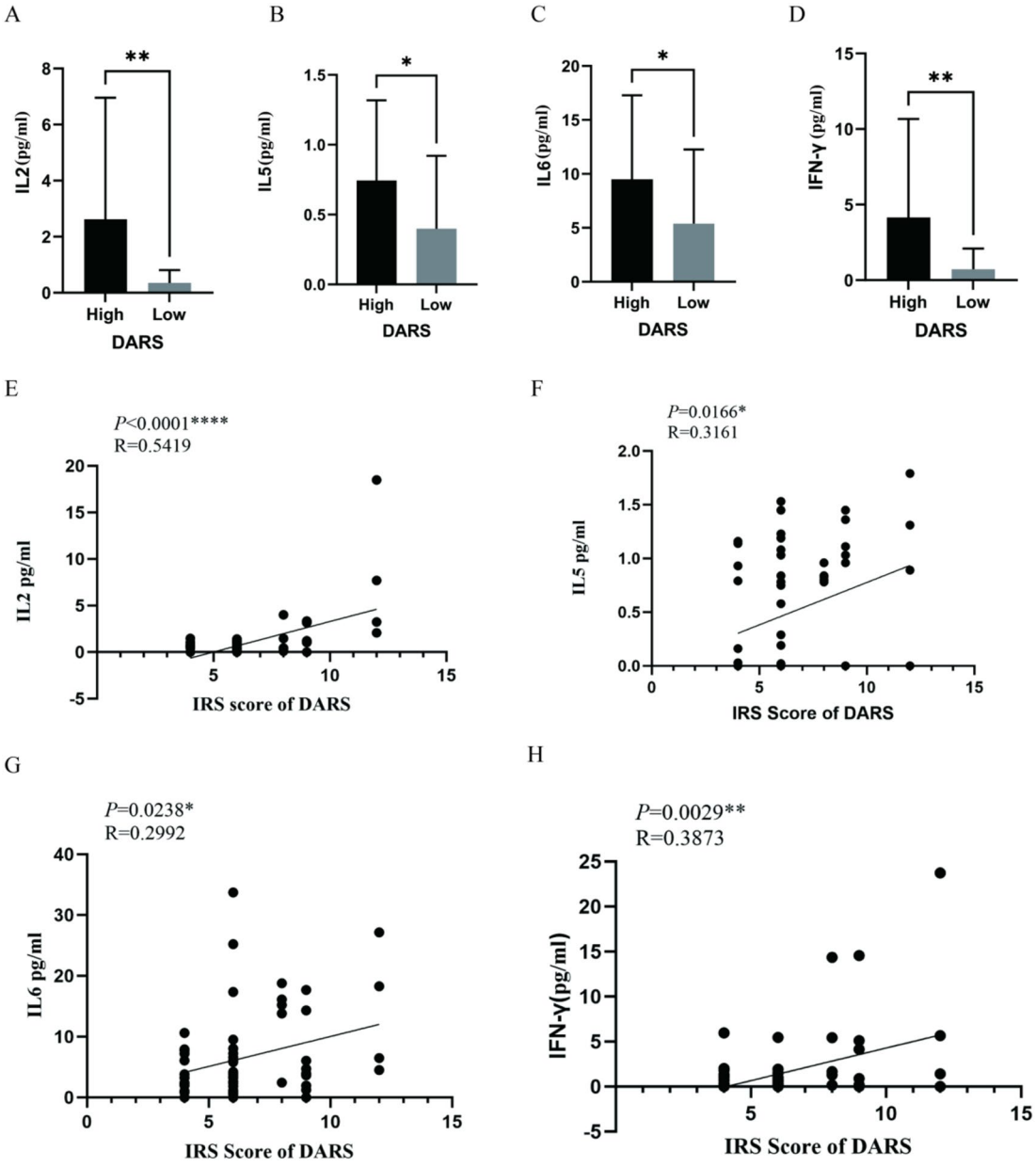

Figure 3. Relationship between DARS expression and circulating cytokines. (**A**–**D**) Based on the IRS score of DARS immunoreactivity, MPN patients were divided into groups with high and low DARS expression, and the differences in IL-2 (pg/ml) (**A**), IL-5 (pg/ml) (**B**), IL-6 (pg/ml) (**C**), and IFN-γ (pg/ml) (**D**) between the two groups were analysed; (**E**–**H**) Based on the IRS score of DARS immunoreactivity, the correlation between DARS expression and IL-2 (pg/ml) (**E**), IL-5 (pg/ml) (**F**), IL-6 (pg/ml) (**G**), and IFN-γ (pg/ml) (**H**) correlations. The numerical results (cytokine plasma levels) are represented as mean ± standard deviation.

CD4+ T levels indicate decreased immune function, which can affect anti-tumor effects and lead to continued tumor progression[40]. The presence of a lower percentage of CD4+ T cells in patients with high expression of *DARS* suggests that aberrantly expressed *DARS* in MPNs may be involved in the depletion of CD4+ T cells. It would be interesting to explore further the relationship between *DARS* expression and CD4+ T cell depletion in MPNs patients and its mechanisms.

Another important immune cell type in the tumor microenvironment is the tumor-associated macrophage, which plays an active role in tumor growth by promoting tumor immune escape[41]. Numerous studies have demonstrated that tumor-associated macrophages are strongly associated with tumor progression and that highly infiltrated TAM is a predictor of poor prognosis in tumor patients[42]. Macrophages were identified primarily by immunohistochemistry with the application of anti-CD68 antibodies[43]. CD68, a 110 kDa transmembrane glycoprotein, is a classical macrophage marker often used as an important indicator of TAM. In this study, MPNs patients with high expression of *DARS* had significantly higher disease loads, and CD68 expression was positively correlated with *DARS* expression. This suggests that tumor-associated macrophages, represented by CD68, may be associated with the progression of MPNs. This is consistent with previous findings on tumor-associated

macrophages in hematological neoplasms such as lymphomas and acute leukemias[44]. It is worth noting that TAM can inhibit the function of CD4+ T and CD8+ T cells through the production of immunosuppressive factors[45,46]. In other words, the high expression of CD68 in MPNs may be associated with the tumor immunosuppressive microenvironment. Combined with our research results that the expression of *DARS* in MPNs patients was positively correlated with the expression of CD68 and negatively correlated with the proportion of CD4+ T cells, we speculated that *DARS* might promote tumor progression by suppressing the anti-tumor response of the tumor immune microenvironment.

Cytokines are involved in regulating the immune system and play a role in tumor progression and prognosis[47]. In MPNs, cytokines are key mediators of amplification and deleterious crosstalk between MPNs clones and the tumor microenvironment, not only play an indispensable role in inflammatory pathology, but are also key immune mediators in myeloproliferative neoplasms' physiology as well as in the disease process, and are inextricably linked to disease progression. In this study, we explored the correlation between common cytokines and the expression of *DARS*. We found that *DARS* was positively correlated with the expression of pro-inflammatory cytokines *IL-2*, *IL-5*, *IL-6*, and *IFN-γ*. Several studies have demonstrated that pro-inflammatory cytokine levels are elevated in all subtypes of MPNs. The pro-inflammatory cytokine *IL-6* can promote cancer growth and spread by causing genetic instability and oxidative stress and by blocking the apoptotic program and cell migration[48]. *IFN-γ* enhances the selective advantage of myeloid malignancy-associated mutant clones[49]. *IL-2/IL-2R* signaling plays an important role in Treg cell biology[50]. The expression of *DARS* was positively correlated with the expression of CD68 and these cytokines that promote tumor cell proliferation and maintain immune tolerance, and negatively correlated with the proportion of CD4+ T cells. These results support the hypothesis that *DARS* acts as a pro-tumorigenic factor in MPNs.

Several strengths of our study are acknowledged: (i) the present study reveals for the first time that aberrant *DARS* may act as an oncogenic factor in *BCR/ABL1*-negative MPNs, although the mechanisms involved remain to be determined; and (ii) the correlation between aberrantly expressed *DARS* and the major immune cells and cytokines in the tumor microenvironment is revealed, which is highly in need of further investigation. However, there are limitations to our study. First, this study included only patients who sent sufficient material to the laboratory, so the number of cases was small, and the study may have been limited due to possible selection bias. Secondly, due to the small number of cases studied, we did not explore the relationship between *DARS* expression and immune cells and cytokines in the three subtypes of MPN, which is another shortcoming of our study. Although we observed a correlation between aberrantly expressed *DARS* and the frequency of CD4+ T cells and tumor-associated macrophages, we did not perform studies on the expression of *DARS* on immune cells. These need to be further validated in subsequent studies.

In conclusion, our study suggests that aberrant expression of *DARS* is present in patients with all subtypes of MPNs, and patients with high expression of *DARS* have a heavy disease load. *DARS* may be a marker for malignant cloning of MPNs myeloid cells. There is a correlation between expression and the frequency of CD4+ T cells, tumor-associated macrophages, and pro-inflammatory cytokines. Further exploration of the relationship between *DARS* and the immune microenvironment of MPNs will be valuable for understanding the immune imbalance in MPNs.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

LSZ and LJL conceived, designed, and supervised the whole study. HX, JML and HTZ performed the analyses. JB, JPZ and YHL interpreted the results, and contributed in this work in study design, data interpretation, and manuscript writing. HX, JML and HTZ wrote the manuscript. All authors provided critical comments and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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