BMC Cancer

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Clinical and immunological characteristics of high-risk double-hit multiple myeloma

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Abstract

At present, the characteristics of double-hit multiple myeloma (DHMM) are unknown. We retrospectively analyzed the clinical data from 433 new diagnosed MM patients and found that DHMM have a higher β2-MG level and percentage of bone marrow plasma cell. Cox regression analysis showed that the prognosis of DHMM was not limited by clinical indicators. The abnormal proliferation of bone marrow in DHMM is obvious, and the proportion of poorly differentiated plasma cell is high. By collecting specimens from our center and performing flow cytometry to analyze the immunophenotypic and functional characteristics of lymphocyte subpopulations, we found that DHMM had a higher ratio of Tregs cells, and the proportion of iTregs cells was also significantly higher than non-DHMM ($P<0.05$). Moreover, DHMM had higher levels of TGF-β1 and IL-10, and TGF-β1 and IL-10 were positively correlated with iTregs ($P<0.05$). In addition, DHMM was highly expressed PD-1 on CD8+T cells and had a higher proportion of $CD38^{high}$Tregs cells. In vitro we have shown that the addition of TGF-β1 antibody or CD38 antibody can effectively inhibit the proportion of $CD38^{high}$ Tregs. This study describes the characteristics of DHMM based on bicentric data, which is helpful to better provide theoretical support for the treatment of DHMM.

Keywords Double-hit multiple myeloma, Bone marrow immune microenvironment, Tregs, TGF-β1, PD-1

Introduction

Survival rates for multiple myeloma (MM) have improved significantly over the past decade; however, they remain critically low for high-risk patients, with rates between 15 and 20% [1]. Cytogenetic alterations play a pivotal role in the pathogenesis of the disease and are considered essential prognostic markers [2]. Recent studies suggest that the presence of multiple concurrent genetic lesions may be more predictive of patient outcomes than individual abnormalities [3, 4]. The latest guidelines from the Mayo Clinic's mSMART 3.0 program (High-Risk Genetic Abnormalities: t(4;14), t(14;16), t(14;20), Del 17p, p53 mutation, RISS Stage 3, High Plasma Cell S-phase, High-Risk Signature, Double Hit Myeloma, Triple Hit Myeloma) propose that double-hit and triple-hit MM, characterized by cytogenetic abnormalities such as Del(17p), t(4;14), t(14;16), t(14;20), Gain(1q), and p53

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mutations, represent particularly high-risk forms of the disease. In this classification, the presence of any two high-risk cytogenetic abnormalities (CAs) defines double-hit multiple myeloma (DHMM), while the presence of three or more CAs constitutes triple-hit MM [2]. Both double-hit and triple-hit MM are associated with significantly poorer prognoses and are classified as high-risk myeloma subtypes.

The bone marrow microenvironment plays a critical role in the proliferation, survival, and migration in MM [5]. An increasing body of research indicates that MM is associated with immune dysfunction [6]. This includes the expression of immune checkpoint ligands on plasma cells, elevated levels of adenosine receptors and adenosine, as well as immunosuppressive mechanisms involving myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) [2, 7–9]. Furthermore, the pathogenesis and progression of MM are believed to involve a complex bidirectional interaction between MM cells, bone marrow stromal cells, the extracellular matrix, and various supportive cells. This interaction induces autocrine and paracrine signaling pathways, which not only regulate tumor growth and development but also transform the bone marrow microenvironment into an immunosuppressive niche [10, 11].

The Transforming Growth Factor-β (TGF-β) signaling pathway serves as a potent modulator of the immune system, exerting immunosuppressive effects primarily through its interactions with T cells. Specifically, TGF-β inhibits the maturation of T cells and prevents naïve T cells from acquiring effector functions [12, 13]. Additionally, TGF-β mediates immunosuppressive effects on T cells via Tregs [13, 14]. Furthermore, TGF-β has been demonstrated to induce the expression of programmed cell death protein 1 (PD-1) on human CD8+and CD4+T cells when stimulated in vitro [15].

Monoclonal antibodies targeting CD38 have been utilized for the treatment of relapsed or refractory MM, with the first such antibody, Daratumumab, receiving approval in 2015 for this indication [16]. Due to the associated increased toxicity of multi-agent combinations, Daratumumab is currently excluded from first-line regimens to maintain a balance between safety and efficacy [17]. In the context of first-line induction therapy, Daratumumab is recommended solely for high-risk patients who are eligible for transplantation [18]. However, it is noteworthy that the mSMART 3.0 guidelines advocate the use of Daratumumab as a first-line treatment for patients who are relapsed and refractory to lenalidomide.

Given that DHMM, which encompasses both initial and/or secondary CAs, represents a relatively advanced stage of tumor development, there is a paucity of studies focusing on double-hit or triple-hit MM. This study aims to analyze the clinical features and microenvironment of DHMM while further exploring the influence of myeloma cells on immune cell populations and the relationship among TGF-β, PD-1, and Tregs in vitro. The objective is to identify potential mechanisms that could enhance the prognosis of patients with DHMM.

Materials and methods

Patients

Patients newly diagnosed with MM between September 2012 and September 2021 at Zhongnan Hospital of Wuhan University and Sun Yat-sen University Cancer Centre in China were retrospectively analysed. All included patients were diagnosed with MM according to the International Myeloma Working Group (IMWG) criteria [19]. Patients with smouldering MM, solitary plasmacytoma, and those without adequate clinical information were excluded. The study was approved by the Institutional Review Boards of all participating institutions. All procedures in the study that involved human participants were performed in accordance with the Declaration of Helsinki.

Data were obtained through a comprehensive review of medical records. The baseline data collected included general demographic information, laboratory results, radiological imaging reports, bone marrow manifestations, immunophenotype, cytogenetics, and administered treatments. CAs were detected using Fluorescence In Situ Hybridization (FISH). The degree of nucleated cell proliferation in the bone marrow was classified into five levels, estimated based on the density of nucleated cells in bone marrow smears or the ratio of nucleated cells to mature red blood cells (RBCs). Detailed records were maintained for the first-line treatment regimens, which encompassed immunomodulatory drug (IMiD)-based regimens, proteasome inhibitor (PI)-based regimens, and combinations of IMiD and PI therapies.

The prognostic evaluation included overall survival (OS) and progression-free survival (PFS). OS was defined as the duration from the date of diagnosis to the last follow-up or death from any cause. PFS was calculated from the date of diagnosis to the occurrence of disease progression, relapse, death from any cause, or the last follow-up. For patients who did not experience any of these events, follow-up data were censored at the date of the last contact. In this study, double-hit and triple-hit multiple myeloma were uniformly classified as DHMM.

Cell lines, medium and reagents

Human multiple myeloma cell lines (MM.1 S) were cultured under mycoplasma-free conditions and maintained in complete culture medium (RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS]) in ventilated tissue culture flasks at 37 °C in a humidified incubator containing 5% CO_2. Peripheral blood (PB) and

bone marrow (BM) samples were collected from the fresh buffy coat of non-tumor donors and (MM patients. Bone marrow mononuclear cells (BMMCs) were expanded in complete culture medium supplemented with 20 IU/mL rIL-2 (PeproTech). Daratumumab was obtained from clinical therapeutic sources, and the TGF-β neutralizing antibody was procured from R&D Systems.

Isolation of peripheral blood mononuclear cells and bone marrow mononuclear cells

Fresh PB and BM samples were collected in heparin-treated tubes from each subject and utilized for the isolation of plasma as well as peripheral blood mononuclear cells (PBMCs) and BMMCs. Following plasma isolation, fresh PB or BM samples were diluted 1:1 with phosphate-buffered saline (PBS) prior to the separation of PBMCs or BMMCs using Ficoll-Hypaque density gradient centrifugation (Tianjin Hao Yang). The specific experimental procedures were conducted in accordance with the manufacturer's protocol. The isolated cells were used immediately for multiparametric flow cytometry or in vitro co-culture experiments.

Ex vivo co-culture

MM cells were co-cultured with BMMCs from either non-tumor donors or MM patients in tissue culture plates at an effector-to-target (E: T) ratio of 10:1. In this context, the E: T ratio denotes the relative proportion of effector cells to target cells. BMMCs alone served as the control group. Serial doses of Daratumumab or TGF-β neutralizing antibody were administered to the co-cultures for a duration of 72 h, after which flow cytometry analysis was conducted to assess the frequency and phenotype of T cells and Tregs. Additionally, supernatants were collected for cytokine assessment.

Multiparametric flow cytometric analysis

Percentages and counts of each lymphocyte subset were determined using Trucount tubes and BD Multitest 6 color TBNK reagent. Gates were established based on the surface markers corresponding to different lymphocyte subsets, with the specific surface markers delineated in Table S2. The ability to detect interferon-γ (IFN-γ) secretion by CD4+T, CD8+T, and NK cells after induction was utilized to assess lymphocyte secretory function.

For surface staining, PBMCs or BMMCs were washed twice in PBS containing 1% FBS (staining buffer), and then were stained with fluorochrome-conjugated monoclonal antibodies (mAbs). Samples were incubated with antibodies for 30 min, then washed with staining buffer. Flow cytometry was performed on a BD FACSCantoII and data were analyzed with FlowJo software version 10 (Treestar).

Antibodies used for flow cytometry were as follows: CD45-Percp-Cy5, CD45-V500, CD3-APC-Cy7, CD3-Percp-Cy5.5, CD4-V450, CD8-Pe-Cy7, CD28-PE, CD45RA-FITC, CD45RO-PE, HLA-DR-APC, CD25-APC, CD127-Pe-Cy7, CD38-PE, PD-1-PE.

Cytokines production assays

Plasm of peripheral blood and bone marrow and cell culture supernatants were collected. The concentration of cytokine including interleukin-6 (IL-6), interleukin-17 A (IL-17 A), tumor necrosis factor-α (TNF-α), IFN-γ, interleukin-12P70 (IL-12P70), interleukin-10 (IL-10) and TGF-β were detected by ELISA according to manufacturer's protocol.

Plasma samples from PB and BM, as well as cell culture supernatants, were collected for analysis. The concentrations of various cytokines, including interleukin-6 (IL-6), interleukin-17 A (IL-17 A), tumor necrosis factor-alpha (TNF-α), IFN-γ, interleukin-12p70 (IL-12p70), interleukin-10 (IL-10), and TGF-β1, were quantified using enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's protocol.

FISH assay

Our centre employs a combination of three FISH assays for diagnosis. For patients with more than 5% myeloma cells in bone marrow smear morphology, the FISH method is applied to the first aspiration of bone marrow smear cells. In patients with BM plasma cell proportions exceeding 20%, the direct FISH method (D-FISH) is used, yielding results comparable to those of cell sorting. The vast majority of patients are tested using the CD138 magnetic bead sorting method (MACS-FISH), which achieves 70-90% plasma cell purity after sorting. This significantly enhances the detection rate of abnormal clones and reduces the false-positive rate [20].

In our center, 20 normal/donor fresh bone marrow cell preparations were firstly used for FISH probe assay; 100 cells were analyzed by two readers in each case, and the percentage of the number of positive signal cells was counted; the cutoff value = mean ($\bar{x}$) ± 3 x standard deviation (SD) [21].

Statistical analysis

Statistical analyses and graphical representations were conducted using IBM SPSS Statistics software version 25.0, GraphPad Prism 7.0, and R software version 3.6.2. For continuous variables in the baseline data, the normality of the distribution was assessed using the Kolmogorov-Smirnov test. Data were presented as either the mean and standard error of the mean (SEM) or the median and interquartile range (IQR). Categorical variables were summarized as counts and percentages. The paired or unpaired t-test and Mann-Whitney U test

were utilized to analyze differences between two distinct groups, applicable for continuous or ordinal categorical variables. The Chi-square test was employed for nominal categorical variables. The correlation between two variables was examined using the Spearman correlation coefficient.

Survival curves for individual factor groupings were estimated using the Kaplan-Meier method, and differences in survival curves between two or more groups were analyzed using the Log-rank test. Univariate and multivariate survival analyses were conducted using the Cox proportional hazards regression model, with risk factors compared through the Wald test. Multivariate analysis was performed using a Cox regression model employing a forward/backward stepwise approach, with entry and removal thresholds set at $P=0.10$ and $P=0.05$, respectively. Results were expressed as hazard ratios with corresponding 95% confidence intervals. All P-values were two-sided, with a significance threshold of 0.05.

Results

Baseline characteristics of a double-hit multiple myeloma

A total number of 443 patients were included for analysis, of which 371 cases were non-DHMM and 72 cases were DHMM. DHMM had high β2-microglobulin (β2-MG) levels and percentages of bone marrow plasma cells, with a statistically significant difference ($P<0.05$) (Fig. 1A, B). Since Revised international staging system (R-ISS) included cytogenetic indicators, the DHMM group had fewer R-ISS I patients and more R-ISS III patients. No other clinical indicators were statistically different except for cytogenetics.

The Del (13q) was not involved in the definition of double-hit cytogenetics in MM, but Fig. 1C showed that the DHMM group had a higher percentage of Del (13q) CA. In Double-hit MM group, which includes 2 or more CA, number of cases of 3 CA were greater than that of cases of two CA, whereas it was not found in the non-Double-hit MM group, namely, more cases in 0 or 1 CA and fewer cases in 2 or 3 CA in non-Double hit MM (Fig. 1D; Table S1). The above results indicate that patients with two or more high-risk cytogenetic abnormalities are more likely to have other CA.

As shown in Fig. 1, the number of cases with any CA was 346 (78.1%) (Fig. 1E), with IgH translocation accounting for the highest proportion (45.8%), followed by Del (13q) (42.9%) and Gain (1q21) (36.6%) (Fig. 1F). Among the high-risk karyotypes for IgH translocation, t (4/14) was the most common, followed by t (14/16) and t (14/20). There were 72 cases of DHMM, accounting for 16.3%, and Gain (1q21) accounted for the highest proportion, followed by IgH translocation (Fig. 1G). Del(17p)+Gain (1q21) had the highest proportion of double-hit CA, followed by Gain(1q21)+t(4/14) (Fig. 1H). Heat maps of each CA in all cases and DHMM were shown in Fig. 1I.

Survival analysis and characteristics of marrow proliferation and plasma cell differentiation

In order to understand the influence and characteristics of clinical factors on the survival of DHMM/non-DHMM, univariate Cox regression analysis was further performed on the two subgroups. As shown in Fig. 2A, for non-DHMM, age, ECOG PS, hemoglobin, albumin, creatinine, β2-MG, LDH, percentage of bone marrow plasma cells, ISS, R-ISS and the initial treatment regimen (PI+IMiD) had a significant effect on survival. For DHMM, only high LDH had a significant effect on survival ($P=0.038$). The remaining clinical characteristics did not differ significantly in survival, and even combined proteasome inhibitors and immunomodulator-based induction therapy did not significantly improve survival in patients with DHMM ($P>0.05$). From the above results, it could be concluded that DHMM had unique clinical characteristics, and its survival prognosis was not limited by clinical indicators, and the impact on survival was independent. Compared with non-DHMM, DHMM patients have worse progression-free survival (PFS) and overall survival (OS)(Figure S1).The K-M survival curves of ISS and primary treatment regimens in DHMM/non-DHMM were shown in Fig. 2(B-E).

In order to understand marrow proliferation and plasma cell differentiation of DHMM/non-DHMM, 21 patients with newly diagnosed MM (10 DHMM and 11 non-DHMM) were analyzed. The proliferation situation was shown in Fig. 2F (left), in DHMM, the number of proliferation extremely reduced case was 1, reduced cases was 2, active case was 1 and obvious active cases was 6. In non-DHMM, the number of proliferation extremely reduced case was 0, reduced cases was 1, active case was 7 and obvious active cases was 3. There was no statistical difference between the two groups. Further defined proliferation active as normal bone marrow proliferation, and extremely reduced, reduced and obvious active as abnormal proliferation. The results showed that there were 9 cases of abnormal proliferation in DHMM and 4 cases of abnormal proliferation in non-DHMM, and there were statistical differences (Fig. 2F right).

For differentiation of plasma cells, there were no statistical differences in proportion of primitive plasma cells, naïve plasma cells, mature plasma cells, and giant malformed plasma cells between DHMM/non-DHMM. Although there was no significant difference, it could be seen that the proportion of primitive plasma cells in the DHMM tended to increase, while the percentage of mature plasma cells was low (Fig. 2G). Primitive plasma cells and giant malformed plasma cells were further defined as poorly differentiated, and naïve plasma cells

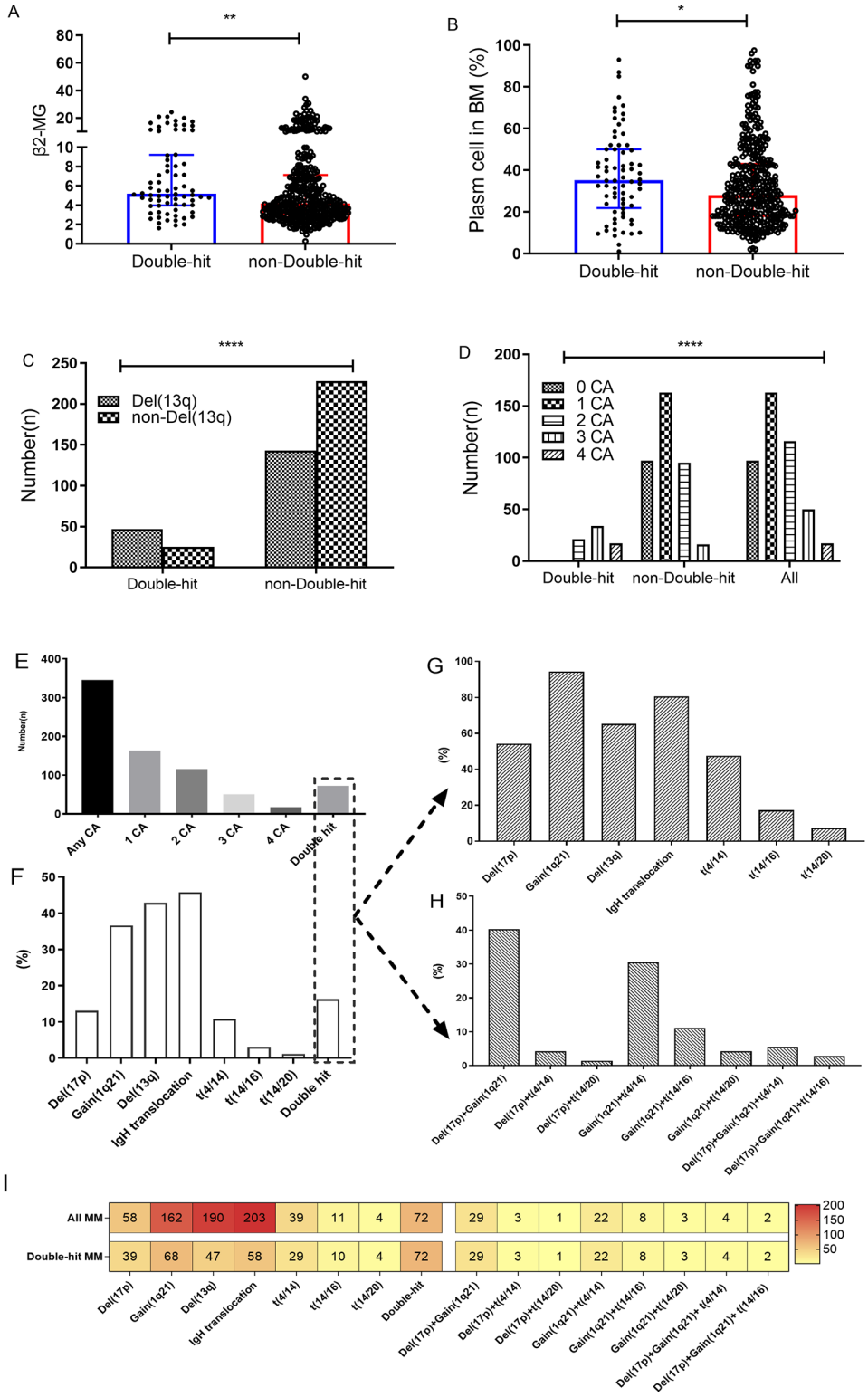

Fig. 1 Comparison of the clinical indicators of DHMM/non-DHMM and cytogenetic features. Distribution of β2-MG in DHMM/non-DHMM (**A**). Distribution of percentage of bone marrow plasma cells in DHMM/non-DHMM (**b**). Distribution of Del(13q) in DHMM/non-DHMM (**C**). Distribution of cytogenetic abnormalities in DHMM/non-DHMM and all patients (**D**). Statistics on the number of cytogenetic abnormalities in multiple myeloma (**E**). Statistics on the percentage of various cytogenetic abnormalities in multiple myeloma (**F**). Statistics on the proportion of various cytogenetic abnormalities in DHMM (**G**). Statistics on the proportion of double-hit cytogenetic abnormalities in DHMM (**H**). The heat map shows the number of cases of various cytogenetic abnormalities in all patients and DHMM (**I**)

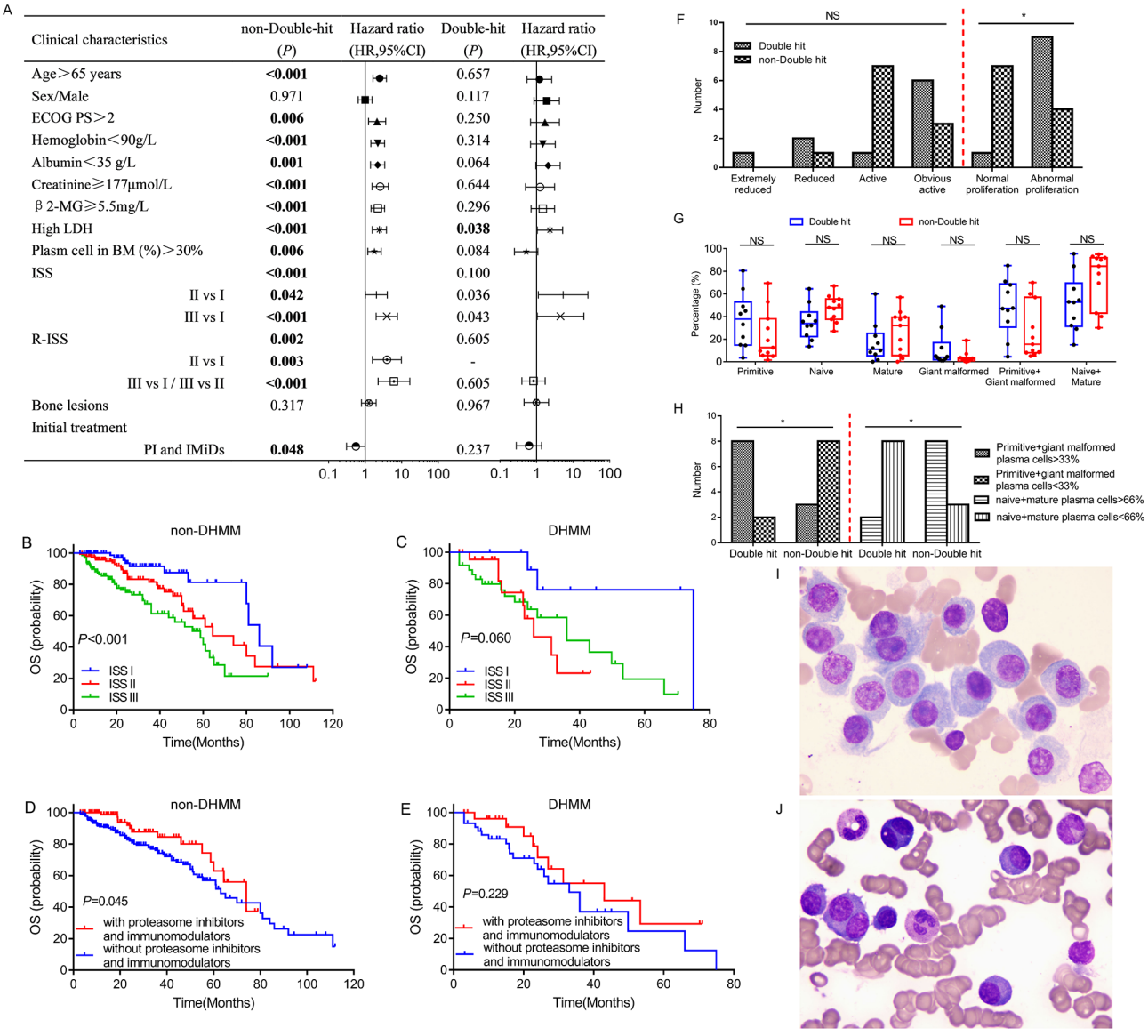

Fig. 2 Survival analysis and characteristics of marrow proliferation and plasma cell differentiation. Univariate Cox analysis of DHMM and non-DHMM (**A**). Stratified capacity of the ISS for non-DHMM (**B**) and DHMM (**C**). Survival analysis of initial treatment regimens in non-DHMM (**D**) and DHMM (**E**). Comparison of marrow proliferation (**F**) and plasma cells morphology (**G**) in DHMM/non-DHMM. Comparison of the degree of plasma cell differentiation in DHMM/non-DHMM (**H**). Demonstration of bone marrow plasma cells differentiation in patients with DHMM (1000x) (**I**) and non-DHMM (1000x) (**J**)

and mature plasma cells were defined as well-differentiated. Although there was no statistically significant difference between DHMM and non-DHMM, but DHMM had a higher proportion of poorly differentiated cells (Fig. 2G). The poorly differentiated type was divided into two groups with a cut of 33%, namely: primitive+giant malformed plasma cells＞33% and primitive+giant malformed plasma cells＜33%, which had a statistically significant difference between the two groups (Fig. 2H). Similarly, the well-differentiated type was divided into two groups with a cut of 66%, and there was a statistical difference. Figure 2I showed plasma cell differentiation in DHMM and non-DHMM.

Immune-microenvironment in double-hit/non-double-hit multiple myeloma

Flow cytometry analyzed the proportion of lymphocyte subsets of bone marrow and peripheral blood in DHMM/non-DHMM, including CD4+T cells, CD8+T cells, NK cells, and B cells. The results showed that, as shown in Figure S2, there was no significant difference in the distribution of peripheral blood lymphocyte subsets between the two groups. The bone marrow lymphocyte subsets showed that no significant difference was observed between the two groups in the subsets of CD4+T cells, CD8+T cells, NK cells and B cells. Significantly, among CD4+T cells, the proportion

of Tregs (CD4+CD25+CD127-/low) cells and iTreg (CD45RO+CD4+CD25+CD127-/low) in patients with DHMM was higher than in patients with non-DHMM (Fig. 3A and B, $P<0.05$). Further analysis of the relationship between lymphocyte subsets found that iTregs was negatively correlated with NK cell (Fig. 3C and D, $P<0.05$). The correlation of Tregs/CD4+T and iTregs/CD4+T cells with clinical features were further analyzed, and no significant correlation were found (Figure S3, $P>0.05$).

Upregulation of cell surface IFN-γ was associated with effector cell-induced cytotoxicity [22]. In peripheral blood and bone marrow, there was no significant difference in T cell and NK cell secretory function between DHMM/ non-DHMM. However, it could be seen that the percentage of IFN-γ on effector cells tended to decrease in DHMM (Figure S4).

Cytokine levels in new-diagnosed DHMM/non-DHMM were detected (Fig. 3E). TGF-β and IL-10 levels in DHMM were significantly higher than in non-DHMM ($P<0.05$). The correlation between cytokines and Tregs was further analyzed, and the results showed that IL-10 and TGF-β were positively correlated with the iTreg/CD4+T ratio ($P<0.05$, Fig. 3F).

PD-1 levels on CD8+T cells and expression of CD38 on Tregs cells

Flow cytometry analysis was conducted to assess PD-1 levels on CD8+T cells in DHMM, non-DHMM, and control cases. The results indicated that PD-1 expression on CD8+T cells was higher in the DHMM group (Fig. 4A). There was a group of CD38-expressing Tregs cells in myeloma cells, and Tregs with high CD38 expression had a stronger immunosuppressive effect [22, 23]. The CD38 expression on Tregs cells in the newly diagnosed MM and control group were further detected. The results were shown that DHMM had a higher proportion of $CD38^{high}$Tregs (Fig. 4B-D, $P<0.05$).

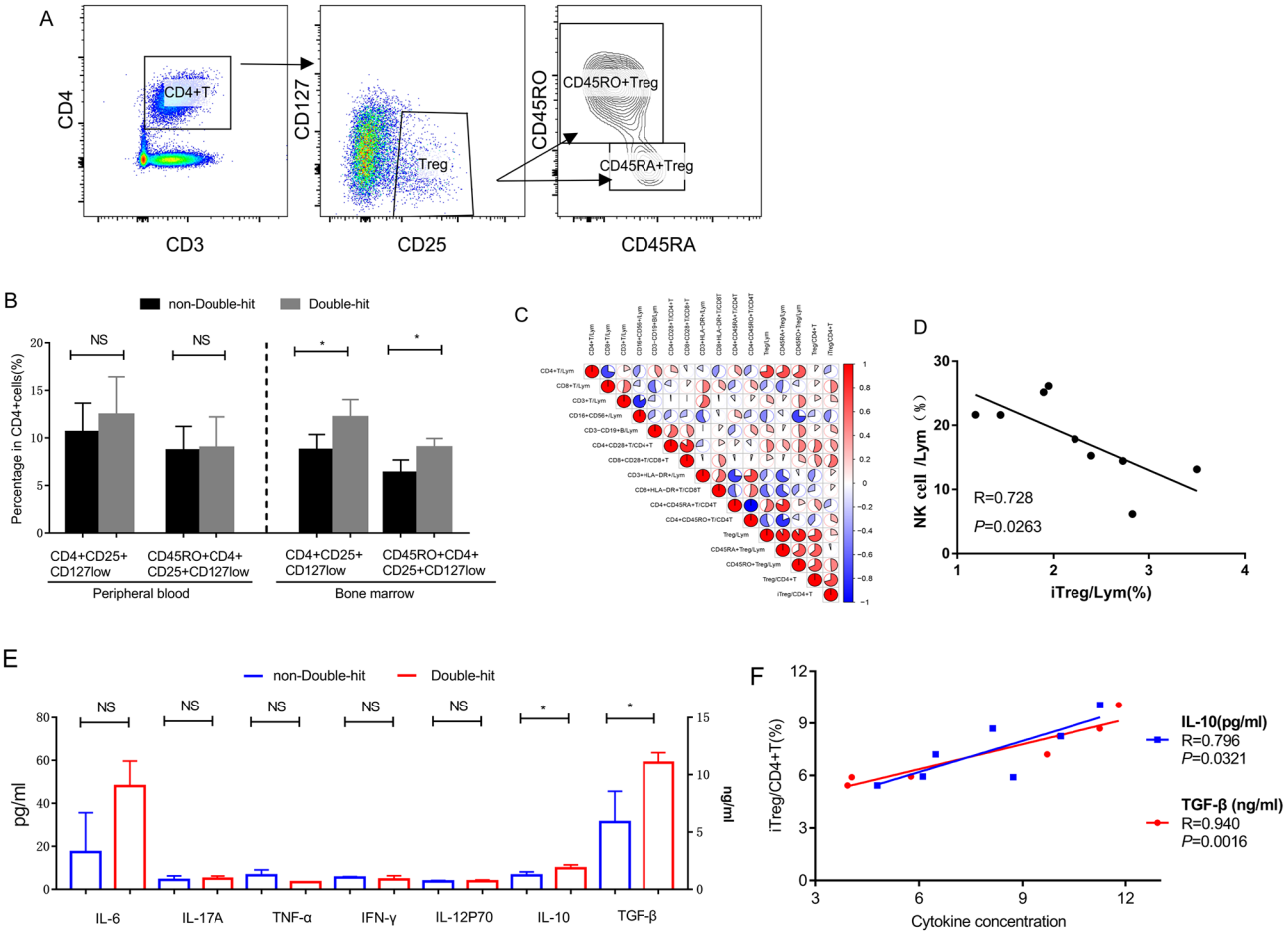

Fig. 3 Immune-microenvironment in double-hit/non-double-hit multiple myeloma. The flow scatter plot shows the gating strategy of Tregs cells and their subpopulations (**A**). Comparison of Tregs/CD4+T and CD45RO+Tregs/CD4+T in peripheral blood and bone marrow in patients with new-diagnosed DHMM/non-DHMM (**B**). Correlation analysis between immunocytes subsets in bone marrow (**C**). iTreg was negatively correlated with NK cells (**D**). Comparison of cytokine levels in bone marrow of new-diagnosed DHMM/non-DHMM (**E**). Correlation analysis of IL-10 and TGF-β with iTreg/CD4+T (**F**)

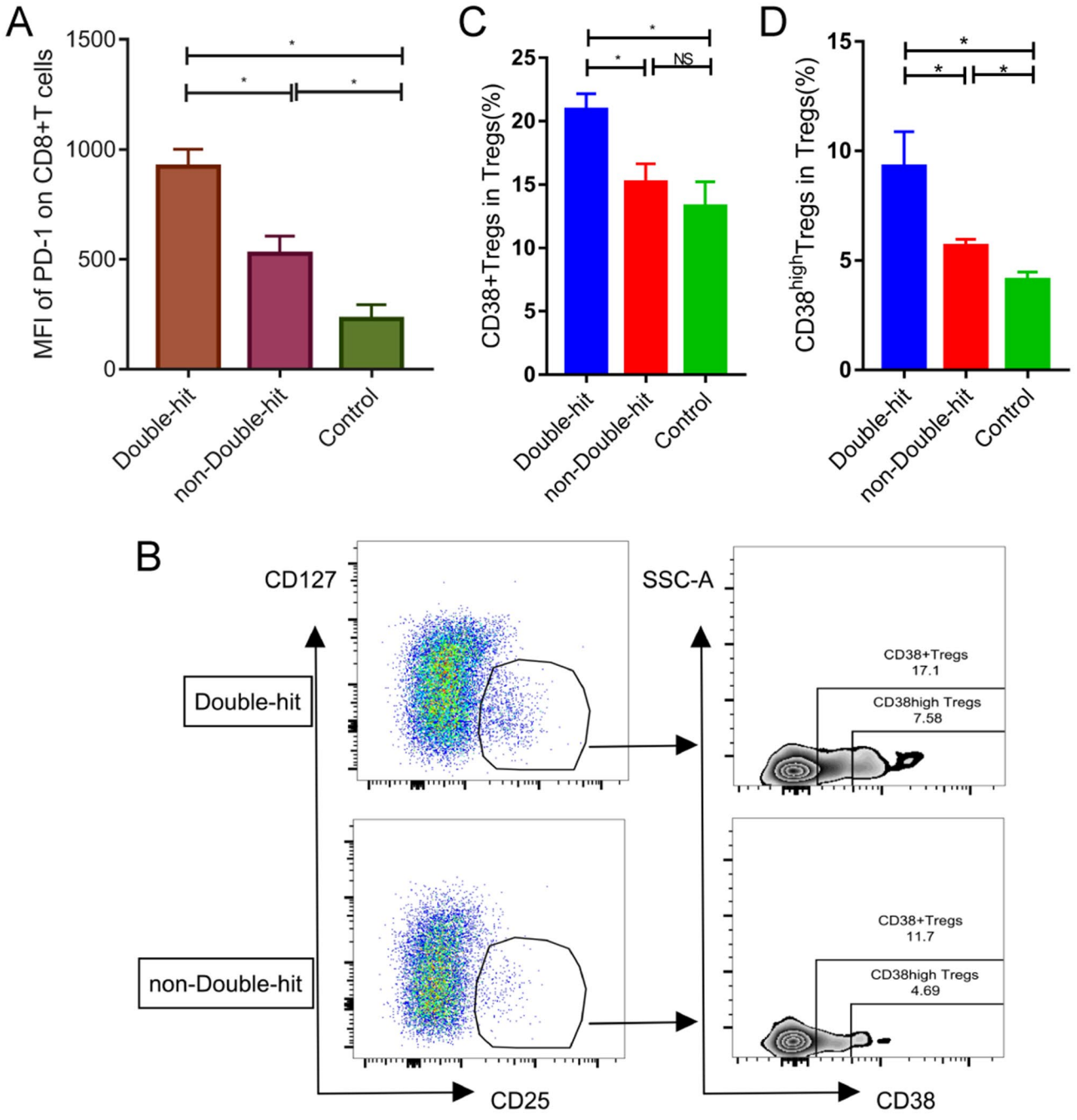

Fig. 4 PD-1 levels on CD8+T cells and expression of CD38 on Tregs cells. PD-1 levels on CD8+T cells in 6 patients (3 DHMM and 3 non-DHMM) and 3 controls (**A**). Tregs and its subpopulations in patients with DHMM and non-DHMM were presented (**B**). CD38+Tregs ratio (**C**). $CD38^{high}$Tregs ratio (**D**)

Effect of TGF-β on lymphocytes

As shown in Fig. 3E, DHMM had higher levels of TGF-β. To further verify the role played by TGF-β in DHMM, we first examined TGF-β levels in BMMCs cells from DHMM patients, myeloma cell line MM.1 S cells and after co-culture of the two cells. As can be seen in Fig. 5A, both DHMM-BMMCs and MM.1 S can secrete TGF-β, which is consistent with previous results in the literature [24], and DHMM-BMMC can produce more TGF-β after the addition of MM cells.

To further verify the role of TGF-β in multiple myeloma-induced immunosuppression, we directly co-cultured healthy donor BMMCs from non-MM patients with MM.1 S and then added with different concentrations of TGF-β neutralizing antibodies. Compared with no TGF-β neutralizing antibodies, no significant changes were found in the proportion of CD4+T cells and

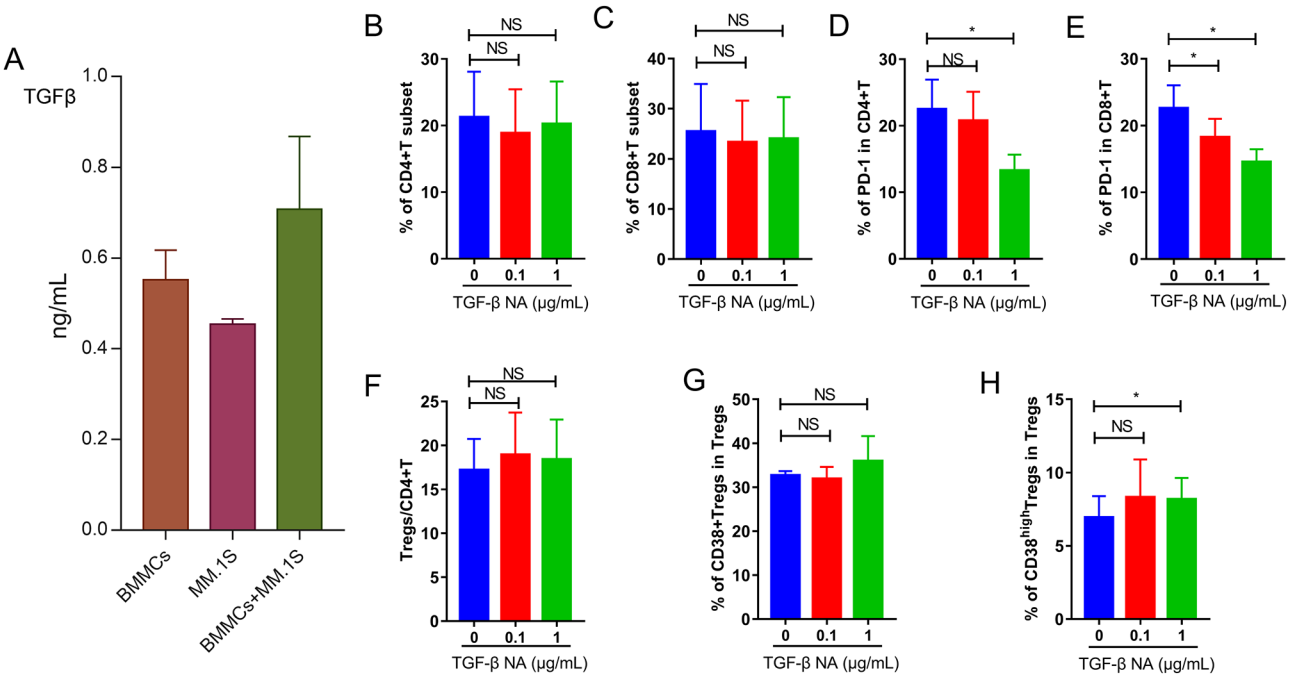

Fig. 5 The secretion of TGF-β and changes of immunocytes when BMMCs and MM.1 S were co-cultured with TGFβ neutralizing antibodies added. The secretion of TGF-β (**A**). The proportions of CD4 + T cells (**B**) and CD8 + T cells (**C**) and the expression of PD-1 on CD4 + T cells (**D**) and CD8 + T cells (**E**) after BMMCs and MM.1 S co-cultured with different concentrations of TGFβ neutralizing antibodies. The proportions of Tregs cells (**F**), CD38-expressing Tregs cells (**G**) and CD38^{high}Tregs cells (**H**) after BMMCs and MM.1 S co-cultured with different concentrations of TGFβ-neutralizing antibodies

CD8 + T cells (Fig. 6B, C), while the expression of PD-1 on T cells decreased (Fig. 5D, E). For Tregs cells, compared with no TGF-β neutralizing antibody, there was no significant difference in the ratio of Tregs/CD4 + T cells with TGF-β neutralizing antibody, but TGF-β antibody significantly increased the ratio of CD38^{high}Tregs cells (Fig. 5F-H).

Effect of CD38 monoclonal antibody on lymphocyte subsets and function

Above we found that TGF-β monoclonal antibody can cause an increase in CD38^{high} Tregs, and we further verified in vitro whether CD38 monoclonal antibody (Daratumumab) affects the growth of Tregs, and we directly co-cultured healthy donor BMMCs from non-MM patients with MM.1 S in the presence of different concentrations of daratumumab.

In the direct co-culture system we found no statistical difference in the proportion of CD4 + T cells to CD8 + T cells compared to the absence of Daratumumab (Fig. 6A, B). No significant difference of PD-1 expression on T cells was found yet (Fig. 6C, D). For Tregs cells, there was no significant difference in ratio of Tregs/CD4 + T cells after adding Daratumumab. The ratio of CD38-expressing Tregs cells and CD38^{high}Tregs cells were significantly reduced at different concentrations of Daratumumab (Fig. 6E-G).

Discussion

In this study, the proportion of DHMM was found to account for approximately 16.3% of cases. Patients with DHMM exhibited a notably elevated percentage of bone marrow plasma cells, and their survival prognosis was not significantly influenced by factors such as age, the International Staging System (ISS), or Eastern Cooperative Oncology Group Performance Status (ECOG-PS). Notably, individuals with multiple high-risk CAs demonstrated a higher likelihood of exhibiting Del(13q) abnormalities ($P<0.05$). Moreover, previous studies have indicated that the frequency of CAs within clonal populations is lower in monoclonal gammopathy of undetermined significance and smoldering multiple myeloma compared to symptomatic multiple myeloma, suggesting a progressive accumulation of genetically abnormal plasma cells during the multi-step evolution of the disease [25]. This finding further supports the notion that DHMM represents a later stage of disease progression and is associated with a poorer prognosis.

In this study, the most prevalent CA identified was IgH rearrangement, which accounted for approximately 45.8% of all newly diagnosed cases of MM. This figure is marginally lower than the report by Lai et al. but exceeds the findings reported by Abdallah and Cao et al. [26–28]. The discrepancies among these studies may be attributable to variations in FISH detection methodologies. IgH rearrangement is regarded as an initial CAs in the

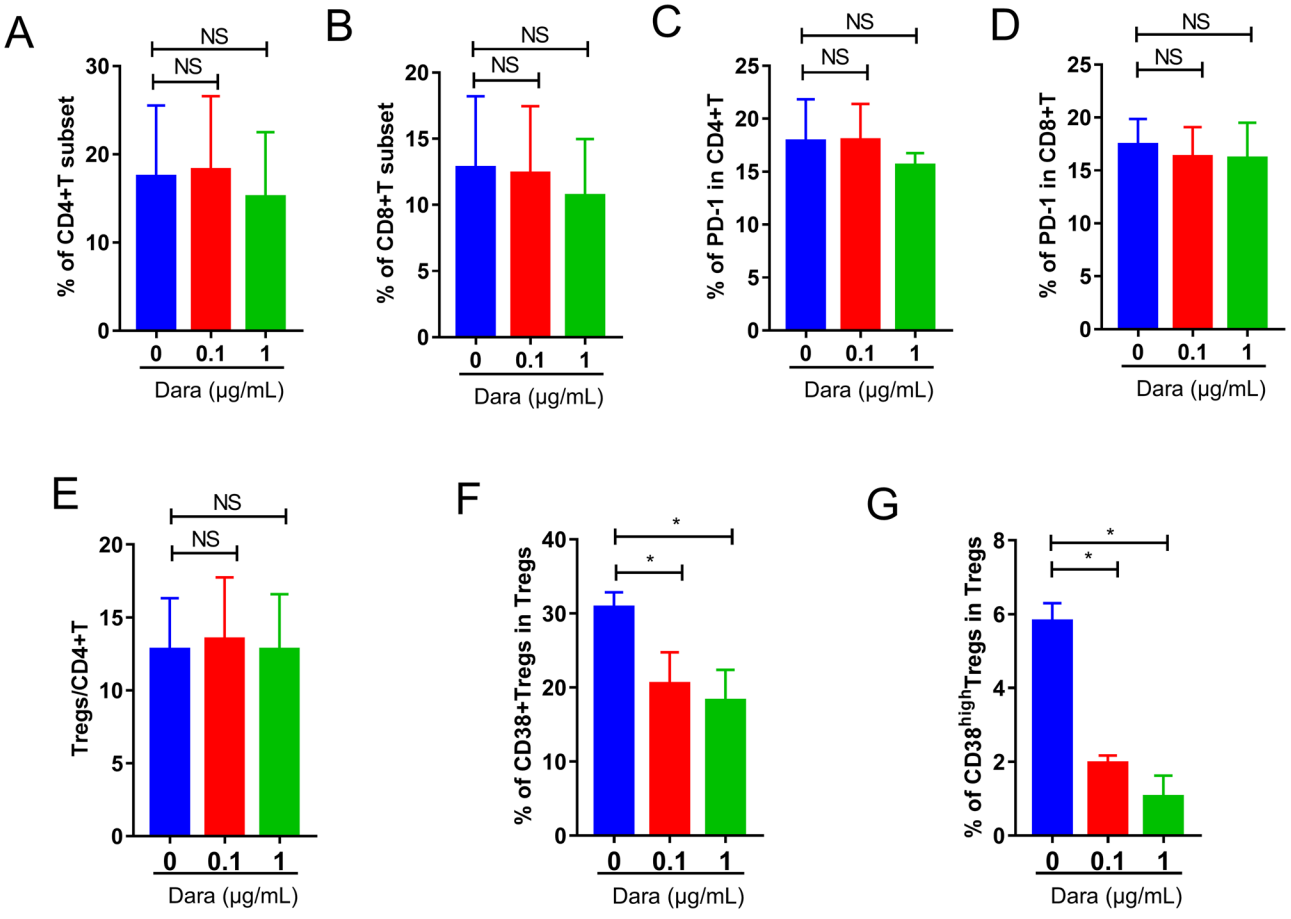

Fig. 6 Changes of immunocytes when BMMCs and MM.1 S were co-cultured with Daratumumab added. The proportions of CD4 + T cells (**A**) and CD8 + T cells (**B**) and the expression of PD-1 in CD4 + T cells (**C**) and CD8 + T cells (**D**) after BMMCs and MM.1 S co-cultured with different concentrations of daratumumab. The proportions of Tregs cells (**E**), CD38-expressing Tregs cells (**F**) and $CD38^{high}$Tregs cells (**G**) after BMMCs and MM.1 S co-cultured with different concentrations of daratumumab

pathogenesis of MM [29, 30]. The Gain (1q21) accounted for the second largest percentage only to IgH rearrangements at about 36.6%, slightly higher than Abdallah's finding (31.0%) [28] and consistent with a meta-analysis of molecular profiles of 1905 trial patients [4]. The prognostic significance of 1q21/CKS1B amplification remains contentious, as isolated gain of 1q21 may have limited prognostic implications. Fonseca et al. indicated that CKS1B gain is typically associated with other high-risk factors, such as t(4;14), as well as high proliferative characteristics of plasma cells [31]. Furthermore, findings by Hao et al. suggested that the prognostic impact of CKS1B amplification on myeloma is influenced by the background karyotype and TP53 status [32]. Consequently, it is imperative to classify patients with high-risk MM by considering additional CAs. Del (17p) continues to be recognized as a high-risk feature in myeloma [33] and has a significant adverse effect on both PFS and OS [34]. Although studies have shown that only TP53 biallele inactivation had extremely poor prognostic [35], Corre et al. also confirmed that Del(17p) itself was still a very high-risk feature, even if it was less unfavorable than TP53's biallele inactivation, but it was still a poor prognostic factor for MM and was a major factor in defining high-risk patients [36].

There is increasing evidence of immune dysregulation in MM, which includes impaired effector function of T cells [37], as well as the accumulation of immunosuppressive cells [38, 39]. Studies have reported alterations in both the phenotype and function of T cells in MM. Firstly, Tregs are known to suppress T cell cytotoxicity, which has been identified as a key contributor to disease progression [40]. Second, cytotoxic T cells appeared to be reduced relative to Tregs [40]. Thirdly, immune checkpoint proteins, such as PD-1, are expressed on T cells in MM patients, while their ligand, PD-L1, is upregulated on malignant plasma cells [41–43]. In the present study, a significant increase in the proportion of iTregs relative to CD4+T cells was observed in cases of DHMM, further supporting the role of immune dysregulation in the pathophysiology of this disease.

Tregs are classified into natural Tregs (nTregs) and iTregs based on their developmental lineage [44]. iTregs mediate immunosuppressive effects through both direct cell-to-cell contact and cytokine-dependent mechanisms [45]. As pivotal modulators of the immune system, Tregs actively inhibit immune responses, facilitating tumor progression by interacting with tumor cells to suppress the function of tumor-specific CD8+and CD4+effector T cells. This interaction depletes effector cells within the tumor microenvironment, further promoting immune evasion by malignant cells [46, 47]. In this study, patients with DHMM exhibited a significantly higher proportion of Tregs, along with increased expression of the immune checkpoint protein PD-1 on CD8+T cells. These findings underscore the existence of an immunosuppressive microenvironment in DHMM, contributing to the progression and poor prognosis of the disease.

Studies conducted by Alrasheed et al. have demonstrated that patients with MM exhibit a significantly higher proportion of bone marrow Treg cells compared to healthy controls. Importantly, those with elevated Treg levels were found to have shorter PFS and distinct immune checkpoint characteristics, such as increased expression of PD-1 and LAG-3 [48], findings that align with the research of Giannopoulos et al. [49]. Other studies further support the notion that elevated Treg levels are frequently associated with increased tumor burden and disease progression [50]. An in vitro study of MM showed an increased proportion of iTregs in the conditioned medium of multiple myeloma cells, indicating the importance of cytokines secreted by multiple myeloma cells in the induction of iTregs. Specifically, when PBMCs were co-cultured with MM cell lines, there was a marked elevation in the levels of TGF-β and IL-10 in the supernatant [22]. These findings suggest that TGF-β and IL-10 may be co-produced by MM cells and Treg cells, creating a reciprocal interaction that promotes immunosuppression within the tumor microenvironment.

TGF-β is widely recognized as a key mediator of immunosuppression within the tumor microenvironment, playing a crucial role in inhibiting anti-tumor immune responses. Specifically, TGF-β exerts direct inhibitory effects on the proliferation and activity of immune effector cells, including macrophages, NK cells, CD4+helper T cells, and CD8+cytotoxic T cells, while simultaneously promoting the proliferation and function of Tregs and MDSCs [51]. Moreover, TGF-β1 has been shown to upregulate the expression of PD-1 on tumor-responsive T cells, thereby further impairing anti-tumor immunity [15]. TGF-β also acts as a potent driver of tumor progression by not only suppressing host immune responses but also inducing tumor cell plasticity, a phenomenon that enables resistance to both immune-based therapies and other treatment modalities [52]. Importantly, combined therapeutic strategies targeting both PD-1/PD-L1 and TGF-β/TGF-βR pathways have demonstrated potential in restoring the balance between CD8+T cells and Tregs [53]. Blocking TGF-β has been shown to significantly enhance the functional recovery of BM CD8+T cells in patients with MM when used in conjunction with anti-PD-1 therapy, highlighting the potential of this dual-inhibition approach in overcoming immune resistance in MM [54].

CD38 is a type II transmembrane protein highly expressed on myeloma plasma cells and various immune cells, including MDSCs and regulatory B cells. These CD38+immunosuppressive cell populations were associated with decreased immune function and disease progression [55–57], showing particularly broad and high levels of expression in MM [58]. Studies had shown that there was a population of CD38-expressing Tregs cells that are more immunosuppressive than CD38-negative Tregs in vitro [23]. In our study, the proportion of $CD38^{high}$ Tregs in DHMM was significantly higher than in non-DHMM. These CD38-expressing Tregs, B cells, and MDSCs have shown sensitivity to daratumumab treatment, a monoclonal antibody targeting CD38 [23]. Drugs targeting CD38 monoclonal antibody could partially overcome immunosuppressive effects, thereby alleviating the immunosuppressive bone marrow microenvironment [22, 23, 39]. Given the poor prognosis associated with DHMM, and the limited effectiveness of proteasome inhibitors combined with immunomodulators in improving outcomes, integrating CD38 monoclonal antibodies into treatment regimens may offer a potential survival benefit for patients with high-risk MM. This therapeutic strategy could enhance immune responses and improve prognosis in these patients.

This study has several limitations. First, the relatively low transplant rates and the exclusion of non-evaluable patients may have contributed to selection bias, making the results less representative of the global MM population. Further studies with more diverse patient groups are needed to better understand the characteristics of DHMM. Second, the analysis of the BM microenvironment was based on a small number of cases, limiting the strength of the conclusions. More clinical samples will be necessary to verify these findings and provide a more robust understanding of the microenvironment's role in DHMM. Additionally, this study did not include long-term patient tracking, which hindered the ability to determine whether changes in the BM microenvironment correlate with treatment efficacy. Future research should involve extended follow-up periods to assess the impact of treatment on the BM microenvironment and its contribution to disease progression and response to therapies. In the in vitro experiments, the specific cell types responsible for secreting TGF-β within BMMCs

were not identified, and the absence of animal models limited our understanding of how MM cells influence the immune microenvironment. The effects of TGF-β on T cell depletion, as well as the influence of CD38 monoclonal antibodies on Tregs, were also not fully explored. Further investigation, including animal studies, is needed to address these gaps and to refine our understanding of the interaction between MM cells and the immune microenvironment.

Through the comprehensive analysis of MM, particularly DHMM, this study identified several unique clinical features of DHMM. First, the survival prognosis of DHMM patients is not constrained by other clinical indicators such as age or performance status, and its impact on survival is independent of these factors. Furthermore, it was observed that standard PIs-based induction chemotherapy combined with immunomodulators did not improve survival in patients with DHMM. DHMM was characterized by significant myelodysplasia and poorer plasma cell differentiation.

The bone marrow of DHMM has elevated immunosuppressive cells and depleted T lymphocytes. At the same time, it was also proved that myeloma cells can change the immune cell subsets and their functions through direct contact or secretion of cytokines and other indirect ways, promoting T cell depletion and Tregs differentiation, thereby causing immune disorders. TGF-β played an important role in immunoregulation, drugs targeting PD-1 or TGF-β inhibitors may improve immune status. Improving immune microenvironment of bone marrow might improve survival.

A critical finding of this study was the identification of a highly immunosuppressive BM microenvironment in DHMM, characterized by an increase in immunosuppressive cells and a depletion of T lymphocytes. This study also demonstrated that myeloma cells can alter immune cell subsets and their functions, both through direct cell-to-cell contact and via the secretion of cytokines, leading to T cell depletion and the differentiation of Tregs, which results in immune dysregulation. TGF-β was found to play a pivotal role in this immunoregulatory process. The study suggests that therapeutic strategies targeting the PD-1 pathway or TGF-β inhibitors may improve the immune status of patients with DHMM, potentially enhancing survival by modifying the bone marrow immune microenvironment. While most studies on DHMM have focused on clinical features, prognosis, and genetic or genomic characteristics [35, 59–61], this study contributes novel insights by characterizing the BM microenvironment in DHMM. It highlights the suppressive nature of the microenvironment in these patients, offering a potential explanation for their poor prognosis. This work provides an important foundation for future studies and serves as literature support for understanding the pathological features of DHMM, thereby contributing to the development of targeted therapies aimed at improving patient outcomes.

Abbreviations

MM	multiple myeloma
DHMM	double-hit multiple myeloma
CA	cytogenetic abnormalities
IMWG	International Myeloma Working Group
FISH	Fluorescence in situ Hybridization
IMiD	immunomodulatory
PI	proteasome-inhibitors
OS	overall survival
PFS	progression-free survival
BMMCs	bone marrow mononuclear cells
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
IL-6	interleukin-6
IL-17A	interleukin-17 A
TNF-α	tumor necrosis factor-α
IFN-γ	interferonγ
IL-12P70	interleukin-12P70
IL-10	interleukin-10
D-FISH	direct FISH
MACS-FISH	magnetic bead sorting FISH
SEM	standard error
IQR	interquartile range
β2-MG	β2-microglobulin
R-ISS	Revised international staging system
CDC	complement dependent cytotoxicity
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-13124-6.

Supplementary Material 1

Acknowledgements

The authors thank the patients who participated in the study.

Author contributions

Y.F.S. designed the research, conducted experiments, analyzed data, drew images and wrote the manuscript, G.P.C. and X.Q.L. designed and conducted experiments, L.L. conducted experiments, Y.X.T. help to design experiments, X.Y.L. provided guidance, H.S., L.L.M., X.Q.T., W.D.W. and X.Q.C. collected the data, R.Y.P organize the content and submit, Z.J.X. and F.L.Z. designed the project, provided professional guidance and revised the manuscript.

Funding

This work was supported by the Zhongnan Hospital of Wuhan University Science, Technology and Innovation Cultivation Fund [Grant Number ZNLH201902].

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The studies involving human subjects were reviewed and approved by the Ethics Committee at Zhongnan Hospital of Wuhan University. The patients provided their written informed consent to participate in this study. All methods were carried out in accordance with declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 19 August 2024 / Accepted: 29 October 2024
Published online: 10 November 2024

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