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IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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ROZPRAWA DOKTORSKA:

Ekspresja NLRs (NOD-like receptors), RLRs (RIG-I-like receptors) i TLRs (Toll-like receptors) w chorobach rozrostowych układu krwiotwórczego.

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Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) in innate immunity. TLRs, NLRs and RLRs ligands as immunotherapeutic agents for hematopoietic diseases. Katarzyna Wicherska-Pawłowska, Tomasz Wróbel, Justyna Rybka. Int.J.Mol.Sci. 2021 Vol.22 no.24 art.13397 [22 s.], tab., bibliogr. 184 poz., summ. DOI: 10.3390/ijms222413397, **IF=6,208**

Infection and expression of Toll-like receptors in lymphoid malignancy patients after autologous stem cell transplantation. Katarzyna Wicherska-Pawłowska, Justyna Rybka, Bożena Jaźwiec, Elżbieta Kalicińska, Aleksandra Bogucka-Fedorczuk, Tomasz Wróbel. Cent.Eur.J.Immunol. 2021 Vol.46 no.4 s.463-469, ryc., tab., bibliogr. 24 poz., summ. DOI: 10.5114/ceji.2021.112071, **IF=1,634**

Polymorphisms in the genes coding for TLRs, NLRs and RLRs are associated with clinical parameters of patients with acute myeloid leukemia. Katarzyna Wicherska-Pawłowska, Katarzyna Bogunia-Kubik, Bartłomiej Kuszczak, Piotr Łacina, Marta Dratwa, Bożena Jaźwiec, Tomasz Wróbel, Justyna Rybka. Int.J.Mol.Sci. 2022 Vol.23 no.17 art.9593 [12 s.], ryc., tab., bibliogr. 31 poz., summ. DOI: 10.3390/ijms23179593, **IF=6,208**

I. STRESZCZENIE

Wstęp:

Układ odporności wrodzonej stanowi pierwszą linię obrony organizmu przed wnikającymi drobnoustrojami chorobotwórczymi. Jednym z elementów tego układu są receptory rozpoznające wzorce molekularne związane z patogenem (pathogen-associated molecular patterns, PAMPs): receptory NOD-podobne (NLRs), receptory RIG-I-podobne (RLRs) i receptory Toll-podobne (TLRs). Są to białka zlokalizowane na powierzchni lub we wnętrzu komórek należących do układu immunologicznego: limfocytów, granulocytów, makrofagów a także komórek spoza układu odpornościowego: komórek endotelialnych, fibroblastów i innych. W odpowiedzi na rozpoznany antygen uruchamiane są wewnątrzkomórkowe szlaki sygnałowe prowadzące do wzmożonej produkcji cytokin prozapalnych i czynników proapoptotycznych służące jak najszybszemu opanowaniu infekcji. Pobudzenie mechanizmów odporności nieswoistej prowadzi do aktywacji układu odporności nabytej i wytworzenia pamięci immunologicznej. Receptory NLRs, RLRs i TLRs rozpoznają także wzorce molekularne związane z uszkodzeniem tkanek (damage-associated molecular patterns, DAMPs), w tym przypadku aktywacja receptorów służy naprawie i regeneracji uszkodzonych struktur organizmu. Przedłużona, patologiczna aktywacja receptorów rozpoznających wzorce prowadzi do występowania przedłużonego stanu zapalnego i rozwoju przewlekłych chorób zapalnych, chorób autoimmunologicznych i karcynogenezy.

Choroby rozrostowe układu krwiotwórczego to heterogenna grupa schorzeń, dotyczących linii mielodalnej i limfoidalnej szpiku kostnego oraz nowotwory układu chłonnego, których przebieg może być ostry lub przewlekły. Rokowanie w tych chorobach jest zależne od wielu czynników: zmian cytogenetycznych, molekularnych, stopnia zaawansowania choroby, stanu ogólnego pacjenta, jego wieku, chorób towarzyszących. Leczenie jest zazwyczaj intensywne a jego przebieg może być powikłany wystąpieniem różnorodnych działań niepożądanych. Najczęściej występują powikłania infekcyjne, które mogą doprowadzić do wystąpienia wstrząsu septycznego i śmierci pacjenta. Stale trwają poszukiwania nowych czynników, które pozwolą na identyfikację pacjentów szczególnie narażonych na powikłania leczenia, czynników korelujących z przebiegiem, rokowaniem i przeżyciem wśród chorych na złośliwe choroby rozrostowe układu krwiotwórczego. Metody leczenia złośliwych chorób szpiku kostnego i układu limfatycznego nadal bazują na chemioterapii choć do leczenia wprowadzane są nowe cząsteczki, których zadaniem jest zwiększenie skuteczności terapii,

zmniejszenie ryzyka wystąpienia działań ubocznych i przedłużenie przeżycia całkowitego pacjentów.

Cel pracy:

Celem pracy była odpowiedź na pytanie w jakim stopniu ekspresja receptorów związanych z wrodzonym układem odpornościowym wpływa na rozwój, przebieg, powikłania leczenia i rokowanie u pacjentów z chorobami rozrostowymi układu krwiotwórczego.

Materiał i metody:

Pierwsza publikacja jest artykułem poglądowym, który opisuje aktualną wiedzę na temat receptorów NOD-podobnych, RIG-I-podobnych i Toll-podobnych. Na podstawie piśmiennictwa dostępnego w bazach publikacji PubMed i Google Scholar podsumowano budowę, funkcje, mechanizm i efekt działania wyżej wymienionych trzech grup receptorów. Opisano trwające badania nad zastosowaniem cząsteczek-agonistów receptorów jako możliwych nowych elementów immunoterapii chorób układu krwiotwórczego.

W kolejnym artykule przedstawiono badanie dotyczące korelacji ekspresji genów dla receptorów TLR2, TLR4 i TLR9, i występowaniem infekcji u pacjentów poddawanych procedurze autoHSCT z powodu złośliwych chorób układu limfatycznego. Grupa badana liczyła 60 chorych z rozpoznaniem szpiczaka plazmocytowego (20 osób), chłoniaków nieziarniczych (20 osób) i chłoniakiem Hodgkina (20 osób). Próbki krwi obwodowej pobierano trzykrotnie: przed rozpoczęciem megachemioterapii, przed wykonaniem ASCT oraz po regeneracji krwiotworzenia. Metodą *real-time* PCR oznaczono ekspresję genów dla TLR2, TLR4 i TLR9, a następnie ustalono związek z częstością występowania infekcji oraz wpływ ekspresji TLRs na regenerację neutrofili po autoprzeszczepieniu. Ponadto oznaczono ekspresję receptorów TLR2, TLR4 i TLR9 w grupie kontrolnej i porównano wyniki z grupą osób chorych.

W trzecim artykule grupę badaną stanowiło 90 pacjentów z rozpoznaniem ostrej białaczki szpikowej, u których oznaczono 15 polimorfizmów pojedynczych nukleotydów w genach dla różnych receptorów NLR, RLR i TLR a następnie opisano związek tych polimorfizmów z różnymi cechami klinicznymi, przebiegiem leczenia i rokowaniem u pacjentów z AML. Materiałem badanym była krew obwodowa pobierana w momencie diagnozy ostrej białaczki szpikowej. Polimorfizmy oznaczano metodą *real-time* PCR.

Wyniki:

Praca poglądowa podsumowuje dotychczasową wiedzę na temat funkcji receptorów NLRs, RLRs i TLRs jako kluczowych elementów układu odporności wrodzonej, wskazuje także na możliwość zastosowania w przyszłości agonistów tych receptorów jako elementów immunoterapii chorób rozrostowych szpiku kostnego.

W kolejnej pracy wykazano, że ekspresja genów dla TLR4 i TLR9 zmienia się po wykonaniu procedury autoHSCT: ekspresja TLR4 jest wyższa przed wykonaniem ASCT niż po wykonaniu procedury a ekspresja TLR9 jest wyższa po ASCT niż przed autotransplantacją. Niska ekspresja receptorów TLR4 i TLR9 przed ASCT ma znaczenie dla wystąpienia powikłań infekcyjnych na dalszych etapach procedury. Ponadto zaobserwowano pozytywną korelację pomiędzy ekspresją TLR9 a szybkością regeneracji neutrofili po ASCT.

W trzeciej publikacji zaobserwowano zależności w występowaniu różnych polimorfizmów pojedynczych nukleotydów w genach *TLR4* rs4986791, *TLR9* rs5743836 i *NOD2* rs2066847 a poziomem CRP w momencie rozpoznania AML oraz *RLR-1* rs10738889 z poziomem LDH w surowicy. Ponadto stwierdzono, że genotyp *TLR3* rs5743305 *AA* występuje częściej u pacjentów z infekcjami. Kolejne opisane zależności dotyczą genotypów *TLR9* rs187084 *C,* który jest związany z lepszym ryzykiem wg ELN (European Leukemia Group) oraz *RLR-1* rs9695310 *GG,* który jest skojarzony ze starszym wiekiem w momencie diagnozy AML. Po podzieleniu grupy badanej na grupę pacjentów młodszych (≤55 lat) i grupę pacjentów starszych (>55 lat) zaobserwowano, że niektóre zależności występują tylko w grupie pacjentów młodszych, inne zaś tylko wśród starszych chorych.

Wnioski:

Elementy wrodzonego układu immunologicznego poza pełnieniem funkcji obronnych mogą mieć wpływ na rozwój wielu nowotworów, w tym chorób rozrostowych układu krwiotwórczego. Poza tym mogą wpływać na przebieg leczenia i rokowanie. Agoniści receptorów NLRs, RLRs i TLRs wykazują potencjał jako forma immunoterapii nowotworów układu krwiotwórczego, jednak ich wprowadzenie do leczenia wymaga dalszych badań. Stopień ekspresji receptorów TLR może być użytecznym biomarkerem do przewidywania wystąpienia powikłań infekcyjnych w trakcie ASCT wykonywanego jako forma leczenia konsolidującego w chorobach rozrostowych układu krwiotwórczego . Zależności pomiędzy poszczególnymi SNP w genach kodujących receptory Toll-podobne, NOD-podobne i RIG-I-podobne a specyficznymi cechami klinicznymi pacjentów z AML mogą wpływać na

rokowanie. Opisane polimorfizmy mogą w przyszłości stać się czynnikami prognostycznymi w ostrej białaczce szpikowej.

II. ABSTRACT

Introduction:

The innate immune system plays a pivotal role in the first line of organism defence against pathogenic microorganisms. Among many of it's elements we distinguish receptors recognizing pathogen-associated molecular patterns (PAMPs) such as: NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). These are proteins located on the surface or inside the immune cells, such as: lymphocytes, granulocytes, macrophages, but also on the surface of endothelial cells, fibroblasts and others. In response of recognized antigen, they activates the intracellular signalling pathways leading to increase production of inflammatory cytokines and proapoptotic factors for rapid infection control. Stimulation of the mechanisms of innate immune system leads to activation of the adaptive immune system and to creation of immunological memory. The NLRs, RLRs and TLRs receptors also recognize the damage-associated molecular patterns (DAMPs). In this case, their activation leads to repairing and regeneration of damaged structures. Prolonged pathological activation of this receptors leads to prolonged inflammation and the development of chronic inflammatory diseases, autoimmune diseases and carcinogenesis.

Hematopoietic diseases are a group of variety of diseases related to the myeloid and lymphoid lines of the bone marrow and neoplasms of the lymphatic system, the course of which may be acute or chronic. Prognosis in this diseases depends on many factors: cytogenetic and molecular changes, general condition of the patient, it's age and comorbidities. Treatment is usually intensive and might be difficult due to presence of various complications, most commonly infectious, which may lead to septic shock and patient's death. Nowadays many ongoing research aims to find the new factors that allows identification of patients which are particularly at the risk of treatment complications and the factors correlating with the course, prognosis and survival among patients with haematopoietic malignancies. Methods of treatment in bone marrow and lymphatic system diseases are still based on chemotherapy, although new molecules are introduced into the treatment. This new therapeutic agents aims to increase the therapy effectiveness, reduce the risk of side effects and extend overall survival of patients.

The aim of the study:

The aim of the study was to answer the question to what extent the expression of receptors related to the innate immune system affects the development, course, treatment complications and prognosis in patients with proliferative hematopoietic diseases.

Material and methods:

The first publication is a review article describing current understanding of NOD-like, RIG-I-like and Toll-like receptors. Based on scientific articles available in PubMed and Google Scholar databases, the structure, functions, mechanism of action and effect of the mentioned three groups of receptors have been summarized. Ongoing research about the use of receptor agonist molecules as possible novel agents in the immunotherapy of haematopoietic diseases has been described.

The next paper presents a study on the correlation of gene expression for TLR2, TLR4 and TLR9 receptors and the incidence of infections in patients undergoing the autoHSCT procedure due to malignant lymphatic system diseases. The study group consisted of 60 patients diagnosed with multiple myeloma (20 patients), non-Hodgkin's lymphoma (20 patients) and Hodgkin's lymphoma (20 patients). Peripheral blood samples were collected three times: before starting mega chemotherapy, before performing ASCT and after hemopoiesis regeneration. The expression of genes for TLR2, TLR4 and TLR9 was determined by the real-time PCR, then the relationship with the frequency of infection and the influence of TLRs expression on the neutrophils regeneration after haematopoietic auto transplantation was established. Moreover, the expression of TLR2, TLR4 and TLR9 receptors was determined in the control group and the results were compared with the study group.

In the third article polymorphism of single nucleotides in receptors genes were examined. The study group consisted of 90 patients with acute myeloid leukemia, in which 15 different variants of single nucleotides polymorphisms in NLR, RLR and TLR receptors were established. Then the relationship of these polymorphisms with various clinical features, treatment course and prognosis in patients with AML was described. Polymorphisms were determined using real-time PCR method from blood obtained from patients during the AML diagnosis.

Results:

The review paper summarizes the current knowledge about the functions of NLRs, RLRs and TLRs as key elements of the innate immune system and indicates the possibility of using agonists of these receptors in the future as elements of immunotherapy in bone marrow proliferative diseases.

In the second study authors prove that the expression of TLR4 and TLR9 genes changes after the autoHSCT procedure: TLR4 expression is higher before ASCT than after the procedure, and TLR9 expression is higher after ASCT than before bone marrow auto transplantation. Low expression of TLR4 and TLR9 receptors before ASCT plays an important role for the occurrence of infectious complications at further stages of the procedure. Moreover, a positive correlation between TLR9 expression and the rate of neutrophil regeneration after ASCT were observed.

In the next publication, the relationship between the occurrence of various single nucleotide polymorphisms in the TLR4 rs4986791, TLR9 rs5743836 and NOD2 rs2066847 genes and the CRP level at the time of diagnosis of AML and RLR-1 rs10738889 with the level of LDH in the serum was observed. In addition, it was found that the TLR3 rs5743305 AA genotype is more common in patients with infections. Authors also described dependencies concern the TLR9 rs187084 C genotypes, which is associated with a greater risk according to ELN (European Leukemia Group) and RLR-1 rs9695310 GG, which is associated with older age at the time of AML diagnosis. After dividing the study group into a group of younger (≤55 years) and older patients (> 55 years), authors observer that some dependencies are only found in the group of younger patients, while others only among elderly group.

Conclusions:

Elements of the innate immune system, apart of their defence functions, may influence on the development of many cancers, including proliferative diseases of the haematopoietic system. Moreover, they might influence the course of treatment and prognosis. The NLRs, RLRs and TLRs agonists have potential as a form of immunotherapy of hematopoietic malignancies, but their introduction to treatment requires further research. The degree of TLR expression might be a useful biomarker for predicting infectious complications during ASCT as a form of consolidation therapy in haematopoietic malignancies. The relationship between particular SNPs in the genes encoding Toll-like, NOD-like, and RIG-I-like receptors and specific clinical features of AML patients may influence prognosis. The described polymorphisms may become prognostic factors in acute myeloid leukemia in the future.

III. WSTĘP

Utrzymanie homeostazy organizmu wymaga sprawnie działającego układu immunologicznego. Mechanizmy układu odpornościowego dzielimy na wrodzone i nabyte. Pierwszą linią obrony organizmu jest układ odporności wrodzonej. Jest on odpowiedzialny za niedopuszczanie do wnikania patogenów wgłąb komórek i tkanek, wykrywanie infekcji drobnoustrojami, produkcję czynników niszczących zakażone komórki oraz aktywację układu odporności nabytej. Do odporności wrodzonej należą takie mechanizmy jak: wielowarstwowy, złuszczający się naskórek i nabłonki wyścielające drogi moczowo-płciowe, pokarmowe i oddechowe, produkcja śliny, łez i soku żołądkowego zawierających substancje przeciwbakteryjne i przeciwwirusowe (lizozym, laktoferyna, defensyny i inne) [1]. Poza tym do układu odporności wrodzonej należą receptory rozpoznające wzorce (pattern recognition receptors, PRRs) zlokalizowane na powierzchni lub na błonach wewnątrzkomórkowych granulocytów, makrofagów, komórek dendrytycznych, komórek NK, ale także na komórkach niewyspecjalizowanych, nie-immunologicznych: endotelialnych, epitelialnych czy fibroblastach. Receptory te rozpoznają wzorce molekularne związane z patogenami (pathogen-associated molecular patterns, PAMPs), którymi mogą być składniki ściany komórkowej, fragmenty materiału genetycznego i inne elementy, które są niezbędne do przeżycia mikroorganizmu, trudno zmienialne w procesie ucieczki przed układem odpornościowym gospodarza [2]. Poza PAMPs receptory rozpoznające wzorce są aktywowane przez wzorce molekularne związane z uszkodzeniem (damage-associated molecular patterns, DAMPs). Są to substancje uwalniane z uszkodzonych komórek i tkanek, jak na przykład: histony, mRNA, kwas moczowy, kwas hialuronowy i inne. Mechanizm uszkodzenia może być różnoraki: niedotlenienie komórek, uszkodzenie mechaniczne, poparzenie, nieprawidłowa budowa czy zainfekowanie komórki. Wykrycie DAMPs prowadzi do naprawy, regeneracji i zastąpienia uszkodzonych tkanek tkanką łączną [3]. Pobudzone receptory uruchamiają wewnątrzkomórkowe szlaki sygnałowe co skutkuje natychmiastową zwiększoną produkcją cytokin prozapalnych: interferonu I (IFN-I), interleukiny 1 (IL-1), interleukiny 6 (IL-6), czynnika martwicy nowotworów (tumor necrosis factor, TNF) oraz innych substancji. Poza produkcją czynników prozapalnych niszczenie zakażonych komórek może się odbywać w mechanizmie aktywacji pyrolizy czy autofagocytozy [4–6].

Właściwa kontrola działania komórek układu immunologicznego i wyciszanie odpowiedzi zapalnej po opanowaniu infekcji jest niezwykle istotną sprawą, gdyż nadmierna lub przedłużona odpowiedź zapalna jest czynnikiem rozwoju wielu przewlekłych chorób

zapalnych, autoimmunologicznych, sprzyja także rozwojowi nowotworów [2,3]. Z drugiej strony, sprawnie działający nadzór immunologiczny gwarantuje odpowiednio szybkie wykrycie i neutralizację komórek zmienionych nowotworowo. Aktualnie dużą wagę przywiązuje się do badania mikrośrodowiska nowotworu (tumor microenvironment, TME). Poza komórkami stromalnymi, endotelialnymi czy fibroblastami w TME znajdują się także komórki układu odpornościowego: makrofagi, komórki dendrytyczne, granulocyty czy komórki NK. Zaburzenie równowagi produkcji cytokin pro- i przeciwzapalnych powoduje niestabilność genetyczną komórek nowotworowych, nabywanie nowych mutacji, ułatwia angiogenezę i tworzenie przerzutów odległych. Ponadto osłabia działanie układu immunologicznego gospodarza i zmniejsza wrażliwość nowotworu na stosowane leczenie [7].

Do chwili obecnej poznano sześć grup rozpoznających wzorce: receptory Toll-podobne (TLRs), NOD-podobne (NLRs), RIG-I-podobne (RLRs), AIM2-podobne (ALRs), receptory lektynowe typu-C (CLRs) i szlak cGAS-STING. TLRs i CLRs są receptorami przezbłonowymi, znajdują się na błonach komórkowych lub błonach wewnątrz komórek (siateczka endoplazmatyczna, ściany lizosomów), pozostałe grupy receptorów to receptory wewnątrzkomórkowe [1,8–10].

Choroby rozrostowe układu krwiotwórczego stanowią dużą i heterogenną grupę chorób szpiku kostnego i układu chłonnego: białaczki, zespoły mielodysplastyczne, zespoły mieloproliferacyjne, zespoły limfoproliferacyjne, w tym chłoniak Hodgkina i chłoniaki nieziarnicze. Na przebieg leczenia, rokowanie i ogólne przeżycie pacjentów ma wpływ wiele czynników: stadium zaawansowania choroby, zmiany cytogenetyczne i molekularne w komórkach, ogólny stan pacjenta, choroby towarzyszące a przede wszystkim poważne powikłania stosowanego leczenia i pierwotna oporność choroby na zastosowaną terapię. Obecnie toczy się wiele badań, które mają na celu opisanie nowych czynników ryzyka, czynników prognostycznych, które ułatwią zaplanowanie odpowiedniego postępowania, identyfikację pacjentów szczególnie wrażliwych na powikłania leczenia i potencjalnie opornych na standardową terapię. Jednocześnie trwają badania nad nowymi cząsteczkami, które poprawią wyniki leczenia, pozwolą na uzyskanie długotrwałych remisji i przedłużą przeżycie całkowite chorych na choroby rozrostowe układu krwiotwórczego. Wysiłki te koncentrują się głównie na immunoterapii nowotworów oraz połączeniu immunoterapii z leczeniem standardowym.

IV. CELE I ZAŁOŻENIA PRACY

Celami niniejszej rozprawy doktorskiej były:

1. Podsumowanie aktualnej wiedzy na temat budowy, funkcji, mechanizmów i efektów działania trzech największych grup receptorów rozpoznających wzorce i związanych z układem odporności nieswoistej. Podsumowanie badań nad zastosowaniem agonistów receptorów jako potencjalnych nowych leków w terapii chorób rozrostowych układu krwiotwórczego.
2. Ocena wpływu ekspresji receptorów na przebieg leczenia i występowanie powikłań infekcyjnych podczas terapii chorób rozrostowych szpiku kostnego.
3. Zbadanie związku pomiędzy polimorfizmami pojedynczych nukleotydów w genach kodujących receptory NLRs, RLRs, TLRs a poszczególnymi cechami klinicznymi oraz powikłaniami leczenia pacjentów z chorobami rozrostowymi układu krwiotwórczego.

V. MATERIAŁ I METODY BADAŃ.

Pierwszy artykuł z cyklu jest pracą poglądową podsumowującą najnowszą wiedzę o budowie, funkcji i mechanizmach funkcjonowania trzech grup receptorów związanych z układem odporności wrodzonej: receptorami Toll-podobnymi, NOD-podobnymi i RIG-I-podobnymi. Na podstawie publikacji naukowych dostępnych w bazach PubMed i Google Scholar opracowano dogłębne podsumowanie aktualnych informacji o budowie, lokalizacji, funkcjach, przebiegu szlaków wewnątrzkomórkowych, efektach działania oraz klinicznych aspektach funkcjonowania TLRs, NLRs i RLRs. Ponadto przedstawiono badania nad możliwościami terapeutycznego zastosowania cząsteczek –agonistów receptorów jako potencjalnie nowych leków w terapii chorób rozrostowych układu krwiotwórczego.

W kolejnej pracy grupę badaną stanowiło 60 pacjentów poddawanych procedurze ASCT jako etapu konsolidacji leczenia z powodu szpiczaka plazmocytowego (MM, 20 chorych), chłoniaka Hodgkina (HL, 20 chorych) oraz chłoniaków nieziarniczych (NHL, 20 chorych). Mediana wieku pacjentów wynosiła 51 lat (zakres 26-64 lata). Grupę kontrolną stanowiło 10 zdrowych osób, ochotników. Materiałem badanym była krew obwodowa, pobierana w grupie badanej trzykrotnie: przed podaniem megachemioterapii okołoprzeszczepowej, przed ASCT oraz po regeneracji krwiotworzenia rozumianej jako liczba neutrofili >500/ul krwi oraz poziom płytek krwi >20 G/L. W pobranych próbkach oceniano ekspresję genów dla receptorów TLR2, TLR4 i TLR9. Jako kondycjonowanie u pacjentów ze szpiczakiem plazmocytowym zastosowano melfalan w dawce 200 mg/m2 lub 140 mg/m2, w zależności od wieku i stanu ogólnego pacjenta, pacjenci z chłoniakami w ramach przygotowania przed ASCT otrzymali chemioterapię wg protokołu BEAM (karmustyna, etopozyd, arabinozyd cytozyny i melfalan). Mediana ilości przetoczonych komórek CD34+ wynosiła 3.38*10^6 komórek/kg masy ciała biorcy. Mediana czasu neutropenii wynosiła 10 dni u pacjentów z MM i 12 dni u pacjentów z HL i NHL. Mediana czasu regeneracji neutrofili wynosiła 14 dni w całej grupie badanej. Wystąpienie gorączki neutropenicznej zaobserwowano u 30 pacjentów, u 24 ustalono czynnik etiologiczny. Ekspresję genów TLR2, TLR4 i TLR9 oceniano metodą *real-time* PCR z użyciem systemu TaqMan Assasays. Beta-glukuronidaza stanowiła kontrolę wewnętrzną. Reakcję PCR przeprowadzono na aparacie 7500 Real Time PCR Instrument z zastosowaniem Gene Expression Master Mix. Analizę statystyczną przeprowadzono przy użyciu programu STATISTICA 12. W grupie badanej dla zmiennych ilościowych obliczono średnią arytmetyczną (X) i odchylenie standardowe (SD) szacowanych wartości. Rozkład zmiennych został zbadany za pomocą testów Lilliefors i Shapiro-Wilk W-

test. Dla zmiennych niezależnych ilościowo o rozkładzie normalnym zastosowano t-test. W przypadku ilościowych zmiennych o rozkładzie innym niż normalny zastosowano test sekwencji par Wilcoxona. W celu zdefiniowania relacji między badanymi zmiennymi przeprowadzono analizę korelacji. Wyniki na poziomie $p<0.001$ uznano za istotne statystycznie.

W trzeciej pracy grupa badana składała się z 90 pacjentów z rozpoznaniem ostrej białaczki szpikowej (AML). Pacjenci byli leczeni w Klinice Hematologii, Nowotworów Krwi i Transplantacji Szpiku Kostnego Wrocławskiego Uniwersytetu Medycznego w latach 2018-2020. W grupie badanej znalazło się 48 kobiet i 42 mężczyzn, mediana wieku wynosiła 61 lat (zakres 21-81 lat), w grupie pacjentów starszych (>55 r.ż.) znajdowało się 54 chorych. Do badań wykorzystano krew obwodową pobieraną od pacjentów w momencie rozpoznania AML. Z komórek jądrzastych wyizolowano DNA przy użyciu zestawów GeneMATRIX Quick Blood DNA Purification firmy EurX (Gdańsk, Polska) i NucleoSpin Blood (MACHEREY-NAGEL GmbH & Co. KG, Dueren, Niemcy). U pacjentów oznaczono polimorfizmy pojedynczych nukleotydów w genach kodujących receptory: DDX58 (rs9695310, rs10738889, rs10813831), NOD1 (rs2075820, rs6958571), NOD2 (rs2066845, rs2066847, rs2066844), TLR3 (rs5743305, rs3775296, 3775291), TLR4 (rs4986791, rs4986790) i TLR9 (rs187084, rs5743836) przy użyciu zestawu LightSNiP (TIB MOLBIOL, Berlin, Niemcy). Reakcje *real-time* PCR przeprowadzono na aparacie LightCycler 480 II (Roche Diagnostics, Rotkreuz, Szwajcaria). Analizę statystyczną przeprowadzono z użyciem programu Real Statistic Resource Pack dla programu Microsoft Excel 2013 (wersja 15.0.5023.1000, Microsoft, Redmont, WA, USA). Do zbadania korelacji między poszczególnymi polimorfizmami pojedynczych nukleotydów (SNP, single nucleotide polymorphisms) a poziomem CRP i LDH w surowicy krwi oraz liczbą blastów w szpiku kostnym wykorzystano U-test Manna-Whitney'a, natomiast do zbadania korelacji miedzy poszczególnymi SNPs a wiekiem w momencie rozpoznania choroby, obecnością ognisk pozaszpikowych, występowaniem infekcji podczas leczenia oraz do stwierdzenia zależności miedzy SNP a ryzykiem wg ELN wykorzystano dokładny test Fishera. Do analizy przeżycia całkowitego wykorzystano krzywe Kaplana-Meiera oraz Gehana-Breslowa-Wilcoxona. Wartość $p<0.05$ ustalono jako istotną statystycznie. Wykresy wykonano za pomocą programu GraphPad Prism (GraphPad Software, La Jolla, CA, wersja 8.0.1).

VI. CYKL PUBLIKACJI

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Review

Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs), and RIG-I-Like Receptors (RLRs) in Innate Immunity. TLRs, NLRs, and RLRs Ligands as Immunotherapeutic Agents for Hematopoietic Diseases

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Abstract: The innate immune system plays a pivotal role in the first line of host defence against infections and is equipped with patterns recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Several classes of PRRS, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) recognize distinct microbial components and directly activate immune cells. TLRs are transmembrane receptors, while NLRs and RLRs are intracellular molecules. Exposure of immune cells to the ligands of these receptors activates intracellular signalling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in the inflammatory and immune responses. The innate immune system also influences pathways involved in cancer immunosurveillance. Natural and synthetic agonists of TLRs, NLRs, or RLRs can trigger cell death in malignant cells, recruit immune cells, such as DCs, CD8+ T cells, and NK cells, into the tumour microenvironment, and are being explored as promising adjuvants in cancer immunotherapies. In this review, we provide a concise overview of TLRs, NLRs, and RLRs: their structure, functions, signalling pathways, and regulation. We also describe various ligands for these receptors and their possible application in treatment of hematopoietic diseases.

Keywords: innate immunity; Toll-like receptors; NOD-like receptors; RIG-I-like receptors; immunotherapy; hematopoietic diseases

1. Introduction

A properly-functioning human immune system is an essential element in maintaining systemic homeostasis. It is responsible for recognizing and controlling infections caused by invasion of pathogenic microorganisms. Immunity is divided into innate and acquired, while non-specific immunity mechanisms constitute the first line of defence of the human organism against pathogens entering the system. Acquired immunity is responsible for fighting infection in its later stages and for producing immune memory [1,2]. The mechanisms of innate immunity activate a specific immune response directed precisely toward the microorganism that caused the disease and toward infected host cells [2].

Pattern recognition receptors (PRRs) play a major role in innate immunity. This is a large group of receptors characterized by several common features, such as recognition of pathogen-associated molecular patterns (PAMPs), continuous expression in the host independent of past infections, and detection of microorganisms regardless of their developmental and life cycle stage. These receptors are localized on the surface or inside of macrophages/monocytes, dendritic cells, NK cells, mast cells, neutrophils, and eosinophils, but also on non-specialized, non-immune epithelial cells, endothelial cells, or fibroblasts [3,4]. PAMPs are building blocks of microorganisms that are essential for their survival and thus are difficult to alter in the process of escaping the host's immune mechanisms. They involve the wall structure of bacteria, fungi, genetic material, and many other substances recognized by specific receptors [5]. In addition to PAMPs, PRRs also recognize damage-associated molecular patterns (DAMPs) that are released from damaged host cells and tissues. DAMPs include hyaluronic acid, histones, mRNAs, cholesterol crystals, and others [5–7].

Six groups of PRRs have been described and they include: Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding domain and leucin-rich repeat-containing receptors (NLRs), C-type lectin receptors (CLRs), cyclic AMPGMP) synthase and stimulator of interferon genes (cGAS-STING pathway), and AIM2-like receptors (ALRs) [8–12]. TLRs and CLRs are transmembrane receptors that are found both on the cell surface and in intracellular membranes, i.e., the endoplasmic reticulum or lysosome wall. The intracellular receptor group includes RLRs, NLRs, and cGAS-STING pathway. Stimulated receptors trigger a cascade of signalling pathways leading to increased production of interferon I (IFN-I), proinflammatory cytokines (interleukin1—IL-1; interleukin-6—IL-6; tumour necrosis factor—TNF), and other proinflammatory substances. The substances released in this process cause an increased influx of immune cells and activation of the nonspecific response system. In addition to stimulating the production of IFN-I and proinflammatory cytokines, another way to fight infection is through direct destruction of infected cells by pyrolysis or autophagocytosis [1,3]. Once DAMPs are recognized, signalling pathways within the cells are also activated, leading to the production of various cytokines and chemokines that ultimately result in the induction of so-called "sterile inflammation". This process plays an important role in the repair and regeneration of damaged tissues [6,7].

Hematopoietic diseases are characterized by heterogeneity and include leukemias, myelodysplastic syndromes, myeloproliferative syndromes, Hodgkin's lymphoma, and a large group of non-Hodgkin's lymphomas (NHL). Currently, numerous studies are underway to develop new therapies that will achieve high response rates, long-term remissions, and improve overall survival of patients with hematologic malignancies, especially in patients who are initially chemo resistant. The direction of this search is mainly focused on immunotherapy and the combination of immunotherapy with conventional treatments [13–15].

In this article, we present a description of the structure, functions, signalling pathways, ways of regulation, and potential use in the treatment of hematopoietic proliferative diseases of three most important groups of PRRs: TLRs, NLRs, and RLRs.

2. Toll-Like Receptors (TLRs)

2.1. Structure, Location, and Functions

Toll-like receptors are the first group included in the broad family of PRRs. The first receptors encoded by the mutated *toll* gene were described in fruit flies (*Drosophila melanogaster*), in which they are responsible for developmental processes and the immune system [16,17]. The discovery of receptors responsible for detecting and controlling fungal infections in fruit flies set off a wave of research looking for similar receptors in other species. Molecular and cytogenetic studies have identified TLRs among all living multicellular organisms that face microbial attack. Toll-like receptors are germline encoded, evolutionarily old proteins [18–20]. Each species has a specific, genetically determined number of TLRs—from 9 in *Drosophila* to 222 in purple sea urchin [21]. In mammals,

13 genes encoding TLRs have been identified so far (TLR1-TLR10 in humans and TLR1TLR13, but without TLR10 in mice) [3,22].

Toll-like receptors are expressed on many cells, both in the immune system—macrophages, dendritic cells (DCs), B cells, NK cells, some T cells, as well as on the surface of epithelial and endothelial cells and fibroblasts. Some TLRs are localized to the cell surface (TLRs 1, 2, 4, 5, 6) and the remaining group of TLRs (TLRs 3, 7, 8, 9) are bound to endosomal membranes intracellularly [3,4,22].

All TLRs show a similar domain organization as they are members of the transmembrane protein I family. TLRs are composed of an extracellular N-terminal leucine-rich repeat (LRR), a single transmembrane domain, and an intracellular Toll/IL-1R receptor, known as a cytoplasmic TIR domain. The LRR domain consists of 16–28 tandem repeats of the LRR motif and is responsible for recognizing ligands such as proteins (e.g., bacterial flagellin), sugars (e.g., fungal zymogen), lipids (bacterial lipopolysaccharide), and nucleic acids (DNA and RNA of viruses). The intracellular domain of TIR consists of approximately 150 amino acids and shows similarity to the cytoplasmic region of the IL-1 receptor. It is essential for activating the signal transduction cascade [12,23–25].

In mammals, Toll-like receptors are synthesized in the endoplasmic reticulum (ER) and then transported to the target site. The endoplasmic reticulum-associated chaperone proteins gp96, PRAT4A, and Unc93B1 play important roles in the generation, maturation, and proper folding of receptors [26–28]. In cells lacking gp96 and PRAT4A, TLRs receptors (except TLR3) do not show their ligand recognition activity leading to a significant reduction in the production of proinflammatory cytokines, IFN I and other chemokines in response to infection [29,30]. Unc93B1 protein is a chaperone protein that binds endoplasmic TLRs (TLR 3, 7, 9) and TLR5 in the cytoplasmic reticulum, facilitating their maturation and proper folding. Once bound to a receptor, it remains bound to it and is essential for maintaining a stable receptor conformation. Unc93B1 accelerates receptor attachment to endosome membranes dissociating from the ER [31,32].
Upon attachment to the endosomal membrane, the LRR domain of TLRs 3, 7, 8, 9, 13 receptors is fragmented by cathepsins. The resulting domains remain connected to each other, allowing receptor dimers to form and function properly. Interestingly, intact receptors can still recognize PAMPs but are unable to trigger the signalling cascade and pass on the threat recognition information [33–37].

Different TLRs recognize different PAMPs and DAMPs in the body. TLR1, TLR2, and TLR6 receptors form heterodimers (TLR1/2, TLR2/6), through which they recognize mainly triacylated lipopeptides from Gram-negative bacteria and diacylated lipopeptides from *Mycoplasma* spp. [38,39] TLR2 itself presents the widest range of detectable ligands, and can respond to the presence of, among others, bacterial proteins (e.g., V-antigen from *Yersinia*), hemagglutinins from smallpox virus, glycolipids, glycopeptides, and lipoproteins from *E. coli*, *B. burgdorferi*, *M. tuberculosis* [40,41]. When combined with a protein known as

dectin-1 (a member of the C-type lectin family), it recognizes zymosan [42]. TLR3 recognizes viral double-stranded RNA (dsRNA) from, for example, reoviruses. In addition, it recognizes dsRNAs arising during replication of single-stranded RNAs (ssRNAs) of viruses, e.g., *West Nile virus, RSV*, or *EMCV* (encephalomyocarditis virus). The ligand for TLR3 can also be a synthetic dsRNA analogue called poly(I:C) [43–45]. TLR4 recognizes lipopolysaccharide (LPS) derived from the wall of Gram-negative bacteria. For proper recognition of LPS, TLR4 requires the formation of a dimer with the membrane protein MD2 [46]. In addition, TLR4 recognizes glycosaminophospholipids from *Trypanosoma*, fusion proteins from *RSV* and also envelope proteins from mouse mammary tumour virus (*MMTV*) [47–50]. In addition, it indirectly or directly detects DAMPs such as heat shock proteins, fibrinogen, hyaluronic acid, beta-defensins, and others [51]. TLR5 recognizes monomers of flagellin, which is a structural protein of bacteria that have the ability to move. TLR5 is abundant on respiratory epithelial cells and in the lamina propria of the small intestine, as detection of bacterial ligands is important in counteracting the adhesion and invasion of microorganisms that cause respiratory tract infections and gastrointestinal infections [52,53]. TLR7, TLR8, and TLR9 are localized to the cell wall of endosomes where they detect nucleic acids, ssRNA (TLR7 and TLR8) [54,55], and unmethylated CpG containing ssDNA (TLR9) from viruses and bacteria [56,57]. Ligands for the human TLR10 receptor have not yet been precisely identified, but some studies suggests that *HIV-1* gp 41 can be TLR10 ligand. A summary of ligands for Toll-like receptors is provided in Table 1.

Table 1. Receptor ligands for TLRs; dsRNA-double-stranded RNA, ssRNA-single stranded RNA, LPS-lipopolisacharide, and dsDNA-double-stranded DNA.

TLR	Location in the Cell	Ligand	Origin of the Ligand
TLR1/2	Cell membrane	Triacylated lipopeptides	Bacteria, Mycobacteria
TLR2	Cell membrane	Hemagglutinins, glycosylphosphatidylinositol, phospholipoman, lipoarabinomannan, peptidoglycans, porins, lipoproteins	Bacteria, Mycobacteria, viruses, fungi, parasites, self
TLR3	Endolysosomal membrane	dsRNA, ssRNA	Viruses
TLR4	Cell membrane	LPS, mannan, inositol phospholipids, envelope proteins	G- bacteria, viruses, self
TLR5	Cell membrane	Flagellin	Bacteria
TLR2/6	Cell membrane	Diacylated lipopeptides, zymosan, lipoteichoic acid	Bacteria, mycobacteria, viruses, fungi
TLR7 (and human TLR8)	Endolysosomal membrane	ssRNA	Viruses, bacteria, fungi
TLR9	Endolysosomal membrane	dsDNA, CpG DNA, hemozoin	Bacteria, viruses, protozoa, self
TLR10	Endolysosomal membrane	HIV-1 gp41	Viruses
TLR11 (mice)	Cell membrane	Profilin-like molecules	Protozoa

2.2. Signal Transmission through TLRs

The studies performed so far have revealed how the ligand recognition signal is transmitted by TLRs. The fundamental step in the initiation of the signalling pathway is the formation of a dimer by the two extramembrane domains followed by the formation of a dimer by the TIR domains. TLR3, 4, 5, 7, 8, 9 receptors form homodimers, while heterodimers are formed by TLR1, 2, 6 (TLR1/2, TLR2/6) [46,58–61]. Each dimer binds different amounts of ligand. The TLR3 dimer binds a single dsDNA molecule, TLR1/2 and TLR2/6 heterodimers also bind one di- or triacetylated lipoprotein molecule each. In contrast, the TLR9

dimer attaches two fragments of CpG-rich DNA and each TLR4 dimer pair attaches two LPS molecules each [58,62].

The exact signalling pathways by which the information of microbial recognition triggers a cellular response and the production of substances to reduce and combat the existing threat are still being studied. Currently, most of the signal transduction pathway, the proteins involved in the signal transduction cascade, and the DNA fragments that respond to given signals have been established, but it is still possible to discover alternative pathways and new participants in this process. As we mentioned earlier, the signalling pathway begins with dimerization of intracellular TIR domains. TIR dimers attach one of the TIR-domain containing adaptor proteins, including MyD88, TRIF, TIRAP/MAL, or TRAM. MyD88 can be used by all TLRs to activate NF-kBs and MAPKs and subsequently stimulate the production of proinflammatory cytokines. TIRAP is a protein that is involved in the attachment of MyD88 to membrane TLRs such as TLR2 and TLR4 and it is directly involved in signal transduction by endosomal TLRs like TLR9. TRIF is attached to TLR3 or TLR4 and promotes an alternative signalling pathway leading to activation of IRF3, NF-kB, and MAPKs, causing increased synthesis of IFN I and proinflammatory cytokines. TRAM is connected to TLR4 as a bridge between TRIF and TLR4 and TLR3 connects directly to TRIF [63,64]. Depending on the adaptor attached, TLRs signalling pathways have been divided into two pathways, MyD88-dependent and TRIF-dependent.

2.2.1. MyD88-Dependent Pathway

When MyD88 binds to TLRs, IRAK family proteins are attached and the resulting complex is called a myddosome. During myddosome formation, IRAK4 activates IRAK1, which binds to the RING-domain of E3 ubiquitin ligase TRAF6. TRAF6 with ubiquitin-conjugating enzyme UBC13 and UEV1A attached to it leads to K63-linked polyubiquitination of its own molecule and TAK1 kinase protein complex formation. TAK1 is a protein belonging to the MAPKKK family of proteins and forms a complex with three regulatory units, TAB1, TAB2, and TAB3, which react with polyubiquitinated chains generated by TRAF6, leading to TAK1 activation. TAK1 then leads to the activation of two pathways: the IKK-complex NF-kB pathway and the MAPK-pathway. TAK1 phosphorylates IKKB of the IKK complex, resulting in the translocation of NF-kB to the cell nucleus and the stimulation of genes responsible for the production of proinflammatory cytokines. TAK1 activation also leads to the activation of MAPK family proteins such as Erk1/2, p38 and JNK, which lead to the activation of AP-1 family transcription proteins which stabilizes mRNA and leads to the regulation of inflammatory response [65–67].

2.2.2. TRIF-Dependent Pathway

TRIF protein interacts with TRAF6 and TRAF3. TRAF6 recruits RIP-1 kinase, which in turn activates the TAK1 complex leading to activation of NF-kB and MAPKs and increased production of proinflammatory cytokines. In addition, TRAF3 recruits the kinases TBK1 and IKKi (related to IKK proteins), which interact with the NEMO protein during IRF3 phosphorylation. IRF3 then forms dimers that travel to the cell nucleus and cause increased gene expression for IFN I [65–67].

Pellino E3 ubiquitin ligases family proteins are also involved in the TRIF-dependent signalling pathway. Pellino-1 is phosphorylated by TBK1/IKKi which accelerates RIP-1 ubiquitination, suggesting that Pellino-1 mediates NF-kB activation in the TRIF-dependent pathway through RIP-1 recruitment [68].

2.2.3. Balance between MyD88- and TRIF-Dependent Pathways

TLR4 activates both MyD88- and TRIF-dependent conduction pathways. Activation of these two pathways is under the control of several proteins that are responsible for an adequate response to detected infection. The balance in the production of proinflammatory cytokines and IFN I may have implications for the development of cancer and inflammatory and autoimmune diseases. TRAF3 is a protein found in both the myddosome and triffosome complex. In the MyD88 complex, TRAF3 is degraded, resulting in

activation of TAK1. Additionally, TRAF3 functions as an inhibitor of the MyD88-dependent pathway. NRDP-1 E3 ubiquitin ligase binds and ubiquitinates MyD88 and TBK1, resulting in MyD88 degradation and TBK1 activation. This results in decreased production of proinflammatory cytokines and increased production of IFN I [69]. MHC class II molecules, which are localized in the endosomes of antigen-presenting cells, cause maintenance of Btk kinase activity. They use CD40 as a co-stimulatory molecule for this task. Activated Btk interacts with MyD88 and TRIF, causing the activation of both MyD88-dependent and TRIF-dependent signalling pathways, leading to enhanced production of proinflammatory cytokines and IFN I, respectively [70].

2.2.4. Intrinsic and Pathogen-Related Negative Regulation of TLR Signalling Pathways

Negative regulation of signalling pathways is necessary to prevent excessive secretion of proinflammatory cytokines and IFN I, both of which may be involved in the development and exacerbation of inflammatory and autoimmune diseases. Targets for molecules that impair signal transduction occur at each key step in the signalling pathway. Activation of the MyD88-dependent pathway is inhibited by ST2825, SOCS-1, and Cbl-b, and the TRIF-dependent pathway is inhibited by SARM and TAG [71,72]. These proteins bind to MyD88 and TRIF preventing them from attaching to TLRs and initiating the signalling pathway. TRAF3 activation is inhibited by SOCS3 and DUBA and TRAF 6 is targeted by numerous inhibitors including A20, USP4, CYLD, and others [73,74]. Additionally, the transcription factor NF-kB can be inhibited by Bcl-3, IkBNS, Nurr1, ATF3, or PDLIM2 [75] and IRF3 activity is attenuated by Pin1 and RAUL [76].

Pathogens also have the ability to impair or inhibit TLR signalling pathways. These strategies can be divided into two categories. The first focuses on early steps in signalling pathways that are unique to a particular TLR and the second involves interference with steps in pathways that are not unique to a particular receptor, such as preventing activation of NF-kBs or MAPKs [75]. Examples include modifications in the conformation of bacterial LPS, whose new conformation is more difficult to recognize by the DC14-MD2-TLR4 complex [77,78]. Staphylococcus aureus changes the peptidoglycan structure to one that is more difficult to recognize in lysosomes [79,80]. Similarly, H. Pylori substitutes the amino acids encoding flagellin making recognition by TLR5 more difficult [52]. An increasing number of microorganisms encode TIR-domain-containing proteins in the genome that inefficiently bind to elements of the myddosome [75]. Some microorganisms produce enzymes that inactivate key signalling pathway proteins: e.g., viral TRIF degrading protease (*HCV, Coxsackie 3 virus*) [81].

3. NOD-Like Receptors (NLRs)

NOD-like receptors are another group of receptors after TLRs that belong to the PRRs family. They are present in cells of invertebrates and vertebrates, in humans 22 receptors of the NLRs family have been described so far [11,82]. These are receptors located in the cytoplasm of cells. The structure of NLRs is characterized by a common domain organization: (i) a centrally located nucleotide-binding NACHT domain (NAIP, HET-E, TP-20) that participates in autooligomerization and is essential for ATP-dependent activation of NLRs, (ii) an N-terminal effector domain that binds to adaptor proteins and downstream effectors to convey receptor excitatory information, and (iii) a C-terminal region that is composed of varying numbers of LRR repeats and is responsible for ligand recognition [83,84]. Human NLRs have been divided into four subgroups, depending on the structure of their N-terminal domain, which may include (i) acidic transactivation domain (AD)-subgroup NLRA, (ii) baculoviral inhibitory repeat-like domain (BIR)-subgroup NLRB, (iii) caspase activation and recruitment domain (CARD)-subgroup NLRC, or (iv) pyrin domain (PYD)-subgroup NLRP [84,85].

The NLRA subgroup includes only one type of receptor called CIITA (Class II Histocompatibility Complex Transactivator). The C domain of this receptor contains four LRR repeats and a GTP-binding domain. GTP attachment accelerates transport of the molecule to the cell nucleus, where it plays a role in activating

gene expression for MHC class II not by binding to DNA but by intrinsic acetyltransferase activity [86–88]. The NLRB-subgroup also includes only one receptor, NAIP (NLR Family Apoptosis Inhibitory Protein) For a long time, NAIP was considered as an anti-apoptotic protein that acts by inhibiting the activity of caspase 3 (CASP3), CASP7 and CASP9, but now, NAIP is consider rather as a sensor of bacterial flagellin or type-3 secretion system components delivered by pathogens leading to NLRC4 inflammasome activation. NAIP mediates also neuronal survival in various pathological states and protects against apoptosis induced by a variety of factors [89–91]. NLRC is a subgroup involving 6 receptors, nucleotide oligomerization domain 1 (NLRC1)

(NOD1), nucleotide oligomerization domain 2 (NLRC2) (NOD2), NLRC3, NLRC4, NLRC5, and NLRX1. NOD1 and NOD2 are considered the two major receptors belonging to the NLRC. They recognize intra-bacterial building blocks that enter the cell through direct bacterial invasion or through other cellular uptake mechanisms [92,93]. The NLRP family includes 14 receptors characterized by the presence of a PYD domain at the N-terminus that is responsible for transmitting an pyroptotic signal or inducing an inflammatory response [94,95]. Interestingly, studies have shown that the NLRP family of receptors plays a significant role in both innate immunity and reproduction in mammals. It has been suggested that NLRPs may play a role in oogenesis and early stages of embryogenesis

(pre-implantation) [96,97].

As PRRs, NLRs recognize a wide range of PAMPs, such as bacterial cell wall components (peptidoglycan, flagellin), microbial secreted toxins, viral RNA, fungal sharps, or even entire microbial and parasitic organisms [10,98–100]. DAMPs that are recognized by NLRs include: ATP, hyaluronic acid, sodium urate (MSU), uric acid, and cholesterol crystals [6,7,101]. Moreover, environmental substances such as asbestos, silicon, aluminium, skin irritants, and UV radiation can cause stimulation of NOD-like receptors. A summary of ligands for NLRs is provided in Table 2. However, not all NLRs function as PRRs as some respond to the presence of cytokines such as interferons.

Table 2. Receptor ligands for NLRs and their functions in the body [84,102]. PRR-pattern recognition receptors, DAPdiaminopimelic acid, MDP-muramyl dipeptide, ROS-reactive oxygen species, and DAMPs-damage associated molecular patterns.

Subgroup	NLR	Ligand/Function
NLRA	CIITA	Regulation of MHC II expression
NLRB	NAIP	PRR for flagellin, pyroptosis, inhibition of apoptosis
NLRC	NOD1	PRR for DAP
	NOD2	PRR for MDP, viral ssRNA, autophagy,
	NLRC3	Negative regulation of T cell activation and TLR activation
	NLRC4	PRR for flagellin, rod proteins, pyroptosis, phagosome formation
	NLRC5	Upregulation of MHC I expression, regulation of innate response
	NLRX1	ROS production, autophagy induced by viral infection
NLRP	NLRP1	PRR for MDP and anthrax toxin
	NLRP2	Negative regulation of NF-kB, embryonic development

NLRP3	PRR for DAMPs
NLRP4	Negative regulation of IFN I, autophagy
NLRP5	embryogenesis
NLRP6	Negative regulation of NF-kB
NLRP7	PRR for lipopeptides
NLRP8	unknown
NLRP9	unknown
NLRP10	Migration of dendritic cells
NLRP11	unknown
NLRP12	Negative regulation of NF-kB
NLRP13	unknown
NLRP14	spermatogenesis

Activated NLRs play various roles that can be divided into four groups: inflammasome formation, signal transduction, stimulation of gene transcription, and autophagy [93,102–106].

3.1. Formation of Inflammasomes

Inflammasomes are multi-molecular complexes that activate caspase-1. Activated caspase-1 leads to the maturation of pro-interleukin-1B (pro-IL-1B) and pro-interleukin-18 (pro-IL-18) into their active forms IL-1B and IL-18 [103,104]. A consequence of inflammasome formation may also be the phenomenon of pyroptosis, a term used to describe inflammatory, programmed cell death dependent on caspase 1/4/5/11. In addition, pyroptosis leads to the release of DAMPs and an enhanced inflammatory response [107,108].

Receptors capable of forming inflammasomes include NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12, and NLRC4 (after activation by NAIP) [103,109]. Inflammasome formation is a response to recognition by PAMPs receptors of such things as bacterial toxins, flagellin proteins, muramyl dipeptide, viral or bacterial RNA and DNA, fungal fragments, fungal mannan, zymosan, and protozoa-derived hemozoin. Sterile activators include DAMPs such as ATP, cholesterol crystals, hyaluronic acid, sodium urate, or amyloid and environmental factors include silicon, aluminium, asbestos, UV radiation, and others [103]. The structure of the inflammasome is composed of a receptor (NLR), an ASC (apoptosis-associated speck-like protein containing CARD) adaptor molecule, and an effector molecule (pro-CASP1). Upon ligand recognition, NLR attaches the ASC molecule via pyrin-pyrin domain binding. Subsequently, pro-CASP1 binds to ASC via the CARD-CARD domain resulting in the formation of a functional inflammasome [110]. NLRP1 has a CARD domain that can directly bind to pro-CASP1 without the involvement of ASC [111,112] protein whereas NLRC4, which lacks the PYD domain, can form two types of inflammasomes. The attachment of ASC protein to NLRC4 results in increased secretion of IL-1B and IL-18 and lack of ASC attachment during inflammasome formation results in pyroptosis [113,114]. The ligand for NLRC4 located in macrophages may be Salmonella rods [115]. NAIP and NLRC4 form the NAIP-NLRC4 inflammasome upon recognition of

the bacterial flagellin and bacterial type III secretion system [114]. NLRP1 inflammasomes are formed after recognition of muramyl dipeptide (MDP), a common bacterial peptidoglycan (produced by G+ and G− bacteria) and after exposure to anthrax toxins [111,112]. NLRP3 forms inflammasomes under the influence of G+ bacteria, viruses, fungi, protozoa, ATP, and ROS. In addition, mitochondrial DNA and RNA can also activate NLRP3 [95,116]. NLRP7 can recognize bacterial lipopeptides [117].

3.2. Signal Transduction

The NOD1 receptor recognizes peptidoglycan derived from G- bacterial D-γ-glutamylmeso-diaminopimelic acid (iE-DAP) and the NOD2 receptor recognizes bacterial G- and G+ MDP [118]. Upon binding to ligand, those NLRs bind to receptor-interacting serine/threonineprotein kinase 2 (RIPK2), resulting in the formation of the RIPK2-IkB complex. Subsequently, RIPK2 causes activation of TAK1 kinase, which is a prerequisite for IKK complex activation and MAPK pathway activation. IKK-dependent dephosphorylation of the NF-kB inhibitor (Ikappa-Balpha) enables its translocation to the cell nucleus and the associated increased transcription of genes for pro-survival cytokines. Activation of the mitogen-activated protein kinase (MAPK) pathway also results in increased secretion of proinflammatory cytokines by the cell [118].

In contrast to NOD1 and NOD2, some NLRs play a role as inhibitors of intracellular signalling pathways mediated by different receptors. Such effects have been described, among others for NRLC3 in T cells, NLRC5 in various non-specific immune cells, NLRP2/NLRP4 which are negative regulators of the NF-kB pathway through TRAF6-modifying effects, NRLP7 inhibits IL-1B secretion and NLRP10 inhibits caspase-1 activation [119–125].

3.3. Activation of Transcription

Another example of the diversity of functions of NLRs in the body is the CIITA receptor (NLRA). Several experiments found that NLRA fully corrected the defect in MHC class II production in cells from patients suffering from bare lymphocyte syndrome (BLS), a severe immunodeficiency. CIITA acts as a transactivator of gene expression for MHC class II. CIITA acts as a scaffold that allows the recruitment of DNA-binding transcription factors and histone-modifying enzymes to the promoters of MHC class II genes [126]. The mechanism of this action is not yet well understood, nor is CIITA's function as a PRR.

Another receptor of the NLRs-NLRC5 family has an important role in the transcription of genes for MHC class I. IFN-γ-treated NLRC5 acts as a transactivator of gene expression by folding regulatory factor X (RFX), c-AMP-responsive-element binding protein 1 (CREB1), activating transcription factor 1 (ATF1), and nuclear transcription factor Y (NFY) into the SXY module in the promoter of genes for MHC class I. (Footnote to NLRC5 in MHC I). Although the expression of genes for MHC class I and II depends on several transcription factors (such as NF-kB, IFN regulatory factor family, RFX, CREB1, ATF1 or NFY), the presence of CIITA and NLRC5 is essential for this process to take place [83].

3.4. Autophagy

Autophagy is one of the primary mechanisms for maintaining homeostasis, in which a cell digests elements of its structure in order to permanently remove them or return certain components to metabolic turnover. Autophagy is also a way to defend the body against intracellular microorganisms including bacteria, viruses, and protozoa, and has a regulatory function in the immune response. The process of autophagy occurs through the formation of unique organelles called autophagosomes. Autophagosomes fuse with lysosomes resulting in the destruction of structures contained in autophagosomes. Many mediator proteins are involved in autophagosome formation and fusion with lysosomes, mammalian target of rapamycin complex 1 (mTORC1), AMP-activated protein kinase (AMPK), serine/threonine protein kinase (ULK1), class III phosphatidylinositol-3-phosphate kinase (PI3K) complex, mammalian homolog of autophagy related proteins (ATG8), and others [105,106,127].

Xenophagy is a type of autophagy in which elements of the autophagosome are intracellular pathogens and other substances foreign to the body. The mechanism of xenophagy is similar to classical autophagy but requires ligand recognition by the PRR. NOD1 and NOD2 recognize bacterial ligands and initiate xenophagy by recruiting ATG16L1. NLRX1, which is localized in mitochondria, regulates autophagy associated with viral infection by interacting with Tu translation elongation factor of mitochondria (TUFM), which later reacts with ATG5-ATG12 and ATG16L1 [105,106,127].

4. RIG-I-Like Receptors (RLRs)

RIG-I-like receptors are cytoplasmic nucleic acid receptors for RNA virions, RNA replication intermediate molecules, and transcription products (footnote to RLRs 1). The RLRs family includes three receptors—retinoid acid inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). They are located in the cytoplasm of most cells in the body, although recent studies report that RLRs may also be located in the cell nucleus [128]. RLRs belong to the DexD/H box helicase family, which is in turn part of the type 2 helicase superfamily. RLRs are made of (i) a conserved helicase core that contains two helicase domains Hel1 and Hel2 separated by a fragment known as Hel2i, (ii) a C-terminal domain (CTD), (iii) RIG-I and MDA5 have an additional two CARD domains. The core domain and CTD interact to detect RNA immunostimulators and CARD is responsible for transducing the ligand recognition signal [9,129]. LGP2, which lacks a CARD domain, is thought to be a receptor that regulates RIG-I and MDA5. It inhibits the RIG-I receptor and enhances the response elicited by

MDA5 [130,131]. It is now known that viral infections caused by the vast majority of viruses are recognized by RLRs. A summary of ligands for RLRs is provided in Table 3.

Table 3. Receptor ligands for RLRs.

Virus Families (Examples)	RLR
Herpesviridae (Herpes simplex virus 1, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus)	RIG-I, MDA5
Poxviridae (vaccinia virus)	RIG-I, MDA5
Adenoviridae (adenoviruses)	RIG-I
Reoviridae (rotavirus)	RIG-I, MDA5
Picornaviridae (rhinovirus, coxsackie B)	RIG-I, MDA5
Flaviviridae (HBV, Zika virus)	RIG-I, MDA5
Coronaviridae (SARS coronavirus)	RIG-I, MDA5
Orthomyxoviridae (Influenza A virus)	RIG-I
Paramyxoviridae (measles virus)	RIG-I, MDA5
Filoviridae (Ebola virus)	RIG-I, MDA5
Retroviridae (HIV)	RIG-I, MDA5
Hepadnavridae (HBV)	RIG-I, MDA5

4.1. Ligands for RLRs

The structures that are recognized by the RIG-I receptor are 5′-triphosphate-RNA (5′-ppp-RNA) and 5′-diphosphate-RNA (5′-pp-RNA) found in both ssRNA and dsRNA, which are not present in most mature cellular RNA. In addition, the 5′-triphosphate and 5′-diphosphate fragments must have adjacent

neighbouring structures paired in conformations such as a hairpin. They are found in the RNA structure of most viruses, e.g., influenza A virus (IAV). Specific sequences such as poly-U or poly-UC present in the genome of, for example, hepatitis C virus (HCV) are also recognized by RIG-I. Studies have shown that RIG-I recognizes genomic RNA as effectively as RNA of damaged interfering Sendai virus and IAV molecules. The ligand for RIG-I may also be the 5′E-region of hepatitis B virus (HBV) pre-genomic RNA [132–138]. Ligands for the MDA5 receptor are much less well understood than those for RIG-I. MDA5 is known to detect large RNA aggregates produced in cells during encephalomyocarditis virus (EMCV) infection. These aggregates contain both ssRNA and dsRNA. Furthermore, ribose 2′-O methylation in the 5′-cup structure avoids MDA5 activation [132,139,140]. Both receptors have the ability to recognize a synthetic dsRNA ligand—polyinosilic-polycytidylic acid (poly(I:C)) but RIG-I recognizes shorter dsRNA fragments and MDA5 responds to high molecular weight poly(I:C)- HMW-poly(I:C) [9,141]. It has recently been elucidated how host cellular RNA can be a ligand for RLRs receptors during viral infection. In studies of cells infected with herpes simplex virus-1 or Kaposi's sarcoma-associated herpesvirus (KSHV), it was found that the RIG-I receptor is associated with pseudogene 5S rRNA-mainly *RNA5SP141* transcription products. The biological role of these 5′-ppp-RNA-containing transcripts is unknown. HSV-1 infection uncovers cellular non-coding RNAs in two ways. First, transcription products of the 5S rRNA pseudogene are inappropriately re-localized to the cytoplasm of the cell. Secondly, HSV-1 infection causes "shutdown" of the mRNA encoding the *RNA5SP141* binding proteins TST and MRPL18. Similar mechanisms may occur with infections with other viruses of the herpesvirus group, such as *Epstein-Barr virus* [142,143].

4.2. Signal Transduction through RLRs

In healthy, uninfected cells, RIG-I and MDA5 receptors are constitutively phosphorylated at several specific serine and threonine residues in the CTD and CARD domains, which keeps them in a state of suppressed signal transduction. Furthermore, RIG-I is maintained in an auto-repressed conformation due to interactions between the helicase domain and the CARD domain, whereas MDA5 presents an open conformation without the presence of foreign RNA [144]. Upon RNA binding, RLRs undergo a conformational change dependent on their ATPase activity a which is stimulated by binding to PACT (protein kinase R activator) [145]. These conformational changes trigger the release of CARD domains, which then bind to regulatory molecules. RIG-I and MDA5 bind to the phosphatase PP1-isoform PP1A or PP1y, which causes dephosphorylation of CARD domains. The RIG-I receptor then attaches TRIM (tripartite motif protein 25) and Riplet (also known as RNF135) proteins, which attach Lys63- linked ubiquitin polymers to the CARDs and C-terminal domain, respectively. The attachment of these polymers is essential for the tetramerization process of the RIG-I receptor and its interaction with the MAVS adaptor protein located on the outer membrane of mitochondria and mitochondria-associates membranes (MAMs) [146]. MDA5 binds dsRNA using Hel-CTD domains and forms filaments along the entire length of dsRNA. Unlike RIG-I, MDA5 does not bind dsRNA ends and filament formation is necessary for stable dsRNA binding and activation of the signal transduction pathway. The filaments formed by MDA5 then bind to MAVS [147]. Once stimulated, MAVS begins to form a prion-like filament structure that is the initiator to the formation of a large signalling complex composed of TRAF (Tumour necrosis factor Receptor-Associated Factors) proteins andTBK1 (or IKKE (IkB kinase-E)) The IKK-a–IKKB–IKK-y triple complex is activated on the ubiquitin chains anchored to the TRAFs. This in turn leads to activation of IFN regulatory factor 3 (IRF3) and/or IRF7 and NF-kB. IRF3, IRF7, and NF-kB, with the participation of AP1 (activator protein 1), lead to increased transcriptional activity of genes for IFNs and other cytokines: TNF, IL-6, and IL-8. IFNa/B secretion then leads to increased transcription of a significant number of ISGs (interferon stimulated genes) resulting in the creation of an "antiviral environment" in virus-infected cells and in adjacent healthy cells [148]. Interestingly, RIG-I and MDA5 have the ability to block viral replication directly by inhibiting the binding of viral proteins to viral RNA [149].

4.3. RLR Regulation

Proper regulation of RLRs is essential for homeostasis—it ensures fast and effective response to viral infection and protects against excessive inflammatory response. Both positive and negative regulatory proteins are involved in the regulation of RIG-I and MDA5 receptors. ZAPS protein binds to RIG-I, causes its oligomerization and enhances its ATPase properties leading to increased IFN I production [150]. TRIM25 accelerates K63-linked polyubiquitination in the CARD domain and enhances the interaction between RIG-I and MAVS. The Riplet protein has a similar effect, but it causes ubiquitination in the CTD domain [146]. Ubiquitin specific protease 15 (USP15) prevents LUBAC-dependent degradation of TRIM25 which also promotes RIG-I signalling pathways [151]. Ubiquitination, which is essential for the early steps of signal transduction by RLRs, is a reversible process. The deubiquitinases USP3 and USP21 inhibit RIG-I activity by removing K63-linked ubiquitin chains. RIG-I is also inhibited by RNF125 and Siglec-G-/SHP2/c-Cbl- mediated K48-linked ubiquitination and protein degradation [152]. Furthermore, RIG-I is inhibited by Ser-Thr phosphorylation in the CARD domain [153]. Much less is known about the regulation of MDA5 activity. DAK, a dihydroacetone kinase, binds to MDA5 (but not to RIG-I) and specifically inhibits MDA5-linked IFN I production [154]. USP3 deubiquitinase reduces MDA5 activity by deubiquitinating its CARD domain [152].

MAVS is a key factor in the signal transduction pathway through RLR receptors. NLRX1 blocks MAVS and RIG-I binding and thus reduces IFN I production. The autophagy proteins Atg5 and Atg12 have an inhibitory effect on the RIG-I signalling pathway through interactions with RIG-I and MAVS. The stability of the MAVS complex is regulated by several E3 ligases. PCBP2 was identified as a MAVS-degrading protein in association with the HECT domain-containing E3 ligase AIP4. Smurf2 and TRIM25 are also negative regulators of the RLRs pathway as they degrade MAVS through K48-linked ubiquitinization [155–157].

5. Clinical Significance of TLRs, NLRs, and RLRs

The effect of over-stimulation of TLRs, NLRs, and RLRs on the development of autoimmune diseases and chronic inflammation has been known for many years. A description of these relationships is beyond the scope of this paper, readers are referred to other studies [4,158,159]. In addition, prolonged inflammation is an ideal environment for the development of cancer. On the other hand, immune surveillance is necessary to detect abnormal, cancerous cells and prevent their proliferation [160]. Nowadays, it is known that in the tumour microenvironment (TME) not only stromal cells, fibroblasts, and endothelial cells are present, but also cells of the immune system. So far, the attention of researchers has been focused mainly on T lymphocytes belonging to the specific immune system. Nowadays, much attention is paid to non-specific immune cells such as macrophages (called tumour-associated macrophages, TAM), dendritic cells, neutrophils, NK cells, myeloid derived suppressor cells (MDSCs), and innate lymphoid cells (ILCs). Proinflammatory substances secreted by these cells contribute to genetic instability, promote angiogenesis, and facilitate metastasis of cancer cells. In addition, they suppress the body's immune response and, once treatment is initiated, cause the tumour to be more resistant to chemotherapy and immunotherapy [14,159,161]. At the same time, increasing sensitivity to tumour cell-derived antigens and activating the nonspecific immune system by ligand-PRR interaction may be a strategy to avoid tumour escape from immune surveillance [162]. Immunotherapy strategies aimed at activating cells of the innate as well as acquired immune system are also effective in eradicating large tumour masses [163,164], not uncommon at diagnosis or recurrence of hematopoietic proliferative disease.

5.1. Ligands for TLRs in the Treatment of Hematopoietic and Lymphatic Diseases

Activation of Toll-like receptors leads to enhanced anti-tumour responses by several mechanisms including increased secretion of proinflammatory cytokines in the tumour microenvironment to induce

immune responses by innate and acquired mechanisms and induction of apoptosis or necrosis of tumour cells [165]. Moreover, activation of TLR signalling pathways leads to maturation of antigen-presenting cells—macrophages and dendritic cells, increased production of IFNs I by these cells, increased expression of CD80, CD86, and CD40 molecules, which subsequently activate other cells of the innate immune system, as well as tumour-specific T-cell responses [166–168]. The cytokines IL-6 and IL-12 are the most important in cancer immunotherapy with TLR receptor ligands. IL-6 enhances antigen-specific T cell activation by inhibiting Treg cells, IL-12 promotes a Th1-directed response profile [169–171].

BCG (Bacillus Calmette-Guerin) is a substance that activates TLR2 and TLR4 through mycobacterium components and TLR9 through the presence of bacterial DNA. It has been used in combination with standard chemotherapy to treat patients with acute myeloid leukemia. Single reports also demonstrate the efficacy of BCG in the monotherapy of AML [172,173]. Poly I:C, poly ICLC molecules that bind to the TLR3 receptor are being investigated as adjuvant molecules in the development of cancer vaccines. In hematological malignancies, the indication is therapy for NHL [9,141,174]. In addition, poly ICLC in combination with radiotherapy is being investigated as a treatment option for cutaneous T-cell lymphoma and in combination with the rhuFlt3L/CDX-301 molecule for the treatment of low-grade B-cell lymphoma [174]. LPS, a TLR4 receptor ligand, is being studied as an ex vivo stimulant for dendritic cells in the treatment of NHL. Another TLR4 ligand-G100-in combination with pembrolizumab is being studied for efficacy in the treatment of follicular lymphoma. TLR7- 852A receptor ligand has been investigated as an immunotherapy for hematological malignancies including AML, ALL, NHL, HL, and MM [175]. The TLR8 receptor binds to a synthetic molecule, VTX-2337, and in combination with radiation therapy may have applications in the treatment of low-grade B-cell lymphomas. The most widely used in immunotherapy of hematopoietic and lymphoid malignancies are CpG ODNs, single-stranded oligodeoxynucleotides, characterized by the presence of repeats containing cytosine and guanine. CpG 7909 is a TLR9 ligand whose efficacy has been studied in the treatment of cutaneous T-cell lymphoma and NHL and in second-line therapy for patients with chronic lymphocytic leukemia [176]. The use of other CpG molecules is being widely studied as a means of immunotherapy for chronic lymphocytic leukemia. CpG-treated leukemic B lymphocytes undergo apoptosis [177,178]. The efficacy of TLR9 ligands in combination with radiotherapy or as part of combination treatment with targeted therapy, monoclonal antibodies or cytokines is under investigation for the treatment of NHL [179]. Most of the studies mentioned are in phase I/II clinical trial [165].

5.2. Ligands for NLRs in the Treatment of Hematopoietic and Lymphoid Malignancies

NLR receptor signalling pathways may be involved in the development of cancer, but also may be used to treat it. In humans, it has been observed that in some solid tumours (e.g., lung cancers, breast cancers, head and neck epithelial cancers), there is a greater expression of NOD1 and NOD2 on tumour cells and greater polymorphism for NOD2 [14,158]. Increased inflammasome activity can also lead to cancer development. Mutations in the gene for NLRP3 that cause persistent stimulation of this receptor have been implicated in melanoma susceptibility, colorectal cancer prognosis, and overall survival in multiple myeloma [180]. The phenomenon of pyroptosis may show a strong correlation with tumour cell proliferation and migration in various types of cancer. Pyroptosis causes inflammatory death of tumour cells, thereby inhibiting tumour growth and its ability to metastasize. Molecules that promote inflammasome formation and pyroptosis phenomenon are currently being investigated for use as part of anticancer treatment. These substances include non-coding RNA and other elements recognized by NLRs receptors [108,181]. An example of an investigational substance recognized by NLRP3 is anthocyanin, showing efficacy in the treatment of hepatocellular carcinoma and oral squamous cell carcinoma [182,183]. However, little is known about their use in the treatment of hematologic malignancies.

5.3. Ligands for RLRs in the Treatment of Hematopoietic and Lymphoid Malignancies

The death of a virus-infected cell is an important mechanism that leads to the elimination of diseased cells and prevents the spread of infection. Activation of RLRs receptors by viral or synthetic ligands leads to production of IFN I and also activation of ISGs and direct cell death or to induction of apoptosis. Furthermore, cancer cells are highly susceptible to RLR-dependent apoptosis while non-cancer cells are resistant to it through endogenous Bcl-xL [184]. Bhoopathi et colleagues in their studies revealed that the immune response against viruses and cancer cells follows similar pathways; therefore, they decided to use RLRs ligands as anticancer substances [184]. Several 5′ppp-siRNA molecules have been developed to activate RLR signalling pathways, and also to simultaneously block oncogenes or immunosuppressive pathways in cancer cells. Application of 5′ppp-siRNA for Bcl-2 in malignant melanoma resulted in inhibition of tumour growth due to, among other things, downregulation of Bcl-2. In contrast, the use of 5′ppp-siRNA for TGF-B has been studied in the treatment of pancreatic cancer. Activation of the MDA5 receptor by the synthetic dsRNA-poly I:C molecule in ovarian cancer resulted in increased cell surface MHC I expression, enhanced secretion of IFN I and other cytokines, and enhanced cell apoptosis [13]. Another important aspect of the use of RLR ligands in cancer therapy is that apoptosis induced by RLRs receptors is independent of the mutational status of the p53 gene, mutations of which are responsible for the resistance of cancer cells to chemotherapy and radiotherapy-induced apoptosis. Research into the use of ligands for RLRs is ongoing among patients with advanced solid tumours refractory to standard treatment. A summary of ligands for PRRs potentially useful in the treatment of hematopoietic diseases is provided in Table 4.

Table 4. Ligands for PRRs in the treatment of hematopoietic diseases. References in the text.

Group of PRRs	PRR	Ligand	Hematopoietic Disease
TLR	TLR2 TLR4 TLR9	BCG	Acute myeloid leukemia
	TLR3	polyI:C, polyICLC	Non-Hodgkin lymphomas, especially cutaneous T-cell lymphoma, low-grade B-cell lymphoma
	TLR4	LPS	Non-Hodgkin lymphomas
	TLR4	G-100	Follicular lymphoma
	TLR7	852A	Acute myeloid leukemia, acute lymphoblastic leukemia, Non-Hodgkin lymphomas, Hodgkin lymphoma, Multiple myeloma
	TLR8	VTX-2337	Low-grade B-cell lymphoma
	TLR9	CpG 7909	Non-Hodgkin lymphoma, especially cutaneous T-cell lymphoma, Chronic lymphocytic leukemia
NLR	NLRP3	anthocyanin	Non-hematological malignancies: hepatocellular carcinoma, oral squamous cell carcinoma
RLR	RIG-I	5’ppp-siRNA for Bcl-2	Non-hematological malignancies: malignant melanoma
	RIG-I	5’ppp-siRNA for TNF-β	Non-hematological malignancies: pancreatic cancer
	MDA5	dsDNA-poly I:C	Non-hematological malignancies: ovarian cancer

BCG- Bacillus Calmette-Guerin, LPS- lipopolysaccharide.

6. Conclusions

Innate immune mechanisms are the body's first line of defense against infection. Over many years of research, the structure, functions, and modes of action of many families of PRRs have been studied in detail. However, their role is still not fully understood, especially in the field of using receptors and their ligands in cancer treatment. So far, it is known that TLRs, NLRs, and RLRs show great potential in immunotherapy of solid organ cancers as well as hematological malignancies. However, further research into their introduction in treatment regimens is warranted.

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Infection and expression of Toll-like receptors in lymphoid malignancy patients after autologous stem cell transplantation

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Abstract

Introduction: *High-dose chemotherapy with autologous stem cell transplantation (ASCT) is one of the main strategies for the treatment of haematological neoplasms. Infections are the most common cause of morbidity and mortality from the ASCT procedure. however, it is challenging to predict when these complications are likely to arise. Toll-like receptors (TLRs) are present on various immune cells and play a broad role in immune surveillance. The aim of the study was to investigate the association between the expression of TLR genes and the occurrence of infections in patients treated with ASCT.*

Material and methods: *TLR expression was analysed in 60 patients who underwent ASCT. The median age was 54 years. blood samples were taken before high-dose chemotherapy and at the time of haematopoietic recovery after ASCT.*

Results: *The expression of Toll-like receptor 4 (TLR4) was significantly higher in patients before ASCT than after transplantation. The expression of Toll-like receptor 9 (TLR9) was significantly higher in patients after ASCT than before transplantation. The expression of TLR9 and TLR4 at the start of the procedure was significantly lower in patients who went on to develop a bacterial infection after ASCT. moreover, we also observed a significant positive correlation between the expression of TLR9 and neutrophil recovery time after ASCT.*

Conclusions: *our findings suggest that TLRs could be useful biomarkers to predict and monitor infections in patients treated with ASCT.*

Key words: *toll-like receptors, stem cell transplantation, haematological malignancies.*

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Introduction

High-dose chemotherapy with autologous stem cell transplant (ASCT) is a widely used therapy in the treatment of haematopoietic neoplasms. However, ASCT is associated with profound deficiencies in the function of the immune system. The proportion of ASCT recipients who go on to develop severe infections and associated complications varies, but infections are still a significant cause of mortality and morbidity. Age, general health, co-morbidities, and the number of previous treatment regimens are some factors known to influence the risk of such complications. In a study by Weaver *et al*., the mortality rate due to infections after high-dose chemotherapy and ASCT was 1.5% [4]. However, new prognostic markers are still being explored, the knowledge of which would allow us to determine the risk of severe complications in individual patients before the start of high-dose chemotherapy.

Toll-like receptors (TLRs) are a group of proteins that are actively involved in the function of the immune system. Most TLRs are expressed on the surface of cells, although TLR3 and TLRs 7-9 are intracellular [1]. TLRs are found on haematopoietic cells, such as B lymphocytes, T lymphocytes, monocytes, macrophages, dendritic cells, and NK cells, as well as on non-haematopoietic cells, including vascular endothelial cells, gastrointestinal and respiratory epithelial cells, adipocytes, cardiomyocytes, and fibroblasts. Their diverse expression means TLRs come into contact with many pathogens attempting to penetrate the body. Their recognition of these pathogens can stimulate the immune system.

One of the mechanisms of TLR function is the activation of the NF-κB transcription factor. The TLRs that are expressed on macrophages and dendritic cells initiate an innate immune response, including the activation of phagocytosis processes and the production of inflammatory chemokines and cytokines such as interleukin (IL)-12, IL-6 and type I interferon (type I IFN). TLRs are often described as a bridge between an innate immune response and an acquired immune response [3].

This study aimed to evaluate the expression of Toll-like receptor genes and their correlation with the incidence of infection after ASCT in patients with haematological malignancies.

Table 1. Patient clinical data

Patient data (*N* = 60)	
Gender	Female: 27 Male: 33
Median age	54 years (range: 25-65)
Diagnosis	MM: 20 nHL: 20 DLBCL – 8 FL – 5 MCL – 7 HL: 20
Disease status	Complete remission: 25 Partial remission: 35
Median neutrophil recovery time	14 days (range: 10-16 days)
Neutropenic fever	30 patients
Infections	24 patients (4 MM, 20 nHL + HL)
Bacterial infection	*Klebsiella pneumoniae:* 10 *enterococcus faecalis hlar:* 8

MM – multiple myeloma, NHL– non-Hodgkin's lymphoma, HL – Hodgkin's lymphoma, DLBCL – diffuse large B-cell lymphoma, FL – follicular lymphoma, MCL – mantle cell lymphoma

Material and methods

Study participants

The expression of Toll-like receptor genes (*TLR2*, *TLR4*, and *TLR9*) was measured in 60 patients undergoing ASCT. The median age of the patients was 54 years (range: 25-65 years). ASCT was performed due to multiple myeloma (MM)

in 20 patients, for non-Hodgkin's lymphoma (nHL) in another 20 patients, and Hodgkin's lymphoma (HL) in 20 patients. The healthy control group include 10 volunteers. The median age of healthy controls was 51 years (range: 26-64 years).

Peripheral blood samples were taken for analysis before the start of high-dose chemotherapy, before ASCT, and at the time of haematopoietic regeneration after stem cell transplantation (median: 14 days; range: 10-16 days after ASCT). In patients with MM, high doses of melphalan were administered (200 mg/m^2 or 140 mg/m^2, depending on the patients' age and general health) during the conditioning regimen before ASCT. Patients with nHL and HL received high-dose chemotherapy according to the BEAM protocol (carmustine, etoposide, cytosine arabinoside, and melphalan).

A median of 3.38×10^6 autologous CD34-positive cells per kilogram of body weight were transplanted. The median duration of neutropenia was 10 days in patients with MM (range: 9-15 days) and 12 days in patients with nHL and HL (range: 11-17 days). The median granulocyte regeneration time (defined as > 500 neutrophils/μl) was 14 days in the entire study population (range: 10-16 days). Febrile neutropenia was observed in 30 patients (8 patients with MM, 12 patients with nHL and 10 patients with HL). Microbiologically confirmed infection was found in 24 cases (4 patients with MM and 20 patients with nHL and HL). The most frequently isolated pathogen was *Klebsiella pneumoniae* (10 cases). Infection was confirmed in stool culture in 8 patients, and in blood culture in 2 patients. In 8 patients, *Enterococcus faecalis* was isolated from the faeces. For prophylaxis of infections, ciprofloxacin, fluconazole, acyclovir and trimethoprim were administered to patients before ASCT. The median response time to antimicrobial treatment in the patients with infection was 4 days (range: 2-10 days).

All patients and healthy volunteers signed informed consent forms in order to participate in the study. The study was approved by the Bioethics Committee of the Medical University of Wroclaw. The clinical data of the patients are presented in Table 1.

Real-time PCR

The relative expression of Toll-like receptors TLR2, TLR4, and TLR9 was assessed by real-time polymerase chain reaction (PCR) using TaqMan Assays (Life Technologies/Thermo Fisher). Beta glucuronidase (GUSB) served as an endogenous control. The reaction was carried out on a 7500 Real-Time PCR instrument (Life Technologies) using Gene Expression Master Mix (Life Technologies/ Thermo Fisher). The comparative CT method was used to compare the expression of patients with healthy controls.

Statistical analysis was conducted using STATISTICA 12 software (StatSoft, Poland). For quantitative variables, arithmetic means (X) and standard deviations (SD) of the estimated parameters were calculated for the study groups. The distribution of variables was tested using Lilliefors and Shapiro-Wilk W tests. In cases of independent quantitative variables with normal distribution, a *t*-test for independent variables was used. In cases of variables with a non-normal distribution, the *t*-test for dependent variables was applied to the quantitative variables of the normal distribution. In cases of quantitative dependent variables with non-normal distribution, Wilcoxon's pair sequence test was applied. In order to define the relationships between the studied variables, correlation analysis was performed. Results at the level of $p < 0.01$ were considered statistically significant.

Results

In comparison to the control group, expression of *TLR4* was decreased in patients (ΔCt TLR4 22.21 ±0.32 vs. 11.91 ±70.22, $p < 0.01$), while *tlr2* gene expression was higher in patients than in healthy individuals (ΔCt TLR2 6.46 ±9.58 vs. 0.98 ±0.43, $p < 0.05$). *TLR9* gene expression was higher in the control group than in haematological patients (ΔCt TLR9 13.65 ±3.29 vs. 3.35 ±1.93, $p < 0.05$).

The expression of *TLR4* was statistically significantly higher in patients who had not yet had transplant-related, high-dose chemotherapy than in those who had undergone ASCT (Ct TLR4 22.93 ±0.93 vs. 22.4 ±0.64). The expression of TLR9 was significantly lower before ASCT than in patients who had undergone ASCT (dCt TLR9 6.18 ±1.34 vs. 7.28 ±1.22, respectively, $p < 0.01$). The results are presented in Table 2 and in Figures 1 and 2.

The expression of *TLR4* and *TLR9* measured before the start of high-dose chemotherapy was significantly lower in patients who presented symptoms of infection in the post-transplant period (Ct TLR4 22.73 ±1.07 vs. 23.07 ±0.81 and Ct TLR9 28.5 ±1.24 vs. 26.71 ±1.18. respectively, $p < 0.01$). In patients with a clinically significant bacterial infection as a post-transplant infectious complication, significantly higher *TLR9* expression was observed compared to patients without any signs of an infection (Ct TLR9 116.34 ±123.67 vs. 105.83 ±98.23). Such a relationship was not observed for *TLR4* and *tlr2* expression. *TLR9* expression and neutrophil cell growth after stem cell transplantation were positively correlated ($r = 0.4075$, $p = 0.023$). We detected no significant differences in TLR expression or infection rate between patients with MM, nHL, and HL. TLR expression was similar in patients with infection and MM, nHL and HL. The results are presented in Table 3.

Table 2. Comparison of TLR values before and after autologous stem cell transplantation (ASCT)

Variable	Parameter	Before ASCT (*N* = 60)	After ASCT (*N* = 60)	*p*-value
TLR2 Ct	*N*	60	60	0.067
	Range	20.61-24.58	19.97-24.39	
TLR2 dCt	*N*	60	60	0.22
	Range	–2.15-2	–2.98-3.09	
TLR2 2exp.dCt	*N*	41	40	0.211
	Range	0.23-4.01	0.13-8.5	
TLR4 Ct	*N*	60	60	0.005
	Range	20.15-25.11	21.14-24.08	
TLR4 dCt	*N*	60	60	0.515
	Range	–2.61-2.79	–1.52-2.42	
TLR4 2exp.dCt	*N*	60	60	0.889
	Range	0.16-6.92	0.35-5.37	
TLR9 Ct	*N*	60	60	0.071
	Range	25.79-30.68	27.04-31.86	
TLR9 dCt	*N*	60	60	0.001
	Range	3.48-8.76	4.16-10.25	
TLR9 2exp.dCt	*N*	41	41	0.008
	Mean (SD)	22.4 (1)	22.06 (0.94)	
	Median (IQR)	22.13 (21.7-23.09)	22.07 (21.59-22.54)	
	Mean (SD)	–0.01 (0.96)	0.28 (1.08)	
	Median (IQR)	–0.18 (–0.68-0.58)	0.33 (–0.25-0.85)	
	Mean (SD)	1.25 (0.97)	1.58 (1.38)	
	Median (IQR)	0.88 (0.62-1.5)	1.26 (0.84-1.8)	
	Mean (SD)	22.93 (0.93)	22.4 (0.64)	
	Median (IQR)	22.83 (22.29-23.37)	22.28 (22.04-22.86)	
	Mean (SD)	0.52 (0.87)	0.64 (0.7)	
	Median (IQR)	0.38 (0.07-0.99)	0.48 (0.26-0.92)	
	Mean (SD)	1.71 (1.18)	1.74 (0.92)	
	Median (IQR)	1.31 (1.05-1.99)	1.39 (1.2-1.9)	

Mean (SD)	28.59 (1.21)	29.05 (1.08)
Median (IQR)	28.7 (27.61-29.24)	29.2 (28.44-29.69)
Mean (SD)	6.18 (1.34)	7.28 (1.22)
Median (IQR)	6.03 (5.21-7.09)	7.27 (6.63-7.91)
Mean (SD)	110.19 (108.14)	216.61 (208.07)
Median (IQR)	65.3 (36.9-136)	154.3 (99.3-240.9)
Range	11.2-433.8	17.8-1221.1

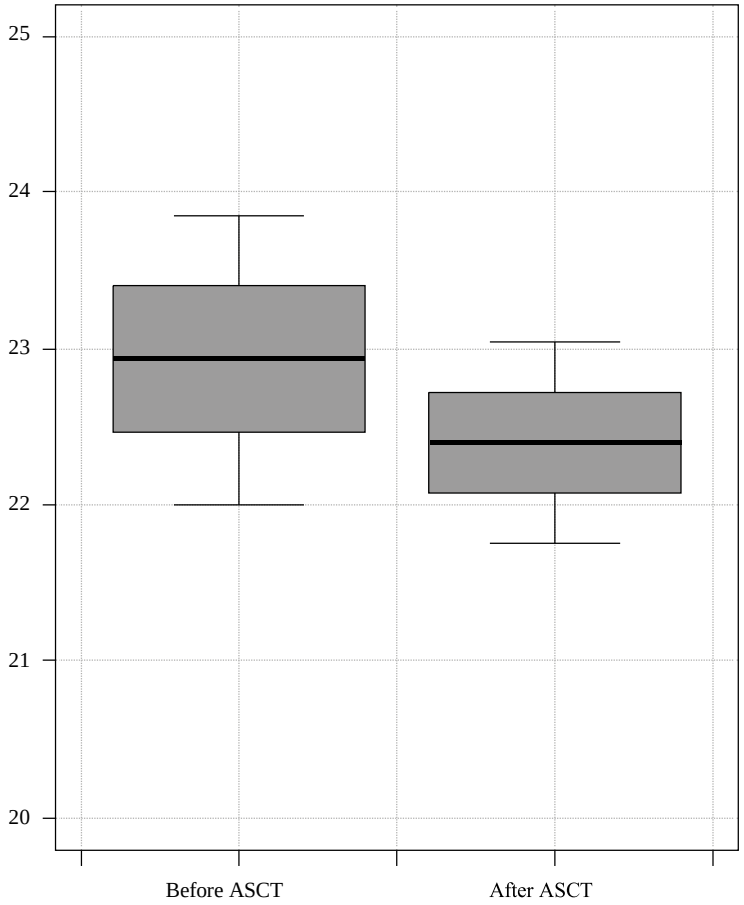

Fig. 1. TLR 4 value before and after autologous stem cell transplantation (ASCT)

Discussion

Infections are a common complication following ASCT. Depending on the period of bone marrow aplasia in which they appear, their course can be severe and can even lead to the death of the patient. Infections with Gram-positive and Gram-negative bacteria occur in about 20% of patients after autotransplantation, including bacteraemia in 7-8% of patients. Viral infections are observed in about 10-11% of patients after ASCT. Invasive mycoses are quite rare [5]. The mortality associated with severe infections after autotransplantation is estimated at around 2-4% of patients [5-7]. Therefore, new factors and prognostic markers that enable the identification of patients with a higher risk of infection are of clinical importance.

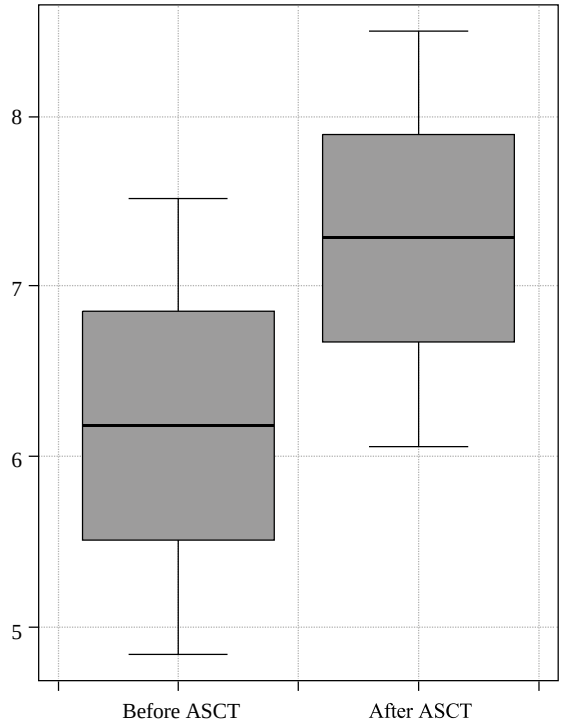

Fig. 2. TLR 9 value before and after autologous stem cell transplantation (ASCT)

The role of TLRs in the post-transplantation course remains unclear. The mechanisms of TLR action in solid organ transplantation have been much more thoroughly examined. Studies have shown that TLR11 plays a crucial role in the development of infections after renal transplantation. The most frequent infection-related complications are asymptomatic bacteriuria, cystitis, and pyelonephritis. These infections increase the risk of early mortality following transplantation [8].

TLR11 acts as a physiological barrier that protects the body against the penetration of uropathogens [8, 9]. Studies on mice have shown that TLR11 is expressed in liver, kidney, bladder, and vascular endothelial cells, and has an affinity with a uropathogenic *E. coli* strain, thereby affecting local immunity in the urogenital tract [10]. It has also been revealed that higher TLR11 expression strengthens immunity against *Toxoplasma gondii* [11]. Finally, Shi *et al*. reported that invasive *Salmonella* infection occurred in mice with diminished TLR11 levels [12].

Innate immunity, of which TLRs are a critical element, plays an essential protective role against pathogens after a liver transplant. This protective role is particularly important when it comes to infections of the hepatitis C virus (HCV). Recurrence of HCV infection after liver transplantation is common [13-15], and HCV reactivation causes aggressive liver fibrosis and graft rejection in approximately 30% of HCV-infected patients [16, 17]. Eid *et al*. demonstrated that the Arg753Gln *tlr2* polymorphism correlates with progressive cirrhosis of the transplanted liver, graft rejection and patient death after liver transplantation due to HCV-dependent cirrhosis [18].

The role of TLRs in infectious complications after stem cell transplantation has not yet been clearly defined. In a prospective analysis, Skert *et al*. assessed the expression of TLRs in T lymphocytes and monocytes in a population of 35 patients after bone marrow stem cell transplantation and its correlation with infectious complications. The authors found that *TLR9* expression in T lymphocytes correlates negatively with bacterial infections. A correlation was also observed between *TLR7* expression and fungal infections in this patient population [19].

In this study, we found that the expression of *TLR4* and *TLR9* before the start of the transplant procedure was statistically significantly lower in patients who had severe infectious complications in the later period. Additionally, *TLR9* expression significantly increased in patients with an active infection compared to patients without infection after transplantation. The physiological function of TLR4 is to stimulate the production of tumour necrosis factor α (TNF-α), interleukin (IL)-1, and IL-12 after binding with the pathogen-associated molecular pattern (PAMP) ligand of the pathogenic agent. The immune system is only activated when TLR4 acts in conjunction with the antigens CD14 and CD11b/CD18 [9, 20]. TLR9 acts by stimulating IL-12, monocytes, macrophages and NK cells. TLR9 is often activated by bacterial DNA, which results in the creation of a natural defence mechanism against bacteria [20, 21]. Consistently, experiments have shown that blocking the gene for the TLR9 receptor results in inhibition of activity against bacterial proteins [23, 24]. Lower expression of TLRs causes reduced synthesis of cytokines and chemokines, which leads to a generalised reduced immune response [22].

We also observed a positive correlation between TLR9 and the number of neutrophils during the period of bone marrow regeneration, which may indicate a role of TLRs in the reconstitution of the immune system following transplantation.

Table 3. Comparison of TLR values before autologous stem cell transplantation (ASCT) in patients with infection and without infection

Variable	**Parameter**	**Without infection after ASCT (*N* = 17)**	**Infection after ASCT (*N* = 24)**	***p*-value**
TLR2 Ct	*N*	24	17	0.1665
	Mean (SD)	22.55 (0.95)	22.18 (1.06)	
	Median (IQR)	22.46 (21.78-23.12)	21.96 (21.58-22.57)	
	Range	20.8-24.24	20.61-24.58	
TLR2 dCt	*N*	24	17	0.1835
	Mean (SD)	0.17 (0.99)	–0.27 (0.89)	
	Median (IQR)	–0.02 (–0.5-0.78)	–0.37 (–0.77-0.17)	
	Range	–1.51-2	–2.15-1.45	
TLR2 2exp.dCt	*N*	24	17	0.1835
	Mean (SD)	1.43 (1.11)	1 (0.67)	
	Median (IQR)	0.99 (0.71-1.72)	0.77 (0.59-1.12)	
	Range	0.35-4.01	0.23-2.74	
TLR4 Ct	N	24	17	0.1492
	Mean (SD)	23.07 (0.81)	22.73 (1.07)	
	Median (IQR)	23.21 (22.45-23.41)	22.51 (22.17-23.18)	
	Range	21.84-25.11	20.15-24.9	
TLR4 dCt	*N*	24	17	0.1492
	Mean (SD)	0.7 (0.78)	0.28 (0.96)	
	Median (IQR)	0.56 (0.15-1.11)	0.17 (–0.05-0.78)	
	Range	–0.49-2.79	–2.61-1.9	
TLR4 2exp.dCt	*N*	24	17	0.1492
	Mean (SD)	1.9 (1.36)	1.44 (0.83)	
	Median (IQR)	1.48 (1.11-2.15)	1.12 (0.97-1.71)	
	Range	0.71-6.92	0.16-3.73	
TLR9 Ct	*N*	24	17	0.005
	Mean (SD)	28.5 (1.24)	26.71 (1.18)	
	Median (IQR)	28.73 (27.46-29.27)	26.63 (27.72-29.24)	
	Range	25.79-30.51	26.96-30.68	
TLR9 dCt	*N*	24	17	0.7835
	Mean (SD)	6.26 (1.39)	6.12 (1.29)	
	Median (IQR)	6.07 (5.01-7.3)	5.77 (5.51-6.83)	
	Range	3.48-8.45	4.6-8.76	
TLR9 2exp.dCt	*N*	24	17	0.003

Mean(SD)	105.83 (98.23)	116.34 (123.67)
Median (IQR)	67.15 (32.25-157.38)	54.7 (45.7-113.7)
Range	11.2-349.2	24.2-433.8

Conclusions

The role of TLRs in the immunological processes which determine immunity against infectious agents is crucial. As demonstrated by the results of our research, and supported by previous publications, lower expression of individual TLRs (especially TLR4 and TLR9) may correlate with a higher risk of infections, especially in immuno-incompetent patients after stem cell transplantation.

Ethics approval and consent to participate

All patients and healthy volunteers signed informed consent forms in order to participate in the study. The study was approved by the Bioethics Committee of the Medical University of Wroclaw.

All methods were carried out in accordance with relevant guidelines and regulations mentioned in the manuscript.

Founding

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The authors declare no conflict of interest.

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Polymorphisms in the Genes Coding for TLRs, NLRs and RLRs Are Associated with Clinical Parameters of Patients with Acute Myeloid Leukemia

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Abstract: Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) are major elements of the innate immune system that recognize pathogen-associated molecular patterns. Single-nucleotide polymorphisms (SNPs) in the TLR, NLR, and RLR genes may lead to an imbalance in the production of pro- and anti-inflammatory cytokines, changes in susceptibility to infections, the development of diseases, and carcinogenesis. Acute myeloid leukemia (AML) is a bone marrow malignancy characterized by uncontrolled proliferation of transformed myeloid precursors. We retrospectively analysed 90 AML patients. We investigated the effect of fifteen SNPs located in the genes coding for RLR1 (rs9695310, rs10738889, rs10813831), NOD1 (rs2075820, rs6958571), NOD2 (rs2066845, rs2066847, rs2066844), TLR3 (rs5743305, rs3775296, 3775291), TLR4 (rs4986791, rs4986790), and TLR9 (rs187084, rs5743836). We observed that *TLR4* rs4986791, *TLR9* rs5743836, and *NOD2* rs2066847 were associated with CRP levels, while *RLR-1* rs10738889 was associated with LDH level. Furthermore, we found *TLR3* rs5743305 *AA* to be more common in patients with infections. We also found *TLR9* rs187084 *C* to be associated with more favourable risk, and RLR-1 rs9695310 *GG* with higher age at diagnosis. In conclusion, the current study showed that SNPs in the genes encoding TLRs, NLRs, and RLRs may be potential biomarkers in patients with AML.

Keywords: single-nucleotide polymorphisms; innate immunity; acute myeloid leukemia

1. Introduction

Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) play essential roles in mechanisms of innate immunity. They detect pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, fungi, protozoa, and other microorganisms that enter the human body. Detection of pathogen invasion activates intracellular transmission pathways, ultimately leading to increased production of proinflammatory cytokines and infected-cell death, as well as activation of adaptive immunity mechanisms [1–3]. In addition, TLRs, NLRs, and RLRs detect host cell damage-associated molecular patterns (DAMPs), which include free nucleic acids, uric acid crystals, heat shock proteins, and many others. Detection of DAMPs leads to the repair and regeneration of damaged tissues [4]. In addition to their involvement in immune mechanisms, these receptors are also involved in cell differentiation, maturation, apoptosis, and angiogenesis. The proper regulation of TLR, NLR, and RLR receptor activity and suppression of inflammatory response after infection is extremely important because chronic overproduction of proinflammatory cytokines is responsible for the development of chronic inflammatory, metabolic, neurodegenerative, and autoimmune diseases and cancers [5–7].

Single-nucleotide polymorphisms (SNPs) in genes encoding TLRs, NLRs, and RLRs can lead to an imbalance in the production of pro- and anti-inflammatory cytokines, changes in susceptibility to certain infections, and the development of allergic and inflammatory diseases, as well as carcinogenesis [8,9]. In addition, some SNPs are associated with increased resistance of cancer cells to treatment and apoptosis [10,11]. The search for associations between the occurrence of specific SNPs and the predisposition to develop various cancers and their impact on disease course and prognosis has been ongoing for several years.

Acute myeloid leukemia (AML) belongs to the group of malignant blood cancers and is the most common acute leukemia among adult patients. It leads to the suppression of normal hematopoiesis in the bone marrow and, therefore, to an increased risk of severe infections, anemia, and thrombocytopenia. AML is heterogeneous and differs in its course, degree of resistance to treatment, ability of blasts to cross the blood–marrow barrier, and formation of extramedullary metastases. Prognosis depends on the presence of specific molecular and cytogenetic mutations in tumour cells, and 5-year patient survival rates range from 20–30% [12]. Infections associated with neutropenia after chemotherapy are a major problem during AML therapy. It is not uncommon for patients to develop sepsis and septic shock that leads to death. Therefore, it is important to identify patients who are at a high risk of severe infections and to apply infection prevention and treatment measures. In order to achieve better AML treatment outcomes, an individualized approach to the patient is necessary, taking into account factors that may affect the course of therapy.

The aim of this study was to present 15 SNPs in TLRs, NLRs, and RLRs encoding genes in patients with de novo diagnosed AML and their association with clinical features such as infection rate, blood CRP level, cytogenetic risk according to the European Leukemia Net (ELN), and tumour burden (number of blasts in the bone marrow and presence of extramedullary metastases, blood LDH levels) at diagnosis, as well as their relationship with patients' age and sex. Due to heterogeneity of the course, response to treatment, prognosis, and the survival of young and elderly patients diagnosed with AML, at a later stage of the study, we analysed the obtained results of two age groups: >55 years of age and ≤55 years of age.

2. Results

2.1. SNPs in Toll-like Receptors Genes

We found that AML patients with favourable or intermediate risk at diagnosis (according to ELN) were more likely to have allele rs187084 *C* in the *TLR9* gene than patients with unfavourable risk ($p = 0.012$), Figure 1. Patients with rs4986791 *T* in the *TLR4* gene had lower CRP levels than CC homozygotes ($p = 0.015$), with rs4986791 *T* in the *TLR4* gene being more common in those with CRP levels < 5 mg/L ($p = 0.028$), Figure 2. Another polymorphism associated with lower CRP levels was rs5743836 *C* in the *TLR9* gene. Carriers of this SNP had lower serum CRP than rs5743836 *TT* homozygotes ($p = 0.048$), Figure 3. Additionally, patients with rs3775291 *G* in the *TLR3* gene tended to have higher LDH levels at diagnosis than rs3775291 *AA* homozygotes ($p = 0.053$). Infectious complications (all types of bacterial, viral, and fungal infections except for SARS-CoV-2 infections) during AML therapy were observed more frequently in *TLR3* rs5743305 *AA* homozygotes ($p = 0.015$), Figure 4. The overall survival (OS) analysis showed no statistically significant differences in OS among patients with *TLR3* rs5743305 *AA* genotype and the *TLR3* rs5743305 *AT* and *TT* genotypes— in the group with infections during AML treatment or in the group without infections (p-value, 0.711 and 0.465, respectively), Figures 5 and 6. Extramedullary metastases of AML tended to be more common in patients who carry rs3775296 *T* in the *TLR3* gene and rs4986790 *G* in the *TLR4* gene ($p = 0.054$ and $p = 0.078$, respectively). Patients who were diagnosed with disease metastases outside the bone marrow at the time of diagnosis tended to be less likely than patients with disease localized to the bone marrow to carry rs3775291 *A* in the *TLR3* gene and rs187084 *C* in the *TLR9* gene ($p = 0.068$ and $p = 0.053$, respectively).

A summary of the correlations between different SNPs in genes coding for TLR3, TLR4, and TLR9 and clinical features in patients with AML is presented in Table 1. For statistically significant results, bold font is used.

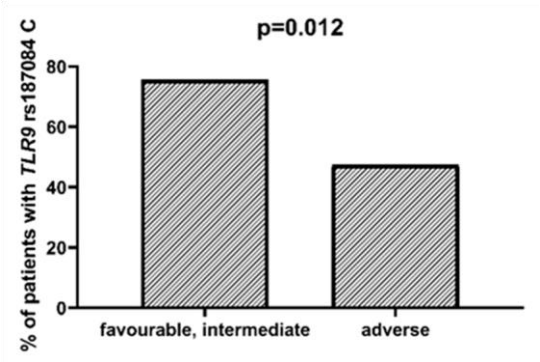

Figure 1. Association between *TLR9* rs187084 *C* and risk according to ELN.

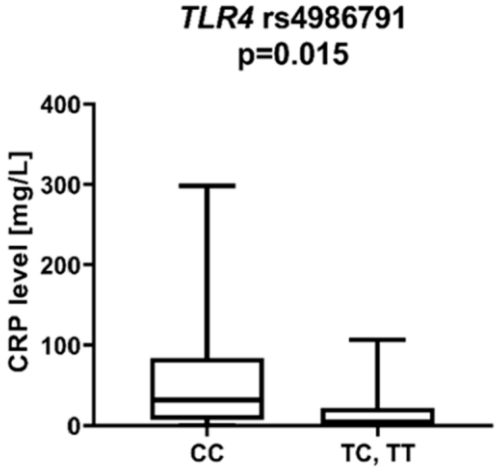

Figure 2. CRP level in patients with and without *TLR4* rs4986791 *T.*

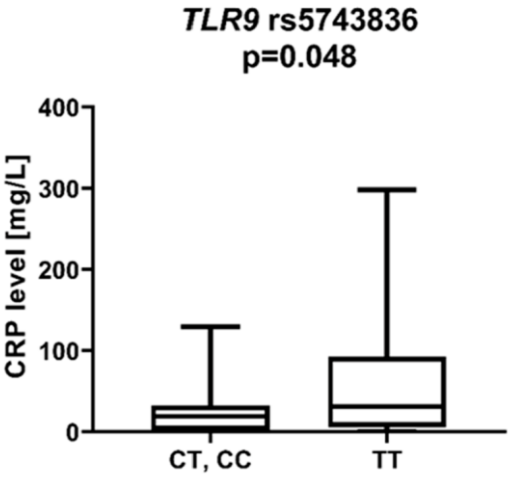

Figure 3. CRP level in patients with and without *TLR9* rs5743836 *C*.

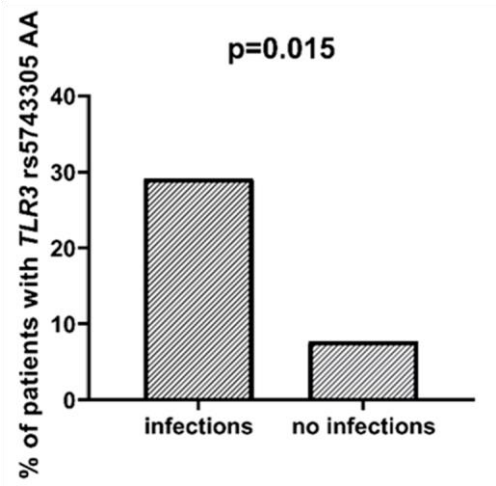

Figure 4. Association between *TLR3* rs5743305 *AA* and incidence of infection.

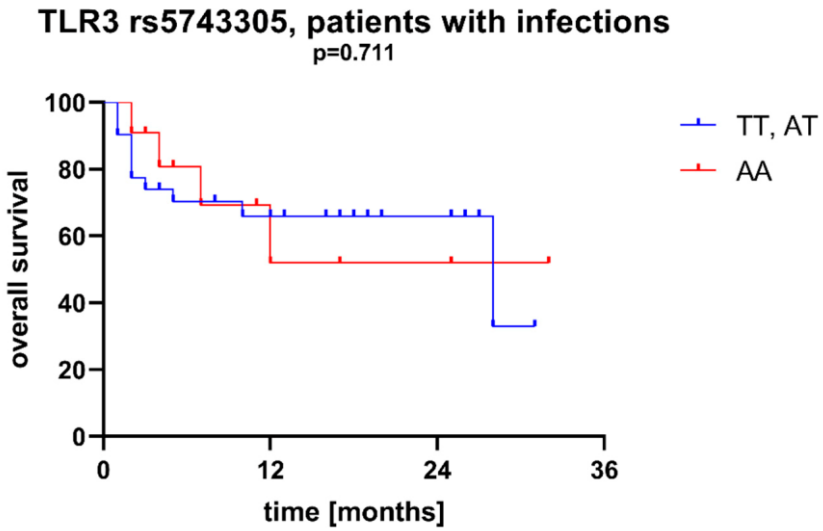

Figure 5. Overall survival of patients with different SNPs in *TLR3* rs5743305—patients with infections during AML therapy.

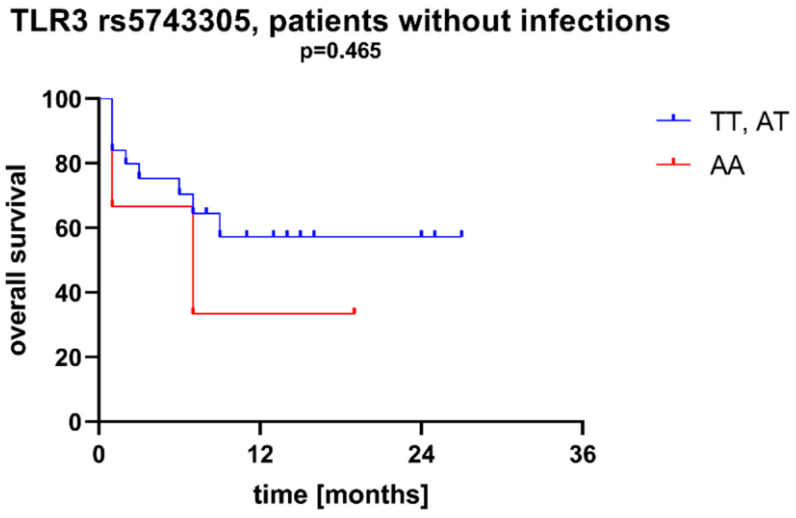

Figure 6. Overall survival of patients with different SNPs in *TLR3* rs5743305—patients without infections during AML therapy.

Table 1. Summary of correlations between SNPs in genes coding for TLR3, TLR4, and TLR9 and different clinical features in patients with AML. For statistically significant results ($p < 0.05$), bold font is used.

	SNPs in Gene for TLR3	SNPs in Gene for TLR4	SNPs in Gene for TLR9
Favorable/intermediate risk according to ELN			**rs187084 C**
Lower CRP level		**rs4986791 T**	**rs5743836 C**
Higher CRP level		**rs4986791 CC**	**rs5743836 TT**
Lower LDH level	rs3775291 A		
Higher LDH level	rs3775291 G		
Higher risk of infection	**rs5743305 AA**		
Extramedullary metastases	rs3775296 T	rs4986790 G	
No extramedullary metastases	rs3775291 A		rs187084 C

2.2. SNPs in NOD-like Receptors Genes

We observed a statistically significant difference in patients with the *NOD2* rs2066847 ins variant (*ins/del, ins/ins*), who showed lower CRP protein levels at diagnosis than patients with rs2066847 *del/del* ($p = 0.045$), Figure 7. There was also a trend for higher LDH levels in patients with *NOD2* rs2066844 *T* compared to patients without the T allele ($p = 0.062$).

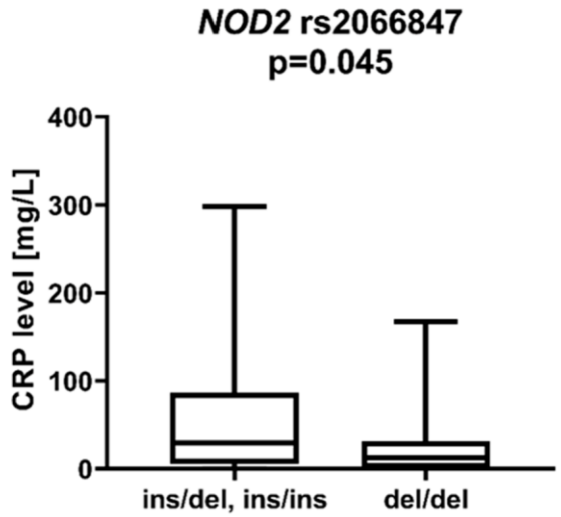

Figure 7. CRP level in patients with and without rs2066847 insertion in *NOD2* gene.

2.3. SNPs in the RIG-I-like Receptors Genes

DDX58 is a gene encoding the DeXD/H-Box Helicase 58 or RIG-I receptor. Statistically significant associations were observed between different SNPs and clinically relevant features of AML. We observed that the rs10738889 *G* allele was associated with lower LDH levels than rs10738889 *AA* ($p = 0.027$, Figure 8). There was also a trend showing a difference in LDH levels in patients with rs10813831 *G* compared to patients with the *AA* genotype ($p = 0.056$). Regarding CRP levels, we observed that *DDX58* rs10813831 *A* tended to be less common in patients with CRP < 5 than in those with CRP > 5 ($p = 0.098$). The rs10738889 *A* allele was more common in male than in female AML patients ($p = 0.033$; Figure 9), and the rs9695310 *C* variant was associated with lower age at diagnosis than *GG*, Figure 10. Furthermore, while not statistically significant, a difference in the number of blast cells in the bone marrow at the diagnosis of AML was observed. A higher percentage of

blast cells was present in patients with the rs10813831 *G* allele than rs10813831 *AA* (p = 0.065), while a lower percentage of blast cells was present in patients with rs10738889 *G* than rs10738889 *AA* (p = 0.082).

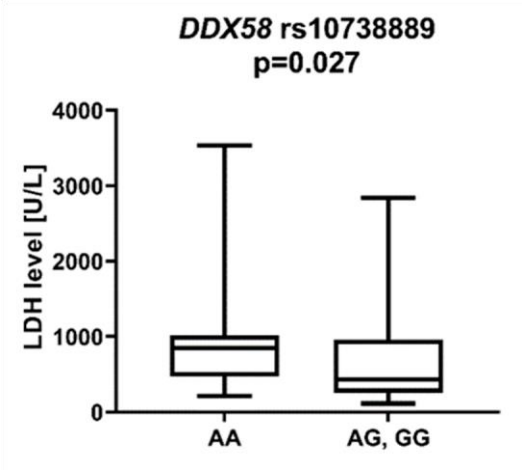

Figure 8. LDH level in patients with *DDX58* rs10738889 *G* allele.

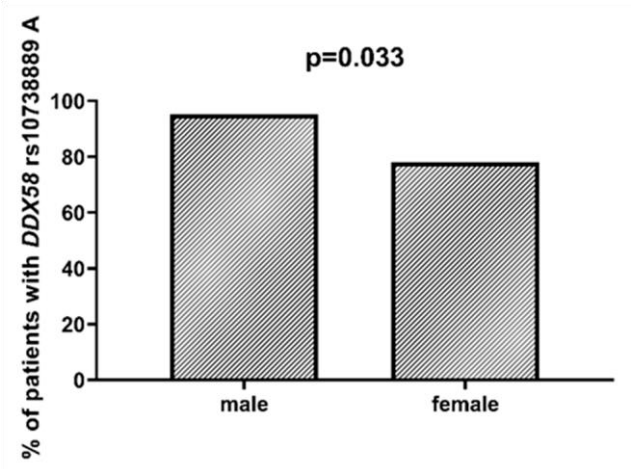

Figure 9. Association between *DDX58* rs10738889 *A* and gender.

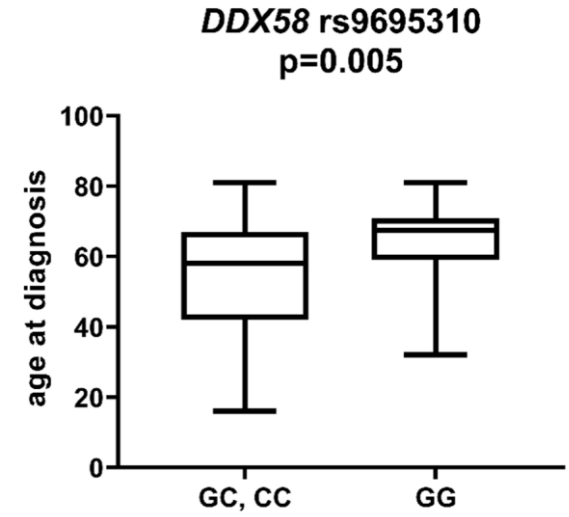

Figure 10. Age at diagnosis in patients with and without *DDX58* rs9695310 *C* allele.

2.4. SNPs in TLRs, NLRs, and RLRs in Groups of Patients >55 yo and ≤55 yo

After dividing the study group into a group of younger (≤55 years old) and older (>55 years old) patients, the statistical analysis showed the following associations: *DDX58* rs10813831 *A* was more common among patients with infections than without infections in the younger ($p = 0.046$) but not in the elderly ($p = 0.566$) group. *TLR4* rs4986791 *T* was associated with a higher LDH level than CC ($p = 0.044$) among young but not in elderly patients ($p = 0.171$). The presence of extramedullary metastases among young patients was associated with a higher prevalence of genotypes *TLR3* rs3775296 *T* ($p = 0.036$) and *TLR4* rs4986790 *G* ($p = 0.020$). These correlations did not occur in the elderly group ($p = 0.593$ and $p = 0.593$, respectively). Additionally, *TLR9* rs187084 *C* was more common in patients without extramedullary lesions in younger patients ($p = 0.035$), but not in older patients ($p = 0.623$). In the group of elderly patients, we showed statistically significant associations: *TLR9* rs187084 *C* was more common among patients with favorable and intermediate risk according to ELN ($p = 0.005$); *TLR4* rs4986791 *T* and *TLR9* rs5743836 *C* were associated with lower CRP level at diagnosis of AML ($p = 0.009$ and $p = 0.003$, respectively). In the group of younger patients, the above associations were not statistically significant ($p = 0.481$, $p = 0.204$, and $p = 0.523$, respectively).

3. Discussion

The results of our study presented here are among the first to describe so extensively the associations between SNPs in genes encoding pattern recognition receptors belonging to the innate immune system and clinical features of patients with AML. Currently, little is known about the impact of SNPs in genes encoding TLRs, NLRs, and RLRs on the course and prognosis of hematologic malignancies. M. Quirino et al. [13] studied the effect of polymorphisms in TLRs genes on the development of myeloproliferative neoplasms (MPNs). In that study, they found that *TLR9*-1486 *CT* may be associated with a lower susceptibility to developing polycythemia vera (PV), and in the haplotype frequency analysis, *TLR9*-1237*T*/-1486*C* was less common in men compared to controls, as well as in men negative for JAK V617F. Y. Zhou et al. [14] showed that inflammasome-related genes (NLRP3, NF-κB1, CARD8, IL-1β, and IL-18) were highly expressed in patients with MPNs and that the NF-κB1 polymorphism (rs28362491) was more common in MPN patients than in the study group. The effects of SNPs on the NLRP3 inflammasome system have also been studied as risk factors for the development and severity of AML. In a study by H. Wang et al. [15], *IL-1B* (rs16944) *GA* was more prevalent in patients with cytogenetic favourable risk, while the number of bone marrow blasts in patients with *IL-18* (rs1946518) *GG* or *GT* genotypes was higher than in *TT* patients. Furthermore, the *IL-18* rs1946518 *GT* genotype was statistically significantly associated with worse AML overall survival.

Patients diagnosed with AML and undergoing chemotherapy are a group of patients at particularly high risk of developing bacterial and fungal infections. Approximately 80% of patients develop neutropenic fever (NF) during AML therapy, with the etiologic agent being detected in less than 50% of cases [16]. The most commonly observed infectious complications are pneumonia, gastrointestinal infections, urinary tract infections, and central vascular catheter-related infections [17]. Invasive fungal infections are a major problem when treating patients with AML. Their occurrence is favoured by prolonged neutropenia, older patient age, and low albumin levels [18]. The treatment of fungal infections in the era of new anticancer drugs poses many difficulties due to the interaction of most new molecules with antifungal drugs [16]. Mechanisms of innate immunity are the body's first line of defense against microbial invasion; their efficient functioning provides protection against the development of severe infections. In the present study, a significant statistical association between the *TLR3* rs5743305 *AA* genotype and an increased frequency of infectious complications (non-SARS-CoV-2 infections) was detected. The TLR3 receptor is responsible for recognizing viral dsRNA in cell endosomes. TLR3 activation leads to the production of increased amounts of interferon type I and the death of infected cells [19]. *TLR3* rs5743305 homozygosity may cause malfunction and make cells much more susceptible to viral infections. Antiviral prophylaxis should be implemented in this group of patients, and they should be closely monitored for the development of viral infections that can lead to the patient's death.

Age at diagnosis is a clinically relevant parameter that indirectly influences the treatment plan of AML patients. In younger patients, intensive chemotherapy protocols are generally possible, whereas in older patients (>65–70 years of age), less intensive regimens are applicable [20]. In a study [21] of 13,283 patients diagnosed with AML, Acharya et al. showed that older patient age and male gender were associated with worse 3-year overall

survival in univariate analysis, and these parameters remained independent prognostic factors in multivariate analyses. In our study group, the mean age was 56 years. Patients with the *DDX58* rs9695310 *CC* genotype had the lowest age at diagnosis (51 years), *GC* heterozygotes 55 years, and *GG* homozygotes 64 years. Polymorphisms in the *NOD1* and *NOD2* genes (rs6958571 *AA* and rs2066847 *ins*, respectively) also showed an association with lower patient age at AML diagnosis, but this value was not statistically significant. Regarding gender, we observed that the *DDX58* rs10738889 *A* allele was more frequently present among male than female patients with AML.

Moreover, we found that the *TLR9* rs187084 *C* allele was less frequent among patients with unfavourable cytogenetic and molecular prognosis than in other risk groups. Patients with unfavourable risk according to ELN are a group that requires particularly intensive treatment, as a resistance to chemotherapy and rapid relapse are more common [20,21].

The number of blasts in the bone marrow at diagnosis of AML does not show a significant impact on disease course, treatment, and prognosis. Nevertheless, we observed an association between individual polymorphisms in the DDX58 gene and a higher percentage of blasts in the bone marrow at diagnosis. Patients with the *DDX58* rs10813831 *G* allele and *DDX58* rs10738889 *AA* genotypes tended to have a higher percentage of tumour cells in the bone marrow than carriers of *DDX58* rs10813831 *AA* and *DDX58* rs10738889 *G*. The DDX58 gene encodes the intracellular receptor RIG-I, which is responsible for detecting foreign double-stranded RNA (dsRNA). Activation of RIG-I leads to the increased production of proinflammatory cytokines (mainly interferon) as well as inflammasome formation and the induction of pyroptosis-induced death of infected cells [22,23]. The aforementioned SNPs in the DDX58 gene may be associated with a predisposition to increased and prolonged production of proinflammatory cytokines, which stimulate bone marrow stem cells to mutate into cancer cells and create the right conditions in the bone marrow microenvironment for mutant cells to proliferate [24,25].

In our present study, AML extramedullary involvement (EMI) metastases were found in 13 patients (14% of all patients). EMI metastases are most commonly found in the skin, central nervous system, lymph nodes, and less commonly, in internal organs and bones. L. Fianchi et al. [26] described factors that increase the risk of extramedullary metastases in AML. EMI was more common in patients with monocytic and myelomonocytic AML subtypes, and blasts were characterized by a lack of CD117 on immunophenotyping; molecularly, MLL gene (mixed-lineage leukemia gene) rearrangement was most commonly diagnosed, and cytogenetically, trisomy of chromosome 8 was most commonly detected. The same study confirmed an unfavourable prognosis in patients with extramedullary metastases with a median survival of 11.6 months. The factors that improve the survival of patients with EMI are intensive chemotherapy treatment, achieving complete remission, and performing an alloHSCT procedure. Our analysis found an association between SNPs in genes encoding TLR3, TLR4, and TLR9 receptors and the frequency of extramedullary metastases. The *TLR3* rs3775296 *T* allele and the *TLR4* rs4986790 *G* allele were more frequent in patients with EMI than in those without extramedullary metastases, whereas the *TLR3* 3775291 *A* allele and the *TLR9* rs187084 *C* allele tended to be less frequent in patients with EMI than in those without extramedullary manifestations of AML. Statistical significance was not demonstrated for all determinations. The study group size would need to be increased to confirm these preliminary correlations.

CRP is an acute-phase protein whose serum levels correlate closely with the severity of the inflammatory response. Inflammation has been a known carcinogen for many years. It contributes to tumour growth, cancer cell invasion, migration, and metastasis [27]. Shrotriya et al. [28] analysed 271 articles on the correlation of CRP levels with prognosis in patients with solid tumours. High CRP levels were associated with increased mortality in 90% of cases, especially among patients with gastrointestinal and renal cancers. In addition, high CRP levels were associated with poor response to treatment and increased relapse rates. We found much less data on the association of CRP levels with prognosis for patients with AML. Gradel et al. [29] found that high CRP levels at the time of AML diagnosis are associated with poorer patient general status. Patients in WHO 3/4 general status had 68 mg/L higher CRP levels than patients in WHO 0 status. We observed associations between the *TLR4* rs4986791 *T* allele and the *TLR9* rs5743836 *C* allele and lower CRP levels compared to *TLR4* rs4986791 *CC* and *TLR9* rs5743836 *TT*. Among NOD-like receptors, a statistically significant correlation was described for

NOD2 rs2066847 *ins*, which was associated with lower CRP than *NOD2* rs2066847 *del/del*. The *DDX58* rs10813831 *A* allele tended to be more frequent in patients with CRP > 5 mg/dL at AML diagnosis, although this was not statistically significant.

Lactate dehydrogenase (LDH) is an enzyme released from ruptured cells. In aggressive diseases with rapid cell turnover, blood LDH levels increase significantly. High LDH levels at diagnosis have been described as a factor for poor prognosis in patients with AML [30,31], correlated with shorter 1-year overall survival and increased 60-day mortality compared to patients with slightly elevated blood LDH levels. In the present study, we found that the *DDX58* rs10738889 *G* allele was associated with lower LDH levels than rs1078889 *AA.* Similarly, *DDX58* rs10738889 *GG* was associated with lower LDH levels than rs10738889 *AG*/*AA*. Furthermore, we observed a trend of higher LDH levels in patients with the *DDX58* rs10813831 *G* allele, *NOD2* rs2066844 *T* allele, and *TLR3* 3775291 *G* allele. Patients with high LDH levels should be closely monitored and may require more intensive treatment protocols.

4. Materials and Methods

4.1. Subjects

In our study, we retrospectively analysed 90 patients diagnosed with AML treated at the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation of Wroclaw Medical University between 2018 and 2020. There were 42 men and 48 women in the study group. The median age was 61 years (range 21–81 years), and the number of patients aged over 55 years was 54. All patients suffered from primary AML. The characteristics of the study group can be found in Table 2. The research was approved by the Bioethics Committee at the Wroclaw Medical University. All participants signed informed consent forms to participate in the study.

4.2. DNA Isolation, Genotyping

Genomic DNA was isolated from peripheral blood taken on EDTA from 90 AML patients using the commercial GeneMATRIX Quick Blood DNA Purification kit from EurX (Gdańsk, Poland) and NucleoSpin Blood kits (MACHEREY-NAGEL GmbH & Co. KG, Dueren, Germany) according to the manufacturers' protocols. DNA concentration and purity were assessed on the DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Isolated DNA was stored at −20 °C for further use. Patients were genotyped for the DDX58 (rs9695310, rs10738889, rs10813831), NOD1 (rs2075820, rs6958571), NOD2 (rs2066845, rs2066847, rs2066844), TLR3 (rs5743305, rs3775296, 3775291), TLR4 (rs4986791, rs4986790), and TLR9 (rs187084, rs5743836) genetic variants using LightSNiP assays (TIB MOLBIOL, Berlin, Germany), and real-time PCR was performed on a LightCycler 480 II device (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturers' instructions.

Table 2. Study group characteristics. LDH—lactate dehydrogenase, ELN—European Leukemia Net.

Characteristic	Study Group (n = 90)
Median age	61 (range 21–81)
Age ≤ 55 yo	36
Age > 55 yo	54
Gender	
Female	48
Male	42
% of blasts in bone marrow at diagnosis	
On average	56
<56	41
≥56	48
LDH (U/L) at diagnosis	
Within normal range (<220 U/L)	8

Elevated (≥220 U/L)	82
Within normal range (<5 mg/L)	21
Elevated (≥5 mg/L)	69
Extramedullary metastases at diagnosis	
Yes	13
No	77
Risk according to ELN	
Favorable/intermediate	52
Unfavorable	38
Infectious complications during treatment	
Yes	50
No	40

4.3. Statistical Analysis

Statistical analysis of the results obtained was performed using the Real Statistics Resource Pack for Microsoft Excel 2013 (version 15.0.5023.1000, Microsoft, Redmont, WA, USA). Mann–Whitney U test was used to determine the associations between individual SNPs and CRP level, LDH level, and blast cell count in the bone marrow, while Fisher's exact test was used to determine the associations between different SNPs and age of onset, presence of extramedullary metastases, presence of infection during treatment, and to illustrate the associations of SNPs with risk groups according to ELN. Kaplan–Meier curves and the Gehan-Breslow-Wilcoxon test were used for overall survival analysis. p-values < 0.05 were regarded as statistically significant. Graphs showing the obtained results were created using GraphPad Prism (GraphPad Software, La Jolla, CA, USA, version 8.0.1).

5. Conclusions

In conclusion, we demonstrated correlations between various SNPs in genes encoding Toll-like, NOD-like, and RIG-I-like receptors and specific clinical features of AML patients that may affect prognosis. These polymorphisms may be used as prognostic markers in AML in the future, but their potential implementation into clinical practice requires further studies on larger groups of patients.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Wroclaw Medical University for studies involving humans (No KB-574/2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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VII. PODSUMOWANIE WYNIKÓW

Receptory Toll-podobne są grupą receptorów starych filogenetycznie, obecnych w każdym wielokomórkowym organizmie, który może zostać zaatakowany przez bakterie i inne drobnoustroje. U człowieka dotychczas oznaczono 10 różnych TLR: TLR1-TLR10. Część z nich jest receptorami przezbłonowymi (TLR 1, 2, 4, 5, 6), pozostałe (TLR 3, 7, 8, 9) są zlokalizowane w błonach wewnątrzkomórkowych, głównie siateczce endoplazmatycznej [11,12]. TLRs występują na komórkach zarówno układu immunologicznego- komórkach dendrytycznych, makrofagach, limfocytach B i innych, ale także na fibroblastach, komórkach epitelialnych i endotelialnych. Rolą receptorów Toll-podobnych jest rozpoznawanie różnorakich PAMPs i DAMPs: receptory TLR1, TLR2 i TLR6 tworzą heterodimery (TLR1/2, TLR2/6) i w tej formie rozpoznają lipopeptydy pochodzące z bakterii Gram-ujemnych oraz *Mycoplasma spp*. TLR2 prezentuje szerokie spektrum rozpoznawanych ligandów: białka bakteryjne (m.in.. *Yersinia spp*.), glikolipidy, glikopeptydy, zymosan czy hemaglutyniny wirusa ospy prawdziwej. TLR3 odpowiada za rozpoznawanie wirusowego dwuniciowego RNA. TLR4 rozpoznaje lipopolisacharyd (LPS) pochodzący ze ścian bakterii Gram-ujemnych. TLR5 wykrywa monomery flagelliny pochodzące z bakterii posiadających umiejętność poruszania się. Receptory TLR7, 8 i 9 są receptorami wewnątrzkomórkowymi, które rozpoznają kwasy nukleinowe bakterii i wirusów[11,13,14]. Kolejną poznaną grupą receptorów rozpoznających PAMPs i DAMPs są receptory NOD-podobne. Obecnie do tej grupy receptorów u człowieka zalicza się 22 wewnątrzkomórkowe białka podzielone na cztery podgrupy: NLRA, NLRB, NLRC i NLRP. NLRs rozpoznają szeroką gamę antygenów pochodzących z bakterii (peptydoglikany, flagellina), wirusów (RNA), grzybów czy pasożytów. Ponadto receptory NOD-podobne biorą udział w regulacji ekspresji MHC I, MHC II oraz procesów takich jak spermatogeneza czy embriogeneza[15–17]. Receptory RIG-I-podobne są receptorami cytoplazmatycznymi odpowiedzialnymi za wykrywanie wirusowego RNA, cząsteczek pośrednich powstających podczas replikacji materiału genetycznego oraz produktów transkrypcji RNA. Do grupy receptorów RIG-I-podobnych należą trzy receptory: RIG-I, MDA5 i LGP2[18–21].

Rozpoznanie ligandu przez wszystkie wymienione receptory prowadzi do uruchomienia kaskad transdukcji sygnałów, które skutkuje ostatecznie zwiększeniem produkcji cytokin prozapalnych (TNF, IL-6, IL-8), IFN-I i innych. Ponadto, w momencie rozpoznania zagrożenia przez mechanizmy odporności nieswoistej uruchamiane są mechanizmy odporności nabytej.

Rola receptorów należących do układu odporności wrodzonej w kontekście nowotworów układu krwiotwórczego jest różnorodna. Po pierwsze, nadmierna aktywacja i brak hamowania działania receptorów z grupy PRRs może prowadzić do rozwoju nowotworów, w tym nowotworów szpiku i układu chłonnego[16]. Z drugiej strony sprawne działanie układu odporności nieswoistej a w nieco późniejszym etapie odporności nabytej ma istotne znaczenie podczas leczenia pacjentów z nowotworami hematologicznymi: częstym skutkiem ubocznym stosowanej terapii jest głęboka neutropenia oraz związane z nią poważne infekcje. Mimo stosowanego leczenia może dojść do rozwoju sepsy, wstrząsu septycznego i śmierci pacjenta. Pacjentami szczególnie narażonymi na infekcje są chorzy poddawani procedurze przeszczepiania komórek krwiotwórczych oraz chorzy na ostre białaczki. Szybkie wykrycie i uruchomienie mechanizmów pierwszej linii obrony organizmu ma kluczowe znaczenie w niedopuszczaniu do rozwoju poważnych, zagrażających życiu ciężkich zakażeń. Z innej jeszcze strony, receptory NLRs, RLRs i TLRs i ich ligandy mogą być wykorzystane jako element immunoterapii w leczeniu chorych na choroby układu krwiotwórczego[22–24]. Ponadto, obecnie wiadomo, że w mikrośrodowisku nowotworu poza komórkami stromalnymi znajdują się także komórki układu immunologicznego: limfocyty T z układu odporności swoistej ale także makrofagi (zwane makrofagami związanymi z nowotworem, tumor-associated macrophages, TAM), komórki dendrytyczne, neutrofile, komórki NK i inne. Substancje prozapalne produkowane przez te komórki powodują niestabilność genetyczną nowotworów, promują angiogenezę oraz ułatwiają przerzutowanie. Dodatkowo hamują odpowiedź immunologiczną organizmu oraz zwiększają oporność nowotworów na stosowane leczenie[25,26]. Obecnie trwają badania ukierunkowane na wykorzystanie ligandów receptorów NLRs, RLRs i TLRs jako elementów immunoterapii chłoniaków nieziarniczych, chłoniaka Hogdkina oraz ostrych i przewlekłych białaczek. Kliniczne zastosowanie tych cząsteczek wymaga jednak dalszych analiz.

Procedura autotransplantacji komórek krwiotwórczych jest uznaną metodą leczenia pacjentów z chorobami układu krwiotwórczego i limfatycznego. Związana jest ona jednak z głębokim upośledzeniem funkcji układu odpornościowego. Infekcje i towarzyszące im powikłania są ważnym czynnikiem zachorowalności i śmiertelności chorych poddawanych ASCT. W pracy pt. "Infection and expression of Toll-like receptors in lymphoid malignancy patients after autologous stem cell transplantation" badano zależność ekspresji receptorów TLR2, TLR4 i TLR9 z częstością występowania infekcji w okresie poprzeszczepowym oraz wpływ tej ekspresji na szybkość regeneracji krwiotworzenia po ASCT. W oznaczeniach

wykonanych u pacjentów oraz w grupie kontrolnej wykazano, że u chorych na nowotwory układu krwiotwórczego ekspresja TLR4 i TLR9 jest niższa niż wśród osób zdrowych, natomiast ekspresja TLR2 była wyższa w grupie badanej. Istotne statystycznie zależności opisano dla ekspresji TLR4 i TLR9 przed i po wykonaniu procedury autotransplantacji komórek krwiotwórczych: ekspresja TLR4 była niższa po wykonaniu ASCT niż przed, odwrotnie, ekspresja TLR9 po ASCT była wyższa niż przed przeszczepieniem. Wśród pacjentów, u których w okresie potransplantacyjnym wystąpiły objawy infekcji ekspresja TLR4 i TLR9 przed podaniem megachemioterapii była niższa, niż u pacjentów, którzy nie mieli infekcji po ASCT. Ekspresja TLR9 była wyższa w okresie poprzeszczepowym u pacjentów, którzy wykazywali cechy infekcji, niż u pacjentów bez powikłań infekcyjnych. Takiej zależności nie opisano dla TLR2 i TLR4. Zaobserwowano także pozytywną korelację między wyższą ekspresją TLR9 a krótszym czasem regeneracji neutrofili po ASCT, co może wskazywać na rolę TLR9 w rekonstytucji układu odpornościowego po transplantacji komórek macierzystych szpiku kostnego. W wykonanych badaniach nie stwierdzono znaczącej różnicy w ekspresji TLR2, TLR4 i TLR9 oraz częstości infekcji wśród chorych z NHL, HL i MM. Ekspresja receptorów była podobna także wśród pacjentów z NHL, HL i MM z objawami infekcji w okresie poprzeszczepowym.

Polimorfizmy pojedynczych nukleotydów w genach kodujących poszczególne receptory z grup TLRs, NLRs i RLRs mogą mieć wpływ na zaburzenia równowagi w produkcji cytokin prozapalnych i przeciwzapalnych, różnej podatności na infekcje oraz rozwój chorób autoimmunologicznych, przewlekłych chorób zapalnych czy karcynogenezę[27–29]. Wpływ polimorfizmów w genach dla receptorów należących do układu odporności wrodzonej na rozwój oraz przebieg naturalny i efekty leczenia chorób rozrostowych układu krwiotwórczego jest nadal bardzo słabo poznany. W pracy pt. „Polymorphisms in the genes coding for TLRs, NLRs and RLRs are associated with clinical parameters of patients with acute myeloid leukemia" opisano zależności pomiędzy 15 różnymi SNP w genach dla TLRs, NLRs i RLRs a czynnikami klinicznymi, przebiegiem leczenia oraz rokowaniem wśród pacjentów cierpiących na pierwotną ostrą białaczkę szpikową. Polimorfizmy pojedynczych nukleotydów w genach kodujących receptory Toll-podobne wykazują zależność z ryzykiem określanym według kryteriów ELN, poziomem CRP i LDH w momencie rozpoznania AML, występowaniem infekcji podczas leczenia oraz obecnością ognisk pozaszpikowych. Pacjenci z ryzykiem korzystnym i pośrednim wg ELN częściej niż pacjenci z ryzykiem niekorzystnym posiadali allel rs187084 *C* w genie dla *TLR9*. Niższe poziomy białka CRP w momencie diagnozy

stwierdzono wśród chorych z rs4986791 *T* w genie dla *TLR4* oraz rs5743836 *C* w genie dla *TLR9*. Tendencję do wyższego poziomu LDH wykazywali pacjenci z rs3775291 *G* w genie dla *TLR3* niż homozygoty rs3775291 *AA*. Infekcje o różnej etiologii (poza infekcjami SARS-CoV-2) podczas terapii ostrej białaczki szpikowej występowały częściej u homozygot *TLR3* rs5743305 *AA*. Analizy przeżycia całkowitego (overall survival, OS) nie wykazały istotnych statystycznie zależności w OS pacjentów z genotypem *TLR3* rs5743305 *AA* oraz *TLR3* rs5743305 *AT* i *TT* – zarówno w grupie z infekcjami w trakcie leczenia jak i bez infekcji. Ogniska pozaszpikowe ostrej białaczki szpikowej występowały częściej u osób z rs3775296 *T* w genie dla *TLR3* oraz rs4986790 *G* w genie dla *TLR4*. Pacjenci ze zmianami chorobowymi poza szpikiem kostnym rzadziej niż chorzy z chorobą zlokalizowaną wyłącznie w szpiku kostnym byli nosicielami *TLR3* rs3775291 *A* oraz *TLR9* rs187084 *C*. W przeprowadzonym badaniu opisano ponadto polimorfizmy pojedynczych nukleotydów w genach dla receptorów NOD-podobnych, które wykazują wpływ na stężenie CRP i LDH w surowicy krwi pacjentów w momencie rozpoznania AML: wariant *NOD2* rs2066847 *ins* (*ins/del*, *ins/ins*) jest skojarzony z niższym poziomem CRP niż *NOD2* rs2066847 *del/del* a wyższe stężenie LDH zaobserwowano u pacjentów z *NOD2* rs2066844 *T* w porównaniu z osobami bez allelu T. *DDX58* jest genem kodującym receptor RIG-I. Polimorfizmy pojedynczych nukleotydów w tym genie mają wpływ na kilka klinicznie ważnych czynników u chorych z ostrą białaczką szpikową. Poziom LDH w momencie diagnozy był niższy u pacjentów z genotypem rs10738889 *G* niż rs10738889 *AA*. Poziom CRP <5 mg/l w momencie rozpoznania choroby rzadziej oznaczano wśród chorych będących nosicielami *DDX58* rs10813831 *A*. Genotyp *DDX58* rs10738889 *A* częściej był skojarzony z płcią męską, zaś warianty rs 9695310 *C* (*CC*, *GC*) były skojarzone z młodszym wiekiem zachorowania niż rs 9695310 *GG*. SNP w genie dla receptora RIG-I wykazywały związek z odsetkiem komórek blastycznych w szpiku: wyższą liczbę blastów AML opisano w materiale pochodzącym od chorych z rs10813831 *G* w porównaniu z rs10813831 *AA* natomiast niższy odsetek komórek blastycznych występował u pacjentów charakteryzujących się genotypem rs10738889 *G* w porównaniu z rs10738889 *AA*. Ciekawych wyników dostarczyła analiza SNP w genach dla TLRs, NLRs i RLRs z podziałem grupy badanej na grupę pacjentów młodszych-≤ 55 roku życia i grupę pacjentów starszych-powyżej 55 roku życia. W przeprowadzonym badaniu wykazano następujące zależności: *DDX58* rs10813831 *A* częściej występował wśród chorych z infekcjami w grupie pacjentów młodszych ale nie starszych. *TLR4* rs4986791 *T* był skojarzony z wyższym poziomem LDH niż *CC* wyłącznie wśród młodszych pacjentów. Obecność ognisk pozaszpikowych pośród młodych chorych była skojarzona z częstszym występowaniem genotypów *TLR3* rs3775296 *T*

i *TLR4* rs4986790 *G*. Tych zależności nie stwierdzono w grupie pacjentów starszych. Ponadto, *TLR9* rs187084 *C* był częściej wykrywany wśród młodych pacjentów bez zmian poza szpikiem kostnym. W grupie pacjentów starszych wykazano istotne statystycznie zależności: *TLR9* rs187084 *C* występował częściej wśród chorych z ryzykiem korzystnym i pośrednim wg ELN; *TLR4* rs4986791 *T* i *TLR9* rs5743836 *C* były skojarzone z niższym poziomem CRP w chwili rozpoznania AML. W grupie pacjentów młodszych powyższa zależności nie wykazywały istotności statystycznej.

VIII. WNIOSKI

1. TLRs, NLRs i RLRs odgrywają istotną rolę w prawidłowym funkcjonowaniu układu odpornościowego. Zaburzenia dotyczące ich aktywacji lub hamowania mogą wpływać na rozwój chorób nowotworowych układu krwiotwórczego.
2. Ekspresja receptorów TLR może być użytecznym biomarkerem ryzyka występowania powikłań infekcyjnych po autoHSCT.
3. TLR9 odgrywa rolę w rekonstytucji układu odpornościowego po ASCT.
4. Polimorfizmy pojedynczych nukleotydów w genach kodujących TLRs, NLRs i RLRs są związane z różnymi cechami klinicznymi pacjentów z AML.
5. Polimorfizmy pojedynczych nukleotydów w genach kodujących TLRs, NLRs i RLRs mają pośredni wpływ na rokowanie pacjentów z AML.

IX. PIŚMIENNICTWO

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X. ZAŁĄCZNIKI