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ZNACZENIE KOMÓREK NKT-LIKE DLA FUNKCJONOWANIA UKŁADU DOKREWNEGO

Rozprawa na stopień naukowy doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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SŁOWA KLUCZOWE

Komórki NKT-like, układ dokrewny, układ immunologiczny, receptor dla tyreotropiny, cukrzyca typu 2, witamina D

NKT-like cells, endocrine system, immune system, thyroid-stimulating hormone receptor, diabetes mellitus type 2, vitamin D

NUMER PROJEKTU BADAWCZEGO

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Spis treści

WYKAZ PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

1. Adamska-Fita E, Śliwka PW, Karbownik-Lewińska M, Lewiński A, Stasiak M. The Absence of Thyroid-Stimulating Hormone Receptor Expression on Natural Killer T Cells: Implications for the Immune-Endocrine Interaction. Int J Mol Sci. 2024 Oct 24;25(21):11434. doi: 10.3390/ijms252111434. **IF: 4,9. MNiSW: 140**

2. Adamska-Fita E, Śliwka PW, Stasiak B, Karbownik-Lewińska M, Lewiński A, Stasiak M. An impact of type 2 diabetes mellitus on NKT-like cell population in humans: a new insight into impaired immune response in hyperglycemia. Front Endocrinol (Lausanne). 2025 Aug 26;16:1641318. doi: 10.3389/fendo.2025. **IF: 4,6. MNiSW: 100**

3. Adamska-Fita E, Śliwka PW, Stasiak B, Karbownik-Lewińska M, Stasiak M. Vitamin D May Increase the Number of CD4-CD8+ NKT-like Cells in Humans-A Novel Insight into Potential Immunomodulatory Action. Nutrients. 2025 Oct 14;17(20):3216. doi: 10.3390/nu17203216. **IF: 5,0. MNiSW: 140**

WYKAZ STOSOWANYCH SKRÓTÓW

- AITD: autoimmune thyroid disease – choroba autoimmunizacyjna tarczycy
- Anti-Tg: thyroglobulin antibodies – przeciwciała przeciw tyreoglobulinie
- Anti-TPO: thyroid peroxidase antibodies – przeciwciała przeciw peroksydazie tarczycowej
- BMI: body mass index – wskaźnik masy ciała
- CD: cluster of differentiation – grupa różnicowania (marker powierzchniowy komórek)
- COVID-19: coronavirus disease 2019 – choroba koronawirusowa 2019
- DCs: dendritic cells – komórki dendrytyczne
- DM2: type 2 diabetes mellitus – cukrzyca typu 2
- DN: double negative – subpopulacja podwójnie ujemna (CD4-CD8-)
- DP: double positive – subpopulacja podwójnie dodatnia (CD4+CD8+)
- ECLIA: electrochemiluminescence immunoassay – elektrochemiluminescencyjna
- FC: flow cytometry – cytometria przepływowa
- FACS: fluorescence-activated cell sorting – sortowanie komórek aktywowanych fluorescencją
- GD: Graves's disease – choroba Gravesa-Basedowa
- HT: Hashimoto's thyroiditis – choroba Hashimoto
- IFN-γ: interferon gamma – interferon gamma
- IL: interleukin – interleukina
- iNKT: invariant Natural Killer T cells –komórki invarant Natural Killer T
- mRNA: messenger ribonucleic acid – informacyjny kwas rybonukleinowy
- NK cells: Natural Killer cells – komórki Natural Killer
- NKT cells: Natural Killer T cells – komórki Natural Killer T
- NKT-like cells: Natural Killer T-like cells – komórki podobne do Natural Killer T
- PBMCs: peripheral blood mononuclear cells – jednojądrzaste komórki krwi obwodowej
- rhTSH: recombinant human thyroid-stimulating hormone – rekombinowana ludzka tyreotropina
- RNA: ribonucleic acid – kwas rybonukleinowy

- RT-PCR: reverse-transcription polymerase chain reaction – reakcja łańcuchowa polimerazy z odwrotną transkrypcją
- STAT6: Signal Transducer and Activator of Transcription 6 – transduktor sygnału i aktywator transkrypcji 6
- TCR T-cell receptor – receptor TCR
- TGF-β: transforming growth factor beta – transformujący czynnik wzrostu beta
- Th1: T-helper type 1 – limfocyty pomocnicze typu 1
- Th2: T-helper type 2 – limfocyty pomocnicze typu 2
- TNF-α: tumour necrosis factor alpha – czynnik martwicy nowotworów alfa
- TRAb: thyroid-stimulating hormone receptor antibodies – przeciwciała przeciw receptorowi TSH
- TSH: thyroid-stimulating hormone – hormon tyreotropowy
- TSHR: thyroid-stimulating hormone receptor – receptor hormonu tyreotropowego
- VDR: vitamin D receptor – receptor witaminy D

OMÓWIENIE CYKLU PUBLIKACJI

Założenia i cele pracy wraz z uzasadnieniem połączenia publikacji w cykl

Układ odpornościowy oraz układ wydzielania wewnętrznego są ściśle ze sobą powiązane i tworząc skomplikowaną sieć regulacyjną, w której interakcje pomiędzy hormonami, cytokinami a komórkami efektorowymi są złożone i nie do końca poznane [1]. Coraz liczniejsze dane naukowe wskazują na obecność receptorów dla hormonów, w tym m.in. tyreotropiny (TSHR) oraz witaminy D (VDR), na powierzchni różnych komórek układu odpornościowego, takich jak limfocyty T i B, monocyty, makrofagi, komórki dendrytyczne (DC), a także komórki Natural Killer (NK) [2–7]. Obecność tych receptorów odgrywa istotną rolę w modulacji funkcji komórek immunokompetentnych, co w konsekwencji może wpływać na regulację odpowiedzi immunologicznej oraz przebieg licznych chorób, w tym autoimmunologicznych, metabolicznych i nowotworowych. Na aktywność i funkcje komórek układu odpornościowego, oddziałują nie tylko czynniki hormonalne, ale również czynniki metaboliczne, wśród których istotną rolę odgrywa hiperglikemia. Utrzymujące się podwyższone stężenie glukozy może wpływać na aktywację, profil wydzielanych cytokin oraz zdolności cytotoksyczne komórek immunokompetentnych, przyczyniając się do zaburzenia równowagi immunologicznej [8-12].

Szczególne znaczenie w kontekście wyżej wymienionych czynników mają komórki NKT-like**,** łączące cechy odporności wrodzonej i nabytej [13]. Komórki NKT-like, wcześniej opisywane w literaturze jako komórki Natural Killer T (NKT), tworzą zróżnicowaną grupę limfocytów T, które posiadają właściwości komórek T (obecność receptora TCR) oraz komórek NK (ekspresja molekuły CD56). Dotychczas przynależność do populacji NKT opierała się na jednoczesnej obecności antygenów CD3 i CD56, jednakże zgodnie z aktualnym stanem wiedzy ekspresję molekuły CD56 uznaje się za wskaźnik aktywnego fenotypu, często powiązanego ze zwiększoną cytotoksycznością i częściowym podobieństwem funkcji do komórek NK. Wraz z odkryciem, że CD56 nie jest markerem swoistym jedynie dla komórek NK, lecz występuje także na aktywowanych limfocytach γδ i αβ, klasyfikacja komórek NKT została zrewidowana celem wyodrębnienia nowej subpopulacji określanej jako NKT-like [14-17]. Komórki NKT-like wykazują zdolność do szybkiej produkcji szeregu cytokin m.in. interleukiny 4 (Il-4), interferonu gamma (IFN-γ) oraz czynnika martwicy nowotworów alfa (TNF-α) przez co wchodzą w sposób pośredni

w interakcje z innymi komórkami układu odpornościowego i regulują odpowiedź zapalą organizmu. Wytwarzają także cytotoksyczne białka tj. perforyny i granzym B, przyczyniające się do bezpośredniej obrony skierowanej przeciw drobnoustrojom oraz komórkom nowotworowym [13,17]. Z uwagi na ich wysoką reaktywność i potencjał immunomodulacyjny, stanowią one innowacyjny cel badań nad wpływem czynników hormonalnych na funkcję układu immunologicznego człowieka. Rozwój immunologii, a w szczególności nowych metod szczegółowej identyfikacji komórek, spowodował konieczność ponownej oceny i udoskonalenia klasyfikacji komórek.

Na podstawie ekspresji molekuł powierzchniowych CD4+ i CD8+ komórki NKT-like możemy podzielić na 4 subpopulacje: CD8+CD4-, CD4+CD8-, CD4-CD8- (double negative; DN), and CD4+CD8+ (double positive; DP) [18]. Co istotne, powyższe subpopulacje różnią się profilem wydzielanych cytokin oraz siłą zdolności cytotoksycznych. Subpopulacja CD4-CD8+ komórek NKT-like wydziela głównie cytokiny typu Th1, takie jak IFN-γ i TNF-α. Z kolei subpopulacja CD4+CD8- wykazuje profil cytokinowy typu Th2, produkując przede wszystkim IL-4, interleukinę 10 (IL-10) oraz interleukinę 13 (IL-13). Subpopulacja DN charakteryzuje się najsilniejszym potencjałem cytotoksycznym spośród wszystkich frakcji NKT-like, natomiast subpopulacja DP jest najmniej liczna i wykazuje mieszany profil wydzielania cytokin Th1/Th2, co świadczy o jej plastyczności funkcjonalnej i zdolności do modulowania odpowiedzi immunologicznej w zależności od kontekstu biologicznego [19,20].

Jak wspomniano powyżej, dotychczasowe badania wykazały obecność licznych receptorów hormonalnych, w tym TSHR na różnych komórkach układu odpornościowego. W modelach doświadczalnych wykazano, że tyreotropina (TSH) zwiększa aktywność fagocytarną mysich DC, a także selektywnie nasila wydzielanie interleukiny 1 beta (IL-1β) i interleukiny 12 (IL-12) w odpowiedzi na stymulację czynnikami aktywującymi fagocytozę [3]. Dodatkowo, w połączeniu z IL-2, TSH zwiększała aktywność komórek NK [21]. Natomiast Adamczewski i wsp. w swojej pracy wykazali istotny wzrost odsetka komórek NKT po podaniu rekombinowanej ludzkiej tyreotropiny (rhTSH) [22], co sugerowało bezpośredni mechanizm działania TSH na te komórki. Podobne przypuszczenia dotyczące takiego mechanizmu zostały wysunięte również w innych badaniach [23]. Oprócz bezpośredniego wpływu na komórki odpornościowe, TSH może również oddziaływać pośrednio, modulując wydzielanie cytokin przez komórki

immunokompetentne oraz modyfikując ich wrażliwość na te cytokiny. Pomimo rosnącej liczby doniesień dotyczących udziału układu wydzielania wewnętrznego w regulacji odpowiedzi odpornościowej, ekspresja receptora TSHR na komórkach NKT-like nie była dotąd przedmiotem badań.

Uwzględniając znaczenie TSH w regulacji funkcji układu odpornościowego, zasadne stało się rozszerzenie analiz o inne czynniki hormonalne o udokumentowanym działaniu immunomodulującym, takie jak witamina D [4-7]. Wykazano, że aktywacja szlaku sygnałowego witamina D – VDR odgrywa istotną rolę w prawidłowym rozwoju komórek invariant Natural Killer T (iNKT). W modelach zwierzęcych niedobór witaminy D w okresie płodowym prowadził do epigenetycznych zmian skutkujących zmniejszeniem liczby tych komórek [24-25]. Udowodniono również, że suplementacja witaminą D zwiększała ilość komórek NKT we krwi obwodowej pacjentów z chorobą koronawirusową (COVID-19) przebywających na oddziale intensywnej opieki medycznej [26]. Pomimo rosnącego zainteresowania rolą witaminy D w regulacji odpowiedzi immunologicznej, jej wpływ na poszczególne subpopulacje komórek NKT-like nie został dotychczas szczegółowo przeanalizowany.

Nie tylko czynniki hormonalne, lecz także metaboliczne i środowiskowe, takie jak stres oksydacyjny czy hiperglikemia, mogą wpływać na funkcjonowanie komórek immunokompetentnych. W badaniu Tang i wsp. [8] wykazano istotne zmniejszenie liczby komórek NKT-like we krwi obwodowej pacjentów z cukrzycą typu 2 (DM2) i współistniejącą ciężką hiperglikemią. Zanotowano również, że hiperglikemia nie tylko modyfikuje profil wydzielanych przez komórki NKT-like cytokin z Th1 na Th2, ale także nasila ich aktywność [9–12]. Mając na uwadze powyższe wyniki, poziom glikemii jest ewidentnie czynnikiem oddziałującym na komórki NKT-like, jednakże w dostępnej literaturze wciąż brakuje szczegółowych danych dotyczących wpływu hiperglikemii na subpopulacje komórek NKT-like.

Zatem celem naszego projektu było w pierwszym jego etapie – określenie ekspresji TSHR na komórkach NKT-like, a w kolejnych etapach – ocena wpływu hiperglikemii i stężenia witaminy D na subpopulacje komórek NKT-like.

W pierwszej z cyklu prac analizowane komórki CD3+CD56+ określono jako komórki NKT, co było zgodnie z ówczesnym stanem wiedzy oraz obowiązującym w literaturze

naukowej nazewnictwem. Takie podejście pozwalało na możliwość porównania z wcześniejszymi badaniami, w których stosowano identyczną metodologię. W kolejnych latach, wraz z rozwojem metod detekcji komórek układu odpornościowego, dokonano aktualizacji terminologii i populację komórek CD3+CD56+, będącą grupą heterogenną limfocytów T o cechach komórek NK, zaczęto określać jako komórki NKT-like, a nazwa komórki NKT została zarezerwowana wyłącznie dla komórek rozpoznających antygeny za pomocą cząsteczki CD1d (invariant NKT, iNKT). W związku z tym, w drugiej i trzeciej publikacji przyjęto już zaktualizowane nazewnictwo, które lepiej odzwierciedla obecny stan wiedzy immunologicznej. Zmiana ta stanowi efekt modyfikacji terminologii i jest skutkiem postępu naukowego, a nie różnic metodologicznych pomiędzy poszczególnymi badaniami. Dla zachowania spójności terminologicznej, w dalszej części pracy komórki o fenotypie CD3+CD56+ będą określane jako NKT-like.

Grupę badaną stanowili pacjenci z cytologicznie łagodnymi zmianami ogniskowymi tarczycy, hospitalizowani w Klinice Endokrynologii i Chorób Metabolicznych Instytutu Centrum Zdrowia Matki Polki w Łodzi lub też diagnozowani w poradni endokrynologicznej Instytutu w latach 2022-2024. Od każdego uczestnika badania pobrano próbki krwi żylnej (2 × 4,9 mL), z których następnie wyizolowano jednojądrzaste komórki krwi obwodowej (PBMC). Z uzyskanej frakcji PBMC wyodrębniono populację komórek NKT-like (CD3+CD56+) przy użyciu technik cytometrii przepływowej (FC). Do pierwszego etapu badań obejmującego analizę ekspresji TSHR, włączono 86 pierwszych przebadanych pacjentów, których podzielono na dwie grupy: 28 pacjentów z autoimmunologicznymi chorobami tarczycy (AITD), w tym 21 z chorobą Hashimoto i 7 z chorobą Gravesa-Basedowa, oraz grupę kontrolną (58 osób) bez AITD. Ekspresję TSHR na powierzchni komórek NKT oceniano z wykorzystaniem techniki sortowania komórek znakowanych fluorescencją (FACS), a następnie uzyskane wyniki potwierdzono metodą reakcji łańcuchowej polimerazy z odwrotną transkrypcją (RT-PCR). Na etapie kolejnej pracy, analizującej wpływ hiperglikemii na rozkład subpopulacji komórek NKT-like, spośród 104 włączonych pacjentów wyodrębniono 24 pacjentów z rozpoznaną cukrzycą typu 2 (DM2) oraz 62 osoby stanowiące grupę kontrolną. Z badania wykluczono osoby ze stanem przedcukrzycowym, chorobami nowotworowymi oraz aktywnymi infekcjami, które mogłyby wpływać na wyniki analiz. Do ostatniej z analiz cyklu, oceniającej wpływ stężenia witaminy D na subpopulację komórek NKT-like zakwalifikowano wyłącznie pacjentów, u których nie stwierdzono schorzeń, ani nie odnotowano przyjmowania leków

wpływających na gospodarkę wapniowo-fosforanową. Większość badanych (71 osób, tj. 82,6%) przyjmowała suplementację cholekalcyferolem w dawkach od 1000 do 4000 IU na dobę. Ponadto 8% uczestników stosowało preparaty magnezu, a 5% – suplementy kwasów omega-3.

Od wszystkich uczestników badania uzyskano pisemną, świadomą zgodę na udział w projekcie po uprzednim przedstawieniu celu, zakresu i założeń badania. Projekt uzyskał pozytywną opinię Komisji Bioetycznej Instytutu Centrum Zdrowia Matki Polki w Łodzi.

O zasadności połączenia trzech publikacji w jeden cykl badawczy decyduje przede wszystkim fakt, iż wszystkie prace koncentrują się na analizie komórek NKT-like. W pierwszej z publikacji oceniano ekspresję TSHR na komórkach NKT-like, porównując dodatkowo pacjentów z AITD z osobami bez cech autoimmunizacji. Druga praca poświęcona była analizie wpływu stężenia glukozy w surowicy krwi obwodowej oraz istniejącego rozpoznania cukrzycy na subpopulacje komórek NKT-like, natomiast trzecia dotyczyła zależności pomiędzy stężeniem witaminy D a immunofenotypowym zróżnicowaniem subpopulacji komórek NKT-like. Poza ścisłym powiązaniem tematycznym i logiczną ciągłością zagadnień, prace te łączy również wspólny zespół badawczy, zaangażowany w opracowanie koncepcji i realizację badań, oraz jednolita metodologia, obejmująca zarówno część laboratoryjną, jak i analizę statystyczną.

Zebrane wyniki badań opublikowano w postaci trzech wzajemnie powiązanych prac oryginalnych, które wspólnie tworzą spójny cykl badawczy, będący źródłem nowych wniosków naukowych.

Cykl obejmuje:

1. Publikację określającą ekspresję receptora dla tyreotropiny (TSHR) na komórkach NKT-like.
2. Publikację oceniającą wpływ hiperglikemii na subpopulacje komórek NKT-like.
3. Publikację oceniającą wpływ stężenia witaminy D na subpopulacje komórek NKT-like.

Omówienie osiągnięć badawczych kandydata opisanych w cyklu publikacji, na tle aktualnego stanu wiedzy.

Cykl publikacji otwiera praca, której celem była analiza ekspresji TSHR na powierzchni komórek NKT-like. Dotychczas wykazano ekspresję TSHR w licznych narządach i tkankach ludzkiego organizmu, m.in. na komórkach układu odpornościowego takich jak limfocyty B, T, makrofagi, DC oraz komórki NK, jednakże ekspresja TSHR na komórkach NKT-like nie była do tej pory przedmiotem badań [2,3].

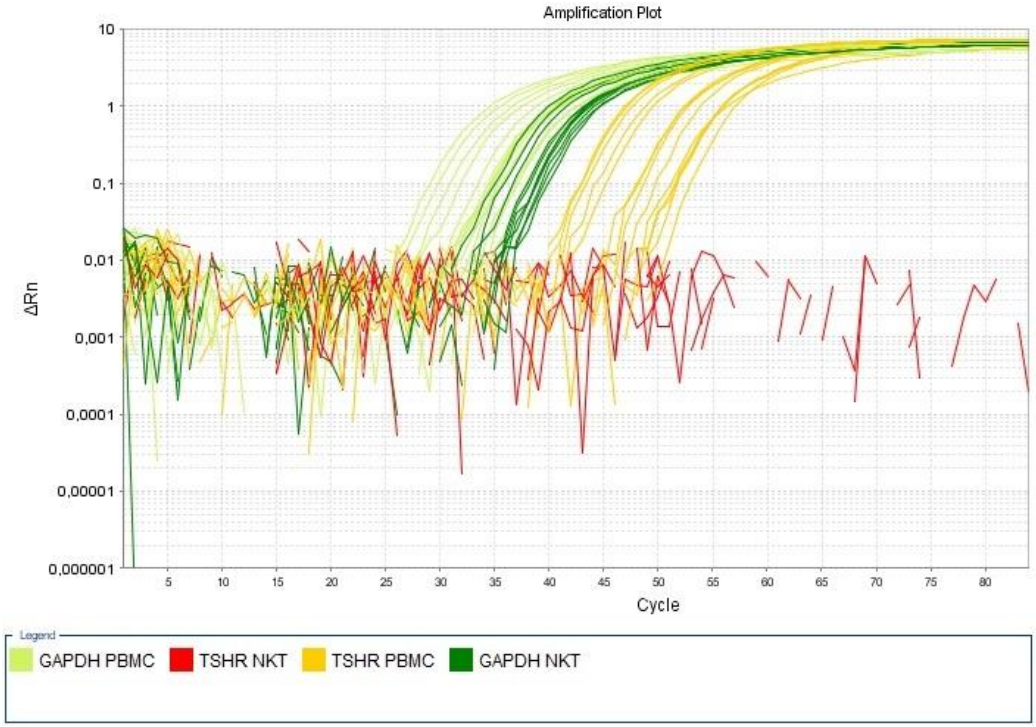
W tej pracy badane komórki CD3+CD56+ określono jako „komórki NKT", co było zgodne z ówczesnym stanem wiedzy. W kolejnych pracach cyklu oraz w dalszej części rozprawy, mając na uwadze aktualne dane literaturowe, przyjęto jednolite określenie „komórki NKT-like", które dokładniej charakteryzuje heterogenną populację limfocytów T o cechach komórek NK. Zmiana ta – jak wspomniano powyżej – była efektem rewizji terminologii i skutkiem postępu naukowego, a nie różnic metodologicznych pomiędzy poszczególnymi badaniami.

Analizą objęto 86 pacjentów z łagodnymi zmianami ogniskowymi tarczycy, w tym 28 osób z AITD oraz 58 bez cech autoimmunizacji. W obu grupach porównano podstawowe parametry kliniczne takie jak wiek, płeć oraz poziomy hormonów i przeciwciał przeciwtarczycowych [tyreotropiny (TSH), wolnej trijodotyroniny (fT3), wolnej tyroksyny (fT4), przeciwciał przeciwko peroksydazie tarczycowej (anty-TPO), przeciwciał przeciwko tyreoglobulinie (anty-Tg), przeciwciał przeciwko receptorowi dla TSH (TRAb)]. Obie grupy nie różniły się istotnie pod względem wieku, płci i czynności tarczycy, natomiast w grupie z AITD stwierdzono znacząco wyższe stężenia przeciwciał anty-TPO i anty-Tg, co potwierdzało aktywny proces autoimmunologiczny. Nie wykazano istotności statystycznej w zakresie TRAb, co można tłumaczyć faktem, że w grupie z AITD jedynie 7 osób chorowało na GD, z czego tylko jedna była pacjentem ze świeżo rozpoznaną postacią choroby i wysokim stężeniem TRAb.

Krew żylną pobierano od uczestników badania o godzinie 6:00, na czczo, a z próbek izolowano jednojądrzaste komórki krwi obwodowej (PBMC) metodą gradientową. Następnie z frakcji PBMC wyodrębniono komórki NKT-like (CD3+CD56+) przy użyciu magnetycznego separatora komórek (Miltenyi Biotec). Uzyskana czystość frakcji NKT-like wynosiła średnio 93,2%, co potwierdzono analizą FACS.

W pierwszym etapie badania ekspresję TSHR na komórkach NKT-like oceniono przy pomocy FACS, wykorzystując przeciwciała sprzężone z fluorochromami przeciwko CD3, CD56 oraz TSHR. Dodatkowo wykonano barwienia kontrolne na całej populacji PBMC, aby potwierdzić wiarygodność zastosowanego przeciwciała anty-TSHR, uzyskując ekspresję TSHR na 2,77% komórek spośród PBMC. W drugim etapie badania celem weryfikacji wyników zastosowano wysokoczułą reakcję łańcuchowej polimerazy z odwrotną transkrypcją (RT-PCR), umożliwiającą ocenę ekspresji genu *TSHR* na poziomie mRNA. Z uwagi na potrzebę uzyskania dużej ilości materiału komórkowego, do analiz RT-PCR wykorzystano kożuszki leukocytarno-płytkowe od zdrowych osób z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi.

Wyniki uzyskane metodą FACS wykazały brak ekspresji receptora TSHR na komórkach NKT-like zarówno u pacjentów z AITD, jak i bez tych chorób. Nie stwierdzono również wpływu leczenia tyreostatycznego ani leczenia lewotyroksyną wynik analizy. Metoda RT-PCR także nie wykazała ekspresji genu *TSHR* w wyizolowanych komórkach NKT-like, podczas gdy w całej frakcji PBMC sygnał ekspresji dla *TSHR* był obecny (mediana $\Delta Ct = 10,3$). Analizy statystyczne z wykorzystaniem testu Fishera oraz testu chi-kwadrat wykazały istotność różnic ($p < 0,0001$), co potwierdziło wiarygodność uzyskanych rezultatów. Wyniki analizy ekspresji genów uzyskane metodą RT-PCR przedstawiono na Rycinie 1.



Rycina 1. Wyniki reakcji łańcuchowej polimerazy z odwrotną transkrypcją (RT-PCR). Krzywe amplifikacji dla genów: dehydrogenazy gliceraldehydu-3-fosforanu (GAPDH) oraz receptora dla tyreotropiny (TSHR), uzyskane z jednojądrzastych komórek krwi obwodowej (PBMC) oraz komórek Natural Killer T-like (NKT-like).

Zgodnie z dotychczasowymi badaniami ekspresja TSHR jest najbardziej nasilona na DC i limfocytach B, natomiast niewielka na komórkach T i NK [2-4]. Adamczewski i wsp. [22] opisali wzrost liczby komórek NKT po podaniu rekombinowanej ludzkiej tyreotropiny (rhTSH), a Miko i wsp. [23] zaobserwowali zwiększenie odsetka tych komórek u kobiet z AITD i zaburzeniami płodności. Wyniki naszej pracy negują możliwość bezpośredniego receptorowego oddziaływania TSH na komórki NKT-like. Wskazują natomiast na pośredni mechanizm regulacyjny, związany prawdopodobnie z udziałem innych komórek immunokompetentnych lub cytokin. Możliwym mechanizmem pośredniczącym w szlaku sygnałowym TSH – komórki NKT jest udział DC, których zwiększoną liczbę wykazano w tkankach tarczycy osób z GD i HT [27-31]. Ponadto, w pracy opublikowanej przez Bessoles i wsp. [32] zaobserwowano, że interleukina 2 (IL-2) może aktywować w komórkach NKT szlak sygnałowy transduktora sygnału i aktywatora transkrypcji 6 (STAT6), prowadząc do sekrecji cytokin zarówno prozapalnych (IFN-γ), jak i przeciwzapalnych (IL-4). W świetle analiz Komorowskiego i wsp. [33,34], którzy opisali

wzrost stężenia IL-2 u chorych z niedoczynnością tarczycy, można przypuszczać, że TSH reguluje funkcję komórek NKT-like pośrednio również poprzez produkcję IL-2.

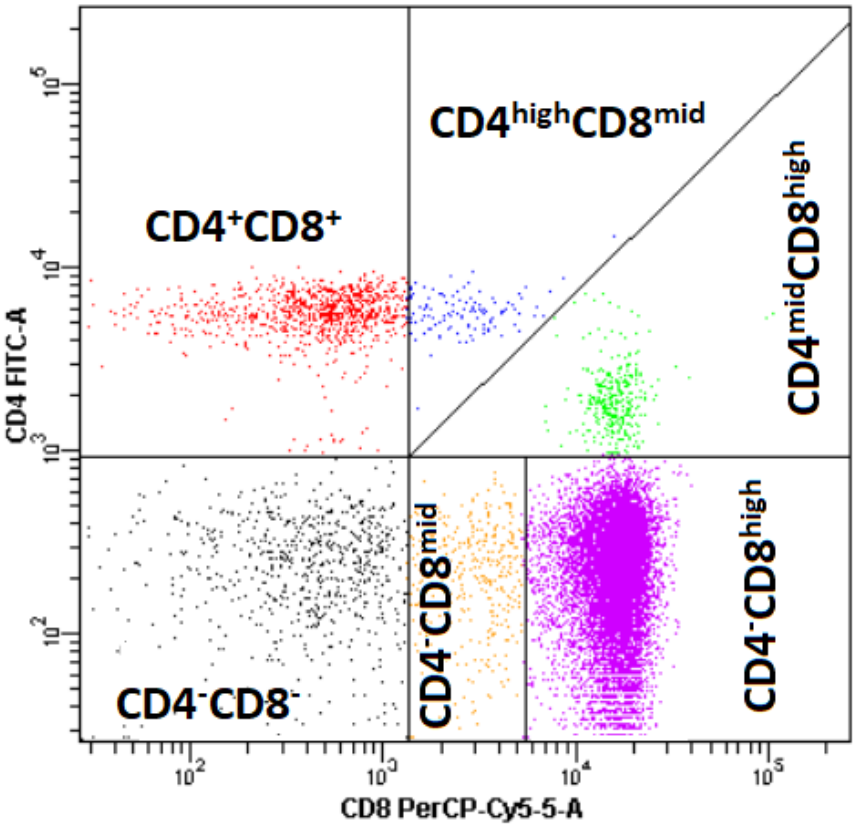
Podsumowując, nasze badanie dostarczyło pierwszych w literaturze dowodów na brak ekspresji TSHR na komórkach NKT-like, zarówno na poziomie białkowym, jak i na poziomie mRNA. Wynik ten wskazuje, że wpływ TSH na komórki NKT-like ma charakter pośredni, związany prawdopodobnie z oddziaływaniem z innymi komórkami układu odpornościowego oraz wydzielanymi przez nie cytokinami. Odkrycie to podważa wcześniejsze hipotezy dotyczące bezpośredniego działania TSH na komórki NKT-like w chorobach autoimmunizacyjnych tarczycy, wskazując jednocześnie nowe kierunki badań nad złożonymi zależnościami między układem dokrewnym a odpornościowym.

Celem drugiej publikacji z omawianego cyklu było przeanalizowanie wpływu hiperglikemii, w tym istniejącej cukrzycy, na subpopulacje komórek NKT-like. W dotychczasowej literaturze wykazano negatywny wpływ hiperglikemii na liczebność i czynność komórek NKT-like, jednakże brakuje badań oceniających wpływ stężenia glukozy na subpopulacje komórek NKT-like [8-12].

Badaniem objęto 24 pacjentów z DM2, natomiast grupę kontrolną stanowiły 62 osoby bez zaburzeń gospodarki węglowodanowej. Kryteriami wykluczenia były: stan przedcukrzycowy, aktywne choroby nowotworowe, infekcyjne lub zapalne, mogące wpływać na stężenie glukozy.

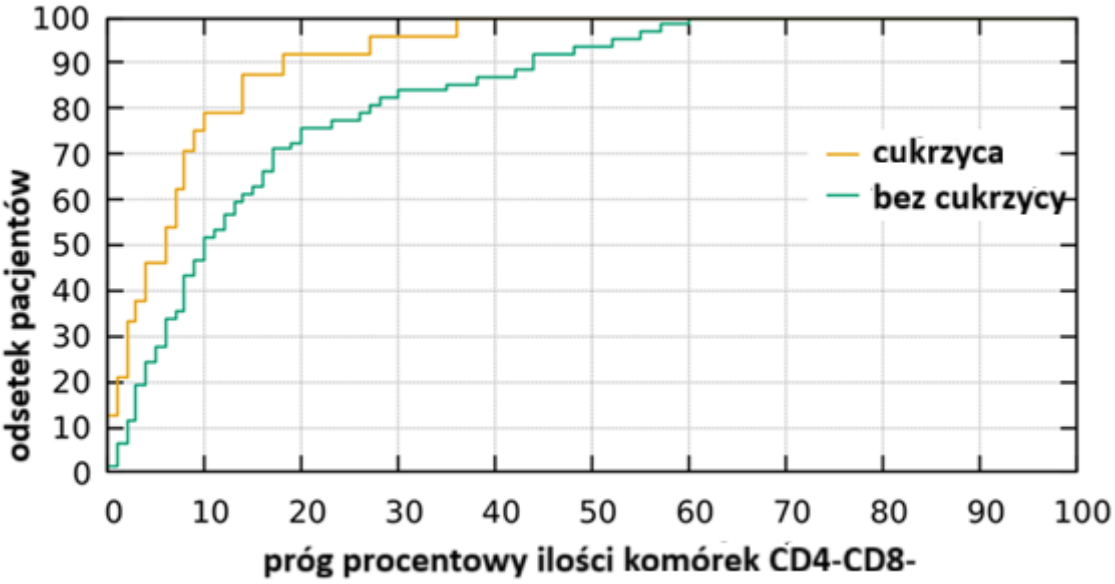
Próbki krwi żylnej pobrano od pacjentów o godzinie 6.00, na czczo. Następnie izolowano PBMC metodą gradientową oraz wyodrębniano komórki NKT-like (CD3+CD56+) za pomocą FC. Uzyskano wysoką czystość populacji, ze średnim odsetkiem 93,2% (zakres 80,1–99,1%). W kolejnym etapie badania dokonano analizy subpopulacji komórek NKT-like. Komórki znakowano przeciwciałami monoklonalnymi sprzężonymi z fluorochromami: CD3 (APC, klon UCHT1), CD56 (PE-Cy7, klon B159), CD4 (FITC, klon SK3) oraz CD8 (PerCP, klon SK1). Dodatkowo zastosowano przeciwciało przeciwko łańcuchowi TCR Va24JaQ (PE, klon 6B11), celem wyodrębnienia populacji komórek iNKT, uzyskując bardzo niską liczebność wymienionej populacji (~1,2% analizowanej populacji). W badaniu nie oceniano ekspresji receptorów TCR αβ i γδ, dlatego dalsze wyniki odnoszą się do heterogennej populacji CD3+CD56+ NKT-like.

Komórki NKT-like podzielono według ekspresji molekuł CD4 i CD8 na cztery podstawowe subpopulacje: CD4-CD8- (double negative, DN), CD4-CD8+, CD4+CD8– oraz CD4+CD8+ (double positive, DP). Dalsza, szczegółowa analiza ujawniła zróżnicowanie w obrębie populacji CD8+ pod względem siły ekspresji tej molekuły oraz molekuły CD4, dlatego wyodrębniono dodatkowe podtypy, t.j. wśród komórek DP: CD4highCD8mid oraz CD4midCD8high, natomiast w grupie CD4-CD8+: CD4-CD8mid i CD4-CD8high. Wprowadzony tutaj podział subpopulacji komórek NKT-like jest całkowicie nowatorski, a w dotychczas opublikowanych pracach nigdy nie wyodrębniono tak szczegółowych podtypów subpopulacji komórek NKT-like. Odsetki poszczególnych subpopulacji wyrażano procentowo względem całkowitej liczby komórek NKT-like oraz, w analizach szczegółowych, względem odpowiedniej populacji nadrzędnej. Strategię ich identyfikacji przedstawiono na Rycinie 2, ilustrującej przykład bramkowania komórek z wykorzystaniem cytometrii przepływowej.



Rycina 2. Przykład ilustrujący strategię identyfikowania subpopulacji komórek NKT-like w analizie z wykorzystaniem cytometrii przepływowej

Średni wiek pacjentów w grupie kontrolnej wynosił 59,7 ± 13,7 lat, natomiast w grupie DM2 – 64,96 ± 11,3 lat. Stężenie glukozy na czczo było istotnie wyższe u pacjentów z cukrzycą (średnio 125,9 mg/dl) niż w grupie kontrolnej (91,1 mg/dl). Analiza nie wykazała wpływu płci na rozkład subpopulacji NKT-like. We wstępnym etapie badania dokonano porównania czterech głównych subpopulacji NKT-like, które ujawniło znamiennie mniejszy odsetek komórek DN u pacjentów z DM2 (8,28 ± 8,76%) w porównaniu do grupy kontrolnej (16,67 ± 15,84%; p = 0,007). Dla pozostałych subpopulacji (CD4-CD8+, CD4+CD8-, CD4+CD8+) nie stwierdzono istotnych różnic. Dodatkowa analiza z wykorzystaniem wartości progowej 9% udziału DN NKT-like wykazała, że częstość cukrzycy była ponad trzykrotnie niższa w grupie z wyższym odsetkiem DN niż wśród osób z jego niskim poziomem (OR = 0,318; p = 0,023). Szczegółowe wyniki analiz progowych przedstawiono na Rycinie 3.



Rycina 3 Odsetek pacjentów z niską (poniżej danego progu) procentową zawartością subpopulacji CD4-CD8- we krwi obwodowej w grupie z cukrzycą typu 2 (DM2 – żółty wykres, n = 24) i w grupie kontrolnej (zielony wykres, n = 62).

Kolejnym etapem badania była ocena zależności pomiędzy odsetkiem poszczególnych subpopulacji komórek NKT-like a stężeniem glukozy i wskaźnikiem masy ciała (BMI). Analiza ta miała na celu ustalenie, czy obserwowany spadek liczby komórek DN NKT-like u pacjentów z DM2 stanowi bezpośrednią konsekwencję hiperglikemii, czy raczej pośredni

skutek zaburzeń immunologicznych związanych z otyłością. Wykazano istotną, ujemną korelację pomiędzy odsetkiem komórek DN NKT-like a poziomem glukozy, natomiast nie stwierdzono zależności między subpopulacjami NKT-like a wartością BMI. Na podstawie powyższych wyników sformułowano hipotezę, w której założono, że hiperglikemia wpływa na subpopulacje komórek NKT-like wykazujące niską ekspresję molekuły CD8 lub jej brak. Przeprowadzono wnikliwą ocenę, która wykazała ujemną korelację pomiędzy stężeniem glukozy a odsetkiem komórek CD4-CD8mid, zarówno względem całkowitej liczby komórek NKT-like jak i wyłącznie wobec subpopulacji CD4-CD8+. Nie stwierdzono natomiast istotnych zależności dla pozostałych podtypów (CD4-CD8high, CD4+CD8+, CD4+CD8-, CD4highCD8mid, CD4midCD8high). Otrzymane wyniki potwierdziły przypuszczenie, iż hiperglikemia może prowadzić do redukcji liczby komórek NKT-like o braku lub niskim poziomie ekspresji molekuły CD8. Nie zaobserwowano natomiast podobnej zależności w odniesieniu do podtypu $CD4^{-}CD8high$.

Uzyskane wyniki pozostają w zgodzie z doniesieniami z pracy Tang i wsp. [8], którzy zaobserwowali zmniejszenie liczby krążących komórek NKT-like u pacjentów z DM2, zwłaszcza u osób z przewlekłą, ciężką hiperglikemią. Natomiast w naszym badaniu oceniano subpopulacje komórek NKT-like pacjentów z umiarkowanie podwyższonym poziomem glukozy, co potwierdza, że nawet niewielkiego stopnia hiperglikemia może prowadzić do zmian w profilu immunologicznym komórek NKT-like.

Ważne wnioski dotyczące zaburzeń w funkcjonowaniu komórek NKT-like w cukrzycy przedstawiła także Dworacka i wsp. [9], wykazując, że w stanie przedcukrzycowym występuje zwiększona liczba aktywowanych komórek NKT-like. natomiast u pacjentów z rozwiniętą cukrzycą ich ilość ulega zmniejszeniu. Również Guo i wsp. [11] analizowali aktywność komórek NKT u pacjentów z nowo rozpoznaną cukrzycą, uzyskując rezultaty odpowiadające wynikom pracy Dworackiej. Z kolei badanie Phoksawat i wsp. [10] wykazało, że u chorych z DM2 komórki NKT-like charakteryzują się nasilonym wytwarzaniem IL-17, co wskazuje na zmianę ich profilu wytwarzanych cytokin w kierunku prozapalnym. Natomiast Gajović i wsp. [12] wykazali, że hiperglikemia może powodować modyfikację profilu cytokinowego NKT w kierunku odpowiedzi typu Th2, z większą produkcją IL-4 i transformującego czynnika wzrostu beta (TGF-β). Autorzy sugerują, że zmiana ta może przyczyniać się do zwiększonej podatności na infekcje i nowotwory u pacjentów z DM2. Wyniki naszej pracy potwierdzają powyższe obserwacje i – co

najważniejsze – dostarczają całkowicie nowatorskich wniosków, iż osłabienie odporności chorych z DM2, może być zależne od redukcji subpopulacji komórek DN NKT-like, odznaczających się szczególnie silnymi właściwościami cytotoksycznymi w obronie przeciwdrobnoustrojowej oraz przeciwnowotworowej. Warto ponownie podkreślić, że zaobserwowane zaburzenia są bezpośrednim skutkiem hiperglikemii, niezależnym od zaburzeń metabolicznych związanych z otyłością.

Kolejna praca, ostania z cyklu publikacji, badała wpływ stężenia witaminy D na subpopulacje komórek NKT-like. Dotychczasowe badania wykazały, że szlak sygnałowy witamina D – VDR niezbędny jest do prawidłowego rozwoju komórek iNKT [24,25]. Udowodniono również, że podawanie witaminy D prowadziło do wzrostu liczby komórek NKT we krwi obwodowej u pacjentów z COVID-19 hospitalizowanych na oddziale intensywnej terapii [26]. Mimo intensywnie rozwijających się badań nad wpływem witaminy D na układ odpornościowy, jej oddziaływanie na poszczególne subpopulacje komórek NKT-like nie było jak dotąd analizowane.

Analizą objęto pacjentów (68 kobiet i 18 mężczyzn) z potwierdzoną cytologicznie łagodną chorobą guzkową tarczycy. Z badania wykluczono osoby ze schorzeniami oraz przyjmowanymi lekami mogącymi wpływać na stężenie wapnia i/lub witaminy D. Większość uczestników (82,6%) suplementowała witaminę D w dawkach 1000–4000 IU/dobę. Średni wiek badanych wynosił 58,1 ± 14,1 lat, a średni wskaźnik BMI 25,3 ± 3,9 kg/m².

Próbki krwi żylnej celem oznaczenia witaminy D oraz wapnia całkowitego pobierano o godzinie 6:00, na czczo. Stężenia witaminy D i wapnia całkowitego oznaczano metodą elektrochemiluminescencyjną (ECLIA, Cobas e601, Roche Diagnostics). PBMC izolowano metodą gradientową, a następnie wyodrębniano komórki NKT-like (CD3+CD56+) przy użyciu FC. W dalszym etapie analizowano subpopulacje NKT-like, wykorzystując znakowanie przeciwciałami monoklonalnymi molekuły: CD3 (APC, klon UCHT1), CD56 (PE-Cy7, klon B159), CD4 (FITC, klon SK3) i CD8 (PerCP, klon SK1). Dodatkowo zastosowano przeciwciało anty-TCR Vα24JαQ (PE, klon 6B11) w celu identyfikacji komórek iNKT, które stanowiły około 1,2% badanej populacji. Z uwagi na brak oceny ekspresji receptorów TCR αβ i γδ, dalsze analizy dotyczyły heterogennej populacji CD3+CD56+ NKT-like. Na podstawie ekspresji CD4 i CD8 wyróżniono cztery główne subpopulacje: CD4-CD8-, CD4-CD8+, CD4+CD8- oraz CD4+CD8+.

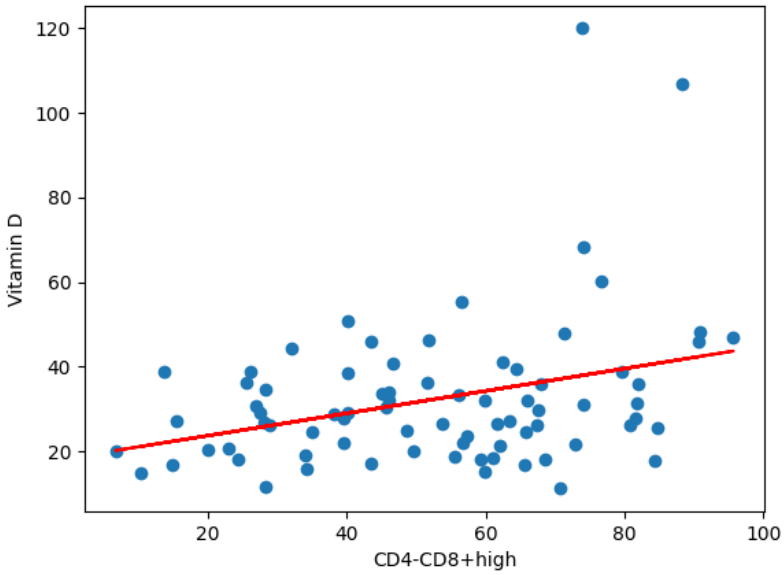
Zastosowano następnie nowatorski podział subpopulacji na podtypy, który wprowadziliśmy w poprzedniej pracy. Ta dodatkowa analiza wykazała zróżnicowanie w obrębie komórek CD8+, co pozwoliło na wyodrębnienie podtypów, t.j. wśród komórek DP: CD4highCD8mid oraz CD4midCD8high, natomiast w grupie CD4-CD8+: CD4-CD8mid i CD4-CD8high. Udziały poszczególnych subpopulacji wyrażano jako procent całkowitej liczby komórek NKT-like oraz, w analizach szczegółowych, względem populacji nadrzędnej.

Średnie stężenie witaminy D w grupie badanej wynosiło 31,7 ± 16,9 ng/mL, a stężenie wapnia całkowitego 2,33 ± 0,15 mmol/L. Nie wykazano istotnej korelacji pomiędzy poziomami obu wymienionych parametrów, co było oczekiwane w grupie bez zaburzeń gospodarki wapniowo-fosforanowej.

Dalsza analiza ujawniła, że spośród czterech głównych subpopulacji komórek NKT-like jedynie komórki CD4-CD8+ wykazywały istotną dodatnią korelację z poziomem witaminy D (współczynnik korelacji Pearsona wynosił 0,312 (p = 0,003)), natomiast współczynnik rang Spearmana (ρ) – 0,225 (p = 0,006). Dla pozostałych populacji nie stwierdzono zależności istotnych statystycznie.

W kolejnym etapie badania, z zastosowaniem bardziej szczegółowego podziału subpopulacji względem siły ekspresji molekuły CD8 wykazano, że podtyp CD4-CD8high również korelował dodatnio z poziomem witaminy D (współczynnik korelacji Pearsona ρ = 0,328, p = 0,004; oraz współczynnik rang Spearmana ρ = 0,262, p = 0,021). Zależności tej nie obserwowano w przypadku komórek CD4-CD8mid oraz – co istotne – efekt ten był niezależny od stężenia wapnia.

Aby potwierdzić powyższe wyniki, przeprowadzono analizę klasterową wyróżniając trzy grupy pacjentów, z których klaster o najwyższym odsetku komórek CD4-CD8+ charakteryzował się istotnie wyższym poziomem witaminy D w porównaniu z klastrem z przewagą komórek CD4+CD8– (p = 0,027, test Manna–Whitneya). Wykonano także analizę progową, dzieląc uczestników na grupy z wyraźnym (>50%) lub mniejszym udziałem komórek CD4-CD8+. Analiza potwierdziła, że osoby z przewagą subpopulacji CD4-CD8+ miały wyraźnie wyższe stężenie witaminy D (34,4 vs 25,2 ng/mL; p = 0,002). Zależność pomiędzy podtypem CD4-CD8high a stężeniem witaminy D przedstawiono na Rycinie 4.



Rycina 4. Zależność pomiędzy poziomem witaminy D a wartością odsetkową komórek NKT-like podtypu CD4-CD8high (na czerwono – prosta regresji liniowej).

Wykazana dodatnia korelacja pomiędzy stężeniem witaminy D a ilością komórek CD4-CD8+ NKT-like, a szczególnie populacji CD8high, wskazuje, że witamina D może promować rozwój lub przeżycie tych komórek. Jest to zgodne z wcześniejszymi obserwacjami z modeli zwierzęcych, gdzie suplementacja witaminą D prowadziła do zwiększenia liczby i aktywności komórek iNKT wyizolowanych z wątroby oraz śledziony [24,25]. Ponadto zaobserwowano, że suplementacja witaminy D może zwiększać odsetek komórek NKT we krwi pacjentów z COVID-19 hospitalizowanych na oddziale intensywnej terapii [26], co istotnie podkreśla potencjał immunostymulujący tej zależności.

Warto zwrócić uwagę, że związek pomiędzy stężeniem witaminy D a subpopulacjami NKT-like nie był dotychczas opisywany. Dostępne dane skupiały się przede wszystkim na jej działaniu wobec całkowitej populacji komórek NKT-like, w kontekście regulacji odpowiedzi immunologicznej w przebiegu chorób autoimmunizacyjnych i nowotworowych [35-37]. Uzyskane w niniejszej pracy wyniki rozszerzają tę wiedzę, pokazując, że witamina D może również modulować rozkład subpopulacji komórek NKT-like promując podtyp CD4-CD8+ wydzielający istotne dla przebiegu wyżej wymienionych schorzeń cytokiny, takie jak IFN-γ and TNF-α. Brak korelacji ze stężeniem wapnia całkowitego potwierdza, że efekt ten wynika z bezpośredniego działania immunomodulującego witaminy D, a nie z jej wpływu na gospodarkę wapniową.

Podsumowanie i wnioski

Komórki NKT-like są tzw. pomostem łączącym odporność wrodzoną i nabytą oraz odgrywają istotną rolę w przebiegu chorób autoimmunizacyjnych, nowotworowych i zakaźnych. Cykl przedstawionych badań obejmował trzy prace oryginalne, które łącznie stanowią istotny wkład w poznanie czynników oddziałujących na komórki NKT-like. Brak ekspresji receptora TSHR na powierzchni komórek NKT-like wyklucza możliwość ich bezpośredniego oddziaływania z TSH i sugeruje pośredni wpływ tego hormonu na ich funkcję, prawdopodobnie poprzez inne komórki układu odpornościowego lub cytokiny. Obserwacja ta zmienia wcześniejsze założenia dotyczące mechanizmu oddziaływania TSH–NKT w patogenezie AITD i wskazuje nowy obszar badań nad interakcjami układu immunologicznego i dokrewnego. Innym czynnikiem modulującym komórki NKT-like, a dokładniej rozkład ich subpopulacji, okazała się hiperglikemia. W kolejnym badaniu wykazano, że nawet umiarkowanie podwyższone stężenie glukozy, niezależnie od wartości wskaźnika BMI, wiąże się ze zmniejszeniem odsetka subpopulacji CD4-CD8- komórek NKT-like, charakteryzującej się wysokim potencjałem cytotoksycznym. Obserwowana zmiana w strukturze subpopulacyjnej tych komórek może stanowić istotny mechanizm immunologiczny, który przyczynia się do zwiększonej podatności pacjentów z DM2 na infekcje oraz rozwój chorób nowotworowych. Liczebność subpopulacji komórek NKT-like kształtowana jest również przez stężenie witaminy D. W ostatniej z cyklu prac zaobserwowano dodatnią korelację pomiędzy stężeniem witaminy D a odsetkiem komórek CD4-CD8+ NKT-like, szczególnie podtypu CD8high. Efekt ten był niezależny od stężenia wapnia, co potwierdza, że zanotowany związek wynika z immunomodulującego działania witaminy D, a nie z jej wpływu na gospodarkę wapniowo-fosforanową. Otrzymane dane wskazują, iż witamina D może odgrywać rolę w utrzymaniu i różnicowaniu subpopulacji CD4-CD8+ komórek NKT-like, odpowiedzialnych za wydzielanie cytokin, takich jak IFN-γ and TNF-α, zaangażowanych w odpowiedź przeciwdrobnoustrojową, przeciwnowotworową oraz w regulację procesów autoimmunizacyjnych. Uzyskane analizy podkreślają znaczenie współdziałania układów odpornościowego oraz dokrewnego w utrzymaniu homeostazy immunologicznej, a także wskazują nowe kierunki badań nad kolejnymi czynnikami regulującymi rozkład subpopulacji komórek NKT-like. Przedstawione wyniki dostarczają nowych informacji o znaczeniu komórek NKT-like, które mogą mieć istotne znaczenie dla codziennej praktyki klinicznej w endokrynologii, diabetologii i immunologii klinicznej.

Piśmiennictwo:

1. Jara, E.L.; Muñoz-Durango, N.; Llanos, C.; Fardella, C.; González, P.A.; Bueno, S.M.; Kalergis, A.M.; Riedel, C.A. Modulating the function of the immune system by thyroid hormones and thyrotropin. Immunol. Lett. 2017, 184, 76–83.
2. Berczi, I. Neuroendocrine regulation of natural immunity. NeuroImmune Biol. 2005, 5, 215–262.
3. Bağriaçik, E.U.; Klein, J.R. The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of CD45RBhigh lymph node T cells: Functional role for thyroid-stimulating hormone during immune activation. J. Immunol. 2000, 165, 6158–6165.
4. Chen, S.; Sims, G.P.; Chen, X.X.; Gu, YYY.; Chen, S.; Lipsky, P.E. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J. Immunol. 2007, 179, 1634–1647.
5. Kongsbak, M.; Levring, T.B.; Geisler, C.; von Essen, M.R. The vitamin D receptor and T cell function. Front. Immunol. 2013, 4, 148.
6. Veldman, C.M.; Cantorna, M.T.; DeLuca, H.F. Expression of 1,25-dihydroxyvitamin D3 receptor in the immune system. Arch. Biochem. Biophys. 2000, 374, 334–338.
7. Cantorna, M.T.; Snyder, L.; Lin, Y.D.; Yang, L. Vitamin D and $1,25(OH)_2D$ regulation of T cells. Nutrients 2015, 7, 3011–3021.
8. Tang, L.; Wang, H.; Cao, K.; Xu, C.; Ma, A.; Zheng, M.; et al. Dysfunction of circulating $CD3^+CD56^+$ NKT-like cells in type 2 diabetes mellitus. Int. J. Med. Sci. 2023, 20, 652–662. doi:10.7150/ijms.83317
9. Dworacka, M.; Wesołowska, A.; Wysocka, E.; Winiarska, H.; Iskakova, S.; Dworacki, G. Circulating $CD3^+CD56^+$ cell subset in pre-diabetes. Exp. Clin. Endocrinol. Diabetes 2014, 122, 65–70. doi:10.1055/s-0033-1363233
10. Phoksawat, W.; Jumnainsong, A.; Leelayuwat, N.; Leelayuwat, C. IL-17 production by NKG2D-expressing $CD56^+$ T cells in type 2 diabetes. Mol. Immunol. 2019, 106, 228–233. doi:10.1016/j.molimm.2018.12.008
11. Guo, H.; Xu, B.; Gao, L.; Sun, X.; Qu, X.; Li, X.; et al. High frequency of activated natural killer and natural killer T cells in patients with new onset of type 2 diabetes mellitus. Exp. Biol. Med. (Maywood) 2012, 237, 556–562. doi:10.1258/ebm.2012.011272

12. Gajović, N.; Zdravković, N.; Jovanović, I.; Jevtić, B.; Lukić, M.L. Diabetes mellitus directs NKT cells toward type 2 and regulatory phenotype. Exp. Appl. Biol. Med. Res. 2016, 17, 35–41. doi:10.1515/sjecr-2016-0005
13. Kaszubowska, L.; Piotrowska, A.; Siedlecka-Kroplewska, K.; Kmiec, Z. NKT cells as a connecting element between innate and adaptive immunity. Postep. Biol. Komorki 2013, 40, 697–724.
14. Doherty, D.G.; Norris, S.; Madrigal-Estebas, L.; McEntee, G.; Traynor, O.; Hegarty, J.E.; O'fArrelly, C. The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns.
15. Godfrey, D.I.; Uldrich, A.P.; McCluskey, J.; Rossjohn, J.; Moody, D.B. The burgeoning family of unconventional T cells. Nat. Immunol. 2015, 16, 1114–1123.
16. Almeida, J.; Ferreira, J.; Gaspar, H.B.; da Silva, J.P. Natural Killer T-like Cells: Immunobiology and Role in Disease. Int. J. Mol. Sci. 2023, 24, 2743.
17. VanAcker,H.H.;Capsomidis, A.; Smits, E.L.; Van Tendeloo, V.F. CD56 in the immunesystem: Morethanamarkerforcytotoxicity? Front. Immunol. 2017, 8, 892.
18. Montoya, C.J.; Pollard, D.; Martinson, J.; Kumari, K.; Wasserfall, C.; Mulder, C.B.; Rugeles, M.T.; Atkinson, M.A.; Landay, A.L.; Wilson, S.B. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. Immunology 2007, 122, 1–14.
19. O'Reilly, V.; Zeng, S.G.; Bricard, G.; Atzberger, A.; Hogan, A.E.; Jackson, J.; Feighery, C.; Porcelli, S.A.; Doherty, D.G. Distinct and overlapping effector functions of expanded human CD4+, CD8α+ and CD4-CD8α- invariant natural killer T cells. PLoS ONE 2011, 6, e28648.
20. Balato, A.; Unutmaz, D.; Gaspari, A.A. Natural killer T cells: An unconventional T cell subset with diverse effector and regulatory functions. J. Investig. Dermatol. 2009, 129, 1628–1642. doi:10.1038/jid.2009.30
21. Provinciali, M., Di Stefano, G., Fabris, N. Improvement in the proliferative capacity and natural killer cell activity of murine spleen lymphocytes by thyrotropin, Int. J. Immunopharmacol. (1992) 14:865–870.
22. Adamczewski, Z.; Stasiołek, M.; Zygmunt, A.; Sliwka, P.W.; Wieczorek-Szukała, K.; Lewiński, A. Recombinant Human Thyroid Stimulating Hormone Increases the

Percentages of Natural Killer T Cells and B Lymphocytes in Human Peripheral Blood In Vivo. Front. Endocrinol. 2020, 11, 543845.

23. Miko, E.; Meggyes, M.; Doba, K.; Farkas, N.; Bogar, B.; Barakonyi, A.; Szereday, L.; Szekeres-Bartho, J.; Mezosi, E. Characteristics of peripheral blood NK and NKT-like cells in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure. J. Reprod. Immunol. 2017, 124, 62–70.
24. Yu, S.; Cantorna, M.T. The vitamin D receptor is required for iNKT cell development. Proc. Natl. Acad. Sci. USA 2008, 105, 5207–5212.
25. Yu,S.; Cantorna, M.T. Epigenetic reduction in invariant NKT cells following in utero vitamin D deficiency in mice. J. Immunol. 2011, 186, 1384–1390.
26. Bychinin, M.V.; Klypa, T.V.; Mandel, I.A.; Yusubalieva, G.M.; Baklaushev, V.P.; Kolyshkina, N.A.; Troitsky, A.V. Effect of vitamin D3supplementation on cellular immunity and inflammatory markers in COVID-19 patients admitted to the ICU. Sci. Rep. 2022, 12, 18604.
27. Leskela, S.; Rodríguez-Munoz, A.; de la Fuente, H.; Figueroa-Vega, N.; Bonay, P.; Martín, P.; Serrano, A.; Sánchez-Madrid, F.; González-Amaro, R.; Marazuela, M. Plasmacytoid dendritic cells in patients with autoimmune thyroid disease. J. Clin. Endocrin. Metab. 2013, 98, 2822–2833.
28. Stasiołek, M.; Sliwka, P.W.; Stasiak, M.; Krawczyk-Rusiecka, K.; Skowrońska-Jóźwiak, E.; Adamczewski, Z.; Lewiński, A. Differences of the Structure of Immune Regulatory Cell Populations between Cellular Material from Sonographically Detected Focal Thyroid Lesions and Peripheral Blood in Humans. Int. J. Mol. Sci. 2019, 20, 918.
29. Quadbeck, B.; Eckstein, A.K.; Tews, S.; Walz, M.; Hoermann, R.; Mann, K.; Gieseler, R. Maturation of thyroidal dendritic cells in Graves' disease. Scand. J. Immunol. 2002, 55, 612–620.
30. Hammerstad, S.S.; Jahnsen, F.L.; Tauriainen, S.; Hyöty, H.; Paulsen, T.; Norheim, I.; Dahl-Jørgensen, K. Inflammation and increased myxovirus resistance protein A expression in thyroid tissue in the early stages of Hashimoto's thyroiditis. Thyroid 2013, 23, 334–341.
31. Hammerstad, S.S.; Jahnsen, F.; Tauriainen, S.; Hyöty, H.; Paulsen, T.; Norheim, I.; Dahl-Jørgensen, K. Immunological Changes and Increased Expression of

Myxovirus Resistance Protein A in Thyroid Tissue of Patients with Recent Onset and Untreated Graves' Disease. Thyroid 2014, 24, 537–544.

32. Bessoles, S.; Fouret, F.; Dudal, S.; Besra, G.S.; Sanchez, F.; Lafont, V. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. J. Leukoc. Biol. 2008, 84, 224–233.
33. Komorowski, J. Increased interleukin-2 level in patients with primary hypothyroidism. Clin. Immunol. Immunopathol. 1992, 63, 200–202.
34. Komorowski, J.; Zyli´nska, K.; Pawlikowski, M.; Stepie´n, H. Stimulatory effect of thyrotropin (TSH) on interleukin-2 (IL-2) release from human peripheral blood lymphocytes. A dose-response study in vitro. Horm. Metab. Res. 1993, 25, 598–599.
35. Zhou,X.; Li, Q.; Li, Y.; Fu, J.; Sun, F.; Li, Y.; Wang, Y.; Jia, Y.; Zhang, Y.; Jia, R.; et al. Diminished natural killer T-like cells correlates with aggravated primary Sjögren's syndrome. Clin. Rheumatol. 2016, 35, 1763–1770.
36. Lin, S.J.; Kuo, M.L.; Hsiao, H.S.; Lee, P.T.; Huang, J.L. Cytotoxic function and cytokine production of natural killer cells and natural killer T-like cells in systemic lupus erythematosus: Regulation with interleukin-15. Mediat. Inflamm. 2019, 2019, 4236562.
37. Yuen, M.F.; Norris, S. Expression of inhibitory receptors in natural killer (CD3−CD56+) cells and CD3+CD56+ cells in peripheral blood lymphocytes and tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. Clin. Immunol. 2001, 101, 276–282.

STRESZCZENIE W JĘZYKU POLSKIM

Tytuł: Znaczenie komórek NKT-like dla funkcjonowania układu dokrewnego

Wstęp: Układ immunologiczny i układ dokrewny tworzą wzajemnie powiązaną sieć, w której hormony, cytokiny oraz komórki efektorowe oddziałują na siebie wielokierunkowo, zarówno w sposób bezpośredni jak i pośredni. Coraz więcej dowodów naukowych potwierdza obecność receptorów hormonalnych, takich jak receptor dla tyreotropiny (TSHR) czy receptora dla witaminy D (VDR), na powierzchni komórek układu odpornościowego, w tym limfocytów T i B, monocytów, komórek dendrytycznych (DC) czy komórek Natural Killer (NK). Odkrycia te podkreślają, że czynniki endokrynne mogą pełnić ważną rolę w kształtowaniu odpowiedzi immunologicznej, co ma znaczenie w patogenezie chorób autoimmunizacyjnych, nowotworowych i zakaźnych. Unikalną grupę komórek o cechach łączących odporność wrodzoną i nabytą stanowią komórki Natural Killer T-like (NKT-like), zdolne do szybkiej produkcji cytokin oraz białek cytotoksycznych. Z uwagi na ich zdolność do modulacji odpowiedzi zapalnej, stanowią one ważny element badań nad mechanizmami współdziałania układu immunologicznego i dokrewnego. Ocena czynników hormonalnych i metabolicznych wpływających na rozkład subpopulacji komórek NKT-like we krwi obwodowej człowieka stanowi istotny krok w zrozumieniu zależności między układem wydzielania wewnętrznego a układem odpornościowym i może mieć znaczenie w rozwoju nowych narzędzi diagnostycznych i terapeutycznych w medycynie klinicznej.

Cele: Celem cyklu prac była analiza ekspresji TSHR na powierzchni komórek Natural Killer T (NKT) oraz ocena czynników hormonalnych i metabolicznych modulujących rozkład subpopulacji komórek NKT-like. Badania miały na celu pogłębienie wiedzy na temat współdziałania układu dokrewnego i odpornościowego w regulacji funkcji tych komórek.

Materiały i metody: Analizą objęto pacjentów diagnozowanych w Klinice Endokrynologii i Chorób Metabolicznych oraz poradni przyklinicznej z powodu cytologicznie łagodnych zmian ogniskowych tarczycy w latach 2022-2024. U wszystkich uczestników wyizolowano metodą gradientową jednojądrzaste komórki krwi obwodowej (PBMC), a następnie identyfikowano komórki NKT-like oraz ich subpopulacje za pomocą cytometrii przepływowej (FC).

W pierwszej z prac, analizującej ekspresję TSHR, w celu potwierdzenia wyników, zastosowano również metodę reakcji łańcuchowej polimerazy z odwrotną transkrypcją (RT-PCR), wykorzystując kożuszki leukocytarno-płytkowe od zdrowych dawców krwi. Poziom glikemii, wapnia całkowitego i witaminy D oznaczano z próbek krwi żylnej pobranych rano, na czczo.

Wyniki: W pierwszej pracy wykazano brak ekspresji receptora TSHR na powierzchni komórek NKT-like, a uzyskane wyniki potwierdzono zarówno na poziomie białkowym, jak i genetycznym, z wykorzystaniem metody sortowania komórek znakowanych fluorescencją (FACS) oraz reakcji łańcuchowej polimerazy z odwrotną transkrypcją (RT-PCR). W drugim badaniu wykazano negatywny, niezależny od BMI, wpływ umiarkowanej hiperglikemii na odsetek subpopulacji CD4-CD8- komórek NKT-like. Zaobserwowano również ujemną zależność między stężeniem glukozy a liczbą komórek CD4-CD8mid, co sugeruje, że nawet umiarkowana hiperglikemia może prowadzić do zaburzeń profilu immunologicznego tych komórek.
W trzecim badaniu stwierdzono dodatnią, niezależną od stężenia wapnia całkowitego korelację pomiędzy poziomem witaminy D a liczebnością subpopulacji CD4-CD8+ komórek NKT-like, szczególnie populacji CD8high, co wskazuje, że witamina D może sprzyjać rozwojowi i przeżyciu tych komórek.

Wnioski: Cykl przedstawionych badań pozwolił na poszerzenie wiedzy dotyczącej czynników wpływających na komórki NKT-like oraz ich subpopulacje. Wykazano, że komórki te nie wykazują ekspresji TSHR, co wyklucza bezpośredni wpływ tyreotropiny (TSH), szczególnie w aspekcie autoimmunizacyjnych chorób tarczycy (AITD). Zaobserwowano także, że nawet umiarkowana hiperglikemia prowadzi do istotnego zmniejszenia odsetka subpopulacji CD4-CD8- komórek NKT-like, co może być przyczyną zwiększonej podatnością pacjentów z cukrzycą typu 2 (DM2) na infekcje oraz choroby nowotworowe. Ponadto zaobserwowana zależność między stężeniem witaminy D a subpopulacją CD4-CD8+ komórek NKT-like, produkujących cytokiny typu Th1, przemawia za tym, że witamina D może uczestniczyć w regulacji aktywności tych komórek. Uzyskane wyniki podkreślają znaczenie czynników hormonalnych i metabolicznych w modulowaniu funkcji układu odpornościowego oraz mogą stanowić podstawę do opracowania nowych strategii diagnostycznych i terapeutycznych w chorobach autoimmunizacyjnych, nowotworowych oraz zakaźnych.

STRESZCZENIE W JĘZYKU ANGIELSKIM

Title: The role of NKT-like cells in endocrine system

Introduction: The immune and endocrine systems form an interconnected network in which hormones, cytokines, and effector cells interact in multiple directions, both directly and indirectly. Increasing scientific evidence confirms the presence of hormonal receptors, such as the thyroid-stimulating hormone receptor (TSHR) and the vitamin D receptor (VDR), on the surface of immune cells, including T and B lymphocytes, monocytes, dendritic cells (DC), and Natural Killer cells (NK). These data confirm that endocrine factors have an important role in modulating the immune response, which is relevant to the pathogenesis of autoimmune diseases, cancers, and infectious diseases. Natural Killer-T like cells (NKT-like) form a unique population that links innate and adaptive immunity and can rapidly produce cytokines and cytotoxic proteins. Due to their ability to modulate inflammatory responses, these cells constitute an important aim of research on the mechanisms integrating immune and endocrine system functions. The assessment of hormonal and metabolic factors influencing the distribution of NKT-like cell subpopulations in human peripheral blood represents a significant step toward understanding the interrelationship between endocrine and immune systems and may contribute to the development of new diagnostic and therapeutic strategies in clinical medicine.

Aim: The purpose of the presented series of articles was to analyze the expression of TSHR on the surface of NKT cells and to assess hormonal and metabolic factors modulating the distribution of NKT-like cell subpopulations. The studies aimed to expand the knowledge on role of the interactions between endocrine and immune systems in regulation of function of these cells.

Materials and Methods: The analysis included patients diagnosed at the Department of Endocrinology and Metabolic Diseases and in an outpatient clinic for benign thyroid nodules between 2022 and 2024. Peripheral blood mononuclear cells (PBMCs) were isolated using the gradient method from all the participants, followed by identification of NKT-like cells and their subpopulations by a flow cytometry (FC) method. In the first study, which analyzed TSHR expression, the results were additionally verified using transverse transcription polymerase chain reaction (RT-PCR) method, performed on leukocyte–platelet buffy coats obtained from healthy blood donors. The levels of glucose,

total calcium, and vitamin D were determined from venous blood samples collected in the morning, after overnight fasting.

Results: In the first study, no expression of TSHR was detected on the surface of NKT cells, and this result was confirmed at both protein and genetic levels using fluorescence-activated cell sorting (FACS) and RT-PCR. In the second study, a negative, BMI-independent, effect of hyperglycemia on the percentage of CD4-CD8- NKT-like subpopulations was demonstrated. A negative correlation between glucose concentration and the number of CD4-CD8mid cells was also observed, suggesting that even moderate hyperglycemia may lead to alterations in the immunological profile of these cells. In the third study, a positive correlation, independent of total calcium levels, was found between vitamin D concentration and the frequency of CD4-CD8+ NKT-like subpopulations, particularly the CD8high subset, indicating that vitamin D may promote the development and survival of these cells.

Conclusions: The presented series of articles broadened the knowledge on factors influencing NKT-like cells and their subpopulations. It was shown that these cells do not express TSHR, which excluded a direct effect of TSH on these cells, particularly in the context of autoimmune thyroid diseases (AITD). It was also demonstrated that even moderate hyperglycemia leads to a significant reduction in the proportion of CD4-CD8- NKT-like subpopulations, which may be associated with increased susceptibility of patients with type 2 diabetes (DM2) to infections and cancers. Moreover, the association between vitamin D levels and the Th1-producing CD4-CD8+ NKT-like subpopulation supports the hypothesis that vitamin D may contribute to regulating the functional activity of these cells. The obtained results emphasize the significance of hormonal factors in modulating immune system functions and may form the basis for developing new diagnostic and therapeutic strategies in autoimmune diseases, cancers, and infectious diseases.

OPUBLIKOWANE PRACE

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The Absence of Thyroid-Stimulating Hormone Receptor Expression on Natural Killer T Cells: Implications for the Immune–Endocrine Interaction

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Abstract: The expression of thyroid-stimulating hormone receptor (TSHR) has been documented on various immune cells, including B lymphocytes, T lymphocytes, Natural Killer (NK) cells, monocytes, and dendritic cells (DCs). Natural Killer T (NKT) cells serve as a crucial link between innate and adaptive immunity, playing significant roles in immunological interactions and autoimmune diseases. The aim of the present study was to evaluate the presence of TSHR on NKT cells. Our research involved patients with thyroid disease, as well as healthy controls. Peripheral blood mononuclear cells (PBMCs) and, thereafter, NKT cells were isolated from 86 patients with benign nodular thyroid disease with and without autoimmune thyroid disease (AITD) (28 and 56 cases, respectively), and TSHR expression was analyzed using fluorescence-activated cell sorting (FACS). In order to confirm the results, the reverse-transcription polymerase chain reaction (RT-PCR) method was used in cells obtained from healthy individuals. Our findings obtained with application of the FACS method revealed that TSHR is not expressed on NKT cells in either AITD or non-AITD patients, though TSHR was detected in the total PBMC population (TSHR+ cells 2.77%). The absence of TSHR on NKT cells was further confirmed with RT-PCR in healthy individuals ($p < 0.0001$). These results questioned the previously suggested direct influence of NKT cells on AITD development.

Keywords: natural killer T cells; thyroid-stimulating hormone receptor; autoimmune thyroid disease

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1. Introduction

The functional interplay between the immune and endocrine systems is complex, with numerous cell types and humoral mediators involved, yet not fully understood [1]. Apart from the well-known direct effects of thyroid hormones on the immune system, alterations in the hypothalamic–pituitary–thyroid axis, particularly changes in thyroid-stimulating hormone (TSH) levels, may be of significance. While the role of thyroid-stimulating hormone receptor (TSHR) in thyroid function is well established, recent studies suggested TSHR expression in certain immune cells, potentially influencing immune responses [2,3]. In studies performed by immunoprecipitation and by flow cytometry, TSHRs were found on B and T lymphocytes, Natural Killer (NK) cells, monocytes, and—in a large amount—on dendritic cells (DCs) [2,3]. Although the impact of TSH levels on the quantity of Natural Killer T (NKT) cells in peripheral blood has been postulated [4,5], no reported studies have explored the expression of TSHR on NKT cells.

NKT cells represent a unique T cell subpopulation characterized by the presence of markers of both NK cells (NK1.1, CD56) and T cells (TCR—T cell receptor). This dual marker expression makes NKT cells essential for bridging innate and adaptive immunity. NKT cells constitute approximately 0.1% of peripheral blood lymphocytes [6]. Unlike

conventional T αβ lymphocytes, NKT cells possess TCR receptors capable of recognizing glycolipid, glycosphingolipid, and lipid structures presented on non-polymorphic CD1d molecules on both professional and non-professional antigen-presenting cells. This unique feature allows NKT cells to recognize both foreign and self-lipid antigens, granting them regulatory and potentially effector roles in various immune responses, including those related to autoimmune diseases, allergies, infections, and cancers [7].

NKT cells are crucial regulators of autoimmune diseases [8,9]. Dysregulation or deficiency of NKT cells is associated with type 1 diabetes, lupus erythematosus, multiple sclerosis, myasthenia gravis, Guillain–Barré syndrome, and autoimmune thyroid diseases (AITDs) [10–14]. AITDs are a group of disorders characterized by an immune system attack against the thyroid gland antigens, including Graves' disease (GD) and Hashimoto's thyroiditis (HT). These diseases involve complex interactions between genetic predispositions and environmental factors, resulting in autoantibody production targeting thyroid-specific proteins, including TSHR [15]. TSHR is part of the G protein-coupled receptor group and is composed of a wide extracellular domain, seven transmembrane passages, and a small intracellular domain [16]. TSHR is mostly expressed in the basolateral membrane of thyrocytes where its stimulation enhances iodine uptake, the synthesis and secretion of thyroid hormones, and the proliferation of thyroid follicular cells, and regulates the expression of thyroid-specific genes like those coding thyroglobulin (Tg), thyroid peroxidase (TPO), and sodium/iodide symporter (NIS) [17–20]. Moreover, several extrathyroidal cell types with expression of TSHR have been revealed, including immune system cells [2,3,21]. Regardless of the low TSHR expression on non-thyroidal cells, the very high binding affinity for TSH contributes to activating the response despite the low density of TSHR on the cellular surface [22]. Many studies have been conducted to test molecular mechanisms involving TSHR in the pathogenesis of AITD. Cuddihy et al. in 1995 discovered the first potential single-nucleotide polymorphism (SNP) of codon 52 of TSHR gene connected with GD in a female population [23]. Recent studies, including three meta-analyses, confirmed important associations between SNPs in intron 1 of the TSHR gene and the risk of GD [24–26]. In a previous study performed in our center, a significant increase in the percentage of NKT cells after administration of recombinant human TSH (rhTSH) was observed [4], suggesting a direct mechanism of TSH action on NKT cells. Such a mechanism was also postulated in other studies [5]. Therefore, in order to evaluate whether the mechanism of TSH action on NKT is direct or indirect, the present study aimed to assess TSHR expression on NKT cells, analyzing NKT cells isolated from the peripheral blood of individuals with and without AITDs.

2. Results

2.1. Comparison of AITD and Non-AITD Groups

The mean age of the patients was 55.54 ± 11.4 and 61.72 ± 14.43 years in the AITD and non-AITD groups, respectively. The non-AITD group included 45 women and 13 men, while the AITD group consisted of 23 women and 5 men, with 2 cases of overt hyperthyroidism, 3 cases of subclinical hyperthyroidism, and 2 cases of subclinical hypothyroidism.

The AITD and non-AITD groups were similar in terms of age and gender. The comparison of both groups revealed statistically significant higher anti-TPO and anti-Tg levels in patients with AITD. TRAb level was higher in the AITD group but the difference did not reach statistical significance, as there was only one patient with newly diagnosed GD and a high TRAb level, while other GD patients were either on thiamazole or had received radioiodine therapy. The characteristics of the AITD and non-AITD groups are presented in Table 1.

Table 1. Clinical characteristics of the study group.

	Patients with AITD Mean ± SD (n = 28)	Patients without AITD Mean ± SD (n = 58)	*p* Value (AITD Versus Non-AITD Group)
Age	55.54 ± 11.4	61.72 ± 14.43	$p = 0.05$
Female/Male (n)	23/5	45/13	$p = 0.63$
fT4 (0.93–1.7 ng/dL)	1.25 ± 0.27	1.27 ± 0.34	$p = 0.9$
fT3 (2–4.4 pg/mL)	2.83 ± 1.01	2.94 ± 0.69	$p = 0.56$
TSH (0.27–4.2 uIU/mL)	1.6 ± 2.39	1.3 ± 1.1	$p = 0.42$
anti-TPO (<34 IU/mL)	157.28 ± 149.04	11.39 ± 4.9	$p < 0.05$ *
anti-Tg (<115 IU/mL)	187.37 ± 193.65	15.5 ± 8.12	$p < 0.05$ *
TRAb (0.8–1.75 IU/L)	2.63 ± 5.17	0.99 ± 0.26	$p = 0.09$

Abbreviations: fT4, free thyroxine; fT3, free triiodothyronine; TSH, thyroid-stimulating hormone; anti-TPO, thyroid peroxidase antibodies; anti-Tg, thyroid antithyroglobulin antibodies; TRAb, thyroid-stimulating hormone receptor antibodies. * indicates statistical significance.

2.2. Absence of TSHR Expression on NKT Cells

No expression of TSHR on the surface of NKT cells was detected by FACS in either the AITD or non-AITD patient group. Neither thyroid function nor thyroid-related pharmacotherapy in AITD group influenced this observation. This finding was further confirmed by an analysis performed with RT-PCR gene assays in the cells obtained from the healthy blood donors.

FACS analysis of NKT cells revealed no TSHR+ NKT cells in any of the patients, showing only neglectable detection of high-autofluorescence individual cells, analogous to the isotype control (Figure 1E). However, positive control staining conducted on seven patients detected TSHR+ cells (2.77%) among the total population of PBMCs (Figure 1F). The difference was statistically significant with $p = 0.018$ (Figure 2).

For further confirmation of the obtained results, a more sensitive method of RT-PCR was used in NKT cells isolated from healthy individuals. To achieve high RNA concentrations, NKT cells were isolated from up to 1×10^8 PBMCs isolated from 10 buffy coats collected from healthy donors. The purity of the NKT cells after magnetic sorting—prior to RNA isolation—was measured by FACS and showed a high (median 94.15%) percentage of NKT cells (Figure 3). During the analysis, one result was excluded due to low amplification of the tested genes.

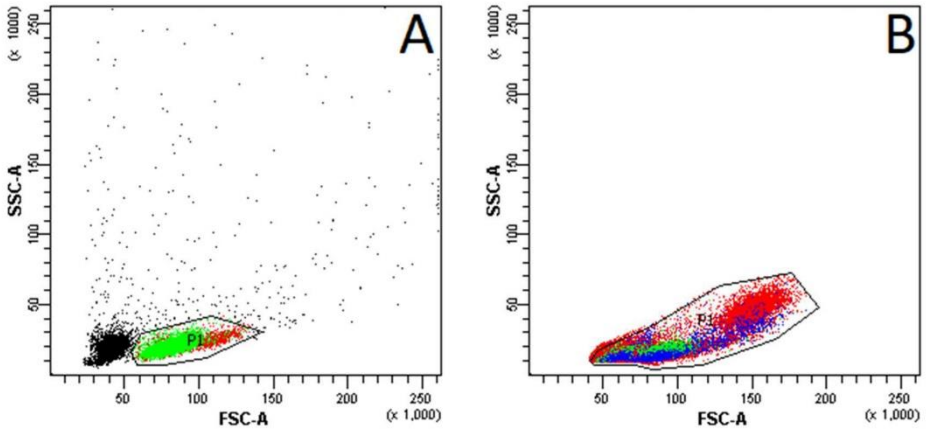

Figure 1. *Cont.*

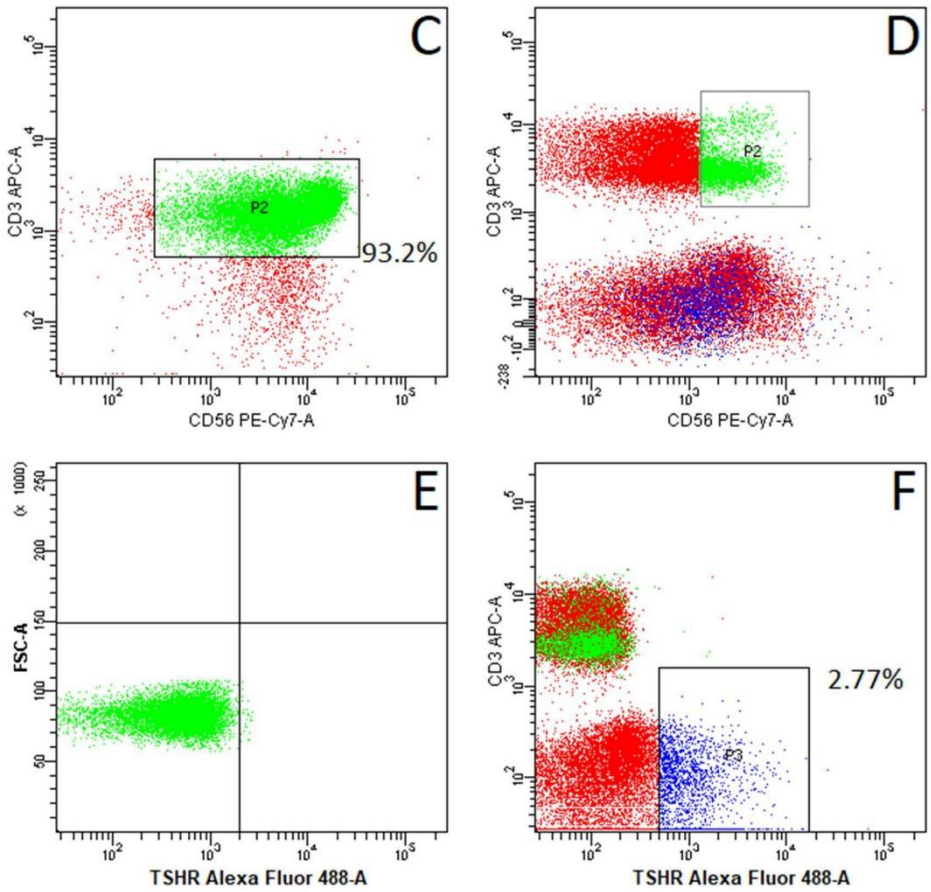

Figure 1. Exemplary plots of flow cytometry fluorescence-activated cell sorting (FACS) analysis showing the gating strategy of analyzed cells: (**A**): Natural Killer T (NKT) cells after magnetic sorting. (**B**): Peripheral blood mononuclear cells (PBMCs). (**C**): CD3+ CD56+ NKT cells after sorting. (**D**): CD3+ CD56+ NKT cells (green) among PBMCs. (**E**): Thyroid-stimulating hormone receptor (TSHR)-expressing NKT cells after sorting. (**F**): TSHR-expressing cells among PBMCs (blue). Black dots represent particles not recognized as single cells. Red dots represent negatively gated cells.

RT-PCR analysis has demonstrated no *TSHR* expression in NKT cells, while *TSHR* expression was present in the total PBMC population (Figure 4A). The results were normalized as positive (1) vs. negative (0) and significant difference was confirmed with Fisher's exact test with $p < 0.00001$, and with chi-square test with $p = 0.0002$. The median value of ΔCt (relative *TSHR* gene expression to housekeeping gene expression) for the *TSHR* gene in PBMCs was 10.3 (Figure 4B).

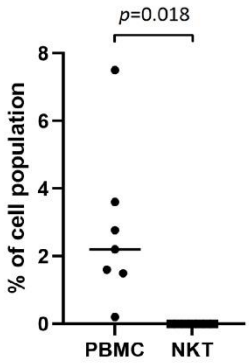

Figure 2. Percentage of cells expressing TSHR among whole PBMC fractions and in NKT cells.

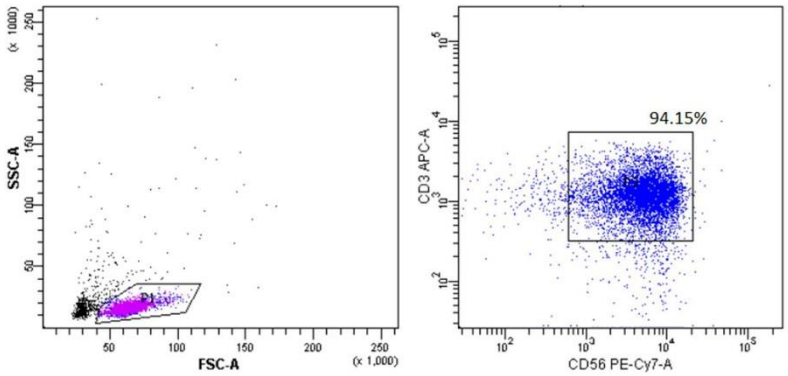

Figure 3. Gating strategy showing the purity of NKT cells isolated from buffy coats.

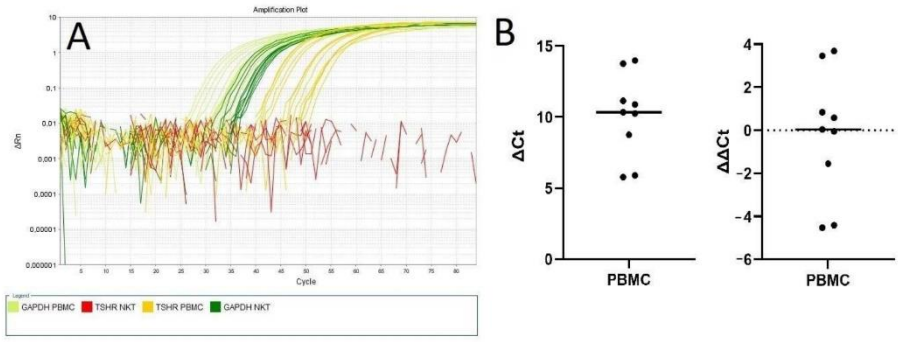

Figure 4. Reverse-transcription polymerase chain reaction (RT-PCR) results: (**A**): Amplification plots of target genes—(glyceraldehyde-3-phosphate dehydrogenase) *GAPDH* and *TSHR* from PBMC and NKT cells. (**B**): All measured ΔCt (relative TSHR gene expression to housekeeping gene expression) and ΔΔCt (ΔCt normalized to ΔCt median) values for *TSHR* gene in PBMC cells. Each dot identifies the mean expression calculated from triplets.

3. Discussion

This study provides the first evidence of the absence of TSHR on the surface of NKT cells in the peripheral blood of both healthy individuals and patients with benign thyroid nodular disease with and without AITD. The use of both FACS and RT-PCR strengthens the validity of our findings. FACS provides a robust method for detecting surface proteins, while RT-PCR allows for the detection of mRNA expression, confirming the absence of TSHR at both the protein and gene levels in NKT cells. The absence of TSHR expression on NKT cells in both AITD and non-AITD patients suggests that NKT cells do not participate directly in the TSHR-mediated pathways involved in thyroid autoimmunity. This finding is significant as it challenges previous assumptions about the involvement of these immune cells in AITD pathophysiology [5,27–29]. However, this does not exclude the involvement of NKT cells in AITD development driven through indirect mechanisms involving other humoral, cellular, or hormonal factors and further modulated by already triggered pathological processes. Additionally, NKT cells are suggested to play a role in AITD development and progression at the local thyroid gland tissue level, through the switch of activating versus inhibiting regulatory roles [29].

Despite the lack of TSHR on NKT cells, the relationship between peripheral blood NKT cell quantity and TSH levels has been previously documented. Adamczewski et al. [4] showed a significant increase in NKT cells after in vivo administration of rhTSH in patients after total thyroidectomy due to differentiated thyroid cancer. Moreover, Miko et al. [5] found a remarkably elevated peripheral NKT level in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure.

The detection of TSHR expression in the total PBMC population, but not in isolated NKT cells, suggests that TSHR expression on immune cells might be restricted to specific PBMC subpopulations. This aligns with previous studies identifying a high expression of TSHR mainly on DCs and B cells but a low expression on T cells or NK cells [2,3]. As mentioned above, NKT cells may be affected by abnormal TSH levels through complex, indirect interactions with other immune cells. One potential intermediary mechanism involving NKT cells in AITD pathophysiology may be related to interactions with antigen-presenting cells (APCs). APCs can capture and transform antigens in order to present them within the context of appropriate major histocompatibility complex (MHC) molecules [30]. NKT cells have a diverse range of TCRs, allowing them to recognize various antigens, though CD1d-restricted invariant NKT (iNKT) cells specifically recognize lipid antigens presented by CD1d molecules [31]. CD1d is a non-polymorphic, MHC class I-like molecule expressed on various APCs, primarily DCs, and to a lesser extent on B cells and macrophages. Its primary function is to present lipid antigens, such as glycolipids and glycosphingolipids, to NKT cells. The interaction between CD1d and the TCR on NKT cells is crucial for their activation. Upon recognizing lipid antigens presented by CD1d, NKT cells rapidly produce a diverse array of cytokines, such as interferon gamma (IFN-γ), interleukin-4 (IL-4), and interleukin-17 (IL-17), modulating the immune response. This activation triggers both direct cytotoxic effects and the recruitment and activation of other immune cells, thereby playing a pivotal role in bridging innate and adaptive immunity. The ability of CD1d to present both self and foreign lipid antigens allows NKT cells to participate in immune surveillance and regulation, contributing to the body's defense mechanisms against infections, tumor surveillance, and the modulation of autoimmune responses [32,33].

A role of DCs, as a population of the most potent APCs, should be considered in the TSH-mediated regulation of NKT cell function. In humans, two main DC groups are known: conventional/myeloid (cDCs) and plasmacytoid (pDCs) [34,35]. Through the expression of numerous co-stimulatory molecules and a unique secretion profile, DCs are involved in cross-interactions with other immune cells. Thus, DCs play a role in stimulating the immune response and ensuring immunological tolerance, making them key subjects in research on the pathogenesis of autoimmune diseases [36]. Interestingly, only a few studies assess DCs' role in human AITD. Leskela et al. [37] revealed a notably elevated amount of pDCs in thyroid tissue compared to peripheral blood in patients with GD and

HT. Stasiołek et al. [38] observed a significantly higher percentage of cDCs in fine-needle aspiration biopsy (FNAB) material than in peripheral blood in a group of AITD patients. Other studies also revealed an increased DC population in thyroid-infiltrating cells in patients with both GD and HT [39–41]. Elevated NKT [5,29] and DC [37–41] levels in AITD patients suggest a complex, indirect, co-stimulatory interaction between NKT cells and DCs as a possible mechanism involving NKT cells in thyroid diseases.

Another potential mechanism involving TSH in correlation with peripheral NKT cells was also postulated. Studies by Bessoles et al. [42] highlighted the complex interaction between glycolipids and environmental signals in regulating cytokine production by NKT cells, thereby modulating immune responses. To elucidate NKT cells' regulatory mechanisms, their activation by interleukin-2 (IL-2) and associated signaling pathways were examined. IL-2 uniquely activates the Signal Transducer and Activator of Transcription 6 (STAT6) pathway in NKT cells, leading to the production of both pro-inflammatory (IFN-γ) and anti-inflammatory (IL-4) cytokines, and to cell proliferation.

Clinical studies by Komorowski et al. [43,44] indicated that patients with primary hypothyroidism and elevated TSH levels exhibited increased IL-2 concentrations in peripheral blood, suggesting that elevated TSH levels may influence IL-2 production. Hence, it can be hypothesized that TSH might modulate NKT cell function indirectly through IL-2 mediation, thereby affecting the immune response. This linkage provides a potential pathway wherein TSH could exert its effects on NKT cells via IL-2, underscoring a novel intersection between endocrine and immune regulation.

This study has potential limitations as we used only two methods for the detection of TSHR expression on NKT cells. The FACS method has been broadly accepted for the detection of TSHR on cells of non-thyroid origin including peripheral blood cells [45–47]. Anti-TSHR antibody (clone C-10) was already proven to sufficiently detect TSHR [48–51]. Additionally, we confirmed that Alexa Fluor-conjugated antibody was sufficient for detecting even low TSHR expression (Figure 1F). However, we are aware that the FACS method is not sufficient to prove the absence of expression of any receptor. Therefore, our findings were verified by a very sensitive method of RT-PCR, which is considered a gold standard in gene expression analysis, including TSHR expression in immune cells [52,53]. Nevertheless, we believe that our results require further confirmation in studies with the application of other methods, including those with the highest available sensitivity (e.g., next-generation sequencing, NGS).

4. Materials and Methods

4.1. Patients

This study involved 86 patients with cytologically benign thyroid nodules (68 and 18 females and males, respectively) treated in the Department of Endocrinology and Metabolic Diseases, Polish Mother's Memorial Hospital—Research Institute, Łódź, Poland. Among the study group, 28 patients were diagnosed with AITD, including 7 cases of Hashimoto's thyroiditis treated with L-thyroxine, 14 cases of euthyroid chronic thyroiditis, and 7 cases of Graves' disease, with 5 patients treated with thiamazole and 2 euthyroid patients who had received radioiodine therapy. The mean age of the study participants was 59.7 ± 13.69 years.

4.2. Inclusion Criteria

AITD diagnosis was based on standard criteria including elevated thyroid peroxidase antibody (anti-TPO) level and/or elevated thyroglobulin antibody (anti-Tg) level, or elevated TSH receptor antibody (TRAb) level [54].

4.3. Biochemical Analysis

Serum levels of TSH, free triiodothyronine (FT3), free thyroxine (FT4), anti-Tg, anti-TPO, and TRAb were measured by the electrochemiluminescence immunoassay (ECLIA) with Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

4.4. NKT Cell Isolation

Peripheral blood samples (2 × 4.9 mL) were collected from each patient via venipuncture into EDTA-containing Blood Collecting Systems (Sarstedt, Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation at 400 g for 30 min using Histopaque®-1077 (Thermo Fisher Scientific, Waltham, MA, USA). NKT cells were then isolated from PBMCs using a CD3+ CD56+ NKT Cell isolation kit with a magnetic bead cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). In each patient, the cell purity was measured and the median NKT cell percentage was 93.2% (range 99.1–80.1%). NKT cells were gated as CD3+ CD56+ cells for further analysis.

4.5. Fluorescence-Activated Cell Sorting (FACS)

To analyze TSHR expression on NKT cells, fluorescence-activated cell sorting (FACS) was employed. Fluorochrome-conjugated antibodies against human CD3 (Becton Dickinson, Franklin Lakes, NJ, USA) and CD56 (Becton Dickinson, NJ, USA) were used to identify NKT cells, and an antibody against human TSHR (Santa Cruz, Santa Cruz, CA, USA) was used to determine TSHR expression on isolated NKT cells. Additional positive staining for antibody validation was conducted on whole blood samples from 7 patients (including 3 patients with AITD and 4 patients without AITD) to recognize TSHR+ cells in the PBMC population (Figure 1A–D). Appropriate isotype controls were used. FACS analyses were conducted using a BD FACSCanto II flow cytometer (Becton Dickinson, NJ, USA).

4.6. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

In order to confirm the obtained results with the application of a different method, TSHR expression was analyzed by reverse-transcription polymerase chain reaction (RT-PCR). To obtain a high number of NKT cells ($0.5–1 \times 10^6$), needed for sufficient RNA isolation, up to 100 mL of peripheral blood would have been needed from each patient. Therefore, for ethical reasons, we obtained a 10 buffy coats from the healthy blood donors registered in the Regional Center of Blood Donation and Treatment. PBMCs and NKT cells were isolated as described above, using a CD3+ CD56+ NKT Cell isolation kit with a magnetic bead cell separator (Miltenyi Biotec, Germany). Total ribonucleic acid (RNA) was extracted from both PBMCs and NKT cells, and *TSHR* expression was analyzed by RT-PCR using TaqMan assays. The *GAPDH* gene was used as a housekeeping gene. Analyses were performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Each measurement was conducted in triplets.

4.7. Statistical Analysis

Descriptive statistics of the collected material contained the mean and standard deviation (SD). For comparisons between the groups, Student's *t*-test for normally distributed variables and the Mann–Whitney U test for the other ones were used. Positive/negative values were tested using Fisher's exact test as well as with the chi-square test with Yates correction. The normality of data distributions was verified by the Shapiro–Wilk test. In all the tests, p-value < 0.05 was considered significant. Statistical Package for the Social Sciences (SPSS 20.0) software for Windows was used for all the calculations.

4.8. Ethics Procedures

Written informed consent was obtained from all patients for the procedures performed after their purpose and course were thoroughly explained. This study was approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland (approval code—41/2021).

5. Conclusions

The present study demonstrated for the first time that TSHR expression was not found on NKT cells. This observation provides new insight on the potential mechanism of TSH's impact on NKT cells, which seems to be indirect. These findings have significant

implications as therapeutic strategies targeting TSHR on immune cells should consider the specific immune cell subtypes involved in the disease development mechanisms. As the expression of TSHR on NKT cells had not been studied before, our findings require further confirmation with the application of different methods.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland (approval code—41/2021).

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

Data Availability Statement: The source data are available on reasonable request from the corresponding author.

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

Abbreviations

AITD	autoimmune thyroid disease
Anti-Tg	thyroid antithyroglobulin antibodies
Anti-TPO	thyroid peroxidase antibodies
APCs	antigen-presenting cells
CD	cluster of differentiation
DCs	dendritic cells
pDCs	plasmocytoid dendritic cells
cDCs	conventional dendritic cells
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescence-activated cell sorting
FNAB	fine-needle aspiration biopsy
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GD	Graves's disease
HT	Hashimoto's thyroiditis
IFN-y	interferon-gamma
IL-2	interleukin-2
IL-4	interleukin-4
IL-17	interleukin-17
iNKT	invariant Natural Killer T cells
MCH	major histocompatibility complex
NK	Natural Killer cells
NKT	Natural Killer T cells
NIS	sodium/iodide symporter
PBMCs	peripheral blood mononuclear cells
mRNA	messenger ribonucleic acid
rhTSH	recombinant human thyroid stimulating hormone
RNA	ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
SNP	single-nucleotide polymorphism
STAT6	Signal Transducer and Activator of Transcription 6

TCR	T-cell receptor
Tg	thyroglobulin
TPO	thyroid peroxidase
TRAb	thyroid-stimulating hormone receptor antibodies
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor

References

1. Jara, E.L.; Muñoz-Durango, N.; Llanos, C.; Fardella, C.; González, P.A.; Bueno, S.M.; Kalergis, A.M.; Riedel, C.A. Modulating the function of the immune system by thyroid hormones and thyrotropin. *Immunol. Lett.* **2017**, *184*, 76–83. [CrossRef] [PubMed]
2. Berczi, I. Neuroendocrine Regulation of Natural Immunity. *NeuroImmune Biol.* **2005**, *5*, 215–262.
3. Bağriaçik, E.U.; Klein, J.R. The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of CD45RBhigh lymph node T cells: Functional role for thyroid-stimulating hormone during immune activation. *J. Immunol.* **2000**, *15*, 6158–6165. [CrossRef] [PubMed]
4. Adamczewski, Z.; Stasiołek, M.; Zygmunt, A.; Śliwka, P.W.; Wieczorek-Szukała, K.; Lewiński, A. Recombinant Human Thyroid-Stimulating Hormone Increases the Percentages of Natural Killer T Cells and B Lymphocytes in Human Peripheral Blood In Vivo. *Front. Endocrinol.* **2020**, *11*, 543845. [CrossRef]
5. Miko, E.; Meggyes, M.; Doba, K.; Farkas, N.; Bogar, B.; Barakonyi, A.; Szereday, L.; Szekeres-Bartho, J.; Mezosi, E. Characteristics of peripheral blood NK and NKT-like cells in euthyroid and subclinical hypothyroid women with thyroid autoimmunity experiencing reproductive failure. *J. Reprod. Immunol.* **2017**, *124*, 62–70. [CrossRef]
6. Kaszubowska, L.; Piotrowska, A.; Siedlecka-Kroplewska, K.; Kmiec, Z. NKT cells as a connecting element between innate and adaptive immunity. *Postepy Biol. Komorki.* **2013**, *40*, 697–724.
7. Bojarska-Junak, A.; Tabarkiewicz, J.; Roliński, J. NKT cells: Their development, mechanisms and effects of action. *Postepy Hig. Med. Dosw.* **2013**, *15*, 65–78. [CrossRef]
8. Hervier, B.; Beziat, V.; Haroche, J.; Mathian, A.; Lebon, P.; Ghillani-Dalbin, P.; Musset, L.; Debré, P.; Amoura, Z.; Vieillard, V. Phenotype and function of natural killer cells in systemic lupus erythematosus: Excess interferon-g production in patients with active disease. *Arthritis Rheum.* **2011**, *63*, 1698–1706. [CrossRef]
9. Rodacki, M.; Svoren, B.; Butty, V.; Besse, W.; Laffel, L.; Benoist, C.; Mathis, D. Altered natural killer cells in type 1 diabetic patients. *Diabetes* **2007**, *56*, 177–185. [CrossRef]
10. Novak, J.; Griseri, T.; Beaudoin, L.; Lehuen, A. Regulation of type 1 diabetes by NKT cells. *Int. Rev. Immunol.* **2007**, *26*, 49–72. [CrossRef]
11. Zarobkiewicz, M.K.; Morawska, I.; Michalski, A.; Roliński, J.; Bojarska-Junak, A. NKT and NKT-like Cells in Autoimmune Neuroinflammatory Diseases-Multiple Sclerosis, Myasthenia Gravis and Guillain-Barre Syndrome. *Int. J. Mol. Sci.* **2021**, *22*, 9520. [CrossRef] [PubMed]
12. Chen, J.; Wu, M.; Wang, J.; Li, X. Immunoregulation of NKT Cells in Systemic Lupus Erythematosus. *J. Immunol. Res.* **2015**, *2015*, 206731. [CrossRef] [PubMed]
13. Watanabe, M.; Nakamura, Y.; Matsuzuka, F.; Takamura, Y.; Miyauchi, A.; Iwatani, Y. Decrease of intrathyroidal CD161Valpha24Vbeta11 NKT cells in Graves' disease. *Endocr. J.* **2008**, *55*, 199–208. [CrossRef] [PubMed]
14. Nagayama, Y.; Watanabe, K.; Niwa, M.; McLachlan, S.M.; Rapoport, B. Schistosoma mansoni and alpha-galactosylceramide: Prophylactic effect of Th1 Immune suppression in a mouse model of Graves' hyperthyroidism. *J. Immunol.* **2004**, *173*, 2167–2173. [CrossRef]
15. McLeod, D.S.; Cooper, D.S. The incidence and prevalence of thyroid autoimmunity. *Endocrine* **2012**, *42*, 252–265. [CrossRef]
16. Rapoport, B.; McLachlan, S.M. The Thyrotropin Receptor in Graves' Disease. *Thyroid. Off. J. Am. Thyroid Assoc.* **2007**, *17*, 911–922. [CrossRef]
17. Vassart, G.; Dumont, J.E. The Thyrotropin Receptor and the Regulation of Thyrocyte Function and Growth. *Endocr. Rev.* **1992**, *13*, 596–611. [CrossRef]
18. Chu, Y.-D.; Yeh, C.-T. The Molecular Function and Clinical Role of Thyroid Stimulating Hormone Receptor in Cancer Cells. *Cells* **2020**, *9*, 1730. [CrossRef]
19. Williams, G.R. Extrathyroidal Expression of TSH Receptor. *Ann. Endocrinol.* **2011**, *72*, 68–73. [CrossRef]
20. Postiglione, M.P.; Parlato, R.; Rodriguez-Mallon, A.; Rosica, A.; Mithbaokar, P.; Maresca, M.; Marians, R.C.; Davies, T.F.; Zannini, M.S.; De Felice, M.; et al. Role of the Thyroid-Stimulating Hormone Receptor Signaling in Development and Differentiation of the Thyroid Gland. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15462–15467. [CrossRef]
21. Wenzek, C.; Boelen, A.; Westendorf, A.M.; Engel, D.R.; Moeller, L.C.; Führer, D. The Interplay of Thyroid Hormones and the Immune System—Where We Stand and Why We Need to Know About It. *Eur. J. Endocrinol.* **2022**, *186*, R65–R77. [CrossRef] [PubMed]
22. Nagayama, Y.; Takeshita, A.; Luo, W.; Ashizawa, K.; Yokoyama, N.; Nagataki, S. High affinity binding of thyrotropin (TSH) and thyroid-stimulating autoantibody for the TSH receptor extracellular domain. *Thyroid* **1994**, *4*, 155–159. [CrossRef] [PubMed]
23. Cuddihy, R.M.; Dutton, C.M.; Bahn, R.S. A polymorphism in the extracellular domain of the thyrotropin receptor is highly associated with autoimmune thyroid disease in females. *Thyroid* **1995**, *5*, 89–95. [CrossRef] [PubMed]

24. Qian, X.; Xu, K.; Jia, W.; Lan, L.; Zheng, X.; Yang, X.; Cui, D. Association between TSHR gene polymorphism and the risk of Graves' disease: A meta-analysis. *J. Biomed. Res.* **2016**, *30*, 466–475. [CrossRef] [PubMed]
25. Gong, J.; Jiang, S.J.; Wang, D.K.; Dong, H.; Chen, G.; Fang, K.; Cui, J.R.; Lu, F.E. Association of polymorphisms of rs179247 and rs12101255 in thyroid stimulating hormone receptor intron 1 with an increased risk of Graves' disease: A meta-analysis. *J. Huazhong Univ. Sci. Technol. Med. Sci.* **2016**, *36*, 473–479. [CrossRef]
26. Xiong, H.; Wu, M.; Yi, H.; Wang, X.; Wang, Q.; Nadirshina, S.; Zhou, X.; Liu, X. Genetic associations of the thyroid stimulating hormone receptor gene with Graves diseases and Graves ophthalmopathy: A meta-analysis. *Sci. Rep.* **2016**, *6*, 30356. [CrossRef]
27. Burek, C.L.; Sharma, R.B.; Rose, N.R. NKT cell regulation of autoimmune thyroiditis. *Autoimmunity* **2003**, *36*, 405–408. [CrossRef]
28. Sharma, R.B.; Fan, X.; Caturegli, P.; Rose, N.R.; Burek, C.L. Invariant NKT Cell Lines Derived from the NOD.H2 Mouse Enhance Autoimmune Thyroiditis. *J. Thyroid Res.* **2011**, *2011*, 895923.
29. Guo, H.; Xu, B.; Yang, X.; Wang, Y.; Liu, X.; Cui, C.; Jiang, Y. A high frequency of peripheral blood NKG2D+NK and NKT cells in euthyroid patients with new onset hashimoto's thyroiditis—A pilot study. *Immunol. Investig.* **2014**, *43*, 312–323. [CrossRef]
30. Savina, A.; Amigorena, S. Phagocytosis and antigen presentation in dendritic cells. *Immunol. Rev.* **2007**, *219*, 143–156. [CrossRef]
31. Bendelac, A.; Savage, P.B.; Teyton, L. The biology of NKT cells. *Annu. Rev. Immunol.* **2007**, *25*, 297–336. [CrossRef] [PubMed]
32. Chaudhry, M.S.; Karadimitris, A. Role and regulation of CD1d in normal and pathological B cells. *J. Immunol.* **2014**, *193*, 4761–4768. [CrossRef] [PubMed]
33. Brigl, M.; Brenner, M.B. CD1: Antigen presentation and T cell function. *Annu. Rev. Immunol.* **2004**, *22*, 817–890. [CrossRef] [PubMed]
34. Shortman, K.; Liu, Y.J. Mouse and Human Dendritic Cell Subtypes. *Nat. Rev.* **2002**, *21*, 151–161. [CrossRef]
35. Reizis, B. Plasmacytoid Dendritic Cells: Development, Regulation, and Function. *Immunity* **2019**, *50*, 37–50. [CrossRef]
36. Turley, S.J. Dendritic cells: Inciting and inhibiting autoimmunity. *Curr. Opin. Immunol.* **2002**, *14*, 765–770. [CrossRef]
37. Leskela, S.; Rodríguez-Munoz, A.; de la Fuente, H.; Figueroa-Vega, N.; Bonay, P.; Martín, P.; Serrano, A.; Sánchez-Madrid, F.; González-Amaro, R.; Marazuela, M. Plasmacytoid dendritic cells in patients with autoimmune thyroid disease. *J. Clin. Endocrin. Metab.* **2013**, *98*, 2822–2833. [CrossRef]
38. Stasiołek, M.; . Śliwka, P.W.; Stasiak, M.; Krawczyk-Rusiecka, K.; Skowrońska-Jóźwiak, E.; Adamczewski, Z.; Lewiński, A. Differences of the Structure of Immune Regulatory Cell Populations between Cellular Material from Sonographically Detected Focal Thyroid Lesions and Peripheral Blood in Humans. *Int. J. Mol. Sci.* **2019**, *20*, 918. [CrossRef]
39. Quadbeck, B.; Eckstein, A.K.; Tews, S.; Walz, M.; Hoermann, R.; Mann, K.; Gieseler, R. Maturation of thyroidal dendritic cells in Graves' disease. *Scand. J. Immunol.* **2002**, *55*, 612–620. [CrossRef]
40. Hammerstad, S.S.; Jahnsen, F.L.; Tauriainen, S.; Hyöty, H.; Paulsen, T.; Norheim, I.; Dahl-Jørgensen, K. Inflammation and increased myxovirus resistance protein A expression in thyroid tissue in the early stages of Hashimoto's thyroiditis. *Thyroid* **2013**, *23*, 334–341. [CrossRef]
41. Hammerstad, S.S.; Jahnsen, F.; Tauriainen, S.; Hyöty, H.; Paulsen, T.; Norheim, I.; Dahl-Jørgensen, K. Immunological Changes and Increased Expression of Myxovirus Resistance Protein A in Thyroid Tissue of Patients with Recent Onset and Untreated Graves' Disease. *Thyroid* **2014**, *24*, 537–544. [CrossRef] [PubMed]
42. Bessoles, S.; Fouret, F.; Dudal, S.; Besra, G.S.; Sanchez, F.; Lafont, V. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *J. Leukoc. Biol.* **2008**, *84*, 224–233. [CrossRef] [PubMed]
43. Komorowski, J. Increased interleukin-2 level in patients with primary hypothyroidism. *Clin. Immunol. Immunopathol.* **1992**, *63*, 200–202. [CrossRef] [PubMed]
44. Komorowski, J.; Zylińska, K.; Pawlikowski, M.; Stepień, H. Stimulatory effect of thyrotropin (TSH) on interleukin-2 (IL-2) release from human peripheral blood lymphocytes. A dose-response study in vitro. *Horm. Metab. Res.* **1993**, *25*, 598–599. [CrossRef] [PubMed]
45. Douglas, R.S.; Afifiyan, N.F.; Hwang, C.J.; Chong, K.; Haider, U.; Richards, P.; Gianoukakis, A.G.; Smith, T.J. Increased generation of fibrocytes in thyroid-associated ophthalmopathy. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 430–438. [CrossRef]
46. Fernando, R.; Placzek, E.; Reese, E.A.; Placzek, A.T.; Schwartz, S.; Trierweiler, A.; Niziol, L.M.; Raychaudhuri, N.; Atkins, S.; Scanlan, T.S.; et al. Elevated Serum Tetrac in Graves' Disease: Potential Pathogenic Role in Thyroid-Associated Ophthalmopathy. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 776–785. [CrossRef]
47. Gillespie, E.F.; Papageorgiou, K.I.; Fernando, R.; Raychaudhuri, N.; Cockerham, K.P.; Charara, L.K.; Goncalves, A.C.; Zhao, S.X.; Ginter, A.; Lu, Y.; et al. Increased expression of TSH receptor by fibrocytes in thyroid-associated ophthalmopathy leads to chemokine production. *J. Clin. Endocrinol. Metab.* **2012**, *97*, E740–E746. [CrossRef]
48. Ma, R.; Gan, L.; Guo, J.; Peng, Z.; Wu, J.; Harrison, A.R.; Qian, J. Insights into Ferroptosis: Targeting Glycolysis to Treat Graves' Orbitopathy. *J. Clin. Endocrinol. Metab.* **2022**, *16*, 1994–2003. [CrossRef]
49. Zulkarnain, Z.; Ulhaq, Z.S.; Sujuti, H.; Soeatmadji, D.W.; Zufry, H.; Wuragil, D.K.; Marhendra, A.P.W.; Riawan, W.; Kurniawati, S.; Oktanella, Y.; et al. Comparative performance of ELISA and dot blot assay for TSH-receptor antibody detection in Graves' disease. *J. Clin. Lab. Anal.* **2022**, *36*, e24288. [CrossRef]
50. Wu, Z.; Xi, Z.; Xiao, Y.; Zhao, X.; Li, J.; Feng, N.; Hu, L.; Zheng, R.; Zhang, N.; Wang, S.; et al. TSH-TSHR axis promotes tumor immune evasion. *J. Immunother. Cancer* **2022**, *10*, e004049. [CrossRef]

51. Lee, S.I.; Kim, D.K.; Seo, E.J.; Choi, E.J.; Kwon, Y.W.; Jang, I.H.; Lee, J.C.; Kim, H.Y.; Shong, M.; Kim, J.H.; et al. Role of Krüppel-Like Factor 4 in the Maintenance of Chemoresistance of Anaplastic Thyroid Cancer. *Thyroid* **2017**, *27*, 1424–1432. [CrossRef] [PubMed]
52. Kim, M.; Oh, S.W.; Youn, H.; Na, J.; Kang, K.W.; Park, D.J.; Park, Y.J.; Jang, J.J.; Lee, K.E.; Jung, K.C.; et al. Thyroid-Related Protein Expression in the Human Thymus. *Int. J. Endocrinol.* **2017**, *2017*, 8159892. [CrossRef]
53. Gao, H.; Lu, X.; Huang, H.; Ji, H.; Zhang, L.; Su, Z. Thyroid-stimulating hormone level is negatively associated with fertilization rate in patients with polycystic ovary syndrome undergoing in vitro fertilization. *Int. J. Gynaecol. Obstet.* **2021**, *155*, 138–145. [CrossRef] [PubMed]
54. Muller, I.; Moran, C.; Lecumberri, B.; Decallonne, B.; Robertson, N.; Jones, J.; Dayan, C.M. 2019 European Thyroid Association Guidelines on the Management of Thyroid Dysfunction following Immune Reconstitution Therapy. *Eur. Thyroid J.* **2019**, *8*, 173–185. [CrossRef] [PubMed]

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An impact of type 2 diabetes mellitus on NKT-like cell population in humans: a new insight into impaired immune response in hyperglycemia

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Background: Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by insulin resistance and pancreatic β-cell dysfunction. T2DM is associated with increased risk of infections and of several malignancies, although the underlying immune mechanisms remain not fully elucidated. Natural Killer T-like cells (NKT-like) belong to a unique subpopulation of T lymphocytes defined by expression of the markers specific to NK (Natural Killer) cells (CD56) and T cells (TCR - T cell receptor). As NKT-like cells possess unique cytotoxic properties, they form a bridge between innate and adaptive immunity. The aim of our study was to assess associations between the presence of T2DM and the profile of NKT-like cell subpopulations.

Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from 86 patients. NKT-like cells were subsequently isolated from the PBMC fraction using a CD3+ CD56+ NKT cell isolation kit in combination with magnetic bead-based separation. To evaluate NKT-like cells subpopulations distribution, flow cytometry (FC) was used. NKT-like cells were categorized into CD4-CD8- (double negative, DN), CD4+CD8-, CD4-CD8+, and CD4+CD8+ (double positive, DP) subpopulations, with further subdivision of DP and CD4-CD8+ subpopulations into CD4highCD8mid/CD4midCD8high and CD4-CD8mid/CD4-CD8high subpopulations, respectively. Associations between NKT-like cells subpopulation and T2DM, and – additionally – correlations between NKT-like and glucose levels and body mass index (BMI) were evaluated.

Results: T2DM group demonstrated a significantly diminished percentage of DN NKT-like cells as compared to control group. A strong negative correlation was observed between DN NKT-like cell levels and glucose concentration, but not BMI. Based on further subdivision of DP and CD4-CD8+ subpopulations a significant negative correlation was also observed between glucose levels and the $CD4^{-}$CD8mid NKT-like cell subpopulation. No such association was detected for the other subpopulations.

Conclusions: Our study demonstrated that DN NKT-like cells, which possess significant cytotoxic activity, are depleted in T2DM patients. These results may explain the novel potential mechanism of increasing susceptibility to infections

and cancers in T2DM and emphasize the need for precise glycemic control. The novel insights into NKT-like cell immunomodulation role in T2DM may open new, targeted therapies in metabolic diseases. Further research in larger cohorts is needed to confirm these pioneering observations.

KEYWORDS

NKT-like cells, CD4-CD8-NKT-like cells, diabetes mellitus, T2DM, glucose, immune response, IFN-γ

1 Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance and progressive pancreatic β-cell dysfunction, leading to hyperglycemia (1). The pathophysiology of T2DM involves a combination of various factors including chronic low-grade inflammation and dysregulation of the immune system (2–4). In 1993, Hotamisligil et al. conducted pioneering research in which a link between inflammation and metabolic dysfunction was found. Authors revealed that tumor necrosis factor alpha (TNF-α), produced by adipose tissue, is a significant mediator of insulin resistance in the context of obesity (5). The underlying mechanisms of T2DM pathogenesis and complications are still incompletely understood, but many studies have proven that immunocompetent cells including T cells, B cells, Natural Killer (NK) cells, and macrophages infiltrate adipose tissue and play a crucial role in adipose inflammation, and in the development of insulin resistance which leads to the onset of T2DM (6–11). However, little is known about the involvement of other immune cells, including Natural Killer T (NKT) cells and Natural Killer T-like (NKT-like) cells, in the pathogenesis of T2DM-related complications.

NKT-like cells, until recently often referred as NKT cells, are a unique, heterogenous subset of T lymphocytes that share properties of both conventional T cells (i.e. T cell receptor - TCR) and NK cells (i.e. cluster of differentiation (CD) 56). Classically, NKT cells were divided into two primary categories: type I NKT cells (invariant NKT - iNKT) and type II NKT cells (variant - vNKT). The classical identification of NKT cells, based on CD3 and CD56 surface antigens, have been reconsidered with the discovery of unspecific expression of CD56 molecule on more broad spectrum of T lymphocytes – mainly activated γδ T lymphocytes and a percentage of αβ T lymphocytes (12). It is, however, strongly considered, that cells expressing CD56 antigen are characterized by higher state of activation, exhibiting some level of cytotoxic properties, therefore, possibly sharing similar properties (13). Unlike conventional T cells that recognize peptide antigens presented by classical major histocompatibility complex (MHC) molecules, iNKT cells recognize lipid antigens presented by the non-classical MHC molecule CD1d (14). Invariant NKT cells are very rare in peripheral blood unlike other subpopulations of NKT-like cells, that occur more frequently in the circulation and their activation is independent of CD1d-mediated antigen presentation. NKT-like cells indicate significant cytotoxic activity and produce proinflammatory cytokines such as IFN-γ and TNF-α, contributing to their anti-tumor and antimicrobial defense (15). NKT-like cells serve as a link between innate and adaptive immunity, exhibiting cytotoxic capabilities through the release of perforin and granzyme B and producing a diverse array of cytokines that modulate inflammatory responses both directly, and indirectly by interacting with various immune cells (16). Recent advances in immunology, particularly in cell identification techniques, have led to ongoing revisions in the classification of iNKT and NKT-like cell subpopulations. Depending on CD4 and CD8 expression, iNKT and NKT-like cells can be categorized into four subpopulations: CD4-CD8+, CD4+CD8, CD4+CD8+ (double positive; DP) and CD4-CD8- (double negative; DN) (17). In the past, CD4-CD8- and CD4-CD8+ NKT cells were considered a part of the same subpopulation (18, 19). However, recent research has demonstrated significant gene expression differences between DN and CD4-CD8+ NKT cells, highlighting their distinct identities (19).

NKT-like cells as extraordinary subset of T lymphocytes characterized by potent cytotoxic activity and their ability to respond rapidly through cytokine production have regulatory effects which makes them a target for new therapeutic interventions related to infections, cancer, and autoimmune disorders (15, 20–23).

In the recent years, significant advancement in immunology is observed, particularly in the characterization of immune cell

Abbreviations: α-GalCer, α-Galactosylceramide; APC, Allophycocyanin; BMI, body mass index; CD, cluster of differentiation; CDF, cumulative distribution function; CG, control group; DMG, diabetes mellitus group; DN, double negative; DP, double positive; FC, Flow cytometry; FITC, Fluorescein isothiocyanate; HbA1c, hemoglobin A1c; IFN-γ, interferon gamma; IL, interleukin; iNKT, invariant Natural Killer T cells; MHC, major histocompatibility complex; NK cells, Natural Killer cells; NKT cells, Natural Killer T cells; NKT-like cells, Natural Killer T-like cells; NOD, nonobese diabetic; PE, Phycoerythrin; PerCP, Peridinin-Chlorophyll-Protein; PeCy7, Phycoerythrin/Cyanine7; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TCR, T cell receptor; TGF-β, transforming growth factor beta; Th1, T-helper type 1; Th2, T-helper type 2; TNF-α, tumor Necrosis Factor alpha; vNKT, variant Natural Killer T cells.

subpopulations. The application of high-dimensional cytometry, single-cell RNA sequencing, and detailed profiling of surface and functional markers has facilitated the identification of novel, functionally distinct subpopulations of immune cells. Data on the impact of T2DM on NKT-like cells are scarce and include only the influence on a total number of NKT-like cells (15, 24, 25). There is a lack of comprehensive study on the impact of NKT-like cell subpopulations on T2DM-related complications. Therefore, the aim of the present study was to evaluate potential association between the presence of T2DM the distribution of CD4/CD8-defined subpopulations within NKT-like cells, as well as correlation between glucose concentration and the distribution of NKT-like subpopulations, in order to find potential mechanisms which can lead to impaired immune response in T2DM via modification of NKT-like cell subpopulation profile.

2 Materials and methods

2.1 Patients

The research included 86 patients (66 women and 20 men) treated at the Department of Endocrinology and Metabolic Diseases of the Polish Mother's Memorial Hospital – Research Institute in Łódź, Poland. The study group included 24 patients with T2DM (DM group, DMG) and 62 persons without glucose metabolism-related disorders (control group, CG). Individuals with prediabetic states, active malignancy, active infection or inflammatory diseases which might have influenced the results were excluded from the study.

2.2 Biochemical analysis

Glucose concentration was assessed using electrochemiluminescence immunoassay (ECLIA) method on the Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

2.3 NKT-like cell isolation

Peripheral blood samples (2 × 4.9 mL) were obtained from each participant into EDTA-coated collection tubes (Sarstedt, Nümbrecht, Germany) using standard venipuncture techniques. Peripheral blood mononuclear cells (PBMCs) were subsequently isolated via density gradient centrifugation with Histopaque®-1077 (Thermo Fisher Scientific, Waltham, MA, USA), performed at 400 × g for 30 minutes at room temperature. In order to isolate NKT-like cells, the PBMCs fraction was subjected to two-step magnetic separation using a CD3+ CD56+ NKT Cell Isolation Kit in combination with a magnetic column system (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was assessed individually for each subject, yielding a median purity of 93.2% across samples, with values ranging from 80.1% to 99.1%. Downstream analyses were conducted on cells gated as CD3+CD56+ to ensure population specificity.

2.4 Flow cytometry

In order to examine the distribution of NKT-like cell subsets, flow cytometry (FC) was employed. Cells were stained with fluorochrome-conjugated monoclonal antibodies specific to human CD3 (APC, clone UCHT1) and CD56 (PE-Cy7, clone B159), (both Becton Dickinson, Franklin Lakes, NJ, USA) for NKT-like cell identification. Anti Vα24JαQ TCR Chain antibody (PE, clone 6B11) was used to determine the percentage of Invariant NK T Cell population resulting in very low (~1.2%) frequency of this cell subtype. We did not analyze the αβ T-cell receptor nor the γδ T-cell receptor on the surface of isolated cells, therefore, according to current standards, we assume that all further subset analysis are performed on heterogenous population of CD3+CD56+ NKT-like cells. Subset differentiation was further refined using antibodies targeting CD4 (FITC, clone SK3) and CD8 (PerCP, clone SK1) surface markers (both Becton Dickinson, NJ, USA). Isotype-matched control antibodies were included in each experiment to verify staining specificity. Flow cytometric analysis was performed on a BD FACSCanto II cytometer (Becton Dickinson, NJ, USA), and data were analyzed using the associated software.

Based on the framework established by Montoya et al. (14) for invariant NKT cells (iNKT), four primary subsets were initially defined: CD4-CD8- (double negative), CD4-CD8+, CD4+CD8-, and CD4+CD8+ (double positive). Subsequent high-resolution analysis revealed notable heterogeneity within the population of cells expressing CD8 molecule. As a result, CD4+CD8+ cells were further stratified into CD4highCD8mid and CD4midCD8high subtypes, while CD4-CD8+ cells were subdivided into CD4-CD8mid and CD4-CD8high subtypes. The detailed gating strategy used for these analyses is shown in Figure 1.

2.5 Statistical analysis

For each patient, the number of NKT-like cells of each type (CD4-CD8-, CD4-CD8+, CD4+CD8-, CD4+CD8+) was obtained, as detailed above, and expressed as a percentage of the total number of NKT-like cells gated as CD3+CD56+ cells. Additionally, the number of CD4highCD8mid cells (and – similarly – of CD4midCD8high cells) was also expressed as a percentage value. In this case, we used two different representations: percentage with respect to the total number of NKT-like cells (as above) and percentage with respect to the number of CD4+CD8+ cells only (note that in this representation CD4highCD8mid sums up to 100% with CD4midCD8high). Similarly, the numbers of CD4-CD8mid and of CD4-CD8high cells were also transformed to percentages in the same two ways: with respect to the total number of NKT-like cells and with respect to the number of CD4-CD8+ cells only. All the analyses reported in the present study were based on thus

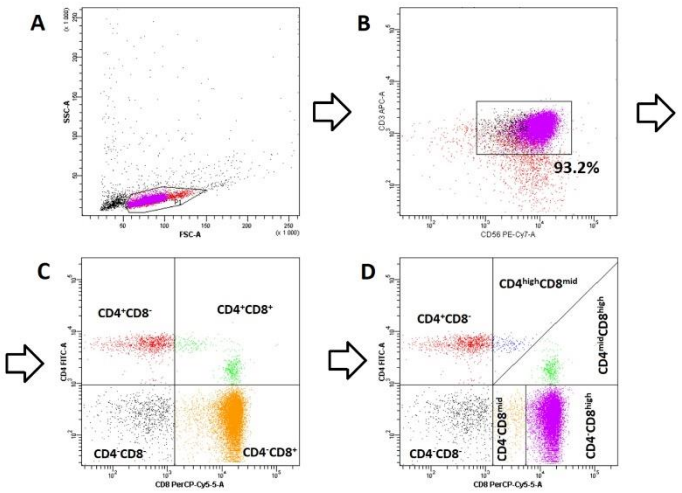

FIGURE 1
Representative flow cytometry (FC) plots illustrating the gating strategy used for cell analysis: **(A)** PBMC-derived population post-magnetic enrichment; **(B)** $CD3^+CD56^+$ NKT-like cells following FC purification; **(C)** Primary gating scheme for initial NKT-like cell identification; **(D)** Refined gating strategy for distinguishing NKT-like cell subpopulations.

defined percentage values (no absolute values of NKT-like cell counts were used).

The NKT-like cell percentages were subjected to statistical analysis including empirical distribution assessment of each NKT-like type/subtype within the DM and control groups. In both groups, summary statistics (mean, standard deviation and order statistics) were computed for all NKT-like types/subtypes and statistical tests were performed to reveal statistically significant differences between DMG and CG. Student's t-test was used for normally distributed variables and Mann-Whitney U-test otherwise. The normality of distribution was assessed with Shapiro-Wilk test. Additionally, for contingency tables obtained from splitting the patients into two groups on the basis of NKT-like cell percentage, chi-squared test was applied. In the case of real-valued variables (glucose level and body mass index) Pearson's linear correlation coefficient and Spearman's rank correlation coefficient with respect to NKT-like cell percentage were computed. All the computations were performed with the use of SciPy library for Python programming language.

2.6 Ethics procedures

All patients provided written informed consent for the procedures after receiving a full explanation of their purpose and course. The study received approval from the Ethics Committee of the Polish Mother's Memorial Hospital – Research Institute in Łódź, Poland (approval code: 41/2021).

3 Results

The study group included 24 patients with T2DM and 62 persons without glucose metabolism-related disorders. The mean age of the included patients was 59.7 ± 13.7 years. The mean age of patients in DMG and CG was 64,96 ± 11,3 and 58,25 ± 14,1 years, respectively. The whole cohort included 66 females and 20 males, and the proportion of males to females in DMG and CG was 9:15 and 11:51 respectively. The mean level of glucose in DMG was 125.88 mg/dl, median 122.5 mg/dl. The mean level of glucose in CG was 91.08 mg/dl, median 90.0mg/dl.

Taking into account the differences between DMG and CG with regard to gender, with significantly higher proportion of males in DMG, we performed an initial analysis of potential association between gender and NKT-like cell subpopulation. No association between gender and the percentage of NKT-like cells was found, with p value of 0.866, 0.826, 0.388 and 0.203 for CD4+CD8-, CD4+CD8+, CD4-, CD8+ and CD4-CD8- subpopulations, respectively, which confirmed the lack of gender-related bias.

In the first part of the main study, we analyzed a possible relationship between our main four NKT-like cell subpopulations and the presence of T2DM. Student t-test and Mann-Whitney test were used to compare the percentage of NKT-like cells (of a given subpopulation) between DMG and CG. This analysis showed significant negative association (p-value = 0.007) between the presence of T2DM and the percentage of CD4-CD8- NKT-like cells (with respect to the total number of NKT-like cells gated as CD3+CD56+ cells, as described above). In the case of the other

TABLE 1 Associations between percentage of NKT-like cell types and T2DM.

		mean ± SD	Median	Min	max	IQR	*p*-value
CD4+CD8-	CG	17.61 ± 15.57	13.4	0.3	62.1	19.7	0.647
CD4+CD8-	DMG	17.08 ± 16.63	10.2	0.6	65.9	22.78	
CD4+CD8+	CG	6.93 ± 9.94	3.25	0.5	52.3	5.68	0.394
CD4+CD8+	DMG	8.23 ± 10.68	5.8	0.3	50.0	7.45	
CD4-CD8-	CG	16.67 ± 15.84	10.3	0.0	60.7	14.6	**0.007**
CD4-CD8-	DMG	8.28 ± 8.76	6.4	0.4	36.0	7.33	
CD4-CD8+	CG	58.81 ± 20.39	62.1	11.2	91.6	28.93	0.112
CD4-CD8+	DMG	66.41 ± 24.36	68.45	7.7	97.3	36.28	

Statistically significant p-values are presented in bold.

NKT-like cell types, no significant association with T2DM was found. Table 1 presents the obtained results (only Mann-Whitney test results are reported, as most of the considered distributions deviated significantly from the normal distribution.

We explored further the dependence between CD4-CD8- and T2DM, by splitting all the patients into two subgroups on the basis of the number of CD4-CD8- cells. We set the percentage threshold to 9%, as this particular value yielded approximately equal-sized groups: 44 patients below the threshold (i.e. having less than 9% of CD4-CD8- cells among all four studied NKT-like cell types gated as CD3+CD56+ cells) and 42 patients over the threshold (with more than 9% of CD4-CD8- cells). The corresponding contingency is presented in Table 2. We performed chi-squared test which clearly confirmed the correlation with T2DM ($\chi^2 = 5.16$, p-value = 0.023). The obtained odds ratio (OR=0.318) indicates that the incidence of T2DM in the group with high CD4-CD8- percentage (7 vs 35) is over three times lower than in the control group (17 vs 27). It should be stressed that these results are stable in a range of threshold values (with the maximum at threshold of 10% which generates groups of 47 and 39 patients respectively, yielding $\chi^2 = 5.56$, p-value = 0.018 and OR=0.293).

Figure 2 shows how many T2DM patients (yellow line) and CG individuals (green line) fall below the threshold, for all possible threshold values. To make comparison more clear, we present the percentage instead of absolute numbers (i.e. the number of T2DM patients below the threshold with respect to all T2DM patients – and similarly for the CG persons). The curves obtained in this way may be interpreted as the estimates of cumulative distribution function (CDF) of CD4-CD8- level in DMG and CG, respectively. For example, all T2DM patients have CD4-CD8- below 37%, so the yellow line hits 100% for the threshold of 37%, while the green line reaches only 85% at this point, as 9 non-DM patients (out of 62) have CD4-CD8- level higher than 37%. It is worth noting that the p-value for this particular case is 0.049.

In the further analysis we evaluated correlation between CD4-CD8- percentage (with respect to the total number of NKT-like cells) and glucose level. Figure 3A shows the scatter plot of glucose level vs CD4-CD8- percentage in our whole study group (DMG + CG). The negative trend is clearly visible here although it is non-linear so we decided to use Spearman's rank correlation coefficient (which yields $\rho = -0.27$ with p-value 0.011) instead of Pearson's ρ. Linear correlation is better visible after logarithmic scaling of both variables (Figure 3B). Pearson's correlation coefficient after this transformation is equal to -0.29 with p-value = 0.007 (Spearman's ρ remains equal to -0.27, due to the rank-preserving monotonicity of the log function).

In the next part of the study, we additionally analyzed correlation of the subtypes of NKT-like cells with glucose level. The analysis revealed negative correlation between glucose and CD4-CD8mid subtype. This correlation was visible irrespective of the method of CD4-CD8mid level representation – either as percentage of all four studied NKT-like types or as percentage of CD4-CD8+ cells only (see Section 2.5). The first case is depicted in Figure 4A (Spearman's $\rho = -0.292$ with p-value = 0.01) and the second one – in Figure 4B ($\rho = -0.25$ with p-value 0.029).

The results for all NKT-like subtypes are presented in Table 3. It should be noted that the results presented in its lower half are obtained for CD4-CD8mid and for CD4-CD8high cells represented as a percentage of CD4-CD8+ cells, as described in Section 2.5. That is why these two results are basically the same (the only difference is the sign of the correlation coefficient), as CD4-CD8mid is simply equal to (100% - CD4-CD8high) in this case. Similarly, CD4highCD8mid and CD4midCD8high are expressed relative to CD4+CD8+ cells in the lower part of Table 3 and therefore their results are also symmetrical (and non-significant, with p-value = 0.554).

In the last step of the study, in order to exclude the potential impact of obesity, we evaluated correlation of NKT-like types with BMI and we did not find any significant correlation (Table 4). Similarly, for NKT-like subtypes no correlation with BMI was found (all p-values for Spearman's correlation coefficient > 0.326).

4 Discussion

The pathogenesis and complications of T2DM are not only related to metabolic disorders, but there is also increasing evidence for the important role of immune system impairment. Studies analyzing the role of NKT-like cells in T2DM etiology and in the development of complications have remained deficient. The currently available literature focused on this issue is scarce and mainly regarded the

TABLE 2 Contingency table for two binary variables: CD4-CD8- (after thresholding) and DM.

	No diabetes	Diabetes
Percentage of CD4-CD8- < 9%	27	17
Percentage of CD4-CD8- ≥ 9%	35	7

absolute counts of circulating NKT-like cells (15, 24, 25) or overall NKT cell population (26, 27), with no analysis of NKT-like types with regard to the CD4 and CD8 molecule expression.

To the best of our knowledge, this study is the first one to assess specific NKT-like cell subpopulations in the peripheral blood of individuals with T2DM in comparison to individuals without glucose-metabolism-related disorders. Our results demonstrated that patients with T2DM had significantly lower percentage of DN NKT-like cells than healthy individuals. In order to further confirm this observation, we searched for a threshold of percentage of DN NKT-like cells which would divide the whole study cohort into two subgroups of approximately equal number of patients. This analysis demonstrated that the proportion of T2DM to healthy subjects in the group with high DN cells percentage was over three times lower than in the group with low DN cells percentage, and that the group with low DN cells percentage included nearly 2.5 times more patients with T2DM than CG. These results suggested that a distribution of NKT-like cell population was significantly different in T2DM as compared with CG and was associated with lower CD4-CD8- percentage.

The next part of the present study aimed to evaluate correlation between the proportion of NKT-like cell types and glucose level as well as BMI, in order to find out whether the observation of decreased percentage of DN NKT-like cells in T2DM should be considered as a direct consequence of hyperglycemia or as an indirect consequence of immune disorders in obesity. The significant negative correlation was confirmed between DN NKT-like cells and glucose level, while no correlation between any of NKT-like cell types and BMI was found.

Having demonstrated that our results are BMI-independent and related to glucose level, we made a hypothesis that hyperglycemia leads to changes in NKT-like cell population towards cells with no or low CD8 expression. In order to investigate this issue, we additionally analyzed correlation of the distribution of subpopulations of NKT-like cells with glucose level. In our study, NKT-like cells were initially subdivided into four main subpopulations: CD4-CD8-, CD4-CD8+, CD4+CD8- and CD4+CD8+. However, advanced research provided a discovery of significant heterogeneity of both CD8+ subpopulations, which led to further subdivision of CD4+CD8+ and CD4-CD8+ subpopulations into CD4highCD8mid/CD4midCD8high and CD4-CD8mid/CD4-CD8high subtypes. To the best of our knowledge, such subclassification of NKT-like cell subpopulations has never been described in the literature. It was used for subclassification of classic CD8 T cells, however, we would like to underline, that our strict gating strategy and high cell isolation purity let us analyze virtually only the cells expressing CD56. Therefore, CD8+ NKT-like cells analyzed in our study cannot be directly compared to total circulating classic CD8+ T cells.

Having applied this subclassification for NKT-like cells, we demonstrated significant negative correlation between glucose level and the percentage of CD4-CD8mid subtype, while no correlation with other subpopulations was found. This observation confirmed our hypothesis that hyperglycemia can be associated with decreased percentage of NKT-like cells both with no expression or low expression of CD8 molecule. No such correlation was found for CD4-CD8high cells if their percentage with respect to the total number of NKT-like cells was analyzed.

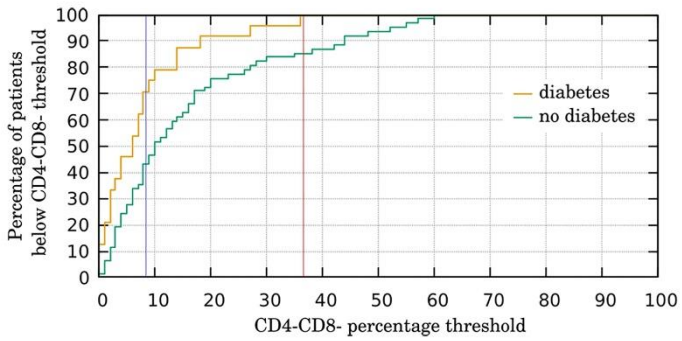

FIGURE 2
The proportion of patients with low CD4-CD8- percentage in peripheral blood in type 2 diabetes group (DMG) (n = 24) and control group (CG) (n = 62). Yellow plot presents how many DMG patients (among all DMG patients) have CD4-CD8- percentage (with respect to all studied NKT-like cell types) lower than a given threshold. Green plot presents how many CG patients (among all CG patients) have CD4-CD8- percentage (with respect to all studied NKT-like cell types) lower than a given threshold. Blue vertical line presents a specific case (approximately equal-sized groups) described in the text and in Table 2; chi-squared test value: 5.16, p-value: 0.023. Red vertical line presents a specific case for the threshold of 37% (all DMG patients have the CD4-CD8- percentage below this threshold) described in the text; chi-squared test value: 3.891, p-value: 0.049).

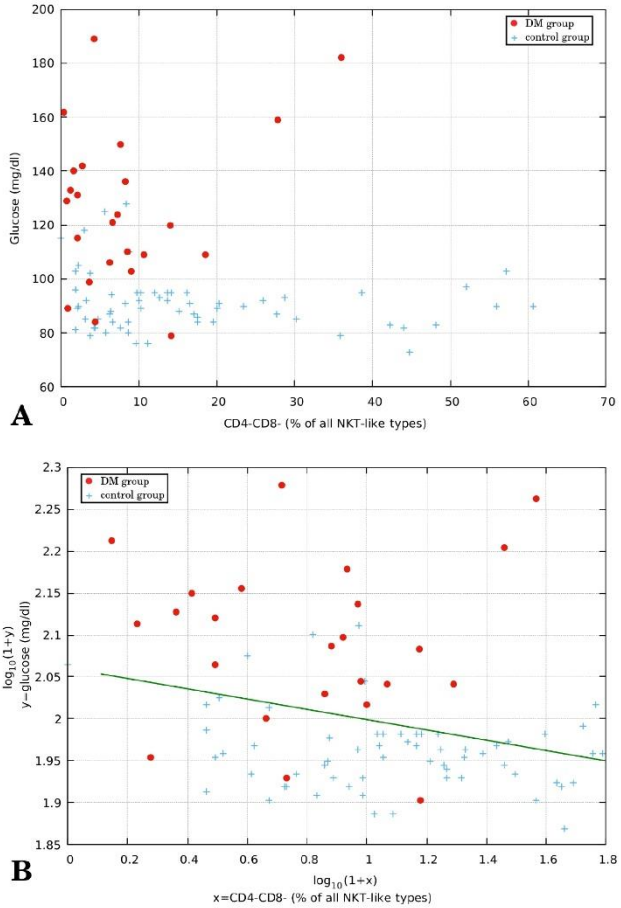

FIGURE 3
(A) Relation between glucose level (mg/dl) and CD4-CD8- percentage in peripheral blood of type 2 diabetes group (n = 24) and control group (n = 62). **(B)** The same relation after logarithmic scaling of both axes. The linear regression parameters are -0.062 and 2.06 (slope and intercept, respectively) and Pearson's ρ: -0.29 with p-value: 0.007 (plot B). Spearman's ρ: -0.27 with p-value: 0.011 (both plots: A and B).

These findings cannot be directly compared to those of other authors due to the absence of similar analyses in the existing studies. However, previous research made attempts to analyze changes of the number of NKT cells and NKT-like cells in T2DM. In the study by Tang et al. the authors found that the frequency and absolute counts of circulating NKT-like cells were significantly lower in patients with T2DM compared to healthy volunteers (15). The study differed significantly from ours, as it included T2DM patients with chronic severe hyperglycemia with a mean HbA1c level exceeding 10%, which indicated a potential presence of multiple T2DM-related complications. In our study, the mean glucose level in T2DM group was 125.88 mg/dl, while in the quoted study it had to be approximately 250 mg/dl (on the basis of HbA1c value). Therefore, our results may be different from those by Tang et al. as very high glucose levels could significantly influence immune responses. We demonstrated that DN NKT-like cell population was depleted, but we did not find a decrease in total number of NKT-like cells. Our study demonstrated that even slight hyperglycemia is associated with impairment of NKT-like cell distribution, and that DN NKT-like cells can be the first or maybe the only population of NKT-like cells depleted in T2DM. In the study by Dworacka et al. (24), no significant difference in the total number of NKT-like cells in the peripheral blood of patients with T2DM compared to healthy controls was found. These results

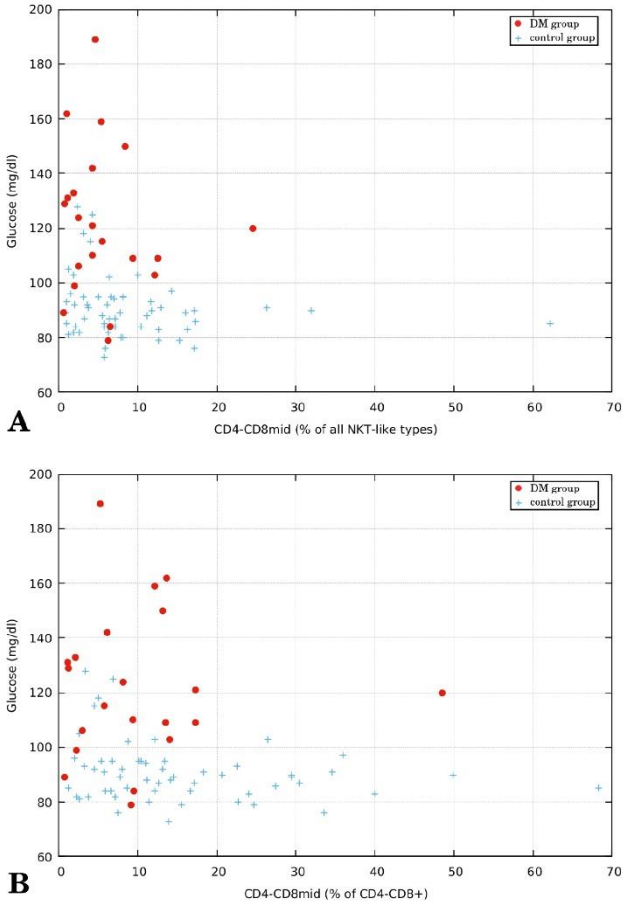

FIGURE 4
Relation between glucose level (mg/dl) and CD4-CD8mid percentage in peripheral blood of type 2 diabetes group (DMG, n = 21) and control group (n = 56). **(A)** CD4-CD8mid percentage represented as % of all studied NKT-like cell types (Spearman's ρ: -0.292 with p-value: 0.01). **(B)** CD4-CD8mid percentage represented as % of CD4-CD8+ cells (Spearman's ρ: -0.25 with p-value: 0.029).

were consistent with the findings by Guo et al. (26) and by Phoksawat et al. (25). Although Guo et al. demonstrated no difference in the total NKT cell number, they observed high level of activated NKT cells in patients with new onset of T2DM and concluded that activated NKT cells played a role in T2DM pathogenesis. Contrary, Dworacka et al. observed that patients with prediabetes presented significantly higher amount of activated NKT-like cells in peripheral blood as compared to both T2DM patients and healthy controls. Moreover, the increased NKT-like cell count in prediabetes was negatively associated with glycated hemoglobin (HbA1c) levels. Authors hypothesized that the diminished number of NKT-like cells in T2DM patients is possibly linked to chronic inflammation and NKT-like cells relocation to vessel walls. On the other hand, Phoksawat et al. (25) did not find any difference in the number of activated NKT-like cells in the peripheral blood of T2DM patients compared to healthy controls. The discrepancies between the studies may be related to a relatively small study groups (24–26) However, the widely observed lack of differences in the total number of NKT-like cells between T2DM and controls may be related to the fact that the changes concern the profile of NKT-like cell subpopulations, as it was demonstrated in our present study, but the total number of cells is not significantly disturbed. In a study conducted by Gajovic et al. (27), oxidative stress caused by hyperglycemia led to a depletion in the amount of

TABLE 3 Correlations between percentage of NKT-like cell subtypes and glucose level.

NKT-like cell subtype (% of all NKT-like cells)	Spearman's ρ	*p*-value
CD4highCD8mid	0.056	0.627
CD4midCD8high	0.209	0.07
CD4-CD8mid	-0.292	**0.01**
CD4-CD8high	0.096	0.407
NKT-like cell subtype (% of CD4+CD8+)		
CD4highCD8mid	-0.068	0.554
CD4midCD8high	0.068	0.554
NKT-like cell subtype (% of CD4-CD8+)		
CD4-CD8mid	-0,249	**0,029**
CD4-CD8high	0,249	**0,029**

Statistically significant p-values are presented in bold.

TABLE 4 Correlation between percentage of NKT-like cell types and body mass index (BMI).

NKT-like cell type	Spearman's ρ (*p*-value)	Pearson's ρ (*p*-value)
CD4+CD8-	0.18 (0.101)	0.14 (0.187)
CD4+CD8+	0.14 (0.197)	0.11 (0.316)
CD4-CD8-	-0.05 (0.681)	-0.05 (0.666)
CD4-CD8+	-0.1 (0.352)	-0.124 (0.256)

splenic NKT cells. Additionally, there was an increase of NKT cells excreting transforming growth factor beta (TGF-β), interleukin (IL)-4, and IL-5. These results led to a hypothesis that T2DM induces a change in cytokine secretion by NKT cells towards a T-helper type 2 (Th2) cytokine profile, which may lead to increased susceptibility of diabetic patients to infections and tumors (27). In this regard, our observations are highly consistent with findings by Gajovic et al., as we demonstrated that T2DM is associated with a decreased percentage of DN NKT-like cells which possess extraordinary cytotoxic properties. Important role in antimicrobial response is connected with innate immunity and production of antimicrobial peptides and cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) by NKT-like cells (28–30). It was also proven that IFN-γ plays a crucial role in bacterial clearance including *L. monocytogenes* and *M. tuberculosis* infections (31–33). Furthermore, TNF-α, IFN- γ and granzyme B produced by NKT-like cells is also involved in antitumor defense (21, 34–36). In the context of the results of the present study, it seems highly important that the cytokine profile of DN NKT-like cells, particularly their production of IFN-γ, TNF-α is essential for their antitumor and antimicrobial activity (21, 28–36). It should be underlined that the association between T2DM and increased risk of cancer was demonstrated for a number of malignancies, including breast, liver, pancreas, colon/rectum, endometrium, and bladder cancer (37, 38). On the other hand, anti-tumor activity of NKT-like cells was proved (21, 34, 35, 39).

The immunoregulatory function of NKT-like cells in metabolic diseases is further supported by research on type 1 diabetes mellitus (T1DM), which was proven to be associated with abnormalities in NKT-like cells and NKT-cells distribution and function (40–43). Among metabolic diseases, obesity seems to became more and more important, especially because it constitutes the main risk factor of T2DM. Although we did not find any association between the distribution of NKT-like cell populations, other authors demonstrated that pathogenesis of obesity is closely linked to the dysregulation of immune system cells, including NKT-like cells In study conducted by Donninelli et al., accumulation of NKT-like cells was observed in the visceral adipose tissue of obese patients (44). This study does not contradict our results, as tissue distribution and function of NKT-like cells may differ. It was previously demonstrated for NKT cells that they can reduce inflammation in adipose tissue, whereas in the liver, their activation may exacerbate it (45, 46). Future studies analyzing adipose-resident NKT-like subpopulations could provide valuable insights into their role in obesity.

Until very recently, most studies analyzing NKT cells focused either on very unique invariant NKT (iNKT) cells based on Vα24-Jα18 TCR antigen, or on broad spectrum of CD3+CD56+ NKT lymphocytes. When discussing any data, obtained from analysis of NKT-like cells, it should be underlined, that raw comparison of results between different studies is still valid, as long as the same method of NKT-like cells identification was used. We acknowledge, however, that new insights into the heterogeneity of CD3+CD56+ NKT-like cells complicates the discussion of results obtained in various studies, including our study on T2DM patients. We are aware, that the differences in the percentage of any NKT-like cells subtype, based on CD4 and CD8 segregation, might be the reflection of any alternations in cell structure. That includes, but is not limited to: changes in only one NKT-like cell subtype – e.g. αβ T-cells vs. γδ T-cells; different state of activation of various subtypes – including changes in CD56 expression, resulting in different cells being sorted as CD3+CD56+ NKT-like cells; or changes affecting every CD3+CD56+ expressing cell. In the aspect of interpretation of our findings in context of T2DM patients, it is important to mention, that cells expressing CD56 antigen are considered to be activated cells exhibiting some level of cytotoxic properties, therefore, possibly sharing similar properties. Statistical significant differences, between healthy individuals and T2DM patients, may indicate their contribution in the disease mechanisms. Further distinguishing NKT-like cells based on CD4/CD8 markers can provide some initial insights into the subtypes of those cells, pointing on potential subpopulations that are more important for the understanding the immunology of T2DM and helping for the design of further studies in the field.

Taking into account all these observations, the results of our study may constitute an important step in broadening the

knowledge on harmful effects of hyperglycemia on immune cells and on mechanisms contributing to the impaired immune defense in T2DM, mainly with regard to increased risk of bacterial infections and complications of infections, as well as of cancer development. The observed depletion represents a novel insight into immune dysregulation in metabolic disease and may serve as a potential therapeutic target. Importantly, this progress would not be possible without detailed analysis of NKT-like subpopulation heterogeneity. The data that NKT-like subpopulations heterogeneity correlate with glucose level suggest that the immune cells subpopulations, rather than total cell counts, should be the core of future studies.

Considering the novelty of our results, the limitations of this study should be taken into account. The main limitation is a relatively small sample size, although it should be noted that no larger cohort has been analyzed with regard to association of NKT-like cell subpopulations and T2DM. Additionally, the DMG included patients treated with different antidiabetic medications, which may influence the results. Moreover, we have not analyzed HbA1c level in our patients and an analysis of correlation of HbA1c and NKT-like cell subpopulations may provide further evidence supporting the results. Our study also did not involve patients with uncontrolled T2DM and extremely high glucose concentrations. Therefore, no conclusion on the impact of severe hyperglycemia on NKT-like cells can be stated. On the other hand, we believe that the strength of this study is the fact that all T2DM patients had similar disease control with only moderately increased glucose level. This fact indicates that even moderate hyperglycemia is associated with changes in NKT-like cell profile with significant decrease in DN subpopulations, which can potentially lead to increased susceptibility to infections as well as increased risk of cancer development. Another strength of our study is the fact that the glucose and NKT-like cell subpopulations were analyzed from the blood collected at the same time point in the same conditions, in order to avoid the impact of other factors, such as meal, physical effort or others.

5 Conclusions

Our study has provided evidence that DN NKT-like cell population is decreased in T2DM, and that this phenomenon is related to increased glucose level but not to BMI. As DN NKT-like cells play important role in antimicrobial and antitumor immune defense, the decreased percentage of these cells may explain one of the mechanisms of increased susceptibility and worse course of infections in hyperglycemic conditions as well as increased malignancy risk in T2DM. It is worth emphasizing, that our study revealed that significant depletion of DN NKT-like cells occurs even if glucose level is only moderately elevated. Therefore, our study provides a new point for strict control of glycemia in T2DM. Taking into account the novelty of our findings and the fact that no other research on associations between NKT-like cell subpopulations has been performed so far, our observations should be considered pioneering and they require further confirmation in larger cohorts. These results shed new light on significance of NKT-like cell subpopulations in T2DM, which – if confirmed – may pioneer the way for new therapeutic strategies targeting immune dysfunction in metabolic disorders.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Polish Mother's Memorial Hospital – Research Institute in Lodz, Poland. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

EA-F: Conceptualization, Validation, Methodology, Investigation, Data curation, Funding acquisition, Resources, Software, Visualization, Writing – original draft. PŚ: Validation, Software, Methodology, Resources, Data curation, Writing – original draft, Conceptualization, Investigation, Visualization. BS: Visualization, Formal Analysis, Writing – review & editing, Validation, Methodology, Writing – original draft, Software. MK-L: Writing – review & editing, Supervision, Project administration, Formal Analysis. AL: Funding acquisition, Project administration, Writing – original draft, Methodology. MS: Project administration, Writing – original draft, Supervision, Methodology, Formal Analysis, Conceptualization, Software, Resources, Writing – review & editing, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of type 2 diabetes mellitus. *Int J Mol Sci.* (2020) 21:6275. doi: 10.3390/ijms21176275

2. Geerlings S, Hoepelman A. Immune dysfunction in patients with diabetes mellitus (DM). FEMS. *Immunol Med Microbiol.* (1999) 26:259–65. doi: 10.1016/s0928-8244(99)00142-x

3. Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, et al. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes.* (2003) 52:1799–805. doi: 10.2337/diabetes.52.7.1799

4. Calle MC, Fernandez ML. Inflammation and type 2 diabetes. *Diabetes Metab.* (2012) 38:183–91. doi: 10.1016/j.diabet.2011.11.006

5. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science.* (1993) 259:87–91. doi: 10.1126/science.7678183

6. Xia C, Rao X, Zhong J. Role of T lymphocytes in type 2 diabetes and diabetes-associated inflammation. *J Diabetes Res.* (2017) 2017:6494795. doi: 10.1155/2017/6494795

7. DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR, et al. B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. *Proc Natl Acad Sci U S A.* (2013) 110:5133–8. doi: 10.1073/pnas.1215840110

8. Meshkani R, Vakili S. Tissue resident macrophages: Key players in the pathogenesis of type 2 diabetes and its complications. *Clin Chim Acta.* (2016) 462:77–89. doi: 10.1016/j.cca.2016.08.015

9. Witcoski Junior L, de Lima JD, Somensi AG, de Souza Santos LB, Paschoal GL, Uada TS, et al. Metabolic reprogramming of macrophages in the context of type 2 diabetes. *Eur J Med Res.* (2024) 29:497. doi: 10.1186/s40001-024-02069-y

10. Kim JH, Park K, Lee SB, Kang S, Park JS, Ahn CW, et al. Relationship between natural killer cell activity and glucose control in patients with type 2 diabetes and prediabetes. *J Diabetes Investig.* (2019) 10:1223–8. doi: 10.1111/jdi.13002

11. Zhou T, Hu Z, Yang S, Sun L, Yu Z, Wang G. Role of adaptive and innate immunity in type 2 diabetes mellitus. *J Diabetes Res.* (2018) 2018:7457269. doi: 10.1155/2018/7457269

12. Doherty DG, Norris S, Madrigal-Estebas L, McEntee G, Traynor O, Hegarty JE, et al. The human liver contains multiple populations of NK cells, T cells, and $CD3^+CD56^+$ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol.* (1999) 163:2314–21.

13. Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF. CD56 in the immune system: more than a marker for cytotoxicity? *Front Immunol.* (2017) 8:892. doi: 10.3389/fimmu.2017.00892

14. Balato A, Unutmaz D, Gaspari AA. Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions. *J Invest Dermatol.* (2009) 129:1628–42. doi: 10.1038/jid.2009.30

15. Tang L, Wang H, Cao K, Xu C, Ma A, Zheng M, et al. Dysfunction of circulating $CD3^+CD56^+$ NKT-like cells in type 2 diabetes mellitus. *Int J Med Sci.* (2023) 20:652–62. doi: 10.7150/ijms.83317

16. Kaszubowska L, Piotrowska A, Siedlecka-Kroplewska K, Kmiec Z. NKT cells as a connecting element between innate and adaptive immunity. *Postepy Biol Komorki.* (2013) 40:697–724.

17. Montoya CJ, Pollard D, Martinson J, Kumari K, Wasserfall C, Mulder CB, et al. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology.* (2007) 122:1–14. doi: 10.1111/j.1365-2567.2007.02647.x

18. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med.* (2002) 195:625–36. doi: 10.1084/jem.20011786

19. Lin H, Nieda M, Hutton JF, Rozenkov V, Nicol AJ. Comparative gene expression analysis of NKT cell subpopulations. *J Leukoc Biol.* (2006) 80:164–73. doi: 10.1189/jlb.0705421

20. Leibinger EA, Pauler G, Benedek N, Berki T, Jankovics I, McNally R, et al. Baseline $CD3^+CD56^+$ (NKT-like) cells and the outcome of influenza vaccination in children undergoing chemotherapy. *Front Immunol.* (2021) 12:690940. doi: 10.3389/fimmu.2021.690940

21. Tao L, Wang S, Kang G, Jiang S, Yin W, Zong L, et al. PD-1 blockade improves the anti-tumor potency of exhausted CD3(+)CD56(+) NKT-like cells in patients with primary hepatocellular carcinoma. *Oncoimmunology.* (2021) 10:2002068. doi: 10.1080/2162402x.2021.2002068

22. Zarobkiewicz MK, Morawska I, Michalski A, Roliński J, Bojarska-Junak A. NKT and NKT-like cells in autoimmune neuroinflammatory diseases-multiple sclerosis, myasthenia gravis and guillain-barre syndrome. *Int J Mol Sci.* (2021) 22:9520–39. doi: 10.3390/ijms22179520

23. Lin SJ, Chen JY, Kuo ML, Hsiao HS, Lee PT, Huang JL. Effect of interleukin-15 on CD11b, CD54, and CD62L expression on natural killer cell and natural killer T-like cells in systemic lupus erythematosus. *Mediators Inflamm.* (2016), 9675861. doi: 10.1155/2016/9675861

24. Dworacka M, Wesołowska A, Wysocka E, Winiarska H, Iskakova S, Dworacki G. Circulating CD3+56+ cell subset in pre-diabetes. *Exp Clin Endocrinol Diabetes.* (2014) 122:65–70. doi: 10.1055/s-0033-1363233

25. Phoksawat W, Jumnainsong A, Leelayuwat N, Leelayuwat C. IL-17 production by NKG2D-expressing CD56+ T cells in type 2 diabetes. *Mol Immunol.* (2019) 106:22–8. doi: 10.1016/j.molimm.2018.12.008

26. Guo H, Xu B, Gao L, Sun X, Qu X, Li X, et al. High frequency of activated natural killer and natural killer T-cells in patients with new onset of type 2 diabetes mellitus. *Exp Biol Med (Maywood).* (2012) 237:556–62. doi: 10.1258/ebm.2012.011272

27. Gajovic N, Zdravkovic N, Jovanovic I, Jevtic B, Lukic ML. Diabetes mellitus directs NKT cells toward type 2 and regulatory phenotype. *Exp Appl BioMed Res.* (2016) 17:35–41. doi: 10.1515/sjecr-2016-0005

28. Jiang Y, Cui X, Cui C, Zhang J, Zhou F, Zhang Z, et al. The function of $CD3^+CD56^+$ NKT-like cells in HIV-infected individuals. *BioMed Res Int.* (2014) 2014:863625. doi: 10.1155/2014/863625

29. Kaplan G, Freedman VH. The role of cytokines in the immune response to tuberculosis. *Res Immunol.* (1997) 147:565–72. doi: 10.1016/S0923-2494(97)85223-6

30. Zganiacz A, Santosuosso M, Wang J, Yang T, Chen L, Anzulovic M, et al. TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. *J Clin Invest.* (2004) 113:401–13. doi: 10.1172/JCI18991

31. Emoto M, Emoto Y. Intracellular bacterial infection and invariant NKT cells. *Yonsei Med J.* (2009) 50:12–21. doi: 10.3349/ymj.2009.50.1.12

32. Gansert JL, Kiessler V, Engele M, Wittke F, Röllinghoff M, Krensky AM, et al. Human NKT cells express granulysin and exhibit antimycobacterial activity. *J Immunol.* (2003) 170:3154–61. doi: 10.4049/jimmunol.170.6.3154

33. Chuang YT, Leung K, Chang YJ, DeKruyff RH, Savage PB, Cruse R, et al. A natural killer T-cell subset that protects against airway hyperreactivity. *J Allergy Clin Immunol.* (2019) 143:565–76.e7. doi: 10.1016/j.jaci.2018.03.022

34. Peng LS, Mao FY, Zhao YL, Wang TT, Chen N, Zhang JY, et al. Altered phenotypic and functional characteristics of $CD3^+CD56^+$ NKT-like cells in human gastric cancer. *Oncotarget.* (2016) 7:55222–30. doi: 10.18632/oncotarget.10484

35. Cao K, Wang X, Wang H, Xu C, Ma A, Zhang Y, et al. Phenotypic and functional exhaustion of circulating $CD3^+CD56^+$ NKT-like cells in colorectal cancer patients. *FASEB J.* (2024) 38:e23525. doi: 10.1096/fj.202301743R

36. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in *vivo*. *J Exp Med.* (2005) 202:1279–88. doi: 10.1084/jem.20050953

37. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. *Diabetes Care.* (2010) 33:1674–85. doi: 10.2337/dc10-0666

38. Ta HDK, Nguyen NN, Ho DKN, Nguyen HD, Ni YC, Yee KX, et al. Association of diabetes mellitus with early-onset colorectal cancer: a systematic review and meta-analysis of 19 studies including 10 million individuals and 30,000 events. *Diabetes Metab Syndr.* (2023) 17:102828. doi: 10.1016/j.dsx.2023.102828

39. Lee JH, Kim SH, Doh J, Cho D, Kim J, Oh SY, et al. Tumor-primed $CD3^+CD56^+$ natural killer T-like cells as an efficient novel cell therapy for relapsed/refractory multiple myeloma. *Blood.* (2024) 144:2057. doi: 10.1182/blood-2024-200559

40. Huang YN, Lin WD, Su PH, Bau DT, Tsai FJ, Huang CC, et al. $CD3^+CD56^+$ T lymphocytes are associated with ER stress and inflammasome activation in type 1 diabetes. *In Vivo.* (2022) 36:2083–91. doi: 10.21873/invivo.12934

41. Piekarski R, Szewczyk L, Bojarska-Junak A, Roliński J. Natural killer T cells (NKT) in children with new onset type 1 diabetes. *Endokrynol Pediatr.* (2013) 12:9–16. doi: 10.18544/ep-01.12.01.1436

42. Gómez-Díaz RA, Aguilar MV, Meguro EN, Márquez RH, Magaña EG, Martinez-Garcia MC, et al. The role of natural killer T (NKT) cells in the pathogenesis of type 1 diabetes. *Curr Diabetes Rev.* (2011) 7:278–83. doi: 10.2174/157339911796397839

43. Novak J, Griseri T, Beaudoin L, Lehuen A. Regulation of type 1 diabetes by NKT cells. *Int Rev Immunol.* (2007) 26:49–72. doi: 10.1080/08830180601070229

44. Donninelli G, Del Cornò M, Pierdominici M, Scazzocchio B, Vari R, Varano B, et al. Distinct blood and visceral adipose tissue regulatory T cell and innate lymphocyte profiles characterize obesity and colorectal cancer. *Front Immunol.* (2017) 8:643. doi: 10.3389/fimmu.2017.00643

45. Wu L, Parekh VV, Gabriel CL, Bracy DP, Marks-Shulman PA, Tamboli RA, et al. Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proc Natl Acad Sci U S A.* (2012) 109:E1143–52. doi: 10.1073/pnas.1200498109

46. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity.* (2012) 37:574–87. doi: 10.1016/j.immuni.2012.06.016

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Article

Vitamin D May Increase the Number of CD4-CD8+ NKT-like Cells in Humans—A Novel Insight into Potential Immunomodulatory Action

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Abstract

Background: Vitamin D has a significant role in immune system regulation due to its profound impact on various immune cells, including Natural Killer T-like (NKT-like) cells. While previous studies have explored the effects of vitamin D on the overall NKT-like cell population, detailed investigations into its impact on specific NKT-like subpopulations are lacking. This study aimed to analyze the correlation between vitamin D levels and NKT-like cell subpopulations (CD4+CD8+; CD4-CD8+; CD4+CD8-; CD4-CD8-) in peripheral blood collected from patients without diseases that can influence vitamin D and/or calcium levels. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from 86 patients. NKT-like cells were separated from PBMCs using a CD3+ CD56+ NKT cell isolation kit and a magnetic bead separator. Flow cytometry (FC) was applied in order to evaluate the distribution of NKT-like cell subpopulations. **Results:** A significant positive correlation between vitamin D levels and the CD4-CD8+ NKT-like cell population, particularly the CD4-CD8high subtype was found. Importantly, this correlation was independent of calcium levels, emphasizing the unique impact of vitamin D on CD4-CD8+ NKT-like cells. **Conclusions:** Our findings suggest that vitamin D concentrations may influence the distribution of NKT-like cell subpopulations in peripheral blood, although further evidence is necessary to confirm this observation. These novel results provide a foundation for elucidating the mechanism underlying the effect of vitamin D on the immune system and may contribute to future therapeutic strategies targeting CD4-CD8+ NKT-like cells in immune and oncological disorders.

Keywords: NKT-like cells; CD4-CD8+ NKT-like cells; vitamin D; 25-hydroxycholecalciferol; immunomodulation; autoimmune disorders; immune response; cancer

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1. Introduction

The role of vitamin D in human physiology has been a focus of scientific interest for over 100 years. The earliest studies linking vitamin D to the prevention of rickets date back to the early 20th century. However, the natural form of vitamin D was not discovered until 1936, and its active form, 1,25-dihydroxyvitamin D (1,25(OH)2D), was identified in 1971 [1].

Vitamin D plays a dual role, functioning both as a vitamin and a hormone, depending on its form and function. It is considered a vitamin because vitamin D is obtained from external sources and is essential for calcium and phosphate homeostasis and bone mineralization. After undergoing 25- and 1α-hydroxylation in the liver and kidneys to its active form (calcitriol), it functions as a hormone, binding to the vitamin D receptor (VDR) in the cellular nucleus [2]. Upon the binding of calcitriol to VDR, a heterodimer is formed with the retinoid X receptor (RXR); this binds to DNA sequences known as vitamin D response elements (VDREs) in the promoter regions of target genes, leading to increased or decreased gene transcription [3]. The VDR belongs to the steroid hormone receptor superfamily and is located in various tissues, including the kidneys, skeletal system, digestive system, endocrine system, and nervous system, as well as being expressed in immune cells such as monocytes, macrophages, T lymphocytes, B lymphocytes, Natural Killer (NK) cells, and Natural Killer T (NKT) cells [4–12].

Previously described in the literature as Natural Killer T (NKT) cells, NKT-like cells form a distinct and diverse group of T lymphocytes that combine functional and phenotypic features of T cells (i.e., T cell receptor (TCR)) and NK cells (i.e., cluster of differentiation (CD)56). The primary classification of NKT cells, which was based on CD3 and CD56 antigen expression, has been changed according due to the finding that CD56 is non-specific and can be detected on a broader spectrum of T lymphocytes, particularly among activated γδ T cells and within populations of αβ T cells [13–15]. Cells expressing CD56 are generally known to have a more activated phenotype that is frequently connected with cytotoxic potential and the partial sharing of functional properties [16]. NKT-like cells act as a bridge between innate and adaptive immunity, as they possess cytotoxic properties due to the production of perforin and granzyme, and produce a broad range of cytokines regulating inflammatory responses through direct and indirect interactions with other cells of the immune system [15,17]. Unlike other subpopulations of NKT-like cells, invariant NKT (iNKT) cells are very rare in peripheral blood; these cells are characterized by recognizing lipid antigens presented by the non-classical MHC molecule CD1d [18,19]. Advances in immunology, with particular emphasis on more detailed cell identification approaches, have resulted in continual reassessment of iNKT and NKT-like cell categorization [13,14]. Based on the expression of CD4 and CD8, iNKT-cells and NKT-like cells can be divided into four subpopulations: CD8+CD4-, CD4+CD8-, CD4-CD8- (double negative; DN), and CD4+CD8+ (double positive; DP) [20]. The CD4-CD8+ subpopulation predominantly releases Th1 cytokines (interferon gamma—IFN-γ; tumor necrosis factor alfa—TNF-α) and exhibits cytotoxic activity, thereby participating in antiviral, antibacterial, and antitumor responses [21].

Numerous studies have evaluated the impact of vitamin D on cancer and autoimmune and infectious diseases in both human and animal models [1,22]. The potential of adequate vitamin D levels to produce a protective effect in autoimmune diseases has been postulated [23]. Vitamin D–VDR signaling is also essential for proper NKT-like cells functioning. In murine models, vitamin D–VDR signaling has been identified as a critical point of iNKT cell maturation and thymic development. Evidence from prenatal vitamin D deficiency showed that improper fetal exposure resulted in epigenetic modifications that persistently reduce iNKT cell populations [11,12]. Undeniably, vitamin D modulates immune system function, including interaction with NKT-like cells [7,9–12]. Previous studies, including our own research on the impact of type 2 diabetes mellitus (T2DM) on NKT-like cell populations, have emphasized the vulnerability of NKT-like cells to metabolic disorders [24–26].

Nevertheless, specific effects of vitamin D on NKT-like subpopulations remain insufficiently characterized. The aim of our study was to analyze the correlation between serum

vitamin D levels and the distribution of NKT-like cell subpopulations in the peripheral blood of patients without parathyroid gland disorders or other diseases that could affect vitamin D or calcium homeostasis.

2. Materials and Methods

2.1. Patients

The research included 86 patients of Caucasian origin (68 women and 18 men) diagnosed in the Department of Endocrinology with thyroid nodular disease, in whom the benign character of the thyroid nodules was cytologically confirmed. Inclusion criteria were as follows: adult patients; benign thyroid nodular disease confirmed cytologically with no other thyroid disease; patient consent for participation in the study; absence of any of the exclusion criteria. The group was selected from outpatient clinic patients living in the same geographical area. Exclusion criteria included presence of any other acute or chronic disease that may influence either vitamin D and/or calcium levels or immune system response and function. Similarly, patients who used any medication that may influence the result were excluded from the study. Seventy-one of the patients (82.56%) took cholecalciferol in doses ranging from 1000 to 4000 IU/day. Other medications used by the patients included magnesium supplements (8%) and omega 3 acid supplements (5%). The mean age of participants was 58.09 ± 14.07 years. The clinical characteristics of the study group, including vitamin D and calcium levels, are presented in Table 1.

Table 1. Clinical characteristics of the study group.

Parameter [unit]	Mean	SD	Median	Reference Range
Vitamin D [ng/mL]	31.7	16.87	28.7	30–50
Calcium	2.33	0.15	2.33	2.2–2.55
CRP [mg/L]	0.64	0.56	0.5	<1.0
ESR [mm/h]	10.94	19.29	2	<15
TSH [mIU/L]	1.41	1.61	1.03	0.27–4.2
FT4 [ng/dL]	1.28	0.31	1.25	0.93–1.7
Creatinine [mg/dL]	0.78	0.17	0.76	0.55–1.2
Glucose [mg/dL]	94.04	11.30	92	70–99
BMI [kg/m^2]	25.29	3.86	24.58	<30

2.2. Biochemical Analysis

For the evaluation of serum vitamin D levels, peripheral venous blood samples were obtained from patients at 6:00 a.m. under fasting conditions, regardless of the time of year. Serum concentrations of vitamin D and total calcium were assessed using the electrochemiluminescence immunoassay (ECLIA) method on the Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

2.3. NKT-like Cell Isolation

Peripheral blood samples (2 × 4.9 mL) were collected into EDTA tubes (Sarstedt, Nümbrecht, Germany) via venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated through gradient centrifugation at 400× *g* for 30 min using Histopaque®-1077 (Thermo Fisher Scientific, Waltham, MA, USA).

NKT-like cells were separated from PBMCs using a CD3+ CD56+ NKT cell isolation kit (No. 130-093-064) and a magnetic bead separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Sequential isolation was performed in two steps following the manufacturer's instructions: first, PBMCs were initially depleted of NK cells and monocytes, which were indirectly magnetically labelled using a cocktail of biotin-conjugated antibodies and Anti-

Biotin MicroBeads. CD3+ CD56+ NKT-like cells are further enriched in the second step of magnetic separation.

2.4. Flow Cytometry (FC)

To characterize the distribution of NKT-like cell subpopulations, flow cytometry (FC) was applied. Cells were labelled using fluorochrome-conjugated monoclonal antibodies directed against human CD3 (APC, clone UCHT1) and CD56 (PE-Cy7, clone B159), both obtained from Becton Dickinson (Franklin Lakes, NJ, USA), to enable identification of NKT-like cells. In addition, an anti-Vα24JαQ TCR chain antibody (PE, clone 6B11) was used to quantify the invariant NKT (iNKT) fraction, which was found to be very low (~1.2%). Since neither αβ nor γδ T-cell receptors were assessed on the isolated cells, all subsequent analyses were carried out on the heterogeneous CD3+CD56+ NKT-like population, in line with current conventions. For subset discrimination, further staining was performed with antibodies recognizing CD4 (FITC, clone SK3) and CD8 (PerCP, clone SK1), also supplied by Becton Dickinson. To confirm the specificity of staining, isotype-matched controls were included in every experiment. Flow cytometric acquisition was performed on a BD FACSCanto II cytometer (Becton Dickinson, NJ, USA), and data processing was carried out with the manufacturer's analysis software (BD FACSDiva Software 6.1.2).

Initially, NKT-like cells were divided into four main subpopulations, CD4-CD8- (double negative), CD4-CD8+, CD4+CD8-, and CD4+CD8+ (double positive), according to the classification described by Montoya et al. [20], which was originally developed for iNKT cells. Further analysis led to the discovery of clear heterogeneity in both CD8+ subpopulations, leading to advanced subdivision of CD4+CD8+ and CD4-CD8+ subpopulations into CD4highCD8mid/CD4midCD8high and CD4-CD8mid/CD4-CD8high, respectively. The detailed gating strategy is presented in Figure 1.

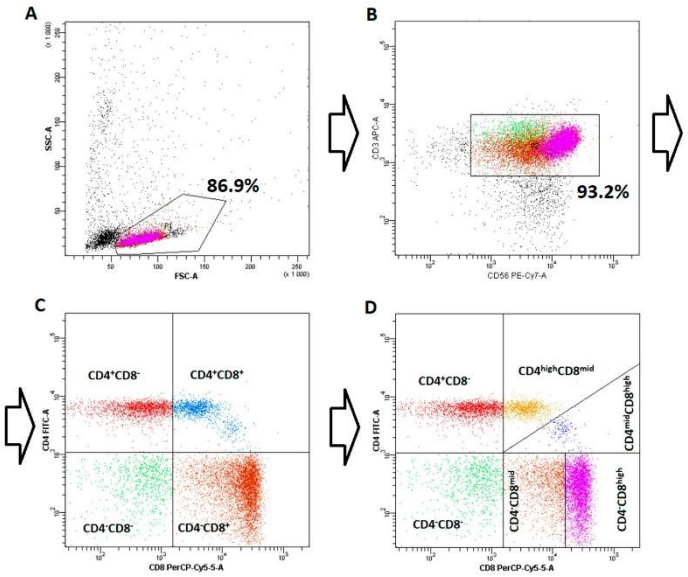

Figure 1. An example of flow cytometry (FC) plots demonstrating the gating strategy used for cell analysis. (**A**) PBMC-derived population post-magnetic enrichment; (**B**) $CD3^{+}CD56^{+}$ NKT-like cells following FC purification; (**C**) Primary gating scheme for initial NKT-like cell identification; (**D**) Refined gating strategy for distinguishing NKT-like cell subpopulations.

2.5. Statistical Analysis

Analysis of the correlation between vitamin D levels and the percentage of individual subpopulations of NKT-like cells—including both the main four subpopulations (henceforth denoted as "types") and the results of the subsequent subdivisions (mid/high subtypes)—was performed on the basis of Pearson's linear correlation coefficient and Spearman's rank correlation coefficient. For the analysis of statistically significant differences between patient groups, Student's *t*-test and the Mann–Whitney U-test were applied, while the normality of parameter distribution within groups was controlled using the Shapiro–Wilk test. For the statistical analysis and plot generation, scipy.stats and matplotlib libraries (version 1.10.1 and 3.7.5, respectively) were used. Patients were clustered into groups with the Expectation-Maximization (EM) algorithm implemented in the Waikato Environment for Knowledge Analysis (Weka, version 3.6.14).

2.6. Ethics Procedures

After receiving a detailed explanation of the purpose and course of the study, all patients signed written informed consent. The study was approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute in Łódź, Poland (approval code: 41/2021).

3. Results

The mean level of vitamin D was 31.7 ±16.87 ng/mL, median 28.7 ± 10.57 ng/mL. The mean level of calcium was 2.33 ± 0.15 mmol/L), median 2.33 ± 0.11 mmol/L. No correlation between vitamin D and calcium level for the whole group was found: Pearson's correlation coefficient was equal to 0.179 (p-value = 0.102), while Spearman's ϱ was 0.183 (p-value = 0.093).

In the first part of the study, we analyzed correlations of the main NKT-like cell types (i.e., CD4-CD8+, CD4-CD8-, CD4+CD8+, CD4+CD8-) with vitamin D level. A significant positive correlation between vitamin D level and CD4-CD8+ NKT-like cells was demonstrated. Pearson's correlation coefficient was equal to 0.312 (p-value = 0.003), while Spearman's ϱ was 0.225 (p-value = 0.006) (Table 2). In the case of other NKT-like cell types, no significant correlation with vitamin D was found (Table 2). Additionally, a slight positive correlation between CD4-CD8+ NKT-like cells and calcium level was demonstrated, with statistical significance reached only for Spearman's rank correlation (ϱ = 0.293, p-value 0.039) (Table 2).

Table 2. Correlations of NKT-like cell type percentage with vitamin D and calcium levels.

NKT-like Cell Subpopulation	Mean ± Std		Correlation Parameter	Pearson's ϱ	p Value	Spearman's ϱ	p Value
CD4+CD8-	17.46% ± 15.68%	vs.	Vitamin D	−0.177	0.103	−0.185	0.089
CD4+CD8-	17.46% ± 15.68%	vs.	Calcium	−0.183	0.093	−0.247	0.022
CD4+CD8+	7.29% ± 10.05%	vs.	Vitamin D	−0.174	0.109	−0.203	0.061
CD4+CD8+	7.29% ± 10.05%	vs.	Calcium	−0.06	0.586	−0.152	0.165
CD4-CD8-	14.32% ± 14.58%	vs.	Vitamin D	−0.141	0.196	−0.182	0.093
CD4-CD8-	14.3%2 ± 14.58%	vs.	Calcium	−0.083	0.452	−0.037	0.736
CD4-CD8+	60.93% ± 21.57%	vs.	Vitamin D	0.312	0.003	0.225	0.006
CD4-CD8+	60.93% ± 21.57%	vs.	Calcium	0.212	0.051	0.293	0.039

In the next part of the study, we analyzed correlations of the subtypes of each type of NKT-like cell (i.e., CD4highCD8mid, CD4midCD8high, CD4-CD8mid, CD4-CD8high) with vitamin D and calcium, respectively. The analysis revealed a significant positive correlation

between vitamin D and the CD4-CD8high subtype (Pearson's $\varrho = 0.328$, with p-value 0.004; and Spearman's $\varrho = 0.262$, with p-value 0.021).

Figure 2 presents the correlation between vitamin D level and the CD4-CD8high NKT-like cell subtype. It should be stressed that removal of the two prominent outliers (with increased vitamin D levels due to excessive supplementation) did not greatly influence the obtained correlation coefficients—for either the types or subtypes of NKT-like cells. For example, the value of Pearson's ϱ for vitamin D vs. CD4-CD8+ after outlier removal was 0.304 (p 0.005).

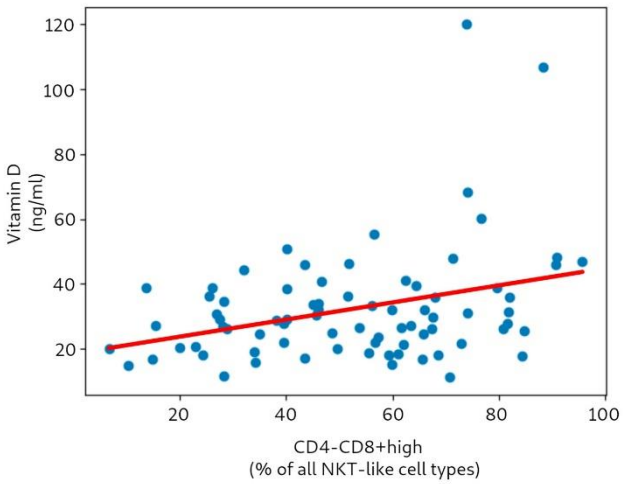

Figure 2. Correlation and linear regression between vitamin D level and the CD4-CD8high subtype ($n = 77$).

In the case of other NKT-like cell subtypes, including the CD4-CD8mid subtype, no significant correlation with vitamin D was found (Table 3). No correlation between the CD4-CD8high subtype and calcium level was found, although significant correlation was demonstrated between the CD4-CD8mid subtype and calcium level (Table 3).

Table 3. Correlations of NKT-like cell subtype percentage with vitamin D and calcium levels.

NKT Cell Subtype (Absolute Percentage)	Mean ± Std		Correlation Parameter	Pearson's ϱ	p Value	Spearman's ϱ	p Value
CD4highCD8mid	6.88% ± 10.26%	vs.	Vitamin D	−0.145	0.208	−0.202	0.079
CD4highCD8mid	6.88% ± 10.26%	vs.	Calcium	−0.113	0.33	−0.135	0.246
CD4midCD8high	1.04% ± 1.18%	vs.	Vitamin D	−0.123	0.289	0.013	0.909
CD4midCD8high	1.04% ± 1.18%	vs.	Calcium	0.103	0.378	0.04	0.73
CD4-CD8mid	8.04% ± 8.71%	vs.	Vitamin D	−0.055	0.635	0.022	0.85
CD4-CD8mid	8.04% ± 8.71%	vs.	Calcium	0.222	0.054	0.25	0.029
CD4-CD8high	52.64% ± 21.74%	vs.	Vitamin D	0.328	0.004	0.262	0.021
CD4-CD8high	52.64% ± 21.74%	vs.	Calcium	0.087	0.454	0.042	0.716

The correlations in Table 3 were calculated for the raw percentages of the NKT-like cells of each subtype. For example, if the CD4+CD8+ type constituted 40% of all NKT-like cells of a patient, divided equally into CD4highCD8mid and CD4midCD8high, both

these subtypes were represented as 20% and 20%, respectively. We additionally tested a hypothesis that it is not the absolute percentage but the relative proportion of the subtypes that should be taken into consideration (so both subtypes would be represented as 50% and 50% in our example, irrespective of the absolute percentage of CD4+CD8+ cells). This hypothesis was not confirmed, as demonstrated in Table 4.

Table 4. Correlations of NKT-like cell subtype related proportion with vitamin D and calcium levels.

NKT-like Cell Subtype (Relative Proportion)	Mean ± Std		Correlation Parameter	Pearson's ϱ	p Value	Spearman's ϱ	p Value
CD4highCD8mid	69.36% ± 27.27%	vs.	Vitamin D	−0.022	0.848	−0.145	0.209
CD4highCD8mid	69.36% ± 27.27%	vs.	Calcium	−0.068	0.562	−0.124	0.284
CD4midCD8high	30.64% ± 27.27%	vs.	Vitamin D	0.022	0.848	0.145	0.209
CD4midCD8high	30.64% ± 27.27%	vs.	Calcium	0.068	0.562	0.124	0.284
CD4-CD8mid	14.06% ± 12.54%	vs.	Vitamin D	−0.129	0.265	−0.099	0.392
CD4-CD8mid	14.06% ± 12.54%	vs.	Calcium	0.173	0.135	0.175	0.13
CD4-CD8CD8high	85.94% ± 12.54%	vs.	Vitamin D	0.129	0.265	0.099	0.392
CD4-CD8CD8high	85.94% ± 12.54%	vs.	Calcium	−0.173	0.135	−0.175	0.13

In the third step of the study, in order to investigate potential relationship between the proportions of individual NKT-like cell types and vitamin D levels, we clustered the patients into three groups using a standard EM approach (Expectation-Maximization [27,28]). The clustering was performed in a four-dimensional space spanned by all four CD4/CD8 NKT-like cell types (CD4+CD8+, CD4+CD8-, CD4-CD8+, CD4-CD8-). Figure 3 shows the obtained groups in some selected subspaces (i.e., in all subspaces involving the CD4-CD8+ NKT-like cell type). The cardinality of the groups, the coordinates of their respective centroids, and standard deviations along individual axes are presented in Table 5.

Table 5. The parameters of the obtained clusters.

Cluster ID		0	1	2
Number of Patients		43	26	17
CD4+CD8-	mean	9.91	35.17	9.11
	std	7.32	15.62	6.01
CD4+CD8+	mean	3.72	16.10	2.68
	std	2.99	14.19	2.07
CD4-CD8-	mean	8.06	8.11	39.19
	std	5.74	5.80	12.37
CD4-CD8+	mean	78.32	40.63	49.03
	std	10.21	16.75	11.03

Figure 3 presents the cluster distribution, with cluster 0 containing the patients with high a percentage of CD4-CD8+ NKT cells; cluster 1 grouping the patients with a low-to-medium percentage of CD4-CD8+, low CD4-CD8-, and rather high CD4+CD8-; and cluster 2 grouping patients with low CD4+CD8+ and CD4+CD8-, medium CD4-CD8+, and high CD4-CD8-.

An important conclusion is that the clustering procedure (assuming the fixed number of three groups) proved quite stable, yielding very similar results for different initial conditions and for a different clustering algorithm based on the standard k-means approach [29].

The results of clustering are presented in Table 6. As for the choice of the test type, we additionally tested for the normality of the vitamin D distribution in each cluster. It revealed that it deviates from normal pdf in cluster 0; therefore, the non-parametric Mann–Whitney test should be used in the first two cases (cluster 0 vs. 1 and 0 vs. 2), which means

that the only comparison result that should be considered statistically significant is cluster 0 (involving the highest percentage of CD4-CD8+ NKT-like cells) vs. cluster 1 (involving the lowest percentage of CD4-CD8+ NKT-like cells and the highest percentage of CD4+ NKT-like cells) (p-value = 0.027) (Table 6).

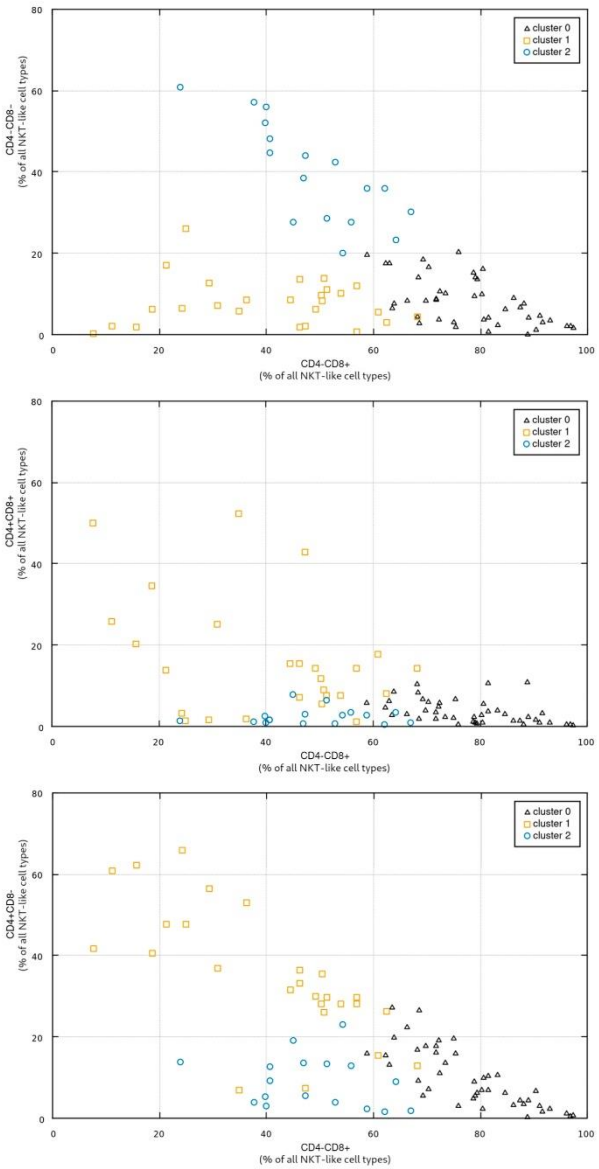

Figure 3. Visualization of NKT-like type clusters (from top to bottom: CD4-CD8- vs. CD4-CD8+; CD4+CD8+ vs. CD4-CD8+; CD4+CD8- vs. CD4-CD8+); number of cases in clusters 0, 1, and 2 was 43, 26, and 17, respectively.

Table 6. The results of vitamin D level comparison between the clusters.

	Cluster 0 vs. Cluster 1	Cluster 0 vs. Cluster 2	Cluster 1 vs. Cluster 2
Student's t	t = 2.729 (p-value = 0.008)	t = 2.049 (p-value = 0.045)	t = −0.624 (p-value = 0.537)
Mann–Whitney	U = 738 (p-value = 0.027)	U = 461.5 (p-value = 0.117)	U = 198.5 (p-value = 0.585)

To further demonstrate the relationship between vitamin D and CD4-CD8+ NKT-like cells, we divided the patients into two groups by thresholding the CD4-CD8+ percentage value. Using the threshold of 50%, we obtained the two groups presented in Table 7.

Table 7. The characteristics of vitamin D parameters in patients with high (>50) and low (≤50) percentages of CD4-CD8+ NKT-like cells.

Group		Vitamin D							
CD4-CD8+	***N***	**Mean**	**Std**	**IQR**	**Min**	**1st Quart.**	**Median**	**3rd Quart.**	**Max**
>50%	61	34.38	18.77	14.90	11.20	24.60	30.00	39.50	120.00
≤50%	25	25.16	8.07	12.70	11.60	18.00	24.70	30.70	38.90

A statistically significant difference between the groups was found (Student's t-test statistic t = 3.185; p-value = 0.002). As the vitamin D distribution in the first group deviated significantly from normal pdf, we repeated the analysis with a Mann–Whitney test, which also confirmed statistical significance (U = 1018; p-value = 0.015). The effect size (rank-biserial correlation) computed for the U statistic is 0.335.

4. Discussion

Research conducted so far emphasizes that vitamin D plays a pivotal role in the development, function, and regulation of NKT-like cells, a crucial component of the immune system [11,12]. Current studies have focused on correlations between vitamin D levels and the overall NKT-like cell population or iNKT cells specifically [11,12]. However, detailed results on the interactions between NKT-like cell subpopulations and vitamin D are still lacking.

To the best of our knowledge, our study is the first one to analyze the correlation between vitamin D level and NKT-like cell subpopulations in the peripheral blood of patients without disorders that may influence calcium and/or vitamin D levels. The lack of correlation between vitamin D and total calcium in the study cohort further confirmed that the results were not influenced by any calcium/vitamin D-related disturbances. Previous studies primarily classified NKT cells into two or three subpopulations: CD4+, CD4-, and CD4-CD8- (double negative) [30–33]. Using fluorescence-activated cell sorting, we categorized NKT-like cells into four subpopulations, CD4+CD8-, CD4-CD8+, CD4-CD8- (double negative, DN), and CD4+CD8+ (double positive, DP), following the classification applied by Montoya et al. [20]. A more detailed analysis revealed significant heterogeneity within the CD8+ subpopulations. Therefore, we further performed an advanced stratification of the CD4+CD8+ and CD4-CD8+ subpopulations. Specifically, CD4+CD8+ and CD4-CD8+ cells were subdivided into CD4highCD8mid/CD4midCD8high and CD4-CD8mid/CD4-CD8high, respectively. Such a stratification of NKT-like cell subpopulations has not been presented in the literature before, but seems highly important for detailed knowledge on the actual role of NKT-like cells, due to the potentially different functions of the NKT-like cell subpopulations, which may not be demonstrated for the whole NKT-like population or even for the CD4+CD8-, CD4-CD8+, CD4-CD8-, CD4+CD8+ subpopulations. Our study

involved detailed subpopulations and subpopulations of the subpopulations to confirm the observed correlation of CD4-CD8+ with vitamin D level.

The current analysis identified a positive correlation exclusively between vitamin D levels and the number of CD4-CD8+ cells. No other significant correlations were observed between vitamin D and the other NKT-like cell subpopulations. To further confirm this correlation, we performed clustering in a four-dimensional space spanned by all four CD4/CD8 NKT cell types (CD4+CD8+, CD4+CD8-, CD4-CD8+, CD4-CD8-). This approach allowed us to demonstrate a significant difference between vitamin D levels in patients belonging to the cluster with the highest percentage of CD4-CD8+ NKT-like cells and the ones belonging to the cluster with the highest percentage of CD8-negative NKT-like cells. As our observation is novel and constitutes an important step in the development of knowledge on vitamin D–immune system interplay, we decided to apply a third different approach to confirm the results. In this analysis, the patients were divided into two groups by thresholding the CD4-CD8+ percentage value at 50%. This approach further confirmed our previous results, as a significantly higher vitamin D level was detected in a group of patients with a domination (above 50%) of CD4-CD8+ cells. Modest simultaneous correlation of total calcium with CD4-CD8+ demonstrated in the first analysis was not confirmed in the further steps of the study, which leads to presumption that the correlation between vitamin D and NKT-like cell subpopulations is calcium-independent, at least in patients without diseases influencing calcium levels.

Our results are difficult to compare with other studies as, to date, no other results regarding the correlation between vitamin D and NKT-like cell subpopulations have been published. Only studies analyzing the total NKT cell population or iNKT cell population are available [11,12,34].

Taking into account the novelty of our results, we decided to perform even more detailed analysis. Initially, NKT-like cells were subdivided into four main subpopulations: CD4-CD8-, CD4-CD8+, CD4+CD8- and CD4+CD8+. However, further analysis led to the discovery of clear heterogeneity in both of the CD8+ subpopulations, leading to advanced subdivision of CD4+CD8+ and CD4-CD8+ subpopulations into CD4highCD8mid/CD4midCD8high and CD4-CD8mid/CD4-CD8high cells, respectively. This subclassification of NKT-like cell subpopulations has never been described before. Our investigation revealed a significant positive relationship between vitamin D and the CD4-CD8high subtype, but no significant associations were found between vitamin D and the other NKT-like cell subtypes, including the CD4-CD8mid subtype, which further indicates the possible exclusive influence of vitamin D on NKT-like cell subpopulations with high CD8 expression. As, conversely, calcium levels were not correlated with CD4-CD8high subtype but with CD4-CD8mid subtype, we can consider this observation as further proof that vitamin D's impact on NKT-like cells is calcium-independent. Studies exploring the connection between NKT-like cells and calcium levels are scarce and primarily concentrated on intracellular calcium regulation mechanisms and their influence on NKT cell activity [35]. Direct studies assessing the correlation between serum calcium concentration and NKT-like cell numbers are lacking.

As mentioned above, our results cannot be directly compared to other authors' observations due to lack of similar analyses in the literature. However, the available results of studies in the field of vitamin D and the immune system provide data that may explain the importance of our findings. Bychinin et al. reported a positive association between vitamin D levels and NKT cells in COVID-19 patients admitted to the intensive care unit (ICU). Their study demonstrated that vitamin D supplementation significantly increased the total number of NKT cells in peripheral blood compared to the placebo group [34]. Additionally, based on murine models, it was revealed that the vitamin D–VDR signal-

ing pathway is essential for the proper maturation and maintenance of adequate NKT cell populations [11,12]. These studies did not analyze the CD4CD8-subpopulations of NKT-like cells, but their results are crucial in underscoring the critical role of sufficient vitamin D levels in the development and maintenance of NKT-like cells. At present, there is a lack of research directly investigating the molecular mechanisms by which vitamin D regulates NKT-like cells. The available literature focuses predominantly on vitamin D–VDR signaling in iNKT cell development [11,12]. Yue et al. demonstrated that the transcriptional co-activator Med1, which interacts with VDR, is essential for iNKT cell development [36]. The analysis of molecular mechanisms was not the aim of our study, and further investigations are required to explain this association.

The impact of vitamin D on the immune system is currently being thoroughly analyzed. The interaction between vitamin D and NKT-like CD4-CD8+ cells is crucial for maintaining human health. Vitamin D has been shown to have a protective effect on many pathological conditions, including allergic diseases such as allergic rhinitis and atopic dermatitis [37–40]. Animal model studies suggest a correlation between serum vitamin D levels and inflammatory markers [37,41]. NKT-like cells are critical regulators of immune responses in allergic diseases due to their ability to secrete both pro-inflammatory and anti-inflammatory cytokines that modulate allergic reactions [15]. IFN-γ, primarily produced by the NKT-like CD4-CD8+ subpopulation, antagonizes Th2 cytokines like IL-4 and IL-13, promoting a more balanced immune response and reducing tissue damage [42]. Our results may provide a potential key mechanism regarding vitamin D's action on NKT-like cells in allergic reactions, i.e., attenuating the Th2-related response by increasing the number CD4-CD8+ NKT-like cells. This hypothesis requires further study to confirm such a causality.

Furthermore, vitamin D has been shown to exert protective effects against autoimmune diseases. The immunomodulatory effect of vitamin D involves, among others, modification of the profile of cytokines produced by NKT-like cells. Increasing the production of anti-inflammatory cytokines can suppress excessive immune responses and tissue injury. The VITAL study revealed that long-term vitamin D supplementation reduced the risk of all autoimmune diseases by 22% compared to the placebo group [23]. Moreover, in an experimental autoimmune encephalomyelitis (EAE) mouse model, Waddell et al. demonstrated that interactions between vitamin D and NKT cells are essential for protection against EAE. In NKT-deficient mice, the protective effects of vitamin D were attenuated [43]. A reduction in the amount NKT-like cells has also been consistently noted in other autoimmune diseases. Zhou et al. [44] found that patients with primary Sjögren's syndrome have significantly diminished circulating NKT-like cells, and this phenomenon correlates with higher disease severity. Also, Lin et al. [45] revealed that NKT-like cell numbers are reduced in systemic lupus erythematosus patients and that NKT-like cells are functionally impaired, with decreased cytotoxicity and IFN-γ production.

Through its influence on gene transcription, vitamin D plays a crucial role in regulating the growth, differentiation, and apoptosis of human cells [46]. Some epigenetic studies have shown that VDR is overexpressed in colorectal, prostate, and ovarian cancers, and increased VDR expression on tumor cells may be associated with a more favorable response to treatment [47–51]. A meta-analysis conducted by Keum et al. suggested that vitamin D reduces cancer mortality [52]. While the underlying mechanism remains unclear, it is likely influenced by the effects of vitamin D on the various types of immune cells, including T lymphocytes, NK cells, macrophages, dendritic cells (DCs), and CD4-CD8+ NKT-like cells. NKT-like CD4-CD8+ cells interact with tumor cells by releasing cytolytic proteins and producing of a wide range of cytokines, such as IFN-γ and TNF-α, affecting other immunocompetent cells, including DCs, T lymphocytes, and NK cells [15,17,53]. Furthermore,

IFN-γ, when secreted in significant amounts by CD4-CD8+ NKT-like cells, inhibits angiogenesis, thereby exhibiting strong antitumor activity [51,54]. Alves et al. demonstrated that NKT-like cells can be expanded ex vivo from patients with ovarian cancer, where they have strong cytotoxicity against tumor cells [55]. Conversely, Yuen et al. [56] showed that tumor-infiltrating NKT-like cells in hepatocellular carcinoma upregulate inhibitory receptors, indicating suppression of effector function within the tumor microenvironment. These findings support our results, suggesting that vitamin D may enhance the antitumor role of CD4-CD8+ NKT-like cells, particularly regarding the novel CD8high subtype.

Vitamin D and NKT-like cells are synergistic elements of antimicrobial defense, with their combined actions strengthening the immune response and modulating its course in a manner beneficial to the organism. Vitamin D plays a significant role in antibacterial and antiviral responses by stimulating the production of antimicrobial peptides, such as cathelicidins and alpha- and beta-defensins, and enhancing the cytotoxic activity of NK cells and macrophages [22,51]. Additionally, vitamin D stimulates CD4-CD8+ NKT-like cells to produce IFN-γ and other cytokines, which in turn increase the activation of macrophages and T lymphocytes—key players in the antimicrobial immune response [15,17]. Moreover, in study conducted by Kokordelis et al. [57], NKT-like cells have been shown to contribute to antiviral defense in patients with acute hepatitis C. While the antimicrobial properties of vitamin D have been extensively studied, the findings remain inconsistent [1,22,58]. Research conducted by Liu et al. demonstrated that vitamin D promotes the expression of cathelicidin LL-37/hCAP-18 in human macrophages, offering protection against M. tuberculosis infection through the activation of TCR 2/1 and VDR [58]. On the other hand, the incidence of respiratory infections occurred at similar levels in placebo and vitamin D groups [1]. Further studies are required to assess the effect of vitamin D on the risk of occurrence and course of infectious diseases, but an adequate supply of vitamin D seems beneficial for the antimicrobial actions of NKT cells and other immune cells [58].

Vitamin D has a significant role in immune system regulation by influencing NKT-like cells, which affect allergic reactions, autoimmune disorders, cancers, and antimicrobial defense. Our study demonstrated a positive correlation between vitamin D and CD4-CD8+ NKT-like cells (further defined as CD4-CD8high) and may constitute the initial step for further explanation of immunomodulatory effect of vitamin D in several disorders and contribute to the future development of novel therapeutic approaches to treat a wide range of immunological and oncological disorders. However, the study has several limitations that should be taken into account. Firstly, the cohort was relatively small, restricted to Caucasian patients, and predominantly included females with benign thyroid nodules. Secondly, we did not analyze the functional properties of NKT-like cells with regard to the observed correlation with vitamin D. Moreover, the majority of participants (82.6%) were receiving vitamin D supplementation, and the impact of supplementation dose and duration on NKT-like cells subpopulations was not analyzed. However, we believe that analysis performed on a population with overt vitamin D deficiency may be less reliable, as different immune response disturbances may result from the vitamin D deficiency. We aimed to analyze whether vitamin levels in patients without overt deficiency may influence the profile of NKT-like cell types and subtypes. Therefore, the choice of a cohort in whom the supplementation was common was intentional.

5. Conclusions

Our study is the first to demonstrate a positive correlation between vitamin D and the number of CD4-CD8+ NKT-like cells, with a more detailed analysis emphasizing the particular effect regarding the CD4-CD8high subtype, which provided further evidence of the immunomodulatory properties of vitamin D. These findings highlight the critical role

of the CD4-CD8+ NKT-like cell subpopulation in the interplay between vitamin D and the immune system. Further studies in larger, multicenter cohorts are warranted to confirm our results and explore the underlying molecular mechanisms. Emphasis should be put on assessment of the functional properties of NKT-like cells, including cytokine production and cytotoxic activity, to support the development of new, targeted therapies in immune and oncological disorders.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute in Łódź, Poland (approval code: 41/2021, approval date 18 May 2021).

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

Data Availability Statement: The data presented in this study are not publicly available due to privacy restrictions but they are available on request from the corresponding author.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

1,25(OH)2D	1,25-dihydroxyvitamin D
α-GalCer	α-galactosylceramide
AITD	autoimmune thyroid disease
anti-Tg	thyroglobulin antibodies
anti-TPO	thyroid peroxidase antibodies
APCs	antigen presenting cells
CD	cluster of differentiation
COVID-19	coronavirus disease 2019
DCs	dendritic cells
DN	double negative
DP	double positive
EAE	experimental autoimmune encephalomyelitis
ECLIA	electrochemiluminescence immunoassay
EDTA	ethylenediamine tetraacetic acid
EM	Expectation-Maximization
FC	flow cytometry
ICU	intensive care unit
Il	interleukin
IFN-y	interferon gamma
iNKT	invariant Natural Killer T cells
MCH	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cells	Natural Killer cells

NKT cells	Natural Killer T cells
NKT-like cells	Natural Killer T-like cells
PBMCs	peripheral blood mononuclear cells
RXR	retinoid X receptor
T2DM	type 2 diabetes mellitus
TCR	T cell receptor
TNF-α	tumor necrosis factor alfa
TRAb	thyroid stimulating hormone receptor antibodies
VDR	vitamin D receptor
VDREs	vitamin D response elements
vNKT	variant Natural Killer T cells

References

1. Gallagher, J.C.; Rosen, C.J. Vitamin D: 100 years of discoveries, yet controversy continues. *Lancet Diabetes Endocrinol.* **2023**, *11*, 362–374. [CrossRef] [PubMed]
2. Ellison, D.L.; Moran, H.R. Vitamin D: Vitamin or Hormone? *Nurs. Clin. N. Am.* **2021**, *56*, 47–57. [CrossRef]
3. Wiecheć, O. The role of vitamin D3 in signaling pathways—Potential anticancer properties of calcitriol and its analogues. *Postep. Hig. Med. Dosw.* **2019**, *73*, 920–936. [CrossRef]
4. Wang, Y.; Zhu, J.; DeLuca, H.F. Where is the vitamin D receptor? *Arch. Biochem. Biophys.* **2012**, *523*, 123–133. [CrossRef]
5. Laśon, W.; Jantas, D.; Leśkiewicz, M.; Regulska, M.; Basta-Kaim, A. The Vitamin D Receptor as a Potential Target for the Treatment of Age-Related Neurodegenerative Diseases Such as Alzheimer's and Parkinson's Diseases: A Narrative Review. *Cells* **2023**, *12*, 660. [CrossRef] [PubMed]
6. Chen, S.; Sims, G.P.; Chen, X.X.; Gu, Y.Y.; Chen, S.; Lipsky, P.E. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J. Immunol.* **2007**, *179*, 1634–1647. [CrossRef]
7. Kongsbak, M.; Levring, T.B.; Geisler, C.; von Essen, M.R. The vitamin D receptor and T cell function. *Front. Immunol.* **2013**, *4*, 148. [CrossRef]
8. Veldman, C.M.; Cantorna, M.T.; DeLuca, H.F. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch. Biochem. Biophys.* **2000**, *374*, 334–338. [CrossRef]
9. Cantorna, M.T.; Snyder, L.; Lin, Y.D.; Yang, L. Vitamin D and 1,25(OH)2D regulation of T cells. *Nutrients* **2015**, *7*, 3011–3021. [CrossRef]
10. Cantorna, M.T. Mechanisms underlying the effect of vitamin D on the immune system. *Proc. Nutr. Soc.* **2010**, *69*, 286–289. [CrossRef]
11. Yu, S.; Cantorna, M.T. The vitamin D receptor is required for iNKT cell development. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5207–5212. [CrossRef]
12. Yu, S.; Cantorna, M.T. Epigenetic reduction in invariant NKT cells following in utero vitamin D deficiency in mice. *J. Immunol.* **2011**, *186*, 1384–1390. [CrossRef]
13. Doherty, D.G.; Norris, S.; Madrigal-Estebas, L.; McEntee, G.; Traynor, O.; Hegarty, J.E.; O'fArrelly, C. The human liver contains multiple populations of NK cells, T cells, and $CD3^{+}CD56^{+}$ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J. Immunol.* **1999**, *163*, 2314–2321. [CrossRef]
14. Godfrey, D.I.; Uldrich, A.P.; McCluskey, J.; Rossjohn, J.; Moody, D.B. The burgeoning family of unconventional T cells. *Nat. Immunol.* **2015**, *16*, 1114–1123. [CrossRef] [PubMed]
15. Almeida, J.; Ferreira, J.; Gaspar, H.B.; da Silva, J.P. Natural Killer T-like Cells: Immunobiology and Role in Disease. *Int. J. Mol. Sci.* **2023**, *24*, 2743. [CrossRef] [PubMed]
16. Van Acker, H.H.; Capsomidis, A.; Smits, E.L.; Van Tendeloo, V.F. CD56 in the immune system: More than a marker for cytotoxicity? *Front. Immunol.* **2017**, *8*, 892. [CrossRef] [PubMed] [PubMed Central]
17. Kaszubowska, L.; Piotrowska, A.; Siedlecka-Kroplewska, K.; Kmiec, Z. NKT cells as a connecting element between innate and adaptive immunity. *Postep. Biol. Komorki* **2013**, *40*, 697–724.
18. Balato, A.; Unutmaz, D.; Gaspari, A.A. Natural killer T cells: An unconventional T-cell subset with diverse effector and regulatory functions. *J. Investig. Dermatol.* **2009**, *129*, 1628–1642. [CrossRef] [PubMed]
19. Jing, Y.; Gravenstein, S.; Chaganty, N.R.; Chen, N.; Lyerly, K.H.; Joyce, S.; Deng, Y. Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. *Exp. Gerontol.* **2007**, *42*, 719–732. [CrossRef]

20. Montoya, C.J.; Pollard, D.; Martinson, J.; Kumari, K.; Wasserfall, C.; Mulder, C.B.; Rugeles, M.T.; Atkinson, M.A.; Landay, A.L.; Wilson, S.B. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology* **2007**, *122*, 1–14. [CrossRef]
21. O'Reilly, V.; Zeng, S.G.; Bricard, G.; Atzberger, A.; Hogan, A.E.; Jackson, J.; Feighery, C.; Porcelli, S.A.; Doherty, D.G. Distinct and overlapping effector functions of expanded human CD4+, CD8α+ and CD4-CD8α- invariant natural killer T cells. *PLoS ONE* **2011**, *6*, e28648. [CrossRef]
22. Ghaseminejad-Raeini, A.; Ghaderi, A.; Sharafi, A.; Nematollahi-Sani, B.; Moossavi, M.; Derakhshani, A.; Sarab, G.A. Immunomodulatory actions of vitamin D in various immune-related disorders: A comprehensive review. *Front. Immunol.* **2023**, *14*, 950465. [CrossRef]
23. Hahn, J.; Cook, N.R.; Alexander, E.K.; Friedman, S.; Walter, J.; Bubes, V.; Kotler, G.; Lee, I.M.; Manson, J.E.; Costenbader, K.H. Vitamin D and marine omega 3 fatty acid supplementation and incident autoimmune disease: VITAL randomized controlled trial. *BMJ* **2022**, *376*, e066452. [CrossRef]
24. Adamska-Fita, E.; Śliwka, P.W.; Stasiak, B.; Karbownik-Lewińska, M.; Lewiński, A.; Stasiak, M. An impact of type 2 diabetes mellitus on NKT-like cell population in humans: A new insight into impaired immune response in hyperglycemia. *Front. Endocrinol* **2025**, *16*, 1641318. [CrossRef] [PubMed]
25. Tang, L.; Wang, H.; Cao, K.; Xu, C.; Ma, A.; Zheng, M.; Xu, Y.; Zhang, M. Dysfunction of circulating $CD3^{+}CD56^{+}$ NKT-like cells in type 2 diabetes mellitus. *Int. J. Med. Sci.* **2023**, *20*, 652–662. [CrossRef] [PubMed]
26. Phoksawat, W.; Jumnainsong, A.; Leelayuwat, N.; Leelayuwat, C. IL-17 production by NKG2D-expressing CD56+ T cells in type 2 diabetes. *Mol. Immunol.* **2019**, *106*, 22–28. [CrossRef]
27. Dempster, A.P.; Laird, N.M.; Rubin, D.B. Maximum likelihood from incomplete data via the EM algorithm. *J. R. Stat. Soc. Ser. B Methodol.* **1977**, *39*, 1–22. [CrossRef]
28. Frank, E.; Hall, M.; Holmes, G.; Kirkby, R.; Pfahringer, B.; Witten, I.; Trigg, L. Weka: A machine learning workbench for data mining. In *The Data Mining and Knowledge Discovery Handbook*; Maimon, O., Rokach, L., Eds.; Springer: Boston, MA, USA, 2005; pp. 1305–1314. [CrossRef]
29. Forgy, E.W. Cluster analysis of multivariate data: Efficiency versus interpretability of classifications. *Biometrics* **1965**, *21*, 768–769.
30. Seino, K.; Taniguchi, M. Functionally distinct NKT cell subsets and subtypes. *J. Exp. Med.* **2005**, *202*, 1623–1626. [CrossRef] [PubMed]
31. Lin, H.; Nieda, M.; Hutton, J.F.; Rozenkov, V.; Nicol, A.J. Comparative gene expression analysis of NKT cell subpopulations. *J Leukoc Biol.* **2006**, *80*, 164–173. [CrossRef]
32. Gumperz, J.E.; Miyake, S.; Yamamura, T.; Brenner, M.B. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* **2002**, *195*, 625–636. [CrossRef]
33. Kronenberg, M.; Gapin, L. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* **2002**, *2*, 557–568. [CrossRef]
34. Bychinin, M.V.; Klypa, T.V.; Mandel, I.A.; Yusubalieva, G.M.; Baklaushev, V.P.; Kolyshkina, N.A.; Troitsky, A.V. Effect of vitamin D3 supplementation on cellular immunity and inflammatory markers in COVID-19 patients admitted to the ICU. *Sci. Rep.* **2022**, *12*, 18604. [CrossRef]
35. Weng, X.; Kumar, A.; Cao, L.; He, Y.; Morgun, E.; Visvabharathy, L.; Zhao, J.; Sena, L.A.; Weinberg, S.E.; Chandel, N.S.; et al. Mitochondrial metabolism is essential for invariant natural killer T cell development and function. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2021385118. [CrossRef]
36. Yue, X.; Izcue, A.; Borggrefe, T. Essential role of Mediator subunit Med1 in invariant natural killer T-cell development. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17105–17110. [CrossRef] [PubMed]
37. Cho, S.W.; Zhang, Y.L.; Ko, Y.K.; Shin, J.M.; Lee, J.H.; Rhee, C.S.; Kim, D.Y. Intranasal treatment with 1,25-dihydroxyvitamin D3 alleviates allergic rhinitis symptoms in a mouse model. *Allergy Asthma Immunol. Res.* **2019**, *11*, 267–279. [CrossRef] [PubMed]
38. Zhu, D.C.; Feng, Y.; Wang, B.Q. Research progress on the relevance between serum vitamin D and IL-33/ST2 levels and allergic rhinitis. *Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* **2019**, *33*, 898–900. [PubMed]
39. Heine, G.; Hoefer, N.; Franke, A.; Nöthling, U.; Schumann, R.; Hamann, L.; Worm, M. Association of vitamin D receptor gene polymorphisms with severe atopic dermatitis in adults. *Br. J. Dermatol.* **2013**, *168*, 855–858. [CrossRef]
40. Hallau, J.; Hamann, L.; Schumann, R.R.; Worm, M.; Heine, G. A promoter polymorphism of the vitamin D metabolism gene Cyp24a1 is associated with severe atopic dermatitis in adults. *Acta Derm. Venereol.* **2016**, *96*, 169–172. [CrossRef]
41. Fang, X.; Xie, Q.; Zhang, X. Serum vitamin D level in mice with allergic rhinitis is correlated with inflammatory factors. *Am. J. Transl. Res.* **2021**, *13*, 3351–3356.
42. Gutiérrez-Vera, C.; García-Betancourt, R.; Palacios, P.A.; Müller, M.; Montero, D.A.; Verdugo, C.; Ortiz, F.; Simon, F.; Kalergis, A.M.; González, P.A.; et al. Natural killer T cells in allergic asthma: Implications for the development of novel immunotherapeutical strategies. *Front. Immunol.* **2024**, *15*, 1364774. [CrossRef]
43. Waddell, A.; Zhao, J.; Cantorna, M.T. NKT cells can help mediate the protective effects of 1,25-dihydroxyvitamin D3 in experimental autoimmune encephalomyelitis in mice. *Int. Immunol.* **2015**, *27*, 237–244. [CrossRef]

44. Zhou, X.; Li, Q.; Li, Y.; Fu, J.; Sun, F.; Li, Y.; Wang, Y.; Jia, Y.; Zhang, Y.; Jia, R.; et al. Diminished natural killer T-like cells correlates with aggravated primary Sjögren's syndrome. *Clin. Rheumatol.* **2016**, *35*, 1763–1770. [CrossRef]
45. Lin, S.J.; Kuo, M.L.; Hsiao, H.S.; Lee, P.T.; Huang, J.L. Cytotoxic function and cytokine production of natural killer cells and natural killer T-like cells in systemic lupus erythematosus: Regulation with interleukin-15. *Mediat. Inflamm.* **2019**, *2019*, 4236562. [CrossRef] [PubMed]
46. Gruber, B.M. The phenomenon of vitamin D. *Postepy Hig. Med. Dosw.* **2015**, *69*, 127–139.
47. Castellano-Castillo, D.; Morcillo, S.; Clemente-Postigo, M.; Crujeiras, A.B.; Fernandez-García, J.C.; Torres, E.; Tinahones, F.J.; Macias-Gonzalez, M. Adipose tissue inflammation and VDR expression and methylation in colorectal cancer. *Clin. Epigenetics* **2018**, *10*, 60. [CrossRef] [PubMed]
48. Kim, J.S.; Roberts, J.M.; Bingman, W.E.; Shao, L.; Wang, J.; Ittmann, M.M.; Weigel, N.L. The prostate cancer TMPRSS2:ERG fusion synergizes with the vitamin D receptor (VDR) to induce CYP24A1 expression-limiting VDR signaling. *Endocrinology* **2014**, *155*, 3262–3273. [CrossRef]
49. Czogalla, B.; Deuster, E.; Liao, Y.; Mayr, D.; Schmoeckel, E.; Sattler, C.; Kolben, T.; Hester, A.; Fürst, S.; Burges, A.; et al. Cytoplasmic VDR expression as an independent risk factor for ovarian cancer. *Histochem. Cell Biol.* **2020**, *154*, 421–429. [CrossRef]
50. Kim, S.H.; Chen, G.; King, A.N.; Jeon, C.K.; Christensen, P.J.; Zhao, L.; Simpson, R.U.; Thomas, D.G.; Giordano, T.J.; Brenner, D.E.; et al. Characterization of vitamin D receptor (VDR) in lung adenocarcinoma. *Lung Cancer* **2012**, *77*, 265–271. [CrossRef]
51. Hendrickson, W.K.; Flavin, R.; Kasperzyk, J.L.; Fiorentino, M.; Fang, F.; Lis, R.; Fiore, C.; Penney, K.L.; Ma, J.; Kantoff, P.W.; et al. Vitamin D receptor protein expression in tumor tissue and prostate cancer progression. *J. Clin. Oncol.* **2011**, *29*, 2378–2385. [CrossRef]
52. Keum, N.; Lee, D.H.; Greenwood, D.C.; Manson, J.E.; Giovannucci, E. Vitamin D supplementation and total cancer incidence and mortality: A meta-analysis of randomized controlled trials. *Ann. Oncol.* **2019**, *30*, 733–743. [CrossRef] [PubMed]
53. Zhu, S.; Zhang, C.; Sun, Q.; Wang, Y.; Yu, W.; Wei, F.; Ren, X. Trained Immunity of IL-12-, IL-15-, and IL-18-Induced CD_3+CD_{56}+ NKT-Like Cells. *J. Oncol.* **2022**, *2022*, 8724933. [CrossRef]
54. Tau, G.; Rothman, P. Biologic functions of the IFN-gamma receptors. *Allergy* **1999**, *54*, 1233–1251. [CrossRef]
55. Alves, P.C.M.; De Angelo Andrade, L.A.L.; Petta, C.A.; Lorand-Metze, I.; Derchain, S.F.; Guimarães, F. Ex vivo expansion of CD56+ NK and NKT-like lymphocytes from peripheral blood mononuclear cells of patients with ovarian neoplasia. *Scand. J. Immunol.* **2011**, *74*, 244–252. [CrossRef]
56. Yuen, M.F.; Norris, S. Expression of inhibitory receptors in natural killer ($CD3^-CD56^+$) cells and $CD3^+CD56^+$ cells in peripheral blood lymphocytes and tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. *Clin. Immunol.* **2001**, *101*, 276–282. [CrossRef]
57. Kokordelis, P.B.; Krämer, B.; Boesecke, C.; Voigt, E.; Ingiliz, P.; Glässner, A.; Wolter, F.M.; Srassburg, C.P.; Spengler, U.; Rockstroh, J.K.; et al. $CD3^+CD56^+$ natural killer-like T cells display anti-HCV activity but are functionally impaired in HIV^+ patients with acute hepatitis C. *J. Acquir. Immune Defic. Syndr.* **2015**, *70*, 338–346. [CrossRef] [PubMed]
58. Liu, P.T.; Stenger, S.; Li, H.; Wenzel, L.; Tan, B.H.; Krutzik, S.R.; Ochoa, M.T.; Schauber, J.; Wu, K.; Meinken, C.; et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **2006**, *311*, 1770–1773. [CrossRef] [PubMed]

OPINIA KOMISJI BIOETYCZNEJ

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Łódź, dnia 18 maja 2021 r.

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Instytutu Centrum Zdrowia Matki Polki w Łodzi

Komisja Bioetyczna przy Instytucie Centrum Zdrowia Matki Polki działając zgodnie z zasadami Dobrej Praktyki Klinicznej na posiedzeniu w dniu 18 maja 2021 r. rozpatrywała wniosek dotyczący pracy:

„Ocena ekspresji receptora tyreotropiny oraz receptorów hormonu tarczycy w komórkach Natural Killer T(NKT)"

Zespół badaczy:
1. Dr hab. n. med., prof. ICZMP Magdalena Stasiak
2. Lek. Emilia Adamska
3. Dr n. med. Przemysław Śliwka

Opinia Nr 41/2021

Komisja Bioetyczna przy Instytucie Centrum Zdrowia Matki Polki zapoznała się z ww. projektem eksperymentu medycznego, przeanalizowała wniosek, wysłuchała opinii recenzenta o przedstawionym projekcie i w wyniku przeprowadzonej dyskusji oraz tajnego głosowania, po rozważeniu kryteriów etycznych oraz celowości i wykonalności projektu pozytywnie zaopiniowała projekt eksperymentu medycznego.

Uchwałę podjęto jednogłośnie.
Uchwałę podjęto przy sprzeciwie .....

Zastępca Przewodniczącej:
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Oświadczam, iż wyrażam zgodę na wykorzystanie przez lek. Emilię Adamską-Fita w postępowaniu o nadanie tytułu doktora nauk medycznych publikacji „The Absence of Thyroid-Stimulating Hormone Receptor Expression on Natural Killer T Cells: Implications for the Immune-Endocrine Interaction" autorów: Emilia Adamska-Fita, Przemysław Wiktor Śliwka, Małgorzata Karbownik-Lewińska, Andrzej Lewiński, Magdalena Stasiak w International Journal of Molecular Sciences (2024 Oct 24;25(21):11434. doi: 10.3390/ijms252111434).

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Publikacje naukowe

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- Adamska-Fita E, Śliwka PW, Stasiak B, Karbownik-Lewińska M, Stasiak M. Vitamin D May Increase the Number of CD4–CD8+ NKT-like Cells in Humans—A Novel Insight into Potential Immunomodulatory Action. Nutrients. 2025;17(20):3216.

- Adamska-Fita E, Śliwka PW, Karbownik-Lewińska M, Lewiński A, Stasiak M. The Absence of Thyroid-Stimulating Hormone Receptor Expression on Natural Killer T Cells: Implications for the Immune-Endocrine Interaction. Int J Mol Sci. 2024;25(21):11434.
- Stasiak M, Witek P, Adamska-Fita E, Lewiński A. Response to Osilodrostat Therapy in Adrenal Cushing's Syndrome. Drug Healthc Patient Saf. 2024;16:35–42.
- Stasiak M, Dedecjus M, Zawadzka-Starczewska K, Adamska E, Tomaszewska M, Lewiński A. Novel germline c.105_107dupGCT MEN1 mutation in a family with newly diagnosed multiple endocrine neoplasia type 1. Genes. 2020.

Streszczenia zjazdowe

- Adamska-Fita E, Śliwka PW, Stasiak B, Karbownik-Lewińska M, Stasiak M. „Immunomodulacyjne właściwości witaminy D: wpływ stężenia witaminy D na subpopulacje komórek NKT-like" XXIII Zjazd Polskiego Towarzystwa Endokrynologicznego, wrzesień 2025r. (Gdańsk), Abstrakt wyróżniony.
- Stasiak M., Adamska-Fita E, Śliwka PW, Zygmunt A., Lewiński A. Lack of Thyroid-Stimulating Hormone Receptor Expression on Natural Killer T Cells: Implications for the Immune-Endocrine Interaction. Endocrine Abstracts 2024; 99: EP697, doi: 10.1530/endoabs.99.EP697. Współautorstwo plakatu prezentowanego na ECE 2024 (Sztokholm)
- Stasiak M, Adamska E, Lewinski A. Treatment with osilodrostat in ACTH-independent Cushing's syndrome. Endocrine Abstracts 2023;90:RC11.4 doi: 10.1530/endoabs.90.RC11.4). Współautorstwo doniesienia ustnego prezentowanego na ECE 2023 (Stambuł)
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