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# Effect of Bortezomib Regimens and Daratumumab Monotherapy on Cellular Immunity in Multiple Myeloma Patients

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## ABSTRACT

**Background:** Treatment with a proteasome inhibitor (bortezomib) and daratumumab monoclonal anti CD38 antibody are effective in patients with multiple myeloma. However, these drugs impair cellular immunity, which may make the patients more prone to infection.

**Objective**: To investigate the effect of bortezomib-based regimens and daratumumab monotherapy on the lymphocyte subpopulation in MM patients.

**Methods:** Peripheral blood samples were collected from 32 patients, 29 were newly diagnosed and treated with bortezomib regimens and 3 patients were relapsed and refractory MM treated with daratumumab as monotherapy. The immunophenotypic analysis was performed by flow cytometry at baseline and during the third cycle of bortezomib regimen and fourth week of daratumumab treatment.

**Results:** In the third cycle of bortezomib, there was a significant decrease in $CD3^+$ T cells, $CD^+4$ T cells, memory T cells ,and natural killer cells (NK cells). However, $CD8^+$ T cells increased dramatically, followed by a significant reduction in the CD4/CD8 ratio. On the other hand, daratumumab led to an increase in the T cell population after four weeks of treatment, with a significant increase in $CD3^+$ T cells as well as $CD4^+$ T cells, while NK cells were dramatically depleted in all patients.

**Conclusion:** Bortezomib hurt subsets of the T cells, while daratumumab positively affected the T cells subsets. In both treatments, NK cells decreased significantly. These results suggested that DARA is more specific to target myeloma cells than bortezomib. Also, DARA enhanced the T cells to extend especially $CD3^+$ T cells and $CD4^+$ T cells.

**Keywords:** Bortezomib, Daratumumab, Lymphocyte population, Multiple myeloma

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## INTRODUCTION

Multiple myeloma (MM) is a genetically heterogeneous disease that presents as the abnormal growth of plasma B cells (1), and it accounts for approximately 10% of hematological malignancies (2). Over the last several years, choices of treatment for MM have expanded considerably with the use of many proteasome inhibitors (PIs), monoclonal antibodies (mAbs), and immunomodulatory drugs (IMiDs). The addition of novel drugs to myeloma therapy has led to an increase in responses to treatment and improved survival, with valuable benefits observed among transplant-eligible patients, and moderate recovery observed in older aged patients(3). However, infections are the leading causes of mortality and morbidity in MM patients (4, 5).

Bortezomib (Velcade) was the first class of PI that demonstrated an effect against MM. Bortezomib was approved by the United States Food and Drug Administration (U.S. FDA) in 2003. Bortezomib has been used in routine clinical practice for over a decade and is considered an essential treatment for individuals in all stages of MM. Bortezomib is recommended for newly diagnosed patients, relapsed or refractory patients ,and renal failure patients in addition to maintenance therapy (6, 7). Bortezomib functions by inhibiting the activation of β1 and β5 subunits of the 20S core of the proteasome, which causes apoptosis in myeloma as well as lymphoma cells, and it also affects the myeloma microenvironment (7, 8). Impressively, myeloma cells seem to be more proteasome-dependent than normal cells, also have more proteasome activity. Additionally, bortezomib inhibited the activity of NF-κB by an accumulation of I-κB in the cytosol (7).

Daratumumab(DARA) was approved by the U.S. FDA in 2015 for MM patients who received at least three previous lines of treatment, including one PI and one IMiD drug, or who were not responding to both. DARA is an IgG1 isotype that targets CD38, which is highly expressed in myeloma cells (9). DARA mediates the death of myeloma cells by different immunological processes, such as antibody-dependent cellular phagocytosis, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and the initiation of apoptosis through Fc mediated crosslinking (10, 11). DARA treatment has displayed clinical potency in relapsed/refractory MM both as a single therapy and in conjunction with other drugs, including lenalidomide, dexamethasone, and bortezomib (12, 13).

Bortezomib and DARA prolonged survival times, and it is a backbone therapy in MM patients, however, infectious complications increase, becoming a life-threatening issue in these patients. We hypothesized the change in lymphocyte count is the main cause of infection in addition to the immunosuppressive effect of the disease. Therefore, in this study by flow cytometry, we investigate the effect of bortezomib-based regimens and DARA monotherapy on the lymphocyte sub-population in MM patients.

## MATERIALS AND METHODS

*Patients and Treatments*

The current study was approved and authorized by the Human Ethics Committee of the College of Science, Salahaddin University, Erbil(Approval No: 3/2/2002 Date: 9/6/2019). For the publication of data in this study, all patients gave written informed consent. This study was conducted from August 2019 to June 2020 in Nanakali Hospital for Blood Diseases and Cancer, Erbil City. Peripheral blood samples were collected from 32 patients with MM; all patients were treated with bortezomib except three patients who were treated with DARA. Most patients were newly diagnosed with MM; only three patients had relapsed and/or were refractory. Of the newly diagnosed patients**,** thirteen received the Vel-TD regimen consisting of bortezomib 1.3 mg/m$^2$ subcutaneously on days 1, 4, 8 , and 11; thalidomide 100 mg

orally daily; and dexamethasone 20mg orally on days 1-2, 4-5,8-9 and 11-12**.** Nine patients were treated with the Vel-D regimen consisting of bortezomib 1.3 mg/$m^2$ subcutaneously on days 1, 4, 8 ,and 11 and dexamethasone 20 mg orally on days 1-2, 4-5,8-9 , and 11-12. Seven patients received the Vel-CD regimen consisting of bortezomib 1.3 mg/$m^2$ subcutaneously on days 1, 4, 8, and 11; and cyclophosphamide 1000 mg/$m^2$ on day1and dexamethasone 20 mg orally on days 1-2, 4-5, 8-9 and 11-12. Of the relapsed and refractory MM patients, three patients used DARA as a monotherapy agent (16 mg/kg, weekly infusion for eight weeks). Also, all patients had been aggressively treated before the introduction of DARA.

*Analysis of Lymphocyte Subpopulation by Flow Cytometry*

The immunophenotypic analysis was performed by using the FACSDiva program of the FACSCanto (Becton Dickinson, USA) flow cytometer device (Figure 1). Peripheral blood samples were collected from the MM patients in the EDTA tube. For differentiation of the lymphocyte subgroups, the cell groups in the samples were firstly labeled with the following fluorescence-labeled-conjugated monoclonal antibodies: CD45RA FITC (Fluorescein isothiocyanate)/CD45RO PE (Phycoerythrin)/ CD3 PerCP (Peridinin-Chlorophyll-Protein)/ CD4 APC (Allophycocyanin), CD8 PE-Cy™7 (Phycoerythrin-Cyanin7), CD56 PE and NKG2A PE ( all from Becton Dickinson, USA). And then incubated at room temperature for 30 minutes in a dark place. Isotype control antibodies ( Mouse IgG1 PE Cy 7, Mouse IgG1 FITC , Mouse IgG2a PE , Mouse IgG1 PerCP ,and Mouse IgG1 APC) were further used to check the background staining. At the end of the incubation, the erythrocytes in the environment were lysed using the "Ammonium Chloride-containing Lysing Solution".

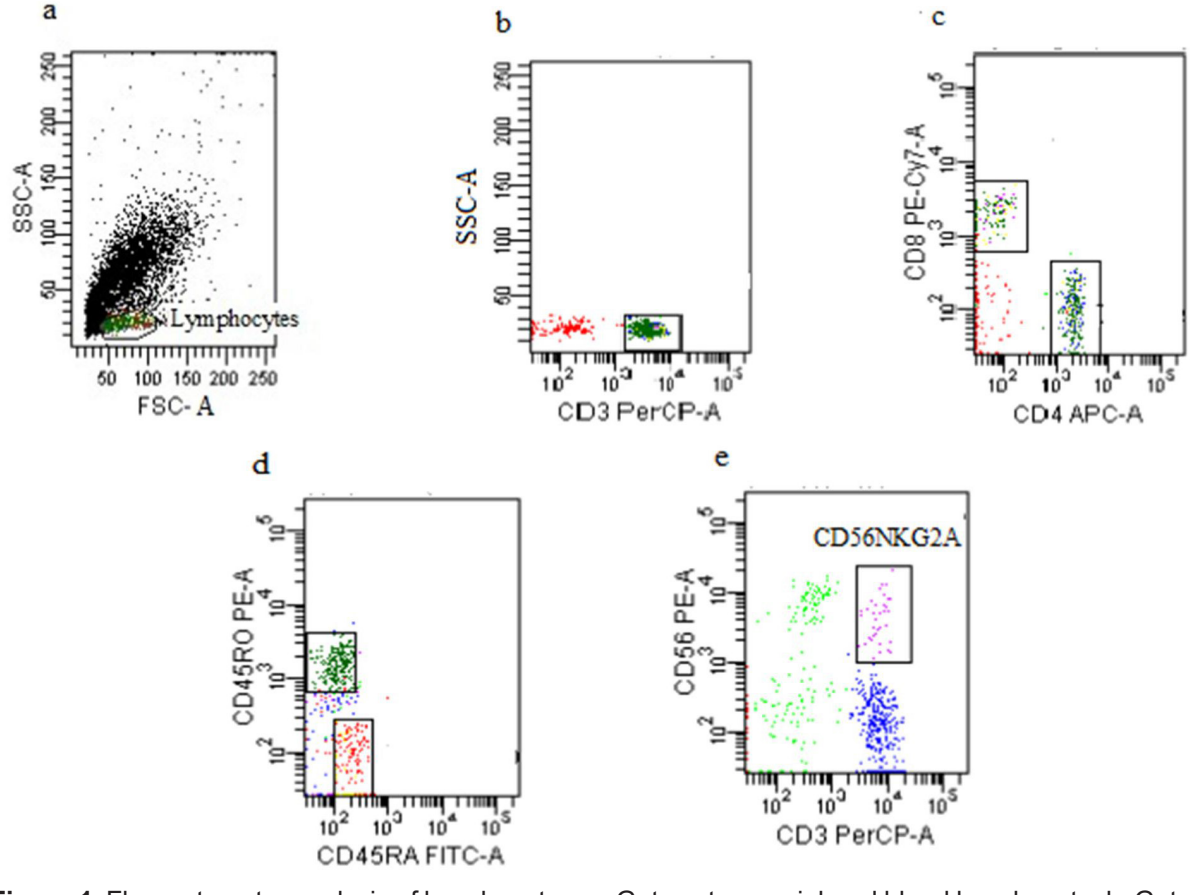


**Figure 1.** Flow cytometry analysis of lymphocytes. a: Gate set on peripheral blood lymphocyte, b: Gate set on $CD3^+$ T cell, c: Gate set on $CD4^+$ Tcell and $CD8^+$ T cell, d: Gate set on memory & naïve T cell, e: Get set on NK cells.

The prepared cells were passed in front of the laser light of the flow cytometer device. The lymphocyte subgroups within the total cell population (10,000) were identified using corresponding monoclonal antibodies to surface expressions of these cells.

*Statistical Analysis*

The statistical analysis and graphs were performed with GraphPad Prism Software (version6.0). D'Agostino-Pearson omnibus test, Shapiro-Wilk normality test, and Kolmogorov-Smirnov test were used to determine whether or not the data were normally distributed. For the comparison of the values before and after treatment, a *t*-test was performed for normally distributed data and presented as means±SE (Standard Error), the Wilcoxon test was applied if the data were not normally distributed and presented as median (range). For comparison among groups, the Kruskall-Wallis test was performed. P values<0.05 were considered statistically significant for all analyses.

## RESULTS

Table 1 summarizes the demographics and baseline characteristics of the patients.

*Lymphocyte Subpopulation within Bortezomib Treatment*

The phenotypic analysis of immune cells in the third cycle of bortezomib-based regimens (Table 2) revealed a decrease in the

**Table 1. Demographic and baseline characteristics of the patients.**

| Variable | Number of patients (%) |
|---|---|
| Total number of patients | 32 |
| Age, mean (range) | 64.5 (41–81) |
| Sex | |
| Male | 19 |
| Female | 13 |
| Myeloma isotype | |
| IgG | 20 |
| IgA | 6 |
| LCMM | 4 |
| NSMM | 2 |
| Laboratory values | |
| Creatinine (mg/dl),median (range) | 1.7 (5.4–0.5) |
| Urea (mg/dl), median (range) | 55 (280–21) |
| Calcium (mg/dl), median range | 8.7 (10.7–7.1) |
| Albumine (g/dl), median (range) | 3.5 ( 4.1–0.4) |
| Haemoglobin (g/dl), median (range) | 9.2 ( 11.9–5.2) |
| $B_2$Microglobulin(mg/l), median(range) | 6.8 ( 21–3) |
| ESR (mm/hr), median (range) | 89.5 (150–20) |
| ISS | |
| I | 10 |
| II | 9 |
| III | 13 |
| Bortezomib Regimens | |
| Vel-TD | 13 |
| Vel-CD | 7 |
| Vel-D | 9 |
| Daratumumb | 3 |

*LCMM* Light chain MM, *NSMM* Nonsecretory MM, *ESR* Erythrocyte sedimentation rate, *ISS* International staging system, *Vel-TD* velcade, thalidomide and dexamethasone, *Vel-CD* Velcade , cyclophosphamide and dexamethasone, *Vel-D* Velcade , and dexamethasone.

**Table 2. Lymphocyte subpopulation in MM patients at baseline and within the third cycle of bortezomib treatment.**

| Lymphocyte | Baseline | Third cycle | P values |
|---|---|---|---|
| **T cells population** | **Median (range)** | **Median (range)** | |
| $CD3^+$ T cell | 66.5 (56–88) | 50 (14–79.4) | 0.001 |
| $CD3^+CD4^+$ % | 58 (40–80) | 40.5 (16–71) | 0.001 |
| $CD3^+CD8^+$% | 38.5 (15.1–71) | 49.2 (24–83) | 0.001 |
| CD4/CD8 ratio | 1.54 (0.2–5.2) | 0.8 (0.2–2.9) | 0.001 |
| $CD4^+CD45RA^+$ % | 1.7 (0.1–27.0) | 1.05 (0.1–30) | 0.82 |
| $CD8^+CD45RA^+$ % | 1.7 (0.2–19) | 1.95(0.1- 24.0) | 0.18 |
| $CD4^+CD45RO^+$ % | 31.2 ( 0.6–53) | 19.9 (0.9–48.9) | 0.001 |
| $CD8^+CD45RO^+$ | 17 (2.1–37) | 12 (0.1–36.4) | 0.001 |
| **NK cells population** | | | |
| $CD3^-CD56^+$ | 13.5 (0.7–41) | 5.9 (0.4–23) | 0.002 |
| $CD16^+CD56^+$ | 11.5 (0.7–30) | 5.9 (0.4–22.1) | 0.001 |
| $CD56^+NKG2a^+$ | 5 (0.2–25) | 3.6 (0.1- 25.3) | 0.001 |

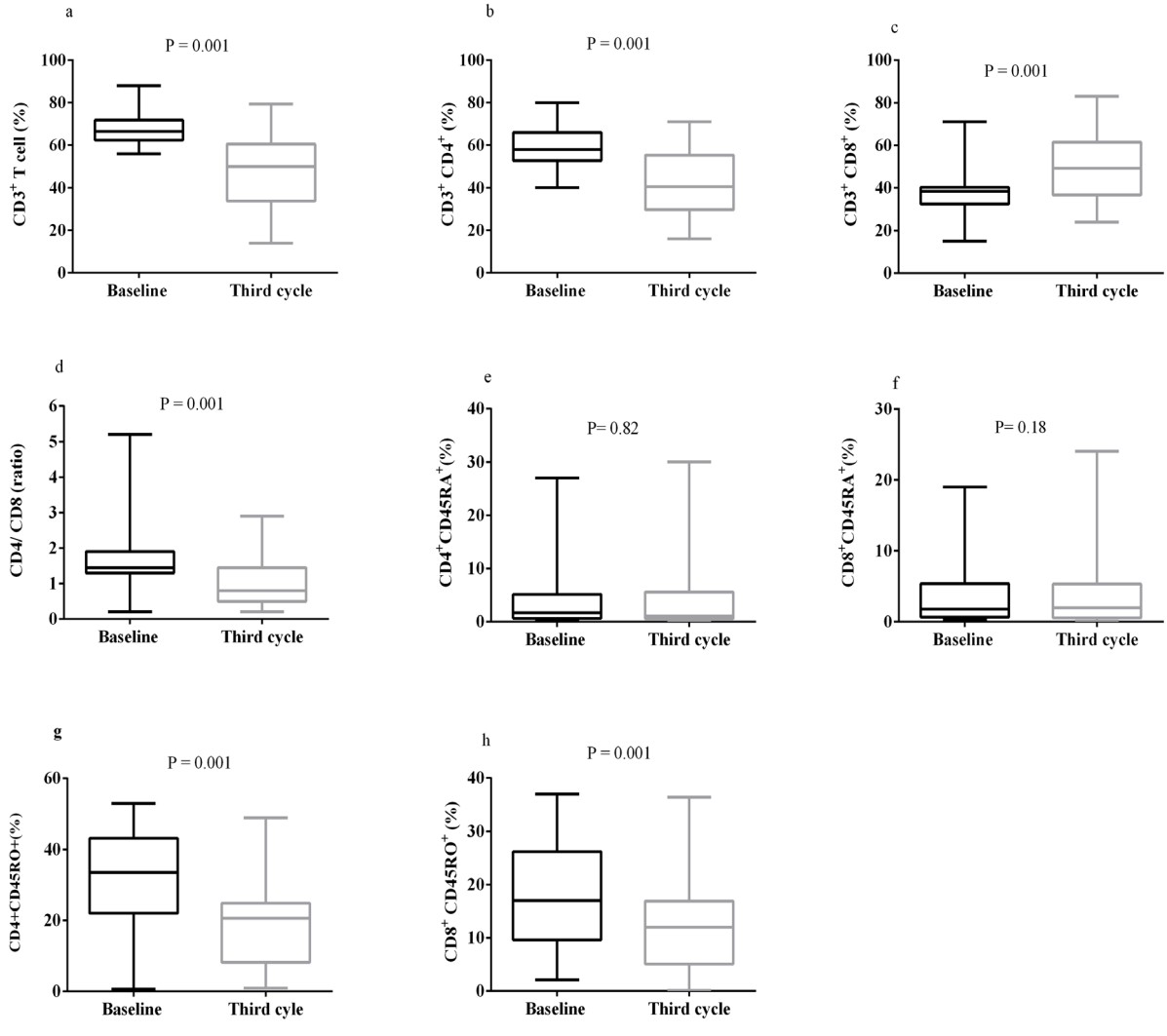


**Figure 2.** The T cells population in MM patients at baseline and within the third cycle of bortezomib treatment, N=29. (a) percentage of $CD3^+$T cells, (b) percentage of $CD4^+$ T cells, (c) percentage of $CD8^+$ T cells, (d) the ratio CD4/CD8, (e-f) percentage of naïve T cell ($CD4^+CD45RA^+$) & ($CD8^+CD45RA^+$), (g-h) percentage of memory ($CD4^+CD45RO^+$) & ($CD8^+CD45RO^+$) T cells.

median percentage of $CD3^+$ T cell by 16.5 % from a median baseline 66.5% to 50% during bortezomib therapy (P=0.001; Figure 2a). This was accompanied by a reduction in $CD4^+$ T cells of 17.5% (median 58% to 40.5%; P=0.001; Figure 2b), whereas the percentage of $CD8^+$ T cells increased by 10.7% during treatment (median 38.5% to 49.2%; P=0.001; Figure 2c). The decrease in the percentage of $CD4^+$ T cells and increase in $CD8^+$ T cells during bortezomib treatment led to a reduction in the CD4/CD8 ratio from a median baseline 1.54 to 0.8 during treatment (P=0.001; Figure 2d). Moreover, there was no significant difference in the median percentage of naïve $CD4^+$ ($CD4^+CD45RSA^+$) cells or naïve $CD8^+$ ($CD8^+CD45RSA^+$) cells during treatment (P=0.82 and P=0.18 respectively; Figures 2e and f).

A reduction in circulating memory $CD4^+$ ($CD4^+CD45RSO^+$) and $CD8^+$ ($CD8^+CD45RSO^+$) cells during treatment with bortezomib was observed in patients. The percentage of memory $CD4^+$ cells decreased by 11.3% (median 31.2% to 19.9%; P=0.001; Figure 2g), and the percentage of memory $CD8^+$ decreased by 5% (median 17% to 12%; P=0.001; Figure 2h). Regarding NK cells, a dramatic reduction in the percentage of circulating NK cells ($CD3^-CD56^+$) during treatment was observed (median 13.5% to 5.9%; P=0.002; Figure 3a). The number of mature NK cells ($CD16^+$ $CD56^+$) decreased by 5.6% during treatment (median 11.5% to 5.9%; P=0.001; Figure 3b), and immature NK cells decreased by 1.4% ($CD56^+$ $NKG2a^+$) during treatment (median 5% to 3.6%; P=0.001; Figure 3c).

A comparison of the three groups receiving the Vel-TD, Vel-CD & Vel-D regimens

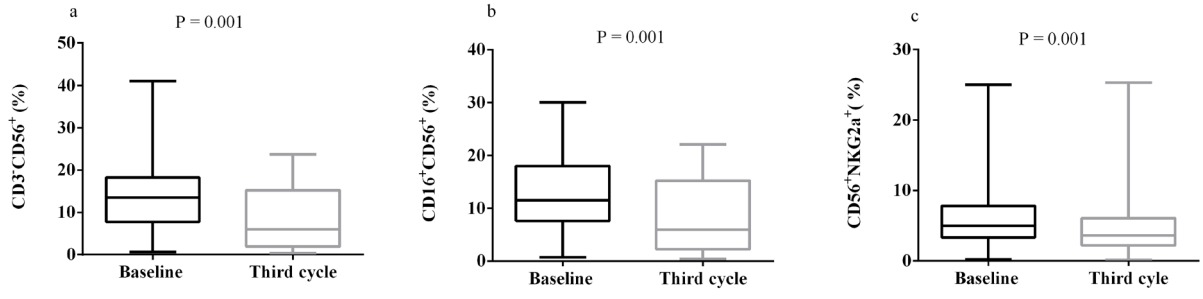


**Figure 3.** NK cells population in MM patients at baseline and within the third cycle of bortezomib treatment, N=29. (a) percentage of total NK cells ($CD3^-CD56^+$), (b) percentage of mature NK cells ($CD16^+CD56^+$) and (c) percentage of immature NK cells ($CD56^+NKG2a^+$).

**Table 3. Lymphocyte subpopulation in MM patients at baseline and within the fourth week of DARA treatment.**

| Lymphocyte | Baseline | Fourth week | P values |
|---|---|---|---|
| T cells population | Mean±SE | Mean±SE | |
| $CD3^+$ T cell | 62±7.3 | 67.7±6.3 | 0.04 |
| $CD3^+CD4^+$ % | 39.3±7.0 | 51.8±5.2 | 0.02 |
| $CD3^+CD8^+$% | 54.1±11.7 | 47.3±9.5 | 0.17 |
| CD4/CD8 ratio | 0.9±0.23 | 1.2±0.34 | 0.9 |
| $CD4^+CD45RA^+$ % | 2.5±0.37 | 2.2±0.15 | 0.54 |
| $CD8^+CD45RA^+$ % | 4.8±1.7 | 4.8±1.4 | 0.97 |
| $CD4^+CD45RO^+$ % | 5.9±2.1 | 7.1±2.7 | 0.35 |
| $CD8^+CD45RO^+$ | 12.5±6.0 | 13.6±6.1 | 0.5 |
| NK cells population | | | |
| $CD3^-CD56^+$ | 9.9±1.4 | 1.0±0.15 | 0.02 |
| $CD16^+CD56^+$ | 10±2.0 | 0.9±0.1 | 0.03 |
| $CD56^+NKG2a^+$ | 4.4±2.0 | 4.5±2.1 | 0.8 |

demonstrated no significant difference.

*Lymphocyte Subpopulation within DARA Treatment*

As part of this study, DARA was administered as a monotherapy for three patients with relapsed and refractory MM. The phenotypic analysis of immune cells in the fourth week of DARA treatment (Table 3) suggested a slight increase in the mean percentage of $CD3^+$ T cells by 5.7%, from a mean baseline 62% to 67.7% during DARA treatment (P=0.04; Figure 4a). $CD4^+$ T cells increased significantly during DARA treatment (mean 39.3% to 51.8%; P=0.02; Figure 4b). $CD8^+$T cells decreased during DARA treatment, but the decrease was not significant (P=0.17; Figure 4c). This was followed by an increase in the CD4/CD8 ratio during treatment that was not significant (P=0.9; Figure 4d). The mean percentage of naïve$CD4^+$ cells decreased slightly during treatment, but the decrease was not statistically significant (P=0.54; Figure 4e). There was no change in the mean percentage of naïve $CD8^+$ cells during treatment (P=0.9; Figure 4f). Furthermore, memory $CD4^+$ cells and memory $CD8^+$ cells were slightly, but not significantly, increased during DARA treatment (P=0.3 and P=0.5 respectively; Figure 4g and h).

There was a drastic reduction in the total number of NK cells during DARA treatment (mean 9.9% to 1%; P=0.02; Figure 5a). The mean percentage of mature NK cells were decreased sharply during DARA treatment as well (mean 10% to 0.9%; P=0.03; Figure 5b). Immature NK cell levels remained constant during treatment (P=0.8; Figure 5c).

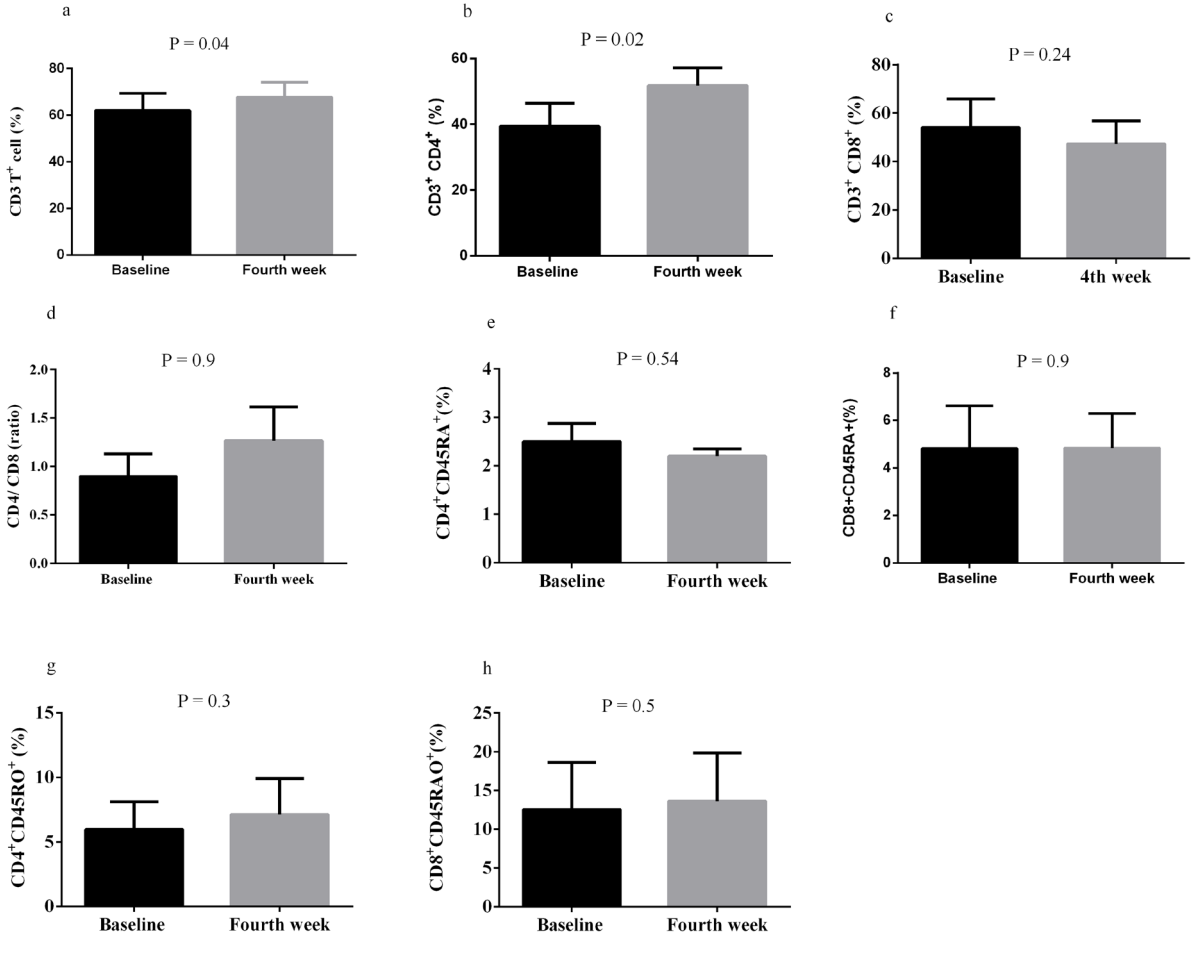


**Figure 4.** T cells population in MM patients at baseline and within the fourth week of DARA treatment, N=3. (a) percentage of $CD3^+$T cells, (b) percentage of $CD4^+$ T cells, (c) percentage of $CD8^+$ T cells, (d) ratio CD4/CD8, (e-f) percentage of naïveT cell ($CD4^+CD45RA^+$) & ($CD8^+CD45RA^+$), (g-h) percentage of memory ($CD4^+CD45RO^+$) & ($CD8^+CD45RO^+$) T cells.

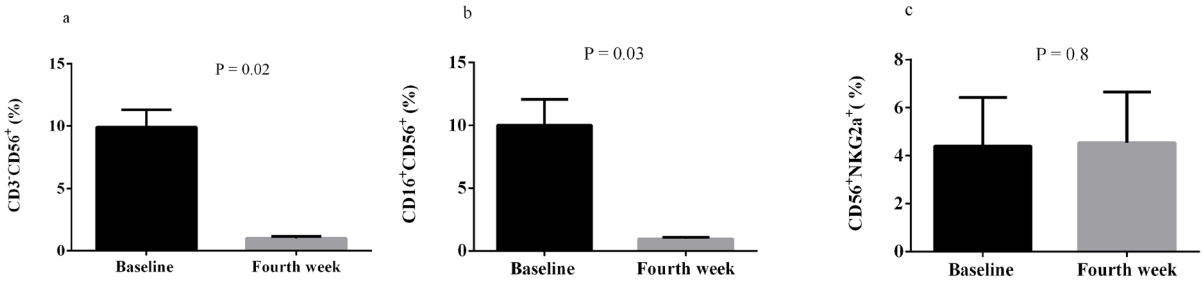


**Figure 5.** NK cells population in MM patients at baseline and within the fourth week of DARA treatment, N=3. (a) percentage of total NK cells ($CD3^-CD56^+$), (b) percentage of mature NK cells ($CD16^+CD56^+$) and (c) percentage of immature NK cells ($CD56^+NKG2a^+$).

## DISCUSSION

Recently, many novel drugs have been used in the treatment of MM. They are different in their modes of action, side effects ,and safety profiles (3). Furthermore, with the introduction of novel, life-prolonging therapies, MM has been transformed into a chronic disease (6).

As was observed in the present study, patients receiving novel agents demonstrated a variety of immunophenotypes. In this study, 29 patients newly diagnosed with MM were treated with bortezomib using different regimens (Vel-TD, Vel-CD ,and Vel-D). The phenotypic analysis of immune cells before the initiation of treatment and during the third cycle of treatment showed a significant reduction in the number of $CD3^+$T cells, $CD4^+$ T cells, the CD4/CD8 ratio, memory T cells , and NK cells population in all protocols. Bortezomib is a potent antimyeloma agent that induces inhibition of the proteasome, resulting in cumulation of malformed proteins and useful proteins that are broken down by the proteasome in the endoplasmic reticulum(ER) lumen and cytoplasm, which promotes many stresses, such as ER excessive load, production of excess oxygen, and functional defect of intracellular proteins, finally formation ER stress-related apoptosis and the death of the cells as a result of DNA damage (8). NF-κB is one type of the proteins controlled by proteasomal activity, which its activation demonstrated survival, proliferation, and drug resistance of MM cells. In addition to promoting the expression of proinflammatory cytokines (Interleukin 6 and Tumor Necrosis Factors-α) (14). When bortezomib inhibits the proteasome, I-κB can not destroy and continues to bind and block NF-κB, causing the death of multiple myeloma cells (7). Also, bortezomib inhibits normal T cells especially, the immune function of $CD4^+$ T cells as well as dendritic cells, NK cells , and B cells (15, 16). An experimental animal study demonstrated that bortezomib-treated mice showed a significant decrease in T cell count (17). Other studies on humans showed that the bortezomib-based regimen decreased lymphocyte count (6, 18).

$CD8^+$ T cells were increased during treatment in all bortezomib regimens. Several studies have indicated that the T cells in cancer patients may have been exposed to chronic antigen stimulation leading to a gradual gathering of late differentiated, antigen-specific, oligoclonal T cells, particularly within the $CD8^+$ T cell compartment (19, 20). In contrast, a previous study showed a decrease in $CD8^+$ T cells during bortezomib treatment (16).

We used bortezomib in combination with other drugs (VTD, VCD ,and VD). All bortezomib regimens decreased lymphocyte count except CD8 T lymphocyte. Statistically, there was no significant difference among bortezomib regimens in effect on a lymphocyte. Thalidomide is an immunomodulatory drug that activates the proliferation of T lymphocytes and induces cytokine production (21). We thought that the addition of thalidomide to bortezomib would

increase the count of lymphocytes. Still, in this study, this combination did not improve lymphocyte count; this means that the drop in lymphocyte count is due to the impact of bortezomib. And we cannot rule out the effect of dexamethasone and cyclophosphamide on lymphocyte decline.

In the current study, DARA was used as monotherapy for three patients. The main observation in the fourth week of DARA treatment was a significant increase in $CD3^+$ T cells as well as $CD4^+$ T cells. As a result, the CD4/CD8 ratio was increased, but the increase was not statistically significant. Moreover, memory T cells were increased slightly but not significantly, while native $CD4^+$ cells decreased slightly and not significantly. A previous study suggested that DARA dramatically increases the count of peripheral total $CD3^+$ T cells, helper and cytotoxic T cells. In addition to enhancing memory T cells, DARA has been shown to reduce naïve T cells (22). In the setting of myeloma, Myeloid-derived suppressor cells and regulatory B cells express CD38 and, were sensitive to DARA therapy. These cells are found in the environment around the tumor and lead to tumor development, metastasis , and immune evasion as well as secretion of immunosuppressive cytokines. Moreover, a novel subpopulation of T regulatory cells ($CD4^+CD25^+$ $CD127^{dim}$) was recognized that also highly expressed $CD38^+$ and was sensitive to DARA treatment. These cells have a superior capacity for autologous T cell suppression. Downregulation of the $CD38^+$ T regulatory cell by DARA may decrease the local immune suppressive effect within the environment of myeloma cells and enhance immune cells to extend and provide an antitumor immune response (22, 23). Specific $CD8^+$ subpopulations decreased during four weeks of DARA treatment, but the decrease was not significant. As would be anticipated for the aggressively pretreated patients, there was wide variability of response to DARA in this population. Additionally, a transient decrease in the $CD8^+$ T cell population, which returned to normal levels at treatment interruption, was observed in another study, which included two patients treated with DARA (24). Because NK cells express high levels of CD38 (22), in the present study, NK cells and mature NK cells drastically decreased in number during DARA therapy. It has been previously reported that NK cell counts declined after dara treatment in a study performed in vitro and patients treated in phase 1 and 2 GEN501 and SIRIUS studies; however, NK cells were not diminished and contributed to antibody-dependent cellular cytotoxicity (25). Many studies have suggested that MM patients treated with bortezomib regimens and daratumumab had a decline in lymphocyte count and a significant increase in the rate of infection (9, 18, 26).

DARA and bortezomib regimens in this study had a reverse effect on the T.cell subset, and this is partly due to the fact that DARA is a monoclonal antibody targeting only the cell that expresses CD38. In addition to improving host-antitumor immune response by removing CD38 positive immune suppressor cells, including regulatory B cells, regulatory T cells ,and myloid-derived suppressor cells (27). On the other hand, proteasome protein is required for maintenance and regulation of essential cellular processes, including regulating substrates that control cell cycles, metabolism, survival, apoptotic, and removing harmful proteins that are toxic to the cells (8). The activity of the proteasome is tightly associated with T cell proliferation and differentiation. Therefore, inhibition of this protein by bortezomib effect myeloma cells and at the same time may affect the T cells count (28).

In conclusion, bortezomib harmed the T cells subset, while DARAregimens had a positive impact on T the cells subset. NK cells decreased significantly in both treatments. These result suggested that DARA is more specific to target myeloma cells than bortezomib. Also, DARA enhanced the T cells to extend especially $CD3^+$ T cells and $CD4^+$ T cells. MM patients should be monitored

closely for a decrease in lymphocyte count, especially when treated with bortezomib regimens. Unfortunately, In this study, we had three patients under Dara's treatment. Therefore, further studies are necessary to know the effect of DARA as monotherapy or in combination with other antimyeloma drugs on a lymphocyte.

## ACKNOWLEDGMENTS

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**Conflicts of Interest:** None declared.

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