lek. Mieszko Krzysztof Lachota

Badanie profilu migracji komórek NK i poszukiwanie metody poprawiającej ich infiltrację w terapii nowotworów litych

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

Promotor: dr hab. n. med. Radosław Zagożdżon

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„Always do what you are afraid to do.”

— Ralph Waldo Emerson

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Wykaz stosowanych skrótów

NK – komórki NK „naturalni zabójcy”, ang. *natural killer cells*

TME – mikrośrodowisko nowotworu, ang. *tumor microenvironment*

MHC I - cząsteczki głównego układu zgodności tkankowej klasy I, ang. *major histocompatibility complex I*

CyTOF - cytometria masowa, ang. *cytometry by time-of-flight*

TCGA – baza danych bioinformatycznych, ang. *The Cancer Genome Atlas*

TARGET - baza danych bioinformatycznych, ang. *Therapeutically Applicable Research to Generate Effective Treatments*

CAR-T - limfocyty T z chimerycznym receptorem antygenowym ang. *chimeric antigen receptor T cells*

B-ALL – białaczka limfoblastyczna z komórek B, ang. *B-cell acute lymphoblastic leukemia*

NKp46, NKp30, NKp44 – receptory komórek NK, ang. *natural cytotoxicity triggering receptors*

NKG2D – receptor aktywujący komórki NK, ang. *natural killer group 2D receptor*

DNAM-1 – receptor aktywujący komórek NK, również znany jako CD226, ang. *DNAX accessory molecule-1*

KIR – receptory komórek NK, ang. *killer cell immunoglobulin-like receptors*

CD94/NKG2A –kompleks receptorów hamujących komórki NK, ang. *CD94/NKG2A*

mbIL-21 – interleukina 21 związana z błoną, ang. *membrane-bound interleukin 21*

4-1BBL – ligand 4-1BB, ang. *4-1BB ligand*

K562-mbIL21-4-1BBL –linia komórkowa modyfikowana genetycznie do ekspresji związanej z błoną interleukiny 21 i ligandu 4-1BB

GvHD – choroba przeszczep przeciw gospodarzowi, ang. *graft-versus-host disease*

HLA – antygeny zgodności tkankowej, ang. *human leukocyte antigens*

AML – ostra białaczka szpikowa, ang. *acute myeloid leukemia*

IFN-γ – interferon γ*, ang. interferon γ*

IL-2 – interleukina 2, ang. *interleukin 2*

IL-15 – interleukina 15, ang. *interleukin 15*

DLBCL – rozlany chłoniak z dużyych komórek B, ang. *diffuse large B-cell lymphoma.*

iPSC – indukowane pluripotencjalne komórki macierzyste, ang. *induced pluripotent stem cells*

Streszczenie w języku polskim

Komórki „naturalni zabójcy" (NK, ang. *natural killer cells*) są obiecującym narzędziem w rozwijaniu nowej generacji terapii przeciwnowotworowych z zastosowaniem inżynierii genetycznej. Nieprzyjazne warunki mikrośrodowiska guzów litych (TME, ang. *tumor microenvironment*) uniemożliwiają komórkom NK pełnienie swojej funkcji, co wymaga odpowiednich modyfikacji poprawiających ich odporność na czynniki obecne w TME. Innym warunkiem, często pomijanym a niezbędnym do przeniesienia sukcesu terapii komórkowych z nowotworów hematologicznych na guzy lite, jest zdolność migracji komórek do organu docelowego. Celem mojej pracy doktorskiej, opartej na trzech publikacjach, jest dogłębna charakteryzacja wzorców migracji komórek NK i identyfikacja strategii poprawiającej ich migrację do guzów litych.

Pierwszą publikacją jest artykuł przeglądowy zatytułowany *Prospects for NK Cell Therapy of Sarcoma*, opublikowany w „Cancers", czasopiśmie otwartego dostępu (ang. *open access*) wydawanym przez MDPI, o zakresie obejmującym biologię i immunologię nowotworów. W artykule omówiono rolę komórek NK w nadzorze immunologicznym mięsaków, jak również mechanizmy unikania przez ten typ nowotworów odpowiedzi immunologicznej hamującej aktywność komórek NK. Mechanizmy te obejmują: zwiększoną ekspresję cząsteczek głównego układu zgodności tkankowej klasy I (MHC, ang. *major histocompatibility complex*), zrzucanie z powierzchni ligandów aktywujących komórki NK, zmiany metaboliczne oraz zwiększone wydzielanie immunosupresyjnych cytokin. Wszystkie powyższe czynniki przyczyniają się do tworzenia nieprzyjaznego mikrośrodowiska, co prowadzi do ograniczenia migracji komórek NK i tłumienia ich cytotoksyczności, a ostatecznie do postępu choroby. Ponadto w artykule przeanalizowano strategie aktywacji i modyfikacji genetycznych komórek NK, które zwiększają ich zdolność do rozpoznawania komórek nowotworowych, migracji do guza i odporność na czynniki hamujące obecne w TME. W artykule podjęto również dyskusję nad możliwościami wyczulenia komórek mięsaków na komórki NK za pomocą przeciwciał monoklonalnych, radioterapii, hipertermii i innych metod.

Druga publikacja to również artykuł przeglądowy opublikowany w czasopiśmie „Cancers", zatytułowany *The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity*. Podsumowano w nim, w jaki sposób mechanizmy unikania odpowiedzi immunologicznej wykorzystywane przez różne guzy lite wpływają na poszczególne

funkcje efektorowe komórek NK, takie jak migracja, formowanie synapsy litycznej i produkcja cytokin. Do wspomnianych mechanizmów należą między innymi hipoksja, zmniejszone pH, stres oksydacyjny, immunosupresyjne cytokiny, ograniczenie dostępności aminokwasów, immunosupresyjne metabolity lipidów czy też metabolity adenozyny. W artykule zostały także omówione strategie mające na celu zwiększenie odporności komórek NK na nieprzyjazne warunki TME, oceniane obecnie w badaniach *in vitro* i *in vivo*, a także w badaniach klinicznych.

Trzecia publikacja to artykuł oryginalny, zatytułowany *Mapping the Chemotactic Landscape in NK Cells Reveals Subset-specific Synergistic Migratory Responses to Dual Chemokine Receptor Ligation*, opublikowany w „eBioMedicine", czasopiśmie otwartego dostępu grupy Lancet, poświęconemu odkrywczym badaniom podstawowym. Przedstawione w artykule badanie bazuje na spostrzeżeniu, że w odróżnieniu od innych typów komórek dojrzałe komórki NK rzadko występują w guzach litych, a ich wzorce migracji są słabo zrozumiane. Dlatego zbadano wzorce migracji ludzkich komórek NK, wykorzystując wieloparametrową cytometrię przepływową, cytometrię masową (CyTOF, ang. *cytometry by time-of-flight*) i sekwencjonowanie RNA na poziomie pojedynczych komórek w połączeniu z testami funkcjonalnymi. W wyniku przeprowadzonych badań odkryto, że repertuar receptorów chemokinowych komórek NK z krwi obwodowej zmienia się w skoordynowany sposób, stając się stopniowo bardziej zróżnicowany w trakcie procesu dojrzewania. Wykazano, że ekspresja receptorów chemokinowych jest ściśle związana ze zdolnością do migracji poszczególnych podtypów komórek NK. Odkryto także, że jednoczesna aktywacja receptorów CXCR1/2 i CX3CR1 prowadzi do synergistycznie wzmożonej migracji. Analiza 9471 litych nowotworów z baz danych TCGA/TARGET ujawniła dziewięć dominujących profili ekspresji chemokin, różniących się w zależności od typu guza. Co istotne, żaden z nich nie miał ligandów dla więcej niż jednego receptora chemokinowego obecnego na dojrzałych komórkach NK. Nasze wyniki pokazują, że rzadkość naturalnie występujących par chemokin-receptorów chemokinowych może tłumaczyć systematyczne wykluczanie komórek NK z TME i reprezentuje niewykorzystany potencjał dla inżynierii nowej generacji terapii przeciwnowotworowych opartych na komórkach NK.

Podsumowując, przeprowadzenie przeglądu literatury pozwoliło na zidentyfikowanie problemu ograniczonej infiltracji guzów litych przez komórki NK oraz niektóre z leżących u jego podstaw mechanizmów. Następnie przeprowadzono

systematyczną charakteryzację profilu migracyjnego podtypów komórek NK, która w połączeniu z analizą bioinformatyczną ponad dziewięciu tysięcy guzów litych dostarczyła dowodów na systematyczne wykluczanie komórek NK z mikrośrodowiska guzów litych poprzez restrykcyjny profil chemokin. Co najważniejsze, wyniki przedstawione w tej rozprawie wskazują, że wyposażenie komórek NK w co najmniej dwa receptory chemokinowe, odpowiadające profilowi chemokin guza, reprezentuje nowe, obiecujące podejście do inżynierii terapii przeciwnowotworowych bazujących na komórkach NK, oparte na odkrytym zjawisku synergii pomiędzy receptorami chemokinowymi.

Streszczenie w języku angielskim

Natural killer (NK) cells hold promise as a highly functional template for genetic engineering in the development of next generation of anticancer cell therapies. The hostile conditions of tumor microenvironment (TME) in solid tumors often preclude NK cells from effectively performing their function, necessitating for modifications improving their resistance to suppressive factors in the TME. Another often overlooked prerequisite for extending the success of the cell therapies against solid tumors is that the cells must reach the intended target organ. The aim of this dissertation, based on three publications, is an in-depth characterization of NK cell migratory patterns and identification of a strategy improving tumor homing of NK cell-based therapies.

The first publication, „*Prospects for NK Cell Therapy of Sarcoma*", is a review article published in *Cancers*, MDPI´s open access journal focused on cancer biology and immunology. In the article, we review the role of NK cells in sarcomas immune surveillance, as well as the immunoevasion mechanisms of sarcomas that affect NK cell function. These include MHC class I upregulation, shedding of NK activating ligands, altered metabolism, and increased secretion of inhibitory cytokines. All of the above-mentioned factors contribute to creating a hostile tumor microenvironment, leading to impaired NK cell homing, cytotoxicity suppression, and ultimately, progression of the disease. We discuss various priming strategies and genetic modifications enhancing cancer cell recognition, tumor homing, and resistance to suppressive factors in the TME. We also discuss the possibility of sensitizing sarcoma cells to NK cell-mediated cytotoxicity by monoclonal antibodies, radiotherapy, hyperthermia, and other modalities.

The second publication, „*The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity*", is also a review article published in *Cancers*. It summarizes how the tumor immune evasion mechanisms employed by different solid tumors, including hypoxia, acidosis, oxidative stress, immunosuppressive cytokines, amino acid deprivation, immunosuppressive lipid, and adenosine metabolites, alter the effector functions of NK cells such as NK cell recruitment, lytic synapse formation and cytokine production. We also discuss strategies aiming to combat the hostile TME conditions, restoring NK cell-mediated immune surveillance, that are evaluated in the *in vitro* and *in vivo* studies as well as clinical trials.

The third publication is an original research article entitled „*Mapping the Chemotactic Landscape in NK Cells Reveals Subset-specific Synergistic Migratory Responses to Dual Chemokine Receptor Ligation*" published in *eBioMedicine*, the *Lancet´s* open access journal for discovery science. This work bases on an observation that in contrast to other cell types, highly differentiated NK cells are rarely found in solid tumors, with their trafficking patterns remaining poorly understood. Therefore, we investigated the trafficking patterns of human NK cells utilizing high-dimensional flow cytometry, mass cytometry by time-of-flight (CyTOF), and single-cell RNA-sequencing combined with functional assays. We found that the chemokine receptor repertoire of peripheral blood NK cells changes in a coordinated fashion becoming gradually more diversified during the differentiation process. The chemokine receptor expression correlated tightly with the migratory response of the distinct NK cell subsets. We also found that simultaneous ligation of CXCR1/2 and CX3CR1 receptors led to a synergistically enhanced migratory response. Investigation of 9471 solid cancer cases in the TCGA/TARGET databases revealed nine predominant chemokine profiles that varied among tumor types, but none of them had ligands for more than one chemokine receptor expressed on mature NK cells. Our results show that the sparsity of naturally occurring pairs of chemokines-chemokine receptors may explain the systematic exclusion of NK cells from the tumor microenvironment and represent an untapped potential for engineering next-generation NK-cell based therapies in oncology.

In summary, through literature review we identified the issue of impaired infiltration of NK cells into solid tumors as well as some of the underlying mechanisms. Then, we performed systematic characterization of NK cell migratory profile in subset resolution, that combined with bioinformatic analysis of over nine thousand solid tumors provided evidence for systematic exclusion of NK cells from the TME by limiting their homing through restrictive chemokine profile. Most importantly, the results presented in this dissertation indicate that arming NK cells with at least two chemokine receptors matching the chemokine profile of the tumor, represents a novel approach for engineering of NK cell-based therapies, based upon the discovered chemokine receptor synergy phenomenon.

1. Wstęp

Nowotwory stanowią drugą najczęstszą przyczynę zgonów na świecie (1). Konwencjonalne metody leczenia, w tym chemioterapia, są często nieskuteczne i obarczone poważnymi skutkami ubocznymi. W odpowiedzi na te problemy na znaczeniu zyskuje immunoterapia komórkowa wykorzystująca cytotoksyczność komórek układu odpornościowego do walki z nowotworem (2). Przykładem jest terapia oparta o genetycznie modyfikowane limfocyty T wyposażone w chimerowe receptory antygenowe (CAR-T, ang. *chimeric antigen receptor T cells*), która odniosła ogromny sukces w leczeniu nowotworów hematologicznych, głównie ostrej białaczki limfoblastycznej z komórek B (B-ALL, ang. *B-cell acute lymphoblastic leukemia*) (3,4). Niestety próby jej wykorzystania w leczeniu nowotworów litych okazały się nieefektywne ze względu na wiele czynników, w tym ucieczkę antygenową i immunosupresyjne mikrośrodowisko nowotworu (TME, ang. *tumor microenvironment*) (3,5).

Komórki „naturalni zabójcy" (NK, ang. *natural killer cells*) stanowią alternatywę i potencjalnie bardziej skuteczne narzędzie immunoterapii nowej generacji. Są one efektorowymi komórkami układu odpornościowego, definiowanymi jako limfocyty $CD3^-$ $CD56^+$, zdolnymi do naturalnej oraz indukowanej przeciwciałami cytotoksyczności komórkowej względem komórek zarażonych wirusowo i/lub transformowanych nowotworowo (6–9). Ich unikatową cechą jest zdolność do spontanicznej cytotoksyczności, bez uprzedniej aktywacji (10). Cytotoksyczność komórek NK jest regulowana poprzez integrację wielu sygnałów przekazywanych przez receptory aktywujące (NKp46, NKp30, NKp44, NKG2D i DNAM), rozpoznające ligandy indukowane stresem na powierzchni komórek nowotworowych oraz poprzez receptory hamujące należące do rodziny KIR lub CD94/NKG2A, rozpoznające własne cząsteczki głównego układu zgodności tkankowej (MHC ang. *major histocompatibility complex*) (6,10–16). Liczne dane literaturowe wskazują na wysokie zróżnicowanie funkcjonalne komórek NK, zależne od ich stadium dojrzewania (17). Ekspresja powierzchniowych receptorów aktywujących i hamujących, cytotoksycznych białek, a także właściwości funkcjonalne komórek, takie jak cytotoksyczność komórkowa, wytwarzanie cytokin, oraz kinetyka tych kluczowych dla odpowiedzi przeciwnowotworowej procesów jest inna dla każdego podtypu komórek NK, co ma związek z odmienną funkcją pełnioną w układzie odpornościowym (17–20).

Zdolność dojrzałych komórek NK do spontanicznej cytotoksyczności względem komórek nowotworowych i indukcji remisji choroby stała się podstawą do podjęcia prób użycia ich jako „żywego leku" (21,22). Terapie adoptywne wykorzystują transfer komórek auto- lub allogenicznych, poddanych namnażaniu w warunkach *in vitro*, często pozwalającego na selektywne namnożenie poszczególnego podtypu komórek i uzyskania homogennego produktu o wysokim potencjale przeciwnowotworowym (21,22). Proces ekspansji komórek NK odbywa się poprzez hodowlę wyizolowanych komórek w obecności mieszanin cytokin oraz tzw. *feeder cells* – napromieniowanych komórek linii nowotworowej K562, zmodyfikowanych genetycznie do ekspresji związanej z błoną interleukiny 21 (mbIL-21, ang. *membrane-bound interleukin 21*) i ligandu 4-1BB (4-1BBL, ang. *4-1BB ligand*) – K562-mbIL21-4-1BBL (21, 22). Przewagą terapii z zastosowaniem komórek NK nad limfocytami T jest brak ryzyka choroby przeszczep przeciwko gospodarzowi (GvHD, ang. *graft-versus-host disease*) oraz uniknięcie konieczności doboru antygenów zgodności tkankowej (HLA, ang. *human leukocyte antigens*) dawcy i biorcy (21,22). Ponadto komórki NK rozpoznają komórki nowotworowe na podstawie integracji wielu sygnałów oraz cząsteczek, co zmniejsza ryzyko ucieczki przed układem odpornościowym (10,21). Inną niewątpliwą zaletą jest ich unikalna zdolność do preferencyjnego rozpoznawania oraz cytotoksyczności względem komórek macierzystych nowotworu, będących przyczyną nawrotów oraz oporności na chemioterapię (23,24). Badania kliniczne z zastosowaniem komórek NK w terapii chorób nowotworowych wykazały się dobrym profilem bezpieczeństwa, małą liczbą działań niepożądanych oraz skutecznością, szczególnie w terapii ostrej białaczki szpikowej (AML, ang. *acute myeloid leukemia*), pozostając jednak nieskuteczne w leczeniu guzów litych odpowiadających za ponad 90% zgonów związanych z nowotworami złośliwymi (1,21,22,25). Zwiększone zapotrzebowanie metaboliczne komórek nowotworowych ogranicza dostępność składników odżywczych w TME i eksponuje naciekające komórki NK na produkty reakcji metabolicznych prowadzące do ich funkcjonalnego wyczerpania (26).

Jedną z najważniejszych, ale często pomijaną przyczyną niepowodzenia terapii adoptywnej komórkami NK, jest niski odsetek migracji przetaczanych komórek do guza nowotworowego (27,28). Aby pełnić swoje funkcje, komórki muszą mieć zdolność do migracji do określonych tkanek. Migracją komórek NK kieruje skomplikowana sieć chemokin, małych (8-14kD) rozpuszczalnych białek wydzielanych w sposób tkankowo- i komórkowo-specyficzny (29). Udowodniono, że odpowiednie nakierowanie komórek, na

przykład poprzez transdukcję plazmidem kodującym receptor chemokinowy CXCR2, zwiększa ich zdolność do migracji w obszar guza, co przekłada się na lepszą odpowiedź przeciwnowotworową (30,31). Takie postępowanie jest umiarkowanie skuteczne, ze względu na niską ekspresję chemokin w nowotworach, która uniemożliwia wykorzystanie pełnego potencjału migracyjnego komórek NK (27,28). Ponadto nowotwór w wyniku presji selekcyjnej może utracić ekspresję ligandów dla użytego receptora.

Mimo podstawowego znaczenia zdolności migracji komórek NK do guzów, wiedza w tym zakresie była dotąd skąpa. Fragmentaryczność informacji na temat ekspresji receptorów chemokinowych na komórkach NK i brak danych na temat związku stopnia ekspresji receptorów chemokinowych z procesem dojrzewania oraz ze zdolnościami do migracji wobec ich ligandów uniemożliwiał zrozumienie biologii migracji tych komórek oraz identyfikację skutecznego sposobu ich ukierunkowania w obszar guza. Zgodnie z tą potrzebą badawczą przedstawiona rozprawa doktorska stanowi monotematyczny cykl trzech publikacji poświęconych migracji komórek NK oraz potencjalnej metodzie poprawiającej zdolności migracji terapii adoptywnych bazujących na komórkach NK, w obszar guza. Doktorant jest pierwszym, wiodącym autorem w dwóch pracach: oryginalnej pt. *Mapping the Chemotactic Landscape in NK Cells Reveals Subset-specific Synergistic Migratory Responses to Dual Chemokine Receptor Ligation* i przeglądowej pt. *Prospects for NK Cell Therapy of Sarcoma* oraz drugim autorem w pracy przeglądowej pt. *The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity*. Łączna wartość współczynnika wpływu (IF, ang. *impact factor*) czasopism, w których opublikowano artykuły wynosi 24.3, a sumaryczna liczba punktów MEiN wynosi 420.

Początkowym celem pracy doktorskiej było opisanie dotychczasowej wiedzy na temat znaczenia infiltracji komórek NK w nowotworach litych z naciskiem na mięsaki. Ponadto praca miała na celu opisanie czynników ograniczających infiltrację komórek NK oraz charakteryzację innych sposobów ucieczki nowotworów spod nadzoru immunologicznego.

Pierwszą publikację cyklu stanowi artykuł pt. *Prospects for NK Cell Therapy of Sarcoma* opublikowany w czasopiśmie „Cancers". Jest to pierwszy obszerny przegląd literatury omawiający rolę komórek NK w mięsakach, nowotworach pochodzących z tkanki łącznej. W artykule przedstawiono dotychczasowy stan wiedzy na temat roli komórek NK w nadzorze immunologicznym mięsaków na różnych etapach ich rozwoju;

inicjacji, progresji i przerzutów. Następnie omówiono wszystkie zidentyfikowane czynniki związane z ucieczką immunologiczną mięsaków spod nadzoru komórek NK, takie jak ograniczenie infiltracji komórek NK przez prostaglandynę E2 oraz niską aktywność szlaku interferonu γ (IFN-γ, ang. *interferon γ*) czy inne, jak np. hipoksja, immunosupresyjne cytokininy i zwiększona odporność na sygnały indukujące apoptozę. Na końcu, bazując na interpretacji obecnego stanu wiedzy i nowych trendów w rozwoju terapii przeciwnowotworowych, omówiono potencjalne strategie poprawiające skuteczność komórek NK i umożliwiające ich zastosowanie w leczeniu tej grupy nowotworów.

Drugą publikacją cyklu jest artykuł przeglądowy pt. *The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity* opublikowany w czasopiśmie „Cancers". W pracy scharakteryzowano biologię procesu infiltracji TME przez komórki NK, sposoby jego ograniczenia wykształcone przez nowotwory oraz potencjalne strategie przeciwdziałania tym czynnikom. Ponadto, zebrano wszystkie zidentyfikowane czynniki mające wpływ na supresję aktywności komórek NK oraz opisano mechanizmy działania każdego z nich na poszczególne funkcje tych komórek. Podsumowany był również związek opisanych czynników i zmian w funkcjonalnym potencjale komórek NK z ograniczeniem ich aktywności przeciwnowotworowej. Na koniec, w świetle opisanych czynników podjęto dyskusję na temat potencjalnych strategii rozwoju terapii przeciwnowotworowych nowej generacji uwzględniających metody uodparniania komórek NK na immunosupresyjne czynniki w TME.

Wyniki prac badawczych uzyskane w ramach projektu doktorskiego zostały przedstawione w trzeciej publikacji, artykule oryginalnym pt. *Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation* opublikowanym w czasopiśmie „eBioMedicine" z grupy Lancet. Pracę rozpoczęto od zbadania ekspresji wszystkich klasycznych i wybranych atypowych receptorów chemokinowych na komórkach NK z krwi obwodowej, na komórkach NK wyizolowanych z krwi po 24-godzinnej hodowli w medium komórkowym, oraz na komórkach NK poddanych stymulacji interleukiną 2 (IL-2, ang. *interleukin 2*) lub interleukiną 15 (IL-15, ang. *interleukin 15*) po 24-godzinnej i 7-dniowej hodowli. Dodatkowo sprawdzono, w jaki sposób ekspansja komórek NK za pomocą komórek K562-mbIL21-4-1BBL oraz ekspansja komórek NK, różnicowanych z indukowanych

pluripotencjalnych komórek macierzystych (iPSC ang. *induced pluripotent stem cells*), wpływa na ich profil migracji.

Po wstępnym fenotypowaniu dogłębnie zbadano wpływ dojrzewania na ekspresję receptorów chemokinowych na podtypach komórek NK za pomocą wieloparametrowej cytometrii przepływowej, cytometrii masowej (CyTOF, ang. *cytometry by time-of-flight*) oraz analizy typu sekwencjonowania RNA pojedynczych komórek. Badanie ujawniło, że ekspresja receptorów charakterystycznych dla nieswoistej odpowiedzi immunologicznej (CX3CR1, CXCR1, CXCR2), występujących na komórkach takich jak neutrofile czy makrofagi, rośnie wraz z dojrzewaniem komórek NK. Odwrotnie – ekspresja receptorów chemokinowych związanych ze swoistą odpowiedzią immunologiczną, takich jak CCR5, CCR7, CXCR3, spada wraz ze wzrostem dojrzałości komórek NK.

Następnie, za pomocą metody Transwell, zbadano odpowiedź migracyjną poszczególnych podtypów komórek NK oraz jej związek z ekspresją odpowiednich receptorów. Wyniki pokazały, że ekspresja receptorów silnie koreluje z odpowiedzią migracyjną wywołaną przez ich ligandy. Ponadto, sprawdzono czy suboptymalne stężenie dwóch chemokin działających na dwa różne receptory (CX3CL1 – CX3CR1, CXCL8 – CXCR1/2) powoduje taką samą odpowiedź jak optymalne stężenie każdej z tych chemokin osobno. Nieoczekiwanie, nasze badania ukazały synergizm działania pomiędzy dwoma różnymi receptorami, a odpowiedź migracyjna była silniejsza zarówno niż optymalne stężenie każdej z tych chemokin osobno, jak i matematyczne dodanie efektów suboptymalnych stężeń tych chemokin osobno. Ta obserwacja skłoniła nas do zbadania wzorców współwystępowania receptorów chemokinowych na komórkach NK. Odkryto, że na podstawie ekspresji receptorów chemokinowych można przewidzieć przynależność do jednego z mniej dojrzałych, klasycznych podtypów komórek NK. Z kolei w miarę dojrzewania komórek NK zwiększa się różnorodność wzorców ekspresji receptorów chemokinowych, uniemożliwiając tego typu predykcje.

Na koniec przeanalizowano dane z sekwencjonowania RNA z 9471 guzów z baz danych TCGA i TARGET pod kątem profilu wydzielanych przez nie chemokin. Zaobserwowano dziewięć głównych profilów wydzielniczych chemokin, z których żaden nie zawierał ligandów dla więcej niż jednego receptora na dojrzałych komórkach NK, stanowiąc jedną z prawdopodobnych przyczyn ich niskiej infiltracji mikrośrodowiska guzów. Dla porównania: wiele z guzów miało ekspresję chemokin będących ligandami dla

trzech lub więcej receptorów na makrofagach, mających działanie wspierające rozwój nowotworu. Dodatkowo nasza analiza wskazała chłoniaka rozlanego z dużych komórek B (DLBCL, ang. *diffuse large B-cell lymphoma*) jako optymalny cel terapeutyczny dla terapii niemodyfikowanymi, namnożonymi komórkami NK ze względu na korespondujący profil chemokin z receptorami chemokinowymi komórek NK poddanych ekspansji komórkami K562-mbIL21-4-1BBL.

Podsumowując, w pracy po raz pierwszy opisany został wpływ dojrzewania komórek NK na ich profil migracji, jak i zależność ekspresji powierzchniowej receptorów chemokinowych z natężeniem migracji indukowanej ich ligandami. W ramach przeprowadzonych badań odkryto również synergistyczny wpływ aktywacji dwóch różnych receptorów chemokinowych, który w połączeniu z analizą profilu ekspresji chemokin w guzach litych nie tylko ukazał mechanizm ograniczania infiltracji komórek NK przez nowotwory, ale reprezentuje także niewykorzystany potencjał dla inżynierii nowej generacji terapii komórkowych opartych na komórkach NK.

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3. Założenia i cel pracy

Istotne właściwości przeciwnowotworowe komórek NK są dobrze znane. Jednakże, wykorzystanie ich w terapii guzów litych jest obecnie mało skuteczne ze względu na niewielkie zdolności infiltracji guzów litych oraz immunosupresyjne warunki TME.

Dlatego, głównym celem niniejszej pracy doktorskiej była identyfikacja sposobów na poprawę zdolności migracji komórek NK w obszar TME poprzez dokładne poznanie biologii migracji komórek NK. Szczegółowe cele pracy obejmowały:

- Opisanie dostępnej wiedzy na temat infiltracji komórek NK w nowotworach litych, ich zalet jako komórek efektorowych w immunoterapii oraz opisanie czynników hamujących zdolność migracji komórek NK do komórek nowotworowych.
- Zbadanie profilu receptorów chemokinowych na komórkach NK we krwi obwodowej bez stymulacji, po stymulacji cytokinami, po ekspansji wykorzystując komórki K562-mbIL21-4-1BBL oraz komórek NK różnicowanych z indukowanych pluripotencjalnych komórek macierzystych (iPSC), przed i po ekspansji.
- Zbadanie wpływu procesu dojrzewania komórek NK na profil ich receptorów chemokinowych.
- Zbadanie związku powierzchniowej ekspresji receptorów chemokinowych ze zdolnością do migracji w odpowiedzi na ich ligandy.
- Zbadanie efektów jednoczasowej stymulacji dwóch różnych receptorów chemokinowych za pomocą suboptymalnych stężeń chemokin.
- Zbadanie współwystępowania receptorów chemokinowych na komórkach NK.
- Charakteryzacja i analiza kompatybilności profilu chemokin ponad 9 tys. guzów litych z profilem receptorów chemokinowych na podtypach komórek NK.

4. Prospects for NK Cell Therapy of Sarcoma

Review

Prospects for NK Cell Therapy of Sarcoma

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Simple Summary: Sarcomas are a group of aggressive tumors originating from mesenchymal tissues. Patients with advanced disease have poor prognosis due to the ineffectiveness of current treatment protocols. A subset of lymphocytes called natural killer (NK) cells is capable of effective surveillance and clearance of sarcomas, constituting a promising tool for immunotherapeutic treatment. However, sarcomas can cause impairment in NK cell function, associated with enhanced tumor growth and dissemination. In this review, we discuss the molecular mechanisms of sarcoma-mediated suppression of NK cells and their implications for the design of novel NK cell-based immunotherapies against sarcoma.

Abstract: Natural killer (NK) cells are innate lymphoid cells with potent antitumor activity. One of the most NK cell cytotoxicity-sensitive tumor types is sarcoma, an aggressive mesenchyme-derived neoplasm. While a combination of radical surgery and radio- and chemotherapy can successfully control local disease, patients with advanced sarcomas remain refractory to current treatment regimens, calling for novel therapeutic strategies. There is accumulating evidence for NK cell-mediated immunosurveillance of sarcoma cells during all stages of the disease, highlighting the potential of using NK cells as a therapeutic tool. However, sarcomas display multiple immunoevasion mechanisms that can suppress NK cell function leading to an uncontrolled tumor outgrowth. Here, we review the current evidence for NK cells' role in immune surveillance of sarcoma during disease initiation, promotion, progression, and metastasis, as well as the molecular mechanisms behind sarcoma-mediated NK cell suppression. Further, we apply this basic understanding of NK–sarcoma crosstalk in order to identify and summarize the most promising candidates for NK cell-based sarcoma immunotherapy.

Keywords: Natural Killer (NK) cells; immunotherapy; sarcoma; cancer; chimeric antigen receptor (CAR); adoptive cell therapy; tumor microenvironment (TME); cell-mediated cytotoxicity; solid tumors

1. Introduction

Natural killer (NK) cells are the first-discovered members of the innate lymphoid cell (ILC) family, providing defense against tumors and pathogen-infected cells [1,2]. They express a remarkably

diverse repertoire of inhibitory and activating surface receptors, regulating their responses [3]. NK cell-activating receptors recognize either stress-induced ligands, virus-encoded proteins, or Ig-coated cells. In contrast, inhibitory receptors contribute to self/non-self-discrimination by recognizing polymorphic major histocompatibility complex (MHC) class I ligands, also known as human leukocyte antigen (HLA) (Table 1) [3–5].

Table 1. A brief summary of natural killer (NK) cell activating and inhibitory receptors.

Receptor	Known Ligands	Molecular Structure	Function
Killer Immunoglobulin-like receptors (KIR)	HLA-A, Bw, C, G	Immunoglobulin Superfamily	Stimulatory (short cytoplasmic tail) or inhibitory (long cytoplasmic tail)
CD16 (FcγRIII)	Fc portion of IgG	Immunoglobulin Superfamily	Stimulatory
CD2 receptor family		Immunoglobulin Superfamily	
2B4 (CD244)	CD48		Stimulatory
DNAM-1 (CD226)	PVR (CD155) and Nectin-2 (CD112)		Stimulatory
NTB-A	Homophilic		Stimulatory
CS1 (CRACC)	Homophilic		Stimulatory
NKG2 receptor family		C-type lectins	
NKG2D	MICA/B, ULBPs		Stimulatory
CD94/NKG2A	HLA-E		Inhibitory
CD94/NKG2C	HLA-E		Stimulatory
CD94/NKG2E	HLA-E		Stimulatory
Natural Cytotoxicity Receptors (NCRs)		Immunoglobulin Superfamily	
NKp30a/b	B7-H6, BAG6		Stimulatory
NKp30c	B7-H6, BAG6		Inhibitory
NKp44	PCNA		Stimulatory
NKp46	Vimentin		Stimulatory
NKp80	AICL		Stimulatory

Different families of NK cell-activating receptors include NK Group 2 (NKG2) receptors, natural cytotoxicity receptors (NCRs), DNAM-1, 2B4, and CD16 (FcRyIIIa). NKG2D is an activating receptor belonging to the NKG2 family, recognizing MHC class I-related chain A/B (MICA/B) and members of the UL-16 binding protein (ULBP) family [6,7]. The NCRs include NKp30, NKp44, NKp46, and NKp80, which bind to B7-H6, AICL, or viral hemagglutinins [8–10]. DNAM-1 recognizes the viral receptors PVR (CD155) and Nectin-2 (CD112). 2B4 binds other SLAM family proteins, whereas CD16 is an Fc receptor for IgG, responsible for mediating antibody-dependent cell cytotoxicity (ADCC) against antibody-opsonized cells [11–13]. The primary NK cell inhibitory receptors are the NKG2A and the long-tailed killer cell immunoglobulin-like receptors (KIRs), which both bind to MHC class I molecules, preventing NK-mediated lysis of cells with normal MHC expression [14]. Inhibitory KIRs are specific for different MHC isotypes [14]. Acquisition of self-MHC class I binding KIRs during cell differentiation tunes NK cells' cytotoxic potential in a process termed education. Repeated interactions of inhibitory KIRs with self-MHC class I molecules allow NK cells to acquire superior cytotoxic properties as well as tolerance to self-MHC expressing cells [15–17]. The dynamic functional tuning of human NK cells during NK cell differentiation and education and the implications for NK cell therapy are discussed in detail in [18].

As their name implies, NK cells can kill transformed or infected cells without the need for earlier priming. Their cytotoxicity is executed either by degranulation, where the directional release of perforin and granzymes induces apoptosis predominantly in a caspase-3-dependent manner, or through death receptor ligands of the tumor necrosis factor (TNF) family, such as TNF, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL), acting primarily through caspase-8 [19,20].

Additionally, NK cells rapidly produce chemokines and cytokines upon activation, including interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10, CCL3, CCL4, CCL5, and CXCL8 that recruit and affect the function of hematopoietic and nonhematopoietic cells in the tumor microenvironment (TME) [21].

A growing amount of evidence suggests that the proper functioning of NK cells plays a significant role in immune surveillance of cancer, prompting researchers to utilize NK cells in cancer treatment [22,23]. Since NK cells do not express rearranged antigen receptors, they can be easily transferred across MHC barriers without causing graft-versus-host disease (GvHD). Lack of MHC restriction and their unique ability of cancer cell recognition through interactions of multiple surface molecules impedes cancer immune evasion by MHC downregulation or a single antigen loss [24]. Because of these potent antitumor properties, intense studies are currently being carried out to use NK cells, induced pluripotent stem cell (iPSC)-derived NK cells, and the NK cell line NK-92 as novel therapeutic tools against cancer [25–27]. However, NK cell therapy faces many challenges, such as inadequate homing properties, hostile TME, or tumor immunoevasion [28].

Notably, one of the most NK cell-sensitive cancer types are sarcomas, a heterogeneous group of aggressive mesenchyme-derived tumors with poor prognosis [29–32]. Sarcomas can originate from different tissues such as bone, cartilage, muscle, adipose tissue, or blood vessels. Sarcoma's yearly incidence is approximately 5 per 100,000, accounting for less than 1% of malignant solid tumors in adults but more than 20% in children [33,34]. The mainstay of sarcoma treatment based on a combination of surgery and radiotherapy (RT) is able to control localized tumors; however, ~40% of the patients experience tumor relapse and distant metastases [29,35]. Unfortunately, current treatment regimens are ineffective in increasing overall survival in metastatic sarcomas, ranging from 11 to 20 months, creating a demand for novel and effective therapies [35,36]. The urgent nature of the demand is further underlined by the fact that irrespectively of the stage, some sarcoma subtypes have very few lines of systemic therapy with a clinically meaningful effect [35]. Both experimental and clinical data support the immune system's involvement in sarcoma tumorigenesis. Spontaneous regressions and efficient immunosurveillance are observed in sarcomas, suggesting the prime role of the immune system in tumor development and prompting researchers to explore the potential use of immunotherapies in sarcoma treatment [22,37–39].

Sarcomas are the first type of cancer for which immunotherapy was effectively applied. William B. Coley injected streptococcal organisms into the tumors, based on observations of tumor regressions in patients with concomitant streptococcal infections in the last decade of the 19th century. More than half of the inoperable sarcoma patients treated by Coley were reported to respond completely [40]. Unfortunately, due to poorly characterized preparation and unpredictable toxicities, "Coley's toxins" never became clinically useful.

Because of the unique relationship between NK cells and sarcomas, we set out to review the intimate crosstalk between NK cells and sarcoma cells during tumor initiation, promotion, progression, and metastasis. Further, we discuss the current knowledge regarding sarcoma immunoevasion and NK cell functionality. Finally, we review NK cell-based therapeutic approaches in sarcoma treatment, tested in both preclinical and clinical settings.

2. NK Cell Immune Surveillance during Distinct Phases of Sarcoma Development

NK cell-mediated immunosurveillance is an important factor in cancer development, especially in metastasis control. Since sarcomas have been identified as one of the most NK-sensitive solid tumors, they are a well-suited model to study NK cell-mediated cancer surveillance and tumor immunoediting [30]. Different combinations of NK cell-activating ligands such as MICA/B ULBP1/2/3/5, CD155, and CD112 are known to be expressed on both primary sarcoma samples and cell lines, allowing for NK cell cytotoxicity [41–45]. The primary pathway of killing sarcoma cells appears to be granule-dependent, with FasL-Fas interactions playing a minor role, possibly due to acquired FasL resistance [43,46,47]. However, the significance of the FasL-Fas pathway may be underestimated

because of technical limitations. A 4-h incubation time during a standard in vitro cytotoxicity assay is insufficient to study death receptor-mediated apoptosis [48,49]. Indeed, NK cells were recently shown to kill target cells by both mechanisms in a sequential manner, starting with granzyme B-dependent killing and then gradually transitioning to death-receptor killing during serial killing events [50].

A decrease in NK cell cytotoxicity in older adults is associated with an increased risk of cancer development [51]. Further, pediatric osteosarcoma (OS) patients have a decreased number of circulating NKs, together implicating NK cells' potential role in controlling tumor initiation and progression [52]. Sarcomas have a scarce immune infiltration compared to other solid tumors [53]. Current evidence of the prognostic role of lymphocyte infiltration in sarcomas is often contradictory, with most of the studies leaning towards the positive effect of immune effector cell infiltration on disease prognosis [54–57]. NK cell abundance in the tumor infiltrate positively correlates with increased overall survival in several sarcoma subtypes [58–60].

Additionally, early lymphocyte recovery after chemotherapy is associated with a better outcome in pediatric OS [61,62]. Combining surgery and polychemotherapy with systemic IL-2 treatment increases NK cell number and activity, with the magnitude of the increase correlating with an improved clinical outcome [63]. Finally, some studies have shown NK cells to be significant contributors to the control of sarcoma metastases [64–68].

The aforementioned clinical data implicate the role of NK cells in controlling sarcomas' growth. To summarize the available knowledge on NK cell-mediated immunosurveillance of sarcomas, we divide the evidence into three sections, categorizing NK cell's role in: initiation and promotion, progression, and metastases (Figure 1).

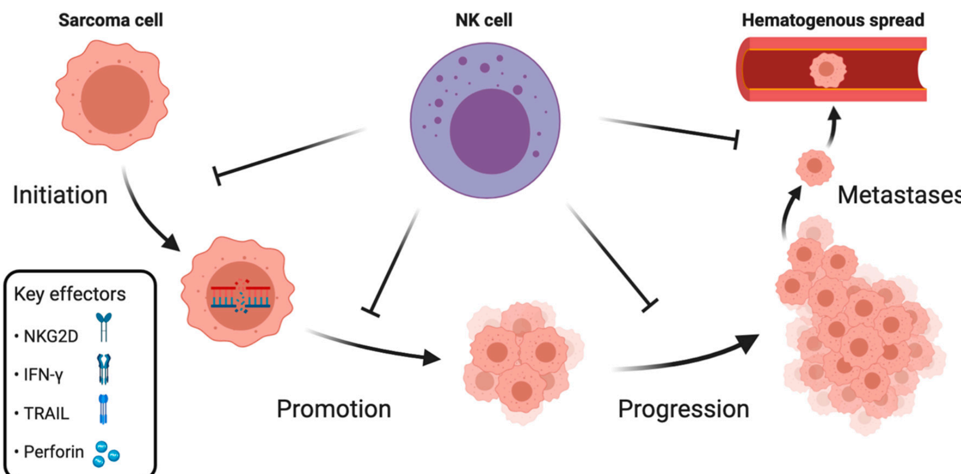

Figure 1. Overview of the central role of natural killer (NK) cells in all stages of sarcoma evolution. Through cell-mediated cytotoxicity, NK cells are able to inhibit tumor initiation, promotion, progression, and development of metastases. The key molecules necessary for NK cell-mediated tumor immunosurveillance are NKG2D, interferon (IFN)-γ, TNF-related apoptosis-inducing ligand (TRAIL), and perforin. Created with BioRender.com.

2.1. Initiation and Promotion

Tumor initiation is the first step of cancer development during which, by rising mutational load, healthy cells transform into cancer cells. It can be followed by tumor promotion, where transformed cells undergo clonal proliferation and form a tumor. During these early stages, it is up to the immune system to eradicate the newly developed neoplastic cells before progression and dissemination occur.

A common carcinogenesis model is based on methylcholanthrene (MCA), which induces chemical mutagenesis and fibrosarcoma development upon inoculation. Smyth et al. evaluated fibrosarcoma formation in mice deficient in NK, Natural Killer T (NKT) cells, or both [69]. Both NK cells and NKT cells seem to be essential for host protection against MCA-induced sarcoma [69]. NK protective function

against sarcoma could be enhanced by IL-12 therapy [69]. Later, the group has confirmed the crucial role of NK cells in preventing the formation of MCA-induced sarcoma and studied the pathways responsible for the recognition of transformed cells. Antibody-mediated neutralization of NK cell-activating receptor NKG2D increased mice susceptibility to MCA-induced sarcoma formation. The importance of the NKG2D was additionally underlined in IFN-$\gamma^{-/-}$ and TRAIL$^{-/-}$ mice, whereas mice depleted of NK cells, T cells, or deficient for perforin did not display any NKG2D-dependent changes in susceptibility. IL-12 therapy augmenting NK cell function and suppressing MCA-induced sarcoma formation was also dependent on the NKG2D pathway. Although NKG2D ligand expression is variable and often not detectable on sarcomas originating in wild type (WT) mice, sarcomas derived from perforin-deficient mice were RAE-1^{+} and immunogenic when transferred into WT syngeneic mice. These findings suggest an essential role of the NKG2D-perforin axis in control and shaping the early events of tumor formation [70]. On the other hand, another NK cell receptor, NKp46, is not associated with the surveillance of MCA-induced fibrosarcoma. However, tumors originating in NKp46$^{-/-}$ mice implanted in WT mice induce a potent immune response suggesting a role of NKp46 in tumor immunoediting [71].

NRLP3 inflammasome, a cellular structure crucial for inducing and sustaining immune response, promotes tumorigenesis in specific cancer types. The deletion of NRLP3 has a protective effect in the MCA-induced fibrosarcoma model, dependent on NK cells and IFN-γ [72]. NRLP3 activation was also shown to impede NK cell antimetastatic function by decreasing NK cell tumor homing [72]. The molecular mechanisms of pro-tumorigenic role of NRLP3 vary between cancer types and are discussed in-depth in [73].

Carcinogenesis is also driven by oncogenic viruses such as HBV, HPV, EBV, and HHV-8 (KSHV). The latter is known to induce Kaposi Sarcoma (KS), with acquired immunodeficiency syndrome (AIDS) immunocompromised individuals being especially prone. Sirianni et al. showed that cells latently infected with KSHV are efficiently lysed by NK cells from healthy individuals [74]. However, the study yields specific limitations as the target cells were carefully selected based on their susceptibility to NK cell cytotoxicity [74]. On the contrary, Matthews et al. reported average levels of classical MHC class I, ICAM-1, HLA-E, and NKG2D ligands on latently infected primary fibroblasts, which caused a limited activation of resting NK cells [75]. Interestingly, infected cells were efficiently cleared by IL-15-primed NK cells [75].

A large study including over 1100 patients investigated the association between the HLA-KIR polymorphism and KSHV and KS status, finding that, in patients with KIR3DS1 plus HLA-B Bw4-80I, the KSHV seroprevalence was 40% lower, but the KS risk was two-fold higher. Similarly, the KSHV seroprevalence was 40% lower, but the KS risk 80% higher with HLA-C group 1 homozygosity. These data suggest that KIR-mediated NK cell activation may decrease KSHV infection's risk but enhance KS progression if infection occurs [76]. Peripheral blood (PB) NK cell counts, on the other hand, do not correlate with the risk of KS development [77].

2.2. Progression

Cancer initiation and promotion can be followed by progression if not controlled by the immune system. Progression is the last phase of localized tumor development, characterized by increased growth speed and acquiring invasive potential.

Mice selectively depleted of NK1.1 positive cells demonstrated more rapid initial growth upon injecting MCA207 sarcoma cells. In addition, large (20 mm) implanted MCA207 sarcomas were rejected following cyclophosphamide and IL-12 treatment, but the time to tumor eradication was significantly longer in mice with depleted NK cells [78]. Other groups confirmed that MCA-induced sarcoma growth could be reduced by IL-12 treatment, with the effect being mediated by NK cells [69]. Takeda et al. provided evidence of NK cells playing a key role in limiting the L929 TRAIL-sensitive fibrosarcoma progression in a subcutaneous murine model. The effect was mediated in a TRAIL and

IFN-γ dependent fashion. The TRAIL pathway's protective effect was dependent on NK cells and IFN-γ, supported by gene knockout experiments [79].

One of the most critical NK cell functions is to enhance the infiltration of other immune cells into the TME. In MCA-induced sarcomas, NK cells are known to infiltrate the developing tumors in early stages, with the semi-mature $CD27^{high}$ NK cells being the predominant subpopulation of NK cells accumulating in the TME. The tumor-infiltrating NK cells display an activated surface phenotype and provide an early source of IFN-γ attracting other immune cells. Interestingly, host IFN-γ is critical for NK cell tumor homing, and, conversely, the tumor-infiltrating NK cells mainly suppressed tumor growth via the IFN-γ pathway. This implicates the importance of IFN-γ as a positive regulatory factor for both NK cell recruitment into the TME and an effective NK antitumor immune response [80]. NK cell-derived IFN-γ can also improve cancer cell recognition and associated NK cell cytotoxicity through ICAM-1 upregulation on cancer cells [81].

Moreover, IFN-γ plays a role in NK cell-mediated sarcoma immunoediting. Tumor cells isolated from immunocompetent mice displayed reduced expression of NKG2D ligand H60 and increased MHC class I expression compared with tumor cells isolated from mice treated with IFN-γ-specific neutralizing monoclonal antibody (mAb) [82]. IFN-γ can also induce programmed cell death ligand 1 (PD-L1) expression on cancer cells, subsequently inhibiting NK cell effector function [83].

Another approach to understanding the drivers of MCA-induced sarcoma progression was taken by O′ Sullivan et al. by comparing the gene expression between unedited and immunoedited tumors [84]. One of the most differentially expressed genes was *IL17D* encoding interleukin 17D (IL-17D), with a significantly increased expression in unedited tumor cells. Overexpression of IL-17D in edited tumor cells induced tumor rejection by stimulating CCL2 production from tumor endothelial cells, leading to an increase in the recruitment of NK cells. IL-17D-induced recruitment attracted mostly $CD27^{high}$ NK cells, a semi-mature population of NK cells participating in IFN-γ-dependent T cell priming and contributing to suppressing tumor progression [84]. These data suggest that NK cells play a role in tumor immunoediting and suppressing sarcoma growth, both directly and indirectly by regulating other immune cells' activity and infiltration.

2.3. Metastases

The metastatic spread of neoplastic cells to distant anatomical regions is a leading cause of death in cancer patients. Metastatic spread is orchestrated by the intrinsic properties of cancer cells, enabling invasion of the local microenvironment and colonization of distant sites through lymphatic or hematogenous spread. Moreover, metastasis is regulated by microenvironmental and systemic processes, such as immunosurveillance.

NK cells are known for their antimetastatic potential [85–88]. Indicators of NK cell function such as high expression of NK cell-activating receptors and high cytotoxic or IFN-γ secreting properties have been linked to decreased metastatic load in multiple cohorts of cancer patients with risk of metastatic disease, suggesting their clinically relevant protective role [87]. High numbers of tumor-infiltrating NK cells have been inversely correlated with the presence of distant metastases in gastrointestinal stromal tumors (GIST), a subtype of sarcomas [64]. Interestingly, the incompatibility of nude mice as hosts for metastatic studies is attributed mainly to their NK cells, which efficiently remove circulating tumor cells [65]. NK cell-protective role against metastases was also recognized in multiple murine sarcoma models, where antibody- or cyclophosphamide-mediated NK cell depletion significantly increased metastatic load [66–68]. Studying the interactions between MHC class I expression, NK cells, and sarcoma metastases provided evidence for the correlation of RCT sarcoma metastatic potential with increased MHC class I expression, which in turn correlated with cancer cell resistance to NK cell lysis [67]. However, others did not observe any simple associations among MHC expression, development of metastases, and NK cells [89].

TNF-α, a highly pro-inflammatory cytokine secreted by effector immune cells, is one of the cytotoxic effector proteins capable of inducing cancer cell apoptosis. Surprisingly, in sarcomas, it was

shown to have an NK cell-dependent prometastatic effect, indicated by selective antibody depletion experiments [86]. TNF-α can also exhibit prometastatic activity on its own through increased production of chemokines inducing angiogenesis and enhancing cancer cell motility, which has been thoroughly reviewed in previous publications [90,91]. Moreover, NRLP3 and IL-1R8 deficiencies were shown to have an antimetastatic effect attributed to enhanced NK cell function [72,92].

NK cells can be successfully used in metastases treatment; K562-expanded NK cells effectively eradicate Ewing sarcoma (EWS) metastases with little effect on the primary tumor in a murine model [93]. Furthermore, allogeneic hematopoietic stem cell transplantation (HSCT) was shown to inhibit the development of sarcoma metastases in an NK cell-dependent manner in clinical trials [94,95].

3. NK Cell Dysfunction in Sarcomas

In the course of cancer microevolution, neoplastic cells undergo a series of metabolic adjustments adapting the cells to increased proliferation. Unfortunately, the shift in cancers metabolic state is accompanied by creating a hostile TME inhibiting the anticancer immune response and promoting homing of immunosuppressive cells such as regulatory T cells (Tregs) or M2 macrophages (Figure 2). Already in 1981, Gerson et al. found that sarcomas substantially inhibit NK cell functions. NK proliferation in response to concanavalin A, macrophage migration inhibitory factor secretion, and cell-mediated cytotoxicity were all suppressed by macrophages infiltrating the sarcoma, with the cytotoxicity being the most preserved function [96].

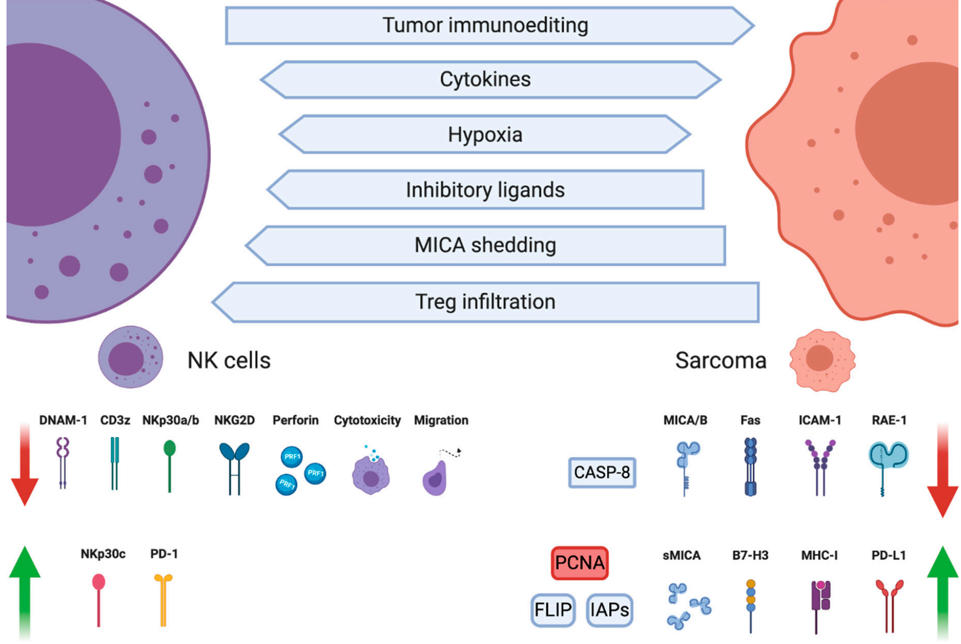

Figure 2. Interactions between natural killer (NK) cells, cancer cells, and tumor microenvironment (TME) shape sarcomas' immunoevasion mechanisms. Tumor immunoediting, cytokines, hypoxia, and cells infiltrating the TME can change the sarcomas cell phenotype into an NK cell-resistant one, characterized by decreased expression of NK cell-activating ligands MHC class I-related chain A/B (MICA/B), retinoic acid early transcript 1 (RAE-1), intercellular adhesion molecule 1 (ICAM-1), and proteins necessary for Fas ligand (FasL)-mediated apoptosis (Fas, caspase-8). Conversely, expression of inhibitory molecules such as major histocompatibility complex (MHC) class I, programmed cell death ligand 1 (PD-L1), B7-H3 (CD276), proliferating cell nuclear antigen (PCNA), and antiapoptotic proteins cellular FLICE-inhibitory protein (c-FLIP), as well as inhibitors of apoptosis (IAPs), is increased. NK cell phenotype and function are also altered in the sarcoma TME by cytokines, hypoxia, and inhibitory ligands, resulting in a disturbed balance between activating and inhibitory receptor expression and associated cytotoxicity impairment. Created with BioRender.com.

Cytotoxicity impairment of PB NK cells was reported in chemotherapy-naïve sarcoma patients, in contrast to NK cells from renal cell carcinoma patients, which displayed normal cytolytic activity [97,98]. The cytotoxic function of NK cells could be restored by IL-2 and Hsp-70-derived TKD peptide. Additionally, NK cells in chemotherapy-naïve sarcoma patients had reduced proportions of mature CD56^{dim} population and slightly increased NKG2D expression compared to age-matched controls. After disease progression or relapse, NK cell phenotypic alterations were more remarkable; progressively reduced CD56^{dim} proportions and decreased expression of NKG2D, CD3ζ, perforin, together with reduced frequencies of differentiated CD57^{+} NK cells were all observed [98].

Suppression of the NK cell compartment increases at the tumor site. A significant decrease in the NK cell proportions is observed in tumor-infiltrating lymphocytes (TILs) compared to matched peripheral blood mononuclear cells (PBMCs). In contrast, no difference was observed between tumor-infiltrating and PB CD3^{+} bulk T cells, CD4^{+}, and CD8^{+} T cells, indicating an impairment in NK cell tumor homing or intratumoral persistence [99]. Profiling of TIL NK cells provided evidence for decreased CD16, KIR2DL1, KIR2DL2/L3, and KIR3DL1 expression in the CD16^{+} KIR^{+} and CD16^{+} KIR^{-} NK cell subsets, compared to NK cells in matched PBMCs. DNAM-1 and NKG2D expression on TIL NK cells was also reduced in the vast majority of the patients compared to matched PBMCs [99]. Significantly, NKG2D and DNAM-1 downregulation might contribute to disease progression in these patients as sarcoma cells are mostly recognized by NKG2D and DNAM-1 receptors [43,97]. In GIST, the NKp30 receptor was preferentially downregulated on tumor-infiltrating NK cells. Interestingly, PB NK cells in GIST patients expressed immunosuppressive NKp30c isoform more frequently with proportionally less NKp30a and -b. The expression of NKp30c isoform was associated with an unfavorable clinical outcome [64].

The co-culture experiments of NK and primary sarcoma cells provided further insights into sarcoma-induced functional impairment. Sarcoma cells caused a decreased expression of NKG2D, DNAM-1, and interfered with IL-15-induced expression of NKG2D, DNAM-1, and NKp30, consecutively inhibiting the cytolytic activity of NK cells. The inhibition was contact-dependent, and the cytotoxicity impairment was directly linked to the downregulation of the respective NK cell-activating receptors. Five days of IL-15 pretreatment was able to increase NK cell resistance to sarcoma suppression. In opposite to the above-mentioned changes in TIL NK cells, CD16 expression and ADCC were not affected by the NK–sarcoma co-cultures [100].

Few reports show no differences in IFN-α signaling, NKG2D expression, and NK cytotoxic properties between PB NK cells from sarcoma patients and healthy donors [43,101]. However, the analyzed patient group was limited to freshly diagnosed patients with a most likely early-stage disease, which indeed can be associated with mild or no functional impairment in PB NK cells [86,98].

3.1. Tumor-Infiltrating Immunosuppressive Cells

Other cells in the sarcoma microenvironment can contribute to creating a suppressive milieu. Tumor stromal cells derived from fresh sarcoma samples display a potent antiproliferative effect on PBMCs, decrease NK cell cytotoxicity and NKp44/46 expression, as demonstrated in co-culture experiments [102]. Through rewiring chemokine and metabolic networks, sarcomas can induce immunosuppressive CD4^{+}CD25^{+} Treg infiltration. Ghiringhelli et al. reported an inverse correlation between NK cell activation and Treg abundance in GIST patients [103]. By expressing membrane-bound transforming growth factor β (TGF-β), Tregs directly inhibit NK cell cytotoxicity, proliferation, and alter NK cell phenotype by downregulating NKG2D receptor expression [103].

3.2. Cytokine-Dependent Inhibition

Cytokines present in the TME can also suppress NK cell function. Hafner et al. showed that TNF-α suppresses NK cell cytotoxicity, consequently impairing NK cell antimetastatic function in murine sarcoma model [86]. The molecular mechanism of TNF-α-mediated NK cell suppression has not been fully elucidated, but new evidence indicates that TNF-α may contribute to NK cell exhaustion in a

TIM-3-dependent manner [104]. The group has also provided insights into time-dependent changes in NK cell cytotoxic function after sarcoma tumor inoculation. At first, the NK cell activity increases, but after quickly reaching its peak it starts to decrease below the initial level [86]. On the other hand, TNF-α was shown to increase human OS cells' susceptibility to NK lysis by CD54 and CD58 upregulation, demonstrating a dual role of TNF-α in NK-sarcoma interactions [105,106]. Notably, CD54 (ICAM-1) expression on cancer cells is essential for NK cell cytotoxicity, and its magnitude directly correlates with OS susceptibility to NK cell lysis [106–108].

Interferons are proinflammatory peptides known for their antiviral properties. One of their mechanisms of action is MHC class I upregulation, aiming to increase the presentation of viral peptides. In murine MCA sarcoma, IFN-γ and IFN-α were shown to reduce expression of NKG2D ligand H60. Downregulation occurred at the transcript level and was STAT1-dependent. IFN-γ-treated MCA sarcomas with initially high levels of H60 were resistant to killing by IL-2-activated NK cells. Resistance was not solely dependent on H60 downregulation but also on IFN-enhanced MHC class I expression [82].

TGF-β is another vital player in sarcoma TME. It regulates the extracellular matrix (ECM) protein composition and induces osteopontin synthesis in OS cells, increasing their malignant potential [109,110]. Additionally, Treg-derived TGF-β can inhibit NK cell effector functions such as cytotoxicity and tumor homing [111–113]. Besides, Gao et al. provided evidence for TGF-β-induced transformation of NK cells into intermediate type 1 innate lymphoid cells (intILC1) and ILC1 in the sarcoma microenvironment. Importantly, intILC1s and ILC1s did not provide sufficient control of local tumor growth and metastasis, whereas NK cells favored tumor immunosurveillance. ILC1-derived TNF-α was suggested to be partially responsible for an escape from the innate immune system [114]. In soft tissue sarcoma patients, high TGF-β1 intratumoral expression is associated with aggressive disease and shorter disease-specific survival [115].

3.3. MHC-Dependent Inhibition

MHC class I molecules serve as ligands for inhibitory KIR and NKG2A receptors. Current evidence shows that chemotherapy can increase classical and nonclassical MHC class I molecule expression in OS cells, consequently inhibiting NK cell activity [43]. One of the critical NK-suppressive MHC molecules is peptide-loaded HLA-E, which can be expressed in different tumor cell types, including sarcomas. It is a potent inhibitor of NK cell activity, acting via NKG2A [116]. Moreover, it has been shown that EWS treated with anti-GD2 chimeric antigen receptor (CAR)-NK cells developed resistance to the treatment in an HLA-G-dependent manner, which was selectively upregulated on tumor cells only in CAR-treated mice. NKG2A knockdown restored CAR-NK lytic function and allowed for effective tumor eradication [117]. In OS patients, the MHC class I expression itself is associated with a better prognosis, most likely due to T cell-mediated immune response [118].

Interestingly, the relationship between NK cell activation and MHC I expression appears to be nonlinear. A moderate increase of MHC class I expression on EWS cells caused a highly NK-resistant phenotype, whereas downregulation of MHC expression did not change the susceptibility, implicating the existence of a threshold. That, in turn, would allow modest changes in the target cell surface phenotype to significantly affect the susceptibility to NK cell-mediated lysis [119]. Not only the surface expression but also the KIR-HLA mismatch degree between NK cells and OS determines their susceptibility to NK cell lysis [120].

3.4. MICA Shedding

Shedding of NK cell-activating ligands can also contribute to sarcoma immunoevasion. NKG2D ligand MICA is shed in a matrix metallopeptidase (MMP)-9-dependent manner in OS. Soluble MICA (sMICA) was shown to cause NKG2D downregulation, impairing NK cell response [121]. High concentrations of sMICA were correlated with poor prognosis in multiple cancer types [122]. In sarcomas, sMICA concentration is increased in advanced disease, downmodulating NKG2D

expression on NK cells. On the contrary, most of the early stage and well-differentiated sarcomas were shown to express MICA on the cancer cell surface, indicating that MICA expression is lost along the disease progression [41]. Therefore, MICA shedding might be an early event in sarcoma immunoevasion, contributing to the disease progression [42,123]. Multiple studies have shown that increased MICA and sMICA expression were associated with decreased NKG2D expression on NK cells and correlated with advanced and metastatic disease [41,42]. Further, MMP-9 and MMP-2 expression is associated with the presence of metastasis and poor survival in OS patients and could be potentially used as a prognostic biomarker [124,125].

3.5. Apoptosis Resistance

Along with the tumor microevolution, neoplastic cells can acquire resistance to cell-mediated cytotoxicity. Resistance to FasL-mediated apoptosis can be mediated through downregulated caspase-8 and increased expression of antiapoptotic proteins such as cellular FLICE-inhibitory protein (c-FLIP) or Inhibitors of apoptosis (IAPs). Such mechanism has been demonstrated to play a role in immunoevasion of rhabdomyosarcoma (RMS) and EWS cell lines, as well as primary EWS samples [46,47]. Notably, FasL is constitutively expressed in the lung, implicating that Fas expressing cancer cells should be eliminated by lung endothelium. However, metastatic OS acquire resistance to Fas-mediated death by Fas downregulation, allowing for lung colonization [126]. Chemotherapeutic agents such as gemcitabine upregulate Fas surface expression and may therefore be an important part of multimodal therapy for OS lung metastases [127]. Cisplatin treatment can overcome FasL resistance by downregulating c-FLIP-L, sensitizing OS cells to FasL mediated apoptosis [128]. Sensitization of OS cells to FasL can also be induced by histone deacetylase inhibitor (HDACi) entinostat, which increases Fas transcription, its localization in membrane lipid rafts and decreases the expression of antiapoptotic c-FLIP [129,130].

3.6. Immune Checkpoints

Immune checkpoints are molecules regulating the activity of immune system, which in physiological setting play a protective role against autoimmunity and overactivation of lymphocytes. Programmed cell death protein 1 (PD-1) is a well-known checkpoint molecule, functioning as a "break" in the immune system [131]. Typically, only a small fraction of PB NK cells express PD-1, but the proportion is increased on NK cells in cancer patients [132]. PB NK cells from KS patients exhibit PD-1 expression in a $CD56^{dim}CD16^{+}$ population with otherwise normal surface phenotype. However, despite the normal phenotype, $PD-1^{+}$ NK cells demonstrated reduced cytotoxicity and IFN-γ production ex vivo following the direct triggering of NKp30, NKp46, CD16, or short stimulation with target cells, suggesting a role of PD-1 in KS-mediated NK cell exhaustion [133]. Moreover, PD-L1 expression in OS cell lines determined their susceptibility to NK cell lysis, shown by mAb blocking experiments [134]. However, in OS patients, intratumoral PD-L1 expression positively correlated with increased immune infiltration including NK cells and, surprisingly, event-free-survival [57].

B7 homolog 3 (B7-H3, CD276) is an immune checkpoint protein of the B7-CD28 family with a vital role in T cell inhibition [135]. B7-H3 overexpression is observed in multiple cancers, including OS, RMS, and EWS [135–138]. B7-H3 is expressed in 91.8% of OS tissues, where it promotes OS cell invasion and is inversely correlated with TIL abundance [136]. High B7-H3 expression is also associated with shorter survival and disease recurrence [136].

Interleukin-1 receptor 8 (IL-1R8) is a member of the IL-1 receptor family, acting as a negative regulator of other IL-1 receptor and Toll-like receptor (TLR) signaling, that has been recently established as a checkpoint molecule in NK cells. Knockout of IL-1R8 has been shown to restore NK cell antitumor function in MCA-induced sarcomas, implicating a role of IL-1R8 in sarcoma-mediated NK cell suppression [92]. Expression of other checkpoint molecules such as T-cell immunoglobulin and mucin domain-3 (TIM-3) ligands and NKp44-inhibiting proliferating cell nuclear antigen (PCNA) was also reported in sarcomas [45,139].

3.7. Altered Oxygen Metabolism

Rapid cell turnover and cancer growth are associated with decreased O_2 gradient as tumors grow beyond their vascular supply. Large murine sarcomas contain a severely hypoxic core, whereas smaller tumors possess hypoxic gradients throughout the tumor mass. Evidence indicates that these hypoxic gradients orchestrate sarcoma cell migration and ECM remodeling, increasing their metastatic potential. Additionally, hypoxia-inducible factor 1-alpha (HIF-1α) increases CXCR4 expression on sarcoma cells, contributing to metastasis development. Notably, in sarcoma patients, increased HIF-1α and CXCR4 expression are associated with advanced disease [140,141].

A hypoxic microenvironment is reported to alter the susceptibility of human OS cells to NK cell-mediated lysis. Different OS cell lines expressed various NKG2D ligands such as MICA, MICB, and ULBP1/2/3, with the MICA being most frequently expressed [44]. In a HIF-1α mediated way, hypoxia decreased cell surface MICA expression without increasing the secretion of soluble MICA, resulting in reduced susceptibility of the OS cells to the NK cell-mediated lysis [44]. Moreover, by inducing HIF-1α, hypoxia impairs NK cell function by inhibiting their response to activating cytokines as well as suppressing cell-mediated cytotoxicity capabilities, except for ADCC [142]. Significantly, in STS tumors, low oxygen content is associated with poor disease-specific and overall survival [143]. Additionally, bone and soft-tissue sarcomas are characterized by increased oxidative stress, which is known to inhibit NK cell effector functions [144,145].

3.8. HIV-KS-NK Cell Axis

NK cell-mediated immunity is significantly impaired in AIDS patients with progressing KS compared to both HIV-negative patients with indolent classic KS and healthy blood donors. The highly active antiretroviral therapy (HAART) is able to rescue impaired NK cell function in AIDS-KS patients, inducing tumor regression and HHV8 clearance. However, AIDS-KS patients with more aggressive disease and no response to therapy had persistent HHV8 viremia and reduced NK cell cytotoxicity. These results suggest a crucial role of NK cells in the control of HHV8 infection and KS tumor, as well as AIDS role in mediating NK cell suppression [74]. Additionally, NKG2C^{+} NKp46^{low} NK cells were discovered to form a novel, poorly functional subset present in AIDS-KS patients [146].

HIV-negative classical KS patients have significantly decreased NK cell cytotoxicity compared to healthy controls, whereas healthy HHV8 carriers have phenotypically impaired NK cells with reduced expression of NKp30, NKp46, and CD161 receptors [147]. Further, KS patients show downmodulation of NKG2D, associated with impaired NK-cell lytic capacity, which could be restored upon KS treatment [148]. Interestingly, KS cells exhibited high expression of NKG2D ligands confirmed in situ by immunohistochemical (IHC) staining of KS biopsies. However, no tumor-infiltrating NK cells were detected, suggesting a defect in NK cell homing or persistence in the KS microenvironment [148]. PGE_2 was identified as a critical inhibitory mediator responsible for impairing NK cell response in KS, acting by down-modulation of NKG2D expression on resting NK cells and impairing IL-15 induced proliferation and phenotypic changes [148]. Other studies have demonstrated that PGE_2 inhibits NK cell migration properties and impairs their accumulation in TME, which could be the reason for the lack of NK cells among the KS TILs [149,150]. Moreover, KSHV proteins K3 and K5 were shown to drastically downregulate ICAM-1, MICA, MICB, and AICL (NKp80 ligand) expression on infected cells, increasing their resistance to NK cells [151–153].

3.9. Iatrogenic NK Cell Suppression

NK cell functional impairment can be iatrogenic. Surgery was shown to transiently impair NK cell cytotoxicity; however, the phenomenon's significance is unclear [154]. While in other cancers chemotherapy is reported to upregulate NK cell-activating ligands, a comparison of pre- and post-chemotherapy OS tissue sections provided evidence for either unaltered or decreased expression of MICA, CD112, and CD155 after chemotherapy [43]. Moreover, depending on the specific agent

and the dose, chemotherapy can also directly suppress NK cell function [154–157]. Zoledronic acid (ZA), tested as maintenance therapy in clinical trials in patients with bone sarcomas (OS and EWS), acts by inhibiting bone resorption and inducing apoptosis in osteoclasts and tumor cells. Its effect on NK cell activity is, however, unfavorable as ZA can impede in vitro NK cell expansion and cytolytic responses to EWS, raising concerns against combining NK cell therapies with ZA in bone sarcoma treatment [158].

4. NK Cell-Based Therapies in Sarcomas

Numerous immunotherapeutic approaches have been tested in patients with sarcomas, and the results have not been as impressive as in the treatment of other solid tumors. In theory, proper management of immune checkpoint signaling by monoclonal antibodies can be one of the modalities to revive the functioning of NK cells within the tumor [159]. Immune checkpoint blockade therapy with antibodies blocking target cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the PD-1/PD-L1 pathway leads to durable clinical responses in an increasing number of solid tumors. Unfortunately, responses in patients with sarcomas have been observed infrequently, except for the TIL-rich undifferentiated pleomorphic sarcoma [160–162]. Preliminary clinical evaluation of PD-1-PD-L1 axis inhibition in angiosarcoma and alveolar soft part sarcoma also shows promising results [163–165].

One of the reasons for the inefficacy of immune checkpoint inhibition therapy is the fact that sarcomas have fewer TILs per gram of tissue and lower ratios of TIL infiltration when compared to other cancers, e.g., melanoma or renal cell carcinoma [53]. However, primary sarcomas and sarcoma cell lines were shown to be one of the most vulnerable tumor types to spontaneous NK cell cytotoxicity, making NK cell-based therapies novel and promising treatment alternative [30–32]. The NK cell-based immunotherapies can be divided into two groups based on their principle of action: strategies augmenting NK cell function and those sensitizing cancer cells to NK cell-mediated lysis (Figure 3).

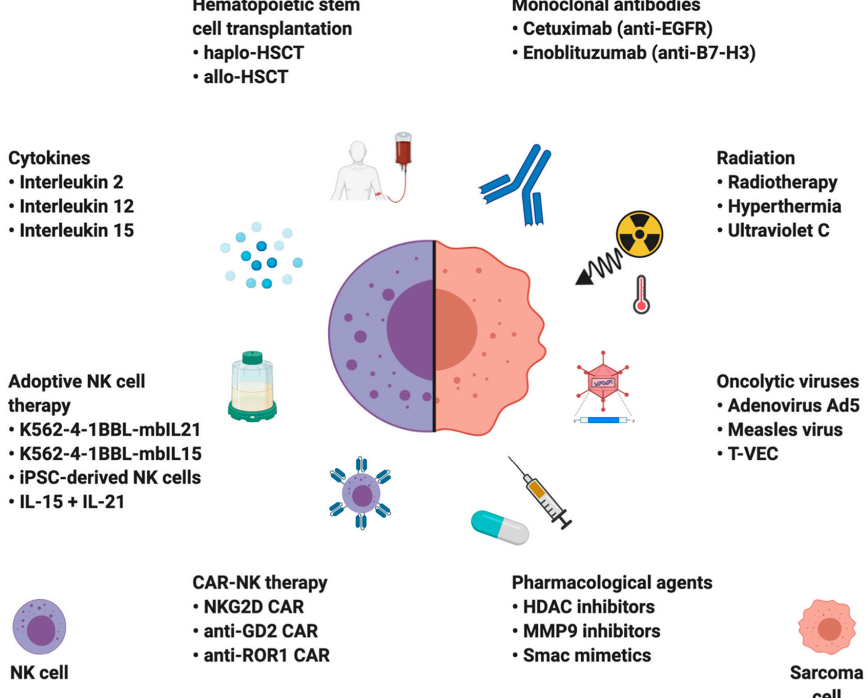

Figure 3. A schematic illustration of selected prospective sarcoma treatment modalities based on: modifying natural killer (NK) cell properties (left); or sensitizing sarcoma cells to NK cell cytotoxicity (right). Created with BioRender.com.

4.1. Hematopoietic Stem Cell Transplantation (HSCT)

HLA-mismatched HSCT combines the effects of chemotherapy and graft-versus-tumor (GvT) phenomenon. HSCT with grafts from haploidentical donors was shown to be safe and beneficial in pediatric solid tumors including sarcomas [166,167]. Already in 1984, a clinical trial in Moscow demonstrated that allogeneic bone marrow transplant suppressed lung metastases development in a group of OS patients after radical surgery. Importantly, all of the treated patients who did not develop metastases had normal NK cytotoxicity levels, whereas, in the metastases group, the NK activity was significantly lower, suggesting a critical role of NK cells in suppressing sarcoma metastases [94]. A clinical trial in pediatric cancer patients, including sarcomas, has shown that HSCT's effects could only be observed in patients with mismatched KIR-HLA [168]. However, the beneficial effect of HLA-mismatched grafts was associated with a higher risk of toxicities [169]. A potential role for NK cells in the graft-versus-tumor effect is supported by in vitro studies showing that the KIR-HLA mismatch degree can predict OS cell lines susceptibility to NK cell lysis [120]. Another study retrospectively investigated allo-HSCT in RMS patients and reported moderate results, prompting further investigations and suggesting the potential use of allo-HSCT as a consolidation therapy [169]. A case report study has also shown haplo-HSCT to be effective in metastases control in two patients with stage IV EWS [95]. Further clinical trials are being carried out to determine the clinical utility of HSCT in high-risk sarcoma patients; however, there are currently no recommendations to use HSCT as a sarcoma therapy irrespectively of the disease stage.

4.2. Cytokines

Because of their potent immunostimulatory properties, cytokines have always raised interest as adjuvant therapies. The antitumor activity of the IFN-α-conjugated antibody against the OS cell line was first reported in 1984 by Flannery et al. The treatment resulted in a modest increase in NK cell cytotoxicity, attributed to IFN-α induced activation [170]. Multiple studies have shown the efficacy of IFN-α, IL-2, IL-12, IL-15, IL-18, and IL-28 in augmenting NK cell functions by increasing their cytotoxicity, rendering resistant to TME-mediated suppression, and altering sarcomas adhesion molecule profile. Such an approach was proven useful through in vivo studies in multiple sarcoma subtypes [43,78,97,171–179]. Because of synergistic effects between cytokines and chemotherapy, they can be used together in sarcoma treatment. A combination of chemotherapy and NK cell-activating cytokines has been shown to induce regression of both, sarcoma primary tumors and lung metastases in murine models [78,175,176]. Importantly, the cytotoxic effect against EWS and RMS cells is almost entirely dependent on NKG2D and DNAM-1 receptors in resting NK cells. However, IL-15-treatment decreases the dependency on NKG2D and DNAM-1, reducing the probability of immune evasion by downregulating their respective ligands [97,174].

Pegylated IFN-α-2b was tested as maintenance therapy in OS in phase III clinical trial NCT00134030. The results show no benefit of IFN-α therapy and poor treatment tolerance due to associated toxicities [180]. IL-2 treatment was also tested in pediatric OS patients [63]. Unfortunately, systemic cytokine therapies are associated with severe side effects [181,182]. An exciting alternative is direct intranasal therapy with either adenoviral or polyethyleneimine vector encoding IL-12, which can locally increase NK cell antitumor potential and lead to the eradication of OS lung metastases [182–184].

A different way of localized cytokine treatment is based on isolated limb perfusion. After sarcoma surgery, blood vessels of the treated limb are being reconnected to form a closed system with a pump and treated with extremely high concentrations of melphalan, TNF-α, and mild hyperthermia [185]. Such an approach has proven to be useful by allowing a higher percentage of limb-sparing surgeries and achieving greater response rates [185].

4.3. Monoclonal Antibodies

Sarcomas can also be targeted by monoclonal antibodies (mAb). Advantages of mAb therapy include the preservation of NK cell ADCC capabilities under hostile sarcoma TME conditions as well as the presence of a synergy between mAb treatment and chemotherapy, which increases ADCC-sensitivity of sarcoma cells [100]. Other agents, such as lenalidomide, can also enhance ADCC against sarcomas [186].

Epidermal growth factor receptor (EGFR) is commonly expressed on OS cell lines and 90% of primary OS samples, with high expression correlated to large tumor volume, prompting to utilize the anti-EGFR monoclonal antibody cetuximab as a potential therapy [187,188]. Unfortunately, no responses to cetuximab were observed in phase II clinical trial in sarcomas indicating that no further trials should follow unless new predictive markers were discovered [189].

Enoblituzumab (MGA271), a humanized IgG1 monoclonal antagonistic B7-H3 antibody, has been studied in phase I clinical trial in patients with refractory B7-H3-expressing neoplasms such as melanoma and advanced solid tumors (NCT01391143). Most of the patients experienced stable disease and significant tumor shrinkage with no dose-limiting toxicity and good tolerance. Another ongoing clinical trial is investigating enoblituzumab in B7-H3-expressing pediatric solid tumors, including neuroblastoma, RMS, OS, EWS, Wilms' tumor, and desmoplastic small round cell tumors (NCT02982941) [190]. A different mAb-based approach is based on insulin-like growth factor receptor (IGFR)-1 antibody-mediated inhibition, which has shown a single-agent efficacy in a subset of EWS patients [191]. Interestingly, IGFR-1 inhibition enhances NK cell expansion without impairing the NK cell-mediated lysis of EWS cells [192].

4.4. Immunomodulation

As discussed above, through NK cell-mediated immunoediting, selective pressure favors persistence of NK cell-resistant sarcoma cells expressing low levels of NKG2D and DNAM-1 ligands and high levels of MHC class I. Increasing the expression of NK cell-activating molecules on cancer cells, thus their sensitivity to NK cell-mediated lysis could serve as an important part of multimodal therapy in future sarcoma treatment. Sodium valproate (VPA), a histone deacetylase inhibitor, increased surface MICA and MICB expression in multiple OS cell lines and, consequently, their susceptibility to NK cell lysis. By inducing acetylation of histones bound to *MICA* and *MICB* gene promoters, VPA increases only the cell-surface but not soluble MICA and B expression [193]. Another study by the same group confirmed this finding and found that VPA synergizes with hydralazine in sensitizing OS cells to NK lysis. The co-treatment additionally upregulated Fas expression, increasing OS cells susceptibility to FasL mediated death [194].

A narrow-spectrum HDACi entinostat has been shown to increase both NKG2D expression on NK cells and MICA/B in multiple tumor target cells, including sarcomas, augmenting NK cell cytotoxicity in vitro and in vivo and consequently suppressing sarcoma lung metastases in mice [195]. Further, entinostat downregulates c-FLIP expression concomitantly redirecting Fas cellular localization to the lipid rafts, sensitizing sarcoma cells to FasL-mediated apoptosis [129,196]. Other groups have shown that entinostat increases the expression of MICA/B, ULBP1/2/5/6, and CD155 in OS, RMS, and EWS cells, enhancing NK cell cytotoxicity [197,198]. However, in a nude mice OS lung metastases model, no significant effects of the treatment were observed. The lack of efficacy was linked with the failure of NK cells to penetrate inside the tumor nodules [197]. Besides, although preliminary studies report entinostat-mediated enhancement of NK cell effector function, both HDAC inhibition and DNA hypomethylation have been linked to NK cell cytotoxicity impairment, raising caution against combining them with NK cell-based therapies [198–200].

NKG2D ligands are upregulated following genotoxic stress induced by, e.g., RT, through activation of ATM and ATR pathways, consecutively alerting the immune system about potentially dangerous transforming cells [201]. Combining radiation and NK cell treatment has shown promising results in preclinical studies targeting sarcomas and sarcoma stem cells. Radiation increases the expression of

NK activating ligands MICA and MICB, as well as Fas, both in vivo and in post-RT patient biopsies. The effects were preferentially observed on cancer stem cells, increasing their sensitivity to NK lysis [202–204]. Another way to increase NKG2D ligand expression is blocking MMP-9-mediated MICA shedding. Using a specific MMP-9 inhibitor might represent a double-benefit therapy, where it can both inhibit tumor invasion and restore NK cell-mediated antitumor immune response [123].

A novel and promising means of cancer therapy are based on infecting tumor cells with modified oncolytic viruses such as adenovirus serotype 5, herpes simplex or measles virus. Such an approach has been shown to increase surface expression of NKG2D ligands in multiple sarcoma cell lines [205,206]. Additionally, viral proteins, e.g., Ad 2/5 E1A, can also be targeted by NK cells [207]. Virus-infected sarcoma cells were shown to induce increased secretion of perforin, granzyme B, IFN-γ, TNF-α, granulysin, and sFasL in NK cells [205]. Phase II clinical trial (NCT03069378) demonstrated antitumor activity of Talimogene Laherparepvec (T-VEC), a genetically engineered oncolytic herpes simplex virus, combined with anti-PD-L1 mAb pembrolizumab in locally advanced or metastatic sarcomas [208].

As discussed above, tumor cells can develop resistance to NK cell-induced apoptosis. Smac mimetics (SM) are small molecules that, by antagonizing IAP proteins, can compensate for their overexpression. In addition to chemotherapeutic agents, SM-treatment was proven to effectively sensitize sarcoma cell lines toward TRAIL-mediated NK cell killing [47,209]. Additionally, SM's presence during IL-2 priming increases NK cell cytotoxic capacity via activation of the TNF-α–NF-kB axis, prompting to use SM as a novel strategy acting through cancer cell sensitization and NK cell functional enhancement [47].

Regional hyperthermia has shown a benefit in certain clinical trials in soft tissue sarcoma treatment as an addition to chemotherapy [210,211]. It has been shown that increased temperature induces HSP72 expression, consecutively increasing OS, EWS, and chondrosarcoma cells susceptibility to NK cell-mediated lysis [212,213]. Other promising immunomodulatory strategies relying on NK cells include ultraviolet c (UVC) irradiation [214], spironolactone [215], tilorone analogs [216], cytokines [217,218], lenalidomide [186], imatinib [219], and low-level γ irradiation [203], which were all proven effective in preclinical settings.

4.5. Adoptive NK Cell Therapies

Adoptive cell-based immunotherapies are based on transferring auto- or allogenic cells capable of eliciting an effective anticancer immune response. The greatest advantage of adoptive therapies is the ability to combine intrinsic cell properties with function-enhancing gene edits and ex vivo cell activation. Because of the NK cell's crucial role in the control of sarcomas, they are intensively investigated as novel means of treatment. Multiple studies have shown that the adoptively transferred NK cells are able to infiltrate sarcoma lung metastases much more effectively than the primary tumor site and mount a potent immune response against metastases, but not the primary tumor [93–95]. Since lung metastases are a primary cause of death among sarcoma patients, NK cell therapy combined with regional tumor management could be effectively used to treat the patients [36,220,221]. Unsatisfying results of HSCT in sarcoma treatment resulted in further attempts at improving the results by administrating IL-15 activated NK cells after haplo-HSCT for pediatric sarcoma treatment. The therapy was safe and most of the patients responded; however, the results were moderate and short-lasting [222].

Autologous or allogeneic NK cells can be expanded to large numbers using K562-based feeder cells expressing 4-1BB-L and membrane-bound (mb) IL-15 or mbIL-21 [223,224]. To generate a sufficient number of cells, the NK cells are expanded for approximately three weeks, depending on the protocol [225]. The doses of expanded NK cells, characterized by potent antitumor activity, range from 10^6 to 10^7 cells/kg [225]. Although there is evidence of in vivo proliferation of transferred cells, multiple infusions in 2–3-day intervals are often used to obtain maximal antitumor effect [225–227]. Additionally, the expansion process allows for genetic modifications of NK cells, which have always been problematic in primary cells [223,228]. Evaluation of expanded NK cells against ex vivo cancer cells isolated from primary pediatric tumors found EWS and RMS especially sensitive to expanded NK cells [30].

Based on these findings, a clinical trial of expanded haploidentical NK cells in the EWS and RMS patients has been started (NCT02409576). Another group has shown promising results in targeting OS with expanded NK cells [215]. A combination of expanded NK cells and RT has proven to be effective in a first-in-dog clinical trial, showing synergy between RT and expanded NK cells. RT increased sarcomas' susceptibility to NK cell cytotoxicity and improved tumor homing of adoptively transferred cells, providing a rationale for testing such combination in clinical settings [229]. In mice, expanded NK cells have been shown to effectively eradicate EWS lung metastases with no significant effect on the primary tumor, urging for advancements in improving primary tumor homing of adoptive NK cells [93]. Metastatic pediatric solid tumors such as OS, neuroblastoma, and glioblastoma can be potentially targeted with expanded NK cells combined with IL-15 superagonist (N-803) and TIM-3 blockage [230]. Overexpression of DNAM-1 in expanded NK cells also yields promising results, due NK cell cytotoxicity's enhancement proven on a dozen sarcoma cell lines [45]. Multimodal immunotherapy based on K562-4-1BBL-mbIL-15-expanded NK cells and an anti-CXCR4 mAb has shown robust antitumor activity in EWS and RMS models. The synergy between NK cell antimetastatic function and inhibition of prometastatic CXCR4 on sarcoma cells resulted in the elimination of primary tumors as well as micro- and macrometastases in mice [231]. Interestingly, NK cell expansion does not have to be limited to feeder cells. Feeder-free two-stage expanded NK cells combined with RT were shown effective in preclinical RMS models [204].

Unfortunately, adoptive therapies with expanded NK cells are not as safe as initially thought. A clinical trial of MHC-matched, T-cell depleted PB stem cell transplantation with donor-derived K562-4-1BBL-mbIL-15-expanded NK cell infusions in pediatric sarcomas (NCT01287104) reported acute GvHD in five of nine participants, attributed to expanded NK cells [232]. Nevertheless, the majority of adoptive NK cell clinical trials demonstrate a very low incidence of GvHD [233]. There is also evidence for the protective role of NK cells against GvHD, mediated through lysis of alloreactive T cells [234].

Chimeric antigen receptors (CARs) are an effective way of targeting tumor cells. Ganglioside GD2 is a commonly expressed antigen among young sarcoma patients. Its expression is stable and unaffected by recurrences, making an anti-GD2 therapy an excellent second-line treatment alternative [235,236]. A GD2 mAb blocking therapy was shown to inhibit tumor viability by itself and enhanced the proapoptotic effects of cisplatin in OS cells [237]. Expression of CARs directed against the GD2 in activated NK cells has proven to increase NK cells' activity against EWS in an antigen-specific manner [117]. Surprisingly, the adoptive transfer of GD2-specific CAR-NK cells failed to eliminate GD2-expressing EWS xenografts due to CAR-NK-induced upregulation of inhibitory HLA-G1 on tumor cells [117]. HLA-E can also exhibit potent immunosuppressive effects on NKG2A^{+} NK cells, prompting to block NKG2A expression on expanded NK cells to enhance their antitumor properties [116]. Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is another potential target tested in the treatment of solid tumors. It is widely expressed across different sarcoma subtypes, and in vitro studies have proven anti-ROR1 CAR NK cells to effectively induce U2OS cell lysis, accompanied by increased IFN-γ secretion [238,239]. Interestingly, a chimeric NKG2D receptor transduced T cells and NK cells were successfully used in preclinical models of EWS and OS [240–242].

Not only NK cells, but also NK cell line NK-92 is being tested as an immunotherapeutic tool in clinical trials in multiple cancer types. In a phase I clinical trial with sarcoma patients, NK-92 therapy was found safe but ineffective, possibly be due to IL-2 dependency of NK-92 cells and associated poor in vivo persistence [243]. On the other hand, a recently published preclinical study has shown that NKG2D and/or DNAM-1 overexpression in NK-92 cells robustly increased their cytotoxicity towards multiple sarcoma explants [99]. A case report of repeated NK-92 cell intratumoral injections combined with systemic chemotherapy in a relapsed multifocal EWS patient reported a moderate antitumor activity limited to the injection site. The authors attributed the effect to the NK-92 cells, as no response was observed in tumor sites where no cells were administered [244].

Sarcoma's tendency to hematogenous spread and lung colonization poses a great therapeutic challenge. A combination of IL-2 in aerosol and NK cell infusion effectively eradicates lung metastases

in a preclinical murine model. Delivery of IL-2 in aerosol selectively increased lung homing of transferred NK cells and lacked systemic toxicities [245]. Another strategy aiming at eradicating sarcoma lung metastases is based on CXCR2 overexpression in the NK-92 cell line, which increased their homing to OS lung metastases and improved therapeutic effect [246].

Finally, iPSC-derived NK cells (iPSC-NK) represent a promising therapeutic modality for the next-generation NK cell adoptive immunotherapy [247]. iPSCs offer a versatile platform to generate unlimited doses of homogenous NK cell products for downstream evaluation in clinical trials. iPSC-NK cells can be fine-tuned by multiple genetic modifications to achieve more potent effector function, introduce tailored specificities, and promote persistence, and they are therefore attractive candidates for off-the-shelf cancer immunotherapies [25].

5. Conclusions

Sarcomas are malignant tumors with poor prognosis. NK cells have a critical role in controlling every phase of the disease, from the early initiation to the metastatic spread. Hence, the breakdown of NK cell-mediated immunosurveillance unleashes the deadly potential of the disease. Immunoevasion mechanisms of sarcomas include a variety of molecular mechanisms such as MHC class I upregulation, shedding of NK activating ligands, altered oxygen metabolism, and increased expression of inhibitory molecules and cytokines. All of the above-mentioned factors contribute to creating a hostile tumor microenvironment, ultimately leading to NK cell suppression, cancer immunoevasion, and subsequent disease progression. Apart from crucial role of NK cells in sarcoma immunosurveillance, other cell types such as NKT cells have also been shown to control sarcoma growth [38,69,248].

Current therapies have shown a limited capacity for improving the survival of patients with advanced disease. Due to the established role of NK cells in sarcoma development and its intrinsic sensitivity to NK cell lysis, sarcomas are a promising target for therapies utilizing NK cells. Augmenting NK cell anticancer properties can be achieved through various priming strategies and genetic modifications, which improve cancer cell recognition, tumor homing, and resistance to suppressive factors in the TME. Further, sarcoma cells can be sensitized to NK cell-mediated cytotoxicity by monoclonal antibodies, radiotherapy, hyperthermia, HDAC inhibitors, and other treatments. Because of complex multifactorial tumor immunoevasion mechanisms, a combination of both types of strategies is likely needed for a successful treatment outcome.

The challenge remains in translating current basic research into novel therapies for sarcoma. Initial NK cell-based therapies have shown promising results in a subset of patients, encouraging further clinical trials. One key task is to implement new insights into the functional diversification of NK cells in terms of refined strategies to expand specific subsets, induce memory-like NK cells, and harness NKG2A or KIR-driven education. Another utterly important task is the identification of factors predicting response to NK cell therapy in patients. Despite all the recent advances, further basic research and clinical studies have to be performed to deepen our understanding of NK cell function in the context of sarcoma immunosurveillance, allowing for a knowledge-driven design of sarcoma therapies that will fully utilize NK cell antitumor potential.

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5. The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity

Review

The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity

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Simple Summary: Natural killer cells are able to effectively eliminate tumor cells without previous sensitization, therefore the interest in their application in tumor immunotherapy has recently increased. However, tumor cells synergize with tumor-associated cells to create a specific immunosuppressive niche, which limits the activity of NK cells. In this review, we provide a detailed description of molecular mechanisms responsible for NK cells' cytotoxic machinery. Moreover, we shortly characterize the tumor microenvironment and summarize how various metabolic factors within the tumor niche modulate antitumor capacity of NK cells. Moreover, we discuss the potential strategies and implications for the novel antitumor therapies augmenting NK cells functions.

Abstract: NK cells have unique capabilities of recognition and destruction of tumor cells, without the requirement for prior immunization of the host. Maintaining tolerance to healthy cells makes them an attractive therapeutic tool for almost all types of cancer. Unfortunately, metabolic changes associated with malignant transformation and tumor progression lead to immunosuppression within the tumor microenvironment, which in turn limits the efficacy of various immunotherapies. In this review, we provide a brief description of the metabolic changes characteristic for the tumor microenvironment. Both tumor and tumor-associated cells produce and secrete factors that directly or indirectly prevent NK cell cytotoxicity. Here, we depict the molecular mechanisms responsible for the inhibition of immune effector cells by metabolic factors. Finally, we summarize the strategies to enhance NK cell function for the treatment of tumors.

Keywords: NK cell; tumor microenvironment; metabolism

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1. Introduction

Natural killer cells (NK cells) have unique capabilities of tumor cells' recognition and killing in a tightly regulated process shaped by a complex balance between inhibitory and activating signals [1]. This special competence of NK cells is gained in the course of development, maturation, and education in the bone marrow (BM) and secondary lymphoid organs, including lymph nodes [2].

NK cells found in the circulation are primarily divided into two subtypes: $CD3^{-}CD56^{dim}CD16^{+}$ and $CD3^{-}CD56^{bright}CD16^{-}$ cells [3] and they represent around 10% (5–20%) of circulating lymphocytes in humans [4]. $CD56^{bright}$ NK cells have a higher capacity for cytokine production; however, they have relatively low cytotoxicity in comparison to $CD56^{dim}$ cells [5].

NK cells develop from $CD34^{+}$ hematopoietic progenitor cells (HPCs) residing in the BM, through a common lymphoid progenitor (CLP) to NK cells precursors (NKP), which are defined by expression of the IL-2/IL-15 receptor β chain (CD122), thus have the capacity for robust differentiation in response to IL-15 [6,7]. From the NKP stage, NK cells mature and begin to express molecules, such as NK1.1, NKp46, CD94 [8], and LFA-1 [9]. NK cells' development, differentiation, functional maturation and survival is crucially dependent on BM stromal cells' cytokines-induced signaling from the joint γ-chain cytokine receptors (IL-2R, IL-7R, IL-15R) (5) and subsequent activation of signal transducer and activator of transcription 5A/B (STAT5A/B) [10]. The mice with genetic defects in the IL-15/IL-15R system are characterized by the deficiency of NK cells [11], while IL-2–deficient mice have impaired NK cell response [12]. In the next stages of NK cells maturation, functional receptors CD161, CD56, NKG2D, and CD16 are expressed [13], while at the final stages of NK cells development CD56 is downregulated. NK cells are educated through the engagement of their inhibitory killer cell immunoglobulin-like receptors (KIRs) with various MHC class I molecules, resulting in the generation of functional NK cells [14]. Developing NK cells that interact with self-ligands through activating receptors become self-tolerant. After leaving the bone marrow, NK cells need to be activated comprehensively in secondary lymphoid organs to fulfil their antitumor role dependent on the release of cytokines and lytic granules. Naive NK cells acquire effector functions after a priming step—an interaction with dendritic cells (DCs) in draining lymph nodes, which results in their mutual regulation [15]. Trans-presentation of IL-15 by IL-15Rα on DCs stimulates the cytotoxic activity of NK cells and their ability to produce interferon-γ (IFN-γ) [16]. On the other side, NK cells activated by tumor cells modulate the adaptive immune response by inducing DCs' maturation and activation. By killing tumor cells and releasing tumor antigens, NK cells stimulate tumor antigens cross-presentation by DCs in MHC class I context [17]. They also prime DCs to produce IL-12, thereby regulating activity and differentiation of functional T helper 1 cells (Th1), which in turn produce IL-21 involved in reciprocal stimulation of NK cells. NK cells can also directly influence T cells activity. CXCR3-dependent migration of NK cells to the lymph node stimulates, IFN-γ production, which successively promotes Th1 polarization [18]. Previous reports have demonstrated that IL-2, IL-12, IL-15, IL-18, and IL-21 play a significant role in NK cells activation and proliferation [19] via the stimulation of JAK/STAT signaling. STAT1 is a crucial regulator of IFN-γ production [20] and is also involved in cell junction formation at the NK cell-lytic synapse [21]. On the other hand, STAT3 has been described as a negative regulator of the NK cells cytotoxicity. The absence of STAT3 correlates with increased levels of perforin, granzyme B, as well as with the higher expression of activating receptor DNAM-1 [22]. STAT5 has been reported to be a master regulator of human and mouse NK cells' activity. STAT5 inhibition in NK cells has been associated with tumor progression in vivo [10,23]. Specifically, IL-2 upon binding to its receptor [24], enhances NK cell response toward cancer cells, through the activation of the JAK-STAT5 signaling pathway. Likewise, NK cells stimulated by IL-15 rapidly increase the granzymes and perforin production, which is strictly regulated by the STAT5A/B activation [23,25] and PI3K-AKT-mTOR pathway [26,27]. It is worth noting that a combination of cytokines can induce synergistic effects on NK cell's effector functions. It has been reported that IL-12 increases IFN-γ production in IL-15 stimulated NK cells [19,25] and together with IL-2 and IL-15 induces activation of STAT4 and STAT5 transcription factors. Also, direct binding of STAT4 to the perforin promoter has been described in IL-12 activated NK cells [28], IL-21 synergizes with IL-2 to augment the expression of NKG2A, CD25, CD86, CD69, and production of perforin and granzyme B [29].

2. Biological Aspects of NK Cell Cytotoxicity

2.1. NK Cells' Metabolism

NK cells utilize glucose to fuel the biosynthesis of amino acids and fatty acids. Resting NK cells are characterized by relatively low rates of glycolysis and oxidative phosphorylation (OXPHOS), which then increases following the stimulation with IL-2, IL-12, or IL-15. Activated NK cells undergo metabolic reprogramming, leading to an increase in glucose uptake through glycolysis, which is supported by increased expression of nutrient receptors such as GLUT1, CD98, and CD71 [30]. As evidenced by the recent report, CD56^{bright} cells are more metabolically active than CD56^{dim} counterparts [31]. Furthermore, CD56^{bright} NK cells, due to their higher metabolic activity, can produce more IFN-γ during an immune response. The mammalian target of rapamycin complex 1 (mTORC1) is critical or NK cells development and maturation [32]. Although, the exact mechanism by which mTORC1 controls NK cells metabolism remains to be elusive. It has been reported that CD56^{bright} NK cells more robustly upregulate the activity of mTORC1 and increase glucose uptake and glycolysis upon cytokine stimulation [31]. Recently, it has been shown that cytokine-induced metabolic reprogramming of NK cells depends on the activity of two transcriptional factors: sterol regulatory element-binding protein (SREBP) and cMYC. SREBP controls elevated metabolism of glucose to cytosolic citrate in the cytokine-stimulated NK cells [33]. In the cytosol, citrate can be cleaved by ATP- citrate lyase (ACLY) to produce acetyl-CoA. SREBP activity, by controlling the expression of SLC25A1 (mitochondrial citrate transporter) and ACLY is essential for increased rates of glucose metabolism and [34]. Thus in NK cells, SREBP is fundamental for the modulation of glycolysis as well as OXPHOS via, regulation of the metabolic switch to citrate-malate-shuttle to fuel OXPHOS [34]. Therefore, SREBP inhibition may result in a reduction of NK cells growth, proliferation, and cytotoxicity against cancer cells by reduction of IFN-γ and granzyme B production [34]. mTOR has been described to have an essential role in promoting glycolytic metabolism in activated NK cells. mTORC1 activity is required for the initial increase in cMYC levels together with IL-2/IL-12 cytokine stimulation [34]. cMYC promotes OXPHOS and glycolysis by the upregulation of the glycolytic enzymes and glucose transporters [35,36]. Moreover, upregulation of cMYC in cytokine-stimulated NK cells heavily depends on the availability of the amino acids, especially glutamine [34,37]. Although glutamine is not an important nutrient for NK cells, cMYC protein expression has been reported to be sensitive to levels of glutamine. cMYC is also regulated by glycogen synthase kinase 3 (GSK-3), which has been shown to target cMYC for proteasomal degradation [36].

2.2. NK Cells Recruitment to the Tumor Site

Although the NK cell infiltrates have been identified in several cancers (e.g., melanomas [38,39] and breast cancers [40,41]), the precise mechanisms underlying trafficking of NK cells to the tumor microenvironment (TME) are yet to be discovered. Noteworthy, the infiltration of tumor sites by highly cytotoxic CD56^{dim} NK cells is often insufficient. Instead, cancer cells produce chemokines that stimulate the influx of less cytotoxic CD56^{bright} NK cells, whereas CD56^{dim} NK cells usually express receptors for chemokines produced at low levels within the tumor site [42,43]. All NK cells receptor chemokines are shown in Table 1, together with their respective ligands present in the tumor microenvironment or lymph node. Moreover, following cytokine stimulation NK cells modify the expression of chemokines and chemokine receptors. Therefore, the subsets composition and the tumor homing of the NK cell infiltrates can vary, depending on cytokines and chemokines present in the tumor microenvironment [44,45]. For example, IL-15 stimulates migration of CD56^{bright} NK cells to the tumor site by increasing the expression of CCR5, while inhibiting trafficking of CD56^{dim} counterparts by decreasing CXCR4 and CX3CR1 expression [46]. IL-2 upregulates chemokine receptors such as CCR1 and CX3CR1, and thus stimulates NK cells migration to the tumor site [47]. Also, it has been shown that IFN-γ stimulates tumor-infiltrating immune cells to release CXCL9-11, chemokines known to recruit CD56^{bright} NK cells [48]. Interestingly, a scarce NK cells infiltration has also been demonstrated

in colorectal cancers despite high levels of NK cell-attracting chemokines within the tumor. Such findings suggest the presence of immune escape mechanisms that impair NK cell migration to the tumor site or decrease their viability within the tumor [49].

Table 1. Summary of chemokine receptors expressed by NK cells subpopulations and their respective ligands expressed by tumor or lymph nodes

Source of Chemokines	Chemokines	Chemokine Receptor	Chemokine Receptor Expression on the NK Cell Population	
			NK^{bright}	NK^{dim}
Tumor	CCL3, CCL5, CCL7, CCL9, CCL14-16, CCL23	CCR1	+	–
	CXCL1-3, CXCL5-8	CXCR2	–	++
	CXCL9-11	CXCR3	++	–
	CXCL12	CXCR4	+	++
	CXCL8	CXCR1	–	++
	CX3CL1	CX3CR1	–	++
	CCL3, CCL4	CCR5	++	+
Lymph nodes	CCL19, CCL21	CCR7	++	–

++ strong expression; + weak expression; – no expression.

2.3. Formation of the Lytic NK-Cell Immunological Synapse

Following NK cells recruitment to the tumor site, the induction of NK cell effector functions requires direct NK cell contact with the tumor cell and formation of the lytic synapse. The formation of NK-cell lytic synapse ensures precise delivery and secretion of cytolytic effector molecules, leading to direct tumor cells death.

2.3.1. Recognition Stage

The formation of a mature and functional NK-cell lytic synapse is a complex process and can be divided into recognition, effector and termination stages. Recognition stage involves lytic synapse formation, which depends on the adherence of the NK cell to target cells. A variety of different molecules participate in lytic synapse formation, which includes NK cell receptors, intracellular signaling molecules, cytoskeletal elements and cellular organelles. A firm adhesion between two cells is established by interactions of high-affinity adhesion molecules, in particular, the NK cells express the intercellular adhesion molecules (ICAM) receptors: LFA1 (CD11a/CD18) [50–53]. After the initial contact of NK cells with the tumor cells, LFA-1 appears to initiate the process of synapse formation. Activation of NK cells cytotoxic activity is controlled by signals integrated from activating and inhibitory receptors (Figure 1) [54–56]. Among activating receptors, NKp30 and NKp46 are expressed on both resting and activated NK cells, whereas NKp44 is upregulated only upon NK cell activation [57,58]. Moreover, NK cells have the Fc receptor Fcγ RIIIa (CD16), which recognizes the Fc proportion of antibodies and triggers NK cell activation in a process termed antibody-dependent cell-mediated cytotoxicity [54]. Another important NK cell-activating receptors are DNAX accessory molecule-1 (DNAM-1) and NKG2D. Most of activating receptors of NK cells signal through the phosphorylation of the key Tyr residues in the cytosolic Tyr-based motifs (ITAMs) motifs. The phosphorylated ITAMs recruit the Tyr kinases ZAP-70 and Syk, which, in turn, phosphorylate transmembrane adaptor proteins, leading to recruitment of several signaling molecules, including the phosphoinositide 3-kinase (PI3K). On the other hand, inhibitory receptors such as KIRs, and C-type lectin inhibitory receptor CD94/NKG2A complex [54,55], inhibit NK cells activity. KIRs acts through immunoreceptor

Tyr-based inhibitory motif (ITIM), which recruit the Tyr phosphatase SHP-1 or SHP-2. Several reports have also reported in NK cells the presence of non-canonical immune checkpoints receptors, such as PD-1 [59], cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [60], T-cell immunoglobulin and mucin-containing domain (TIM-3) [61], T cell immunoglobulin and ITIM domain (TIGIT) [62], CD96 [63], and lymphocyte activation gene 3 (LAG-3) [64]. PD-1 is highly expressed on activated T cells, but its presence was also reported in NK cells, mainly in CD56^{dim} NK cells subset [65]. PD-1 directly participates in NK cell exhaustion, limiting their cytotoxic activity and cytokines production [59]. Influence of CTLA-4 on the NK cells dysfunction remains very poorly understood. CTLA-4 might be involved in the inhibition of IFN-γ production by NK cells induced by DCs [60]. Additionally, it has been shown that IL-2-driven NK cells activation triggers CTLA-4 upregulation [60]. The role of TIM-3 in NK cells cytotoxicity is unclear, as both activating and inhibitory properties were described. On the one hand, TIM-3 blockade reduces NK cells cytotoxicity [66]; whereas, on the other side, high TIM-3 expression determines the subset of exhausted NK cells [67]. TIGIT and CD96 compete with activating receptor DNAM-1 for binding to CD112 and CD155 [68]; also both of them contain ITIM motifs in their cytoplasmic fragments, through which they deliver inhibitory signals to the target cell [69]. TIGIT is mainly upregulated on T cells, however higher expression of TIGIT has also been found on NK cells in intratumoral regions in colon cancer. Furthermore, blockade of TIGIT prevents NK cell exhaustion and promotes their cytotoxicity [70]. Moreover, LAG-3 expression has been observed only on the activated NK cells [71]. LAG-3 has been reported to be a NK cell negative regulator [69]; however, the direct impact of LAG-3 on NK cells function remains elusive. More study is needed, as currently only one group showed that inhibition of LAG-3 increases production of INF-γ, TNF-α, CCL3, and CCL4 [72].

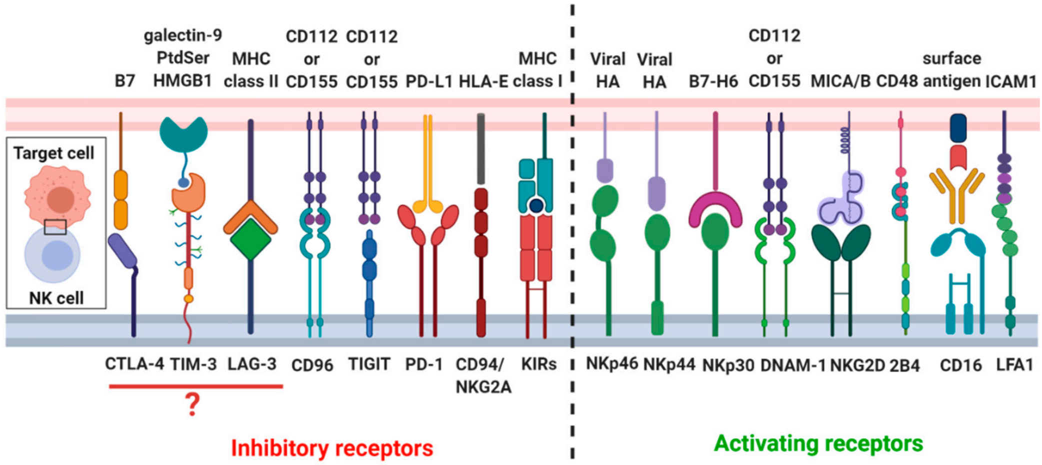

Figure 1. Overview of the NK cell receptors and their respective ligands. NK cells' cytotoxicity is tightly regulated through the complex balance between inhibitory and activating signals originating from different NK cell receptors.

2.3.2. Effector Stage

At the effector stage, throughout synapse maturation, filamentous actin (F-actin) and adhesion receptors form a ring in the peripheral supramolecular activation cluster (pSMAC) zone, which is responsible for lytic granule secretion [53,73,74]. In the next stage, the lytic granules of NK cells dock onto the microtubule-organizing center (MTOC) in a process termed granule convergence. Then, the MTOC, along with the docked lytic granules, is polarized towards the NK-target cell synapses [52,75,76] by dynein–dynactin motor complex followed by docking of secretory lysosomes with the plasma membrane. The interaction between two cellular membranes, NK and target cell, is catalyzed by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Degranulation of NK cells is not only associated with pronounced cytoskeletal rearrangements but also is accompanied by mobilization of intracellular calcium and alterations of intracellular pH [77,78].

Stable NK-cell-target-cell conjugate enables secretion of preformed lytic granules, which are armed with perforin, serine proteases termed granzymes, and cathepsins [53,79]. An increase in pH and calcium concentrations triggers perforin release, its polymerization, as well as its subsequent binding and insertion into the target cells membrane, while granzymes induce target cells' apoptosis [80]. Perforin is synthesized as premature, non-active form, which undergoes a multistep cleavage in acidic secretory lysosomes, leading to the formation of active perforin, which easily incorporates into target cell's plasma membrane [81]. Granzymes A and B are the most abundant granzymes in NK cells. Similarly to perforin, in secretory lysosomes, granzymes are bind to the proteoglycans in the mechanism dependent on low pH. When the propeptides are removed, the inactive progranzymes become active proteases [82]. Human NK cells also produce high amounts of cathepsins, lysosomal peptidases involved in the regulation of effector stage of NK cells' cytotoxicity by the processing of perforin, and granzymes.

2.3.3. Termination Stage

The cleft that is formed at the lytic synapse between the NK cell and the target cell creates a protected pocket, which probably remains intact during a period of relative inactivity after the release of granules [83,84]. In this way, the concentration of the lytic effector molecules that are delivered to the target cell can increase while protecting neighboring cells from exposure to the cytotoxic molecules. At the termination stage, activating receptors previously recruited to the synapse, such as NKG2D and CD16, are downregulated [53]. Moreover, membrane flipping of the target cell-induced by perforin results in phosphatidylserine exposure on its external surface [80], which is recognized by ITIM-containing molecule CD300a [85] and provides a signal to terminate NK response. Once the NK cell has carried out its cytolytic function, it detaches from the target cell and restores its ability to kill another susceptible cell by generating new lytic granules and re-expressing activating receptors. The termination stage was shown to play a critical role in the process defined as 'serial killing'. Termination stage is compulsory for NK cells to reinitiate a subsequent recognition and killing of a target cell [86].

2.4. NK Cells' Cytokine Production

Although perforin/granzymes-mediated cytotoxicity is the most effective way of killing tumor cells, NK cells are now also known to promote slower receptor-mediated apoptosis and to produce cytokines. By secretion of IFN-γ and TNF-α, which activate resident inflammatory cells and recruit other cytotoxic immune cells, NK cells induce tumor cell death [87]. Production of IFN-γ by NK cells can be influenced by a cell to cell contact or by stimulation with IL-2, IL-12, or IL-15. It has been demonstrated that cytokine secretion by NK cells occurs independently from cytolytic granules release. In contrast to the release of cytolytic granules into the synaptic space, IFN-γ and TNF-α are delivered through multiple sites on the NK cells surface in a largely non-polarized mode [88]. The separation of these two effector pathways is an important mechanism allowing NK cells to simultaneously kill target cells and recruit other immune cells in antitumor response.

3. Characteristics of the Tumor Microenvironment

Tumor mass consists not only of malignant cells by also of stromal cells, infiltrating immune cells, blood vessels and a variety of extracellular factors, which constantly interact and shape tumor microenvironment (TME) during all stages of cancer development and progression [89]. Although the exact role of stromal cells in cancer development is context and cancer type-dependent in general, the overall function of stromal cells is beneficial for cancer cell survival and metastasis [90]. Within the tumor microenvironment, there are many types of stromal cells; however, the three major stromal cells populations are; cancer-associated fibroblasts (CAFs) [91], mesenchymal stem cells (MSCs) [92], and tumor associated macrophages (TAMs) [93]. Stromal cells together with cancer cells secret multiple factors to the extracellular space (Figure 2), which may have the immunosuppressive effect on the

immune effector cells, thus limiting the efficacy of various immunotherapies, including NK cell-based therapeutic modalities. Nevertheless, it is worth mentioning that some of the systemic disorders, including obesity [94] and diabetes [95], may also influence tumor microenvironment. For example, during weight gain, adipocyte accumulates more lipids and undergo cellular hypertrophy and die [96]. This phenomenon results in increased concentration of numerous cytokines, such as IL-6, IFN-γ, and TNF-α, within the tumor microenvironment and subsequent cancer progression [95]. Therefore, in this part of the review, we summarize factors present in the tumor environment, which may shape tumor metabolism, development, and progression.

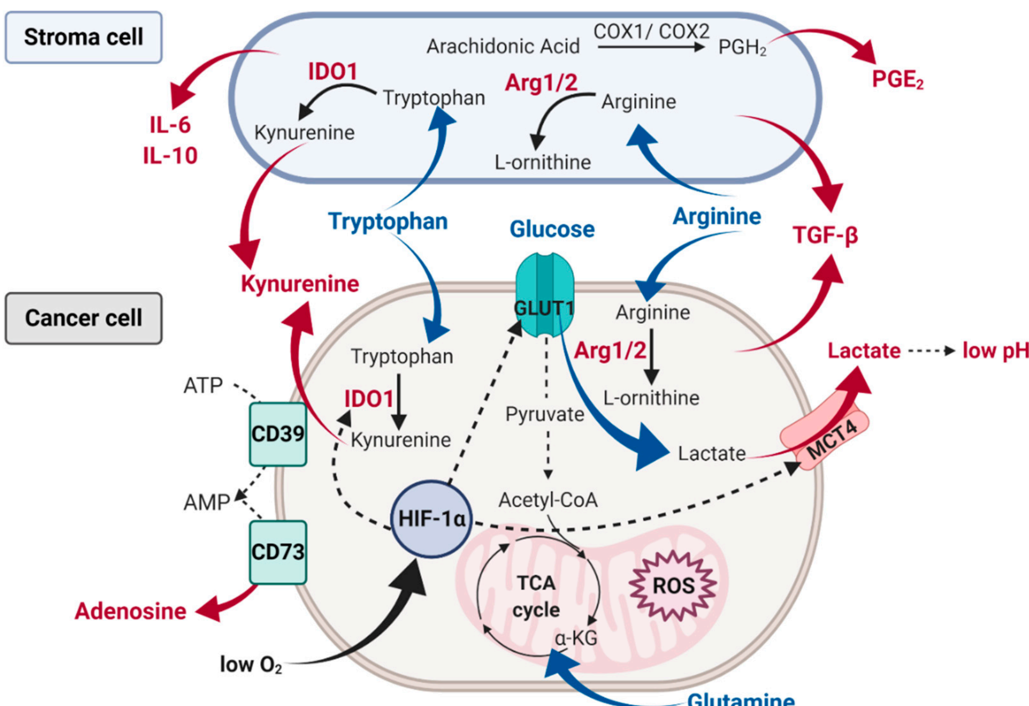

Figure 2. A schematic characteristic of the tumor microenvironment metabolites and other factors impacting NK cell effector function. Within the tumor microenvironment cancer cells consume large amounts of glucose and produce lactate and subsequent extracellular acidosis. Glycolytic conversion of glucose into pyruvate also stimulates the production of ROS. Tumor cells, as well as stromal cells, compete for nutrients such as glucose, glutamine, and amino acids. Thus, cancer cells together with stroma cells increase amino acids consumption and upregulate key amino acid metabolism enzymes, such as IDO and Arg1/2, leading to the accumulation of amino acids' immunosuppressive metabolites, such as kynurenine. Tumor cells also generate extracellular adenosine through the ectonucleotidases CD39 and CD73. Moreover, high oxygen consumption by tumor cells can cause hypoxic conditions, which sustains HIF-1α, which in turn promotes glycolytic metabolism by upregulation of GLUT1 and lactate production by modulation of lactate transporters expression. Moreover, tumor and tumor-associated cells secrete factors, which prevent NK cell activation, such as TGF-β, IL-6, IL-10, and PGE_2.

3.1. Tumor Hypoxia and Acidosis

In the majority of solid tumors, there are regions permanently or transiently subjected to the abnormally low level of oxygen. Environmental hypoxia results from inadequacies between the tumor microcirculation and the oxygen demands of the growing tumor mass. A central role in cellular adaptation to lower oxygen partial pressure is played by a hypoxia-inducible family of transcription factors (HIFs), comprising three members HIF-1, -2, and -3. Mechanistically, hypoxic conditions prevent HIF-1α from degradation by impairing its hydroxylation by prolyl hydroxylase-domain

enzymes (PHDs) and subsequent polyubiquitination by von Hippel Lindau (VHL) tumor suppressor protein. Activation of HIF-1α leads to its accumulation, dimerization with HIF-β (aryl hydrocarbon receptor nuclear translocator) and subsequent nuclear translocation. In the nucleus, HIF-1 binds to the core DNA-binding sequence hypoxia response element (HRE) in the promoter regions and activates transcription of target genes. Among others, HIF-1 activation causes the upregulation of glucose transporters (GLUTs) and induces the transcription of glycolytic enzymes, particularly hexokinase, pyruvate dehydrogenase (PDH), and lactate dehydrogenase A (LDHA) [97]. This ultimately results in the ability of tumor cells to upregulate aerobic glycolysis, known as the Warburg effect. Glycolysis generates several intermediate products such as pyruvate, which can be transported to the mitochondria and used in the tricarboxylic acid (TCA) cycle. A large proportion of pyruvate is also converted in tumor cells to lactate by LDHA [98].

Moreover, a hypoxic TME is associated with a metabolic switch toward glycolysis and subsequent extracellular acidosis (pH < 6.8) and high (up to 40 mM) extracellular lactate (Figure 2). Under normal conditions, adenosine triphosphate (ATP) is generated from glucose slowly and efficiently by OXPHOS. However, tumor cells upregulate glucose uptake and lactate fermentation to sustain their increased metabolic demands [99]. Noteworthy, glycolysis produces ATP faster yet less efficiently than OXPHOS, therefore forcing tumor cells to consume much more glucose to maintain their high proliferative status [100]. Extremely low glucose, increased lactate and glycolytic intermediates concentrations are found in the tumor microenvironment (TME) in tumors of various origin [101]. When intracellular levels of lactate become too high, the proton-linked monocarboxylate transporters (MCT) pump lactate and protons outside the cell [102], leading to the further acidification of the tumor microenvironment. Likewise, when lactate is exported into the circulation, both local and distant tissues can utilize it as a fuel source. MCT are key players in this process: low-affinity lactate transporter MCT4 remove lactate from tumor cells and high-affinity transporter MCT1 ensures lactate uptake [103].

3.2. Oxidative Stress

Glycolytic conversion of glucose into pyruvate may stimulate reactive oxygen species (ROS) production in a mechanism dependent on mitochondrial membrane hyperpolarization [104,105]. Noteworthy, ROS regulate multiple cellular functions and can act as second messengers. For example, H_2O_2 modulates activation of signaling cascades of several growth factors and it can either induce cell death or proliferation depending on the dose [106]. Other active radicals, including nitric oxide (NO) belonging to reactive nitrogen species, owing to their high chemical activity, interact with multiple target molecules and play a pleiotropic role in cancerogenesis (summarized elsewhere [107]). Mitochondria-associated ROS are generated by electron transport chain, different isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and other enzymes including xanthine oxidase, lipoxygenase, or cyclooxygenase, as well as cytochrome P450 [108]. Under normal conditions, cells maintain a tight balance between ROS production and scavenging by cellular antioxidant enzymes [109]. Increase of glucose uptake by cancer cells is frequently observed under oxidative stress, a condition characterized by the imbalance between the generation of ROS and the antioxidant defense mechanisms [110]. Oxidative stress activates AMP-activated protein kinase (AMPK), which is meant to promote glucose-sparing oxidative metabolism, rather than aerobic glycolysis [111]. AMPK in turn activates pyruvate dehydrogenase complex (PDHc), a rate-limiting enzyme directly controlling pyruvate influx to the TCA cycle, which maintains TCA cycle and supports cancer metastasis [112]. Moreover, AMPK activation may occur under metabolic stress, such as glucose deprivation. AMPK increases cellular levels of NADPH, which subsequently neutralizes cellular ROS levels via NADPH-dependent synthesis of glutathione (GSH) and thus promotes tumor cell survival [113]. What is more, during hypoxia ROS levels increase and lead to the HIF-1α stabilization through PHDs inhibition [114–116]. In addition, HIF-1α can also be stabilized by phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK p38) activated by hypoxia-derived ROS [117] and TGF-β [118]. Furthermore, TGF-β increases the production of ROS by impairing

mitochondrial function and inducing NOXs activity. TGF-β also suppresses antioxidant systems, leading to oxidative imbalance [119].

3.3. Cytokines

Many cell types within tumor mass are described to contribute to the generation of an immunosuppressive tumor microenvironment by secretion of various cytokines. Accumulated in TME ROS participate in the regulation of downstream TGF-β signal transduction which involves SMADs, MAPKs, and NF-κB. In human cancer, TGF-β acts both as a tumor suppressor and as a promoter of tumor growth. The tumor suppressive effect includes inhibition of cell proliferation and induction of cancer cell apoptosis at the early stage of cancerogenesis [120]. On the other hand, tumor promoting effect includes induction of epithelial-to-mesenchymal transition (EMT), migration, and metastasis observed in aggressive and invasive tumors. In addition to cancer cells, a substantial source of TGF-β in the TME are TAMs [104,105,108] and neutrophils (TANs) [109], regulatory T cells (Tregs) [110], as well as myeloid-derived suppressor cells (MDSCs) [121,122]. These cells also secrete other cytokines, such as IL-6 [123] and IL-10 (Figure 2) [124]. Binding IL-6 and IL-10 to its receptors activate STAT3 tyrosine phosphorylation and subsequent transcription of target genes that support the tumorigenesis and maintain immunosuppression through MDSCs and TAMs [125]. Cancer cells can also produce IL-6 acting in an autocrine manner, in this way they do not depend on the paracrine release of IL-6 by stromal cells. Within the tumor microenvironment, various molecules support cancer cell growth and aggressive phenotype. However, it has been also shown that stressful and oncogenic stimuli, including cytotoxic agents and ionizing radiation, can induce senescence in cancer cells [126]. Unlike apoptotic cells, senescent cells are viable and secrete a wide array of immunomodulatory factors, including cytokines, growth factors and metalloproteinases, a phenomenon collectively termed as senescent associated secretory phenotype (SASP) [127]. Although NK cells are involved in the clearance of senescent cells [128], SASP can contribute to their inhibition in a mechanism involving metalloproteinase-mediated shedding of MICA and MICB, thus preventing activation of NKG2D receptors.

3.4. Amino Acid Deprivation

Close metabolic requirements of both immune and cancer cells for amino acids, like tryptophan (Trp), arginine (Arg), and glutamine (Gln) lead to the metabolic competition between them, resulting in amino acid depletion within the tumor niche [129]. Furthermore, under hypoxia glutamine consumption in cancer cells is elevated, which provides energy for cell survival. Glutamine promotes the TCA cycle and ATP production by being converted into α-ketoglutarate [130]. Importantly, glutaminolysis products activate mTORC1, hence promoting cell proliferation [131]. Amino acids can also help to counteract the negative influence of ROS within TME. For example, cancer cells depend on the glutamine for the synthesis of glutathione, which acts as an essential antioxidant in the cancer cells and maintains the redox homeostasis in the tumor niche [132].

On the contrary, glutamine and tryptophan deprivation leads to decreased mTOR activity resulting in inhibition of cancer cells growth. Furthermore, depletion of tryptophan, which is an essential amino acid for T-cell proliferation, depends on the indoleamine-2,3-dioxygenase (IDO) activity. IDO converts tryptophan into kynurenines (Kyn), which inhibit T and NK cells functions. IDO expression can be stimulated by various cytokines, including TNF-α, TGF-β, IL-6, and IFN-ɣ. In many cancer models, IL-6 was noted to modulate IDO expression through STAT3 phosphorylation [133]. What is more, IFN-ɣ can also promote IDO expression in DCs and MDSCs via STAT1 and STAT3 activity [134]. Nevertheless, tumoral expression of IDO can be inhibited by hypoxia and nitric oxide (NO) [135,136]. Although the low activity of IDO during hypoxia promotes the activation of immune cells [137], hypoxic conditions can also augment the secretion of IFN-ɣ, which in turn upregulates IDO mRNA expression [138]. Noteworthy, immunosuppressive properties of IDO can be further potentiated by PGE_2, an arachidonic acid metabolite [139]. Other immunoregulatory enzymes within TME include arginase 1 (Arg1) and

arginase 2 (Arg2), which catalyze the degradation of arginine. Both enzymes hydrolyze arginine into urea and L-ornithine, the main substrates for the production of polyamines required for cell cycle progression. Quantification of the murine tumor interstitial fluid metabolites revealed that arginine is one of the strongly depleted amino acids within the tumor microenvironment [140]. Noteworthy, there are several plausible explanations for this phenomenon. First of all, tumor-associated stromal cells seem to be the primary source of arginase within the tumor microenvironment [141,142]. They also express cationic amino acid transporter 2B (CAT2B), the transporter responsible for the rapid influx of Arg into the tumor-associated myeloid cells responsible for the depletion of extracellular arginine [143]. On the other hand, arginine may also be depleted by cancer cells [144–146], which overexpress Arg1 to sustain their rapid proliferation dependent on polyamine production. Moreover, some studies suggest that high Arg1 expression in TAMs is associated with enhanced tumor proliferation [147]. Arginine can also be metabolized by inducible nitric oxide synthase (NOS) to produce citrulline and NO, which are essential factors in tumor vascularization [143]. Reduced NO formation caused by arginine depletion may enhance ROS generation, which can further inhibit the activity of immune effector cells [148].

3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism

Arachidonic acid (AA) belongs to polyunsaturated fatty acids and is converted to various lipid-derived immune-mediators, including prostaglandins. The product of AA, prostaglandin H2 (PGH2) serves as the substrate for the isomerization to PGE_2 [149]. The conversion is carried out by COX-1 and COX-2 enzymes. COX-1 is believed to be constitutively expressed in all tissues with the potential to induce an acute inflammatory response, whereas COX-2 is induced upon malignant transformation. Interestingly, H_2O_2 has been shown to induce COX-2 expression in a mechanism dependent on the inactivation of protein phosphatases activity and subsequent increased protein tyrosine kinases phosphorylation [150]. Prostaglandins and prostacyclins have a documented role in the modulation of the immune response. Secretion of PGE_2 was confirmed in many cancer types and was associated with tumor progression and metastasis [151,152]. Within the tumor microenvironment, MDSC were also identified as the primary source of PGE_2 and IDO1 (Figure 2) [153].

Adenosine (ADO), purine nucleoside, present at immunosuppressive concentrations within the solid tumor microenvironment, may also play a key role in immune evasion [154]. Two ectoenzymes-CD39 that hydrolyses ATP to AMP and CD73 that dephosphorylates AMP to adenosine constitute the main source of ADO in TME. Adenosine kinase is a cytosolic enzyme which controls ADO levels. Inhibition of adenosine kinase can effectively increase ADO extracellular concentrations. What is more, oxygen deprivation can also increase extracellular concentrations of ADO in tumor niche, since HIF-1 activation is responsible for the increased expression of CD73 [155,156].

4. How Tumor Microenvironment Factors Inhibit NK Cells

As previously described, induction of the NK cells' cytotoxic activity involves several distinct stages, starting from the initiation of contact with a target cell to the directed delivery of lytic granules to the target cell. In this part of the review, we discuss how the tumor microenvironment could alter the balance between activating and inhibitory signals of NK cells, and also how it could shape NK cells' priming and metabolism, which are essential to display full effector functions. We also review the effect of the TME factors on NK cells' migration to the tumor site, degranulation, and expression of lytic granules' enzymes.

4.1. NK Cells' Metabolism

Activated NK cells upregulate glycolysis and OXPHOS in order to facilitate their cytotoxic function [157]. The primary regulators of the NK cell' metabolism are mTOR, cMYC and SREBP (Figure 3A), which activity strongly depends on the nutrient availability within the tumor niche. Accelerated glucose metabolism in tumor cells is among others related to the reduced glucose availability, which represents a considerable obstacle for NK cells cytotoxic activity. When glucose

levels are low, mTORC1 is inhibited, in turn leading to the repression of numerous anabolic processes. Impairment of mTORC1-maintained glycolysis in NK cells diminishes their cytotoxicity by inhibition of IFN-γ production and granzyme B expression [158,159]. Moreover, since cancer and immune cells compete for glucose, it seems that reduced glucose availability within the tumor microenvironment may represent one of the cancer strategies to suppress immune effector cells. For instance, Cascone et al. showed decreased T cell infiltration in tumors with high glycolytic rates [160,161]. However, whether glucose deprivation inhibits NK cells infiltration remains still an open question. Increased lactate uptake by NK cells can lead to their intracellular acidification, as evidenced by the ATP drop, indicating impaired NK cells' energy potential [162]. Additionally, Harmon et al. have also shown that liver-resident NK cells treated with tumor conditioned medium (TCM) underwent apoptosis, which was associated with elevated lactate concentration within TCM [162]. Moreover, it has been demonstrated that lowering pH from 6.8 to 6.0 results in a significant decrease in NK cell activity [163]. It has been noticed that lactate accumulation and acidification of the extracellular environment cause dysfunction of NK cells by interfering with mTOR signaling, as shown in Figure 3B [159]. Also, it has been shown that lactate pretreatment inhibits the cytotoxic function of both human and mouse NK cells. Intracellular lactate decreases the intracellular pH and reduces ATP generation (Figure 3B) [164], which may cause ROS accumulation and mitochondrial stress with subsequent apoptosis of liver- resident NK cells [162]. It is worth noting that it has been shown that reduced lactate production by tumor cells results in slower tumor growth [165,166]. Likewise, a lactic acid concentration above 20 mM was shown to induce NK cells apoptosis, which might explain a smaller proportion of NK cells in tumors with a higher concentration of lactate, such as melanoma [162,167]. Moreover, under hypoxia, glutamine levels within TME are decreased due to elevated consumption by cancer cells. It has been noticed that glutamine withdrawal or SLC7S5 blockade results in the rapid loss of cMYC protein level and impaired NK cells cytotoxic response [34]. In the absence of cMYC, activated NK cells produced fewer IFN-γ and had reduced granzyme B expression [34]. Likewise, Cong et al. noticed that NK cells at later tumor stages were found in lower numbers and that loss of the antitumor effect of NK cells was closely associated with tumor progression [168]. Secondly, their transcriptome analysis of tumor-associated NK cells showed a strong upregulation of fructose- 1,6- bisphosphatase (FBP1) expression, a rate-limiting enzyme in gluconeogenesis (Figure 3B). Cong et al. showed that tumor-associated NK cells dysfunction and lower viability was strongly related to FBP1- mediated inhibition of NK cells glycolysis [168]. Additionally, short term hypoxia acts synergistically with IL-15 priming to induce the upregulation of genes involved in the glycolytic pathway [169]. However, the role of HIF-1α in NK cells effector functions and its effect on glycolytic activity remains unclear. In addition, the hypoxia-driven activity of CD73 impairs NK cells metabolism by generation of the highly immunosuppressive metabolite adenosine. ADO suppresses NK cells metabolic activity by inhibition of mitochondrial respiration and their proliferation through the mTOR pathway (Figure 3B) [170]. Likewise, besides cancer cells, NK cells themselves also can synthesize and secrete ADO within the tumor microenvironment. CD56^{bright} NK cells produce ADO via CD38 and CD203a, thus regulating other lymphocytes, while CD56^{dim} cells express lower levels of CD39 and CD73 [171]. Also, within the tumor microenvironment, amino acids deprivation may contribute to evasion of the anticancer immune response. For example, leucine depletion in tumor media was shown to inhibit mTORC1-dependent NK cell stimulation [172].

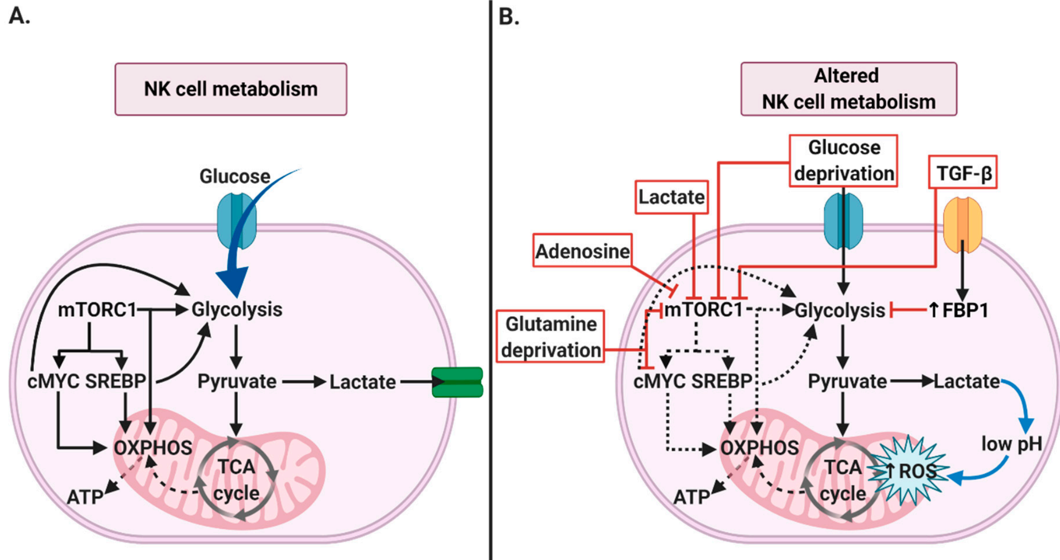

Figure 3. NK cell metabolism. (**A**) Key regulators of NK cells metabolism. Activated NK cells are characterized by increased glucose uptake and OXPHOS. mTORC1 is the key factor, controlling NK cell metabolism by upregulation of NK cells' glycolysis and OXPHOS. mTORC1 is also involved in activation of cMYC and SREBP, which may further modulate glycolysis and OXPHOS. (**B**) Mechanisms disrupting NK cell metabolism in cancer. Many factors within the tumor microenvironment, can directly impact rates of glycolysis and OXPHOS by interfering with mTORC1, cMYC, or FBP1 activity. Moreover, mitochondrial dysfunction through ROS accumulation can be induced by intracellular lactate accumulation.

4.2. NK Cells Recruitment to the Tumor Site

According to literature data, the process of NK cells adhesion to endothelial cells in hypoxic conditions remains unchanged. However, in murine models, hypoxic mammary tumor cells support metastatic growth by secretion of cytokines and growth factors, which attract a specific subset of myeloid cells (CD11b+/Ly6Cmed/Ly6G+), as well as NK cells with reduced cytotoxicity [173]. Very recently, HIF-1α deficiency has been linked to reduced tumor growth, which was associated with non-productive angiogenesis and increased NK cell activity [174,175]. It has also been demonstrated that hypoxic conditions differently influences chemotactic responses of two functionally distinct human NK cell subsets in vitro. In tumors, CXCR4/CXCR7/CXCL12 pathway is involved in the complex scenario of tumor progression, including invasion, chemotaxis, and angiogenesis [174,176]. CXCL12 (a ligand for both CXCR4/CXCR7) promoter contains two HIF-1α binding sites. CXCL12, significantly upregulated in the tumor microenvironment, increases adhesion, migration, and homing of CXCR4-positive progenitor cells to ischemic tissues requiring regeneration and neovascularisation. Paroid et al. showed that hypoxia-induced CXCR4 and CXCR7 upregulation on $CD56^{bright}$ NK cell population, thus induce migration to CXCL12 positive cells [177]. Therefore, the hypoxic environment may profoundly influence the nature of the NK cell infiltrate and its effects on immune-mediated responses within tumor tissues by promoting the accumulation of poorly cytotoxic $CD56^{bright}$ NK cells [178]. It has also been shown that NK cells are a critical source of sVEGFR1 and thereby negatively regulate VEGF bioavailability in the tumor microenvironment. Deletion of HIF-1α in NK cells inhibits their ability to infiltrate tumor site and by increasing the VEGF availability and supporting non-functional tumor angiogenesis inhibits tumor growth [175].

Moreover, hypoxia also can limit the ability of NK cells to release chemokines involved in recruitment, differentiation, proliferation, and activation of APCs, Th1 lymphocytes, and NK cells; such as GM-CSF, CCL3, and CCL5 [177,179]. Likewise, TGF-β has been shown to modulate chemokine receptors repertoire, it downregulates the expression of CX3CR1 [180], but also increase expression of

CXCR4 [181] (Figure 4). It has been noticed that extracellular messengers, such as ROS, could also guide NK cells to their destination. Previous studies have shown that H_2O_2 can recruit leukocytes to wounded sites [182] or oncogene-transformed cells [183]. Oxidative stress can also affect NK cell tumor infiltration. In gastric and esophageal cancer, H_2O_2 produced within tumor microenvironments inversely correlates with the infiltration of $CD56^{dim}$ NK cells [184]. Several tumors, such as breast cancer [178] or non-small cell lung cancer [185], are characterized by the infiltration of $CD56^{bright}$, poorly cytotoxic NK cells. NK cells decrease in number during lung cancer progression, and their intracellular ROS level is increased in the lung cancer microenvironment [168]. Moreover, ADO further potentiates the NK cell function inhibition. Among four adenosine receptors (A1, A2a, A2b, A3), A2a receptor (A2aR) is the most abundantly expressed on human NK cells and is responsible for the majority of immunosuppressive effects. Moreover, CD39 and CD73 were shown to interfere with the trafficking and cytotoxic activity of NK cells into solid tumor site through the heterologous desensitization of chemokine receptors [170]. NK cell homing to tumor tissue is also substantially changed by PGE_2 [186,187]. It has been demonstrated that PGE_2 inhibits migration of NK cells in response to SDF1a, MIP1a, ITAC, and CCL21 [151,188,189]. PGE_2 also alters the NK cell chemokine receptor profile [187]. Within the tumor microenvironment PGE_2 interferes with the production of CCL5 and CCL27 [187–193]. Since NK cells are responsible for dendritic cell recruitment to tumor tissue, PGE_2-mediated inhibition of cytokine and chemokine production disrupts the NK-DC axis, causing a domino-like effect that impairs NK cell recruitment and other components of the anti-cancer immune response, leading to immune evasion and disease progression [187]. Trafficking of NK cells to inflamed tissues and tumor microenvironment was also shown to be affected by IDO metabolites [194–197].

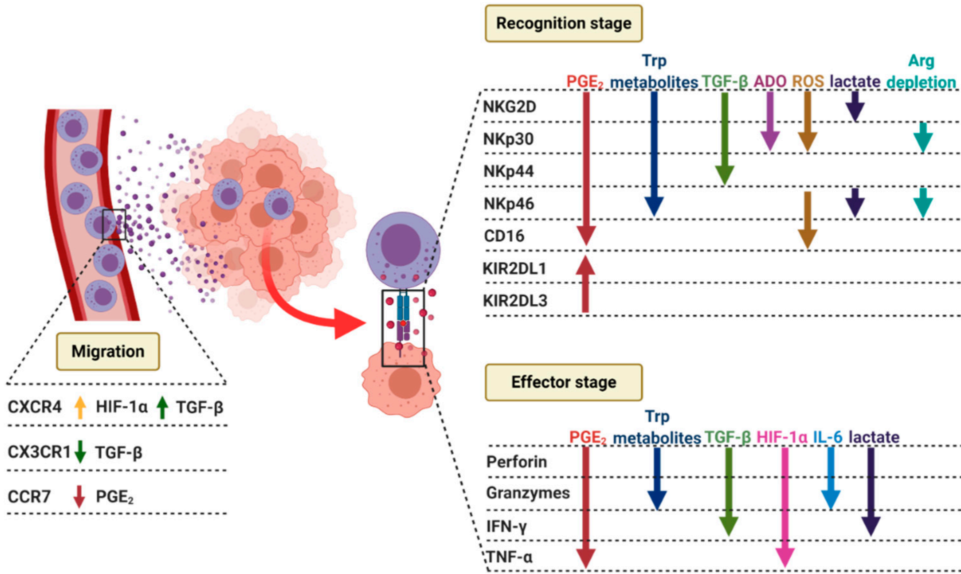

Figure 4. Tumor microenvironment shapes NK cells' migration to the tumor site by upregulation of CXCR4 receptor through HIF-1α and TGF-β. It also downregulates CX3CR1 expression by TGF-β and CCR7 levels by PGE_2, thus limiting their recruitment to the tumor sites. The formation of NK-cell lytic synapse can be divided into recognition, effector and termination stages. Within the tumor microenvironment factors such as PGE_2, tryptophan metabolites, TGF-β, ADO, ROS, and lactate can downregulate NK cells activating receptors, including NKp46, NKp44, NKp30, and CD16 and inhibitory receptors, such as KIR2DL1 and KIR2DL3. On the other hand, during the effector stage, the same metabolites can decrease expression of the lytic granule molecules, such as perforin and granzymes. Also, they can influence NK cells' ability for cytokine production, including IFN-γ and TNF-α.

4.3. NK Cells' Lytic Synapse

4.3.1. Recognition Stage

As described in detail before, tumor cells consume large amounts of glucose and produce lactate, which can accumulate in the TME and limit NK cells antitumor response. It has been observed that lactate-treated NK cells are characterized by decreased expression of activating receptor NKp46 as compared with the untreated cells, with no change in the level of NKp30, NKp44, and NKG2D [163]. In addition, the expression of NK cells surface molecules, such as CD18, CD54, and CD56, is reduced by acidic pH. While NK stimulation with IL-2 at neutral pH increased the expression of these molecules, in pH below 6.8 IL-2 lost its ability to modulate surface markers expression and did not affect the number of $CD56^+$ NK cells [99]. Likewise, Crane et al. showed that LDH isoform 5 (LDH5) secreted by glioblastoma cells and detectable in sera from glioblastoma patients caused downregulation of NKG2D on NK cells via induction of NKG2D ligands on myeloid cells. Lower expression of NK cells activating NKG2D receptor decreased NK cells antitumor effect [198]. In another study, it has been shown that LDHA-deficient tumors are more responsive to anti-PD1 treatment. Similarly, LDHA blockade was shown to increase infiltration by NK cells characterized by increased IFN-γ production and higher granzyme B expression [199]. Moreover, targeting tumor acidosis has also been shown to increase the effectiveness of checkpoint inhibitors, including ant-PD-1 and anti-CTLA-4 [200]. Although PD-1 and CTLA-4 blockade is shown to increase mainly T cell activity, one can speculate that it presumably also enhances NK cells cytotoxicity [201]. Furthermore, within tumor microenvironment hypoxia is another factor which significantly reduces the expression of the activating receptors on NK cells. It has been demonstrated that in hypoxic environment NK cells lose their ability to upregulate the expression of NKG2D, NKp46, NKp30 and NKp44 in response to IL-2, IL-15, IL-12, and IL-21 [202]. Correspondingly, hypoxia has also been reported to increase the shedding [203] or downregulate the expression of an NKG2D ligand, the major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA), which correlates with a decreased susceptibility of tumor cells to NK cell-mediated cytotoxicity [204]. Interestingly, hypoxia does not significantly alter the surface density and the function of the FcγRIIIA receptor CD16, thus allowing NK cells to destroy target cells under hypoxic conditions via antibody-dependent cellular cytotoxicity [202]. Furthermore, downmodulation of NK cell-activating receptors, such as NKp46 and NKG2D, can also be triggered by ROS derived from phagocytic cells (Figure 4) [205]. However, this observation was limited only to the NK $CD56^{dim}$ subset, while no changes were observed in $CD56^{bright}$ cells, more resistant to redox stress. It has been shown that NKp46 is also downregulated in H_2O_2-treated $CD56^{dim}$ cells [206]. It remains unresolved whether the down-modulation of activating NK receptors is related to the initiation of NK cell apoptosis by ROS or results from the direct effect of ROS on NK cell receptor expression. Interestingly, incubation of NK cells with PARP-1 inhibitor prevents ROS-induced NK cell apoptosis [207], which is accompanied by significant downregulation of NKp46 and CD16 with a modest decrease in NKp80 and DNAM-1 expression [208]. Downregulation of NKG2D, NKp30, NKp44 can also be a result of the production of high quantities of TGF-β in the tumor microenvironment (Figure 4). TGF-β also disrupts the NK cells ability to perform ADCC by CD16 downregulation [209]. Noteworthy, negative regulation of NKG2D by TGF-β- depends on the downregulation of DAP10 mRNA expression, which is an adaptor protein that stabilizes NKG2D on the cell surface and also transmits the phosphorylation events occurring upon NKG2D ligation [210]. ADO is also known to downregulate the expression of activating receptors NKG2D and NKp30, though their expression could be rescued by IL-2 priming [170]. Furthermore, recent studies have shown that Trp metabolites can decrease not only the expression of NKG2D but also NKp46, NKp44, NKp30, granzyme B, perforin, and CD69 in a kynurenine-dependent way (Figure 4). Interestingly, whereas Trp metabolites downregulate NKG2D, NKp46, TNF-α, and inhibit cytotoxicity in peripheral blood NK cells, they do not have any effect on decidual NK cells, suggesting a potential resistance of tissue-resident NK cells to kynurenine [211,212]. The abovementioned effects of Trp metabolites on

NK cells are mediated via STAT1 and STAT3 pathways. First, kynurenine enters NK cells via the aryl hydrocarbon receptor (AhR) on the surfaces of the NK cells. Then, by disrupting STAT1 and STAT3 pathways with JNK inhibition being the critical event, it alters the NK cell phenotype [213]. It has also been shown that L-arginine deprivation within the tumor microenvironment reduces the expression of NKp30 and NKp46 and thus modulates functional properties of NK cells [214]. The cytotoxicity of NK cell was also shown to be inhibited by PGE_2 [151,188,189,192,215–224]. The effect was dose-dependent and led to increased tumor burden in in vivo models [151,188,218,221,225–227]. The suppression of natural cytotoxicity was linked to PGE_2-induced NKG2D downregulation [228]. Moreover, signaling from other receptors such as NKp30, NKp44, NKp46, CD16, as well as expression of Granzyme B and perforin is inhibited via PGE_2 [192,215,216,222,224,228,229] (Figure 4). Additionally, PGE_2 increases the expression of inhibitory receptors, such as KIR2DL1 and KIR2DL3 [216]. Since PGE_2 suppresses NK cells within the tumor microenvironment, it also might inhibit DCs recruitment and subsequent NK-DC crosstalk.

4.3.2. Effector Stage

A critical step in the establishment of the immunological synapse between tumor cells and NK cells is the translocation of the microtubule-organizing center and granules in NK cells toward the cell–cell contact region. In the target cells, actin cytoskeleton-dependent tethering of ICAM-1 and -2 (LFA-1 ligands), is required for proper integrin signaling in NK cells [230]. It has been shown that depolymerization of actin filaments in tumor cells inhibits the formation of immunological synapse mediated by LFA-1 and results in the impaired polarization of the granules. In hypoxia, remodeling of the actin cytoskeleton in the tumor cells can promote resistance to NK cell-mediated killing. Actin cytoskeleton remodeling in breast cancer cells has been linked to escape from NK-mediated cytotoxicity [231]. Specifically, hypoxia has been described to increase the expression and activity of actin-binding proteins and Rho GTPases (RhoA, RAc1, Cdc42). Subsequent stimulation of Rho GTPases-mediated actin and adhesion signaling pathways in tumor cells help them potentially to escape from the immune system control. It is also well documented that cancer cells can adapt to hypoxic stress through the activation of autophagy, a process responsible for the degradation of proteins and cytoplasmic organelles in well-characterized structures known as autophagosomes. It has also been reported that autophagy activation in breast cancer cells under hypoxia induces their resistance to NK-mediated killing.

Mechanistically, the resistance of hypoxic cancer cells to NK-mediated killing is related to selective degradation of NK-derived granzyme B in autophagosomes in target cells [232]. Intracellular granzyme B and perforin levels were also reported to be downregulated in NK cells in hypoxia (Figure 4); however, the exact mechanism of this phenomenon remains to be elucidated. Moreover, the decrease of granzyme B and perforin mRNA in NK cells can also be induced by lactate and low pH. Some reports suggest that the lactate may interfere with the secretory pathway of NK cytolytic machinery [163,233]. Diminished expression of granzymes A and B [234] and perforin can also be a consequence of long-term/chronic exposition of NK cells to cytokines, such as TGF-β or IL-6. IL-6 added to the human NK cells culture trigger the downregulation of perforin and granzyme B; however, a high level of IL-6 does not obscure the degranulation process performed by NK cells. The addition of tocilizumab, an IL-6 receptor blocker, rescues the expression of perforin and granzyme B and promotes cytotoxicity of NK cells [123].

4.4. NK Cells' Cytokines and Chemokines Production

Hypoxia has been shown to change NK cells expression of proinflammatory cytokines, chemokines, and chemokine-receptors. In a comprehensive transcriptome analysis of hypoxic NK cells, Parodi et al. observed the downregulation of IFN-γ and several members of the TNF family, including TNF-α, LTA, LTB, TNFSF14, TNFSF10, and TNFSF11, which are involved in triggering tumor immunogenicity and decreasing tumor proliferation [177]. Recently, it has been shown that conditional deletion of HIF-1α in NK cells inhibits tumor growth, the phenomenon dependent on elevated expression of

activation markers, effector molecules and an enriched NF-κB pathway in tumor-infiltrating NK cells. Accordingly, HIF-1α inhibitor increased human NK cell activity, and low HIF-1α expression was associated with high expression of IFN-ɣ in human tumor-infiltrating NK cells [174]. Moreover, lactate has been reported to impair the cytotoxic activity of PMA/Ionomycin-stimulated NK cells by inhibition of IFN-γ production, which likely promotes tumor immune evasion and growth. A possible explanation for the lower IFN-ɣ production may be the downregulation of nuclear factor of activated T cells (NFAT), key IFN-ɣ transcription factor [167,235]. On the other hand, the TGF-β family of cytokines orchestrate the multistep cascade of events resulting in the downregulation of the IFN-ɣ gene expression. The signaling of TGF-β is transmitted by TGF-β I and II transmembrane receptors and is associated with the phosphorylation cascade of serine and threonine kinases that mediate downstream SMAD2 and SMAD3 phosphorylation [236]. SMAD2/3 activation results in the direct downregulation of the IFN-ɣ gene as well as downregulation of the T-bet or E4BP4 transcription factors governing IFN-γ expression [237,238]. Another member of the TGF-β family, Activin- A, was also shown to activate SMAD2/3 pathway but by an alternative route —mainly by binding to ALK4 receptor. Similarly to TGF-β, activin-A can trigger the downregulation of transcription factor T-bet and subsequent IFN-ɣ. IL-10, at first, was shown to suppress NK cells expression of INF-ɣ and TNF-α in vitro [239]. However, in the light of recent findings, the influence of IL-10 in TME seems to be beneficial for NK cells, as it was shown to enhance the expression of genes engaged in NK cytotoxic and migratory activity [240]. Also, IL-2-induced IFN-ɣ, TNF-α, and GMCSF production were shown to be inhibited in NK cells by IDO [192,211,241]. Moreover, ADO inhibits TNF-α release from IL-2 stimulated NK cells. It has also been observed that in IL-15 stimulated NK cells. ADO increased the level of IFN-γ both in $CD56^{bright}$ and $CD56^{dim}$ NK cells [170]. Within the tumor microenvironment PGE_2 also interferes with NK cells cytokines production. Disruption of IFN-ɣ, GM-CSF, and TNF-α synthesis was reported in multiple studies [187–193]. PGE_2 in cAMP-dependent mechanism suppresses CCL5, CCL19, CXCL10, IL-12, IL-18 secretionand expression of ICAM-1 on dendritic cells, what was linked to decreased NK cell activation. Also, PGE_2 inhibits NK cells ability to produce cytokines, such as IFN-γ [242] and TNF-α [216].

5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

Although NK cells, representing the first line of defense against the tumor, are able to recognize and rapidly eliminate aberrant cells, TME constitutes one of the critical barriers to their activity (summarized in [236]). A complex interplay between tumor cells and surrounding TME cells occurring in the tumor microenvironment (TME) promotes the immune escape of tumor cells from NK cell-mediated surveillance, thus contributing to tumor progression. Suppression of NK cells within the tumor site is orchestrated by a variety of stromal, myeloid, and lymphoid cells, immunosuppressive cytokines, intratumoral nutrient availability, engagement of checkpoint molecules, and metabolic changes. Increased metabolic demands of tumor cells limit nutrient availability and expose tumor-infiltrating NK cells to various products of metabolic reactions that drive their functional exhaustion. Therefore, to overcome the NK cell limitations in their fight against tumor cells, several strategies have been recently introduced and explored at both preclinical and clinical levels [243]. The currently investigated main NK cell-based immunotherapeutics include the monoclonal antibodies (mAbs) neutralizing the immune checkpoint molecules, the adoptive transfer of ex vivo activated and expanded NK cells or chimeric antigen receptor (CAR)-modified NK cells (summarized elsewhere) [243]. Furthermore, understanding the metabolic changes and the immune suppression mechanisms present in TME has led to the development of new promising therapeutic agents and strategies. Here, we summarize the strategies targeting tumor cell metabolism designed specifically to support NK cells in hash TME by increasing nutrient availability to immune cells, decreasing acidity and hypoxia and reducing the production of immunosuppressive metabolites (Table 2).

Table 2. Strategies to overcome the inhibitory effects of TME.

TME Factor	Strategies	Examples of Clinical Trials (NCT: ClinicalTrials.gov Identifier)
Hypoxia	Priming of NK cells with IL-2 increases the expression of activating receptors and thus overcomes the inhibitory effects of hypoxia [244,245].	Natural Killer Cells Plus IL-2 Following Chemotherapy to Treat Advanced Melanoma or Kidney Cancer NCT00328861 Intraperitoneal Delivery of Adaptive Natural Killer (NK) Cells (FATE-NK100) With Intraperitoneal Interleukin-2 in Women with Recurrent Ovarian, Fallopian Tube, and Primary Peritoneal Cancer NCT03213964
	Modification of NK cells to increase ADCC potential and activity—high-affinity NK cells (haNK) expressing CD16 and IL-2 are resistant to acute hypoxia [246].	Phase 1 Study of haNK™ for Infusion in Subjects with Metastatic or Locally Advanced Solid Tumors NCT03027128
	Inhibition of HIF-1α (either by genetic modifications or small molecular HIF-1α inhibitor) enhances effector functions of activated NK cells (degranulation, production of IFN-γ and TNF-α [247].	None
Lactic acid/Low pH	Genetic blockade of LDHA (mice with LDHA deficiency) heightens infiltration of NK cells in the melanoma tumors. Infiltrated NK cells have an elevated production of IFN-γ and granzyme B [199]. Novel LDHA inhibitor reduces lactate production, thus decrease TME acidity [248].	None
	Bicarbonate monotherapy neutralises tumor acidity and increases effector cells infiltration [249,250].	Extended Use of Sodium Bicarbonate in Patients with Cancer NCT02531919
	Blocking the mitochondrial ROS accumulation to prevent NK cells mitochondria dysfunction and apoptosis [162].	None
Glucose depletion	FBP1 inhibition during tumor promotion, but not tumor progression, can restore NK cell function [168].	None
	GSK-3 inhibitors, CHIR99021, blocks proteasomal degradation of cMYC and thus promotes glucose consumption in NK cells [251]. CHIR99021 was shown to improve NK-cells function in ovarian cancer [251]. Moreover, other GSK-3 inhibitors, including LY-2090314 were shown to augment NK cells cytotoxicity in AML patients [252].	Phase 1 trials evaluating the application of NK-cells expanded ex-vivo and pre-treated with CHIR99021 in patients with AML (NCT03081780), ovarian cancer (NCT03213964) and other solid tumors (NCT03319459) Phase 2 trial of LY2090314 and Chemotherapy in Participants With Metastatic Pancreatic Cancer (NCT01632306)

Table 2. *Cont.*

TME Factor	Strategies	Examples of Clinical Trials (NCT: ClinicalTrials.gov Identifier)
ROS	Superoxide dismutase and other SOD-mimicking substances partially restore the NK-cell mediated killing of YAC-1 cells inhibited by superoxide [253].	None
	Histamine reverses granulocyte-induced inhibition of human NK-cell mediated killing of K562 cells [254]. Serotonin restores NK cell-mediated killing of K562 cells inhibited by mononuclear phagocytes [255]	Maintenance Therapy With Ceplene® (Histamine) and IL-2 on Immune Response and MRD in Acute Myeloid Leukemia NCT01347996 -A Study of HDC/IL-2 Treatment in Chronic Myelomonocytic Leukemia (CMML) NCT03040401
	Catalase protects human NK cells from H_2O_2 induced apoptosis [206,253,256].	None
	Genetic inhibition of NOX2 (Nox2$^{-/-}$ mice that lack the myeloid gp91^{phox} subunit of NOX2) or NOX2 inhibitor HDC reduces melanoma metastasis in a murine NK cell-dependent model of melanoma metastasis [257]. NOX2 inhibitors HDC and diphenylene iodonium chloride (DPI) play a protective role from monocyte-derived ROS-dependent NK cell apoptosis and mostly restore NK cell-mediated ADCC of primary CLL cells. NOX2 inhibitor HDC promotes degranulation of NK cells toward CMML cells in ADCC process and reverses CMML-induced NK cell apoptosis [208,256,257].	None
	ERK1/2 inhibitor PD98059 protects NK cell from H_2O_2-induced or monocyte-dependent apoptosis [207].	None
TGF-β	Chemical inhibitors- TGF-β receptor kinase inhibitor, galunisertib (LY2157299) improves the activity of NK cells in metastatic colon cancer mouse model [209].	A Study of Galunisertib on the Immune System in Participants with Cancer NCT02304419 ExIST Study of LY2157299 (Galunisertib) in Rectal Cancer NCT02688712
	anti-TGF-β antibodies- are shown to restore NK cells degranulation and cytokine release [258].	-Anti-TGF Monoclonal Antibody (GC1008) in Relapsed Malignant Pleural Mesothelioma NCT01112293 -Safety and Efficacy Study of GC1008 to Treat Renal Cell Carcinoma or Malignant Melanoma NCT00356460
	Genetic modification strategies- TGF-β dominant-negative receptor knockout receptor coupled to NK-activating domains (DAP12 or synNotch-RELA) enhance the cytotoxic activity of NK cells (particularly with DAP12 domain) [259]	None

Table 2. *Cont.*

TME Factor	Strategies	Examples of Clinical Trials (NCT: ClinicalTrials.gov Identifier)
Glutamine depletion	CB-839 It has been reported that glutaminolysis can be inhibited without reducing NK cell functional responses [34].	Study of the Glutaminase Inhibitor CB-839 in Solid Tumors NCT02071862
Tryptophan metabolites	IDO1 inhibition restores NKG2D expression on NK cells and promotes their proliferation [260,261]. IDO pathway inhibition enhances NK cell tumor infiltration and antitumor activity [262].	-Intraperitoneal Natural Killer Cells and INCB024360 for Recurrent Ovarian, Fallopian Tube, and Primary Peritoneal Cancer NCT02118285 -NLG802 Indoleamine 2,3-Dioxygenase (IDO) Inhibitor in Advanced Solid Tumors NCT03164603
	AHR antagonism increases cancer cell susceptibility to NK cell-mediated cytotoxicity and enhances NK cell-mediated ADCC [263].	-A First-in-Humans Dose Finding Study for an Aryl Hydrocarbon Receptor Inhibitor (AhRi) in Patients with Advanced Cancer NCT04069026 -IK-175 in Patients with Advanced or Metastatic Solid Tumors and Urothelial Carcinoma NCT04200963
	IL-18 treatment reversed IDO-mediated NK cell inhibition by upregulating NKG2D receptor [213].	None
Adenosine	A3R agonists: C1-IB-MECA increases activation and NK cells infiltration of B16-F10 melanoma. CF101 potentiation of NK cells' activity [264–266].	None
	A2aR antagonists: SCH58261- enhances NK cells maturation, cytokine production, cytotoxic function against tumor cell lines, increases expression of granzyme B and reduces metastasis in a perforin-dependent manner. Increases NK cells infiltration of BRAF^{V600E}-mutant melanoma. Promotes mouse NK cells proliferation and differentiation of human CD56^{bright} into CD56^{dim} mature NK cells. ZM241385- restores the cytotoxic function of IL-2 activated NK cells and cytokines production [267–273].	-A Study to Evaluate Immunotherapy Combinations in Participants with Gastrointestinal Malignancies NCT03720678 -A Study to Evaluate the Safety and Tolerability of Immunotherapy Combinations in Participants with Advanced Malignancies NCT03629756 - A Study to Evaluate Safety/Tolerability of Immunotherapy Combinations in Participants with Triple-Negative Breast Cancer or Gynecologic Malignancies NCT03719326
	CD73 inhibitor: APCP- reduces metastasis trough decreased A2aR-mediated suppression of NK cell-mediated cytotoxicity. Improves lytic activity of NK cells [270,273].	A Study of the CD73 Inhibitor LY3475070 Alone or in Combination with Pembrolizumab in Participants with Advanced Cancer NCT04148937
	anti-mouse CD73 antibody: TY/23- enhances anti-metastatic activity derived by NK cells [272]. Anti-human CD73 antibody increases the cytotoxicity of NK cells against ovarian cancer cell lines overexpressing CD73 [274].	A Study of AK119 (Anti-CD73) in Combination with AK104 (PD-1/CTLA-4) in Subjects with Advanced Solid Tumors -Study of GS-1423 (Anti-CD73-TGFβ-Trap Bifunctional Antibody) in Participants with Advanced Solid Tumors NCT03954704

Table 2. *Cont.*

TME Factor	Strategies	Examples of Clinical Trials (NCT: ClinicalTrials.gov Identifier)
	Anti-human CD39 antibody increases the cytotoxicity of NK cells against ovarian cancer cell lines overexpressing CD39 [274].	- Study of SRF617 (anti-CD39 antibody) in Patients with Advanced Solid Tumors NCT04336098 - TTX-030 (anti-CD39 antibody) Single Agent and in Combination With Immunotherapy or Chemotherapy for Patients With Advanced Cancers NCT03884556
	CD39 inhibitors: Polyoxometalate-1 (POM-1)- reverses Treg-mediated suppression of NK cells cytotoxicity and enhances their anti-metastatic activity. ARL67156- enhances the lytic activity of polyclonal NK cells [270,275,276].	None
Arginine	MDSCs upregulate arginase and catabolise arginine to NO. It has been found that NO impairs NK cell antibody-dependent cellular cytotoxicity and that the inhibition of iNOS can rescue this function [277].	
	Inhibition of the arginase activity by CB-1158 reduces tumor growth and increases tumor-infiltrating NK cells in vitro and in vivo [278]. OATD-02, another arginase inhibitor, has been shown to delay cancer progression [279,280].	Arginase Inhibitor INCB001158 as a Single Agent and in Combination with Immune Checkpoint Therapy in Patients with Advanced/Metastatic Solid Tumors NCT02903914
Arachidonic acid metabolites	Selective COX-2 inhibitors increase cancer cell sensitivity to NK cell-mediated lysis [281].	-Perioperative Administration of COX 2 Inhibitors and Beta Blockers to Women Undergoing Breast Cancer Surgery NCT00502684 -Perioperative Intervention to Reduce Metastatic Processes in Pancreatic Cancer Patients Undergoing Curative Surgery (BC-PC) NCT03838029
	EP2 antagonists restore tumor NK cell-mediated lysis [216]. EP4 antagonists restore NK cell antitumor activity cytokine production and migratory potential. Also, they decrease MHC I expression on cancer cells rendering them more sensitive to NK cell-mediated cytotoxicity [151,226].	Phase 1a/1b Study of TPST-1495 (EP2/EP4 antagonist) Alone and With Pembrolizumab in Subjects with Solid Tumors

6. Conclusions

In the immunosuppressive tumor microenvironment, NK cell priming, metabolism, and antitumor responses can be impaired by numerous factors. First of all, tumor cells compete with stromal cells and immune cells for the supply of glucose, glutamine and amino acids. In addition to reduced glucose availability, lactate accumulation and decreased pH within the TME also have a substantial impact on NK cell functionality. Therefore, targeting cancer metabolism represents a reasonable approach to improve the efficacy of NK cell-based therapies. Although most of the currently developed immunotherapies rely on the genetic modification of the cancer cells, strategies targeting cancer microenvironment offer

novel and exciting possibilities. For example, CB-839, which is known to inhibit glutaminolysis without impairing NK cell cytotoxicity, could be utilized to augment immunotherapies. Additionally, NK cell cytotoxicity can be strengthened by targeting adenosine present in the tumor microenvironment, as few A2aR antagonists are currently tested. These compounds could potentially enhance NK cells maturation, activation and cytokine production. Similarly, NK cells' cytotoxicity could be potentially strengthened by anti-CD73 antibodies. Furthermore, stimulation with different cytokine combinations can upregulate the expression of activating receptors and enhance NK cells lytic activity. Noteworthy, IL-2, IL-15, or IL-18 can also increase the expression of amino acid and glucose transporters. Therefore, it is necessary to continue exploring NK cell activity and to understand how it could be modified to resist the metabolically restrictive TME and preserve the effector functions, leading to the improvement in various immunotherapies.

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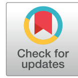
6. Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation

Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation



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Summary

Background Natural killer (NK) cells have a unique capability of spontaneous cytotoxicity against malignant cells and hold promise for off-the-shelf cell therapy against cancer. One of the key challenges in the field is to improve NK cell homing to solid tumors.

Methods To gain a deeper understanding of the cellular mechanisms regulating trafficking of NK cells into the tumor, we used high-dimensional flow cytometry, mass cytometry, and single-cell RNA-sequencing combined with functional assays, creating a comprehensive map of human NK cell migration phenotypes.

Findings We found that the chemokine receptor repertoire of peripheral blood NK cells changes in a coordinated manner becoming progressively more diversified during NK cell differentiation and correlating tightly with the migratory response of the distinct NK cell subsets. Simultaneous ligation of CXCR1/2 and CX3CR1, synergistically potentiated the migratory response of NK cells. Analysis of 9471 solid cancers from publicly available TCGA/TARGET repositories revealed dominant chemokine patterns that varied across tumor types but with no tumor group expressing ligands for more than one chemokine receptor present on mature NK cells.

Interpretation The finding that chemokine stimulation can elicit a synergistic migratory response in NK cells combined with the identified lack of naturally occurring pairs of chemokines-chemokine receptors in human cancers may explain the systematic exclusion of NK cells from the tumor microenvironment and provides a basis for engineering next-generation NK cell therapies against malignancies.

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Keywords: NK cells; Natural killer cells; Cancer; Immunology; Migration; Chemokine receptors; Synergy; Differentiation; Immunotherapy; Cell therapy; TCGA; Adoptive therapy

Introduction

Natural killer (NK) cells are members of the innate lymphoid cell (ILC) family that provide host defense against tumors and pathogen-infected cells.[1–3] Unlike T and B cells, they can recognize and kill target cells without prior sensitization, due to a diverse repertoire of germline-encoded inhibitory and activating receptors on their cell surface.[1,4–11] One important mode of target recognition is mediated by the FcγRIII (CD16) triggering antibody-dependent cellular cytotoxicity (ADCC).[12,13] NK cells can kill transformed or infected cells by the release of perforin and granzymes or by utilizing effector

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Research in context

Evidence before this study
Natural killer (NK) cells hold promise as a highly functional template for genetic engineering in the development of next generation off-the-shelf cell therapies. However, a prerequisite for extending the success of cell therapy to solid tumors is that the cells reach the intended target organ. In contrast to T cells, highly differentiated NK cells are rarely found in solid tumors and trafficking patterns of NK cells remain poorly understood. Furthermore, there is limited information on the dynamic regulation of chemokine receptor expression during NK cell differentiation and *ex vivo* expansion for cell therapy.

Added value of this study
In this study, we investigated the trafficking patterns of human NK cells utilizing high-dimensional flow cytometry, mass cytometry, and single-cell RNA-sequencing combined with functional assays. We found that the chemokine receptor repertoire of peripheral blood NK cells changes in a coordinated fashion becoming gradually more diversified during the differentiation process. The chemokine receptor expression correlated tightly with the migratory response of the distinct NK cell subsets. We also found that simultaneous ligation of CXCR1/2 and CX3CR1 receptors led to a synergistically enhanced migratory response. Investigation of 9471 solid cancer cases in the TCGA/TARGET databases revealed nine predominant chemokine profiles that varied among tumor types, but none of them had ligands for more than one chemokine receptor expressed on mature NK cells.

Implications of all the available evidence
We here report a comprehensive study of the chemokine receptor landscape and the migratory behavior of human NK cells. Our results show that chemokine stimulation can elicit a synergistic migratory response in NK cells. We speculate that the sparsity of naturally occurring pairs of chemokines-chemokine receptors may explain the systematic exclusion of NK cells from the tumor microenvironment and represent an untapped potential for engineering next-generation NK-cell based therapies in oncology.

molecules of the tumor necrosis factor (TNF) family, such as TNF, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL).[14–16] Additionally, upon activation, NK cells rapidly produce chemokines and cytokines, including interferon (IFN)-γ, and GM-CSF, that recruit and affect the function of hematopoietic and non-hematopoietic cells in the tumor microenvironment.[17–20] These potent effector functions allow them to play an important role in various diseases, including cancer and infectious diseases.[3,21]

The repertoire of human NK cells is functionally diversified through a tightly regulated differentiation process characterized by an early transition from $CD56^{bright}$ to $CD56^{dim}$ NK cells, followed by coordinated changes in expression of inhibitory receptors, including NKG2A and killer cell immunoglobulin-like receptors (KIRs).[22–28] Along the process, NK cells gradually progress from an immunoregulatory phenotype to highly cytotoxic and mature cells capable of effective immune surveillance of cancer.[22–24,26,29,30] The role of NK cells in cancer is supported by the positive prognostic value of mature NK cell infiltration in a variety of tumors including melanoma, renal cell carcinoma, sarcoma, lung, liver, and breast cancer.[2,31–34] Although NK cell differentiation is a critical determinant of the cytotoxic potential of these cells, little is known about how these events shape the migratory behavior of NK cells.

In order to perform their intended function, NK cells must possess the ability to migrate to different target tissues.[35–39] This process is mediated by a family of small, secreted cytokines, known as chemokines. By binding to their specific receptor, chemokines guide cellular migration. Trafficking is also facilitated by interactions between adhesion molecules and membrane-bound integrins. Altogether, the expression of all these trafficking molecules can be used as an indicator of cellular localization, activation status, and function of immune effector cells.[35] The significance of the chemokine-chemokine receptor axis is gaining an increasing recognition in immuno-oncology.[36–39] The infiltration of solid tumors by NK cells is relatively low when compared to T cells or macrophages.[34,40] Thus, enhancing NK cell trafficking into the TME is one of the main challenges in improving the effectiveness of NK cell therapy against solid tumors.

Here, we have used a combination of high-dimensional flow cytometry, time-of-flight mass cytometry (CyTOF) and single-cell RNA sequencing to map chemokine repertoires and functional migratory profiles in resting and activated primary NK cells as well as induced pluripotent stem cell (iPSC)-derived NK cells (iNK). Our results delineate the natural variation in NK cell chemokine receptor repertoires and provide a guide for engineering the migratory properties of NK cells.

Methods

Sample collection and PBMC isolation

Buffy coats from random healthy blood donors were obtained from the Oslo University Hospital Blood bank with written informed consent. The samples were anonymized prior to NK cell isolation. The approval was obtained from the regional committees for medical and health research ethics in Norway: 2018/2485. Peripheral blood mononuclear cells were separated from buffy coats by density gravity centrifugation (Lymphoprep;

Axis-Shield) using fretted spin tubes (SepMate; Stemcell Technologies). After isolation, the cells were resuspended in MACS buffer (PBS supplemented with 1% BSA and 2 mM EDTA) and stained or transferred into cell culture medium within 1 h.

Cell isolation, culture, and stimulation

NK cells were purified from PBMC using negative selection with an AutoMACS Pro Separator (Miltenyi) using NK cell isolation kits (Miltenyi, Cat# 130-092-657). The purity of isolated NK cells was over 90%. A panel of CD56, CD3, CD14, CD19, and a dead cell stain was used for each flow cytometry profiling of stimulated NK cells to ensure both adequate NK cell purity and that the chemokine receptors were profiled exclusively on live NK cells.

Isolated NK cells were cultured in R10 medium (RPMI 1640, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptavidin, 2 mM L-glutamine, 20 mM HEPES, pH 7.0). For experiments with stimulation, 1 or 10 ng/ml of IL-15 (Miltenyi, Cat# 130-095-765) or 100 IU/ml IL-2 (Miltenyi, Cat# 130-097-744) was added to R10 NK cell culture medium. NK cells were stimulated either for 24 h or 7 days. For NK cell expansion experiments, cells were expanded from CD3/CD19-depleted PBMC through co-culture with 200 Gy irradiated K562 feeder cells transfected with a lentiviral construct to express high levels of membrane-bound IL-21 and 4-1BBL, kindly provided by Dr. Dean A. Lee (Nationwide Children's Hospital, Columbus, OH). NK cells were expanded with K562-feeder cells in G-Rex24 (Wilson Wolf) at a 1:2 ratio with a total cell number of $0.5 \times 10^6/\text{cm}^2$. Cells were cultured in GMP-grade Stem Cell Growth Medium (SCGM, CellGenix) supplemented with 100 IU/ml human recombinant IL-2 (Proleukin) for 11 days with 60% medium exchange on day 7, and IL-2 replenishment days 4, 7 and 10. The percentage of live $CD56^+$ $CD3^-$ $CD14^-$ $CD19^-$ cells after stimulation was over 90% in 24 h experiments and over 80% in longer experiments.

Derivation and expansion of iNK cells

Human iPSC were first differentiated into hematopoietic progenitors and then into NK cells, as previously described.[41] The iPSC cell line characterized in this study was previously described in a recent paper by Zhu et al.[41] Fully differentiated iNK cells were kindly provided to us by Fate Therapeutics.

Briefly, human iPSCs were differentiated into hematopoietic progenitor cells by spin EB formation, in a 10-day process, as previously described.[41–44] Then, $CD34^+$ cells were subsequently enriched prior to differentiation into iNK cells. At the beginning of the iNK cell differentiation culture, $CD34^+$ hematopoietic progenitors were plated on OP9 cells in B0 media containing a 2:1 mixture of Dulbecco modified Eagle medium/Ham F12 (Thermo Fisher Scientific, Cat# 11965092, Cat# 11765054), 2 mM L-glutamine (Thermo Fisher Scientific, Cat# 25030081), 1% penicillin/streptomycin (Thermo Fisher Scientific, Cat# 25030081), 25 μM β-mercaptoethanol (Gibco, Cat# M3148), 10% heat-inactivated human serum AB (Sigma, Cat# H3667-100M), 5 ng/ml sodium selenite (Merck Millipore, Cat# S5261), 50 μM ethanolamine (Sigma, Cat# E0135), 20 mg/ml ascorbic acid (Merck Millipore, Cat# A4544), interleukin-3 (IL-3, R&D, Cat# 203-IL); for first week only), stem cell factor (SCF; R&D, Cat# 7466-SC), interleukin-15 (IL-15; R&D, Cat# 247-ILB), Fml-like tyrosine kinase 3 ligand (FLT3L; R&D, Cat# 207-IL) to support NK cell differentiation from hematopoietic progenitors, as previously described.[41,45,46] The cells were left in these conditions for 20 days receiving weekly media changes until they had developed into $CD45^+CD56^+CD33^-CD3^-$ cells as determined by flow cytometry. The percentage of $CD45^+$ $CD56^+$ $CD33^-$ $CD3^-$ cells was above 80%. At this point, iNK cells were cryopreserved and kindly provided to us by FATE. We then expanded iNK cells in our laboratory using the irradiated K562 cells with transduced membrane-bound IL-21 and 4-1BBL constructs in supplemented B0 media supplemented with 10 mM Hepes and 100 IU/ml IL-2 for 7 days, in agreement with the ethical approval, REK 2019/333.[41,47] K562 cells were propagated in R10 medium (RPMI 1640, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptavidin, 2 mM L-glutamine, 20 mM HEPES, pH 7.0).[47]

Flow cytometry

Cells were stained for flow cytometric analysis using an appropriate combination of antibodies for 15 min in FACS buffer (phosphate-buffered saline (PBS) pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA) in the dark, at room temperature. For intracellular staining with anti-granzyme B-AF700 (clone GB11), cells were fixed and permeabilized using a fixation/permeabilization kit (BD Bioscience Cytofix/Cytoperm) after surface staining. Finally, cells were washed twice. Samples were acquired using BD LSR II or FACSymphony A5 equipped with HTS (BD Biosciences), and the data was analyzed using FlowJo 10.6.1 (BD Biosciences) and CytoExploreR (1.1.0).[48]

The list of used fluorochrome-labelled antibodies can be found in the Supplementary Table S1. All mAbs were titrated and used at dilutions ensuring saturated staining of 1×10^6 cells. Dead cells were labelled using Viobility™ 405/520 Fixable Dye (Miltenyi). Biotin-conjugated antibodies were visualized using streptavidin-Brilliant Violet 605 or 785 (Biolegend). The gates for chemokine receptors and adhesion molecules were set using appropriate FMO-1 controls in each experiment. The chemokine receptor expression score used for linear regression analysis to assess the relationship between chemokine receptor expression and chemokine-induced migratory response was calculated

as follows: *log2(MFI of the positive population + 1) * Percentage of Chemokine Receptor Expression*.

Mass cytometry

PBMCs collected from 20 healthy donors were frozen in 10% DMSO and 90% fetal calf serum and stored in liquid nitrogen. The day before acquisition, PBMCs were thawed, counted, and stained with Cell-ID Intercalator-Rh103 (Fluidigm) for viability testing, followed by Fc blocking reagent and a cocktail of surface antibodies. The list of used antibodies can be found in the Supplementary Table S2. Subsequently, cells were fixed in PBS (without calcium and magnesium) with 2% paraformaldehyde, permeabilized, barcoded using the Cell-ID 20-Plex Barcoding Kit (Fluidigm) and pooled. Samples were then transferred to methanol and stored at −20 °C. On the acquisition day, cells were stained with an intracellular antibody cocktail and labelled with Cell-ID Intercalator-Ir. Samples were supplemented with EQ Four Element Calibration Beads (Fluidigm) and acquired on a CyTOF 2 (Fluidigm) equipped with a SuperSampler (Victorian Airship) at an event rate below 350 events per second. Samples were analyzed in a single run. Antibodies were either obtained pre-labelled from Fluidigm or conjugated with metal isotopes using Maxpar X8 antibody labeling kits (Fluidigm). FCS files were normalized and debarcoded using the R package CATALYST (1.21.1).[49] Then, the gating and downstream analysis were performed in CytoExploreR (1.1.0).[48] All CyTOF data was transformed using arcsinh (x/5).

Chemotaxis assay

To measure NK cell migration, we used Corning® Transwell® plates with inserts (pore size 5.0 μm, Cat# CLS3421). 650 μl R10 medium containing recombinant chemokines manufactured by Peprotech including CCL5 (Cat# 300-06), CCL19 (Cat# 300-29B), CXCL8 (Cat# 200-08M), CXCL10 (Cat# 300-12), CXCL12 (Cat# 300-28A), or CX3CL1 (Cat# 300-31) was placed in the lower chamber of a 24-well Transwell plate (Corning). Each chemokine was titrated to determine the optimal concentration; the range tested was from 0.1 ng/ml to 1000 ng/ml. NK cells (5×10^5) were added in 100 μl R10 medium to the upper chamber (5-μm pore size), and the plates were incubated for 1 h at 37 °C. Then, the cells that migrated to the lower chamber were mixed with flow cytometry counting beads (Invitrogen, Cat# PCB100), stained with antibody cocktail as in section Cell isolation, culture, and stimulation, and acquired. The results are presented as migration index which was calculated as a ratio of cells that have migrated to the bottom well during chemokine-induced migration compared to the cells that have spontaneously migrated to the bottom well, without any chemoattractant present. The migration index was then normalized by counting beads (Invitrogen, Cat# PCB100). The calculations were performed separately for each subset of NK cells.

Data mining

The single-cell RNA sequencing data from peripheral blood NK cells were obtained from the GEO database, accession number GSE130430.[50] The count data was then filtered for peripheral blood samples from healthy donors only (2 donors). The bulk gene expression profiles of primary solid tumors were obtained from the combined TCGA, TARGET and GTEx cohort downloaded from the UCSC Xena, filtered for primary solid tumors. The downloaded data was of RSEM tpm type.

Data analysis

Both single-cell and bulk RNA-seq data were processed using the Seurat (v4.1.0) package in R (v4.1.2).[51,52] In the single-cell RNA-seq data, we filtered out the cells that expressed <200 genes or >2500 genes, as well as the cells with >5% mitochondrial transcripts content. Gene expression values for each cell were log normalized and scaled by a factor of 10,000. To avoid biasing the clusters by cell library size or mitochondrial transcript content, gene expression values were scaled based on the number of UMIs in each cell and the cell mitochondrial transcript content. We then combined cells derived from peripheral blood. Naive clustering of the cells into sub-populations was then conducted using Seurat's implementation of a shared nearest neighbor modularity optimization-based clustering algorithm (Louvain's original algorithm). Based on the PCElbowPlot, we selected a certain number of principal components (PCs) for the clustering analysis when that number reached the baseline of the standard deviation of PC. Cell clusters were visualized using UMAP. In order to predict cellular differentiation, cells were ordered in pseudotime using monocle3 (v1.2.9) through SeuratWrappers (v0.3.0).[53,54] The diversity of chemokine receptor-based clusters in classical clusters was calculated with Shannon diversity index in vegan (v2.6-2) package.[55] Then, the plots were created using Seurat (v4.1.0), dittoSeq (v1.6.0) and Nebulosa (v1.4.0) packages.[51,56–58]

Bulk RNA-seq data was first filtered for primary solid tumors and loaded into the Seurat. Then, naive clustering of the cells into sub-populations was conducted using Seurat's implementation of a shared nearest neighbor modularity optimization-based clustering algorithm (Louvain's original algorithm). Based on the PCElbowPlot, we picked a certain number of principal components (PCs) for the clustering analysis when that number reached the baseline value of the standard deviation of PC. Cell clusters were visualized using UMAP. Then, the graphs were created with Complexheatmap (v.2.10.0) and plotly (v4.10.0) packages.[58–60] Tidyverse packages were used throughout the analysis.[61,62]

Statistical methods

Simple linear regression was used to examine the relationship between the migration index and the

chemokine receptor expression score. As described above, the migration index was calculated as a ratio of cells that have migrated to the bottom well during chemokine-induced migration compared to the cells that have spontaneously migrated to the bottom well, without any chemoattractant present, normalized by counting beads. The chemokine receptor expression score was calculated as follows: *log2(MFI of the positive population + 1) * Percentage of Chemokine Receptor Expression*. R^2 was used as a measure of goodness of fit. P values were calculated using Wald's test.

Differences between the migration index curves from chemokine-induced Transwell migration experiments were assessed using two-way analysis of variance (ANOVA) with post-hoc Dunnett's test.

Role of the funding source
The Polish Ministry of Science and Higher Education (DI2018 020548), the National Science Centre, Poland (2020/37/B/NZ6/02191), the Norwegian Cancer Society (grant no 223310), the Research Council of Norway (grant no 275469), the South-Eastern Norway Regional Health Authority (grant no 2021073) and the National Cancer Institute of the National Institutes of Health (Award Number P01CA111412) supported this study. The funders have no role in the study design, the data collection, data analysis, interpretation, and the writing of the report. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

Results

Profiling the chemokine receptor repertoire of NK cells by flow cytometry
To get a comprehensive view of chemokine receptor profile in NK cells, we examined the expression of all classical chemokine receptors and several non-classical chemotactic receptors by flow cytometry in NK cells from PBMC (PB NK), the K562-4-1BBL-mbIL21- or cytokine-expanded NK cells, and iNK cells (Fig. 1). The representative chemokine receptor staining plots as well as the chemokine receptor expression for each donor and each stimuli representing donor-to-donor variability can be found in Supplementary Figures S1 and S2. Flow cytometry analysis of PB NK cells revealed a consistent picture of the chemokine receptor profile, with CXCR1, CXCR2, CXCR3, CXCR4, CX3CR1, and atypical chemotactic receptor CMKLR1, as the most highly expressed (>50%). Additionally, the expression of CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8 and CXCR6 was also detected, but only on a small fraction of NK cells. A 24-h culture of isolated NK cells without any stimulation slightly altered the profile of chemokine receptors, resulting in the decreased expression of CCR4, CCR6, and CXCR1 and a slight increase in CXCR3 and CXCR4 expression. CCR5, CCR7, CXCR2, CX3CR1 and CMKLR1 receptors showed no significant changes in expression after 24-h culture. We did not detect CCR3, CCR9, CCR10, CXCR5, XCR1 or CCRL2 on PB NK cells.

To explore dynamic changes of the chemokine receptor repertoire, we phenotyped NK cells upon exposure to cytokines (IL-2 or IL-15) associated with the development, differentiation, and acquisition of effector functions by NK cells. Isolated NK cells were cultured and stimulated with two concentrations of IL-15 (1 and 10 ng/ml) or with IL-2 (100 IU/ml) for either 24 h or 7 days. Short-term cytokine stimulation had minimal impact on surface expression of chemokine receptors. However, long-term (7-day) cytokine stimulation resulted in a decreased expression of CXCR1, CXCR2, CX3CR1, CMKLR1, and a marked increase in the expression of CCR5. Although these changes were more pronounced for IL-15 compared to IL-2, stimulation with either of them resulted in the same overarching change in chemokine receptor repertoires (Fig. 1).

We next evaluated series of NK cell-based products with potential clinical application, and we observed a profoundly altered chemokine receptor profile in NK cells subjected to a 11-day expansion protocol using K562-4-1BBL-mbIL21 cells.[47] Compared to PB NK cells, expanded cells acquired high expression of CCR4, CCR5, CCR7, CCRL2, and especially CXCR3, while losing CXCR1, CXCR2, CX3CR1 and CMKLR1 (Fig. 1). Mature resting and short term expanded iNK cells were characterized by the most unique chemokine receptor signature among the cells tested, with high expression of CXCR2, CXCR3, CCR1, CCR6 and S1P5R, low levels of CXCR1 and CXCR4, and modest expression of CX3CR1 (Fig. 1). Based on this initial screening, we excluded CCR3, CCR9, CCR10, CXCR5, XCR1 or CCRL2 from further experiments.

Effects of differentiation on chemokine receptor expression and migratory potential of NK cells
We evaluated the expression of selected chemokine receptors on different NK cell subsets in healthy donors with an extended high-dimensional flow cytometry panel. Based on the surface expression of CD56, CD57, NKG2A, NKG2C and four KIRs, NK cells were divided into 6 different subsets, and ordered accordingly to their differentiation status: 1) $CD56^{bright}$, 2) $CD56^{dim}$ $NKG2A^{+}$ $CD57^{-}$ KIR^{-}, 3) $CD56^{dim}$ $NKG2A^{+}$ $CD57^{-}$ KIR^{+}, 4) $CD56^{dim}$ $NKG2A^{-}$ $CD57^{-}$ KIR^{+}, 5) $CD56^{dim}$ $NKG2A^{-}$ $CD57^{+}$ self-KIR^{+}, and 6) $CD56^{dim}$ $NKG2A^{-}$ $CD57^{+}$ self-KIR^{+} $NKG2C^{+}$.

We found that the differentiation process was associated with a gradual change in the expression of most of the studied receptors. Both percentage of positive cells and median fluorescent intensity (MFI) of CCR5, CCR7, CCR8 and CXCR3 progressively decreased along with the differentiation (Fig. 2a and b, Supplementary Figure S3). Notably, expression of these chemokine

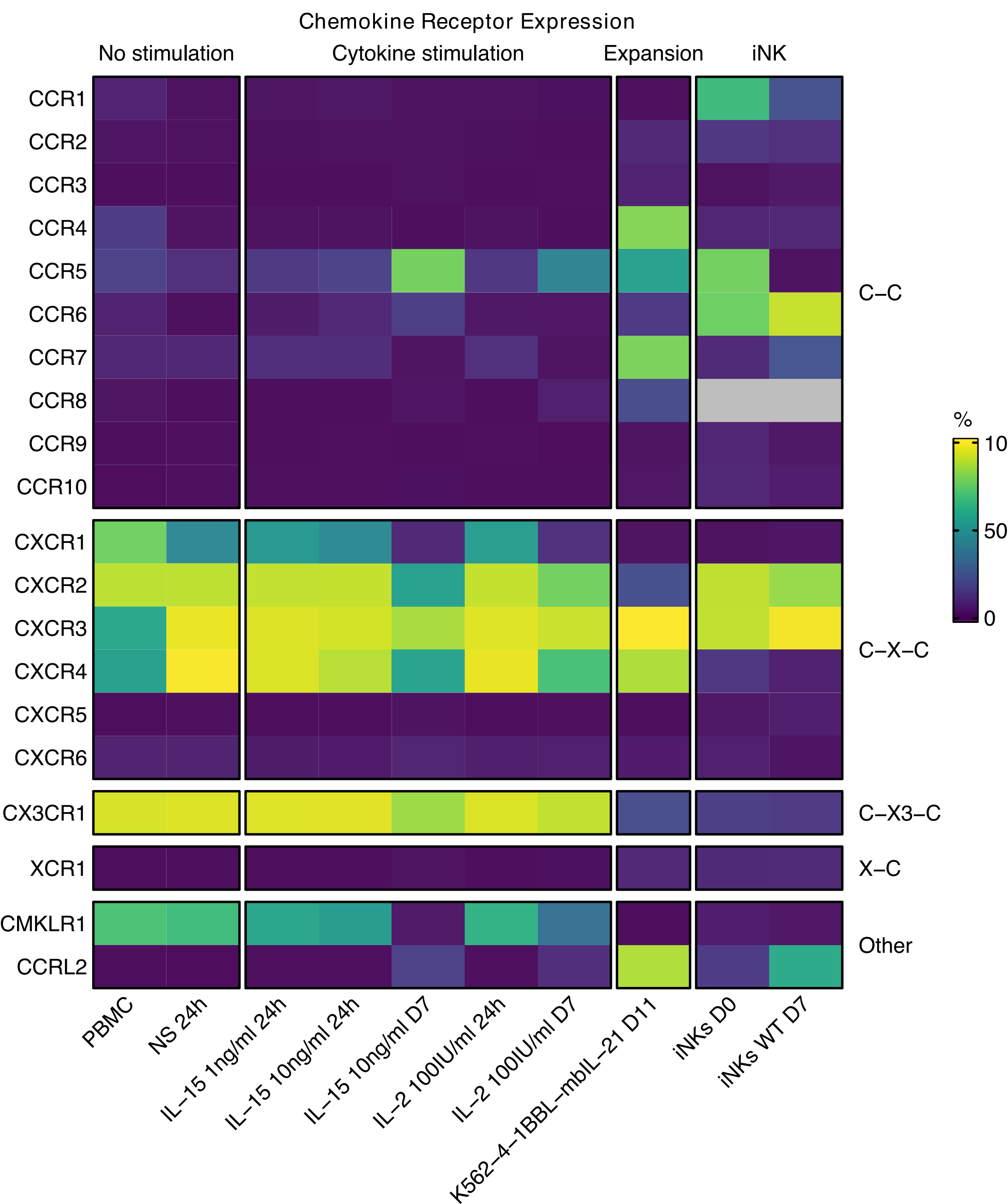

***Fig. 1:* Chemokine receptor expression in NK cells.** Chemokine receptor expression represented as percentage of cells expressing given chemokine receptor. Missing data is marked with gray. Vertically, chemokine receptors are grouped based on their structure (C–C, CX–C, CX3–C, X–C, and other chemotactic receptors). Horizontally, the NK cells are divided based on the condition (no stimulation, cytokine stimulation, 11-day feeder cell expansion of PBMC-derived NK cells, 7-day expansion of iPSC-derived NK cells). A panel of CD56, CD3, CD14, CD19, and a dead cell stain was used for each flow cytometry profiling to ensure adequate NK cell purity and that the chemokine receptors were profiled exclusively on live NK cells. Each experiment (n = 3) was performed on independent donors in two technical replicates. Data represents means.

receptors increased slightly in adaptive NK cells compared to the most differentiated but non-adaptive CD56^{dim} NKG2A^{-} CD57^{+} Self-KIR^{+} NKG2C^{-} cells. Conversely, CX3CR1, CXCR1, CXCR2, and CMKLR1 expression levels increased during NK cell differentiation, rising from less than 5% to almost 100% positive cells (Fig. 2a and b, Supplementary Figure S3). Again, the trend was inversed in adaptive NK cells, as their expression levels were slightly lower than in non-adaptive mature NK cells. CCR1, CCR6 and CXCR6 were primarily expressed by NKG2A^{+} positive CD56^{dim} NK cells (Supplementary Figure S3) while CXCR4 expression followed a sinusoidal pattern across all subsets (Fig. 2a and b). Expression of CCR4 was uniform in all subsets, while CCR2 was characterized by low expression, highest in the least mature subsets (Supplementary Figure S3). This data was verified in CyTOF experiment using a panel of chemokine receptors (CCR2, CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, CX3CR1) in 20 healthy PBMC donors (Supplementary Figure S4).

To confirm the relationship between differentiation and chemokine receptor expression, we analyzed single-cell RNA-seq data from PB NK cells from two healthy donors. First, cells were clustered and ordered accordingly to the differentiation status through trajectory analysis (Supplementary Figure S5). Then, the expression levels of chemokine receptors were projected on UMAP plots. The mRNA expression of all analyzed chemokine receptors showed subset-specific expression patterns that closely matched those observed at the protein level (Fig. 2a–c, Supplementary Figure S3A–S3C).

Next, we analyzed the expression of L-selectin (CD62L), along with other adhesion molecules: P-selectin glycoprotein ligand-1 (Psgl-1, CD162), Integrin B2 (CD18), Integrin B7 chain, ICAM-3 (CD50), and Integrin α M (CD11b) in a similar manner. Likewise, the expression of the adhesion molecules was also modulated by differentiation. The expression of L-selectin and CD44 decreased with differentiation, whereas CD162, CD11b, CD18, and ICAM-3 were upregulated (Supplementary Figure S6).

Based on these experiments, we selected a set of classical chemokine receptors that were expressed on >50% of at least one subpopulation of unstimulated PB NK cells for further characterization and downstream functional experiments. The receptors matching these criteria were CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1.

Effects of differentiation on migratory potential

To determine whether the observed differences in chemokine receptor expression between distinct subsets of NK cells affect their migratory potential, we employed the Transwell system. Solely the ligands of previously selected chemokine receptors (CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1) were included in these functional experiments. CX3CL1- and CXCL8-induced migration capability correlated with the differentiation status of NK cells, as did the expression pattern of their corresponding receptors (Fig. 3a). Conversely,

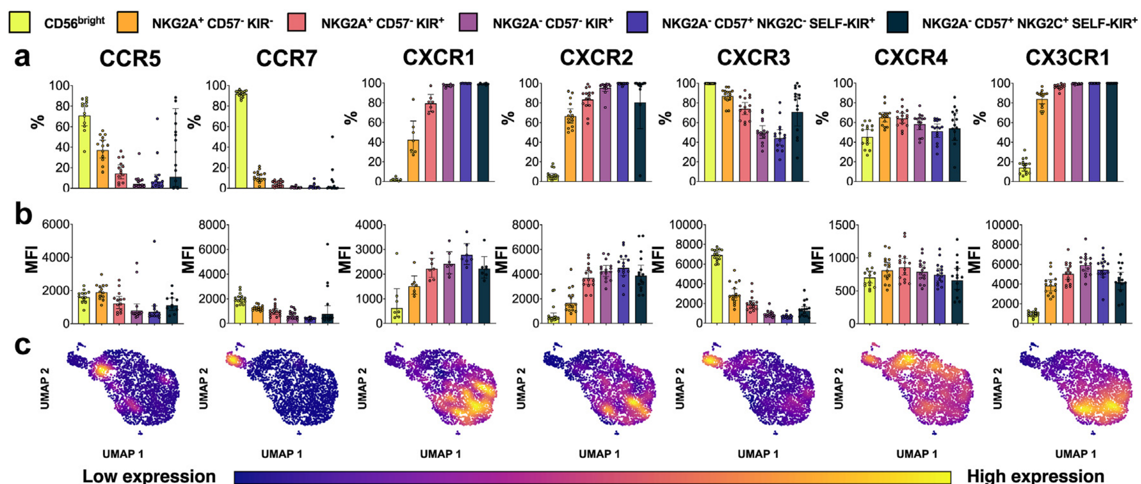

Fig. 2: **The effects of differentiation on NK cell chemotactic landscape.** NK cell subsets were identified by CD56, NKG2A, CD57, NKG2C, and KIR expression, and ordered accordingly to their differentiation status. Each heading applies to the whole column. The legend applies to sections (a and b). In (a and b) each dot represents an individual donor. Bars represents geometric mean with 95% confidence interval. Two technical replicates were assessed in each donor. The chemokine receptor expression is presented as (a) percent of cells expressing chemokine receptor, (b) median fluorescent intensity of chemokine receptor in positive population, and (c) chemokine receptor mRNA expression derived from single-cell RNA-seq data. The single-cell RNA-seq clustering and pseudotime analysis is presented in Supplementary Figures S5 and S9. The cells are positioned from the least mature (left, top) to most mature (right, bottom).

CCL19, CCL5, and CXCL10-induced migration capability decreased with NK cell differentiation, which corresponded to a decrease in receptor expression (Fig. 3a). In general, we observed that the chemokine-induced migration capability of each subset of NK cells reflected the expression level of the corresponding chemokine receptor. To assess the relationship between chemokine receptor protein levels and functional migration, we performed linear regression analysis which showed very high coefficients of determination (R^2) for nearly all chemokines tested (CCL5, CCL19, CXCL8, CXCL12, and CX3CL1) with R^2 ranging from 0.7495 to 0.9917, indicating that the surface expression of chemokine receptor is a critical factor determining migratory response to its ligand (Fig. 3b). We found that the optimal formula representing chemokine receptor expression, closely correlating with migratory response was *log2(MFI of the positive population + 1) * Percentage of Chemokine Receptor Expression*. In this context, adaptive NKG2C$^+$ NK cells stood out from other subsets, as they had a unique chemokine repertoire and migratory profile, with higher intrinsic migratory potential despite lower expression of chemokine receptors such as CXCR1, CXCR2, CXCR4, and CX3CR1, when compared to non-adaptive mature NK cells. We also found CXCR4 expression to be inversely correlated with CXCL12-induced migration due to high CXCL12-induced migratory response and relatively low CXCR4 expression in CD56^{bright} NK cells. This is likely due to higher intrinsic migratory potential of CD56^{bright}

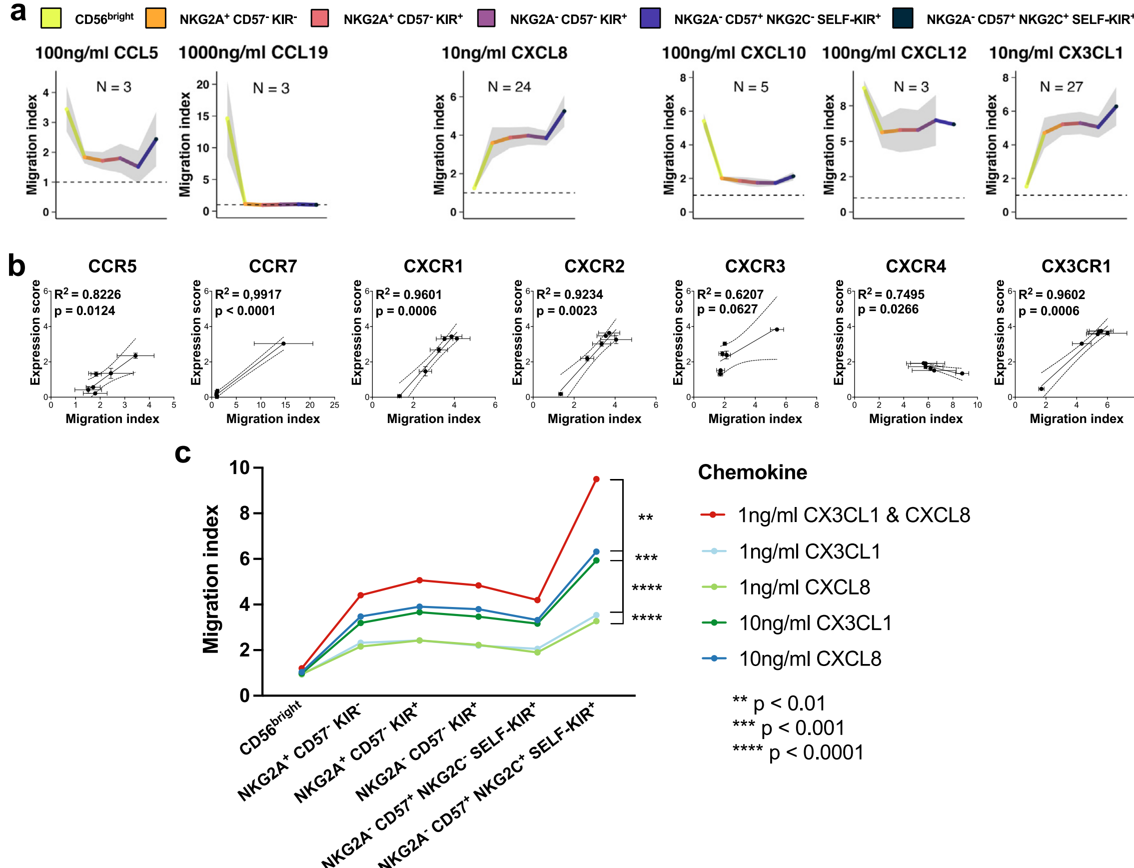

***Fig. 3:* NK cell chemokine-induced migratory responses in subset resolution.** NK cell subsets were identified by CD56, NKG2A, CD57, NKG2C, and KIR expression, and ordered accordingly to their differentiation status. (a) Transwell analysis of chemokine induced migration in a subset resolution. Migration index was calculated as a ratio of cells that have migrated to the bottom well during chemokine-induced migration compared to the cells that have spontaneously migrated to the bottom well, without any chemoattractant present. Data represents means ± SEM (gray area). Some chemokines (CX3CL1, CXCL8) were evaluated in more donors as they were used for the further experiments evaluating potential synergies (c). (b) A linear regression analysis between migration index and respective chemokine receptor protein expression (Fig. 2a and b). The chemokine receptor expression score was calculated as follows: *log2(MFI of the positive population + 1) * Percentage of Chemokine Receptor Expression*. P values were calculated by Wald's test. (c) Transwell analysis of combined effects between two chemokines in subset resolution. The experiment was performed on 10 donors. Data represents geometric mean. The differences between the curves were assessed using two-way ANOVA with post-hoc Dunnett's test.

NK cells and is in agreement with the literature reporting accumulation of $CD56^{bright}$ NK cells in tissues with high CXCL12 expression.[63–65]

Our flow cytometry data indicated that some NK cell subsets express multiple chemokine receptors. Therefore, we examined whether simultaneous ligation of multiple chemokine receptors would provide synergistic effects on NK cell migration. After titration experiments performed to find the concentrations needed to achieve peak migratory responses (Supplementary Figure S7), we stimulated the NK cells with optimal (10 ng/ml) or suboptimal (1 ng/ml) concentrations of CX3CL1 or CXCL8, as well as suboptimal concentration of both chemokines simultaneously (1 ng/ml CX3CL1 with 1 ng/ml CXCL8). Within all $CD56^{dim}$ subsets of NK cells, the combination of both chemokines induced a stronger migratory response than both suboptimal and optimal concentrations of each chemokine, confirming the presence of a synergistic effect between chemokine receptor stimulation in $NKG2C^{+}$ $CD57^{+}$ adaptive NK cells and additive effect in other $CD56^{dim}$ NK cell subsets, emphasizing the importance of studying the entire chemokine-chemokine receptor landscape (Fig. 3c, Supplementary Figure S8).

Exploring chemokine receptor co-expression patterns

Discovering synergistic effects of simultaneous chemokine receptor ligation prompted us to conduct a more detailed mapping of chemokine diversity within the NK cell repertoire. We designed a mass cytometry (CyTOF) panel to perform a parallel assessment of CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1 in the NK cell compartment. This chemokine-centric approach revealed that co-expression patterns of chemokine receptors alone could accurately identify classical NK cell subsets, such as $CD56^{bright}$, based on high CXCR3 and CCR7 expression, and $CD56^{dim}$ $NKG2A^{+}$ based on high expression of CXCR4 and modest expression of CXCR3 (Fig. 4). Interestingly, in more differentiated NK cell subsets (4,5,6,7) the expression patterns of chemokine receptors showed increasing complexity, suggesting an additional layer of functional diversification in subsets with identical expression of NK cell receptors and canonical markers (Fig. 4).

To corroborate our observation of increased chemokine receptor diversity in more differentiated NK cell subsets, we re-clustered single-cell RNA-seq data based on the expression of CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1. We identified 8 clusters with unique chemokine receptor combinations (Supplementary Figure S9A). These clusters were plotted onto the previously created UMAP plot (Supplementary Figure S5A and S9B) with a known differentiation trajectory (Supplementary Figure S9C). We noticed a substantial heterogeneity of chemokine receptors in NK cells, which was progressively increasing along with differentiation. This observation was further verified by the Shannon diversity index calculated for each of the "classical" clusters associated with NK differentiation (Supplementary Figure S9D). Our data indicates that NK cell differentiation not only alters chemokine receptor profile in a coordinated fashion but also does it in a way that progressively increases the overall diversity of chemokine receptors in NK cells.

Chemokine-based clustering of malignant tumors

The detailed profiling and functional assessment of primary PB NK cells in terms of chemokine receptor landscape suggested an increasing complexity associated with NK cell differentiation, along with a broad overlap in the expression of chemokine receptors across distinct subsets. We next addressed whether and how the underlying diversity in the chemokine repertoire could explain the sparsity of NK cell in solid tumors or identify suitable targets for NK cell therapy. To this end, we analyzed the chemokine landscape in solid tumors by mining RNA-seq data from TCGA and TARGET databases. Using harmonized RNA-seq data from TCGA and TARGET cohorts, we clustered 9471 primary solid tumors based on chemokine expression. Louvain algorithm-based clustering implemented in Seurat revealed 9 distinct patterns of chemokine co-expression within the tumors (Fig. 5a and b). Further, to assess the composition of each cluster, we calculated the proportion of cluster assignment for each tumor (Fig. 5c). The results showed that some clusters e.g., cluster 7 or 8, comprised a single histological tumor type (hepatocellular carcinoma (LIHC) and thymoma (THYM), respectively), whereas clusters 2 and 4 represented a significant fraction of more than 10 different histological tumor types. Strikingly, none of the clusters showed expression of ligands for more than one chemokine receptor on mature NK cells. The chemokine expression profile of each cluster allowed us to identify cluster 3 as the one most favoring the infiltration of expanded NK cells and potentially enhancing the efficacy of NK cell therapy, because of high CXCR3 and CCR4 expression on expanded NK cells. Since nearly 60% of DLBCL tumors have a chemokine profile of cluster 3, this tumor would appear to be a suitable target for evaluation of unmodified expanded NK cell therapy.

Discussion

NK cells are important innate effector cells in the human immune system.[3] Their function far exceeds the role initially attributed to them as a sentinel patrolling the human body. They are a group of cells residing in both lymphoid and non-lymphoid tissues and performing additional functions such as participating in the normal development of spiral vessels during pregnancy or modulating T-cell polarization through the secretion of cytokines.[3,66–69] The multifaceted role of NK cells and

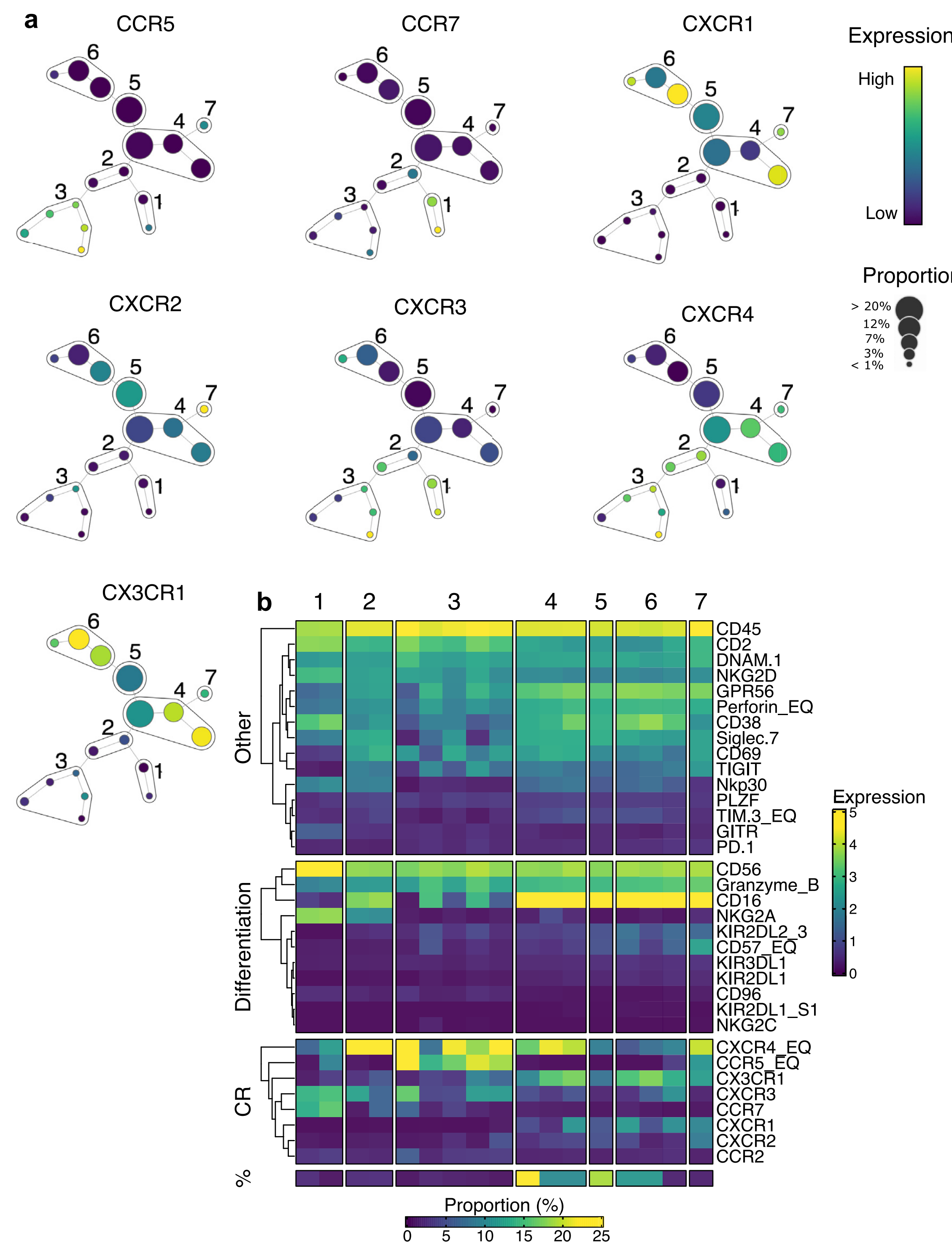

Fig. 4: **Mass cytometry profiling of chemokine receptor co-expression on peripheral blood NK cells.** (a) Results of SPADE clustering of peripheral blood NK cells based on expression of 7 chemokine receptors: CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4, CX3CR1. The size of the circles reflects number of cells in the group. The color scale reflects the chemokine receptor expression. (b) Expression levels (MFI) of chemokine receptors, NK cell differentiation markers and other NK cell receptors in SPADE clusters (1–7). The bottom row of the heatmap shows the frequency of each cluster in bulk peripheral blood NK cells. The experiment was performed on samples from 20 healthy donors.

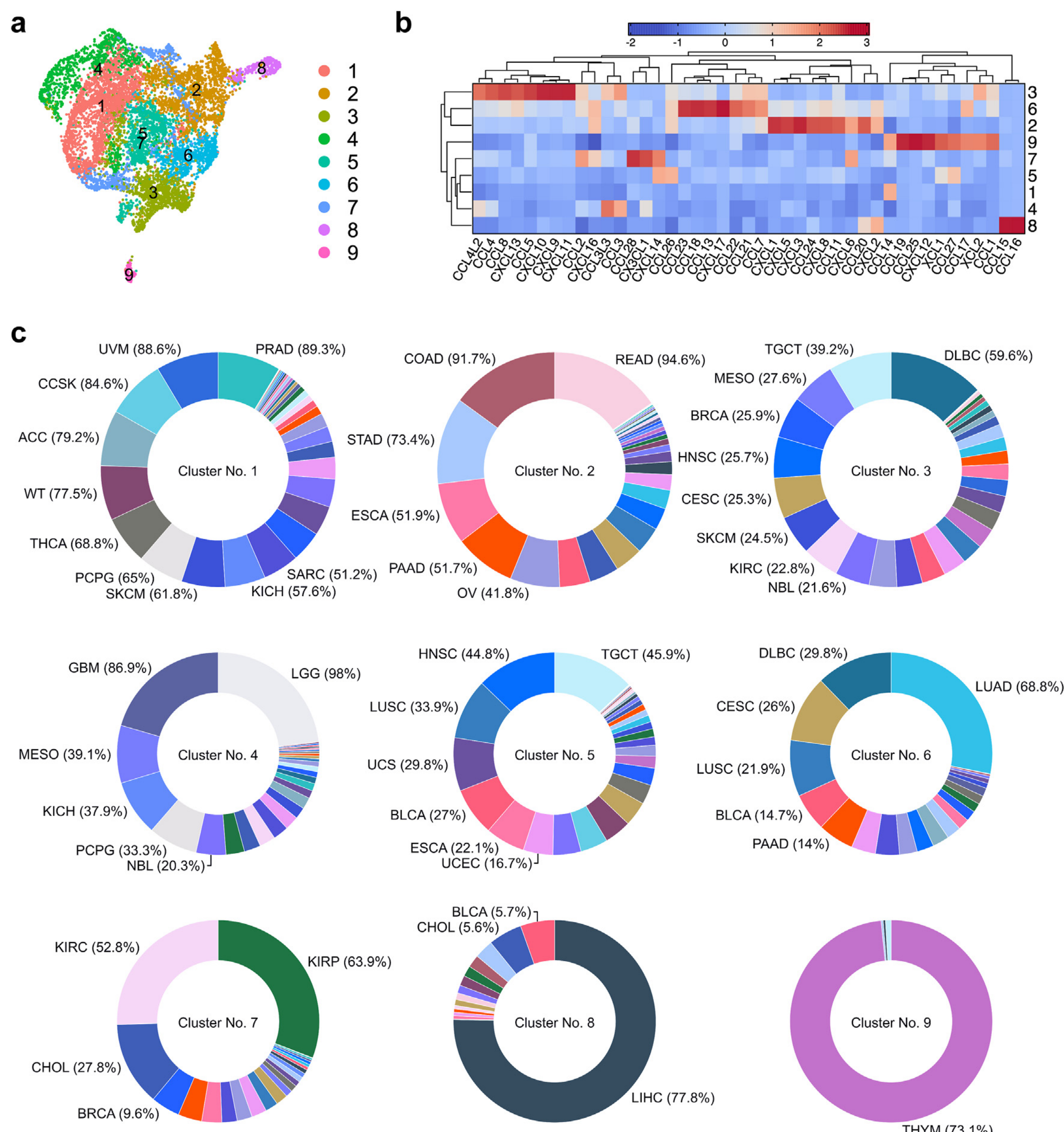

Fig. 5: **Chemokine landscape of 9471 primary solid tumors from TCGA and TARGET cohorts.** (a) UMAP plot visualizing the results of clustering 9471 primary solid tumors from TCGA and TARGET cohorts based on chemokine expression. (b) Expression of chemokines across nine identified clusters. (c) Proportion of cluster assignment for each tumor visualized as donut plots. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; LGG, brain lower grade glioma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; CCSK, clear cell sarcoma of the kidney; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; NBL, neuroblastoma; OV, Ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THYM, thymoma; THCA, thyroid carcinoma; UCS, uterine carcinosarcoma; UCEC, uterine corpus endometrial carcinoma; UVM, uveal melanoma; WT, wilms tumor.

the high phenotypic and functional diversity of this population requires adequate trafficking capability, which is controlled by a specific repertoire of chemotactic receptors, responding to environmental signals mediated by chemokines.[70] While the effects of differentiation on NK cell cytotoxicity, cytokine secretion, proliferation and metabolism are well documented, previous studies provide limited and fragmented information regarding the expression of chemokine receptors among different NK cell subsets, without concluding whether it is regulated by mechanisms related to the functional maturation of these cells and the stimulatory effects of cytokines.[22,69,71] Using high-dimensional flow and mass cytometry, we corroborate and extend previous studies, showing that NK cells express a wide range of chemotactic receptors, identifying CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 receptors among those most abundantly expressed on the surface of NK cells.[72,73] Importantly, multi-parameter profiling of PB NK cells by mass cytometry revealed distinct and coordinated chemokine repertoires associated with NK cell differentiation process.

Each chemokine has its own specific set of functions in the immune system, based on which it can be classified as mostly related to innate immunity, adaptive immunity, or both.[74] In this study, we found that receptors associated with adaptive immunity, characteristic for T cells, such as CCR5, CCR7 and CXCR3, were most prominently expressed in immature $CD56^{bright}$ NK cells, while receptors associated with innate immunity (neutrophils, macrophages) were expressed in mature NK cells. This implies vast differences in functionality of NK cell subsets and supports the notion that immature $CD56^{bright}$ NK cells are not a dysfunctional subset being solely the precursor to mature NK cell subsets, rather they are an important self-standing component of the human immune system. $CD56^{bright}$ NK cells are considered efficient cytokine producers with immunoregulatory properties.[69] In this context, our data indicates that the chemokine receptor profile of $CD56^{bright}$ NK cells is essential for their function, by facilitating their migration to lymph nodes where upon endogenous T cell-derived IL-2 or dendritic cell-derived IL-12, $CD56^{bright}$ NK cells secrete T helper type 1 (Th1) cytokines, such as IFN-γ and TNF-α, that favor Th1 T cell polarization and influence the developing antigen-specific immune response.[69,75,76]

Mature NK cells are on the other side of the functionality spectrum. While under certain conditions they can also be potent producers of cytokines, their main proposed function is to directly kill virus-infected cells and tumor cells through cell-mediated cytotoxicity.[71] Here, we demonstrate that their chemokine receptor repertoire ($CXCR1/2^{+}$, $CX3CR1^{+}$) is similar to that of patrolling monocytes and neutrophils, indicating that their role is likely to involve patrolling peripheral tissues.[77,78] The repertoire of neutrophil-like chemokine receptors may also be important for neutrophil-NK cell crosstalk, which has recently been shown to be an important process in NK cell maturation.[79]

As chemokine receptors cooperate with adhesion molecules such as selectins and integrins to guide cell migration, we analyzed the expression of L-selectin (CD62L), P-selectin glycoprotein ligand-1 (Psgl-1, CD162), Integrin B2 (CD18), Integrin B7 chain, ICAM-3 (CD50), and Integrin α M (CD11b) in NK cell subsets. Similarly to chemokine receptors, we found that their levels were modulated by differentiation as the expression of L-selectin and CD44 decreased with NK cell differentiation, while CD162, CD11b, CD18, and ICAM-3 were upregulated. The decline in CD62L in more mature NK cells was previously reported.[80] Notably, functionally related chemokine receptors and adhesion molecules, e.g., CD62L and CCR7 (secondary lymphoid organ homing via HEV), CX3CR1-CD162 (extravasation), followed the same expression pattern in subsets at different stages of NK cell differentiation.

The profile of chemokine receptors among NK cells, although relatively stable, is not fixed and can change dynamically. In this study, we found that external stimuli affect its composition both in the short and long timeframe. Culture of isolated PB NKs without additional stimulation can alter the quantitative expression of the major receptors, leading to a decrease in CXCR1 and CXCR2 with an increase in CXCR3 and CXCR4 expression. Stimulation with both IL-2 and IL-15 in long-term cultures (7-day) resulted in a complete loss of CXCR1 expression, downregulation of CXCR2, CXCR4, CX3CR1 and CMKLR1 with a marked upregulation of CCR5 expression. The plasticity and responsiveness of NK cells to environmental stimuli, as reflected by profound changes in profile of chemokine receptors, is also indicated by the results of NK cells undergoing expansion with K562-based feeder cells with membrane-bound IL-21 and 4-1BBL, which shifts NK cells toward responsiveness to chemokines associated with adaptive immunity. Since physiological concentrations of cytokines modulate chemokine receptor expression and therefore affect NK cell migration, they may be used to enhance the efficacy of cell therapies. However, our study also highlights the risk for diverting their homing away from the intended target organ.

To assess whether differences in the expression of surface chemokine receptors correspond to the chemokine responsiveness of different subsets of NK cells, we used the Transwell system coupled with high-dimensional flow cytometry. We observed that the migratory response was closely associated with the expression of the corresponding chemokine receptor, indicating that it is the most important factor determining chemokine responsiveness. Furthermore, we investigated whether simultaneous ligation of multiple chemokine receptors would provide synergistic effects

allowing to bypass the limitations of a single ligand-receptor chemokine pair. To this end, we stimulated NK cells with optimal and suboptimal concentrations of CXCL8 and CX3CL1, as well as a combination of suboptimal concentrations of both chemokines. We found that simultaneous ligation with suboptimal concentrations of both chemokines resulted in a stronger migratory response than both suboptimal and optimal concentration of each chemokine separately. Thus, equipping NK cells for adoptive therapy with more than one chemokine receptor to guide NK cells can enhance tumor infiltration compared to the use of a single chemokine receptor. Such an approach would also reduce the chance of tumor immune evasion through loss of expression of one of the targeted chemokines.

The observation of synergistic effects following simultaneous chemokine receptor ligation prompted us to carry out a more detailed mapping of chemokine receptor co-expression using mass cytometry. Clustering of PB NK cells based on chemokine receptor expression revealed that co-expression patterns alone could accurately identify immature classical NK cell subsets, such as CD56^{bright} and CD56^{dim} NKG2A^{+} cells. However, in differentiated NK cell subsets, the expression patterns of chemokine receptors were more diverse. This finding adds another layer to the functional diversification of NK cells based on migratory potential. In this context, the most mature adaptive NKG2C^{+} cells showed a unique chemokine receptor profile, with high expression of receptors typical of both mature and immature NK cells. Additionally, despite lower expression of CXCR1, CXCR2, CXCR4 and CX3CR1 than CD56^{dim} CD57^{+} NKG2A^{-} KIR^{+} NK cells, their migratory response was more potent. This indicates that other important factors, besides chemokine receptor expression, come into play and influence the strength of chemokine-induced NK cell migration.

The ability of cells to infiltrate a tumor is a fundamental prerequisite for anti-tumor immunity.[35,39,81] The chemokine receptor co-expression patterns and their synergistic action, prompted us to search for potential reasons explaining the low abundance of mature NK cells in solid tumors, as well as for appropriate tumor types that could be effectively targeted by adoptive NK cell therapy.[32,35] In the context of chemokine receptor synergy following simultaneous ligation, we classified primary solid tumors by re-clustering 9471 primary solid tumors (35 different tumor types) from the TCGA and TARGET cohorts based solely on chemokine expression. We identified nine clusters with distinct chemokine expression patterns. Strikingly, none of them expressed chemokine ligands for more than one chemokine receptor expressed on mature NK cells (CXCR1/2, CXCR4, CX3CR1). On the other hand, the chemokine receptor profile (CCR5^{+}, CCR7^{+}, CXCR3^{+}) of Th1 T cells allows for dual chemokine receptor ligation in clusters 3, 6, and 9.[82–86] Myeloid cells are even more compatible with the chemokine milieu of solid tumors, with four clusters (3, 4, 6, 7) ligating more than one chemokine receptor expressed on classical monocytes (CCR1, CCR2, CX3CR1).[87–89] Thus, the selection pressure during early stages of tumor microevolution appears to favor infiltration of immunosuppressive cells while excluding mature NK cells with antitumor potential, possibly through avoidance of NK cell chemokine receptor synergy. Importantly, we consider CXCR1 and CXCR2 as one as they are naturally co-expressed in immune cells and share CXCL8 as their main ligand.[90] We speculate that the lack of synergistic chemokine input may be one factor contributing to the sparse infiltration of mature NK cells in solid tumors. Further work is needed to explore how these distinct chemokine networks correlate with NK cell infiltration in more complex *in vitro* and *in vivo* models and ultimately how this relate to clinical outcomes.

Two of the discovered clusters of primary tumors (cluster 3 and 6) showed expression of multiple ligands for chemokine receptors on expanded NK cells. Cluster 3 was characterized by high expression of CCL3, CCL4, CCL4L2, CCL5 (CCR5 ligands), CXCL9, CXCL10, CXCL11 (CXCR3 ligands) and moderate expression of CCL21 (CCR7 ligand) and CCL22 (CCR4 ligand), while cluster 6 expressed high levels of CCL21 (CCR7 ligand) and CCL22 (CCR4 ligand). Together, almost 90% of DLBCL tumors belong to one of these clusters, indicating that diffuse large B-cell lymphoma may be a permissive target for adoptive feeder cell-based NK cell therapy in terms of its chemokine milieu. As the role of infiltrating NK cells in DLBCL is already partially established as high circulating NK cell count is associated with improved outcomes, it further reinforces the idea of NK cell-based immunotherapy.[91,92] Aside from the importance of natural cytotoxicity, NK cell-mediated ADCC plays a crucial role in improving treatment outcomes of patients treated with rituximab using R-CHOP regimen.[91,92]

Another promising therapeutic approach is iPSC-derived NK cells. iNKs can be fine-tuned through numerous genetic modifications to achieve stronger effector function, introduce tailored specificities and promote persistence, and are therefore attractive candidates for cancer immunotherapy.[93] Profiling of the chemokine receptor repertoire on one example of a multi-edited iNK line showed that the chemokine receptor profile shared similarities with PB NK cells, e.g., high CXCR2 and CXCR3 expression, but also had some unique features e.g., high expression of CCR1 and CCR6. These data need to be interpreted with some caution given that different iNK cell clones may vary in their receptor repertoire. Given the unique possibility to perform multiple gene edits in the iNK cell platform, adding one or more homing receptors to promote synergistic signaling and homing to the tumor type seems

like an attractive approach to enhance iNK-cell based immunotherapy.

In summary, our data provide a comprehensive map of the chemokine receptor landscape in NK cells and reveal an unexpected diversity in the chemokine receptor co-expression that collectively control migratory responses to chemokine gradients. Our observations may also serve as a basis for engineering next-generation adoptive NK cell therapies against hematological malignancies and solid tumors.

Contributors

Conceptualization–M.L., K.-J.M., data curation–M.L., K.-J.M., formal analysis–M.L., K.-J.M., funding acquisition–M.L., K.-J.M., R.Z., investigation–M.L., K.Z., D.P., M.K., H.J.H., M.T.W., methodology–M.L., M.K., H.J.H., M.T.W., project administration–M.L., L.K., R.Z., K.-J.M., resources–M.L., K.Z., W.H., R.Z., K.-J.M., software–M.L., supervision–R.Z., K.-J.M., validation–L.P., L.K., verification of the underlying data–M.L., K.-J.M., visualization–M.L., K.-J.M., writing—original draft–M.L., K.-J.M., and writing—review & editing–M.L., K.Z., W.H., R.Z., K.-J.M. All authors have read and approved the final version of the manuscript.

Data sharing statement

The datasets used and/or analyzed during the current study available from the corresponding authors on reasonable request.

Declaration of interests

Karl-Johan Malmberg is a consultant and has research grants from Fate Therapeutics Inc., and is a member of the advisory board at Vycellix. Radoslaw Zagozdzon is an ad hoc scientific consultant for Pure Biologics S.A. (Wroclaw, Poland) and 4Cell Therapies S.A. (Gliwice, Poland). The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104811.

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Mapping the Chemotactic Landscape in NK Cells Reveals Subset-specific Synergistic Migratory Responses to Dual Chemokine Receptor Ligation

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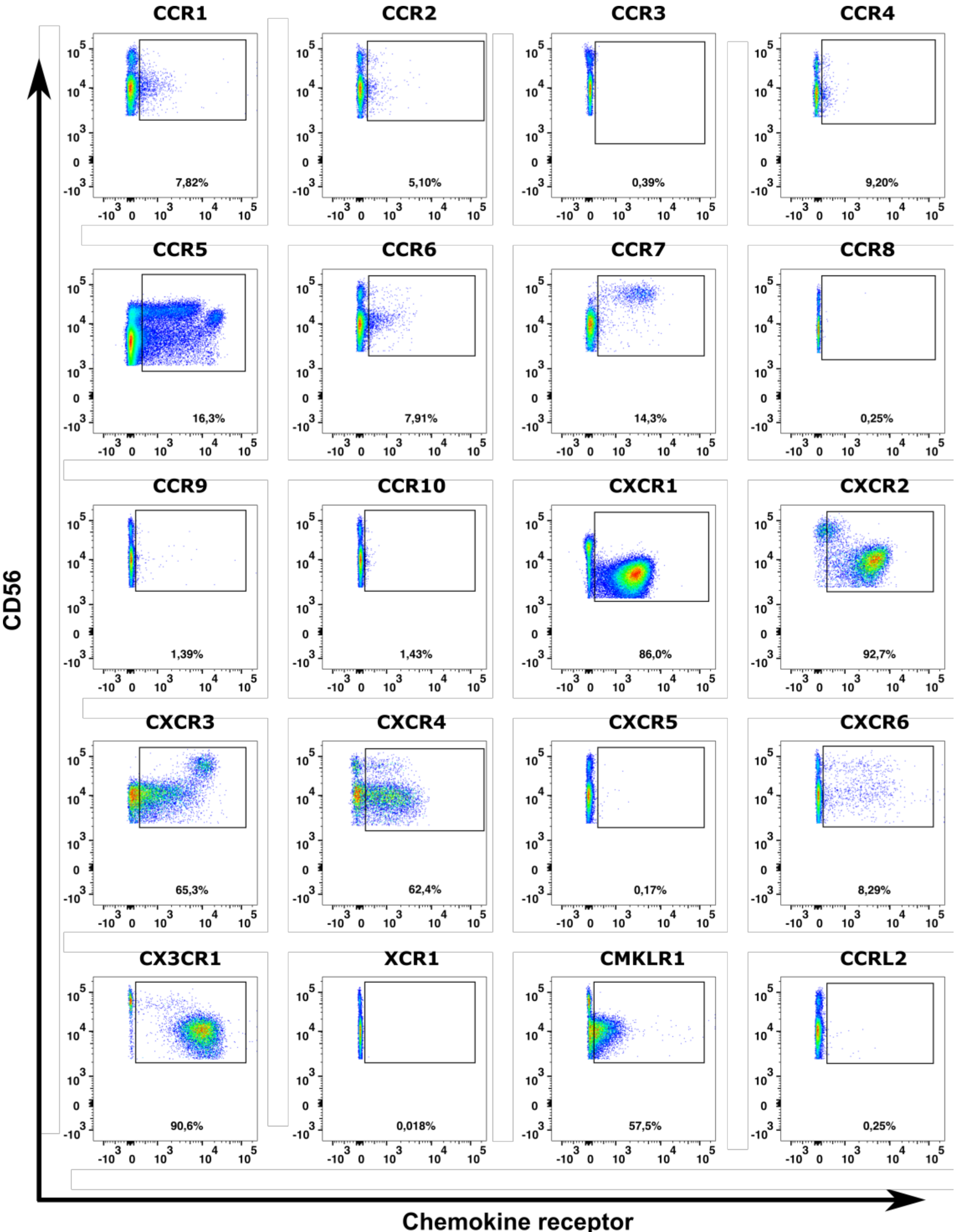
Supplementary Data

Fluorochrome	Antigen	Clone	Dilution	Vendor
V500	CD14	M5E2	1:50	BD Bioscience
V500	CD19	HIB19	1:50	BD Bioscience
VioGreen	CD3	REA613	1:50	Miltenyi
Biotin	CD56	REA196	1:50	Miltenyi
APC-Vio770	CD57	REA769	1:50	Miltenyi
BV786	NKG2A	131411	1:50	BD Bioscience
APC	NKG2A	Z199	1:25	Beckman Coulter
AF700	NKG2C	134591	1:50	R&D
PE	NKG2C	REA205	1:50	Miltenyi
PerCp	KIR2DL3	REA147	1:50	Miltenyi
FITC	KIR2DL1	REA284	1:50	Miltenyi
BV421	KIR3DL1	REA1005	1:50	Miltenyi
PerCp	KIR2DL2/L3/S2	GL183	1:25	Beckman Coulter
PE-Vio770	KIR2DL1/S1	REA1010	1:10	Miltenyi
AF700	Granzyme B	GB11	1:100	BD Bioscience
PE	CD62L	DREG-56	1:50	Biolegend
PE	CD162	KPL-1	1:50	Biolegend
PE	CD50	CRB-IC3/1	1:50	Biolegend
PE	Integrin B71	FIB504	1:50	Biolegend
PE	CD11b	M1/70	1:50	Biolegend
PE	CD18	TS1/18	1:50	Biolegend
APC	CCRL2	152254	1:50	Novus
PE & APC	CCR1	REA158	1:50	Miltenyi
PE & APC	CCR2	REA264	1:50	Miltenyi
PE & VioBlue	CCR3	REA574	1:50	Miltenyi
PE & APC	CCR4	REA279	1:50	Miltenyi
PE & APC	CCR5	REA245	1:50	Miltenyi
PE & APC	CCR6	REA190	1:50	Miltenyi
PE & APC	CCR7	REA108	1:50	Miltenyi
PE	CCR8	L263G8	1:50	Biolegend
PE & APC	CCR9	REA469	1:50	Miltenyi
PE & APC	CCR10	REA326	1:50	Miltenyi
PE & VioBlue	CXCR1	REA958	1:50	Miltenyi
PE & APC	CXCR2	REA208	1:50	Miltenyi
PE & APC	CXCR3	REA232	1:50	Miltenyi
PE & APC	CXCR4	REA649	1:50	Miltenyi
PE & APC	CXCR5	REA103	1:50	Miltenyi
PE & APC	CXCR6	REA458	1:50	Miltenyi
PE & APC	CX3CR1	2A9-1	1:25	Miltenyi
PE & APC	CMKLR1	REA455	1:50	Miltenyi
PE	XCR1	1097A	1:50	R&D

Supplementary Table S1. The list of antibodies used in flow cytometry experiments.

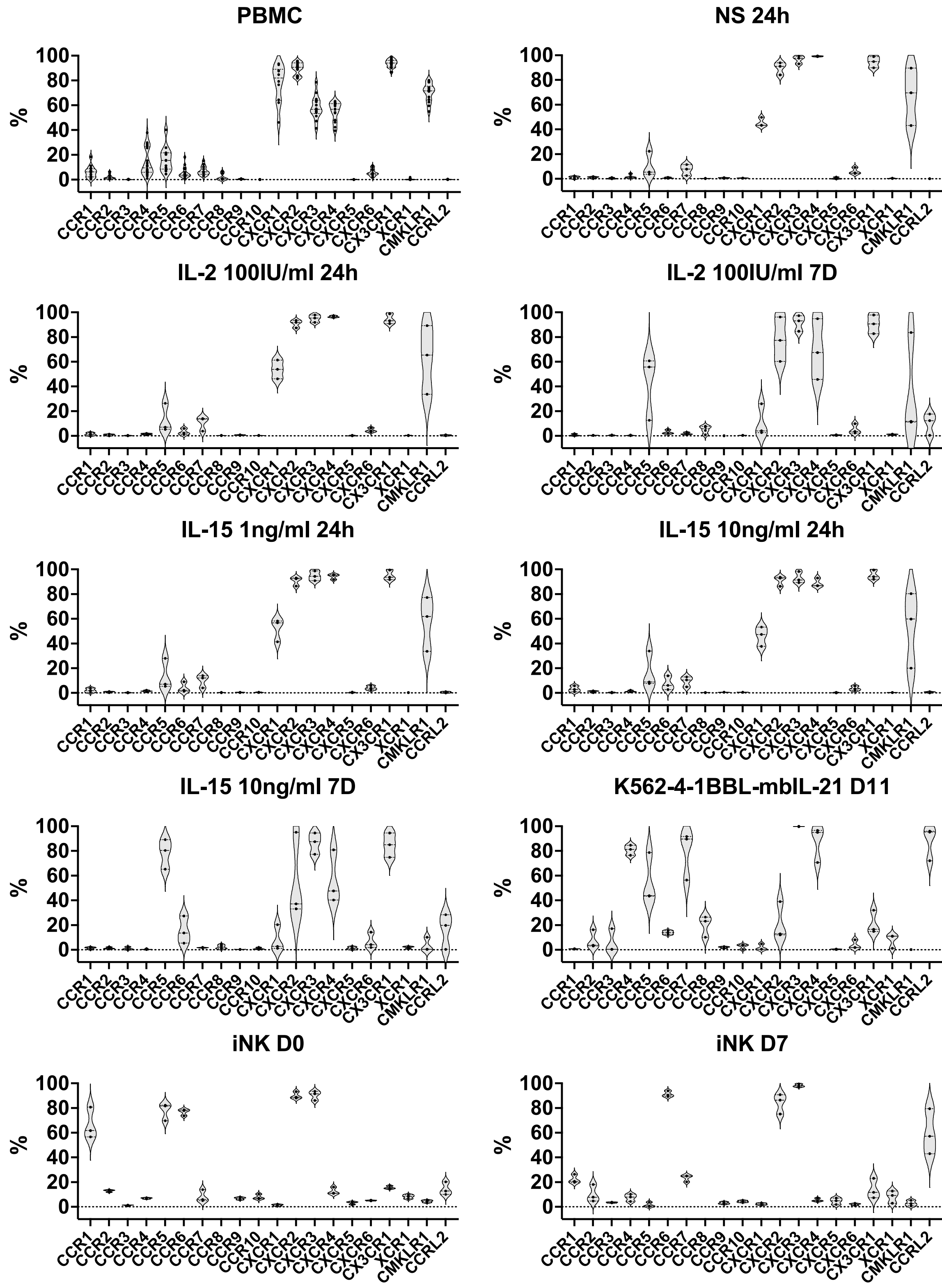
Mass	Antigen	Clone	Dilution	Vendor
89Y	CD45	HI30	1:400	Fluidigm
141Pr	CX3CR1	REA385	1:100	Miltenyi
142Nd	CD57	HCD57	1:1600	Fluidigm
143Nd	CD2	TS1/8	1:800	Biolegend
144Nd	CD38	REA572	1:800	Miltenyi
145Nd	CXCR3	REA232	1:50	Miltenyi
146Nd	CCR2	REA538	1:50	Miltenyi
147Sm	CD96	NK92.39	1:100	Biolegend
148Nd	CD3	OKT3	1:800	Biolegend
148Nd	CD14	RMO52	1:400	Fluidigm
148Nd	CD19	HIB19	1:400	Biolegend
149Sm	FasL	NOK-1	1:100	Biolegend
150Nd	LAG3	11C3C65	1:200	Fluidigm
151Eu	CXCR4	REA649	1:200	Miltenyi
152Sm	Siglec-7	194211	1:100	Fluidigm
153Eu	TIM-3	F38-2E2	1:100	Fluidigm
154Sm	NKG2C	REA205	1:1600	Miltenyi
155Gd	CXCR1	REA958	1:200	Miltenyi
156Gd	KIR2DL1/S1	11PB6	1:100	Miltenyi
158Gd	KIR2DL1	REA284	1:50	Miltenyi
159Tb	GITR	108-17	1:200	Biolegend
160Gd	GPR56	CG4	1:600	Biolegend
161Dy	PLZF	17.10,.17	1:100	Biolegend
162Dy	CD69	FN50	1:200	Fluidigm
163Dy	KIR2DL2/S2/L3	GL183	1:400	Beckman Coulter
164Dy	TIGIT	MBSA43	1:200	Thermo Fisher
165Ho	CCR5	REA245	1:800	Miltenyi
166Er	NKG2D	ON72	1:100	Fluidigm
167Er	CCR7	REA108	1:100	Miltenyi
168Er	NKp30	P30-15	1:100	Miltenyi
169Tm	NKG2A	Z199	1:200	Fluidigm
170Er	CXCR2	REA208	1:50	Miltenyi
171Yb	DNAM-1	DX11	1:200	Fluidigm
172Yb	KIR3DL1	DX9	1:800	R&D
173Yb	Granzyme B	GB11	1:400	Fluidigm
174Yb	PD-1	EH12.2H7	1:100	Fluidigm
175Lu	Perforin	B-D48	1:400	Fluidigm
176Yb	CD56	NCAM16.2	1:400	Fluidigm
209Bi	CD16	3G8	1:200	Fluidigm

Supplementary Table S2. The list of antibodies used in mass cytometry experiment.



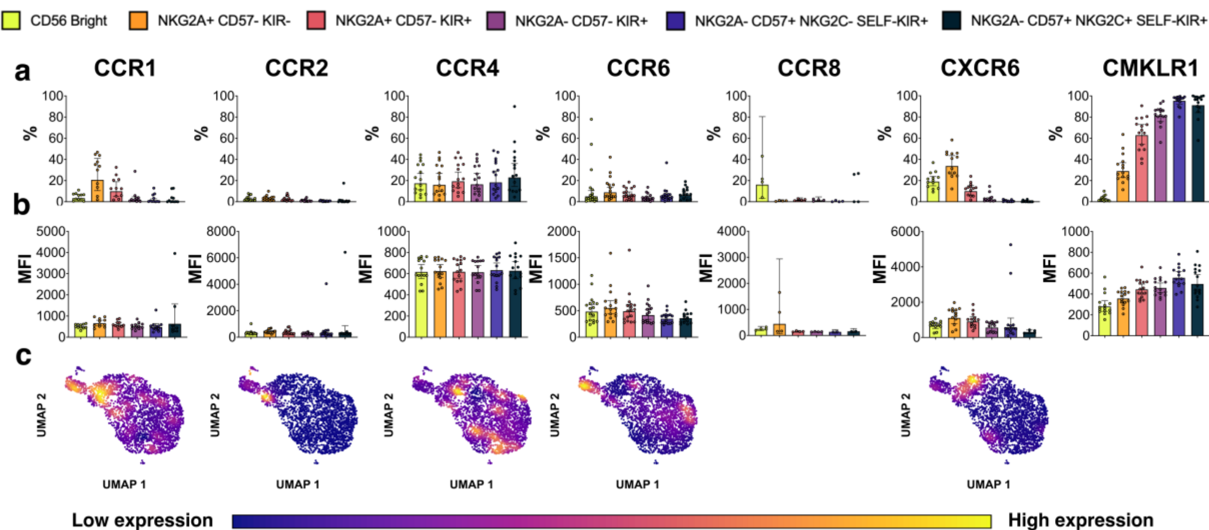
Supplementary Figure S1. Representative staining of chemokine receptors on peripheral blood NK cells.

NK cells were identified as live, single, CD56+ CD3-, CD14-, CD19- cells in peripheral blood mononuclear cells isolated from healthy donors. Each plot represents expression of chemokine receptor (X-axis) and CD56 (Y-axis).



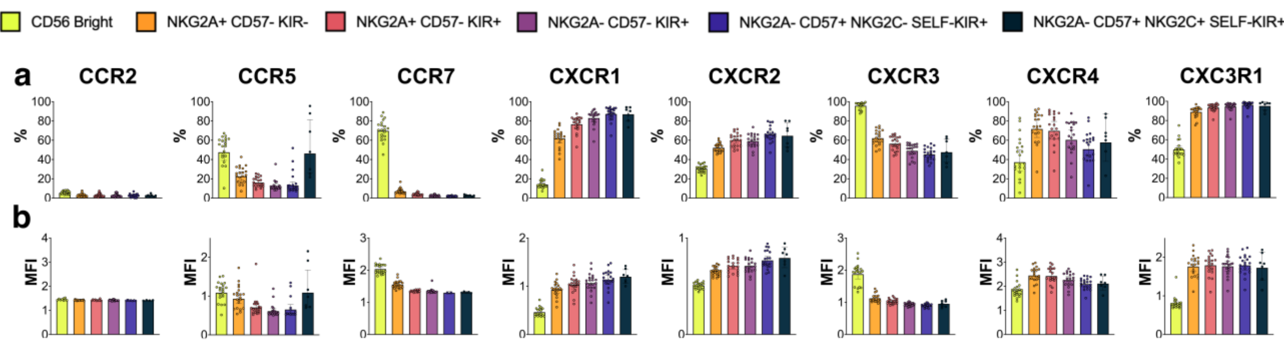
Supplementary Figure S2. Chemokine receptor expression in NK cells.

NK cells in PBMC, after isolation, with and without different stimuli were profiled for chemokine receptor expression. Chemokine receptor expression is represented as percentage of cells expressing given chemokine receptor. Each experiment (n=3) was performed on independent donors. Each dot represents each experiment/donor.



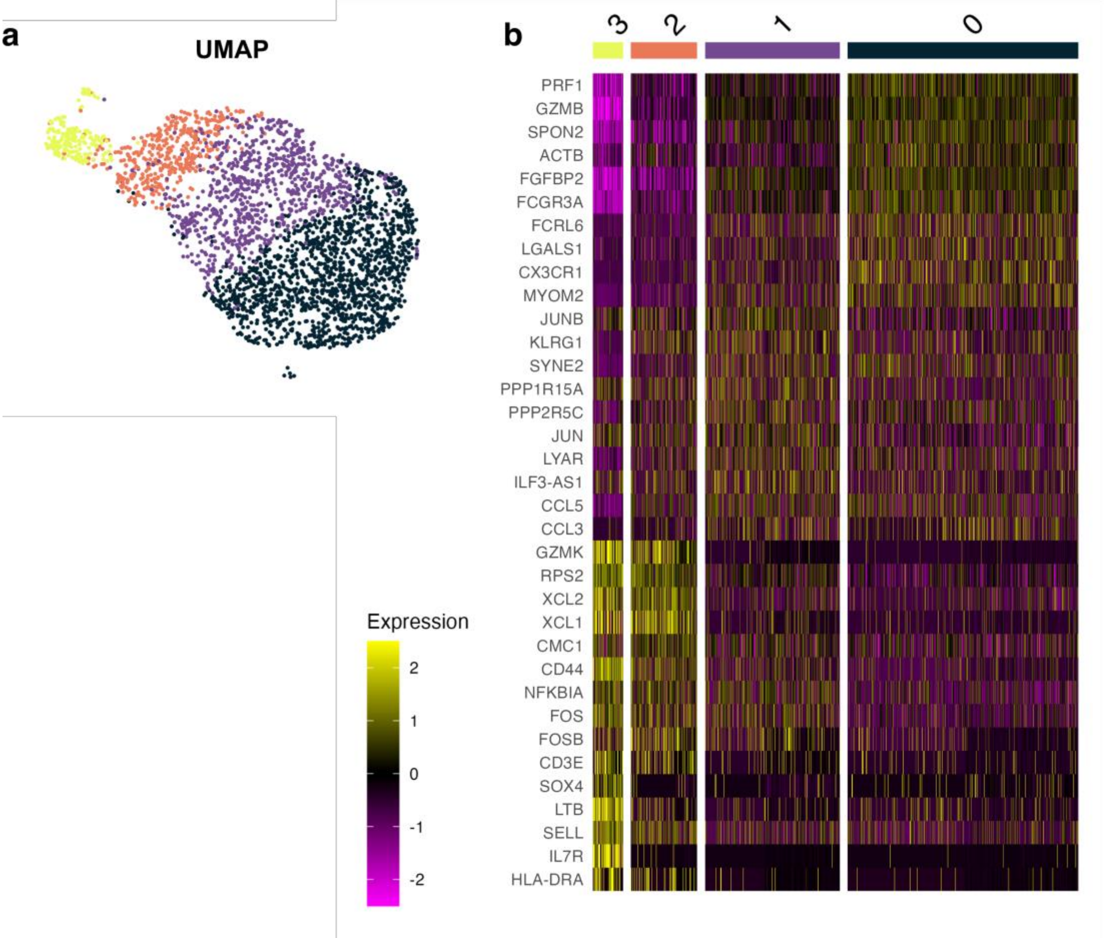
Supplementary Figure S3. The effects of NK cell differentiation on expression of other chemokine receptors.

NK cell subsets were identified by CD56, NKG2A, CD57, KIR and NKG2C expression and ordered accordingly to their differentiation status. Each heading applies to the whole column. The legend applies to sections A and B where each dot represents an individual donor. Bars in (**A**) and (**B**) represent geometric mean with 95% confidence interval. Two technical replicates were assessed in each donor. The chemokine receptor expression is presented as (**A**) percent of cells expressing a marker (**B**) median fluorescent intensity (**C**) chemokine receptor mRNA expression derived from single-cell RNA-seq data. The single-cell RNA-seq clustering and pseudotime analysis is presented in Supp. Fig. 5 & 9. The cells are positioned from the least mature (left, top) to most mature (right, bottom).



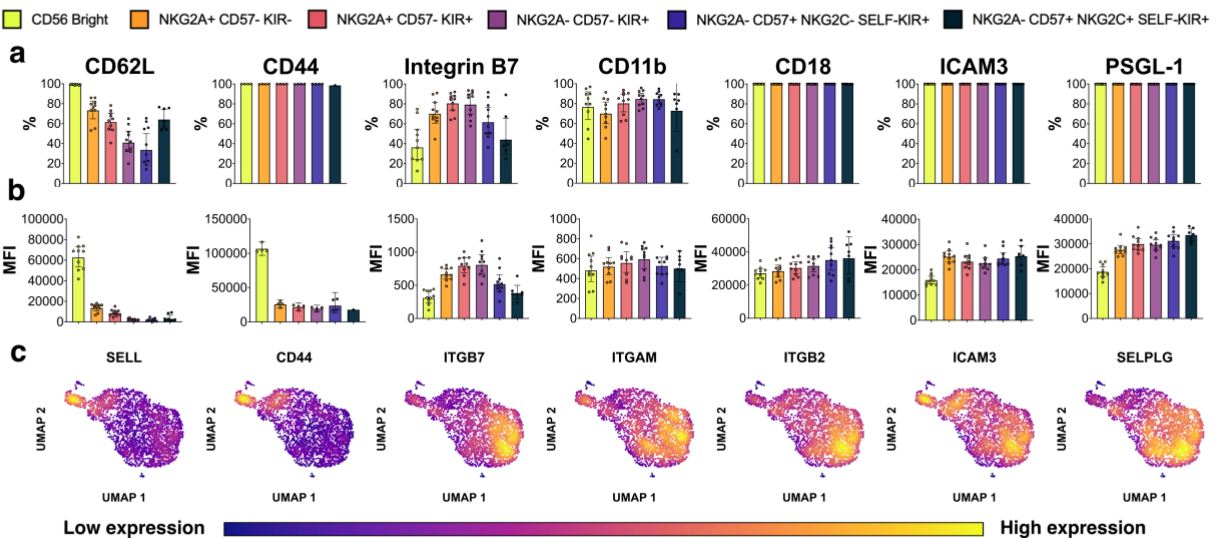
Supplementary Figure S4. The effects of NK cell differentiation on expression of chemokine receptors verified by mass cytometry.

The experiment was performed on cryopreserved PBMC samples from 20 healthy donors. NK cell subsets were identified in the same gating scheme as in Figure 2. Briefly, NK cell subsets were identified by CD56, NKG2A, CD57, KIR and NKG2C expression and ordered accordingly to their differentiation status. Each heading applies to the whole column. The chemokine receptor expression is presented as (**A**) percent of cells expressing a marker (**B**) median staining intensity. Each dot represents an individual donor. Bars represent geometric mean with 95% confidence interval.



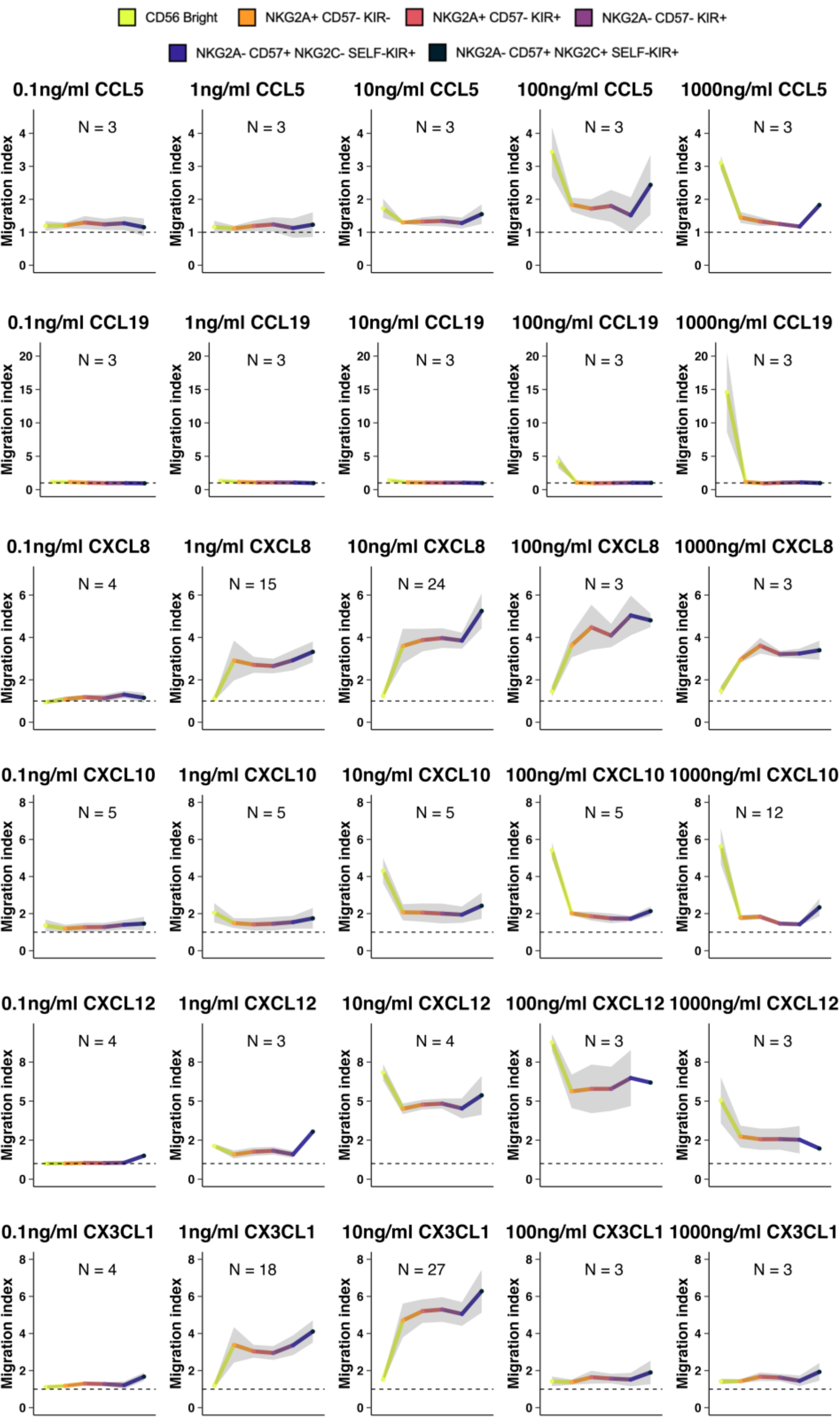
Supplementary Figure S5. Unbiased clustering of human peripheral blood NK cells.

A. Four distinct human PB NK clusters were numbered and displayed with an UMAP plot **B.** Top 10 up-regulated DEGs (ranked by log fold change) of each cluster are presented as a heatmap.



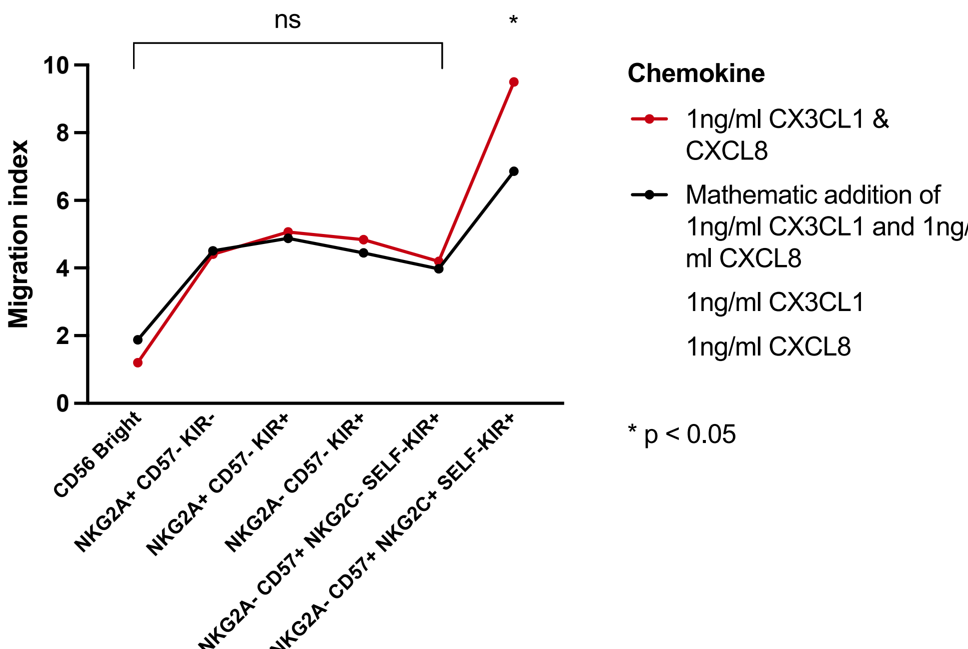
Supplementary Figure S6. The effects of differentiation on NK cell adhesion molecule expression.

NK cell subsets were identified by CD56, NKG2A, CD57, KIR and NKG2C expression and ordered accordingly to their differentiation status. Each heading applies to the whole column. The legend applies to sections A and B where each dot represents an individual donor. Bars in (**A**) and (**B**) represent geometric mean with 95% confidence interval. Two technical replicates were assessed in each donor. The adhesion molecule expression is presented as (**A**) percent of cells expressing a marker (**B**) median fluorescent intensity (**C**) adhesion molecule mRNA expression derived from single-cell RNA-seq data. The single-cell RNA-seq clustering and pseudotime analysis is presented in Supp. Fig. 5 & 9. The cells are positioned from the least mature (left, top) to most mature (right, bottom).


CD56 Bright
NKG2A+ CD57- KIR-
NKG2A+ CD57- KIR+
NKG2A- CD57- KIR+
NKG2A- CD57+ NKG2C- SELF-KIR+
NKG2A- CD57+ NKG2C+ SELF-KIR+
0.1ng/ml CCL5
1ng/ml CCL5
10ng/ml CCL5
100ng/ml CCL5
1000ng/ml CCL5
0.1ng/ml CCL19
1ng/ml CCL19
10ng/ml CCL19
100ng/ml CCL19
1000ng/ml CCL19
0.1ng/ml CXCL8
1ng/ml CXCL8
10ng/ml CXCL8
100ng/ml CXCL8
1000ng/ml CXCL8
0.1ng/ml CXCL10
1ng/ml CXCL10
10ng/ml CXCL10
100ng/ml CXCL10
1000ng/ml CXCL10
0.1ng/ml CXCL12
1ng/ml CXCL12
10ng/ml CXCL12
100ng/ml CXCL12
1000ng/ml CXCL12
0.1ng/ml CX3CL1
1ng/ml CX3CL1
10ng/ml CX3CL1
100ng/ml CX3CL1
1000ng/ml CX3CL1
Migration index
N = 3
N = 4
N = 15
N = 24
N = 5
N = 12
N = 18
N = 27

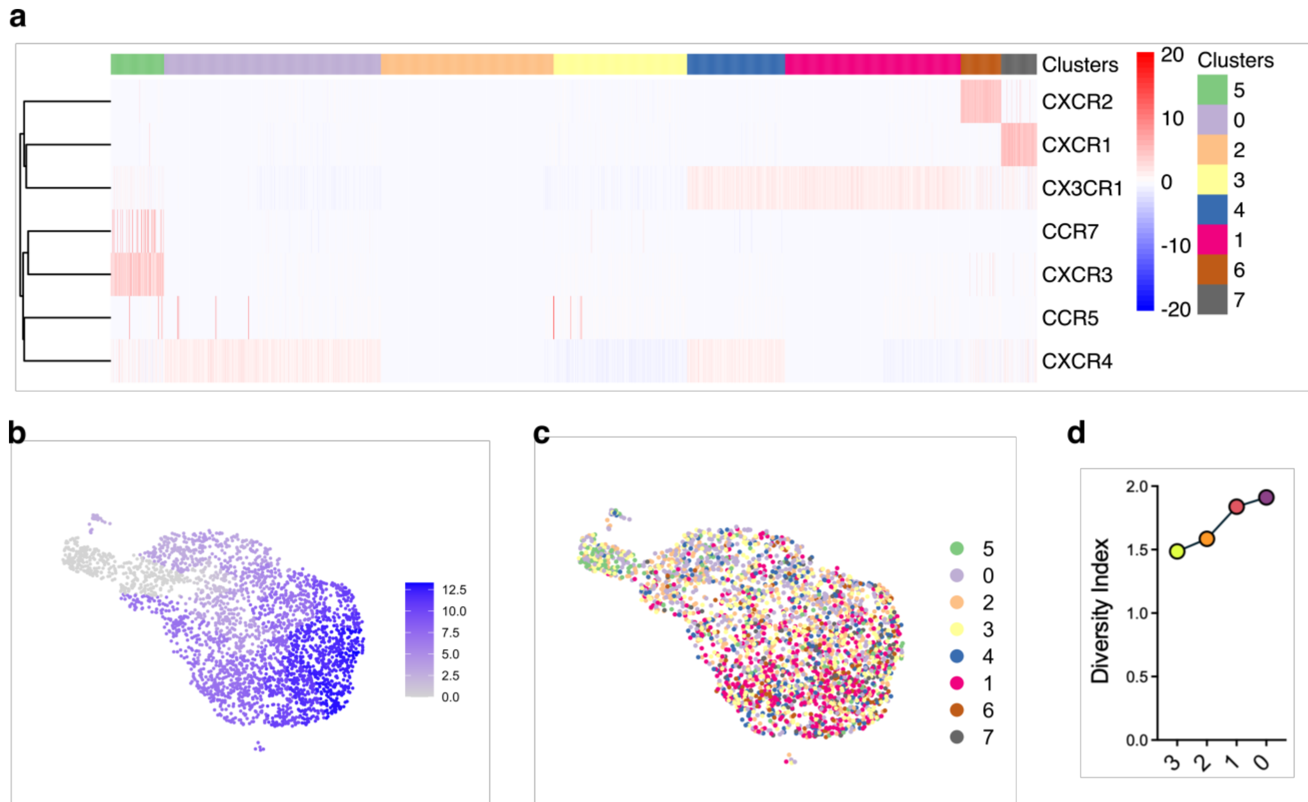
Supplementary Figure S7. Chemokine titration in NK cell subsets

A transwell-based titration of different chemokines in a subset resolution. Migration index was calculated as a ratio of cells that have migrated to the bottom well during chemokine-induced migration compared to the cells that have spontaneously migrated to the bottom well, without any chemoattractant present. NK cell subsets were identified by CD56, NKG2A, CD57, NKG2C, and KIR expression, and ordered accordingly to their differentiation status. "N" specifies number of donors in each condition. Data represents means ± SEM (gray area). Some chemokines (e.g., CX3CL1, CXCL8) in some concentrations (e.g., 1ng/ml, 10ng/ml) were evaluated in more donors as they were used for the further experiments evaluating potential synergies (Figure 3).



Supplementary Figure S8. Synergistic or additive effects of chemokine receptor simultaneous ligation?

Analysis of synergistic effects between two chemokines at the subset resolution. The migration index was calculated as a ratio of cells that have migrated to the bottom well during chemokine-induced migration compared to the cells that have spontaneously migrated to the bottom well, without any chemoattractant present. NK cell subsets were identified by CD56, NKG2A, CD57, NKG2C, and KIR expression, and ordered accordingly to their differentiation status. The experiment was performed on ten donors. Data represents geometric mean. The differences between the curves representing 1ng/ml CX3CL1 & 1ng/ml CXCL8 and the mathematic addition of migration index of 1ng/ml CX3CL1 and 1ng/ml CXCL8 was assessed for each subset using the Wilcoxon test, which revealed statistically significant difference in adaptive $CD56^{dim}$ $NKG2A^{-}$ $CD57^{+}$ $NKG2C^{+}$ self-KIR^{+} NK cell subset.



Supplementary Figure S9. Chemokine receptor-based clustering of peripheral blood NK cells.

The results of clustering peripheral blood NK cells based on expression of 7 chemokine receptors: CCR5, CCR7, CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1. **A.** Distribution of chemokine receptors in each cluster. **B.** Pseudotime visualizing developmental trajectories assigned to each cell in UMAP plot. **C.** Chemokine-receptor clustering results visualized as identity-colored UMAP plot. **D.** Chemokine receptor diversity calculated with Shannon index in NK cell differentiation-based clusters.

7. Podsumowanie i wnioski

Komórki NK są obiecującym narzędziem w rozwijaniu nowej generacji terapii komórkowych w onkologii. Warunkiem przeniesienia sukcesu terapii komórkowej z nowotworów hematologicznych na guzy lite jest zdolność migracji komórek do TME. W przeciwieństwie do innych komórek, dojrzałe komórki NK są rzadko znajdowane w guzach litych, a wzorce migracji komórek NK były dotąd słabo poznane.

W przedstawionej pracy, podsumowano obecny stan wiedzy na temat infiltracji komórek NK w nowotworach litych, ich zalet jako komórek efektorowych w immunoterapii oraz czynników hamujących zdolność migracji komórek NK do komórek nowotworowych. Następnie, zbadano wzorce migracji komórek NK za pomocą cytometrii przepływowej, cytometrii masowej (CyTOF) i sekwencjonowania RNA pojedynczych komórek, w połączeniu z testami funkcjonalnymi. Odkryto, że profil receptorów chemokinowych komórek NK z krwi obwodowej zmienia się w skoordynowany sposób podczas procesu dojrzewania oraz w wyniku stymulacji cytokinami lub tzw. „feeder cells". Ponadto, wykazano, że ekspresja receptorów chemokinowych jest ściśle skorelowana z odpowiedzią migracyjną różnych podtypów komórek NK. Co więcej, jednoczesna ligacja receptorów CXCR1/2 i CX3CR1 ma działanie synergistycznie, znacznie zwiększając odpowiedź migracyjną w porównaniu do stymulacji tylko jednego z receptorów. Badanie danych sekwencjonowania RNA 9471 nowotworów litych w bazach danych TCGA i TARGET ujawniło dziewięć dominujących profili chemokin, które różniły się między typami nowotworów, ale żaden z nich nie miał ligandów dla więcej niż jednego receptora chemokinowego na dojrzałych komórkach NK. Tym samym, cele pracy zostały osiągnięte.

Nasze wyniki sugerują, że brak naturalnie występujących par chemokiny-receptory chemokinowe może wyjaśniać systematyczne wykluczenie komórek NK z mikrośrodowiska guzów litych i reprezentować znaczący potencjał dla inżynierii nowej generacji terapii komórkowych opartych na komórkach NK.

Oświadczenia współautorów publikacji

Warszawa, 22.09.2023
(miejscowość, data)

Mieszko Lachota
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 73%.

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(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Marianna Vincenti
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 73%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

Marianna Vincenti
....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Magdalena Winiarska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 73%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

MWiniarska
....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Kjetil Boye
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 73%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Radosław Zagożdżon
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w pisaniu artykułu i jego redakcja przed wysłaniem, zapewnienie finansowania.

Mój udział procentowy w przygotowaniu publikacji określam jako 10%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 73%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Karl-Johan Malmberg
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Prospects for NK Cell Therapy of Sarcoma" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania, konceptualizację pracy, udział w przeglądzie literatury, udział w pisaniu artykułu i jego redakcja przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 73%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Joanna Domagała
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Konceptualizacja pracy, przegląd literatury, przygotowanie figur i przygotowanie rozdziałów:

1. Introduction
2. Biological Aspects of NK Cell Cytotoxicity
2.1. NK Cells' Metabolism
2.3. Formation of the Lytic NK-Cell Immunological Synapse
    2.3.1. Recognition Stage
    2.3.2. Effector Stage
    2.3.3. Termination Stage
3. Characteristics of the Tumor Microenvironment
3.1. Tumor Hypoxia and Acidosis
4. How Tumor Microenvironment Factors Inhibit NK Cells
4.1. NK Cells' Metabolism
4.2. NK Cells Recruitment to the Tumor Site
4.3. NK Cells' Lytic Synapse
    4.3.1. Recognition Stage
    4.3.2. Effector Stage
4.4. NK Cells' Cytokines and Chemokines Production
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions
6. Conclusions

Mój udział procentowy w przygotowaniu publikacji określam jako 60 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

....

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Mieszko Lachota
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Mój udział procentowy w przygotowaniu publikacji określam jako 7.5 %.

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Marta Kłopotowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury i w przygotowaniu rozdziałów:

3.2. Oxidative Stress

4.1. NK Cells' Metabolism

Mój udział procentowy w przygotowaniu publikacji określam jako 7.5 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,
(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty
(imię i nazwisko kandydata do stopnia)

Marta Kłopotowska
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Agnieszka Graczyk-Jarzynka
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury i w przygotowaniu rozdziałów:

3.3. Cytokines

4.4. NK Cells' Cytokines and Chemokines Production

5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions Mój udział procentowy w przygotowaniu publikacji określam jako 5.5 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

Warszawa, 19.09.2023 (miejscowość. data)

Antoni Domagała
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury i w przygotowaniu rozdziałów:

2.4. NK Cells' Cytokine Production

3.4. Amino Acid deprivation

5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

Mój udział procentowy w przygotowaniu publikacji określam jako 5.5 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

Antoni Domagała
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Andriy Zhylko
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury i w przygotowaniu rozdziałów:

3.4. Amino Acid deprivation

5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

Zhylko Andriy ....

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Karolina Soroczyńska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Udział w przeglądzie literatury i w przygotowaniu rozdziałów:

3.4. Amino Acid deprivation

5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

Karolina Soroczyńska

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 19.09.2023 (miejscowość, data)

Magdalena Winiarska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Tumor Microenvironment—A Metabolic Obstacle to NK Cells' Activity" Activity oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Konceptualizacja pracy, przegląd literatury i udział w przygotowaniu rozdziałów:

1. Introduction
2. Biological Aspects of NK Cell Cytotoxicity
3. Characteristics of the Tumor Microenvironment
4. How Tumor Microenvironment Factors Inhibit NK Cells
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions
6. Conclusions

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.

Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 7.5 %,

(imię i nazwisko kandydata do stopnia)

obejmował on:

Udział w przeglądzie literatury, redakcja pracy przed wysłaniem, udział w przygotowaniu rozdziałów:

2.2. NK Cells Recruitment to the Tumor Site
3.5. Alterations in the Key Enzymes of Lipid and Adenosine Metabolism
4.2. NK Cells Recruitment to the Tumor Site
5. Strategies to Overcome the Inhibitory Effects of TME on NK Cell Functions

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty

(imię i nazwisko kandydata do stopnia)

....

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Mieszko Lachota
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 70%.

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Katarzyna Zielniok
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w eksperymentach badających profil receptorów chemokinowych na komórkach NK i ich zdolność do migracji. Redakcja artykułu przed wysłaniem.

-

Mój udział procentowy w przygotowaniu publikacji określam jako 3.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

Katarzyna Zielniok
....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Daniel Palacios
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w eksperymentach badających profil receptorów chemokinowych na komórkach NK i ich zdolność do migracji. Redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 2%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

Daniel Palacios O.
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Minoru Kanaya
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Ekspansja komórek iNK.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

Minoru Kanaya
....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Leena Peena
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Weryfikacja danych.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Hanna Julie Hoel
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Udział w optymalizacji panelu do cytometrii mas (CyTOF).

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,

(imię i nazwisko kandydata do stopnia)

obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.

(imię i nazwisko kandydata do stopnia)

Hanna Julie Hoel
....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Merete Thune Wiiger
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Ekspansja komórek NK za pomocą komórek K562-mbIL21-4-1BBL.

Mój udział procentowy w przygotowaniu publikacji określam jako 1%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,

(imię i nazwisko kandydata do stopnia)

obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.

(imię i nazwisko kandydata do stopnia)

Merete Thune Wiiger
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Lise Kveberg
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Weryfikacja danych.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

Lise Kveberg

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Wojciech Hautz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 0.5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 22.09.2023
(miejscowość, data)

Radosław Zagożdżon
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania, nadzór nad projektem i redakcja artykułu przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 5%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Oslo, 22.09.2023
(miejscowość, data)

Karl-Johan Malmberg
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zapewnienie finansowania, konceptualizacja pracy, nadzór nad projektem, weryfikacja danych, udział w pisaniu artykułu i jego redakcja przed wysłaniem.

Mój udział procentowy w przygotowaniu publikacji określam jako 15%.
Wkład lek. Mieszka Lachoty w powstawanie publikacji określam jako 70%,
(imię i nazwisko kandydata do stopnia)
obejmował on: konceptualizację pracy, zapewnienie finansowania, przegląd literatury, opracowanie metodyki badania, przeprowadzenie eksperymentów, analiza i interpretacja zebranych danych, przygotowanie figur, napisanie artykułu i jego redakcja przed wysłaniem.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Mieszka Lachoty.
(imię i nazwisko kandydata do stopnia)

....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Analiza bibliometryczna dorobku publikacyjnego

BIBLIOTEKA UCZELNIANA

Nr referencyjny
BIBG/Punktacja/ 663 /2023/KK **Warszawa, 09.10.2023**

Sz. Pan
Mieszko Lachota

ANALIZA BIBLIOMETRYCZNA CAŁOKSZTAŁTU DOROBKU PUBLIKACYJNEGO
PANA MIESZKA LACHOTY,
W POSTĘPOWANIU O NADANIE STOPNIA NAUKOWEGO DOKTORA

Lp.	Opis bibliograficzny	Impact Factor	MEiN
I. Artykuły opublikowane w czasopismach naukowych lub w recenzowanych materiałach z konferencji międzynarodowych ujętych w aktualnym wykazie MEiN[1]			
1.	**Lachota M**, et al. Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation. EBioMedicine. 2023;96:1-16 [Rodzaj publikacji: praca oryginalna]	11,100	140
2.	Krysa K, Kowalczyk E, Borysowski J, **Lachota M**, Pasierski T. Exclusion of older adults from clinical trials in cancer-related pain. Frontiers in Medicine. 2022;9:1-10 [Rodzaj publikacji: praca oryginalna]	3,900	70
3.	Kłopotowska M, Bajor M, Graczyk-Jarzynka A, Kraft A, Pilch Z, (...), **Lachota M**, et al. PRDX-1 supports the survival and antitumor activity of primary and CAR-modified NK cells under oxidative stress. Cancer Immunology Research. 2022;10(2):228-244 [Rodzaj publikacji: praca oryginalna]	10,100	200
4.	**Lachota M** [aut. koresp.], Lennikov A, Malmberg K, Zagożdżon R. Bioinformatic Analysis Reveals Central Role for Tumor-Infiltrating Immune Cells in Uveal Melanoma Progression. Journal of Immunology Research. 2021;2021:1-18 [Rodzaj publikacji: praca oryginalna]	4,493	100
5.	Kaleta B, **Lachota M**, et al. Osteopontin Gene Polymorphisms rs1126616 C>T and rs1126772 A>G are Associated with Atopic Dermatitis in Polish Population. Application of Clinical Genetics. 2021;2021(14):417-425 [Rodzaj publikacji: praca oryginalna]	-	200

[1] Wykaz sporządzony zgodnie z przepisami wydanymi na podstawie art. 267 ust. 2 pkt 2 lit. b Ustawy z dnia 20 lipca 2018 r. - Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2022 r., poz. 574 z późn. zm.). Wykaz stanowi załącznik do komunikatu MEiN z 17 lipca 2023 r. w sprawie wykazu czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych.

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02-091 Warszawa
www.biblioteka.wum.edu.pl

tel.: +48 22 116 60 11
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6.	Hikage F, Lennikov A, Mukwaya A, **Lachota M**, et al. NF-κB activation in retinal microglia is involved in the inflammatory and neovascularization signaling in laser-induced choroidal neovascularization in mice. Experimental Cell Research. 2021;403(1):1-12 [Rodzaj publikacji: praca oryginalna]	4,145	100
7.	**Lachota M**, et al. Prospects for NK Cell Therapy of Sarcoma. Cancers. 2020;12(12):1-31 [Rodzaj publikacji: praca poglądowa]	6,639	140
8.	Domagała J, **Lachota M**, Kłopotowska M, Graczyk-Jarzynka A, Domagala A, Zhylko A, Soroczyńska K, Winiarska M. The Tumor Microenvironment-A Metabolic Obstacle to NK Cells' Activity. Cancers. 2020;12(12):1-36 [Rodzaj publikacji: praca poglądowa]	6,639	140
9.	Mukwaya A, Lennikov A, Xeroudaki M, Mirabelli P, **Lachota M**, et al. Time-dependent LXR/RXR pathway modulation characterizes capillary remodeling in inflammatory corneal neovascularization. Angiogenesis. 2018;21(2):395-413 [Rodzaj publikacji: praca oryginalna]	5,894	40
10.	Lennikov A, Mirabelli P, Mukwaya A, Schaupper M, Thangavelu M, **Lachota M**, et al. Selective IKK2 inhibitor IMD0354 disrupts NF-κB signaling to suppress corneal inflammation and angiogenesis. Angiogenesis. 2018;21(2):267-285 [Rodzaj publikacji: praca oryginalna]	5,894	40
	Łącznie:	58,804	1170
II. Artykuły opublikowane przed 1.01.2019 r. w czasopismach ujętych w wykazie czasopism MNiSW z dnia 25.01.2017 r., o ile czasopismo uzyskało co najmniej 10 pkt.			
	Łącznie:	-	-
III. Pozostałe artykuły			
	Łącznie:	-	-
	Łącznie (cz. I- III):	**58,804**	**1170**
IV. Monografie naukowe/rozdziały w monografiach wydane przez wydawnictwa ujęte w wykazie MEiN[2] lub jednostki organizacyjne podmiotów, których wydawnictwa są ujęte w tym wykazie			
brak			
V. Pozostałe monografie lub rozdziały w monografiach			
brak			
VI. Patenty			
brak			

p.o. DYREKTOR
Biblioteki Uczelnianej
Warszawskiego Uniwersytetu Medycznego
mgr Agnieszka Czarnecka

[2] Wykaz sporządzony zgodnie z przepisami wydanymi na podstawie art. 267 ust. 2 pkt 2 lit. a Ustawy z dnia 20 lipca 2018 r. - Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2022 r., poz. 574 z późn. zm.). Wykaz ogłoszony komunikatem MEiN z dnia 22 lipca 2021 r. w sprawie wykazu wydawnictw publikujących recenzowane monografie naukowe.

WARSZAWSKI UNIWERSYTET MEDYCZNY

BIBLIOTEKA UCZELNIANA

Nr referencyjny
BIBG/Punktacja/ 664 /2023/KK

Warszawa, 09.10.2023

Sz. Pan
Mieszko Lachota

ANALIZA BIBLIOMETRYCZNA PUBLIKACJI
PANA MIESZKA LACHOTY,
WCHODZĄCYCH W SKŁAD CYKLU PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

Lp.	Opis bibliograficzny	Impact Factor	MEiN
Artykuły			
1.	**Lachota M**, et al. Prospects for NK Cell Therapy of Sarcoma. Cancers. 2020;12(12):1-31 [Rodzaj publikacji: praca poglądowa]	6,639	140
2.	Domagala J, **Lachota M**, Kłopotowska M, Graczyk-Jarzynka A, Domagala A, Zhylko A, Soroczyńska K, Winiarska M. The Tumor Microenvironment-A Metabolic Obstacle to NK Cells' Activity. Cancers. 2020;12(12):1-36 [Rodzaj publikacji: praca poglądowa]	6,639	140
3.	**Lachota M**, et al. Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation. EBioMedicine. 2023;96:1-16 [Rodzaj publikacji: praca oryginalna]	11,100	140
	Łącznie:	**24,378**	**420**
Książki			
1.	-		
Rozdziały w książkach			
1.	-		

p.o. DYREKTOR
Biblioteki Uczelnianej
Warszawskiego Uniwersytetu Medycznego
mgr Agnieszka Czarnecka

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WARSZAWSKI
UNIWERSYTET
MEDYCZNY

BIBLIOTEKA UCZELNIANA

Nr referencyjny
BIBG/Punktacja/ 665 /2023/KK

Warszawa, 09.10.2023

Sz. Pan
Mieszko Lachota

ANALIZA BIBLIOMETRYCZNA PUBLIKACJI
PANA MIESZKA LACHOTY,
STANOWIĄCYCH PODSTAWĘ DO WYRÓŻNIENIA ROZPRAWY DOKTORSKIEJ

Lp.	Opis bibliograficzny	MEiN	IF	Kwartyl*
	Artykuły			
1.	**Lachota M**, et al. Prospects for NK Cell Therapy of Sarcoma. Cancers. 2020;12(12):1-31 [Rodzaj publikacji: praca poglądowa]	140	6,639	Q1 (Oncology)
2.	**Lachota M**, et al. Mapping the chemotactic landscape in NK cells reveals subset-specific synergistic migratory responses to dual chemokine receptor ligation. EBioMedicine. 2023;96:1-16 [Rodzaj publikacji: praca oryginalna]	140	11,100	Q1 (Medicine, Research & Experimental)
	Łącznie:	**280**	**17,739**	
Książki				
1.	-			
Rozdziały w książkach				
1.	-			

* Kwartyl z roku publikacji, według Impact Factor.

p.o. DYREKTOR
Biblioteki Uczelnianej
Warszawskiego Uniwersytetu Medycznego
mgr Agnieszka Czarnecka

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