SZKOŁA DOKTORSKA
BioMedChem
Uniwersytetu Łódzkiego
i Instytutów Polskiej
Akademii Nauk w Łodzi

INSTYTUT ERCE BiolMed

PODPIS ZAUFANY
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06.11.2025 20:52:01 GMT+1
Dokument podpisany elektronicznie
podpisem zaufanym

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Praca doktorska:

Szczepionka BCG jako immunomodulator wrodzonej odpowiedzi odpornościowej dzieci na antygeny wirusowych patogenów dróg oddechowych

Doctoral thesis:

BCG vaccine as an immunomodulator of children's innate immune response to the antigens of viral respiratory pathogens.

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Łódź, 2025

Pragnę wyrazić głęboką wdzięczność
Pani dr hab. Magdalenie Druszczyńskiej, prof. UŁ,
za inspirujące rozmowy oraz nieustające wsparcie merytoryczne,
które miały ogromny wpływ na kształt i realizację niniejszej pracy doktorskiej.

Spis treści

PUBLIKACJE STANOWIĄCE PODSTAWĘ ROZPRAWY DOKTORSKIEJ

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2. **Godkowicz, M.,** & Druszczyńska, M. (2022). NOD1, NOD2, and NLRC5 Receptors in Antiviral and Antimycobacterial Immunity. Vaccines, 10(9), 1487. doi: 10.3390/vaccines10091487.

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   IF: 3,3; MNiSW: 100

Sumaryczny IF = 12,9

Łączna liczba punktów MNiSW = 480

Wartość IF oraz punktację MNiSW podano zgodnie listą obowiązującą w roku publikacji

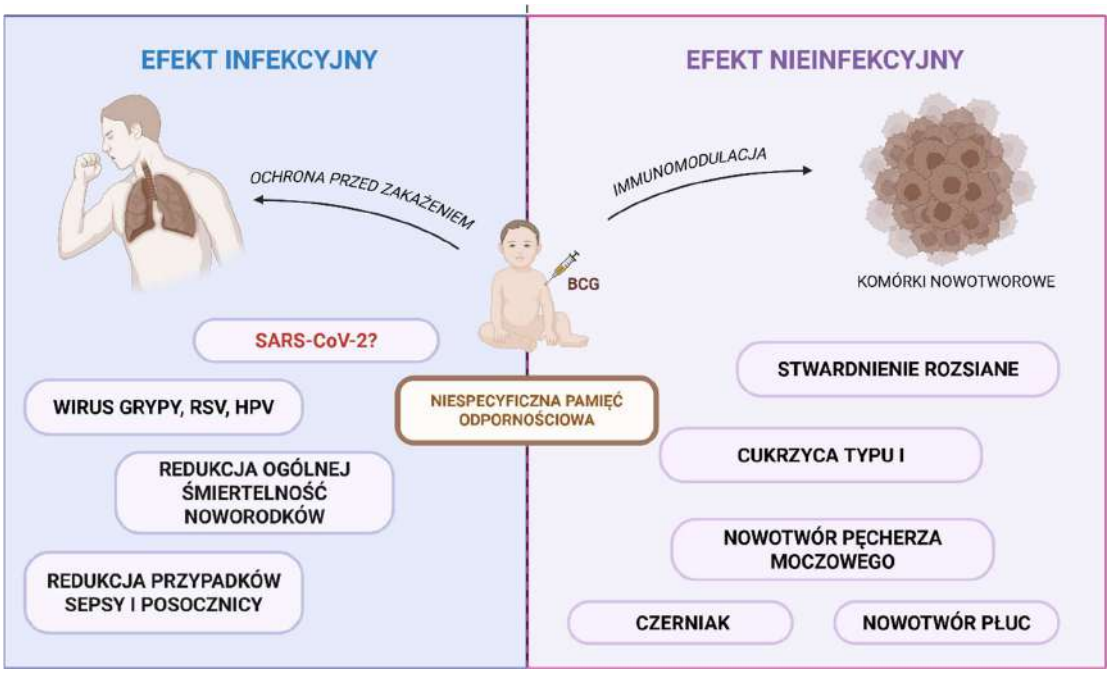
WSTĘP

Szczepionka BCG (Bacillus Calmette–Guérin), opracowana w latach 20. XX wieku, pozostaje jedyną zarejestrowaną szczepionką przeciwko gruźlicy, jednej z najpoważniejszych chorób zakaźnych na świecie. Mimo że jej skuteczność w zapobieganiu gruźlicy płucnej u dorosłych jest ograniczona, od ponad stu lat stanowi podstawowe narzędzie profilaktyki ciężkich postaci gruźlicy u dzieci, w tym gruźliczego zapalenia opon mózgowo-rdzeniowych i gruźlicy rozsianej (Du i in. 2023).

Rosnąca liczba badań wskazuje jednak, że szczepionka BCG wywołuje także nieswoiste efekty odpornościowe, wykraczające poza klasyczne działanie przeciwgruźlicze. Już w latach 70. XX wieku zaobserwowano, że dzieci szczepione BCG w krajach o wysokiej śmiertelności wykazywały niższą ogólną umieralność, nawet w przypadku braku kontaktu z prątkami gruźlicy. Badania epidemiologiczne potwierdziły, że szczepienie BCG wiąże się z redukcją zapadalności i śmiertelności z powodu różnych infekcji wirusowych i bakteryjnych, co sugeruje immunomodulacyjny charakter tego preparatu (Aaby i in. 2011; Biering-Sørensen i in. 2012; Prentice i in. 2021; Wilkie i in. 2022). Mechanizmem leżącym u podstaw tego zjawiska jest nieswoista aktywacja układu odpornościowego poprzez przeprogramowanie komórek wrodzonej odporności, takich jak monocyty, makrofagi czy naturalne komórki zabijające – NK (ang. *natural killer*). Efekty te nabrały szczególnego znaczenia w kontekście zagrożeń wirusowych, m.in. pandemii COVID-19 (ang. *Coronavirus Disease 2019*) wywołanej przez SARS-CoV-2 (ang. *severe acute respiratory syndrome coronavirus 2*) (Amirlak i in. 2021; Hilligan i in. 2021; Kaufmann i in. 2022; Kim i in. 2021; Rey-Jurado i in. 2017; Stensballe i in. 2005).

W odróżnieniu od odporności nabytej, opartej na swoistości i długotrwałej pamięci immunologicznej z udziałem limfocytów T i B, odporność wrodzona przez długi czas była postrzegana jako mechanizm niespecyficzny i pozbawiony zdolności do pamięci immunologicznej. W klasycznym ujęciu komórki takie jak: makrofagi, monocyty, granulocyty czy komórki NK miały odpowiadać w sposób szybki, ale krótkotrwały, bez możliwości adaptacji do kolejnych wyzwań immunologicznych. Jednak obecne dane istotnie zmieniły to podejście, wskazując na istnienie zjawiska wytrenowanej odporności (trained immunity), które redefiniuje tradycyjny podział na odporność wrodzoną i nabytą (Netea i in. 2011; Trunk i in. 2023). Mechanizm ten polega na funkcjonalnym przeprogramowaniu komórek wrodzonego układu odpornościowego — przede wszystkim

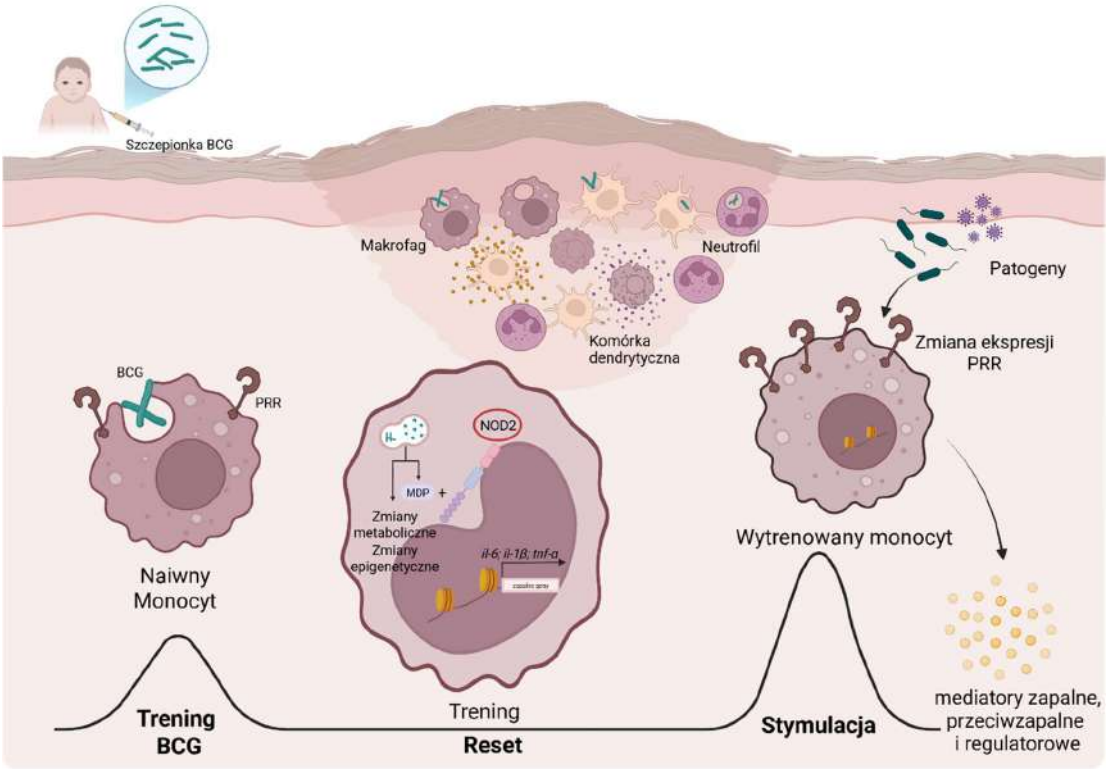
makrofagów, monocytów i komórek NK — prowadząc do szybszej i bardziej wydajnej odpowiedzi na kolejne bodźce patogenne, również niespokrewnione z pierwotnym czynnikiem infekcyjnym inicjującym te zmiany (Netea i in. 2011; Trunk i in. 2023). Podstawą wytrenowanej odporności są trwałe zmiany epigenetyczne, takie jak modyfikacje histonów (np. acetylacja H3K27, trimetylacja H3K4) oraz zmiany w strukturze chromatyny, które zwiększają dostępność promotorów i wzmacniają ekspresję genów prozapalnych (Kleinnijenhuis i in. 2012; Saeed i in. 2014). Procesom tym towarzyszą zmiany metabolizmu komórkowego w postaci promowania glikolizy tlenowej, zwiększenia aktywności szlaku pentozofosforanowego czy aktywacja szlaku mewalonianowego – co sprzyja szybszej syntezie mediatorów zapalnych i efektywnej odpowiedzi przeciwdrobnoustrojowej (Arts i in. 2016; Bekkering i in. 2016; Cheng i in. 2014). Szczepionka BCG jest najlepiej poznanymi najczęściej stosowanym w badaniach induktorem wytrenowanej odporności. Wykazano, że podanie BCG prowadzi nie tylko do aktywacji komórek efektorowych krążących we krwi, ale także do epigenetycznej i metabolicznej przebudowy hematopoetycznych komórek szpiku kostnego będących prekursorami komórek immunokompetentnych – zarówno w modelu mysim, jak i u ludzi, co skutkuje długofalową modyfikacją odpowiedzi odpornościowej z udziałem całej populacji komórek odporności wrodzonej (Cirovic i in. 2020; Kaufmann i in. 2018). Zatem wytrenowana odporność nie ogranicza się tylko do zwiększonej reaktywności prozapalnej komórek. Mechanizm ten może prowadzić do bardziej zrównoważonej regulacji odpowiedzi immunologicznej – z jednej strony wzmacniając ochronę przed infekcjami bakteryjnymi i wirusowymi, a z drugiej redukując ryzyko nadmiernej, patologicznej reakcji zapalnej. Wskazuje to na potencjał BCG nie tylko jako szczepionki przeciwgruźliczej, lecz także jako uniwersalnego narzędzia terapeutycznego w profilaktyce i leczeniu szerokiego spektrum chorób – od infekcji wirusowych, przez choroby nowotworowe, aż po choroby autoimmunologiczne (Kamat i in. 2015; Kühtreiber i in. 2018; Ristori i in. 2018) (Ryc.1). Szczegółowe omówienie nieswoistych efektów szczepionki BCG, podkreślające jej potencjał jako możliwego narzędzia terapeutycznego mobilizującego aktywność układu odpornościowego, zostało przedstawione w Publikacji 1 (Jurczak i Druszczynska 2025), wchodzącej w skład niniejszej rozprawy doktorskiej.



Ryc.1. Nieswoiste efekty szczepionki BCG. Dwutorowe działanie szczepionki BCG, obejmujące zarówno efekty związane z ochroną przed zakażeniem, jak i chorobami nieinfekcyjnymi. Po stronie efektu infekcyjnego przedstawiono, że szczepionka BCG zapewnia redukcję przypadków sepsy i posocznicy, co wiąże się ze zmniejszoną śmiertelnością noworodków; chroni przed zakażeniami wirusowymi takimi jak: wirus grypy, RSV, HPV oraz potencjalnie SARS-CoV-2. Po stronie efektu nieinfekcyjnego zaznaczono rolę szczepionki BCG w chorobach nowotworowych takich jak: czerniak, nowotwór pęcherza moczowego lub płuc, a także w chorobach autoimmunologicznych (stwardnienie rozsiane, cukrzyca typu I). Informację o niespecyficznym działaniu szczepionki BCG przedstawiono w Publikacji 1 (Jurczak i Druszczynska 2025).
Skróty: Bacillus Calmette-Guérin (BCG), Drugi koronawirus ciężkiego ostrego zespołu oddechowego (SARS-CoV-2), wirus brodawczaka ludzkiego (HPV), Syncytialny wirus oddechowy (RSV).

Kluczowymi komponentami komórek odporności wrodzonej umożliwiającymi zależną od BCG nieswoistą aktywację tych komórek są receptory rozpoznające wzorce molekularne – PRR (ang. *pattern recognition receptors*), które stanowią podstawę wczesnej odpowiedzi odpornościowej. Wśród nich szczególną rolę przypisuje się receptorowi NOD2 (ang. *nucleotide-binding oligomerization domain-containing protein 2*), zdolnemu do rozpoznawania muramylo-dipetydu (MDP) – fragmentu peptydoglikanu ściany komórkowej prątków (Publikacja 2, Godkowicz i Druszczyńska 2022). Aktywacja NOD2 przez komponenty BCG inicjuje w komórkach kaskady sygnałowe prowadzące do aktywacji czynników transkrypcyjnych, takich jak czynnik jądrowy NF-κB (ang. *nuclear factor kappa B*) i białko aktywujące 1 - AP-1 (ang. *activating protein 1*), co skutkuje indukcją ekspresji

licznych genów determinujących odpowiedź odpornościową (Wannigama i Jacquet 2020). Zjawisko to wykracza jednak poza klasyczne, krótkotrwałe mechanizmy odpowiedzi zapalnej. Wykazano, że aktywacja NOD2 prowadzi do powstawania trwałych zmian epigenetycznych w komórkach wrodzonej odporności, obejmujących m.in. acetylację histonów (H3K27ac) oraz metylację (H3K4me3) w promotorach genów cytokin, co skutkuje łatwiejszym i szybszym dostępem czynników transkrypcyjnych przy kolejnym kontakcie z patogenem (Kleinnijenhuis i in. 2012). Równolegle dochodzi do przeprogramowania metabolicznego monocytów i makrofagów – z dominacji fosforylacji oksydacyjnej w kierunku glikolizy tlenowej (efekt Warburga), a także do zwiększonego wykorzystania szlaku glutaminolizy i cholesterogenezy, co zapewnia substraty energetyczne i biosyntetyczne niezbędne dla wzmożonej odpowiedzi immunologicznej tych komórek (Arts i in. 2016). Co istotne, mechanizmy te wykraczają poza czas bezpośredniej stymulacji BCG, nadając komórkom wrodzonej odporności „pamięć funkcjonalną" umożliwiającą silniejszą i szybszą odpowiedź na kolejne bodźce – zarówno bakteryjne, jak i wirusowe. W badaniach na ludzkich komórkach nabłonka płuc A549 człowieka wykazano, że modulacja aktywności NOD2 odgrywa istotną rolę nie tylko w odpowiedzi przeciwprątkowej, ale także w kształtowaniu odporności przeciwwirusowej, poprzez wpływ na odpowiedź interferonową i regulację wydzielania cytokin pro- i przeciwzapalnych (Sabbah i in. 2009). Tym samym NOD2 można uznać za jeden z kluczowych molekularnych mediatorów wytrenowanej odporności indukowanej przez BCG, łączącym rozpoznawanie komponentów bakteryjnych z przebudową epigenetyczną i metaboliczną komórek odporności wrodzonej (Ryc. 2). Szczegółowe omówienie roli receptorów NOD, w tym NOD2, w odpowiedzi przeciwwirusowej i przeciwprątkowej zawarto w Publikacji 2 (Godkowicz i Druszczyńska 2022), stanowiącej część niniejszej dysertacji.



Ryc. 2. Mechanizm wytrenowanej odporności indukowanej za pośrednictwem receptora NOD2. Komponenty szczepionki BCG takie jak MDP zostają rozpoznane przez receptory dla wzorców molekularnych patogenów (PRR), w tym NOD2, co prowadzi do aktywacji szlaków sygnałowych w komórkach odporności wrodzonej m.in. monocytach, czego efektem są zmiany metaboliczne i epigenetyczne w tych komórkach, które prowadzą do ich przeprogramowania w kierunku szybszej i silniejszej odpowiedzi na patogeny, niezależnie od ich rodzaju, co wiąże się z wydzielaniem mediatorów zapalnych, przeciwzapalnych i regulatorowych. Taki efekt określa się jako stan „wytrenowanej odporności".
Skróty: *Bacillus Calmette-Guérin (BCG), Interleukina (IL), muramylo dipeptyd (MDP), receptory rozpoznające wzorce molekularne – (PRR) (ang. pattern recognition receptors), czynnik martwicy nowotworów (TNF-α) (ang. tumor necrosis factor α).*

W ostatnich latach, szczególnie w okresie pandemii COVID-19, zainteresowanie immunomodulacyjnym działaniem szczepionki BCG wyraźnie wzrosło. Wstępne analizy epidemiologiczne sugerowały, że kraje utrzymujące obowiązkowe szczepienia BCG notowały niższe wskaźniki zachorowalności i śmiertelności z powodu SARS-CoV-2, co może wskazywać na potencjalny ochronny efekt nieswoisty tej szczepionki (Junqueira-Kipnis i in. 2020; Miyasaka 2020; Yitbarek i in. 2020). Hipoteza ta doprowadziła do licznych badań klinicznych, w których oceniano, czy immunizacja BCG może zmniejszać ryzyko ciężkiego przebiegu COVID-19 i innych infekcji wirusowych. Pierwsze wyniki badań randomizowanych u dorosłych pracowników ochrony zdrowia wykazały, że szczepionka BCG może redukować częstość występowania ostrych infekcji dróg oddechowych, w tym zakażeń wirusowych oraz łagodzić ich przebieg (Curtis i in. 2020;

Tsilika i in. 2022). W części populacji efekt ten obejmował również zmniejszone ryzyko hospitalizacji z powodu COVID-19 (Rivas i in. 2021). Jednocześnie należy podkreślić, że dane dostępne w literaturze naukowej są niejednoznaczne. W wielu badaniach klinicznych i metaanalizach jak dotąd nie udało się potwierdzić istotnego związku między szczepieniem BCG a zmniejszoną zapadalnością lub śmiertelnością z powodu COVID-19. Wykazano, że odsetek hospitalizacji i ciężkich przebiegów były zbliżone w grupach szczepionych i placebo (Czajka i in. 2022; Inauen i in. 2025; Madsen i in. 2024; Xia i in. 2025). Podobne rozbieżności wyników badań dotyczą również syncytialnego wirusa oddechowego - RSV (ang. *respiratory syncytial virus*), jednego z najważniejszych patogenów układu oddechowego wieku dziecięcego (Stensballe i in. 2005). Choć niektóre dane eksperymentalne i kliniczne wskazują, że mechanizmy wytrenowanej odporności indukowane przez BCG mogą wspierać szybszą i bardziej zrównoważoną odpowiedź przeciwwirusową na zakażenie RSV (Bueno i in. 2008; Wang i in. 2023), to badań dotyczących tej tematyki jest niewiele, a ich wyniki są często niespójne. W badaniach kohortowych wykazywano, że dzieci szczepione BCG rzadziej chorują na infekcje dróg oddechowych, także te wywoływane wirusami (Aaby i in. 2011), jednak brak jednoznacznych danych dotyczących pacjentów zakażonych wirusem RSV pozostaje poważną luką badawczą. Wobec powyższego oraz ograniczonej liczby badań pediatrycznych, szczególnie w zakresie zakażeń wirusowych układu oddechowego, ocena zdolności szczepionki BCG w modulowaniu odporności u dzieci nabiera wyjątkowego znaczenia. Analiza nieswoistych efektów tej szczepionki może nie tylko pomóc w zrozumieniu mechanizmów wytrenowanej odporności w młodym wieku, lecz także stanowić podstawę dla opracowania strategii immunoprofilaktyki w obliczu nowych zagrożeń infekcyjnych.

HIPOTEZA BADAWCZA

Zgodnie z koncepcją wytrenowanej odporności szczepionka BCG może prowadzić do przeprogramowania komórek wrodzonego układu odpornościowego, które działają w sposób nieswoisty, zwiększając ich gotowość do odpowiedzi na różne patogeny bakteryjne i wirusowe. **W oparciu o tę koncepcję wysunięto hipotezę, że immunizacja dzieci w okresie noworodkowym prątkami szczepionkowymi BCG o właściwościach immunomodulujących powoduje trwałe zmiany w komórkach odporności wrodzonej, które mogą ujawniać się i być wykrywalne wiele lat później, w wieku szkolnym.**

Założono, że takie zmiany mogą zachodzić w komórkach leukocytów krwi obwodowej dzieci na poziomie regulacji genowej (ekspresja mRNA) i funkcjonalnym (sekrecja cytokin pro- i przeciwzapalnych oraz interferonów, a także ekspresja wybranych receptorów powierzchniowych odzwierciedlających stan aktywacji i zdolności efektorowe komórek), po ekspozycji na antygeny czynników zakaźnych zarówno homologicznych (prątki BCG) jak i heterologicznych (antygeny wirusów RSV lub SARS-CoV-2), w hodowlach *in vitro*. Charakterystyka komórek odporności wrodzonej dzieci szczepionych BCG pod względem efektywności przebytego treningu odpornościowego w modelu *in vitro* może dostarczyć cennych informacji na temat gotowości immunologicznej tych komórek do zwalczania różnych zagrożeń infekcyjnych.

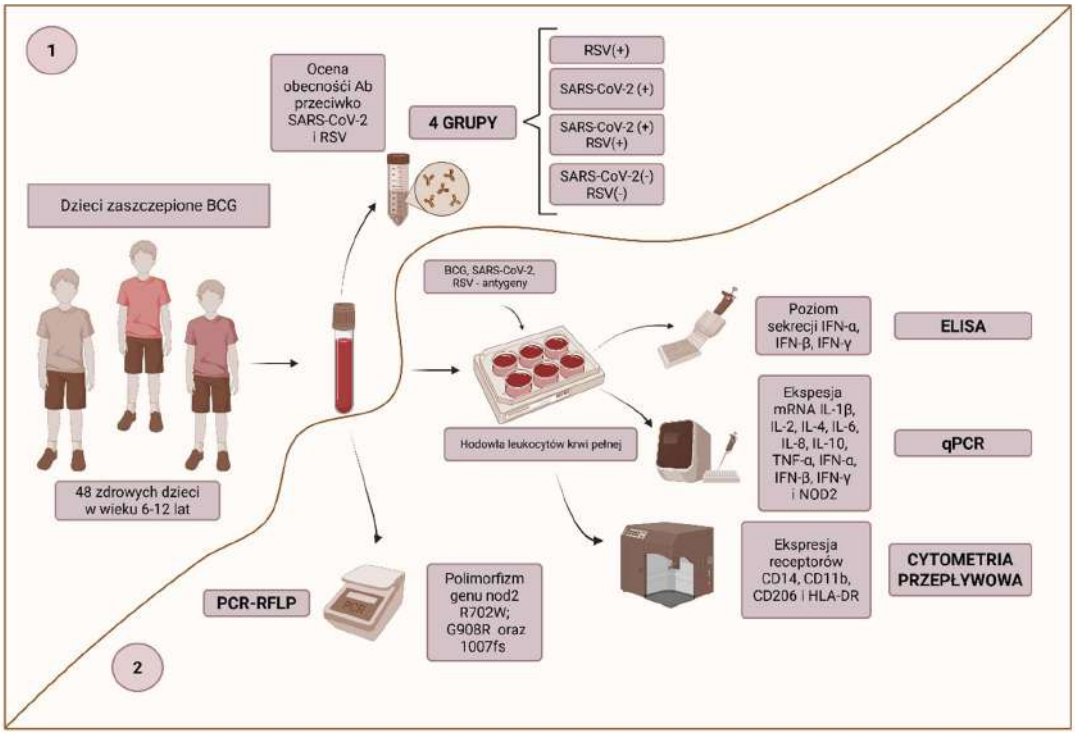
CEL PRACY

Biorąc pod uwagę stawianą hipotezę, celem rozprawy doktorskiej była ocena nieswoistej odpowiedzi odpornościowej u dzieci w wieku 6–12 lat, zaszczepionych BCG w okresie noworodkowym, z uwzględnieniem indukowanych przez BCG mechanizmów wytrenowanej odporności weryfikowanych na podstawie odpowiedzi leukocytów pełnej krwi obwodowej na bodziec homologiczny (prątki BCG) lub heterologiczny (antygeny wirusów RSV i SARS-CoV-2), w hodowlach *in vitro*.

Do realizacji założonego celu zastosowano model hodowli komórkowej *in vitro* oparty na stymulacji leukocytów pełnej krwi obwodowej dzieci prątkami szczepionkowymi BCG lub antygenami wirusów oddechowych RSV lub SARS-CoV-2, niezależnie lub łącznie. W badaniu uwzględniono status serologiczny dzieci w zakresie przeciwciał przeciwko RSV i/lub SARS-CoV-2. Jako wyznaczniki odpowiedzi odpornościowej nieswoistej u badanych dzieci przyjęto: zdolność leukocytów do wytwarzania kluczowych cytokin pro- (IL-1β, IL-2, IL-6, IL-8, TNF-α) (interleukina) i przeciwzapalnych (IL-4, IL-10), odpowiedź interferonową w zakresie wytwarzania IFN-α, IFN-β (odpowiedź przeciwwirusowa), IFN-γ (działanie immunomodulujące), nasilenie ekspresji receptorów powierzchniowych na monocytach, w tym CD14 (receptor typu PRR), CD11b (adhezyna powierzchniowa), HLA-DR (cząsteczka biorąca udział w prezentacji antygenów), CD206 (receptor mannozy) oraz receptor NOD2 pełniący m.in. rolę sensora komponentów ściany komórkowej prątków. Uwzględniono także ocenę zmienności genetycznej receptora NOD2, która może wpływać na przebieg odpowiedzi immunologicznej, zakładając że może być to pomocne w wyjaśnieniu różnic w aktywności komórek immunokompetentnych badanych osób. Takie podejście łączy możliwość oceny nieswoistych efektów szczepienia BCG z badaniem indywidualnych determinant odporności wrodzonej, istotnych w epidemiologii zakażeń wirusowych u dzieci. Zastosowany model komórkowy *in vitro* umożliwił przeprowadzenie oceny wybranych molekularnych i komórkowych wyznaczników odpowiedzi immunologicznej, w warunkach kontrolowanych, pozwalając ocenić zależność pomiędzy immunizacją BCG a odpowiedzią na antygeny wirusowe, co stanowi unikalny aspekt w badaniach nad nieswoistymi efektami szczepionki BCG w populacji pediatrycznej.

W pracy wyznaczono następujące cele szczegółowe, których realizację zobrazowano na Ryc. 3.

1. Charakterystyka odpowiedzi leukocytów krwi obwodowej dawców immunizowanych prątkami BCG, seronegatywnych pod względem przeciwciał przeciwko RSV i SARS-CoV-2, na antygeny prątków (czynnik homologiczny) na podstawie ekspresji mRNA cytokin i powierzchniowych receptorów komórkowych.
2. Ocena odpowiedzi leukocytów krwi obwodowej dzieci immunizowanych prątkami BCG, seronegatywnych w zakresie przeciwciał anty-RSV i/lub anty-SARS-CoV-2, na antygeny prątków (czynnik homologiczny) i/lub antygeny RSV/SARS-CoV-2 (czynnik heterologiczny) na podstawie ekspresji mRNA cytokin, w tym interferonów i powierzchniowych receptorów komórkowych.
3. Wyznaczenie profilu odpowiedzi cytokinowej leukocytów krwi obwodowej dzieci immunizowanych prątkami BCG, seropozytywnych pod względem przeciwciał przeciwko RSV i/lub SARS-CoV-2.
4. Ocena zależności między szczepieniem BCG w okresie noworodkowym a nieswoistą odpowiedzią immunologiczną na antygeny wirusowe (SARS-CoV-2 i/lub RSV).
5. Ocena związku między ekspresją receptora NOD2 indukowaną przez prątki BCG a odpowiedzią leukocytów na antygeny SARS-CoV-2 i/lub RSV.



Ryc. 3. Zestawienie przeprowadzonych badań.

Krew pełna, która została pobrana od grupy zdrowych dzieci w wieku 6-12 lat posłużyła do założenia hodowli leukocytów pełnej krwi obwodowej w obecności żywych prątków *Mycobacterium bovis* BCG i/lub antygenów SARS-CoV-2 lub RSV. Na podstawie oceny obecności przeciwciał przeciwko SARS-CoV-2 oraz RSV wyróżniono cztery grupy badane: RSV(+), SARS-CoV-2(+), SARS-CoV-2(+) RSV(+) oraz SARS-CoV-2(-) RSV(-). Supernatanty z hodowli leukocytów krwi obwodowej posłużyły do oceny poziomy sekrecji IFN-α, IFN-β, IFN-γ, a osady komórek z tej hodowli wykorzystano do oceny ekspresji mRNA IL-1β, IL-2, IL-4, IL-6 IL-8, IL-10, TNF-α, IFN-α, IFN-β i IFN-γ (reakcja qPCR) oraz ekspresji receptorów powierzchniowych monocytów CD14, CD11b, CD206 oraz HLA-DR (cytometria przeplywowa). Do oceny polimorfizmu genu *nod2* R702W, G908R oraz 1007fs wykorzystano pobraną krew pełną i zastosowano technikę PCR-RFLP z elektroforezą w żelu poliakrylamidowym.

Skróty: Bacillus Calmette-Guérin (BCG), Interleukina (IL), Interferon (IFN), NOD2 (ang. nucleotide-binding oligomerization domain containing 2), Polimorfizm długości fragmentów restrykcyjnych (ang. restriction fragments length polymorphism) (RFLP) reakcja łańcuchowej polimerazy (PCR) (ang. polymerase chain reaction), Drugi koronawirus ciężkiego ostrego zespołu oddechowego (SARS-CoV-2), Syncytialny wirus oddechowy (RSV), czynnik martwicy nowotworów (TNF-α) (ang. tumor necrosis factor α), RSV(+)– grupa dzieci po przebytym zakażeniu RSV, seropozytywnych pod względem przeciwciał przeciwko RSV, SARS-CoV-2(+) – grupa dzieci po przebytym zakażeniu SARS-CoV-2, SARS-CoV-2(+)RSV(+) – grupa dzieci po przebytym zakażeniu SARS-CoV-2 i RSV, seropozytywnych pod względem przeciwciał przeciwko SARS-CoV-2 i RSV, SARS-CoV-2(-)RSV(-) – grupa dzieci seronegatywnych pod względem przeciwciał przeciwko SARS-CoV-2 i RSV, seropozytywnych pod względem przeciwciał przeciwko SARS-CoV-2, ilościowa reakcja łańcuchowa polimerazy - qPCR (ang. quantitative polymerase chain reaction).

METODYKA PRACY

Badaniem objęto 48 zdrowych dzieci w wieku od 6 do 12 lat, które otrzymały szczepionkę *Mycobacterium bovis* BCG Moreau (Biomed Lublin, Polska), w pierwszej dobie życia, zgodnie z kalendarzem polskiego narodowego programu szczepień. Wśród dzieci wyróżniono cztery grupy badane na podstawie historii zakażenia RSV i SARS-CoV-2:

- Grupa 1: RSV(-)SARS-CoV-2(-) – grupa dzieci seronegatywnych pod względem przeciwciał przeciwko RSV i SARS-CoV-2.
- Grupa 2: RSV(+) – grupa dzieci po przebytym zakażeniu RSV, seropozytywnych pod względem przeciwciał przeciwko RSV.
- Grupa 3: SARS-CoV-2(+) – grupa dzieci po przebytym zakażeniu SARS-CoV-2, seropozytywnych pod względem przeciwciał przeciwko SARS-CoV-2.
- Grupa 4: RSV(+)SARS-CoV-2(+) – grupa dzieci po przebytym zakażeniu RSV i SARS-CoV-2, seropozytywnych pod względem przeciwciał przeciwko RSV i SARS-CoV-2.

Wszystkie dzieci, za zgodą prawnych opiekunów, zostały zakwalifikowane do badania przez konsultantów chorób zakaźnych, w tym Wojewódzkiego Konsultanta ds. Pulmonologii Dziecięcej w Wojewódzkim Specjalistycznym Szpitalu Gruźlicy, Chorób Płuc i Rehabilitacji w Łodzi. Protokół badania został zatwierdzony przez Komisję Bioetyczną Uniwersytetu Medycznego w Łodzi (Nr RNN/122/22/KE).

Badania obejmowały:

1. Hodowlę prątków *Mycobacterium bovis* BCG (Biomed Lublin, Polska) na podłożu płynnym Middlebrooka 7H9 (Becton Dickinson, Sparks, USA) z dodatkiem 5% Tween 80 oraz ODAC w temperaturze 37°C, w atmosferze 5% CO_2.
2. Spektrofotometryczną ocenę gęstości zawiesiny bakterii przy długości fali λ = 600 nm.
3. Ocenę obecności i miana surowiczych przeciwciał przeciwko SARS-CoV-2 z wykorzystaniem testu DiaSorin LIASON® SARS-CoV-2 TrimericS IgG (DiaSorin, Stillwater, MN, USA).
4. Ocenę obecności i miana surowiczych przeciwciał przeciwko RSV z wykorzystaniem testu Respiratory Syncytial Virus IgG ELISA kit (Serion-Diagnostics, Würzburg, Niemcy).

5. Prowadzenie hodowli leukocytów pełnej krwi obwodowej w obecności żywych prątków *Mycobacterium bovis* BCG (Biomed Lublin, Polska), antygenów SARS-CoV-2 (PepTivator SARS-CoV-2, MiltenyiBiotec, Niemcy) lub RSV (PepTivator RSV, MiltenyiBiotec, Niemcy) (48 godzin, 37°C, 5% CO_2).
6. Ocenę poziomu cytokin - IL-1β, IL-2, IL-4, IL-6 IL-8, IL-10, TNF-α, IFN-α, IFN-β i IFN-γ w surowicach oraz supernatantach pohodowlanych w teście immunoenzymatycznym ELISA, z wykorzystaniem zestawów DuoSet® ELISA (R&D Systems, Minneapolis, MN, USA), QuantiFERON – TB Gold Plus ELISA (Qiagen, Hilden, Niemcy) oraz techniki multipleks ELISA Bio-Plex Pro Human Cytokine 8-plex Assay (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF) (Bio-Rad, Hercules, CA, USA).
7. Izolację genomowego DNA z komórek krwi obwodowej dzieci z wykorzystaniem zestawu EXTRACTME DNA BLOOD KIT (Blirt, Gdańsk, Polska) oraz ocenę stężenia i jakości uzyskanych izolatów DNA metodą spektrofotometryczną (NanoDrop, Holliston, MA, USA).
8. Ocenę wariantów polimorficznych genu *nod2* - R702W (rs2066844), G908R (rs2066845) oraz 1007fs (rs5743293) techniką PCR-RFLP w połączeniu z elektroforezą w żelu poliakrylamidowym (Heliö i in. 2003).
9. Izolację RNA z hodowli leukocytów krwi obwodowej dawców, które prowadzono w obecności prątków BCG i/lub antygenów SARS-CoV-2/RSV z wykorzystaniem zestawu QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Niemcy), ocenę stężenia RNA metodą spektrofotometryczną (NanoDrop, Holliston, MA, USA) i stopnia jego degradacji w elektroforezie, w żelu agarozowym.
10. Syntezę cDNA metodą odwrotnej transkrypcji z wykorzystaniem zestawu iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).
11. Ocenę ekspresji mRNA IL-1β, IL-2, IL-4, IL-6 IL-8, IL-10, TNF-α, IFN-α, IFN-β oraz IFN-γ w hodowlach komórek krwi obwodowej dawców, które prowadzono w obecności prątków BCG i/lub antygenów SARS-CoV-2/RSV z wykorzystaniem techniki qPCR.
12. Ocenę ekspresji powierzchniowych receptorów CD14, HLA-DR, CD-11b oraz CD206 na komórkach monocytów krwi obwodowej dawców, które pochodziły z hodowli prowadzonych w obecności prątków BCG i/lub antygenów SARS-CoV-2/RSV metodą cytometrii przepływowej (FAC Symphony™ A1 , BD Biosciences, Franklin Lakes, NJ, USA).

WYNIKI

Uzyskane wyniki i ich interpretacja zostały szczegółowo przedstawione w publikacji nr 3 (Jurczak i in. 2025a) i nr 4 (Jurczak i in. 2025b).

1. Charakterystyka odpowiedzi leukocytów krwi obwodowej dawców immunizowanych prątkami BCG, seronegatywnych pod względem przeciwciał przeciwko RSV i SARS-CoV-2, na antygeny żywych prątków (czynnik homologiczny) na podstawie ekspresji mRNA cytokin i powierzchniowych receptorów komórkowych.

W pierwszym etapie badań, w celu oceny, czy szczepionka BCG pełni funkcję modulatora wrodzonej odporności, zwiększając gotowość komórek immunokompetentnych do reakcji prozapalnej i przeciwwirusowej przy jednoczesnej kontroli tych aktywności (działanie przeciwzapalne), oceniono odpowiedź leukocytów krwi obwodowej dzieci seronegatywnych w zakresie przeciwciał przeciwko–SARS-CoV-2 i RSV SARS-CoV-2(-)RSV(-), w hodowlach *in vitro* prowadzonych w obecności żywych prątków BCG, jako homologicznego stymulatora antygenowego komórek immunokompetentnych. Wykazano, że stymulacja leukocytów prątkami szczepionkowymi BCG w warunkach hodowli prowadziła do znaczącego wzrostu ekspresji mRNA cytokin prozapalnych, w tym IL-1β, IL-2, IL-6 i TNF-α, a także przeciwzapalnej IL-4 oraz interferonów typu I i II (IFN-α, IFN-β, IFN-γ) przy braku istotnych zmian w poziomie ekspresji mRNA prozapalnej IL-8 i regulatorowej IL-10 (Jurczak i in. 2025a; Jurczak i in. 2025b).

Uzyskane wyniki wskazują na jednoczesną aktywację ścieżek odpowiedzi zapalnej i przeciwwirusowej przy zachowaniu mechanizmów regulacyjnych, ograniczających ryzyko nadmiernej aktywacji komórek odpornościowych, ale jej nie blokując. Świadczyć może o tym m.in. brak nasilenia ekspresji mRNA, odpowiednio IL-8, kluczowej cytokiny o działaniu chemotaktycznym i IL-10, która wykazuje działanie regulatorowe.

Analiza wyników badań dotyczących fenotypu monocytów wykazała istotny wzrost powierzchniowej ekspresji receptorów CD14 i HLA-DR, co może odzwierciedlać zwiększoną zdolność rozpoznawania wzorców molekularnych czynników zakaźnych przez te komórki i ich gotowość do prezentacji antygenów limfocytom, a zatem do inicjacji odpowiedzi adaptacyjnej. Zaobserwowany brak zmian w ekspresji cząsteczek powierzchniowych CD11b i CD206 na monocytach może sugerować, że stymulacja BCG nie prowadzi do ich polaryzacji, co jest korzystne ze względu na zachowanie zdolności tych komórek do reagowania na różne bodźce środowiskowe, w tym infekcyjne.

Ponadto, w hodowlach leukocytów stymulowanych prątkami BCG wykazano wzrost ekspresji mRNA NOD2, co wskazuje na możliwość sygnalizacji wtórnej w tych komórkach i nasilenie odpowiedzi wrodzonej.

Analiza wyników badań w odniesieniu do wieku i płci badanych dzieci nie wykazała istotnych różnic, co wskazuje, że obserwowane zmiany były raczej efektem działania BCG, a nie czynników demograficznych.

Uzyskane wyniki wskazują, że **szczepionka BCG wywołuje u osób immunizowanych w okresie noworodkowym trwałe zmiany w komórkach odporności wrodzonej. Zmiany te manifestują się zwiększoną reaktywnością leukocytów na antygeny homologicznego czynnika infekcyjnego (prątki BCG) w hodowlach *in vitro***.

Wyniki te wspierają koncepcję, że immunizacja prątkami szczepionkowymi BCG w okresie noworodkowym pozostawia długotrwały „ślad immunologiczny". Oznacza to gotowość komórek immunokompetentnych do efektywnego i zrównoważonego reagowania na późniejsze wyzwania infekcyjne, co może mieć istotne znaczenie w kontekście profilaktyki chorób zakaźnych w populacji pediatrycznej. Wyniki badań przedstawiono w publikacjach nr 3 (Jurczak i in. 2025a) i nr 4 (Jurczak i in. 2025b).

2. Ocena odpowiedzi leukocytów krwi obwodowej dzieci immunizowanych prątkami BCG, seronegatywnych w zakresie przeciwciał anty-RSV i/lub anty-SARS-CoV-2, na antygeny prątków (czynnik homologiczny) i/lub antygeny RSV/SARS-CoV-2 (czynnik heterologiczny) na podstawie ekspresji mRNA cytokin, w tym interferonów oraz powierzchniowych receptorów komórkowych

W celu odpowiedzi na pytanie, czy „wytrenowana odporność" indukowana w komórkach odporności wrodzonej przez immunizację BCG może modulować odpowiedź przeciwwirusową, oceniono aktywność leukocytów ko-stymulowanych podczas hodowli prątkami szczepionkowymi BCG łącznie z antygenami RSV i/lub SARS-CoV-2. Wykazano, że prątki BCG pełnią funkcję nieswoistego modulatora odpowiedzi przeciwwirusowej, jakkolwiek charakter tej modulacji zależy od rodzaju wirusa. W hodowlach ko-stymulowanych prątkami BCG i antygenami RSV wykazano istotny wzrost ekspresji mRNA cytokin prozapalnych, w tym IL-1β, IL-2, IL-6 i cytokiny przeciwzapalnej IL-4, a także interferonów typu I (IFN-α, IFN-β) oraz sekrecje interferonów typu II (IFN-γ), w porównaniu do hodowli prowadzonych tylko w środowisku antygenów RSV. Towarzyszył temu wzrost

ekspresji mRNA NOD2 oraz markera powierzchniowego CD14 na monocytach, co wskazuje na wzmocnienie przez prątki BCG mechanizmów wrodzonej odporności i jej potencjału przeciwwirusowego w odpowiedzi na antygeny RSV.

Natomiast w hodowlach kostymulowanych prątkami BCG i antygenami SARS-CoV-2 wykazano głównie wzrost ekspresji mRNA TNF-α, IL-1β oraz sekrecje interferonu typu II (IFN-γ), a także IL-10, ale nie IFN-α w porównaniu do hodowli stymulowanych tylko antygenami SARS-CoV-2. Ten profil odpowiedzi leukocytów pozwala sugerować, że SARS-CoV-2 może częściowo ograniczać aktywację szlaku sygnałowego prowadzącego do wytwarzania przeciwwirusowego IFN-α, wpływając na zmniejszenie potencjału synergistycznego wytrenowanej odporności indukowanej przez BCG. Jakkolwiek, biorąc pod uwagę silne działanie prozapalne SARS-CoV-2 *in vivo,* nie można wykluczyć, że aktywność leukocytów wytrenowanych przez BCG w kierunku nasilenia ekspresji IL-10, po ekspozycji na prątki BCG i antygeny SARS-CoV-2 łącznie, może wskazywać na znaczenie BCG w negatywnej modulacji odpowiedzi pro-zapalnej leukocytów w środowisku antygenów SARS-CoV-2. **Uzyskane wyniki sugerują, że BCG działa jako nieswoisty modulator odpowiedzi przeciwwirusowej, zdolny zarówno do wzmacniania mechanizmów wrodzonej odporności, jaki i do ich modulowania w zależności od wtórnego infekcyjnego bodźca.** Różnice w odpowiedzi leukocytów na antygeny RSV lub SARS-CoV-2 wskazują, że rodzaj patogenu może w znacznym stopniu determinować efekt wytrenowanej odporności, inicjowanej przez prątki szczepionkowe BCG.

3. Wyznaczenie profilu odpowiedzi cytokinowej leukocytów krwi obwodowej dzieci immunizowanych prątkami BCG, seropozytywnych pod względem przeciwciał przeciwko RSV i/lub SARS-CoV-2.

Aby ocenić wpływ wcześniejszej ekspozycji wirusowej na modulację wytrenowanej odporności indukowanej przez żywe prątki BCG prześledzono profil odpowiedzi odpornościowej leukocytów izolowanych od dzieci seropozytywnych w zakresie przeciwciał przeciwko SARS-CoV-2 i/lub RSV.

Jak wykazano w poprzednim rozdziale, w grupie dzieci seronegatywnych pod względem przeciwciał przeciwko obu wirusom SARS-CoV-2(-)/RSV(-), kostymulacja leukocytów prątkami BCG oraz antygenami RSV prowadziła do wyraźnego wzrostu ekspresji mRNA IL-1β, IL-2, IL-6, IL-4, a także IFN-α, IFN-β oraz sekrecji IFN-γ, a po kostymulacji

prątkami BCG i antygenami SARS-CoV-2, IL-1β, TNF-α i IL-10 oraz sekrecji IFN-γ, co może odzwierciedlać „uaktywnienie" ścieżki odpowiedzi prozapalnej i/lub przeciwwirusowej komórek poddanych treningowi odpornościowemu przez immunizację BCG w pierwszej dobie życia.

Leukocyty dzieci RSV(+) po kostymulacji w hodowli *in vitro* prątkami szczepionkowymi BCG łącznie z antygenami RSV wykazywały nasiloną ekspresję mRNA IL-6, ale też IL-2, w porównaniu do hodowli stymulowanych wyłącznie antygenami RSV, co może wskazywać na współdziałanie mechanizmów wytrenowanej odporności nieswoistej z pamięcią adaptacyjną limfocytów T, będących głównym źródłem IL-2.

Dla porównania, profile cytokinowe w hodowlach leukocytów pochodzących od dzieci SARS-CoV-2(+) były bardziej zróżnicowane i obejmowały m.in. wzrost ekspresji mRNA IL-10 oraz sekrecji IFN-γ, w hodowlach kostymulowanych prątkami BCG i antygenami SARS-CoV-2, w porównaniu do hodowli stymulowanych wyłącznie antygenami SARS-CoV-2. Sugeruje to, że podczas infekcji SARS-CoV-2 *in vivo* komórki immunokompetentne wytrenowane podczas immunizacji BCG mogły być kierowane w stronę równoważenia reakcji zapalnej za pośrednictwem IL-10. Ten efekt może wskazywać, że rodzaj wirusa wywołującego infekcję może mieć znaczenie dla kształtowania profilu aktywności wytrenowanych komórek odporności wrodzonej.

W hodowlach leukocytów krwi pełnej izolowanych od dzieci RSV(+) lub RSV(+)SARS-CoV-2(+) kostymulacja komórek prątkami BCG i antygenem RSV prowadziła do zwiększonego wydzielania przez nie IFN-α i IFN-γ, w porównaniu do komórek stymulowanych wyłącznie białkami wirusowymi - RSV. Analiza wyników dotyczących ekspresji mRNA interferonów po kostymulacji leukocytów BCG i RSV lub SARS-CoV-2 wykazała nasilenie ekspresji badanego mRNA w grupach dzieci seropozytywnych, chociaż nie zawsze przekładało się to na podwyższony poziom wydzielanych cytokin. To sugeruje udział mechanizmów potranskrypcyjnych i regulacyjnych w odpowiedzi cytokinowej leukocytów, umożliwiających ograniczenie nadmiernej odpowiedzi zapalnej indukowanej przez wirusy.

W celu porównania, w poszczególnych grupach badanych, aktywacji receptorów powierzchniowych monocytów, oceniono ekspresję wybranych receptorów po stymulacji leukocytów prątkami BCG i antygenami RSV/SARS-CoV-2. Wykazano, zwiększoną ekspresję receptora CD14 na leukocytach stymulowanych antygenami BCG i RSV/SARS-CoV-2,

w porównaniu do komórek stymulowanych wyłącznie antygenami wirusowymi. Ponadto, w grupach dzieci po przebytym zakażeniu RSV lub RSV i SARS-CoV-2 odnotowano podwyższoną ekspresję receptora HLA-DR na leukocytach stymulowanych BCG i RSV/SARS-CoV-2, w porównaniu do hodowli stymulowanych wyłącznie antygenami wirusowymi, co wskazuje na wyższy poziom aktywacji monocytów w kontekście wcześniejszego szczepienia i kontaktu z RSV i/lub SARS-CoV-2 *in vivo*. Nasilenie ekspresji receptora CD14 może oznaczać zwiększoną gotowość rozpoznawania wzorców PAMP, natomiast nasilenie ekspresji HLA-DR zwiększoną zdolność prezentacji antygenów. Co ciekawe, w żadnej z badanych grup nie wykazano wpływu prątków BCG na ekspresję CD11b lub CD206 w odpowiedzi na RSV i/lub SARS-CoV-2, co może wskazywać, że prątki BCG nie powodują utrwalonej polaryzacji monocytów.

Uzyskane wyniki wskazują, że ekspozycja organizmu gospodarza na RSV i/lub SARS-CoV-2 *in vivo* może mieć wpływ na aktywność komórek odporności wrodzonej, które podlegały treningowi odpornościowemu wskutek immunizacji BCG. Aktywność prozapalna i przeciwwirusowa takich komórek może ulegać nasileniu lub być nakierowana na równoważenie odpowiedzi zapalnej i przeciwwirusowej w zależności od rodzaju wirusa oraz statusu immunologicznego gospodarza.

4. Ocena korelacji pomiędzy ekspozycją mRNA cytokin i interferonów u leukocytów izolowanych od dzieci badanych grup, po ekspozycji na prątki BCG i/lub antygeny RSV/SARS-CoV-2 w hodowlach *in vitro.*

W prezentowanej pracy przeprowadzono analizę korelacji między poziomem ekspresji mRNA poszczególnych cytokin u leukocytów izolowanych od dzieci z poszczególnych grup badanych po stymulacji komórek w hodowlach *in vitro* BCG i/lub RSV/SARS-CoV-2 (wyniki nieopublikowane).

W grupie SARS-CoV-2(-)RSV(-) wykazano istotną dodatnią korelację między ekspresją mRNA IL-2 oraz mRNA IFN-β, w hodowlach leukocytów stymulowanych prątkami BCG w porównaniu z hodowlami stymulowanymi tylko antygenami SARS-CoV-2, co może wskazywać na ukierunkowaną aktywację szlaków proliferacyjnych (IL-2) oraz przeciwwirusowych (IFN-β) pod wpływem BCG, w warunkach braku wcześniejszego zakażenia RSV i/lub SARS-CoV-2. Z kolei ujemna korelacja w ekspresji mRNA IL-2 pomiędzy komórkami stymulowanymi prątkami BCG w porównaniu do komórek eksponowanych na

antygeny RSV może wskazywać, że BCG wpływa na ograniczenie proliferacyjnej odpowiedzi limfocytów indukowanej przez antygeny RSV. Może to świadczyć o selektywnej regulacji intensywności podziałów komórkowych w zależności od rodzaju bodźca. Obserwowane różnice sugerują, że w układzie odpornościowym dzieci SARS-CoV-2(-)RSV(-) prątki BCG mogą inicjować mechanizmy wytrenowanej odporności prowadzące do zwiększonej gotowości do odpowiedzi przeciwwirusowej, bez jednoczesnej nadmiernej aktywacji prozapalnej.

W grupie dzieci SARS-CoV-2(+), w hodowlach leukocytów stymulowanych prątkami BCG wykazano dodatnie korelacje w ekspresji mRNA TNF-α, IFN-α, IFN-β oraz IFN-γ, w porównaniu z hodowlami stymulowanymi antygenami SARS-CoV-2. Natomiast w porównaniu z hodowlami stymulowanymi antygenami RSV — w ekspresji mRNA IL-4, IFN-β oraz IFN-γ. Takie profile korelacji wskazują na współaktywację mechanizmów prozapalnych, przeciwwirusowych i regulatorowych, co sugeruje, że u dzieci szczepionych BCG z nabytą pamięcią immunologiczną po przebyciu zakażenia SARS-CoV-2 prątki BCG nadal mogą modyfikować odpowiedź komórek odpornościowych, wzmacniając ich reaktywność w sposób zrównoważony i zależny od cech czynnika infekcyjnego.

W grupie RSV(+) wykazano dodatnie korelacje w ekspresji mRNA IFN-α, IFN-β, IFN-γ oraz IL-10 między leukocytami stymulowanymi prątkami BCG a leukocytami eksponowanymi na antygeny SARS-CoV-2. Natomiast w hodowlach leukocytów stymulowanych tylko antygenami RSV – korelacje dodatnie wykazano dla ekspresji mRNA IFN-β i IFN-γ. Obserwacje te sugerują, że wcześniejsza ekspozycja gospodarza *in vivo* na RSV może utrwalać mechanizmy wytrenowanej odporności poprzez indukcję interferonów typu I i II oraz równoległą aktywację IL-10 o właściwościach immunoregulacyjnych. Może to odzwierciedlać zjawisko „adaptacyjnej regulacji" wytrenowanej odporności, w którym równowaga między mediatorami prozapalnymi a przeciwzapalnymi minimalizuje ryzyko nadmiernej reakcji zapalnej podczas kolejnych ekspozycji na antygeny wirusowe.

W grupie SARS-CoV-2(+)/RSV(+) wykazano dodatnie korelacje w ekspresji mRNA IFN-β i IFN-γ u leukocytów stymulowanych antygenami SARS-CoV-2 w porównaniu do leukocytów stymulowanych prątkami BCG. Natomiast, w porównaniu do leukocytów stymulowanych antygenami RSV, korelacje dodatnie wykazano w ekspresji mRNA IL-6, IL-8 oraz IFN-β, przy jednoczesnej ujemnej korelacji dla mRNA IFN-α. Uzyskane wyniki sugerują współdziałanie mechanizmów prozapalnych i przeciwwirusowych w warunkach ekspozycji

na wirusy RSV i SARS-CoV-2 *in vivo*, co może wskazywać na plastyczność odpowiedzi wytrenowanej odporności oraz jej dynamiczną regulację w zależności od historii immunologicznej gospodarza.

Zaobserwowane zależności między ekspresją mRNA cytokin po stymulacji prątkami BCG lub antygenami wirusowymi w hodowlach *in vitro* wskazują, że szczepienie BCG w okresie noworodkowym może trwale modulować funkcjonalny potencjał komórek odpornościowych. Mechanizm ten obejmuje zarówno wzmacnianie odpowiedzi przeciwwirusowej (poprzez indukcję interferonów), jak i kontrolę odpowiedzi zapalnej poprzez cytokiny regulatorowe, takie jak IL-4 i IL-10. Zależność profilu cytokinowego od statusu serologicznego gospodarza wobec RSV i SARS-CoV-2 sugeruje, że wcześniejsze infekcje mogą mieć wpływ na kierunek działania wytrenowanej odporności.

Uzyskane wyniki wskazują, że ekspozycja gospodarza *in vivo* na zakażenie RSV i/lub SARS-CoV-2 może mieć wpływ na kierunek odpowiedzi komórek immunokompetentnych dzieci immunizowanych BCG, potwierdzając plastyczność mechanizmów wytrenowanej odporności oraz ich odziaływania z mechanizmami odporności adaptacyjnej swoistej.

5. Ocena związku między ekspresją receptora NOD2 indukowaną przez prątki BCG a odpowiedzią leukocytów na antygeny SARS-CoV-2 i/lub RSV.

W celu zbadania udziału receptora NOD2 w odpowiedzi odpornościowej indukowanej przez szczepienie BCG oceniono ekspresję mRNA tego receptora w leukocytach krwi obwodowej dzieci należących do badanych grup, różniących się statusem serologicznym w zakresie przeciwciał anty-RSV i/lub anty-SARS-CoV-2. Ekspresję mRNA oceniono w komórkach po ekspozycji na prątki BCG i/lub antygeny RSV/SARS-CoV-2, a uzyskane wyniki zestawiono z profilem cytokinowym tych komórek.

Wykazano, że u leukocytów od dzieci SARS-CoV-2(-)RSV(-) ko-stymulowanych prątkami BCG i antygenami RSV, w porównaniu do leukocytów stymulowanych wyłącznie antygenami RSV lub leukocytów hodowanych w samym podłożu dochodziło do istotnego wzrostu ekspresji mRNA NOD2, co może wskazywać na aktywację ścieżek sygnałowych w tych komórkach. Pobudzenie NOD2 przez komponenty ściany komórkowej prątków BCG stwarza warunki do wzmocnienia nieswoistej odpowiedzi wobec antygenów wirusowych. W grupie dzieci RSV(+) odnotowano wyższą ekspresję mRNA NOD2 u leukocytów ko-

stymulowanych prątkami BCG i antygenami RSV w porównaniu do leukocytów w hodowli zawierającej wyłącznie antygeny RSV, co może świadczyć o zdolności prątków BCG do modulowania odpowiedzi odpornościowej również u dzieci immunizowanych BCG z pamięcią immunologiczną wobec RSV, po przebytym zakażeniu. Wysoki poziom ekspresji mRNA NOD2 wykazano w grupie dzieci SARS-CoV-2(+) po stymulacji leukocytów samymi antygenami RSV, w porównaniu do hodowli z prątkami BCG lub prątkami BCG i antygenami RSV. Wynik ten sugeruje, że wcześniejsza ekspozycja na SARS-CoV-2 może modulować odpowiedź na kolejny bodziec wirusowy, co pozostaje w związku z nasileniem ekspresji NOD2 po stymulacji RSV. Jednakże osłabienie ekspresji mRNA NOD2 w hodowlach leukocytów dawców SARS-CoV-2(+) po ekspozycji na prątki BCG lub prątki BCG i antygeny RSV może wskazywać z jednej strony na właściwości regulacyjne prątków BCG wobec leukocytów tych dawców lub przeciwnie na hamowanie reaktywności takich leukocytów wobec prątków BCG.

Analiza statystyczna wyników badań wykazała, że ekspresja mRNA NOD2 była powiązana z poziomem ekspresji mRNA wybranych cytokin w sposób zależny od statusu serologicznego dzieci. W grupie RSV(+) wykazano dodatnią korelację między ekspresją mRNA NOD2 a mRNA IL-4 w hodowlach leukocytów stymulowanych antygenami RSV i SARS-CoV-2, co może wskazywać na udział NOD2 w rozwoju odpornościowych mechanizmów zależnych od limfocytów pomocniczych typu Th2 oraz w regulacji odpowiedzi immunologicznej. W grupie SARS-CoV-2(+) wykazano dodatnią korelację między ekspresją mRNA NOD2 a mRNA IL-6 po stymulacji leukocytów antygenami RSV, co sugeruje utrzymującą się w komórkach aktywność szlaków prozapalnych zależnych od NOD2. Z kolei ujemna korelacja między ekspresją mRNA NOD2 a mRNA IL-1β po stymulacji leukocytów antygenami SARS-CoV-2 może odzwierciedlać regulacyjną funkcję NOD2 w ograniczaniu nadmiernej odpowiedzi zapalnej. W grupie SARS-CoV-2(+)RSV(+) odnotowano negatywną korelację między ekspresją mRNA NOD2 a mRNA IL-8 w hodowlach leukocytów stymulowanych antygenami SARS-CoV-2, co dodatkowo potwierdza potencjalną rolę NOD2 w tłumieniu nadmiernej reakcji zapalnej.

Uzyskane wyniki wskazują, że receptor NOD2 jest zaangażowany w kształtowanie odpowiedzi odpornościowej na prątki BCG i antygeny SARS-CoV-2 i RSV. Wykazane zależności pomiędzy ekspresją mRNA NOD2 a mRNA badanych cytokin: IL-1β, IL-2, IL-4, IL-6, IL-10 i TNF-α sugerują, że NOD2 może działać jako regulator kaskad

cytokinowych, wpływając na odpowiedź zapalną i regulacyjną. W zależności od wcześniejszej ekspozycji gospodarza na zakażenie wirusem SARS-CoV-2 lub RSV, NOD2 obecny w leukocytach może wspierać aktywację szlaków prozapalnych (np. poprzez IL-6), promować regulacyjne mechanizmy ograniczające nadmierną odpowiedź (np. poprzez IL-10), co pozostaje w związku z hamowaniem nadmiernego wydzielania cytokin prozapalnych (np. IL-1β, IL-8). Tym samym NOD2 może stanowić istotny element komórkowy modulujący wytrenowaną odporność indukowaną przez BCG, w sposób zależny od statusu immunologicznego gospodarza. Publikacja nr 4 (Jurczak i in. 2025b).

PODSUMOWANIE

Podsumowanie głównych rezultatów badań przedstawiono poniżej.
W przedstawionej pracy doktorskiej:

1. Opracowano model hodowli leukocytów pełnej krwi obwodowej dzieci immunizowanych w okresie noworodkowym szczepionką BCG umożliwiający wstępną ocenę efektów treningu odpornościowego leukocytów po ich ekspozycji *in vitro* na homologiczny (*M. bovis* BCG) lub heterologiczny bodziec infekcyjny (antygeny RSV i/lub SARS-CoV-2).
2. Wykazano, że szczepienie BCG w okresie noworodkowym może prowadzić do rozwoju wytrenowanej odporności wrodzonej kształtującej profil reakcji komórek immunokompetentnych na antygeny czynników zakaźnych takich jak: RSV i SARS-CoV-2. Charakter tej modulacji zależy od statusu serologicznego dziecka: u dawców seronegatywnych w zakresie przeciwciał przeciwko SARS-CoV-2 i RSV dominowały mechanizmy przeciwwirusowe charakteryzujące się wzrostem ekspresji mRNA IFN-β i IL-2, natomiast u dawców seropozytywnych obserwowano jednoczesną aktywację odpowiedzi prozapalnej, (wzrost ekspresji mRNA IL-1β, IL-6, TNF-α) oraz regulatorowej (wzrost ekspresji mRNA IL-4 i IL-10), co wskazuje na synergistyczne współdziałanie wytrenowanej odporności z nabytą pamięcią immunologiczną.
3. Wykazano wzrost ekspresji receptora NOD2, w leukocytach szczególnie po ich kostymulacji *in vitro* prątkami BCG i antygenami wirusów RSV/SARS-CoV-2, korelujący z poziomem mRNA cytokin zarówno pro-, jak i przeciwzapalnych. Pozwala to sugerować, że receptor NOD2 może wpływać na przebieg odpowiedzi zapalnej, modulując jej charakter w zależności od wcześniejszych ekspozycji na czynniki zakaźne takie jak: RSV/SARS-CoV-2.
4. Stwierdzono zwiększoną ekspresję receptorów monocytów (CD14, HLA-DR), po stymulacji prątkami BCG i/lub antygenami wirusowymi, co potwierdza utrzymywanie się efektów wytrenowanej odporności wrodzonej. Jednocześnie zróżnicowanie ekspresji receptorów CD11b i CD206 sugeruje, że BCG może modulować kierunek aktywacji monocytów – od prozapalnej do regulacyjnej, w zależności od wcześniejszej ekspozycji na antygeny wirusowe, co wskazuje na dynamiczny i złożony charakter odpowiedzi odpornościowej indukowanej przez szczepienie BCG.

Warto podkreślić, że uzyskane wyniki otwierają możliwości do dalszych badań porównawczych, które mogłyby obejmować populację dzieci nieszczepionych BCG. Tego typu analizy pozwoliłyby bardziej jednoznacznie określić udział szczepienia BCG w kształtowaniu obserwowanych efektów immunologicznych. Choć wszystkie dzieci uczestniczące w badaniu pochodziły z populacji objętej obowiązkowym programem szczepień BCG, co odzwierciedla realny kontekst epidemiologiczny w Polsce, to dalsze prace mogłyby koncentrować się na porównaniach międzynarodowych, uwzględniających różnice socjalno-ekonomiczne, osobnicze oraz ekspozycję na prątki środowiskowe. Tego typu podejście pozwoliłoby pogłębić zrozumienie mechanizmów wytrenowanej odporności oraz lepiej określić jej znaczenie w odporności populacyjnej.

WNIOSEK

Uzyskane wyniki sugerują, że prątki BCG nie tylko wzmacniają mechanizmy przeciwwirusowe leukocytów, ale także stymulują rozwój odpowiedzi prozapalnej i ograniczają nadmierną reakcję zapalną, co przejawia się poprzez modulację ekspresji NOD2 w porównaniu z ekspresją cytokin pro- lub przeciwzapalnych. Taki „ślad immunologiczny" pozostawiony po szczepieniu BCG podkreśla znaczenie wytrenowanej odporności jako mechanizmu wspierającego odporność populacyjną wobec różnych patogenów oraz wskazuje na potencjał tej szczepionki jako narzędzia immunomodulacyjnego w profilaktyce chorób zakaźnych.

STRESZCZENIE

Szczepionka BCG (Bacillus Calmette-Guérin) zawierająca atenuowany szczep *Mycobacterium bovis (M. bovis)*, stanowi jedyną dostępną szczepionkę przeciwgruźliczą na świecie. Pomimo ponad stuletniego stosowania, jej skuteczność w ochronie przed *Mycobacterium tuberculosis* (*M. tuberculosis*) pozostaje ograniczona, szczególnie w przypadku dorosłych. U dzieci zapewnia ona skuteczną ochronę przed ciężkimi postaciami gruźlicy, takimi jak gruźlicze zapalenie opon mózgowo-rdzeniowych czy gruźlica rozsiana. Poza ochroną swoistą przeciwko gruźlicy, szczepionka BCG wywiera istotny wpływ na układ odpornościowy, wywołując nieswoiste efekty ochronne wobec innych niż prątki patogenów. Mechanizmem leżącym u podstaw tego zjawiska jest tzw. wytrenowana odporność (ang. *trained immunity*) wynikająca z epigenetycznego przeprogramowania komórek odporności wrodzonej, która może prowadzić do ich zwiększonej gotowości do odpowiedzi na kolejne bodźce patogenne, również niespokrewnione z pierwotnym czynnikiem infekcyjnym inicjującym te zmiany.

Celem niniejszej rozprawy doktorskiej była ocena nieswoistej odpowiedzi odpornościowej u dzieci w wieku 6-12 lat, szczepionych BCG w okresie noworodkowym, z uwzględnieniem indukowanych przez BCG mechanizmów wytrenowanej odporności weryfikowanych na podstawie odpowiedzi leukocytów pełnej krwi obwodowej na bodziec homologiczny (prątki BCG) lub heterologiczny (antygeny syncytialnego wirusa oddechowego - RSV (ang. *respiratory syncytial virus*) i SARS-CoV-2 (ang. *severe acute respiratory syndrome coronavirus 2*)), w hodowlach *in vitro*. Do realizacji założonego celu zastosowano model hodowli komórkowej *in vitro* oparty na stymulacji leukocytów pełnej krwi obwodowej dzieci prątkami szczepionkowymi BCG lub antygenami wirusów oddechowych RSV lub SARS-CoV-2, niezależnie lub łącznie. W badaniu uwzględniono status serologiczny dzieci w zakresie przeciwciał przeciwko RSV i/lub SARS-CoV-2. Jako wyznaczniki odpowiedzi odpornościowej nieswoistej u badanych dzieci przyjęto: zdolność leukocytów do wytwarzania kluczowych cytokin pro- (IL-1β, IL-2, IL-6, IL-8, TNF-α) i przeciwzapalnych (IL-4, IL-10), odpowiedź interferonową w zakresie wytwarzania IFN-α, IFN-β, IFN-γ, nasilenie ekspresji receptorów powierzchniowych na monocytach, w tym CD14, CD11b, HLA-DR, CD206 oraz receptora NOD2 pełniącego m.in. rolę sensora komponentów ściany komórkowej prątków. Uwzględniono także ocenę zmienności

genetycznej receptora NOD2, która może wpływać na przebieg odpowiedzi immunologicznej badanych dzieci.

Wyniki badań wskazują, że szczepienie BCG w okresie noworodkowym może prowadzić do rozwoju wytrenowanej odporności wrodzonej kształtującej profil reakcji komórek immunokompetentnych na antygeny czynników zakaźnych, takich jak RSV i SARS-CoV-2. Charakter tej modulacji zależy od statusu serologicznego dziecka - u dawców seronegatywnych w zakresie przeciwciał przeciwko SARS-CoV-2 i RSV dominowały mechanizmy przeciwwirusowe charakteryzujące się wzrostem ekspresji mRNA IFN-β i IL-2, natomiast u dawców seropozytywnych obserwowano jednoczesną aktywację odpowiedzi prozapalnej, (wzrost ekspresji mRNA IL-1β, IL-6, TNF-α) oraz regulatorowej (wzrost ekspresji mRNA IL-4 i IL-10), co wskazuje na synergistyczne współdziałanie wytrenowanej odporności z nabytą pamięcią immunologiczną. Ponadto wykazano wzrost ekspresji receptora NOD2, w leukocytach szczególnie po ich kostymulacji *in vitro* prątkami BCG i antygenami wirusów RSV/SARS-CoV-2, korelujący z poziomem mRNA cytokin zarówno pro- jak i przeciw-zapalnych, co pozwala sugerować, że receptor NOD2 może wpływać na przebieg odpowiedzi zapalnej, modulując jej charakter w zależności od wcześniejszych ekspozycji na czynniki zakaźne, takie jak RSV/SARS-CoV-2. Dodatkowo obserwowano zwiększoną ekspresję receptorów monocytów (CD14, HLA-DR), po stymulacji prątkami BCG i/lub antygenami wirusowymi, co może potwierdzać utrzymywanie się efektów wytrenowanej odporności wrodzonej. Jednocześnie zróżnicowanie ekspresji receptorów CD11b i CD206 sugeruje, że BCG może modulować kierunek aktywacji monocytów – od prozapalnej do regulacyjnej, w zależności od wcześniejszej ekspozycji na antygeny wirusowe, co wskazuje na dynamiczny i złożony charakter odpowiedzi odpornościowej indukowanej przez szczepienie BCG.

Uzyskane wyniki sugerują, że prątki BCG nie tylko wzmacniają mechanizmy przeciwwirusowe leukocytów, ale także stymulują rozwój odpowiedzi prozapalnej i ograniczają nadmierną reakcję zapalną, co przejawia się poprzez modulację ekspresji NOD2 w porównaniu z ekspresją cytokin pro- lub przeciwzapalnych. Taki „ślad immunologiczny" pozostawiony po szczepieniu BCG podkreśla znaczenie wytrenowanej odporności jako mechanizmu wspierającego odporność populacyjną wobec różnych patogenów oraz wskazuje na potencjał tej szczepionki jako narzędzia immunomodulacyjnego w profilaktyce chorób zakaźnych.

SUMMARY

The BCG (Bacillus Calmette-Guérin) vaccine, containing an attenuated strain of Mycobacterium bovis (M. bovis), is the only tuberculosis vaccine available worldwide. Despite more than a century of use, its effectiveness in protecting against Mycobacterium tuberculosis (M. tuberculosis) remains limited, especially in adults. In children, it provides effective protection against severe forms of tuberculosis, such as tuberculous meningitis and disseminated tuberculosis. In addition to specific protection against tuberculosis, the BCG vaccine has a significant effect on the immune system, inducing non-specific protective effects against pathogens other than mycobacteria. The mechanism underlying this phenomenon is known as trained immunity, resulting from the epigenetic reprogramming of innate immune cells, which can lead to their increased readiness to respond to subsequent pathogenic stimuli, including those unrelated to the original infectious agent that initiated these changes.

The aim of this doctoral dissertation was to evaluate the non-specific immune response in children aged 6-12 years who were vaccinated with BCG in the neonatal period, taking into account BCG-induced mechanisms of trained immunity verified on the basis of the response of peripheral blood leukocytes to a homologous (BCG bacilli) or heterologous (respiratory syncytial virus (RSV) and SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antigens) in in vitro cultures. To achieve the set goal, an in vitro cell culture model was used, based on the stimulation of peripheral blood leukocytes of children with BCG vaccine bacilli or RSV or SARS-CoV-2 respiratory virus antigens, independently or in combination. The study took into account the serological status of children in terms of antibodies against RSV and/or SARS-CoV-2. The following were adopted as determinants of the non-specific immune response in the children studied: the ability of leukocytes to produce key pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, TNF-α) and anti-inflammatory cytokines (IL-4, IL-10), the interferon response in terms of the production of IFN-α, IFN-β, IFN-γ, the intensity of surface receptor expression on monocytes, including CD14, CD11b, HLA-DR, CD206, and the NOD2 receptor, which acts as a sensor for mycobacterial cell wall components, among other things. The assessment of genetic variability of the NOD2 receptor, which may influence the course of the immune response in the studied children, was also included.

Research results indicate that BCG vaccination in the neonatal period may lead to the development of trained innate immunity shaping the response profile of immunocompetent cells to infectious agents such as RSV and SARS-CoV-2. The nature of this modulation depends on the serological status of the child—in seronegative donors for SARS-CoV-2 and RSV antibodies, antiviral mechanisms characterized by increased expression of IFN-β and IL-2 mRNA predominated, while in seropositive donors, simultaneous activation of pro-inflammatory (increased expression of IL-1β, IL-6, TNF-α mRNA) and regulatory (increased expression of IL-4 and IL-10 mRNA) responses was observed, indicating synergistic interaction between trained immunity and acquired immune memory. In addition, an increase in NOD2 receptor expression was demonstrated in leukocytes, particularly after their in vitro costimulation with BCG bacilli and RSV/SARS-CoV-2 virus antigens, correlating with the level of both pro- and anti-inflammatory cytokine mRNA, suggesting that the NOD2 receptor may influence the course of the inflammatory response by modulating its nature depending on previous exposure to infectious agents such as RSV/SARS-CoV-2. In addition, increased expression of monocyte receptors (CD14, HLA-DR) was observed after stimulation with BCG bacilli and/or viral antigens, which may confirm the persistence of trained innate immunity effects. At the same time, the differentiation in the expression of CD11b and CD206 receptors suggests that BCG may modulate the direction of monocyte activation – from pro-inflammatory to regulatory, depending on previous exposure to viral antigens, which indicates the dynamic and complex nature of the immune response induced by BCG vaccination.

The results suggest that BCG bacilli not only strengthen the antiviral mechanisms of leukocytes, but also stimulate the development of a pro-inflammatory response and limit excessive inflammatory reactions, which manifests itself through the modulation of NOD2 expression compared to the expression of pro- or anti-inflammatory cytokines. Such an “immunological footprint” left after BCG vaccination emphasizes the importance of trained immunity as a mechanism supporting population immunity against various pathogens and indicates the potential of this vaccine as an immunomodulatory tool in the prevention of infectious diseases.

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PUBLIKACJE STANOWIĄCE PODSTAWĘ ROZPRAWY DOKTORSKIEJ

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Review

Beyond Tuberculosis: The Surprising Immunological Benefits of the Bacillus Calmette–Guérin (BCG) Vaccine in Infectious, Auto-Immune, and Inflammatory Diseases

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Abstract: The Bacillus Calmette–Guérin (BCG) vaccine, best known for its role in preventing tuberculosis, has recently garnered attention for its broader immunomodulatory effects. By inducing trained immunity, BCG reprograms innate immune cells, enhancing their responses to various pathogens. This process, driven by epigenetic and metabolic reprogramming, suggests that BCG may have therapeutic potential far beyond tuberculosis. Emerging evidence points to its potential benefits in conditions such as autoimmune diseases, cancer, and viral infections. Furthermore, by modulating immune activity, BCG has been proposed to reduce chronic inflammation and promote immune tolerance. This review delves into the multifaceted role of BCG, highlighting its potential as a versatile therapeutic tool for managing a wide range of diseases.

Keywords: Bacillus Calmette–Guérin; non-specific BCG effect; trained immunity

Academic Editors: Hinh Ly and Hwa-Jung Kim

Received: 27 November 2024
Revised: 8 January 2025
Accepted: 14 February 2025
Published: 15 February 2025

Citation: Jurczak, M.; Druszczynska, M. Beyond Tuberculosis: The Surprising Immunological Benefits of the Bacillus Calmette–Guérin (BCG) Vaccine in Infectious, Auto-Immune, and Inflammatory Diseases. *Pathogens* **2025**, *14*, 196. https://doi.org/10.3390/pathogens14020196

1. Introduction

Recently, the protective, non-specific effects of the Bacillus Calmette–Guérin (BCG) vaccine have been recognised in the treatment of viral, autoimmune, and inflammatory diseases. The broad non-specific effects of BCG have attracted the attention of many scientists. As a result, research has begun to focus on developing new therapeutic approaches using the BCG vaccine. The mechanism responsible for the non-specific effects of the BCG vaccine is trained immunity, during which innate immune cells exhibit increased activity against pathogens other than *Mycobacterium* [1–3].

To explore the implications of this connection, we conducted a systematic review of studies published predominantly between 2020 and 2024, using keywords such as "trained immunity", "BCG vaccine", "non-specific BCG effect", and "immunotherapy". The studies were selected based on their critical importance in understanding the mechanisms of trained immunity, which play a significant role in the development of new therapeutic approaches.

The purpose of this review is to present current information on the BCG vaccine, with a focus on its underlying mechanisms, epidemiological evidence, and potential clinical applications. This review highlights recent reports on the potential use of the BCG vaccine in the treatment of viral infections, cancers, and autoimmune diseases. The goal of this

study is to elucidate the broader health impacts of BCG vaccination, particularly in the context of emerging infectious diseases and evolving public health challenges.

2. The BCG Vaccine: Historical Context

The BCG vaccine was developed over a century ago by Dr. Albert Calmette and veterinarian Georges Guérin at the Pasteur Institute in Lille, France. After 13 years of cultivating *Mycobacterium bovis* (*M. bovis*) through 230 passages on a specialised potato–glycerol medium with 5% bile, the scientists obtained a strain that retained its antigenic properties while losing its original virulence [4]. In 1921, the BCG vaccine was administered for the first time to a human subject, a newborn whose mother had succumbed to tuberculosis. This pioneering procedure was soon followed by the widespread introduction of BCG vaccinations across Europe. After the Second World War, the World Health Organisation (WHO) expanded its vaccination recommendations globally [5]. Despite the long history of the tuberculosis vaccine, it remains the most widely used vaccine worldwide, with approximately 120 million newborns vaccinated annually. The cultivation of the original BCG strain on various microbiological media over time has led to numerous genetic alterations, resulting in the emergence of multiple genetically distinct sub-strains [6]. Genetic analyses, which have identified differences in deletions and insertions among the BCG strains, have classified these strains into four groups. Group 1, which shows the greatest similarity to the original BCG strain, includes BCG Russia, BCG Moreau, and BCG Japan. Group 2, which lacks the IS6110 gene located upstream of the phoP gene, includes BCG Sweden and BCG Birkhaug. Group 3 (BCG Glaxo, BCG Prague, and BCG Danish) emerged after 1931. The most recent group, designated as Group 4, includes the BCG TICE, BCG Frappier, BCG Phipps, BCG Connaught, and BCG Pasteur 1173 passaged strains [7].

The global incidence of tuberculosis has been significantly reduced due to the introduction of the BCG vaccination program. Moreover, scientific studies have begun to establish links between BCG vaccination and protection not only against tuberculosis but also against other pathogens. In 1927, Carl Näslund was the first to demonstrate that infants vaccinated with BCG had lower mortality rates compared to those who were not vaccinated [4]. A year later, Pearl (1928) discovered that tuberculosis patients had a lower incidence of cancer. Subsequent studies have shown that the BCG vaccine can also be effective in reducing child mortality [8–10].

In the following years, new findings emerged regarding the non-specific protective effects of BCG, which appeared to enhance host resistance to other infections. Following the implementation of the BCG vaccination program, a 40% reduction in mortality was observed in African countries, alongside a decline in the prevalence of respiratory diseases, malaria, sepsis, and leprosy [11–14]. The discovery of BCG's broader protective effects prompted scientists to investigate the mechanisms underlying these non-specific reactions. In 2011, Netea et al. proposed the term "trained immunity" to describe this phenomenon [15]. Since then, research into the non-specific effects of BCG has gained renewed momentum.

3. Trained Immunity

The phenomenon in which the innate immune system exhibits immunological memory following repeated exposure to a pathogen is known as "immune training". This concept is particularly intriguing, as it challenges the long-standing assumption that only the adaptive immune system could generate immunological memory, while innate immunity was traditionally considered incapable of forming a lasting imprint.

It is important to note, however, that the immune memories formed by these two systems are fundamentally different. Adaptive memory, mediated by long-lived T and B

lymphocytes, arises from interactions between peptide-major histocompatibility complex (MHC) molecules and T and B cell receptors. This results in a highly specific and long-lasting response tailored to the encountered pathogen. In contrast, innate immune memory, or "trained immunity", is rapidly induced following exposure to certain vaccines and antigens. These antigens are recognised by pattern recognition receptors (PRRs) on innate immune cells, triggering the reprogramming of myeloid cells, including macrophages, monocytes, natural killer (NK) cells, and dendritic cells (DCs) [16]. This reprogramming enables a heightened state of readiness upon subsequent encounters with pathogens, albeit in a less specific manner compared to adaptive memory.

The discovery of trained immunity not only broadens our understanding of immune system plasticity but also opens up potential therapeutic avenues. Harnessing this phenomenon could improve vaccine efficacy and offer novel strategies for combatting infections and even certain non-infectious diseases, such as cancer and autoimmune disorders.

3.1. Metabolic and Epigenetic Changes

One of the most effective inducers of trained immunity is the BCG vaccine, which has the capacity to reprogram innate immune cells. This reprogramming is achieved through metabolic and epigenetic modifications that enhance the functionality of these cells during non-specific infections (Figure 1). The BCG vaccine induces trained immunity by binding to PRRs. In both in vivo and in vitro studies, Kleinnijenhuis et al. demonstrated that the BCG vaccine interacts with Nucleotide-binding Oligomerisation Domain 2 (NOD2), leading to an enhanced innate immune response. This interaction results in NOD2-dependent trimethylation of histone 3 at lysine 4 (H3K4me3) in the promoters of inflammatory cytokine genes [17]. The induction of trained immunity also involves the activation of the mevalonate pathway, which is further potentiated by the insulin-like growth factor I (IGF-I) pathway. Notably, the synthesis of both cholesterol and the metabolite mevalonate is essential for the induction of trained immunity [18]. These alterations are linked to post-translational modifications of histones within promoter regions and enhancer sequences. In response to training signals, histones undergo acetylation and methylation, leading to chromatin remodelling and gene expression changes that produce a stronger immune response. Furthermore, the stimulation of immune cells with BCG results in the induction of glycolysis and an increase in oxygen consumption, which are crucial for the full training of the immune system. When glucose metabolism is shifted from lactate production to the tricarboxylic acid (TCA) cycle, cytokine secretion is inhibited. The inhibition of glycolytic enzymes has been shown to prevent BCG-induced epigenetic alterations, such as H3K4me3 and histone 3 lysine 9 trimethylation (H3K9me3), indicating that enhanced glycolysis is a crucial component of trained immunity [19]. These metabolic and epigenetic modifications are regulated by the Akt/mTOR pathway [20]. Recent studies have further elucidated the role of metabolic pathways in trained immunity. For instance, research has shown that activating transcription factor 4 (ATF4) plays a significant role in regulating glucose metabolism in macrophages during sepsis. Knockdown of ATF4 impairs glycolysis and mediates immune tolerance by targeting hexokinase II (HK2) and hypoxia-inducible factor 1-alpha (HIF-1α) ubiquitination. This finding underscores the importance of glycolytic pathways in modulating immune responses [21].

Additionally, the concept of trained immunity has been explored in the context of other vaccines. For example, the diphtheria–tetanus–pertussis whole-cell (DTPw) vaccine has been shown to induce different programs of trained immunity in mice, suggesting that various vaccines may have distinct effects on the innate immune system [22].

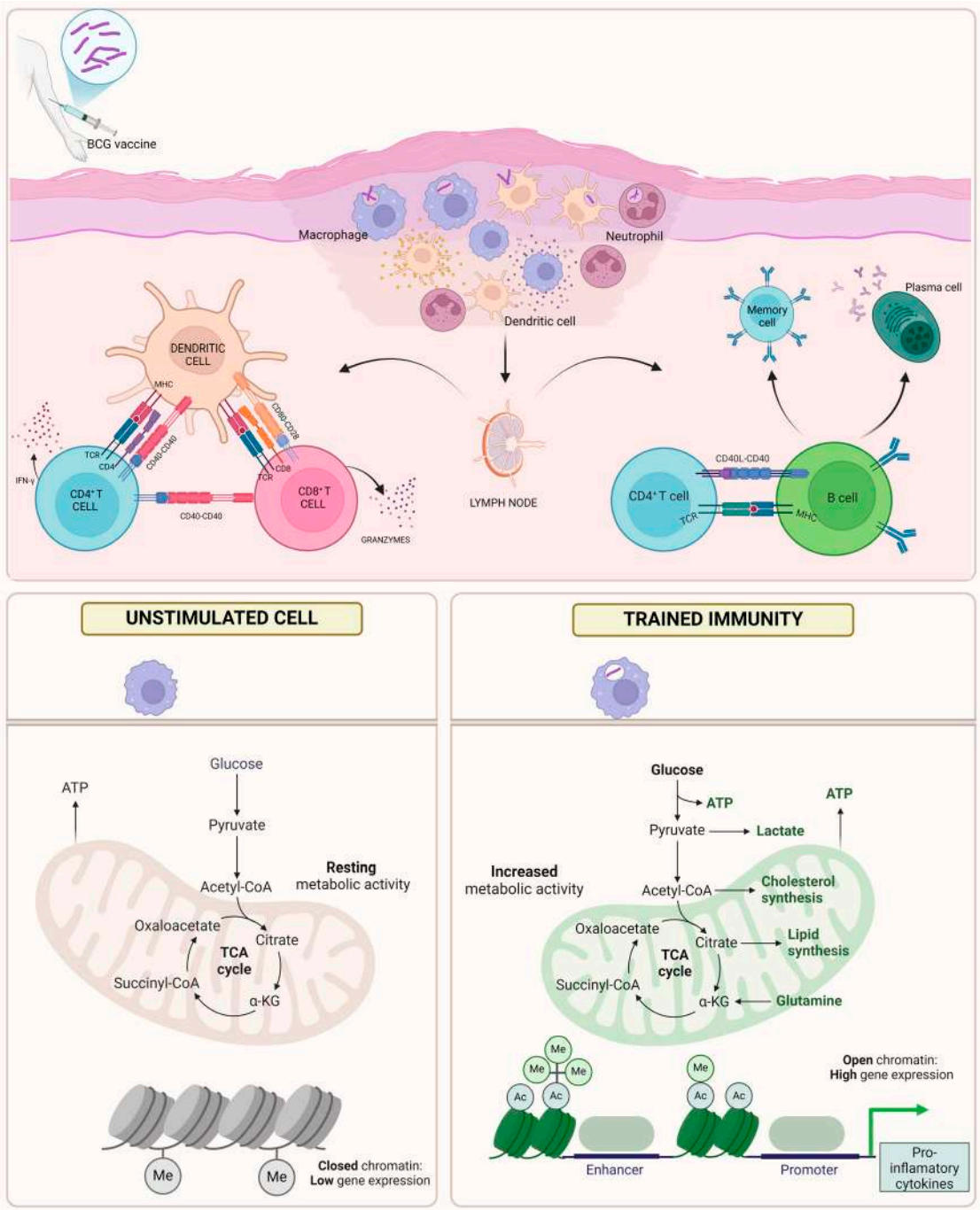

Figure 1. BCG-induced immunity training. The administration of the BCG vaccine causes the activation of macrophages, neutrophils, and dendritic cells. DCs then present antigens to $CD4^+$ and $CD8^+$ T lymphocytes, initiating an adaptive response, and $CD4^+$ T lymphocytes stimulate B lymphocytes to produce memory cells and plasma cells. BCG vaccination induces epigenetic reprogramming in innate immune cells, such as monocytes and macrophages. Chromatin remodelling opens promoter regions, leading to high expression of pro-inflammatory cytokines. This response includes an increased production of cytokines like IL-1, TNF, and IL-6, which significantly strengthen the immune defence. Additionally, BCG induces metabolic reprogramming that elevates glycolysis, supplying the energy required for sustained cellular activation. Unlike adaptive immunity, BCG imparts a form of non-specific memory to the innate immune system, enabling it to mount faster and stronger responses to a variety of pathogens. Abbreviations: (Ac) acetylation, (Me) methylation, (ATP) adenosine triphosphate, (BCG) Bacille Calmette–Guérin, (TCA) tricarboxylic acid cycle, Acetyl-CoA (acetyl coenzyme A), (Succinyl-CoA) Succinyl-coenzyme A, interleukin (IL).

These insights into the metabolic and epigenetic mechanisms underlying trained immunity highlight the complex interplay between cellular metabolism and immune function, offering potential avenues for therapeutic interventions in infectious and inflammatory diseases.

3.2. Cell Populations That Mediate Trained Immunity

The characteristics of trained immunity have been observed in numerous cells of innate immunity, including monocytes/macrophages, neutrophils, DCs, NK cells, innate lymphoid cells, haematopoietic stem cells (HSCs), microglia, and gamma delta (γδ) T cells [23–28]. Researchers have demonstrated that the immune-trained phenotype of monocyte-derived macrophages results in an elevated production of pro-inflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumour necrosis factor (TNF) [29]. This regulation is associated with increased levels of H3K27 acetylation (H3K27ac) following BCG vaccination, specifically on the promoters of genes encoding cytokines involved in the inflammatory response [30]. Additionally, polymorphisms in the IL-1β gene have been observed to correlate with the intensity of the immune training response. Consequently, IL-1β has been identified as a key marker of BCG-induced innate memory in macrophages [30,31].

NK cells represent a vital element of the innate immune system, serving as a primary line of defence against pathogens. NK cells also possess the capacity to generate immune memory in response to a range of stimuli, including specific and non-specific inducers such as cytomegalovirus (CMV) infection, malaria, influenza vaccination, and BCG [26,32–35]. Studies have demonstrated that individuals who received the BCG vaccine and were subsequently stimulated with Mycobacterium tuberculosis, Staphylococcus aureus, or Candida albicans exhibited an elevated secretion of the pro-inflammatory cytokines TNF, IL-6, and IL-1β by NK cells. This phenomenon persisted for up to three months [17]. Furthermore, Suliman et al. observed that the re-administration of the BCG vaccine induced a prolonged NK cell response [36].

The induction of long-term innate immune memory is attributed HSCs, which exhibit greater longevity and intensified proliferation in the bone marrow compared to other innate immune cells. BCG vaccination has been shown to alter the transcriptional profile of HSCs, favouring myelopoiesis over lymphopoiesis. This modification results in the epigenetic reprogramming of macrophages [37]. Additionally, Cirovic et al. demonstrated that BCG vaccination in healthy individuals induces long-lasting transcriptomic alterations in hematopoietic stem and progenitor cells (HSPCs), further promoting myelopoiesis [38]. Recent studies have expanded on these findings. For instance, it has been observed that the modulation of HSC transcriptional profiles by BCG vaccination not only enhances myelopoiesis but also improves the resilience of macrophages against inflammatory and infectious challenges. Furthermore, the interplay between HSC reprogramming and metabolic pathways, such as glycolysis and oxidative phosphorylation, has been identified as a critical determinant of the trained immunity phenotype [39]. Novel insights into the role of chromatin remodelling complexes, such as the SWItch/sucrose non-fermentable (SWI/SNF) family, have elucidated their contribution to the stable epigenetic changes observed in innate immune cells post-BCG vaccination [40].

The capacity of the BCG vaccine to reprogram HSCs and subsequently enhance innate immune responses provides a promising foundation for the advancement of clinical vaccines and the therapeutic targeting of trained immunity. These findings open new avenues for the development of interventions aimed at improving immune responses in vulnerable populations and combatting a variety of infectious and non-infectious diseases. Further research is required to optimise vaccine strategies and fully elucidate the mech-

anisms underlying trained immunity, particularly in the context of varying genetic and environmental influences.

4. Non-Specific Effect

The BCG vaccine, originally developed for tuberculosis prevention, has demonstrated significant non-specific effects that extend beyond its primary purpose. Unlike many other vaccines, the BCG vaccine primarily activates cellular immunity, a mechanism that can also induce a phenomenon known as "trained immunity". The components of the BCG vaccine, particularly its cell wall components, are recognised by PRRs, including Toll-like receptors (TLRs), NOD2, and C-type lectin receptors (CLRs). These interactions initiate a cascade of immune signalling pathways, leading to the production of pro-inflammatory cytokines, chemokines, and enhanced phagocytosis. This broad activation of the immune system is believed to contribute to the vaccine's non-specific protective effects against various infections and even certain malignancies, including potential roles in modulating the immune environment in cancers such as lung cancer. Emerging research also highlights the potential of the BCG vaccine in immunotherapy, where its ability to stimulate innate and adaptive immune responses could complement checkpoint inhibitors or other targeted therapies. These findings underscore the versatility of the BCG vaccine and its broader implications for immune modulation and disease prevention [1].

4.1. Antiviral Effects

In recent years, an increasing number of novel respiratory pathogens have emerged, driving the occurrence of new pandemics and epidemics. As a result, the potential therapeutic application of immune training induced by the BCG vaccine is gaining attention as a strategy to combat emerging infections. It is hypothesised that the trained immunity elicited by the BCG vaccine could serve as an effective immunoprophylactic tool, enhancing the innate immune system's response to viral infections. Current research is actively exploring the prophylactic effects of the BCG vaccine against various viral pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), respiratory syncytial virus (RSV), and influenza [41–43].

This line of investigation not only offers hope for mitigating the impact of respiratory viruses but also underscores the broader potential of trained immunity in public health. By harnessing the non-specific immune-boosting properties of vaccines like BCG, researchers aim to develop adjunct strategies that complement existing antiviral therapies and vaccination programs. However, while promising, the efficacy and safety of this approach require rigorous validation in diverse populations and across different viral contexts.

4.1.1. SARS-CoV-2

The SARS-CoV-2 virus infects the respiratory tract, leading to acute disease. As of January 2025, it has caused approximately 704,753,890 cases worldwide, resulting in 7,010,681 deaths [44,45]. At the onset of the SARS-CoV-2 pandemic, scientists considered the BCG vaccine, known for inducing non-specific host responses to certain viral infections, as a potential prophylactic against SARS-CoV-2. This hypothesis was initially supported by observations of lower infection rates in countries with mandatory tuberculosis vaccination policies. Several epidemiological studies provided evidence supporting this idea. However, subsequent experimental studies questioned the protective effect of the BCG vaccine against SARS-CoV-2 infection [46].

In animal models, Zhang et al. demonstrated that intravenous BCG vaccination did not prevent infection but reduced SARS-CoV-2 replication and the pro-inflammatory response by activating the glycolysis pathway and promoting myeloid cell polarisation [47].

Similarly, Singh et al. found that BCG vaccination had a beneficial impact on SARS-CoV-2 infection in animals, inducing reprogramming of myeloid cells and lymphocytes, thereby preventing virus-induced lymphopenia [41]. Hilligan et al. reported that intravenous BCG vaccination reduced pulmonary pathology, including granuloma formation in alveolar septa [48]. In contrast, Kaufmann et al., 2022, observed no significant protective effect of BCG vaccination against SARS-CoV-2, suggesting that differences in BCG strains and administration routes might explain these discrepancies [43]. The route of BCG vaccine administration appears to influence its efficacy. In mice, subcutaneous administration did not affect the course of SARS-CoV-2 infection, whereas intravenous administration increased the likelihood of a protective effect by activating $CD4^+$, $CD8^+$, and NKT cells [48]. Retrospective and ecological studies on the association between BCG vaccination and SARS-CoV-2 incidence have yielded inconclusive results. For instance, a study in the United Arab Emirates found that healthcare workers who received a second BCG dose did not contract SARS-CoV-2, while non-vaccinated individuals did [49]. Conversely, the BCG-CORONA study in South Africa indicated that BCG revaccination was associated with an elevated risk of severe SARS-CoV-2 infections compared to a placebo [50]. A Brazilian study found that the revaccination of healthcare workers with the BCG-Moscow vaccine did not provide protection against SARS-CoV-2 infection [51].

Some researchers have explored the potential of BCG vaccination as an adjuvant to SARS-CoV-2 vaccines. It has been observed that administering the BCG vaccine prior to anti-SARS-CoV-2 vaccination results in a superior immune response, characterised by elevated antibody titres and increased levels of pro-inflammatory cytokines, including TNF and IL-1β [52,53]. The inconsistency in study results regarding the protective effect of BCG vaccination can be attributed to variables such as gender distribution, age, participant numbers, BCG strains, administration methods, dosage, and timing relative to pathogen exposure. Additionally, the protective effect of BCG may vary depending on the specific pathogen. Kaufmann et al., 2022, noted that while BCG vaccination did not protect against SARS-CoV-2, it reduced morbidity and mortality associated with influenza A virus [43]. Notably, aerosol administration of the BCG-Danish vaccine to rhesus macaques resulted in an increased production of IL-6, IL-1β, and TNF as well as elevated numbers of circulating monocytes and activated γδ T cell populations, suggesting that BCG aerosol vaccination may induce innate immune mechanisms and prepare unconventional T-cell populations [54].

Recent investigations have highlighted the potential role of the BCG vaccine in mitigating the effects of COVID-19. A study by Jalalizadeh et al., 2025, explored the impact of BCG vaccination administered during the active phase of COVID-19. The findings suggest that the vaccine may help prevent the development of long COVID by enhancing trained immunity mechanisms that reduce systemic inflammation and promote faster immune recovery. This study emphasises the potential of BCG vaccination as a therapeutic intervention during COVID-19 infection to address lingering symptoms and complications associated with long COVID [55]. In addition, the preliminary findings of the randomised controlled trial Bacillus Calmette–Guérin Vaccination as Defense Against SARS-CoV-2 (BADAS), which aimed to assess the efficacy of BCG vaccination in preventing SARS-CoV-2 infection and reducing disease severity among high-risk individuals, highlighted the importance of BCG-induced immune training as a cost-effective and broadly applicable strategy for pandemic preparedness [56]. Both studies contribute valuable insights into the broader applications of the BCG vaccine in managing not only SARS-CoV-2 infection but also its long-term effects, emphasising the need for further research to confirm these findings and optimise intervention strategies.

4.1.2. RSV

RSV is a leading causative agent of lower respiratory tract infections and is responsible for a substantial burden of hospitalisations among newborns and young children worldwide. The clinical spectrum of RSV infection ranges from mild to severe, with manifestations including alveolitis, bronchiolitis, and pneumonia [57]. Globally, RSV is estimated to cause approximately 33.1 million cases of acute respiratory infections in children under five years of age annually, with more than 3.6 million cases requiring hospitalisation and over 100,000 fatalities [58].

Epidemiological studies have revealed that BCG vaccination of newborns provides protection not only against tuberculosis but also against other respiratory infections, including RSV [42]. Research has demonstrated that BCG vaccines, particularly when engineered to express RSV antigens, show promising outcomes in conferring immunity against RSV. Recombinant BCG strains expressing RSV antigens (rBCG-N-hRSV) have been shown to promote protective Th1-type immunity in mice, characterised by a robust activation of RSV-specific T cells that produce IFN-γ and IL-2 [59]. Studies by Cautivo et al. and Céspedes et al. further highlighted that rBCG immunisation enhances the early recruitment of $CD4^+$ and $CD8^+$ T cells to the lungs alongside the secretion of IFN-γ and IL-17, which are critical in controlling RSV infection [57,60]. Espinoza et al. observed that immunisation with rBCG-N-hRSV also confers neuroprotective effects by preventing RSV-related central nervous system complications in murine models [61]. Additionally, Wang et al. demonstrated that administering the BCG vaccine at birth, followed by a formalin-inactivated RSV vaccine, reduces the severity of RSV-related respiratory disease and enhances long-term protection through trained immunity and balanced Th immune memory [62]. Importantly, rBCG-N-hRSV has been found to induce not only cellular but also humoral immunity, with the production of antibodies targeting RSV proteins [63]. This dual immune response is particularly significant, as rBCG-N-hRSV is currently the only RSV vaccine designed specifically for newborns—a demographic that represents the majority of RSV-related hospitalisations, especially infants under six months of age [63]. These findings highlight the importance of integrating novel recombinant vaccines into immunisation programs to mitigate the global burden of RSV, especially in vulnerable populations.

4.1.3. Influenza Virus

Influenza is a major global health concern, with approximately one billion cases reported worldwide each year [64]. Of these cases, an estimated 3 to 5 million people develop severe influenza, resulting in a mortality rate of 290,000 to 650,000 deaths annually. Influenza A virus, the causative agent of acute respiratory infections, is highly contagious, spreading through direct or indirect contact. Clinical manifestations of infection typically include cough, fever, sore throat, body aches, and fatigue [64].

Recent research has explored the protective effects of the BCG vaccine in response to viral infections, including the influenza A virus. Kaufmann et al., 2022, demonstrated that the BCG vaccine provides significant protection against influenza A virus infection in animal models. Specifically, Syrian hamsters vaccinated with BCG showed a marked reduction in viral titres in the lungs following infection with the influenza A virus [43]. Further advancements have focused on developing vaccines utilising the BCG cell wall skeleton (BCG-CWS) as an adjuvant. Studies have revealed that BCG-CWS induces robust humoral and cellular immune responses, characterised by elevated levels of immunoglobulin G (IgG)1 and IgG2a antibodies. Additionally, BCG-CWS has been shown to regulate pathological inflammation and suppress excessive Th1 cell proliferation during influenza virus exposure [65].

Building upon these findings, recent studies corroborate the protective effects of the BCG vaccine. Tran et al. highlighted that intravenous administration of the BCG vaccine conferred substantial protection against subsequent influenza A virus infection in mice [66]. A critical mechanism underlying this protection involves the enrichment of conventional αβ $CD4^+$ effector memory T cells in circulation and lung parenchyma. These cells express high levels of the CX3C chemokine receptor 1 ($CX3CR1^{hi}$), playing a pivotal role in mitigating the early stages of viral infection independently of an antigen. The enhanced efficacy of these $CX3CR1^{hi}$ T cells is attributed to their intensive production of interferon-γ, which activates alveolar macrophages and promotes sustained antimicrobial activity. This interplay between innate and adaptive immune responses underscores the broad-spectrum protection afforded by the BCG vaccine [67].

These findings collectively highlight the potential of the BCG vaccine and its derivatives as effective tools not only for preventing tuberculosis but also for combatting viral infections such as influenza A. Future research should prioritise clinical trials to validate these effects in humans and explore the utility of BCG-based strategies in pandemic preparedness and respiratory virus management.

4.2. Immunotherapy for Cancer

Cancer remains the leading cause of mortality in humans, with an estimated 20 million new cases and 9.7 million deaths reported in 2022 [68]. The anti-cancer effects of BCG were first investigated as early as 1929, when researchers observed a reduced incidence of cancer in autopsy cases of individuals with tuberculosis [69]. Initially, it was hypothesised that the BCG vaccine induced an intracellular inflammatory response, resulting in tumour shrinkage. The utilisation of immunotherapy in cancer treatment has emerged as a promising strategy for inhibiting tumour growth and metastasis by harnessing the immune system's response [70]. However, traditional cancer therapies often rely heavily on T-cell activation, which may prove insufficient in certain cases due to the suppressive nature of the tumour microenvironment, which inhibits robust immune responses [71]. This limitation highlights the potential of "trained immunity", a phenomenon where the innate immune system develops a form of immunological memory [72]. BCG vaccination, whether used as monotherapy or in combination with other treatments, can reprogram the tumour microenvironment, transforming it into a setting more conducive to effective anti-tumour activity. This approach fosters pro-inflammatory conditions, which support the mobilisation of immune responses and sustain the activity of tumour-targeting T cells [73].

By leveraging both the direct and indirect effects of BCG vaccination, cancer immunotherapy may overcome the barriers posed by the tumour microenvironment, offering a novel pathway for improved clinical outcomes in cancer management.

4.2.1. Trained Immunity in Cancer Immunotherapy

The training of the immune system is influenced by various factors, notably the BCG vaccine, which has been shown to enhance the innate immune response by strengthening NK cells and activating T lymphocytes. Trained innate immune cells, including macrophages, monocytes, and NK cells, exhibit heightened reactivity and an increased capacity to respond swiftly to stimuli, such as signals from cancer cells. This enhanced responsiveness may improve the efficacy of therapies like chimeric antigen receptor (CAR)-T cell therapy, which aims to activate T lymphocytes or NK cells against tumour cells [74]. Epigenetic modifications, particularly histone alterations, are closely associated with immune training. Specific pharmacological agents can influence histone methylation, thereby augmenting anti-tumour responses. For instance, lysine demethylase 6B (KDM6B), an enzyme responsible for histone demethylation, can support a pro-inflammatory response,

potentially enhancing the effectiveness of anti-cancer therapies [75]. Moreover, the phenomenon of trained immunity depends on a fully functional autophagy mechanism. The inhibition of autophagy has been observed to impede epigenetic modifications, thereby limiting the development of trained immunity. This is exemplified by the reduced responsiveness of patients with mutations in autophagy-related genes, such as autophagy-related 2B (ATG2B), to BCG immunotherapy for bladder cancer [76]. Understanding these intricate mechanisms offers promising avenues for enhancing cancer immunotherapy by modulating trained immunity, epigenetic landscapes, and autophagic pathways.

4.2.2. Bladder Cancer

Bladder cancer represents the 9th most common cancer worldwide, with a higher prevalence in males than females. The majority of patients are diagnosed with non-muscle invasive bladder cancer (NMIBC); however, approximately 20% present with muscle-invasive bladder cancer (MIBC). Treatment typically includes endoscopic tumour removal, intravesical adjuvant therapy, radiotherapy, and chemotherapy [77].

In 1929, Pearl's observations led to the development of the BCG vaccine, which has demonstrated efficacy against bladder cancer [78]. BCG immunotherapy is now considered the gold standard for the adjunctive treatment of NMIBC. This therapy involves the intravesical administration of a suspension of live attenuated BCG bacteria at a specific concentration, with dosage and duration tailored to the patient's condition [79]. Research indicates that BCG immunotherapy not only stimulates the immune system to suppress neoplastic cells but also offers superior protection against disease recurrence [80]. Despite extensive studies over several decades, the precise mechanisms remain unclear. It is hypothesised that intravesical BCG administration triggers both local and systemic immune responses, activating urothelial cells, macrophages, dendritic cells, lymphocytes, and neutrophils [70]. Furthermore, it was noted that combination treatment against PD-1 in patients with NMIBC bladder cancer could potentially increase the number of patients responding to treatment with the BCG vaccine [81]. In contrast, Patwardhan et al., 2024, showed that glutathione-S-transferase Theta 2 (Gstt2) expression was associated with a better response to BCG intravesical immunotherapy in patients with NMIBC [82]. In vitro studies have shown that BCG can increase the production of cytokines such as IL-6, IL-8, and granulocyte–macrophage colony-stimulating factor (GM-CSF) o [83–85]. In vivo studies in humans have observed that BCG administration induces the production of neutrophils, macrophages, monocytes, NK cells, and T and B lymphocytes, accompanied by elevated levels of IL-1β, IL-8, IL-15, IL-18, and CXC chemokine ligand (CXCL)10, CCL2, and CCL3. The rationale for using cytokines in the treatment of bladder cancer with BCG is known to be based on their ability to induce a Th1-type immune response, crucial for the elimination of cancer cells. Cytokines such as IL-2, IL-12, IL-18, TNF, GMCSF, and IFN-α work together to stimulate the production of IFN-γ to enhance the immune response. The synergistic effect of cytokines supports the development of an immune response and improves the efficacy of treatment [86–88].

Despite its effectiveness, BCG therapy is associated with certain limitations, including significant variability in patient response and the risk of recurrence. Addressing these challenges requires further research to unravel the complex interplay between host immunity, tumour biology, and the mechanisms of BCG action. Such insights could pave the way for more personalised and effective therapeutic strategies in bladder cancer management.

4.2.3. Melanoma

Malignant melanoma, the most aggressive form of skin cancer, is being diagnosed in an increasing number of individuals annually. In 2024, there were 100,640 cases reported

globally, including 8290 deaths [89]. An early diagnosis of melanoma significantly improves a patient's prognosis. However, approximately 10% of cases are diagnosed at an advanced stage, characterised by metastases and inoperability [90].

The first attempts to use the BCG vaccine in the treatment of melanoma date back to the 20th century, when Morton et al. observed tumour regressions in cancer patients treated with BCG [91]. Several therapeutic approaches utilising the BCG strain have since been explored for melanoma treatment. These include the administration of the BCG vaccine alone, in combination with autologous tumour cells, or via direct injection into tumour lesions [92]. However, combining the BCG vaccine with cytotoxic drugs, cancer vaccines, or cytokine therapies has not demonstrated significant benefits for melanoma patients [93,94]. Conversely, Faries et al. reported that co-administration of BCG with the anti-tumour vaccine Canvaxin elicited a robust anti-tumour response and prolonged patient survival [95]. Furthermore, promising results have been observed in melanoma patients treated with BCG combined with TLR agonists [96]. Nishida et al. focused on the BCG-CWS, which acts as a potent immune adjuvant due to its abundance of TLR2 ligands. Their study demonstrated an increased production of monocytes and neutrophils in response to BCG-CWS. Notably, some patients exhibited $CD4^+$ T cell activation and differentiation into memory phenotypes, enhancing their anti-tumour response [97].

The use of BCG as an immunomodulator in melanoma therapy leads to the production of inflammatory cytokines (IFN-γ, TNF, TNF-β, IL-15, IL-1β, IL-6, and IL-32) and chemokines (CCL2, CCL18, CXCL9, CXCL10, and CXCL11). Additionally, stress-induced molecules such as Butyrophilin Subfamily 3 Member A1 (BTN3A1) and MHC class I-related chain B (MICB) are induced, resulting in melanoma metastasis regression [98]. BCG-induced anti-tumour responses, while effective, remain poorly understood at the immune mechanism level. Borges et al. investigated the immune profile of B16-F10 mouse melanoma cells and highlighted the pivotal role of MyD88 signalling in cytokine production during BCG immunotherapy. Their findings revealed that MyD88 promotes the recruitment and activation of various immune cells, including M1-like tumour-associated macrophages, neutrophils, dendritic cells, $CD4^+$ and $CD8^+$ T cells, NK cells, and NKT cells, within the tumour microenvironment [99].

Recent studies have explored the potential of combining BCG with immune checkpoint inhibitors, such as anti-PD-1/PD-L1 antibodies, to enhance the therapeutic efficacy in melanoma patients. This combinatorial strategy aims to overcome resistance mechanisms associated with advanced melanoma and has shown promising preclinical results [94]. Additionally, there is growing interest in the use of BCG-derived exosomes as carriers for tumour-specific antigens. These exosomes can potentiate the immune response against melanoma, providing a novel strategy for delivering targeted immunotherapy [94].

Emerging research continues to uncover novel insights into the immunological mechanisms underlying BCG-mediated anti-tumour responses, opening new avenues for its integration into advanced melanoma immunotherapies.

4.2.4. Lung Cancer

Lung cancer is the second most common cancer worldwide, diagnosed in 2,480,675 individuals in 2022 [100]. Unfortunately, it has a low survival rate and accounts for the highest number of cancer-related deaths due to its poor prognosis and challenges in early detection [100]. In 1976, McKneally et al. conducted the first study investigating the use of BCG in lung cancer treatment. They demonstrated that a single postoperative intrapleural dose of BCG was well tolerated at restricted doses in patients following lung resection and improved outcomes in patients with stage I lung cancer [101]. Subsequent studies focused on the BCG cell wall, showing that BCG-CWS alleviated lymphocyte suppression

in lung cancer patients and prolonged survival in stage III and IV cases [65]. However, conflicting reports have emerged about the positive effects of the BCG vaccine, with some suggesting that BCG might stimulate tumour growth in lung cancer [102–104]. On the other hand, Moreo et al. demonstrated in mouse models that intravenously administered BCG induced $CD8^+$ T cell and NK cell responses against lung tumour cells [105]. Furthermore, the combination of BCG therapy with an immune checkpoint blockade (ICB) showed beneficial effects. The enhancement of systemic tumour cell-specific cytotoxic responses was shown [105]. Supporting this, Lapieza et al. provided strong evidence that the intravenous administration of BCG in mice activates robust NK and $CD8^+$ T cell responses, which significantly enhances anti-tumour immunity in the lungs and effectively overcomes resistance to existing immunotherapies. Their findings suggest a promising role for BCG in combination strategies to enhance the efficacy of checkpoint inhibitors and other immune-targeted therapies [106].

Other studies have investigated the use of mitumomab (BEC-2), a murine monoclonal antibody targeting ganglioside GD3, in combination with the BCG vaccine for treating small-cell lung cancer. Unfortunately, phase III clinical trials showed no significant benefit for patients receiving mitumomab and BCG [107,108].

4.3. Immunotherapy for Autoimmune Diseases

The prevalence of autoimmune diseases is steadily increasing, currently affecting approximately 3–8% of the global population [109]. This rise is attributed to a variety of environmental factors, including pollution, changes in dietary habits, and possibly the increasing prevalence of sedentary lifestyles and stress. These factors contribute to a heightened immune dysregulation, triggering autoimmune responses in susceptible individuals. Since research has shown that the BCG vaccine enhances glycolysis in trained human monocytes—a metabolic reprogramming process that strengthens immune memory and functionality—it has sparked significant interest in its potential application as a therapeutic tool for autoimmune diseases [110]. By reprogramming innate immunity, fostering an anti-inflammatory environment, and potentially restoring immune balance, the BCG vaccine represents a novel and promising approach to addressing the dysregulated immune response characteristics of autoimmune disorders.

4.3.1. Multiple Sclerosis (MS)

An estimated 2.9 million people worldwide are affected by multiple sclerosis (MS), a chronic demyelinating and inflammatory disease of the central nervous system (CNS) characterised by damage to nervous tissue. This condition disrupts communication between the brain and other parts of the body, leading to a wide range of neurological symptoms and progressive disability [111].

Studies on animal models of MS, such as experimental autoimmune encephalomyelitis (EAE), have demonstrated that the administration of a killed Mycobacterium strain can inhibit demyelination and reduce disease severity. These promising preclinical findings have sparked interest in the potential therapeutic effects of the Bacillus Calmette–Guérin (BCG) vaccine for MS [112]. Clinical investigations have provided encouraging results. A pivotal study by Ristori et al. demonstrated that the intradermal administration of the BCG vaccine to patients with relapsing–remitting MS (RRMS) reduced the number of active lesions visible on magnetic resonance imaging (MRI) without causing adverse effects [113]. Follow-up studies by the same group revealed that the early administration of BCG in the disease course could delay MS progression. Additionally, BCG vaccination appeared to block tissue damage, promote neuroinflammation repair, and reduce the risk of new MRI lesions in patients with clinically isolated syndrome (CIS), a condition that often precedes

MS [114,115]. The exact mechanisms through which BCG exerts its protective effects in MS remain unclear. However, recent research has highlighted the vaccine's ability to induce metabolic and immunological changes beneficial in MS. BCG vaccination has been shown to upregulate glycolysis and modulate cellular respiration pathways, processes often impaired in patients with relapsing–remitting multiple sclerosis (RRMS). This metabolic reprogramming enhances immune cell functionality, potentially fostering a better regulation of neuroinflammatory responses [116]. Furthermore, BCG's role in "trained immunity", which involves epigenetic reprogramming of innate immune cells, may significantly impact its therapeutic potential. This mechanism has been shown to promote balanced immune responses and reduce autoreactive activity driving CNS inflammation in MS. Notably, monocytes and macrophages subjected to BCG-induced trained immunity exhibit enhanced glycolysis, increased cytokine production, and improved immune tolerance—all factors potentially contributing to CNS repair [117]. Recent studies have suggested potential interactions between trained immunity induced by BCG and cytokines from the IL-1 family, which play a central role in modulating inflammation in autoimmune diseases. The broader benefits of BCG-induced metabolic switching, such as promoting aerobic glycolysis, have been discussed in autoimmune and neurodegenerative diseases. This metabolic adaptation, transitioning from oxidative phosphorylation to glycolysis, may underlie the improved immune regulation and neuroprotection observed in MS patients [118]. These findings collectively emphasise the potential of BCG as a safe and cost-effective therapeutic option for MS, particularly in the early stages of the disease. However, larger randomised controlled trials are essential to confirm these benefits, unravel the underlying mechanisms, and optimise treatment protocols.

4.3.2. Type 1 Diabetes (T1D)

Type 1 diabetes (T1D) is a chronic autoimmune disease affecting approximately 9.4 million individuals worldwide, with projections indicating that this number will rise to 17.4 million by 2040. This condition is characterised by the targeted destruction of pancreatic β-cells by autoreactive $CD8^+$ T cells, leading to insulin deficiency and hyperglycaemia. Despite extensive research, the precise aetiology of T1D remains elusive. Current evidence suggests that the disease arises from a complex interplay of genetic predisposition and environmental triggers, including infections, diet, and microbiome composition [119]. Interestingly, epidemiological observations have revealed a lower incidence of T1D among individuals vaccinated with BCG. These findings spurred preclinical studies using the non-obese diabetic (NOD) mouse model. In these studies, the administration of complete Freund's adjuvant (which contains heat-killed BCG) or the BCG vaccine alone significantly reduced the development of diabetes [120]. These results laid the foundation for subsequent investigations into the immunomodulatory effects of BCG in T1D.

One of the proposed mechanisms involves the induction of TNF, which selectively eliminates insulin-autoreactive T cells while sparing healthy T cells [121–123]. Additionally, TNF exposure has been shown to enhance the proliferation of regulatory T cells (Tregs), which suppress autoreactive immune responses. Beyond immune modulation, BCG-induced TNF has been implicated in pancreatic regeneration, mediated by increased levels of C-peptide, a marker of endogenous insulin production and β-cell function [115,124]. Further research has highlighted the role of BCG vaccination in altering metabolic pathways within the immune system. Patients with T1D who received BCG vaccinations demonstrated significant reductions in haemoglobin A1c levels, bringing them closer to normal ranges. Mechanistically, this improvement was linked to a shift in immune cell metabolism from oxidative phosphorylation (OxPhos) to aerobic glycolysis, a phenomenon often referred to as the "Warburg effect." This metabolic reprogramming may enhance

immune tolerance and β-cell resilience [118,125]. More recent studies have underscored the potential of BCG as a therapeutic agent in T1D, not only for its immune-regulatory effects but also for its ability to stabilise blood glucose levels and improve overall metabolic health. The vaccine's broad effects on innate immune training, particularly through epigenetic and transcriptional reprogramming, may also contribute to its therapeutic benefits. This expanding body of evidence positions BCG as a potential adjunct therapy for T1D, warranting further clinical trials to optimise dosing regimens and evaluate long-term safety and efficacy [124–126] (Table 1).

Table 1. Clinical studies on the protective effect of the BCG vaccine.

	ClinicalTrials.govID	Disease Entity	Study	Location Countries	Phase	Study Start	References
ANTIVIRAL EFFECTS	NCT03213405	RSV	Assess Safety, Tolerability and Immunogenicity of the Live Attenuated hRSV Vaccine rBCG-N-hRSV (EVA-VRS01)	Chile	Phase 1	2017	[127]
	NCT04659941	SARS-CoV-2	Use of BCG Vaccine as a Preventive Measure for COVID-19 in Health Care Workers	Brazil	Phase 2	2020	[128]
	NCT04648800	SARS-CoV-2	Effect of BCG Vaccination on the Incidence and Severity of SARS-CoV-2 Infections Among Healthcare Professionals During the COVID-19 Pandemic	Poland	Phase 3	2020	[129]
	NCT04439045	SARS-CoV-2	Efficacy and Safety of VPM1002 (rBCG) in Reducing SARS-CoV-2 (COVID-19) Infection Rate and Severity	Canada	Phase 3	2020	[130]
	NCT02114255	Influenza	Effects of BCG on Influenza Induced Immune Response	Netherlands	Phase 2 Phase 3	2014	[131]
CANCER	NCT06462001	Bladder Cancer	Adding Mitomycin C to BCG in High-risk, Non-muscle-invasive Bladder Cancer	United Kingdom	Phase 3	2020	[132]
	NCT06441110	Bladder Cancer	Effectiveness and Safety of Instillation of BCG afor Intermediate and High-risk Non-muscle Invasive Bladder Cancer	China	Phase 3	2023	[132]
CANCER	NCT01838200	Melanoma	Intralesional BCG Followed by Ipilimumab in Advanced Metastatic Melanoma	Australia	Phase 1	2014	[133]
	NCT00671554	Melanoma	Melaxin Cancer Vaccine Plus BCG to Treat Malignant Melanoma	United States	Phase 1 Phase 2	2008	[134]
	NCT00006352	Lung Cancer	Monoclonal Antibody Therapy Plus BCG in Treating Patients With Limited-Stage Small Cell Lung Cancer	Netherlands	Phase 3	1999	[107]
	NCT00037713	Lung Cancer	Survival Patients With Limited Disease (LD) Small Cell Lung Cancer Vaccinated With Adjuvant BEC2 and BCG	Not provided	Phase 3	1998	[135]
AUTOIMMUNE DISEASES	NCT00202410	Multiple Sclerosis	Efficacy of Anti-Tubercular Vaccination in Multiple Sclerosis	Italy	Phase2 Phase 3	2001	[136]
	NCT05866536	Type 1 diabetes	Repeat BCG Vaccinations for the Treatment of NewOnset Type 1 Diabetes in Children	United States	Phase 2	2023	[137]
	NCT05180591	Type 1 diabetes	Repeat BCG Vaccinations For The Treatment Of Pediatric Type 1 Diabetes	United States	Phase 2	2022	[138]
	NCT02081326	Type 1 diabetes	Repeat BCG Vaccinations for the Treatment of Established Type 1 Diabetes	United States	Phase 2	2015	[139]

5. Conclusions

The trained immunity induced by the BCG vaccine represents a promising avenue for advancing our understanding of the innate immune response and its potential for therapeutic applications. Originally developed to prevent tuberculosis, the BCG vaccine has been observed to elicit a broad spectrum of non-specific protective effects. These effects are attributed to epigenetic and metabolic reprogramming in innate immune cells, such as monocytes and macrophages, which enhance their ability to respond to subsequent infections. Remarkably, these effects can persist for months or even years, positioning BCG as a unique and efficacious immunomodulatory agent. Studies have demonstrated that BCG vaccination is associated with a reduction in the incidence and severity of viral infections, including respiratory illnesses. This is likely mediated through the activation and enhanced functional capacity of macrophages and monocytes, enabling them to effectively neutralise viral antigens. Moreover, BCG has shown promise as an immunotherapy for certain cancers, particularly bladder cancer. The vaccine's ability to modulate the tumour microenvironment facilitates the recruitment and activation of immune cells that directly target and destroy cancer cells. In addition to its direct cytotoxic effects on pathogens and tumour cells, trained immunity induced by BCG plays a critical role in fine-tuning the inflammatory response. By regulating the activation of innate immune cells, BCG can mitigate excessive inflammation, which is a hallmark of many chronic and infectious diseases. This dual capacity to enhance host defence while limiting hyperinflammatory responses underscores the therapeutic versatility of the vaccine.

Despite significant advancements in understanding the mechanisms underlying BCG-induced trained immunity, several questions remain. Key areas for future research include the durability of these immunological effects, the potential variability in response among individuals, and the long-term safety of repeated or off-label BCG administration. Furthermore, exploring the interplay between trained immunity and adaptive immune responses could open new frontiers in vaccine development and immunotherapy. BCG's non-specific benefits highlight its broader significance beyond tuberculosis prevention, offering insights that may inform strategies for combatting emerging infectious diseases, inflammatory conditions, and cancer. As our understanding of trained immunity deepens, BCG serves as a valuable model for harnessing the innate immune system in innovative and sustainable ways.

Author Contributions: Conception, M.J. and M.D.; writing—original draft preparation, M.J. and M.D.; writing—review and editing, M.J. and M.D.; supervision, M.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available under reference numbers.

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

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Review

NOD1, NOD2, and NLRC5 Receptors in Antiviral and Antimycobacterial Immunity

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Abstract: The innate immune system recognizes pathogen-associated molecular motifs through pattern recognition receptors (PRRs) that induce inflammasome assembly in macrophages and trigger signal transduction pathways, thereby leading to the transcription of inflammatory cytokine genes. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) represent a family of cytosolic PRRs involved in the detection of intracellular pathogens such as mycobacteria or viruses. In this review, we discuss the role of NOD1, NOD2, and NLRC5 receptors in regulating antiviral and antimycobacterial immune responses by providing insight into molecular mechanisms as well as their potential health and disease implications.

Keywords: NLR; antimicrobial immunity; ssRNA virus

Citation: Godkowicz, M.; Druszczyńska, M. NOD1, NOD2, and NLRC5 Receptors in Antiviral and Antimycobacterial Immunity. *Vaccines* **2022**, *10*, 1487. https://doi.org/10.3390/vaccines10091487

Academic Editor: Anke Osterloh

Received: 2 August 2022
Accepted: 1 September 2022
Published: 7 September 2022

1. Introduction

Pattern recognition receptors (PRRs) play an important role in non-specific immunity. In a sense, these receptors provide a universal alarm signal that enables the rapid transmission of danger information and thus an immediate host defense. PRRs recognize not only pathogen components, such as lipopolysaccharide, glycolipids, and nucleic acids, but also toxic metabolic products. An important class of intracellular PRRs is the NOD-like receptor (NLR) family [1,2].

2. The NLR Family

In humans, the NLR family includes at least 23 receptors that share a similar structure. Most proteins belonging to the NLR family contain three domains: an N-terminal signaling domain (pyrin domain (PYD) or caspase recruitment containing domain (CARD)), central nucleotide binding and oligomerization domain (NOD1 or NOD2), and C-terminal agonist sensing/binding domain (Leucine Rich Repeat (LRR)) [3]. Based on the N-terminal region, this family has been divided into four groups: (1) NLRP (NOD-like receptor P; P for PYD), (2) NLRC (NOD-like receptor C; C for CARD), (3) NLRA (NOD-like receptor A; A for the acidic transactivating domain), (4) NLRB (NOD-like receptor B; B for the baculovirus inhibitor of apoptosis (BIR)) (Table 1).

NLR receptors play a key role in regulating the host innate immune response. They recognize a variety of ligands from microbial pathogens (peptidoglycan, viral RNA, flagellin), host cells (cholesterol crystals, uric acid), and environmental sources (asbestos, silica, alum).The majority of NLRs function as PRRs, detecting specific ligands and inducing inflammatory reactions, however some NLRs do not function as PRRs but respond to cytokines, such as interferons. Inflammasome platforms formed by NLRs, which function as scaffold proteins, stimulate mitogen-activated protein kinase (MAPK) and nuclear factor-κβ (NF-κβ) signaling pathways and control the activation of inflammatory caspases. In this review, we discuss the role of three NLR molecules, NOD1, NOD2, and NLRC, in antiviral

and antimicrobial immune responses, providing insight into the molecular mechanisms and their potential implications for health and disease.

2.1. NLRP

The most abundant group of NLRs is the NLRP subfamily, consisting of 14 proteins that are mostly composed of a PYD effector domain, a central NOD domain, and a C-terminal LRR domain. NLRP1 and NLRP10 have a different structure. NLRP1 protein has an additional finder function domain (FIND) and a caspase activation and recruitment domain (CARD), whereas NLRP10 lacks the LRR domain [4]. Recent studies have shown that autolytic proteolysis within FIND is non-specific for inflammasome activity [5], but the specific function of this domain is unknown. In contrast, CARD has the ability to attach caspases involved in inflammation and apoptosis (caspase 1) [6,7]. Many receptors in this subfamily (NLRP1, NLRP3, NLRP6, NLRP7, NRLP10, and NRLP12) are involved in inflammasome formation [8].

2.2. NLRC

The NLRC subfamily consists of six proteins, NOD1, NOD2, NLRC3, NLRC4, NLRC 5, and NLRX1—also named NOD5 or NOD9—and their common feature is the presence of the LRR and NOD domains. NLRX1 is unique because of the fact that, unlike the other NLRs, it is located in the mitochondria. The protein is referred to as NLRX1 since its N-terminus has not been completely defined [9]. There are different receptor structures in the NLRC subfamily. NOD1 and NLRC4 have one CARD domain and NOD2 has two CARD domains, while NLRC3, NLRC5, and NLRX1 still do not have an identified NH2 domain [10]. The best known proteins are NOD1 and NOD2, which recognize different bacterial and viral components [11]. NOD1 recognizes gamma-glutamyl diaminopimelic acid (iE-DAP), a peptidoglycan product of Gram-negative bacteria. In contrast, NOD2 recognizes muramyl dipeptide (MDP) present in the peptidoglycans of most bacteria [12,13]. Recent studies have shown that NLRs can also recognize some viruses through NOD2, which reacts with viral single-stranded RNA (ssRNA), leading to type I interferon production [14,15].

Table 1. Structures of the NLR subfamilies.

NLR Family	Member	Stucture	Reference
NLRA	CIITA		[16,17]
NLRB	NAIP		[16,17]
NLRC	NOD1		[9,10]
	NOD2		[9,10]
	NLRC4		[9,10]
	NLRC3/C5/X1		[9,10]
NLRP	NLRP1		[4]

Table 1. *Cont.*

NLR Family	Member	Stucture	Reference
	NLRP2-9, 11-14		[4]
	NLRP10		[4]

Abbreviations: AD, acidic activation domain; BIR, baculovirus inhibitor of apoptosis; CARD, caspase recruitment containing domain; CIITA, class II transactivator; FIND, finder function domain; NAIP, neuronal apoptosis inhibitory protein, NLRA, NOD-like receptor A; NLRB, NOD-like receptor B; NLRC, NOD-like receptor C; NLRP, NOD-like receptor P; NOD, nucleotide-binding oligomerization domain; LRR, leucine rich repeat; PYD, pyrin domain.

2.3. NLRA and NLRB

In the other two subfamilies (NLRA, NLRB), one member of each has now been identified, a class II transactivator (CITTA) and a neuronal apoptosis inhibitory protein (NAIP), respectively. What distinguishes these receptors from the others is the presence of an acidic activation domain (AD) in NLRA and BIR in NLRB [16,17]. CIITA activation is regulated by a number of post-translational modifications, such as acetylation, phosphorylation, and ubiquitination. CIITA activates MHC class II gene expression in different populations of antigen-presenting cells (APCs). In the absence of CIITA expression, MHC class II activation does not occur. CIITA is responsible for the expression of accessory proteins required for proper peptide presentation through MHC class II [16,18,19]. NAIP member, NLRB, is involved in host defence and cell survival. The characteristic feature of this protein is the inhibition of apoptosis by suppressing the activities of caspase (CASP) 3 (CASP3), CASP7, and CASP9; the autocleavage of pro-CASP9; and the cleavage of pro-CASP3 by CASP9 [16,20].

3. Structure and Expression of NOD1 and NOD2

Through sequence-homology searches, we were able to identify NOD1 and NOD2 as the first members of the NLR family. The NOD1 receptor consists of 953 amino acids and is located on chromosome 7p14-p15, while NOD2 contains 1040 amino acids and is located on human chromosome 16p21 [21–23]. While NOD1 shows a ubiquitous expression pattern, NOD2 is more strongly expressed in macrophages, dendritic cells, paneth cells, keratinocytes, epithelial intestinal cells, lung epithelial cells, oral epithelial cells, and osteoblasts ([24]). Both receptors recognize peptidoglycan residues, with NOD1 reacting with the peptidoglycan (PG) group containing gamma-glutamyl diaminopimelic acid (iE-DAP), which is mainly found in Gram-negative bacteria [13,25,26]. The NOD2 receptor senses MDP structures found in both Gram-positive and Gram-negative bacteria [12,27]. Recent studies suggest that NOD2 directly binds MDP with high affinity [28], and N-glycolyl MDP more strongly modulates the host response compared to N-acetyl MDP [29]. An increasing number of studies suggest that, in addition to peptidoglycan recognition, NOD1 and NOD2 may respond to danger signals or damage-associated molecular patterns (DAMPs). Endoplasmic reticulum (ER) stress contributes to inflammatory diseases such as Crohn's disease and type 2 diabetes. ER stress induced by various microbial infections can be interpreted as a danger signal recognized by NOD1 and NOD2. *Brucella abortus* infection was shown to induce ER stress, which promoted NOD1/2-dependent inflammation and IL-6 production [30]. Furthermore, bacterial effector proteins can activate NOD1 and NOD2. *Shigella flexneri* effector proteins, such as OspB and IpgB2, and *Salmonella enterica* serotype, Typhimurium SipA and SopE, induce NF-κB activation in a peptidoglycan-independent, but NOD1- or NOD2-dependent, manner [31–33]. Recent studies have shown that NLRs can also recognize certain viruses through NOD2, which reacts with viral ssRNA, leading to type I interferon production [14,15]. Finally, NOD1 and NOD2 signaling leads to the activation of host defense mechanisms, such as the production of pro-inflammatory cytokines and antimicrobial molecules [34,35]. NOD1 and NOD2 are

cytoplasmic receptors, but their activation depends on their location in endosomes and in the plasma membrane [36–38]. The recruitment of NOD1 and NOD2 to the cell membrane occurs through interaction with the actin cytoskeleton [39,40]. Rho GTPases, which are responsible for regulating the actin cytoskeleton, are known to be able to interact with and activate NOD1 [32]. Furthermore, NOD2 binds to regulators of the actin cytoskeleton, GTPase Arf [41]. Moreover, transport to the cell membrane requires lipid modification (S-palmitoylation) for recruitment to the cell membrane [42].

Upon recognition of the appropriate agonist by NOD1 and NOD2 receptors, activation of pathways whose primary targets are MAPK, caspase-1, and NF-κB occurs. These activations lead to the transcription of relevant genes and the production of inflammatory mediators. The activation of NOD1 and NOD2 signaling pathways is regulated by post-translational modifications, such as phosphorylation and ubiquitination. The recognition of iE-DAP and MADP by NOD1 and NOD2, respectively, leads to a change in receptor conformation and exposure of the CARD domain, which recruits and activates receptor-interacting serine/threonine kinase 2 (RIP2) [21,43,44]. The activation of RIP2 kinase is necessary to activate the inflammatory signaling pathway associated with NOD1 and NOD2 receptors [45]. In the next step, RIP2 can lead to the ubiquitination of the essential modulator of NF-κB (NEMO) and activation of the IKK (IκB kinase) complex. The IKK complex phosphorylates the inhibitor of kappaB (IκBα), which is then degraded, and the released NF-κB is translocated to the cell nucleus. In the cell nucleus, NF-κB dimers bind to kappaB (κB) elements, activating pro-inflammatory cytokines and factors responsible for immune cells, among others [46–49] (Figure 1).

The formation of nodosome complexes can also lead to the activation of one of the three major MAP kinase families: p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). As a result, the MAPK pathway is activated, and p38, JNK, and ERK translocate to the nucleus and phosphorylate AP-1 transcription factors responsible for the expression of pro-inflammatory cytokines and chemokines [34,50,51] (Figure 1).

In addition to the pathways mentioned above, NOD 1 and NOD2 receptors can also activate the interferon signaling pathway. Sabbah et al. showed that NOD2 not only recognizes MDP, but also interacts with viral ssRNA [14]. The researchers revealed that RSV activates NOD2 through the mitochondrial antiviral signaling protein (MAVS), which leads to the activation of the regulatory transcription factor, interferon 3 (IRF3). In subsequent steps, IRF3 migrates to the nucleus and activates type I IFN (IFNα/β) genes. Interestingly, the IFN pathway is also activated during bacterial infections. The mechanism is not fully understood, but it has been shown that upon binding of bacterial ligands, conformational changes of NOD1 and NOD2 occur, followed by the formation of the RIP2-TRAF3 complex, which recruits TANK shunt kinase 1 (TBK1) and the inhibitor of nuclear factor kappaB kinase (IKKε). These two kinases form a TBK1-IKKε complex that drives IRF3-dependent expression of IFNβ and type I IFN genes [52,53] (Figure 1).

It is still not entirely clear how the NOD1 and NOD2 signaling pathways are regulated. Several studies have shown that certain molecules can positively or negatively affect NOD1 and NOD2 activation pathways. GRIM-19 protein and vimentin have been described as positive regulators of this process. They are required for NF-κB activation after MDP recognition by NOD2 [54,55]. A negative regulator of NOD signaling is the Erbin protein, which, when bound to NOD2, inhibits MDP-induced signaling [56]. Moreover, overexpression of Centaurin β-1 (CENTβ1), a GTPase-activating protein, inhibits NOD1- and NOD2-dependent expression of NF-κB [57].

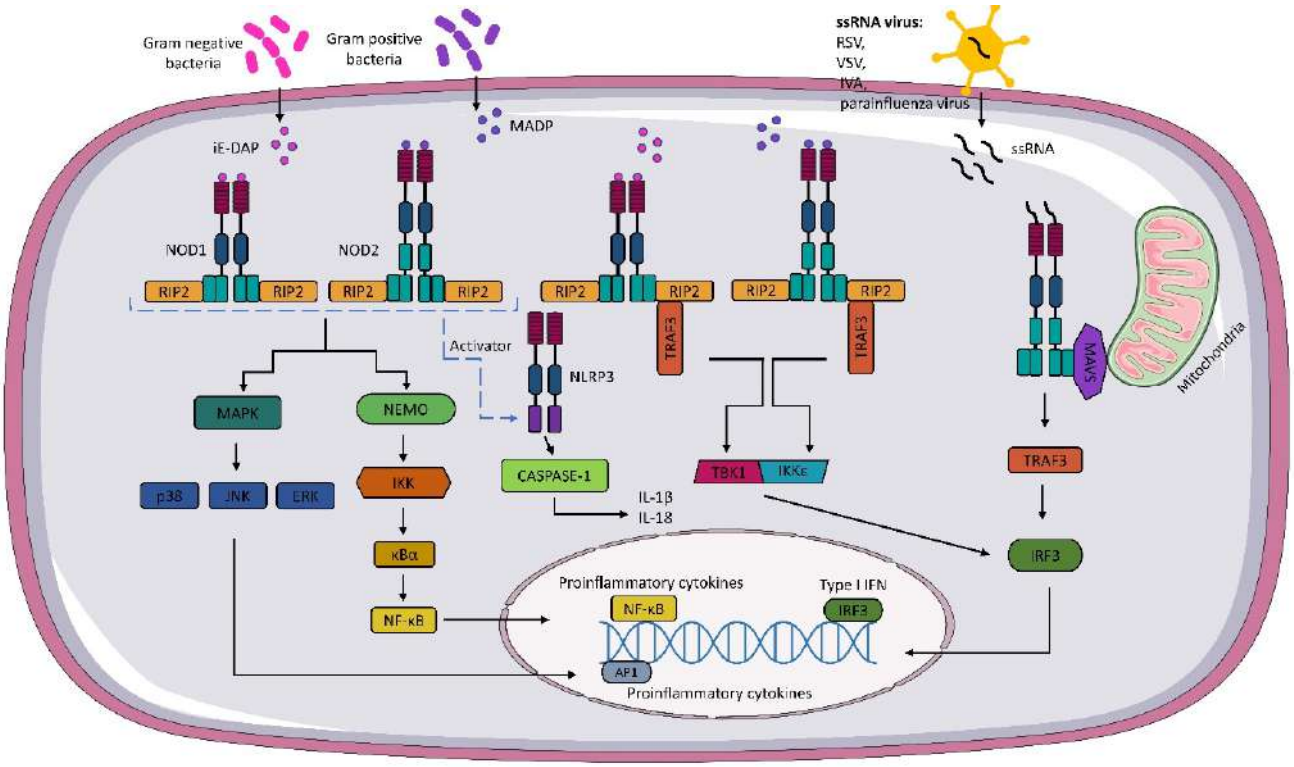

Figure 1. NOD1 and NOD2 signaling pathway. Gamma-glutamyl diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) activate the nucleotide-binding oligomerization domains 1/2 (NOD1) and (NOD2), respectively. Activation of NODs leads to the recruitment of receptor-interacting serine/threonine kinase (RIP2). In the next step, activated RIP2 can lead to ubiquitination of the essential modulator of NF-κB (NEMO) and activation of the IKK (IκB kinase) complex. The activated IKK complex phosphorylates the inhibitor of kappaB (IκBα). The activated IKK complex phosphorylates the kappaB inhibitor (IκBα), leading to the release of NF-κB, which, after translocation to the cell nucleus, binds to kappaB (κB) elements, thereby activating pro-inflammatory cytokines. NOD1 and NOD2 also activate mitogen-activated protein kinases (MAPKs), such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). NOD1 and NOD2 also interact with the NLRP3 inflammasome, which leads to caspase-1 activation and IL-18 and IL-1β production. Activation of NOD1 and NOD2 results in the formation of a TBK1 and the inhibitor of the nuclear factor kappaB kinase (IKKε) complex, which leads to the expression of type I IFNs. As well, through the interaction of NOD2 with mitochondrial antiviral signaling protein MAVS, the expression of IFN I genes occurs.

4. Structure and Expression NLRC5

Among NLR members, NLRC5 contains the largest number of LRRs, consisting of 713 amino acids, making it the largest protein in this receptor family [58]. The complete human NLRC5 gene consists of 1866 amino acids and is located at locus 16q13 [59]. NLRC5 responds to viral components and lipopolysaccharides, and is highly sensitive to interferon γ (IFN-γ) [58,60,61]. NLRC5 has a structure similar to the other receptors belonging to the NLR family; however, the N-terminal CARD of NLRC5 is characterized by the folding of the death domain, making it an atypical CARD domain [62]. Furthermore, the NOD domain of the NLRC5 receptor contains the Walker A and Walker B regions, which are required for the binding and hydrolysis of nucleotide triphosphate, respectively. The Walker A motif is essential for NLRC5 migration into the nucleus and, consequently, for the transactivation activity of major histocompatibility complex (MHC) class I genes [63,64]. The structure of NLRC5 is highly conserved in many mammalian species; the homology of human NLRC5 to mice NLRC5 is 64% [65], suggesting that NLRC5 shows similar functions

in different organisms.NLRC5 is highly expressed in immune cells and tissues, such as the spleen, lymph nodes, bone marrow, thymus, intestine, lung, and bone marrow [59,60,65].

NLRC5 has different functions depending on its localization. In the cytoplasm, NLRC5 can inhibit nuclear factor kappa B (NF-κB) signaling by interacting with IκB kinase alpha/beta (IKKα/β) so that NEMO does not bind to IKK and autophosphorylation and kinase activity are inhibited [59,65]. NLRC5 in the cytoplasm is involved in the regulation of type I IFN, but data on the effect of this receptor on IFN I are conflicting. Some studies suggest that NLRC5 interacts with RIG-I and MAVS, resulting in an enhanced IFN I response. (Figure 2) However, there are also reports suggesting that NLRC5 inhibits the IFN I response [65–67]. The formation of the inflammasome is important in the recognition of the infectious agent and in modulating the host immune response. It has been speculated that NLRC5 may lead to the activation of the inflammasome and even to the formation of its own inflammasome [68]. NLRC5 is involved in the activation of the NLRP3 inflammasome. The overexpression of NLRC5 leads to the increased activation of caspase-1, which converts pro-IL-1β into active IL-1β [67,68] (Figure 2). Tong et al. and Kumar et al. observed similar levels of IL-1β secretion in wild-type and NLRC5-/- mice [67,69]. Recent studies suggest that NLRC5 interacts with tumors through multiple pathways, such as β-catenin, TGF-β, and Akt [70–72]. It is worth noting that most of the presented functions of NLRC5 are still unclear and debatable, so further studies on this receptor are needed.

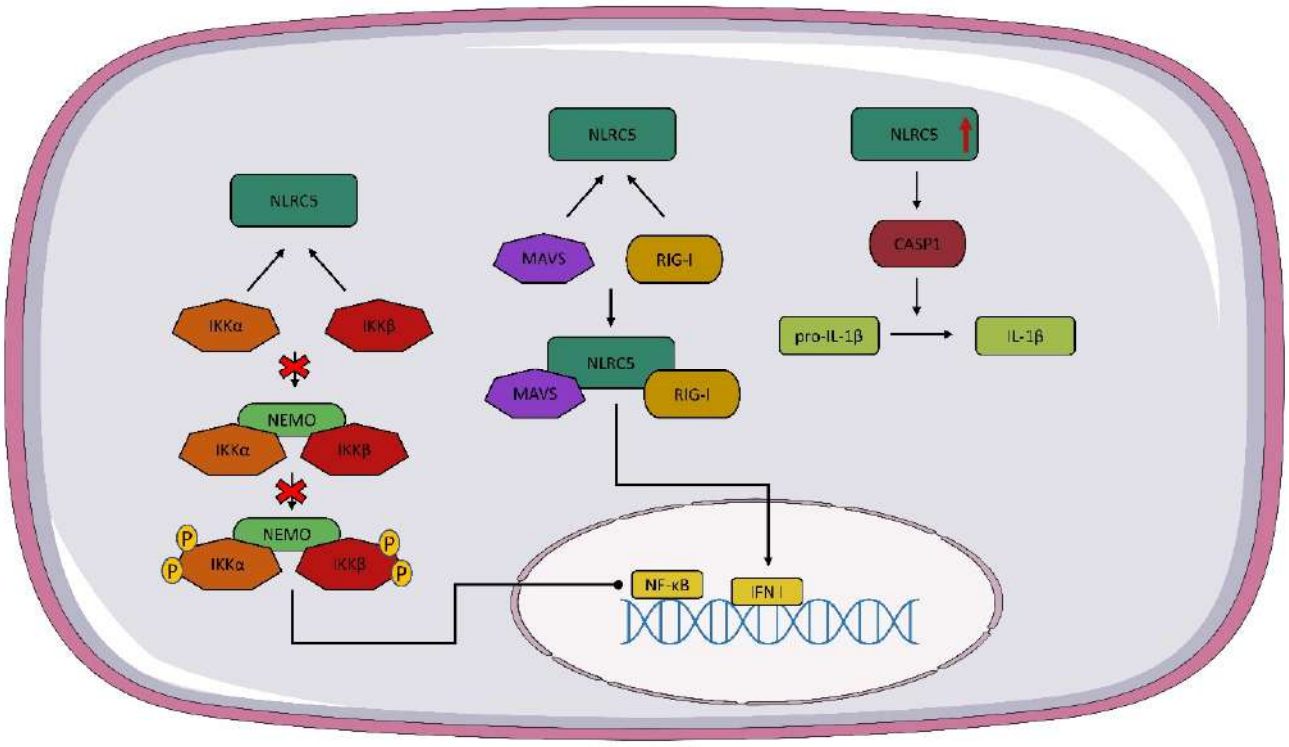

Figure 2. NLRC5 signaling pathway. As a result of the interaction of NOD-like receptor C5 NLRC5 with IκB kinase alpha/beta (IKKα/β), the NEMO IKKα/β complex is not formed and nuclear factor kappa B (NF-κB) expression is stopped. The interaction of NLRC5 with mitochondrial antiviral signaling protein (MAVS) and RIG-I leads to the formation of a complex of NLRC5 plus MAVS and RIG-I, which contributes to the expression of IFN I. Overexpression of NLCR5 activates caspase 1 (CASP1), which converts pro-IL-1β to active IL-1β.

5. Interaction of NOD1, NOD2, and NLRC5 with Viruses

Recent studies have shown that recognition of viral ssRNA by NOD2 leads to the activation of interferon regulatory factors (IRF) 3 and IRF 7, and the induction of type I-mediated antiviral responses [14]. Due to the lack of structural similarities between bacterial MDP and viral ssRNA motifs, the molecular recognition and signaling of the

NOD2 receptor are still unclear. NOD2 is thought to be activated by direct interaction with the viral genome or viral proteins [14,73]. Several studies have revealed that in the absence of NOD2, a strong antiviral response does not occur and the control of infection and viral replication is impaired [14,74]. Therefore, it is believed that NOD2 along with the other NLRs (e.g., NOD1 and NLRC5) may promote inflammation and an antiviral response [74].

NOD2 has been shown to be involved in host defense against viral infections caused by the respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), influenza A virus (IAV), parainfluenza virus 3, and Middle East respiratory syndrome (MERS-CoV) [14,15] (Table 2). RSV infection has been found to increase the expression of NOD2, which plays a key role in the induction of IFN-β production in cells [14,75]. Once the viral ssRNA is recognized, NOD2 uses the adaptor protein, MAVS (mitochondrial antiviral signaling), to activate IRF3 (interferon regulatory factor-3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), leading to IFN production. Mice lacking NOD2 do not produce IFN efficiently and show increased susceptibility to RSV-induced pathogenesis, suggesting that this receptor plays a key role in the antiviral immune response [14]. Another study by Gao et al. revealed that RSV infection leads to the induction of NLRC5, an intracellular NOD-like protein containing the CARD domain, which has recently been identified as a transactivator of major tissue compatibility complex (MHC) class I. RSV infection of pulmonary epithelial cells was found to increase retinoic acid-inducible gene I (RIG-I) expression, resulting in NLRC5 induction and subsequent upregulation of MHC class I molecules. It is interesting to note that NLRC5 induction came after IFN-β induction and RIG-I upregulation because RIG-I suppression inhibited the expression of both NLRC5 and MHC-I [76].

The activation of the NOD2 signaling pathway is required for the production of type I IFN after infection with three other ssRNA viruses: VSV, IVA, and parainfluenza virus. Recent data suggest that direct interaction between ssRNA and NOD2 activates MAVS, leading to IRF3 activation and IFN-beta secretion [14]. During IAV infection, NOD2-/- mice were found to exhibit reduced IFN-β levels and a lower activity of dendritic cells, which were more susceptible to lung cell death. In addition, CD8+ T cell production and activation, as well as IFN-γ secretion, were shown to be lower in NOD-/- mice [77]. The results of this study suggest that NOD2 plays a key role in inducing both innate and adaptive immune responses that are required to control IVA infection. We also demonstrated an altered CD8+ T cell response during IAV infection in NLRC5-/- mice. Although reduced CD8+ T cell production was not observed in NLRC5-/- mice, IAV-specific CD8+ T cells exhibited impaired effector functions, which may contribute to the impaired clearance of viral infection [78].

The role of NOD2 in SARS-CoV2 recognition is not yet fully understood, but it is worth noting that there are reports that another coronavirus, MERS-CoV, is able to reduce NOD2 expression in macrophages [15]. Furthermore, infection of human fetal brain cells with Zika virus induces NOD2 expression [79]. At present, there is very little information on the role of NOD2 in SARS-CoV-2 infection. A bioinformatics analysis revealed the differential expression of NOD2 in COVID-19 patients. NOD2 expression was downregulated in patients with mild COVID-19 compared to healthy individuals. However, higher levels of NOD2 expression were observed in individuals with severe forms of the disease [80]. Interestingly, SARS-CoV-2 infection can activate NOD1 (a receptor for Gram-negative bacteria), leading to the activation of the NF-κB pathway and IFN secretion [81].

A great number of studies have suggested that NLRC5 may enhance the antiviral signaling that is involved in the modulation of the innate antiviral immune response [66]. It has been shown that NLRC5 efficiently inhibits viral infection by preventing the activation of RIG-I and MDA5, as well as the generation of type I IFN [82]. Nevertheless, in vitro studies have shown that NLRC5 can regulate antiviral type I IFN production either negatively or positively, suggesting a contradictory antiviral function of NLRC5 [58,60,65]. NLRC5 is an important regulator of MHC class I expression that mediates antiviral activity, SARS-CoV-2, like other viruses, has evolved mechanisms for targeting the MHC class I

pathway to evade host immunity. In nasopharyngeal samples from SARS-CoV-2 patients, it was observed that NLRC5 expression was downregulated or not induced at all. The Open Reading Frame 6 (ORF6) SARS-CoV-2 protein has been shown to reduce IFN-γ-mediated NLRC5 expression and inhibit nuclear transport of NLRC5 [83].

Table 2. Viruses stimulating NOD1, NOD2, or NLRC5 signaling.

Viruses	Receptor	Potiential Signaling Pathways	Refs
Respiratory Syncytial Virus	NOD2	MAVS-IRF3-IFN pathway	[14]
Parainfluenza Virus 3	NOD2	MAVS-IRF3-IFN pathway	[14]
Influenza A Virus	NOD2	MAVS-IRF3-IFN pathway	[14]
	NLRC5	NLRC5-RFX-CREB1-ATF1-NF-Y-X1/2-Y-MHCI pathway	[77]
Vesicular Stomatitis Virus	NOD2	MAVS-IRF3-IFN pathway	[14]
Middle East Respiratory Syndrome	NOD2	MAVS-IRF3-IFN pathway	[15]
Zika Virus	NOD2	Unknown	[79]
SARS-CoV-2	NOD1	LGP2+MDA5-MAVS-IRF3/5-IFN pathway	[81]
	NLRC5	STAT1-IRF1-NLRC5 pathway	[83]

Abbreviations: ATF1, activating transcription factor 1; CREB1, cAMP responsive element binding protein 1; IFN, interferon; IRF3, regulatory transcription factor interferon 3; NF-Y, nuclear transcription factor Y; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; MHC I, major histocompatibility complex class I; RFX, regulatory factor X.

6. Interaction of NOD1, NOD2, and NLRC5 with Mycobacteria

As receptors that recognize peptidoglycan fragments, NOD1 and NOD2 are known to be involved in the response to many bacterial infections. NOD1 aids in the immune clearance of *Shigella flexneri*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Legionella pneumophila*, *Clostridium difficile*, and *Haemophilus influenzae*, leading to the activation of the NF-κB pathway [84–90]. NOD2 is involved in the recognition of bacterial pathogens such as *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Salmonella enterica* Typhimurium, *Shigella flexneri*, and *Mycobacterium tuberculosis* [34,91–93]. There are increasing numbers of reports on the role of NOD1 and NOD2 in activating the adaptive immune response. NOD2 stimulation by MDP has been shown to trigger a strong antigen-specific Th2 immune response, resulting in IL-4 and IL-5 production and IgG1 responses [94].

NOD2 plays a key role in host innate immunity to bacterial lung infection [95–98]. Interactions between pathogenic mycobacterial cell elements and NOD2 receptors play a key role in the course of infection. NOD2 regulates the production of inflammatory mediators in response to MDP, which is present in the *Mycobacterium* cell wall [12,29]. The role of NOD2 in mycobacterial infection has been mainly studied in *Mycobacterium tuberculosis*(*M.tb*) and *Mycobacterium bovis* BCG (BCG) infection models. Previous studies have shown that impaired NOD2 signaling results in inappropriate recognition of mycobacteria in vitro by macrophages [91,99–101]. The stimulation of human macrophages with NOD2 ligands led to the secretion of more TNF-α and IL-1β in response to *M.tb* and BCG [102]. Furthermore, NOD-deficient mice showed reduced TNF-α and NO production in response to *M.tb* [91,99]. It is supposed that if the initial host defense mechanisms fail, then NOD2 is a secondary line of defense for macrophages and, more importantly, mediates iNOS production, which leads to NO secretion in response to *M.tb* [103]. NOD1-deficient mice were shown to respond normally to *M.tb* infection, and NOD1 alone could not compensate for the lack of NOD2 [91]. Little is known about the role of NOD2 in adaptive immunity to mycobacteria,

but Divangahi et al. demonstrated that T cell-dependent immunity was reduced in NOD2-deficient mice after mycobacterial infection of both the lung and skin [104]. In addition, we observed that the stimulation of NOD2 by MDP in human alveolar macrophages infected with *M.tb* led to the increased intracellular control of *M.tb* growth and transport to the autophagosome of proteins that are associated with autophagy [98]. Interestingly, three common non-synonymous polymorphisms in the NOD2 gene were shown to be associated with genetic susceptibility to tuberculosis in African Americans [105].

The activation of NOD1 and NOD2 can also lead to the initiation of autophagy, which relies on cooperation with the autophagy-related 16-like 1 (ATG16L1) protein. Autophagy is a highly-conserved process of cellular homeostasis, during which damaged organelles, protein aggregates, and various pathogens are removed. It has been shown that *M.tb*-infected human follicular macrophages leads to NOD2 activation, which contributes to the recruitment of the autophagy markers IRGM(immunity-related GTPase family M protein), LC3 (microtubule-associated protein 1A/1B-light chain 3), and ATG16L1 (autophagy related 16 like 1) to *M.tb*-containing vesicles. This finding demonstrates that the active NOD2 receptor in human alveolar macrophages may represent an early innate control of primary *M.tb* infections [98]. NOD1 and NOD2 are also involved in the formation of reactive oxygen species (ROS). Dual oxidase 2 (DUOX2) protein during bacterial infection has been shown to be involved in NOD2-dependent ROS production [106].

Referring to the study by Kleinnijenhuis et al. it can be speculated that NOD2 mediates TB resistance by modulating the innate immune response to the Bacillus Calmette-Guérin (BCG) vaccine. In this study, BCG was shown to induce NOD2-dependent epigenetic reprogramming of monocytes [107]. Arts et al. confirmed the induction of trained immunity through NOD2 receptor activation in response to γ-irradiated BCG (γBCG). NOD2-deficient patients exhibited impaired induction of trained immunity [108]. Wannigama and Jacquet suggested that BCG induces NOD2- dependent trained immunity, which may influence the course of SARS-CoV-2 infection [109]. IFN-I is known to be an important mediator of the antiviral response; however, a delayed IFN-I response may be associated with a severe course of COVID-19 disease [110]. The early induction of IFN-I and the other cytokines, followed by a slow-down in this antiviral response, may impact disease outcome. Through NOD2 signaling, BCG induces the secretion of IFN-I [111]. Hilligan et al. showed that prior intravenous injection of BCG to mice inhibited the SARS-CoV-2-induced cytokine storm. [112];this suggests that pro-inflammatory cytokine signaling might be a mechanism by which intravenous BCG injection provides protection against SARS-CoV-2. This study revealed a non-specific protective effect of the BCG vaccine against SARS-CoV-2.

Kang and Chae investigated the role of NOD receptors in *M. leprae* infection. They showed that *M. leprae* stimulated NF-κB activation and IL-1β and TNF-α expression in cells transfected with a NOD1 or NOD2 expression plasmid. A minimal response to *M. leprae* was observed in NOD1-transfected cells, which is in contrast to NOD2-transfected cells where the response was much higher [113]. This suggests that the host response to *M. leprae* is dependent on both NOD1 and NOD2. It is worth noting that *M. leprae* has a unique MDP structure, which differs from that of other mycobacteria [114]. The unique structure of *M. leprae* has been shown to induce NOD2-dependent IL-32 production and dendritic cell differentiation [115].

Lee et al. evaluated the role of NOD2 in innate host immunity to *Mycobacterium abscessus* infection. They demonstrated that NOD2 plays a key role during clearance of *M. abscessus* bacteria. NOD2-/- mice exhibited a higher bacterial load and reduced levels of IL-6, TNF-α, and IL-1β. Similar to *M. tuberculosis* infections, NOD2 was also involved in iNOS expression and NOD production in *M. abscessus*-infected macrophages [116]. In the subsequent study, Ahn et al. showed that NOD2 mediated the expression of type I IFN, which mediates NO production in *M. abscessus*-infected macrophages to provide intracellular control of mycobacterial growth [117].

Carvalho et al. investigated the role of NOD2 during infection with *Mycobacterium avium*. They found that NOD2 was not required for host immunity to *M. avium* infection, al-

though reduced IL-12 and TNF-α secretion was observed in NOD2-deficient macrophages. Furthermore, during chronic infection, NOD2-/- mice were characterized by impaired IFN-γ production and reduced size and number of lymphocyte/granulocyte liver granulomas [118]. Ferwerda et al. first demonstrated that NOD2 was involved in the recognition of *M. avium* subsp. *paratuberculosis* [119].

In vivo studies have revealed that NLRC5 plays an important role in host defence against intracellular pathogens. It has been shown that the absence of NLRC5 reduces MHC class I expression, but does not affect MHC class II levels. In mice infected with *Listeria monocytogenes* with an NLRC5 defect, lack of activation of CD8+ T cells was observed, leading to increased bacterial load [120,121].

7. NOD1, NOD2, and NLRC5 Gene Polymorphisms and Disease Predisposition

Many pathological processes originate in defective PRR signaling, which is caused by mutations within the encoding genes. Dysregulation of NLR receptor function has been described in many diseases, including chronic inflammation, auto-immunity, and cancer [122,123]. NOD2 is one of the NLR genes that have been extensively studied in relation to the innate immune response. Polymorphisms in the gene encoding NOD2 have been linked to a growing number of chronic inflammatory disorders, such as Crohn's disease, Blau syndrome, and early-onset sarcoidosis [124–126]. Three major NOD2 variants, R702W, G908R, and L1007fsinsC, and some minor variants in the C-terminal LRR region and the helix domain 2 (HD2) have been found to be associated with the development of Crohn's disease in both European and American populations [124,125,127]. The R702W and G908R polymorphisms are single amino acid changes within the LRR domain, while the L1007fsinsC variant is caused by a deletion that leads to the loss of 33 amino acids. The relative risk of developing Crohn's disease in homozygous or heterozygous individuals has been estimated to be between 10- and 40-times higher than that in the general population. [128]. The loss of NOD2 function resulting from these mutations enables bacterial invasion and an abnormal mucosal immune response is the underlying cause of Crohn's disease. The inability of MDP to activate forms of NOD2 carrying mutations associated with Crohn's disease has been observed in both in vitro and in vivo studies [124,125,129–131]. Mutations associated with Blau syndrome are located in the NOD domain of NOD2, and at least 17 different mutations have been identified, with the following missense mutations being the most abundant: R334Q, R334W, and L469F, which together account for 80% of cases, and the E383K variant accounting for 5% of cases [132]. These mutations have been found to cause excessive activation of NF-κB and MAPK compared to the wild-type NOD2, leading to the hypothesis that Blau syndrome is the result of gain-of-function NOD2 [126]. Genetic studies also indicate the importance of NOD2 polymorphic alleles in association with the risk of tuberculosis caused by mycobacterium tuberculosis infection [105,133]. In an African-American study, Austin et al. observed that the three common non-synonymous single-nucleotide NOD2 polymorphisms, Pro268Ser, Arg702Trp, and Ala725Gly, were significantly associated with susceptibility to active tuberculosis [105]. Moreover, in the Chinese Han population, another genetic polymorphism, Arg587Arg (CGT → CGG), located in exon 4 of the NOD2 gene, has been shown to be a possible risk factor for tuberculosis [134]. Leprosy caused by *M. leprae* is the most common mycobacterial disease associated with NOD2 polymorphisms [96,135–138]. During genome analysis of leprosy patients, Zhang et al. showed a single nucleotide polymorphism in the NOD2 gene that is associated with susceptibility to *M. leprae* infection [135]. In a Chinese population, Pan et al. also observed that variants in the NOD2 gene were significantly associated with susceptibility to leprosy [136]. Leturiondo et al. confirmed that NOD2 plays a major role in resistance against *M. leprae* in an Amazonian admixed ethnic population [138]. Interestingly, Crohn's disease susceptibility genes (*NOD2*,*TNSF15*) have been shown to be associated with leprosy in a Vietnamese population [137].

It has been revealed that certain genetic NOD1 polymorphisms are associated with a susceptibility to bronchial asthma, atopic dermatitis, and inflammatory bowel disease.

An insertion-deletion polymorphism (ND1+32,656) near the start of intron IX has been found to be associated with high IgE levels, while the deletion allele of a complex functional NOD1 indel polymorphism (ND1+32,656 * 1) is significantly correlated with early-onset inflammatory bowel disease [139,140].

A few studies have shown that polymorphisms in the NLRC5 gene can affect susceptibility to infection or be a determinant of chronic inflammation status [141,142]. Zupin et al. observed that the rs289723 polymorphism, located in NLRC5 gene, was closely associated with an increased risk of chronic mild periodontitis and chronic local periodontitis [141]. In addition, Zhong et al. found an association between NLRC5 allelic variants and susceptibility to pulmonary aspergillosis [142].

8. Potential Use of NOD1 and NOD2 Signaling Modulation in Therapy

As one of the main functions of NLRs is to detect pathogens and activate signaling pathways involved in immune processes and tissue repair, they play a key role in maintaining health and resistance to infection. Therefore, controlled modulation of the innate immune response triggered by these molecules may be a potential target for therapeutic interventions. Since NOD1 and NOD2 directly activate the NF-κB pathway, NOD1 and NOD2 signaling inhibition may be useful in treating a variety of acute and chronic disorders where the suppression of the pro-inflammatory response is desirable. NOD proteins can be modulated by several natural agents with anti-inflammatory properties. The polyphenol curcumin, which is found in the Curcuma longa plant, has been demonstrated to inhibit both lauric acid- and MDP-induced NOD2 signaling, which, in turn, inhibits NF-κB activation and IL-8 production [143]. Similar inhibitory effects on NF-κB activation have also been found for parthenolide, helenalin, and several other sesquiterpene lactones, but their mechanism of action has yet to be elucidated [144,145]. Docosahexaenoic acid and eicosapentaenoic acid are the polyunsaturated fatty acids that have the largest inhibitory effects on NOD1 and NOD2 by preventing NOD2 from self-oligomerizing, which suppresses NF-κB activation and IL-8 production [146]. In the search for small molecule NOD inhibitors, a thorough high-throughput screening (HTS) program was carried out under the supervision of the Molecular Libraries Probe Production Center Network [147,148]. Using cell-based NF-κB driven luciferase reporter gene activity to measure NOD1 modulation, several active scaffolds were found from a library of 290,000 compounds from the Molecular Libraries Small Molecule Repository of the NIH (National Institutes of Health). The indoline, tetrahydroisoquinoline, and benzimidazole scaffolds met the screening criterion of the half-maximal inhibitory concentration (IC50) values below 10 μM. Two molecules, ML130 (CID-1088438) and ML146 (CID-5310346), were identified as active NOD1-selective inhibitors, whereas a benzimidazole diamide compound designated GSK669 selectively inhibited an MDP-stimulated, NOD2-mediated IL-8 response without directly inhibiting RIP2 kinase activity [147–149]. In most cases, it is still unclear how these small molecules influence NOD1 and NOD2 signaling. It is possible that they act directly to affect NOD protein stability, ligand recognition, ATP binding, and hydrolysis activity, or target any of the numerous regulatory proteins in signaling pathways [150]. Although NOD1 and NOD2 are among the most thoroughly studied proteins in the NLR family, their therapeutic potential for modulation of NOD1 and NOD2 has remained largely untapped. Developing pharmacological treatments that target NOD signaling require further in-depth understanding of the mechanisms underlying NOD activation and modulation.

9. Future Directions and Conclusions

Although NOD-like receptors were first identified more than a decade ago, research on this specific family of microbial detectors continues to be conducted to better understand the mechanisms underlying ligand detection, signal transduction, and activation of inflammatory signaling pathways. Of great interest is the development of small-molecule antagonists that inhibit NLR activation by directly targeting specific receptor domains, such as the CARD domains in NOD1 and NOD2. New specific modulators of NLR signaling

are urgently needed, as therapeutic intervention of clinical NLR dysfunction is currently only possible through the administration of potent anti-inflammatory drugs with a wide range of serious side effects. However, it must be emphasized that the strategy of using NLR signaling modulation to treat pro-inflammatory diseases and cancer requires intensive research aimed at determining the negative and positive effects of targeting receptor pathways to balance the inhibition of harmful inflammation with immune suppression and susceptibility to infection. The number of NLRs and their diversity testify to the enormous potential of these structures, which are, so far, completely untapped in animal and human clinics. The apparent rapid increase in the number of studies on NOD1, NOD2, and NLRC5 receptors in recent years suggests that the NLR family of proteins represents a huge cargo of potential biological novelties and applications.

Author Contributions: Conception, M.G. and M.D.; writing—original draft preparation, M.G. and M.D., writing—review and editing, M.G. and M.D., supervision, M.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Since the paper is a review, it did not require the consent of the local Ethics Committee.

Informed Consent Statement: Since the paper is a review, there was no need for informed consent of subjects.

Data Availability Statement: The data presented in this study are openly available under reference numbers.

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

Abbreviations

AD: activation domain; APC, antigen presenting cells; BCG, Bacillus Calmette-Guerin; CARD, caspase recruitment containing domain; CASP, caspase; CENTβ1, centaurin β-1; CIITA, class II transactivator; DAMP, damage-associated molecular patterns; ERK, extracellular signal-regulated kinase; FIND, finder function domain; iE-DAP, gamma-glutamyl diaminopimelic acid; IκBα, inhibitor of kappa B; IKK, IκB kinase; IKKε, inhibitor of nuclear factor kappaB kinase; IRF3, transcription factor interferon 3; IVA, influenza A virus; JNK, Jun N-terminal kinase; LRR, leucine rich repeat; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; MERS-CoV, Middle East respiratory syndrome; MHC, major histocompatibility complex; *M.tb*, *Mycobacterium tuberculosis*; NAIP, neuronal apoptosis inhibitory protein; NF-κβ, nuclear factor-κβ; NLR, NOD-like receptor; NLRX1, NLR family member X1; ORF6, open reading frame 6; PG, peptidoglycan; PRR, pattern recognition receptor; PYD, pyrin domain; ssRNA, single stranded RNA; RIP2, receptor-interacting serine/threonine kinase; RSV, respiratory syncytial virus; TNSF15, tumor necrosis factor ligand superfamily member 15; VSV, vesicular stomatitis virus.

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Archivum Immunologiae et Therapiae Experimentalis
73, 7 (2025), DOI: 10.2478/aite-2025-0007

ORIGINAL

Immunomodulatory Effect of the Bacillus Calmette–Guérin (BCG) Vaccine on the *In Vitro* Interferon Response Induced by Respiratory Syncytial Virus (RSV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigens

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Abstract
Studies on the bacillus Calmette–Guérin (BCG) vaccine, traditionally used against tuberculosis, indicate its potential benefit in protecting against infections. The vaccine's ability to broadly activate the immune system suggests its potential to bolster non-specific immunity, which could be crucial for combating respiratory pathogens. This study aimed to evaluate the messenger RNA (mRNA) expression of interferon (IFN)-α, IFN-β, and IFN-γ as well as the secretion of these cytokines in whole blood co-stimulated cultures with BCG and antigens of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or respiratory syncytial virus (RSV) from BCG-vaccinated Polish children who have been infected or uninfected with RSV and/or SARS-CoV-2. Significant differences were observed in the secretion and mRNA expression of IFN-α and IFN-γ in response to RSV antigens in all groups of children studied. When cultures were conducted in the presence of SARS-CoV-2 antigens, live BCG did not induce increased IFN-α secretion compared with cultures stimulated with these antigens alone. However, enhanced secretion was observed for IFN-γ, and no such relationship was observed for mRNA expression. Furthermore, discrepancies between IFN-β secretion and mRNA expression were observed, suggesting that IFN protein secretion can also be controlled at the translational or posttranslational level. The data from our studies indicate that BCG vaccination may modulate the IFN response to viral challenges with SARS-CoV-2 and RSV, suggesting a potential immunoregulatory role.

Keywords
Bacillus Calmette–Guérin • Interferon • Severe acute respiratory syndrome coronavirus 2 • Respiratory syncytial virus

Received: 21 October 2024 / Accepted: 29 January 2025/

1. Introduction

The bacillus Calmette–Guérin (BCG) vaccine, which contains a live, attenuated strain of *Mycobacterium bovis*, has been a cornerstone of tuberculosis prevention for decades. Despite its wide use, much remains unknown about its mechanisms of action. Historically, BCG was thought to induce primarily a heterologous immune response involving memory T and B cells, which develop over several weeks and do not contribute to immediate perinatal immunity (Biering-Sørensen et al. 2012). However, recent research proposes an additional mechanism called "trained immunity," in which innate immune cells such as macrophages, natural killer cells, dendritic cells (DCs), and hematopoietic stem cells in the bone marrow are trained (Netea et al. 2011). This training enables these cells to perform memory-like behavior, potentially explaining the short-term actions of immune cells that gain long-term memory capacity (Kaufmann et al. 2018; Mitroulis et al. 2018; Liu et al. 2024b). Epigenetic modifications, in particular histone modifications, play a key role in the process of trained immunity (Sviridov et al. 2022). After BCG vaccination, an increase in H3K4me3 methylation is observed in the promoters of key pro-inflammatory cytokine genes, increasing their activation (Kleinnijenhuis et al. 2012; Arts et al. 2015). The vaccine also affects cellular metabolic pathways, including glycolysis, oxidative phosphorylation, and glutamine catabolism, which are essential for the growth and function of trained immune cells. This metabolic shift toward glycolysis further promotes histone modification and BCG-induced trained immunity.

The benefits of the BCG vaccine extend beyond tuberculosis, as evidenced by its protective effect against a variety of nonspecific infections, thereby reducing morbidity and mortality, especially in children (Uthayakumar et al. 2018). The vaccine is effective against bacterial and viral pathogens, including herpes virus, influenza virus, and respiratory syncytial virus (RSV) (Starr et al. 1976; Stensballe et al. 2005; Mukherjee et al. 2017). Studies have shown that BCG vaccination modifies the effectiveness of antiviral vaccines and improves the immune response to viral infections. Recent studies are even exploring the potential of BCG in the development of new vaccines, such as the recombinant BCG vaccine against RSV, which has shown promising results in animal models (Arts et al. 2016). Kaufmann et al.

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(2022) observed a reduction in morbidity and mortality due to influenza A virus in a mouse model administered the BCG vaccine. Moreover, monocytes from BCG-vaccinated people showed an increased cytokine response. It is worth noting that BCG has been shown to not only enhance the body's response to viral infections but also increase the effectiveness of antiviral vaccines. For example, a study in a group of healthy volunteers showed that BCG vaccination enhances the effect of the influenza vaccine by increasing the production and persistence of interferon (IFN)-γ (Leentjens et al. 2015). Studies conducted in Guinea-Bissau also indicate that young children who received the BCG vaccine had a lower incidence of acute respiratory infections caused by RSV (Stensballe et al. 2005). Additionally, current research efforts are focused on developing a vaccine against RSV using the BCG strain. In an animal study in newborn calves, a recombinant BCG vaccine expressing hRSV nucleoprotein (rBCG-N-hRSV) was shown to induce both humoral and cellular responses against RSV (Díaz et al. 2021). Additionally, a study on adult volunteers confirmed the safety, protective properties, and increased production of interleukin-2 and IFN-γ of this vaccine (Abarca et al. 2020).

In viral infections, the innate immune response is the main line of defense, and IFNs are the key cytokines produced. IFNs help block virus binding to host cells, inhibit nucleocapsid release from the viral envelope, limit viral messenger RNA (mRNA) translation and viral protein synthesis, and promote immune cell activation and recruitment (Kaur and Secord 2021; Liu et al. 2024a). Type 1 IFN (including IFN-α and IFN-β) and type 3 IFN (IFN-λ) are particularly associated with the antiviral response (Chong et al. 2022; Ogger et al. 2022). The role of type 2 interferon (IFN-γ) during viral infections such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has not yet been fully elucidated. Although IFN-γ, IFN-α, and IFN-β, are critical in combating RSV infection, research indicates that type I IFNs may play a limited role in responding to RSV infection (Blasius and Beutler 2010). Furthermore, Marr et al. (2014) observed an increase in IFN-α production in RSV-infected primary plasmacytoid DCs, which becomes more pronounced with age.

In light of these findings, our study examined the effect of the BCG vaccine on the IFN response to SARS-CoV-2 and RSV antigens *in vitro*, to understand how BCG may enhance immune defense against these viruses. Our study aimed to investigate whether the BCG vaccine modulates the antiviral response of blood cells from BCG-vaccinated individuals upon co-stimulation with viral agents. While vaccination with BCG vaccine during viral infection is not the standard practice, we aimed to investigate the potential for the long-term immunological effects of BCG vaccine. Considering that early exposure to the pathogen may influence the intensity of the immune response, our study examined in detail the effect of BCG mycobacteria on IFN-α, IFN-β, and IFN-γ mRNA expression and protein secretion in whole blood cultures from BCG-vaccinated seropositive seronegative RSV/SARS-CoV-2 Polish children. An additional aim of our study was to elucidate the potential modulating role of early viral exposure on the BCG-induced IFN response. The *in vitro* model used in our study represents a controlled method to evaluate the effects of the BCG vaccine in combination with viral antigens. Although this approach does not fully replicate the physiological conditions, it provides a valuable framework for analyzing the immunological and molecular mechanisms. This model enables a detailed exploration of vaccine interactions and immune responses, offering insights that might not be feasible *in vivo*. Understanding these mechanisms has the potential to inform clinical strategies, particularly in optimizing vaccine design and combination therapies.

2. Materials and Methods

2.1. Study population

The study group consisted of 40 healthy children, aged 6–12 years, vaccinated on the first day of life with *M. bovis* BCG Moreau (Biomed Lublin, Lubin, Poland) according to the Polish national vaccination program. Furthermore, four study groups were distinguished on the history of RSV and coronavirus disease 2019 (COVID-19) infection. Group 1: RSV(+) – children seropositive for RSV; Group 2: SARS-CoV-2(+) – children seropositive for SARS-CoV-2; Group 3: RSV(+) SARS-CoV-2(+) – children seropositive for RSV and SARS-CoV-2; Group 4: RSV(–) SARS-CoV-2(–) – children seronegative for RSV and SARS-CoV-2. The characteristics of each study group are shown in Table 1. All volunteers were examined and diagnosed by infectious disease consultants, including the provincial consultant for pediatric pulmonology at the Regional Specialized Hospital of Tuberculosis, Lung Diseases and Rehabilitation in Lodz, Poland. The study protocol was approved by the Research Ethics Committee of the Lodz Medical University (no. RNN/122/22/KE). SARS-CoV-2 virus infection was confirmed with chemiluminescence immunoassay method using the DiaSorin LIASON® SARS-CoV-2 TrimericS IgG test (DiaSorin, Stillwater, MN, USA), and the Respiratory Syncytial Virus IgG kit (Serion-Diagnostics, Wurzburg, Germany) was used to assess the RSV infection (Serion-Diagnostics). The study groups were similar in terms of age and gender (Table 1). Moreover, there were no statistically significant differences in blood morphotic parameters between the studied groups (Table 1). In addition, IFN levels in cultures stimulated with peptides were compared with cultures not stimulated with these peptides.

There were no statistically significant differences between the study groups with regard to age (ANOVA with Dunn's

73, 7 (2025), DOI: 10.2478/aite-2025-0007

Table 1. *The characteristics of the groups of the study*

Parameter	Groups			
	RSV(+)	SARS-CoV-2(+)	RSV(+) SARS-CoV-2(+)	RSV(−) SARS-CoV-2 (−)
N	14	17	11	6
Sex M/F	6/8	12/5	5/6	2/4
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Age median	9	9	8	8
Age range	7–12	6–12	7–12	7–12
BCG vaccination	100%	100%	100%	100%
Leukocytes (tys/μL)	8.27	7.55	8.64	9.42
Erythrocytes (mln/μL)	4.75	4.83	4.81	5.26
Hemoglobin (g/dL)	13.29	12.76	13.52	13.43
Hematocrit (%)	39.00	38.93	39.50	39.15
MCV (fl)	82.21	77.76	82.36	74.50
MCH (pg)	28.00	28.04	28.18	25.50
MCHC (g/dL)	34.06	50.62	34.21	34.17
Platelets (tys/μL)	306.29	304.71	333.18	424.83
RDW-SD (fl)	38.82	36.04	38.90	37.02
RDW-CV (%)	13.16	12.18	13.15	14.03
PDW (fl)	11.57	12.46	11.41	10.22
MPV (fl)	10.06	10.15	10.04	9.25
P-LCR (%)	25.64	25.98	25.47	19.40
PCT (%)	0.34	0.32	0.35	0.40
Neutrophils (%)	49.23	45.74	51.69	52.48
Lymphocytes (%)	35.66	35.96	34.43	33.68
Monocytes (%)	9.45	8.63	8.65	6.82
Eosinophils (%)	5.18	7.79	4.75	5.30
Basophils (%)	0.36	0.55	0.35	0.58

BCG, bacillus Calmette–Guérin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; N, number; PCT, procalcitonin; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; RDW-CV, red blood cell distribution width – coefficient of variation; RDW-SD, red blood cell distribution width-standard deviation; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

post-test test, $p > 0.05$), sex (Chi-square test or Fisher's exact test, $p > 0.05$), and blood morphotic parameters (ANOVA with Dunn's post-test, $p > 0.05$).

2.2. Whole blood cultures

Whole blood cultures were conducted using peripheral blood (7 mL) in 24-well culture plates (Nunc, Roskilde, Denmark; 1000 μL/well) for 48 h at 37°C with 5% CO_2. Two types of whole blood culture were developed: (1) blood cell cultures stimulated with Peptivator SARS-CoV-2 virus peptides from MiltenyiBiotec (Bergisch Gladbach, Germany; 1 mg/well) and live *M. bovis* BCG Moreau mycobacteria (Biomed Lublin; 10^6 cells/culture) conducted in parallel with cultures stimulated only with SARS-CoV-2 antigens or live *M. bovis* BCG and (2) blood cell cultures stimulated with PepTivator® RSV peptides from MiltenyiBiotec (Bergisch Gladbach; 1 μg/well) and live *M. bovis* BCG mycobacteria conducted in parallel with cultures stimulated with RSV peptides only or live BCG (Thieme et al. 2020; Sir Karakus et al. 2021). In addition, culture controls were carried out in RPMI 1640 (Sigma-Aldrich, Gillingham, UK) and PHA (10 μg/well) medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Whole blood cells were stimulated with peptides for 48 h, then harvested and frozen at –80°C until mRNA expression and protein secretion were measured.

2.3. Measurement of IFN-α2 and IFN-β in serum and supernatants of peripheral whole blood cultures

The IFN-α2 and IFN-β concentrations in serum and supernatants were measured using a Human IFN-α2/IFN-β

DuoSet® Enzyme-Linked Immunosorbent Assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples of supernatants were diluted at a ratio of 1:5 in assay diluent (10% bovine serum albumin in phosphate buffered saline) to optimize the expected IFN-α2/IFN-β concentrations to the range of the standard curve and tested in duplicate. In summary, 50 µL of a diluted Human IFN-α2/IFN-β Capture Antibody (2 µg/mL) was added to each well and incubated overnight at room temperature. After washings, 150 µL of assay diluent was added to block the plates and then incubated for 2 h at room temperature. The plates were washed and then 50 µL of the recombinant Human IFN-α2/IFN-β standard in the concentration range of 200–3.13 pg/mL for IFN-α2 and 500–7.81 pg/mL for IFN-β or serum/diluted supernatants was added to the wells and incubated for 2 h at room temperature. After washing, 50 µL of Human IFN-α2/IFN-β Detection Antibody (125 ng/mL, 250 ng/mL) was added and incubated for 2 h at room temperature. In the next step, 50 µL/well of streptavidin coupled with horseradish peroxidase (Streptavidin-HRP, R&D Systems, Minneapolis, MN, USA), diluted at 1:40 in the assay diluent, was added and incubated for 20 min at room temperature. In the final step of the assay, 50 µL each of substrate mixtures A and B were added to the wells of the plate in a 1:1 ratio. The enzymatic reaction was stopped by adding 25 µL of 1M H_2SO_4 solution to the wells. The optical densities were read at 450 nm in 10 min using an ELISA plate reader (Multiskan EX, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.4. Measurement of IFN-γ in serum and supernatants from peripheral whole blood cultures

The IFN-γ concentrations in serum and supernatants were measured using a QuantiFERON – TB Gold Plus ELISA (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, in the first step of the assay, 50 µL of a diluted solution of mouse anti-human IFN-γ antibody conjugate with horseradish peroxidase (HRP) was applied to the wells, and then 50 µL of each dilution of the IFN-γ standard or tested samples (serum and supernatants diluted 1:5). After application, the plate was covered with a lid, shaken on a microplate shaker for 1 min, and then incubated at room temperature and in the dark for 2 h. After washing the wells 6 times with wash buffer, 100 µL of Enzyme Substrate Solution was added to each well, and the plates were incubated for 30 min at room temperature in the dark. After this time, 50 µL of Enzyme Stopping Solution was added to each well, and then the optical density of each sample was measured using an ELISA plate reader (Multiskan EX, Thermo Fisher Scientific) equipped with a 450 nm filter.

2.5. RNA isolation

Isolation of RNA obtained from whole blood culture pellets was carried out using a commercial QIAamp® RNA Blood Mini kit (Qiagen). The isolation process was fully compliant with the manufacturer's guidelines. Genetic material was isolated from 500 µL of culture sediment. Part of the extracted RNA was used to obtain complementary DNA (cDNA) immediately after the isolation process; the rest of the genetic material was stored at –80°C until analyzed.

2.6. Spectrophotometric evaluation of isolated RNA and gel visualization

To assess the quality and quantity of nucleic acids obtained, RNA concentration was measured after the isolation procedure using a NanoDrop device (BioDrop, Holliston, Massachusetts, USA) preparing an additional blank containing water to calibrate the device. To visualize the RNA, electrophoresis was carried out in a 1.2% agarose gel in Tris-Acetate-EDTA (TAE) buffer at 90 V for 60 min, and the images were then documented on a Gel-Doc 2000 system (Bio-Rad, Hercules, California, USA) equipped with Quantity One software (Bio-Rad).

2.7. Reverse transcription

The cDNA was synthesized using the iScript® cDNA Synthesis Kit (Bio-Rad). In the first step of the reverse transcription reaction, 1 µg of matrix RNA, previously checked for quality and integrity, was transferred from the isolated sample into 0.2 µL Eppendorf tubes. Then the reagents necessary for cDNA synthesis were added according to the manufacturer's proportions, resulting in a mixture with a final volume of 10 µL. The reverse transcription reaction was carried out in a Biometra UNO II Thermocycler (Analytik Jena, Germany) under conditions according to the test manufacturer's guidelines. The resulting cDNA was stored at –20°C until analysis.

2.8. quantitative polymerase chain reaction (qPCR) reaction

The quantitative polymerase chain reaction (qPCR) reaction was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The reaction mixture contained 5 µL of iTaq universal SYBR Green Supermix, 1 µL of primer (concentration), 3.5 mL of nuclease-free water, and 1 µL of cDNA. SYBR® Green Assay IFNA5, IFNG, IFNB (Bio-Rad) primers were used. The primer sequences were selected to ensure the specificity of the amplification reaction and were purchased from Bio-Rad. Amplification was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad), using the following

cycles: initial activation step (95°C for 2 min), followed by 40 cycles of denaturation at 95°C for 5 s, annealing/extension (60°C for 30 s). A cycle of the dissociation step (65°C for 5 s, followed by 0.5°C for 5 s to 95°C) was added to the melting curve analysis. We selected *GAPDH* and *HPRT1* as our reference genes for this study. These genes demonstrated the lowest *M* values, which indicates their high stability in gene expression, making them the most reliable internal controls for accurate normalization of data (Wawrocki et al. 2020). All qRT-PCR experiments were performed in three technical replicates. Gene expression analysis was performed using the comparative method (ΔΔCt) to determine the relative expression levels of selected mRNAs. This method is based on calculating the differences in the expression levels of the test gene and the reference gene. The calculation uses the qPCR reaction's threshold cycle (Ct) values. Ct values were determined for both test and reference genes in the test samples, for which the differences between each Ct value (ΔCt) were then calculated.

2.9. Statistical analysis

The expression of genes between the study groups was compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. The correlation between mRNA expression and IFN level was analyzed using the Spearman's correlation test. A *p*-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) software.

3. Results

3.1. The serum level of IFN-α, IFN-β and IFN-γ in the study groups

Figure 1 shows the average levels of IFN-α, IFN-β, and IFN-γ in the sera of each study group. The highest mean level of IFN-α (9.26 ± 7.22 pg/mL) was observed in children seropositive for SARS-CoV-2 (SARS-CoV-2(+)). This level was significantly higher than the serum IFN-α levels observed in the other study groups (Figure 1A). Similarly, the serum concentrations of IFN-γ were significantly higher in the SARS-CoV-2(+) group (32.23 ± 19.99 pg/mL) compared with the RSV(+) (12.15 ± 10.37) or RSV(+)SARS-CoV-2(+) (11.84 ± 7.34 pg/mL) groups (Figure 1C). On the contrary, the average concentrations of IFN-β observed in the studied groups were very similar to each other, with no significant differences (Figure 1B).

3.2. Levels of IFN-α, IFN-β, and IFN-γ in the whole blood culture supernatants

The mean concentrations of IFN-α, IFN-β, and IFN-γ in each study group were assessed in supernatants collected from whole peripheral blood cultures stimulated with, respectively: (1) live *M. bovis* BCG mycobacteria (BCG), (2) SARS-CoV-2 virus proteins (SARS-CoV-2), (3) RSV virus proteins (RSV), (4) live BCG mycobacteria and SARS-CoV-2 virus proteins (BCG + SARS-CoV-2), and (5) BCG mycobacteria and RSV virus proteins (BCG + RSV).

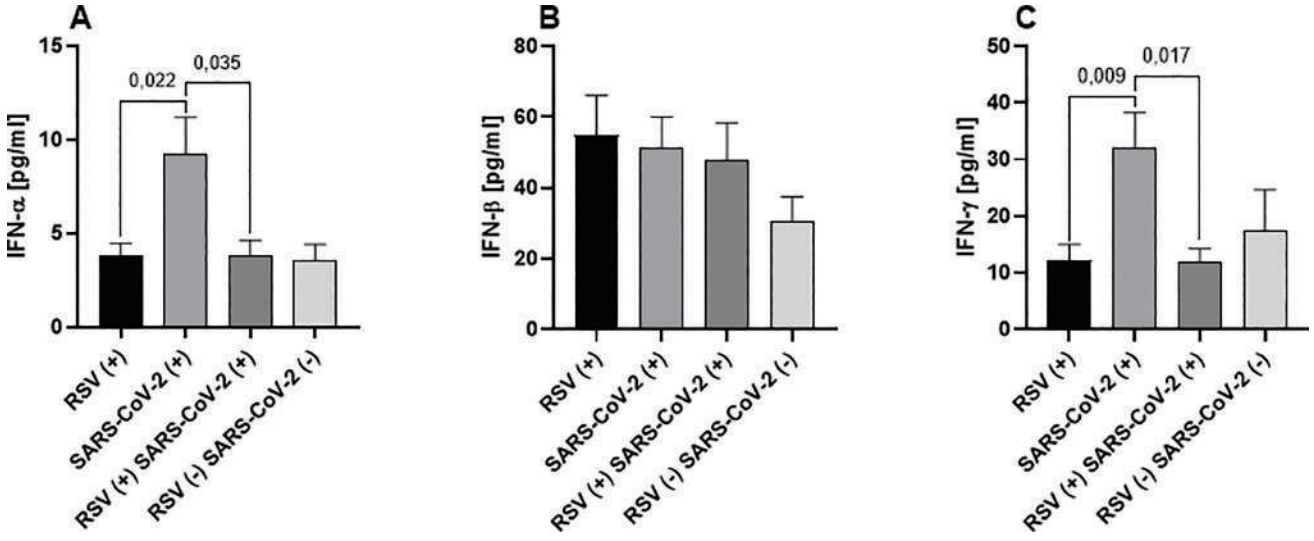

Fig 1. *The serum level of IFN-α (**A**), IFN-β (**B**), and IFN-γ (**C**) in the study groups. Differences in the levels of IFN-α, IFN-β, and IFN-γ concentrations between the studied groups were compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. A p-value was considered significant if <0.05. A result of > 0.05 was not statistically significant. IFN, interferon; RSV(-) SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV(+) SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(+), group seropositive for RSV infection; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.*

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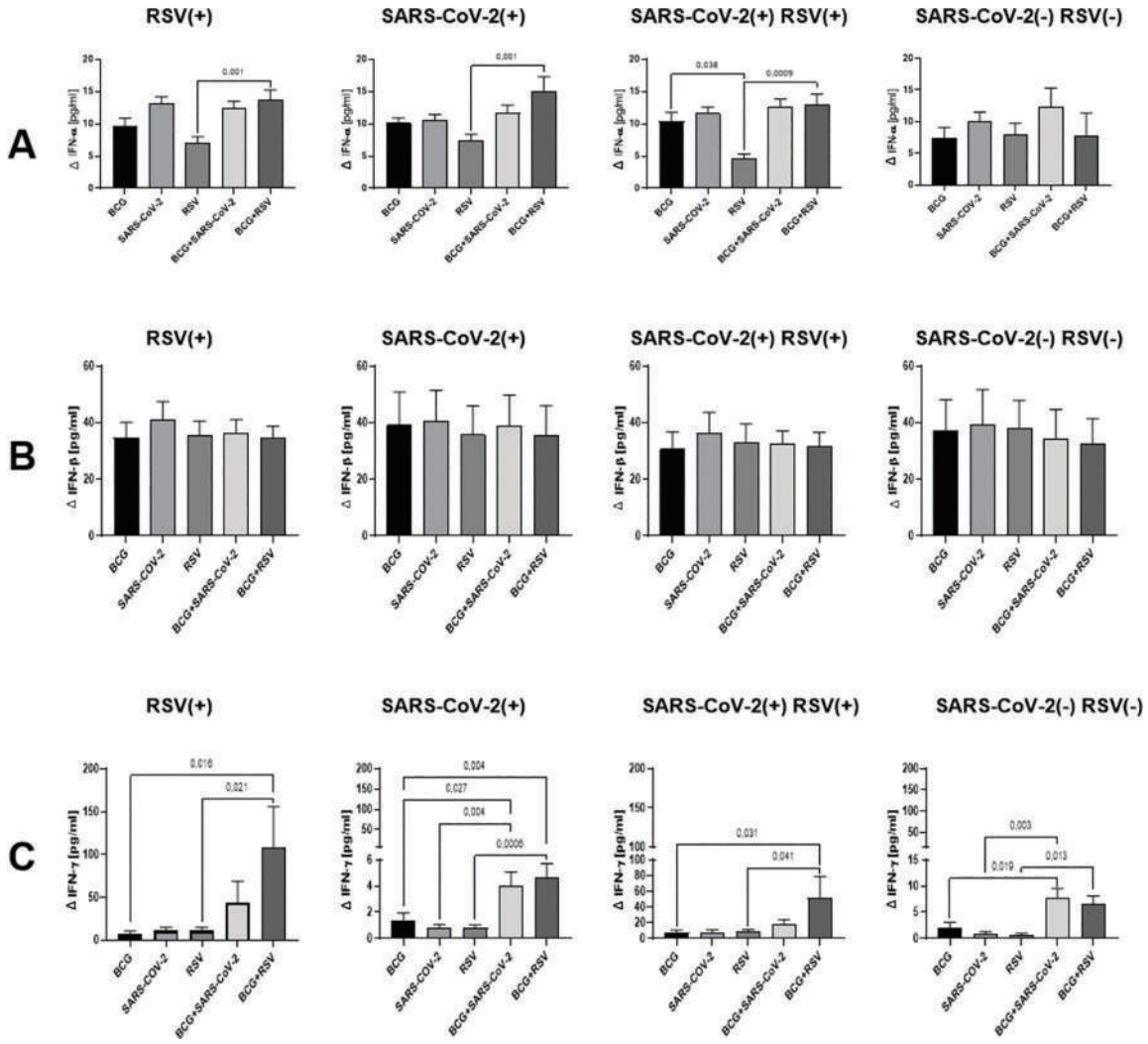

Fig 2. *Levels of IFN-α (**A**), IFN-β (**B**), and IFN-γ (**C**) in cell culture supernatants across study groups. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures. The value on the y-axis is the delta value relative to the unstimulated control sample. Differences in the levels of IFN-α, IFN-β, and IFN-γ concentrations between the studied groups were compared using the non-parametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. A p-value was considered significant if <0.05. BCG, bacillus Calmette–Guérin; IFN, interferon; RSV(-) SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV(+) SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(+), group seropositive for RSV infection; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.*

Additionally, each experimental setup included a control, where the blood culture was carried out without any stimulators. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures.

3.2.1. IFN-α levels in whole blood culture supernatants

Figure 2A illustrates the average IFN-α concentrations in the cell culture supernatants across different study groups. For the study groups with RSV or/and SARS-CoV-2 infection (RSV(+); SARS-CoV-2(+); RSV(+)SARS-CoV-2(+)), the highest mean IFN-α levels were observed in supernatants from BCG-co-stimulated cultures with RSV peptides (13.77 ± 5.42 pg/mL; 14.94 ± 9.63pg/mL; 12.97 ± 5.33pg/mL, respectively). Conversely, in the group without RSV and SARS-CoV-2 infections (RSV(-)SARS-CoV-2(-)), the highest mean IFN-α concentration was noted in supernatants from BCG-co-stimulated cultures with SARS-CoV-2 peptides (12.32 ± 7.28 pg/mL). Notably, live BCG mycobacteria significantly increased IFN-α production induced by RSV antigens in the three infected groups. However, in the group of children without RSV and SARS-CoV-2 infections, although there was an elevation in IFN-α levels in cultures with live

BCG mycobacteria and SARS-CoV-2 antigens, the differences were not statistically significant.

3.2.2. IFN-β levels in whole blood culture supernatants

Figure 2B shows the mean levels of IFN-β measured in the cell culture supernatants from each of the four study groups. The analysis revealed that the IFN-β levels are consistent across all groups, with no statistically significant differences observed in the measured values.

3.2.3. IFN-γ levels in whole blood culture supernatants

Figure 2C shows the average concentrations of IFN-γ in the cell culture supernatants from different study groups. Among subjects seropositive for RSV/SARS-CoV-2, the highest IFN-γ levels were observed in cultures stimulated with both live BCG and RSV antigens, with concentrations as follows: RSV(+) Group: 107.30 ± 53.18 pg/mL; SARS-CoV-2(+) Group: 4.66 ± 3.34 pg/mL; and RSV(+) SARS-CoV-2(+) Group: 51.51 ± 67.56 pg/mL. Conversely, in the group of children without RSV and SARS-CoV-2 infections, the peak levels of IFN-γ were detected in cultures stimulated with both BCG and SARS-CoV-2 antigens, registering at RSV(-) SARS-CoV-2(-) Group: 6.49 ± 3.25 pg/mL. Across all groups, cultures exposed to live BCG mycobacteria along with RSV antigens consistently showed a significantly enhanced production of IFN-γ. Additionally, co-stimulated cultures with BCG and SARS-CoV-2 antigens exhibited significantly higher IFN-γ production compared with those stimulated solely with SARS-CoV-2 antigens in both the groups with seropositive for SARS-CoV-2 infection and in the group seronegative for virus. Moreover, significantly elevated IFN-γ production was noted in co-stimulated cultures with both BCG and RSV compared with cultures with only live BCG mycobacteria in all groups seropositive for both viruses. This trend was also observed in the group seropositive for SARS-CoV-2 and in the group seronegative for both viruses, where IFN-γ levels were notably higher in co-stimulated cultures with BCG and SARS-CoV-2 antigens compared with those stimulated with BCG alone.

3.3. mRNA expression of IFN-α, IFN-β, and IFN-γ

The relative mRNA expression levels of IFN-α, IFN-β, and IFN-γ were assessed in each study group using sediments from whole peripheral blood cultures stimulated with, respectively: (1) live *M. bovis* BCG mycobacteria (BCG), (2) SARS-CoV-2 virus proteins (SARS-CoV-2), (3) RSV virus proteins (RSV), (4) live BCG mycobacteria and SARS-CoV-2 virus proteins (BCG + SARS-CoV-2), and (5) BCG mycobacteria and RSV virus proteins (BCG + RSV). In each experimental setup, a control was included where the whole blood culture was conducted without any stimulators. The data presented in the graphs illustrate the differences in relative mRNA expression levels between the stimulated and unstimulated cultures.

3.3.1. mRNA expression of IFN-α

Figure 3A depicts the relative mRNA expression levels of IFN-α in cultured cell pellets. Notably, the highest expression levels of IFN-α mRNA were found in the SARS-CoV-2(+), RSV(+)SARS-CoV-2(+), and RSV(−)SARS-CoV-2(−) groups in co-stimulated cultures of live mycobacteria BCG and RSV antigens (60.97 ± 97.53; 6.19 ± 14.01; 128.50 ± 26.42, respectively). A significant elevation in IFNA5 expression was observed in co-stimulated cultures with both BCG and RSV antigens compared with those stimulated solely with RSV in the groups positive for SARS-CoV-2 and negative for both RSV and SARS-CoV-2. Conversely, in children seropositive for RSV infection, the highest relative expression levels of IFNA5 were observed in co-stimulated cultures of live mycobacteria BCG with SARS-CoV-2 antigens, which were significantly elevated compared with cultures stimulated with only SARS-CoV-2 antigens. Additionally, in the SARS-CoV-2 seropositive group, a marked increase in IFNA5 production was noted in co-stimulated cultures with both BCG and RSV compared with those stimulated with BCG alone.

3.3.2. mRNA expression of IFN-β

Figure 3B illustrates the relative expression levels of IFN-β mRNA in cultured cell pellets. Among all study groups, the highest relative expression levels of IFN-β mRNA were observed in co-stimulated cultures with both BCG mycobacteria and RSV antigens (respectively: 19.84 ± 34.29; 23.97 ± 39.47; 4.11 ± 10.79; 34.15 ± 21.63). Specifically, in the groups with either RSV infection, SARS-CoV-2 infection, or neither (RSV(+); SARS-CoV-2(+) and RSV(−)SARS-CoV-2(−)), there was a significant enhancement of IFN-β mRNA expression in co-stimulated cultures with both BCG and RSV compared with cultures stimulated with BCG or RSV antigens alone. However, in the group of children co-infected with RSV(+) and SARS-CoV-2(+), no statistically significant differences in IFN-β mRNA expression levels were observed.

3.3.3. mRNA expression of IFN-γ

Figure 3C shows the relative expression levels of IFN-γ mRNA in cultured cell pellets. Across all study groups, the highest relative levels of IFN-γ mRNA expression were observed in co-stimulated cultures with both mycobacteria BCG and RSV antigens (15.91 ± 20.73; 14.98 ± 19.33; 17.19 ± 23.13; 26.02 ± 16.20). Notably, in groups seropositive for both

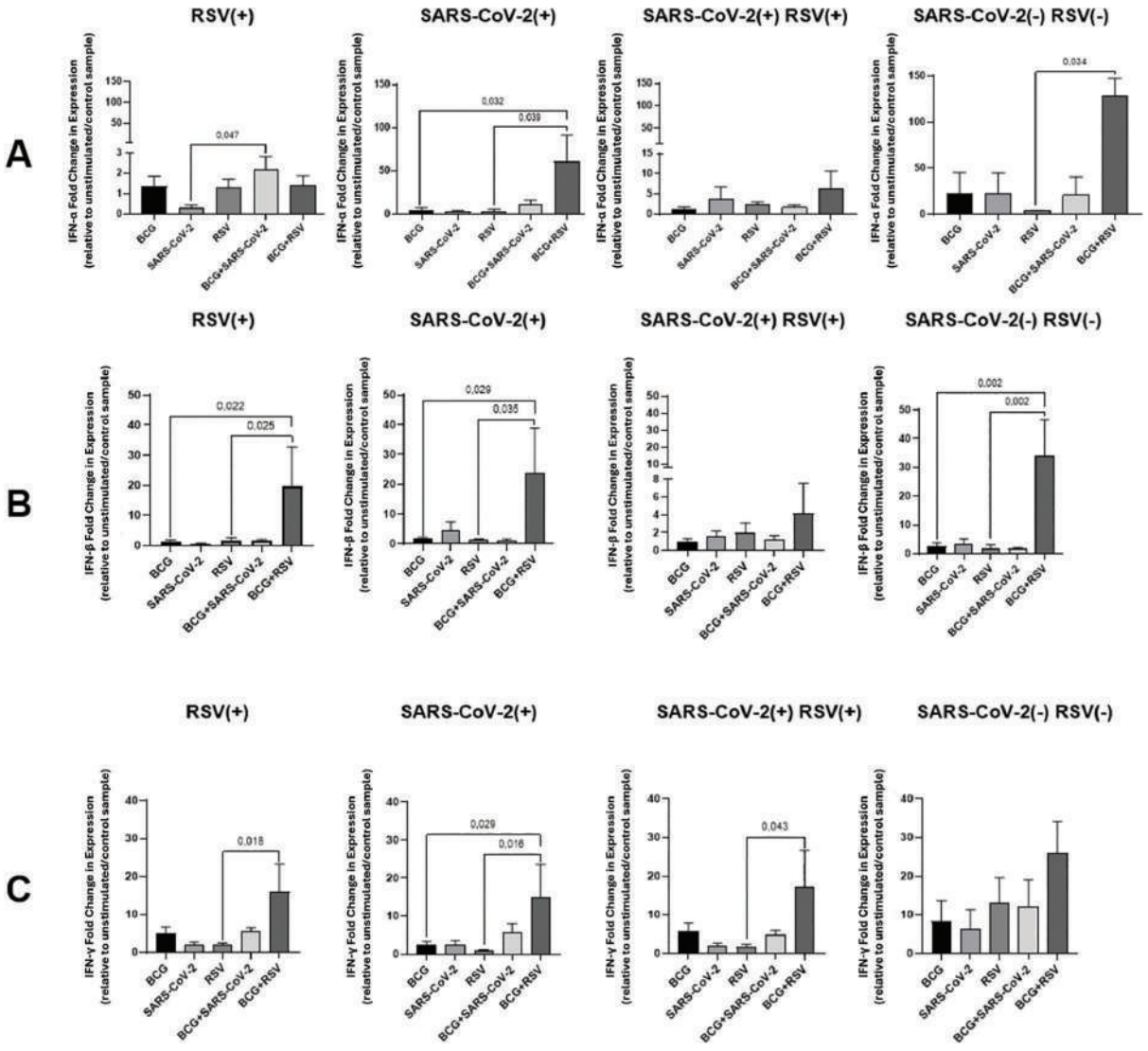

Fig 3. *Differential expression of IFN-A5 mRNA (**A**), IFN-β mRNA (**B**), and IFN-γ mRNA (**C**) in co-stimulated cultures with BCG and viral antigens among study groups. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures. The value on the y-axis is the fold change from the unstimulated control. Differences in the levels of IFN-γ concentrations between the studied groups were compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. A p-value was considered significant if <0.05. BCG, bacillus Calmette–Guérin; IFN, interferon; IFNA5, interferon-alpha 5; RSV(+), group seropositive for RSV infection; RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(-)SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.*

viruses, there was a significantly higher relative expression of IFN-γ mRNA in co-cultures stimulated with both BCG and RSV antigens compared with those stimulated with only RSV antigens. Additionally, in the group of children seropositive for SARS-CoV-2, a significantly higher relative expression of IFN-γ mRNA was found in BCG + RSV co-stimulated cultures than in cultures stimulated with BCG alone. However, in the groups of children seronegative for RSV or SARS-CoV-2 (RSV(-)SARS-CoV-2(-)), no statistically significant differences in IFN-γ mRNA expression were observed.

3.4. Relationship between mRNA expression and IFNs level in response to BCG and viral antigens

Our studies indicate that the correlation between mRNA expression and IFNs level varies depending on the type of IFN and the stimulus used (Figure 4). In stimulated cultures, IFN-γ levels consistently show a positive correlation with mRNA expression. We observed a statistically significant correlation for IFN-γ for cultures stimulated with BCG (Figure 4K) and RSV (Figure 4M) antigens. For IFN-β, a negative correlation was noted in most of

73, 7 (2025), DOI: 10.2478/aite-2025-0007

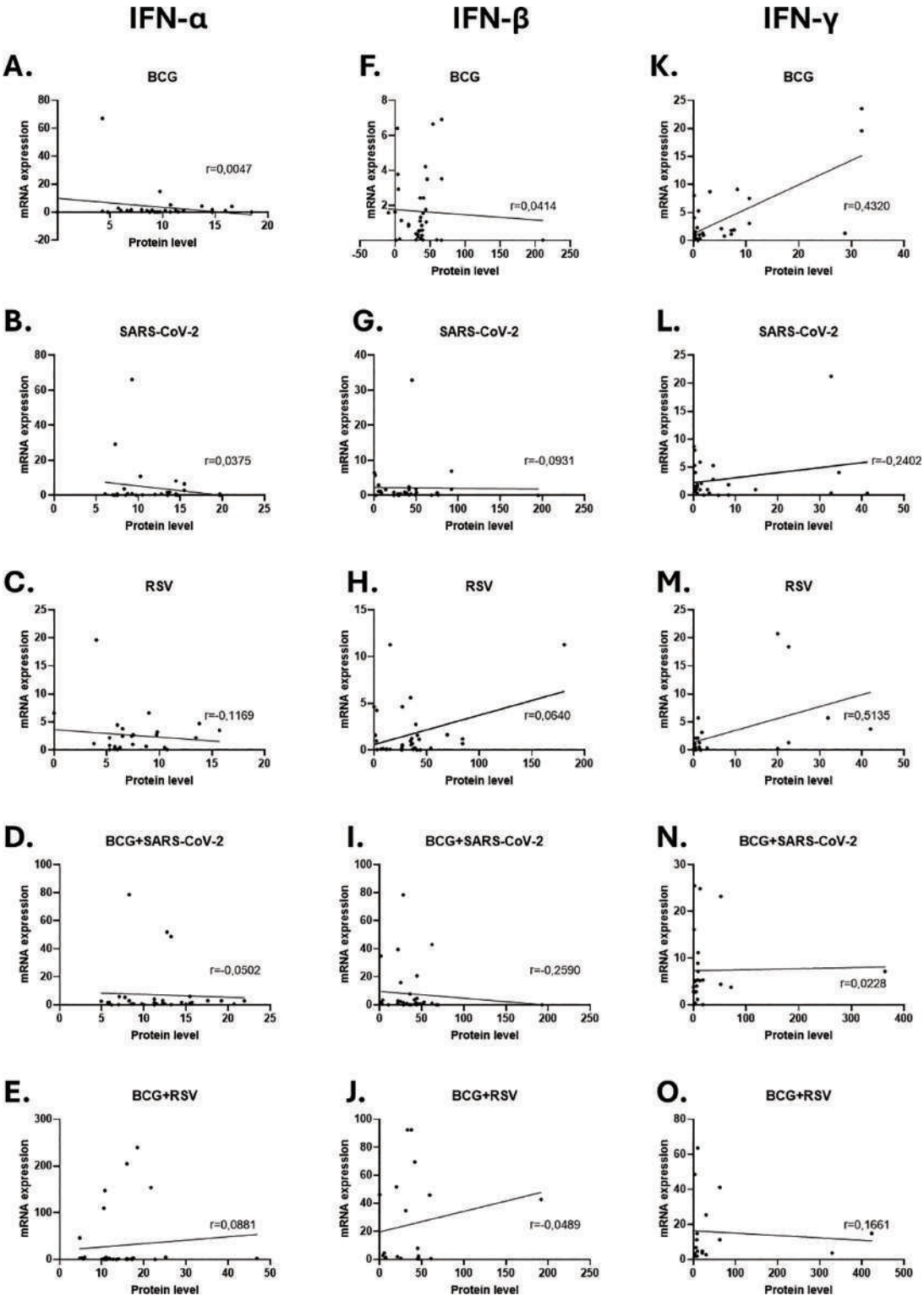

Fig 4. *Correlation between mRNA expression and IFNs level in co-stimulated cultures with BCG and viral antigens among study groups. The correlation between mRNA expression and IFN level was analyzed using the Spearman's correlation test. BCG, bacillus Calmette–Guérin; IFN, interferon; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. IFN-α mRNA expression in whole blood cultures stimulated with antigens: A-BCG, B-SARS-CoV-2, C-RSV, D-BCG+SARS-CoV-2, E-BCG+RSV. IFN-β mRNA expression in whole blood cultures stimulated with antigens: F-BCG, G-SARS-CoV-2, H-RSV, I-BCG+SARS-CoV-2, J-BCG+RSV. IFN-γ mRNA expression in whole blood cultures stimulated with antigens: K-BCG, L-SARS-CoV-2, M-RSV, N-BCG+SARS-CoV-2, O-BCG+RSV.*

the variants tested (Figure 4G, I, and J), suggesting a potential mechanism to inhibit protein secretion when mRNA expression increases. In other cases, no relationship was seen between mRNA expression and protein secretion (Figure 4F and H). In the case of IFN-α cultures stimulated with BCG, SARS-CoV-2 and BCG + RSV antigens showed no correlation between mRNA expression and protein secretion (Figure 4A, B, and E). However, a negative correlation between mRNA expression and IFN-α secretion was observed in cultures stimulated with RSV and BCG + SARS-CoV-2 (Figure 4C and D).

4. Discussion

To elucidate the role of the BCG vaccine in modulating the immune response to viral infections, it is important to examine the multifaceted aspects of immune system activation, training, and response modulation that BCG offers. Trained immunity through BCG involves epigenetic and metabolic reprogramming of innate immune cells, especially monocytes and macrophages, resulting in increased and more rapid production of pro-inflammatory cytokines during subsequent infections. These long-lasting effects may provide nonspecific protection against a variety of pathogens beyond *Mycobacterium tuberculosis* (Moorlag et al. 2019; Faustman et al. 2022). Furthermore, research suggests that BCG may alter the bone marrow microenvironment, enhancing the functionality of hematopoietic stem cells and progenitors, and thereby expanding the scope of immune defense (Arts et al. 2018).

In particular, our research focused on the effect of the BCG vaccine on the production of IFNs, which play a key role in the antiviral response. Type I interferon, which includes IFN-α, is known to be a key element involved in virus clearance and regulation of the host immune response. Several studies have shown that a strong IFN-α response is associated with protection and a milder course of RSV infection in infants (Hijano et al. 2019). Unfortunately, it is known that the innate immune response, especially the production of type I interferons, is suppressed by RSV in infants, which can lead to serious disease progression (Ramaswamy et al. 2006). In our study, stimulation with RSV peptides alone resulted in detectable levels of IFN-α, which were higher than those observed in unstimulated cultures. However, the magnitude of this induction was relatively low compared with other stimulation conditions, suggesting that RSV peptides alone have a limited capacity to induce IFN-α production. This may indicate that additional signals or co-stimulated cultures with other immune activators are necessary to elicit a more robust IFN-α response. These findings underscore the complexity of IFN induction pathways and highlight that RSV peptides alone may not fully engage the immune mechanisms required for strong type I interferon production. Upon assessing the effect of BCG mycobacteria on the production of type I interferon (IFN-α) in response to RSV and SARS-CoV-2 virus antigens, significant discrepancies were observed in culture supernatants. It was found that the administration of live BCG bacilli increases the production of IFN-α in response to RSV antigens in all groups except the uninfected cohort. A particularly marked difference in the production of IFN-α was observed in blood cultures of children from the SARS-CoV-2(+) and SARS-CoV-2(-) RSV(-) groups. This indicates that the BCG vaccine can specifically modulate the immune response. The literature suggests that RSV can hinder signaling of the IFN-α synthesis, potentially reducing its production (Ramaswamy et al. 2006). Conversely, when cultures were performed in the presence of SARS-CoV-2 virus antigens, live BCG mycobacteria did not induce an increase in IFN-α levels compared with cultures stimulated with antigens alone. While BCG triggers immune responses to various pathogens, our data on IFN-β and IFN-γ indicate that these mechanisms are not distinctly different for each pathogen.

Interferons play a crucial role in defending against SARS-CoV-2. It has been shown that IFN-α can inhibit viral replication, but SARS-CoV-2 may suppress IFN-α signaling, and genetic variations in the *IFNRA2* gene are linked to disease severity (Vanderheiden et al. 2020; Bessière et al. 2021; Akter et al. 2022; Znaidia et al. 2022). However, SARS-CoV-2 has developed strategies to evade host responses by suppressing type I interferon signaling (Beyer and Forero 2022). For example, angiotensin-converting enzyme 2, the entry receptor of SARS-CoV-2, can interfere with the IFN-α signaling pathway, inhibiting its production and thus facilitating viral infection (Chen et al. 2022). Although the benefits of prophylactic administration of IFN-α in the early stages of the disease are clear, questions remain regarding the feasibility of using IFN-α in patients with severe COVID-19. Despite initial reports indicating decreased IFN production, emerging evidence suggests that people with severe COVID-19 have a sustained response to type I IFN. This is in contrast to the delayed and potentially suppressed response to IFN observed in the early stages of infection. The study found no significant differences in IFN-α levels between cultures stimulated with SARS-CoV-2 antigens alone and cultures stimulated with live BCG bacilli and SARS-CoV-2 antigens. However, in the group seropositive for RSV, the expression of IFNA5 mRNA was significantly higher in cultures stimulated with live BCG bacilli and SARS-CoV-2 antigens compared with cultures stimulated with SARS-CoV-2 antigens alone. The lack of differences in IFN-α secretion may suggest post-transcriptional mechanisms regulating the secretion or translation of this IFN. This phenomenon may explain the observed blocking of the secretion of larger amounts of IFN-α protein, despite higher expression of IFN-α mRNA. Additionally, the immune system may have regulatory mechanisms that limit excessive production of IFN-α despite elevated mRNA levels to prevent an excessive inflammatory response that may be

harmful. The results indicate that stimulation of *IFN-α* gene transcription by live BCG mycobacteria in combination with SARS-CoV-2 antigens is due to higher mRNA expression. However, alternative mechanisms at the post-transcriptional level may impede both the production and secretion of the IFN-α protein. It is noteworthy that the significant increase in mRNA levels for the three IFNs tested after stimulation with BCG and RSV antigens provides insight into transcriptional activation of immune pathways. The discrepancy between mRNA levels and protein production highlights a potential role for post-transcriptional regulation, such as mRNA stability, translation efficiency, or protein degradation. Further analysis of post-transcriptional mechanisms and secretion studies may help to clarify these issues.

Previous studies have shown that IFN-β is a key component of the immune response to RSV infection, modulating immunity and inhibiting viral replication (Antunes et al. 2019; Hijano et al. 2019). Notably, Lo et al. (2005) demonstrated that RSV develops mechanisms to attenuate the host response to IFN-β. RSV unstructured proteins (NS1 and NS2) have been observed to downregulate Stat2, a key player in the IFN-α/β signaling pathway, thereby interfering with the IFN response and facilitating host evasion. The results of our study support this hypothesis, as IFN-β secretion levels in our experimental cultures were low and did not differ significantly from those in the control groups. This indicates that the stimulation conditions used in our experiments may not have been sufficient to elicit a robust IFN-β response or that variability among replicates may have obscured any potential differences. These findings suggest that additional factors or alternative experimental approaches might be required to better characterize IFN-β production under these conditions. Moreover, in the context of SARS-CoV-2 infection, IFN-β plays a pivotal role in the host immune response. A study by Li et al. (2020) showed that the ORFF6 and ORF8 proteins, as well as the nucleocapsid proteins of the SARS-CoV-2 virus, can inhibit the IFN-β signaling pathways, thereby enhancing the infection. In the present study, no significant differences were observed in the levels of IFN-β secretion and expression across the experimental conditions. This could suggest the involvement of regulatory mechanisms that limit IFN-β production, such as feedback inhibition by suppressor of cytokine signaling proteins or the action of regulatory cytokines, which are known to modulate IFN responses. Alternatively, it is possible that the stimulation conditions used, including the duration or type of stimuli, were not sufficient to robustly induce IFN-β expression, allowing such inhibitory mechanisms to dominate. These findings highlight the complexity of IFN-β regulation and suggest that further investigation is needed to elucidate the specific factors influencing its production in this context.

The BCG vaccine has gained attention for its potential role in enhancing antiviral immunity, particularly through modulating the IFN-γ response (Weir et al. 2006; Lalor et al. 2010, 2011; Corral-Fernández et al. 2016). Our study showed that BCG increased the production of IFN-γ in co-stimulated cultures with RSV or SARS-CoV-2 antigens. This aligns with previous research indicating that BCG boosts IFN-γ production even against non-mycobacterial infections (Kleinnijenhuis et al. 2012, 2014). IFN-γ stimulates antigen presentation by inducing the expression of major histocompatibility complex (MHC) molecules and promotes the cytotoxic activity of virus-specific Natural killer (NK) cells and T cells. The early IFN-γ response plays a key role in influencing the course of viral infection. Consequently, decreased production of IFN-γ during RSV infection may serve as a determinant of disease severity. Bont et al. (2001) showed that the severe course of the RSV virus is associated with reduced secretion of IFN-γ in the blood. Furthermore, Aberle et al. (1999) observed a reduction in IFN mRNA expression in the context of RSV-induced bronchiolitis. Our study also demonstrated the lack of strong IFN-γ secretion and IFN-γ mRNA expression in whole blood cultures stimulated with isolated RSV antigens. However, in co-stimulated cultures, live BCG mycobacteria significantly increased IFN-γ production in response to RSV antigens, which may indicate that the BCG vaccine may specifically modulate the immune response and provide protection against RSV. It is worth noting the potential interaction between prior RSV infection and the immunomodulatory effects of BCG vaccination. One possible explanation is that RSV, as a respiratory virus with distinct immunological signatures, may elicit a more robust activation of trained immunity pathways when combined with BCG-induced immune modulation. This is supported by the observed increases in interferon production, particularly IFN-γ, which is known to play a key role in antiviral defenses. The study by Eichinger et al. (2015) in an animal model provides evidence that treatment with IFN-γ protects against RSV infection. This shows that IFN-γ is of great importance in reducing viral load and preventing serious disease progression.

BCG has also been proposed to prevent SARS-CoV-2 infection due to its immunostimulatory properties. Studies show that BCG enhances IFN secretion, potentially benefiting COVID-19 patients (Nguyen et al. 2021; van Laarhoven et al. 2021). Hilligan et al. observed that intravenous BCG vaccination in mice leads to a sustained strong IFN-γ response in the lungs, which may promote early control of SARS-CoV-2 replication (Hilligan et al. 2022, 2023). Similarly, our study found higher IFN-γ levels in BCG + SARS-CoV-2 co-stimulated cultures, indicating that BCG stimulates T cells to produce IFN-γ, counteracting SARS-CoV-2's suppression of IFN responses. Differences in IFN-γ levels between RSV/SARS-CoV-2 seropositive and RSV seropositive groups suggest complex interactions in co-infections. The observed differences are consistent across independent experimental replicates, suggesting a reproducible pattern rather than

random variation. Small changes in the levels of cytokines, such as IFN-γ, can have a disproportionately large effect on the immune response. Experimental conditions designed to simulate viral infection scenarios can amplify subtle changes in cytokine expression, highlighting a context-dependent significance that requires further study. It is conceivable that an increased IFN response may have a beneficial effect on the response to viral infections, but it is important to remember that an excessive pro-inflammatory response, including the production of type I IFN, can be detrimental, causing a cytokine storm. Our findings suggest that co-infection with RSV and SARS-CoV-2 affects the BCG-induced immune response, particularly IFN-γ production. We observed lower IFN-γ levels in the RSV/SARS-CoV-2 co-infected group compared with the RSV-only group. One possible explanation for this observation is immune interference between the two viruses. SARS-CoV-2 may alter the immune response to RSV, potentially through immune exhaustion, which can impair the ability to respond effectively to both pathogens. Alternatively, BCG-induced responses may redirect focus in the presence of dual infections, reducing IFN-γ production for RSV. These mechanisms warrant further investigation, especially for vaccine-based therapies like BCG in the context of co-infections.

Our results showed significantly higher serum levels of IFN-α and IFN-γ in the SARS-CoV-2 seropositive group, suggesting a preactivated immune state. The elevated levels of IFN-α and IFN-γ in the serum of SARS-CoV-2(+) individuals likely reflect the heightened immune activation associated with the infection. IFN-α is a type I interferon known to play a key role in the antiviral response by promoting viral clearance and activating innate immune cells, while IFN-γ, a type II interferon, is primarily produced by activated T cells and NK cells to enhance adaptive immunity and macrophage activation. The observed increase in these cytokines may be attributed to the host's attempt to control viral replication and modulate the immune response during infection. However, persistent elevation of these cytokines has also been associated with hyperinflammatory states and disease severity in COVID-19. This dual role highlights the complexity of IFN responses in SARS-CoV-2 infection. Differences in the immune response to RSV and SARS-CoV-2 have been observed in different age groups. Children generally show a stronger immune response to SARS-CoV-2 than adults. This may be influenced by the initial immune training following BCG vaccination, which is common in many countries for children. The distinct pathophysiological characteristics of these viruses, with RSV having a more pronounced impact on the lower respiratory tract in young children than SARS-CoV-2, may be influenced in part by the trained immune profile established by BCG vaccination early in life. The results of our study indicate that the BCG vaccine may have an immunomodulatory effect on the IFN response induced by SARS-CoV-2 and RSV viruses. The different magnitudes of the observed responses may indicate the existence of separate immunological mechanisms stimulated by BCG in response to different pathogens. Further research on broad population groups is necessary to clarify the molecular basis of the observed phenomenon and its potential clinical significance, especially in the context of using the BCG vaccine as a regulator of the immune response to viral infections. Our *in vitro* model provides a controlled environment to evaluate the effects of the BCG vaccine in combination with viral antigens. While it does not entirely mimic actual physiological conditions, it enables a focused analysis of molecular and immunological mechanisms underlying vaccine–antigen interactions. These insights could have significant implications for clinicians, particularly in designing effective immunotherapeutic strategies and understanding immune modulation induced by such vaccine combinations. By delineating the immune pathways activated in this controlled setting, the study lays the groundwork for translating these findings into clinically relevant applications. Our study does not recommend BCG vaccination during active viral infection, but rather investigates the immunomodulatory potential of BCG-trained immunity *in vitro* as a proof-of-concept.

The observed uniformity in cytokine production patterns across seropositive and seronegative individuals suggests that in our *in vitro* experimental settings, cytokine responses were more dependent on the stimuli used than on immune memory or prior exposure history. This result aligns with the hypothesis that innate immune responses, such as those triggered *in vitro*, may overshadow or mask the contributions of adaptive immune memory in this context. The controlled environment of our experiments and the specific nature of the stimuli (RSV or SARS-CoV-2 antigens) may not have been sufficient to differentiate the nuanced memory responses that might occur *in vivo*. Additionally, innate immune responses, such as those associated with trained immunity or cross-reactivity, may have overshadowed adaptive memory effects in our assays. It is reasonable to hypothesize that individuals with prior exposure (seropositive) might exhibit either a quantitative or qualitative difference in cytokine response compared with seronegative individuals due to immune memory. One might expect higher cytokine production in RSV(+) individuals, particularly in responses involving memory T cells or other adaptive immune components. However, it is also possible that memory-driven responses would differ in their cytokine profile rather than magnitude, depending on the nature and strength of the prior immune encounter. Similarly, we might anticipate heightened cytokine responses in SARS-CoV-2(+) individuals, reflecting the presence of adaptive immune memory. However, the magnitude and profile of the response could vary based on the time elapsed since infection, the antigen used in the assay, and the individual's immune status.

The exclusion of unvaccinated children in our study is a significant limitation that hinders the generalization of our findings to the broader population. Our conclusions are focused solely on the immune mechanisms in BCG-vaccinated children, which may not reflect the immune responses in the general population. Due to the retrospective nature of data collection, information on the sequence of infections was not available for all participants, highlighting the need for further prospective studies that account for these variables. Further research is warranted to explore how the immunomodulatory properties of BCG can be optimized for current and emerging viral threats. To fully realize the potential of the BCG vaccine in modulating immunity against viruses, it is necessary to consider both its proven benefits and the challenges associated with its wider use. The vaccine's affordability and existing supply chains make it a valuable tool in low-resource settings, potentially offering a cost-effective strategy to enhance global health security against viral epidemics. However, variability in immune responses due to genetic differences between populations poses a significant challenge, highlighting the need for targeted research to enhance BCG's effectiveness in different demographic groups. Ongoing global trials are examining the effectiveness of BCG in preventing infections other than tuberculosis (Kleinnijenhuis et al. 2014). It is worth noting that we only included one time-point in our study, 48 h after stimulation, which is a limitation in terms of fully understanding the dynamics of cytokine expression. It is known that there is a difference in the kinetics of mRNA and protein expression of cytokines, with mRNA expression often peaking much earlier than protein levels. Therefore, while a time-point of 48 h may be appropriate to assess the peak of cytokine expression at the protein level, it may be too late to capture changes at the mRNA level that may occur earlier. Furthermore, mRNA analysis allows measurement of the transcript at one specific time-point, which may not reflect the full dynamics of the transcription process. In contrast, measuring protein in the supernatant provides information on cytokine accumulation or consumption at a specific time-point, which may be more optimal for the long-term cellular response. These studies are of great importance in determining the role of the vaccine in the broader context of treating infectious diseases. They may also guide future vaccination strategies to include BCG as a permanent part of vaccination schedules around the world, especially in regions most vulnerable to emerging pathogens. It is critical to remove potential barriers, including regulatory approvals and public perception, to facilitate the successful repositioning of BCG. Additionally, public health communication must evolve to support the understanding of new and potential uses of BCG. This is essential to ensure that communities are informed and open to revised vaccination protocols.

In summary, while the BCG vaccine shows promise in enhancing the immune response to a range of viral infections, fully realizing this potential will require a coordinated approach across research, health policy, and community engagement. Results from ongoing research will be critical in shaping the future of infectious disease prevention and management by using an old vaccine to address new public health challenges.

5. Conclusions

The conducted studies indicate that the BCG vaccine may have an immunomodulatory effect on the IFN response induced by SARS-CoV-2 and RSV infections. Differences in the intensity of this response may suggest the existence of distinct immune mechanisms stimulated by BCG in reaction to various pathogens. The observed variability in the immune response highlights the complexity of BCG's action and underscores the need for further comprehensive investigations. Additional research on large and diverse population groups is necessary to elucidate the molecular basis of the observed phenomenon and its potential clinical significance. Such studies should identify specific biomarkers and genetic factors that contribute to the differential immune responses. Understanding these mechanisms is crucial, especially in the context of utilizing the BCG vaccine as a potential regulator of the immune response to a broad spectrum of viral infections. Moreover, these insights could pave the way for new therapeutic strategies and improve the efficacy of existing vaccines against emerging viral threats.

Funding

This research was supported by the resources for scientific activities of doctoral students at the Bio-Med-Chem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, Lodz, Poland.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of the Medical University in Lodz, Poland (No. RNN/122/22/KE).

Informed Consent Statement

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

Data Availability Statement

Not applicable.

Author Contributions

Conceptualization – MD and MJ; methodology – MD and MJ; software – MJ; validation – MJ; formal analysis – MK-P; investigation – MJ; resources – MK-P; data curation – JK; writing and original draft preparation – MJ, MD; writing – review and editing – MJ, MD; visualization – MJ; supervision – MD, MK-P; project administration – MJ; funding acquisition – MJ. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli

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Abstract

The Bacillus Calmette-Guérin (BCG) vaccine confers broad, non-specific immunity that may bolster defenses against respiratory viruses. While NOD2 (nucleotide-binding oligomerization domain-containing protein 2)-driven pathways are central to innate immune responses, the contribution of surface receptor modulation on monocytes to shaping these responses remains underexplored. We analyzed whole-blood cultures from BCG-vaccinated Polish children, stratified by serostatus to SARS-CoV-2 and RSV, and stimulated for 48 h with live BCG, purified viral antigens, or both. RT-qPCR quantified mRNA levels of NOD2 and key cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF), while flow cytometry assessed CD14, HLA-DR, CD11b, and CD206 expression. Co-stimulation with BCG + RSV elicited the strongest transcriptional response, notably a 2–4-fold upregulation of NOD2, IL-1β, and IL-6 versus RSV alone. In SARS-CoV-2(+) donors, RSV alone induced higher NOD2 expression than BCG or BCG + RSV, while IL-2 peaked following BCG + SARS-CoV-2. Across conditions, NOD2 positively correlated with IL-4 and IL-6 but negatively correlated with IL-1β in SARS-CoV-2 cultures. Viral antigens increased CD14 and HLA-DR on monocytes, suggesting activation; CD206 rose only in dual-seropositive children. Our findings indicate that BCG stimulation affects pediatric antiviral immunity through NOD2-related cytokine production and induction of a $CD14^{+}HLA\text{-}DR^{+}$ phenotype, supporting its potential role in boosting innate defenses against respiratory pathogens.

Keywords: Bacillus Calmette-Guérin; respiratory syncytial virus; severe acute respiratory syndrome coronavirus 2

Academic Editor: Moriya Tsuji

Received: 12 June 2025
Revised: 4 July 2025
Accepted: 8 July 2025
Published: 11 July 2025

Citation: Jurczak, M.; Kaczmarek, J.; Kowalewska-Pietrzak, M.; Stelmach, P.; Druszczynska, M. NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. *Pathogens* **2025**, *14*, 683. https://doi.org/10.3390/pathogens14070683

1. Introduction

Bacille Calmette-Guérin (BCG) is a live attenuated vaccine containing *Mycobacterium bovis*, mainly used against tuberculosis, but also provides non-specific protection against various pathogens, including respiratory viruses [1]. This phenomenon is referred to as 'trained immunity', in which innate immune cells are reprogrammed, leading to an

enhanced response to subsequent infection, mediated by various signaling pathways, including those involving nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [2,3]. NOD2 recognizes muramyl dipeptide (MDP) of bacteria and ssRNA of viruses [4,5]. NOD2 can detect peptidoglycan fragments of mycobacteria, such as MDP, initiating an immune response, particularly in the context of infection and vaccination with BCG [6]. Importantly, the BCG vaccine has been shown to engage the NOD2 receptor, leading to the epigenetic reprogramming of monocytes, during which the histone modification of histone 3 at lysine 4 (H3K4me3) enhances the expression of pro- and anti-inflammatory cytokine genes [7]. In addition, metabolism is activated by the Akt/mTOR pathway, resulting in a change in metabolism to glycolysis [8]. This reprogramming enhances the production of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) during subsequent encounters with unrelated pathogens, which is a characteristic of trained immunity [1].

Studies suggest that BCG can enhance immune responses against influenza, human papillomavirus (HPV) and herpes simplex virus (HSV), respiratory syncytial virus (RSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [9,10]. A recent study showed that mice previously vaccinated with BCG and then infected with the influenza virus and SARS-CoV-2 were better at eliminating viruses and showed less weight loss. This effect was linked to a two-step innate response and a strong type 1 helper T lymphocyte (Th1) response in the lungs. And the presence of $CD4^+$ lymphocytes producing interferon-gamma (IFN-γ) was important for long-term antiviral immunity, demonstrating the synergy of innate and adaptive immunity in BCG-induced non-specific protection [9]. In addition, a recombinant BCG vaccine expressing the RSV nucleoprotein (rBCG-N-hRSV) has been developed and shows promising immunogenic and protective properties. This vaccine induces both cellular and humoral responses [10]. In animal models, the administration of rBCG-N-hRSV has been shown to prevent lung damage associated with RSV infection and reduce inflammation. The protective mechanism of the vaccine includes the early recruitment of CD4+ and CD8+ T lymphocytes to the lungs and increased secretion of the cytokines IFN-γ and IL-17, which play a key role in the control of infection [11].

Given the role of the NOD2 receptor in recognizing bacterial and viral components and orchestrating downstream immune responses, we hypothesize that whole-blood stimulation in BCG-vaccinated children leads to an enhanced immune response to viral antigens (RSV, SARS-CoV-2), driven by NOD2-dependent mechanisms. Specifically, we propose that BCG vaccination primes innate immune cells to mount a more robust and flexible antiviral response upon secondary exposure to viral antigens, potentially counteracting immune-evasion strategies employed by SARS-CoV-2 and RSV. This effect may be mediated through the upregulation of NOD2 expression and context-dependent modulation of inflammatory cytokine production, including IL-1β, IL-2, IL-4, and IL-6, ultimately supporting more effective viral clearance.

In our study, using an in vitro whole blood stimulation model, we analyze the immune responses in children vaccinated with BCG, taking into account their serological status against RSV and SARS-CoV-2. We further hypothesize that the modulation of monocyte surface markers such as CD14, HLA-DR, and CD206 contributes to this enhanced responsiveness. In addition, we are investigating whether previous exposure to viruses such as RSV or SARS-CoV-2 is associated with differential innate response characteristics in children after BCG vaccination. Elucidating these interactions may offer novel insights into innate immune reprogramming and support the development of BCG-based immunomodulatory strategies against respiratory viral infections in pediatric populations.

2. Materials and Methods

2.1. Study Population

The study included 48 healthy children between the ages of 6 and 12 who had received the *Mycobacterium bovis* BCG Moreau vaccine (Biomed, Lublin, Poland) on the first day of life, in accordance with the Polish national vaccination program. No deviations from the Polish National Immunization Program were noted, and no child had received additional non-mandatory or experimental vaccines beyond those included in the standard schedule. While it is recognized that some vaccines may exert non-specific immunomodulatory effects, the standardized nature of vaccination coverage across all participants in our study limits the likelihood that such differences could introduce meaningful bias. Since all children were comparably immunized according to national guidelines, any minor variation in timing or formulation (e.g., different vaccine brands) is unlikely to have had a systematic effect on the immunological markers that we assessed. The inclusion criteria were as follows: (I) age between 6 and 12 years at the time of enrolment, (II) documented receipt of BCG Moreau vaccine on the first day of life, in accordance with the Polish national immunization schedule, (III) general good health status, with no current or chronic illnesses, as confirmed by a pediatric infectious disease specialist and a provincial pediatric pulmonology consultant, and (IV) informed written consent obtained from legal guardians. The exclusion criteria were as follows: (I) history of chronic respiratory diseases, including asthma, cystic fibrosis, or a previous diagnosis of tuberculosis, (II) any form of immunodeficiency (primary or secondary), including children on immunosuppressive therapy, (III) acute infection or antibiotic therapy within 30 days prior to sample collection, (IV) previous hospitalization for severe RSV or SARS-CoV-2 infection, and (V) a lack of compliance with the Polish vaccination schedule or missing documentation of BCG immunization. Based on the serostatus against RSV and SARS-CoV-2, participants were classified into four groups: (1) RSV-seropositive (RSV(+)), (2) SARS-CoV-2-seropositive (SARS-CoV-2(+)), (3) RSV- and SARS-CoV-2-seropositive (RSV(+) SARS-CoV-2(+)), and (4) RSV- and SARS-CoV-2-seronegative (RSV(−) SARS-CoV-2(−)) (Table 1). The children's health was assessed by infectious disease consultants, including the provincial representative for pediatric pulmonology employed at the Provincial Specialist Hospital for Tuberculosis, Lung Diseases and Rehabilitation in Lodz. The Research Ethics Committee of the Medical University of Lodz approved the study protocol (No RNN/122/22/KE). SARS-CoV-2 antibodies were assessed using the DiaSorin LIASON® SARS-CoV-2 TrimericS IgG assay on the LIASON® XL analyzer (DiaSorin, Stillwater, MN, USA), with a diagnostic cut-off of $\geq$33.8 BAU/mL for positivity. According to the manufacturer, this assay demonstrates 98.7% sensitivity ($\geq$15 days post symptom onset) and 99.5% specificity, with no significant cross-reactivity reported for seasonal coronaviruses, influenza A/B, RSV, or adenovirus. RSV infection was confirmed using the Respiratory Syncytial Virus IgG ELISA kit (Serion-Diagnostics, Würzburg, Germany), calibrated to WHO standards. Results $\geq$16 U/mL were considered positive (8–16 U/mL equivocal, <8 U/mL negative). The test's reported sensitivity and specificity are 96.2% and 98.4%, respectively, with <1% cross-reactivity with human metapneumovirus or parainfluenza viruses.

Table 1. The characteristics of the groups in the study.

Parameter	Total	Groups			
		RSV(+)	**SARS-CoV-2(+)**	**RSV(+) SARS-CoV-2(+)**	**RSV(−) SARS-CoV-2(−)**
Ethnicity	**Caucasia**	**Caucasian**	**Caucasian**	**Caucasian**	**Caucasian**
N	48	14	17	11	6
Sex Girls, *n* (%) Boys, *n* (%)	 23 (48%) 25 (52%)	 8 (57%) 6 (43%)	 5 (30%) 12 (70%)	 6 (55%) 5 (45%)	 4 (66%) 2 (33%)
Age median	8	9	9	8	8
Age range	6–12	7–12	6–12	7–12	7–12
BCG vaccination	100%	100%	100%	100%	100%
Compliance with the Polish vaccination schedule	Yes	Yes	Yes	Yes	Yes
General health status	Good	Good	Good	Good	Good
Chronic respiratory diseases	No	No	No	No	No
Form of immunodeficiency	No	No	No	No	No
Acute infection <30 days prior to blood collection	No	No	No	No	No
Antibiotics <30 days prior to blood collection	No	No	No	No	No
Hospitalized for severe RSV or SARS-CoV-2	No	No	No	No	No

Abbreviations: RSV—respiratory syncytial virus; SARS-CoV-2—severe acute respiratory syndrome coronavirus 2. The differences between the groups studied did not reach statistical significance with regard to age (ANOVA with Dunn's post-test test, $p > 0.05$) and sex (Chi-square test or Fisher's exact test, $p > 0.05$).

2.2. Whole-Blood Cultures

Whole blood (7 mL) was collected into heparinized tubes (Sarstedt, Germany) and maintained at room temperature. All samples were processed within 2 h of collection. Leukocyte viability was assessed using the trypan blue exclusion method, and only samples with ≥95% viable cells were included in the analysis. For stimulation, whole blood was plated directly into 24-well culture plates (Nunc, Denmark) at 1 mL per well without the prior separation of peripheral blood mononuclear cells. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h. The first involved the stimulation of blood cells with SARS-CoV-2 virus peptides (1 µg/well) (Peptivator, MiltenyiBiotec, Bergisch Gladbach, Germany), in combination with live *Mycobacterium bovis* BCG strain Moreau (Biomed, Lublin, Poland; 10^6 cells per culture). These cultures were compared with cultures stimulated with SARS-CoV-2 peptides alone or with BCG mycobacteria alone.

The second type of culture involved the stimulation of blood cells with RSV antigens (1 µg/well) (PepTivator RSV, MiltenyiBiotec, Germany), also in combination with live *M. bovis* BCG mycobacteria. Again, parallel control cultures were conducted in which cells were stimulated with RSV peptides alone or with mycobacteria BCG alone [12,13]. Control conditions included cultures stimulated with BCG alone, with viral peptides alone, or with phytohemagglutinin (PHA; 10 µg/well; Thermo Fisher Scientific, Waltham, MA, USA) and unstimulated cultures in RPMI-1640 medium (Sigma-Aldrich, Gillingham, UK). After 48 h, culture supernatants were collected, centrifuged (1000× *g* for 10 min), aliquoted, and stored at −80 °C until cytokine quantification. The cell cultures were also frozen at −80 °C and used for cytometric analysis at a later date. Samples with visible hemolysis or low viability (<90%) were excluded.

2.3. Isolation of DNA and Its Spectrophotometric Evaluation

DNA was isolated from peripheral blood collected from all volunteers using EDTA tubes and the BD Vacutainer® blood collection system. Isolation was performed within no more than 2 h of sample collection using the commercial EXTRACTME DNA BLOOD KIT (Blirt, Gdansk, Poland). To 200 µL of blood, 200 µL of erythrocyte lysis buffer was added, and samples were centrifuged for 4 min at 8600× *g*. After centrifugation, the supernatant was carefully removed from the white cell pellet and the cells were washed with 375 µL of BL buffer. Then, 6 µL of Proteinase K was added, and samples were incubated at 55 °C for 10 min. After the incubation was completed, 400 µL of buffer BB was added and the entire lysate was transferred to a DNA purification column. The samples were centrifuged for 60 s at 11,000× *g*. The column was then transferred to a new collection tube and washed twice: first with 600 µL of buffer BW1 and then with 400 µL of buffer BW2, each time centrifuging for 30 s at 11,000× *g*. In order to completely remove the alcohol from the column before elution, the samples were subjected to an additional centrifugation for 2 min at 21,000× *g*. Finally, DNA was eluted by adding 50 µL of elution buffer directly to the membrane of the purification column, incubating for 2 min and centrifuging for 60 s at 11,000× *g*. BioDrop µLITE (Holliston, MA, USA) was used to assess the quality and quantity of isolated DNA. The extracted DNA was stored at −80 °C until analyzed.

2.4. Assessment of the NOD2 Gene Polymorphism

To determine the genotypes, the isolated DNA was analyzed via PCR-RFLP. For the determination of R702W (rs2066844) variants, the following primer pairs were used: 5′-AGATCACAGCAGCCTTCCTG-3′, 5′-CACGCTCTCTTGGCCTCACC-3′. The 185 bp PCR product was digested at 37 °C for 1 h with 2 U MspI (EURx). The following fragments could be obtained by obtaining the following fragments: 20, 35, 54, and 76 bp in R702R homozygotes; 20, 35, 54, 76, and 130 bp in R702W heterozygotes; and 20, 35, and 130 bp in W702W homozygotes [14]. For the G908R (rs2066845) mutation assay, the primers 5′-CTCTTGGCCTTTCAGATTCTG-3′ and 5′-CAGCTCCTCCCTCTTCACCT-3′ were used. The 163 bp PCR product was digested at 37 °C for 1 h with 2 U HhaI (EURx). The following fragments could be obtained by obtaining the following fragments: 163 bp in G908G homozygotes; 27, 136, and 163 in G908R heterozygotes; and 27 and 136 bp in R908R homozygotes [14]. For the 1007fs (rs5743293) mutation assay, the primer pairs 5′-GGCAGAAGCCCTCCTGCAGGCC-3′ and 5′-CCTCAAAATTCTGCCATTCC-3′ were used. The 151 bp PCR product was digested for 1 h at 24 °C with 2 U ApaI (EURx). The following fragments could be obtained by obtaining the following fragments: 151 bp for Leu1007Leu homozygotes; 20, 131, and 151 bp in Leu1007Pro heterozygotes; and 20 and 131 bp in Pro1007Pro homozygotes [14].

A gradient thermocycler (BioRad, Hercules, CA, USA) was used to determine appropriate reaction temperatures and primer concentrations. The PCR reaction conditions are shown in Table 1. To visualize the digestion products (R702W, G908R), electrophoresis was run in a 12% polyacrylamide gel, and, for the 1007fs mutation, electrophoresis was run in a 2% agarose gel in TBE buffer at 70 V for 90 min, and the image was then documented on a ProXima 2750 system (Isogen Life Science, Utrecht, The Netherlands).

2.5. Isolation of RNA and Its Spectrophotometric Evaluation

The QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to isolate RNA from culture sediments. For erythrocyte lysis, 1 mL of the cultured pellet was mixed with 5 mL of EL buffer and incubated at 4 °C for 15 min. After incubation, the cells were centrifuged for 10 min at 400× *g* at 4 °C. The pellet was then resuspended in 2 mL of EL buffer and centrifuged again for 10 min at 400× *g* at 4 °C. The resulting leukocyte pellet was resuspended in 350 µL of RTL buffer, which disrupts the cells. The entire lysate was transferred onto a QIAshredder column and centrifuged at maximum speed for 2 min to ensure homogenization. Subsequently, 350 µL of 70% ethanol was added to the homogenized lysate and carefully transferred onto a QIAamp column, followed by centrifugation for 15 s at 8000× *g*. The column was washed twice: first with 700 µL of RW1 buffer, then with 500 µL of RPE buffer, each time centrifuged for 15 s at 8000× *g*. An additional 500 µL of RPE buffer was added, and the sample was centrifuged for 3 min at 20,000× *g*. To completely remove any remaining RPE buffer, the column was subjected to an additional centrifugation step for 1 min at 20,000× *g*. Finally, RNA was extracted by adding 30 µL of RNase-free water directly onto the column and centrifuging for 1 min at 8000× *g*. To assess the quality and concentration of the extracted nucleic acids, RNA levels were measured using a BioDrop µLITE spectrophotometer (Holliston, MA, USA). A water sample free of the analyzed substances was used for calibration. Additionally, RNA integrity was assessed via electrophoresis on a 1.2% agarose gel in TAE buffer, run at 90 V for 60 min. The Gel-Doc 2000 system (Bio-Rad, Hercules, CA, USA) and Quantity One software (Version 4.6.8) were used to document gel images. The extracted DNA was stored at −80 °C until analyzed.

2.6. Reverse Transcription

A commercial iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) was used for cDNA synthesis. The synthesis of cDNA was performed according to the manufacturer's instructions, using 1 µg of previously assessed RNA for integrity and quality as a template. Reverse transcription was performed using a Biometra UNO II thermocycler (Analytik, Jena, Germany), using parameters according to the kit manufacturer's instructions. The resulting cDNA was stored at −20 °C until analysis.

2.7. qPCR Reaction

The reaction mixture for the IL-1B, IL-8, TNF, and IL-10 primers (BioRad, Hercules, CA, USA) contained 5 µL of iTaq universal SYBR Green Supermix, 0.5 µL of primer (20 µM), 3 µL of nuclease-free water, and 1 µL of cDNA, while for IL-2, IL-4, and IL-6 primers, it contained 5 µL of iTaq universal SYBR Green Supermix, 0.5 µL of each primer (25 µM), 3 µL of nuclease-free water, and 1 µL of cDNA (Table 2). Amplification was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The protocol included a pre-activation step at 95 °C for 2 min, followed by 40 cycles comprising denaturation (95 °C for 5 s) and annealing and elongation (60 °C for 30 s). An additional dissociation step was used to analyze the melting curve: initially, 65 °C for 5 s, followed by a temperature increase of 0.5 °C every 5 s until 95 °C was reached. GAPDH and HPRT1 were selected as reference genes due to their lowest M values, which

indicate high expression stability. This makes them reliable internal controls, enabling precise normalization of the results [15]. The qRT-PCR reactions were performed in three technical replicates. The ΔΔCt method was used to analyze gene expression levels, enabling the assessment of relative amounts of selected mRNA transcripts. This method involves comparing the expression of the target gene to that of a reference gene, using cycle threshold (Ct) values obtained from the qPCR reaction. In the first step, Ct values were determined for both target and reference genes in the tested samples, followed by the calculation of differences between these values (ΔCt).

Table 2. Starters and temperatures of annealing selected for expression analysis.

	Sequence	Temperature of Annealing	Source [Reference]
R702W	forward 5′-AGATCACAGCAGCCTTCCTG-3′, reverse 5′-CACGCTCTCTTGGCCTCACC-3′	62	[14]
G908R	forward 5′-CTCTTGGCCTTTCAGATTCTG-3′ reverse 5′-CAGCTCCTCCCTCTTCACCT-3	54	[14]
1007fs	forward 5′-GGCAGAAGCCCTCCTGCAGGCC-3′ reverse 5′-CCTCAAAATTCTGCCATTCC-3′	60	[14]
IL-2	forward 5′-AGAATCCCAAACTCACCAGGA-3′ reverse 5′-TGCTGATTAAGTCCCTGGGT-3′	60	[16]
IL-4	forward 5′-GCAGTTCTACAGCCACCATG-3′ reverse 5′-ACTCTGGTTGGCTTCCTTCA-3′	60	[16]
IL-6	forward 5′-CCTTCCAAAGATGGCTGAAA-3′ reverse 5′-CAGGGGTGGTTATTGCATCT-3′	60	[16]
IL-1B	qHsaCID0022272	60	Bio-Rad
IL-8	qHsaCED0046633	60	Bio-Rad
TNF	qHsaCED0037461	60	Bio-Rad
IL-10	QHsaCED0044704	60	Bio-Rad

2.8. Peripheral Blood Leukocyte (PBL) Harvest and Flow Cytometry

After 48 h of whole blood culture, culture supernatants were removed and EDTA in PBS (2 mM) was added to each well to pellet adherent cells. The cells were then incubated with 5 times the volume of RBCL lysis buffer (A&A biotechnology, Gdańsk, Poland) on ice for 15 min. After erythrocyte lysis, PBLs were harvested and resuspended in fetal bovine serum with 10% dimethylsulfoxide and frozen in liquid nitrogen. For cytometric analysis, cells were thawed in a water bath at 37 °C, and then, warm RPMI 1640/10%FBS/EDTA (2 mM) was added to wash out DMSO and detach adherent cells. PBLs were washed in warm RPMI 1640/10%FBS and incubated for 1 h for cell regeneration. After incubation, cells were harvested and resuspended in HBSS/1%BSA and stained for 30 min at 4 °C with the following antibodies: CD14-FITC; HLA-DR-Bv605; CD-11b-APC; CD206-PE (all from BD Biosciences, Franklin Lakes, NJ, USA). The measurement was performed using a FAC Symphony™ A1 (BD Biosciences, Franklin Lakes, NJ, USA). Compensation was performed

using BD™ CompBead Plus (BD Biosciences, Franklin Lakes, NJ, USA) compensation particles stained separately with each antibody conjugate.

2.9. Statistical Analysis

The correlation between mRNA expression levels and NOD2 protein concentrations was assessed using Spearman's nonparametric rank correlation test. Results with a *p*-value less than 0.05 were considered statistically significant. Due to multiple comparisons, the Benjamini-Hochberg procedure was used to control the false discovery rate (FDR). The correction was performed globally, i.e., collectively for all tests performed as part of the cytokine analysis, which allowed for consistent control of the risk of false positive results across the entire set of analyses. Results with an adjusted *p*-value < 0.05 were considered statistically significant. A post-hoc power analysis was performed using GPower 3.1 [17]. A one-way ANOVA was used to assess statistical power across the four independent groups: RSV(+) (n = 14), SARS-CoV-2(+) (n = 17), RSV(+)SARS-CoV-2(+) (n = 11), and RSV(−)/SARS-CoV-2(−) (n = 6), with a total sample size of N = 48. Assuming a medium effect size (f = 0.25) and a significance level of $\alpha = 0.05$, the achieved statistical power was 0.69. These results indicate limited power to detect moderate between-group differences and a potential risk of type II errors, especially in comparisons involving the smallest group. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA). FlowJo™ 10.2 software (FlowJo LLC, Beaverton, OR, USA) was used to analyze the flow cytometry data after prior generation of the compensation matrix. Monocytes were selected based on the expression of CD14 markers and their characteristics in terms of size and granularity.

3. Results

3.1. Assessment of the NOD2 Gene Polymorphism

The distribution of the G908R, R702W, and 1007fs (Figures S1–S3) genotypes in the study group is presented in Table 3. The R702R homozygous variant was observed in 100% of the children studied, as was the case for G908G and Leu1007Leu, suggesting that these variants may be predominant in the Polish pediatric population. The high frequency of homozygosity for R702R, G908G, and Leu1007Leu suggests the potential genetic stability of these variants within the Polish pediatric population.

Table 3. Genotype distribution of the G908R, R702W, and 1007fs polymorphisms in the NOD2 gene.

NOD2	Genotyping	N (%)
G908R	G/G	40 (100)
	R/R	0 (0)
	G/R	0 (0)
R702W	R/R	40 (100)
	W/W	0 (0)
	R/W	0 (0)
1007fs	Leu/Leu	40 (100)
	Pro/Pro	0 (0)
	Leu/Pro	0 (0)

3.2. mRNA Expression of NOD2

Figure 1 demonstrates a comparison of NOD2 gene mRNA expression in cultured whole blood. The highest NOD2 mRNA expression was observed in the RSV(+) and

RSV(−)/SARS-CoV-2(−) groups co-stimulated with *Mycobacterium bovis* BCG and RSV antigens (14.02 ± 17.83 and 4.27 ± 0.09, respectively). In RSV-seropositive and RSV-seronegative groups, as well as in SARS-CoV-2 groups, a statistically significant increase in NOD2 gene expression was observed in cells co-stimulated with BCG and RSV antigens, compared to cells stimulated with RSV antigens alone. Furthermore, in the SARS-CoV-2-seropositive group, *NOD2* expression was significantly higher in cultures stimulated with RSV antigens alone compared to those stimulated with BCG alone or with the combination of BCG and RSV antigens alone.

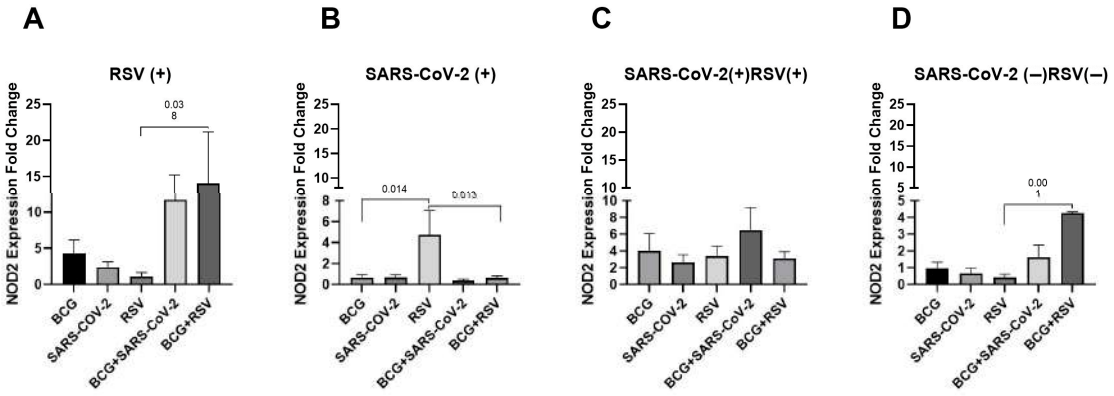

Figure 1. Changes in NOD2 gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The *Y*-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; NOD2, nucleotide-binding oligomerization domain containing 2; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2; BCG, bacillus Calmette-Guérin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare NOD2 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.3. mRNA Expression of IL-1β

Figure 2 illustrates the mRNA expression levels of IL-1β in cultured cell sediments. In the RSV(+) and SARS-CoV-2(+) RSV(+) groups, the highest IL-1β mRNA expression levels were observed in cultures stimulated with both *Mycobacterium bovis* BCG and RSV antigens (156.71 ± 149.06; 111.29 ± 78.81, respectively). In contrast, in the SARS-CoV-2(+) and SARS-CoV-2(−)/RSV(−) groups, the highest expression levels were detected in those co-stimulated with BCG and SARS-CoV-2 antigens (227.69 ± 204.67; 11.6 ± 4.44, respectively). A significant increase in IL-1β expression was observed in all study groups after co-stimulation with BCG and RSV antigens, compared to groups stimulated with RSV antigen alone. Conversely, in the SARS-CoV-2(+) and SARS-CoV-2(−)/RSV(−) groups, IL-1β mRNA expression was significantly higher in those co-stimulated with BCG and SARS-CoV-2 antigens compared to those stimulated with SARS-CoV-2 antigens alone. Additionally, a significant difference in IL-1β mRNA levels was observed in the RSV(+) group between cultures stimulated with BCG alone versus those co-stimulated with BCG and RSV antigens. Furthermore, a significant difference in IL-1β expression was noted in the SARS-CoV-2-seropositive group between cultures stimulated with BCG alone and those stimulated with BCG and SARS-CoV-2 antigens.

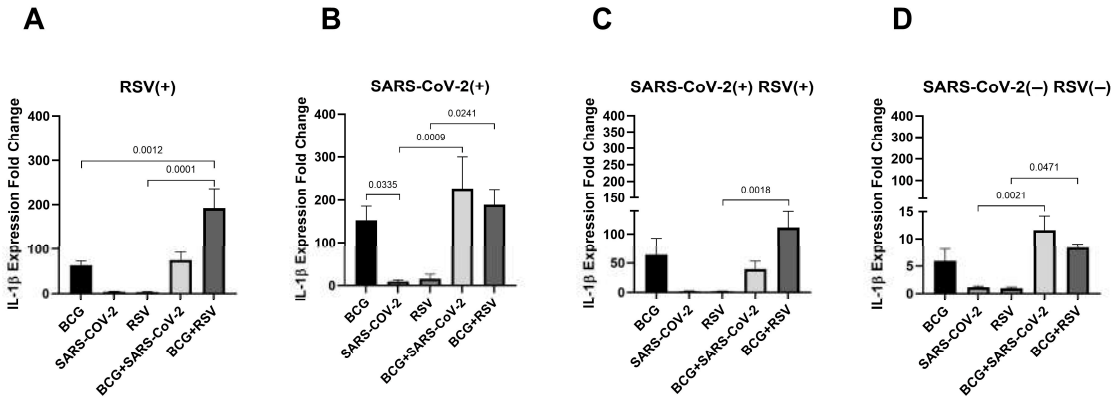

Figure 2. Changes in IL-1β gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-1β concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.4. mRNA Expression of IL-8

Figure 3 depicts the mRNA expression levels of IL-8 in cultured cell sediments across all study groups. The highest IL-8 mRNA expression was observed in the SARS-CoV-2(−)/RSV(−) group in co-stimulated with both *Mycobacterium bovis* BCG and RSV antigens. However, no significant differences in IL-8 mRNA expression were detected between culture conditions in any of the study groups.

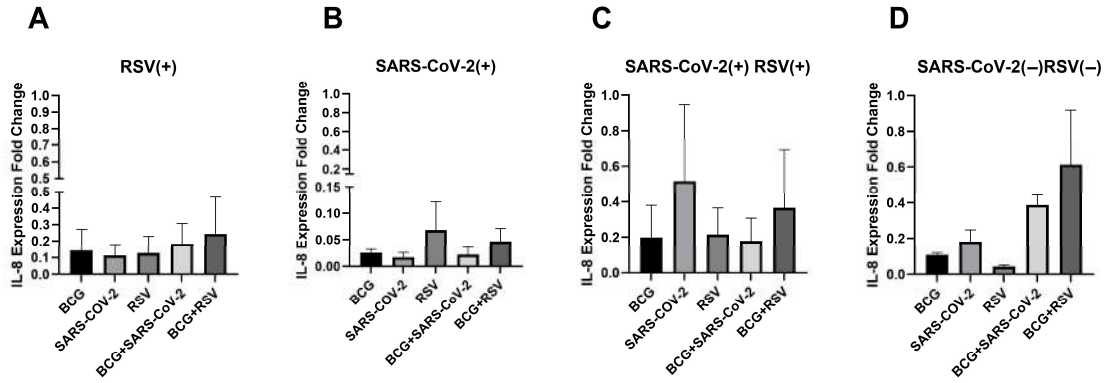

Figure 3. Changes in IL-8 gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-8 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.5. mRNA Expression of IL-4

As shown in Figure 4, IL-4 mRNA expression levels in cultured cell sediments were analyzed across all study groups. The highest expression levels were observed in those co-stimulated with both *Mycobacterium bovis* BCG and RSV antigens, in both seropositive and seronegative groups for RSV and SARS-CoV-2 (9.96 ± 9.33; 3.88 ± 0.66, respectively). These levels were significantly higher compared to cultures stimulated with RSV antigens alone or BCG alone. In contrast, no significant differences in IL-4 expression levels were detected between the RSV(+) and SARS-CoV-2(+) groups.

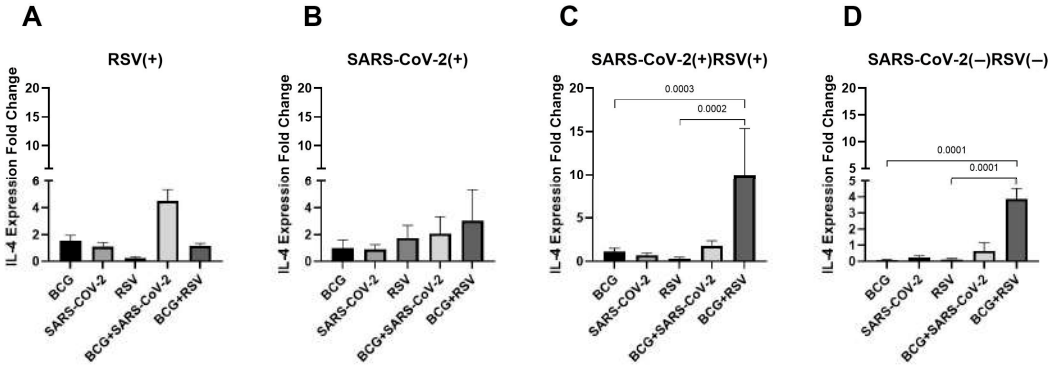

Figure 4. Changes in IL-4 gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-4 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.6. mRNA Expression of TNF

Figure 5 illustrates the changes in TNF mRNA expression levels in cultured cell sediments. In the group seronegative for SARS-CoV-2 and RSV, the highest TNF mRNA expression levels were observed in cultures stimulated with both *Mycobacterium bovis* BCG and SARS-CoV-2 antigens (61.53 ± 10.31). Only in this group were statistically significant differences in TNF mRNA expression observed between cultures stimulated simultaneously with BCG and SARS-CoV-2 and those exposed to one of these antigens. No such differences were observed in other groups.

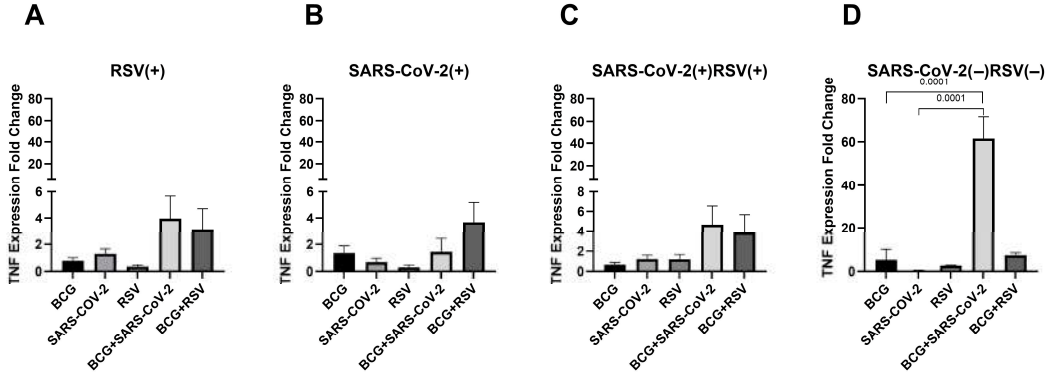

Figure 5. Changes in TNF gene mRNA expression in different groups of subjects who were simultan-

-eously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare TNF concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.7. mRNA Expression of IL-10

Figure 6 presents the mRNA expression levels of IL-10 in cultured cell sediments. In the SARS-CoV-2(+) and SARS-CoV-2(−)/RSV(−) groups, the highest IL-10 mRNA expression was observed in cells co-stimulated with both *Mycobacterium bovis* BCG and SARS-CoV-2 antigens (6.27 ± 4.53; 8.86 ± 4.49, respectively). In the case of the other research groups, the highest level of expression occurred after the simultaneous use of BCG and RSV antigens (7.81 ± 3.48). A significant increase in IL-10 expression was observed in those co-stimulated with both BCG and SARS-CoV-2 antigens compared to those stimulated with either SARS-CoV-2 or BCG alone in the SARS-CoV-2(+) and SARS-CoV-2(−)/RSV(−) groups. Significantly higher IL-10 expression was detected in the co-stimulated condition (BCG + RSV) than with the RSV-only stimulation, specifically in the group seropositive for both viruses.

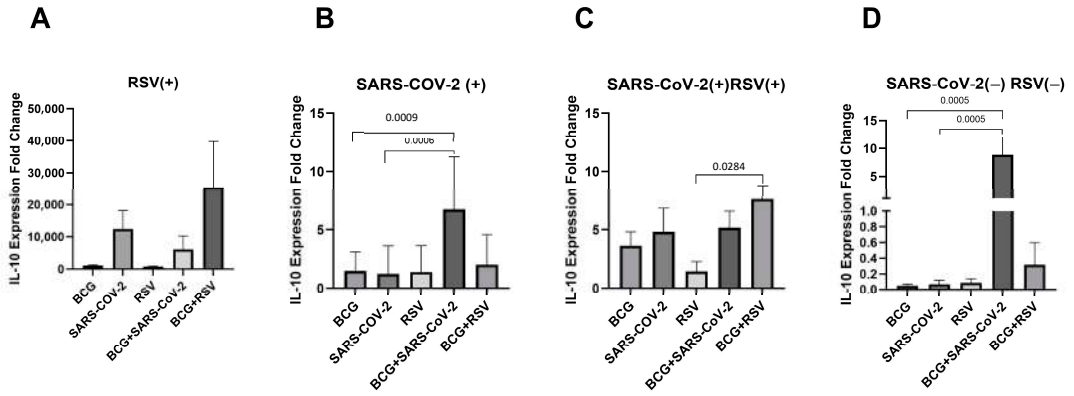

Figure 6. Changes in IL-10 gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-10 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.8. mRNA Expression of IL-6

Figure 7 illustrates the changes in IL-6 mRNA expression across all study groups. In the RSV(+), SARS-CoV-2(+)/RSV(+), and SARS-CoV-2(−)/RSV(−) groups, the highest IL-6 expression levels were observed in cultures stimulated with both *Mycobacterium bovis* BCG and RSV antigens (12.24 ± 7.99; 10.00 ± 7.33; 33.46 ± 6.41, respectively). In all study groups, IL-6 mRNA expression was significantly higher in BCG+RSV co-stimulated groups compared to cultures stimulated with either RSV antigens or BCG alone. Furthermore,

in the RSV(+) and SARS-CoV-2(+)/RSV(+) groups, a significant increase in IL-6 mRNA expression was observed in BCG+SARS-CoV-2 co-stimulated groups compared to those stimulated with either SARS-CoV-2 antigens or BCG alone.

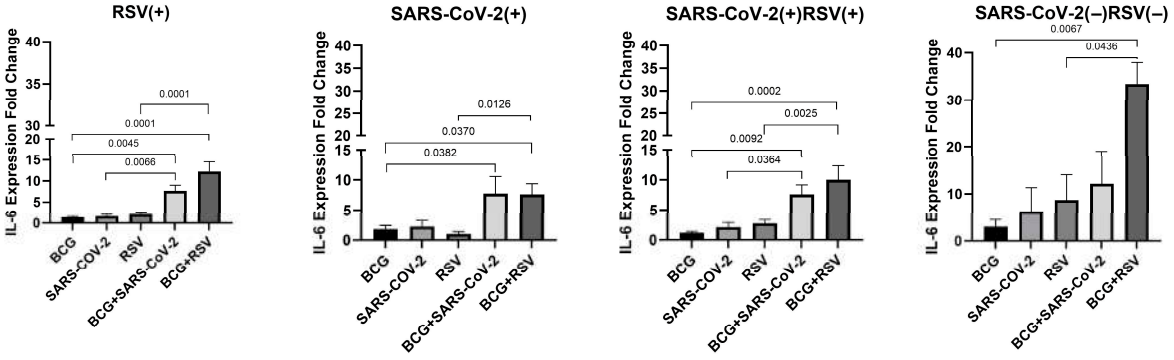

Figure 7. Changes in IL-6 gene mRNA expression in different groups of subjects who were simultaneously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; RSV(+), group seropositive for RSV infection; SARS-CoV-2(+), group seropositive for SARS-CoV-2; RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-6 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.9. mRNA Expression of IL-2

Figure 8 illustrates the changes in IL-2 mRNA expression levels across all study groups. The highest expression levels were observed in the RSV(+) and SARS-CoV-2(−)/RSV(−) groups in cultures stimulated with both *Mycobacterium bovis* BCG and RSV antigens (7.27 ± 7.09; 108.63 ± 26.28, respectively), while in the SARS-CoV-2-seropositive group, the highest expression was detected in BCG+SARS-CoV-2 co-stimulated groups (16.15 ± 17.74). In the RSV(+), SARS-CoV-2(+), and SARS-CoV-2(−)/RSV(−) groups, IL-2 mRNA expression was significantly higher in BCG+RSV co-stimulated groups compared to those stimulated with either BCG or RSV antigens alone. Additionally, in the RSV(+) and SARS-CoV-2(+) groups, IL-2 mRNA expression was significantly elevated in BCG+SARS-CoV-2 co-stimulated groups compared to cultures stimulated with either BCG alone or SARS-CoV-2 antigens alone.

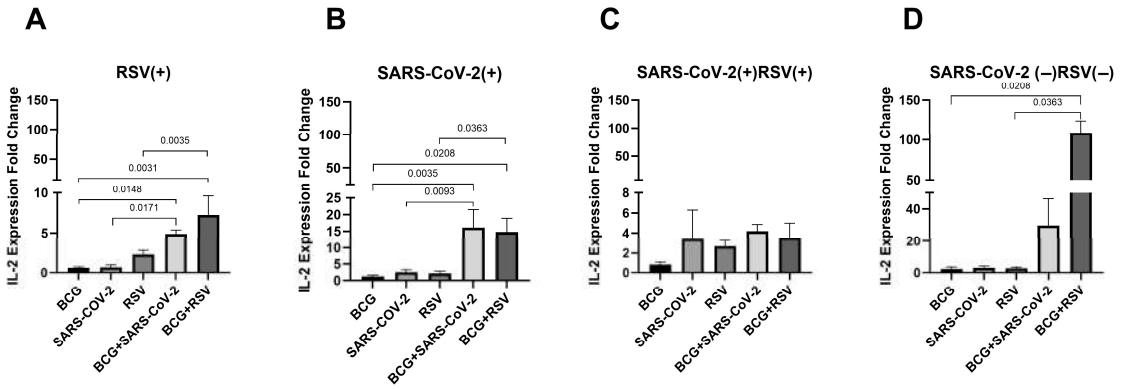

Figure 8. Changes in IL-2 gene mRNA expression in different groups of subjects who were simulta-

-neously stimulated with BCG bacilli and viral antigens. The data presented in the graphs reflect differences in cytokine levels between stimulated and unstimulated cultures. The Y-axis shows the relative change in expression compared to the control, expressed as a multiple relative to the unstimulated sample; (**A**). RSV(+), group seropositive for RSV infection; (**B**). SARS-CoV-2(+), group seropositive for SARS-CoV-2; (**C**). RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; (**D**). RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. To compare IL-2 concentrations between individual study groups, a two-factor nonparametric Kruskal-Wallis analysis of variance was used, supplemented by Dunn's post hoc test. Results with a *p*-value lower than 0.05 were considered statistically significant.

3.10. Relationship Between NOD2 and Cytokine Expression in Whole Blood Cultures Stimulated with Mycobacterium bovis BCG and Viral Antigens

As illustrated in Figure 9(A1–A5), a range of correlation plots are provided, which demonstrate the relationship between NOD2 and IL-1β mRNA expression levels under various stimulation conditions. In whole blood cultures stimulated with SARS-CoV-2 (Figure 9(A2)), a moderate negative correlation was observed (R = −0.3454, $p = 0.0285$). Conversely, in those co-stimulated with BCG and RSV antigens (Figure 9(A5)), a moderate positive correlation was detected (R = 0.3270, $p = 0.0241$). In turn, Figure 9(B1–B5) illustrate the correlation between NOD2 and IL-2 mRNA expression levels. In RSV-stimulated cultures (Figure 9(B3)), a moderate positive correlation (R = 0.3827, $p = 0.0097$) was observed. In the other groups, there were no correlations. The relationship between NOD2 and IL-4 mRNA is shown in Figure 9(C1–C5). A strong positive correlation was observed in SARS-CoV-2-stimulated cultures (Figure 9(C2), R = 0.6512, $p = 0.0001$). Similarly, under RSV stimulation (Figure 9(C3)), a very strong positive correlation was detected (R = 0.7503, $p < 0.0001$). In those co-stimulated with BCG+SARS-CoV-2 (Figure 9(C4)), a moderate positive correlation was found (R = 0.3089, $p = 0.0378$). In contrast, in BCG-only (Figure 9(C1)) and BCG+RSV-stimulated cultures (Figure 9(C5)), there were no correlations. The correlation between NOD2 and IL-6 mRNA expression is presented in Figure 9(D1–D5). A moderate positive correlation (R = 0.3191, $p = 0.0375$) was identified in the SARS-CoV-2-antigen-stimulated cultures (Figure 9(D2)). Similarly, a moderate correlation (R = 0.3467, $p = 0.0413$) was observed in the BCG + SARS-CoV-2-co-stimulated group (Figure 9(D4)). In the other groups, no correlation was observed between mRNA expression levels of NOD2 and IL-6. Figure 9(E1–E5) show the correlation between NOD2 mRNA and IL-10 expression. No correlation between NOD2 mRNA and IL-10 was observed in any culture. In turn, Figure 9(F1–F5) show the relationships between NOD2 mRNA levels and IL-8. A positive correlation (R = 0.4064, $p < 0.05$) was observed in whole-blood cultures stimulated with RSV antigens only (Figure 9(F5)), while in the other cases, no correlation was observed between NOD2 mRNA levels and IL-8. Figure 9(F1–F5) show the correlations between *NOD2* and *IL-8* mRNA expression. Finally, Figure 9(G1–G5) illustrate the correlation between *NOD2* and *TNF* mRNA expression under different stimulation conditions. A positive correlation (R = 0.4350, $p = 0.0081$) was solely observed within the context of the RSV-antigen-stimulated culture (Figure 9(G3)).

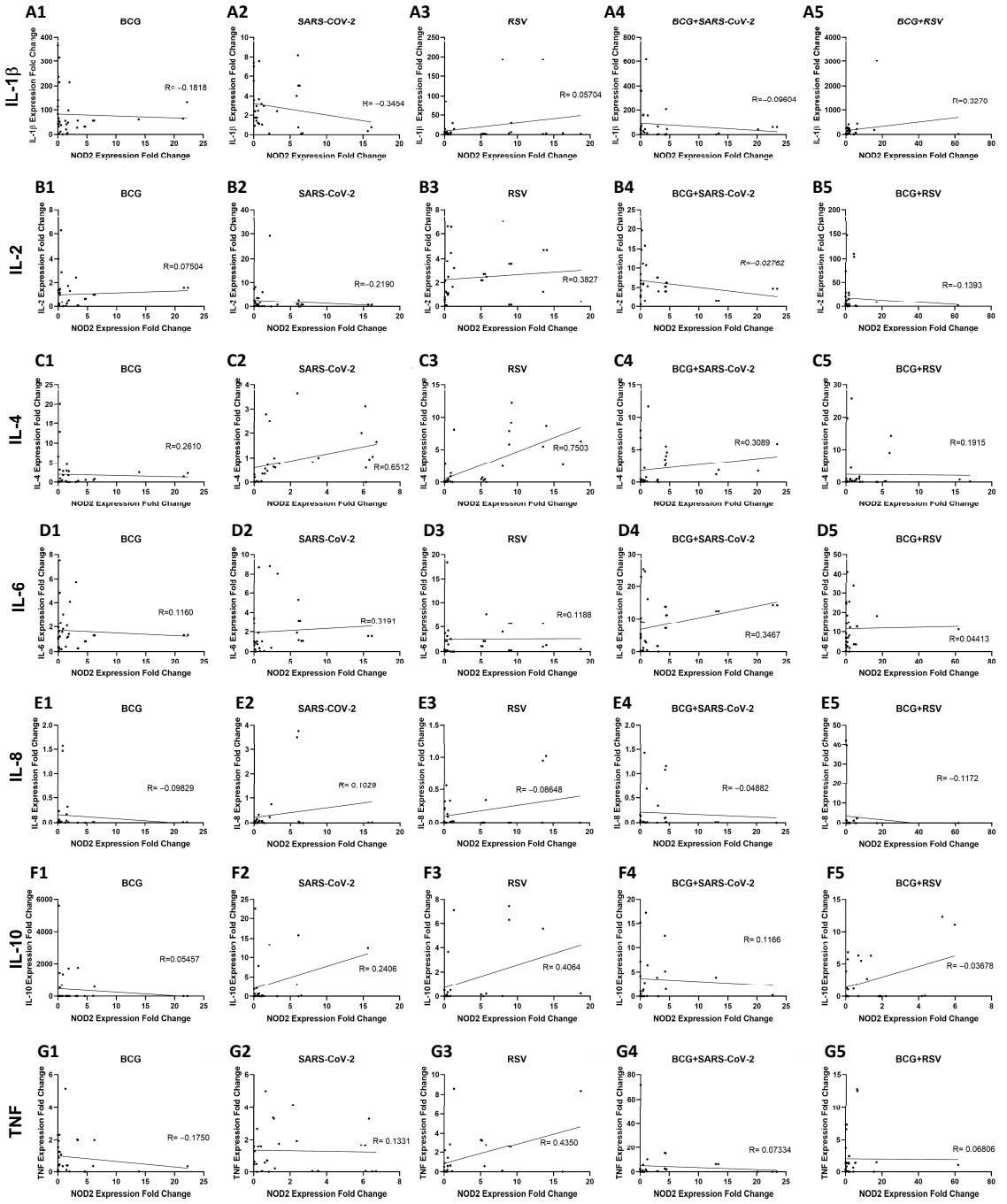

Figure 9. Correlation between NOD2 receptor expression and cytokine expression in whole-blood cultures stimulated with BCG and SARS-CoV-2 and RSV virus antigens; RSV(+), group seropositive for RSV infection; ((**A**): IL-1β; (**A1**)-BCG, (**A2**)-SARS-CoV-2; (**A3**)-RSV; (**A4**)-BCG+SARS-CoV-2; (**A5**)-BCG+RSV. (**B**): IL-2; (**B1**)-BCG, (**B2**)-SARS-CoV-2; (**B3**)-RSV; (**B4**)-BCG+SARS-CoV-2; (**B5**)-BCG+RSV. (**C**): IL-4; (**C1**)-BCG, (**C2**)-SARS-CoV-2; (**C3**)-RSV; (**C4**)-BCG+SARS-CoV-2; (**C5**)-BCG+RSV. (**D**): IL-6; (**D1**)-BCG, (**D2**)-SARS-CoV-2; (**D3**)-RSV; (**D4**)-BCG+SARS-CoV-2; (**D5**)-BCG+RSV. (**E**): IL-8; (**E1**)-BCG, (**E2**)-SARS-CoV-2; (**E3**)-RSV; (**E4**)-BCG+SARS-CoV-2; (**E5**)-BCG+RSV. (**F**): IL-10; (**F1**)-BCG, (**F2**)-SARS-CoV-2; (**F3**)-RSV; (**F4**)-BCG+SARS-CoV-2; (**F5**)-BCG+RSV. (**G**): TNF; (**G1**)-BCG, (**G2**)-SARS-CoV-2; (**G3**)-RSV; (**G4**)-BCG+SARS-CoV-2; (**G5**)-BCG+RSV). SARS-CoV-2(+), group seropositive for SARS-CoV-2; RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. Differences in the levels of CD14, HLA-DR, CD11b, and CD206 concentrations between the studied groups were compared using the Spearman correlation. A *p*-value was considered significant if <0.05.

3.11. Expression Levels of CD14, HLA-DR, CD11b, and CD206 on Monocytes

To assess monocyte activation and its association with altered cytokine and chemokine responses to non-specific stimuli in BCG-vaccinated children, we performed flow cytometric analysis of peripheral blood leukocytes (PBLs) isolated from 48 h diluted whole-blood stimulation assays. Cells were stimulated with RSV or SARS-CoV-2 antigens and subsequently stained for surface markers indicative of the monocyte phenotype and activation status. In line with the BCG-induced enhancement of broad cytokine and chemokine responses upon stimulation with heterologous antigens, we observed upregulated expression of CD14 across all child cohorts (Figure 10). Furthermore, increased HLA-DR expression was noted in monocytes from children seropositive for RSV (RSV(+)) and those co-exposed to both RSV and SARS-CoV-2 (RSV(+)SARS-CoV-2(+)), suggesting an elevated state of monocyte activation in the context of prior viral exposure and BCG priming. Interestingly, no significant BCG-related modulation was detected in the expression of CD11b across any of the study groups in response to these non-specific stimuli (Figure 10). In contrast, although overall group differences in CD206 expression did not reach statistical significance, a trend toward increased CD206 expression was evident in the RSV(+)SARS-CoV-2(+) group. This may reflect a more nuanced, stimulus-dependent modulation of monocyte function, possibly involving alternative activation pathways.

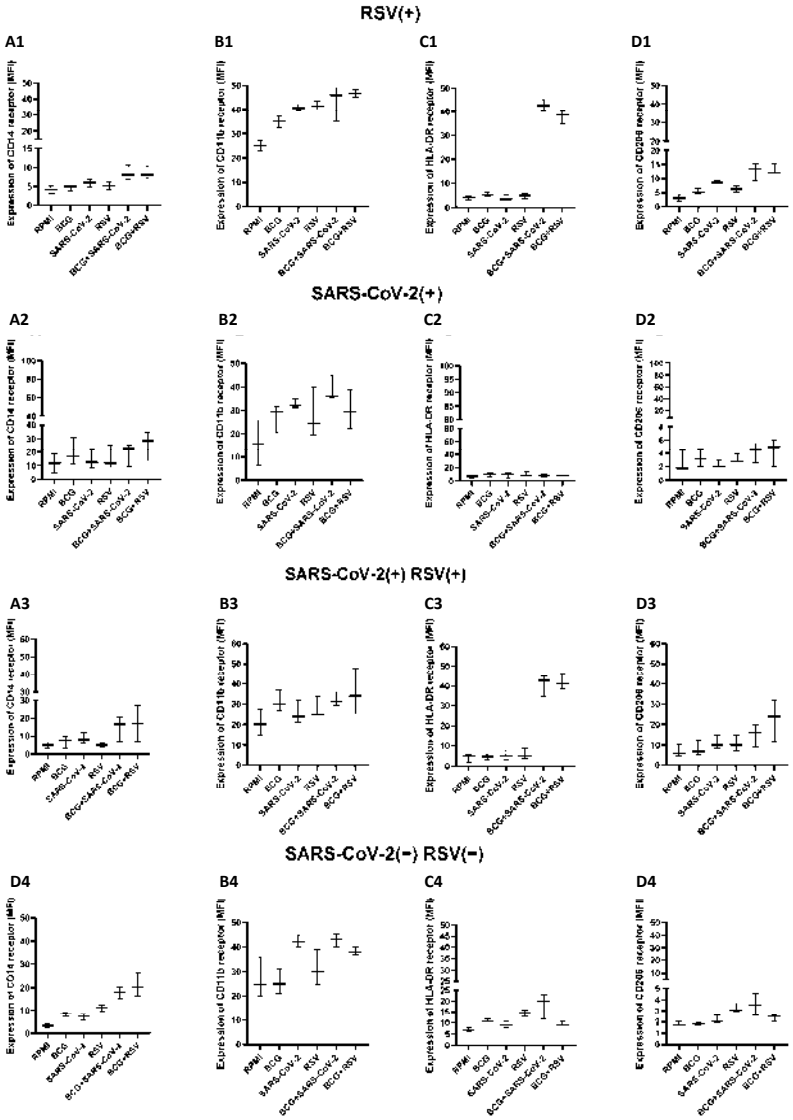

Figure 10. Density levels of CD14 (panel (**A1**–**A4**)), HLA-DR (panel (**B1**–**B4**)), CD11b (panel (**C1**–**C4**)),

and CD206 (panel (**D1**–**D4**)) receptors on monocytes after 48 h stimulation of diluted whole-blood samples from BCG-vaccinated children; RSV(+), group seropositive for RSV infection; SARS-CoV-2(+), group seropositive for SARS-CoV-2; RSV(+)SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(−)SARS-CoV-2(−), group seronegative for RSV and SARS-CoV-2, BCG, bacillus Calmette-Guérin, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RSV, respiratory syncytial virus. Differences in the levels of CD14, HLA-DR, CD11b, and CD206 concentrations between the studied groups were compared using the non-parametric Kruskal-Wallis Two-way ANOVA with Dunn's post-test. A *p*-value was considered significant if <0.05.

4. Discussion

In this study, we investigated how BCG vaccination modulates immune responses in children with varying serostatuses for SARS-CoV-2 and RSV, using an in vitro whole-blood stimulation model. We assessed the mRNA expression of key cytokines (IL-2, IL-6, IL-1β, IL-8, TNF, IL-4, IL-10), as well as the pattern recognition receptor NOD2, following stimulation with viral antigens, either alone or in combination with live BCG. Our findings emphasize the complex and dynamic nature of BCG-induced immunomodulation, demonstrating that prior viral exposure markedly influences cytokine profiles. This effect appears to be at least partly mediated through NOD2-dependent pathways, suggesting that BCG may reprogram innate immune responses in a context-specific manner.

The BCG vaccine, traditionally used against tuberculosis, has shown potential benefits in protecting against various infections through broad activation of the immune system [1]. The ability of BCG to enhance non-specific immunity may be crucial in the control of respiratory pathogens [18]. In agreement with previous studies [3,7], we observed that co-stimulation with BCG and RSV antigens led to the significant upregulation of NOD2 mRNA expression, particularly in children who were RSV-seropositive and SARS-CoV-2-seronegative/RSV-seronegative. Interestingly, we found that children with prior SARS-CoV-2 exposure (SARS-CoV-2 seropositive) exhibited the highest NOD2 expression following RSV antigen stimulation alone. This suggests that previous SARS-CoV-2 infection may alter NOD2 responsiveness to subsequent microbial stimuli, potentially shaping the immune system's ability to respond to new infections. This observation is in line with the study by [19], which demonstrated that aerosol-delivered BCG vaccination in rhesus macaques resulted in increased cytokine responses following SARS-CoV-2 challenge. Moreover, these findings corroborate reports from [20], who described altered cytokine profiles in individuals with severe COVID-19, further supporting the notion that prior viral infections may modulate immune system responses.

Cytokine expression patterns were markedly influenced by BCG vaccination. Our study showed the differential expression of key pro-inflammatory cytokines (IL-1β, IL-6, TNF) depending on the individuals' serological status and BCG stimulation, suggesting an important role for this vaccine in shaping the immune response against viral pathogens. Notably, we observed a significant increase in IL-1β and IL-6 mRNA levels in SARS-CoV-2-seropositive children following co-stimulation with BCG and SARS-CoV-2 antigens, suggesting that BCG vaccination enhances innate immune responses to SARS-CoV-2. These findings are in agreement with those of [19], who showed the similar upregulation of pro-inflammatory cytokines in rhesus macaques vaccinated with BCG before SARS-CoV-2 exposure. IL-6, in particular, was upregulated across all groups, reflecting its dual role in both pro-inflammatory and anti-inflammatory responses [21]. This observation corresponds with earlier studies indicating that IL-6 plays a critical role in both protection and pathology during respiratory viral infections [20,22]. IL-6 is rapidly produced in the lungs and airways following RSV infection, with its levels often remaining elevated throughout the course of the disease [22,23] (McNamara et al., 2003; Levitz et al., 2012). As a key regulator of the inflammatory response, IL-6 promotes the maturation of regulatory T

cells (Tregs) and limits excessive Th1 activity [24]. Through these mechanisms, IL-6 may reduce the severity of RSV-induced pathology while maintaining effective viral clearance. Previous studies have demonstrated that the early depletion of IL-6 during infection leads to an increased viral load, more severe inflammatory responses, and greater lung damage [25]. Furthermore, Besteman et al. (2022) examined the impact of IL-6 on RSV infection and found that CD14-deficient patients showed reduced IL-6 expression, resulting in the inadequate control of RSV infection [26]. In our earlier research, we showed that the stimulation of cells with BCG antigens can affect the interferon response to RSV antigens, revealing significant differences in the secretion and mRNA expression of IFN-α and IFN-γ in response to both RSV antigens and the BCG vaccine [27]. Our current results build upon these studies, demonstrating the effect of BCG stimulation on additional cytokines. Specifically, we observed increased IL-6 expression in cell cultures co-stimulated with BCG and RSV. This effect was especially pronounced in seronegative patients. In the SARS-CoV-2(−)RSV(−) group, BCG stimulation, in the absence of prior viral exposure, modulated the pro-inflammatory response without attenuating it. Elevated IL-6 expression in these cases may aid in controlling RSV infection by enhancing regulatory mechanisms within the inflammatory response. The elevated IL-2 levels in BCG+SARS-CoV-2-co-stimulated cultures further corroborate the ability of BCG to induce robust immune responses, as seen in other studies on trained immunity [28]. A particularly noteworthy observation was the significant increase in IL-10 expression, especially in cultures co-stimulated with BCG and SARS-CoV-2. IL-10 is a well-known regulatory cytokine that plays a crucial role in limiting excessive inflammation while promoting antiviral responses [29]. This suggests that stimulation with the BCG vaccine not only boosts immune activation but also helps regulate the immune response to prevent immunopathology, particularly during viral infections. This observation is consistent with findings from [19] and studies on the role of IL-10 in mitigating inflammation during viral infections.

An imbalance between Th1 and Th2 cells is thought to be responsible for the severity of disease in RSV-infected individuals [30]. A dominant Th2 response marked by elevated levels of IL-4, IL-5, IL-6, and IL-10 has been associated with ineffective viral clearance and more severe disease symptoms. In turn, Th1 cells, which produce IFN-γ, TNF, and IL-2, promote the activity of cytotoxic T cells and NK cells, enabling effective viral clearance [31,32]. It is known that a severe course of RSV infection is associated with an excessive Th2 (IL-4) and a reduced Th1 (IFN-γ) response [33,34]. In earlier studies, we showed that the stimulation of whole-blood cells with the BCG vaccine can modulate the interferon (IFN-γ) response during viral infections [27]. However, our current data suggest a more complex effect of BCG stimulation on immunity against RSV, involving both protective (Th1) and potentially damaging (Th2) pathways. We observed that co-stimulation with BCG and RSV antigens led to increased mRNA expression of both cytokines associated with Th1-type responses (e.g., IL-2) and cytokines specific to Th2-type responses (e.g., IL-4, IL-10, and IL-6). Elevated levels of IL-2 and IL-4 may suggest that the BCG vaccine promotes the simultaneous activation of Th1 and Th2 responses, which would control inflammation while eliminating the virus. However, it must be borne in mind that simultaneous increases in both cytokines do not always imply a perfect balance between these types of responses. Interestingly, the observed simultaneous increase in IL-2 and IL-4 following combined BCG + RSV stimulation suggests an integrated, rather than polarized, T helper response. IL-2 is central to Th1-driven T cell proliferation and cytotoxic function, while IL-4 promotes Th2 differentiation and humoral immunity. The concurrent upregulation of both cytokines may represent an intermediate or transitional state, enabling a balanced immune response that leverages Th1-mediated cellular defenses (crucial for intracellular pathogens like RSV) alongside Th2 mechanisms that modulate inflammation or prime the system for future

antigen exposure. This pattern corroborates findings from other contexts, where high doses of BCG have induced mixed Th1/Th2 responses in mice (e.g., lower doses favor a Th1 bias, but higher doses shifted toward mixed responses) [35]. Moreover, heterologous priming with BCG has been shown to produce the context-dependent activation of both innate and adaptive immunity, supporting trained immunity and broad immunological adaptability. Notably, studies of recombinant BCG-based RSV vaccines in humans indicate that such platforms can elicit robust IFN-γ and IL-2 secretion alongside a measured IL-4 release, reflecting similarly balanced immune activation. Thus, our data align with mounting evidence that BCG does not strictly skew immunity toward Th1 responses but can facilitate versatile and heterogeneous cytokine milieus, tailored to the pathogen context. Future work should dissect the T cell subset dynamics to clarify the downstream effects of this mixed cytokine profile.

Interestingly, we observed stronger IL-2 and IL-6 mRNA expression in the seronegative groups (SARS-CoV-2(−)/RSV(−)) after stimulation with BCG and RSV antigens. The seronegativity to these viruses indicates that the immune system of these individuals had no previous contact with these viruses. Consequently, their immune cells may be more reactive to new antigens, resulting in stronger cytokine mRNA expression. This finding aligns with the concept of "trained immunity" [36], in which prior exposure to one pathogen, such as through BCG vaccination, can enhance the immune system's response to novel pathogens.

Our study suggests the central role of NOD2 in shaping immune responses to BCG and viral antigens. We observed that NOD2 expression was positively correlated with the production of several cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF (Figures S1–S10), indicating that NOD2 may act as a key upstream regulator of cytokine responses [4]. NOD2 has previously been implicated in the modulation of both innate and adaptive immune responses to infections [3,7]. In this context, our data suggest that the NOD2-dependent regulation of cytokine production plays a pivotal role in the heterologous immunity induced by BCG stimulation. Interestingly, our results indicate that NOD2 may have a distinct role depending on the serological status of the individuals. In the SARS-CoV-2-seropositive group, NOD2 expression correlated positively with IL-4 and IL-6 after SARS-CoV-2 stimulation, indicating that NOD2 may influence both inflammatory and regulatory pathways in previously infected individuals. Conversely, in the seronegative children, we observed an inverse correlation between NOD2 and TNF, suggesting a potential regulatory role for NOD2 in preventing excessive inflammation in naïve immune systems. These observations are in line with previous research, which showed altered NOD2 expression in response to COVID-19 severity [37], although our study did not detect significant differences in NOD2 mRNA expression in SARS-CoV-2-stimulated cultures. Interestingly, our results indicate that NOD2 may have a distinct role depending on the serological status of the individuals. For example, in the SARS-CoV-2(+) group, NOD2 expression correlated positively with IL-4 and IL-6 after SARS-CoV-2 stimulation, suggesting that NOD2 may influence both inflammatory and regulatory pathways in previously infected hosts. Conversely, in seronegative children, we observed an inverse correlation between NOD2 and TNF, which may indicate a regulatory role for NOD2 in preventing excessive inflammation in naïve immune systems. This aligns with findings by [37], who observed altered NOD2 expression in response to COVID-19 severity, although our study did not detect significant differences in NOD2 mRNA expression in SARS-CoV-2-stimulated cultures. The role of NOD2 in immune regulation was further supported by our observation that NOD2 expression was correlated with IL-10 levels, particularly in cultures co-stimulated with BCG and RSV antigens. This suggests that NOD2 may help balance the immune response, promoting anti-inflammatory cytokine

production to limit immunopathology during viral infections. This is particularly relevant as excessive inflammation, as observed in diseases like COVID-19 [20], can lead to severe disease outcomes.

The findings also suggest that prior viral exposure, whether from RSV or SARS-CoV-2, significantly influences immune responses to BCG antigen stimulation. For instance, in the RSV-seropositive group, we observed elevated IL-1β and IL-10 mRNA levels following co-stimulation with BCG and RSV, which mirrors earlier findings by [38], showing enhanced cytokine production following RSV infection. These results imply that viral exposure may "prepare" the immune system, priming it for a more robust inflammatory response upon subsequent stimulation, a concept that aligns with the idea of immune memory and trained immunity [3]. Interestingly, we observed that seronegative children (SARS-CoV-2(−)/RSV(−)) showed stronger IL-2 and IL-6 mRNA responses to BCG and viral antigen co-stimulation. This suggests that the immune system of seronegative individuals, without prior viral exposure, may respond more vigorously to new antigens, reflecting the enhanced capacity of trained immunity to prime naïve immune systems [36]. These findings also complement our previous observations of differential interferon responses to RSV antigens in BCG-vaccinated individuals [27].

The differential cytokine profiles observed in this study, which varied with prior viral exposure and antigenic stimulation in BCG-vaccinated children, highlight the complex interactions between past infections and immune responses to respiratory pathogens. The ability of BCG to induce both pro-inflammatory and regulatory cytokines highlights its complex role in immune regulation. This dual effect may provide a mechanism to boost antiviral defenses while minimizing the risk of immunopathology, a critical factor in managing diseases like COVID-19. Furthermore, the insights gained from this study pave the way for designing novel immunomodulatory strategies to enhance protection against respiratory infections, especially in the context of emerging pathogens such as SARS-CoV-2.

In our study, we did not detect any significant differences in the polymorphism of the NOD2 gene, which may limit the interpretation of the differential patterns of immune responses observed in children vaccinated with BCG. Similarly, the absence of mutations in the NOD2/CARD15 gene was also reported in a study by Yamazaki et al. among 483 Japanese patients with Crohn's disease [39]. Thus, the mechanism of impaired immunity associated with the 3020insC mutation is unlikely to play an important role in the immune response of children in this age group in Poland. The potential influence of genetic variations in NOD2 on immune responses, particularly in relation to BCG-induced immune modulation, has been well-documented in previous studies. For instance, research by [40] emphasizes that genetic variations in NOD2 may influence both innate and adaptive immune responses to stimuli such as BCG, suggesting a potential interaction between genetic factors and vaccine-induced immune training. The study demonstrated that γ-irradiated BCG had long-term effects on immune responses, with the significant modulation of cytokine profiles in both in vitro and in vivo models. However, this modulation was not solely attributed to NOD2 polymorphisms, suggesting that other factors, including epigenetic changes and broader immune system reprogramming, may also play crucial roles in BCG's effectiveness. The findings from Arts et al. [40] further highlight the intricate and multifaceted nature of BCG-induced immunity. They found that BCG vaccination can have lasting effects on the immune system, extending well beyond the direct response to *Mycobacterium tuberculosis*. Their work demonstrated that the immune system's responsiveness to secondary pathogens, such as viruses, was enhanced after BCG vaccination, a phenomenon attributed to the induction of trained immunity [40]. This concept, which involves the long-term reprogramming of innate immune cells to respond more robustly to subsequent infections, aligns with our observation that BCG stimulation affects cytokine ex-

pression in children with varying serostatuses to SARS-CoV-2 and RSV. Although our study did not observe significant differences in NOD2 gene polymorphisms in BCG-vaccinated children, it cannot be excluded that subtle genetic differences may shape patterns of the immune response to BCG stimulation and viral stimuli. It is worth noting that NOD2 is a key player in innate immunity, particularly in the recognition of bacterial components and the activation of downstream signaling pathways, such as the NF-κB pathway, which modulates the production of pro-inflammatory cytokines. Given the central role of NOD2 in maintaining immune homeostasis and its involvement in recognizing pathogens like RSV, as well as modulating responses to BCG vaccination, the absence of genetic differences in our study suggests that other factors may be more prominent in shaping the immune response. Future research focused on exploring the impact of NOD2 polymorphisms in more detail, particularly in relation to trained immunity and vaccine responses, would be valuable in further understanding the genetic underpinnings of immune responses to BCG and its potential role in mitigating respiratory infections like RSV and SARS-CoV-2.

The present data confirm that BCG-primed children mount a quantitatively and qualitatively altered monocyte response when confronted with heterologous viral antigens. The increased expression of CD14 and HLA-DR observed in children after RSV or SARS-CoV-2 infections may be indicative of prolonged monocyte activation and persistent changes in the non-specific response, dependent on previous exposure to pathogens. The similar upregulation of CD14, TLR4, and CD11b was first described three months after BCG vaccination in adults, where it was shown to depend on the NOD2-driven epigenetic remodeling of monocytes. More recently, Ahmed et al. demonstrated that BCG revaccination boosts pro-inflammatory cytokine production by HLA-DR^{+}CD14+ CD16^{-} cells in adults, further corroborating the link between BCG and sustained monocyte activation. Our findings extend those observations to the pediatric setting and indicate that prior infection by common respiratory viruses provides an additional layer of stimulus-specific imprinting [41]. Interestingly, we did not observe the significant upregulation of CD11b on monocytes following BCG or RSV stimulation, despite clear evidence of activation through increased cytokine production and elevated expression of CD14 and HLA-DR. This finding contrasts earlier studies in adults, where CD11b upregulation was reported following BCG vaccination. Several factors may explain this discrepancy. Firstly, age-dependent kinetics are likely to play a role. Our study focused on pediatric participants whose samples were collected relatively soon after BCG vaccination in newborns, whereas studies involving adults typically included longer intervals between vaccination and analysis. It is possible that CD11b expression peaks at different times in infants and may have been missed in our sampling window. It is also known that CD11b undergoes rapid internalization upon activation, particularly in response to viral ligands. This internalization may reduce surface detectability via flow cytometry, even if the receptor is functionally active. Additionally, the heterogeneity of monocyte subsets and the nature of the stimulus (e.g., live virus vs. vaccine) may influence the degree and timing of CD11b expression. Context-dependent differences in marker expression have been observed in other models. For example, in macaque infants, BCG administered to newborns selectively enhanced cytokine-rich responses without causing a uniform increase in all canonical activation markers, including CD11b. This suggests that BCG and RSV may activate monocytes through pathways that do not strongly induce CD11b or that regulatory feedback mechanisms limit its surface expression to prevent excessive inflammation [42]. Future studies incorporating kinetic analyses and subset-specific phenotyping will be valuable to clarify the role of CD11b in monocyte activation under these conditions.

The tendency toward higher CD206 (mannose receptor) expression in the RSV(+)SARS-CoV-2(+) subgroup adds nuance to this picture. RSV infection of a three-dimensional

human lung model was recently shown to drive the differentiation of blood monocytes toward a CD206+ HLA-DR+ phenotype that retains antiviral functionality while acquiring tissue-resident features [43]. Considering that BCG is capable of priming both classical and alternative activation pathways, the modest CD206 upregulation observed may indicate a transition toward a reparative or immunoregulatory state, apparent only in children who have undergone sequential viral exposures.

Taken together, our results support a model in which BCG-induced innate immunity does not merely amplify baseline immune signaling but reprograms monocytes to respond in a stimulus- and history-dependent manner. The upregulation of CD14 and HLA-DR reflects a shared activation phenotype, while the differential expression of CD11b and CD206 suggests more nuanced modulation shaped by the timing of BCG exposure and the nature of subsequent viral encounters. These receptor-level adaptations likely act in concert with epigenetically imprinted transcriptional changes, enabling a broad yet highly calibrated immune response. Such reprogramming may underlie the heterologous protection observed after BCG vaccination against unrelated respiratory pathogens, including RSV and SARS-CoV-2 [41].

This study has several important limitations that should be considered when interpreting the results. First, all participants were BCG-vaccinated children, and we did not include a BCG-unvaccinated control group due to national vaccination policies and ethical constraints. Therefore, it is not possible to definitively attribute the observed immune response patterns solely to BCG vaccination. Second, multiple cytokines were tested simultaneously, which increases the risk of false positive results. We addressed this by applying the Benjamini-Hochberg correction to control the false discovery rate. However, this approach may also increase the chance of false negatives, which should be considered in the interpretation of non-significant findings. Third, as with any observational study, potential confounding variables such as environmental exposures, the genetic background, and health status could influence immune responses. Although we controlled for major confounders where possible, unmeasured factors may have contributed to the observed variability. Finally, an important limitation of this study is the relatively small and uneven sample size across the four groups, which may reduce the statistical power. The analysis indicated that with our sample size ($N = 48$) and assuming a medium effect size ($f = 0.25$), the achieved power was 69%. This increases the risk of type II errors, particularly for smaller subgroups, and may limit the detection of biologically relevant effects. While we applied non-parametric methods and implemented several analytical controls, we recognize that these findings should be interpreted with caution. This study is exploratory in nature, and the results should be validated in larger, prospectively powered cohorts. Nevertheless, the effect size estimates generated here may serve as a basis for sample size planning in future studies.

5. Conclusions

In summary, our results indicate that in BCG-vaccinated children, immune responses to respiratory viruses are associated with differential NOD2-dependent cytokine expression, which may reflect the influence of both prior viral exposures and antigenic stimulation. The induction of both pro-inflammatory and regulatory cytokines in response to RSV and SARS-CoV-2 antigens in BCG-vaccinated children suggests a balanced immune profile, potentially shaped by prior antigenic exposures and immune training. These results underscore the therapeutic potential of BCG in strengthening host immunity against respiratory infections and offer valuable insights for the development of future vaccine strategies and immunomodulatory interventions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens14070683/s1, Figure S1: Genotyping 1007fs; Figure S2: Genotyping G908R; Figure S3: Genotyping R702W; Figure S4: Interdependence of NOD2 and IL-1β expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S5: Interdependence of NOD2 and IL-2 expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S6: Interdependence of NOD2 and IL-4 expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S7: Interdependence of NOD2 and IL-6 expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S8: Interdependence of NOD2 and IL-8 expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S9: Interdependence of NOD2 and IL-10 expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens; Figure S10: Interdependence of NOD2 and TNF expression in whole-blood cultures following stimulation with BCG, SARS-CoV-2, and RSV antigens.

Author Contributions: Conceptualization, M.D. and M.J.; methodology, M.D. and M.J.; software, M.J.; validation, M.J. and P.S.; formal analysis, M.K.-P.; investigation, M.J.; resources, M.K.-P.; data curation, J.K.; writing—original draft preparation, M.J. and M.D.; writing—review and editing, M.J. and M.D.; visualization, M.J.; supervision, M.D. and M.K.-P.; project administration, M.J.; funding acquisition, M.J. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by funding allocated for scientific research conducted by doctoral students at the Bio-Med-Chem Doctoral School, University of Lodz, and the Lodz Institutes of the Polish Academy of Sciences, Lodz, Poland.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of the Medical University in Lodz, Poland (no. RNN/122/22/KE).

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

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corre-sponding author(s).

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

Abbreviations

The following abbreviations are used in this manuscript:

BCG	Bacillus Calmette-Guérin
SARS-CoV-2	Directory of open access journals
RSV	Three letter acronym
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
H3K4me3	Histone 3 at lysine 4
IL	Interleukin
HPV	Human papillomavirus
HSV	Herpes simplex virus
Th1	Type 1 helper lymphocyte
IFN	Interferon
rBCG-RSV	Recombinant BCG vaccine expressing the RSV nucleoprotein
N	number

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WYKAZ POZOSTAŁEGO DOROBKU NAUKOWEGO

PUBLIKACJE NAUKOWE

Godkowicz, M., & Rudnicka, K. (**2021**). Usefulness of microbial cytotoxins in the diagnosis of selected bacterial infections. *Advancements of Microbiology, 60*(3), 211–222. https://doi.org/10.21307/PM-2021.60.3.17

IF: 1,118; MNiSW: 20

Druszczyńska, M., **Godkowicz, M**., Kulesza, J., Wawrocki, S., & Fol, M. **(2022)**. Cytokine Receptors—Regulators of Antimycobacterial Immune Response. *International Journal of Molecular Sciences*, *23*(3), 1112. https://doi.org/10.3390/ijms23031112

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Godkowicz, M., & Druszczynska, M. (**2022**). Biological functions and diagnostic implications of microRNAs in *Mycobacterium tuberculosis* infection. *Asian Pacific Journal of Tropical Biomedicine, 12*(1), 1–8. https://doi.org/10.4103/2221-1691.333208

IF: 1,545; MNiSW: 100

Druszczynska, M., Seweryn, M., Sieczkowska, M., Kowalewska-Pietrzak, M., Pankowska, A., **Godkowicz, M**., & Szewczyk, R. (**2022**). Targeted metabolomics analysis of serum and *Mycobacterium tuberculosis* antigen-stimulated blood cultures of pediatric patients with active and latent tuberculosis. *Scientific Reports, 12*, 4131. https://doi.org/10.1038/s41598-022-08201-4

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Druszczynska, M., Seweryn, M., Wawrocki, S., Pankowska, A., **Godkowicz, M**., & Kowalewska-Pietrzak, M. **(2023)**. The interferon-gamma release assay versus the tuberculin skin test in the diagnosis of *Mycobacterium tuberculosis* infection in BCG-vaccinated children and adolescents exposed or not exposed to contagious TB. *Vaccines, 11*(2), 387. https://doi.org/10.3390/vaccines11020387

IF: 3,4; MNiSW: 140

Fol, M., Karpik, W., Zablotni, A., Kulesza, J., Kulesza, E., **Godkowicz, M**., & Druszczynska, M. (2024). Innate lymphoid cells and their role in the immune response to infections. *Cells, 13*(4), 335. https://doi.org/10.3390/cells13040335

IF: 5,2; MNiSW: 140

Druszczynska, M., Seweryn, M., Wawrocki, S., Pankowska, A., Kulbachko, A., **Jurczak, M**., & Kowalewska-Pietrzak, M. (2025). Interferon (IFN)-gamma (γ) inducible protein 10 (IP-10) in

the diagnosis of latent and active tuberculosis in Bacille Calmette Guerin (BCG)-vaccinated pediatric population. *PLoS ONE,* *20*(1), e0314400. https://doi.org/10.1371/journal.pone.0314400

IF: 3,24; MNiSW: 100

Kozłowska, E., Agier, J., Różalska, S., **Jurczak, M**., Góralczyk-Bińkowska, A., & Żelechowska, P. (2025). Fungal β-Glucans Shape Innate Immune Responses in Human Peripheral Blood Mononuclear Cells (PBMCs): An In Vitro Study on PRR Regulation, Cytokine Expression, and Oxidative Balance. *International Journal of Molecular Sciences*, *26*(13), 6458. https://doi.org/10.3390/ijms26136458

IF: 4,9; MNiSW: 140

Podsumowanie całego dorobku Sumaryczny

IF = 43,507

Łączna liczba punktów MNiSW = 1400

Wartość IF oraz punktację MNiSW podano zgodnie listą obowiązującą w roku publikacji

KOMUNIKATY ZJAZDOWE

Godkowicz M., Kowalewska-Pietrzak M., Pankowska A., Druszczyńska M.. *Stężenie cytokin profilu Th1, Th2, Th17 oraz poziom przeciwciał klasy IgG i IgA anty-BCG w surowicach zdrowych dzieci poddanych szczepieniu przeciwgruźliczemu we wczesnym dzieciństwie*. V Sesja Mikrobiologów Środowiska Łódzkiego – Łódź – 10.06.2022

Godkowicz M., Kowalewska-Pietrzak M., Pankowska A., Druszczyńska M. *The comparison study between the interferon-gamma release assay and tuberculin skin test in healthy children and adolescents vaccinated with BCG.* FEMS Conference on Microbiology – Belgrad, Serbia – 30.06–02.07.2022

Godkowicz M., Kowalewska-Pietrzak M., Kaczmarek J., Druszczyńska M. *microRNAs and cytokines involved in T lymphocyte functions in BCG-vaccinated children.* SIICA Congress 2023 – 22–25.05.2023

Godkowicz M., Kowalewska-Pietrzak M., Kaczmarek J., Druszczyńska M. *Immunomodulujący wpływ szczepionki Bacillus Calmette-Guérin (BCG) na odpowiedź cytokinową in vitro wzbudzoną przez antygeny koronawirusa ciężkiego ostrego zespołu oddechowego-2 (SARS-CoV-2) i syncytialnego wirusa oddechowego (RSV).* Ogólnopolska Konferencja „Biotechnology, new perspectives for a better future" – 09.09.2023

Godkowicz M., Kowalewska-Pietrzak M., Kaczmarek J., Druszczyńska M. *Optymalizacja warunków analizy polimorfizmu długości fragmentów restrykcyjnych (RFLP) w ocenie mutacji domeny oligomeryzacji wiążącej nukleotydy (NOD2).* Ogólnopolska Konferencja „Biotechnology, new perspectives for a better future" – 09.09.2023

Jurczak M., Kaczmarek J., Kowalewska-Pietrzak M., Druszczyńska M. *Immunomodulujący wpływ szczepionki BCG na odpowiedź interferonową in vitro wzbudzoną przez antygeny wirusowe*. VII Sesja Młodych Mikrobiologów Środowiska Łódzkiego – Łódź – 14.06.2024

Jurczak M., Kaczmarek J., Kowalewska-Pietrzak M., Druszczyńska M. *Wpływ szczepionki Bacillus Calmette-Guérin (BCG) na odpowiedź interferonową wzbudzoną przez antygeny wirusowe*. XXIII Konferencja Naukowo-Szkoleniowa Alergia Astma Immunologia Kliniczna – Łódź – 13–15.06.2024

Jurczak M., Kaczmarek J., Kowalewska-Pietrzak M., Druszczyńska M. *Bacillus Calmette-Guérin (BCG) Vaccine's role in modulating immune responses against severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) and respiratory syncytial virus (RSV) in children*. ECI 2024 European Congress of Immunology – Dublin, Irlandia – 01–04.09.2024

Jurczak M., Kaczmarek J., Kowalewska-Pietrzak M., Druszczyńska M. *Wpływ receptora NOD2 na produkcję cytokin i ekspresję receptorów powierzchniowych monocytów w odpowiedzi na BCG (Bacillus Calmette-Guérin) i antygeny wirusowe.* XXIV Konferencja Naukowo-Szkoleniowa Alergia Astma Immunologia Kliniczna 2025 – 05–07.06.2025

Jurczak M., Kaczmarek J., Kowalewska-Pietrzak M., Druszczyńska M. *Wpływ prątków BCG na odpowiedź immunologiczną dzieci wobec antygenów wirusów oddechowych – Nagroda za najlepszy plakat*. VIII Sesja Młodych Mikrobiologów Środowiska Łódzkiego – Łódź – 13.06.2025

OŚWIADCZENIA WSPÓŁAUTORÓW O UDZIALE W PUBLIKACJACH

Oświadczenie o udziale w publikacjach

Mgr Magdalena Jurczak

Jurczak, M., & Druszczynska, M. (2025). Beyond Tuberculosis: The Surprising Immunological Benefits of the Bacillus Calmette–Guérin (BCG) Vaccine in Infectious, Auto-Immune, and Inflammatory Diseases. Pathogens, 14(2),196.

*Oświadczam, że mój udział w ww. publikacji wynosi **70%,** który obejmował współudział w tworzeniu koncepcji pracy i przygotowaniu manuskryptu.*

.....
Podpis współautora

Godkowicz, M., & Druszczyńska, M. (2022). NOD1, NOD2, and NLRC5 Receptors in Antiviral and Antimycobacterial Immunity. Vaccines, 10(9), 1487.

*Oświadczam, że mój udział w ww. publikacji wynosi **70%,** który obejmował współudział w tworzeniu koncepcji pracy i przygotowaniu manuskryptu.*

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Podpis współautora

Jurczak, M., Kaczmarek, J., Kowalewska-Pietrzak, M. & Druszczynska, M. (2025). Immunomodulatory Effect of the Bacillus Calmette–Guérin (BCG) Vaccine on the In Vitro Interferon Response Induced by Respiratory Syncytial Virus (RSV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigens. Archivum Immunologiae et Therapiae Experimentalis, 73(1).

*Oświadczam, że mój udział w ww. publikacji wynosi **55%,** który obejmował współudział w tworzeniu koncepcji pracy, wykonaniu doświadczeń, analizie wyników i przygotowaniu manuskryptu.*

.....
Podpis współautora

Jurczak, M., Kaczmarek, J., Kowalewska-Pietrzak, M., Stelmach, P., & Druszczynska, M. (2025). NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. Pathogens, 14(7), 683.

*Oświadczam, że mój udział w ww. publikacji wynosi **55%,** który obejmował współudział w tworzeniu koncepcji pracy, wykonaniu doświadczeń, analizie wyników i przygotowaniu manuskryptu.*

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Podpis współautora

Oświadczenie o udziale w publikacjach

Dr hab. Magdalena Druszczyńska, prof. UŁ

Jurczak, M., & **Druszczynska, M.** (2025). Beyond Tuberculosis: The Surprising Immunological Benefits of the Bacillus Calmette–Guérin (BCG) Vaccine in Infectious, Auto-Immune, and Inflammatory Diseases. Pathogens, 14(2),196.

*Oświadczam, że mój udział w ww. publikacji wynosi **30%,** który obejmował współudział w tworzeniu koncepcji pracy i przygotowaniu manuskryptu.*

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Podpis współautora

Godkowicz, M., & **Druszczyńska, M.** (2022). NOD1, NOD2, and NLRC5 Receptors in Antiviral and Antimycobacterial Immunity. Vaccines, 10(9), 1487.

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Podpis współautora

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*Oświadczam, że mój udział w ww. publikacji wynosi **20%,** który obejmował współudział w tworzeniu koncepcji pracy, nadzór nad realizacją pracy, ocenę postępów pracy, uczestnictwo w interpretacji wyników i przygotowaniu manuskryptu.*

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Podpis współautora

Jurczak, M., Kaczmarek, J., Kowalewska-Pietrzak, M., Stelmach, P., & **Druszczynska, M.** (2025). NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. Pathogens, 14(7), 683.

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Podpis współautora

Oświadczenie o udziale w publikacjach

Dr Joanna Kaczmarek

Jurczak, M., **Kaczmarek, J.,** Kowalewska-Pietrzak, M., Stelmach, P., & Druszczyńska, M. (2025). NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. *Pathogens*, *14*(7), 683.

*Oświadczam, że mój udział w ww. publikacji wynosi **15%,** który obejmował pozyskiwanie materiału biologicznego i klasyfikację pacjentów.*

.....

Podpis współautora

*Jurczak, M., **Kaczmarek, J.,** Kowalewska-Pietrzak, M. & Druszczyńska, M. (2025). Immunomodulatory Effect of the Bacillus Calmette–Guérin (BCG) Vaccine on the In Vitro Interferon Response Induced by Respiratory Syncytial Virus (RSV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigens. Archivum Immunologiae et Therapiae Experimentalis, 73(1).*

*Oświadczam, że mój udział w ww. publikacji wynosi **15%,** który obejmował pozyskiwanie materiału biologicznego i klasyfikację pacjentów.*

.....

Podpis współautora

Oświadczenie o udziale w publikacjach

Dr Magdalena Kowalewska-Pietrzak

Jurczak, M., Kaczmarek, J., **Kowalewska-Pietrzak, M.,** Stelmach, P., & Druszczyńska, M. (2025). NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. *Pathogens*, *14*(7), 683.

Oświadczam, że mój udział w ww. publikacji wynosi ***10%,*** *który obejmował pozyskiwanie materiału biologicznego i klasyfikację pacjentów.*

M. Kowalewska-Pietrzak

Podpis współautora

Jurczak, M., Kaczmarek, J., ***Kowalewska-Pietrzak, M.*** *& Druszczyńska, M. (2025). Immunomodulatory Effect of the Bacillus Calmette–Guérin (BCG) Vaccine on the In Vitro Interferon Response Induced by Respiratory Syncytial Virus (RSV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigens. Archivum Immunologiae et Therapiae Experimentalis, 73(1).*

Oświadczam, że mój udział w ww. publikacji wynosi ***10%,*** *który obejmował pozyskiwanie materiału biologicznego i klasyfikację pacjentów.*

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Mgr Paulina Stelmach

Jurczak, M., Kaczmarek, J., Kowalewska-Pietrzak, M., **Stelmach, P.,** & Druszczynska, M. (2025). NOD2 (Nucleotide-Binding Oligomerization Domain-Containing Protein 2)-Mediated Modulation of the Immune Response Induced by BCG (Bacillus Calmette-Guérin) Bacilli. Pathogens, 14(7), 683.

Oświadczam, że mój udział w ww. publikacji wynosi ***1%,*** *który obejmował współudział w wykonaniu doświadczeń.*

Stelmach

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Podpis współautora