UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

ANNA KULIKOWSKA de NAŁĘCZ

Ekspresja supresorowych antygenów BTLA, PD-1 i CTLA-4 w populacji efektorowych i regulatorowych limfocytów krwi obwodowej u chorych na szpiczaka plazmocytowego

ROZPRAWA DOKTORSKA

Promotor: dr hab. n. med. Agata Kosmaczewska

Wrocław 2023

PODZIĘKOWANIA

Serdeczne podziękowania Pani Promotor dr hab. n. med. Agacie Kosmaczewskiej za podzielenie się ze mną swoją ogromną wiedzą, nieocenioną pomoc, wskazówki i cierpliwość.

Podziękowania Pani Prof. dr hab. n. med. Irenie Frydeckiej za słowa wsparcia.

Podziękowania mężowi za cierpliwość i wsparcie w chwilach zwątpienia.

SPIS TREŚCI

I. LISTA PUBLIKACJI SKŁADAJĄCYCH SIĘ NA CYKL:

The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. Agata Kosmaczewska, Anna Masternak, Katarzyna Kościow. OA Immunology 2013 Vol.1 no.1 art.7, ryc., tab., bibiogr. 45 poz., summ. DOI:10.13172/2052-9295-1-1-1018

Deregulated expression of immune checkpoints on circulating CD4 T Cell may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients. Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka, Agata Kosmaczewska. Int. J. Mol. Sci. 2021 Vol.22 no.17 art.9298 [19 s.], ryc., tab., bibliogr. 57 poz., summ. DOI: 10.3390/ijms22179298; **IF - 6,208**

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors. Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka, Agata Kosmaczewska. Int. J. Mol Sci. 2023 Vol.24 no.6 art.5730 [17 s.], ryc., tab., bibliogr. 69 poz., summ. DOI: 10.3390/ijms24065730; **IF- 6,208**

II. STRESZCZENIE

Wstęp:

Szpiczak plazmocytowy (multiple myeloma, MM) jest nowotworem hematologicznym B-komórkowym charakteryzującym się głębokimi zaburzeniami immunologicznymi postępującymi wraz z progresją choroby. Dlatego poszukiwanie skutecznych leków oddziałujących na elementy tego układu, takich jak inhibitory punktów kontrolnych nadzoru immunologicznego, w celu poprawy immunologicznej odpowiedzi przeciwnowotworowej wydaje się mieć szczególne znaczenie kliniczne. Immunologicznymi punktami kontrolnymi (immune checkpoint, IC) nazywane są cząsteczki, które regulują mechanizmy nadzoru immunologicznego i są zaangażowane w rozwój tolerancji immunologicznej, m.in. badane w niniejszej rozprawie doktorskiej cząsteczki supresorowe PD-1, CTLA-4, BTLA występujące na powierzchni limfocytów T i uczestniczące w procesie hamowania aktywacji tych komórek. Poziom ekspresji tych supresorowych cząsteczek ma zasadnicze znaczenie dla utrzymywania homeostazy układu immunologicznego, tolerancji własnych antygenów tkankowych oraz niszczenia komórek nowotworowych. Zaburzenia ekspresji punktów kontrolnych mogą przyczyniać się do zachwiania równowagi immunologicznej i rozwoju procesów autoimmunizacyjnych (przy obniżonej ekspresji) lub sprzyjać rozwojowi nowotworów (przy zwiększonej ekspresji punktów kontrolnych). Wprowadzenie do terapii przeciwnowotworowej inhibitorów punktów kontrolnych układu immunologicznego (immune checkpoint inhibitor, ICI) poprawiło znacząco wyniki leczenia wielu nowotworów litych oraz chłoniaka Hodgkina, co zachęciło do podjęcia badań klinicznych z użyciem przeciwciał blokujących cząsteczki supresorowe PD-1/PD1-L i CTLA-4 również w innych rozrostach hematologicznych. Przeprowadzone dotąd badania kliniczne z zastosowaniem blokady osi PD-1/PD1-L i/lub CTLA-4 u chorych na MM przyniosło rozczarowanie z powodu niskiego wskaźnika odpowiedzi klinicznych (overall response rate, ORR) przy stosunkowo dużej liczbie poważnych powikłań głównie o charakterze autoimmunizacyjnym. Mechanizmy leżące u podłoża oporności na terapeutyczne inhibitory punktów kontrolnych obserwowanej u przeważającego odsetka pacjentów z MM pozostają nadal niewyjaśnione.

Cel pracy:

Celem niniejszej pracy było zbadanie ekspresji immunologicznych punktów kontrolnych BTLA, PD-1 i CTLA-4 na limfocytach T CD4 krwi obwodowej u chorych na MM w celu określenia znaczenia poszczególnych cząsteczek w rozwoju immunosupresji

systemowej w różnych fazach choroby, a także określenie ich użyteczności klinicznej jako celu terapii z zastosowaniem inhibitorów punktów kontrolnych, oraz zbadanie związku ekspresji PD-1 i CTLA-4 z przebiegiem klinicznym MM celem oznaczenia ich potencjalnej wartości predykcyjnej i/lub rokowniczej.

Materiał i metody:

Pierwsza praca jest artykułem przeglądowym podsumowującym wiedzę na temat biologii MM, wpływu mikrośrodowiska na rozwój choroby i odpowiedź na leczenie. Na podstawie publikacji naukowych dostępnych w bazach PubMed i Google Scholar przedstawiono zależności elementów komórkowych, macierzy mikrośrodowiska i cytokin obecnych w przestrzeni pozakomórkowej w rozwoju i przerzutowaniu MM.

Kolejne dwie prace oryginalne (powstałe w kontynuacji) prezentują wyniki badań przeprowadzonych w grupie obejmującej 40 pacjentów z aktywnym MM w różnych fazach choroby: 26 pacjentów z nowo rozpoznanym MM (newly diagnosed MM, NDMM) oraz 14 pacjentów z nawrotowym/opornym MM (relapsed/refractory MM, RRMM) uprzednio leczonych chemioterapią, lekami immunomodulującymi (immunomodulatory drugs, IMID), inhibitorami proteasomu (proteasome inhibitor, PI). Grupę kontrolną stanowiło 20 zdrowych ochotników odpowiadających wiekiem i płcią grupie badanej (healthy controls, HC). Badania przeprowadzono po uzyskaniu pisemnej zgody od wszystkich uczestników. Materiał do badań stanowiły komórki jednojądrzaste wyizolowane z krwi obwodowej (peripheral blood mononuclear cells, PBMCs). Metodą cytometrii przepływowej oznaczano ekspresję jakościową i ilościową badanych ICs (PD-1, CTLA-4 i BTLA) w populacjach limfocytów T CD4+, CD4+CD127+ (T effector cells, Teff) oraz CD4+CD127- (T regulatory cells, Treg). Stan systemowej aktywacji określano poprzez określenie ekspresji markera aktywacji CD69 w populacji limfocytów T CD4. Ponadto badano populację limfocytów efektorowych: Th1 (CD3+CD8-IFN-γ+) i Th17 (CD3+CD8-IL-17+) oraz subpopulacje limfocytów regulatorowych Treg (CD4+CD25+CD127, CD4+CD25+FOXP3+, CD4+FOXP3+CD127-), a także ich wzajemne proporcje w postaci stosunku Th1/Treg i Th17/Treg w różnych fazach choroby. Przedmiotem badania była także wielkość obwodowej populacji starzejących się autoreaktywnych limfocytów T CD4+CD28-. Dodatkowo korelowano ekspresję białek supresorowych PD-1 i CTLA-4 na limfocytach T CD4 z czynnikami niekorzystnego przebiegu klinicznego, a także z czasem do wystąpienia progresji (time to progression, TTP) i czasem całkowitego przeżycia (overall survival, OS) pacjentów.

Wyniki:

Praca poglądowa podsumowuje dotychczasową wiedzę nt. roli mikrośrodowiska szpiku kostnego w rozwoju i progresji MM podkreślając sieć wzajemnych oddziaływań między elementami komórkowymi (uwzględniając również komórki układu immunologicznego) z elementami macierzy pozakomórkowej, które razem tworzą immunosupresyjne środowisko kreujące warunki do transformacji nowotworowej plazmocytów. Przenoszenie się plazmocytów do krwi obwodowej prowadzi do osadzania się komórek nowotworowych poza szpikiem kostnym, a tym samym do progresji choroby.

W drugiej publikacji z cyklu o charakterze oryginalnej pracy badawczej wykazano, że limfocyty T CD4 w NDMM prezentują cechy immunostarzenia (immunosenescence), obejmujące deficyt ilościowy ekspresji immunologicznych punktów kontrolnych PD-1, BTLA i CTLA-4 oraz relatywnie zwiększoną sekrecję cytokin prozapalnych IFN-γ i IL-17. Natomiast progresja MM (RRMM) przebiega z częściowym przywróceniem ekspresji cząsteczek supresorowych uczestniczących w nadzorze immunologicznym, co koresponduje z pogłębieniem dysfunkcji sekrecyjnej IFN-γ i IL-17, i odpowiada fenotypowi komórek wyczerpanych (exhausted). Obserwowano także, że PD-1 jest jedynym receptorem supresorowym występującym na większym odsetku limfocytów T CD4 u wszystkich chorych na MM niezależnie od fazy choroby, którego ekspresja koreluje z niekorzystnym przebiegiem klinicznym i tendencją do skróconego OS.

W kolejnej pracy oryginalnej (będącej kontynuacją poprzedniej), poza potwierdzeniem związku suboptymalnej ekspresji immunologicznych punktów kontrolnych z różnymi etapami choroby (NDMM i RRMM), zaobserwowano także obwodową ekspansję limfocytów T CD4+CD28- u wszystkich pacjentów (największą w grupie NDMM), których fenotyp i funkcjonalna charakterystyka odpowiada autoreaktywnym limfocytom cytotoksycznym (noszącym cechy immunostarzenia). Ciekawą obserwacją było wykazanie związku poziomów ekspresji cząsteczek supresorowych z TTP; niski poziom CTLA-4 w grupie NDMM i wysoka ekspresja PD-1 w grupie RRMM korelują z krótszym TTP.

Wnioski:

Zwiększona dystrybucja PD-1 i związek z niekorzystnym przebiegiem klinicznym wskazuje na nadrzędną rolę PD-1 w sieci supresorowych oddziaływań jako przyczynę zaburzonej odporności komórkowej w MM. Mechanizm immunosupresji w MM w

początkowych etapach choroby wiąże się z nasilonym immunostarzeniem limfocytów T, natomiast w czasie progresji MM jest związany głównie z wyczerpaniem funkcjonalnym tych komórek; jedną z cech różniących oba dysfunkcjonalne stany (tj. immunostarzenie i wyczerpanie) jest różny stopień deficytu poziomu ekspresji immunologicznych punktów kontrolnych PD-1, CTLA-4 i BTLA zależny od etapu choroby; suboptymalna ekspresja punktów kontrolnych w MM może istotnie obniżać skuteczność terapii z użyciem inhibitorów tych cząsteczek i stanowić jeden z mechanizmów oporności na tę formę immunoterapii w tej chorobie. Ponadto ekspansja obwodowa starzejących się limfocytów T CD4+CD28- (o właściwościach autoreaktywnych komórek cytotoksycznych) wraz z obniżonym poziomem ekspresji PD-1 i CTLA-4 obserwowanym u pacjentów z MM (szczególnie w grupie NDMM) może świadczyć o zwiększonym potencjale do rozwoju procesów autoimmunizacyjnych zarówno w przebiegu MM, jak i w czasie immunoterapii z użyciem terapeutycznych inhibitorów punktów kontrolnych. Związek niskiego poziomu ekspresji CTLA-4 z istotnie krótszym TTP w grupie NDMM oraz negatywny wpływ ekspresji PD-1 na przebieg kliniczny, OS i TTP w grupie RRMM wskazuje, że stosowanie inhibitorów badanych punktów kontrolnych jest niekorzystną strategią leczniczą dla chorych z NDMM, natomiast pacjenci w grupie RRMM z progresją choroby stanowią grupę chorych, która może odnieść korzyść kliniczną z tej formy terapii.

III. ABSTRACT

Introduction:

Multiple myeloma (MM) is a B-cell haematological malignancy characterized by profound and progressing immune alterations. Therefore, the efficacy of drugs targeting the elements of immune environments (such as immune checkpoint inhibitors) in order to improve anti-tumor response is of particular clinical importance. Immune checkpoints (ICs) are molecules that regulate immune surveillance mechanisms and are involved in the development of immune tolerance (e.g. inhibitory PD-1, CTLA-4, and BTLA molecules examined in the current dissertation); they are expressed on the surface of immune cells (including T cells) and involved in inhibition of cell activation. The optimal level of ICs expression is essential for maintaining homeostasis of the immune system including tolerance toward tissue self-antigens and eradication of cancer cells. Alterations in the expression of ICs may contribute to the immune imbalance and the development of autoimmune responses (at reduced ICs expression) or promote the development of cancer (when ICs expression is increased). The introduction of ICs inhibitors (ICIs) into cancer therapy significantly improved the results of treatment of many solid cancers and Hodgkin's lymphoma, what prompted physicians to administration of ICIs into clinical trials in other hematological malignancies. However, the clinical trials conducted so far with the use of antibodies blocking the PD-1/PD1-L axis and CTLA-4 in patients with MM have shown disappointing results due to the unfavorable benefit-to-risk profile with low overall response rate (ORR) and a much higher frequency and severity of immune-related adverse events (iRAEs). The mechanisms underlying the resistance to ICIs observed in the vast majority of MM patients remain still unresolved.

The aim of the study:

The purpose of this study was to assess the expression of ICs (BTLA, PD-1 and CTLA-4) on CD4 T lymphocytes in order to determine the involvement of particular receptors in the development of systemic immunosuppression in MM patients at different stages of the disease (at the time of diagnosis and during progression), determination of their usefulness as a target of therapy with ICIs, as well as examination of their relationship with the clinical course of MM in order to determine their potential predictive and prognostic significance.

Material and methods:

The first paper is a review article summarizing the knowledge on the biology of MM, the influence of the microenvironment on both the development of the disease and response to treatment. On the basis of scientific publications available in the PubMed and Google Scholar databases, the relationships between cellular elements, microenvironmental matrix and cytokines present in the extracellular space in the development and metastasis of MM have been shown.

The next two original papers demonstrate the results of research conducted (as a consistent continuation) in a study group of 40 patients with active MM in the different clinical stages of the disease: 26 patients with newly diagnosed MM (NDMM) and 14 patients with relapsed/refractory MM (relapsed/refractory MM, RRMM) previously treated with chemotherapy, immunomodulatory drugs (IMIDs), proteasome inhibitors (PIs). The control population compromised 20 healthy volunteers matched for age and sex (HC). The study was conducted after obtaining written consent from all participants. The study material consisted of mononuclear cells isolated from peripheral blood (PBMCs). The qualitative and quantitative expression of the examined ICs (PD-1, CTLA-4 and BTLA) in the populations of CD4+, CD4+CD127+ (Teff) and CD4+CD127- (Treg) lymphocytes was determined by flow cytometry. Systemic activation status was tested by determining the expression of CD69 activation marker in the CD4 T cell population. In addition, the population of effector lymphocytes: Th1 (CD3+CD8-IFN-γ+) and Th17 (CD3+CD8-IL-17+) and subpopulations of Treg regulatory lymphocytes (CD4+CD25+CD127), CD4+CD25+FOXP3+, CD4+FOXP3+CD127-), as well as the relationship between them (Th1/Treg and Th17/Treg) in different phases of the disease were examined as well. Peripheral expansion of senescent autoreactive CD4+CD28- cells was also studied in all subjects. In addition, the expression of PD 1 and CTLA-4 molecules on CD4 T cells was correlated with clinic and laboratory indices of adverse clinical course, as well as with time to progression (TTP) and patient overall survival (OS).

Results:

The review paper summarized the current knowledge on the role of the bone marrow microenvironment in the development and progression of MM, emphasizing the network of interactions between cellular elements (including immune cells) with elements of the extracellular matrix, which together create an immunosuppressive environment creating

conditions for malignant transformation of plasma cells. The transfer of plasma cells to the peripheral blood leads to the deposition of tumor cells outside the bone marrow, and thus to the progression of the disease.

The second study demonstrated that CD4 T cells in NDMM displayed features of immunosenescence (i.e. quantitative deficit of IC expression and relatively increased secretion of pro-inflammatory IFN-γ and IL-17 cytokines), while MM progression is accompanied by a reversion of ICs expression, which corresponds to pronounced IFN-γ and IL-17 secretory dysfunction, consistently with an exhausted phenotype. It was also observed that PD-1 is the only IC expressed on a higher percentage of CD4 T cells in all MM patients, regardless of the stage of the disease, and its expression correlates with an unfavorable clinical course and a tendency to shorten OS.

In the next study (which is a continuation of the previous one), apart from confirming the relationship between suboptimal expression of ICs and different stages of the disease (NDMM and RRMM), expansion of CD4+CD28- cells was also observed in all patients (more pronounced in the NDMM group), whose phenotype and functional characteristics correspond to autoreactive lymphocytes (exerting features of immunosenescence). An interesting observation was also the relationship between ICs expression levels and TTP; low CTLA-4 levels in the NDMM group and high expression of PD-1 in the RRMM group correlate with shorter TTP.

Conclusions:

The increased distribution of PD-1 in the T cell compartment and its association with the unfavorable clinical course of MM may indicate the superior role of PD-1 in the network of suppressor interactions as the cause of impaired cellular immunity in MM. The mechanism of immunosuppression in MM in the early stages of the disease (NDMM) is associated with increased immunosenescence of lymphocytes, while during MM progression (RRMM) it is mainly associated with functional exhaustion of T lymphocytes; one of the features differentiating the two dysfunctional states (namely immunosenescence and exhaustion) is varying degrees of the suboptimal expression level of ICs depending on the stage of the disease, which may significantly reduce the effectiveness of ICIs therapy and constitute one of the mechanisms of resistance to this form of immunotherapy in MM. Peripheral expansion of senescent CD4+CD28- lymphocytes (with autoreactive cytotoxic properties) observed in patients with MM (especially in the NDMM group) may indicate an increased potential for

the development of autoimmune responses both in the course of MM and during immunotherapy with ICIs. The relationship of the low CTLA-4 expression with significantly shorter TTP in the NDMM group and the negative impact of PD-1 expression on the clinical course, OS and TTP in the RRMM group indicate that ICI therapy is an unfavorable treatment strategy for patients with NDMM, while in RRMM patients with disease progression this form of therapy could be beneficial.

IV. WSTĘP

Szpiczak plazmocytowy (multiple myeloma, MM) jest nowotworem hematologicznym B-komórkowym charakteryzującym się proliferacją i akumulacją w szpiku kostnym klonalnych komórek plazmatycznych produkujących białko M[1]. Biologiczna właściwością MM jest postępujący rozwój zaburzeń immunologicznych, które prowadzą do ucieczki nowotworowych komórek plazmatycznych spod nadzoru immunologicznego, wzrostu masy nowotworu i klinicznych komplikacji w postaci niewydolności nerek, osteolizy kostnej i nawracających infekcji[2]. Patogeneza dysregulacji immunologicznej w przebiegu MM jest złożona i wynika z procesu rozrostowego w szpiku kostnym, który obejmuje m.in. elementy układu odpornościowego, ale może być również skutkiem leczenia (chemio- i immunoterapii). W wyniku narastających zaburzeń immunologicznych dochodzi do rozwoju immunosupresji, co oprócz osłabienia odpowiedzi przeciwnowotworowej zwiększa istotnie ryzyko rozwoju infekcji o ciężkim przebiegu. W szczególności, rozwój MM charakteryzuje postępująca dysfunkcja prawidłowych plazmocytów sukcesywnie wypieranych przez klonalne komórki plazmatyczne z następową hypogammaglobulinemią, a także zaburzenia odporności komórkowej związane z dysfunkcją limfocytów T, komórek dendrytycznych (dendritic cells, DC) i komórek NK (natural killer)[3]. Pomimo postępu w immunoterapii MM związanego z wprowadzeniem do leczenia inhibitorów proteasomu (proteasome inhibitors, PI), leków immunomodulujących (immunomodulatory drugs, IMID), terapii limfocytami T ze zmodyfikowanym (chimerycznym) receptorem antygenowym (CAR-T cells), MM nadal pozostaje nowotworem nieuleczalnym. Dysfunkcja układu immunologicznego, w szczególności obniżenie aktywności i funkcji efektorowych limfocytów T (T effector cells, Teff), przyczynia się do nieskutecznego niszczenia komórek szpiczakowych i rozczarowujących wyników leczenia MM[2, 3].

Osłabienie mechanizmów nadmiernie rozwiniętej tolerancji immunologicznej oraz odwrócenie hyporeaktywności limfocytów T w MM wydaje się mieć zasadnicze znaczenie dla optymalizacji i poprawy skuteczności stosowanych strategii immunoterapeutycznych. Wprowadzenie do terapii nowotworowej inhibitorów punktów kontrolnych w nadzorze immunologicznym (immune checkpoint inhibitors, ICI), czyli przeciwciał blokujących cząsteczki supresorowe PD-1 i/lub CTLA-4, poprawiło istotnie wyniki leczenia guzów litych[4], a także chłoniaka Hodgkina[5], co dało nadzieję i asumpt do podjęcia prób ich wykorzystania w terapii nowotworów hematologicznych. Jednak zastosowanie inhibitorów cząsteczek supresorowych w badaniach klinicznych u chorych na MM przyniosło rezultaty

niezgodne z oczekiwaniami w postaci niskiego wskaźnika odpowiedzi klinicznych (overall response rate, ORR) - jedynie w kombinowanych strategiach leczniczych u chorych z progresywnym/nawrotowym MM (RRMM)[6-8]. Niespełniające oczekiwań wyniki stosowania blokady punktów kontrolnych w MM mogą wskazywać na zróżnicowany udział poszczególnych cząsteczek supresorowych zaangażowanych w hamowanie odpowiedzi immunologicznej [8], a także skłaniają do poszukiwania mechanizmów odpowiadających za postępującą immunosupresję u chorych na MM oraz wskazują na potrzebę identyfikacji tych pacjentów, którzy mogą odnieść korzyść kliniczną z terapii blokującej cząsteczki supresorowe.

Dostępna literatura wskazuje, że zaburzenia funkcji limfocytów T w przebiegu MM są bardziej pogłębione w porównaniu do innych nowotworów hematologicznych B-komórkowych, a mechanizmy leżące u ich podłoża związane są głównie z immunostarzeniem (immunosenescence) i/lub wyczerpaniem funkcjonalnym limfocytów T (exhaustion)[9-12], prowadzących w różny sposób do postępującej hyporeaktywności tych komórek i systemowej supresji immunologicznej[13-15]. Identyfikacja mechanizmu leżącego u podłoża immunosupresji w MM wydaje się mieć implikacje kliniczne w postaci doboru właściwego sposobu odwrócenia supresji immunologicznej za pomocą dostępnych metod terapeutycznych. Jak wskazują liczne badania, immunostarzenie limfocytów T jest spowodowane wewnątrzkomórkowymi sygnałami indukowanymi przez uszkodzenia DNA lub inne czynniki związane z nasilonym stresem komórkowym, i może być odwrócone jedynie metodami farmakologicznymi oddziałującymi na poziomie sygnałów wewnątrzkomórkowych[16]. Natomiast wyczerpanie limfocytów T jest konsekwencją czynników zewnątrzkomórkowych w postaci przewlekłej stymulacji antygenowej (w tym przypadku antygenami związanymi z komórkami szpiczaka) i ligacji receptorów supresorowych (tj. PD-1, CTLA-4)[17]. Wyczerpanie limfocytów T jest dysfunkcją, która jest potencjalnie odwracalna po zastosowaniu zewnątrzkomórkowej blokady tych receptorów (czyli za pomocą inhibitorów immunologicznych punktów kontrolnych)[17]. Oba stany dysfunkcjonalne limfocytów T (czyli immunostarzenie oraz wyczerpanie) mają wiele cech wspólnych (jak np. zahamowanie cyklu komórkowego, zaburzenia funkcji efektorowych, ekspresja cząsteczek supresorowych), co utrudnia ich identyfikację[16-18]. Warto jednak zaznaczyć, że wyniki najnowszych badań nad zaburzeniami dotyczącymi limfocytów T w przebiegu MM (w tym zawarte w obu niniejszych pracach) przyniosły postęp w postaci znalezienia różnicy w poziomie ekspresji cząsteczek supresorowych PD-1 i CTLA-4 w limfocytach T CD4 u chorych z MM w różnych etapach

choroby[13-15], co ułatwia odróżnienie limfocytów T starzejących się od wyczerpanych i dobór adekwatnej metody leczniczej prowadzącej do przywrócenia funkcji tych komórek. Z tego powodu ważne klinicznie wydaje się określenie charakteru dysfunkcji dominującej w limfocytach T CD4 u pacjentów z aktywnym MM w zależności od etapu choroby (NDMM i RRMM) na podstawie różnic związanych z cechami fenotypowymi (ekspresja markerów supresji i aktywacji: PD-1, CTLA-4, CD69, CD28) i funkcjonalnymi (zdolność do sekrecji cytokin zapalnych IFN-γ, IL-17) charakterystycznymi dla immunostarzenia i wyczerpania limfocytów T CD4.

V. CELE I ZAŁOŻENIA PRACY

Celami niniejszej rozprawy doktorskiej było:

1. Podsumowanie wiedzy na temat funkcjonalnego znaczenia mikrośrodowiska szpiku kostnego w rozwoju i progresji MM.

2. Badanie ekspresji cząsteczek supresorowych PD-1, CTLA-4 i BTLA w limfocytach T CD4 u pacjentów z MM celem identyfikacji dominującego mechanizmu immunosupresji (immunostarzenie vs. wyczerpanie komórek) w różnych etapach choroby dla określenia możliwości zastosowania terapii z użyciem inhibitorów immunologicznych punktów kontrolnych.

3. Badanie związku ekspresji cząsteczek supresorowych PD-1 i CTLA-4 z przebiegiem klinicznym MM (tj. z czynnikami niekorzystnego przebiegu, OS, TTP) w celu określenia ich ewentualnego znaczenia predykcyjnego i/lub prognostycznego.

VI. MATERIAŁ I METODY BADAŃ

Pierwszy artykuł z cyklu jest pacą poglądową podsumowującą wiedzę na temat MM, wpływu mikrośrodowiska na rozwój choroby i odpowiedź na leczenie. Na podstawie publikacji naukowych dostępnych w bazach PubMed i Google Scholar opisano zależności elementów komórkowych, macierzy mikrośrodowiska i cytokin obecnych w przestrzeni pozakomórkowej w rozwoju i przerzutowaniu MM.

W kolejnych dwóch pracach oryginalnych grupę badawczą stanowiła grupa 40 pacjentów z aktywnym MM, w tym 26 pacjentów ze świeżo rozpoznanym MM (newly diagnosed MM, NDMM) i 14 pacjentów z nawrotowym/opornym na leczenie MM (relapsed/refractory MM, RRMM). Wśród pacjentów 21 osób stanowiły kobiety. Pacjenci rekrutowani byli w Klinice Hematologii we Wrocławiu oraz w Oddziale Hematologii w Opolu u których rozpoznanie choroby postawione było zgodnie z kryteriami Międzynarodowej Grupy Roboczej ds. Szpiczaka (International Myeloma Working Group, IMWG)[19]. Stadium choroby zostało określone w momencie włączenia pacjenta do badania na podstawie Międzynarodowego Systemu Stopniowania dla Szpiczaka (International Staging System, ISS)[20]. Pacjenci z RRMM w przeszłości byli leczeni chemioterapią, lekami immunomodulującymi, inhibitorami proteasomu; kryterium wyłączającym było zastosowanie w pierwszej linii leczenia transplantacji komórek macierzystych (stem cell transplantation, SCT). Mediana wieku pacjentów wynosiła 69 lat (zakres 59 - 76 lat). Grupę kontrolną stanowiło 20 zdrowych osób (ochotników) odpowiadających pod względem płci i wieku grupie badanej, którzy nie byli leczeni lekami wpływającymi na układ immunologiczny przez okres 6 miesięcy przed pobraniem materiału. Z badania wyłączono także osoby ze współistniejącymi chorobami o podłożu zapalnym, cukrzycą, chorobami autoimmunologicznymi i towarzyszącymi nowotworami. Materiałem do badań była krew obwodowa pobrana w każdej grupie jednorazowo; w grupie badanych pacjentów przed rozpoczęciem chemioterapii.

Po uzyskaniu zgody uczestnikom badania pobrano krew obwodową w celu izolacji komórek jednojądrzastych (peripheral blood mononuclear cells, PBMC) na gradiencie stężeń Lymphoflotu (Bio-Rad Medical Diagnostics GmbH, Dreieich, Germany). Następnie wyizolowane komórki PBMC inkubowano z kombinacjami przeciwciał monoklonalnych sprzężonych z florochromami celem przeprowadzenia analizy cytometrycznej (barwienie bezpośrednie). Dla określenia szlaków supresorowych w obrębie limfocytów T CD4+ i w

subpopulacjach Teff i Treg (fenotypowanych wg Liu et al.[21] odpowiednio jako komórki CD4+CD127+ i CD4+CD127-), wykonano barwienie powierzchniowe (używając standardowych protokołów) następujących białek: CD4, CD69, BTLA, PD-1, CTLA-4 i CD127. Analizę populacji regulatorowych limfocytów T CD4 (Treg) o fenotypach: CD4+CD25+CD127-, CD4+CD25+FOXP3+ i CD4+FOXP3+CD127- przeprowadzono barwiąc komórki PBMC bezpośrednio po izolacji z krwi obwodowej przeciwciałami skierowanymi przeciwko antygenom powierzchniowym CD4, CD25, CD127. Następnie po inkubacji z w/w przeciwciałami, komórki zostały poddane fiksacji i permeabilizacji za pomocą zestawu Fix/Perm Buffer Set (eBioscience, San Diego, CA, USA) z następowym wewnątrzkomórkowym barwieniem przeciwciałem skierowanym przeciwko czynnikowi transkrypcyjnemu FOXP-3. W celu oznaczenia wewnątrzkomórkowego poziomu cytokin zapalnych IFN-γ i IL-17, komórki PBMC zostały poddane 4 h hodowli z dodatkiem poliklonalnych stymulatorów (PMA + jonomycyna) i brefeldyny A (inhibitora wewnątrzkomórkowego transportu cytokin) w temperaturze 37^{o}C w atmosferze 5% CO_2. Po zakończeniu hodowli, komórki zostały poddane barwieniu przeciwciałami skierowanymi przeciwko antygenom powierzchniowym CD3 i CD8 w celu identyfikacji populacji komórek CD4+ (poprzez określenie fenotypu CD3+CD8- z uwagi na nasilenie internalizacji receptora CD4 w czasie stymulacji z PMA i zmniejszenia jego ekspresji powierzchniowej)[22]. Po przeprowadzeniu permeabilizacji, komórki zostały poddane inkubacji z przeciwciałami monoklonalnymi skierowanym przeciwko cytokinom (IFN-γ lub IL-17).

Bezpośrednio po przeprowadzeniu powyższych barwień zewnątrz- i wewnątrzkomórkowych, komórki zostały poddane analizie cytometrycznej przy użyciu cytometru przepływowego FASCalibur (Becton Dickinson, BD Biosciences, San Diego, CA, USA). Fenotypowano następujące populacje komórkowe: CD3+CD4+, CD4+CD127+PD-1, CD4+CD127+CTLA-4+, CD4+CD127+BTLA, CD4+CD127-PD-1, CD4+CD127-CTLA-4+, CD4+CD127-BTLA, CD3+CD4+CD69+, CD4+CD28-, CD3+CD8-IFN-γ+ (Th1), CD3+CD8-IL-17+ (Th17), CD4+CD25+FOXP-3+, CD4+CD25+CD127-, CD4+FOXP3+CD127-. Wyniki przedstawiono jako odsetek komórek o określonym fenotypie (ekspresja jakościowa CD69, PD-1, CTLA-4, BTLA, IFN-γ, IL-17). Oceniano także ekspresję ilościową w/w cząsteczek na podstawie intensywności fluorescencji przeciwciała specyficznie związanego z badanym białkiem; wyniki przedstawiono jako średni kanał fluorescencji cząsteczki w określonej populacji komórkowej (mean fluorescence intensity, MFI) i jej wartość przedstawiono w jednostkach arbitrarnych (arbitrary units, AU). Kontrole izotopowe

sprzężone z fluorochromami zostały zastosowane w każdym przypadku do potwierdzenia specyficzności ekspresji badanych białek. Badanie związku ekspresji PD-1 i CTLA-4 z długością czasu do wystąpienia progresji (time to progression, TTP) przeprowadzono na różnych etapach MM (NDMM i RRMM). W tym celu dokonano podziału grup pacjentów z NDMM i RRMM na 2 podgrupy: z niską i wysoką ekspresją PD-1 lub CTLA-4 (na wykresach oznaczone odpowiednio jako grupy low i high), a granicą podziału była wartość mediany ekspresji ocenianej jakościowo - jako wartość odsetkowa limfocytów T CD4+, CD4+CD127+ i CD4+CD127- z ko-ekspresją określonego receptora lub ilościowo – jako intensywność fluorescencji badanego receptora w poszczególnych subpopulacjach komórkowych limfocytów T CD4+ CD4+CD127+, CD4+CD127-. Następnie określano i porównywano długość czasu jaki upłynął od rozpoczęcia leczenia do wystąpienia progresji (TTP) w każdej podgrupie pacjentów (low < mediany vs. high > mediany).

Analizę statystyczną przeprowadzono w oparciu o program Statistica 7.1 (TIBCO Software Inc., Palo Alto, CA, USA). Kliniczne parametry zostały przedstawione jako liczby absolutne oraz jako wartość odsetkowa (częstotliwość występowania w grupie). W grupie badanej dla zmiennych ilościowych obliczono wartość mediany (oraz wartość 25 i 75 kwartyla). Rozkład zmiennych został zbadany za pomocą testów Lilliefors i Shapiro-Wilk-W. Wszystkie zgromadzone zmienne wykazywały rozkład inny niż normalny, dlatego do obliczenia różnic pomiędzy badanymi grupami zastosowano testy nieparametryczne dla powiązanych (testy Friedmana, Wilcoxona) i niepowiązanych (testy Kruskala-Wallisa, Manna-Whitneya-U) zmiennych. W celu zdefiniowania relacji między badanymi zmiennymi przeprowadzono analizę korelacji za pomocą testu korelacji tau Kendalla. Do analizy przeżycia (całkowitego OS i czasu do progresji TTP) wykorzystano krzywe Kaplana-Meiera, a różnice między badanymi grupami były badane przy użyciu testu log-rank. Wieloczynnikowa analiza została przeprowadzona przy użyciu modelu proporcjonalnego hazardu Coxa po włączeniu do niej wszystkich istotnych statystycznie (oraz granicznych) wartości zmiennych objaśniających z analiz jednoczynnikowych Coxa. Wyniki na poziomie wartości $p \leq 0.05$ uznano za istotne statystycznie. Wykresy wykonano za pomocą programu GraphPad Prism (GraphPad software, San Diego, CA, USA, wersja 8.0.1).

VII.CYKL PUBLIKACJI

OA Immunology

Critical review

Tumour Immunology & Immune Tolerance

The functional significance of the bone marrow microenvironment in multiple myeloma development and progression

A Kosmaczewska[1]*, A Masternak[2], K Kosciow[2]

Abstract

Introduction

Multiple myeloma is one of the most common haematological malignancies, occurring mainly in men over 60 years of age. Despite significant therapeutic progress and a twofold increase in overall survival, multiple myeloma is still an incurable disease. The reason for the relatively poor prognosis for multiple myeloma patients lies in the biology of this tumour, the progressive development of which is closely dependent on the bone marrow microenvironment. The conditions in the bone marrow, in particular, the presence of growth factors for multiple myeloma cells (including interleukin 6, insulin-like growth factor-1, and vascular endothelial growth factor) secreted by different cells, promote their survival and proliferation in the bone marrow niches. Migration and expansion of malignant plasma cells and their mobilisation to/from the peripheral blood, characteristic of myeloma progression, are mainly due to disruption of the stromal cell-derived factor-1/CXCR4 axis caused by numerous molecular extracellular factors. It has been shown that the formation of premetastatic niches in the bone marrow, which are indicative of progression, occurs even before the first metastatic cells, home to the bone marrow, and is affected by cellular and extracellular components of the bone marrow. This close interaction between malignant plasma cells and the bone marrow microenvironment should determine appropriate therapeutic management focused on all elements of this complex biological system for a real improvement in prognosis. This paper reviews the current literature describing the participation of myeloma cells and the bone marrow microenvironment in disease development and progression.

Conclusion

Novel therapeutic approaches should target not only the malignant plasma cell, but also its interaction with the bone marrow microenvironment to sufficiently prevent disease progression. Despite administration of several immunomodulators and proteasome inhibitors, other therapies are still under active investigation.

* Corresponding author
Email: kosmaczi@iitd.pan.wroc.pl

[1] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland
[2] Department of Haematology, Regional Hospital, Opole, Poland

Introduction

Multiple myeloma is a B-cell malignancy caused by uncontrolled clonal proliferation of plasma cells in the bone marrow or outside it (affecting the liver and spleen). Myeloma plasma cells are similar to long-lived normal plasma cells and, similarly, they show a strong dependence on the bone marrow microenvironment. Probably in the process of malignant transformation, not fully known epigenetic processes are activated and they alter the surface expression of certain antigens, such as CD19, CD45 and CD56[1,2], owing to which it is possible to distinguish normal from malignant plasma cells. The phenotype of normal plasma cells is defined as CD38+CD138+CD19+CD45+CD56. In contrast, cancer cells in multiple myeloma exhibit positive expression of CD38 and CD138, but as many as 90% of them are CD19-, 99% CD45- or CD45 low, and 70% CD56+[1,2].

The clinical course of multiple myeloma is characterised by frequent occurrence of numerous complications including the presence of osteolytic lesions in bones, anaemia, and immune disorders with reduced levels of serum immunoglobulins. The presence of monoclonal protein produced by malignant plasma cells in serum and/or urine of patients, which often leads to renal dysfunction, is also very characteristic[3]. Myeloma accounts for approximately 1% of all malignant tumours and approximately 14% of haematological malignancies. The annual incidence in Europe is approximately 4.5/100 thousand. It has been shown that more men than women suffer from the disease, with a ratio of 3:2. The incidence of MM increases with age, with peak incidence in the seventh decade of life (median age, 65 years). Despite the significant progress in MM treatment and longer median lifespan of MM patients (from 3 to 6 years), it is still an incurable disease[3]. The aim of this review was to discuss the functional significance of the bone marrow microenvironment in multiple myeloma development and progression.

Discussion

The role of the cellular compartment in multiple myeloma development and progression

The bone marrow microenvironment plays an essential role in multiple

For citation purposes: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Immunology
Open Access

myelomadevelopmentandprogression, and in the development of cytostatic drug resistance[4]. Conditions of the bone marrow determine both maturation and maintenance of stem cells and blood precursor cells under physiological conditions and in malignancies such as multiple myeloma. Studies on the biology of MM clearly indicate that its induction probably results from engraftment of long-lived normal plasma cells, produced in the germinal centres of peripheral lymphoid tissues in the bone marrow. It has been shown that the bone marrow environment *per se* may promote carcinogenesis, inducing malignant transformation of normal plasma cells[5]. In addition, conditions in the bone marrow, in particular, the presence of a number of myeloma cell growth factors, promote their survival and proliferation in the bone marrow niches.

The role of stromal cells

In the process of carcinogenesis within bone marrow stromal elements, structural and functional changes occur and they cause an imbalance between factors that stimulate and inhibit the growth and differentiation of progenitor blood cells. In MM patients, there are changes in interactions between malignant plasma cells derived from multiple myeloma stem cells and the microenvironment. These interactions play a significant role in the proliferation, migration, and survival time of myeloma cells and the development of cytostatic drug resistance. Interactions between myeloma cells, stromal cells and elements of the extracellular matrix (ECM) occur directly via cell receptors and adhesion molecules, such as integrins, proteoglycans, immunoglobulins, cadherins, selectins, and syndecans, which are present on the surface of myeloma cells. Plasma cell adhesion to extracellular matrix proteins (e.g. collagen (mainly type I and VI), fibronectin, laminin, vitronectin) activates signalling pathways in plasma cells, which affects their proliferation and survival, development of drug resistance, as well as synthesis and secretion of urokinase-type plasminogen activator and metalloproteinases[6]. Some of the adhesion molecules, such as cadherins, facilitate intercellular binding and participate in the formation of functional multicellular structures in the bone marrow by anchoring to the actin cytoskeleton of cell through catenins. Free catenins which accumulate in plasma cells in high concentrations are involved in Wnt signal transduction pathways, activating certain oncogenes (e.g. c-Myc) or cyclin D1, and thereby contributing to the development of cancer.

In the process of adhesion of myeloma cells to stromal cells, an important role is also played by extracellular factors, such as cytokines, chemokines, and growth factors, which have the ability to activate multiple signalling pathways (such as NF-kB and Notch), and increase the expression of cell cycle regulatory proteins (D-type cyclins) and Bcl-2 family anti-apoptotic proteins in both stromal and myeloma cells[7]. The activation of these cells leads to secretion of factors that are of particular importance for the proliferation and survival of myeloma cells, especially IL-6, VEGF, and IGF-1, to the environment and intensification of chemotherapy resistance[8,9]. It has also been confirmed that factors such as basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and interleukin 1 (IL-1), secreted by stromal cells of patients with MM, affect the growth of myeloma cells[10]. Recently, it has been reported that there is a novel mechanism of intercellular transfer of genetic information which involves stromal cell-derived exosomes, which, after entering myeloma cells, modulate their growth mediated by specific miRNAs[11].

Stromal cells are also a source of chemokines – CCL2 (MCP-1), CCL8 (MCP-2), and CCL7 (MCP-3) – involved in the migration and homing of myeloma cells to the bone marrow due to the presence of chemokine receptor CCR2 on their surface. However, a special role in this process is attributed to the SDF-1 (CXCL12)/CXCR4 axis[3]. Stromal cells also have the ability to secrete CCL3 chemokine (MIP-1α), affecting the severity of adhesion and interaction between the integrins of myeloma plasma cells and vascular cell adhesion protein 1 (VCAM-1) molecules on the surface of endothelial cells, which in turn promotes neovascularisation in the bone marrow of MM patients. Further expansion of plasma cells in the bone marrow stroma is promoted by cytokines, growth factors, and metalloproteinases (especially MMP-9, MMP-2, IL-6, VEGF, TNFα, SDF-1, MIP-1α, IGF-1, IL-1, IL-3, IL-10 and IL-15), secreted by a variety of bone marrow cells[3].

The role of endothelial cells and angiogenesis

Clear intensification of angiogenesis in samples from patients with MM, measured by microvessel density (MVD), is observed in patients with active disease as compared to those with inactive MM or monoclonal gammopathy of undetermined significance (MGUS). Bone marrow angiogenesis in multiple myeloma is a determinant of tumour cell growth and disease progression, and a process partially regulated by pro-angiogenic factors released by plasma cells, stromal cells, and osteoclasts. Among these factors, the most important role is attributed to VEGF, bFGF, MMP, IL-6, TNFα, HGF, and chemokines IGF-1, MIP-1, MCP-1, and SDF-1, the secretion of which is a consequence of interaction between stromal cells and myeloma plasma cells[12]. Constitutive secretion of VEGF and bFGF by myeloma cells may also result from the activation of oncogenes and/or genetic mutations[13].

FOR CITATION PURPOSES: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Immunology

Critical review

In order to identify the vascular mechanism underlying metastasis and disease progression, expression profiling of genes involved in the regulation of ECM formation and bone marrow remodelling, angiogenesis, cell cycle regulation, chemotaxis, cell adhesion, and resistance to apoptosis, or processes which promote disease progression, was performed in the endothelial cells of MM patients, showing aberrant expression of 22 genes assayed[14]. These studies have highlighted the role of the microenvironment in the induction of bone marrow neovascularisation, encouraging development of tumour cells and MM progression. This study clearly shows that the use of anti-angiogenic factors in multiple myeloma therapy can significantly improve the prognosis[14].

The role of osteoclasts and osteoblasts

The observed severe bone destruction in the immediate vicinity of myeloma cells indicates their participation in the process of osteoclastogenesis. In particular, it has been shown that the interaction of myeloma cells with stromal cells in the bone marrow leads to increased expression of RANKL (receptor activator of NF-kB ligand) on myeloma cells, thus generating a signal for the activation of osteoclast precursors that express the cell-surface receptor RANK[15]. The RANKL and OPG (osteoprotegerin) molecules, which are their competitive receptors, are assigned the most important role in the regulation of bone resorption[16]. In physiological conditions, a dynamic balance between RANKL and OPG develops, and in the course of multiple myeloma it shifts towards higher RANKL expression in the bone marrow microenvironment, contributing to increased osteoclast activation and bone osteolysis. RANK ligand (RANKL) promotes osteoclastogenesis also via inhibition of osteoclast apoptosis, which significantly prolongs their survival. Blocking RANKL with a soluble form of RANKL modulates bone resorption and inhibits MM progression[17]. The osteoclast activity and bone destruction are also enhanced by MIP- 1α, IL-1, IL-3, IL-6, IL-11, TGFα, MMP-9, and TNFα[18]. In turn, the process of osteoclastogenesis is inhibited by OPG and TGF-beta. It has been shown that osteoclasts are also a constant source of osteopontin, which is a known pro-angiogenic factor[19], promoting the formation of an environment conducive to the development and progression of the disease.

It has been shown that both disease progression and the resulting increased destruction of bone tissue can result from impaired osteoblast activity, which depends on the activity of the inhibitor DKK1[20]. DKK1 is a Wnt antagonist, secreted by myeloma cells, which inhibits differentiation of precursor cells to osteoblasts. In MM patients with osteolytic foci detected in the bones, significantly increased DKK1 expression on the surface of malignant plasma cells has been found. The importance of DKK1 for MM development and progression has been demonstrated in studies which involved blocking of DKK1, subsequent inhibition of bone osteolysis and tumour weight reduction[21-23]. In contrast, despite the loss of their osteogenic function, osteoblasts maintain their ability to produce and secrete OPG, which binds to the TRAIL receptor on the surface of myeloma cells and is responsible for their prolonged survival by blocking apoptosis signal transduction[24]. Osteoblasts are also an additional source of IL-6, a recognised MM cell growth factor, as confirmed in the culture of MM cell lines[25].

The impact of the extracellular environment on multiple myeloma development

Complex positive and negative interactions mediated by various adhesion molecules, cytokines, and their receptors occur between individual bone marrow cells and myeloma cells. Some of the growth and survival factors for myeloma cells, such as IL-6, are produced by many types of bone cells (osteoblasts, osteoclasts, stromal cells) and the MM cells themselves. Additional external factors such as hypoxia or internal signals generated by a deregulated c-Myc oncogene in myeloma cells lead to hypoxia-inducible factor 1-α (HIF-1α) activation and VEGF secretion by myeloma cells[26]. VEGF, in turn, stimulates endothelial cells to secrete IGF-1, which induces proliferation of myeloma cells. Thus, interleukin-6, VEGF, and IGF constitute a network of factors essential for MM development and progression.

Interleukin-6

Interleukin-6 (IL-6) is a key growth and survival factor for multiple myeloma cells, originally produced by stromal cells, osteoclasts, and malignant plasma cells, and affecting tumour growth by autocrine and paracrine mechanisms[27,28]. IL-6 secretion by medullary stromal cells is regulated directly by adhesion to myeloma cells, and indirectly by soluble factors secreted by these cells (TNFα, VEGF, IL-1β, TGFβ), which lead to activation of the transcription factor NF-kB in plasma cells[29]. Thus, a cross-regulation network develops between the tumour cells and the microenvironment, which promotes myeloma progression. After binding to its receptor on myeloma cells, IL-6 induces a signalling pathway that leads to activation and proliferation of plasma cells, inhibits the activity of p27 and p21 inhibitors, and activates anti-apoptotic proteins (Mcl-1 and Bcl-x) and c-Myc[28]. It has been observed that a high level of IL-6 in the serum of MM patients is correlated with poor prognosis and an increased percentage of proliferating myeloma cells in the peripheral blood[30].

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

For citation purposes: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

OA Immunology
Open Access

Critical review

Insulin-like growth factor 1
Insulin-like growth factor 1 (IGF-1) is a factor promoting the carcinogenesis of many cancers[31]. IGF-1 is secreted by stromal cells, endothelial cells, and bone marrow osteoblasts, and in the paracrine mechanism, it is conducive to MM development by binding to its receptor on tumour cells. This interaction leads to a shift in the balance of factors associated with apoptosis towards cell death inhibitors in myeloma cells[32].

Vascular endothelial growth factor
It has been shown that increased bone marrow vascularisation is positively correlated with a poor prognosis of cancer patients[12]; the growth of new blood vessels significantly improves the conditions for nutrient and oxygen transport to cells, facilitating further growth of the tumour. Vascular endothelial growth factor (VEGF), as a known pro-angiogenic factor in a number of haematological malignancies including multiple myeloma, is clearly associated with disease progression[12,33]. Secreted by stromal and myeloma cells under the influence of cytokines and other growth factors, such as IL-6, bFGF, TGFβ, and TNFα, present in the bone marrow of MM patients, it enhances the development of a favourable microenvironment conducive to gradual progression of the disease.

Multiple myeloma progression
In most cases, myeloma cells develop primarily in the bone marrow, although in aggressive forms of the disease, malignant plasma cells can also be home to extramedullary sites, such as spleen, liver, and body fluids. Symptomatic MM progression is associated with further expansion of myeloma cells within the bone marrow and spreads to secondary sites in bone marrow by the bloodstream. Myeloma cell migration from the blood to the bone marrow (called homing) is a multi-stage process actively managed by several interactions in the bone marrow, primarily initiated by the activation of selectins. The next stages of homing, such as adhesion and extravasation, when myeloma cells exit the capillaries, occur by the activation of integrins LFA-1 and VLA-4 on their surface[34]. However, substantial mobilisation of malignant plasma cells from the peripheral blood to the bone marrow occurs due to activity of the SDF-1/CXCR4 axis. SDF-1 chemokine, which induces homing of myeloma cells into the bone marrow, is secreted by stromal cells (e.g. endothelial cells, mesenchymal stem cells), and binds specifically to the cell-surface receptor CXCR4 expressed on plasma cells. Worth mentioning is the large diversity of CXCR4 expression on myeloma cells, confirmed on the surface of 10% to 100% of the cells. It has been found that blocking CXCR4 inhibits migration of myeloma cells to SDF-1 chemokines in the bone marrow[35]. In turn, migration and expansion of myeloma cells in the bone marrow and their mobilisation or egress into the peripheral blood occur due to disruption of the SDF-1/CXCR4 axis, which involves weakening of SDF-1 expression under the influence of bone marrow environment proteases and intensification of CXCR4 expression following hypoxic myeloid niches (1%–2% O_2)[36,37].

Nevertheless, the bone marrow microenvironment is not sufficiently prepared for metastatic myeloma cell engraftment. The requirement for MM dissemination within the bone marrow is the adaptation of myeloid niches to conditions that enable further development of MM, which is mediated by ECM components, stroma, endothelial cells, cytokines, chemokines, and growth factors, in particular, IL-6, IGF-1, and APRIL. It has been demonstrated that myeloma cells can invade large areas of the bone marrow with particular ease by entering into abnormal interactions with both ECM proteins and bone marrow stromal cells, on which they are closely dependent. The formation of premetastatic niches in the bone marrow, which are an expression of a pathological response of the bone marrow environment to the presence of myeloma cells, occurs even before the first arrival of metastatic cells and substantially facilitates their distribution, thus promoting the creation of new malignant foci[5]. This results in characteristic premetastatic disorders in the said components of the bone marrow microenvironment, which together facilitate the growth and survival of the metastasizing myeloma cells. This specific bone marrow microenvironment remodelling is also affected by increased osteoclast activity mediated by RANKL/RANK and MIP-1alpha, osteoblastogenesis attenuated by DKK1 and IL-3, as well as the previously mentioned intensive neoangiogenesis. It is believed that myeloma cells circulating in the peripheral blood and molecular factors secreted by them, including metalloproteinases, are also of particular importance in the phenomenon of MM bone marrow metastases[38]. Metalloproteinases (MMPs) have the ability to degrade ECM, and at the same time, they stimulate angiogenesis, which promotes the spread of malignant cells[39]. Among the various MMPs, considerable importance in MM progression is attributed mainly to gelatinases MMP-2 and MMP-9, which degrade type IV collagen, the major component of the basal membrane of cells, and thus affect their ability to spread myeloma[40–43]. Among other factors involved in premetastatic bone marrow remodelling, there are also adhesion molecules, characterised by increased VLA-4 expression on the surface of myeloma cells, and exosomes which induce neoangiogenesis in metastatic lesion locations[38,44,45]. Additionally, cells of haematopoietic origin that exhibit the expression of fibronectin (VLA-4) and VEGF (VEGFR1) receptors on their surface allow implantation

FOR CITATION PURPOSES: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

OA Immunology

Critical review

of new myeloma cells into the bone marrow[38].

Conclusion

The recent studies clearly emphasize a strong interaction between myeloma cells and elements of the bone marrow microenvironment both in MM development and progression. It even seems that the role of each component of the tumour-host interactions is equivalent in MM pathogenesis. Novel therapeutic approaches should target not only the malignant plasma cell, but also its interaction with the bone marrow microenvironment, to sufficiently prevent disease progression. Despite administration of several immunomodulators and proteasome inhibitors, other therapies are still under active investigation.

Abbreviations list

Ang-1, angiopoietin-1; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; HGF, hepatocyte growth factor; HIF-α, hypoxia-inducible factor 1-α; IGF-1, insulin-like growth factor 1; IL-1, interleukin 1; IL-6, interleukin-6; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; MVD, microvessel density; PDGF, platelet-derived growth factor; RANKL, receptor activator of NF-kB ligand; TGF-beta, transforming growth factor beta; VCAM-1, vascular cell adhesion protein 1.

References

1. Pérez-Persona E, Vidriales MB, Mateo G, García-Sanz R, Mateos MV, de Coca AG, et al. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. Blood. 2007 Oct;110(7):2586–92.
2. Raja KR, Kovarova RL, Hajek R. Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders. Br J Haematol. 2010 May;149(3):334–51.
3. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011 Mar;364(11):1046–160.
4. Mahindra A, Laubach J, Raje N, Munshi N, Richardson PG, Anderson K. Latest advances and current challenges in the treatment of multiple myeloma. Nat Rev Clin Oncol. 2012 Feb;9(3):135–43.
5. Ghobrial IM. Myeloma as a model for the process of metastasis: implications for therapy. Blood. 2012 Jul;120(1):20–30.
6. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer. 2003 Jun;3(6):453–8.
7. Hideshima T, Bergsagel P, Kuehl W, Anderson K. Advances in biology of multiple myeloma: clinical applications. Blood. 2004 Aug;104(3):607–18.
8. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene of tumour suppressor. Nat Rev Cancer. 2003 Oct;3(10):756–67.
9. Nefedova Y, Cheng P, Alsina M, Dalton W, Gabrilovich D. Involvement of Notch-1 signaling in bone-marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood. 2004 May;103(9):3503–10.
10. Giuliani N, Storti P, Bolzoni M, Palma B, Bonomini S. Angiogenesis and multiple myeloma. Cancer Microenviron. 2011 Dec;4(3):325–37.
11. Roccaro AM, Sacco A, Maiso P, Azab AK, Tai YT, Reagan M, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. J Clin Invest. 2013 Apr;123(4):1542–55.
12. Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood. 2005 Feb;105(40):1383–95.
13. Rajkumar SV, Witzig TE. A review of angiogenesis and antiangiogenic therapy with thalidomide in multiple myeloma. Cancer Treat Rev. 2000 Oct;26(5):351–62.
14. Ria R, Todoerti S, Berardi S, Coluccia AM, De Luisi A, Mattioli M. Gene expression profiling of bone marrow endothelial cells in patients with multiple myeloma. Clin Cancer Res. 2009 Sep;15(17):5369–78.
15. Ehrlich LA, Roodman GD. The role of immune cells and inflammatory cytokines in Paget's disease and multiple myeloma. Immunol Rev. 2005 Dec;208:252–66.
16. Emery JG, Mc Donnell P, Burke MB, Deen KC, Lyn S, Silverman C, et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem. 1998 Jun;273(23):14363–7.
17. Yaccoby S, Pearse RN, Johnson CL, Barlogie B, Choi Y, Epstein J. Myeloma interacts with the bone marrow microenvironment to induce ostoclastogenesis and is dependent on osteoclast activity. Br J Haematol. 2002 Feb;116(2):278–90.
18. Roodman GD. Pathogenesis of myeloma bone disease. Leukemia. 2009 Mar;23:435–41.
19. Tanaka Y, Abe M, Hiasa M, Oda A, Amou H, Nakano A, et al. Myeloma cell-osteoclast interaction enhances angiogenesis together with bone resorption: a role for vascular endothelial cell growth factor and osteopontin. Clin Cancer Res. 2007 Feb;13(3):816–23.
20. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, Shaughenessy JD Jr. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. New Engl J Med. 2003 Dec;349(26):2483–94.
21. Yaccoby S, Ling W, Zhan F, Walker R, Barlogie B, Shaughnessy JD Jr. Antibody-based inhibition of DKK1 suppresses tumor-induced resorption and multiple myeloma growth in vivo. Blood. 2007 Mar;109(5):2106–11.
22. Heath DJ, Chantry AD, Buckle CH, Coulton L, Shaughnessy J Jr, Evans H, et al. Inhibiting Dickopff-1 (Dkk1) removes suppression of bone formation and prevents the development of osteolytic bone disease in multiple myeloma. J Bone Miner Res. 2009 Mar;24(3):425–36.
23. Zhou F, Meng S, Song H, Claret FX. Dickoppf-1 is a key regulator of myeloma bone disease: Opportunities and challenges for therapeutic intervention. Blood Rev. 2013 Nov;27(6):261–7.
24. Kawano Y, Ueno S, Abe M, Kikukawa Y, Yuki H, Iyama K, et al. TRAIL produced from multiple myeloma cells is associated with osteolytic markers. Oncol Rep. 2012 Jan;27(1):39–44.
25. Noll JE, Williams SA, Tong CM, Wang H, Quach JM, Purton LE, et al. Myeloma plasma cells alter the bone marrow microenvironment by stimulating the proliferation of mesenchymal stromal cells. Haematologica. 2013 Jan;99(1):161–71.
26. Hu J, Handisides DE, Van Valckenborgh E, De Raeve H, Menu E, Van de Broek I, et al. Targeting the multiple myeloma hypoxic niche with TH-302, a

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript.
All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

For citation purposes: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

OA Immunology
Open Access

Critical review

hypoxia-activated prodrug. Blood. 2010 Sep;116(9):1524–7.
27. Ishikawa H, Tsuyama N, Abroun S, Liu S, Li FJ, Otsuyama K, et al. Interleukin-6, CD45 and the src-kinases in myeloma cell proliferation. Leuk Lymphoma. 2003 Sep;44(9):1477–81.
28. Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequel of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene. 2001 Sep;20(42):5991–6000.
29. Salem K, Brown CO, Schibler J, Goel A. Combination chemotherapy increases cytotoxicity of multiple myeloma cells by modification of nuclear factor (NF)-kB activity. Exp Hematol. 2013 Feb;41(2):209–18.
30. Ludwig H, Nachbaur D, Fritz E, Krainer M, Huber H. Interleukin-6 is a prognostic factor in multiple myeloma. Blood. 1991 Jun;77(12):2794–5.
31. Westley RL, May FE. A twenty-first century cancer epidemic caused by obesity: the involvement of insulin, diabetes, and insulin-like growth factors. Int J Endocrinol. 2013;2013:632461.
32. Huo J, Xu S, Lin B, Chng WJ, Lam KP. Fas apoptosis inhibitory molecule is upregulated by IGF-1 signaling and modulates Akt activation and IRF4 expression in multiple myeloma. Leukemia. 2013 Apr;27(5):1165–71.
33. Podar K, Anderson K. Inhibition of VEGF signaling pathways in multiple myeloma and other malignancies. Cell Cycle. 2007 Mar;6(5):538–42.
34. Sanz-Rodriguez F, Hidalgo A, Teixidó J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. Blood. 2001 Jan;97(2):346–51.
35. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. Blood. 2007 Apr;109(7):2708–17.
36. Cipolleschi MG, Sbarba P, Olivotto M. The role of hypoxia in the maintenance of hematopoietic stem cells. Blood. 1993 Oct;82(7):2031–7.
37. Manier S, Sacco A, Leleu X, Ghobrial IM, Roccaro AM. Bone marrow microenvironment in multiple myeloma progression. J Biomed Biotechnol. 2012;2012:157496.
38. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005 Dec;438(7069):820–7.
39. Hutchinson CA, Basnayake K, Cockwell P. Serum free light chain assessment in monoclonal gammopathy and kidney disease. Nat Rev Nephrol. 2009 Nov;5(11):621–8.
40. Barillé S, Akhoundi C, Collette M, Mellerin M, Rapp M, Harousseau J, et al. Metalloproteinases in multiple myeloma: Production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. Blood. 1997 Aug;90(4):1649–55.
41. Corre J, Mahtouk K, Attal M, Gadelorge M, Huynh A, Fleury-Cappellesso S, et al. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia. 2007 May;21(5):1079–88.
42. Jezierska A, Motyl T. Matrix metalloproteinase-2 involvement in breast cancer progression: a mini-review. Med Sci Monit. 2009 Feb;15(2):RA32–40.
43. Matsumoto H, Ishibashi Y, Ohtaki T, Hasegowa Y, Koyama C, Inoue K. Newly established murine pituitary folliculostellate-like cell line (TtT/GF) secretes potent pituitary glandular cell survival factors, one of which corresponds to metalloproteinase inhibitor. Biochem Biophys Res Commun. 1993;194:909–15.
44. Sleeman JP. The metastatic niche and stromal progression. Cancer Metastasis Rev. 2012 Dec;31(3–4):429–40.
45. Zhang H, Grizzle W. Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. Am J Pathol. 2014 Jan;184(1):28–41.

For citation purposes: *Kosmaczewska A, Masternak A, Kosciow K.* The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.

International Journal of
Molecular Sciences

MDPI

Article

Deregulated Expression of Immune Checkpoints on Circulating CD4 T Cells May Complicate Clinical Outcome and Response to Treatment with Checkpoint Inhibitors in Multiple Myeloma Patients

Anna Kulikowska de Nałęcz [1], Lidia Ciszak [2], Lidia Usnarska-Zubkiewicz [3], Irena Frydecka [2], Edyta Pawlak [2], Magdalena Szmyrka [4] and Agata Kosmaczewska [2,*]

[1] Hematology and Hematological Oncology Department, Provincial Hospital, 45-372 Opole, Poland; a_mastemak@poczta.fm
[2] Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland; lidia.ciszak@hirszfeld.pl (L.C.); irena.frydecka@umed.wroc.pl (I.F.); edyta.pawlak@hirszfeld.pl (E.P.)
[3] Department and Clinic of Haematology, Blood Neoplasms, and Bone Marrow Transplantation, Wroclaw Medical University, 50-367 Wroclaw, Poland; lidia.zubkiewicz@gmail.com
[4] Department of Rheumatology and Internal Diseases, Wroclaw Medical University, 50-367 Wroclaw, Poland; magdalena.szmyrka@umed.wroc.pl
* Correspondence: agata.kosmaczewska@hirszfeld.pl

check for updates

Citation: Kulikowska de Nałęcz, A.; Ciszak, L.; Usnarska-Zubkiewicz, L.; Frydecka, I.; Pawlak, E.; Szmyrka, M.; Kosmaczewska, A. Deregulated Expression of Immune Checkpoints on Circulating CD4 T Cells May Complicate Clinical Outcome and Response to Treatment with Checkpoint Inhibitors in Multiple Myeloma Patients. *Int. J. Mol. Sci.* 2021, 22, 9298. https://doi.org/10.3390/ijms22179298

Academic Editor: Mariele F. Fransen

Received: 23 July 2021
Accepted: 24 August 2021
Published: 27 August 2021

Abstract: Unlike solid-tumor patients, a disappointingly small subset of multiple myeloma (MM) patients treated with checkpoint inhibitors derive clinical benefits, suggesting differential participation of inhibitory receptors involved in the development of T-cell-mediated immunosuppression. In fact, T cells in MM patients have recently been shown to display features of immunosenescence and exhaustion involved in immune response inhibition. Therefore, we aimed to identify the dominant inhibitory pathway in MM patients to achieve its effective control by therapeutic interventions. By flow cytometry, we examined peripheral blood (PB) CD4 T cell characteristics assigned to senescence or exhaustion, considering PD-1, CTLA-4, and BTLA checkpoint expression, as well as secretory effector function, i.e., capacity for IFN-γ and IL-17 secretion. Analyses were performed in a total of 40 active myeloma patients (newly diagnosed and treated) and 20 healthy controls. At the single-cell level, we found a loss of studied checkpoints' expression on MM CD4 T cells (both effector (Teff) and regulatory (Treg) cells) primarily at diagnosis; the checkpoint deficit in MM relapse was not significant. Nonetheless, PD-1 was the only checkpoint distributed on an increased proportion of T cells in all MM patients irrespective of disease phase, and its expression on CD4 Teff cells correlated with adverse clinical courses. Among patients, the relative defect in secretory effector function of CD4 T cells was more pronounced at myeloma relapse (as seen in declined Th1/Treg and Th17/Treg cell rates). Although the contribution of PD-1 to MM clinical outcomes is suggestive, our study clearly indicated that the inappropriate expression of immune checkpoints (associated with dysfunctionality of CD4 T cells and disease clinical phase) might be responsible for the sub-optimal clinical response to therapeutic checkpoint inhibitors in MM.

Keywords: multiple myeloma; CD4 T cells; PD-1; CTLA-4; checkpoint inhibitors; clinical outcome

1. Introduction

Multiple myeloma (MM) is an incurable hematologic malignancy characterized by proliferation and accumulation of clonal plasma cells producing M-protein in the bone marrow (BM) [1]. A biologic property of MM is progressive development of immune deficiency that leads to tumor escape, disease growth, and clinical complications, such as bone disease or recurrent serious infections [2]. The pathogenesis of the immune

dysregulation in MM is complex and includes disease- and treatment-related factors, thus resulting in cumulative immunosuppression and increased risk of severe infections. The immune dysfunction in MM is associated with the inhibition of normal plasma cells with subsequent hypogammaglobulinemia as well as impaired cellular immunity, including dysfunction of T cells, dendritic cells (DCs), and NK cells [2,3]. The profound T cell alterations in MM include a rapid loss of effector function and an increase in the abundance of immunosuppressive Tregs in the BM [2]. A major role in the development of the immunosuppressive state in MM has recently been attributed to the immune checkpoints, such as PD-1, CTLA-4, and BTLA, expressed on T cells isolated from the BM of patients [4]. These molecules play an essential role in the loss of immune surveillance by regulating T cell activation and maintaining peripheral tolerance, and their significance for the development of solid tumors and hematologic malignancies has been well documented [5].

Impaired tumor immunity is suggested to be responsible for the very limited effectiveness of anti-myeloma immunotherapies in refractory MM [3]. Reversing tumor-mediated immune tolerance in MM seems to be a critical therapeutic goal in the development or optimization of new immunotherapeutic strategies. The introduction of inhibitors targeting the immune checkpoints remarkably shifted the paradigm in the treatment of solid tumors and hematologic malignancies with impressive single-agent responses for PD-1/PD-L1 axis inhibitors in Hodgkin's lymphoma [6–10]. However, unlike solid-tumor patients, only a minor subset of MM patients treated with checkpoint inhibitors have been shown to derive clinical benefits, primarily after combined therapy, thus suggesting differential participation of inhibitory receptors or different categories of inhibitory pathways involved in tumor immunity [11]; in fact, it has recently been reported that T cells in MM patients display features of immunosenescence and exhaustion, and, notably, these dysfunctional states may coexist in time [5,12,13]. Although both senescence and the exhaustion of T cells are associated with compromised immune responses, they substantially differ in their phenotypic and functional characteristics, as well as underlying mechanisms [14]. Available data demonstrate that immunosenescence is caused by intrinsic signals induced by DNA damage or other stresses and can be reversed pharmacologically, while exhaustion is a consequence of ligation of inhibitory receptors and is reversible upon external receptor blockade [15]. Therefore, it is crucial to resolve immunosuppressive mechanisms by identifying the dominant inhibitory pathway in MM patients to achieve their effective control with therapeutic interventions.

Herein, we extended and completed our preliminary data to explore mechanisms underlying the systemic CD4 T cell-related immunosuppression according to the disease course to identify a target group suitable for therapeutic use of immune checkpoint inhibitors [16]. Therefore, we aimed to examine phenotypic and functional characteristics of CD4 T cells assigned to cell senescence or exhaustion, considering PD-1, CTLA-4, and BTLA checkpoint expression, as well as secretory effector function, including capacity for IL-17 and IFN-γ production. We assessed CD4 T cells from peripheral blood (PB) of active MM patients at disease onset and relapse, as well as healthy age-matched donors. Our study demonstrated that CD4 T cell senescence (associated with defective checkpoint expression in MM [13]) might play a role in supporting myeloma growth, whereas T cell exhaustion (characterized by recovery of checkpoint expression) is a predominant dysfunctional state at disease relapse, which may affect the clinical response to therapeutic checkpoint inhibitors in MM.

2. Results

2.1. CD4 T Cells from PB of MM Patients Are Maximally Stimulated In Vivo and Possess Strong Potential for Inhibition of the Immune Response

Having demonstrated that immune checkpoints function as negative feedback to regulate the ongoing immune responses and their dysregulated expression may be a consequence of altered in vivo stimulation [17], we analyzed the state of systemic activation and the capacity for re-stimulation of MM CD4 T cells.

While we noted an increased proportion of $CD4^+CD69^+$ T cells in the PB of all patients (as shown in Table 1), a statistically significant difference was found only between the RRMM group and healthy controls ($p = 0.027$); in the newly diagnosed (NDMM) group, the increase in $CD69^+$ cell expansion was of borderline significance ($p = 0.06$). As demonstrated in Table 2, the median fluorescence intensity of CD69 was the highest in CD4 T cells from RRMM patients, but it remained at a statistically similar level compared to corresponding healthy cells. In contrast, CD4 T cells from NDMM patients exhibited markedly lower amounts of CD69 than those from the controls ($p = 0.017$), which did not differ significantly in comparison to the CD69 levels found in the RRMM group. The in vitro re-stimulation revealed that patients' cultured CD4 T cells from both studied groups exhibited a significantly lower proportion of $CD69^+$ cells than the corresponding healthy cells (44.29% (26.22–54.72%) vs. 55.73% (53.00–73.16%), $p = 0.044$) under the same stimulation conditions.

Table 1. Immune checkpoint and CD69 expression (%) in PB CD4 T cell subsets in different MM phases and healthy controls (HC).

T-Cell Subset (%)	At Diagnosis (NDMM) (n = 26)	At Relapse (RRMM) (n = 14)	HC (n = 20)	*p* Value
$CD3^+CD4^+PD\text{-}1^+$	15.33 (12.62–18.52)	17.72 (11.75–19.86)	12.55 (8.37–15.29)	(a) ns (b) 0.02 (c) 0.04
$CD4^+CD127^+PD\text{-}1^+$	23.53 (16.42–29.99)	25.00 (12.96–31.13)	16.87 (10.52–21.75)	(a) ns (b) 0.088 * (c) 0.090 *
$CD4^+CD127^-PD\text{-}1^+$	9.70 (6.78–11.94)	14.79 (5.31–26.50)	6.14 (3.90–7.62)	(a) 0.037 (b) 0.041 (c) 0.0001
$CD3^+CD4^+BTLA^+$	28.64 (20.04–43.21)	23.85 (15.06–30.73)	19.03 (13.25–37.52)	(a) ns (b) 0.018 (c) ns
$CD4^+CD127^+BTLA^+$	38.87 (26.26–50.23)	40.30 (26.08–47.58)	23.62 (10.85–40.79)	(a) ns (b) ns (c) ns
$CD4^+CD127^-BTLA^+$	3.98 (2.54–6.29)	7.70 (5.21–11.35)	2.47 (1.24–5.15)	(a) ns (b) ns (c) 0.010
$CD3^+CD4^+CTLA\text{-}4^+$	1.91 (0.78–3.37)	1.28 (0.89–1.88)	1.27 (0.78–2.00)	(a) ns (b) ns (c) ns
$CD4^+CD127^+CTLA4^+$	3.23 (2.21–7.66)	3.42 (1.68–4.36)	3.55 (1.64–4.22)	(a) ns (b) ns (c) ns
$CD4^+CD127^-CTLA4^+$	1.93 (0.47–2.67)	1.68 (1.45–2.19)	1.15 (0.57–1.38)	(a) ns (b) ns (c) 0.031
$CD3^+CD4^+CD69^+$	0.58 (0.23–1.09)	0.70 (0.36–0.95)	0.34 (0.22–0.44)	(a) ns (b) 0.060 * (c) 0.027

(a) NDMM vs. RRMM; (b) NDMM vs. HC; (c) RRMM vs. HC; *—trend; ns—not statistically significant. Differences in fluorescence intensity of immune checkpoints between examined groups were evaluated using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test).

Table 2. Immune checkpoint and CD69 expression (MFI, mean fluorescence intensity) in PB CD4T cells in different MM phases and healthy controls (HC).

Fluorescence Intensity	At Diagnosis (NDMM) (n = 26)	At Relapse (RRMM) (n = 14)	HC (n = 20)	*p* Value
PD-1 in $CD4^+$	72.99 (67.04–86.64)	79.41 (56.39–111.33)	83.04 (71.73–105.09)	(a) ns (b) 0.063 * (c) ns
PD-1 in $CD4^+CD127^+$	67.19 (52.34–84.45)	85.34 (57.28–112.96)	90.27 (60.38–105.84)	(a) ns (b) 0.085 * (c) ns
PD-1 in $CD4^+CD127^-$	68.47 (54.77–93.55)	73.92 (59.29–113.76)	102.13 (79.79–127.27)	(a) ns (b) 0.016 (c) ns
BTLA in $CD4^+$	169.60 (146.40–184.92)	184.12 (109.50–196.04)	215.18 (192.79–289.32)	(a) ns (b) 0.0002 (c) 0.002
BTLA in $CD4^+CD127^+$	150.75 (142.37–167.89)	186.19 (149.70–241.82)	240.20 (166.25–304.14)	(a) ns (b) 0.001 (c) ns
BTLA in $CD4^+CD127^-$	147.00 (120.49–168.15)	192.21 (154.61–235.12)	240.60 (167.13–292.31)	(a) 0.033 (b) 0.001 (c) ns
CTLA-4 in $CD4^+$	44.95 (38.52–63.20)	76.21 (63.09–129.84)	65.40 (60.10–111.23)	(a) 0.002 (b) 0.005 (c) ns
CTLA-4 in $CD4^+CD127^+$	36.22 (33.40–41.87)	55.58 (49.57–95.97)	80.96 (51.93–90.08)	(a) 0.0002 (b) 0.0005 (c) ns
CTLA-4 in $CD4^+CD127^-$	34.13 (30.84–40.13)	53.69 (43.86–66.96)	62.97 (41.04–74.55)	(a) 0.008 (b) 0.0008 (c) ns
CD69 in $CD4^+$	53.64 (47.87–58.82)	75.69 (49.52–94.75)	70.81 (55.55–123.46)	(a) ns (b) 0.017 (c) ns

(a) NDMM vs. RRMM; (b) NDMM vs. HC; (c) RRMM vs. HC; *—trend; ns—not statistically significant. Differences in the proportions of PD-1, BTLA, and CTLA-4 expressing $CD4^+$ T cells between examined groups were evaluated using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test).

This part of the data shows that PB CD4 T cells in MM were maximally activated in vivo, but hypo-responsive and failed to respond to polyclonal re-stimulation. Among patients, a lower level of systemic CD4 T cell activation was observed at MM diagnosis.

2.2. *Expression of Immune Checkpoints in Myeloma CD4 T Cell Subsets Is Clearly Impaired, Especially in Newly Diagnosed Patients*

It is well established that immune checkpoint receptors play an essential role in immune surveillance and tumor immunity by inhibiting T-cell immune responses [5]. We and others have previously demonstrated the altered expression of inhibitory receptors CTLA-4, PD-1, and BTLA in tumors [4,18–26]. As recent clinical trials with administration of the immune checkpoint inhibitors in MM showed real disappointment, we aimed to verify whether the onset and/or exacerbation of MM is accompanied by alterations in the immune checkpoints' expression, thereby affecting their usefulness as targets for therapeutic inhibitors. Therefore, we assessed PD-1, BTLA, and CTLA-4 checkpoint expression in PB $CD4^+$ T cell subsets in MM patients both at disease diagnosis and relapse.

As shown in Figure 1A,D,E, and Table 1, a comparison with healthy controls demonstrated an increasing median proportion of CD4 T cells expressing PD-1 checkpoint in all MM patients regardless of cell subsets (both Teff and Treg cells defined as $CD4^+CD127^+$ and $CD4^+CD127^-$ T cells, respectively) ($p < 0.05$). Although the expansion of PD-1+ Teff cells was similar in all patients, Treg cells from RRMM patients expressed the PD-1 molecule on a significantly higher proportion of cells than in the NDMM group ($p = 0.037$).

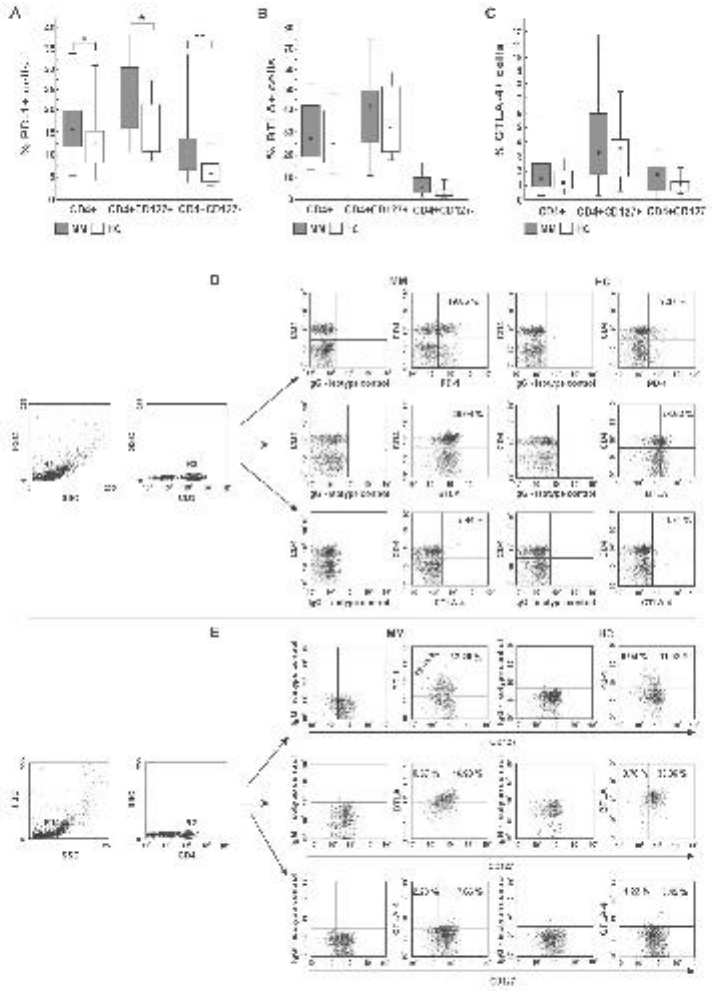

Figure 1. Distribution of PD-1 (**A**), BTLA (**B**), and CTLA-4 (**C**) immune checkpoints in different PB CD4 T-cell subsets. Among studied inhibitors, significant differences between myeloma patients (MM) and healthy controls (HC) were found in PD-1 expression only ($p < 0.05$). The frequency of $BTLA^+$ and $CTLA\text{-}4^+$ cells did not significantly differ between MM and HC ($p > 0.05$). Boxes and whiskers 25th–75th interquartile range and minimum-maximum, respectively; the median is the central square in each box. ** represents $p < 0.01$ and * represents $p < 0.05$. (**D**,**E**) Representative dot plots show PD-1, BTLA, and CTLA-4 expression in PB CD4 T cells. Numbers on dot plots represent the frequency of $PD\text{-}1^+$, $BTLA^+$, or $CTLA\text{-}4^+$ cells within the examined subsets. The percentages of cells expressing the immune checkpoint receptors were determined using isotype control IgG. The statistical analysis was performed using the Mann-Whitney U-test.

A quantitative analysis of PD-1 expression showed its lower levels in NDMM patients compared with healthy controls (Table 2). Whereas PD-1 deficit was observed in the whole population of NDMM CD4 T cells, including both Teff and Treg subsets, its loss was more pronounced in Treg cells ($p = 0.016$); in Teff cells, the decrease of PD-1 was of borderline significance ($p = 0.08$). Likewise, in the RRMM patients, PD-1 expression was also defective (primarily in the Treg subset); however, its median values were statistically comparable to those observed in corresponding healthy cells. Similarly, the differences in PD-1 expression between patient groups, although apparent, were not statistically significant (Table 2).

As demonstrated in Figure 1B,D,E, and Table 1, regarding BTLA expression, we found no significant differences in the percentages of $BTLA^+$ cells within CD4 T cells and their subsets (both Teff and Treg cells) between patients and controls, except for the higher proportion of $CD4^+$ and Treg cells co-expressing BTLA in the NDMM and RRMM groups, respectively ($p = 0.018$ and $p = 0.01$, respectively). In addition, compared with healthy cells, a decrease in the MFI values of BTLA in the MM CD4 T cells, more profound in NDMM patients, was observed ($p < 0.002$). BTLA levels in the Treg subset in NDMM patients were also lower than those observed in RRMM patients ($p = 0.001$) (Table 2).

Likewise, we found no significant differences in the proportion of CTLA-4 expressing cells within the examined subsets between participants studied, except for the higher abundance of $CTLA\text{-}4^+$ Treg cells in RRMM patients compared with healthy controls ($p = 0.031$) (Table 1). Remarkably, its quantitative estimation showed that the only group exhibiting substantially down-regulated levels of CTLA-4 on both Teff and Treg cells was the NDMM patient group ($p \leq 0.008$ and $p \leq 0.005$, respectively) contrasting of the normal levels in corresponding cells from the RRMM and healthy groups (Table 2).

Taken together, these data clearly show that PD-1 is the only T cell inhibitory receptor widely distributed within PB CD4 T subsets in patients at every stage of MM and increasing within Treg population during disease progression. Nonetheless, myeloma CD4 T cells had significantly defective levels of all studied checkpoints, primarily at myeloma diagnosis, which may be insufficient for appropriate blockade with therapeutic inhibitors.

2.3. *Dysfunctional Characteristics of PB CD4 T Cells Depend on Myeloma Stage*

As altered expression of immune checkpoints is one of the features of cell senescence or exhaustion observed in MM [5,12,13], we wanted to assess whether it corresponds with the other dysfunctional characteristics of these two states, i.e., aberrant and opposed capacity for inflammatory IFN-γ and IL-17 cytokine secretion [27,28].

In the pooled MM group, we observed significantly diminished proportions of the CD4 T cells with capacity for IFN-γ secretion (Th1 subset) compared with healthy donors ($p < 0.001$) (as shown in Figure 2A,B, and Table 3). Moreover, we found markedly lower values of IFN-γ fluorescence intensity in the CD4 T cells than those seen in controls (31.86 (20.72–37.10) vs. 58.79 (36.41–69.87), $p = 0.01$). Although we did not find any significant differences in the abundance of Th1 cells in PB between patients regarding treatment state or ISS stage, a pronounced deficit was observed in the RRMM group and patients with higher tumor stage (Table 3 and Table S1). Likewise, while a substantial decline in the Th1/Treg cell ratio was observed in both groups of patients irrespective of MM phase ($p = 0.00007$ for NDMM patients, $p = 0.00003$ for RRMM patients), and patients at MM relapse exhibited the lowest Th1/Treg rate (Figure 3A).

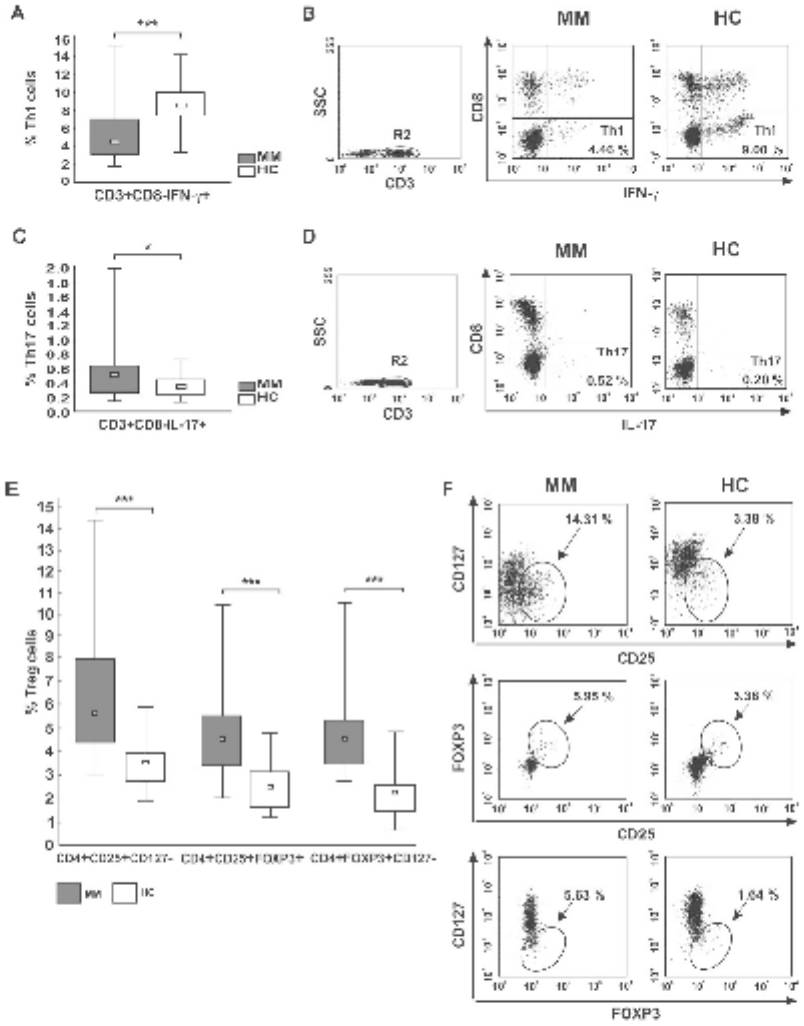

Figure 2. CD4 T cell capacity for cytokine secretion (IFN-γ, IL-17) and peripheral distribution of Treg cells. (**A**,**B**) IFN-γ- (Th1) and (**C**,**D**) IL-17-secreting cells (Th17) were phenotyped by flow cytometry as $CD3^+CD8^-IFN\text{-}\gamma^+$ and $CD3^+CD8^-IL\text{-}17^+$ cells, respectively. (**E**,**F**) Treg cells were identified as the following subsets: $CD4^+CD25^+CD127^-$, $CD4^+CD25^+FOXP3^+$, and $CD4^+FOXP3^+CD127^-$ cells. Boxes and whiskers 25th–75th interquartile range and minimum-maximum, respectively; the median is the central square in each box. *** represents $p < 0.001$ and * represents $p < 0.05$. Numbers on dot plots represent the percentage of Th1, Th17, and Treg cells within PBMCs in MM patients and healthy subjects (HC). Significant decreases in PB Th1 and increases in both Th17 and Treg cells among patients were found in all analyses using the Mann-Whitney U-test.

Table 3. Frequency of CD4 T cells secreting inflammatory cytokines and Treg cells in different MM phases and healthy controls (HC).

T-Cell Subset (%)	At Diagnosis (NDMM) (n = 26)	At Relapse (RRMM) (n = 14)	HC (n = 20)	*p* Value
$CD3^+CD8^-IFN\text{-}\gamma^+$	4.79 (3.34–7.03)	3.91 (3.01–6.40)	9.00 (7.66–10.07)	(a) ns (b) 0.005 (c) 0.0002
$CD3^+CD8^-IL\text{-}17^+$	0.51 (0.24–0.59)	0.56 (0.29–1.08)	0.32 (0.22–0.45)	(a) ns (b) 0.048 (c) 0.045
$CD4^+CD25^+CD127^-$	5.02 (4.40–6.02)	6.61 (5.70–8.98)	3.69 (2.72–3.90)	(a) 0.076 * (b) 0.0001 (c) 0.0001
$CD4^+CD25^+FOXP3^+$	4.30 (3.43–5.34)	4.58 (3.88–5.60)	2.51 (1.66–3.19)	(a) ns (b) 0.0001 (c) 0.001
$CD4^+ FOXP3^+CD127^-$	4.52 (3.79–5.35)	4.35 (3.42–6.17)	2.34 (1.49–2.67)	(a) ns (b) 0.0001 (c) 0.001

(a) NDMM vs. RRMM; (b) NDMM vs. HC; (c) RRMM vs. HC; *—trend; ns—not statistically significant. Differences in Th1, Th17, and Treg cell subsets between examined groups were evaluated using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test).

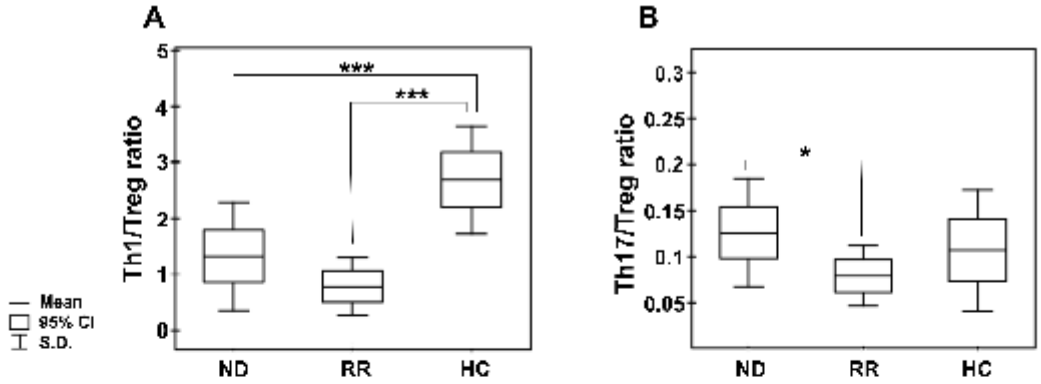

Figure 3. Peripheral blood Th1/Treg and Th17/Treg cell ratios in the different clinical phases of MM and healthy controls. (**A**) shows the ratio of the numbers of circulating IFN-secreting CD4 T cells (Th1) to regulatory $CD4^+CD25+CD127^-$ T cells (Treg); (**B**) shows the ratio of circulating IL-17-secreting CD4 T cells (Th17) to regulatory $CD4^+CD25^+CD127^-$ T cells. Boxes and whiskers show confidence interval and standard deviation (S.D.) respectively; the mean is the central square in each box. *** represents $p < 0.001$ and * represents $p < 0.05$.

Additionally, we assessed the level of PB $CD4^+$ T cells capable of inflammatory IL-17 cytokine synthesis (Th17 cells). In patients, the frequencies of Th17 cells were significantly higher than in controls ($p < 0.05$) (Figure 2C,D), especially those at ISS stage I/II (Table S1). Nonetheless, the MFI values of IL-17 in the Th17 subpopulation were comparable to those observed in controls (23.39 (13.14–39.00) vs. 21.98 (16.37–52.11), $p > 0.05$, respectively). Although no significant difference in Th17 cell levels between patient groups was found (Table 3), we clearly observed that NDMM patients exhibited a markedly increased Th17/Treg ratio compared with those with RRMM ($p = 0.047$), as shown

in Figure 3B. While the Th17/Treg ratio in RRMM was the lowest, it did not significantly differ to that observed in healthy controls.

This part of the data demonstrates that CD4 T cells from MM patients are functionally impaired but secrete more inflammatory cytokines during disease development than those at myeloma progression, which may imply different functional characteristics corresponding with, respectively, senescence or exhaustion depending on disease stage.

2.4. Expansion of PB Treg Cells Is Most Pronounced at Less Advanced MM, Which May Create Conditions Promoting Disease Development

Having ascertained that Treg cells might be involved in T cell senescence during tumor induction [29,30], we evaluated the abundance of PB Treg cells in the different clinical phases of MM. We determined the following Treg cell subsets: $CD4^+CD25^+CD127^-$, $CD4^+CD25^+FOXP3^+$, and $CD4^+FOXP3^+CD127^-$ cells.

The median percentages of all studied Treg subtypes were significantly higher at every clinical stage of MM compared with controls ($p \leq 0.004$) (Table 3; Figure 2E,F). Our cohort of active MM patients (both NDMM and RRMM) exhibited statistically comparable proportions of Treg cells; however, the $CD4^+CD25^+CD127^-$ Treg subset was the only regulatory cell population found to tend to increase after therapy ($p = 0.076$). We also surprisingly noted that Treg levels were higher at stage I/II compared with the values observed at stage III, and the differences reached statistical significance for $CD4^+CD25^+FOXP3^+$ and $CD4^+FOXP3^+CD127^-$ subsets (both $p = 0.02$) (Table S1). Furthermore, among examined Treg subtypes, the abundance of $CD127^-$ Treg cells (both $CD4^+CD25^+CD127^-$ and $CD4^+FOXP3^+CD127^-$ phenotypes) negatively correlated with ISS stage ($r = -0.24$, $p = 0.04$ and $r = -0.49$, $p = 0.006$, respectively) (Figure S1).

This part of our data clearly shows an increase in the circulating Treg cell compartment irrespective of treatment state, although more pronounced at less-advanced stages of myeloma. PB Treg enrichment observed at tumor induction may create conditions supporting CD4 T cell senescence-mediated systemic immune suppression.

2.5. Markers of T Cell Exhaustion Are Associated with Adverse MM Clinical Behavior

Since the impact of checkpoints' up-regulated expression on the clinical outcome of neoplastic diseases has been demonstrated [4,18–25], we wanted to find out whether T cell inhibitors might be associated with clinical characteristics of MM as well. The associations between immune characteristics and both MM clinical variables and patient survival are summarized in Tables 4 and 5, respectively.

As shown in Table 4, the patients with adverse clinical features, such as higher levels of β2-microglobulin (β2M) (≥3.5 mg/dL), IgA myeloma subtype, ISS stage > 2, and lower albumin level (≤3.5 g/dL) had higher expression of PD-1 checkpoint ($p = 0.06$, $p = 0.01$, $p = 0.02$, and $p = 0.05$, respectively), while patients with hypercalcemia (≥10 mg/dL of calcium) exhibited elevated levels of CTLA-4 on the pooled CD4 T cells and their subpopulations studied (Teff and Treg) ($p = 0.02$, $p = 0.0006$, and $p = 0.002$, respectively). Additionally, we noted an association between anemia (Hb ≤ 12 g/dL) and higher concentrations of β2M with increased frequencies of $CD4^+CD69^+$ T cells ($p = 0.08$ and $p = 0.02$, respectively).

Next, we aimed to evaluate whether any of the immune checkpoints associated with an unfavorable clinical course of MM might possess prognostic significance for overall survival (OS). We stratified the results obtained for low and high expression of each immune checkpoint according to the median split. Similar analysis was performed regarding clinicopathological variables known to be involved in MM progression and prognosis. The median follow-up of our cohort of MM patients was 27 months (range: 0–86 months).

Table 4. Association of immune features with clinical characteristics of multiple myeloma.

Cell Subsets	Clinical Features	Median (IQ Range)	*p* Value
$CD4^+CD127^+PD\text{-}1^+$ (%)	<3.5 mg/L of β2M ≥3.5 mg/L of β2M	18.66 (13.76–24.29) 23.99 (16.42–32.32)	0.06 *
PD-1 in $CD4^+$ (MFI)	≤3.5 g/dL of albumin >3.5 g/dL of albumin	84.42 (72.42–129.93) 69.05 (55.08–94.03)	0.05
PD-1 in $CD4^+CD127^+$ (MFI)	IgA IgG	107.59 (99.75–121.15) 63.85 (52.34–84.45)	0.01
PD-1 in $CD4^+CD127^-$ (MFI)	IgA IgG	123.41 (104.10–149.45) 62.38 (54.77–85.70)	0.01
PD-1 in $CD4^+CD127^-$ (MFI)	Stage ≤ 2 Stage > 2	59.49 (53.62–85.70) 96.70 (69.07–113.76)	0.02
CTLA-4 in $CD4^+$ (MFI)	<10 mg/dL of serum calcium ≥10 mg/dL of serum calcium	44.48 (39.87–62.12) 72.71 (45.56–98.68)	0.01
CTLA-4 in $CD4^+CD127^+$ (MFI)	<10 mg/dL of serum calcium ≥10 mg/dL of serum calcium	35.87 (32.94–39.74) 51.21 (44.45–72.31)	0.0006
CTLA-4 in $CD4^+CD127^-$ (MFI)	<10 mg/dL of serum calcium ≥10 mg/dL of serum calcium	33.79 (27.96–35.29) 53.69 (40.72–66.97)	0.002
$CD4^+CD69^+$ (%)	≤12 g/dL of hemoglobin >12 g/dL of hemoglobin	0.69 (0.56–1.09) 0.37 (0.21–0.91)	0.08 *
$CD4^+CD69^+$ (%)	<3.5 mg/L of β2M ≥3.5 mg/L of β2M	0.30 (0.19–0.68) 0.69 (0.37–1.09)	0.02

MFI—mean fluorescence intensity; IQ—interquartile; *—trend.

Regarding clinical characteristics (as illustrated in Figure S2), a log-rank test showed that high β2M (Figure S2A), low albumin (Figure S2B), ISS stage > 2 (Figure S2C), and to a lesser extent anemia (Figure S2D), high creatinine levels (Figure S2E), and older age (Figure S2F) predicted shorter OS of patients ($p = 0.0004$, $p = 0.003$, $p = 0.01$, $p = 0.06$, $p = 0.08$, $p = 0.09$, respectively). There was no significant correlation between myeloma isotype, serum calcium concentration, circulating plasmocytes, or lactate dehydrogenase (LDH) level and patient OS (Figure S3).

Among immune features studied, only increased frequencies of both $CD69^+$ (Figure S2G) and $PD\text{-}1^+$ CD4 Teff cells (Figure S2H) predicted with borderline significance shortened patient OS (both $p = 0.06$); we observed that the intensity of PD-1 expression in CD4 T cells may have a minor effect on patient survival ($p = 0.14$) (Figure S2I).

In univariate Cox analyses (Table 5), clinical variables including low albumin, high creatinine and β2M levels, and anemia correlated with worse OS ($p = 0.005$, $p = 0.04$, $p = 0.05$, and $p = 0.08$, respectively); ISS stage > 2 and older age were also shown to associate with shortened OS to some extent ($p = 0.11$ and $p = 0.10$, respectively). In turn, no correlation was observed between patient OS and serum calcium level, LDH, and platelet and plasmocyte counts. Among the immune parameters studied, only the percentage of $PD\text{-}1^+$ CD4 Teff cells was found to tend to slightly increase the risk of death ($p = 0.10$).

A multivariate Cox regression model was built including clinical prognostic factors and frequency of $PD\text{-}1^+$ CD4 Teff cells and CTLA-4 level in $CD4^+$ T cells (reaching p values ≤ 0.11 in univariate analysis). This multivariate analysis allowed the independent prognostic value for OS to be retained only for albumin level, anemia, and age ($p = 0.001$, $p = 0.05$, and $p = 0.05$, respectively) (Table 5).

Table 5. Univariate and multivariate Cox regression analysis.

Prognostic Factors	Univariate	Multivariate	
	p Value	HR (95% CI)	*p* Value
Age > 65 years	0.10 *	9.74 (0.94–100.33)	0.05
ISS stage > 2	0.11 *		
Albumin < 3.5 g/dL	0.005 *	0.04 (0.01–0.29)	0.001
β2-microglobulin ≥ 3.5 mg/L	0.05 *	21.65 (0.65–714.02)	0.08
Creatinine ≥ 2.0 mg/dL	0.04 *		
Serum calcium ≥ 10 mg/dL	0.91		
LDH > 190 U/L	0.96		
Hemoglobin ≤ 12 g/dL	0.08 *	0.16 (0.02–1.04)	0.05
Platelets < 100,000/mm^3	0.43		
Plasmocytes > 5 %	0.69		
CD4$^+$CD127$^+$PD-1$^+$ > median (%)	0.10 *		
PD-1 in CD4$^+$ > median (MFI)	0.56		
PD-1 in CD4$^+$CD127$^+$ > median (MFI)	0.38		
PD-1 in CD4$^+$CD127$^-$ > median (MFI)	0.34		
CTLA-4 in CD4$^+$ > median (MFI)	0.11 *		
CTLA-4 in CD4$^+$CD127$^+$ > median (MFI)	0.65		
CTLA-4 in CD4$^+$CD127$^-$ > median (MFI)	0.58		
CD4+CD69$^+$ (%) > median (MFI)	0.20		

MFI—mean fluorescence intensity; HR—hazard ratio; CI—confidence interval; *—selected for multivariate analysis.

Taken together, these data suggest that no immune feature could be added to the clinical scoring system in MM; however, CD4 T cells with predominance of the activated and exhausted phenotype are involved in adverse clinical behavior.

3. Materials and Methods

3.1. Samples and Patient Characteristics

The study group of patients consisted of a total of 40 active myeloma patients (26 newly diagnosed and 14 relapsed/refractory (RR)) (21 female). Patients were recruited in the Department of Hematology and Bone Marrow Transplantation at Wroclaw Medical University and the Department of Hematooncology at the Provincial Hospital in Opole, and diagnosed based on criteria from the International Myeloma Working Group (IMWG) [31]. The disease stage was determined according to the International Staging System (ISS) at the study entry [32]. Relapsed/refractory MM patients (RRMM) were treated with chemotherapy, immunomodulatory drugs, and proteasome inhibitor; no patient enrolled in the study received prior treatment with stem cell transplantation (SCT) or immune checkpoint inhibitors. The baseline characteristics of the patients are shown in Table 6. The control population comprised 20 healthy individuals matched for age and sex; they had been without any treatment affecting the immune system for 6 months before entering the study. Patients with simultaneous active or chronic infection, diabetes, autoimmune disease, or with a history of other malignancies or connective tissue diseases were excluded from the study. Blood samples from all participants were collected after informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Local Research Bioethics Committee at Wroclaw Medical University.

Table 6. Patient demographics and characteristics.

Characteristic	Newly Diagnosed (NDMM)	Relapsed/Refractory (RRMM)	Total
Number of patients, *n* (%)	26 (65%)	14 (35%)	40 (100%)
Sex (female)	17 (65.5%)	4 (28.5%)	21 (52.5 %)
Age of sampling (median, range)	66 (50–76)	72 (65–75)	69 (59–76)
ISS			
I	5 (19%)	1 (7%)	6 (15.0 %)
II	10 (38.5%)	6 (43%)	16 (40.0 %)
III	11 (42.5%)	7 (50%)	18 (45.0 %)
Myeloma isotype			
IgG	18 (69%)	9 (64%)	27 (67.5 %)
IgA	3 (11.5%)	3 (21.5%)	6 (15.0 %)
Light chain only	5 (19.5%)	2 (14.5%)	7 (17.5 %)
Type of Ig light chain (serum)			
Kappa	16 (61.5%)	7 (50%)	23 (57.5 %)
Lambda	9 (34.5%)	7 (50%)	16 (40.0 %)
None	1 (4%)	0 (0%)	1 (2.5 %)
Osteolytic bone lesion/s, *n* (%)	15 (60.0%)	13 (92.8%)	28 (70.0 %)
Laboratory values			
β2-microglobulin ≥ 3.5 mg/L	18 (69%)	9 (64%)	27 (67.5 %)
Creatinine ≥ 2.0 mg/dL	9 (34.5%)	4 (28.5%)	13 (32.5 %)
LDH > 190 U/L	6 (23%)	2 (14%)	8 (20.0 %)
Serum calcium ≥ 10 mg/dL	9 (34.5%)	11 (78.5%)	20 (50.0 %)
Hemoglobin ≤ 12 g/dL	23 (88.5%)	8 (57%)	31 (80.0 %)
Platelets < 100,000/mm^3	2 (7.5%)	1 (7%)	3 (7.5 %)
Prior treatment			
1–3 therapy lines	0 (0%)	8 (57%)	8 (20.0 %)
≥4 therapy lines	0 (0%)	6 (43%)	6 (15.0 %)
BTZ-based therapy	0 (0%)	12 (85.5%)	12 (30.0 %)
IMiD therapy	0 (0%)	11 (78.5%)	11 (27.5 %)
No therapy	26 (100%)	0 (0%)	26 (65.0 %)

Abbreviations: BTZ, bortezomib; LDH, lactate dehydrogenases; ISS, International Staging System; IMiD, immunomodulatory drug; UNV, upper normal values.

3.2. *Cell Isolation from Peripheral Blood*

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoflot (Bio-Rad Medical Diagnostics GmbH, Dreieich, Germany) density gradient centrifugation from venous blood samples of patients with MM and healthy donors, and then cryopreserved. Recovery rates from frozen T cells were above 85%.

3.3. *Secretory Effector Function, Immunofluorescence Staining, and Flow Cytometric Analysis*

Peripheral blood mononuclear cells (PBMCs) were stained with several combinations of anti-human fluorochrome-conjugated monoclonal antibodies (mAbs) for multi-color analysis. For assessment of the dominant inhibitory pathway in the pooled $CD4^+$ T cells as well as their subsets (both Treg and Teff cells, phenotyped according to Liu et al. [33] as $CD4^+CD127^-$ and $CD4^+CD127^+$ cells, respectively), surface staining of CD4, CD69, BTLA, PD-1, CTLA-4, and CD127 was performed by standard protocols. The following mAbs were used in this procedure: CD3-FITC (Pharmingen, San Diego, CA, USA), CD4-PerCP (Pharmingen, USA), BTLA-PE (Becton Dickinson, Biosciences, San Diego, CA, USA), PD-1-PE (Pharmingen, San Diego, CA, USA), CTLA-4-PE (Pharmingen, San Diego, CA, USA), CD69-PE (Pharmingen, San Diego, CA, USA), and CD127-FITC (Pharmingen, San Diego, CA, USA).

For analysis of the regulatory T cell (Treg) subpopulations phenotyped as $CD4^+CD25^+CD127^-$, $CD4^+CD25^+FOXP3^+$, and $CD4^+CD127^-FOXP3^+$ cells, PBMCs were aliquoted into

tubes directly after isolation for further staining with the following mAbs: anti-CD4-PerCP (BD Biosciences, San Diego, CA, USA), anti-CD25-FITC (BD Biosciences, San Diego, CA, USA), and anti-CD127-PE (BioLegend, San Diego, CA, USA), respectively. For intracellular staining, the cells were then fixed and permeabilized with the Fixation/Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions with subsequent incubation with anti-human FOXP3-PE (BD Biosciences, San Diego, CA, USA) mAbs for 30 min at room temperature in the dark.

For assessment of the secretory effector function of $CD4^+$ T cells, staining of intracellular cytokines IL-17 and IFN-γ was performed. Percentages of cytokine-producing T cells (Th1 and Th17) were calculated after stimulation of refrozen PBMCs with 25 ng/mL phorbol 12-myristate 23-acetate (PMA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 1 µg/mL ionomycin (Ion) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany)h) in the presence of 10 µg/mL brefeldin A (BFA, protein transport inhibitor) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. Because incubation with PMA triggers internalization and degradation of the CD4 molecule, which would affect the identification of Th1 (phenotyped as $CD4^+IFN\text{-}\gamma^+$) and Th17 cells (characterized as $CD4^+IL\text{-}17^+$) [34], we decided to identify both subpopulations as $CD3^+CD8^-IFN\text{-}\gamma^+$ and $CD3^+CD8^-IL\text{-}17^+$, respectively. Directly after PMA+Ion stimulation, PBMCs were surface-stained with anti-CD3-PerCP (BD Biosciences, San Diego, CA, USA) and anti-CD8-PE mAbs (BD Biosciences, San Diego, CA, USA). Then, after fixation and permeabilization with the Fixation/Permeabilization Buffer Set (eBioscience, San Diego, CA, USA), the cells were incubated with anti-IFN-γ-FITC (BD Biosciences, San Diego, CA, USA) or anti-IL-17-FITC (BioLegend, San Diego, CA, USA) mAbs for 30 min at room temperature in the dark.

Directly after immunostaining, the cells were washed and analyzed by flow cytometry using a FACScan cytometer (Becton Dickinson, San Diego, CA, USA) equipped with Cell Quest software (BD Biosciences, San Diego, CA, USA). Appropriate fluorochrome-labeled isotypic controls were used to confirm expression specificity and for gate settings in each case. A total of 100,000 events were recorded for each sample before any electronic gate setting. Data were analyzed by Cell Quest software.

The results were expressed as the proportions of $CD3^+CD4^+$ (CD4 T cells), as well as $CD4^+CD127^-$ and $CD4^+CD127^+$ cells (Treg and Teff, respectively) co-expressing inhibitory receptors BTLA, PD-1, or CTLA-4. The percentages of $CD3^+CD8^-$ co-expressing IFN-γ (Th1 subset) or secreting IL-17 (Th17 subset) were also examined. In addition, we studied the frequencies of $CD4^+CD25^+$ cells with the presence of the FOXP3 transcription factor and/or without or with low expression of CD127 antigen, thus defining the different subsets of Tregs. In order to demonstrate quantitative expression of studied molecules at the single-cell level, the results are shown as the mean fluorescence intensity (MFI) values and expressed in arbitrary units (AU).

3.4. *Statistical Analysis*

Statistical analysis was performed using the package Statistica 7.1 (TIBCO Software Inc., Palo Alto, CA, USA) and GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA). Clinical parameters were presented as absolute numbers and percentages for frequencies. For all other analyzed variables, the median values and 25th and 75th interquartile ranges (IQ ranges) were calculated. As collected data were not normally distributed and/or had heterogeneous variances, differences between examined groups were evaluated using nonparametric tests for paired (Friedman, Wilcoxon) and unpaired (Kruskal-Wallis, Mann-Whitney U) data. The relationship between the ISS stage and other analyzed variables was evaluated by Kendall's tau coefficient analysis. Kaplan-Meier curves were generated to present the survival time of the two groups, and the differences were assessed by the log-rank test. Multivariate analyses were performed with the Cox proportional hazards model by including all statistically significant covariates from univariate Cox models. A p value ≤ 0.05 was considered significant.

4. Discussion

The results of the present study clearly support an important role of the immune checkpoints in the development of systemic T cell immune dysregulation in active myeloma. Our study strengthens the suggestion that myeloma growth disrupts both the qualitative and quantitative expression of immune checkpoints in the PB $CD4^+$ T cell subsets, which may complicate the clinical response to therapeutic checkpoint inhibitors. Here, we observed that among the studied immune checkpoints, PD-1 was the only inhibitory receptor found in higher proportions of PB Teff and Treg cells and correlated with adverse MM clinical outcomes. This observation is consistent with the report of Rosenblatt et al. [4], who observed an increased frequency of PB $CD4^+$PD-1+ T cells in myeloma patients with advanced active disease as a result of chronic antigen stimulation, thus contributing to tumor-induced suppression of T cell responses. A reduction of PD-1^+ T cell frequency in patients who achieve a minimal disease state following chemotherapy strongly supports an association of PD-1 expression with exposure to the tumor antigens and stimulation in vivo. Consistent with tumor antigen exposure, we observed increased frequencies of in vivo-stimulated MM $CD4^+CD69^+$ T cells, although exhibiting lower potential to respond to further in vitro polyclonal stimulation, thus indicating a dysfunctional phenotype of PB $CD4^+$ T cells. This notion together with the increased expression of PD-1 within the CD4 T cell subset and the severely impaired Th1 response seems to reflect an in vivo-stimulated and most likely exhausted phenotype of $CD4^+$ T cells in our cohort of active patients, especially those with relapsed MM. In fact, increased expression of inhibitory receptors PD-1, CTLA, LAG-3, and TIM-3, together with defective effector functions, is regarded as a hallmark of T cell exhaustion [25,26,28]. The influence of MM therapy on quantitative and functional characteristics of circulating CD4 T cells has consistently been reported by Batorov et al. [35]. While it has been found that in the course of MM T cell exhaustion occurs predominantly in the myeloma BM, PB T cells also exhibit an abrogated function, albeit to a minor extent [5,25]. Our study suggests that MM relapse (and treatment refractoriness) is associated with an increasing population of activated and exhausted PB CD4 T cells, which may clearly affect the clinical outcome, as shown by the correlation with hypercalcemia, high β2M levels, low albumin levels, a possible association with anemia, and shortened survival.

Remarkably, we also observed that systemic checkpoints' expression examined at a single-cell level on the different types of CD4 T cells was clearly impaired mainly at diagnosis of MM. This notion is in line with recent studies by Suen et al. [12,13], who reported decreased levels of PD-1 and CTLA-4 on clonal T cells in MM patients as a feature of telomere-independent immunosenescence rather than exhaustion. Likewise, we found that the CD4 T cell compartment in patients with disease onset was characterized by relatively higher capacity for the secretion of inflammatory IL-17 and IFN-γ cytokines compared with patients with relapsed and advanced disease, which may be a characteristic of the senescent-associated secretory effector phenotype (SASP) [27]. T-cell senescence is believed to be an alternative mechanism of immune evasion utilized by malignant cells for tumor development [36–38], as senescent T cells were shown to be an important source of immunosuppressive cytokines, such as IL-10 and TGF-β [30]. It is also postulated that Treg cells are involved in conversion of normal T cells into senescent cells [29,30]. Our finding of a negative correlation of enriched PB Treg cells with MM stage may correspond with their role in systemic CD4 T cell senescence supporting myeloma growth. There is increasing evidence that senescence and exhaustion of CD4 T cells represent two different categories of inhibitory pathways leading to functional immune suppression [14]. Therefore, our study indicated that development and relapse of MM are likely related to dynamic changes in dysfunctional characteristics of PB CD4 T cells and confirmed recent data showing that immunomodulatory drugs and chemotherapy of MM are preferentially able to delete senescent T cells while retaining checkpoint inhibitory molecule expression [5]. Distinguishing between senescent and exhausted T cells, and targeting both types of cells in MM, may be of great clinical relevance, since reversion of these two dysfunctional states require different

therapeutic approaches, among which checkpoint blockade has been reported to reverse only T cell exhaustion. We believe that an assessment of the level of immune checkpoints on T cell subsets may facilitate the identification of the predominant dysfunctional state of T cells in MM to improve therapeutic efficacy.

In accordance with the results of our quantitative analysis, Lee et al. [39] reported different expression levels of PD-1 regarding the clinical course of MM; patients in a refractory state exhibited markedly higher PD-1 amounts on T cells compared with those at diagnosis. Likewise, the CTLA-4 expression was also recently found to be lower and increasing with MM progression [39], an observation consistent with our finding of the significant increase in CTLA-4 fluorescence intensity on CD4 T cells (both Teff and Treg) to normal levels in patients with refractory disease. Although the majority of available data demonstrated an increase in the immune checkpoints' expression in MM T cells, one should emphasize that they were based on qualitative assessment only [9,10,39,40]. A few recent reports [12,13,39] demonstrating the involvement of the quantitative alterations of immune checkpoints' expression in pathogenesis and the clinical course of myeloma are similar to the results of our study, and point to the importance of their estimation at the quantitative level as well. The inappropriate checkpoint levels in MM T cell subsets observed in our study, primarily in newly diagnosed patients, might explain the sub-optimal clinical responses in clinical studies using checkpoint inhibitors and the real disappointment with this therapeutic modality in MM [41,42]. This is in sharp contrast to the impressive response to blockade of CTLA-4, PD-1, and PD-L1 seen in a broad variety of cancers of different origin [43], and strengthens the suggestion of the requirement for a relevant expression level of checkpoints on T cells. Consistently, we previously reported that CTLA-4 blocking antibody might be a beneficial form of immunotherapy for a subset of chronic lymphocytic leukemia (CLL) patients depending on the level of CTLA-4 expression on leukemic cells [23].

The reason for the down-regulation of the checkpoints' expression level in CD4 T cells in a proportion of MM patients is still unresolved, although in light of the higher CD69 values seen in our study, insufficient in vivo stimulation of MM T cells should be excluded. The influence of the transcription factors (such as Blimp or T-bet) that have been demonstrated to control the checkpoint expression might also be considered [44,45]. In addition, recent research performed on MM, including from our group, demonstrated that genetic variations of genes encoding the immune checkpoints, primarily PD-1 and CTLA-4, may affect their protein expression level as well [46–48]. While Katsumoto et al. [45] stated that PD-1 high-expression haplotype is implicated in susceptibility to MM, we previously reported that polymorphisms in the *CTLA-4* gene associated with lower CTLA-4 protein expression significantly increase the risk of developing MM in the Polish population [47]. Similarly, Zheng et al. [48] found that an (AT)n microsatellite polymorphism within the 3′-untranslated region (UTR) of exon 3 of the *CTLA-4* gene might represent a susceptibility locus for MM, as the increased frequencies of the alleles containing extended AT repeats seen in MM patients are associated with lower CTLA-4 mRNA stability and protein expression.

Herein, we confirmed the independent prognostic value of age, albumin, hemoglobin, and β2M levels, thus indicating the clinical representativeness of patients enrolled in the study. Yet, the only immune characteristic found to predict a poor clinical outcome in MM was the PD-1 checkpoint expressed on PB CD4 Teff cells; patients with higher expression of PD-1 had an unfavorable clinical course and tended to live shorter. Our observation is in line with the report by Alrasheed et al. [49], who reported independent prognostic significance of high abundance of PD-1^{+}CD4 Teff cells in the prediction of early relapse of MM. The relatively small cohort of patients included in the current analysis might weaken the significance of Cox regression analysis with regards to examined immune parameters. Likewise, clinical studies showing that among therapeutic checkpoint inhibitors, only the anti-PD-1 antibody revealed a clinical response, although sub-optimal, in a proportion of MM patients when administered in a combined therapy only, might strengthen the possible

contribution of PD-1 to prognosis in MM [50,51]. Further studies including larger cohorts of MM cases are required to verify our findings.

It is worth noting that among the immune checkpoints studied, PD-1 expression was found to be the most deregulated, when considering co-existence of qualitative PD-1 overexpression at every stage of MM with quantitative impairment of PD-1 at disease development. While CTLA-4 expression was found to be associated with hypercalcemia, our observation of the PD-1 expression increasing with several other features of adverse clinical courses, such as advanced ISS stage, higher level of β2M, and decreased albumin levels and anemia, emphasizes a superior role of the PD-1 inhibitory receptor in the development of systemic immune suppression and myeloma progression. It has been reported that the widespread expression of PD-1L on neoplastic plasma cells and dendritic cells (DC) facilitates interaction with PD-1 on the marrow-infiltrating lymphocytes (MILs), and strongly restricts anti-tumor T cell responses within the BM microenvironment, thereby allowing for the tumor escape [52,53]. This is in accordance with the demonstration that PD-1 enhances regulatory properties in Treg cells and inhibition of anti-tumor activity of CD4 Teff cells in MM, indicating a role of PD-1 in the MM clinical outcome [49]. In fact, it has been found that the PD-1$^+$ Treg subset is the main population participating in immune deficiency during tumor progression [24,52]. Additionally, a role of PD-1 in conversion of Th1 into Treg cells was recently demonstrated [54], thus emphasizing the significance of the PD-1 checkpoint for a shift of the immune balance towards immune suppression due to a decline in the Th1/Treg ratio [49]. Our observation on the enrichment of PB Treg cells in all MM patients is in line with a role of PD-1 in Treg expansion. Although we observed a decrease in FOXP3$^+$Tregs at stage III, we noted that their values still remained increased in the periphery irrespective of tumor stage. At this point of the study, we cannot completely explain the FOXP3+Treg decrease in the most advanced MM (ISS stage III), but our findings confirmed the similar former observation [55]. Infiltration of the BM by Tregs should be taken into consideration, since these cells have been shown in MM to acquire chemokine receptors promoting trafficking to the tumor site. In fact, Tregs accumulate in the BM primarily in the most advanced disease, whereby they become capable of creating a highly immunosuppressive microenvironment supporting tumor growth [5,56,57].

The decrease in the PB CD4 T cell compartment secreting IFN-γ, a Th1 cytokine involved in tumor immunity, observed in our cohort of patients seems to reflect severe inhibition of anti-tumor effector functions of these cells in MM. The deficit in the Th1 type response observed in our study is likely associated with tumor progression, as we observed that patients with refractory advanced MM exhibited the lowest Th1 cell level and Th1/Treg ratio. The impact of the treatment-induced increase in PD-1 level on the compromised Th1/Treg ratio observed in the present study is consistent with recent observations [35] and may reflect the deterioration of T-cell-tumor immunity despite the treatment. Nonetheless, normalization of checkpoint levels on CD4 T cells in treated patients, despite development of refractoriness, appears to open an avenue for the reactivation of the immune responses after therapeutic use of checkpoint inhibitors in the combined modality. In conclusion, although the contribution of PD-1 to MM clinical outcomes is suggestive, our study clearly indicated that inappropriate expression of immune checkpoints (associated with the dysfunctionality of CD4 T cells and disease stage) might be responsible for the sub-optimal clinical response to checkpoint inhibitors in MM. Our data demonstrating defective levels of PD-1 and CTLA-4 within the CD4 T cell population in newly diagnosed patients suggest that immune checkpoints are not appropriate targets for therapeutic inhibitors at disease onset. This study also showed that chemo- and/or immunotherapy of MM, despite a risk of the development of refractoriness, is capable of reinforcing checkpoint expression and T cell reactivity of PB CD4 T cells, making them more attainable to therapeutic inhibitors in relapsed MM patients only.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22179298/s1.

Author Contributions: Conceptualization and design of the study, A.K.d.N., A.K.; methodology, A.K.d.N., A.K.; data curation, A.K.d.N., L.U.-Z., I.F.; cell culture, fluorescence staining, cytometric analysis (investigation), L.C., E.P., A.K.; analysis or interpretation of data, A.K.d.N., L.C., E.P., M.S., A.K.; statistical analysis and validation, L.C., E.P.; visualization, L.C., M.S.; manuscript writing—original draft preparation, A.K.d.N., A.K.; writing—review and editing, A.K.d.N., L.C., L.U.-Z., I.F., E.P., M.S., A.K.; supervision, A.K.; funding acquisition, A.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a grant from the Wroclaw Centre of Biotechnology, the Leading National Research Centre (KNOW) program for the years 2014–2018 (grant no. 132/2017/KNOW/IITD to A.K.).

Institutional Review Board Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (permission no. KB-528/2017 from the Wroclaw Medical University) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analyzed during this study are available on request from the corresponding author.

Acknowledgments: We are grateful to our patients and healthy subjects for blood donation and agreement to participate in this study. We also acknowledge Aleksandra Szteblich for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kyle, R.A.; Rajkumar, S.V. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. *Leukemia* **2008**, *23*, 3–9. [CrossRef]
2. Díaz-Tejedor, A.; Lorenzo-Mohamed, M.; Puig, N.; García-Sanz, R.; Mateos, M.-V.; Garayoa, M.; Paíno, T. Immune System Alterations in Multiple Myeloma: Molecular Mechanisms and Therapeutic Strategies to Reverse Immunosuppression. *Cancers* **2021**, *13*, 1353. [CrossRef] [PubMed]
3. Tamura, H. Immunopathogenesis and immunotherapy of multiple myeloma. *Int. J. Hematol.* **2018**, *107*, 278–285. [CrossRef]
4. Rosenblatt, J.; Glotzbecker, B.; Mills, H.; Vasir, B.; Tzachanis, D.; Levine, J.D.; Joyce, R.M.; Wellenstein, K.; Keefe, W.; Schickler, M.; et al. PD-1 Blockade by CT-011, Anti-PD-1 Antibody, Enhances Ex Vivo T-cell Responses to Autologous Dendritic Cell/Myeloma Fusion Vaccine. *J. Immunother.* **2011**, *34*, 409–418. [CrossRef]
5. Zelle-Rieser, C.; Thangavadivel, S.; Biedermann, R.; Brunner, A.; Stoitzner, P.; Willenbacher, E.; Greil, R.; Jöhrer, K. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. *J. Hematol. Oncol.* **2016**, *9*, 1–12. [CrossRef]
6. Hallett, W.H.; Jing, W.; Drobyski, W.R.; Johnson, B.D. Immunosuppressive Effects of Multiple Myeloma Are Overcome by PD-L1 Blockade. *Biol. Blood Marrow Transplant.* **2011**, *17*, 1133–1145. [CrossRef]
7. Xing, J.; Lu, G.; Liu, G.Q.; Xu, M.; Zhao, X.; Han, E.; Wang, L.; Ding, H.F. Imbalance of treg/th17 in bone marrow of patients with multiple myeloma. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **2014**, *22*, 1321–1325. [CrossRef]
8. Atanackovic, D.; Luetkens, T. Biomarkers for checkpoint inhibition in hematologic malignancies. *Semin. Cancer Biol.* **2018**, *52*, 198–206. [CrossRef]
9. Kearl, T.J.; Jing, W.; Gershan, J.A.; Johnson, B.D. Programmed Death Receptor-1/Programmed Death Receptor Ligand-1 Blockade after Transient Lymphodepletion Therapy Treat Myeloma. *J. Immunol.* **2013**, *190*, 5620–5628. [CrossRef]
10. Ansell, S.M.; Lesokhin, A.M.; Borrello, I.; Halwani, A.; Scott, E.C.; Gutierrez, M.; Schuster, S.J.; Millenson, M.M.; Cattry, D.; Freeman, G.J.; et al. PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *N. Engl. J. Med.* **2015**, *372*, 311–319. [CrossRef]
11. Thanendrarajan, S.; Puryear, J.; Schinke, C.D.; van Rhee, F.; Zangari, M.; Mathur, P.; Mohan, M.; Susanibar, S.; Kamimoto, J.J.; Hoque, S. Nivolumab for treatment of advanced, refractory, high-risk multiple myeloma. *Blood* **2017**, *130*, 1858. [CrossRef]
12. Suen, H.; Brown, R.F.; Yang, S.; Ho, P.J.; Gibson, J.; Joshua, D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study. *Leukemia* **2015**, *29*, 1621–1622. [CrossRef]
13. Suen, H.; Brown, R.; Yang, S.; Weatherburn, C.; Ho, P.J.; Woodland, N.; Nassif, N.; Barbaro, P.; Bryant, C.; Hart, D.; et al. Multiple myeloma causes clonal T-cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* **2016**, *30*, 1716–1724. [CrossRef]
14. Zhao, Y.; Shao, Q.; Peng, G. Exhaustion and senescence: Two crucial dysfunctional states of T cells in the tumor microenvironment. *Cell. Mol. Immunol.* **2019**, *17*, 27–35. [CrossRef]
15. Pawelec, G. Is There a Positive Side to T Cell Exhaustion? *Front. Immunol.* **2019**, *2019 10*, 111. [CrossRef]

16. Kosmaczewska, A.; Masternak, A.; Kosciow, K.; Ciszak, L.; Usnarska-Zubkiewicz, L.; Szteblich, A.; Potoczek, S.; Frydecka, I. PD-1 overexpression determines the disproportion of circulating Th1/Th17/Treg cells and clinical outcome of multiple myeloma. *Res. Sq.* **2019**. [CrossRef]
17. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* **2012**, *12*, 252–264. [CrossRef]
18. Kosmaczewska, A.; Frydecka, I.; Boćko, D.; Ciszak, L.; Teodorowska, R. Correlation of blood lymphocyte CTLA-4 (CD152) induction in Hodgkin's disease with proliferative activity, interleukin 2 and interferon-gamma production. *Br. J. Haematol.* **2002**, *118*, 202–209. [CrossRef]
19. Frydecka, I.; Kosmaczewska, A.; Bocko, D.; Ciszak, L.; Wolowiec, D.; Kuliczkowski, K.; Kochanowska, I. Alterations of the expression of T-cell related costimulatory CD28 and down regulatory CD152 (CTLA-4) molecules in patients with B-cell chronic lymphocytic leukemia. *Br. J. Cancer* **2004**, *90*, 2042–2048. [CrossRef] [PubMed]
20. Kosmaczewska, A.; Ciszak, L.; Suwalska, K.; Wolowiec, D.; Frydecka, I. CTLA-4 overexpression in $CD19^+/CD5^+$ cells correlates with the level of cell cycle regulators and disease progression in B-CLL patients. *Leukemia* **2004**, *19*, 301–304. [CrossRef]
21. Ciszak, L.; Frydecka, I.; Wolowiec, D.; Szteblich, A.; Kosmaczewska, A. CTLA-4 affects expression of key cell cycle regulators of G0/G1 phase in neoplastic lymphocytes from patients with chronic lymphocytic leukaemia. *Clin. Exp. Med.* **2016**, *16*, 317–332. [CrossRef]
22. Karabon, L.; Partyka, A.; Ciszak, L.; Pawlak-Adamska, E.; Tomkiewicz, A.; Bojarska-Junak, A.; Roliński, J.; Wołowiec, D.; Wrobel, T.; Frydecka, I.; et al. Abnormal Expression of BTLA and CTLA-4 Immune Checkpoint Molecules in Chronic Lymphocytic Leukemia Patients. *J. Immunol. Res.* **2020**, *2020*, 1–12. [CrossRef]
23. Ciszak, L.; Frydecka, I.; Wolowiec, D.; Szteblich, A.; Kosmaczewska, A. Patients with chronic lymphocytic leukaemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: The possible implications for immunotherapy with CTLA-4 blocking antibody. *Tumour Biol.* **2016**, *37*, 4143–4157. [CrossRef]
24. Thommen, D.S.; Schreiner, J.; Müller, P.; Herzig, P.; Roller, A.; Belousov, A.; Umana, P.; Pisa, P.; Klein, C.G.; Bacac, M.; et al. Progression of Lung Cancer Is Associated with Increased Dysfunction of T Cells Defined by Coexpression of Multiple Inhibitory Receptors. *Cancer Immunol. Res.* **2015**, *3*, 1344–1355. [CrossRef]
25. Tan, J.; Chen, S.; Huang, J.; Chen, Y.; Yang, L.; Wang, C.; Zhong, J.; Lu, Y.; Wang, L.; Zhu, K.; et al. Increased exhausted $CD8^+$T cells with programmed death-1, T-cell immunoglobulin and mucin-domain-containing-3 phenotype in patients with multiple myeloma. *Asia-Pacific J. Clin. Oncol.* **2018**, *14*, e266–e274. [CrossRef]
26. Arai, Y.; Saito, H.; Ikeguchi, M. Upregulation of TIM-3 and PD-1 on $CD4^+$ and $CD8^+$ T Cells Associated with Dysfunction of Cell-Mediated Immunity after Colorectal Cancer Operation. *Yonago Acta Med.* **2012**, *55*, 1–9.
27. Coppé, J.-P.; Desprez, P.-Y.; Krtolica, A.; Campisi, J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu. Rev. Pathol. Mech. Dis.* **2010**, *5*, 99–118. [CrossRef]
28. Pauken, K.E.; Wherry, E.J. Overcoming T cell exhaustion in infection and cancer. *Trends Immunol.* **2015**, *36*, 265–276. [CrossRef]
29. Liu, X.; Mo, W.; Ye, J.; Li, L.; Zhang, Y.; Hsueh, E.C.; Hoft, D.F.; Peng, G. Regulatory T cells trigger effector T cell DNA damage and senescence caused by metabolic competition. *Nat. Commun.* **2018**, *9*, 1–16. [CrossRef]
30. Ye, J.; Huang, X.; Hsueh, E.C.; Zhang, Q.; Ma, C.; Zhang, Y.; Varvares, M.A.; Hoft, D.F.; Peng, G. Human regulatory T cells induce T-lymphocyte senescence. *Blood* **2012**, *120*, 2021–2031. [CrossRef]
31. Rajkumar, S.V.; Dimopoulos, M.A.; Palumbo, A.; Blade, J.; Merlini, G.; Mateos, M.-V.; Kumar, S.; Hillengass, J.; Kastritis, E.; Richardson, P.; et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* **2014**, *15*, e538–e548. [CrossRef]
32. Greipp, P.R.; Miguel, J.S.; Durie, B.G.; Crowley, J.J.; Barlogie, B.; Bladé, J.; Boccadoro, M.; Child, J.A.; Avet-Loiseau, H.; Kyle, R.A.; et al. International Staging System for Multiple Myeloma. *J. Clin. Oncol.* **2005**, *23*, 3412–3420. [CrossRef]
33. Liu, W.; Putnam, A.L.; Xu-Yu, Z.; Szot, G.L.; Lee, M.R.; Zhu, S.; Gottlieb, P.A.; Kapranov, P.; Gingeras, T.R.; Fazekas de St Groth, B.; et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD^{4+} T reg cells. *J. Exp. Med.* **2006**, *203*, 1701–1711. [CrossRef]
34. Petersen, C.; Christensen, E.; Andresen, B.; Møller, B. Internalization, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. *Exp. Cell Res.* **1992**, *201*, 160–173. [CrossRef]
35. Batorov, E.V.; Aristova, T.A.; Sergeevicheva, V.V.; Sizikova, S.A.; Ushakova, G.Y.; Pronkina, N.V.; Shishkova, I.V.; Shevela, E.Y.; Ostanin, A.A.; Chernykh, E.R. Quantitative and functional characteristics of circulating and bone marrow PD-1- and TIM-3-positive T cells in treated multiple myeloma patients. *Sci. Rep.* **2020**, *10*, 1–12. [CrossRef]
36. Montes, C.L.; Chapoval, A.I.; Nelson, J.; Orhue, V.; Zhang, X.; Schulze, D.H.; Strome, S.E.; Gastman, B.R. Tumor-Induced Senescent T Cells with Suppressor Function: A Potential Form of Tumor Immune Evasion. *Cancer Res.* **2008**, *68*, 870–879. [CrossRef] [PubMed]
37. Ye, J.; Peng, G. Controlling T cell senescence in the tumor microenvironment for tumor immunotherapy. *Oncoimmunology* **2015**, *4*, e994398. [CrossRef]
38. Ye, J.; Ma, C.; Hsueh, E.C.; Dou, J.; Mo, W.; Liu, S.; Han, B.; Huang, Y.; Zhang, Y.; Varvares, M.A.; et al. TLR8 signaling enhances tumor immunity by preventing tumor-induced T-cell senescence. *EMBO Mol. Med.* **2014**, *6*, 1294–1311. [CrossRef]
39. Lee, M.Y.; Park, C.-J.; Cho, Y.-U.; Jang, S. PS1426 IMMUNE CHECKPOINT (PD-1, PD-L1, PD-L2, AND CTLA-4) EXPRESSION IN PLASMA CELL MYELOMA. *HemaSphere* **2019**, *3*, 656–657. [CrossRef]

40. Simonetta, F.; Pradier, A.; Bosshard, C.; Masouridi-Levrat, S.; Dantin, C.; Koutsi, A.; Tirefort, Y.; Roosnek, E.; Chalandon, Y. Dynamics of Expression of Programmed Cell Death Protein-1 (PD-1) on T Cells After Allogeneic Hematopoietic Stem Cell Transplantation. *Front. Immunol.* **2019**, *10*, 1034. [CrossRef]
41. Lesokhin, A.M.; Ansell, S.M.; Armand, P.; Scott, E.C.; Halwani, A.; Gutierrez, M.; Millenson, M.M.; Cohen, A.D.; Schuster, S.J.; Lebovic, D.; et al. Preliminary Results of a Phase I Study of Nivolumab (BMS-936558) in Patients with Relapsed or Refractory Lymphoid Malignancies. *Blood* **2014**, *124*, 291. [CrossRef]
42. Lesokhin, A.M.; Ansell, S.M.; Armand, P.; Scott, E.C.; Halwani, A.; Gutierrez, M.; Millenson, M.M.; Cohen, A.D.; Schuster, S.J.; Lebovic, D.; et al. Nivolumab in Patients With Relapsed or Refractory Hematologic Malignancy: Preliminary Results of a Phase Ib Study. *J. Clin. Oncol.* **2016**, *34*, 2698–2704. [CrossRef]
43. Webb, E.S.; Liu, P.; Baleeiro, R.; Lemoine, N.R.; Yuan, M.; Wang, Y.-H. Immune checkpoint inhibitors in cancer therapy. *J. Biomed. Res.* **2017**, *32*, 317–326. [CrossRef]
44. Kao, C.; Oestreich, K.J.; Paley, M.A.; Crawford, A.; Angelosanto, J.M.; Ali, M.A.; Intlekofer, A.M.; Boss, J.M.; Reiner, S.L.; Weinmann, A.S.; et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat. Immunol.* **2011**, *12*, 663–671. [CrossRef]
45. Lu, P.; Youngblood, B.; Austin, J.W.; Mohammed, A.U.R.; Butler, R.; Ahmed, R.; Boss, J.M. Blimp-1 represses CD8 T cell expression of PD-1 using a feed-forward transcriptional circuit during acute viral infection. *J. Exp. Med.* **2014**, *211*, 515–527. [CrossRef] [PubMed]
46. Kasamatsu, T.; Awata, M.; Ishihara, R.; Murakami, Y.; Gotoh, N.; Matsumoto, M.; Sawamura, M.; Yokohama, A.; Handa, H.; Tsukamoto, N.; et al. PDCD1 and PDCD1LG1 polymorphisms affect the susceptibility to multiple myeloma. *Clin. Exp. Med.* **2019**, *20*, 51–62. [CrossRef] [PubMed]
47. Karabon, L.; Pawlak-Adamska, E.; Tomkiewicz, A.; Jedynak, A.; Kielbinski, M.; Woszczyk, D.; Potoczek, S.; Jonkisz, A.; Kuliczkowski, K.; Frydecka, I. Variations in Suppressor Molecule CTLA-4 Gene Are Related to Susceptibility to Multiple Myeloma in a Polish Population. *Pathol. Oncol. Res.* **2011**, *18*, 219–226. [CrossRef]
48. Zheng, C.; Huang, D.; Liu, L.; Björkholm, M.; Holm, G.; Yi, Q.; Sundblad, A. Cytotoxic T-lymphocyte antigen-4 microsatellite polymorphism is associated with multiple myeloma. *Br. J. Haematol.* **2001**, *112*, 216–218. [CrossRef]
49. Alrasheed, N.; Lee, L.; Ghorani, E.; Henry, J.Y.; Conde, L.; Chin, M.; Galas-Filipowicz, D.; Furness, A.J.; Chavda, S.J.; Richards, H.; et al. Marrow-Infiltrating Regulatory T Cells Correlate with the Presence of Dysfunctional CD4+PD-1+ Cells and Inferior Survival in Patients with Newly Diagnosed Multiple Myeloma. *Clin. Cancer Res.* **2020**, *26*, 3443–3454. [CrossRef]
50. Usmani, S.Z.; Schjesvold, F.; Rocafiguera, A.O.; Karlin, L.; Rifkin, R.M.; Yimer, H.A.; Leblanc, R.; Takezako, N.; McCroskey, R.D.; Suzuki, K.; et al. A phase 3 randomized study of pembrolizumab (pembro) plus lenalidomide (len) and low-dose dexamethasone (Rd) versus Rd for newly diagnosed and treatment-naive multiple myeloma (MM): KEYNOTE-185. *J. Clin. Oncol.* **2018**, *36*, 8010. [CrossRef]
51. Mateos, M.-V.; Blacklock, H.; Schjesvold, F.; Rocafiguera, A.O.; Simpson, D.; George, A.; Goldschmidt, H.; LaRocca, A.; Sherbenou, D.W.; Avivi, I.; et al. A phase 3 randomized study of pembrolizumab (Pembro) plus pomalidomide (Pom) and dexamethasone (Dex) for relapsed/refractory multiple myeloma (RRMM): KEYNOTE-183. *J. Clin. Oncol.* **2018**, *36*, 8021. [CrossRef]
52. Tamura, H.; Ishibashi, M.; Sunakawa-Kii, M.; Inokuchi, K. PD-L1–PD-1 Pathway in the Pathophysiology of Multiple Myeloma. *Cancers* **2020**, *12*, 924. [CrossRef]
53. Ishibashi, M.; Tamura, H.; Sunakawa, M.; Kondo-Onodera, A.; Okuyama, N.; Hamada, Y.; Moriya, K.; Choi, I.; Tamada, K.; Inokuchi, K. Myeloma Drug Resistance Induced by Binding of Myeloma B7-H1 (PD-L1) to PD-1. *Cancer Immunol. Res.* **2016**, *4*, 779–788. [CrossRef]
54. Amarnath, S.; Mangus, C.W.; Wang, J.C.M.; Wei, F.; He, A.; Kapoor, V.; Foley, J.E.; Massey, P.R.; Felizardo, T.C.; Riley, J.; et al. The PDL1-PD1 Axis Converts Human TH1 Cells into Regulatory T Cells. *Sci. Transl. Med.* **2011**, *3*, 111ra120. [CrossRef]
55. Prabhala, R.H.; Pelluru, D.; Fulciniti, M.; Prabhala, H.K.; Nanjappa, P.; Song, W.; Pai, C.; Amin, S.; Tai, Y.-T.; Richardson, P.G.; et al. Elevated IL-17 produced by Th17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. *Blood* **2010**, *115*, 5385–5392. [CrossRef]
56. Braga, W.M.; da Silva, B.R.; de Carvalho, A.C.; Maekawa, Y.H.; Bortoluzzo, A.B.; Rizzatti, E.G.; Atanackovic, D.; Colleoni, G.W. FOXP3 and CTLA4 overexpression in multiple myeloma bone marrow as a sign of accumulation of CD4(+) T regulatory cells. *Cancer Immunol Immunother.* **2014**, *63*, 1189–1197. [CrossRef]
57. Favaloro, J.; Brown, R.; Aklilu, E.; Yang, S.; Suen, H.; Hart, D.; Fromm, P.; Gibson, J.; Khoo, L.; Ho, P.J.; et al. Myeloma skews regulatory T and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. *Leuk. Lymphoma* **2013**, *55*, 1090–1098. [CrossRef]

Table S1. Patient demographics and characteristics.

Characteristic	Number/frequency
Number of patients, n (%)	40
Gender (female)	21 (52.5 %)
Age of sampling (median, range)	69 (59–76)
ISS	
I	6 (15.0 %)
II	16 (40.0 %)
III	18 (45.0 %)
Myeloma isotype	
IgG	27 (67.5 %)
IgA	6 (15.0 %)
Light chain only	7 (17.5 %)
Type of Ig light chain (serum)	
Kappa	23 (57.5 %)
Lambda	16 (40.0 %)
None	1 (2.5 %)
Osteolytic bone lesion/s, n (%)	28 (70.0 %)
Laboratory values	
β2-microglobulin ≥ 3.5 mg/l	27 (67.5 %)
Creatinine ≥ 2.0 mg/dl	13 (32.5 %)
LDH > 190 U/L	8 (20.0 %)
Serum calcium ≥ 10 mg/dl	20 (50.0 %)
Hemoglobin ≤ 12 g/dl	31 (80.0 %)
Platelets < 100,000/mm³	3 (7.5 %)
Prior treatment	
1-3 therapy lines	8 (20.0 %)
≥ 4 therapy lines	6 (15.0 %)
BTZ based therapy	12 (30.0 %)
IMiD therapy	11 (27.5 %)
No therapy	26 (65.0 %)

Abbreviations: BTZ, bortezomib; LDH, lactate dehydrogenases; ISS, International Staging System; IMiD, immunomodulatory drugs; UNV, upper normal values

Table S2. Immune checkpoint and CD69 expression (%) in PB CD4 T cell subsets in MM stages and controls (HC).

T-cell subset (%)	Stage I/II of MM (n = 22)	Stage III of MM (n = 18)	HC (n =20)	*P* - value
$CD3^+CD4^+PD\text{-}1^+$	16.8 (11.68-19.86)	14.7 (12.18-18.87)	12.54 (8.37-15.29)	a) ns b) 0.012 c) 0.08*
$CD4^+CD127^+PD\text{-}1^+$	23.4 (16.57-30.05)	22.2 (13.97-31.42)	16.8 (10.52-21.75)	a) ns b) 0.023 c) 0.027
$CD4^+CD127^-PD\text{-}1^+$	9.70 (6.44-13.98)	11.49 (7.97-12.80)	6.14 (3.90-7.62)	a) ns b) 0.023 c) 0.027
$CD3^+CD4^+BTLA^+$	27.38 (2.46-4.76)	26.45 (2.38-5.58)	23.85 (17.04-40.16)	a) ns b) ns

				c) ns
$CD4^+CD127^+BTLA^+$	23.30 (13.29-49.21)	24.78 (15.60-51.60)	32.50 (20.75-54.43)	a) ns b) ns c) ns
$CD4^+CD127^-BTLA^+$	5.10 (2.06-7.60)	4.05 (3.72-7.35)	2.87 (1.85-3.40)	a) ns b) ns c) ns
$CD3^+CD4^+CTLA\text{-}4^+$	1.61 (1.00-2.51)	1.17 (0.55-3.04)	1.27 (0.78-2.00)	a) ns b) ns c) ns
$CD4^+CD127^+CTLA4^+$	3.28 (2.21-6.00)	3.84 (1.47-6.07)	3.55 (1.64-4.22)	a) ns b) ns c) ns
$CD4^+CD127^-CTLA4^+$	1.57 (0.75-2.20)	1.99 (0.88-2.49)	1.14 (0.56-1.37)	a) ns b) ns c) ns
$CD3^+CD4^+CD69^+$	0.56 (0.22-0.83)	0.94 (0.35-1.21)	0.42 (0.23-0.44)	a) 0.08* b) ns c) 0.006

a) Stage I/II vs. stage III
b) Stage I/II vs. HC
c) Stage III vs. HC
*trend; ns – not statistically significant

Differences in the proportions of PD-1, BTLA, and CTLA-4 expressing CD4+ T cells between examined groups were evaluated using nonparametric tests (Kruskal-Wallis and Mann-Whitney U-test).

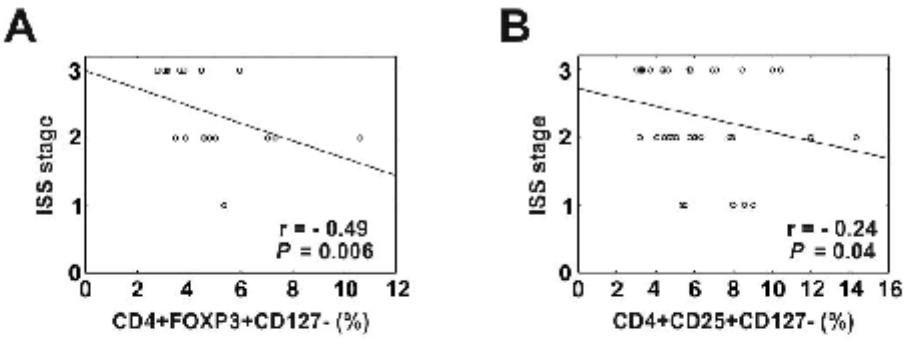

FIGURE S1: Correlation of CD127- Treg subsets with clinical stages of myeloma. We found negative associations between the abundance of PB Treg cells and MM stage.

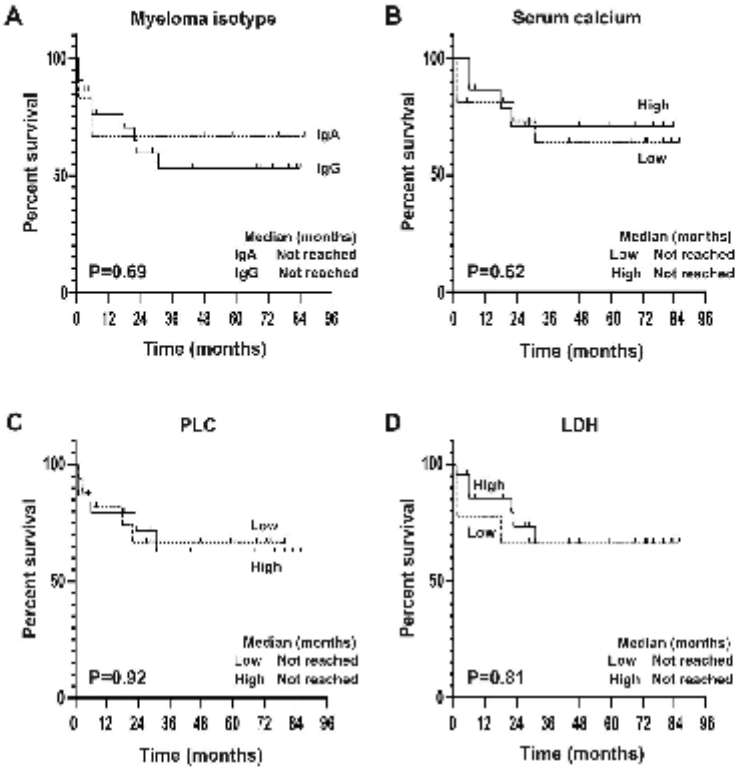

FIGURE S2: Effect of clinical features on patient survival. The cohort of studied patients was divided into high and low expressors of clinical indices according to the median split. We found no significant influence of (A) myeloma isotype, (B) serum calcium level, (C) circulating plasmocytes (PLC), or (D) LDH activity on patient survival ($p > 0.05$). We used the log-rank test in statistical comparison.

International Journal of
Molecular Sciences

Article

Inappropriate Expression of PD-1 and CTLA-4 Checkpoints in Myeloma Patients Is More Pronounced at Diagnosis: Implications for Time to Progression and Response to Therapeutic Checkpoint Inhibitors

Anna Kulikowska de Nałęcz [1], Lidia Ciszak [2], Lidia Usnarska-Zubkiewicz [3], Edyta Pawlak [2], Irena Frydecka [2], Magdalena Szmyrka [4] and Agata Kosmaczewska [2,*]

[1] Hematology and Hematological Oncology Department, Provincial Hospital, 45-061 Opole, Poland
[2] Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland
[3] Department of Hematology, Blood Neoplasms, and Bone Marrow Transplantation, Wroclaw Medical University, 50-367 Wroclaw, Poland
[4] Department of Rheumatology and Internal Diseases, Wroclaw Medical University, 50-556 Wroclaw, Poland
* Correspondence: agata.kosmaczewska@hirszfeld.pl

Abstract: Multiple myeloma (MM) is a hematologic malignancy characterized by severely profound immune dysfunction. Therefore, the efficacy of drugs targeting the immune environments, such as immune checkpoint inhibitors (ICIs), is of high clinical importance. However, several clinical trials evaluating ICIs in MM in different therapeutic combinations revealed underwhelming results showing a lack of clinical efficacy and excessive side effects. The underlying mechanisms of resistance to ICIs observed in the majority of MM patients are still under investigation. Recently, we demonstrated that inappropriate expression of PD-1 and CTLA-4 on CD4 T cells in active MM is associated with adverse clinical outcomes and treatment status. The aim of the current study was to determine the usefulness of immune checkpoint expression assessment as a predictive biomarker of the response to therapeutic inhibitors. For this purpose, along with checkpoint expression estimated by flow cytometry, we evaluated the time to progression (TTP) of MM patients at different clinical stages (disease diagnosis and relapse) depending on the checkpoint expression level; the cut-off point (dividing patients into low and high expressors) was selected based on the median value. Herein, we confirmed the defective levels of regulatory PD-1, CTLA-4 receptors, and the CD69 marker activation in newly diagnosed (ND) patients, whereas relapsed/refractory patients (RR) exhibited their recovered values and reactivity. Additionally, substantially higher populations of senescent $CD4^+CD28^-$ T cells were found in MM, primarily in NDMM subjects. These observations suggest the existence of two dysfunctional states in MM CD4 T cells with the predominance of immunosenescence at disease diagnosis and exhaustion at relapse, thus implying different responsiveness to the external receptor blockade depending on the disease stage. Furthermore, we found that lower CTLA-4 levels in NDMM patients or higher PD-1 expression in RRMM patients may predict early relapse. In conclusion, our study clearly showed that the checkpoint level in CD4 T cells may significantly affect the time to MM progression concerning the treatment status. Therefore, when considering novel therapies and potent combinations, it should be taken into account that blocking PD-1 rather than CTLA-4 might be a beneficial form of immunotherapy for only a proportion of RRMM patients.

Keywords: multiple myeloma; PD-1; CTLA-4; immune checkpoint inhibitors; time to progression; clinical response

check for updates

Citation: Kulikowska de Nałęcz, A.; Ciszak, L.; Usnarska-Zubkiewicz, L.; Pawlak, E.; Frydecka, I.; Szmyrka, M.; Kosmaczewska, A. Inappropriate Expression of PD-1 and CTLA-4 Checkpoints in Myeloma Patients Is More Pronounced at Diagnosis: Implications for Time to Progression and Response to Therapeutic Checkpoint Inhibitors. *Int. J. Mol. Sci.* 2023, *24*, 5730. https://doi.org/10.3390/ijms24065730

Academic Editors: Sibylle Mittnacht and Pierosandro Tagliaferri

Received: 30 November 2022
Revised: 24 February 2023
Accepted: 15 March 2023
Published: 17 March 2023

1. Introduction

Despite the current advances in treatment seen with the inclusion of proteosome inhibitors (PIs), immunomodulatory drugs (IMIDs), and chimeric T cell therapy (CAR-T)

multiple myeloma (MM) remains an almost universally incurable malignancy. Along with reducing the bulk of myeloma cells by conventional therapy, host factors including the cytotoxic capacity of activated T cells is essential for tumor eradication and clinical outcomes of MM therapy. Accumulating evidence has shown that tumor-induced immune dysfunction in MM patients seems to be greater than in other B cell malignancies [1–3], and includes dysfunction of dendritic and T cells [4], accumulation of suppressive cell types [2,4], cytotoxic T cell/Treg and Th17/Treg imbalance [5,6], and T cell hyporesponsiveness [7,8].

The dysfunction of T cells in cancer patients is characterized by anergy, senescence, and/or exhaustion [9–11], namely the states sharing expression of the multiple inhibitory molecules implicated in the impaired effector functions and hyporesponsiveness. Overcoming the hyporesponsiveness of T cells could reinvigorate the host's immune response and restore antitumor immunity. Available data, including ours, demonstrates that T cells in patients with MM display features of exhaustion and senescence [6,12]. It is interesting to note that both dysfunctional states may exist at the same disease stage. Determination of the extent of senescence and/or exhaustion within the T cell compartment remain confusing due to several similarities. While both states share overlapping functional and phenotypic features including the expression of regulatory receptors (e.g., PD-1 and CTLA-4), increased cell cycle arrest, and affected effector functions, they also have distinct regulatory and molecular mechanisms controlling their development and disturbed antitumor functions [13–15]. Another difference is the potential for reversion as a consequence of modulation of regulatory pathways after external receptor blockade, a feature attributed only to exhaustion [13,16]. An increasing body of evidence suggests that the extent of the clinical response to checkpoint inhibitors might be closely related to the level of checkpoint expression on T cells [6,16]. Therefore, the identification of dysfunctional states predominating in MM patients regarding the clinical stage could help determine if T cell hyporesponsiveness is reversible by manipulating the immune checkpoint blockade, thus predicting the clinical response to this therapeutic approach.

Accordingly, our recent study was conducted to assess the usefulness of immune checkpoints as predictive biomarkers of the response to therapeutic checkpoint inhibitors at different clinical stages, namely NDMM and RRMM patients [6]. In the current study, we confirmed the suboptimal level of CTLA-4 and PD-1 in circulating MM CD4 T cells, primarily in NDMM patients. Furthermore, we observed that the level of checkpoint expression may predict the clinical outcome, when considering TTP, in relation to the treatment status; lower CTLA-4 expression at disease diagnosis or higher PD-1 levels in RRMM patients might predict early relapse. Our results clearly indicate that blocking immune checkpoints might be a beneficial form of immunotherapy for only a proportion of RRMM patients.

2. Results

2.1. Circulating Myeloma CD4 T Cells Contain Lower Levels of Immune Checkpoints at Diagnosis

As recent clinical trials of the administration of immune checkpoint inhibitors (ICIs) in MM showed real disappointment [16–20], we wanted to verify whether the onset and/or exacerbation of MM is accompanied by alterations in the expression of immune checkpoints, thereby affecting their usefulness as targets for therapeutic inhibitors. Therefore, we assessed PD-1 and CTLA-4 expression in the peripheral blood (PB) $CD4^+$ T cell subsets involved in the antitumor response in MM patients at disease diagnosis and relapse.

In our study, as is shown in Figure 1a, a median proportion of $CD4^+$ T cells expressing the PD-1 checkpoint was found to increase in all MM patients regardless of disease stage when compared to controls ($p < 0.05$). While an expansion of PD-1^+ Teff cells was similar in all patients, Treg cells from RRMM patients expressed the PD-1 molecule on a significantly higher proportion of cells than in the NDMM group ($p = 0.037$) (Figure 1a).

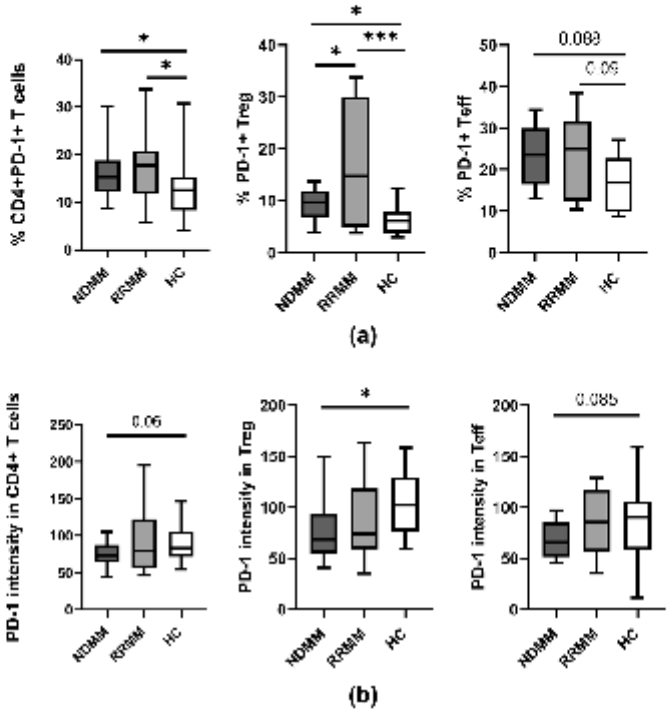

Figure 1. PD-1 expression on PB CD4 T cell subtypes of MM patients in the different clinical stages and healthy controls. (**a**) Frequency of PD-1-expressing $CD4^+$ T cells (identified as $CD3^+CD4^+$), Treg (identified as $CD4^+CD127^-$), and Teff (identified as $CD4^+CD127^+$) cells in healthy controls (HC) (n = 20) and patient subgroups (NDMM and RRMM) (n = 26 and n = 14, respectively). (**b**) PD-1 level (determined as mean fluorescence intensity) in $CD4^+$ T cells, Treg, and Teff cells in HC (n = 20), NDMM (n = 26), and RRMM (n = 14) patients. Boxes and whiskers show 25th and 75th interquartile range and minimum–maximum, respectively; the median is the central line in each box. The differences between the studied groups were statistically evaluated using Kruskal–Wallis, ANOVA, and Mann–Whitney U tests. * $p < 0.05$, *** $p < 0.001$.

Remarkably, a quantitative analysis of PD-1 expression on PB $CD4^+$ T cells showed lower levels in NDMM patients compared to healthy controls ($p = 0.06$) (Figure 1b). While a decline of PD-1 was observed in the whole population of NDMM $CD4^+$ T cells, including both Teff and Treg subsets, the loss of PD-1 was more pronounced in Treg cells ($p = 0.016$) (Figure 1b); in Teff cells, a decrease of PD-1 was of borderline significance ($p = 0.08$) (Figure 1b). In RRMM patients, PD-1 expression was also down-regulated primarily in the Treg subset; however, its median values were statistically comparable to those observed in corresponding healthy cells (Figure 1b). Although some differences in PD-1 expression between patient groups were observed, they did not reach statistical significance.

As is demonstrated in Figure 2a, we also found no significant differences in the proportion of CTLA-4 expressing cells within the examined subsets between the participants studied, except for the higher abundance of CTLA-4^+ Treg cells in RRMM patients when compared to healthy controls ($p = 0.031$). Remarkably, similarly to PD-1 expression, a quantitative estimation of CTLA-4 showed that the only group exhibiting a markedly down-regulated level of CTLA-4 on CD4 T cells, including Teff and Treg cells, was NDMM patients ($p \leq 0.008$ and $p \leq 0.005$, respectively) compared to normal levels in corresponding

cells from the RRMM and healthy groups (Figure 2b). Additionally, there were no differences in CTLA-4 expression between RRMM patients and controls regarding the studied $CD4^+$ T cell subsets (Figure 2b).

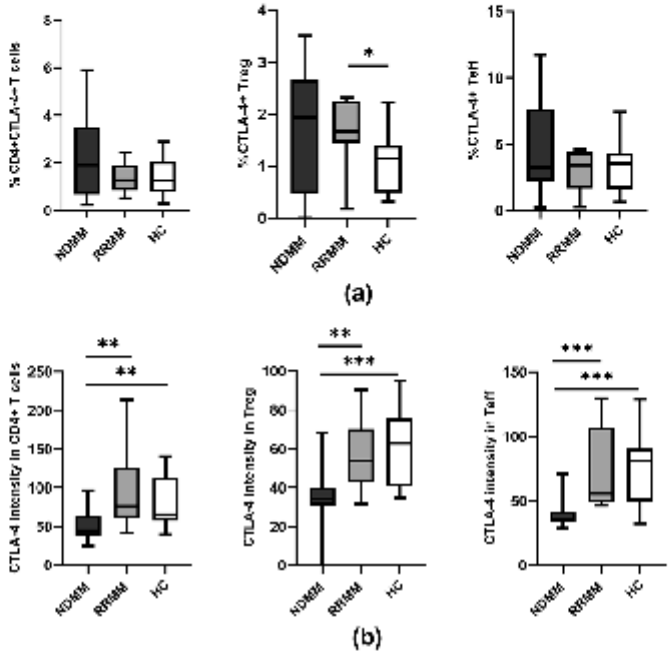

Figure 2. CTLA-4 expression on PB CD4 T cell subtypes of MM patients in the different clinical stages and healthy controls. (**a**) Frequency of CTLA-4 expressing $CD4^+$ T cells (identified as $CD3^+CD4^+$), Treg (identified as $CD4^+CD127^-$), and Teff (identified as $CD4^+CD127^+$) cells in healthy controls (HC) (n = 20) and patient subgroups (NDMM and RRMM) (n = 26 and n = 14, respectively). (**b**) CTLA-4 level (determined as mean fluorescence intensity) in $CD4^+$ T cells, Treg, and Teff cells in HC (n = 20), NDMM (n = 26), and RRMM (n = 14) patients. Boxes and whiskers show 25th and 75th interquartile range and minimum–maximum, respectively; the median is the central line in each box. The differences between the studied groups were statistically evaluated using Kruskal–Wallis, ANOVA, and Mann–Whitney U tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

From our results it seems that the lower levels of both immune checkpoints in MM CD4 T cells suggest they are not appropriate targets for therapeutic inhibitors in MM, at least at disease onset, and, consequently, should not to be considered in first-line treatment settings. Furthermore, chemo- and/or immunotherapy of MM, despite the risk of the development of refractoriness, is capable of reinforcing both PD-1 and CTLA-4 checkpoint expression, making them more attainable to the therapeutic inhibitors in RRMM.

2.2. *Lower CTLA-4 Levels at Myeloma Diagnosis Predispose to a Shortened TTP*

Next, we analyzed whether the observed alterations in PD-1 and CTLA-4 expression may influence the clinical outcome of MM. Therefore, we assessed the TTP of MM patients at the different clinical stages (disease onset and relapse/refractoriness) depending on the expression of both immune checkpoints on CD4 T cells. The cut-off point was selected based on the median value of the checkpoint expression (measured at qualitative and quantitative levels). The statistically significant associations are shown in Figure 3.

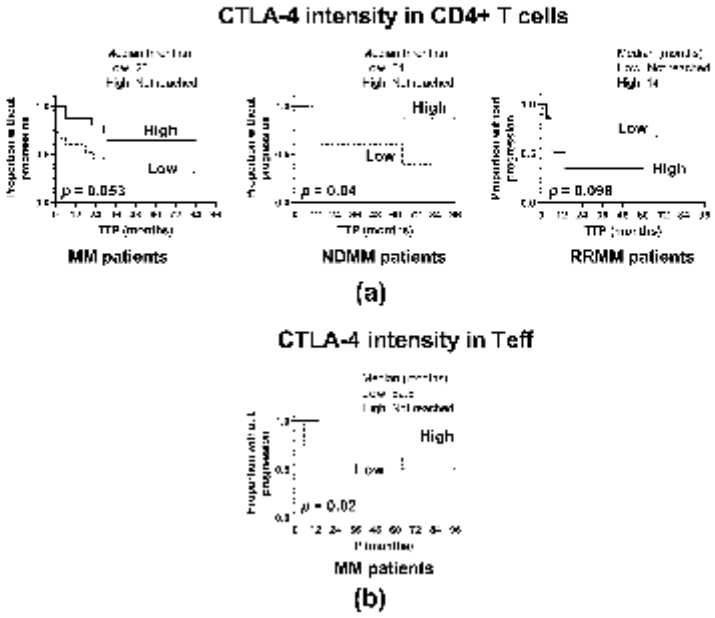

Figure 3. Influence of CTLA-4 expression on the time to progression (TTP). (**a**) TTP in patients with high and low CTLA-4 expression in CD4+ T cells (shown as CTLA-4 fluorescence intensity) identified as >median and ≤median values, respectively, in the whole patient cohort (MM) (n = 40) and patient subgroups (NDMM and RRMM) (n = 26 and n = 14, respectively). (**b**) TTP in patients with high and low CTLA-4 expression in Teff cells (shown as CTLA-4 fluorescence intensity) identified as >median and ≤median values, respectively, in the whole patient cohort (MM) (n = 40). The log-rank test was performed for the Kaplan–Meier curves.

For the whole MM patient cohort, regardless of CD4+ T cell subtypes, a weak positive association of CTLA-4 level on CD4+ T cells with TTP was indicated ($p = 0.053$) (Figure 3a); however, this relationship was found to be strengthened only with regards to the Teff subset ($p = 0.02$) (Figure 3b), and CTLA-4 expression on Treg was not associated with TTP. Furthermore, clinical subanalysis revealed that the above association might be assigned to NDMM patients (Figure 3a); patients with a CTLA-4 level below the median value had significantly shorter TTP than those with a higher CTLA-4 expression ($p = 0.04$). In contrast, RRMM patients exhibited no statistically significant association of CTLA-4 fluorescence intensity with TTP ($p = 0.098$) (Figure 3a). Likewise, no impact was found of changes in CTLA-4 distribution within CD4+ T cells on MM progression in either group of patients.

Our study demonstrating the association of CTLA-4 loss in CD4 T cells with shortened TTP seen in NDMM patients points to the possibility that blockade of CTLA-4 in MM may be an unfavorable strategy at diagnosis.

2.3. *Higher PD-1 Expression May Predict Early Relapse in RRMM Patients*

Given it is noted that MM patients at diagnosis exhibited an impaired PD-1 level, we next examined its association with clinical outcomes in terms of TTP as well.

For the whole patient cohort, as is demonstrated in Figure 4, several inverse associations of TTP with the frequency of PD-1-expressing CD4+ T cells ($p = 0.057$) (Figure 4a) have been found: PD-1+ Treg cells ($p = 0.07$) (Figure 4b), PD-1+ Teff ($p = 0.043$) (Figure 4c), and the level of PD-1 in Treg and Teff cells ($p = 0.046$ and $p = 0.035$, respectively) (Figure 4b and 4c, respectively). Together these indicate that high-PD-1 expressors experienced an MM relapse

significantly earlier than those with PD-1 expression below the median values. However, patient analysis based on the clinical division according to MM stage revealed that the above observation was primarily assigned to the RRMM group, where patients with percentages of CD4+PD-1+ T cells over the median value had markedly shorter TTP compared to those with a lower frequency of these cells ($p = 0.033$) (Figure 4a); of note, such an association was not shown for NDMM patients ($p = 0.41$) (Figure 4a).

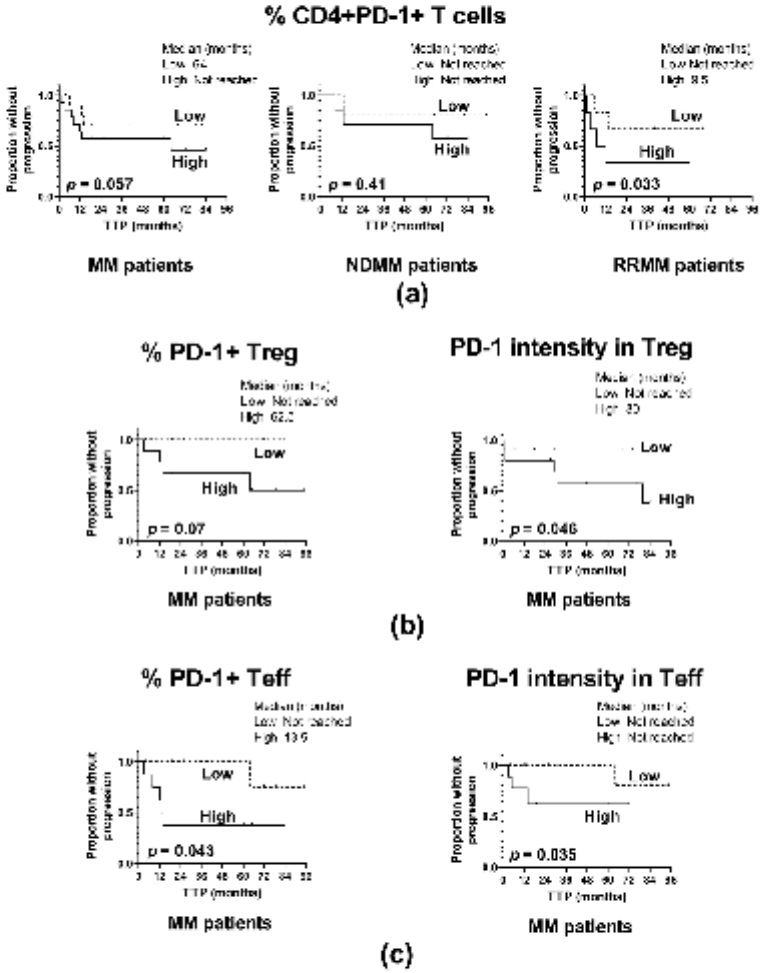

Figure 4. Influence of PD-1 expression on the time to progression (TTP). (**a**) TTP in patients with high and low frequency of PD-1 positive $CD4^+$ T cells identified as >median and ≤median values, respectively, in the whole patient cohort (MM) (n = 40) and patient subgroups (NDMM and RRMM) (n = 26 and n = 14, respectively). (**b**) TTP in patients with high and low PD-1 expression in Treg cells (shown as the frequency of PD-1^+ Treg cells and as PD-1 fluorescence intensity in Treg), identified as >median and ≤median values, respectively, in the whole patient cohort (MM) (n = 40). (**c**) TTP in patients with high and low expression of PD-1 in Teff cells (shown as frequency of PD-1^+ Teff and as PD-1 fluorescence intensity in Teff) identified as >median and ≤median values, respectively, in the whole patient cohort (MM) (n = 40). The log-rank test was performed for the Kaplan–Meier curves.

From the above data it appears that the administration of PD-1 inhibitors might be a beneficial form of therapy for a proportion of RRMM patients, particularly those exhibiting higher PD-1 expression within $CD4^+$ T cells.

2.4. CD4 T Cells from NDMM Patients Retain In Vivo Lower Reactivity to Stimuli

Having demonstrated that the dysregulated expression of immune checkpoints on T cells may be a consequence of altered in vivo stimulation, we also analyzed the systemic activation of CD4 T cells in all individuals studied. The assessment of T cell reactivity is important in predicting the biological effectiveness of the checkpoint blockade [13,21].

While we noted an increased proportion of $CD4^+CD69^+$ T cells in the PB of all patients ($p < 0.06$), statistically significant differences were found only between the RRMM group and healthy controls ($p = 0.027$) (Figure 5a). Remarkably, the median fluorescence intensity of the activation marker CD69 was the lowest in the $CD4^+$ T cells from NDMM patients and it significantly differed from that obtained in corresponding healthy cells ($p = 0.017$). Although the difference in CD69 expression was also observed in comparison to the RRMM group, it remained at a statistically similar level (Figure 5a).

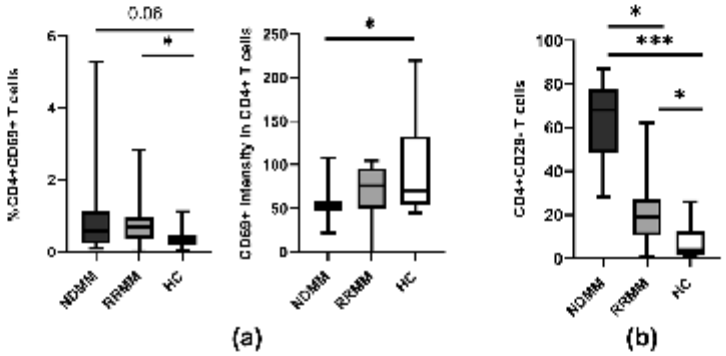

Figure 5. Markers of CD4 T cell reactivity. (**a**) Expression of the CD69 activation marker on $CD4^+$ T cells in PB of healthy controls (HC) (n = 20) and patient subgroups (NDMM and RRMM) (n = 26 and n = 14, respectively). (**b**) Frequency of CD28 lacking $CD4^+$ T cells at the different MM stages. Boxes and whiskers show 25th and 75th interquartile range and minimum–maximum, respectively; the median is the central line in each box. The differences between the studied groups were statistically evaluated using Kruskal–Wallis, ANOVA, and Mann–Whitney U tests. * $p < 0.05$, *** $p < 0.001$.

The above results indicate that the level of immune checkpoint expression in MM corresponds with the suboptimal activation of T cells. Therefore, inappropriate expression of checkpoints may not be responsible for MM T cell hyporesponsiveness observed primarily at disease onset.

2.5. CD28 Loss Related to Cell Senescence Is More Pronounced at Myeloma Diagnosis

Despite the altered pattern of immune checkpoint expression, the lack of CD28 could also help to identify a predominant dysfunctional state in MM CD4 T cells related to immune suppression. Given that it has been demonstrated that the lack of CD28 is one of the constant features of senescent T cells, CD28 negativity has been proposed as a surrogate senescence marker of T cells [13,22]. Therefore, we analyzed the frequency of pathogenic PB $CD4^+CD28^-$ T cells at different MM stages in order to predict a clinical response to ICIs.

We found a substantial increase in the proportion of $CD4^+CD28^-$ T cells in circulation among all MM patients compared to the controls ($p < 0.0001$). However, detailed analysis revealed a significant difference in the abundance of these pathogenic cells among patients regarding disease stage, with the predominance of senescent $CD4^+CD28^-$ T cells evident in the NDMM group when compared to RRMM patients ($p < 0.001$) (Figure 5b).

Our data clearly showed that MM immunopathology is characterized by a higher frequency of $CD4^+CD28^-$ T cells, which is more pronounced in NDMM patients. This may clearly indicate prevalence of the senescent state in MM and points toward the suggestion that reversion of hyporesponsiveness by administrating therapeutic checkpoint inhibitors in MM could be associated with an increasing risk of ineffectiveness.

2.6. The Significance of Clinico-Pathological Features in Prediction of Myeloma Early Relapse

In order to determine the representativeness of our cohort of MM patients, we assessed the impact of the clinico-pathological features of patients on the MM clinical outcome regarding the time to disease relapse. Among the clinical characteristics studied, including patient age, the International Staging System (ISS), anemia, and the levels of β2-microglobulin, albumin, creatinine, plasmacytes, lactate dehydrogenase (LDH), and serum calcium, we found that hyper-β2-microglobulinemia, anemia, and the higher abundance of circulating plasmacytes shortened the time to MM progression ($p = 0.009$, $p = 0.02$, and $p = 0.08$, respectively), thus indicating their predictive significance and enrollment of suitable patients (Figure 6).

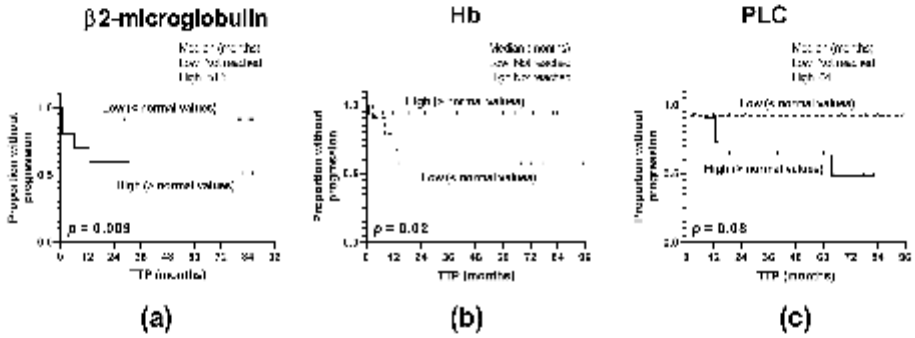

Figure 6. Predictive significance of the clinico-pathological characteristics of MM patients. (**a**) Levels of β2-microblobulin, (**b**) anemia, and (**c**) percentages of circulating plasmacytes (PLCs) affect the time to progression (TTP) of MM patients (n = 40). The log-rank test was performed for the Kaplan–Meier curves.

3. Discussion

The reversal of T cell hyporesponsiveness plays an essential role in myeloma immunotherapy aimed at the restoration of the host's immune response. Growing evidence has shown that defective tumor immunity contributes to compromised effectiveness of anti-myeloma therapy applied so far mainly in advanced high-risk RRMM [23]. The available research demonstrated that MM T cells, depending on the clinical stage, may display features of immune senescence and/or functional exhaustion [6,12,24]. In order to resolve the mechanism responsible for the hyporesponsiveness of MM T cells, it is crucial to distinguish between both dysfunctional states to achieve their adequate and effective control with the appropriate therapeutic approaches.

Herein, we report that in patients with active MM the alterations of the immune checkpoint level depend on the clinical stage of the disease associated with treatment status, and correspond to the extent of the systemic activation of circulating CD4 T cells. In particular, defected levels of PD-1 and CTLA-4 checkpoints, as well as CD69 were observed in MM primarily at diagnosis. Furthermore, we noted that patients, although relapsed, also exhibited inappropriate checkpoint expression, thus confirming the recent suggestion that immune checkpoints are not responsible for T cell hyporesponsiveness in MM [6,24]. The above observation points to the possibility that immune checkpoints might not be appropriate targets for therapeutic inhibitors in MM, and indicates that T

cell-related immune suppression in MM cannot be effectively reversed by manipulating extrinsic regulatory pathways. Remarkably, our observation on an association of the lower CTLA-4 expression on CD4 T cells with shortened TTP in NDMM patients confirms the suggestion that blocking CTLA-4 in MM may be an unfavorable therapeutic strategy for a proportion of MM patients, primarily at diagnosis. This finding is consistent with former observations, including ours, on the impact of genetic variations of the *CTLA-4* gene involved in lower CTLA-4 protein expression with increasing susceptibility to MM development [25–27]. Similar dependence of the response to a CTLA-4 blockade on the level of CTLA-4 expression was previously observed in chronic lymphocytic leukemia by our group [28].

Moreover, inappropriate levels of PD-1 and CTLA-4 in MM CD4 T cells together with the increased subset of $CD4^+CD28^-$ T cells found in our patient cohort have been demonstrated as features of senescent T cells, thus indicating senescence as a predominant dysfunctional state in MM [24]. Our observation is in accordance with a study by Zeller-Riese et al. [12], who recently found that T cells from MM patients express several molecules associated with either T cell exhaustion (e.g., PD-1 and CTLA-4) or T cell senescence (a lack of CD28). They also reported, however, that the enhancement of the senescent CD28-negative T cell population was more pronounced in NDMM patients in comparison to RRMM subjects. These results clearly correspond with our recent study performed in the same patient cohort [6], demonstrating the expansion of CD4 T cells exhibiting a senescence-associated secretory effector phenotype (SASP) [29], primarily in NDMM patients. This is suggestive of the significance of immune senescence in myeloma development, rather than anergy as a dominant functional state of hyporesponsiveness, and emphasizes a role for determining the extent of T cell senescence [6]. Remarkably, the data available have shown that T cell senescence is caused by intrinsic signals induced by DNA damage and can be reversed pharmacologically, but not with an external receptor blockade [13,22,30,31], thereby minimizing the role for checkpoint inhibitors in MM immunotherapy. It should be emphasized, however, that T cell clones in MM have recently been demonstrated not to be related to shortened telomeres, which may imply that their senescence is potentially reversible in MM, if the underlying mechanism is elucidated [24]. Nonetheless, estimation of the senescent T cell population in MM may be of clinical relevance in terms of predicting the clinical response to therapeutic checkpoint inhibitors.

The defective levels of immune checkpoints observed in the present study, more pronounced in the Treg subset, may also imply the inappropriate prevention of autoimmunity [32]. Likewise, in the same patient cohort, we recently found a higher population of CD4 T cells with capacity for inflammatory IL-17 and IFN-γ cytokine secretion with an increased Th17/Treg ratio when compared to the values obtained in the RRMM group [6]. Although the Treg cell population was found to be expanded in all studied patients, the current analysis revealed that this subset is mostly affected by impaired expression levels of PD-1 and CTLA-4 at disease diagnosis, which might compromise the Treg regulatory function. Given that checkpoint expression in Treg cells is associated with suppressive activity [33–35], the decreased level of checkpoints in the Treg population is suggestive of the shift in the immune balance toward autoimmune inflammatory conditions, especially in NDMM patients. In fact, the augmented population of $CD4^+CD28^-$ T cells that we found in all MM patients was previously shown to secrete inflammatory cytokines, which corresponds with the phenotypic and functional characteristics of autoreactive cytotoxic $CD4^+$ T cells [6,36–38]. The notion that among MM patients, the $CD4^+CD28^-$ T cell population is significantly enriched, primarily at disease onset, is in line with the current phenotypic analysis of Tregs and strongly indicates the possibility of a higher incidence of autoimmune responses in MM. In fact, a personal history of autoimmune disease was found to be associated with a significantly higher risk of the monoclonal gammopathy of undetermined significance (MGUS) and MM, indicating a common genetic susceptibility between autoimmunity and plasma cell disorders [39,40]. It has also been demonstrated that co-morbidity of the autoimmune disease might be prognostic of a worse survival rate

in MM patients [39], and suboptimal immune checkpoint expression cannot be excluded when considering the underlying mechanism. Our finding that the lower CTLA-4 expression on CD4 T cells may predict early relapse in NDMM patients also seems to confirm these associations. Therefore, an increasing risk of exaggeration of autoimmune events should be taken into account when considering blocking immune checkpoints in MM [41]. A case of an NDMM patient who developed a lethal immune-related myocarditis after a single dose of pembrolizumab (a PD1-L inhibitor) in combined therapy with lenalidomide and dexamethasone seems to strengthen the above concern, and indicates that therapeutic checkpoint inhibitors should be administered in MM with extreme caution, if at all, especially in first-line treatments [42].

Interestingly, our present study also showed that chemo- and/or immunotherapy of MM, despite the well-known risk of the development of refractoriness [43], is capable of reinforcing both PD-1 and CTLA-4 checkpoint expression, and this notion is consistent with other studies [6,12,44]. The post-treatment ligation of regulatory receptors resulting in the increase and recovery of T cells found in this study has previously been demonstrated as a key phenotypic feature of cell exhaustion, which is also a dysfunctional state related to MM T cell hyporesponsiveness [12]. It should be emphasized, however, that unlike the cell senescence predominant in NDMM patients, the exhaustion of T cells observed mainly in relapse has been shown to be reversible upon inhibitory receptor blockade [13,23]. Here we report that an increase in immune checkpoint expression seen in RRMM patients and accompanied by CD69 reversion is suggestive of the treatment-related tendency to the restoration of reactivity in CD4 T cells, thus making them more attainable for therapeutic inhibitors in RRMM. Of note, exhaustion has been proposed as a mechanism of the prevention of T cell loss and the retention of T cell clones required for immune surveillance and tumor immunity [13]. In fact, expanded clones of cytotoxic T cells exist in patients with MM and other hematologic malignancies [35,45–51]. Although exhausted T cells are hyporesponsive in vitro, their presence in the blood of MM patients is related to better prognosis, probably due to their potential to reverse cell dysfunction [45,46,52,53]. However, our current study, showing that high-PD-1 expressors (primarily within the Teff population) in RRMM patients exhibit a shortened TTP, is indicative of the possibility that a PD-1 blockade might be a beneficial form of immunotherapy for a subset of RRMM patients depending on their PD-1 expression level. This notion is in line with a recent study by Alrasheed et al. [54], who demonstrated that a high level of PD-1$^+$ Teff cells predicts early progression in MM. In our recent study [6], we consistently found an association of high PD-1 expression in Teff and Treg cells in MM with an adverse clinical outcome. Genetic susceptibility to MM development regarding *PD-1* gene polymorphisms also indicated a significant association with a high PD-1 expression haplotype [25], which is in opposition to the *CTLA-4* gene, as its lower expression level has been reported to be involved in MM development [26,27]. These results together with our findings might suggest that, among immune checkpoints, PD-1 rather than CTLA-4 could have potential as a target for therapeutic blockade in a subset of RRMM patients, if the relevant expression of T cells is observed.

Although a weakness of our study is that the relatively small size of the patient cohort inhibits our ability to make strong conclusions, noteworthy is its accordance with the results of two recent phase III clinical trials (namely KEYSTONE-183 and KEYSTONE-185 conducted in RR and ND MM patients, respectively) [17,18]. Both trials showed an unfavorable benefit-to-risk profile after administration of pembrolizumab (a PD-1 inhibitor) in combination with dexamethasone and immunomodulatory drugs (IMIDs). While these trials failed to show higher overall response rate (ORR) or TTP/progression-free-survival (PFS) in the experimental arms, they also demonstrated a much higher frequency and severity of immune-related adverse events (iRAEs) compared to those observed in a checkpoint blockade in other malignancies [17,18]. Moreover, regarding the clinical stages of MM, iRAEs were more pronounced in NDMM patients than in RRMM subjects, probably due to a less exhausted immune system at disease onset [19] and inappropriate prevention of autoimmunity, which clearly confirms the findings presented in our current work. In

addition, we displayed a predictive value of the clinico-pathological characteristics of MM patients enrolled in the study, demonstrating the impact of β2-microglobulin, plasmacyte, and hemoglobin levels on the time to relapse, thus indicating the representativeness of our cohort of patients.

In the immunotherapy of MM patients, except pembrolizumab, several other ICIs have been administered, including nivolumab (anti-PD-1 mAb) and atezolizumab (anti-PD-L1 mAb) alone, or in combination with conventional chemotherapeutics and/or IMIDs, which are showing a disappointing clinical response in the majority of cases [23,55]. Available data showed that the low efficacy of conventional checkpoint inhibitors may be caused by the existence of compensatory inhibitory mechanisms related to the up-regulation of the other checkpoints, such as VISTA or TIM-3 [56,57]. Remarkably, our study clearly showed that an inappropriate level of PD-1 and CTLA-4 checkpoint expression in CD4 T cells in MM patients may also be responsible for the failure of ICIs, and in a proportion of NDMM patients, therapy with ICIs might even be deleterious by shortening the TTP. Our finding implies the significance of checkpoint level assessment in MM patients for predicting the clinical response rate to ICIs and for determining effective therapeutic strategies. Therefore, recently discovered and developed small-molecule inhibitors (SMIs) of checkpoint receptors provide an alternative and promising approach to the immunotherapy of cancers, and are of growing interest due to several desirable benefits they offer [58]. One of these benefits is the capability of SMIs to target more than one checkpoint protein, and the selectivity against other immune checkpoints and enzymes involved in the transcription of genes engaged in tumor suppression [58,59]. In consequence, SMIs reveal the potency for inducing a greater clinical response rate compared to conventional ICIs [58–60]. This pleiotropic activity of SMIs seems to be an attractive property for the area of MM immunotherapy aimed at breaking immune suppression, and appears to be of special interest, especially in view of our notion that PD-1 and CTLA-4 could be less accessible for ICIs in MM. Having ascertained that reversion of both the checkpoint levels and T cell reactivity observed in our cohort of RRMM patients might improve the clinical response to therapeutic inhibitors in terms of delayed relapse, the development of methods targeting the restoration of PD-1 expression in MM T cells, for example, SMIs modulating PD-1 gene transcription, seems to be desirable. In fact, epigenetic small-molecule modulators of PD-L1 and PD-L2 genes' transcription have been shown to up-regulate the expression of these ligands, thus making them more amenable for effective inhibition of the PD-1/PD-L interaction when combined with a PD-1 blocking antibody in mice [61]. This is in accordance with reports showing that PD-L1 expression in cancers might predict a better response to ICIs and improve survival [62]. In cancer treatment, SMIs may act alone or in combination with approved therapies, namely chemo-, radio-, or immunotherapy, including monoclonal antibodies directed toward PD-1/PD-L1 or CTLA-4, such as nivolumab, pembrolizumab, atezolizumab, or ipilimumab (anti-CTLA-4 mAb) [63–67]. Several epigenetic SMIs have also recently been suggested as potent adjuvant agents for combined treatment in numerous types of advanced cancers [58]. Of these, entinostat, panobinostat, and azacitidine are currently in clinical trials in combined therapy with pembrolizumab or ipilimumab in immunogenic cancer patients [63,66,67]. Encouraging results in pre-clinical studies on PD-1-derived CA170 (a small molecule dually targeting PD-L1 and VISTA pathways) reporting high efficacy in the suppression of tumor growth at well-tolerated doses has prompted the advancement of CA170 to clinical trials [59].

In conclusion, the results of our study together with the impressive clinical response to the checkpoint blockade seen in patients with solid tumors, expressing regulatory receptors at a relevant level [16], strengthens the suggestion of a predictive role of checkpoint expression in this therapeutic approach in MM. Therefore, considering novel therapies and potent combinations, estimation of the immune checkpoint levels in T cells before the administration of inhibitors in the different clinical stages of MM is warranted for excluding patients with checkpoint suboptimal levels in order to avoid very limited effectiveness

of the anti-myeloma response, early relapse, and/or severe aggravation of autoimmune adverse events.

4. Materials and Methods

4.1. Samples

The study group of patients studied consisted of a total of 40 active myeloma patients (26 newly diagnosed (NDMM) and 14 relapsed/refractory (RRMM)). Patients were recruited in two centers: the Department of Hematology and Bone Marrow Transplantation at Wroclaw Medical University and the Department of Hematological Oncology at the Provincial Hospital in Opole. The diagnosis of MM was based on the criteria set out by the International Myeloma Working Group (IMWG) [68]. The disease stage was determined according to the International Staging System (ISS) upon entry into the study [69]. RRMM patients were treated with chemotherapy, IMIDs, and a proteasome inhibitor; no patient enrolled in the study received prior treatment with stem cell transplantation (SCT) or ICIs.

The control population comprised 20 healthy individuals matched for age and sex; they had been without any treatment affecting the immune system for six months before entering the study. Patients with a simultaneous active or chronic infection, diabetes, autoimmune disease, or with a history of other malignancies or connective tissue diseases were excluded from the study. Blood samples from all participants were collected after informed consent in accordance with the Declaration of Helsinki and approval from the Institutional Local Research Bioethics Committee at Wroclaw Medical University.

4.2. Clinical and Laboratory Characteristics of Patients

The main characteristics of MM patients are summarized in Table S1. Of the 26 NDMM patients, the majority were women (n = 17, 65.4% vs. n = 9, 34.6%). The median age at diagnosis was 66.0 years old (range 50–76). According to the ISS, 5 patients (19.0%) were classified as stage I, 10 (38.5%) were classified as stage II, and 11 (42.5%) were classified as stage III. The immunoglobulin subtype was IgG for 18 patients (69.0%), and IgA for 3 patients (11.5%). Five patients (19.5%) had light chain only. Of the NDMM patients, the majority (n = 16, 61.5%) had light chain kappa, 9 patients (34.5%) had light chain lambda. A majority of the NDMM patients presented with osteolytic bone lesions (n = 15, 60.0%), had a serum β2-microglobulin level $\geq$ 3.5 mg/L (n = 18, 69.0%), and a hemoglobin value $\leq$ 12 g/dL (n = 23, 88.5%). A creatinine level $\geq$ 2.0 mg/dL and a serum calcium value $\geq$ 10 mg/dL were present in 9 (34.5%) NDMM patients. An LDH level higher than 190 U/L and a platelet value less than 100,000/mm^3 was present in 6 (23.0%) and 2 (7.5%) patients, respectively.

Of the 14 RRMM patients, 10 were men (71.4%) and 4 were women (28.6%). The median age at diagnosis was 72.0 years old (range 65–75). According to the ISS, 1 patient (7.0%) was classified as stage I, 6 (43.0%) were classified as stage II, and 7 (50.0%) were classified as stage III. The immunoglobulin subtype was IgG for 9 patients (64.0%), and IgA for 3 patients (21.5%). Two patients had light chain only (14.5%). Seven RRMM patients had light chain kappa (50.0%) and 7 had light chain lambda. The majority of the RRMM patients (n = 13, 92.8%) presented with osteolytic bone lesions. Of the RRMM patients, a majority had a serum β2-microglobulin level $\geq$ 3.5 mg/L (n = 9, 64.0%), a serum calcium level $\geq$ 10 mg/dL (n = 11, 78.5%), and a hemoglobin value $\leq$ 12 g/dL (n = 8, 57.0%). Four RRMM patients (28.5%) had a creatinine level $\geq$ 2.0 mg/dL. An LDH level higher than 190 U/L and a platelet value less than 100,000/mm^3 was present in 2 (14.0%) patients and 1 (7.0%) patient, respectively. Of the RRMM patients, 12 subjects (85.5%) received bortezomib (BTZ)-based therapy, and 11 patients (78.5%) were treated with IMiDs. The majority of RRMM patients (n = 8, 57.0%) received 1–3 therapy lines, while the remainder (n = 6, 43.0%) received $\geq$ 4 therapy lines.

4.3. Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral venous blood from MM patients and healthy individuals was collected in tubes containing lithium heparin as anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated by buoyant density-gradient centrifugation on Lymphoflot (Bio-Rad Medical Diagnostics GmbH, Dreieich, Germany). After centrifugation, the PBMCs were washed three times in sterile phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}), and then suspended in 95% fetal calf serum (CytoGen GmbH, Sinn, Germany) containing 5% DMSO (Sigma-Aldrich, St. Gallen, Switzerland) and cryopreserved prior to use.

4.4. Determination of Immune Checkpoints (PD-1 and CTLA-4), CD28 and CD69 Expression

Multicolor flow cytometry was used to analyze the PBMCs of MM patients and healthy controls for the expression of PD-1 and CTLA-4 proteins in pooled $CD4^+$ T cells as well as their subsets: Treg cells ($CD4^+CD127^-$ cells) and Teff cells ($CD4^+CD127^+$ cells). The expression of CD28 and CD69 molecules was detected on gated $CD4^+$ T cells. According to standard protocols, isolated PBMCs were stained with several combinations of fluorochrome-conjugated monoclonal primary antibodies (mAbs) purchased from Pharmingen (Pharmingen, BD Biosciences, San Diego, CA, USA): FITC anti-human CD3 (Catalog #555332), FITC anti-human CD127 (Catalog # 560549), PerCP anti-human CD4 (Catalog #566924), PE anti-human PD-1 (Catalog #560795), PE anti-human CTLA-4 (Catalog #555853), PE anti-human CD69 (Catalog #555531), and PE anti-human CD28 (Catalog #555729). Appropriate fluorochrome-labeled isotype control antibodies included from Pharmingen (Pharmingen, BD Biosciences, San Diego, CA, USA) were used to confirm expression specificity and for gate settings in each case (FITC mouse IgG1: Catalog #555748, PE mouse IgG1: Catalog #554680, PE mouse IgG2a: Catalog #555574, PE mouse IgG1: Catalog #555749). Briefly, refrozen PBMCs were washed twice in PBS, divided into tubes at a concentration of 5×10^5 cells per tube, and incubated with appropriate antibodies for 30 min at 4 °C in the dark. Excess unbound antibodies were removed by two washes with PBS. Following washing, the cells were fixed in PBS containing 1.5% paraformaldehyde (Catalog #P6148, Sigma-Aldrich, St. Gallen, Switzerland) and analyzed by flow cytometry. Finally, a total of 100,000 events per sample were acquired using a FACSCalibur flow cytometer (Becton-Dickinson, BD Biosciences, San Diego, CA, USA) equipped with Cell Quest software 3.3 (Becton-Dickinson, BD Biosciences, San Diego, CA, USA). Data were analyzed by Cell Quest software and the results were expressed as the proportions of $CD3^+CD4^+$ ($CD4^+$ T cells) as well as $CD4^+CD127^-$ (Treg) and $CD4^+CD127^+$ (Teff) cells co-expressing PD-1 or CTLA-4. The percentages of $CD4^+$ T cells co-expressing CD69 and the frequencies of $CD4^+CD28^-$ T cells were also examined. The gating strategy is presented in Figure S1 in supplementary materials. In order to demonstrate quantitative expression of immune checkpoints as well as CD69 protein at the single-cell levels, the results are shown as mean fluorescence intensity (MFI) values and expressed in arbitrary units (AU).

4.5. Statistical Analysis

Statistical analyses of the clinical data and laboratory findings were performed using the Statistica 7.1 package (TIBCO Software Inc., Palo Alto, CA, USA) and GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA). Clinical parameters were presented as absolute numbers and percentages for frequencies. For all other analyzed variables, the median values and 25th and 75th interquartile ranges (IQ ranges) were calculated. As collected data were not normally distributed and/or had heterogeneous variances, differences between examined groups were evaluated using a one-way analysis of variance (ANOVA), with the Kruskal–Wallis or the Mann–Whitney U test as nonparametric alternatives. The Kaplan–Meier method was used to plot survival curves and the difference between curves was calculated by the log-rank test. A p value ≤ 0.05 was considered significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24065730/s1.

Author Contributions: Conceptualization and design of the study, A.K.d.N. and A.K.; methodology, A.K.d.N. and A.K.; data curation, A.K.d.N., L.U.-Z. and I.F.; cell culture, fluorescence staining, and cytometric analysis (investigation), L.C., E.P. and A.K.; analysis or interpretation of data, A.K.d.N., L.C., E.P., M.S. and A.K.; statistical analysis and validation, L.C. and E.P.; visualization, L.C. and M.S.; manuscript writing—original draft preparation, A.K.d.N. and A.K.; writing—review and editing, A.K.d.N., L.C., L.U.-Z., I.F., E.P., M.S. and A.K.; supervision, A.K.; funding acquisition, A.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a grant from the Wroclaw Centre of Biotechnology, the Leading National Research Centre (KNOW) program for the years 2014–2018 (grant no. 132/2017/KNOW/IITD to A.K.).

Institutional Review Board Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (permission no. KB-528/2017 from Wroclaw Medical University) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analyzed during this study are available on request from the corresponding author.

Acknowledgments: We are grateful to our patients and healthy subjects for their blood donations and agreement to participate in this study. We also acknowledge Aleksandra Szteblich for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Favaloro, J.; Brown, R.; Aklilu, E.; Yang, S.; Suen, H.; Hart, D.; Fromm, P.; Gibson, J.; Khoo, L.; Ho, P.J.; et al. Myeloma skews regulatory and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. *Leuk. Lymphoma* **2015**, *55*, 1090–1098. [CrossRef] [PubMed]
2. Favaloro, J.; Liyadipitiya, T.; Brown, R.; Yang, S.; Suen, H.; Woodland, N.; Nassif, N.; Hart, D.; Fromm, P.; Weatherburn, C.; et al. Myeloid derived suppressor cells are numerically, functionally, and phenotypically different in patients with multiple myeloma. *Leuk. Lymphoma* **2014**, *55*, 2893–2900. [CrossRef]
3. Brown, R.; Suen, H.; Favaloro, J.; Yang, S.; Ho, P.J.; Gibson, J.; Joshua, D. Trogocytosis generates acquired regulatory T cells adding further complexity to the dysfunctional immune response in multiple myeloma. *Oncoimmunology* **2012**, *1*, 1658–1660. [CrossRef]
4. Brown, R.D.; Pope, B.; Murray, A.; Esdale, W.; Sze, D.M.; Gibson, J.; Ho, P.J.; Hart, D.; Joshua, D. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-β_1 and interleukin-10. *Blood* **2001**, *98*, 2992–2998. [CrossRef]
5. Bryant, C.; Suen, H.; Brown, R.; Yang, S.; Favaloro, J.; Aklilu, E.; Gibson, J.; Ho, P.J.; Iland, H.; Fromm, P.; et al. Long-term survival in multiple myeloma is associated with a distinct immunological profile, which includes proliferative cytotoxic T cell clones and a favourable Treg/Th17 balance. *Blood Cancer J.* **2013**, *3*, e148. [CrossRef] [PubMed]
6. Kulikowska de Nalecz, A.; Ciszak, L.; Usnarska-Zubkiewicz, L.; Frydecka, I.; Pawlak, E.; Szmyrka, M.; Kosmaczewska, A. Deregulated Expression of Immune Checkpoints on Circulating CD4 T Cells May Complicate Clinical Outcome and Response to Treatment with Checkpoint Inhibitors in Multiple Myeloma Patients. *Int. J. Mol. Sci.* **2021**, *22*, 9298. [CrossRef]
7. Dosani, T.; Carlsten, M.; Maric, I.; Landgren, O. The cellular immune system in myelogenesis: NK and T cells in the development of myeloma and their uses in immunotherapies. *Blood Cancer J.* **2015**, *5*, e306. [CrossRef]
8. Kawano, Y.; Zavidij, O.; Park, J.; Moschetta, M.; Kokubun, K.; Mouhieddine, T.H.; Manier, S.; Mishima, Y.; Murakami, N.; Bustoros, M.; et al. Blocking IFNAR1 inhibits multiple myeloma-driven Treg expansion and immunosuppression. *J. Clin. Investig.* **2018**, *128*, 2487–2499. [CrossRef] [PubMed]
9. Doering, T.A.; Crawford, A.; Angelosanto, J.M.; Paley, M.A.; Ziegler, C.G.; Wherry, E.J. Network analysis reveals centrally connected genes and pathways involved in $CD8^+$ T cell exhaustion versus memory. *Immunity* **2012**, *37*, 1130–1144. [CrossRef]
10. Wherry, E.J.; Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* **2015**, *15*, 486–499. [CrossRef]
11. Suen, H.; Brown, R.; Yang, S.; Ho, P.J.; Gibson, J.; Joshua, D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase I study. *Leukemia* **2015**, *29*, 1621–1622. [CrossRef] [PubMed]
12. Zeller-Rieser, C.; Thangavadivel, S.; Biedermann, R.; Brunner, A.; Stoitzner, P.; Willenbacher, E.; Greil, R.; Jöhrer, K. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. *J. Hematol. Oncol.* **2016**, *9*, 116. [CrossRef] [PubMed]
13. Pawelec, G. Is there a positive side to T cell exhaustion? *Front. Immunol.* **2019**, *10*, 111. [CrossRef]

14. Reiser, J.; Banerjee, A. Effector, memory, and dysfunctional CD8+ T cell fates in the antitumor immune response. *J. Immunol. Res.* **2016**, *2016*, 8941260. [CrossRef] [PubMed]
15. Akbar, A.; Henson, S. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat. Rev. Immunol.* **2011**, *11*, 289–295. [CrossRef]
16. Webb, E.S.; Liu, P.; Baleerino, R.; Lemoine, N.R.; Yuan, M.; Wang, Y. Immune checkpoint inhibitors in cancer therapy. *J. Biomed. Res.* **2018**, *32*, 317–326.
17. Mateos, M.V.; Blacklock, H.; Schjesvold, F.; Rocafiguera, A.O.; Simpson, D.; George, A.; Goldschmidt, H.; LaRocca, A.; Sherbenou, D.W.; Avivi, I.; et al. A phase 3 randomized study of pembrolizumab (Pembro) plus pomalidomide (Pom) and dexamethasone (Dex) for relapsed/refractory multiple myeloma (RRMM): Keynote-183. *J. Clin. Oncol.* **2018**, *36*, 8021. [CrossRef]
18. Usmani, S.Z.; Schjesvold, F.; Rocafiguera, A.O.; Karlin, L.; Rifkin, R.M.; Yimer, H.A.; Leblanc, R.; Takezako, N.; McCroskey, R.D.; Suzuki, K.; et al. A phase 3 randomized study of pembrolizumab (pembro) plus lenalidomide (len) and low-dose dexamethasone (Rd) versus Rd for newly diagnosed and treatment-naïve multiple myeloma (MM): Keynote-185. *J. Clin. Oncol.* **2018**, *36*, 8010. [CrossRef]
19. Bertamini, L.; Gay, F. Checkpoint inhibitors and myeloma: Promises, deadlocks and new directions. *Ann. Transl. Med.* **2020**, *8*, 777. [CrossRef]
20. Alkharabsheh, O.; Trisel, Z.; Badami, S.; Aljama, M.A.; Sidiqi, M.H. Checkpoint inhibitors in multiple myeloma: Intriguing potential and unfulfilled promises. *Cancers* **2022**, *14*, 113. [CrossRef]
21. Zhao, Y.; Shao, Q.; Peng, G. Exhaustion and senescence: Two crucial dysfunctional states of T cells in the tumor microenvironment. *Cell. Mol. Immunol.* **2020**, *17*, 27–35. [CrossRef] [PubMed]
22. Lanna, A.; Henson, S.M.; Escors, D.; Akbar, A.N. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. *Nat. Immunol.* **2014**, *15*, 965–972. [CrossRef] [PubMed]
23. Tamura, H. Immunopathogenesis and immunotherapy of multiple myeloma. *Int. J. Hematol.* **2018**, *107*, 278–285. [CrossRef] [PubMed]
24. Suen, H.; Brown, R.; Yang, S.; Weatherburn, C.; Ho, P.J.; Woodland, N.; Nassif, N.; Barbaro, P.; Bryant, C.; Hart, D.; et al. Multiple myeloma causes clonal T cell immunosenescence: Identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* **2016**, *30*, 1716–1724. [CrossRef]
25. Kasamatsu, T.; Awata, M.; Ishihara, R.; Murakami, Y.; Gotoh, N.; Matsumoto, M.; Sawamura, M.; Yokohama, A.; Handa, H.; Tsukamoto, N.; et al. PDCD1 and PDCD1LG1 polymorphisms affect the susceptibility to multiple myeloma. *Clin. Exp. Med.* **2020**, *20*, 51–62. [CrossRef]
26. Karabon, L.; Pawlak-Adamska, E.A.; Tomkiewicz, A.; Jedynak, A.; Kielbinski, M.; Woszczyk, D.; Potoczek, S.; Jonkisz, A.; Kuliczkowski, K.; Frydecka, I. Variations in suppressor molecule CTLA-4 gene are related to susceptibility to multiple myeloma in a Polish population. *Pathol. Oncol. Res.* **2011**, *18*, 219–226. [CrossRef]
27. Zheng, C.; Huang, D.; Liu, L.; Bjorkholm, M.; Holm, G.; Yi, Q.; Sundblad, A. Cytotoxic T-lymphocyte antigen-4 microsatellite polymorphism is associated with multiple myeloma. *Br. J. Haematol.* **2001**, *112*, 216–218. [CrossRef]
28. Ciszak, L.; Frydecka, I.; Wolowiec, D.; Szteblich, A.; Kosmaczewska, A. Patients with chronic lymphocytic leukemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: The possible implications for immunotherapy with CTLA-4 blocking antibody. *Tumor Biol.* **2016**, *37*, 4143–4157. [CrossRef]
29. Coppe, J.P.; Desprez, P.Y.; Krtolica, A.; Campisi, J. The senescence-associated secretory phenotype: The dark side of tumor suppression. *Annu. Rev. Pathol.* **2010**, *5*, 99–118. [CrossRef]
30. Lanna, A.; Gomes, D.C.; Muller-Durovic, B.; McDonnell, T.; Escors, D.; Gilroy, D.W.; Lee, J.H.; Karin, M.; Akbar, A.N. A sestrin-dependent Erk-Jnk-p38 MAPK activation complex inhibits immunity during aging. *Nat. Immunol.* **2017**, *18*, 354–363. [CrossRef]
31. Liu, X.; Mo, W.; Ye, J.; Li, L.; Zhang, Y.; Hsueh, E.C.; Hoft, D.F.; Peng, G. Regulatory T cells trigger effector T cell DNA damage and senescence caused by metabolic competition. *Nat. Commun.* **2018**, *9*, 249. [CrossRef] [PubMed]
32. Paluch, C.; Santos, A.M.; Anzilotti, C.; Cornall, R.J.; Davis, S.J. Immune checkpoints as therapeutic targets in autoimmunity. *Front. Immunol.* **2018**, *9*, 2306. [CrossRef] [PubMed]
33. Asano, T.; Meguri, Y.; Yoshioka, T.; Kishi, Y.; Iwamoto, M.; Nakamura, M.; Sando, Y.; Yagita, H.; Koreth, J.; Kim, H.T.; et al. PD-1 modulates regulatory T-cell homeostasis during low-dose interleukin-2 therapy. *Blood* **2017**, *129*, 2186–2197. [CrossRef] [PubMed]
34. Francisco, L.M.; Salinas, V.H.; Brown, K.E.; Vanguri, V.K.; Freeman, G.J.; Kuchroo, V.K.; Sharpe, A.H. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* **2009**, *206*, 3015–3029. [CrossRef] [PubMed]
35. Stathopoulou, C.; Gangaplara, A.; Mallett, G.; Flomerfelt, F.A.; Liniany, L.P.; Knight, D.; Samsel, L.A.; Berlinguer-Palmini, R.; Yim, J.J.; Felizardo, T.C.; et al. PD-1 inhibitory receptor downregulates asparaginyl endopeptidase and maintains Foxp3 transcription factor stability in induced regulatory T cells. *Immunity* **2018**, *49*, 247–263.e7. [CrossRef]
36. Thewissen, M.; Somers, V.; Hellings, N.; Fraussen, J.; Damoiseaux, J.; Stinissen, P. $CD4^+CD28^{null}$ T cells in autoimmune disease: Pathogenic features and decreased susceptibility to immunoregulation. *J. Immunol.* **2007**, *179*, 6514–6523. [CrossRef]
37. Kosmaczewska, A.; Ciszak, L.; Stosio, M.; Szteblich, A.; Madej, M.; Frydecka, I.; Wiland, P.; Szmyrka, M. $CD4^+CD28^{null}$ T cells are expanded in moderately active systemic lupus erythematosus and secrete pro-inflammatory interferon gamma, depending on the Disease Activity Index. *Lupus* **2020**, *29*, 705–714. [CrossRef]

38. Perez-Andres, M.; Almeida, J.; Martin-Ayuso, M.; Moro, M.J.; Martin-Nunez, G.; Galende, J.; Hernandez, J.; Mateo, G.; San Miguel, J.F.; Orfao, A. Characterization of bone marrow T cells in monoclonal gammopathy of undetermined significance, multiple myeloma, and plasma cell leukemia demonstrates increased infiltration by cytotoxic/Th1 T cells demonstrating a squed TCR-Vβ repertoire. *Cancer* **2006**, *106*, 1296–1305. [CrossRef]
39. Lindqvist, E.K.; Landgren, O.; Lund, S.H.; Turesson, I.; Hultcrantz, M.; Goldin, L.; Björkholm, M.; Kristinsson, S.Y. History of autoimmune disease is associated with impaired survival in multiple myeloma and monoclonal gammopathy of undetermined significance: A population-based study. *Ann. Hematol.* **2017**, *96*, 261–269. [CrossRef]
40. Shimanovsky, A.; Alvarez, A.J.; Murali, S.; Dasanu, C.A. Autoimmune manifestations in patients with multiple myeloma and monoclonal gammopathy of undetermined significance. *BBA Clin.* **2016**, *6*, 12–18. [CrossRef]
41. Salik, B.; Smyth, M.J.; Nakamura, K. Targeting immune checkpoints in hematological malignancies. *J. Hematol. Oncol.* **2020**, *13*, 111. [CrossRef] [PubMed]
42. Martínez-Calle, N.; Rodriguez-Otero, P.; Villar, S.; Mejías, L.; Melero, I.; Prosper, F.; Marinello, P.; Paiva, B.; Idoate-Gastearena, M.; Miguel, J.S. Anti-PD-1 associated fulminate myocarditis after a single pembrolizumab dose: The role of occult pre-existing autoimmunity. *Haematologica* **2018**, *103*, e318. [CrossRef] [PubMed]
43. Kumar, S.K.; Rajkumar, S.V.; Dispenzieri, A.; Lacy, M.Q.; Hayman, S.R.; Buadi, F.K.; Zeldenrust, S.R.; Dingli, D.; Russell, S.J.; Lust, J.A.; et al. Improved survival in multiple myeloma and the impact of novel therapies. *Blood* **2008**, *111*, 2516–2520. [CrossRef]
44. Batorov, E.V.; Aristova, T.A.; Sergeevicheva, V.V.; Sizikova, S.A.; Ushakova, G.A.; Pronkin, N.V.; Shishkova, I.V.; Shevela, E.Y.; Ostanin, A.A.; Chernykh, E.R. Quantitative and functional characteristics of circulating and bone marrow PD-1- and TIM-3-positive T cells in treated multiple myeloma patients. *Sci. Rep.* **2020**, *10*, 20846. [CrossRef] [PubMed]
45. Brown, R.; Yuen, E.; Nelson, M.; Gibson, J.; Joshua, D. The prognostic significance of T cell receptor beta gene rearrangements and idiotype-reactive T cells in multiple myeloma. *Leukemia* **1997**, *11*, 1312–1317. [CrossRef]
46. Joshua, D.; Brown, R.; Sze, D.M.; Raitakari, M.; Gibson, J.; Ho, P.J.; Yang, S.; Yuen, E.; Murray, A. The role of T cells in myeloma. *Hematol. J.* **2003**, *4*, S26–S28.
47. Li, Y.; Yang, L.J.; Chen, S.H.; Zhang, Y.P.; Zhang, X.L.; Luo, G.X. T cell receptor Vbeta repertoire usage and clonal expansion of T cells in chronic myelogenous leukemia. *Chin. Med. J.* **2004**, *117*, 840–843.
48. Mustjoki, S.; Ekblom, M.; Arstila, T.P.; Dybedal, I.; Epling-Burnette, P.K.; Guilhot, F.; Hjorth-Hansen, H.; Hoglund, M.; Kovanen, P.; Laurinolli, T.; et al. Clonal expansion of T/NK-cells during tyrosine kinase inhibitor dasatinib therapy. *Leukemia* **2009**, *23*, 1398–1405. [CrossRef]
49. Li, J.; Sze, D.M.-Y.; Brown, R.D.; Cowley, M.J.; Kaplan, W.; Mo, S.-L.; Yang, S.; Aklilu, E.; Kabani, K.; Loh, Y.S.; et al. Clonal expansions of cytotoxic T cells exist in the blood of patients with Waldenstrom macroglobulinemia but exhibit anergic properties and are eliminated by nucleoside analogue therapy. *Blood* **2010**, *115*, 3580–3588. [CrossRef]
50. Epling-Burnette, P.; Painter, J.S.; Rollison, D.E.; Ku, E.; Vendron, D.; Widen, R.; Boulware, D.; Zou, J.X.; Bai, F.; List, A. Prevalence and clinical association of clonal T-cell expansion in Myelodysplastic Syndrome. *Leukemia* **2007**, *21*, 659–667. [CrossRef]
51. Vries, A.D.; Langerak, A.W.; Verhaaf, B.; Niemeyer, C.M.; Stary, J.; Schmiegelow, K.; Van Wering, E.R.; Zwaan, C.M.; Beishuizen, A.; Pieters, R.; et al. T-cell receptor Vβ CDR3 oligoclonality frequently occurs in childhood refractory cytopenia (MDS-RC) and severe aplastic anemia. *Leukemia* **2008**, *22*, 1170–1174. [CrossRef] [PubMed]
52. Brown, R.; Spencer, A.; Ho, P.J.; Kennedy, N.; Kabani, K.; Yang, S.; Sze, D.M.; Aklilu, E.; Gibson, J.; Joshua, D.E. Prognostically significant cytotoxic T cell clones are stimulated after thalidomide therapy in patients with multiple myeloma. *Leuk. Lymph.* **2009**, *50*, 1860–1864. [CrossRef] [PubMed]
53. Sze, D.M.-Y.; Giesajtis, G.; Brown, R.D.; Raitakari, M.; Gibson, J.; Ho, J.; Baxter, A.; Groth, B.F.D.S.; Basten, A.; Joshua, D.E. Clonal cytotoxic T cells are expanded in myeloma and reside in the $CD8^+CD57^+CD28^-$ compartment. *Blood* **2001**, *98*, 2817–2827. [CrossRef] [PubMed]
54. Alrasheed, N.; Lee, L.; Ghorani, E.; Henry, J.Y.; Conde, L.; Chin, M.; Galas-Filipowicz, D.; Furness, A.J.; Chavda, S.J.; Richards, H.; et al. Marrow infiltrating regulatory T cells correlate with the presence of dysfunctional $CD4^+PD\text{-}1^+$ cells and inferior survival in patients with newly diagnosed multiple myeloma. *Clin. Cancer Res.* **2020**, *26*, 3443–3454. [CrossRef]
55. Tan, S.; Day, D.; Nicholls, S.J.; Segelov, E. Immune checkpoint inhibitor therapy in oncology: Current uses and future directions. *J. Am. Coll. Cardiol. Cardio Onc.* **2022**, *4*, 579–597.
56. Koyama, S.; Akbay, E.A.; Li, Y.Y.; Herter-Sprie, G.S.; Buczkowski, K.A.; Richards, W.G.; Gandhi, L.; Redig, A.J.; Rodig, S.J.; Asahina, H.; et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat. Commun.* **2016**, *7*, 10501. [CrossRef]
57. Gao, J.; Ward, J.F.; A Pettaway, C.; Shi, L.Z.; Subudhi, S.K.; Vence, L.M.; Zhao, H.; Chen, J.; Chen, H.; Efstathiou, E.; et al. VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer. *Nat. Med.* **2017**, *23*, 551–555. [CrossRef]
58. Smith, W.M.; Purvis, I.J.; Bomstad, C.N.; Labal, C.M.; Velpula, K.K.; Tsung, A.J.; Regan, J.N.; Venkataraman, S.; Vibhakar, R.; Asuthkar, S. Therapeutic targeting of immune checkpoints with small molecule inhibitors. *Am. J. Transl. Res.* **2019**, *11*, 529–541.
59. Sasikumar, P.G.; Sudarshan, N.S.; Adurthi, S.; Ramachandra, R.K.; Samiulla, D.S.; Lakshminarasimhan, A.; Ramanathan, A.; Chandrasekhar, T.; Dhudashiya, A.A.; Talapati, S.R.; et al. PD-1 derived CA-170 is an oral immune checkpoint inhibitor that exhibits preclinical anti-tumor efficacy. *Commun. Biol.* **2021**, *4*, 699. [CrossRef]

60. Guo, L.; Overholser, J.; Darby, H.; Ede, N.J.; Kaumaya, P.T.P. A newly discovered PD-L1 B-cell epitope peptide vaccine (PDL1-Vaxx) exhibits potent immune responses and effective anti-tumor immunity in multiple syngeneic mice models and (synergizes) in combination with a dual HER-2 B-cell vaccine (B-vaxx). *Oncoimmunology* **2022**, *11*, e2127691. [CrossRef]
61. Woods, D.M.; Sodré, A.L.; Villagra, A.; Sarnaik, A.; Sotomayor, E.M.; Weber, J. HDAC inhibition upregulates PD-1 ligands in melanoma and augments immunotherapy with PD-1 blockade. *Cancer Immunol. Res.* **2015**, *3*, 1375–1385. [CrossRef] [PubMed]
62. Sabatier, R.; Finetti, P.; Mamessier, E.; Adelaide, j.; Chaffanet, m.; Ali, H.R.; Viens, P.; Caldas, C.; Birnbaum, D.; Bertucci, F. Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget* **2015**, *6*, 5449–5464. [CrossRef] [PubMed]
63. Terranova-Barberio, M.; Thomas, S.; Munster, P.N. Epigenetic modifiers in immunotherapy: A focus on checkpoint inhibitors. *Immunotherapy* **2016**, *8*, 705–719. [CrossRef] [PubMed]
64. Frys, S.; Simons, Z.; Hu, Q.; Barth, M.J.; Gu, J.J.; Mavis, C.; Skitzki, J.; Song, L.; Czuczman, M.S.; Hernandez-Ilizaliturri, F.J. Entinostat, a novel histone deacetylase inhibitor is active in B-cell lymphoma and enhances the anti-tumour activity of rituximab and chemotherapy agents. *Br. J. Haematol.* **2015**, *169*, 506–519. [CrossRef]
65. Orillion, A.; Hashimoto, A.; Damayanti, N.; Shen, L.; Adelayie-Ogala, R.; Arisa, S.; Chintala, S.; Ordentlich, P.; Kao, C.; Elzey, B.; et al. Entinostat neutralizes myeloid-derived suppressor cells and enhances the anti-tumor effect of PD-1 inhibition in murine models of lung and renal cell carcinoma. *Clin. Cancer Res.* **2017**, *23*, 5187–5201. [CrossRef] [PubMed]
66. Azad, N.S.; El-Khoueiry, A.; Yin, J.; Oberg, A.L.; Flynn, P.; Adkins, D.; Sharma, A.; Weisenberger, D.J.; Brown, T.; Medvari, P.; et al. Combination epigenetic therapy in metastatic colorectal cancer (mCRC) with subcutaneous 5-azacitidine and entinostat: A phase 2 consortium/stand up 2 cancer study. *Oncotarget* **2017**, *8*, 35326–35338. [CrossRef]
67. Chiappinelli, K.B.; Zahnow, C.A.; Ahuja, N.; Baylin, S.B. Combining epigenetic and immunotherapy to combat cancer. *Cancer Res.* **2016**, *76*, 1683–1689. [CrossRef]
68. Rajkumar, S.V.; Dimopoulos, M.A.; Palumbo, A.; Blade, J.; Merlini, G.; Mateos, M.V.; Kumar, S.; Hillengass, J.; Kastritis, E.; Richardson, P.; et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* **2014**, *15*, e538–e548. [CrossRef]
69. Greipp, P.R.; San Miguel, J.; Durie, B.G.; Crowley, J.J.; Barlogie, B.; Bladé, J.; Boccadoro, M.; Child, J.A.; Avet-Loiseau, H.; Kyle, R.A.; et al. International staging system for multiple myeloma. *J. Clin. Oncol.* **2005**, *23*, 3412–3420. [CrossRef]

Table S1. Patient clinical and laboratory characteristics.

Parameters	Newly Diagnosed (NDMM)	Relapsed/Refractory (RRMM)	Total
Number of patients, n (%)	26 (65%)	14 (35%)	40 (100%)
Age of patients, years			
median (range)	66 (50-76)	72 (65-75)	69 (59-76)
Gender , n (%)			
Female	17 (65.38%)	4 (28.57%)	21 (52.5%)
Male	9 (34.62%)	10 (71.43%)	19 (47.5%)
ISS stage at diagnosis, n (%)			
I	5 (19.23%)	1 (7.14%)	6 (15.00%)
II	10 (38.46%)	6 (42.86%)	16 (40.00%)
III	11 (42.31%)	7 (50.00%)	18 (45.00%)
Type of myeloma, n (%)			
IgG	18 (69.23%)	9 (64.28%)	27 (67.50%)
IgA	3 (11.54%)	3 (21.43%)	6 (15.00%)
Light chain disease	5 (19.23%)	2 (14.29%)	7 (17.50%)
Type of Ig light chain (serum), n (%)			
Kappa	16 (61.54%)	7 (50.00%)	23 (57.50%)
Lambda	9 (34.62%)	7 (50.00%)	16 (40.00%)
Unknown	1 (3.84%)	0 (0.00%)	1 (2.50%)
Osteolytic bone lesion/s, n (%)			
Present	15 (57.69%)	13 (92.86%)	28 (70.00%)
Absent	11 (42.31%)	1 (7.14%)	12 (30.00%)
Morphological indicators, n (%)			
Hemoglobin ≤ 12 g/dL	23 (88.47%)	8 (57.14%)	31 (77.50%)
Platelets < 100,000/mm^3	2 (7.69%)	1 (7.14%)	3 (7.50%)
Biochemical indicators, n (%)			
β-2 microglobulin ≥ 3.5 mg/L	18 (69.23%)	9 (64.29%)	27 (67.50%)
Creatinine ≥ 2.0 mg/dL	9 (34.62%)	4 (28.57%)	13 (32.50%)
LDH > 190 U/L	6 (23.08%)	2 (14.29%)	8 (20.00%)
Serum calcium ≥ 10 mg/dL	9 (34.62%)	11 (78.57%)	20 (50.00%)
Prior treatment, n (%)			
1-3 therapy lines	0 (0%)	8 (57.14%)	8 (20.00%)
≥ 4 therapy lines	0 (0%)	6 (42.86%)	6 (15.00%)
BTZ based therapy	0 (0%)	12 (85.71%)	12 (30.00%)
IMiD therapy	0 (0%)	11 (78.57%)	11 (27.50%)
No therapy	26 (100%)	0 (0%)	26 (65.00%)

Abbreviations: BTZ, bortezomib; LDH, lactate dehydrogenases; ISS, International Staging System; IMiD, immunomodulatory drug.

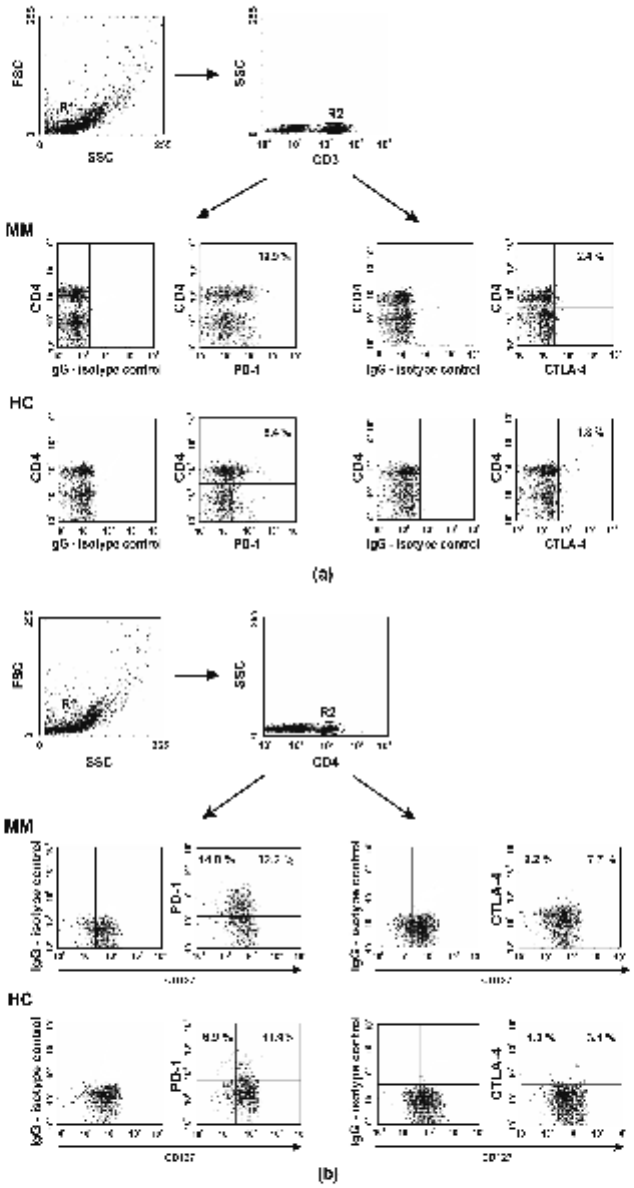

Figure S1. Representative dot plots from MM patient and healthy control (HC). Dot plots demonstrate the gating strategy for assessing the proportion of $CD4^+$ T cells ($CD3^+CD4^+$), Treg ($CD4^+CD127^-$) and Teff ($CD4^+CD127^+$) cells co-expressing PD-1 or CTLA-4 protein. (a) Dot plots show the method for analyzing PD-1 or CTLA-4 positive $CD4^+$ T cells. Lymphocytes were gated (R1) based on their FSC/SSC properties, and then T cells were identified on the SSC/CD3 profile (R2). The R2 gated events were next analyzed for CD4 and PD-1 or CTLA-4 staining. Numbers on dot plots show the percentage of PD-1 or CTLA-4 positive $CD4^+$ T cells. (b) Dot plots show the method for analyzing PD-1 or CTLA-4 positive Treg and Teff cells. The R1 gated lymphocytes were subsequent gated on SSC/CD4 (R2) to identify $CD4^+$ cells. The R2 gated populations were then analyzed for CD127 and PD-1 or CTLA-4 staining. Numbers on dot plots show the percentage of PD-1 or CTLA-4 positive Treg and Teff cells.

VIII. PODSUMOWANIE WYNIKÓW

Zaburzenia ekspresji immunologicznych punktów kontrolnych mogą stanowić cechę fenotypową komórek starzejących się lub wyczerpanych i, jak wykazano we wcześniejszych badaniach, limfocyty T pacjentów z MM mogą wykazywać cechy charakterystyczne dla obu tych stanów funkcjonalnych jednocześnie[13-15]. Mimo że starzenie oraz wyczerpanie funkcjonalne limfocytów T przyczynia się istotnie do osłabienia odpowiedzi immunologicznej i rozwoju immunosupresji systemowej, warto zaznaczyć występowanie istotnych różnic w charakterystyce fenotypowej, funkcjonalnej oraz mechanizmach prowadzących do rozwoju obu dysfunkcji, uwzględniając różnicę w poziomie ekspresji cząsteczek supresorowych (głównie PD-1 i CTLA-4) i w konsekwencji różny potencjał do uzyskania poprawy odpowiedzi przeciwnowotworowej po zastosowaniu przeciwciał blokujących te receptory[13-15]. Identyfikacja dominującego stanu dysfunkcjonalnego w limfocytach T w różnych fazach MM z uwzględnieniem ekspresji punktów kontrolnych może mieć implikacje z punktu widzenia przewidywania odpowiedzi klinicznej na immunoterapię z zastosowaniem przeciwciał blokujących immunologiczne punkty kontrolne, która może być różna w określonej fazie choroby.

W pracy pt. „Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhbititors in multiple myeloma patients” oceniano ekspresję receptorów supresorowych pełniących rolę immunologicznych punktów kontrolnych w nadzorze immunologicznym, czyli PD-1, CTLA-4 i BTLA, na powierzchni obwodowych limfocytów T CD4 – zarówno w subpopulacji komórek efektorowych (Teff), jak i regulatorowych (Treg) - u pacjentów z aktywnym MM w różnych fazach choroby: w chwili zdiagnozowania (NDMM) i w czasie nawrotu choroby (RRMM). Analizie poddano też związek ekspresji badanych supresorów z cechami funkcjonalnymi limfocytów T CD4 charakterystycznymi dla immunostarzenia i wyczerpania. Zaobserwowano, że PD-1 jest jedynym białkiem inhibitorowym występującym w istotnie statystycznie wyższym odsetku limfocytów T CD4 chorych na MM na każdym etapie choroby. Wyraźne nasilenie dystrybucji wszystkich badanych punktów kontrolnych w obrębie populacji limfocytów Treg w RRMM wskazuje, że progresji choroby towarzyszy większa zdolność do nabywania ekspresji cząsteczek supresorowych, co może odpowiadać fenotypowi komórek wyczerpanych[23]. Z kolei analiza ilościowa pozwoliła zauważyć deficyt ekspresji PD-1, CTLA-4 i BTLA w limfocytach T CD4 u pacjentów, najbardziej wyraźny w grupie z NDMM. Jak wskazuje literatura, obniżony poziom ekspresji białka PD-1 i CTLA-4

zaobserwowany u chorych na MM stanowi cechę charakterystyczną dla starzenia się komórek immunologicznych, przy czym proces ten w MM jest niezależny od długości telomerów[13-15]. W przeprowadzonym badaniu potwierdzono, że etap rozwoju MM jest wyraźnie związany z utratą ekspresji PD-1, CTLA-4 i BTLA na limfocytach T CD4. Wykazano bowiem, że poziom badanych supresorów w grupie NDMM był statystycznie istotnie niższy w porównaniu do grupy kontrolnej osób zdrowych (HC), a w przypadku CTLA-4 również w porównaniu do grupy chorych z RRMM. Suboptymalny poziom ekspresji punktów kontrolnych na limfocytach T CD4 w MM może okazać się niewystarczający do uzyskania efektywnej poprawy odpowiedzi przeciwnowotworowej po zastosowania terapeutycznych inhibitorów PD-1 i/lub CTLA-4, szczególnie w grupie NDMM, co obserwowano w badaniach klinicznych u przeważającego odsetka chorych na MM[6, 7]. Równoczesna analiza ekspresji markera aktywacji CD69 w limfocytach T CD4 wykazała zwiększony odsetek komórek T CD4+CD69+ *in vivo* (głównie u chorych z RRMM) z jednoczesnym obniżonym jego poziomem w komórkach w grupie NDMM w porównaniu do grupy kontrolnej (HC). Dalsza stymulacja poliklonalna komórek w warunkach hodowli *in vitro* potwierdziła hyporeaktywność limfocytów T CD4 u wszystkich pacjentów z MM.

Ciekawych obserwacji dostarczyły również badania funkcjonalne, które pokazały nie tylko istotnie zmniejszoną u wszystkich pacjentów wielkość populacji efektorowych limfocytów Th1, ale również obniżoną w nich zdolność do syntezy IFN-γ. Pomimo braku statystycznie istotnych różnic wśród chorych (pomiędzy grupami NDMM i RRMM), warto podkreślić, że defekt populacji Th1 oraz korespondujący obniżony istotnie stosunek odsetkowy limfocytów Th1/Treg były najbardziej widoczne w grupie pacjentów z progresją MM (RRMM). Obserwacja ta wskazuje wyraźnie na postępujące w przebiegu MM upośledzenie odpowiedzi przeciwnowotworowej zależnej od IFN-γ[23]. Interesujących wyników dostarczyła również ocena wewnątrzkomórkowego stężenia IL-17, cytokiny o działaniu prozapalnym i niejasnej roli w patogenezie MM, w obrębie populacji limfocytów T CD4. Wykazano, że populacja efektorowych limfocytów Th17 u wszystkich pacjentów niezależnie od statusu leczenia (NDMM vs. RRMM) była istotnie większa niż w grupie kontrolnej osób zdrowych (HC). Warto zauważyć, że najwyższy odsetek Th17 obserwowano w grupie chorych z I/II stadium zaawansowania klinicznego (wg ISS), czyli w okresie rozwoju choroby. Również analiza porównawcza stosunku limfocytów Th17/Treg pomiędzy poszczególnymi grupami chorych (NDMM vs RRMM) pozwoliła wyciągnąć podobny wniosek, gdyż najwyższa jego wartość dotyczyła pacjentów z nowo rozpoznanym MM

(NDMM), a różnica w stosunku do grupy RRMM była istotna statystycznie. Przedstawione w pracy wyniki badań funkcjonalnych jasno wskazują, że progresja MM przebiega z osłabieniem funkcji efektorowych limfocytów T CD4, co może odpowiadać funkcjonalnej charakterystyce komórek wyczerpanych (exhausted). Stan wyczerpania charakteryzuje się również narastaniem ekspresji punktów kontrolnych[23-25], co może przywrócić reaktywność limfocytów T na stosowanie zewnątrzkomórkowej blokady sygnałów supresorowych. To ważna i przydatna z klinicznego punktu widzenia obserwacja, gdyż może skutkować poprawą odpowiedzi klinicznej pacjentów z RRMM na terapeutyczną blokadę punktów kontrolnych[17]. Z kolei limfocyty T CD4 w grupie NDMM są funkcjonalnie zaburzone w sposób prowadzący do relatywnie zwiększonej (w porównaniu do grupy RRMM) sekrecji cytokin prozapalnych (IFN-γi IL-17), co może odpowiadać sekrecyjnemu efektorowemu fenotypowi związanemu ze starzeniem komórek, czyli SASP (senescence-associated secretory effector phenotype)[26]. Immunostarzenie limfocytów obserwowane w większym nasileniu w przebiegu procesów rozrostowych jest uważane za alternatywny sposób ucieczki komórek nowotworowych spod nadzoru immunologicznego [27], gdyż wykazano, że starzejące się limfocyty T wydzielają poza cytokinami prozapalnymi również czynniki immunosupresyjne, m.in. IL-10 i TGF-β[28]. Wyraźny wzrost odsetkowy populacji obwodowych limfocytów Treg obserwowany w pracy u wszystkich chorych (i dodatkowo skorelowany negatywnie ze stadiami zaawansowania klinicznego wg ISS) uważany jest za czynnik kreujący środowisko sprzyjające konwersji limfocytów T do komórek starzejących się, w ten sposób promując rozwój nowotworu[28, 29]. Wykazanie dynamicznej zmiany w fenotypowej i funkcjonalnej charakterystyce obwodowych limfocytów T CD4 w różnych etapach MM, tj. w czasie rozwoju (NDMM) i progresji (RRMM) przedstawione w niniejszej rozprawie, wydają się potwierdzać wcześniejszą obserwację, że cytostatyki i leki IMID stosowane w konwencjonalnej terapii MM w preferencyjny sposób usuwają starzejące się limfocyty T przy jednoczesnym zachowaniu/przywracaniu ekspresji immunologicznych punktów kontrolnych[13].

Ponadto u badanych pacjentów z wybranymi czynnikami niekorzystnego przebiegu klinicznego (β2-mikroglobuliny ≥ 3,5 mg/l, izotyp IgA, ISS > 2, albumina < 3,5 g/dl, Ca w surowicy > 10 mg/dl, hemoglobina ≤ 12 g/dl) obserwowano statystycznie istotnie wyższe poziomy ekspresji markerów aktywacji i wyczerpania komórek w limfocytach T CD4 (PD-1, CTLA-4, CD69). Uwzględniając cechy kliniczne niekorzystnego przebiegu i związane z nimi czynniki immunologiczne badano następnie ich wpływ na całkowity czas przeżycia pacjentów (overall survival, OS). Test log-rank pozwolił potwierdzić w badanej grupie

chorych związek czynników złej prognozy, takich jak β2-mikroglobulina > 3 mg/dl, albumina < 3,5 g/dl, ISS > 2, hemoglobina ≤ 12 g/dl, kreatyniny ≥ 2,0 g/dl, wiek > 65 r.ż. z krótszym OS. Nie znaleziono istotnego wpływu izotypu IgA, poziomu Ca, liczby krążących plazmocytów czy aktywności LDH na OS pacjentów. Spośród czynników immunologicznych zaangażowanych w niekorzystny przebieg kliniczny MM, jedynie wysoki odsetek CD4+CD69+ i PD-1+Teff był związany z krótszym OS w postaci tendencji statystycznej. W jednoczynnikowej analizie regresji Coxa znaleziono istotny związek albuminy < 3,5 g/dl, kreatyniny ≥ 2,0 g/dl, β2-mikroglobuliny ≥ 3,5 mg/l, hemoglobiny ≤ 12 g/dl z krótszym OS, natomiast korelacje: hemoglobina ≤ 12 g/dl, ISS > 2, wiek > 65 r.ż. ze złym rokowaniem miały istotność graniczną. Nie znaleziono związku między OS a surowiczym poziomem Ca, LDH, liczbą krążących plazmocytów. Spośród parametrów immunologicznych jedynie wyższy odsetek komórek PD-1+Teff wykazywał tendencję do zwiększania ryzyka zgonu, w związku z czym ten parametr immunologiczny został uwzględniony również w wieloczynnikowej analizie regresji Coxa (zbudowanej zasadniczo w oparciu o zidentyfikowane kliniczne czynniki prognostyczne). Na jej podstawie potwierdzono w badanej grupie pacjentów niezależne znaczenie prognostyczne poziomu albuminy, anemii i starszego wieku pacjentów. Wprawdzie żadnemu z badanych czynników immunologicznych (związanych z ekspresją punktów kontrolnych) nie udało się przypisać znaczenia rokowniczego, należy podkreślić wykazanie w niniejszej pracy związku zwiększonej dystrybucji obwodowej limfocytów T o fenotypie komórek wyczerpanych PD-1+Teff z niekorzystnych przebiegiem klinicznym i tendencją do skróconego OS, co jest zgodne z wcześniejszą obserwacją[30], i sugeruje nadrzędną rolę PD-1 w sieci immunosupresyjnych oddziaływań i rozwoju zaburzonej odporności komórkowej w MM.

Kolejnym etapem badań, których wyniki ukazały się w pracy pt.„Innapropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patients is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors", było określenie znaczenia poziomu ekspresji cząsteczek PD-1 i CTLA-4 w obwodowych limfocytach T CD4 jako potencjalnego czynnika predykcji odpowiedzi na terapeutyczne inhibitory PD-1/PD1-L i CTLA-4. Ocenie poddano TTP, czyli czas przeżycia od momentu rozpoczęcia leczenia do rozpoznania laboratoryjnych i/lub klinicznych cech progresji choroby z ocenzurowanymi (wyeliminowanymi) zgonami z przyczyn innych niż progresja MM. W tym celu rozszerzono analizę kliniczną pacjentów z aktywnym MM o oszacowanie TTP w obu badanych grupach pacjentów (NDMM i RRMM) w zależności od ekspresji PD-1 i

CTLA-4 w obwodowych limfocytach T CD4 (Teff i Treg). W badaniu tym potwierdzono ponadto obserwowany defekt ilościowy ekspresji PD-1 i CTLA-4 oraz CD69 na limfocytach T CD4 w grupie NDMM w porównaniu do kontrolnej grupy osób zdrowych (HC), a w przypadku CTLA-4 również w stosunku do grupy RRMM. Następnie dokonano podziału obu grup pacjentów NDMM i RRMM na 2 podgrupy: z niską lub wysoką ekspresją PD-1 lub CTLA-4. Granicą podziału była wartość mediany ekspresji (określanej jakościowo - jako wartość odsetkowa limfocytów z ekspresja receptora lub ilościowo - jako intensywność fluorescencji badanego receptora MFI). Nie znaleziono zależności TTP od jakościowej ekspresji CTLA-4 w limfocytach T CD4 (mierzonej jako odsetek limfocytów z ekspresją CTLA-4). W grupie wszystkich pacjentów z MM wykazano natomiast wyraźny pozytywny związek poziomu ekspresji (intensywności fluorescencji) CTLA-4 w limfocytach T CD4+ z długością TTP (bardziej wyraźny w subpopulacji Teff). Jednak szczegółowa analiza kliniczna uwzględniająca etap choroby (NDMM i RRMM) pozwoliła zaobserwować, że zależność ta występuje jedynie w grupie NDMM; pacjenci z NDMM z poziomem ekspresji CTLA-4 w limfocytach T CD4+ poniżej mediany mają statystycznie istotnie skrócony TTP w przeciwieństwie do grupy RRMM, w której takiej zależności nie obserwowano. Powyższa obserwacja sugeruje, że blokowanie CTLA-4 może być niekorzystną strategią terapeutyczną w grupie pacjentów z NDMM. W odniesieniu do PD-1, znaleziono szereg negatywnych związków pomiędzy długością TTP a ekspresją PD-1 (zarówno ocenianą jakościowo - jako odsetek komórek PD-1+, jak i ilościowo - jako intensywność fluorescencji PD-1 w komórkach) w całej grupie pacjentów z MM; zauważono, że TTP jest skrócony u pacjentów z wyższym odsetkiem limfocytów CD4+PD-1+, PD-1+Treg, PD-1+Teff oraz fluorescencją (poziomem) PD-1 w komórkach Treg i Teff. Wyniki te kompleksowo i zgodnie z badaniem przeprowadzonym przez Alrasheed et al.[30] wskazują, że pacjenci z wysoką ekspresją PD-1 w limfocytach T CD4 doświadczają progresji MM wcześniej niż pacjenci z niską ekspresja PD-1, i różnice te mają cechy istotności lub tendencji statystycznej. Szczegółowa analiza kliniczna przeprowadzona w niniejszej pracy pozwoliła wskazać, że powyższe zależności dotyczą tylko grupy pacjentów z nawrotową postacią MM (RRMM). W grupie NDMM powyższej zależności nie obserwowano, co łącznie wskazuje, że zastosowanie terapeutycznych przeciwciał blokujących PD-1 może być korzystną klinicznie formą terapii MM dla pacjentów z RRMM, szczególnie tych z wysoką ekspresją PD-1. W pracy tej potwierdzono także suboptymalny poziom aktywacji *in vivo* limfocytów T CD4 w grupie NDMM określony na podstawie ilościowej ekspresji markera aktywacji CD69 w porównaniu do korespondujących komórek osób zdrowych (HC). Pomimo że zauważono również różnicę

w porównaniu do ekspresji CD69 w limfocytach pacjentów RRMM, to nie była ona istotna statystycznie. Ocena reaktywności limfocytów T poprzez określenie ekspresji CD69 ma również wartość predykcyjną w kontekście możliwości zastosowania terapii blokującej punkty kontrolne nadzoru immunologicznego[17, 31]. Obniżony poziom CD69 *in vivo* (oraz stwierdzona w poprzedniej pracy hyporeaktywność limfocytów T CD4 w badaniach *in vitro*) korespondujące dodatkowo z deficytem ekspresji receptorów supresorowych PD-1 i CTLA-4, obserwowane w obu niniejszych pracach u pacjentów z NDMM, sugerują, że hyporeaktywność komórkowa i immunosupresja w czasie rozwoju MM nie wynika z ekspresji badanych punktów kontrolnych.

Ciekawą obserwacją przedstawioną w niniejszej pracy było również wykazanie, że limfocyty T CD4 wszystkich chorych na MM mają statystycznie istotnie zwiększoną populację obwodowych limfocytów CD4+CD28- (z negatywną ekspresją CD28). Wprawdzie ekspansja komórek CD4+CD28- stwierdzana w grupie RRMM była istotnie większa w porównaniu do tej obserwowanej w grupie kontrolnej (HC), to jednocześnie była wyraźnie mniejsza niż w grupie pacjentów z NDMM, co razem wskazuje, że początkowy etap choroby cechuje najwyższy odsetek limfocytów T CD4+CD28-. Podobną obserwację przedstawiono też w innej pracy u pacjentów z MM[13]. Biorąc pod uwagę, że brak ekspresji antygenu CD28 uważany jest za jedną z fenotypowych oznak immunostarzenia komórek[17, 32], stwierdzenie wyraźnie większego odsetka limfocytów T CD4+CD28- w MM potwierdza, że immunostarzenie limfocytów T w przebiegu MM jest dysfunkcjonalnym stanem dominującym we wczesnych etapach choroby (NDMM), co może istotnie obniżać skuteczność kliniczną terapeutycznych inhibitorów punktów kontrolnych w grupie pacjentów z NDMM. Z drugiej strony, literatura wskazuje, że starzejące się limfocyty CD4+CD28- mają właściwości komórek autoreaktywnych i ich ekspansja obwodowa cechuje procesy autoimmunizacyjne, które u pacjentów z MM są stwierdzane statystycznie częściej i w istotny sposób pogarszają rokowanie[33-35].

Wykazanie dodatkowo związku czynników niekorzystnego przebiegu klinicznego ze skróceniem TTP w badanej grupie chorych pozwoliło potwierdzić reprezentatywność kliniczną pacjentów włączonych do badań immunologicznych. W szczególności spośród badanych czynników złej prognozy (wiek > 65 r.ż., ISS > 2, hemoglobina ≤ 12 g/dl, β2-mikroglobulina ≥ 3,5 mg/l, albumina < 3,5 g/dl, kreatynina ≥ 2,0 g/dl, plazmocyty > 5%, LDH > 190 U/l, poziom Ca w surowicy ≥ 10 mg/l) wykazano istotny związek zwiększonego

poziomu β2-mikroglobuliny i krążących plazmocytów oraz anemii z wcześniejszym wystąpieniem progresji MM.

IX. WNIOSKI

1. Ilościowy defekt ekspresji immunologicznych punktów kontrolnych stwierdzony w limfocytach T CD4 u chorych na MM polegający na wyraźnym obniżeniu ich poziomu w grupie NDMM oraz częściowym przywróceniu ekspresji u chorych z RRMM może być niewystarczający do uzyskania istotnej poprawy odpowiedzi przeciwnowotworowej po zastosowaniu terapeutycznych inhibitorów, i stanowić jedną z przyczyn osłabionej odpowiedzi klinicznej na tę formę terapii w MM.

2. Suboptymalny poziom ekspresji badanych punktów kontrolnych z towarzyszącą ekspansją obwodową autoreaktywnych komórek CD4+CD28- stwierdzany u chorych na MM (najbardziej wyraźne w grupie NDMM) mogą świadczyć o upośledzonej tolerancji immunologicznej i nasilonym potencjale do procesów autoimmunizacyjnych zarówno w przebiegu MM, jak i w czasie terapii z użyciem przeciwciał blokujących PD-1 i/lub CTLA-4.

3. Wykazanie związku obniżonego poziomu ekspresji CTLA-4 w limfocytach T CD4 ze skróceniem czasu do progresji (TTP) w grupie NDMM wskazuje, że blokowanie CTLA-4 za pomocą terapeutycznych przeciwciał może być niekorzystną strategią leczniczą dla pacjentów dotychczas nieleczonych (NDMM).

4. Spośród badanych immunologicznych punktów kontrolnych jedynie PD-1 jest stwierdzany na istotnie większym odsetku limfocytów T CD4 niezależnie od etapu klinicznego MM, co może wskazywać na nadrzędną rolę PD-1 w zaburzeniach odporności komórkowej i rozwoju immunosupresji związanej z tą chorobą.

5. Wprawdzie żadnemu z badanych parametrów immunologicznych (związanych z ekspresją punktów kontrolnych) nie udało się przypisać znaczenia rokowniczego, jednak wykazano istotny związek zwiększonego odsetka limfocytów efektorowych T (Teff) ko-ekspresjonujących PD-1 (Teff-PD-1+) z niekorzystnym przebiegiem klinicznym i tendencją do skrócenia całkowitego przeżycia (OS).

6. Stwierdzenie związku zwiększonej ekspresji PD-1 ze skróceniem czasu do progresji (TTP) w grupie RRMM sugeruje, że jest to jedyna grupa pacjentów z MM, w której zastosowanie terapeutycznych inhibitorów osi PD-1/PD1-L może przynieść korzyść

kliniczną, i wskazuje na zasadność oznaczania ekspresji PD-1 przed rozpoczęciem tej formy terapii w nawrotowym MM.

X. WYKAZ STOSOWANYCH SKRÓTÓW

AU	*arbitrary unit,* jednostka arbitrarna
BTLA	*B and T lymphocyte activation attenuator*, strażnik aktywacji limfocytów B i T
Ca	*calcium,* wapń
CAR-T cell	*chimeric antigen receptor T-cell*, limfocyt T z chimerycznym receptorem
CD	*cellular differentiation antigen*, antygen różnicowania komórkowego
CTLA-4	*cytotoxic T cell-associated antygen-4*, antigen-4 cytotoksycznych limfocytów T
DC	*dendritic cell*, komórka dendrytyczna
DNA	*deoxyribonucleic acid*, kwas dezoksyrybonukleinowy
HC	*healthy control*, osoba zdrowa (z grupy kontrolnej)
IC	*immune checkpoint*, immunologiczny punkt kontrolny
ICI	*immune checkpoint inhibitor,* inhibitor immunologicznego punktu kontrolnego
IFN	*interferon*, interferon
IL	*interleukin*, interleukina
IMID	*immunomodulatory drug*, lek immunomodulujący
IMWG	*International Myeloma Working Group*, Międzynarodowa Grupa Robocza ds. Szpiczaka
iRAE	*immune-related adverse event*, autoimmunizacyjny objaw niepożądany
ISS	*International Staging System*, Międzynarodowego Systemu Stopniowania (Szpiczaka)
LDH	*lactate dehydrogenase*, dehydrogenaza mleczanowa
MFI	*mean fluorescence intensity*, średnia intensywność fluorescencji
MM	*multiple myeloma*, szpiczak plazmocytowy
NDMM	*newly diagnosed MM*, nowo rozpoznany MM
NK	*natural killer*, naturalny zabójca
ORR	*overall response rate,* wskaźnik odpowiedzi klinicznej
OS	*overall survival*, czas całkowitego przeżycia
PBMC	*peripheral blood mononuclear cell*, jednojądrzasta komórka krwi obwodowej
PD-1	*programmed cel death-1*, receptor-1 programowanej śmierci komórki,

PI	*proteasome inhibitor*, inhibitor proteasomu
PMA	*phorbol 12-myristate 23-acetate*
RRMM	*relapsed/refractory MM*, nawrotowy/oporny MM
SASP	*senescence-associated secretory effector phenotype*, sekrecyjny efektorowy fenotyp związany ze starzeniem
SCT	*stem cell transplantation*, transplantacja komórek macierzystych
Teff	*T effector cell*, efektorowy limfocyt T
TGF	*tumor growth factor*, czynnik wzrostu nowotworów
Th	*T helper cell*, pomocniczy (efektorowy) limfocyt T
Treg	*T regulatory cell*, regulatorowy limfocyt T
TTP	*time to progression*, czas do wystąpienia progresji

XI. PIŚMIENNICTWO

1. Kyle: R.A.; Rajkumar, S.V. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. *Leukemia* **2009**, *23*, 3-9.
2. Diaz-Tejedor, A.; Lorenzo-Mohamed, M.; Puig, N.; Garcia-Sanz, R.; Mateos, M.-V.; Garayoa, M.; Paino, T. Immune system alterations in multiple myeloma: molecular mechanisms and therapeutic strategies to reverse immunosuppression. *Cancers (Basel)* **2021**, *13*, 1353.
3. Tamura, H. Immunopathogenesis and immunotherapy of multiple myeloma. *Int. J. Hematol.* **2018**, *107*, 278-285.
4. Webb, E.S.; Liu, P.; Baleeiro, R.; Lemoine, N.R.; Yuan, M.; Wang, Y. Immune checkpoint inhibitors in cancer therapy. J. Biomed. Res. **2018**, 32, 317-326.
5. Ansell, S.M.; Lesokhin, A.M.; Borrello, I.; Halwani, A.;, Scott, E.C.; Gutierrez, M.; et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N. Engl. J. Med.* **2015**, *372*, 311-319.
6. Mateos, M.V.; Blacklock, H.; Schjesvold, F.; Rocafiguera, A.O.; Simpson, D.; George, A.; et al. A phase 3 randomized study of pembrolizumab (Pembro) plus pomalidomide (Pom) and dexamethasone (Dex) for relapsed/refractory multiple myeloma (RRMM): Keynote-183. J. Clin. Oncol. **2018**, 36, 8021.
7. Usmani, S.Z.; Schjesvold, F.; Rocafiguera, A.O.; Karlin, L.; Rifkin, R.M.; Yimer, H.A.; et al. A phase 3 randomized study of pembrolizumab (pembro) plus lenalidomide (len) and low-dose dexamethasone (Rd) versus Rd for newly diagnosed and treatment-naïve multiple myeloma (MM): Keynote-185. J. Clin. Oncol. **2018**, 36, 8010.
8. Thanendrarajan, S.; Puryear, J.; Schinke, C.D.; van Rhee, F.; Zangari, M.; Mathur, P.; et al. Nivolumab for treatment of advanced, refractory, high-risk multiple myeloma. *Blood* **2017**, *130* (suppl. 1), 1858.
9. Favaloro, J.; Brown, R.; Aklilu, E.; Yang, S.; Duen, H.; Hart, D.; et al. Myeloma skews regulatory and pro-inflammatory T helper 17 cell balance in favor of a suppressive state. Leuk. Lymphoma **2015**, 55, 1090-1098.
10. Dosani, T.; Carlsten, M.; Maric, I.; Landgren, O. The cellular immune system in myelogenesis: NK and T cells in the development of myeloma and their uses in immunotherapies. Blood Cancer J. **2015**, 5, e306.

11. Doering T.A.; Crawford A; Angelosanto J.M.; Paley M.A.; Ziegler C.G.; Wherry E.J. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity **2012**, 37, 1130-1144.
12. Wherry E.J.; Kurachi, M. Molecular and cellular insights into T cell exhaustion. Nat. Rev. Immunol. **2015**, 15, 486-499.
13. Zeller-Rieser, C.; Thangavadivel, S.; Biedermann, R.; Brunner, A.; Stoitzner, P.; Willenbacher, E.; et al. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. *J. Hematol. Oncol.* **2016**, *9*, 116.
14. Suen, H.; Brown, R.; Yang, S.; Ho, P.J.; Gibson, J.; Joshua, D. The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase I study. *Leukemia* **2015**, *29*, 1621-1622.
15. Suen, H.; Brown, R.; Yang, S.; Weatherburn, C.; Ho, P.J.; Woodland, N.; et al. Multiple myeloma causes clonal T cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade. *Leukemia* **2016**, *30*, 1716-1724.
16. Zhao, Y.; Shao, Q.; Peng, G. Exhaustion and senescence: two crucial dysfunctional states of T cells in the tumor microenvironment. *Cell. Mol. Immunol.* **2020**, *17*, 27-35.
17. Pawelec, G. Is there a positive side to T cell exhaustion? *Front. Immunol*. **2019**, *10*, article 111.
18. Akbar A.; Henson S. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? Nat. Rev. Immunol. **2011**, 11, 289-295.
19. Rajkumar, S.V.; Dimopoulos, M.A.; Palumbo, A.; Blade, J.; Merlini, G.; Mateos, M.V.; et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. **2014**, 15, e538-e548.
20. Greipp, P.R.; San Miguel, J.; Durie, B.G.; Crowley, J.J.; Barlogie, B.; Blade, J.; et al. International staging system for multiple myeloma. J. Clin. Oncol. **2005**, 23, 3412-3420.
21. Liu, X.; Mo, W.; Ye, J.; Li, L.; Zhang, Y.; Hsueh, E.C.; et al. Regulatory T cells trigger effector T cell DNA damage and senescence caused by metabolic competition. *Nat. Commun.* **2018**, *9*, 249.
22. Ye, J.; Huang, X.; Hsueh, E.C.; Zhang, Q.; Ma, C.; Zhang, Y.; et al. Human regulatory T cells induce T-lymphocyte senescence. *Blood* **2012**, *120*, 2021-2031.
23. Batorov, E.V.; Aristova, T.A.; Sergeevicheva, V.V.; Sizikova, S.A.; Ushakova, G.A.; Pronkin, N.V.; et al. Quantitative and functional characteristics of circulating and bone

marrow PD-1- and TIM-3-positive T cells in treated multiple myeloma patients. *Sci. Rep.* **2020**, 10, 20846.

24. Karabon, L.; Partyka, A.; Ciszak, L.; Pawlak-Adamska, E.; Tomkiewicz, A.; Bojarska-Junak, A.; et al. Abnormal expression of BTLA and CTLA-4 immune checkpoint molecules in chronic lymphocytic leukemia patients. *J. Immunol. Res.* **2020**, *2020*, article ID 6545921.
25. Thommen, D.S.; Schreiner, J.; Muller, P.; Herzig, P.; Roller, A.; Belousov, A.; et al. Progression of lung cancer is associated with increased dysfunction of T cells defined by coexpression of multiple inhibitory receptors. *Cancer Immunol. Res.* **2015**, *3*, 1344-1355.
26. Ciszak, L.; Frydecka, I.; Wolowiec, D.; Szteblich, A.; Kosmaczewska, A. Patients with chronic lymphocytic leukemia (CLL) differ in the pattern of CTLA-4 expression on CLL cells: the possible implications for immunotherapy with CTLA-4 blocking antibody. *Tumour Biol.* **2016**, *37*, 4143-4157.
27. Ye, J.; Peng, G. Controlling T cell senescence in the tumor microenvironment for tumor immunotherapy. Oncoimmunology **2015**, 4, e994398.
28. Arai, Y.; Saito, H.; Ikeguchi, M. Upregulation of TIM-3 and PD-1 on CD4+ and CD8+ T cells associated with dysfunction of cell-mediated immunity after colorectal cancer operation. *Yonago Acta Med.* **2012**, *55*, 1-9.
29. Tan, J.; Chen, S.; Huang, J.; Chen, Y.; Yang, L.; Wang, C.; et al. Increased exhausted $CD8^+$ T cells with programmed death-1, T-cell immunoglobulin and mucin-domain-containing-3 phenotype in patients with multiple myeloma. *Asia Pac. J. Clin. Oncol.* **2018**, *14*, e266-e274.
30. Alrasheed, N.; Lee, L.; Ghorani, E.; Henry, J.Y.; Conde, L.; Chin, M.; et al. Marrow infiltrating regulatory T cells correlate with the presence of dysfunctional CD4+PD-1+ cells and inferior survival in patients with newly diagnosed multiple myeloma. *Clin. Cancer Res.* **2020**, 26, 3443-3454.
31. Zhao, Y.; Shao, Q.; Peng, G. Exhaustion and senescence: two crucial dysfunctional states of T cells in the tumor microenvironment. Cell. Mol. Immunol. **2020**, 17, 27-35.
32. Lanna, A.; Henson, S.M.; Escors, D.; Akbar, A.N. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. Nat. Immunol. **2014**, 15, 965-972.

33. Thewissen, M.; Somers, V.; Hellings, N.; Fraussen, J.; Damoiseaux, J.; Stinissen, P. CD4+CD28null T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. J. Immunol. **2007**, 179, 6514-6523.
34. Lindqvist, E.K.; Landgren, O.; Lund, S.H.; Turesson, I.; Hultcrantz, M.; Goldin, L.; et al. History of autoimmune disease is associated with impaired survival in multiple myeloma and monoclonal gammopathy of undetermined significance: a population-based study. Ann. Hematol. **2017**, 96, 261-269.
35. Shimanovsky, A.; Alvarez, A.J.; Murali, S.; Dasanu, C.A. Autoimmune manifestations in patients with multiple myeloma and monoclonal gammopathy of undetermined significance. BBA Clinical **2016**, 6, 12-18.

XII.ZAŁĄCZNIKI

1.OŚWIADCZENIA WSPÓŁAUTORÓW

Agata Kosmaczewska

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy przeglądowej:

The functional significance of the bone marrow microenvironment in multiple myeloma development and progression

Agata Kosmaczewska, Anna Masternak (Kulikowska de Nałęcz), Katarzyna Kosciow

OA Immunology 2013 Aug 01;1(1)7

DOI: 10.13172/2052-9295-1-1-1018

Published online: 2013 Aug 1

mój udział polegał na zbieraniu i krytycznej analizie piśmiennictwa, pisaniu manuskryptu.

dr hab. n. med. Agata Kosmaczewska
lekarz chorób wewnętrznych
specjalista pielęgniarstwa żywienia i dietetyki
1491500

Anna Kulikowska de Nałęcz Wrocław, 28/04/2023

Szpital Wojewódzki w Opolu
Oddział Kliniczny Hematologii, Onkologii Hematologicznej i Chorób Wewnętrznych

OŚWIADCZENIE

Oświadczam, że w pracy przeglądowej:

The functional significance of the bone marrow microenvironment in multiple myeloma development and progression

Agata Kosmaczewska, Anna Masternak (Kulikowska de Nałęcz), Katarzyna Kosciow
OA Immunology 2013 Aug 01;1(1)7
DOI:10.13172/2052-9295-1-1-1018
Published online: 2013 Aug 1

mój udział polegał na przeglądzie i krytycznej analizie piśmiennictwa, pisaniu manuskryptu oraz pisaniu ostatecznej wersji artykułu.

Anna Kulikowska de Nałęcz

Katarzyna Kościów

Opolskie Centrum Onkologii
im. prof. T. Koszarowskiego
Klinika Onkologii

Opole, 28/04/2023

OŚWIADCZENIE

Oświadczam, że w pracy przeglądowej:

The functional significance of the bone marrow microenvironment in multiple myeloma development and progression

Agata Kosmaczewska, Anna Masternak (Kulikowska de Nałęcz), Katarzyna Kosciow

OA Immunology 2013 Aug 01;1(1)7

DOI:10.13172/2052-9295-1-1-1018

Published online: 2013 Aug 1

mój udział polegał na zbieraniu i przeglądzie piśmiennictwa oraz krytycznej analizie manuskryptu.

Katarzyna Kościów

Anna Kulikowska de Nałęcz Wrocław, 28/04/2023

Szpital Wojewódzki w Opolu
Oddział Kliniczny Hematologii, Onkologii Hematologicznej i Chorób Wewnętrznych

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska
Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]
DOI: 10.3390/ijms22179298
Published online: 2021 Aug 27

mój udział polegał na opracowaniu koncepcji pracy, opracowaniu metodologii badawczej, zbieraniu materiału, analizie i interpretacji danych klinicznych pacjentów włączonych do badania, analizie i interpretacji danych pozyskanych w toku badania, zbieraniu piśmiennictwa, pisaniu manuskryptu oraz pisaniu ostatecznej wersji artykułu.

Anna Kulikowska de Nałęcz

Agata Kosmaczewska

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kalikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]

DOI: 10.3390/ijms22179298

Published online: 2021 Aug 27

mój udział polegał na pozyskaniu środków do realizacji badania, współopracowaniu metodologii badania, interpretacji danych, krytycznej analizie artykułu.

lek. Agata Kosmaczewska
lekarz chorób wewnętrznych
specjalista hematologii
1461490

Prof. dr hab. n. med. Irena Frydecka

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]

DOI: 10.3390/ijms22179298

Published online: 2021 Aug 27

mój udział polegał na zbieraniu danych klinicznych pacjentów i interpretacji danych.

prof. dr hab. Irena Frydecka
Klinika Chorób Wewnętrznych
i Hematologii
51-602 Wrocław, ul. Koszarowskiego 8
tel. 561317652 5621490

Prof. dr hab. n. med. Lidia Usnarska-Zubkiewicz

Wrocław, 28/04/2023

Klinika Hematologii, Nowotworów Krwi
i Transplantacji Szpiku
Uniwersyteckiego Szpitala Klinicznego
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]
DOI: 10.3390/ijms22179298
Published online: 2021 Aug 27

mój udział polegał na kwalifikacji pacjentów do badań, zbieraniu próbek krwi oraz analizie danych klinicznych pacjentów włączonych do badania.

Prof. dr hab. n. med. L. Usnarska-Zubkiewicz
Specjalista [illegible]
Specjalista [illegible]
Specjalista [illegible]
Specjalista transplantolog
8474971

INSTYTUT IMMUNOLOGII
I TERAPII DOŚWIADCZALNEJ
im. Ludwika Hirszfelda
Polskiej Akademii Nauk
ZAKŁAD TERAPII DOŚWIADCZALNEJ
LABORATORIUM IMMUNOPATOLOGII
53-114 Wrocław, ul. Rudolfa Weigla 12
NIP: 896-000-56-96

Edyta Pawlak

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kalikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]

DOI: 10.3390/ijms22179298

Published online: 2021 Aug 27

mój udział polegał na wykonaniu barwienia fluorescencyjnego, analizie statystycznej, interpretacji i wizualizacji danych oraz krytycznej analizie artykułu.

Laboratorium Immunopatologii
dr hab. n. biol. Edyta Pawlak, prof. UMW
Kierownik

Magdalena Szmyrka

Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu
Klinika Reumatologii i Chorób Wewnętrznych

Wrocław, 28/01/2023

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]

DOI: 10.3390/ijms22179298

Published online: 2021 Aug 27

mój udział polegał na interpretacji i wizualizacji danych oraz krytycznej analizie artykułu.

dr hab. n. med. Magdalena Szmyrka specjalista reumatologii specjalista chorób wewnętrznych 6363508

Lidia Ciszak

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

Wrocław, 28/01/2023

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Irena Frydecka, Edyta Pawlak, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol. Sci. 2021 Vol. 22(17), art. 9298 [19 s.]

DOI: 10.3390/ijms22179298

Published online: 2021 Aug 27

mój udział polegał na wykonaniu hodowli komórkowych, barwieniu fluoresceencyjnym, analizie cytometrycznej, analizie statystycznej, interpretacji i wizualizacji danych.

Ciszak

Anna Kulikowska de Nałęcz Wrocław, 28/04/2023

Szpital Wojewódzki w Opolu
Oddział Kliniczny Hematologii, Onkologii Hematologicznej i Chorób Wewnętrznych

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]
DOI: 10.3390/ijms24065730
Published online 2023 Mar 17

mój udział polegał na opracowaniu koncepcji pracy, opracowaniu metodologii badawczej, zbieraniu materiału, analizie i interpretacji danych klinicznych pacjentów włączonych do badania, analizie i interpretacji danych pozyskanych w toku badania, zbieraniu piśmiennictwa, pisaniu manuskryptu oraz pisaniu ostatecznej wersji artykułu.

Anna Kulikowska de Nałęcz

Agata Kosmaczewska

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na pozyskaniu środków do realizacji badania, współopracowaniu metodologii badania, interpretacji danych, krytycznej analizie artykułu.

dr hab. n. med. Agata Kosmaczewska
lekarz chorób wewnętrznych
specjalista pielęgniarstwa i położnictwa
1451400

Prof. dr hab. n. med. Irena Frydecka

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

Wrocław, 28/04/2023

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kusmierczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na zbieraniu danych klinicznych pacjentów włączonych do badania, interpretacji wyników

prof. dr hab. Irena Frydecka
specj. chorób wewnętrznych
i hematologii
51-502 Wrocław ul. Kochanowskiego 3
tel. 661317632 8621490

Prof. dr hab. n. med. Lidia Usnarska-Zubkiewicz

Klinika Hematologii, Nowotworów Krwi
i Transplantacji Szpiku
Uniwersyteckiego Szpitala Klinicznego
we Wrocławiu

Wrocław, 28/04/2023

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na zbieraniu materiału, analizie i interpretacji danych klinicznych pacjentów oraz krytycznej analizie artykułu .

Prof. dr hab. n. med. Lidia Usnarska-Zubkiewicz
Specjalista chorób wewnętrznych
Specjalista hematolog
Specjalista transplantolog
8174971

INSTYTUT IMMUNOLOGII
I TERAPII DOŚWIADCZALNEJ
im. Ludwika Hirszfelda
Polskiej Akademii Nauk
ZAKŁAD TERAPII DOŚWIADCZALNEJ
LABORATORIUM IMMUNOPATOLOGII
53-114 Wrocław, ul. Rudolfa Weigla 12
NIP: 896-000-56-96

Edyta Pawlak

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na analizie statystycznej, interpretacji i wizualizacji danych, zbieraniu piśmiennictwa oraz analizie artykułu.

Laboratorium Immunopatologii
dr hab. n med. Edyta Pawlak, prof. PAN
Kierownik

Magdalena Szmyrka

Wrocław, 28/04/2023

Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu
Klinika Reumatologii i Chorób Wewnętrznych

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na analizie i wizualizacji danych oraz zbieraniu piśmiennictwa.

M. Szmyrka

lek. n. med. Magdalena Szmyrka
st. asystent reumatologii
specjalista reumatologii

Lidia Ciszak

Wrocław, 28/04/2023

Instytut Immunologii i Terapii Doświadczalnej
im. Ludwika Hirszfelda Polskiej Akademii Nauk
we Wrocławiu

OŚWIADCZENIE

Oświadczam, że w pracy badawczej oryginalnej:

Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patient is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors

Anna Kulikowska de Nałęcz, Lidia Ciszak, Lidia Usnarska-Zubkiewicz, Edyta Pawlak, Irena Frydecka, Magdalena Szmyrka and Agata Kosmaczewska

Int. J. Mol Sci. 2023 Vol. 24(6), art. 5730 [17 s.]

DOI: 10.3390/ijms24065730

Published online 2023 Mar 17

mój udział polegał na wykonaniu hodowli komórkowych, barwieniu fluorescencyjnym, analizie cytometrycznej, analizie statystycznej, interpretacji i wizualizacji danych.

2.NOTA BIOGRAFICZNA

Lek. med. Anna Kulikowska de Nałęcz de domo Masternak
Urodzona 10. 07. 1973 r. w Kłobucku

Wykształcenie:
1992-1998 r. studia na Wydziale Lekarskim Akademii Medycznej w Łodzi
1997 r. sześciomiesięczne studia w ramach planu studiów na Wydziale Lekarskim w Uniwersytecie Claude'a Bernard w Lyonie
Specjalizacja z chorób wewnętrznych ukończona w 2007 r.
Specjalizacja z hematologii ukończona 2012 r.
Studia podyplomowe „Zarządzanie i finanse w ochronie zdrowia", Uniwersytet Ekonomiczny we Wrocławiu, ukończone 2008 r.

Doświadczenie:
1998-1999 r. Oddział Chorób Wewnętrznych Wojewódzkiego Szpitala Zespolonego w Częstochowie, stanowisko młodszego asystenta
1999-2001 r. Zespół Wyjazdowy Oddział Pomocy Doraźnej Zespołu Opieki Zdrowotnej w Kłobucku, kierownik zespołu wyjazdowego, lekarz ambulatorium
2001-2003 r. Kliniczny Oddział Chorób Wewnętrznych Wojewódzkiego Szpitala Specjalistycznego w Sosnowcu, stanowisko młodszego asystenta
2003-2006 r. Klinika Chorób Wewnętrznych i Chemioterapii Onkologicznej ŚAM w Katowicach, stanowisko młodszego asystenta
Od 2006 r. Poradnia POZ GOBL we Wrocławiu, a następnie ZPZOZ w Bierutowie
Od 2008 r. Oddział Kliniczny Hematologii, Onkologii Hematologicznej i Chorób Wewnętrznych, Poradnia Hematologiczna Szpitala Wojewódzkiego w Opolu, stanowisko starszego asystenta

Przynależność do towarzystw naukowych: Polskie Towarzystwo Hematologów i Transfuzjologów

3.WYKAZ PUBLIKACJI AUTORA

Anna Kulikowska de Nałęcz

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1.	Kosmaczewska Agata, Bońko Dorota, Ciszak Lidia, Włodarska-Polińska Iwona, Kornafel Jan, Szteblich Aleksandra, **Masternak Anna**, Frydecka Irena: Dysregulated expression of both the costimulatory CD28 and inhibitory CTLA-4 molecules in PB T cells of advanced cervical cancer patients suggests systemic immunosuppression related to disease progression, Pathology & Oncology Research, 2012, vol. 18, nr 2, s. 479-489	1,555	20
2.	Walter-Croneck Adam, Grzasko Norbert, Soroka-Wojtaszko Marianna, Jurczyszyn Artur, Torosian Tigran, Rymko Marcin, Nowicki Adam, Druzd-Sitek Agnieszka, Lech-Marańda Ewa, Madro Elżbieta, Zielińska Patrycja, Grygoruk-Wiśniowska Iwona, Błońska Danuta, Usnarska-Zubkiewicz Lidia, Potoczek Stanisław, Iskierka Elżbieta, **Masternak Anna**, Hołojda Jadwiga, Dawidowska Dorota, Gawron Ludmiła, Barchnicka Agnieszka, Olszewska-Szopa Magdalena, Rybicka Malwina, Gontarska Agnieszka, Jachalska Anna, Rzepecki Piotr, Subocz Edyta, Boguradzki Piotr, Charliński Grzegorz, Dzierzak-Mietla Monika, Wiśniewska-Piąty Katarzyna, Świstek Wojciech, Kopacz Agnieszka, Blajer-Olszewska Beata, Świderska Alina, Dmoszyńska Anna: Case-adjusted bortezomib-based strategy in routine therapy of relapsed/refractory multiple myeloma shown to be highly effective - a report by Polish Myeloma Study Group, Leukemia Research, 2014, vol. 38, nr 7, s. 788-794, DOI:10.1016/j.leukres.2014.04.011	2,351	25
3.	Hus Iwona, Walter-Croneck Adam, **Masternak Anna**, Jurczyszyn Artur, Usnarska-Zubkiewicz Lidia, Bołkun Łukasz, Druzd-Sitek Agnieszka, Rymko Marcin, Łętowska Jadwiga, Lech-Marańda Ewa, Pasiarski Marcin, Dmoszyńska Anna: Real-life experience with bortezomib-based regimens in elderly patients with newly diagnosed multiple myeloma and comorbidities: a Polish retrospective multicenter study, Polskie Archiwum Medycyny Wewnętrznej, 2017, vol. 127, nr 11, s. 765-774, DOI:10.20452/pamw.4099	2,658	30
4.	Jurczyszyn A, Radocha J, Davila J, Fiala MA, Gozzetti A, Grząśko N, Robak P, Hus I, Waszczuk-Gajda A, Guzicka-Kazimierczak R, Atilla E, Mele G, Sawicki W, Jayabalan DS, Charliński G, Szabo AG, Hajek R, Delforge M, Kopacz A, Fantl D, Waage A, Avivi I, Rodzaj M, Leleu X, Richez V, Knopińska-Posłuszny W, **Masternak A**, Yee AJ, Barchnicka A, Druzd-Sitek A, Guerrero-Garcia T, Liu J, Vesole DH, Castillo JJ. Prognostic indicators in primary plasma cell leukaemia: a multicentre retrospective study of 117 patients. Br J Haematol. 2018 Mar;180(6):831-839. doi: 10.1111/bjh.15092	5,206	40

5.	Olszewska-Szopa Magdalena, Sobas Marta, Laribi Kamel, Bao Perez Laura , Drozd-Sokołowska Joanna, Subocz Edyta, Joks Monika, Zduniak Krzysztof, Gajewska Małgorzata, **Kulikowska de Nalecz Anna** , Romejko-Jarosińska Joanna, Kumiega Beata, Waszczuk-Gajda Anna, Wróbel Tomasz, Czyż Anna: Primary cutaneous indolent B-cell lymphomas - a retrospective multicenter analysis and a review of literature, Acta Oncologica, 2021, vol. 60, nr 10, s. 1361-1368, DOI:10.1080/0284186X.2021.1956689	4,311	100
6.	Mądry Krzysztof, Lis Karol, Tukiendorf Andrzej, Szwedyk Paweł, Kapelko-Słowik Katarzyna, Subocz Edyta, Gołos Aleksandra, Makowska Wioletta, **Masternak Anna**, Kopińska Anna, Czemerska Magdalena, Zawadzka-Leska Sara, Rusicka Patrycja, Drozd-Sokołowska Joanna, Wiater Elżbieta, Hołojda Jadwiga, Pogłódek Bartłomiej, Centkowski Piotr, Waszczuk-Gajda Anna, Machowicz Rafał, Hałka Janusz, Czerw Tomasz, Basak Grzegorz, Dwilewicz-Trojaczek Jadwiga: Low serum albumin level deteriorates prognosis in azacitidine-treated myelodysplastic syndromes patients - results of the PALG study 'PolAZA', Hematology, 2021, vol. 26, nr 1, s. 556-564, DOI:10.1080/16078454.2021.1956182	2,264	40
7.	**Kulikowska de Nałęcz Anna**, Ciszak Lidia, Usnarska-Zubkiewicz Lidia, Frydecka Irena, Pawlak Edyta, Szmyrka Magdalena, Kosmaczewska Agata: Deregulated expression of immune checkpoints on circulating CD4 T cells may complicate clinical outcome and response to treatment with checkpoint inhibitors in multiple myeloma patients, International Journal of Molecular Sciences, 2021, vol. 22, nr 17, art.9298 [19 s.], DOI:10.3390/ijms22179298	6,208	140
8.	Grzasko Norbert, Charliński Grzegorz, Morawska Marta, Kicinski Pawel, Waszczuk-Gajda Anna, Drozd-Sokołowska Joanna, Subocz Edyta, Błońska Danuta, Razny Małgorzata, Druzd-Sitek Agnieszka, Holojda Jadwiga, Swiderska Alina, Usnarska-Zubkiewicz Lidia, **Masternak Anna**, Giannopoulos Krzysztof: Bendamustine-based regimens as salvage therapy in refractory/relapsed multiple myeloma patients: a retrospective real-life analysis by the Polish Myeloma Group, Journal of Clinical Medicine, 2021, vol. 10, nr 23, art.5504 [12 s.], DOI:10.3390/jcm10235504	4,964	140
9.	Barbui Tiziano, Carobbio Alessandra, Ghirardi Arianna, Iurlo Alessandra, Sobas Marta Anna, Elli Elena Maria, Rumi Elisa, De Stefano Valerio, Lunghi Francesca, Marchetti Monia, Daffini Rosa, Gasior Kabat Mercedes, Cuevas Beatriz, Fox Maria Laura, Andrade-Campos Marcio Miguel, Palandri Francesca, Guglielmelli Paola, Benevolo Giulia, Harrison Claire, Foncillas Maria-Angeles, Bonifacio Massimiliano, Alvarez-Larran Alberto, Kiladjian Jean-Jacques, Bolaños Calderón Estefanía, Patriarca Andrea, Quiroz Cervantes Keina, Griesshammer Martin, Garcia-Gutierrez Valentin, Marin Sanchez Alberto, Magro Mazo Elena, Carli Giuseppe, Hernandez-Boluda Juan Carlos, Osorio Santiago, Carreno-Tarragona Gonzalo, Sagues Serrano Miguel, Kusec Rajko, Navas Elorza Begona, Angona Anna, Xicoy Cirici Blanca, Lopez Abadia Emma, Koschmieder Steffen, Cattaneo Daniele, Bucelli Cristina, Cichocka Edyta, **Kulikowska de Nałęcz Anna**, Cavalca Fabrizio, Borsani Oscar, Betti Silvia, Bellini Marta, Curto-Garcia Natalia, Rambaldi Alessandro, Vannucchi Alessandro Maria: Determinants of early triage for hospitalization in myeloproliferative neoplasm (MPN) patients with COVID-19 [letter to the editor], American Journal of Hematology, 2022, vol. 97, nr 12, E470-E473, DOI:10.1002/ajh.26732	13,268*	140

10.	Sobas Marta, Kiladjian Jean-Jacques, Beauverd Yan, Curto-Garcia Natalia, Sadjadian Parvis, Shih Lee Yung, Devos Timothy, Krochmalczyk Dorota, Galli Serena, Bieniaszewska Maria, Seferynska Ilona, McMullin Mary Frances, Armatys Anna, Spalek Adrianna, Waclaw Joanna, Zdrenghea Mihnea, Legros Laurence, Girodon François, Lewandowski Krzysztof, Angona Figueras Anna, Samuelsson Jan, Abuin Blanco Aitor, Cony-Makhoul Pascale, Collins Angela, James Chloé, Kusec Rajko, Lauermannova Marie, Noya Maria Sol, Skowronek Malgorzata, Szukalski Lukasz, Szmigielska-Kaplon Anna, Wondergem Marielle, Dudchenko Iryna, Gora Tybor Joanna, Laribi Kamel, **Kulikowska de Nalecz Anna**, Demory Jean-Loup, Le Du Katell, Zweegman Sonja, Besses Raebel Carlos, Skoda Radek, Giraudier Stéphane, Griesshammer Martin, Harrison Claire N., Ianotto Jean-Christophe: Real-world study of children and young adults with myeloproliferative neoplasms: identifying risks and unmet needs, Blood Advances, 2022, vol. 6, nr 17, s. 5171-5183, DOI:10.1182/bloodadvances.2022007201	7,642*	40
11.	Barbui Tiziano, Carobbio Alessandra, Ghirardi Arianna, Iurlo Alessandra, De Stefano Valerio, Sobas Marta, Rumi Elisa, Elli Elena Maria, Lunghi Francesca, Gasior Kabat Mercedes, Cuevas Beatriz, Guglielmelli Paola, Bonifacio Massimiliano, Marchetti Monia, Alvarez-Larran Alberto, Fox Laura, Bellini Marta, Daffini Rosa, Benevolo Giulia, Carreno-Tarragona Gonzalo, Patriarca Andrea, Al-Ali Haifa Kathrin, Andrade-Campos Maria Marcio Miguel, Palandri Francesca, Harrison Claire, Foncillas Maria Angeles, Osorio Santiago, Koschmieder Steffen, Magro Mazo Elena, Kiladjian Jean-Jacques, Bolaños Calderón Estefanía, Heidel Florian H., Quiroz Cervantes Keina, Griesshammer Martin, Garcia-Gutierrez Valentin, Sanchez Alberto Marin, Hernandez-Boluda Juan Carlos, Lopez Abadia Emma, Carli Giuseppe, Sagues Serrano Miguel, Kusec Rajko, Xicoy Cirici Blanca, Guenova Margarita, Navas Elorza Begona, Angona Anna, Cichocka Edyta, **Kulikowska de Nałęcz Anna**, Cattaneo Daniele, Bucelli Cristina, Betti Silvia, Borsani Oscar, Cavalca Fabrizio, Carbonell Sara, Curto-Garcia Natalia, Benajiba Lina, Rambaldi Alessandro, Vannucchi Alessandro Maria: Breakthrough infections in MPN-COVID vaccinated patients [letter to the editor], Blood Cancer Journal, 2022, vol. 12, nr 11, art.154 [6 s.], DOI:10.1038/s41408-022-00749-8	9,812*	140
12.	Stuckey Ruth, Ianotto Jean-Christophe, Santoro Marco, Czyż Anna, Perez Encinas Manuel M., Gómez-Casares María Teresa, Noya Pereira Maria Soledad, **Kulikowska de Nałęcz Anna**, Gołos Aleksandra, Lewandowski Krzysztof, Szukalski Łukasz, González-Martín Jesús M., Wróbel Tomasz, Sobas Marta Anna: Validation of thrombotic risk factors in 1381 patients with essential thrombocythaemia: a multicentre retrospective real-life study, British Journal of Haematology, 2022, vol. 199, nr 1, s. 86-94, DOI:10.1111/bjh.18387	8,615*	140

13.	Barbui Tiziano, Iurlo Alessandra, Masciulli Arianna, Carobbio Alessandra, Ghirardi Arianna, Carioli Greta, Sobas Marta Anna, Elli Elena Maria, Rumi Elisa, De Stefano Valerio, Lunghi Francesca, Marchetti Monia, Daffini Rosa, Kabat Mercedes Gasior, Cuevas Beatriz, Fox Maria Laura, Andrade-Campos Marcio Miguel, Palandri Francesca, Guglielmelli Paola, Benevolo Giulia, Harrison Claire, Foncillas María Ángeles, Bonifacio Massimiliano, Alvarez-Larran Alberto, Kiladjian Jean-Jacques, Calderón Estefanía Bolaños, Patriarca Andrea, Cervantes Keina Quiroz, Griessammer Martin, Garcia-Gutierrez Valentin, Sanchez Alberto Marin, Mazo Elena Magro, Ruggeri Marco, Hernandez-Boluda Juan Carlos, Osorio Santiago, Carreño-Tarragona Gonzalo, Serrano Miguel Sagues, Kusec Rajko, Elorza Begona Navas, Angona Anna, Cirici Blanca Xicoy, Abadia Emma Lopez, Koschmieder Steffen, Cattaneo Daniele, Bucelli Cristina, Cichocka Edyta, **Anna Masternak Kulikowska de Nałęcz**, Cavalca Fabrizio, Borsani Oscar, Betti Silvia, Benajiba Lina, Bellini Marta, Curto-Garcia Natalia, Rambaldi Alessandro, Vannucchi Alessandro Maria: Second versus first wave of COVID-19 in patients with MPN [letter to the editor], Leukemia, 2022, vol. 36, nr 3, s. 897-900, DOI:10.1038/s41375-022-01507-2	12,897*	200
14.	**Kulikowska de Nałęcz Anna**, Ciszak Lidia, Usnarska-Zubkiewicz Lidia, Pawlak Edyta, Frydecka Irena, Szmyrka Magdalena, Kosmaczewska Agata: Inappropriate expression of PD-1 and CTLA-4 checkpoints in myeloma patients is more pronounced at diagnosis: implications for time to progression and response to therapeutic checkpoint inhibitors, International Journal of Molecular Sciences, 2023, vol. 24, nr 6, art.5730 [17 s.], DOI:10.3390/ijms24065730	6,208*	140
	Podsumowanie	**87,959**	**1335**

IF 2021

1.2 Publikacje w czasopiśmie bez IF

Lp	Opis bibliograficzny	Punkty
1	Frydecka Irena, Kosmaczewska Agata, Ciszak Lidia, **Masternak Anna**, Woszczyk Dariusz: A blockade of the inhibitory CTLA-4 molecule - a novel strategy for the immunotherapy of neoplastic diseases, Acta Haematologica Polonica, 2011, vol. 42, nr 2, s. 181-191	6
2	**Masternak A**, Woszczyk D.: Redukcja dawki inhibitorów kinazy tyrozynowej jako problem terapeutyczny leczenia przewlekłej białaczki szpikowej Acta Haematologica Polonica 2011, 42, nr 3, str. 573-575	6
3	Kosmaczewska A, **Masternak A**, Kosciow K. The functional significance of the bone marrow microenvironment in multiple myeloma development and progression. OA Immunology 2013 Aug 01;1(1)7.	-
4	**Masternak A**, Woszczyk D.: Skuteczność terapii ruksolitynibem u pacjentki z mielofibrozą wtórną do czerwienicy prawdziwej. Hematologia 2017;supl. C: C5-C8	8
	Podsumowanie	**20**

3. Abstrakty

Lp	Opis bibliograficzny
1	Frydecka Irena, Kosmaczewska Agata, Ciszak Lidia, **Masternak Anna**, Woszczyk Dariusz: A blockade of the inhibitory CTLA-4 molecule - a novel strategy for the immunotherapy of neoplastic diseases, Acta Haematologica Polonica, 2011, vol. 42, nr supl., 25 poz.W-23, [XXIV Zjazd Polskiego Towarzystwa Hematologów i Transfuzjologów. Lublin, 16-18 wrzesień 2011 r. Streszczenia]
2	Ianotto Jean-Christophe, Kiladjian Jean-Jacques, Sobas Marta, Sadjadian Parvis, Shih Lee-Yung, Bieniaszewska Maria, Seferyńska Ilona, McMullin Mary Frances, Wącław Joanna, Legros Laurence, Lewandowski Krzysztof, Zdrenghea Mihnea, Blanco Aitor Abuin, Armatys Anna, Samuelsson Jan, Spałek Adrianna, Figueras Anna Angola, Lauermannova Marie, Noya Maria-Soledad, Czyż Jarosław, Szmigielska-Kapłon Anna, Kusec Rajko, Góra-Tybor Joanna, **Masternak Anna**, Michiels Jan J., Skowronek Małgorzata, Demory Jean-Loup, Le Du Katell, Zweegman Sonja, Besses Raebel Carlos, Giraudier Stephane, Griesshammer Martin, Harrison Claire N.: Myeloproliferative neoplasms in patients below 25 years old at diagnosis: a retrospective international cooperative work, Blood, 2018, vol. 132, nr suppl.1, poz.1759, [60th ASH Annual Meeting and Exposition. San Diego, CA, December 1-4, 2018. Abstracts], DOI:10.1182/blood-2018-99-112374
3	Sobas Marta, Andrasiak I., Lewandowski K., Narkun M., Czyż J., Szukalski L., Nierychlewska P., Czyż Anna, Gołos A., **Masternak A.**, Blanco A. Abuin, Encinas M. Perez, Casares M.T. Gomez, Pereira M.S. Noya, Marczyk J., Roczek Karolina, Wróbel Tomasz: Analysis of IPSET-thrombosis vs R-IPSET-thrombosis in a population of 776 essential thrombocythemia patients - a multicenter retrospective study, HemaSphere, 2018, vol. 2, nr suppl.1, 267 poz.PF630, [23rd Congress of the European Hematology Association. Stockholm, Sweden, June 14-17, 2018], DOI:10.1097/HS9.0000000000000060
4	Mądry Krzysztof, Lis Karol, Tukiendorf Andrzej, Szwedyk Paweł, Kapelko-Słowik Katarzyna, Subocz Edyta, Halka Janusz, Gołos Aleksandra, Makowska Wioletta, **Masternak Anna**, Kopińska Anna, Czemerska Magdalena, Zawadzka-Leska Sara, Rusicka Patrycja, Drozd-Sokołowska Joanna, Wiater Elżbieta, Hołojda Jadwiga, Pogłódek Bartłomiej, Centkowski Piotr, Waszczuk-Gajda Anna, Machowicz Rafał, Basak Grzegorz W., Dwilewicz-Trojaczek Jadwiga: Low serum albumin level predicts the risk of azacitidine early discontinuation in MDS, CMML and 20-30% AML patients- results from the Polaza, the retrospective study of the Polish Adult Leukemia Group, Blood, 2019, vol. 134, nr suppl.1, poz.5421, [61st ASH Annual Meeting and Exposition. Orlando, FL, December 7-10, 2019. Abstracts], DOI:10.1182/blood-2019-125632
5	Charlinski Grzegorz, Tyczyńska Agata, Zaucha Jan M, Czyz Adriana, Czyż Jarosław, Dytfeld Dominik, Malecki Bartosz, Gil Lidia, Fornagiel Szymon, Grosicki Sebastian, Barchnicka Agnieszka, Holojda Jadwiga, Kolkowska Agnieszka, Hus Iwona, Helbig Grzegorz, Kopinska Anna, **Masternak Anna**, Woszczyk Dariusz, Markiewicz Miroslaw, Piszcz Jaroslaw, Kloczko Janusz, Usnarska-Zubkiewicz Lidia, Wróbel Tomasz, Robak Pawel J., Robak Tadeusz, Waszczuk-Gajda Anna, Basak Grzegorz, Jurczyszyn Artur: Early mortality in patients with multiple myeloma treated with novel agents - analysis from Polish Myeloma Study Group, Blood, 2020, vol. 136, nr suppl.1, s. 36-37, [62nd ASH Annual Meeting. December 5-8, 2020], DOI:10.1182/blood-2020-143003
6	Olszewska-Szopa Magdalena, Sobas Marta, Wróbel Tomasz, Drozd-Sokołowska J., Subocz E., Zduniak Krzysztof, Gajewska M., **Masternak A.**, Romejko-Jarosińska J., Waszczuk-Gajda A., Wołowiec Dariusz, Kumiega B., Laribi K., Bao-Perez L., Joks M., Czyż Anna: Primary cutaneous B-cell indolent lymphomas - retrospective multicentric analysis, HemaSphere, 2020, vol. 4, nr suppl.1, 983 poz.PB2182, [25th Congress of the European Hematology Association Virtual Edition, 2020. Abstract book], DOI:10.1097/HS9.0000000000000404

7	Barbui Tiziano, Iurlo Alessandra, Masciulli Arianna, Carobbio Alessandra, Ghirardi Arianna, Carioli Greta, Sobas Marta, Elli Elena Maria, Rumi Elisa, De Stefano Valerio, Lunghi Francesca, Marchetti Monia, Daffini Rosa, Gasior Kabat Mercedes, Cuevas Beatriz, Fox Maria Laura, Andrade Marcio, Palandri Francesca, Guglielmelli Paola, Benevolo Giulia, Harrison Claire N., Foncillas Maria Angeles, Bonifacio Massimiliano, Alvarez-Larran Alberto, Kiladjian Jean-Jacques, Bolaños Estefanía, Patriarca Andrea, Quiroz Keina, Griesshammer Martin, Garcia Gutierrez Valentín, Sanchez Alberto Marin, Magro Elena, Ruggeri Marco, Boluda Juan Carlos Hernandez, Osorio Santiago, Gomez-Tarragona Gonzalo Carreño, Sagüés Miguel, Kusec Rajko, Navas Begoña, Angona Anna, Xicoy Blanca, Lopez Abadia Emma, Koschmieder Steffen, Cattaneo Daniele, Bucelli Cristina, Cichocka Edyta, **Masternak Anna**, Cavalca Fabrizio, Borsani Oscar, Betti Silvia, Bellini Marta, Curto-Garcia Natalia, Rambaldi Alessandro, Vannucchi Alessandro: Second versus first wave of COVID-19 in patients with MPN, Blood, 2021, vol. 138, nr suppl.1, poz.315, [63rd ASH Annual Meeting. [Atlanta, GA], December 11-14, 2021. Abstracts], DOI:10.1182/blood-2021-146134
8	Stuckey R., Ianotto J.-C., Santoro M., Wróbel Tomasz, Pérez Encinas M. M., Gómez-Casares M. T., Noya Pereira M. S., **Kulikowska De Nałęcz A.**, Gołos A., Lewandowski K., Szukalski L., González-Martín J. M., Sobas Marta: Analysis of thrombotic and bleeding complications in 1381 essential thrombocythemia patients assessed by R-IPSET thrombosis risk score: a multicenter retrospective study, HemaSphere, 2021, vol. 5, nr suppl.2, 522 poz.EP1091, [EHA2021 Virtual Congress. Abstract book]
9	Barbui Tiziano, Carobbio Alessandra, Ghirardi Arianna, Iurlo Alessandra, De Stefano Valerio, Sobas Marta Anna, Rumi Elisa, Elli Elena Maria, Lunghi Francesca, Gasior Kabat Mercedes, Cuevas Beatriz, Guglielmelli Paola, Bonifacio Massimiliano, Marchetti Monia, Alvarez-Larran Alberto, Fox Laura Maria, Bellini Marta, Daffini Rosa, Benevolo Giulia, Carreño Gonzalo, Patriarca Andrea, Al-Ali Haifa Kathrin, Andrade Marcio, Palandri Francesca, Harrison Claire, Foncillas Maria Angeles, Osorio Santiago, Koschmieder Steffen, Magro Elena, Kiladjian Jean-Jacques, Bolaños Estefanía, Heidel Florian H., Quiroz Keina, Griesshammer Martin, García Gutiérrez Valentín, Marin Sanchez Alberto, Hernandez Boluda Juan Carlos, Lopez Abadia Emma, Carli Giuseppe, Sagüés Miguel, Kusec Rajko, Xicoy Blanca, Guenova Margarita, Navas Begoña, Angona Anna, Cichocka Edyta, **Masternak Anna**, Cattaneo Daniele, Bucelli Cristina, Betti Silvia, Borsani Oscar, Cavalca Fabrizio, Carbonell Sara, Curto-Garcia Natalia, Benajiba Lina, Rambaldi Alessandro, Vannucchi Alessandro M.: Protection against breakthrough Delta/Omicron variants in vaccinated patients with myeloproliferative neoplasms (MPN), Blood, 2022, vol. 140, nr suppl.1, s. 6815-6818, DOI:10.1182/blood-2022-158902
10	Barbui T., Carobbio A., Masciulli A., Iurlo A., Sobas Marta, Elli E. M., Rumi E., De Stefano V., Lunghi F., Marchetti M., Daffini R., Gasior Kabat M., Cuevas B., Fox M. L., Andrade Campos M. M., Palandri F., Guglielmelli P., Benevolo G., Harrison C., Foncillas M. A., Bonifacio M., Alvarez-Larran A., Kiladjian J.-J., Bolanos Calderon E., Patriarca A., Quiroz Cervantes K. S., Griesshammer M., Garcia-Gutierrez V., Marin Sanchez A., Magro Mazo E., Ruggeri M., Hernandez-Boluda J. C., Osorio S., Carreno-Tarragona G., Sagues Serrano M., Kusec R., Navas Elorza B., Angona A., Xicoy Cirici B., Lopez Abadia E., Koschmieder S., Cichocka E., **Kulikowska de Nałęcz A.**, Bellini M., Cattaneo D., Bucelli C., Cavalca F., Borsani O., Betti S., Curto-Garcia N., Carbonell S., Benajiba L., Rambaldi A., Vannucchi A. M.: Predictors of hospitalization and severe outcomes in patients with MPN AND COVID-19, HemaSphere, 2022, vol. 6, nr suppl.3, 899-900 poz.P1009, [EHA2022 Hybrid Congress : 27th Congress of the European Hematology Association. Vienna, June 9-17. Abstract book], DOI:10.1097/01.hs9.0000846904.31214.37

Impact factor: **87,959**

Punktacja ministerialna: **1355,0**

24.04.2023

Piotr Owsiak

4.OPINIA KOMISJI BIOETYCZNEJ

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 528/2017

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 78/XV R/2014 z dnia 26 listopada 2014 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

prof. dr hab. Maciej Bagłaj (chirurgia, pediatria)
prof. dr hab. Karol Bal (filozofia)
dr hab. Jacek Daroszewski (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód (duchowny)
mgr Luiza Müller (prawo)
prof. dr hab. Krystyna Orzechowska-Juzwenko (farmakologia kliniczna, choroby wewnętrzne)
prof. dr hab. Zbigniew Rudkowski (pediatria)
dr hab. Sławomir Sidorowicz (psychiatria)
Danuta Tarkowska (położnictwo)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.:

„Ekspresja supresorowych antygenów BTLA, PD-1 i CTLA-4 w populacji efektorowych i regulatorowych limfocytów krwi obwodowej u chorych na szpiczaka plazmocytowego"

zgłoszonym przez **lek. Annę Masternak** zatrudnioną w Oddziale Hematologii i Onkologii Hematologicznej Szpitala Wojewódzkiego w Opolu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Klinice Hematologii, Nowotworów Krwi i Transplantacji Szpiku SPSK Nr1 we Wrocławiu; Oddziale Hematologii i Onkologii Hematologicznej Szpitala Wojewódzkiego w Opolu oraz Laboratorium Immunopatologii Instytutu Immunologii i Terapii Doświadczalnej PAN we Wrocławiu pod nadzorem dr hab. Agaty Kosmaczewskiej, prof. nadzw. **pod warunkiem zachowania anonimowości uzyskanych danych.**

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu.

Opinia powyższa dotyczy projektu badawczego będącego podstawą rozprawy doktorskiej.

Wrocław, dnia 3 sierpnia 2017 r.

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kotwica

EZ