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Detection of senescent CD8+ T-lymphocyte in newly diagnosed and relapsed/refractory multiple myeloma using CD28 and CD57

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Abstract:

BACKGROUND: Multiple myeloma is a clonal B-cell malignancy characterized by proliferation of plasma cells that secrete a complete and/or partial monoclonal immunoglobulin protein. It induces dysfunction of cytotoxic T cells that may be responsible for immune evasion and therapeutical failure of immunotherapies. T cells during senescence tend to lose co-stimulatory molecules such as CD27 and CD28 while expressing killer cell lectin-like receptor subfamily G (KLRG-1) and CD57. Therefore, enhanced knowledge about the actual status of T cells in myeloma patients is needed.

OBJECTIVES: The aims of this study were to detect the senescent cytotoxic T-cell (CD8+) in patients with refractory/resistance multiple myeloma, and to compare it with newly diagnosed multiple myeloma patients, and their implications for cellular therapies.

MATERIALS AND METHODS: This is a cross sectional study performed, from January to October 2021. Sixty multiple myeloma patients were sequentially chosen, thirty of them were newly diagnosed patients and another thirty were relapse/refractory who progress after receiving 2 lines of chemotherapy containing bortezomib and immunomodulators. And/or patients progressing after autologous stem cell transplantation. Seventeen apparently healthy age and gender matched adults were enrolled as a control group. Multicolor flow cytometry was utilized for the analyses of surface molecules CD 28 and CD57 on CD 8 positive T-lymphocyte using peripheral blood, subsequently the percentage of T cells senescent were estimate in CD 8+ T cells with CD28- and CD57+.

RESULTS: The mean percentage of senescent CD8 positive T lymphocyte (negative CD28 and positive CD57) were significantly higher in multiple myeloma patients than control (P<0.001). There was no statistically significant difference in percentage of CD8+ T cells between newly diagnosed multiple myeloma and relapsed /refractory cases. However higher percentage of senescent T cells was found in relapsed cases. No significant correlation was found between percentage of CD8 +T cells and patients age, or duration of disease.

CONCLUSION: Percentage of senescent CD 8+ T cells with CD28- and CD 57+were significantly higher in MM patients compared to healthy controls. Percentage of senescent CD 8+ T cells with CD28- and CD 57+were lower in newly diagnosed MM patients compared to relapsed patients but the difference was statistically not significant

Keywords:

CD28, CD57, myeloma, senescent, T-lymphocytes

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Introduction

Multiple myeloma (MM) is a malignancy of plasma-cell, characterized by the expansion of malignant plasma cells in the

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bone marrow (BM), leading to destruction and loss in the major organ functions.[1]

It constitutes 1% of all malignancies and around 10% of all hematologic malignancies.[2]

Virtually, all patients with MM develop from a premalignant asymptomatic stage called monoclonal gammopathy of undetermined significance. It is present in over 3% of the population above the age of 50, and the average rate of progression to MM is 1%/year.[2,3]

Patients with MM have a significant reduce in the function of T-cells, in with changes in T-cell immune response and loss of antigen-specific T-cell function associated with disease progression. In MM, several mechanisms involved associated reductions in T-cell responsiveness including T-cell exhaustion, energy, and/or senescence.[4]

Immunosenescence is the main feature of T-cells' dysfunctional activity in MM characterized by maintained functional activity but limited proliferative capacity, with characteristic phenotypic changes including positive CD57/CD160/killer cell lectin-like receptor (KLRG1$^+$), negative CD28, with low programmed death 1 (PD1), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expression, suggesting that in MM the T-cells have a molecular signature of senescence instead of exhaustion phenotype.[5]

Patients, Materials, and Methods

This study is a case–control study done in Baghdad Teaching Hospital, Hematology Department in Medical City, Baghdad, Iraq, from January to October 2021. From all patients, written informed consent was taken, and the collection of data was done according to a questionnaire form which is collected directly from patients and their data records.

The study was approved by review ethical committee of Pathology Scientific Council of Iraqi Board for Medical Specializations.

A sample of 60 adult patients with multiple myeloma has been chosen, 30 with newly diagnosed MM, and others 30 with relapsed/refractory MM patients diagnosed by workup of both consultant clinical hematologists and hematopathologists dependent on International Myeloma Working Group criteria for MM diagnosis, which was updated in 2016.[6]

Eighteen of them had relapsed after receiving therapeutic protocol containing bortezomib and/or immune-modulating drugs, whereas the remaining twelve had relapsed after autologous stem cell transplant (ASCT).

Seventeen healthy individuals' age and gender-matched were involved in the study as the control group (six males and eleven females) the shortage in markers supply during study II was behind his limited number.

Materials include fluorochrome-conjugated monoclonal antibodies for gating and surface staining of CD8 and CD28 obtained from BioLegend USD and from Biosciences (BD Pharmingen™, USA), for CD57. Monoclonal antibodies for surface staining and gating which include: (anti-CD8: Brilliant Violet 421™.– anti-CD28: phycoerythrin and anti-CD57: allophycocyanin.[7-9]

Blood sampling

Under the aseptic technique, 3 ml of venous blood from the antecubital fossa was taken from each patient included in this study. These 3 ml of blood were added to K3-EDTA tubes and taken for flow cytometric evaluation of the expression surface CD28, and CD57 in CD8+, and estimation percentage of CD28$^-$, CD57$^+$ cells out of CD8$^+$ T-lymphocytes.[10-12] The sample analysis, percentage of expression, and data acquisition were achieved by using eight color FCM (BD FACSCanto™ II) USA and device software based on (BD FACSDiva).

Principles of the procedure of estimating senescent T-lymphocyte

Incubate whole blood with fluorochrome-conjugated monoclonal antibody reagents at room temperature in a dark place, the antibodies will bind specifically to the appropriate leukocyte antigens on the cell surface.

Gating on events with low SSC and CD8 T-lymphocyte, then the percentage of senescent T-cell estimated (CD28 – and CD57$^+$).

Stain-lyse-wash method was used to detect the surface markers.

Data acquisition and sample analysis

Data acquisition and sample analysis were performed in a BD FACSCanto™ II (8-color, Becton Dickinson, USA), using BD FACSDiva software.

The gating strategy for cytotoxic CD8$^+$ T-cell depends on FSC/SSC and using dot plots of CD8 expression versus intracellular complexity (SSC) SSC/CD8. A total of 100,000 events were acquired at the gate. Both CD28 and CD57 antigens are surface markers and are considered to be a positive expression in T-cell CD8$^+$ if ≥20% [Figure 1].

Table 1: Demographic characteristics of studied groups

Variables	Newly diagnosed MM (*n*=30)	Relapsed MM (*n*=30)	Control (*n*=17)	*P*
Age (years), mean±SD	65.1±9.1	68.9±6.2	63.8±7.4	0.063*
Gender, *n* (%)				
Male	14 (46.7)	13 (43.3)	6 (35.3)	0.74**
Female	16 (53.3)	17 (56.7)	11 (64.7)	
Total	30	30	17	

*ANOVA test, **Chi-square test, significant ≤0.05. SD=Standard deviation, MM=Multiple myeloma

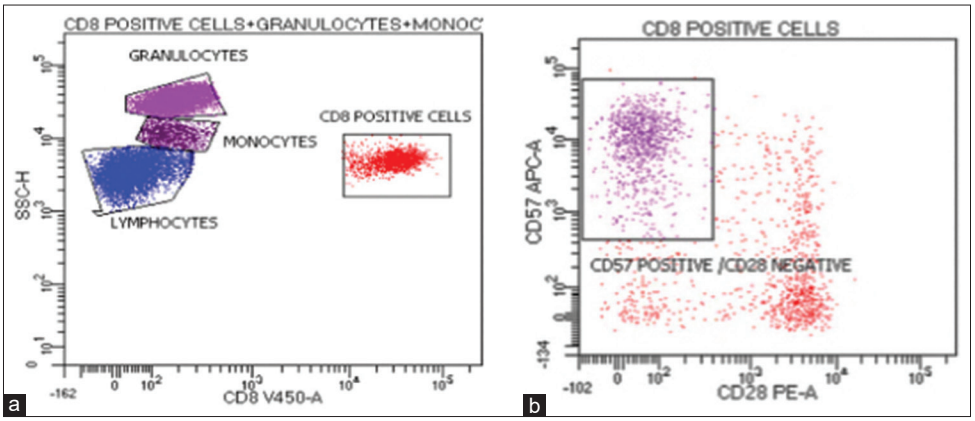

Figure 1: Dot plot showing: (a) CD8 versus side scatter. (b). The expression of CD28 and CD57. SSC-H = Side scatter, PE-A = Phycoerythrin

Statistical analysis

The studied sample data were entered and analyzed using the Statistical Package for the Social Sciences (SPSS) version 26 Released 2015.(IBM SPSS Staticsfor Windows,Armonk,NY:IBM Crop). Descriptive statistics were presented as frequencies, proportions (%), means, and standard deviations. Analytic statistics such as the Chi-square test were used to estimate the association between two categorical variables, ANOVA test, and student's *t*-test were used to find the association between categorical variables and continuous variables, and significant results in ANOVA test are analyzed by Tukey's *post hoc* test to detect which groups have a significant results. A Pearson's correlation test was used to find the correlation between two continuous variables, where a correlation coefficient between 0.2 and 0.29 mean weak correlation, 0.3–0.39 mean moderate correlation, , 0.4–0. 69 mean strong correlation, and when a correlation coefficient ≥0.7 mean very strong correlation. The $P \leq 0.05$ was considered to be statistically significant.

Results

Steady-state samples were collected from 30 patients who are newly diagnosed with MM, 30 patients with relapsed MM, and 17 normal subjects; the demographic characteristics of the studied groups are shown in Table 1, the samples were homogenous regarding age and gender ($P = 0.063, 0.74$).

The hematological parameters (h*emoglobin* [Hb], *white blood cell* [WBC], and platelet) showed a significant difference between patient groups ($P \leq 0.001, 0.035$, and 0.021 respectively), [Table 2].

There was no significant difference in biochemical tests (blood urea and *serum* creatinine, *serum* calcium, and albumin level) between newly diagnosed and relapsed cases ($P = 0.27, 0.93, 0.2$, and 0.13 respectively), [Table 2].

The mean percentage of $CD28^{-}/57^{+}$ $CD8^{+}$ T-cells was significantly different between the studied groups ($P < 0.001$) with the highest mean found in relapsed cases and lowest mean found in normal group, [Table 3].

The *post hoc* analysis of Table 3 shows a significant difference in the percentage of the senescent $CD8^{+}$ T cells between newly diagnosed MM cases and control group, and between relapsed MM cases and control group, but shows no statistically significant difference between new and relapsed MM cases, [Table 4].

In new cases of MM, there was no correlation between the percentage of senescent $CD8^{+}$ T-cells and age ($r = 0.23$, $P = 0.204$), and also there was no significant correlation between BM plasma cell % and percentage of senescent $CD8^{+}$ T-cells ($r = 0.25$, $P = 0.17$). In relapsed cases of MM, there was no correlation between the percentage of $CD8^{+}$ T-cells and age ($r = 0.23$, $P = 0.21$), and there was no significant correlation between BM plasma % and percentage of + $CD8^{+}$ T cells ($r = -0.16$, $P = 0.38$), and no correlation with disease duration [$r = 0.37$, $P = 0.84$, Table 5].

Table 2: Difference in hematological and biochemical tests between patients groups

Lab test	Mean±SD		*P*
	Newly diagnosed MM	Relapsed MM	
Hb	9.3±1.2	8.3±0.9	0.001*
WBC	5.7±1.7	4.8±1.6	0.035*
Platelet	190.4±69.2	154.6±45.8	0.021*
Blood urea	80.7±77.2	105.6±94.8	0.27
Serum creatinine	1.3±0.7	1.4±0.8	0.93
Serum calcium (mg/dL)	8.80±1.9	9.4±2.1	0.2
Albumin (g/dL)	3.5±0.7	3.39±0.5	0.13

*Student's t-test, significant ≤ 0.05. WBC=White blood cell, SD=Standard deviation, MM=Multiple myeloma, Hb=Hemoglobin

Table 3: Difference in percentage of $CD28^-/57^+$senescent $CD8^+$T-cells among studied groups

Variable Participants	Percentage of $CD28^-/57^+CD8^+$T-cells			*P*
	Mean±SD	Maximum	Minimum	
New cases	49.3±13.5	78.2	27	<0.001*
Relapsed case	55±10	73.6	32.1	
Normal	35.1±7.9	52	23.8	

*ANOVA test, significant ≤ 0.05. SD=Standard deviation

Table 4: *Tukey's *post hoc* test

Participant	*Significance	95% CI	
		Lower bound	Upper bound
New cases			
Relapsed cases	0.127*	−12.5302*	1.2168
Control	0.000	7.6968	23.0665
Relapsed cases			
New cases	0.127*	−1.2168*	12.5302
Control	0.000	13.3535	28.7232
Control			
New cases	0.000	−23.0665*	−7.6968*
Relapsed cases	0.000	−28.7232*	−13.3535*

*Tukey honestly significance difference test at the 0.05 level, CI=Confidence interval

Table 5: Correlation between studied marker, age, and bone marrow plasma percentage in cases of multiple myeloma group

Variable	Percentage of senescent $CD8^+$T-cells new cases		Percentage of senescent $CD8^+$T-cells relapsed cases	
	Correlation coefficient	*P*	Correlation coefficient	*P*
Age	0.23	0.204*	0.23	0.21*
BM plasma (%)	0.25	0.17*	0.16	0.38*
Duration of disease	-	-	0.37	0.84*

*Pearson's correlation test, significant ≤ 0.05. BM=Bone marrow

Discussion

MM is a malignancy of clonal plasma cells, recently the outcomes for patients with MM have considerably improved due to introduce of approval novel agents and high-dose therapy followed by ASCT. However, MM remains incurable, with a high frequency of disease progression and relapsing.

In this study, the mean age of newly diagnosed MM patients was 65.1 ± 9.1 with a range of 47–80 years This result was comparable with other Iraqi studies done by Alwan[10] Badi *et al*.[11] and with a Saudi Arabian study,[12] and the mean age of relapsed MM patients included was 68.9 ± 6.2 with a range of 59–80 years. These results were comparable with American studies of Katodritou *et al*.[13] and Kumar *et al*.[14] but it differs from the Indian study of Jasrotia *et al*.[15]

The mean of Hb in newly diagnosed patients was 9.3 ± 1.2 g/dl which is comparable to what was reported by other studies of El-Naby *et al*.[16] and Kumar *et al*.[17]

However, the mean level of Hb in relapsed MM patients was 8.3 ± 0.9. This is lower than other studies done by Kumar *et al*.[14] and Richardson[18] and this may be due to the late presentation of our patients and delay in the diagnosis of relapses.

The mean level of WBCs count and platelets count is lower in relapse cases than in newly diagnose although both of them are still within the normal range. In newly diagnosed cases, the mean of WBCs count was 5.7 ± 1.7 × 109/L, and mean of the platelets count was 190.4 ± 69.2 × 109/L. These were consistent with what was reported by Alwan[10] Yassin[19] Sultan *et al*.[20]

While in relapsed cases, mean WBCs was 4.8 ± 1.6 × 109/L and mean platelets count was 154.6 ± 45.8 × 109/L which is comparable to what is reported by Richardson[18] Lonial *et al*.[21]

The percentage of senescent $CD8^+$ T-lymphocytes was found to be higher in MM cases compared to the normal control group. This result is supported by another study done by Suen *et al*.[22] who evaluated the dysfunctional activity of clonal T-cells in MM cases and confirmed that immunosenescence is the main phenotype of $CD8^+$ T-lymphocytes. These senescent T-lymphocytes carry effector phenotype characterized by positive KLRG-$CD57^+/CD160^+/CD28-$ and low PD1 and CTLA-4 expression, suggesting that T-cells in MM have a molecular signature of senescence.[22] The importance of this senescent phenotype is the possibility of a link with therapy failure, and further studies are needed to clarify the effect of the senescent phenotype on treatment decisions.

In addition, Zelle-Rieser *et al*.[23] evaluated the expression of inhibitory molecules PD-1, CTLA-4, 2B4, CD160, and senescence markers CD57, and CD28 on T-lymphocyte of untreated and treated MM cases in both the BM

and peripheral blood. He defined that T-cells from the MM cases were impaired in the BM more than in the peripheral blood. He also established that at the tumor site (BM); the proliferation of $CD8^+$ T cells in MM cases was more lower when compared to the healthy control group. These $CD8^+$ T-cells carry some molecular markers related to T-cell exhaustion such as PD1 and CTLA-4, and also negative for CD28 and are positive for CD57; a senescence phenotype, the percentage of senescent $CD57^+$ CD28 – $CD8^+$ T cells were more lower in treated myeloma cases in related to untreated cases associated with a reduction in both proliferative capacity and function.[23] However, the current study did not include treated MM patients who are in remission, it included groups of newly diagnosed MM and patients who are relapsed MM or refractory MM patients and all these patients are having active MM. This can explain the expansion of senescent T-cells in both groups which seems to parallel disease activity rather than refractoriness of disease.

The comparison between newly diagnosed and relapsed MM showed no significant difference in the mean percentage of $CD28^-/57^+$ in $CD8^+$ T-cells, with the highest mean percentage found in relapsed cases. This result was seen in another study done by Lee *et al*.[24] who found a subset of relapsed patients with a significant increase in the markers of senescence in the peripheral $CD8^+$ T cells.[24]

Furthermore, Raitakari *et al*.[25] showed progressive elevation in the percentage of $CD8^+$ T-lymphocytes with positive CD57 from patients with Stage I to those with Stage III MM. Concurrently, decrease in the percentage of lymphocytes expressing CD28. While Chung *et al*.[26] showed that patients with relapsed disease, post-ASCT show elevation in the percentage of $CD8^+$ CD28 – $CD57^+$ cells when compared to healthy donors' age-matched or cases in a complete remission post-ASCT.

The mean percentage of senescent T-cells was not different between post-ASCT-relapsed subgroup (who are 12 patients) and chemotherapy-resistant subgroup (who are 18 patients), both subgroups showed a high percentage of senescent T-cells. Sample size can explain this result in which further study including a larger number of patients is needed. Whatever the study was done by Chung *et al*.[26] showed that relapsing cases have a higher population of $CD8^+$ CD28 – T-cells after transplant by 3 months or more, but before detection of clinical relapsed disease, representing their applicability in recognizing patients at higher risk of relapse, so the senescent phenotype of T-lymphocyte may help in predicting early relapsing diseases, and support the introduction of immunotherapy to stimulate antitumor immunity post-ASCT.

Correlation between the mean percentage of senescent CD8+ T-cells with patients' age, % plasma cells in BM aspirate, and duration of disease showed nonsignificant results, this result disagreed with a study done by Lee *et al*.[24] who found older patients, and those treated for a longer period before relapsing found to have higher percentage of senescent T-cell, this can be explained by the sample size or characteristics of the population in the area involved in the study.

In the study of Dosani *et al*.,[27] he reported that smoldering MM cases had lymphocytes with lower expression of negative CD57 (with an increase in positive CD57 expression) in patients who were more likely to progress to MM, proposing that CD57 expression may be associated with increasing disease stage.[27]

Till now there is no clear clinical implication of the effects of the senescent phenotype of $CD8^+$ T-lymphocyte but further studies may disclose the exact etiology and significance of senescent T-cell in MM and if reversal of this phenomenon may add to treatment lines of MM.

Other factors may contribute to a high percentage of T-cells senescent in newly cases, both aging and human cytomegaly virus infection are believed to affect $CD8^+$ T-cells phenotype and may lead to immunosenescence of these cells.[28,29]

The percentage of senescent T-cells is higher in the elderly, with the most phenotypical changes occurring in the $CD8^+$ T-cells subset isolated from the elderly, with a progressive loss of CD28 expression, and increase expression of CD57 in $CD8^+$ T-cells.[28-30]

The studies reported by Strioga[31] and Mojumdar.[32] found a high correlation between human cytomegalovirus (HCMV) and T-cells senescence. HCMV infection gives rise to the higher percentage of long-term memory cells carrying mature phenotype with low CD27/CD28, high CD57 expression, and often perforin positive with T-cells.[31,32]

In an Iraqi study, the detection of anti-cytomegalovirus antibody was conducted on 472 healthy blood donors using enzyme-linked immunosorbent assay test. The study found that 31.36% of the donors had$^+$ ve anti-CMV Immunoglobulin G (IgG) Nanakaly and Hussen,[33] whereas another Iraqi study found that 46.6% of blood donors out of 120 samples had + ve anti-CMV IgG antibody Yasir and Majhol.[34]

Both studies showed that our population has a significant percentage of infection with CMV, which may affect the percentage of senescent T-cell, However, more sensitive

methods, for example, polymerase chain reaction (PCR) is needed for better correlation.[33,34]

Conclusion

The percentage of senescent $CD8^+$ T-cells with CD28 – and $CD57^+$ was significantly higher in MM cases related to healthy control group. The percentage of senescent $CD8^+$ T cells with CD28 – and $CD57^+$ was lower in recently diagnosed MM cases related to relapsed cases but the difference was statistically not significant.

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Conflicts of interest
There are no conflicts of interest.

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