

MASARYKOVA UNIVERZITA

Lékařská fakulta

**Změny v zastoupeních a funkcích T- a B-lymfocytů a
neutrofilů u imunodeficitních a
imunokompromitovaných pacientů**

**Changes in the Distribution and Function of T and B
Lymphocytes and Neutrophils in Immunodeficient
and Immunocompromised Patients**

Habilitační práce

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Abstrakt

Průtoková cytometrie, vyvinutá v 60. letech minulého století, se stala nepostradatelnou metodou v imunologickém výzkumu i klinické diagnostice. Umožňuje detailní charakterizaci leukocytárních populací a jejich funkčních vlastností. Díky technologickému pokroku se její využití rozšířilo od studia primárních imunodeficiencí až po monitorování imunitního stavu u pacientů v kritických stavech. Metoda však i nadále naráží na limity spojené s interpretací rozsáhlých datových souborů a nutností jejich standardizace.

Autorka se dlouhodobě věnuje imunofenotypizaci T- a B-lymfocytů u pacientů s běžným variabilním imunodeficitem (CVID) a selektivním deficitem IgA. Tyto studie přispěly k lepšímu porozumění lymfocytárním abnormalitám u těchto pacientů. V rámci mezinárodní studie EUROclass se autorka podílela na hodnocení pacientů z našeho pracoviště podle jednotného protokolu a následně spolupracovala na přípravě publikace, která se stala základem klasifikace CVID pacientů. Na tuto práci navázal výzkum zaměřený na detailní analýzu vývojových stadií B buněk pomocí polychromatické průtokové cytometrie a na studium T-lymfocytárních poruch.

Další část práce byla zaměřena na myeloidní buňky, zejména neutrofilů a granulocytární myeloidní supresorové buňky (gMDSC), jejichž podíl na dysregulaci imunitní odpovědi u CVID pacientů byl dosud podceňován. V posledních letech se výzkum orientuje také na neutrofilní subpopulace u pacientů po závažném traumatu, kde tyto buňky hrají klíčovou úlohu v rozvoji prozánětlivých procesů a oslabení imunity.

Habilitační práce je zpracována jako soubor komentovaných publikací, které dokumentují autorčinu vědeckou činnost v oblasti imunofenotypizace a funkční charakterizace leukocytů metodou průtokové cytometrie.

Klíčová slova: průtoková cytometrie, CVID, IgAD, T-lymfocyty, B-lymfocyty, neutrofilů

Abstract

Flow cytometry, developed in the 1960s, has become an indispensable method in both immunological research and clinical diagnostics. It enables detailed characterization of leukocyte populations and their functional properties. With technological advances, its applications have expanded from the study of primary immunodeficiencies to the monitoring of immune status in critically ill patients. Nevertheless, the method still faces limitations related to the interpretation of large data sets and the need for their standardization.

The author's research has long focused on the immunophenotyping of T- and B-lymphocytes in patients with common variable immunodeficiency (CVID) and selective IgA deficiency. These studies have contributed to a better understanding of lymphocytic abnormalities in these patients. Within the international EUROclass study, the author participated in the evaluation of patients from her institution according to a standardized protocol and subsequently contributed to the preparation of a publication that laid the foundation for a widely used classification of CVID patients. This work was followed by research aimed at a detailed analysis of B-cell developmental stages using polychromatic flow cytometry, as well as the study of T-lymphocyte abnormalities.

Another part of the research focused on myeloid cells, particularly neutrophils and granulocytic myeloid-derived suppressor cells (gMDSC), whose role in immune dysregulation in CVID patients had been underestimated. In recent years, the research has further expanded to neutrophil subpopulations in patients after severe trauma, where these cells play a crucial role in driving pro-inflammatory processes and immune compromise.

This habilitation thesis is presented as a collection of commented publications documenting the author's scientific work in the field of immunophenotyping and functional characterization of leukocytes using flow cytometry.

Keywords: flow cytometry, CVID, IgAD, T-lymphocytes, B-lymphocytes, neutrophils

Poděkování

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..... podpis autora

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1 Seznam zkratek

Ag	Antigen (antigen)
ALPS	Autoimmune Lymphoproliferative Syndrome (autoimunitní lymfoproliferativní syndrom)
BCR	B Cell Receptor (receptor B-lymfocytů pro antigen)
Bin	(v kontextu probability binning) diskrétní interval datového souboru, do kterého jsou řazeny cytometrické hodnoty; jednotka pro rozdělení dat v multidimenzionálním prostoru
Breg	regulatory B cells (regulační B-lymfocyty, charakterizované schopností produkovat IL-10)
B ⁺	skupina pacientů s absolutním počtem B-lymfocytů ≥ 1 %
B ⁻	skupina pacientů s absolutním počtem B-lymfocytů < 1 %
CARS	Compensatory Anti-Inflammatory Response Syndrome (kompenzační anti-inflamatorní odpověď)
CCL-2 / MCP-1	C-C motif chemokine ligand 2 / Monocyte Chemoattractant Protein 1 (chemokin přitahující monocyty)
CD	Cluster of Differentiation (klasifikační systém povrchových molekul leukocytů, např. CD3, CD4, CD8, CD16, CD19, CD21, CD56)
CD10	Common Acute Lymphoblastic Leukemia Antigen / Neprilysin (marker zralosti neutrofilů, snižuje se se stárnutím nebo senescencí)
CD11b	(Mac-1 integrin, $\alpha M\beta 2$) – adhezní molekula a receptor pro komplement, marker aktivace neutrofilů
CD16 (Fc γ RIII)	Fc gamma receptor III (marker maturace a aktivace neutrofilů)
CD21 ^{low}	subpopulace B-lymfocytů s nízkou expresí CD21
CD21 ^{norm}	subpopulace B-lymfocytů s normální expresí CD21
CD25	Interleukin-2 Receptor alpha chain (IL-2R α ; receptorová podjednotka pro IL-2, marker aktivace i regulačních T buněk)
CD28	kostimulační receptor T-lymfocytů, jeho ztráta je znakem imunosenescence
CD40	kostimulační receptor na B-lymfocytech, klíčový pro jejich aktivaci a diferenciaci
CD40L	CD40 Ligand (ligand pro CD40, exprimovaný aktivovanými T-lymfocyty)
CD45RA	izoforma tyrosinfosfatázy CD45, typická pro naivní T-lymfocyty
CD45RO	izoforma tyrosinfosfatázy CD45, typická pro paměťové T-lymfocyty

CD57	marker pozdní diferenciacie a senescence T-lymfocytů
CD62L	L-selectin (adhesní molekula, spojená s homingem naivních T-lymfocytů do lymfatických uzlin)
CD66b	marker degranulace neutrofilů
CK	Creatine Kinase (enzym, marker poškození svalů a rabdomyolýzy)
CMV	Cytomegalovirus
CR2	Complement Receptor 2 (alternativní označení pro CD21)
CR3	Complement Receptor 3 (v textu označen jako další receptor spolu s CD21i)
CVID	Common Variable Immunodeficiency (běžný variabilní imunodeficit)
CXCL-10 / IP-10	C-X-C motif chemokine ligand 10 / Interferon gamma-induced Protein 10 (chemokin indukovaný IFN- γ , chemoatraktant T buněk)
DNT	Double Negative T cells (dvojitě negativní T-lymfocyty, CD4 ⁻ CD8 ⁻)
Eotaxin (CCL11)	chemokin z rodiny C-C, přitahující eosinofily
EUROclass	mezinárodní klasifikační studie pacientů s CVID, založená na imunofenotypizaci B- lymfocytů
Fmlf	N-Formylmethionyl-leucyl-phenylalanine
FOI	Follicular I B cells (folikulární B buňky typu I)
FOII	Follicular II B cells (folikulární B buňky typu II)
FOXP3	Forkhead box P3 (transkripční faktor, hlavní marker Treg buněk)
G-CSF	Granulocyte Colony-Stimulating Factor (cytokin stimulující granulopoézu)
gMDSC	granulocytic Myeloid-Derived Suppressor Cells (granulocytární myeloidně odvozené supresorové buňky)
HIV	Human Immunodeficiency Virus (virus lidské imunodeficiency)
HLA-DR	Human Leukocyte Antigen – DR isotype (MHC II molekula)
IFN- γ	Interferon gamma
IgA	Immunoglobulin třídy A
IgD	Imunoglobulin třídy D
IgG	Imunoglobulin třídy G
IgG2	podtřída Imunoglobulinu G2
IgG4	podtřída Imunoglobulinu G4
IgM	Imunoglobulin třídy M)
Ig α / Ig β	Immunoglobulin alpha / beta chains (signální podjednotky asociované s BCR)

IL-1	Interleukin 10
IL-12 (p40)	Interleukin 12 subunit p40 (podjednotka heterodimerického cytokinu IL-12)
IL-1R	Interleukin 1 Receptor
IL-6	Interleukin 6
iLDN	immature Low-Density Neutrophils (nezralé nízkodenzitní neutrofily)
ISS	Injury Severity Score (skóre závažnosti poranění na základě anatomických kritérií)
IVIg	Intravenous Immunoglobulin (intravenózní imunoglobulin)
LDN	Low-Density Neutrophils (nízkodenzitní neutrofily, přítomné ve frakci PBMC)
LPS	Lipopolysaccharide (endotoxin gramnegativních bakterií, aktivátor TLR4)
MDSC	Myeloid-Derived Suppressor Cells (myeloidní supresorové buňky)
mLDN	mature Low-Density Neutrophils (zralé nízkodenzitní neutrofily)
MPO	Myeloperoxidase (enzym neutrofilních granulí)
MyD88	Myeloid Differentiation Primary Response 88 (adaptorová molekula v signalizaci TLR a IL-1R)
NAD ⁺	Nicotinamide Adenine Dinucleotide
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NK	Natural Killer (přirozený zabíječ, NK buňky)
PBMC	Peripheral Blood Mononuclear Cells (mononukleární buňky periferní krve, obsahují lymfocyty a monocyty)
PD-1	Programmed Death 1 (inhibiční receptor T buněk)
PD-L1	Programmed Death-Ligand 1 (inhibiční molekula, ligand receptoru PD-1)
r	Pearsonův korelační koeficient (statistická míra lineární závislosti mezi dvěma proměnným)
PMA	Phorbol Myristate Acetate
RA	Rheumatoid Arthritis (revmatoidní artritida)
ROS	Reactive Oxygen Species (reaktivní formy kyslíku)
sCD14	soluble CD14 (solubilní forma CD14)
sCD25	soluble CD25 (solubilní IL-2 receptor α)
SCID	Severe Combined Immunodeficiency (těžký kombinovaný imunodeficit)
SHM	Somatic Hypermutation (somatická hypermutace)
slgAD	Selective Immunoglobulin A Deficiency (selektivní deficit IgA)
SIRS	Systemic Inflammatory Response Syndrome (syndrom systémové zánětlivé odpovědi)

SLE	Systemic Lupus Erythematosus (systémový lupus erythematosus)
smB	switched memory B cells (izotypově přepnuté paměťové B-lymfocyty)
Tc	T cytotoxic (cytotoxické T-lymfocyty, CD3 ⁺ CD8 ⁺)
TCR	T Cell Receptor (T-buněčný receptor)
Tfh	T follicular helper cells (folikulární pomocné T-lymfocyty,)
Th	T helper cells (pomocné T-lymfocyty, CD3 ⁺ CD4 ⁺)
Th1	T helper 1 cells (podskupina pomocných T-lymfocytů produkujících IFN- γ)
Th17	T helper 17 cells (podskupina pomocných T-lymfocytů produkujících IL-17)
TLR4	Toll-Like Receptor 4 (receptor rozpoznávající LPS)
TLR7	Toll-Like Receptor 7 (receptor rozpoznávající jednovláknovou RNA)
TNF- α	Tumor Necrosis Factor alpha
TP1	Time Point 1 (časový bod odběru vzorků pacientů s traumatem, při přijetí)
TP2	Time Point 2 (časový bod odběru vzorků pacientů s traumatem, 5.–7. den po traumatu)
Treg	Regulatory T cells (regulační T-lymfocyty)
TRISS	Trauma and Injury Severity Score (kombinované skóre pro predikci přežití po traumatu)
Tr ^{hi}	transitional B cells high (subpopulace se zvýšeným zastoupením tranzientních B-lymfocytů, >9 %)
Tr ^{norm}	transitional B cells normal (subpopulace s normálním zastoupením tranzientních B-lymfocytů)

2 Úvod

2.1 Vrozené poruchy imunity a průtoková cytometrie

Vrozené poruchy imunity představují dědičné poruchy různých složek imunitního systému, které se obvykle klinicky manifestují opakovanými, často závažnými nebo život ohrožujícími infekcemi. U řady pacientů se dále objevují autoimunitní projevy nebo zvýšená náchylnost k rozvoji malignit (Leonardi et al., 2020). V posledních dvou dekádách došlo k výraznému rozšíření klinického spektra vrozených poruch imunity i k identifikaci genetického základu většiny z nich. V závislosti na konkrétním genetickém defektu může být určitá část imunitního systému zcela chybějící, snižena, nebo funkčně narušená. Přibližně 60-65 % pacientů má postižení lymfoidní linie zahrnující B- a/nebo T-lymfocyty, často v kombinaci s dalšími buněčnými typy.^{1,2}

Včasná a přesná diagnostika vrozených poruch imunity je zásadní pro nasměrování pacientů do specializovaných center, zahájení cílené terapie a poskytování genetického poradenství pacientům i jejich rodinám. Diagnostický proces bývá náročný a vyžaduje komplexní imunologické vyšetření včetně identifikace genetických příčin. V tomto ohledu se průtoková cytometrie stala klíčovým nástrojem: umožňuje detailní imunofenotypizaci, analýzu povrchových markerů i funkčních vlastností leukocytů, což napomáhá rozpoznání specifických defektů leukocytů a pochopení klinické heterogenity onemocnění. Flowcytometrické výsledky navíc často směřují další funkční testování a genetickou analýzu, čímž významně přispívají k diagnostice, rozhodování o terapii a sledování její účinnosti, včetně funkční validace nově identifikovaných genetických defektů.

Vývoj průtokové cytometrie v posledních desetiletích umožnil posun od základního diagnostického screeningu k detailnějšímu hodnocení imunitních buněk. Původně se vycházelo z kvantifikace B-, T- a NK buněk pomocí kombinace monoklonálních protilátek značených dvěma fluorochromy. K rozlišení hlavních populací se využívaly zejména následující kombinace: CD3 a CD19 pro stanovení T- a B-lymfocytů, CD3 a CD16/CD56 pro odlišení T-lymfocytů od NK buněk, CD3 a CD4 pro identifikaci pomocných Th lymfocytů, a CD3 spolu s CD8 pro určení cytotoxických Tc lymfocytů. Stanovení těchto populací bylo využito ke klasifikaci těžkého kombinovaného imunodeficitu SCID, či diagnostického screeningu vrozené agamaglobulinémie, založený pouze na stanovení koncentrace imunoglobulinů a následném vyšetření počtu B lymfocytů, přes klasifikaci běžné variabilní imunodeficiency (CVID) podle podílu transientních, naivních, izotypově přesmyknutých a CD21^{low} B-lymfocytů umožněnou 5-ti barvenou průtokovou cytometrií, až po deseti či více barevných panely používané při podrobné typizaci subpopulací T- či B-lymfocytů u kombinovaných imunodeficitů. V dnešní době cytometrické protokoly umožňují detailní stanovení lymfocytárních subpopulací zároveň s funkčními vlastnostmi těchto buněk, jako je produkce cytokinů, proliferace či exprese aktivačních markerů.

Tak jako mnoho jiných center, i naše pracoviště v průběhu doby postupně vyvinulo a používá některé vlastní vícebarevné průtokové cytometrické panely protilátek pro diagnostiku a klasifikaci vrozených poruch imunity. Existence mezinárodních studií byly často směřovány na panely protilátek pro identifikaci závažných defektů v B a T lymfocytech, NK-buňkách a/nebo diagnostický screening specifické dědičné poruchy či specifické podskupiny poruch. Příkladem může být klasifikace CVID na základě počtu jednotlivých populací B-lymfocytů,^{3,4} nebo kvantifikace CD4-CD8- (dvojitě negativních - DNT) TCRαβ⁺ T-lymfocytů pro screening autoimunitního lymfoproliferativního syndromu (ALPS).⁵

Výzkum na našem pracovišti je dlouhodobě zaměřen na studium imunologických mechanismů spojených s vrozenými poruchami imunity, zejména humorálními imunodeficiencemi a to na pacienty s běžnou variabilní imunodeficiencí (CVID) a selektivním deficitem IgA (SIgAD). Spolu s vývojem průtokové cytometrie se náš odborný zájem postupně vyvíjel od detailní fenotypizaci B-lymfocytárních subpopulací, přes charakterizaci T-lymfocytárních abnormalit, až k analýze funkčních a regulačních aspektů vrozené imunity – konkrétně neutrofilních granulocytů – v kontextu imunodysregulace a zánětlivých stavů.

3 Deficit IgA a CVID

3.1 Deficit IgA

Z humorálních vrozených poruch imunity se nejčastěji vykytuje selektivní deficit IgA (SIgAD), jehož výskyt se u bělošské populace pohybuje mezi 1 : 300 až 1 : 1200.⁶ Genetická příčina onemocnění u těchto pacientů dosud nebyla přesně identifikována. Klinicky se může projevit častějšími infekcemi, alergiemi a autoimunitními chorobami. Zvýšený výskyt autoimunitních onemocnění a infekcí je zaznamenán zejména u pacientů se SIgAD, u nichž byly popsány změny ve fenotypu B-lymfocytárních paměťových subpopulací.⁷

Vývoj onemocnění u pacientů s deficitem IgA je velmi heterogenní — někteří pacienti jsou zcela bez příznaků, zatímco u jiných může dojít k progresi do závažnější formy známé jako běžný variabilní imunodeficit.⁸ Často se také objevuje kombinace deficitu IgA s deficitem podtříd IgG, zejména IgG2 a IgG4.⁹

3.2 Běžný variabilní imunodeficit – Common Variable Immunodeficiency (CVID)

CVID je charakterizován nízkými hladinami IgG a IgA, a/nebo IgM v séru. Může se vyvinout z dříve diagnostikovaného deficitu IgA, jak už bylo popsáno výše, ale častěji vzniká samostatně, bez předchozí známky imunodeficitu.¹⁰ Nejčastěji se manifestuje ve druhé až třetí dekádě života, nicméně může být diagnostikován již v dětství nebo až ve vyšším věku. Prevalence CVID se v bělošské populaci pohybuje mezi 1 : 10 000 až 1 : 50 000.¹¹ Příčiny onemocnění zůstávají stále nejasné, s výjimkou několika geneticky definovaných případů. S ohledem na heterogenitu pacientů s CVID se liší i klinický průběh a laboratorní nálezy. U některých pacientů se vyskytují lymfoproliferativní, granulomatózní a autoimunitní onemocnění, což naznačuje odlišné klinické podskupiny v rámci této diagnózy.¹² Laboratorně u pacientů nacházíme kromě hypogamaglobulinémie, časté změny ve složení a funkci leukocytů, především T- a B-lymfocytů.

4 B-lymfocyty

4.1 Vývojová stadia B-lymfocytů v periferní krvi

Chybění imunoglobulinů je u pacientů s CVID dáno chybou v diferenciaci B-lymfocytů a projevuje se sníženým počtem dlouhodobě žijících plazmatických buněk — konečného vývojového stadia B-lymfocytů a dále snížením počtu paměťových B-lymfocytů.

Vývoj B buněk v kostní dřeni probíhá v přesně regulovaných krocích, řízených expresí a funkcí receptoru B buněk (BCR). Pro uvolnění B-lymfocytů do periferní krve je nezbytné úspěšné postupné přeskupení těžkého a později lehkého řetězce do podoby funkčního BCR, schopného rozpoznat antigen.¹³ Z kostní dřene migrují B buňky ve stadiu tzv. přechodných (transientních) B-lymfocytů, které již exprimují funkční BCR asociovaný s heterodimerem Igα–Igβ. Transientní B-lymfocyty představují mezistupeň mezi nezralými a zralými naivními B-lymfocyty a nacházejí se jak v kostní dřeni, tak v periferní krvi. Po opuštění kostní dřene, kdy transientní B-lymfocyty jako BCR exprimují IgM, pokračují v maturaci ve slezině a dalších sekundárních lymfatických orgánech, kde zvyšují expresi IgD i CD21 a dozrávají do naivních B-lymfocytů.¹⁴ Transientní B buňky představují nejhojnější populaci u novorozenců, avšak jejich zastoupení se s věkem snižuje a v dospělosti tvoří přibližně 3 % z celkové populace B-lymfocytů.¹⁵

Zralé cirkulující B-lymfocyty zahrnují naivní B buňky, které se dosud nesetkaly s antigenem, B-lymfocyty marginální zóny, izotypově přepnuté paměťové B-lymfocyty a plazmablasty. Naivní B-lymfocyty představují nejpočetnější populaci B-lymfocytů v periferní krvi. Osídlují primární lymfoidní folikuly v lymfatických uzlinách a slezině. Pokud se zde setkají se svým specifickým antigenem, který se naváže na jejich BCR, začíná další fáze jejich vývoje v zárodečných centrech. Antigenem aktivované naivní B buňky proliferují, vytvářejí primární folikul, později pak germinální centra, kde je spuštěna mutace genů imunoglobulinů tzv. somatické hypermutace (SHM) a z nově vznikajících mutovaných buněk jsou selektovány takové, jež mají vysokou afinitu k danému antigenu, neboť pouze vysoce afinitní B buňky se stávají buď paměťovými B-lymfocyty, nebo plazmatickými buňkami.¹⁶

B-lymfocyty marginální zóny představují heterogenní populaci složenou z vrozených (přirozených) paměťových B buněk a IgM paměťových B-lymfocytů, které již byly aktivovány a prošly v germinálních centrech somatickou hypermutací. Vrozené paměťové B buňky^{17,18} se vyvíjejí bez účasti germinálních center a T buněk. Podílejí se na extrafolikulárních T-nezávislých odpovědích a produkují přirozené protilátky. I tyto buňky však mohou být sekundárně remodelovány v germinálních centrech a podílet se na adaptivní imunitní odpovědi.¹⁹ IgM paměťové B-lymfocyty působí jako první obranná linie proti infekcím. Díky svému původu, funkci a lokalizaci ve slezině jsou IgM paměťové B buňky považovány za lidský ekvivalent myších B-lymfocytů marginální zóny či B-1 buněk. Bylo prokázáno, že paměťové B buňky, ať již IgM či izotypově přepnuté, jsou uloženy především ve slezině, následkem čehož je populace IgM paměťových B buněk výrazně redukována u asplenických pacientů.²⁰

B buněčná odpověď představuje klíčový prvek obrany proti širokému spektru patogenů. Aktivace B-lymfocytů antigenem (Ag) probíhá ve dvou hlavních formách: odpovědi závislé na T buňkách, které tvoří přibližně 80 % celkové B buněčné odpovědi, a odpovědi nezávislé na T buňkách, podílející se na celkové B buněčné odpovědi zhruba 20 %.^{21,22} V T-dependentních reakcích jsou B-lymfocyty aktivovány kombinací rozpoznání specifického antigenu a interakce CD40 na jejich povrchu s CD40 ligandem exprimovaným folikulárními T-lymfocyty v lymfoidních tkáních.²³ Následná aktivace, modulovaná aktuálním cytokinovým prostředím, otevírá B buňkám několik diferenciačních drah. Mohou se přeměnit na krátce žijící plazmatické buňky produkující protilátky v časně fázi infekce, nebo vstoupit do zárodečného centra, kde podstupují klonální expanzi, somatickou hypermutaci a izotypový přesmyk. Výsledkem je vznik dlouze žijící plazmatických buněk, které přetrvávají zejména v kostní dřeni. Další možností je vznik izotypově přepnutých paměťových B-lymfocytů s dlouhou životností, přítomných v periferní krvi, lymfatických orgánech i kostní dřeni.²⁴ Tyto izotypově přepnuté paměťové B-lymfocyty jsou při opětovném setkání se svým specifickým Ag schopny rychlé aktivace a následné diferenciace do stádia dlouhodobě žijících plazmatických buněk. Tento proces znovu probíhá za již výše popsané pomoci folikulárních T-lymfocytů, kdy po aktivaci paměťové B-lymfocyty v germinálním centru mohou znovu podstoupit SHM zajišťující další zvýšení specifity nově vzniklých protilátek a může také docházet k dalšímu izotypovému přesmyku.²⁵ Plazmablasty představují prekurzory plazmatických buněk a tvoří přibližně 3–5 % CD19-pozitivních B-lymfocytů v periferní krvi zdravých

dospělých. Jsou to proliferující buňky, které díky nepřetržité sekreci protilátek již zajišťují ochrannou imunitu. Následně dozrávají v dlouhověké či krátce žijící plazmatické buňky, jež představují terminálně diferencované efektorové buňky linie B-lymfocytů.²²

V současné diagnostické praxi se u pacientů s podezřením na imunodeficit v B-lymfocytární linii rutinně hodnotí zastoupení tranzientních, naivních a paměťových B-lymfocytů včetně buněk marginální zóny, dále populace CD21^{low} B-lymfocytů a plasmablastů.

U jednotlivých pacientů s CVID se nálezy analyzující B-lymfocytární subpopulace liší: u některých pacientů nacházíme chybění plasmablastů včetně paměťových forem B-lymfocytů, zatímco jiní pacienti mají počet paměťových B-lymfocytů v nezměněn a chybí jim nebo mají silně snížené počty plasmablastů.²⁶

U některých pacientů s CVID byly nalezeny zvýšené počty CD21^{low} B-lymfocytů.²⁷ Znak CD21, známý také jako CR3 receptor, funguje jako kostimulační receptor BCR. Starší literatura uvádí, že vazba antigenu opsonizovaného C3d složkou komplementu současně na BCR a CD21 vede k zesílení aktivace B-lymfocytu.²⁸ To se však ukázalo platné pouze u myši: Autoři Kovacs et al., studovali vliv shlukování BCR a CR2 na uvolnění intracelulárního volného Ca²⁺ a expresi časného aktivačního markeru CD69, uvolňování cytokinů, proliferaci a produkci protilátek lidskými B lymfocyty a zjistili, že při společném zapojení CR3 a BCR na lidských B-lymfocytech se zvyšuje odpověď Ca²⁺, zatímco exprese CD69, produkce cytokinů, proliferace a produkce protilátek jsou inhibovány.²⁹ Pro populaci CD21^{low} B-lymfocytů je typická nejen nízká exprese CD21, ale také snížená exprese CD38.³ CD38, receptor a enzym přítomný na povrchu B-lymfocytů i dalších imunitních buněk, se podílí na buněčné signalizaci, adhezi, metabolismu NAD⁺ a regulaci intracelulárních hladin vápníku. Jeho exprese se mění během vývoje B-lymfocytu a roste při jeho aktivaci.³⁰

U zdravých dospělých je zastoupení CD21^{low} B buněk nízké, avšak expanze této populace byla zaznamenána u řady autoimunitních onemocnění, včetně systémového lupusu erythematos (SLE), revmatoidní artritidy (RA) či primárního Sjögrenova syndromu. Zvýšený výskyt byl popsán i u chronických infekcí, jako je HIV, malárie či infekce cytomegalovirem (CMV), kde bývá spojen s nárůstem patogenně specifických B buněk. Zatímco expanze autoantigenně specifických CD21^{low} B buněk u autoimunitních onemocnění naznačuje jejich přímý podíl na patogenezi autoimunity, jejich zvýšené zastoupení při infekčních chorobách odráží zapojení do fyziologických imunitních odpovědí proti určitým patogenům.³¹ Jakou roli hrají CD21^{low} B-lymfocyty u pacientů s CVID nebylo objasněno.

5 Klasifikace pacientů s CVID

Vzhledem k výrazné klinické a imunologické heterogenitě pacientů s CVID bylo nutné vytvořit klasifikační systémy, které by umožnily jejich stratifikaci do homogennějších podskupin. Již v 90. letech byla publikována **klasifikace dle Bryantové**,³² která rozdělovala pacienty podle schopnosti tvorby protilátek po stimulaci B buněk. Kvůli nízké mezi-laboratorní reprodukovatelnosti však tato metoda nenašla širší uplatnění.

5.1 B-lymfocytární klasifikace CVID

5.1.1 Freiburská klasifikace

Výraznější průlom v klasifikaci pacientů s CVID přinesl pokrok v průtokové cytometrii, který umožnil detailní analýzu vývojových stadií B-lymfocytů. V roce 2002 byla vytvořena tzv. **Freiburská klasifikace**,

která rozděluje pacienty na základě procentuálního zastoupení izotypově přesmyknutých paměťových B-lymfocytů (tzv. switched memory B cells, smB: IgD⁺IgM⁺CD27⁻) a CD21^{low} B buněk.³

Freiburská klasifikace definovala tři hlavní skupiny:

Skupina I: nízké zastoupení izotypově přesmyknutých paměťových smB buněk (<2 %), dále děleno na **Ia** (CD21^{low} >10 %) a **Ib** (CD21^{low} ≤10 %),

Skupina II: normální zastoupení smB buněk,

Skupina III: celkové B-lymfocyty <1 %.

Pacienti ze skupiny Ia vykazovali vyšší výskyt autoimunitních komplikací, splenomegalii a granulomatózních postižení.

5.1.2 EUROclass

Na Freiburskou klasifikaci navázala rozsáhlá evropská studie **EUROclass**,⁴ které se naše pracoviště aktivně účastnilo a jsme v ní uvedeni mezi spoluautory.

V rámci této studie jsme u pacientů s CVID hodnotili zastoupení izotypově přepnutých paměťových B-lymfocytů (IgD⁻IgM⁻CD27⁺), naivních B-lymfocytů (IgD⁺CD27⁻), B buněk marginální zóny (IgD⁺CD27⁺), transientních B buněk (CD24⁺⁺CD38⁺⁺) a CD21^{low} B-lymfocytů (CD21⁻CD38⁻).

Na základě procentuálního zastoupení výše uvedených subpopulací B-lymfocytů byli pacienti s CVID v klasifikaci EUROclass nejprve rozděleni podle absolutního počtu B-lymfocytů na skupinu B⁻ s počtem B-lymfocytů <1 % a B⁺ s počtem B-lymfocytů ≥1 %). Skupina B⁻ zahrnuje pacienty s zřejmě s nedokončeným vývojem B-lymfocytů v kostní dřeni, které nemohou přejít do periferie, nebo takové, u kterých byla zásoba zárodečných buněk pro B-lymfocyty vyčerpána. Skupina B⁺ byla dále dělena podle zastoupení izotypově přepnutých paměťových B buněk na smB⁻ (<2 %) a smB⁺ (≥2 %). V rámci skupiny smB⁻ byla rozlišena podskupina smB⁻Tr^{hi}, u které počet transientních B buněk přesahoval 9 % a která zahrnuje pacienty se závažnými defekty časně diferenciací B buněk, a podskupina smB⁻Tr^{norm} s jejich normálním zastoupením.

V dalším kroku byli pacienti ze skupin smB⁺ i smB⁻ rozděleni podle podílu CD21^{low} B-buněk na podskupiny s jejich vysokým (CD21^{low}>10%) nebo normálním zastoupením CD21^{low} B-buněk (CD21^{norm}). Tím vznikly skupiny jako smB⁺CD21^{low}, smB⁺CD21^{norm}, smB⁻CD21^{low} a smB⁻CD21^{norm}.

Do studie bylo zařazeno spolu s našimi pacienty celkem 303 CVID pacientů z celé Evropy a výsledná klasifikace korelovala s některými klinickými ukazateli tj., například pacienti skupiny smB⁻Tr^{hi} častěji trpěli lymfadenopatií, zatímco zvýšený počet CD21^{low} B-lymfocytů byl spojen s vyšším výskytem splenomegalie a granulomatózních lézí, viz článek The EUROclass trial: defining subgroups in common variable immunodeficiency.

6 Původní práce autorky

6.1 Podrobná charakteristika B-lymfocytárních populací pacientů s CVID

V letech 2005–2010 došlo k výraznému rozvoji průtokové cytometrie a příchodu přístrojů schopných měřit až 10 parametrů současně. Tuto technologii jsme využili k podrobnější charakterizaci B-lymfocytů u pacientů s CVID. Vytvořili jsme sedmibarevný panel zahrnující CD19, IgD, IgM, CD21, CD24, CD27 a CD38, který umožňoval v jediné zkumavce analyzovat všechny hlavní B-lymfocytární subpopulace: naivní, paměťové (IgM⁺CD27⁺, IgM⁻CD27⁺), transientní (CD24⁺⁺CD38⁺⁺), plazmatické (CD27⁺⁺CD38⁺⁺CD21⁻) i CD21^{low} buňky (CD21⁻CD38⁻).

Analýzou subpopulací B-lymfocytů jsme zjistili, že u pacientů s CVID byly CD21^{low} buňky více zastoupeny mezi naivními IgM⁺CD27⁻ B-lymfocyty, zatímco u zdravých osob převažovaly mezi CD27⁺ paměťovými B buňkami. Naše výsledky byly v roce 2015 potvrzeny studií autorů K. Thorarinsdottir et al.,³³ kteří ukázali, že CD21^{low} B-lymfocyty tvoří přibližně 5 % B buněk zpravidla se vyskytujících v subpopulacích paměťových buněk u zdravých dospělých, ale nevyskytují se v pupečnickové krvi.³³ Dále jsme popsali, že populace IgM⁺CD21^{low} B-lymfocytů má zvýšenou expresi CD19 a je tvořena dvěma subpopulacemi s rozdílnou expresí znaku CD24: CD21^{low}CD24⁺ a CD21^{low}CD24⁻. Obě populace byly detekovány i u zdravých dárců, avšak v nižším zastoupení. Analýza jejich replikační historie ukázala, že obě tyto subpopulace odpovídají spíše paměťovým buňkám. CD21^{low}CD24⁻ buňky, přestože jsou CD27⁻, nesou znaky opakovaného buněčného dělení v porovnání se zdravými kontrolami. CD21^{low}CD24⁻ B-buňky jsou méně stimulovatelné než CD21^{low}CD24⁺ buňky, které reagují na TLR7 stimulaci podobně jako zdravé CD27⁺ paměťové B-lymfocyty, což bylo později také potvrzeno studií Thorarinsdottir et al.³⁴

U naivních B-lymfocytů jsme popsali dvě subpopulace: FOI buňky s nižší expresí IgM a CD24 v porovnání s FOII B-lymfocyty s vyšší expresí IgM a CD24 v porovnání s FOI buňkami analogicky ke studiím na myších,³⁵ u kterých FOI buňky dávají vznik izotypově přepnutým paměťovým buňkám a FOII buňky B-lymfocytům marginální zóny. Naše výsledky ukázaly, že pacienti se se zvýšeným výskytem CD21^{low} B-lymfocytů měli vyšší zastoupení FOII buněk. Dále jsme zjistili, že FOII B-lymfocyty měly u pacientů s CVID vyšší replikační aktivitu než u zdravých kontrol. Podobně jako u myší i námi zkoumané lidské FOI B-lymfocyty reagovaly na stimulaci BCR, zatímco FOII B-buňky vykazovaly vyšší aktivaci po stimulaci TLR7.

Zvýšené zastoupení FOII buněk u pacientů CVID může být výsledkem snahy imunitního systému o náhradu chybějících paměťových B-buněk. To je v souladu s výsledky studie Pillai et al., kde bylo prokázáno, že buňky s vyšší expresí IgM mají lepší stimulační odpověď a vyšší schopnost diferenciaci do plazmatických buněk.³⁶

Naše výsledky publikované v článku: Characterization of lymphocyte subsets in patients with common variable immunodeficiency reveals subsets of naive human B cells marked by CD24 expression, byly potvrzeny i dalším výzkumem, neboť novější studie u myší ukázaly přednostní diferenciaci FOII buněk do CD21^{low} B buněk charakteristických pro autoimunitní stavy.^{37,38} To je způsobeno tím, že IgM-BCR častěji rozpoznává autoantigeny než IgD-BCR. CD21^{low} B buňky jsou u pacientů s autoimunitními chorobami někdy popisovány jako izotypově přepnuté preplasmablasty, které jsou po stimulaci

schopny produkovat protilátky.³⁴ U pacientů s CVID však z důvodu poruchy diferenciacie a produkce imunoglobulinů zůstává tato odpověď nedokončena.

Characterization of lymphocyte subsets in patients with common variable immunodeficiency reveals subsets of naive human B cells marked by CD24 expression. **Vlková M**, Fronková E, Kanderová V, Janda A, Ruzicková S, Litzman J, Sedivá A, Kalina T. J Immunol. 2010 Dec 1;185(11):6431-8. Epub 2010 Nov 1.

Characterization of Lymphocyte Subsets in Patients with Common Variable Immunodeficiency Reveals Subsets of Naive Human B Cells Marked by CD24 Expression

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Increased proportions of naive B cell subset and B cells defined as CD27^{neg}CD21^{neg}CD38^{neg} are frequently found in patients with common variable immunodeficiency (CVID) syndrome. Current methods of polychromatic flow cytometry and PCR-based detection of κ deletion excision circles allow for fine definitions and replication history mapping of infrequent B cell subsets. We have analyzed B cells from 48 patients with CVID and 49 healthy controls to examine phenotype, frequency, and proliferation history of naive B cell subsets. Consistent with previous studies, we have described two groups of patients with normal (CVID-21^{norm}) or increased (CVID-21^{lo}) proportions of CD27^{neg}CD21^{neg}CD38^{neg} B cells. Upon further analyses, we found two discrete subpopulations of this subset based on the expression of CD24. The B cell subsets showed a markedly increased proliferation in CVID-21^{lo} patients as compared with healthy controls, suggesting developmental arrest rather than increased bone marrow output. Furthermore, when we analyzed CD21^{pos} naive B cells, we found two different subpopulations based on IgM and CD24 expression. They correspond to follicular (FO) I and FO II cells previously described in mice. FO I subset is significantly underrepresented in CVID-21^{lo} patients. A comparison of the replication history of naive B cell subsets in CVID patients and healthy controls implies refined naive B cell developmental scheme, in which human transitional B cells develop into FO II and FO I. We propose that the CD27^{neg}CD21^{neg}CD38^{neg} B cells increased in some of the CVID patients originate from the two FO subsets after loss of CD21 expression. *The Journal of Immunology*, 2010, 185: 6431–6438.

Common variable immunodeficiency (CVID) is a clinically heterogeneous group of primary Ab immunodeficiency diseases with an estimated prevalence of 1 in 30,000 with unclear etiology (1). It is characterized by decreased serum levels of IgG, IgA, and occasionally IgM and impaired Ab response to Ag stimulation and/or vaccination as well; it is usually accompanied by recurrent bacterial infections, Splenomegaly, granuloma formation, autoimmune disorders, and increased risk of malignancy are common complications (1, 2).

Although various abnormalities in number and function of T cells (3–7), B cells (8–13), and dendritic cells (14–16) have been described, the immunopathological mechanism leading to disturbed Ab production in CVID patients has not yet been defined.

Previous studies have shown abnormalities in B cell subset composition – a diminution in memory B cells and increase in naive follicular (FO) B cells (5). Naive FO B cells IgM^{pos}CD27^{neg} (17, 18) constitute a major B cell subset in the peripheral blood of patients with CVID. In some CVID patients, increased numbers of transitional B cells and CD21^{neg} B cells were observed (11, 19, 20). The CD21^{neg} B cell subpopulation was originally described as CD19^{high}CD21^{neg} B cells and has since developed into the present characterization CD21^{neg}CD38^{neg}CD19^{high}CD27^{neg} to distinguish this population from other CD21^{neg} B cell populations: CD21^{neg} plasmablast, CD21^{neg} transitional B cells. Expansion of the CD21^{neg} B cell subset has been found in patients suffering from systemic lupus erythematosus or HIV infection (21, 22). In patients with systemic lupus erythematosus, the CD21^{neg} subpopulation represents activated B cells, as these cells express activation markers CD86 and CD95 (21, 23). In CVID patients, CD21^{neg} B cells were shown to have increased expression of these activation markers as well as low expression of CD24. Although CD21^{neg} B cells were found among CD27^{neg} naive B cells in CVID patients, recent results showed that CD21^{neg} B cells in CVID patients were preactivated, polyclonal, partially autoreactive B cells that home to peripheral tissues (23).

The heterogeneity of naive CD27^{neg} B cell subpopulations has not been thoroughly studied. The CD21^{neg} B cell subpopulation forms <10% of all B cells in healthy controls (5, 20). The CD21^{pos} naive CD27^{neg} B cell subset can be further divided into two groups, as follows: 1) transitional immature B cells (IgM^{high}CD24^{high}CD38^{high}) making up ~3% of B cells (24), and 2) the remaining CD27^{neg} B cells that are comprised of mature IgM^{pos}CD24^{pos}CD38^{int}CD27^{neg} FO B cells, which reside in the spleen and lymph nodes and also circulate through the body. FO B cells are primed by T cells to generate class-switched memory and plasma cells producing high-affinity Abs. Two FO B cell recirculating popu-

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CVID, common variable immunodeficiency; FC, flow cytometry; FO, follicular; GC, germinal center; KREC, κ -deleting recombination excision circle; MFI, mean fluorescence intensity.

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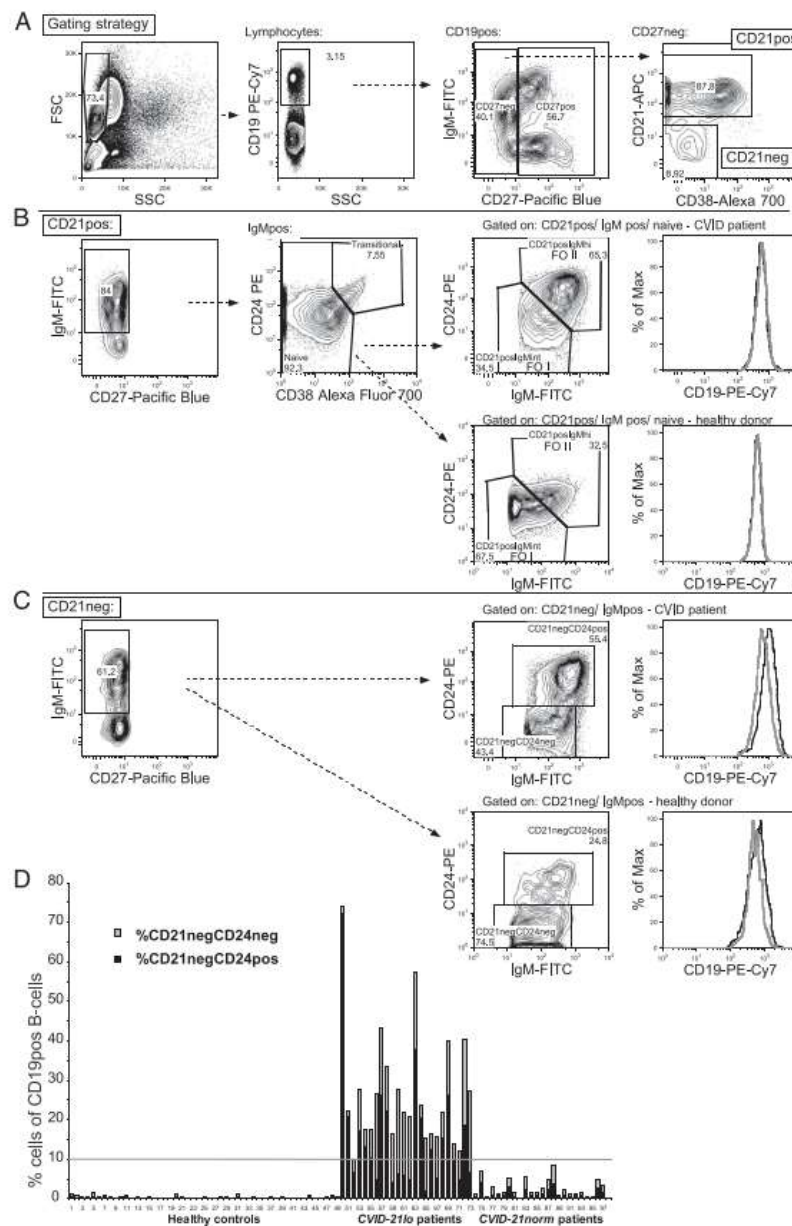


FIGURE 1. Scheme for gating B cell subsets. A, Gating of B cells is shown for a representative healthy donor. Lymphocytes were gated on forward and side light scatter and then on CD19 positivity, after which the remaining cells were divided into CD21^{pos} and CD21^{neg} subsets. Furthermore, naive B cells were defined as IgM^{pos} and CD27^{neg}. The CD21^{pos}IgM^{pos}CD27^{neg} cells were subdivided into transitional and naive subsets. The CD21^{neg}IgM^{pos}CD27^{neg} subset was increased in some CVID patients. The expression of IgM and CD24 defined two subsets of naive CD21^{pos} (B) and CD21^{neg} (C) B cells. Both subsets were observed in a CVID patient and in a healthy control. Overlays of CD19 histograms show differences between CD21^{neg}CD24^{neg} (black) and CD21^{neg}CD24^{pos} (gray) cells, whereas no difference was found between CD21^{pos}CD24^{neg} (black) and CD21^{pos}CD24^{pos} (gray) cells. D, Bar graph presenting the frequency of CD21^{neg}CD24^{pos}/CD21^{neg}CD24^{neg} cells in individual patients and controls.

Table 1. Characterization of frequencies of basic B lymphocyte subpopulations and expression levels of CD19 for each examined group of CVID patients and healthy controls

Subpopulations of Naive B cells	Healthy Controls (n = 49)	CVID-21lo Patients (n = 24)	CVID-21norm Patients (n = 24)
Percentage from CD19 ^{pos} B cells			
IgM ^{pos} CD27 ^{neg}	44.1 ± 11.1	75.0 ± 15.1*	71.1 ± 13.9*
CD21 ^{pos} IgM ^{pos} CD27 ^{neg}	39.9 ± 10.7	28.8 ± 13.0* ^{***}	59.8 ± 12.4*
CD21 ^{neg} CD38 ^{neg} IgM ^{pos} CD27 ^{neg}	3.8 ± 2.6	27.0 ± 15.8* ^{***}	3.7 ± 2.3
Transitional B cells	1.5 ± 1.1	5.6 ± 6.8	7.5 ± 7.5*
Expression level of CD19 (MFI)			
CD21 ^{pos} IgM ^{pos} CD27 ^{neg}	557.1 ± 59.5	583.2 ± 119.7	571.1 ± 57.2
CD21 ^{neg} CD38 ^{neg} CD24 ^{pos}	554.4 ± 119.9	676.9 ± 205.1 ^{***}	649.8 ± 118.6 ^{***}
CD21 ^{neg} CD38 ^{neg} CD24 ^{neg}	828.6 ± 186.0*	975.5 ± 293.3 ^{***}	1011.6 ± 190.6 ^{***}

Percentage from CD19^{pos} B cells: Results are expressed as mean ± SD. Statistically different from healthy controls (* $p < 0.00001$; ** $p \leq 0.0002$). Statistically different from CVID-21norm patients (^{***} $p \leq 0.00003$). Expression level of CD19 (MFI): Results are expressed as mean ± SD. Statistically different from CD21^{neg}CD38^{neg}CD24^{neg} B cell subpopulation (* $p \leq 0.00003$; ** $p = 0.00052$). Statistically different from healthy controls (^{***} $p \leq 0.008$). MFI, mean fluorescence intensity.

lations, the IgD^{high}IgM^{low}CD21^{int}CD24^{int} and the IgD^{high}IgM^{high}CD21^{int}CD24^{high}, designated FO I and FO II B cells, respectively, were recently described in mice (25). FO I B cells require Ag-derived signals and make up the majority of cells in the recirculating FO B cell pool. FO II B cells can develop in the absence of Ag into marginal zone B cells (26). To date, no corresponding B cell subpopulations have been described in humans.

We focused on a detailed analysis of naive CD27^{neg} B cells using polychromatic flow cytometry (FC) in healthy human donors and in CVID patients. Consistent with previous studies, we describe two groups of patients with normal (CVID-21norm) or increased (CVID-21lo) proportions of CD27^{neg}CD21^{neg}CD38^{neg} B cells. The CVID-21lo group contains both the smB^{21low} (decreased number of CD27^{pos}IgD^{neg}IgM^{neg} switched memory B cells and increased number of CD21^{neg} B cells) and smB^{21norm} (normal number of switched memory B cells and increased number of CD21^{neg} B cells) patients, as defined by Wehr et al. (20), whereas the CVID-21norm group comprises smB^{21norm} (decreased number of switched memory B cells and normal number of CD21^{neg} B cells) and smB^{21norm} (normal number of switched memory B cells and CD21^{neg} B cells) patients. To assess the developmental

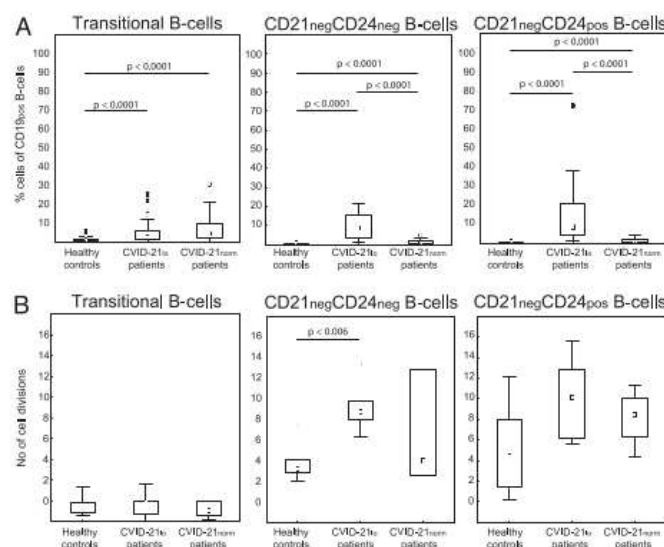
relationships between subsets identified by FC, we performed a replication history analysis in these cells by means of κ -deleting recombination excision circles (KRECs) (27). We describe two phenotypically distinct subpopulations of CD21^{neg} B cells that are expanded in CVID patients, but can be also identified in healthy controls. Furthermore, we identified cells in human peripheral blood that correspond to mouse FO I and FO II B cells. The frequencies, replication history, and mutual proportions of these populations differ between healthy controls and CVID patients. Based on these findings, we propose a revised developmental scheme of human naive B cell development.

Materials and Methods

Patients and healthy donors

We examined 48 CVID patients (25 females, 23 males; mean of age 43.4 ± 15.3 y, range 12–74 y) and 49 healthy donors (26 females, 23 males; mean of age 42.6 ± 13.6 y, range 20–78 y). In all CVID patients, B cells constituted >1% lymphocytes in the peripheral blood. The presence of polymorphisms in the *TNFR family member transmembrane activator and cyclophilin ligand interactor* gene (*TNFRSF13B*) was analyzed in all patients. The p.C104R polymorphism was found in two patients, and the

FIGURE 2. The frequencies (A) and replication history (B) of naive B cell subpopulations for each examined group of CVID patients and healthy donors. Transitional B cells, IgM^{pos}CD27^{neg}CD24^{high}CD38^{high}, CD21^{neg}CD24^{neg}, IgM^{pos}CD27^{neg}CD38^{neg}CD21^{neg}CD24^{neg} B cells; CD21^{neg}CD24^{pos}, IgM^{pos}CD27^{neg}CD38^{neg}CD21^{neg}CD24^{pos} B cells. The number of cell divisions is expressed relative to the transitional B cell value in a healthy control sample (set as 0). Results are expressed as the median, the 25th–75th percentiles, and the outlier range. ○, Outlier values; *, extreme values.



p.187N polymorphism was found in one patient; all three of the identified polymorphic genes were heterozygous. In 15 patients, ICOS gene mutations were analyzed with negative results.

The patients were on regular i.v. or s.c. Ig substitution. For patients who received i.v. Ig treatment, blood samples were always collected before the treatment. Informed consent was approved by the Medical Ethics Committee of St. Anne's Faculty Hospital and was obtained before blood sampling.

CVID patients were divided into two groups based on the expression patterns of CD21 and CD38 on the patients' B cells. Twenty-four patients fell into the *CVID-2/lo* group, which was characterized by an increased proportion of B cells with low expression of CD21 and CD38 (>10%). The remaining 24 patients were placed in the *CVID-2/norm* group, which was characterized by a normal frequency (<10%) of CD21^{low}CD38^{low} B cells.

The raw FC data from these patients and the control cohort were used in a previously published technical study describing probability binning algorithms for the evaluation of multicolor FC data (28). FC data analysis was performed using the FlowJo 7.5 or 8.8.7 software (Tree Star, Ashland, OR).

B cell staining and FC

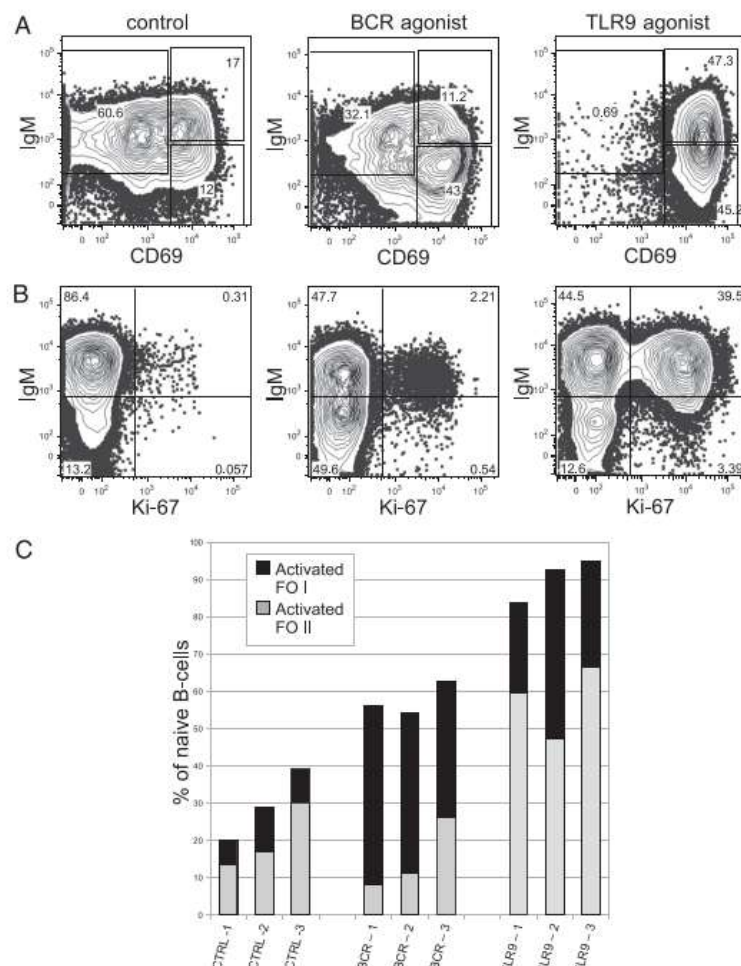
PBMCs were isolated from 10 ml EDTA blood by density gradient centrifugation using a Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden), and the isolated PBMCs were washed twice with PBS that was

supplemented with 1% BSA. The PBMCs were incubated for 15 min in the dark with anti-CD27 Pacific Blue, anti-CD38 Alexa-Fluor 700 (Exbio Praha a.s., Prague, Czech Republic), anti-human IgM FITC or anti-human IgM biotin (BD Pharmingen, San Jose, CA), followed by streptavidin-Qdot 605 (Invitrogen, Carlsbad, CA), anti-CD21 allophycocyanin (BD Pharmingen), anti-CD24 PE, and anti-CD19 PC7 (Immunotech, Marseille, France). After a final wash with PBS, two million PBMCs from each sample were acquired using a Cyan ADP flow cytometer (Dako, Glostrup, Denmark), as described in Kalina et al. (28).

B cell response to a TLR9 agonist or a BCR agonist

B lymphocytes were isolated from peripheral blood using the RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies, Rockford, IL) and Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) density gradient centrifugation. Cells were stimulated with the TLR9 agonist CpG oligodeoxynucleotide 2006 at 2.5 µg/ml (Imgenex, San Diego, CA) with recombinant protein L at 10 µg/ml (Thermo Fisher Scientific, Rockford, IL) or with a negative control in RPMI 1640 medium that contained L-glutamine and 25 mM HEPES (Lonza, Basel, Switzerland) and supplemented with 10% heat-inactivated FBS (HyClone, South Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (Lonza Walkersville), and 10 ng/ml human rIL-4 (R&D Systems, Minneapolis, MN) at a starting concentration of 5×10^6 cells/ml.

FIGURE 3. Responses of FO B cells to BCR and TLR9 agonists reveal activated FO I and FO II phenotypes. Enriched B cells were stimulated in vitro with a BCR agonist (protein L) or a TLR9 agonist (CpG oligodeoxynucleotide 2006). A, Activation of B cells led either to a dominant FO II phenotype (IgM high) or to a combined FO II/FO I phenotype (IgM high and low) after 24 h. B, Proliferation was more pronounced in TLR9 agonist-stimulated B cells and was restricted to cells with the FO II phenotype after 3 d. C, Proportion of activated FO I or FO II cells in samples from three healthy donors in the presence of IL-4. No stimulation (CTRL), BCR agonist stimulation (BCR), or TLR9 agonist stimulation (TLR9).



Proliferation was detected with anti-Ki-67 PE (BD Biosciences, San Jose, CA) after permeabilization with BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2, according to the manufacturer's protocol (BD Biosciences), 3 d after in vitro stimulation.

The apoptosis after stimulation was assessed using annexin V Dy647 (Exbio Praha a.s.), and cell activation was detected by anti-CD69 PerCP-Cy5.5 (clone FN50; BioLegend, San Diego, CA).

Investigation of B cell replication history

B cell subsets were purified from the blood samples of 16 CVID patients (8 CVID-21lo, 8 CVID-21norm). To ensure that we had sufficient numbers of cells belonging to subpopulations that were not expanded in healthy controls, we used cells that were sorted from 11 buffy coats (instead of peripheral blood) as control subpopulations. The cells were sorted using a FACS Aria flow cytometer (BD Biosciences) using the same set of mAbs that were described above. DNA from the sorted cell populations was extracted using the QIAamp DNA Blood Micro Kit (Qiagen, Hilden, Germany). We used the KREC detection method that was described previously (27) with in-house modifications, as follows. Unlike the method that was described by van Zelm et al. (27), we used a δ cycle threshold method with a calibration sample consisting of sorted transitional B cells from a healthy donor that was split into two quantitative PCR reactions. The normalized δ cycle threshold (KREC) values indicated the number of cell divisions in a given subpopulation in relation to the calibration sample. If the KREC amplification produced no signal within the sensitivity limit of the quantitative PCR reaction (e.g., the KRECs in a given sample were too dilute), we used the lowest possible number of cell divisions that was calculated using the number of intronR55-KDE rearrangements in the sample.

Statistical analysis

The numerical differences between the subgroups were evaluated using the nonparametric Mann-Whitney *U* test and Wilcoxon test. In all statistical analyses, $p < 0.05$ was considered statistically significant. Student's *t* test and Fisher's exact test were used when appropriate. Statistical software STATISTICA (StatSoft, Tulsa, OK) version 7 was used.

Results

Clinical characteristics of the CVID patient groups

The following parameters were assessed for both patient groups: age, age of first symptoms, duration of the disease, presence of bronchiectasis, obstructive lung disease, chronic diarrhea, splenomegaly, lymphadenopathy, granulomatous disease, levels of Igs at the time of diagnosis, and presence of *TNFRSF13B* polymorphisms (Supplemental Table I). The only significant differences that we found were between the age of the patients (CVID-21lo: mean = 52.5 y, SD = 11.5; CVID-21norm: mean = 35.5 y, SD = 13.9, $p < 0.001$), the age of the first symptoms (CVID-21lo: mean = 33.5 y, SD = 10.8; CVID-21norm: mean = 18.5 y, SD = 15.5, $p < 0.01$), and the presence of bronchiectasis (CVID-21lo: 12 patients [52%]; CVID-21norm: 6 patients [26%], $p < 0.05$). No other differences were found.

The CD27^{neg}CD21^{neg}CD38^{neg} B cell subset is composed of two cell types that are defined by CD24 expression

For cytometric analysis, all B cells were divided into naive and memory B cell populations, based on the expression of CD27 (Fig. 1A). We confirmed the previously described increased frequency of naive IgM^{pos}CD27^{neg} B cells that was observed in subgroups of CVID patients (Table I). In CVID-21lo patients, the total B cell population contained an increased percentage of CD21^{neg}CD38^{pos} B cells, which was expected, given that this patient group was defined by this characteristic (Table I).

Unlike other groups (23), we found that the CD27^{neg}CD21^{neg}CD38^{neg} B cell subset is composed of two cell types that are defined by CD24 expression: CD27^{neg}CD21^{neg}CD38^{neg}IgM^{pos}CD24^{neg} cells (referred to in this study as CD21^{neg}CD24^{neg} cells) and CD27^{neg}CD21^{neg}CD38^{neg}IgM^{pos}CD24^{pos} cells (referred to in this study as CD21^{neg}CD24^{pos} cells) (Fig. 1C). Both of these cell types contributed to the increased frequency of CD27^{neg}CD21^{neg}CD38^{neg} B cells, but their relative contributions varied among patients (Fig. 1D).

Previous studies found elevated expression levels of CD19 in the CD21^{neg} naive B cell subset, compared with total B cells (11, 19, 20, 23). Our findings show that this elevation was restricted to the CD21^{neg}CD24^{neg} subset in both healthy controls and CVID patients, although the elevation was more prominent in CVID patients (Fig. 1C, Table I).

Although CVID-21lo patients displayed relatively increased frequencies of naive IgM^{pos}CD27^{neg} B cells, the increase was caused primarily by higher numbers of CD21^{neg}CD38^{neg} B cells. The frequency of naive B cells with the typical CD21^{pos} phenotype was actually decreased in CVID-21lo patients compared with the frequency of naive CD21^{pos} B cells in healthy controls, whereas naive CD21^{pos} B cells were significantly increased in CVID-21norm patients (Table I). The proportion of transitional B cells was higher in both groups of CVID patients compared with healthy controls (Fig. 2A), as already reported (20).

Replication history of the naive B cell subset (excluding transitional B cells) showed a higher number of cell divisions in CVID patients

The analysis of KRECs in transitional B cells showed that the number of cell divisions in both of the CVID subgroups was similar to that in healthy donors. However, in CVID-21lo patients, the CD21^{neg}CD24^{neg} subset was found to undergo a higher number of divisions than in controls (Fig. 2B). The same pattern (although not significant) was found for the CD21^{neg}CD24^{pos} B cell subset.

Subsets of naive B cells corresponding to mouse FO I and FO II were found in humans and were underrepresented in CVID-21lo patients

Two FO B cell populations, CD21^{int}IgM^{low}IgD^{high}CD24^{pos} and CD21^{int}IgM^{high}IgD^{high}CD24^{high}, that were designated FO I and FO

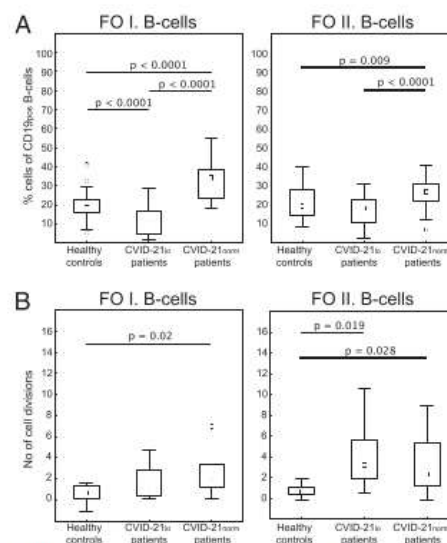


FIGURE 4. The frequencies (A) and replication history (B) of B cell subpopulations for each examined group of CVID patients and healthy donors. FO I, IgM^{int}CD24^{int}CD38^{int}CD27^{neg}; FO II, IgM^{high}CD24^{pos}CD38^{int}CD27^{neg}. The number of cell divisions is expressed relative to the transitional B cell value in a healthy control sample (set as 0). Results are expressed as the median, the 25th–75th percentiles, and the outlier range. ○, Outlier values.

II, respectively, were recently described in mice. Cariappa et al. (25) distinguished mouse FO II cells from FO I cells according to the enhanced expression of IgM in FO II B cells. Both subpopulations showed increased expression of CD24. When we analyzed CD21^{pos}CD27^{neg}CD38^{int} B cells, we found two subpopulations of cells: IgM^{high}CD21^{pos}CD27^{neg}CD38^{int}CD24^{pos} and IgM^{int}CD21^{pos}CD27^{neg}CD38^{int}CD24^{int}. We observed both subpopulations in human healthy donors and in CVID patients. The FO I/FO II difference was marked by enhanced CD24 expression on FO II-like cells in CVID patients, whereas in healthy individuals, the CD24 expression was only moderately increased on FO II cells (Fig. 1B). In mice, the distinction between FO subsets from marginal zone B cells is possible because of the enhanced expression of CD21 on marginal zone B cell subsets. Both subpopulations that we found in human peripheral blood had identical CD21 expression levels. The expression of CD21 was higher than it was in marginal zone-like B cells (IgM^{pos}CD27^{pos}CD38^{neg}CD24^{high}) (Supplemental Fig. 1). Because we observed two subpopulations with uniform expression of CD21, we assumed that the IgM^{high}CD27^{neg}CD38^{int}CD24^{pos} subset corresponds to FO II B cells and that the IgM^{int}CD27^{neg}CD38^{int}CD24^{int} subset corresponds to FO I B cells.

Different responses to activation stimuli by FO I and FO II cells in human B cells

The mouse model that was reported by Cariappa et al. (25, 26) suggests that FO I B cells develop from FO II B cells after strong BCR stimulation, and that FO II B cells can give rise to MZ-

like cells. To support the phenotype distinction between FO I and FO II B cells in human B cells using functional data, we stimulated human B cells from healthy donors in vitro using a TLR9 agonist and a BCR agonist. We chose the *Peptostreptococcus magnus* protein L superantigen that binds to conserved κ L chains (29) as the BCR agonist because anti-IgM stimulation does not allow for a surface IgM-based definition of the responding B cells. The FO I and FO II definition was based solely on surface IgM in this experiment because CD24 is rapidly downregulated after activation by BCRs (30).

Both agonists triggered cell activation leading to CD69 upregulation and cell proliferation (Fig. 3A, 3B), whereas apoptosis was always below 9% and did not differ between stimulated or control cells at 2 h, 24 h, 48 h, or 3 d after stimulation (Supplemental Fig. 2). In line with the data for the mouse model, the BCR agonist led to the activation of ~55% of the naive B cells (corresponding to the proportion of κ L chain-positive B cells) that had acquired a predominantly FO I phenotype (Fig. 3A, 3C). In contrast, the TLR9 agonist activated all naive B cells and forced ~40% of cells to proliferate by day 3 after stimulation. Of note, all proliferating B cells displayed the FO II phenotype (Fig. 3B), which is in line with the lack of cell divisions that was documented in the FO I compartment ex vivo (Fig. 3C).

The FO I compartment is disrupted in CVID patients

The naive CD21^{pos} B cell compartment is composed of FO I, FO II, and transitional B cells. The CVID-21lo patients had an

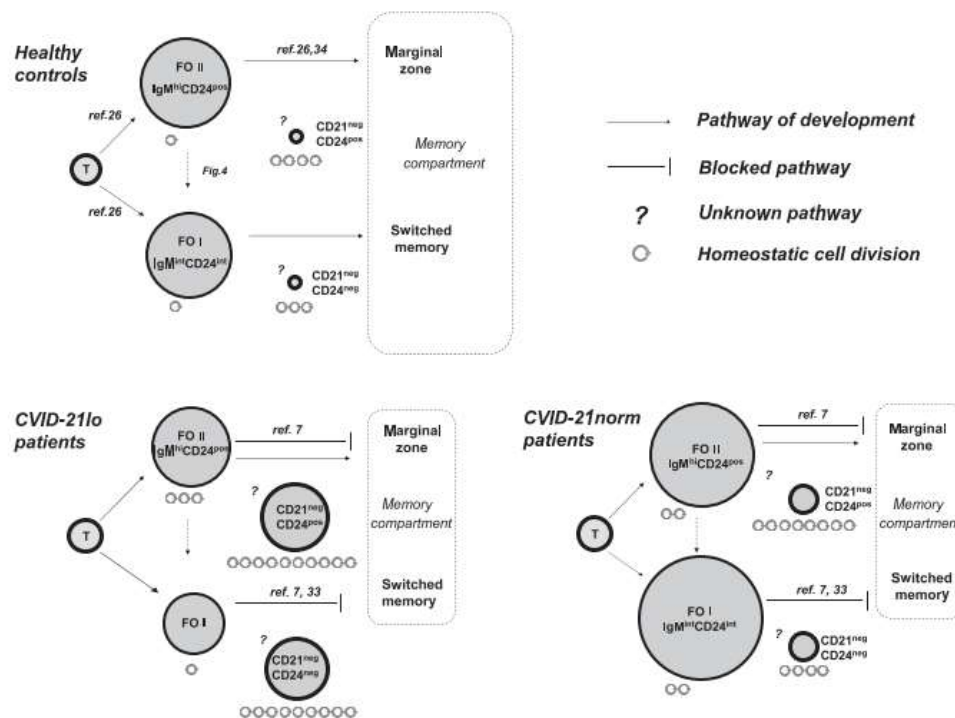


FIGURE 5. Proposed scheme of peripheral naive B cell development in healthy controls and in CVID patients. In healthy donors, transitional B cells are assumed to mature into FO I and FO II B cells. FO I B cells are primed by T cells to generate class-switched memory cells and plasma cells producing high-affinity Abs. FO II B cells can develop into marginal zone B cells. The developmental pathway of CD21^{neg} B cells is to date unclear. Frequency alterations in CVID patients are represented by the size of the full circles, and the median number of homeostatic cell divisions is shown as a circular arrow below each circle.

increased percentage of CD21^{neg} B cells by definition, and thus had reciprocally decreased percentages of CD21^{pos} B cells. We also found an elevated frequency of transitional B cells, but the frequency of FO II cells was similar to that in healthy controls. Fig. 4A shows that the FO I subset of cells formed a significantly lower proportion of B cells in *CVID-21lo* patients than in healthy donors. Thus, the decrease of FO I B cells accounts for the diminution of CD21^{pos} B cells in *CVID-21lo* patients.

CVID-21norm patients presented with a higher frequency of CD21^{pos} B cells, and both of the FO subsets were relatively increased (Fig. 4A).

The number of cell divisions for FO I and FO II cells in healthy controls was similar to those of transitional B cells (Fig. 4B). However, in *CVID-21norm* patients, the FO I and FO II cells divided more often than the FO B cells from healthy controls. FO II B cells also divided more often in *CVID-21lo* patients.

Discussion

Based on the combined analyses of healthy donors and CVID patients, we identified new subsets of human naive B cells that are defined by IgM and CD24 expression. First, we found subsets corresponding to mouse FO I and FO II cells (25, 31) in both healthy donors and CVID patients. Second, when analyzing the expanded CD27^{neg}CD21^{neg}CD38^{neg} B cell subset in *CVID-21lo* patients, we were able to distinguish between two distinct populations, based on CD24 expression. A detailed analysis of peripheral blood using sensitive six-color FC also revealed that both CD21^{neg}CD24^{pos} and CD21^{neg}CD24^{neg} subsets were present in healthy donors, although they were present in low numbers. This finding was in contrast to a previous study by Rakhmanov et al. (23), which showed that the entire CD21^{neg} population was also CD24^{neg}. This was presumably caused by the fact that in that study, CD21^{neg} cells were gated as CD19^{high}, whereas we observed that the expression of CD19 in CD21^{neg}CD24^{pos} B cells was similar to that of naive and memory B cells, that is, lower than in CD21^{neg}CD24^{neg} B cells. CD24 represents the first pan-B cell molecule, and this GPI-anchored protein belongs to the heat-stable protein family. CD24 is downregulated after BCR activation, but its complete physiological function in B cells is unknown (32). Further studies will be needed to determine whether this molecule plays an essential role in naive B cell homeostasis. Currently, CD24 is used in FC as a marker of the different stages of naive B cell development.

Low numbers of cell divisions in the FO II (IgM^{high}CD24^{pos}) and FO I (IgM^{int}CD24^{int}) subpopulations from healthy control individuals indicate that these cells mature from transitional B cells in the absence of proliferation, whereas the CD21^{neg}CD24^{pos} and CD21^{neg}CD24^{neg} subpopulations showed a higher number of cell divisions (Fig. 5). The median numbers of divisions in the latter subsets correspond to the number of divisions in the population of naive B cells, defined as CD19^{pos}IgD^{pos}CD27^{neg} by van Zelm et al. (27). In contrast to healthy donors, the *CVID-21lo* and *CVID-21norm* patients displayed not only an altered distribution of naive B cells, but also elevated numbers of cell divisions in this compartment (Figs. 2, 5). We also found that the percentage of FO I B cells was strongly reduced in *CVID-21lo* patients. This may be explained by deficient responses to BCR signaling.

A marked decrease or absence of switched memory B cells and an increased frequency of naive B cells (e.g., CD21^{neg}CD38^{neg} in a subgroup of patients), together with an increased division rate, suggests a developmental arrest in normal bone marrow migration. Recent studies of a large cohort of patients showed that there was an increase in autoimmune phenomena in the *CVID-21lo* patient group (20). This may be a consequence of an oligoclonal expansion

in the naive B cell compartment occurring during prolonged homeostatic proliferation or during proliferation in response to non-BCR stimuli, such as the TLR9 agonist that is exemplified in Fig. 3.

In contrast to the proportions of the examined B cell subsets in *CVID-21lo* patients, the proportions in *CVID-21norm* patients were inflated in all naive CD21^{pos} B cell compartments. FO I and FO II B cell subsets were found to undergo a higher number of divisions than controls. This result may be due to prolonged homeostatic proliferation, as described above.

Of note, both groups of CVID patients had an increased proportion of transitional B cells compared with healthy donors (20). However, these cells did not show significantly increased numbers of cell divisions compared with control transitional B cells. Thus, we speculate that increased bone marrow migration represents an attempt to compensate for the reduced numbers of mature B cells in the periphery of these patients.

Replication history data from healthy donors, together with the findings from Cariappa et al. (25), point to a sequence of subset development from transitional B cells to FO II (IgM^{high}CD24^{pos}) cells, and later, to FO I (IgM^{int}CD24^{int}) cells in the absence of cell division. The two later stage cell types can presumably give rise to the CD21^{neg}CD24^{pos} and CD21^{neg}CD24^{neg} subsets, respectively, as indicated by the fact that there are more cell divisions at these stages (Fig. 2B). Naive B cells in CVID patients fail to undergo the germinal center (GC) reaction and fail to enter the switched memory pool. These naive B cells were, however, shown to react to stimulation with anti-IgM plus IL-2, as demonstrated by the downregulation of CD24 (33), but the upregulation of the costimulatory molecules CD70 and CD86 was impaired. It is reasonable to suggest that the physiological (and likely repeated) stimulation of naive B cells by Ag and/or proinflammatory signals in CVID patients may lead to proliferation, after which these cells lose CD21 and become CD21^{neg}CD24^{pos} or CD21^{neg}CD24^{neg} cells (with added cell divisions) instead of undergoing the GC reaction. CD21^{neg} cells cannot participate in the GC reaction because CD21 expression is essential for GC formation, B cell FO dendritic cell contact, and B cell survival during the GC reaction (34).

In conclusion, we used IgM and CD24 surface expression patterns to refine our knowledge of naive peripheral B cell development, which is disrupted in CVID patients. We propose that cell proliferation without developmental progression is responsible for the accumulation of CD21^{neg}CD38^{neg} cells in a subset of CVID patients.

Disclosures

The authors have no financial conflicts of interest.

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6.2 Profilování dat polychromatické průtokové cytometrie na B-lymfocytech odhaluje skupiny pacientů s běžnou variabilní imunodeficiencí

Vzhledem k rostoucímu počtu měřených parametrů v průtokové cytometrii se ukázalo, že manuální gatování, tedy vymezení buněčných populací na základě jejich fenotypu, je časově náročné a částečně subjektivní. Tato omezení vedla k vývoji metod automatizovaného vyhodnocení dat. Pro dosažení objektivních a reprodukovatelných výsledků je však nezbytné zajistit konzistenci měření pomocí vnitřních standardů, jednotné přípravy vzorků a používání stejných protilátek.

V rámci naší studie jsme využili metodu „probability binning“ k analýze šestibarevného cytometrického panelu B-lymfocytárních subpopulací u pacientů s CVID a zdravých kontrol. Tato metoda rozdělila naše naměřená data o B-lymfocytech do 1024 binů, přičemž každý bin představoval specifickou oblast v šesti-rozměrném parametrovém prostoru. Takto vzniklé mapy buněčných populací jednotlivých vzorků byly následně porovnávány pomocí Pearsonova korelačního koeficientu a klasifikovány do skupin podle podobnosti.

Analýza vedla k rozdělení našeho souboru do 12 klastrů. Šest klastrů bylo tvořeno převážně zdravými kontrolami, zatímco tři specifické klastry sdružovaly pacienty s CVID podle EUROclass klasifikace: $smB^{-}CD21^{low}$, $smB^{-}CD21^{norm}$ a $smB^{+}CD21^{low}$. Zbylé klastry byly smíšené. Tato metoda nám umožnila identifikovat parametry, které nejvíce přispívaly k odlišnosti mezi jednotlivými klastry, a tím i specifické subpopulace, v nichž byly pozorovány významné rozdíly.

Výsledky jsme následně ověřili manuálním gatováním, které potvrdilo správnost automatizovaného přístupu. Tím jsme prokázali použitelnost této metody pro efektivní a objektivní vyhodnocování rozsáhlých a složitých cytometrických dat, a zároveň validovali klasifikace pacientů podle systému EUROclass.⁴

Multiparametrová průtoková cytometrie významně rozšiřuje možnosti analýzy zastoupení leukocytárních populací, funkčních profilů i vzorců imunitní odpovědi. Lze očekávat, že její využití se stane klíčovým při definování korelátů ochrany účinnosti vakcín i při monitorování imunitního systému, včetně imunoterapie u onemocnění, jako je například HIV. Podobný význam nabývá průtoková cytometrie také v diagnostice hematologických malignit, kde se pro detailnější charakterizaci nádorových buněk kombinuje stále větší počet markerů. Do budoucna lze předpokládat, že víceparametrové cytometrické testy se stanou standardem při screeningu širokého spektra imunologických onemocnění. S tímto vývojem však roste i potřeba rychlých a intuitivních analytických metod, které překračují rámec jednoduchého hodnocení pozitivních či negativních výsledků. Moderní aplikace průtokové cytometrie vyžadují schopnost rychle extrapolovat informace, a proto by cílem automatizovaných analytických nástrojů měla být identifikace podobností i rozdílů mezi vzorky. Algoritmy sice nemohou plně nahradit odbornost zkušeného operátora, zejména při detekci specifických subpopulací, avšak jejich využití v kombinaci s odbornou interpretací a standardizací přístrojů nepochybně povede k dalšímu prohloubení znalostí v oblasti základního i translačního výzkumu.

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Profiling of Polychromatic Flow Cytometry Data on B-Cells Reveals Patients' Clusters in Common Variable Immunodeficiency

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• Abstract

The aim of this study was to find an objective computational approach for phenotype analysis of common variable immunodeficiency (CVID) patients that describes all differences in the six-color space and to form groups of patients using computational methods. CVID is a heterogeneous primary immunodeficiency disorder where molecular defect is recognized in <10% of the cases and is unknown in the majority of patients. The current CVID classification, EUROClass, is based on quantification of selected B-cell subsets. Using six-color polychromatic flow cytometry, we analyzed B-cell phenotypes in a cohort of 48 CVID patients and 49 healthy donors. We used a "probability binning" algorithm to create 1,024 bins (each bin is a six-color gate) that covered the cells' distribution within the entire B-cell compartment. A matrix file recording cellular content in all the bins was made. The hierarchical clustering of the individual samples was analyzed using a Pearson correlation of the bins' values. The Cut tree algorithm found 12 clusters. In six clusters, healthy individuals predominated; in one cluster, smB+CD21low (CVID patients by EUROClass) cells prevailed; in one cluster, smB-CD21norm cells prevailed; in one cluster, smB+CD21low cells prevailed; the remaining cluster was mixed. The overall reproducibility of probability binning clustering was confirmed by matching of replicates to the original cohort using the similarity matrix of the Pearson correlation, 15 replicates matched the same individual, three replicates matched a different individual within the same cluster, and three replicates matched to a different cluster. We were able to define B-cell subsets over- or under-represented in a particular cluster and display them back in the flow cytometry software. We describe a new analytical approach that enables a search in an objective computational environment for patient cohorts that are defined by similar B-cell profiles and thus contribute to the description of differences between CVID patient groups. © 2009 International Society for Advancement of Cytometry

• Key terms

B-lymphocyte subsets; common variable immunodeficiency; flow cytometry; probability binning; B-lymphocyte profiling

THE peripheral blood B-cell compartment is a dynamic complex of B-cell subsets that undergo maturational steps, antigen exposure, memory cell formation, memory maintenance, and effector functions (antibody secretion). Polychromatic flow cytometry offers an extraordinary tool to investigate such a complex set of cell types. It allows for the definition and proportional assessment of B-cell subsets and also subdivides into less well-characterized intermediates. However, the analysis of complex data obtained from the six-color space is difficult. The standard analysis strategy uses the creation of series of two-dimensional gates, which makes the resulting analysis subjective, and "a priori" unknown small subsets are difficult to discover. Analysis of

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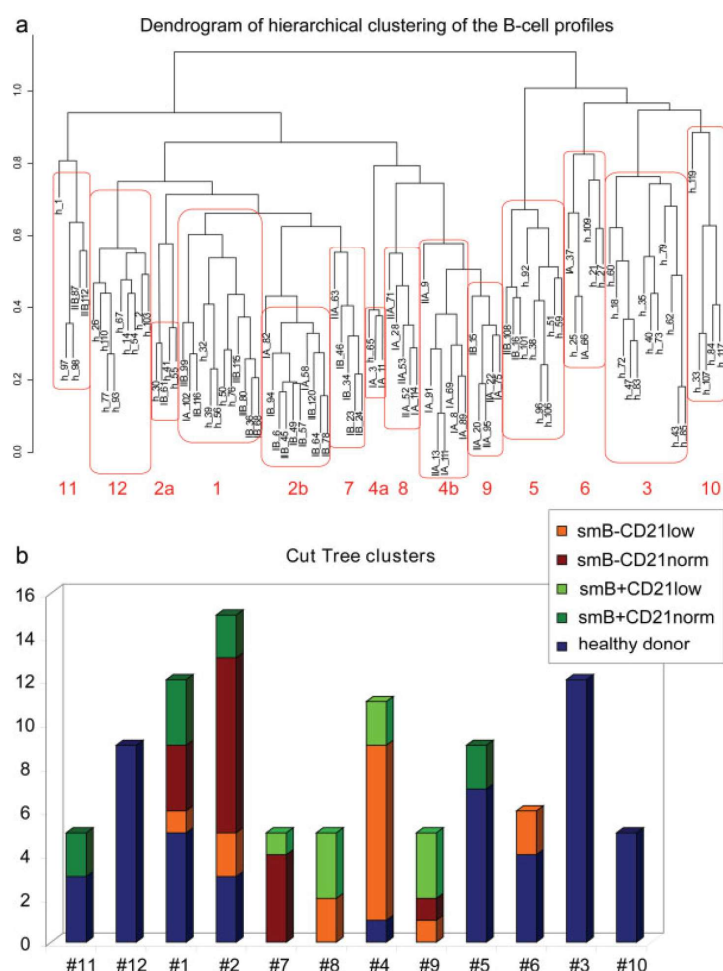


Figure 1. a) Dendrogram of the hierarchical clustering of all individuals in the study. For short, EUROClass groups are coded as follows: IA (smB-CD21low), IB (smB-CD21norm), IIA (smB+CD21low), and IIB (smB+CD21norm). Clusters created by Tree Cut algorithm are depicted by rectangles. b) Bar chart summarizing the content of clusters: Healthy controls in blue, “smB-CD21low” in orange, “smB-CD21norm” in dark red, “smB+CD21low” in light green, and “smB+CD21norm” in dark green. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

largest variance, and divides the events in symmetrical halves (bins) based on the median value of that parameter and is repeatedly applied until the desired number of bins is created (7). When 1,024 bins were created, we ran all individual samples through the same network of bins in six-parameter space and obtained a table with each patient in one column and each bin in a row containing a number of cells in each respective bin.

For each bin we take expected number of cells (E),
 $E = (\text{total number of cells in sample})/1,024$ (throughout the samples in the experiment $E \approx 20$).

Taking the measured number of cells in the bin (M), we assign to each bin a value (F),

$$F = M/E - 1.$$

Thus, if the number of cells in the bin was equal to the reference dataset of healthy controls, the value F was zero, if

the bin was “over-fed” with more cells, $F > 0$, and “under-fed” bins were assigned values $F < 0$. We considered this data set to represent a B-cell profile and we further analyzed it by applying approaches used in expression profiling.

This approach enables six-color flow cytometry data to be evaluated without any bias introduced by subjective choice of a position and sequence of bi-dimensional gates made by an expert. Moreover, it takes into account the complete cellular information contained in the six-color space.

B-Cell Profile Data Analysis and Hierarchical Clustering

The dataset was ordered as 97 columns (one column per individual) and 1,024 rows (one row per bin). The dataset was analyzed by tools developed for the DNA microarray analysis. Hierarchical clustering was performed in the Bioconductor package of the R-project (Available at: <http://www.r-project.org/>). The Dynamic Tree Cut (dynamicTreeCut R-package) (8) algorithm was used to define clusters. The clusters were validated using the internal and the stability measures designed in clValid R-package (9). The nonrandomness of patient distribution among clusters was tested by Fisher's Exact Test with simulated P -values (10^7 replicates).

Evaluation and Visualization of Significantly Different Cell Subsets Between Clusters

Selected clusters were compared with each other to find significantly different bins in a search for cellular subsets that would serve as hallmark of the cluster. Multiple testing procedure (MTP) (10) was used in the search for differently “fed” bins, which is designed for testing a hypothesis with unknown dependence structures among variables.

MTP was set to find significantly different bins at the level of $P < 0.01$.

An output table with significant bins represented by artificial events (i.e., each significant bin was filled by 100 random events generated from uniform distribution on the bin) was analyzed in MeV-TM4 software (11) by principal component analysis (PCA) (12) to reveal the spatial relationship of “over-fed” and “under-fed” bins. Thus, for each cluster comparison, we generated a PCA with a graphical representation and eigenvectors describing the values of each component. Eigenvectors were used to construct a polyvariate plot (“PV plot” in FlowJo) on the original CD19+ gated listmode data of patients and healthy donors. Over- or under-fed spaces displayed by PCA were gated on the PV plot.

RESULTS

Clusters of Patients

The B-cell profile of the whole cohort of CVID patients and healthy controls was analyzed by hierarchical clustering using a Pearson correlation. Clusters were defined by dynamic branch cutting method implemented in R-package (8). We allowed only groups of five or more patients to be considered as a cluster. The hierarchical clustering algorithm on this dataset was validated by clValid R-package (9). This approach uses all information contained in all six parameters from 1,024

Table 1. Replicates of blood drawn from 17 patients and four healthy controls were obtained to test the reproducibility of the B-cell profile (Pearson correlation)

REPLICATE CODE	ORIGINAL SAMPLE CODE	TIME TO REPLICATE (DAYS)
Match to the replicate		
IA_3n→	IA_3	167
IA_8n→	IA_8	126
IIA_13n→	IIA_13	329
IIA_11n→	IIA_11	309
h_117n→	h_117	147
IIB_45n→	IIB_45	336
IB_49n→	IB_49	336
IIA_71n→	IIA_71	336
IA_75n→	IA_75	139
IA_82n→	IA_82	336
IIB_87n→	IIB_87	330
IA_89n→	IA_89	323
IA_91n→	IA_91	153
IA_105n→	IA_105	126
IA_114n→	IA_114	309
Match within cluster		
IB_6n→	IB_57	309
IIA_20n→	IB_15	168
h_30n→	h_117	99
Match to different cluster		
IB_17n→	IB_34	98
h_1n→	h_59	103
h_41n→	h_43	156

The closest match for the replicate in the original cohort is listed in the second column. Time (in days) from the original draw to the replicate draw is shown.

bins to create a similarity matrix. The cluster dendrogram is shown in Figure 1a, with the 12 clusters depicted in red.

The objective computational method was able to cluster together healthy controls (in clusters 3, 5, 6, and 10–12). Furthermore, the composition of clusters in relation to the EUROClass CVID classification (5) has a nonrandom pattern (Fisher's Exact Test $P < 10^{-6}$; Fig. 1b), where the most of the switched memory B-cell lacking (smB–CD21norm) patients are within clusters 7 and 2, smB+CD21low patients are in clusters 8 and 9 and most of the smB–CD21low patients are in cluster 4. Surprisingly, although patients with switched memory B cells are scattered in many clusters, the smB+CD21low and smB+CD21norm patients never locate to a common cluster. SmB+CD21norm patients frequently locate with healthy control subjects, which is in line with the preservation of some B-cell function in this patient group.

Replicate Samples from Patients are Properly Assigned as the Closest Match

To test the reliability of this method, we obtained samples from 21 donors repeatedly 98–336 days apart. This approach tests the whole procedure including sampling, antibody staining, acquisition by flow cytometry, and the data binning, and

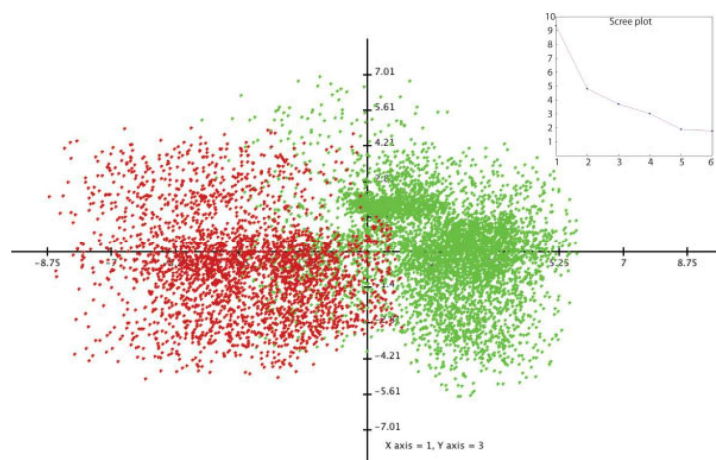


Figure 2. Visual representation of significantly “fed” bins between cluster 7 and controls. Representations of significant bins were subjected to PCA; over-fed bins are in red, and under-fed bins are in green. Each significant bin is represented by 100 dots distributed uniformly in each bin. X-axis Principal Component 1, Y-axis Principal Component 2. Eigenvectors of component 1: IgM -0.605; CD24 -0.158; CD19 0.005; CD27 0.33; CD21 0.017; CD38 -0.7. Eigenvectors of component 2: IgM 0.22; CD24 -0.38; CD19 0.03; CD27 -0.67; CD21 0.41; CD38 -0.41. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

similarity matrix generation. When we matched the B-cell profile of replicates to the original cohort using the similarity matrix of the Pearson correlation, 15 of replicates matched to the same individual, three replicates matched to a different individual within the same cluster, and three replicates matched to a different cluster (Table 1).

Thus, we conclude that the procedure is highly accurate and reproducible when comparing cellular profiles in complex six-parameter flow cytometry data.

B-Cell Phenotype Stability

The replicate sample analysis also revealed unprecedented stability of the B-cell profile among CVID patients. Although the time between replicates was longer for the CVID patients than for the healthy controls (mean 249 vs. 126 days), 14 of 17 of the CVID patients’ replicates (82%) matched to their original samples, whereas only one of four healthy controls matched exactly (25%) Table 1. This observation suggests a tight regulation of the abnormal B-cell compartment in CVID patients.

Principal Component Analysis Reveals Significantly Different Cell Subsets

To reduce the data back to a level where significantly different B-cell subsets could be visualized by the common flow cytometry software tools, we set out to find significant differences in the cell composition between the CVID clusters and healthy controls. Thus, we tested clusters 2b, 4, 7, 8, and 9 against controls (controls were all healthy donors from groups 3, 5, 6, and 10). Using the MTP procedure, we selected differ-

entially filled bins. Bins were annotated as “over-fed” or “under-fed” as compared with controls. We received a list of 15–311 different bins for each group. Because it is impossible to plot six parameters in two-dimensional plots, the coordinates of the “over-fed” or “under-fed” bins’ means were subjected to PCA to find the distinct portions of the six-parameter space where the “over-” or “under-fed” bins reside. PCA was able to display distinct spaces of “over-” or “under-fed” bins (e.g., Cluster 7 vs. controls on Fig. 2). Moreover, PCA computed the best resolving composition of components values (eigenvectors), which could be used to create a “PV plot” in FlowJo software. However, when we displayed differentially filled bins for cluster 12 versus controls, the PCA did not allow spatial separation of the “over-fed” and “under-fed” bins (Supporting Information 2).

Differentially Represented Cell Subsets can be Visualized and Verified in the Original Listmode Data

Using the same eigenvectors, a PV plot was created in the FlowJo software using the original listmode data of donors in cluster 7 and healthy controls (Fig. 3a). The PV plot enabled a display of the major differences between the two clusters, similar to the PCA of the clusters’ differences, but also allowed for further analysis of each individual CVID patient back in the listmode file. When we gated on the prominent PCA (7) over-fed region, we could track the significantly different over-represented cells as transitional B-cells, Figure 3b. The percentage of the PV plot gated cells (cluster 7 med = 21.6%, SD = 8.6%; healthy med = 2.35%, SD = 2.6) and classically gated transitional B-cells (cluster 7 med = 13.4%, SD = 7%; healthy

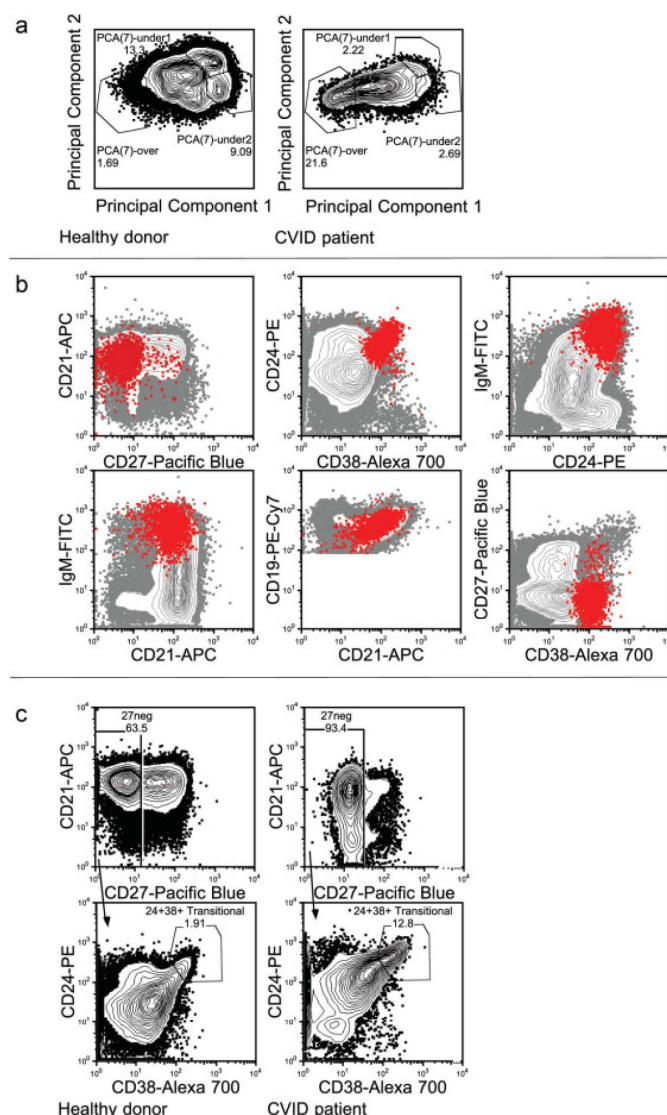


Figure 3. a) Example PV plot of CD19+ cells from a healthy control (left) and from a CVID patient from cluster 7 (right). PCA gates were created based on the PCA clusters from Fig 2. Note that in contrast to the PCA in Fig 2, where only significant bins are displayed, all CD19+ cells from a FCS file are displayed in Fig 3. b) Backtracking of the PCA (7)-over gate to a series of 2-D plots revealed that this PCA gate contains primarily transitional B-cells. Overlay of normal donor CD19+ gated B-cells (grey contour plot) with the PCA(7)-over gate (depicted in red). c) Classical gating of Transitional B-cells in a healthy donor (left) and a CVID patient from cluster 7 (right). Plots are gated on CD19+ lymphocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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Table 2. Location of different bins tracked to the six-dimensional space by PCA

PCA GATE	IgM	CD24	CD19	CD27	CD21	CD38	B-CELL SUBSET
PCA(2)-over	+ OR ++	+ OR ++	+	— OR +	+	— OR +	Transitional
PCA(2)-under	—	— OR +	+	— OR +	— OR +	— OR + OR ++	
PCA(4)-over	+	+	+	+	—	—	
PCA(7)-over	++	++	+	—	+	++	
PCA(8)-under	—	— OR +	+	—	—	— OR +	
PCA(8)-over	+	—	+	—	—	—	
PCA(9)-over	+	+	+	+	+	—	
PCA(9)-under	— OR +	— OR +	+	—	+	— OR +	

Cluster number is shown in parenthesis. “over” and “under” denotes over- and under-fed bins. Symbols “—” or “+” or “++” stand for the level of expression on the subset (negative or dim or bright). Expression range “—” or “+” means the bin spanned across from negative to positive cells. Only the PCA(7)-over gate was clearly overlapping with the known subset of transitional B-cells. Compare with Figure 3b and Supporting Information 3.

med = 1.5%, SD = 2.3) was higher in cluster 7 patients than in the healthy donors ($P < 0.01$ for both comparisons). Classical gating is shown in Figure 3c.

Cellular subsets that were hallmarks of the defined clusters were tracked back. However, the majority of them did not correspond with the B-cell subsets described so far (13–17), (Table 2, Supporting Information 3).

DISCUSSION

This is, to our knowledge, the first attempt to use unsupervised computational methods to define clusters of individuals with similar immunophenotype cellular profiles and to apply the methodology for the analysis of the B-cell compartment of CVID patients. Furthermore, we were able to prove the reproducibility of this approach by analysis of biological replicates. The acquired number of B-cells in a sample (20,000) related to the number of bins (1,024) created was sufficient for the description of the overall B-cell profile, and also for the definition of small hallmark subsets that comprised only 1.5% of the healthy control B-cells (transitional B-cells). While we have evaluated the usefulness of the matrix composed of 64–4,096 bins, clustering according to the profile was very similar between 256 and 4,096 bins (data not shown). We decided to use 1,024 bins to ensure the recognition of small subsets (the number of bins was such that one bin contained 0.1% of the B-cells). A crucial precondition of the described approach is the long-term stability of the fluorescence measurements. Typically, defocusing of the alignment of the lasers was the major cause of our instrument stability failure, which occurred about once a month. Careful instrument stability monitoring ensured early detection of technical problems and setting the target MFI on the brightest peak of Rainbow beads was instrumental to restore the fluorescence measurements to the standardized level. Whether or not such an approach can be adopted for a multicenter study remains to be evaluated. We can foresee that some adaptations of this process might be necessary; for example, the use of a percentile scale for bin definition would reflect distribution of a signal measured on an individual instrument.

The amount and quality of information gained by this approach is critically dependent on the composition of the measured markers and also on a careful fluorochrome choice that ensures sufficient sensitivity for each marker. Selected markers were tested to be able to recognize important known B-cell subsets before sample acquisition in this study. Using the six-color panel, we were able to define: transitional B-cells as CD19+CD27–CD24++CD38++ (14), follicular B cells as CD19+CD38+CD24+CD21+CD27–IgM+ (16), marginal zone-like B-cells as CD19+CD38–CD24++CD21int CD27+IgM++ (17), class switched memory B cells as CD19+CD38+CD24++CD21+CD27+IgM– (15), and plasmablasts as CD19+CD38+++CD24–CD27++ (13).

A factor limiting the number of markers used in this study was the availability of key B-cell markers conjugated to less common fluorochromes (Pacific Orange, PE-TxRed, PerCP, and APC-H7) that would be expressed strongly enough for a meaningful analysis.

Unsupervised hierarchical clustering analysis enabled the definition of clusters of individuals with similar B-cell profiles. We chose the Pearson correlation as a distance metric to recognize trends (correlations) in the individual profiles. On the contrary, Euclidian distance evaluates the absolute value of the distance without regard to the direction of change from normality. Both methods yielded meaningful results, but Euclidian distance was better in organizing the healthy controls together and separating healthy controls from CVID patients. The Pearson correlation enhanced formation of CVID patient clusters. The differences are logical as we have chosen the value $F = 0$ for the “average” or “normal” profile. Thus, healthy controls have low absolute distance from each other, but can be negatively correlated.

The described approach allows for a search of common features of each cluster. Although the cohort of patients is too small to reveal common blocks in B-cell maturation in such a highly heterogeneous syndrome, we were able to prove the principle and show the capacity for its future use. We have shown that there is an incomplete overlap between the EURO-Class trial classification and unsupervised clustering. When we reduce the information again in search of a cluster’s hallmarks, we can find well defined subsets (e.g., “over-fed” transitional

B-cell subset in cluster 7) or less well described subsets (see Table 2, Supporting Information 3). This opens a way for functional characterization of the revealed subsets and the determination of their role in the CVID pathogenesis. A larger study of B-cell profile, T-cell profile, and clinical course of CVID patients is underway.

The major contribution of this study is that it exploits advanced technology (polychromatic flow cytometry) and combines it with new approaches in high-throughput data analysis (well-known in the field of expression profile microarray analysis) to analyze a syndrome that goes unresolved despite several decades of research effort.

There have been some pilot studies of novel approaches in data analysis going in a similar direction; notably, Pedreira et al. (18) used computational approaches to define minimal residual disease in B-cell chronic lymphoproliferative disorders and Steinbrich-Zollner et al. (19) classified the pattern of inflammatory diseases. However, while Pedreira et al. use the whole profile for PCA analysis, they do not use polychromatic data and they do not aim to form clusters of similar profiles. Steinbrich-Zollner et al. perform clustering the results of analysis but necessitate manual expert gating of the listmode data.

Although expression profiling (also known as DNA microarrays) provide information about tens of thousands genes, they suffer from problems arising from the mixed cell populations they analyze. Thus, small cell subsets, if not purified, will not change the expression profile and will go unnoticed. Strengths of the presented “cellomics” approach lay in the analysis of single cells and their surface protein expression, the ease of use, and the rather wide availability of the 3-laser flow cytometry instrumentation. The pitfalls are in the proper choice of defining antigens. The presented proof of principle study sets the stage for more focused, in depth studies of B-cell functional subsets in CVID patients and is applicable to many other research questions in hematopoietic cell development.

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7 Změny v T-lymfocytárních populacích pacientů s CVID

Jak už bylo uvedeno výše, primárním znakem pacientů s CVID je snížená sekrece imunoglobulinů zapříčiněná selháním funkcí B-lymfocytů, nicméně byla popsána i řada abnormalit v zastoupení a funkci T-lymfocytů, včetně redukce naivních CD4⁺ T-lymfocytů, snížené proliferací a cytokinové odpovědi na mitogeny, redukovaný počet T-regulačních buněk u vybraných pacientů.³⁹ Další výzkumy ukázaly, že změny v zastoupení B buněk, popisované ve Freiburské i EUROclass klasifikaci souvisejí s již dříve popisovanými změnami v zastoupení T-lymfocytárních populací nalezených u pacientů s CVID, což také dokládá naše studie analyzující změny T-lymfocytů u pacientů s CVID rozdělených podle Freiburské klasifikace (viz 4.4.1).

7.1 Věková závislost a vzájemné vztahy abnormalit T a B lymfocytů u pacientů s běžnou variabilní imunodeficiencí

V této studii jsme pacienty rozdělili do skupin dle Freiburské klasifikace. Srovnání výsledků mezi jednotlivými skupinami ukázalo, že aktivace CD4⁺ T-lymfocytů byla výrazná zejména ve skupině Ia, což se projevilo zvýšením exprese CD29, HLA-DR a také se ukázal posun těchto buněk do paměťových a efektorových forem (zvýšení CD45RO, snížení CD27, CD62L, CD45RA). Naproti tomu změny pozorované u CD8⁺ T-lymfocytů (např. zvýšená exprese HLA-DR, CD57, CD38, CD27, CD28 a snížená exprese CD27, CD28) byly nezávislé na klasifikační skupině.

Podrobnější analýza B-lymfocytů dále odhalila, že s věkem klesá zastoupení naivních B-buněk (IgM⁺CD27⁻), zatímco stoupá počet B-buněk marginální zóny (IgM⁺CD27⁺). Rovněž u CD4⁺ T-lymfocytů jsme zaznamenali rychlejší snižování počtu naivních CD27⁺CD45RA⁺ a nárůst CD45RO⁺ paměťových T buněk s věkem vyšetřovaných pacientů ve srovnání se zdravými kontrolami. Exprese markerů CD27, CD45RA, CD45RO a HLA-DR na CD4⁺ T-lymfocytech korelovala nejen s věkem, ale i s podílem CD21^{low} B-lymfocytů. Podobné změny T-lymfocytárních populací byly popsány u zdravých starších osob ve věku 75–84 let.^{40,41} U pacientů jsme rovněž našli zvýšené zastoupení CD28⁻CD8⁺ T-lymfocytů, což je rovněž považováno za znak imunosenescence.⁴² Výsledky naší práce ukázaly, že změny v zastoupení subpopulací T- a B-lymfocytů nejsou u CVID pacientů neměnné, ale dynamicky se vyvíjejí s věkem.

Age dependency and mutual relations in T and B lymphocyte abnormalities in common variable immunodeficiency patients. **Vlková M**, Thon V, Sárkyová M, Bláha L, Svobodník A, Lokaj J, Litzman J. Clin Exp Immunol. 2006 Feb;143(2):373-9.

Age dependency and mutual relations in T and B lymphocyte abnormalities in common variable immunodeficiency patients

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Summary

Common variable immunodeficiency (CVID) is primary hypogammaglobulinaemia with an unknown aetiopathogenesis. Although various abnormalities of T and B cells have been described, their pathogenetic roles are unclear. We determined T and B lymphocyte subsets known to be abnormal in CVID in order to disclose possible relations between numerical abnormalities in those cells. Markers associated with B cell development (CD21, CD27, IgM, IgD) were determined on B lymphocytes (CD19⁺); T lymphocyte development (CD45RA, CD45RO, CD62L) and activation markers (CD25, CD27, CD28, CD29, CD38, CD57, HLA-DR) were determined on CD4⁺ and CD8⁺ T lymphocytes in 42 CVID patients and in 33 healthy controls. Abnormalities in CD4⁺ T lymphocyte activation markers (increase in CD29, HLA-DR, CD45RO, decrease in CD27, CD62L, CD45RA) were observed particularly in patients with a decreased number of memory (CD27⁺) and mature (CD21⁺) B cells (group Ia according to the Freiburg group's classification), while abnormalities observed in CD8⁺ cells (increase in CD27 and CD28 and decrease in HLA-DR, CD57 and CD38) did not depend upon grouping patients together according to B lymphocyte developmental subpopulations. We observed correlations between immature B cells (IgM⁺CD21⁻) and expression of CD27, CD62L, CD45RA, CD45RO and HLA-DR on CD4⁺ T cells in CVID patients but not in the control group. The expression of CD27 and CD45RA on CD4⁺ T lymphocytes, such as the percentage of IgD⁺CD27⁻ and IgD⁺CD27⁺ cells in B lymphocytes, showed age dependency to be more significant than in the control group. Our study demonstrates that T and B lymphocyte abnormalities in CVID are partially related to each other. Some of those abnormalities are not definite, but may evolve with age of the patient.

Keywords: age factor, B lymphocyte subsets, common variable immunodeficiency, differentiation antigens, T lymphocyte subsets

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Introduction

Common variable immunodeficiency (CVID) is a primary humoral immunodeficiency disease characterized by impaired antibody response; immunodeficiency manifests in people older than 2 years of age. Decreased levels of IgG, IgA and sometimes also IgM are observed in these patients [1]. They suffer from recurrent and complicated infections, specifically of the respiratory tract. Other frequent complications include autoimmune diseases (22% in one study) [2]. The incidence of malignancies is also increased with a standardized incidence ratio for all malignancies of 1.8 [3]. The

aetiopathogenetic mechanisms leading to the disease are probably heterogeneous, causing variable clinical and laboratory patterns in affected individuals.

Previous studies have shown various abnormalities in both T and B lymphocytes. Defective T cell activation [4–6] and impaired cytokine production have been described [7–9]. A plethora of T subpopulation abnormalities, including reduced expression of CD45RA and CD62L or increased expression of CD45RO, HLA-DR and CD57, have been reported [10–13].

Determination of B lymphocyte subpopulations showed that the number of circulating B cells was normal or reduced.

Recent results have shown that CVID patients have a reduced number of memory CD27⁺ IgD⁺ IgM⁺ B cells [14,15]. A new classification of CVID based on flow-cytometric analysis of B cell subpopulations was proposed by the Freiburg group [16], who divided CVID patients into four groups and subgroups: group I comprises patients with a decreased number of switched memory B cells [below 0.4% of total peripheral blood lymphocytes (PBLs)] and group II includes all patients with a normal number of switched memory B cells (> 0.4 of PBLs). Group I was subdivided into group Ia with an increased number of immature B cells (> 20% of CD19 number IgM⁺ CD21⁺) and group Ib with a normal number of IgM⁺ CD21⁺ immature B cells. Group III had less than 1% B cells of PBLs. This approach was accepted by other groups [17].

The above-mentioned abnormalities of both T and B lymphocytes are not present in all CVID patients. Very little is known about relations between T and B cell abnormalities in CVID. Only Piqueras *et al.* have shown that T lymphocyte markers associated with chronic stimulation of CD4⁺ cells (increase in HLA-DR, CD95 and decrease in CD45RA), such as expansion of HLA-DR and CD57 on CD8⁺ cells, were less up-regulated in CVID patients with a normal number of memory (IgD⁺ CD27⁺) cells compared to those with a decreased proportion of CD27⁺ cells (both IgD⁺ and IgD⁺) [18]. It is not clear which of the T and B cell abnormalities are possibly causative, which are secondary, and which are only epiphenomena. A more detailed analysis of mutual relations between numbers of T and B cells in various developmental stages, together with markers associated with different stages of activation, might help in elucidating mechanisms leading to T–B cells imbalance in CVID. For this purpose we determined those T and B cell subpopulations that were most frequently abnormal in CVID patients.

Patients and methods

Patients and controls

B and T cell subpopulations of 42 CVID patients (aged 10–75 years, mean 43.7 ± 15 years; 28 females, 13 males) and 33 healthy controls (aged 21–59 years, mean 37.5 ± 10.7; 7 males, 26 females) were examined. Twenty-three patients fulfilled the European Society for Immunodeficiencies (ESID) diagnostic criteria for CVID [1], including low isohaemagglutinin levels or decreased response to specific antigen stimulation. In 19 patients, namely those whose treatment was initiated before 1996 (the introduction of relevant tests in our laboratory), the diagnosis was made by low immunoglobulin levels, clinically significant immunodeficiency and exclusion of other causes of hypogammaglobulinaemia. The patients were on regular immunoglobulin substitution by intravenous immunoglobulin (i.v. IG) in 38 patients, dose 250–520 mg/kg/3–4 weeks; four patients were on intramuscular immunoglobulin at a dose of 1.2 g/1–2 weeks.

After informed consent was obtained, blood samples were collected before the i.v. IG infusion was given. Healthy donors were studied in parallel with the patients.

B cell analysis

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of heparinized blood by density gradient centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway), washed twice with phosphate-buffered saline and resuspended in RPMI-1640 (Sigma R0883) supplemented with 10% fetal calf serum.

T cell analysis

Five millilitres of peripheral blood were taken into ethylenediamine tetraacetic acid (EDTA) tubes and stained using a standard procedure by direct immunofluorescence. In brief, 50 µl of the blood was treated with 5–10 µl of the appropriate antibodies for 30 min at laboratory temperature in the dark. The stained blood was then processed by the Multi-Q-Prep workstation (Beckman Coulter, Miami, FL, USA) for automated lysis, stabilization and fixation of the sample.

Monoclonal antibodies and flow cytometry

Two commercial cocktails of monoclonal antibodies (mAb) for basic lymphocyte population analysis were used: (1) anti-CD45FITC, anti-CD4PE, anti-CD8ECD and anti-CD3PC5; and (2) anti-CD45FITC, anti-CD56PE, anti-CD19ECD, and anti-CD3PC5; (Cyto-Stat tetraChrome, Beckman Coulter). Monoclonal antibody anti-CD16PE (Immunotech, Marseille, France) was added to the second cocktail.

In addition, the following mAbs directed against leucocyte antigens were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD25, CD27, CD28, CD29, CD57, CD62L, phycoerythrin (PE)-conjugated anti-CD38, CD45RA, CD45RO, R-Phycoerythrin-Cyanine 5 (PC5)-conjugated anti-CD3 (all from Dako A/S, Glostrup, Denmark); PC5-conjugated anti-CD27, ECD (phycoerythrin-Texas Red X)-conjugated anti-CD4, CD8, CD19 (all from Beckman Coulter); FITC-conjugated anti-CD38, PE-conjugated anti-IgD and anti-CD21, PC5-conjugated anti-IgM (all from Pharmingen International, San Diego, CA, USA) and PE-conjugated anti-HLA DR (Becton Dickinson, San Jose, CA, USA).

Phenotyping was performed by four-colour cytometry Epics MCL (Beckman Coulter).

Statistical analysis

The differences between the groups were evaluated by analysis of variance (ANOVA) followed by Dunnett's (comparison between patients and control group) or Newman–Keul's (general comparison between groups of patients) contrasts.

Table 1. Percentage of B lymphocyte subpopulations in various subgroups of common variable immunodeficiency (CVID) patients; for definition of CVID subgroups see Introduction.

	CVID with B cells (mean $\pm$ s.d.)	Ia (mean $\pm$ s.d.)	Ib (mean $\pm$ s.d.)	II (mean $\pm$ s.d.)	III	Control (mean $\pm$ s.d.)
No. of patients	38	11	15	10	6	33
%CD19	9.4 $\pm$ 5.6	6.7 $\pm$ 3.3	9.4 $\pm$ 5.0	12.9 $\pm$ 6.9	< 1	9.5 $\pm$ 3.7
%IgD ⁺ CD27 ⁻ of CD19 ⁺	75.7 $\pm$ 18.0§	81.0 $\pm$ 10.8§	87.1 $\pm$ 7.2§	56.4 $\pm$ 16.1	n.a.	57.4 $\pm$ 13.3
%IgD ⁺ CD27 ⁺ of CD19 ⁺	13.0 $\pm$ 13.1§	10.5 $\pm$ 9.5	6.5 $\pm$ 4.1§	25.5 $\pm$ 17.7	n.a.	18.1 $\pm$ 6.8
%IgD ⁻ CD27 ⁺ of CD19 ⁺	4.7 $\pm$ 7.2§	1.6 $\pm$ 1.6§	1.8 $\pm$ 2.0§	9.0 $\pm$ 2.8§	n.a.	18.7 $\pm$ 6.9
%IgM ⁺ CD21 ⁻ of CD19 ⁺	20.4 $\pm$ 15.2§	35.8 $\pm$ 13.0§	10.2 $\pm$ 4.2	20.4 $\pm$ 15.0§	n.a.	6.4 $\pm$ 3.3

§Statistically significant difference from the control group ($P < 0.05$) shown in bold type; n.a. = not applicable.**Table 2.** Clinical characteristics of common variable immunodeficiency patients.

	Ia	Ib	II	III
Number of patients	11	15	10	6
Male/female	4/7	2/13	6/4	2/4
Age	50.2 $\pm$ 11.4	35.8 $\pm$ 13.5	48.3 $\pm$ 18.0	44.7 $\pm$ 17.8
Age of diagnosis	30.4 $\pm$ 12.9	22.0 $\pm$ 13.7	33.0 $\pm$ 18.6	31.0 $\pm$ 12.0
Splenomegaly	10	9	4	5
Lymphadenopathy	4	8	4	1
Autoimmunity	2	1	3	3

The results were confirmed using non-parametric Mann–Whitney test. P -values less than 0.05 were considered statistically significant in all statistical analyses. Student's t -test and Fisher's exact test were used when appropriate.

Correlations between variables were evaluated using Spearman's correlation coefficient (R_s) and the results were revised by Bonferroni's correction for each group of lymphocytes: CD4⁺, CD8⁺ and CD19⁺ [19]. The statistical package STATISTICA (StatSoft, Inc., Tulsa, OK, USA) version 7 was used.

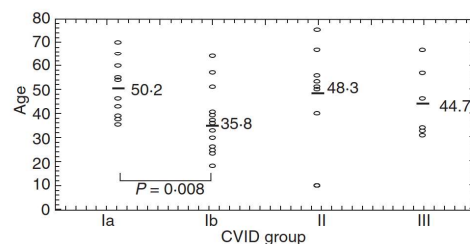
Results

Fluorescence-activated cell sorter (FACS)-based classification of CVID patients according to B cells

We have classified our patients into four groups in accordance with the Freiburg group's classification [17]. Six patients had B cells less than 1% of PBLs (group III). The remaining 36 patients were divided into the following groups: 11 patients belonged to group Ia, 15 to group Ib and 10 to group II (Table 1). The clinical characteristics of patients belonging to these subpopulations are given in Table 2. We observed a higher incidence of splenomegaly in groups Ia and III compared with group II (Ia *versus* II: $P = 0.03$, III *versus* II: $P = 0.04$, Fisher's exact test). We found significant differences in the patients' age in groups Ia and Ib ($P = 0.008$, Student's t -test and $P = 0.009$, Mann–Whitney test) (Fig. 1). We did not find any significant differences in age between the remaining groups.

Age-dependent variation of lymphocyte subsets in CVID

Because of the above-mentioned differences in the age of patients in groups Ia and Ib, correlations between T lymphocyte and B lymphocyte markers and the age in both patient and control groups were performed. The percentage of naive B cells (IgD⁺ CD27⁻) and IgM memory B cells (IgD⁺ CD27⁺) was age-dependent in the CVID group, such as the percentage of CD27⁺, CD45RA⁺ and CD45RO⁺ in CD4⁺ cells. The only significant age-dependent correlation in the control group was the percentage of CD45RO⁺ in CD4⁺ cells. Figure 2 shows that although the regression line both in patients and controls always has the same direction, in CVID patients the slopes are greater.

**Fig. 1.** Age distribution in common variable immunodeficiency subgroups.

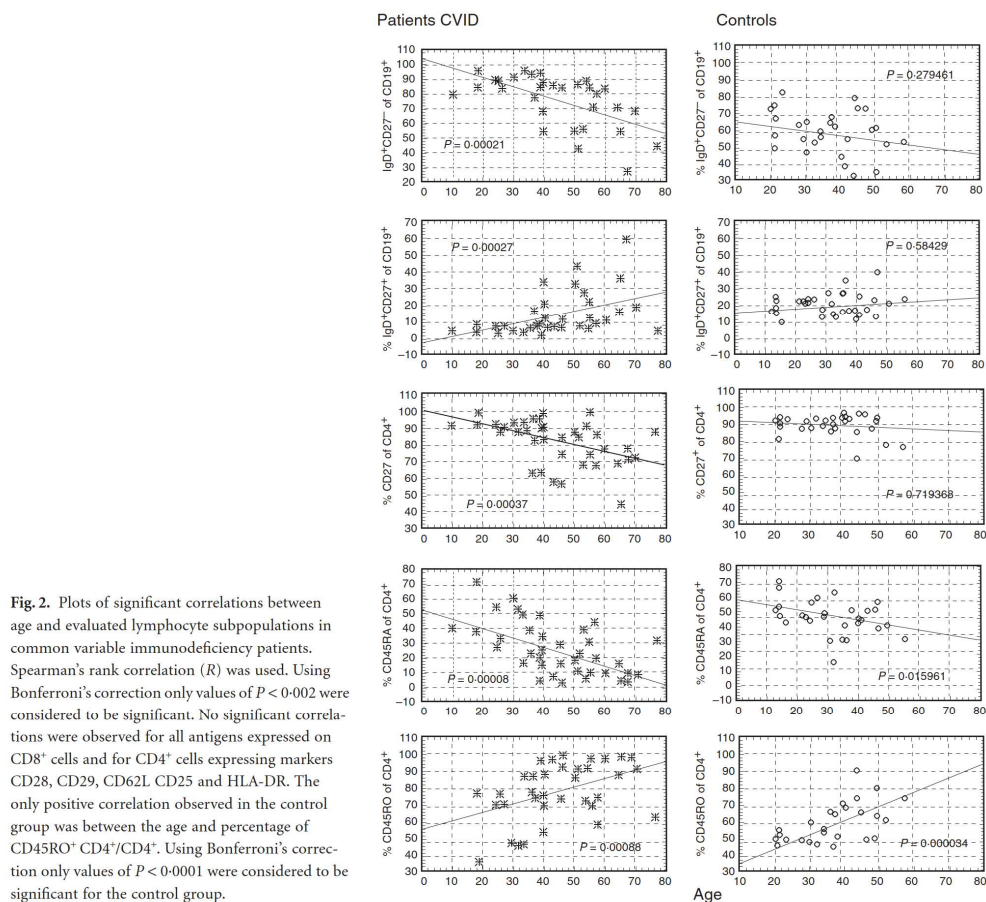


Fig. 2. Plots of significant correlations between age and evaluated lymphocyte subpopulations in common variable immunodeficiency patients. Spearman's rank correlation (R) was used. Using Bonferroni's correction only values of $P < 0.002$ were considered to be significant. No significant correlations were observed for all antigens expressed on CD8⁺ cells and for CD4⁺ cells expressing markers CD28, CD29, CD62L CD25 and HLA-DR. The only positive correlation observed in the control group was between the age and percentage of CD45RO⁺ CD4⁺/CD4⁺. Using Bonferroni's correction only values of $P < 0.0001$ were considered to be significant for the control group.

T lymphocytes

T lymphocyte subpopulations in various CVID subgroups are shown in Table 3. When analysing significant differences in T lymphocyte subpopulations in CVID subgroups compared to controls, we found that the abnormalities in CD8⁺ subpopulations scarcely depended on the CVID subgroups, as abnormalities in the percentage of CD27⁺, CD28⁺, CD38⁺ and CD57⁺ cells in the CD8⁺ subpopulation were observed in almost all groups investigated.

Conversely, the CD4⁺ subpopulation abnormalities differed according to CVID subgroups. Although the increase in CD45RO⁺ activated/memory CD4⁺ T cells was documented in all CVID subgroups, abnormalities in other T lymphocyte activation markers (increased in CD29, HLA-DR, decreased in CD27, CD62L, CD45RA) were observed specifically in group Ia and also the differences from the

control group were more significant in the Ia subgroup than in the remaining groups.

Comparing the frequency of T cell abnormalities in various CVID subgroups, no differences were observed in subpopulations CD4⁺, CD28 of CD4⁺ or CD25 of CD4⁺. Statistically significant differences were observed most frequently comparing groups Ia and Ib; comparing other groups, the differences were relatively rare (see Table 4).

Relations between T and B lymphocyte subpopulations in CVID patients

No significant correlations were observed between T cell activation markers and the percentage of memory CD27⁺ IgD⁺ B cells. When analysing relations between immature IgM⁺ CD21⁻ B cell and T lymphocyte activation markers, we showed a correlation between CD27⁺, CD62L⁺,

Table 3. T lymphocyte subpopulations and expression of activation markers in various subgroups of common variable immunodeficiency (CVID) patients; see Introduction for definition of CVID subgroups.

	CVID with B cells (mean $\pm$ s.d.)	Ia (mean $\pm$ s.d.)	Ib (mean $\pm$ s.d.)	II (mean $\pm$ s.d.)	III (mean $\pm$ s.d.)	Controls (mean $\pm$ s.d.)
CD4 ⁺	37.2 $\pm$ 9.6§	40.3 $\pm$ 11.0	36.8 $\pm$ 10.0§	34.2 $\pm$ 6.5§	36.4 $\pm$ 8.9§	47.2 $\pm$ 7.7
%CD27 ⁺ of CD4 ⁺	81.0 $\pm$ 13.6§	68.1 $\pm$ 15.2§	88.0 $\pm$ 7.2	84.5 $\pm$ 9.0	80.0 $\pm$ 9.7§	89.6 $\pm$ 6.3
%CD28 ⁺ of CD4 ⁺	93.4 $\pm$ 5.9§	92.7 $\pm$ 5.9	93.6 $\pm$ 4.4	93.8 $\pm$ 8.1	92.1 $\pm$ 8.3	96.7 $\pm$ 4.7
%CD29 ⁺ of CD4 ⁺	64.0 $\pm$ 17.0§	72.0 $\pm$ 21.7§	58.4 $\pm$ 15.4	63.6 $\pm$ 10.0	58.0 $\pm$ 17.7	49.9 $\pm$ 11.0
%CD62L ⁺ of CD4 ⁺	64.0 $\pm$ 19.5	45.8 $\pm$ 18.6§	73.7 $\pm$ 14.6	69.5 $\pm$ 12.6	72.2 $\pm$ 17.5	74.2 $\pm$ 10.9
%CD45RA ⁺ of CD4 ⁺	24.9 $\pm$ 17.2§	8.1 $\pm$ 6.9§	37.3 $\pm$ 15.7	24.7 $\pm$ 10.8§	21.1 $\pm$ 17.2§	46.2 $\pm$ 13.9
%CD45RO ⁺ of CD4 ⁺	77.4 $\pm$ 16.7§	92.5 $\pm$ 7.6§	67.9 $\pm$ 16.7§	75.1 $\pm$ 12.3§	76.5 $\pm$ 18.5§	55.7 $\pm$ 14.3
%CD25 ⁺ of CD4 ⁺	4.1 $\pm$ 2.4§	3.9 $\pm$ 2.7	4.4 $\pm$ 2.6§	3.8 $\pm$ 1.8§	2.0 $\pm$ 1.0	1.9 $\pm$ 0.8
%HLA DR ⁺ of CD4 ⁺	15.0 $\pm$ 9.3§	23.3 $\pm$ 10.5§	10.7 $\pm$ 5.9	12.4 $\pm$ 6.3§	11.1 $\pm$ 6.0§	5.3 $\pm$ 3.0
CD8 ⁺	32.1 $\pm$ 10.1§	36.8 $\pm$ 9.0§	32.7 $\pm$ 10.2§	25.9 $\pm$ 8.8§	43.9 $\pm$ 11.4§	18.3 $\pm$ 5.1
%CD27 ⁺ of CD8 ⁺	46.3 $\pm$ 21.7§	31.3 $\pm$ 16.0§	52.6 $\pm$ 22.1§	53.4 $\pm$ 19.8	29.2 $\pm$ 16.7§	68.9 $\pm$ 18.0
%CD28 ⁺ of CD8 ⁺	38.9 $\pm$ 20.5§	24.4 $\pm$ 11.8§	43.9 $\pm$ 21.7§	47.6 $\pm$ 19.0§	22.7 $\pm$ 12.8§	68.0 $\pm$ 19.4
%CD29 ⁺ of CD8 ⁺	78.8 $\pm$ 23.2§	90.8 $\pm$ 9.9	65.9 $\pm$ 26.7§	85.0 $\pm$ 19.6	85.5 $\pm$ 21.7	95.5 $\pm$ 4.3
%CD62L ⁺ of CD8 ⁺	37.7 $\pm$ 19.1	30.5 $\pm$ 15.4	41.1 $\pm$ 23.1	40.8 $\pm$ 15.5	24.4 $\pm$ 15.1	42.7 $\pm$ 16.7
%CD45RA ⁺ of CD8 ⁺	53.4 $\pm$ 14.6	47.5 $\pm$ 14.5	61.2 $\pm$ 12.3	48.2 $\pm$ 13.5	50.6 $\pm$ 20.5	59.0 $\pm$ 14.1
%CD45RO ⁺ of CD8 ⁺	43.8 $\pm$ 13.6	49.4 $\pm$ 12.0	36.7 $\pm$ 11.9	48.2 $\pm$ 13.9	46.5 $\pm$ 19.9	39.8 $\pm$ 13.3
%HLA DR ⁺ of CD8 ⁺	21.3 $\pm$ 15.8§	26.6 $\pm$ 7.9§	19.0 $\pm$ 21.9	18.8 $\pm$ 10.5§	23.6 $\pm$ 12.5§	7.1 $\pm$ 6.9
%CD38 ⁺ of CD8 ⁺	29.8 $\pm$ 16.6§	34.1 $\pm$ 14.6§	30.6 $\pm$ 19.3§	23.7 $\pm$ 13.7§	31.8 $\pm$ 13.1§	11.3 $\pm$ 6.1
%CD57 ⁺ of CD8 ⁺	32.8 $\pm$ 14.3§	41.2 $\pm$ 8.2§	28.5 $\pm$ 16.8§	29.8 $\pm$ 12.7§	49.1 $\pm$ 10.2§	16.2 $\pm$ 12.9

§Statistically significant difference from the control group ($P < 0.05$) shown in bold type.

CD45RA⁺, CD45RO⁺ and HLA-DR⁺ of the CD4⁺ cells. Concerning CD8⁺ T cells, we detected correlations between IgM⁺ CD21⁺ B cell and CD57⁺, such as HLA-DR⁺ subpopulations (see Table 5).

Discussion

The goal of our study was to disclose relations between T and B cell abnormalities in CVID patients. Some lymphocyte abnormalities were observed in the majority of CVID patients almost independently of B cell-based (Freiburg group) classification: the CD8⁺ cells were not only increased in number, but were also activated as seen by increased expression of CD38, CD57 and HLA-DR and decreased expression in CD27 and CD28. CD8⁺ cells expressing CD57 and CD38 without expression of CD27 and CD28 were described as effector cells in viral infections [20–22].

Although, with the exception of chronic enteroviral meningoencephalitis, no well-defined viral infection was proved to be increased in CVID [23], perhaps common respiratory viruses [24] cause this stimulation.

A different situation was found in CD4⁺ T cells. We observed that these cells were activated predominantly in patients with decreased activated memory (CD27⁺) and increased immature B cells (IgM⁺ CD21⁺) (group Ia according to the Freiburg group classification) [17]. The patients from this subgroup also had the sharpest change in T lymphocyte subpopulations compared to controls: decreased in CD27, CD62L, CD45RA and increased in CD29, HLA-DR, CD45RO of CD4⁺ cells. The change in these CD4⁺ lymphocyte markers indicates a shift to activated and memory phenotypes. Our observation is in accordance with the study by Piqueras *et al.*, who showed that patients with normal numbers of memory (IgD⁺CD27⁺) cells up-regulate lymphocytes

Table 4. Statistical comparison of differences between common variable immunodeficiency subgroups as determined by two-sided binomial testing expression of defined T lymphocyte activation markers. The numbers in the table represent P -values for the difference in proportion of patients with abnormal values of particular T lymphocytes. Statistically significant difference between groups is shown in bold type.

	Ia/Ib	Ia/II	Ia/III	Ib/II	Ib/III	II/III
%CD27 of CD4 ⁺	<0.001	0.027	0.061	0.14	0.142	0.902
%CD29 of CD4 ⁺	0.032	0.136	0.083	0.572	0.876	0.571
%CD62L of CD4 ⁺	0.013	0.056	0.083	0.643	0.815	0.884
%CD45RA of CD4 ⁺	0.002	0.027	0.495	0.307	0.023	0.171
%CD45RO of CD4 ⁺	0.002	0.010	0.024	0.572	0.534	0.902
%HLA-DR of CD4 ⁺	0.043	0.137	0.186	0.626	0.680	1.000

Table 5. Correlations between the expression of maturation/activation markers on B and T lymphocytes. Spearman's rank correlation (*R*) was used. Using Bonferroni's correction only the values $P < 0.0028$ and $P < 0.004$ for CD4⁺ and CD8⁺ subpopulations (subsequently) were considered as significant (shown in bold type).

	IgM ⁺ 21 ^{low} of CD19		IgD ⁺ 27 ⁺ of CD19	
	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>
CD4	-0.063582	0.712574	0.091912	0.610953
%CD27 of CD4 ⁺	-0.690907	0.000003	-0.213235	0.233467
%CD28 of CD4 ⁺	-0.313791	0.062366	-0.065508	0.717207
%CD29 of CD4 ⁺	0.498359	0.001979	0.167447	0.351638
%CD62L of CD4 ⁺	-0.735440	0.000000	-0.107955	0.549846
%CD45RA of CD4 ⁺	-0.643027	0.000023	-0.187578	0.295874
%CD45RO of CD4 ⁺	0.628869	0.000040	0.191025	0.286926
%CD25 of CD4 ⁺	-0.130768	0.447141	-0.180329	0.315266
% HLA-DR of CD4 ⁺	0.545981	0.000572	0.009358	0.958778
CD8	0.220592	0.196068	-0.047794	0.791687
%CD27 of CD8 ⁺	-0.486744	0.002610	0.109291	0.544886
%CD28 of CD8 ⁺	-0.404118	0.014509	-0.080214	0.657229
%CD29 of CD8 ⁺	0.336165	0.045009	0.008356	0.963191
%CD62L of CD8 ⁺	-0.292149	0.083813	-0.115976	0.520408
%CD45RA of CD8 ⁺	-0.245560	0.148860	-0.266377	0.134019
%CD45RO of CD8 ⁺	0.216988	0.203660	0.202540	0.258305
% HLA-DR of CD8 ⁺	0.524067	0.001036	0.027409	0.879654
%CD38 of CD8 ⁺	0.185586	0.278514	-0.075535	0.676107
%CD57 of CD8 ⁺	0.589356	0.000156	-0.017380	0.923524

less with activation markers associated with chronic stimulation of CD4⁺ cells (increased in HLA-DR, CD95 and decreased in CD45RA) and CD8⁺ cells (increased in HLA-DR and CD57) compared to patients with a decreased proportion of CD27⁺ cells [18].

However, CD4⁺ T lymphocyte abnormalities were also seen in other subgroups of CVID patients. CD25 expression on T lymphocytes was increased in groups Ib and II, while there were no significant changes in CD62L and CD29 antigen expression. CD25 is an early activation marker whose expression begins 4 h after activation [25], while CD29 is positive mainly on memory T cells long after previous activation [26]. CD62L (L-selectin) initiates entry of T cells into the peripheral lymph nodes; it is shed from the cell surface following lymphocyte activation [27]. Taken together, the early activation pattern in particular is seen on CD4⁺ lymphocytes in groups Ib and II.

We observed several correlations between CD4⁺ cell activation markers and differentiating markers of B lymphocytes. They were limited to the IgM⁺ CD21⁻ subpopulation of immature B cells, which correlated with the expression of several T cell markers (decreased in CD27, CD62L, CD45RA and increased in CD29, HLA-DR, CD45RO). This shows that the extent of activation of CD4⁺ T cells in group Ia may be related to the number of immature B cells. These results can be explained by a more significant regulatory defect or by secondary stimulation of the immune system by the antigen load in immunodeficient patients.

Surprisingly, when comparing T lymphocyte abnormalities in various CVID subgroups we observed most

differences between subgroups Ia and Ib, both of which are characterized by decreased numbers of memory (CD27⁺) B cells; moreover, the subgroup Ia was more similar to subgroups II (normal B cell subpopulations) and III (decreased number of B cells) than to Ib. We also showed that subgroup Ib was more similar to normal donors than the other subgroups. Because the number of IgM⁺ CD21⁻ (immature B cells) is also increased in subgroup II, it seems that increased T cell activation accompanies disturbed maturation of B cells from immature to mature cells in CVID patients.

We showed that the decrease in memory-switched cells in CVID patients may be age-dependent, because the number of CD27⁺ IgM⁺ naive B cells decreased with age, while CD27⁺ IgM⁺ non-switched memory cells increased. This phenomenon was not observed either in our control group or in healthy people in another study [28]. Evaluating T lymphocyte subpopulations, only an age-dependent shift from CD45RA into CD45RO in CD4⁺ populations was observed in both CVID patient and control groups, while the decrease in CD27 in CD4⁺ populations was seen in CVID patients but not in controls. In another study a similar decrease in the proportion of CD4⁺ CD27⁺ in CD4⁺ cells was observed in older people (75–84 years) compared to young people (19–28 years) [29]. In our study the proportion of CD27⁺ CD4⁺ cells in the control group was similar, but the proportion of CD27⁺ CD4⁺ cells in middle-aged CVID patients was comparable to healthy older patients [29]. The increase in CD45RO⁺ and decrease in CD45RA⁺ CD4⁺ cells was more extensive in our CVID patients than in older people [28]. However, in other studies we found that the defect in

functional activation of T cells [5,7] and the cytokine release (e.g. interleukin-4) in CVID patients was different from the outcome of the study of older people [29]. This shows that the observed subpopulation changes in CVID may not only be the results of ageing of the immune system.

Future studies of T and B cell subpopulations might help to define more effectively subgroups of CVID patients and disclose aetiopathogenetic mechanisms leading to a blockade of antibody production in various CVID subgroups.

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Naše výsledky publikované v článku *Age dependency and mutual relations in T and B lymphocyte abnormalities in common variable immunodeficiency patients* potvrdila řada dalších nezávislých studií. Fenotypy T-lymfocytů byly zkoumány v řadě kohort CVID, přičemž redukce naivních CD4⁺ T lymfocytů byla nejkonzistentnějším výsledkem.⁴³ Publikována byla také T-lymfocytární klasifikace CVID.⁴⁴ V ní bylo navrženo rozdělení pacientů CVID podle počtu naivních CD4⁺ T-lymfocytů. Rozlišeny byly tři skupiny: (1) pacienti s výrazně sníženým počtem a vysokou aktivací naivních CD4⁺ T-lymfocytů, často se sklonem k apoptóze, změnám v TCR repertoáru, splenomegalií a závažnými komplikacemi; (2) pacienti s podobným, ale mírnějším profilem; (3) pacienti s normálním počtem naivních CD4⁺ T-lymfocytů, ale se změnami v populaci CD8⁺ T-lymfocytů a mírnějším průběhem. Následné studie prokázaly silnou korelaci mezi počtem naivních CD4⁺ T-lymfocytů a klinickými projevy.⁴⁵ Pacienti s autoimunitními cytopeniemi a orgánově specifickými autoimunitami mají nejnižší hladiny naivních CD4⁺ T-lymfocytů, následují pacienti s polyklonální lymfoproliferací.⁴⁶ Studie zahrnující 238 pacientů prokázala, že podíl naivních CD4⁺ T-lymfocytů pod 10 % je citlivým indikátorem buněčné imunodeficiency a je spojen s vyšším výskytem komplikací, včetně lymfomů a úmrtí.^{47,48} Dysfunkce T-lymfocytů u pacientů s CVID a autoimunitními komplikacemi dokládá, že patogeneze tohoto onemocnění přesahuje poruchy B-buněčné imunity. Tito pacienti mají nižší celkový počet CD4⁺ i CD8⁺ T-lymfocytů než jedinci s CVID bez autoimunitních projevů,⁴⁹ přičemž nízké počty CD8⁺ T buněk predikují vyšší riziko autoimunitních manifestací.^{43,50} Autoimunita je dále spojena s úbytkem naivních a nárůstem terminálně diferencovaných forem CD8⁺ T-lymfocytů. Typickým rysem je rovněž snížený počet regulačních T-buněk (Treg) se sníženou expresí FOXP3 a oslabenou supresivní funkcí.^{51–53} U těchto pacientů byl také zaznamenán zvýšený počet prozánětlivých Th1 a folikulárních T-helper (Tfh) buněk, jejichž vyšší zastoupení v periferní krvi odpovídá expanzi zárodečných center.^{54,55} Vzhledem k tomu, že CVID je vždy provázen nízkými hladinami IgA, může být zvýšený počet Tfh reakcí částečně důsledkem endotoxemie způsobené mukózní deficiencí IgA a následnou mikrobiální translokací.⁵⁶ U pacientů s autoimunitní formou CVID byla navíc popsána expanze Th17 lymfocytů.^{57,58} Tyto nálezy ukazují, že pro CVID s autoimunitními komplikacemi je charakteristická dysregulace T-lymfocytů, zejména úbytek Treg a nárůst efektorových a paměťových prozánětlivých subpopulací.

7.2 Srovnání změn T- a B-lymfocytárních populací u pacientů s CVID a se selektivním deficitem IgA (slgAD)

CVID a deficiencie IgA (IgAD) jsou považovány za různé fenotypové projevy podobného nebo souvisejícího genetického pozadí, o čemž svědčí jak popsání případy přechodu pacientů z IgAD do CVID, tak i výskyt rodin, u nichž se obě poruchy vyskytují současně.^{8,59} Z tohoto důvodu byl náš výzkum zaměřen také na analýzu T- a B-lymfocytárních populací u pacientů se slgAD. Z našich výsledků vyplynulo, že u pacientů s IgA deficitem nebyly v B-buněčné populaci zjištěny významné změny v zastoupení naivních a paměťových subsetů, pouze mírné zvýšení počtu CD21^{low} B-lymfocytů. U těchto pacientů jsme pozorovali u T-lymfocytů vzestup absolutního počtu CD8⁺ T-lymfocytů a relativní pokles CD4⁺ T buněk. Podrobnější analýza diferenciačních stadií T-lymfocytů podle exprese CD45RO a CD27 nám ukázala, že u pacientů se slgAD dochází ke snížení podílu naivních CD4⁺ T buněk a nárůstu pozdně diferencovaných paměťových buněk v populaci jak pomocných, tak cytotoxických T-lymfocytů. Tyto změny byly u CVID vyjádřeny výrazněji a statisticky významně a u pacientů s CVID jsme navíc zaznamenali pokles časně diferencovaných CD4⁺ T buněk a snížení podílu naivních CD8⁺ T buněk. I v jiných studiích byly potvrzeny naše výsledky: u pacientů s CVID byl opakovaně popsán zvýšený absolutní počet CD8⁺ cytotoxických T-lymfocytů a snížený počet CD4⁺ pomocných T buněk.^{43,44}

U CD4⁺ T buněk jsme našli pokles exprese HLA-DR a nárůst CD25⁺ buněk, zatímco u CD8⁺ T buněk jsme zaznamenali sníženou expresi CD29. Snížená exprese CD29 může znamenat oslabenou schopnost aktivace CD8⁺ T buněk, neboť bylo publikováno, že CD29⁺ cytotoxické T-lymfocyty vykazují vyšší produkci IFN- γ , granzymu a perforinu než CD29⁻ buňky, což svědčí o jejich významné efektorové funkci (Nicolet et al., 2020). Nižší výskyt těchto buněk, který jsme našli u pacientů se slgAD by mohl souviset s vyšší aktivitou regulačních T buněk, což podporuje námi popsáný zvýšený počet CD4⁺CD25⁺ buněk, podobně jako publikovali Raziuddin et al.⁶⁰ Vzhledem k tomu, že CD25 je exprimován i na aktivovaných T-lymfocytech, nebylo možné bez dalších markerů z našich výsledků jednoznačně odlišit regulační buňky. Absence zvýšené exprese HLA-DR na CD4⁺ T-lymfocytech však spíše naznačuje, že nejde o aktivované efektorové buňky.

Úbytek CD4⁺ T-lymfocytů u pacientů s CVID tedy souvisí především se ztrátou naivních buněk, zatímco nárůst CD8⁺ T buněk odráží expanzi časně a pozdně diferencovaných efektorových populací. Tento posun T-lymfocytárních subsetů směrem k paměťovým a efektorovým formám podporuje hypotézu o předčasném stárnutí imunitního systému (immunosenescence) u pacientů s CVID. U pacientů se selektivním deficitem IgA jsou obdobné změny přítomny také, avšak v méně vyjádřené podobě.

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T and B lymphocyte subpopulations and activation/differentiation markers in patients with selective IgA deficiency

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Summary

Selective deficiency of immunoglobulin A (IgAD) and common variable immunodeficiency (CVID) are genetically closely related diseases, both of unknown pathogenesis. A plethora of abnormalities in lymphocyte subpopulations and expression of activation markers were repeatedly documented in CVID patients, while almost no data are available about lymphocyte subpopulations in IgAD patients. We determined basic lymphocyte subpopulations and those subpopulations that were reported to be abnormal in CVID patients (CD25, human leucocyte antigen (HLA)-DR CD45RA, CD45RO, CD27, CD28 and CD29 on both CD4⁺ and CD8⁺ cells, CD57 and CD38 on CD8⁺ cells, CD21, CD27, IgM, IgD on B lymphocytes) in 85 patients with IgAD, 47 patients with CVID and in 65 healthy controls. Statistical analysis was performed by the Mann–Whitney *U*-test; significant *P*-values were determined by means of Bonferroni's correction. Our results showed an increase in the relative number of CD8⁺ cells and a decrease in the absolute number of CD4⁺ cells compared to healthy people, but similar abnormalities in CVID patients were much more expressed. IgAD patients had significantly decreased expression of HLA-DR and increased expression of CD25 on CD4⁺ lymphocytes, also CD29 expression was decreased on CD8⁺ cells, while other activation/differentiation markers on T cells (including the expression of CD45RA and CD45RO antigens) were not changed. There were no statistically significant abnormalities in B lymphocyte developmental stages in IgAD patients compared to healthy controls. Our observation showed that the majority of T and B lymphocyte subpopulation abnormalities described previously in CVID are not present in IgAD patients.

Keywords: common variable immunodeficiency, B lymphocyte subsets, differentiation antigens, IgA deficiency, T lymphocyte subsets

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Introduction

Selective deficiency of immunoglobulin A (IgAD) is the most frequent, but usually asymptomatic, abnormality of immunoglobulin production, with a frequency ranging from 1 : 278 to 1 : 3040 in Caucasians [1]. Despite its high frequency, very little is known about the aetiopathogenic mechanisms leading to this immunological abnormality.

IgAD is linked to a more severe immunoglobulin production disease – common variable immunodeficiency (CVID), which is characterized by decreased IgG and IgA levels, while IgM levels are variable. Clinically significant immunodeficiency evolves at any age after apparently normal function of the immune system [2]. Familial

occurrence of both diseases has been documented repeatedly [3,4]. Deficiency of transmembrane activator and CALM interactor (TACI) was observed both in CVID and IgAD patients [5,6]. All these results show that IgAD and CVID may be different phenotypic manifestations of the same, or similar, genetic background. Repeatedly documented progression of IgAD into CVID [7] shows that IgAD may be a developmental stage in evolving CVID.

Although the pathogenesis of CVID has not yet been elucidated, many abnormalities in the number or function of immune cells were described in CVID patients. Great attention has been given to abnormalities in lymphocyte subpopulations. Defective activation of T lymphocytes

[8–10] and impaired cytokine production [11–13] were observed in CVID patients. Various T subpopulation abnormalities including a decreased number of CD4⁺ cells [14–16], reduced expression of CD45RA [15] and CD62L [17] or increased expression of CD45RO [15] on CD4⁺ cells, human leucocyte antigen (HLA)-DR [16] and CD57 on CD8⁺ [18] were reported. Also, decreased expression of CD27, CD28, CD29 on both CD4⁺ and CD8⁺ cells and increased expression of CD38 and CD57 on CD8⁺ cells were observed [19]. When evaluating B lymphocyte subpopulations, a decrease in the number of memory CD27⁺ IgD⁺ IgM B cells has been described repeatedly [20–24].

Despite the assumed relation of CVID and IgAD, there are almost no data available about T and B lymphocyte subpopulations in IgAD. Previous studies performed on limited numbers of patients showed a possible decrease of the proportion of CD4⁺ cells and increase of proportion of CD8⁺ cells [25–27], while Klemola *et al.* [28] did not observe any significant abnormalities in relative numbers of CD3⁺, CD4⁺ and CD8⁺ cells. Because of the above-mentioned similar genetic background of CVID and IgAD, assessing those abnormalities in IgAD may help to elucidate whether lymphocyte abnormalities in CVID are primary, inherited or whether they evolve secondarily during the development of the diseases, or are even a consequence of immunodeficiency in CVID.

In our study we have evaluated lymphocyte subpopulations most frequently known to be abnormal in CVID patients in a group of 85 patients with IgAD, 65 controls and 47 patients with CVID.

Patients and methods

Eighty-five patients with IgAD fulfilling diagnostic criteria of IgAD [29] (48 females, 37 males) aged 18–72 years were included in the study. These patients were referred most frequently for immunological investigation because of apparently increased frequency of mild respiratory tract infections; none of them suffered from clinically significant immunodeficiency or clinically apparent autoimmune disease. Lymphocyte subpopulations were also determined in 47 patients with CVID (30 females, 17 males) aged 10–75 years. A detailed analysis of relations of lymphocyte subpopulations in the CVID subgroup has been published previously [19]. As a control group, 65 healthy people with normal serum IgA levels (35 females, 30 males) aged 22–66 years were used. Controls were recruited from hospital staff and medical students. All blood samples were obtained in an acute infection-free period; in CVID patients on IVIG treatment, blood samples were obtained prior to immunoglobulin infusion.

The study was approved by the Ethics Committee of the Faculty of Medicine of Masaryk University in Brno. Informed consent was obtained from all investigated individuals before the samples were drawn.

Lymphocyte subpopulation determination

All blood samples were taken between 8 and 11 a.m. to exclude diurnal variation of lymphocyte subsets. B lymphocyte subpopulations were analysed from density gradient separated cells while T lymphocyte subpopulations were determined from non-separated blood, as described previously [19]. Immunophenotyping of lymphocytes was performed by four-colour cytometry Epics MCL (Beckman Coulter Miami, FL, USA).

Major lymphocyte populations were determined by two cocktails of monoclonal antibodies (MoAb): 1, anti-CD45 fluorescein isothiocyanate (FITC), anti-CD4 phycoerythrin (PE), anti-CD8 phycoerythrin-Texas red X (ECD) and anti-CD3PC5; 2, anti-CD45FITC, anti-CD56PE, anti-CD19ECD, anti-CD3 r-phycoerythrin-cyanine 5 (PC5); (Cyto-Stat tetraCHROME; Beckman Coulter, Miami, FL, USA) and anti-CD16PE (Immunotech, Marseille, France). In addition, the following MoAbs were used: PE-conjugated anti-CD38, CD45RA, CD45RO, FITC-conjugated anti-CD25, CD27, CD28, CD29, CD57, CD62L, PC5-conjugated anti-CD3 (all from Dako A/S, Glostrup, Denmark); PC5-conjugated anti-CD27, ECD-conjugated anti-CD4, CD8, CD19 (all from Beckman Coulter); FITC-conjugated anti-CD38, PE-conjugated anti-IgD and anti-CD21, PC5-conjugated anti-IgM (all from Pharmingen International, San Diego, CA, USA) and PE-conjugated anti-HLA-DR (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Normality of the obtained lymphocyte subpopulations was assessed by the Kolmogorov–Smirnov test. Because the majority of the obtained values showed a distribution different from normal, a non-parametric Mann–Whitney *U*-test was used. Critical values for statistical significance were determined by Bonferroni's correction [30] for each group of lymphocytes: absolute and relative numbers of major lymphocyte subpopulations, CD4⁺ activation/differentiation markers, CD8⁺ activation/differentiation markers, absolute and relative numbers of B cell activation states. Statistical package STATISTICA (StatSoft, Inc.) version 7 was used.

Results

The absolute and relative numbers of CD3⁺, CD4⁺ and CD8⁺ T lymphocytes in IgAD patients, healthy controls and CVID patients are shown in Tables 1 and 2. As can be seen, the absolute number of CD4⁺ cells was decreased and the relative number of CD8⁺ cells was increased in IgAD patients compared to healthy controls. Comparing CVID patients with IgAD patients, the relative numbers of CD3⁺, CD8⁺ cells were increased in CVID patients while CD4⁺ and natural killer (NK) (CD16/56⁺CD3⁺) cells were decreased compared to IgAD patients. Evaluating absolute numbers, the number of

Table 1. Relative numbers of basic lymphocyte subpopulations in IgAD, CVID and controls. Results are expressed as median $\pm$ standard deviation (s.d.). Lymphocytes are expressed as percentage of CD45⁺ cells, lymphocyte subpopulations as percentage of lymphocytes.

	Controls (n = 65)	IgAD (n = 85)	CVID (n = 47)	P, IgAD controls	P, IgAD CVID
Lymphocytes (%)	31.0 $\pm$ 7.8	29.0 $\pm$ 7.5	24.0 $\pm$ 10.7	0.084	0.007*
CD3 ⁺ (%)	74.0 $\pm$ 7.5	73.0 $\pm$ 7.1	79.0 $\pm$ 9.1	0.799	< 0.001*
CD4 ⁺ (%)	45.9 $\pm$ 8.5	42.7 $\pm$ 8.2	37.8 $\pm$ 9.5	0.015	< 0.001*
CD8 ⁺ (%)	21.8 $\pm$ 7.4	25.9 $\pm$ 5.8	36.0 $\pm$ 11.4	< 0.001*	< 0.001*
CD19 ⁺ (%)	11.09 $\pm$ 3.1	12.0 $\pm$ 4.0	10.0 $\pm$ 7.3	0.094	0.099
CD16/56 ⁺ CD3 ⁻ (%)	15.0 $\pm$ 7.8	13.0 $\pm$ 6.4	8.0 $\pm$ 7.1	0.422	0.004*

*Indicates statistically significant difference using critical value after Bonferroni's correction: 0.008. CVID: common variable immunodeficiency; IgAD: selective deficiency of immunoglobulin A.

CD4 cells and NK cells was also decreased significantly in CVID patients compared to IgAD patients.

Assessing the expression of activation/differentiation markers on CD4⁺ cells, a decreased expression of HLA-DR on CD4⁺ cells and an increased expression of CD25 on CD4⁺ cells was observed in IgAD patients compared to controls; this increase was also significant when comparing IgAD patients with CVID patients. No other significant differences were observed when comparing IgAD and controls. When comparing IgAD patients with CVID patients, an increase in the expression of CD45RO and a decrease in the expression of CD45RO and HLA-DR was observed in IgAD patients. Also, the number of CD28⁺ cells was increased and that of

CD29⁺ cells decreased in IgAD compared to CVID patients (see Table 3).

When assessing activation/differentiation markers on CD8⁺ cells, only a decrease in CD29⁺ expression was observed comparing IgAD and healthy people or CVID patients, while almost in all parameters significant differences were observed between CVID and IgAD patients (decrease in CD45RO, CD57, CD38, HLA-DR and increase in CD25, CD27 and CD28 when comparing IgAD to CVID patients) (see Table 4).

There were no significant abnormalities in the absolute and relative numbers of B lymphocyte differentiation stages when comparing IgAD and controls, while in CVID patients typical abnormalities described previously by others [20–24]

Table 2. Absolute numbers of basic lymphocyte subpopulations in IgAD, CVID and controls. Results are expressed as median $\pm$ standard deviation (s.d.).

	Controls (n = 65)	IgAD (n = 85)	CVID (n = 47)	P, IgAD controls	P, IgAD CVID
Leucocytes (10 ⁹ /l)	6.1 $\pm$ 1.5	6.1 $\pm$ 1.6	6.3 $\pm$ 2.3	0.962	0.483
Lymphocytes (10 ⁹ /l)	1.89 $\pm$ 0.12	1.77 $\pm$ 0.12	1.61 $\pm$ 0.25	0.170	0.186
CD3 ⁺ (10 ⁹ /l)	1.32 $\pm$ 0.49	1.25 $\pm$ 0.43	1.22 $\pm$ 0.77	0.231	0.483
CD4 ⁺ (10 ⁹ /l)	0.89 $\pm$ 0.36	0.75 $\pm$ 0.28	0.54 $\pm$ 0.32	0.006*	< 0.001*
CD8 ⁺ (10 ⁹ /l)	0.39 $\pm$ 0.32	0.43 $\pm$ 0.20	0.58 $\pm$ 0.50	0.143	0.023
CD19 ⁺ (10 ⁹ /l)	0.21 $\pm$ 0.09	0.20 $\pm$ 0.11	0.15 $\pm$ 0.18	0.905	0.023
CD16/56 ⁺ CD3 ⁻ (10 ⁹ /l)	0.24 $\pm$ 9.20	0.22 $\pm$ 0.13	0.14 $\pm$ 0.11	0.117	0.003*

*Indicates statistically significant difference using critical value after Bonferroni's correction: 0.007. CVID: common variable immunodeficiency; IgAD: selective deficiency of immunoglobulin A.

Table 3. Activation/differentiation markers on CD4⁺ cells on IgAD, CVID and controls. Results are expressed as median $\pm$ standard deviation (s.d.).

	Controls (n = 65)	IgAD (n = 85)	CVID (n = 47)	P, IgAD controls	P, IgAD CVID
CD4 ⁺ CD45RA ⁺ /CD4 ⁺ (%)	44.9 $\pm$ 16.2	45.0 $\pm$ 14.4	24.3 $\pm$ 16.3	0.775	< 0.001*
CD4 ⁺ CD45RO ⁺ /CD4 ⁺ (%)	49.7 $\pm$ 15.1	54.1 $\pm$ 15.1	75.4 $\pm$ 18.9	0.597	< 0.001*
CD4 ⁺ HLA-DR ⁺ /CD4 ⁺ (%)	6.9 $\pm$ 6.4	3.9 $\pm$ 3.6	10.2 $\pm$ 8.2	< 0.001*	< 0.001*
CD4 ⁺ CD25 ⁺ /CD4 ⁺ (%)	3.5 $\pm$ 3.1	9.7 $\pm$ 4.0	6.5 $\pm$ 7.9	< 0.001*	< 0.001*
CD62L ⁺ CD4 ⁺ /CD4 ⁺ (%)	75.3 $\pm$ 11.8	74.6 $\pm$ 11.2	71.9 $\pm$ 18.4	0.887	0.131
CD4 ⁺ CD27 ⁺ /CD4 ⁺ (%)	89.0 $\pm$ 8.5	86.3 $\pm$ 7.9	85.9 $\pm$ 12.8	0.039	0.314
CD4 ⁺ CD28 ⁺ /CD4 ⁺ (%)	98.3 $\pm$ 4.6	98.3 $\pm$ 3.6	95.6 $\pm$ 5.9	0.868	< 0.001*
CD4 ⁺ CD29 ⁺ /CD4 ⁺ (%)	50.4 $\pm$ 12.5	47.2 $\pm$ 13.4	65.3 $\pm$ 16.6	0.197	< 0.001*

*Indicates statistically significant difference using critical value after Bonferroni's correction: 0.006. CVID: common variable immunodeficiency; IgAD: selective deficiency of immunoglobulin A.

Table 4. Activation/differentiation markers on CD8⁺ cells of IgAD, CVID and control. Results are expressed as median $\pm$ standard deviation (s.d.).

	Controls (<i>n</i> = 65)	IgAD (<i>n</i> = 85)	CVID (<i>n</i> = 47)	<i>P</i> , IgAD controls	<i>P</i> , IgAD CVID
CD8 ⁺ CD45RA ⁺ /CD8 ⁺ (%)	62.4 $\pm$ 13.5	58.5 $\pm$ 14.8	54.8 $\pm$ 16.2	0.197	0.131
CD8 ⁺ CD45RO ⁺ /CD8 ⁺ (%)	34.5 $\pm$ 13.6	28.9 $\pm$ 13.1	41.2 $\pm$ 14.2	0.010	< 0.001*
CD8 ⁺ CD57/CD8 ⁺ (%)	17.3 $\pm$ 15.0	18.5 $\pm$ 12.9	34.7 $\pm$ 14.7	0.965	< 0.001*
CD8 ⁺ CD38/CD8 ⁺ (%)	13.3 $\pm$ 8.3	10.0 $\pm$ 7.1	23.8 $\pm$ 17.0	0.014	0.001*
CD8 ⁺ CD25 ⁺ /CD8 ⁺ (%)	1.1 $\pm$ 3.2	1.7 $\pm$ 5.6	0.6 $\pm$ 5.4	0.346	< 0.001*
CD8 ⁺ HLA-DR ⁺ /CD8 ⁺ (%)	6.2 $\pm$ 6.2	2.4 $\pm$ 4.6	14.5 $\pm$ 15.8	0.280	0.005*
CD62L ⁺ CD8 ⁺ /CD8 ⁺ (%)	40.3 $\pm$ 17.5	39.2 $\pm$ 15.5	34.5 $\pm$ 19.1	0.603	0.151
CD8 ⁺ CD27 ⁺ /CD8 ⁺ (%)	69.2 $\pm$ 19.2	58.0 $\pm$ 15.6	42.5 $\pm$ 22.4	0.022	0.001*
CD8 ⁺ CD28 ⁺ /CD8 ⁺ (%)	65.7 $\pm$ 20.4	58.8 $\pm$ 17.9	35.6 $\pm$ 20.4	0.030	0.002*
CD8 ⁺ CD29 ⁺ /CD8 ⁺ (%)	93.4 $\pm$ 7.4	79.1 $\pm$ 12.8	92.4 $\pm$ 21.7	< 0.001*	< 0.001*

*Indicates statistically significant difference using critical value after Bonferroni's correction: 0.005. CVID: common variable immunodeficiency; IgAD: selective deficiency of immunoglobulin A.

were observed when compared to IgAD patients (Table 5). When assessing B lymphocyte numbers according to the Freiburg [22] classification of CVID, no major abnormalities were observed. Only one IgAD patients and one control person fell into group Ib.

Discussion

The goal of our study was to determine whether lymphocyte subpopulation abnormalities observed in CVID patients were also present in a genetically related disorder – IgAD. The results showed that the longest known abnormalities in CVID – the decrease in CD4⁺ cells and the increase in the relative number of CD8⁺ cells – can also be documented in IgAD patients although, in our results, these abnormalities were demonstrated only in absolute numbers of CD4⁺ cells and in relative numbers of CD8⁺ cells. The decrease in CD4⁺ cells was ascribed to the decrease in CD45RA⁺ cells [15], but we could not detect this abnormality in our IgAD patients; the percentage of CD45RA and also CD45RO was normal both in CD4⁺ and CD8⁺ cells.

A surprising observation was that of a decrease in the expression of HLA-DR on CD4⁺ cells of IgAD patients compared to both CVID patients and healthy controls, and an increase in the CD25 expression on CD4⁺ cells, again compared to both CVID and IgAD patients. Razziudin *et al.* [25] also described increased expression of Tac (CD25) antigen on peripheral blood lymphocytes in two of three IgAD patients; these individuals had an increased proportion of OKDR (HLA-DR) and OKIa1 (HLA-DR)-positive peripheral lymphocytes. HLA-DR and CD25 are both activation markers of T lymphocytes; CD25 is an earlier activation marker compared to HLA-DR [31]. We cannot explain the cause of possible activation of CD4⁺ cells in our IgAD patients because they were investigated in an infection-free period and none of them had clinically apparent autoimmune disease at the time of investigation. Surprisingly, no other activation abnormalities were observed in IgAD patients compared to controls. As well as on the activated lymphocytes, CD25 is also expressed on T regulated (T_{reg}) cells; we cannot exclude the possibility that this subpopulation abnormality influences the above-mentioned difference

Table 5. Absolute and relative numbers of differentiation stages of B cells. CVID patients with B lymphocytes < 1% were excluded. Naive B lymphocytes were defined as: CD19⁺ CD27⁺ IgM⁺ IgD⁺ cells, IgM memory as: CD19⁺ CD27⁺ IgM⁺ IgD⁺ cells, switched memory as: CD19⁺ CD27⁺ IgM⁺ IgD⁺ cells, immature as CD19⁺ IgM⁺ CD21⁺.

	Controls (<i>n</i> = 65)	IgAD (<i>n</i> = 85)	CVID (<i>n</i> = 40)	<i>P</i> , IgAD controls	<i>P</i> , IgAD CVID
Naive (10 ⁹ /l)	0.099 $\pm$ 0.053	0.111 $\pm$ 0.072	0.142 $\pm$ 0.139	0.308	0.271
IgM memory (10 ⁹ /l)	0.046 $\pm$ 0.025	0.043 $\pm$ 0.031	0.015 $\pm$ 0.043	0.902	< 0.001*
Switched memory (10 ⁹ /l)	0.034 $\pm$ 0.038	0.028 $\pm$ 0.024	0.004 $\pm$ 0.012	0.108	< 0.001*
IgM ⁺ CD21 ⁺ (10 ⁹ /l)	0.009 $\pm$ 0.011	0.012 $\pm$ 0.015	0.029 $\pm$ 0.064	0.034	< 0.001*
Naive/B cells (%)	52.8 $\pm$ 12.5	56.8 $\pm$ 14.5	83.3 $\pm$ 15.8	0.135	< 0.001*
Naive/PBL (%)	1.8 $\pm$ 1.1	1.6 $\pm$ 1.1	0.2 $\pm$ 0.6	0.203	< 0.001*
IgM memory/B cells (%)	22.6 $\pm$ 7.4	20.5 $\pm$ 10.4	8.0 $\pm$ 12.3	0.520	< 0.001*
Switched memory/B cells (%)	19.6 $\pm$ 7.5	14.9 $\pm$ 7.5	1.8 $\pm$ 3.8	0.039	< 0.001*
IgM ⁺ CD21 ⁺ /B cells (%)	4.8 $\pm$ 4.3	6.7 $\pm$ 4.2	14.8 $\pm$ 14.8	0.025	< 0.001*

*Indicates statistically significant difference using critical value after Bonferroni's correction: 0.006. CVID: common variable immunodeficiency; IgAD: selective deficiency of immunoglobulin A.

between IgAD and controls and also CVID patients. Unfortunately, there are no data available about the number of T_{reg} cells in IgAD patients. Similar changes as in CD4⁺ cells were observed in CD8⁺ lymphocytes, although they were less intensive, not reaching statistical significance when comparing IgAD and controls.

Unlike in CVID, we could not document any statistically significant abnormalities in the differentiation stages of B cells, only a tendency to a decreased number of switched memory cells and a mild increase in IgM⁺ CD21⁺ immature cells was present, but these abnormalities were much less extensive than those observed in CVID patients. In IgAD patients, a reduced but still significant number of IgA⁺ B cells can be documented [28]. On a molecular level, an α germ-line transcript expression was reduced and α circle transcripts were not detected in three IgAD patients [32]. Another study showed normal α germ-line transcription in four IgAD patients, but in two of them the S μ -S α switch recombination was impaired while in the remaining two decreased secreted and membrane C α mRNA was markedly decreased in IgA switched B cells [33]. All these results show a probable heterogeneity in the B cell defect of IgAD patients, but according to our results, unlike the situation in CVID, such abnormalities do not affect maturation of the majority of B cells, as reflected by lymphocyte activation marker determination in blood.

In conclusion, our study showed that, with the exception of abnormalities in the numbers of CD4⁺ and CD8⁺ cells, no major abnormalities of lymphocyte subpopulations or expression of activation/differentiation markers were observed in IgAD patients. These results may show that lymphocyte subpopulation abnormalities seen in CVID are probably not a consequence of an inborn genetic defect but that they evolve as a part, or as a consequence, of immunodeficiency in CVID.

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Terminally differentiated memory T cells are increased in patients with common variable immunodeficiency and selective IgA deficiency

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Abstract

Introduction: Previous studies showed that several lymphocyte abnormalities seen in the most frequent symptomatic immunoglobulin deficiency, common variable immunodeficiency (CVID), were also observed in a genetically related asymptomatic disorder – selective IgA deficiency (IgAD). In this study we searched for abnormalities in the differentiation stages of T cells as well as for similarities of these abnormalities in CVID and IgAD patients.

Material and methods: Using flow cytometry in 80 patients with IgAD, 48 patients with CVID, and 80 control persons we determined T-lymphocyte subsets: both CD4 and CD8 were divided into the naïve CD45RO⁺CD27⁺, early differentiated CD45RO⁺CD27⁺, late differentiated CD45RO⁺CD27⁺ and fully differentiated effector CD45RO⁺CD27⁺ memory T cells, as well as Treg cells, defined as CD4⁺CD25^{high}CD127^{low} T cells.

Results: An increase of CD4⁺ and CD8⁺ late differentiated memory cells was observed comparing CVID patients to controls, as well as comparing IgAD patients to controls. In CVID patients an increase of CD4⁺ early differentiated memory cells, a decrease of CD8⁺ intermediate memory cells, and CD4⁺ and CD8⁺ naïve cells were found as well. The abnormalities in IgAD patients might be explained by higher CMV seropositivity observed in our IgAD. We confirmed the repeatedly published decrease of Treg cells in CVID patients, while Treg cells in IgAD patients were increased compared to controls.

Conclusions: Our results show T-cell activation not only in CVID, but also in IgAD patients. The increase in IgAD patients may be influenced by a more frequent CMV infection in our group of IgAD patients.

Key words: IgA deficiency, common variable immunodeficiency, T-lymphocyte subpopulations, CMV.

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Introduction

The pathogenesis of selective IgA deficiency (IgAD, serum IgA < 0.07 g/l, and normal other immunoglobulin isotype levels), the most frequent immunoglobulin deficiency in Caucasians, is unknown [1]. Patients with IgAD are asymptomatic in most cases, or they may suffer from recurrent infections of the respiratory tract; also the frequency of autoimmune diseases is increased in IgAD [2]. Compared to this, in the most frequent symptomatic hypogammaglobulinaemia: common variable immunodeficiency (CVID), besides IgA, also IgG and frequently IgM serum levels are decreased [3]. The clinical manifestation of CVID is diverse,

the patients suffer from recurrent and complicated infections mainly of the respiratory tract, but also from autoimmune diseases, splenomegaly, gastrointestinal diseases, or systemic granulomatous disease [4-6]. CVID seems to be a group of different diseases with similar clinical and laboratory manifestation, and we cannot exclude a similar situation in IgAD. However, the occurrence of both diseases in affected families and the repeatedly described progression of IgAD to CVID suggest a similar genetic background of IgAD and CVID, at least in some cases [7, 8].

Various abnormalities in the number or function of T and B cells were described in patients with CVID [9-15].

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Assessing T cells, a decrease in CD4⁺ and an increase in CD8⁺ T cells were repeatedly described, also an increase in memory CD4⁺CD45RO⁺ [9, 11, 12], activated CD4⁺CD25⁺ [11, 12], CD8⁺HLA-DR⁺ [9, 11-13], CD8⁺CD38⁺ [11, 12], terminally differentiated CD8⁺CD57⁺ [9, 12, 13], or a decrease in, naïve CD4⁺CD45RA⁺CD27⁺ [11, 12], and non-effector CD4⁺CD27⁺ [11], CD8⁺CD27⁺ [11, 12], or regulatory (T_{reg}) T cells [14, 16] were reported. Although with a markedly less significant expression, some of these abnormalities in T [11, 12] and B [10-12] cells were described in IgAD as well, showing that the immunopathogenesis of both diseases may be similar. The elevated numbers of activated CD4⁺ and CD8⁺ lymphocytes, but also higher serum neopterin [17] or soluble CD25 (sCD25) levels [18], point to the activation of the immune system in patients with CVID and possibly IgAD.

The development and maturation of T cells in periphery is a complex process, and various schemes are used to describe the differentiation stages of T lymphocytes in peripheral blood [19-21]. In our study, we intended to determine the T-cell differentiation stages spectrum in CVID and IgAD patients. For the differentiation stages of T cells we used the scheme described by Maenette [22] based on the determination of CD45RO and CD27. Because our previous studies showed that many abnormalities in B-cell subsets seen in CVID were also observed in IgAD patients [10-12], we have also searched for similarities and dissimilarities in T-cell differentiation stages in CVID and IgAD.

Material and methods

Peripheral blood was obtained from 80 patients with IgAD (27 males and 53 females; aged 18-70, median 33 years), 48 patients with CVID (19 males and 29 females; aged 19-75, median 45 years), and 80 healthy controls (27 males and 53 females; aged 18-74, median 32 years).

The control group was mainly represented by relatives of hospital employees and students of medicine. The patients fulfilled the European Society for Immunodeficiencies (ESID) and the Pan-American Group for Immunodeficiency's (PAGID) diagnostic criteria for CVID and IgAD [23].

More detailed characteristics of these groups were described previously [10].

T-cell analysis

Five millilitres of peripheral blood taken in ethylenediaminetetraacetic acid (EDTA) was stained with monoclonal antibodies and analysed using flow cytometry methods. Forty-five microlitres of the blood was treated with 5–10 µl of the appropriate antibodies for 30 minutes at laboratory temperature in the dark. The blood was then processed for automated lysis, stabilisation, and fixation of the sample by a TQ-PREP workstation (Beckman Coulter, Miami, USA).

Flow cytometry analysis

Immunophenotyping was performed by flow cytometric analysis (five-colour flow cytometer FC-500; Beckman Coulter with CXP software).

For the staining of T-lymphocyte differentiation stages the following antibodies were used: R-Phycoerythrin-Cyanine 7 (PC7)-conjugated anti-CD4, anti-CD8, Phycoerythrin-Texas red X (ECD)-conjugated anti-CD8, anti-CD45RO; PC5-conjugated anti-CD27, and anti-CD25 (all from Beckman Coulter). For the staining of T_{reg} we used anti-CD4 PC7, anti-CD127 PE, and anti-CD25 PC5 (Beckman Coulter).

T lymphocytes, both CD4 and CD8, were divided into naïve CD45RO⁺CD27⁺, early differentiated (ED) CD45RO⁺CD27⁺, late differentiated (LD) CD45RO⁺CD27⁺, and fully differentiated effector (FD) CD45RO⁺CD27⁺ memory T cells. In CD8⁺ cells a unique CD45RO⁺CD27^{dim} intermediate population was determined as well [22] (Fig. 1). T_{reg} cells were defined as CD4⁺CD25^{high}CD127^{low} [24] in accordance with recent studies that demonstrated that the description of T_{reg} as a CD4⁺CD25^{high}CD127^{low} phenotype correlates well with FoxP3 expression [25, 26].

Autoantibodies

Enzyme-linked immunosorbent assay (ELISA) was used for the determination of antibodies: anti-thyroid peroxidase (AESKULISA a-TPO, Aesku.Diagnostics, Wendelsheim, Germany), anti-thyroglobulin (AESKULISA a-TG, Aesku.Diagnostics), anti-tissue transglutaminase antibodies (AESKULISA tTg-G, AESKULISA tTg-A, Aesku.Diagnostics), and the rheumatoid factor (AESKULISA RF-AGM, Aesku.Diagnostics). Anti-nuclear antibodies were tested by indirect immunofluorescence (IIF) using HEp-2 cells (HEp-2 kit, Euroimmun, Lübeck, Germany); anti-gastric parietal cells, anti-reticulon, and anti-smooth muscle antibodies were determined using the rat liver/kidney/stomach IIF kit (Autoantibodies RL/RK/RS kit, Orgentec, Mainz, Germany).

Detection of CMV-specific antibodies

IgG anti-CMV antibodies were determined by ELISA using the EIA CMV IgG kit (Test-Line, Brno, Czech Republic). The cut-off of positivity was used upon the manufacturer's instruction.

Statistical analysis

Statistical significance was determined using a non-parametric Mann-Whitney test for comparisons of two independent samples. A standard level of statistical significance $p = 0.05$ was used. The results are expressed as median (fifth to 95th percentile). All analyses were performed using Statistica 9.1 software (StatSoft Inc., Tulsa, USA).

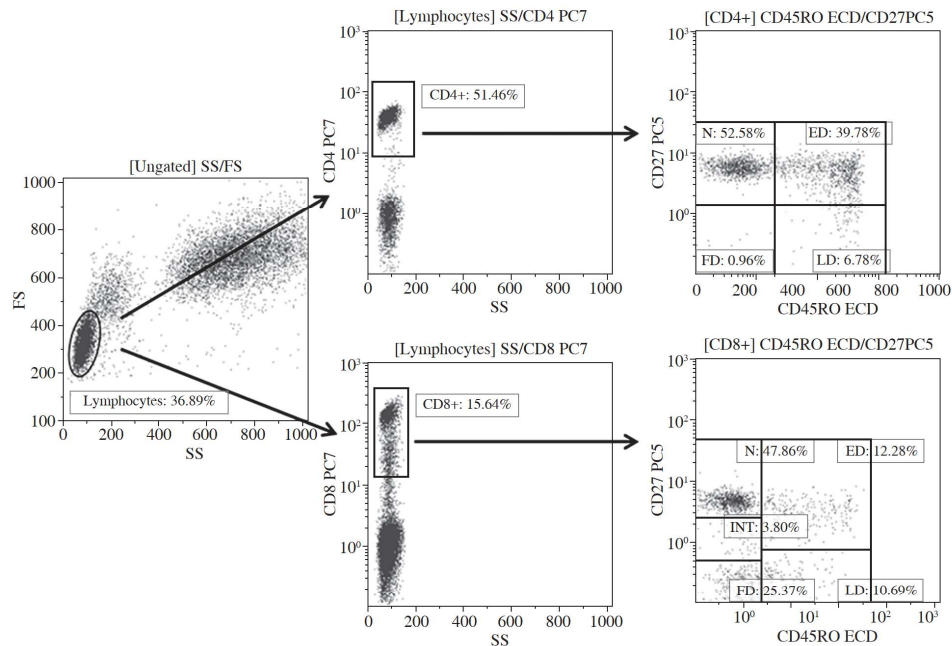


Fig. 1. Gating strategy for T-cell subsets. Gating is shown for a representative healthy donor. N naïve cells (CD45RO⁻CD27⁺), ED early differentiated (CD45RO⁺CD27⁺), LD late differentiated (CD45RO⁺CD27⁻), FD fully differentiated effector (CD45RO⁻CD27⁻), INT intermediate (CD45RO⁻CD27^{dim}) memory T cells

and GraphPad Prism 5.04 software (GraphPad software Inc., La Jolla, USA).

The study was approved by the Ethics Committee of St. Anne's Faculty Hospital in Brno. Informed consent was obtained from all investigated individuals before the samples were drawn.

Results

Late T-lymphocyte differentiation stages are increased in IgAD and CVID patients

Comparing CVID patients to control persons, an increase of CD4⁺ and CD8⁺ late differentiated memory cells ($p < 0.001$ and $p < 0.001$, respectively), CD4⁺ early differentiated memory cells ($p < 0.001$), and a decrease of CD8⁺ intermediate memory cells ($p = 0.001$) and also of CD4⁺ and CD8⁺ naïve cells ($p < 0.001$ and $p < 0.001$, respectively) was found. In patients with IgAD a significant increase of both CD4⁺ and CD8⁺ late differentiated memory cells ($p = 0.006$ and $p < 0.001$, respectively) and a decrease in

CD4⁺ naïve cells ($p = 0.026$) were documented as compared to healthy control persons (Fig. 2).

Frequency of CMV-specific antibodies is increased in IgAD patients

Since previous studies showed increased numbers of terminally differentiated memory T cells in patients with CMV infection [27, 28], we determined CMV IgG antibodies in the sera of control persons and patients with IgAD. Using IgG as a marker of CMV positivity, 40 IgAD patients (aged 18-70, median 37.5 years) and 27 control persons (aged 19-74, median 39 years) were seropositive while 38 IgAD patients (aged 18-54, median 26.5 years) and 52 controls (aged 18-71, median 28 years) were seronegative, the difference between IgAD patients and controls being significant ($p = 0.030$, chi-square test).

We observed an increase in late differentiated CD8⁺ cells in seropositive IgAD patients compared to seropositive control persons ($p = 0.005$), while there were no significant differences when comparing other subsets in seropositive IgAD patients to seropositive controls. When

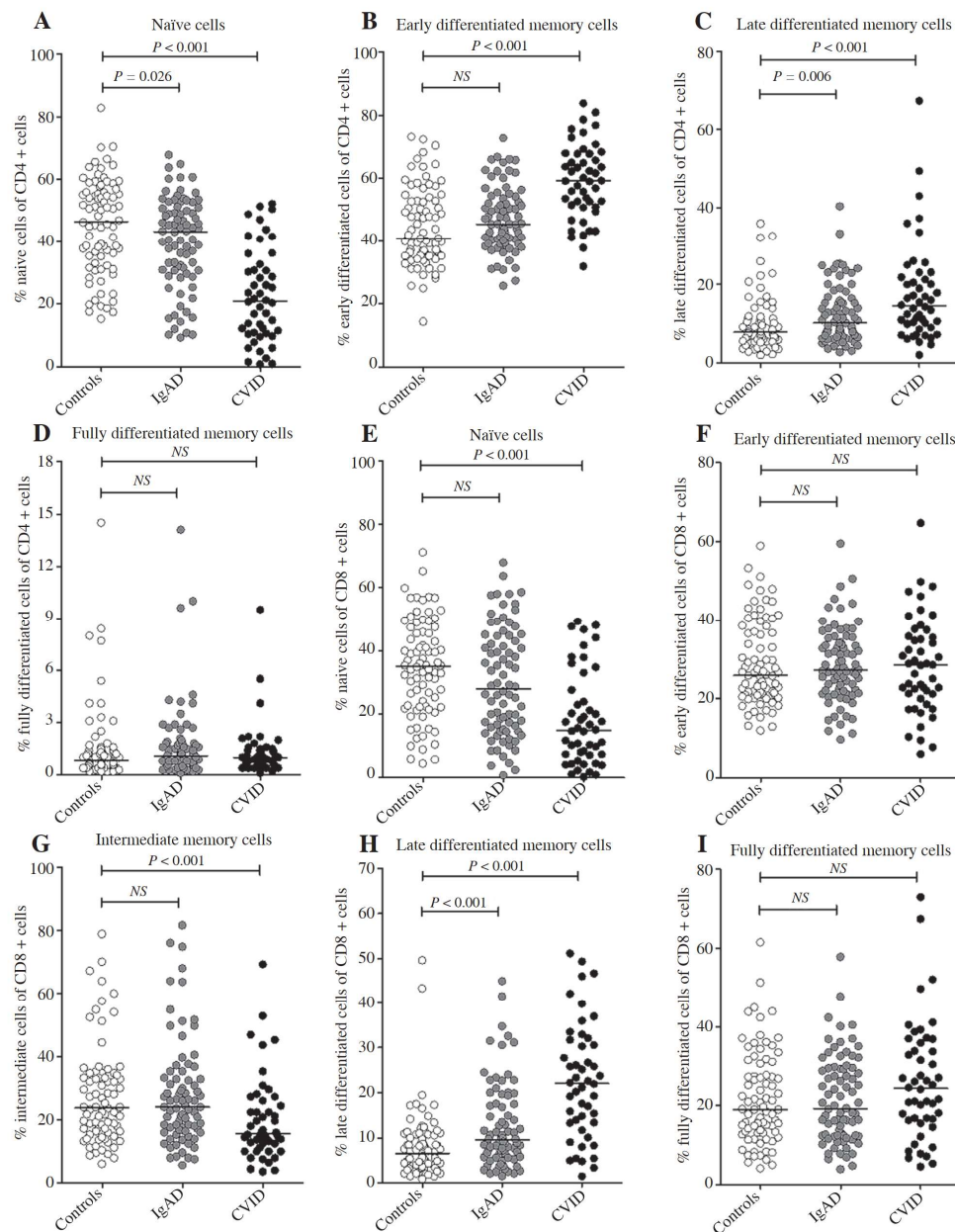


Fig. 2. Distribution of T-lymphocyte differentiation subsets in control persons and patients with IgAD and CVID. Naïve (CD45RO⁺CD27⁺) CD4⁺ and CD8⁺ cells (A, E), early differentiated memory (CD45RO⁺CD27⁺) CD4⁺ and CD8⁺ cells (B, F), intermediate memory (CD45RO⁺CD27^{dim}) CD8⁺ cells (G), late differentiated memory (CD45RO⁺CD27⁻) CD4⁺ and CD8⁺ cells (C, H), fully differentiated memory (CD45RO⁺CD27⁻) CD4⁺ and CD8⁺ cells (D, I). Horizontal lines indicate group medians; intergroup differences were analysed using Mann-Whitney test

we compared all CD4⁺ and CD8⁺ subsets in seronegative IgAD patients to the seronegative control group, we found only a very slight decrease of CD8⁺ fully differentiated memory cells ($p = 0.042$) in IgAD patients; no significant changes in other subsets were shown. When we compared CMV-seropositive to CMV-seronegative IgAD patients, we found a decrease of both CD4⁺ and CD8⁺ naïve cells (in both cases $p < 0.001$), a decrease in CD8⁺ early differentiated memory cells ($p = 0.030$), an increase in CD4⁺ and CD8⁺ late differentiated (in both cases $P < 0.001$) and fully differentiated ($p = 0.038$; $p < 0.001$, respectively) memory cells. Similar findings were obtained when comparing seropositive to seronegative controls: a decrease of both CD4⁺ and CD8⁺ naïve cells ($p = 0.041$; $p = 0.013$, respectively), and an increase in CD4⁺ and CD8⁺ late differentiated ($p < 0.001$; $p < 0.001$, respectively) and fully differentiated ($p = 0.002$; $p = 0.045$, respectively) memory cells in seropositive controls (Table 1).

Age dependence of T-cell differentiation stages

Correlating age and T-cell differentiation stages in control persons, we observed a decrease of CD4⁺ and CD8⁺ naïve cells with age ($r = -0.326$, $p = 0.003$, $r = -0.445$, $p < 0.001$, respectively), while CD4⁺ early differentiated ($r = 0.271$, $p = 0.015$) and CD8⁺ late differentiated ($r =$

0.335 , $p = 0.002$) memory cells increased. These changes were more pronounced in IgAD patients with a decrease of both CD4⁺ and CD8⁺ naïve ($r = -0.598$, $p < 0.001$; $r = -0.632$, $p < 0.001$, respectively) and an increase of late differentiated memory cells ($r = 0.4653$, $p < 0.001$; $r = 0.405$, $p < 0.001$, respectively) CD4⁺ and CD8⁺ cells, CD4⁺ early differentiated ($r = 0.537$, $p < 0.001$) and CD8⁺ fully differentiated ($r = 0.472$, $p < 0.001$) memory cells. In CVID patients a significant age-dependent correlation was observed in CD4⁺ naïve and late differentiated cells ($r = -0.468$, $p < 0.001$; $r = 0.401$, $p = 0.005$, respectively) and CD8⁺ naïve cells ($r = -0.461$, $p = 0.001$).

T_{reg} cells are decreased in CVID but not IgAD patients

The frequency of T_{reg} cells is shown in Fig. 3. We documented a decrease of T_{reg} cells in patients with CVID compared to controls ($p < 0.001$). Interestingly, we observed an increase of T_{reg} cells in IgAD patients compared to control persons ($p = 0.039$). We did not observe any significant changes in the frequency of T_{reg} cells when we compared 28 IgAD patients with the presence of autoantibodies to 52 patients without the presence of autoantibodies ($p = 0.912$) or to 31 healthy donors with autoantibodies

Table 1. Frequency of T-cell subsets in CMV⁺ and CMV⁻ IgAD patients and healthy donors

	Controls CMV ⁺ age (median) = 39 <i>n</i> = 27	Controls CMV ⁻ age (median) = 28 <i>n</i> = 52	IgAD CMV ⁺ age (median) = 37.5 <i>n</i> = 40	IgAD CMV ⁻ age (median) = 26.5 <i>n</i> = 38	CMV ⁺ IgAD vs. C <i>p</i>	CMV ⁻ IgAD vs. C <i>p</i>	IgAD CMV ⁺ vs. CMV ⁻ <i>p</i>	C CMV ⁺ vs. CMV ⁻ <i>p</i>
CD4 ⁺ CD45RO ⁻ CD27 ⁺ (naïve)	38.5 (17.4-61.6)	51.2 (21.1-66.4)	32.7 (10.2-53.5)	48.5 (15.7-63.7)	0.086	0.590	< 0.001	0.041
CD4 ⁺ CD45RO ⁺ CD27 ⁺ (ED)	45.4 (31.1-60.5)	40.3 (29.1-68.2)	47.4 (31.0-65.8)	42.8 (31.0-66.0)	0.133	0.452	0.132	0.967
CD4 ⁺ CD45RO ⁺ CD27 ⁻ (LD)	11.0 (4.8-32.5)	7.0 (2.8-14.0)	15.3 (7.1-29.2)	7.0 (3.0-18.2)	0.143	0.407	< 0.001	< 0.001
CD4 ⁺ CD45RO ⁻ CD27 ⁻ (FD)	1.2 (0.4-8.0)	0.7 (0.2-4.1)	1.3 (0.4-9.8)	1.0 (0.2-2.7)	0.740	0.155	0.038	0.002
CD8 ⁺ CD45RO ⁻ CD27 ⁺ (naïve)	26.5 (5.6-50.8)	37.1 (15.0-57.0)	17.4 (3.1-47.7)	40.1 (8.5-63.6)	0.065	0.551	< 0.001	0.013
CD8 ⁺ CD45RO ⁻ CD27 ^{dim} (INT)	6.8 (2.3-13.5)	5.6 (2.4-16.0)	5.2 (2.0-15.3)	6.6 (2.1-16.0)	0.292	0.354	0.163	0.563
CD8 ⁺ CD45RO ⁺ CD27 ⁺ (ED)	25.5 (13.1-47.6)	26.8 (15.7-51.0)	26.7 (12.3-43.1)	32.4 (18.8-50.5)	0.919	0.325	0.030	0.469
CD8 ⁺ CD45RO ⁺ CD27 ⁻ (LD)	10.7 (4.8-43.3)	5.2 (1.5-11.6)	17.2 (6.9-38.2)	6.8 (2.0-14.9)	0.005	0.156	< 0.001	< 0.001
CD8 ⁺ CD45RO ⁻ CD27 ⁻ (FD)	26.5 (7.3-43.7)	17.0 (6.2-43.8)	27.1 (10.9-45.0)	12.8 (4.7-32.6)	0.650	0.042	< 0.001	0.045

CMV⁺ seropositive (CMV⁺), CMV⁻ seronegative (CMV⁻). N naïve cells (CD45RO⁻CD27⁺), ED early differentiated (CD45RO⁺CD27⁺), LD late differentiated (CD45RO⁺CD27⁻), FD fully differentiated effector (CD45RO⁺CD27⁻), INT intermediate (CD45RO⁺CD27^{dim}) memory T cells. Healthy donors (C). The results are expressed as median (5th to 95th percentiles). The *p* value was obtained by the Mann-Whitney U test

($p = 0.509$) or the whole control group (with and without presence of autoantibodies) ($p = 0.152$).

Discussion

Due to the previously described activation of the T-lymphocytes immune system in patients with CVID we determined the degree of T-cell activation by the presence of differentiation stages of T-lymphocyte memory cells. The same T-cell differentiation stages were also determined in IgAD patients.

In IgAD patients we found a higher frequency of late differentiated memory cells, in both CD4⁺ and CD8⁺ cells. In patients with CVID the changes were more extensively expressed. Comparing CVID patients to controls, besides the increase of both CD4⁺ and CD8⁺ late differentiated cells, an increase of early differentiated CD4⁺ cells and a decrease of both CD4⁺ and CD8⁺ naïve cells were observed. Loss of both CD4⁺ and CD8⁺ naïve cells (defined as CD45RA⁺CCR7⁺ or CD45RA⁺CD62L⁺) had also been described by Bateman *et al.* [29] and Giovannetti *et al.* [30], who proposed that the level of CD4⁺ naïve T cells might be considered as a parameter essential for classifying CVID due to its strong correlation with the severity of the disease and association with the “Freiburg” classification [31] based on B-cell differentiation markers.

This study showed in both IgAD and CVID patients an increase of terminally differentiated CD8⁺ cells, which are also supposed as a marker of virus infection-associated activation and memory maturation [22, 32, 33]. The link between CD8⁺ memory differentiation and immune activation was previously described [34]. This points to similarities between CVID and IgAD as observed in B-cell subpopulations [10]. This result is in contrast to the study by Bateman *et al.* [29], who did not find significant differences in any differentiated subset of T cells in the selective IgA-deficient group.

Several studies published an increase of terminally differentiated T cells in patients with HIV, leukaemia, or CVID, who suffered from CMV infection [22, 28, 33]. As our study showed an increase in the late differentiated memory cells in IgAD patients, we aimed to answer the question of whether the presence of CMV infection in IgAD patients was responsible for the above-mentioned observation. Therefore, we compared both CMV-seronegative and CMV-seropositive IgAD patients to CMV-seronegative and CMV-seropositive control persons, and found only an increase of CD8⁺ late differentiated memory cells in CMV-seropositive IgAD patients. With the exception of a slight decrease of fully differentiated memory cells in IgAD patients, all other comparisons of the differentiated subsets between seropositive or seronegative groups were not significant. We suggest that the differences between the IgAD and the control groups can be, at least partially, associated with the increased CMV seropositivity in IgAD patients.

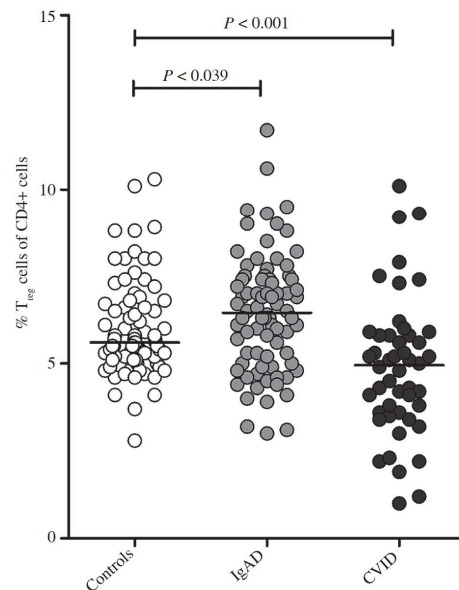


Fig. 3. Frequency of Treg cells in control persons and patients with IgAD and CVID. Horizontal lines indicate group medians; intergroup differences were analysed using Mann-Whitney test

The observation of the increase of CMV positivity in our IgAD patients was unexpected and, to our knowledge, no similar study was published. Although the groups of IgAD patients and control persons examined in our study were very similar as regards their age and sex, we cannot exclude a bias due to different socioeconomic status of the patients and control persons [35, 36] because our healthy donors were obtained mainly from the hospital staff, students of medicine, and relatives of the staff of our department, while the IgAD patients can be considered as a general, highly unselected, population. This finding deserves further confirmation.

T_{reg} cells play a critical role in suppressing the response of the immune system to self-antigens [37]. Previous studies showed decreased numbers of T_{reg} in CVID [14, 16], mainly in patients with autoimmune diseases [38]. We confirmed the decrease of T_{reg} cells in patients with CVID described previously. In contrast to CVID, surprisingly, there were increased numbers of T_{reg} in IgAD patients compared to control persons. Our observation is in agreement with the similar tendency recently published for IgAD patients by Bateman *et al.* [29] but is contrary to Soheili *et al.* [39], who described a decrease of T_{reg} cells in 26 paediatric pa-

tients of Iranian origin. Based on the previously observed expansion of T_{reg} cells in patients with chronic infections [40, 41], we suggest that the increase of T_{reg} may be associated with chronic immune activation in IgAD patients. You *et al.* in a previous study [42] described a decrease in the frequency of T_{reg} cells in CVID patients with autoimmunity compared both to patients without autoimmunity and healthy donors. Using this approach, we did not observe any similar findings in IgAD patients.

Our observation shows that terminally differentiated memory T cells are increased not only in CVID but also in IgAD patients. The increase in IgAD patients may be related to a different frequency of CMV infection in these persons. The observation of a higher frequency of CMV infection in IgAD patients deserves further study.

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The authors declare no conflict of interest.

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8 Funkční změny T- a B-lymfocytárních populací u pacientů s CVID

U pacientů s CVID byly zaznamenány nejen změny v počtech T a B buněk, ale také významná dysfunkce těchto buněk. Zvýšené hladiny aktivačních markerů na CD4⁺ T-lymfocytech se projevují ve zvýšené produkci IFN- γ .^{61,62} U některých pacientů bylo navíc zjištěno zvýšení hladin TNF- α , přičemž oba tyto cytokiny jsou typické pro Th1 odpověď.⁶³ V roce 2010 studie výzkumné skupiny profesorky Mauri et al.⁶⁴ prokázala, že subpopulace CD19⁺CD24⁺⁺CD38⁺⁺ obsahuje nejvyšší podíl B-lymfocytů, které po stimulaci přes komplex CD40-CD40L produkují IL-10. Tyto buňky zároveň potlačují produkci Th1-prozánětlivých cytokinů TNF- α a IFN- γ efektorovými CD4⁺ T-lymfocyty a byly označeny jako regulační B-lymfocyty (Breg). Vzhledem k popsáným odchylkám v diferenciaci a funkci B-lymfocytů a zvýšené Th1 odpovědi u pacientů s CVID proto vyvstala otázka, zda se tato porucha neprojevuje také na úrovni Breg buněk. Tomu se věnovala naše studie, publikovaná v roce 2015 pod názvem: Regulatory B cells in CVID patients fail to suppress multifunctional IFN- γ ⁺TNF- α ⁺ CD4⁺ T cells differentiation.

Naše výsledky ukázaly, že subpopulace B-reg (CD24^{hi} CD38^{hi}), charakterizovaná produkcí IL-10, je u pacientů s CVID významně snížena ve schopnosti produkovat IL-10, a to bez souvislosti s klinickými projevy či zařazením do EUROclass klasifikace.⁴ Metoda stanovení funkce Bregulačních lymfocytů je založena na stimulaci izolovaných PBMC anti-CD3 buněk s následnou aktivací PMA/ionomycinem. Při ní dochází k aktivaci T- a následně i B- lymfocytů. B-lymfocyty pacientů s CVID vytvářely standardní počet aktivovaných B buněk CD24^{hi} CD38^{hi}, ale jejich schopnost tvořit IL-10 byla ve srovnání s kontrolní skupinou snížena. To vedlo k omezené schopnosti potlačit produkci IFN- γ a TNF- α ze strany CD4⁺ T-lymfocytů pacientů s CVID. Souvislost mezi nízkou IL-10 produkcí a vyšší produkcí prozánětlivých cytokinů byla u pacientů potvrzena negativní korelací mezi procentem CD24^{hi} CD38^{hi} B-lymfocytů a IFN- γ ⁺TNF- α ⁺CD4⁺ T-buňkami. To naznačuje poruchu už ve fázi vývoje B-regulačních lymfocytů, což může přispívat k jejich snížené schopnosti diferenciaci do plazmatických buněk a tím také do tvorby imunoglobulinů, ale také jako regulační buňky tlumit diferenciaci prozánětlivých efektorových CD4⁺ T lymfocytů.

Regulatory B cells in CVID patients fail to suppress multifunctional IFN- γ ⁺TNF- α ⁺CD4⁺ T cells differentiation. **Vlkova M**, Ticha O, Nechvatalova J, Kalina T, Litzman J, Mauri C, Blair PA. Clin Immunol. 2015 Oct;160(2):292-300.doi: 10.1016/j.clim.2015.06.013.



Regulatory B cells in CVID patients fail to suppress multifunctional IFN- γ ⁺TNF- α ⁺CD4⁺ T cells differentiation



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ABSTRACT

Common variable immunodeficiency (CVID) refers to primary hypogammaglobulinemia with unknown pathogenesis. Although there is evidence for intrinsic B cell defects in some CVID patient groups, various abnormalities in cytokine production by T cells in CVID patients are frequently observed. Here, we demonstrate a relationship in the production of pro-inflammatory Th1 cytokines and regulatory B cells producing IL-10 between CVID patients and healthy controls. We describe CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ regulatory B cells generated after T cell stimulation of human peripheral blood lymphocytes *ex vivo* are able to suppress IFN- γ ⁺TNF- α ⁺ producing CD4⁺ T cells. This process is impaired in CVID patients, who present with both low numbers of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells and increased numbers of IFN- γ ⁺TNF- α ⁺CD4⁺ T cells. Disruption of the regulatory B cell response to T cell stimulation explains the excessive T cell activation regarded as an immunoregulatory abnormality that is a frequent finding in CVID patients.

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1. Introduction

Common variable immunodeficiency (CVID) encompasses a clinically heterogeneous group of primary antibody immunodeficiency diseases [1]. It is characterized by decreased serum levels of IgG, IgA and/or IgM and impaired antibody responses to antigen stimulation and/or vaccination [2]. Clinically it is accompanied by recurrent or chronic bacterial infections, inflammatory and autoimmune disorders and increased risk of malignancy [1].

Although various abnormalities in the number and function of lymphocytes and dendritic cells [3,4] have been described, the immunopathological mechanism leading to disturbed antibody production in CVID patients has not yet been elucidated fully. Several studies have shown alterations and defects in T cells: reduced proportion of naive CD4⁺CD45RA⁺ T cells, increased number of CD4⁺CD45RO⁺ memory T cells and increased number of CD4⁺HLA-DR⁺ activated T cells, defects in TCR repertoire, and changes in regulatory T cells [5–10]. The change in CD4⁺ T lymphocyte markers indicates a shift to activated and memory phenotypes. The increase in activation of CD4⁺ T cells is also reflected by the increase in production of interferon (IFN)- γ by CD4⁺ T cells in CVID patients upon CD3 or PMA/PMH stimulation after

24 h and upon phorbol myristate acetate (PMA)/Ionomycin stimulation after 12 h [11,12]. Increased levels of (tumor necrosis factor) TNF- α and also IFN- γ after stimulation of T-cells were reported in several studies in CVID patients [13]. IFN- γ and TNF- α are both considered crucial Th1 cytokines [10]. By contrast, B cells in CVID patients have increased number of naive follicular B cells but low numbers of memory B cells and plasmablasts [14]. A subset of CVID patients presents with markedly increased transitional B cells [15]. A significant portion of the B lymphocytes in CVID patients are anergic cells [16]. The alterations found in the B-cell compartment are unexpectedly stably detected over 4–11 months in CVID patients [15].

Apart from antibody production and antigen presentation, B lymphocytes also secrete cytokines. Similarly to T regulatory cells, a distinct subpopulation of B lymphocytes, now called regulatory B cells (B_{reg}), can produce anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)- β and can express inhibitory molecules that suppress T cells and autoreactive B cells in a cell-to-cell contact-dependent manner [17]. The first observations of B cell regulatory function in experimental autoimmune encephalomyelitis in mice were carried out in 1996 by Janeway and colleagues [18]. The term “regulatory B cells” was first “popularized” by Mizoguchi and Bhan, or Ono and Hamaoka in 2000 for a mouse B cell subset that secretes IL-10 and suppresses progression of inflammation [19,20]. In recent years, regulatory B cells have been intensively studied in various models of inflammation, cancer, transplantation and in human autoimmune diseases [21–24]. Various populations of B cells producing IL-10 have been

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detected among transitional 2-marginal zone precursor cells (T2-MZP), follicular B cells (FO), marginal zone B cells (MZ) and plasma cells [24]. In 2010, Mauri and colleagues demonstrated that the CD19⁺CD24^{hi}CD38^{hi} subset of human B cells comprises the highest fraction of IL-10-producing B cells upon CD40 stimulation in human peripheral blood from healthy individuals [17]. CD19⁺CD24^{hi}CD38^{hi} B cells have a phenotype that was previously assigned to immature transitional B cells [25]. These B cells were shown to suppress the production of pro-inflammatory cytokines (TNF- α and IFN- γ in CD4⁺ T cells) [17].

Although an increase in Th1 cytokine production in CVID patients was described [10], regulatory B cells in CVID patients have not yet been studied. Here we focused on a detailed analysis of the interaction between regulatory B cells and CD4⁺ T cells in peripheral blood in CVID patients and compared it to healthy donors.

2. Material and methods

2.1. Patients and controls

PBMCs from 26 CVID patients were examined. All patients were Caucasians of Czech origin and fulfilled the PAGID (Pan-American Group for Immunodeficiency)/ESID (European Society for Immunodeficiencies) diagnostic criteria for CVID [2]. Basic characteristics of the patients are given on Table 1. In one patient the p.C104R mutation of the *TNFRSF13B* gene (coding for TACI) was observed; no mutations in the genes *ICOS* (performed in 14 patients) or *TNFRSF13C* (performed in 2 patients) were recorded.

The patients were on regular intravenous (IVIG, given once in 3–4 weeks) or subcutaneous (given 1–2 times per week) immunoglobulin replacement treatment (see Table 1).

In case of patients on IVIG, blood samples were collected before the IVIG infusion was given. Healthy donors were studied in parallel with the patients.

All samples were collected during apparent acute infection-free period defined as worsening cough, rhinitis, or presence of new symptoms suspicious of respiratory, gastrointestinal or urinary tract infections or significant increase in CRP above the levels typically observed in the given patient. One patient was treated by methylprednisolone 4 mg/day, and leflunomide 20 mg/day because of arthritis and demyelinating disease. No patient was under cytostatic treatment at the time of the study.

2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood by density gradient (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) and washed twice with phosphate-buffered saline. Isolated PBMCs were re-suspended in fetal calf serum with 5% dimethyl sulfoxide at the concentration of 1×10^7 /ml before cryopreservation in liquid nitrogen.

2.3. Cell culture

2.3.1. Stimulation with plate-bound CD3 mAb

Thawed PBMCs were stimulated with 0.5 μ g/ml purified plate-bound CD3 monoclonal antibody – mAb (Hit-3a) (BioLegend, San Diego, USA) for 72 h. This stimulation led to activation and expression of CD154 on CD4⁺ T-cells. The interaction between CD40 on B cells and CD154 on CD4⁺ T cells resulted in the production of IL-10 in B regulatory cells [17]. For costimulation and detection of

Table 1
Clinical and laboratory characteristics of evaluated CVID patients.

Patient No.	Sex	Age (years)	EUROclass classification	Absolute B cells count (10^6 /ml)	%CD19 ⁺ CD38 ^{hi} /CD19 ⁺ ex vivo	%CD19 ⁺ CD38 ^{hi} /CD19 ⁺ after stimulation	%IL-10 ⁺ /CD19 ⁺ CD38 ^{hi} after stimulation	IFN- γ /TNF- α /CD4 ⁺	Immunoglobulin dose (mg/kg/4 weeks)	Route of administration	IgG at diagnosis (g/l)	IgA at diagnosis (g/l)	IgM at diagnosis (g/l)	Splenomegaly*	Autoimmunity	Bronchiectasis	Malignancy
1	M	61	smB-/21low	0.128	4.9	31.7	5.5	23.2	394	iv	0.42	<0.02	0.33	Yes	No	Yes	No
2	M	55	smB-/21low	0.012	12.8	27.1	10.6	21.5	480	iv	1.15	0.08	0.21	Yes	No	Yes	Hodgkin lymphoma**
3	F	80	smB-/21low	0.595	0.3	39.0	8.0	34.8	373	iv	0.59	<0.05	0.07	No	Pernicious anemia	Yes	No
4	F	65	smB-/21low	0.182	13.0	9.8	8.9	12.6	232	iv	0.75	<0.08	0.13	Yes	Thrombocytopenia	Yes	No
5	M	47	smB-/21low	0.039	5.3	30.3	19.4	36.2	385	iv	1.39	<0.01	<0.05	Yes	No	No	No
6	F	50	smB-/21low	0.057	8.4	38.7	12.6	13.3	458	iv	3.36	<0.01	0.32	Yes	No	No	No
7	F	56	smB-/21low	0.143	6.8	21.8	10.4	6.1	600	iv	1.84	<0.08	0.27	Yes	Thrombocytopenia	Yes	No
8	F	33	smB-/21low	0.141	6.9	62.0	15.2	8.5	471	iv	0.08	0.04	<0.05	Yes	No	No	No
9	F	68	smB-/21low	0.054	2.6	32.9	27.8	6.9	381	iv	4.34	0.09	0.08	No	SLE, vitiligo, DM-I	Yes	Basalioma**
10	F	49	smB-/21low	0.14	6.3	39.0	24.6	11.0	472	iv	1.13	<0.08	<0.08	No	No	Yes	No
11	F	50	smB-/21norm	0.202	3.8	21.7	4.2	20.4	424	sc	1.73	<0.08	0.08	Yes	No	No	No
12	M	65	smB-/21norm	0.175	6.4	48.3	13.5	15.9	220	iv	2.33	0.11	0.09	Yes	No	No	No
13	M	27	smB-/21norm	0.253	8.8	50.3	12.6	23.7	323	iv	1.74	<0.01	0.22	Yes	No	No	No
14	F	67	smB-/21norm	0.117	7.5	34.3	15.3	38.8	301	iv	1.99	0.14	<0.04	No	No	No	No
15	M	36	smB-/21norm	0.198	23.6	30.5	27.8	7.9	360	iv	3.19	<0.08	0.38	Yes	No	Yes	No
16	F	36	smB-/21norm	0.372	6.2	51.6	16.7	18.9	360	sc	4.01	<0.01	<0.08	No	No	No	No
17	F	45	smB-/21norm	0.271	7.4	44.7	21.3	14.3	444	iv	1.68	<0.08	0.23	Yes	No	No	No
18	F	26	smB-/21norm	0.039	2.6	30.7	17.4	6.4	481	iv	0.66	<0.01	0.13	No	Hypothyreosis	No	No
19	M	40	smB-/21norm	0.066	16.6	38.3	25.0	7.5	280	iv	2.35	<0.01	0.06	No	No	No	No
20	M	40	smB-/21norm	0.129	3.0	30.7	31.1	11.8	350	sc	1.51	<0.01	0.09	Yes	No	No	No
21	M	51	smB-/21low	0.033	3.5	21.1	5.1	23.8	357	iv	1.72	0.34	0.11	No	Pernicious anemia	No	No
22	M	61	smB-/21low	0.043	4.8	25.3	5.6	33.7	420	iv	0.85	<0.08	<0.08	Yes	No	No	No
23	M	33	smB-/21low	0.198	3.0	7.2	5.4	27.9	409	iv	2.51	0.39	<0.05	No	No	No	No
24	F	75	smB-/21low	0.049	5.1	71.2	6.8	26.5	329	iv	2.17	0.20	0.01	Yes	Pernicious anemia	No	Gastric cancer**
25	F	61	smB-/21low	0.11	3.4	42.3	15.7	22.2	270	iv	4.48	<0.01	0.06	No	No	No	No
26	F	30	smB-/21low	0.141	8.1	13.9	18.7	8.7	320	sc	2.05	0.08	0.23	No	Arthritis, demyelination, hypothyreosis	No	No

*Defined as spleen length <11 cm on sonography.

**In remission in the time of the study.

SLE: systemic lupus erythematosus, DM-I: Diabetes mellitus type 1.

cytokines, 50 ng/ml of phorbol myristate acetate (PMA) (Sigma, Missouri, USA), 0.25 µg/ml of Ionomycin (Calcium Ionophore) (Sigma) and 10 µg/ml of Brefeldin A (Sigma), were added for the last 4 h.

2.3.2. Blockade of IL-10 production

For inhibitory testing, thawed PBMCs were stimulated in the presence of 0.5 mg/ml plate-bound CD3 mAb and in the presence of anti-IL-10 mAb and anti-IL-10 receptor mAb (BioLegend, San Diego, USA) were used in the same concentration of 10 µg/ml. PMA + Ionomycin were added for the last 4 h of culture.

2.4. Assessment of B cell and T cell subsets

For determination of T cells, monoclonal antibodies were used in the following combinations: CD4 PerCP – Cy5.5, (BD Biosciences, San Jose, CA, USA), CD154 PE, CD3 PC-7, CD8 APC Alexa Fluor 750, CD45-Krome Orange (Beckman Coulter, Marseille, France). For B-cell staining, monoclonal antibody combinations of CD19-PE-Cy7, CD24-APC A750 (Beckman Coulter), CD38-FITC (BD Biosciences) were applied. The PBMCs were incubated for 30 min in the dark. Samples were acquired using a Navios flow cytometer (10 colors, 3 Laser) (Beckman Coulter, Inc., Miami, FL, USA) and cytometry data (LMD files) were analyzed with Kaluza software (Beckman Coulter). For detection of live and dead cells, aqua fluorescent reactive dye (LIVE/DEAD Fixable dead Cell stain Kit, Invitrogen, California, USA) was used.

2.5. Cytokine detection

Cells were washed, fixed, permeabilized by Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, California, USA)

and stained with IFN-γ BV421 and TNF-α APC (BioLegend, San Diego, USA) and IL-10-PE mAbs (BD Biosciences) for the detection of intracellular cytokines.

2.6. Calculation of percent inhibition (PI) of IFN-γ⁺TNF-α⁺ in CD4⁺ T cells

To calculate inhibitory effects we used a formula that expresses the percentage growth of IFN-γ⁺TNF-α⁺ in CD4⁺ T cells after inhibition against IFN-γ⁺TNF-α⁺ in CD4⁺ T cells after stimulation:

$$PI = 100 - (100 \times \text{frequency of IFN-}\gamma^+ \text{ TNF-}\alpha^+ \text{ in CD4}^+ \text{ T cells after stimulation} / \text{frequency of IFN-}\gamma^+ \text{ TNF-}\alpha^+ \text{ in CD4}^+ \text{ T cells after inhibition}).$$

2.7. Statistical analysis

Data were analyzed using Mann–Whitney rank sum, Spearman rank order correlation tests and Wilcoxon signed-rank tests. The statistical software GraphPad Prism 5 was used (GraphPad Software Inc. California, USA). A standard level of statistical significance, $\alpha = 0.05$, was used.

2.8. Ethics

The study was approved by the Ethics committee of St Anne's University Hospital in Brno under protocol No. 11G/2009.

Patients' and control persons' blood was collected after informed consent was signed.

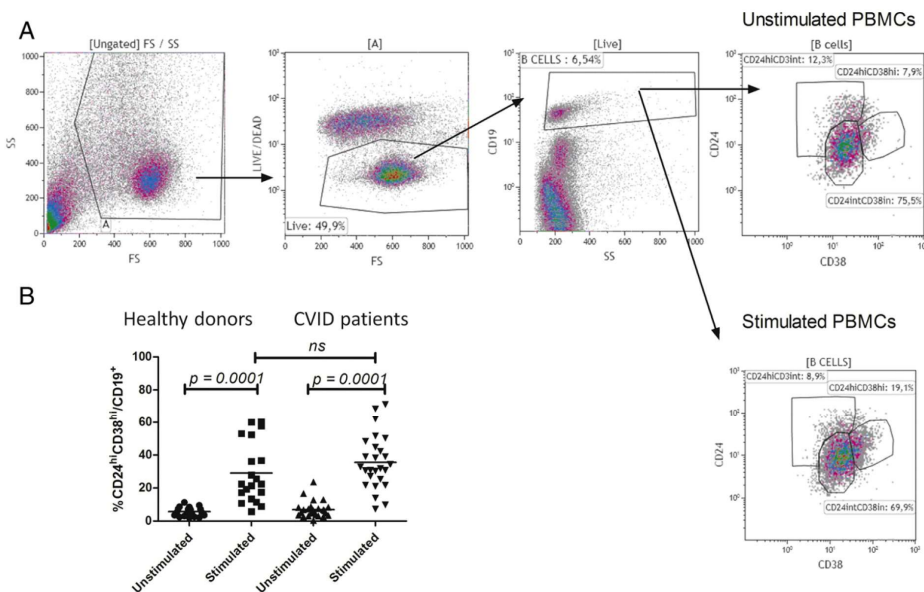


Fig. 1. A: Gating strategy for B cell subsets. Gating of B cells is shown for a representative healthy donor. Lymphocytes were gated on forward and side light scatter, live cells and then on CD19 positivity, after which the remaining cells were divided into CD19⁺CD24^{hi}CD38^{int} B cells (transitional B-cell phenotype), CD19⁺CD24^{int}CD38^{int} (follicular B cell phenotype) and CD19⁺CD24^{hi}CD38^{hi} (memory B cell phenotype). Representative dot plots show increased frequency of CD19⁺CD24^{hi}CD38^{hi} B cells after anti-CD3mAb stimulation. B: Proportion of newly formed activated CD19⁺CD24^{hi}CD38^{hi} B cells in CVID patients and healthy donors. The difference between CVID patients and control subjects was assessed using Mann–Whitney rank sum test. Wilcoxon signed-rank tests were used for comparison of the frequency of CD19⁺CD24^{hi}CD38^{hi} B cells before and after stimulation both for healthy donors and CVID patients.

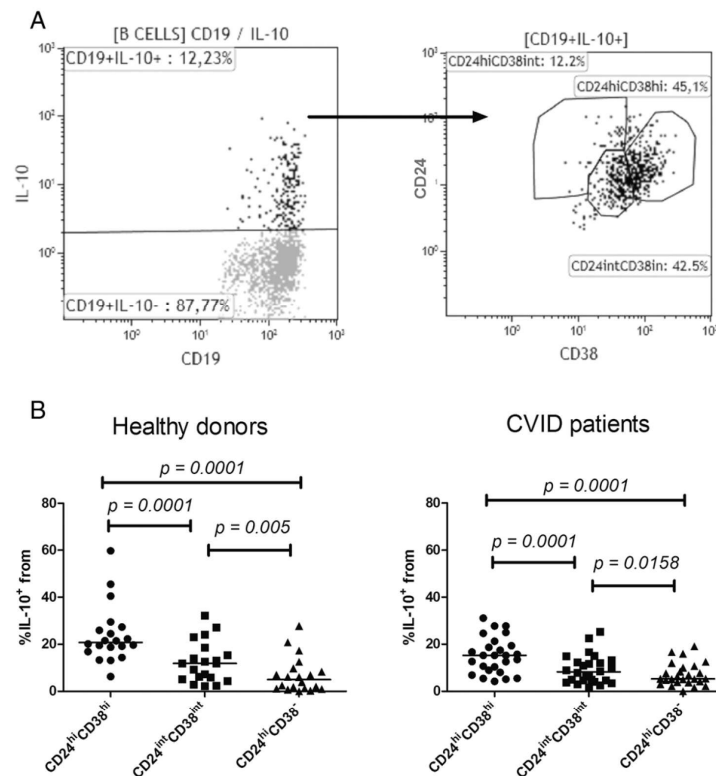


Fig. 2. IL-10⁺ producing B-cells were present in the CD19⁺CD24^{hi}CD38^{hi} compartment. A: Representative dot plots show that the majority of IL-10⁺ producing B-cells were in the CD19⁺CD24^{hi}CD38^{hi} compartment. B: Proportion of IL-10⁺ cells among CD19⁺CD24^{hi}CD38^{hi}, CD19⁺CD24^{hi}CD38^{int} and CD19⁺CD24^{int}CD38^{hi} B cell phenotypes in CVID patients and healthy donors. Wilcoxon signed-rank test was used for comparison of the frequency of IL-10 cells in defined B cell subpopulations for both healthy donors and CVID patients.

3. Results

3.1. IL-10⁺ B regulatory cells are less responsive to T cell stimulation in CVID patients

Regulatory B cells are largely defined as IL-10 producing B cells [17] with a phenotype resembling transitional CD19⁺CD24^{hi}CD38^{hi} B cells; therefore, the percentage of CD19⁺CD24^{hi}CD38^{hi} B cells (transitional B-cell phenotype), CD19⁺CD24^{int}CD38^{int} (follicular B cell phenotype) and CD19⁺CD24^{hi}CD38^{int} (memory B cell phenotype) [25] (Fig. 1A) was measured in PBMCs with stimulation (in the presence of 0.5 mg/ml plate-bound CD3 mAb, PMA + Ionomycin were added for the last 4 h of culture) or without stimulation (PBMCs were cultured in medium only). After 72 h of stimulation, we observed a significant rise in the frequency of CD19⁺CD24^{hi}CD38^{hi} B cells in both the CVID patients and the healthy donors (Fig. 1B). We did not find a difference between the CVID patients and healthy controls in the frequency of activated CD19⁺CD24^{hi}CD38^{hi} B cells after stimulation. In both the healthy donors and the CVID patients, the initial number of CD19⁺CD24^{hi}CD38^{hi} B cells (transitional B cell phenotype) failed to correlate with newly formed activated CD19⁺CD24^{hi}CD38^{hi} B cell numbers, nor did the initial number of transitional phenotype B cells correlate with the frequency of IL-10 in CD19⁺CD24^{hi}CD38^{hi} B cells (Supl. Fig. 1).

To determine IL-10⁺ B cells, we measured the production of IL-10 in B cells after stimulation in CVID patients and healthy donors. In both healthy subjects and CVID patients, we confirmed that the majority of IL-10⁺ producing B cells were in the CD19⁺CD24^{hi}CD38^{hi} compartment (Fig. 2). Even though the frequency of activated CD19⁺CD24^{hi}CD38^{hi} B cells was not different in CVID patients compared with healthy donors, we repeatedly found a significantly lower frequency of IL-10⁺ cells among total B cells in CVID patients (Fig. 3). Next, we looked for IL-10 producing cells in the three major B cell compartments (activated CD19⁺CD24^{hi}CD38^{hi} like – transitional B cells, follicular and memory), and we found a significantly lower frequency of IL-10⁺ cells in CVID patients (as compared to healthy controls) only in the activated CD19⁺CD24^{hi}CD38^{hi} like – transitional B cell compartment (Fig. 3).

Production of IL-10 by PBMCs other than B cells was negligible (Supl. Fig. 2).

3.2. Expression of CD154⁺ is higher in CD4⁺ T cells in CVID patients after T cell stimulation

CD40–CD154 interaction is crucial in T cell/B cell interactions. It is essential for B cell maturation and for the formation of regulatory B cells [17,26]. We evaluated the expression of CD154⁺ on CD4⁺ T cells in CVID patients and healthy controls after stimulation as described in Method Section 2.3.1. We expected that low expression of CD154⁺ in

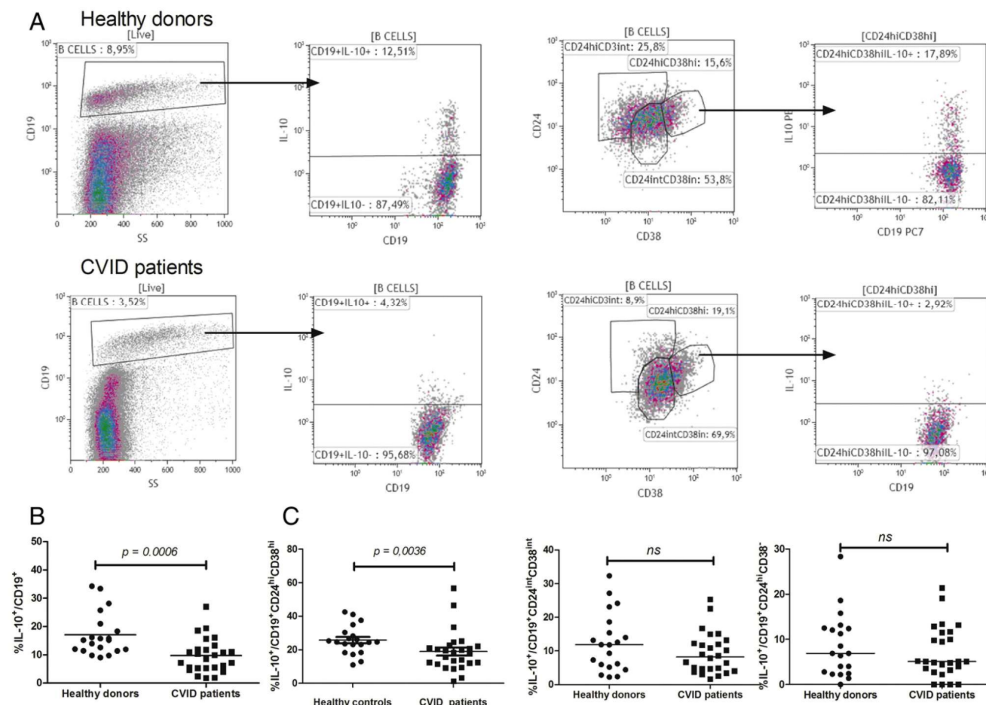


Fig. 3. The production frequency of IL-10⁺ cells from B cells after anti-CD3mAb stimulation. A: Representative dot plots show decreased production of IL-10 in B cells and decreased production of IL-10 in CD19⁺CD24^{hi}CD38^{hi} B cells in CVID patients compared to healthy donors. B: Proportion of IL-10⁺ cells among B cells. C: Proportion of IL-10⁺ cells among CD19⁺CD24^{hi}CD38^{hi}, CD19⁺CD24^{hi}CD38^{int} and CD19⁺CD24^{hi}CD38^{int} B cells. The differences between CVID patients and control subjects were assessed using the Mann-Whitney rank sum test.

CD4⁺ T cells in CVID patients might explain the observed low frequency of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells in some CVID patients. However, we found the opposite: CD4⁺ T cells in CVID patients responded with increased expression of CD154 after T cell stimulation (Fig. 4). Thus, our data indicated that diminution of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells was not caused by deficient expression of CD154⁺ in CD4⁺ T cells.

3.3. Production of Th1 cytokines in CVID patients increases after T cell stimulation

Regulatory B cells are known to inhibit production of these particular cytokines in CD4⁺ T cells [17]. In the next step, we tested the intrinsic regulatory function of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells by measuring production of the pro-inflammatory cytokines IFN- γ and TNF- α in CD4⁺ T cells after stimulation as described in Section 2.3.1. Detailed analysis of the cytokine-producing CD4⁺ T cell subsets showed that there are three subpopulations defined by TNF- α and IFN- γ (co) expression: CD4⁺ T cells that produce only TNF- α (IFN- γ ⁻ TNF- α ⁺); CD4⁺ T cells that produce only IFN- γ (IFN- γ ⁺ TNF- α ⁻) and CD4⁺ T cells that produce both IFN- γ and TNF- α (IFN- γ ⁺ TNF- α ⁺). We found elevated frequencies of IFN- γ ⁻ TNF- α ⁺ cells and even more IFN- γ ⁺ TNF- α ⁺ cells among CD4⁺ T cells in CVID patients compared with healthy donors after T cell stimulation (Fig. 5A). There was no correlation between the absolute number of B-cells in patients' peripheral blood and the proportion of IFN- γ + TNF- α + in CD4⁺ T cells ($r = -0.051$, $p = 0.802$).

3.4. IL-10⁺ regulatory B cells counterbalance CD4⁺ T cell pro-inflammatory cytokines in CVID patients

CVID is a heterogeneous group of mostly undefined molecular defects with common presentation (lack of memory antibody response). We observed variable numbers of both IL-10⁺ regulatory B cells and CD4⁺ effector cytokine producing T cells among individual patients. Thus, we asked whether there is a relationship between the number of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells and the increased frequency of both IFN- γ ⁻ TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ CD4⁺ effector T cells across the CVID patients' dataset.

We observed an inverse correlation between the frequency of IFN- γ ⁺ TNF- α ⁺ cells in CD4⁺ T cells and the frequency of IL-10⁺ cells in CD19⁺CD24^{hi}CD38^{hi} B cells ($r = -0.6314$; $p = 0.0012$) (Fig. 5B) in CVID patients but not in healthy subjects, suggesting an important regulatory role of the IL-10⁺ B reg cells in CVID patients.

Next, we verified whether the increased number of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells was related to IL-10 production by B reg cells. We cultured patients' PBMCs in the presence of 0.5 mg/ml plate-bound CD3 mAb, anti-IL-10 and IL-10 receptor inhibitory antibodies for 72 h, PMA + Ionomycin were added for the last 4 h of culture. We detected an increased frequency of IFN- γ ⁺ TNF- α ⁺ producing CD4⁺ T cells after IL-10 blockade in CVID patients as well as in healthy donors (Fig. 6).

To confirm that CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B reg cells were linked to IL-10⁺ mediated inhibition of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells, we evaluated the percentage of inhibition (PI) as described in the Methods Section 2.6. We found a correlation between the frequency of IFN- γ ⁺ TNF- α ⁺ in CD4⁺ T cells after IL-10 blockade and the frequency of

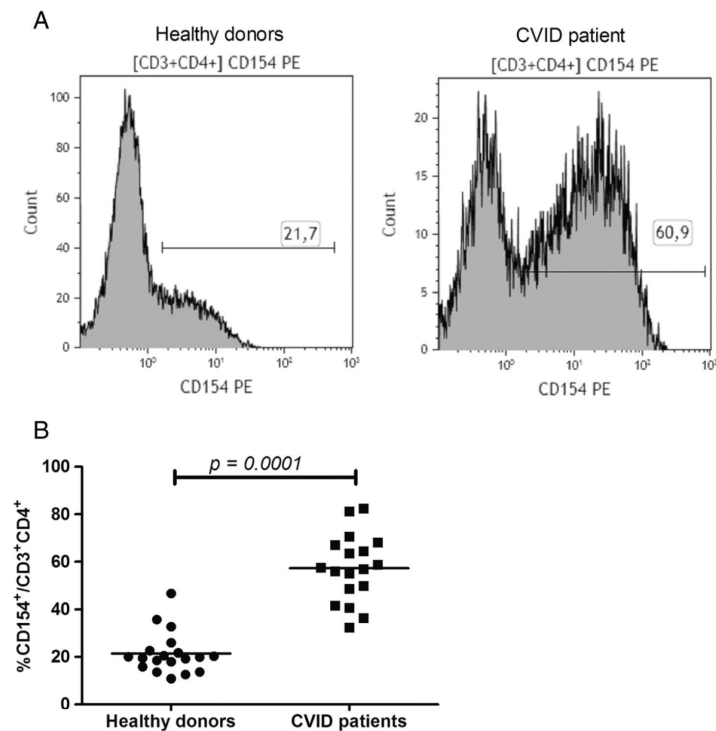


Fig. 4. The expression frequency CD154⁺ on CD4⁺ T cells. A: Representative histograms show increased production of CD154⁺ cells from CD4⁺ T cells in CVID patients compared to healthy donors. B: Proportion of CD154⁺ cells among CD4⁺ T cells. The differences between CVID patients and control subjects were assessed using the Mann–Whitney rank sum test.

CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B reg cells ($r = 0.7727$; $p = 0.0074$) in CVID patients but not in healthy donors (Fig. 6). This correlation showed that those CVID patients who have high or normal numbers of B regulatory cells can partially regulate the production of TNF- α ⁺IFN- γ ⁺ cells in CD4⁺ T cells. In contrast, blockade of IL-10 has only a small effect on the frequency of IFN- γ ⁺TNF- α ⁺ cells in CD4⁺ T cells in the group of patients who had a lower frequency of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B reg cells.

3.5. B regulatory cells are independent of EUROClass classification

We did not show any difference in the proportion of CD19⁺CD24^{hi}CD38^{hi} B-reg cells ($p = 0.092$, Kruskal–Wallis test), CD154⁺ in CD4⁺ T cells ($p = 0.386$) or IFN- γ ⁺TNF- α ⁺ CD4⁺ T cells ($p = 0.110$) comparing patients from the subgroups smB+/CD21low, smB-/CD21low, smB-/CD21norm (no patient fell into the smB+/CD21norm subgroup) according to EUROClass classification.

4. Discussion

Recently published reports show that human regulatory B cells play an important role in the regulation of Th1 cell cytokine production [24, 27–31]. Our aim was to measure the number and function of regulatory B cells producing IL-10 generated after stimulation of peripheral blood B cells from patients with CVID and from healthy donors. We found an increase in the frequency of CD19⁺CD24^{hi}CD38^{hi} peripheral blood B cells in response to T cell stimulation in mixed PBMC cultures, probably via CD40–CD40L, and that this activated B cell subpopulation produces

the highest number of IL-10⁺ cells of all cell populations studied. Surprisingly, the frequency of activated CD19⁺CD24^{hi}CD38^{hi} B cells and also of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells after activation was independent of the original frequency of transitional or naive B cells before the stimulation. This finding can mean that regulatory B cells in patients with CVID as well as healthy donors do not form from the transitional B lymphocytes only, but can be formed after activation via CD40 from other B cells subpopulations. These results are in concordance with the observation of Blair et al. [17] who described similar total numbers of IL-10⁺ B cells in the CD24^{hi}CD38^{hi} and CD24^{int}CD38^{int} B cell subsets.

Because CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells develop after T cell stimulation, we investigated CD154 (CD40L) expression by T cells after stimulation in patients with CVID [5]. Contrary to our expectation, we found an increased frequency of CD4⁺CD154⁺ T cells in patients with CVID, so the lack of CD40L cannot be the reason for the decrease in CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells. However, we cannot rule out that in these patients there is a CD40 signaling pathway disorder in B cells, which is also manifested in the inability to generate immunoglobulins [32,33].

The increased numbers of CD154⁺CD4⁺ T cells detected herein conflict with the findings by Ravanbakhsh M et al. [34] who described normal expression of CD154 after a six-hour stimulation with PMA and IM, and with those of Pons et al. [35] who also measured expression of CD154 comparable with that of a healthy population after a 48-hour stimulation with anti-CD3. A possible reason for these conflicting findings may be the different stimulation conditions used in our tests (cells were cultured for 72 h with 0.5 mg/ml plate-bound CD3 mAb. PMA + Ionomycin were added for the last 4 h of culture).

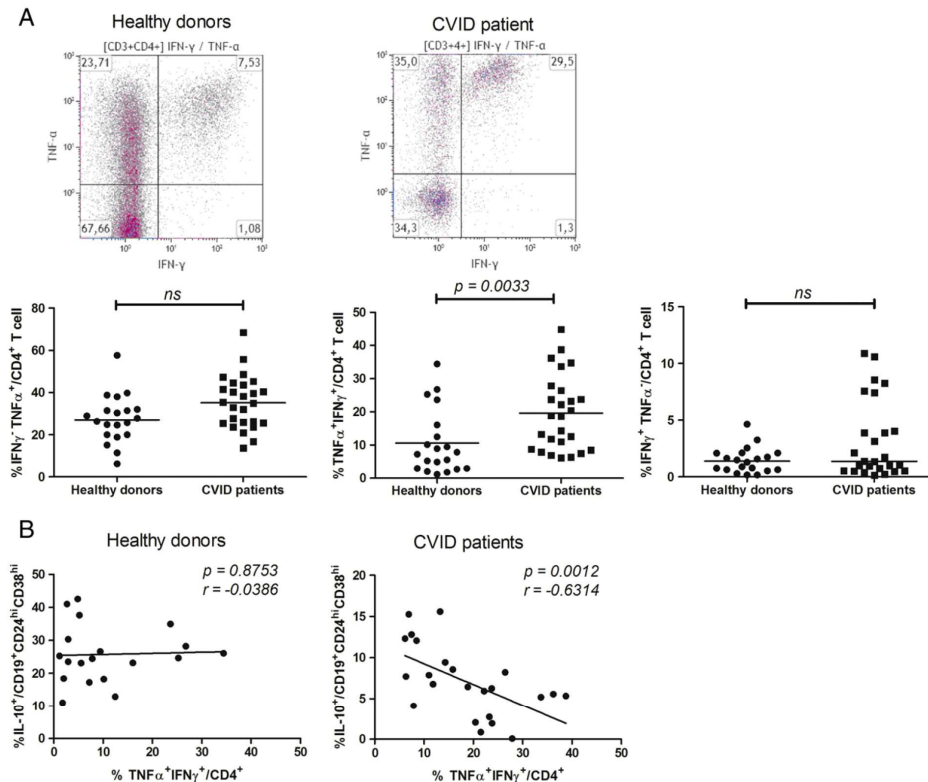


Fig. 5. The production of TNF- α and IFN- γ in CD4⁺ T cells. **A:** Representative dot plots show increased frequency of IFN- γ ⁺ TNF- α ⁺ cells and IFN- γ ⁺ TNF- α ⁺ cells in CD4⁺ T cells in CVID patients after anti-CD3 mAb stimulation. Proportion of IFN- γ ⁺ TNF- α ⁺, IFN- γ ⁺ TNF- α ⁺ and IFN- γ ⁺ TNF- α ⁺ cells in CD4⁺ T cells. The differences between CVID patients and control subjects were assessed using the Mann-Whitney rank sum test. **B:** Correlation of the frequency of IFN- γ ⁺ TNF- α ⁺ in CD4⁺ T cells and the frequency of IL-10 in CD19⁺ CD24^{hi} CD38^{hi} B reg cells (Spearman rank order correlation tests).

In 2010, Blair et al. [17] described that peripheral blood contains a subpopulation of CD19⁺CD24^{hi}CD38^{hi} B cells that produce increased levels of IL-10 after separation and subsequent stimulation with CD40 and that inhibits differentiation of pro-inflammatory cytokines. Therefore, we investigated whether the CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells generated after T cell stimulation have the same regulatory potential as regulatory B cells generated from an isolated subpopulation of “transitional CD19⁺CD24^{hi}CD38^{hi} B cells”. For this reason we also studied the production of IFN- γ ⁺ and TNF- α ⁺ in CD4⁺ T cells, which is efficiently inhibited by regulatory B cells generated from transitional cells [17,28,36]. A detailed analysis using eight-color flow cytometry showed that patients with CVID generate an increased frequency of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells after stimulation, i.e., cells with the ability to produce both cytokines at the same time. Although IFN- γ ⁺ CD4⁺ T cells or TNF- α ⁺ CD4⁺ T cells belong to the category of less differentiated CD4⁺ T cells, IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells are referred to as multifunctional CD4⁺ T cells [37] and belong to the category of highly immunocompetent memory or effector memory T cells [38,39]. In patients with CVID, these CD4⁺ T cells were never described because the last intracellular measurement of these cytokines in patients with CVID was published in 1996, where cells were stimulated with PMA and Ionomycin using a three-color flow cytometry that did not allow for their simultaneous measurement [12]. Nevertheless, even in this measurement North et al. found increased proportions of IFN- γ ⁺ CD4⁺

and TNF- α ⁺ CD4⁺ T cells. The increased occurrence of these multifunctional CD4⁺ T cells may be due to the increased ratio of CD4⁺ memory T cells that is found in patients with CVID [6].

We also observed that patients with the highest frequency of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells also had low numbers of CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells. These findings were later confirmed by the negative correlation between the frequency of IFN- γ ⁺ TNF- α ⁺ cells in CD4⁺ T cells and CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ B cells in patients with CVID. This result is in concordance with the finding of Blair et al. [17] who confirmed that the inhibitory effect of CD19⁺CD24^{hi}CD38^{hi} B cells is cell dose dependent. On the other hand, five CVID patients with CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ regulatory B cells above the >10% (which we can suppose as the “normal range” of our control persons) show low proportion of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells (below 15%). However in CVID patients with CD19⁺CD24^{hi}CD38^{hi}IL-10⁺ regulatory B cells below 10% both patients with low or high number of activated IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells can be observed. This shows that the number of CD19⁺CD24^{hi}CD38^{hi}IL-10 B reg cells is not the only factor influencing activation of IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells in our experimental system (See Fig. 5B).

We verified that IL-10 inhibition together with inhibition of the receptor for IL-10 led to an increase in IFN- γ ⁺ TNF- α ⁺ cells in CD4⁺ T cells in both the healthy control group and patients with CVID, thus confirming the regulatory role of IL-10 in multifunctional CD4⁺ T cell

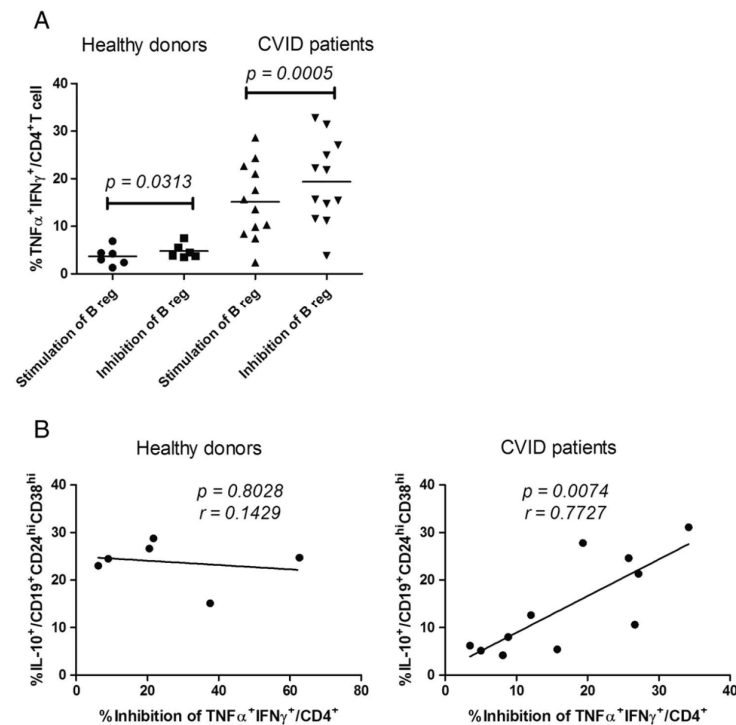


Fig. 6. The frequency of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells increased after the inhibition of IL-10 in B reg cells. A: Proportion of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ cells among CD4^+ T cells after blockade of the IL-10 suppressor function of $\text{CD19}^+ \text{CD24}^{\text{hi}} \text{CD38}^{\text{hi}} \text{IL-10}^+$ B cells. PBMCs were cultured in the presence of 0.5 mg/ml plate-bound CD3 mAb, in the presence of anti-IL-10 and IL-10 receptor inhibitory antibodies, PMA + iono was added for the last 4 h of culture. The Wilcoxon signed-rank test was used for comparison of the frequency of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells after stimulation and after inhibition of B reg cells both for healthy donors and CVID patients. B: The frequency of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cell inhibition correlated with the frequency of IL-10 in $\text{CD19}^+ \text{CD24}^{\text{hi}} \text{CD38}^{\text{hi}}$ B reg cells in CVID patients but not in healthy donors (Spearman rank order correlation tests).

development. Indeed, we observed the highest effect of IL-10 blockade occurred in patients who had the highest numbers of $\text{CD19}^+ \text{CD24}^{\text{hi}} \text{CD38}^{\text{hi}} \text{IL-10}^+$ B cells. In contrast, there was no further increase in the frequency of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells in patients with low numbers of $\text{CD19}^+ \text{CD24}^{\text{hi}} \text{CD38}^{\text{hi}} \text{IL-10}^+$ B cells and high proportions of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells.

These findings were also confirmed by the negative correlation between the ratio of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cell inhibition and the frequency of IL-10^+ cells from $\text{CD19}^+ \text{CD24}^{\text{hi}} \text{CD38}^{\text{hi}}$ B cells in a cohort of patients with CVID. Although we have indeed observed IL-10 regulated $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells in the control group, the frequency of $\text{IFN-}\gamma^+ \text{TNF-}\alpha^+$ in CD4^+ T cells was generally higher in CVID patients.

Because no other cells producing IL-10 were found in our experiments, we can conclude that the suppression of multifunctional T cells is mediated by regulatory B cells through IL-10 secretion.

Regulatory B cells are dynamically generated in response to T cell stimulation, however the number of IL-10 producing B regulatory cells is compromised in CVID patients. Our study shows for the first time a defect in a regulatory circuit (involving B cell response) that explains the immunoregulatory abnormalities observed in CVID patients. Failure of regulatory B cell responses would explain the T cell defects leading to abnormal (chronic) T cell activation, pointing to B cells as the culprit in both the immunodeficiency and dysregulation characteristics of CVID.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2015.06.013>.

Acknowledgments

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9 Neutrofilní dysregulace u CVID: od supresivních funkcí po biomarkery aktivace

9.1 Suprese T-lymfocytů zprostředkovaná neutrofily a granulocytárními myeloidními supresorovými buňkami u pacientů s CVID

Zatímco B- a T-lymfocytární poruchy představují klíčový rys běžné variabilní imunodeficiency (CVID), imunodysregulace u těchto pacientů často přesahuje rámec adaptivní imunity. Výrazné autoimunitní projevy, lymfoproliferace či granulomatózní záněty svědčí o hlubokém narušení rovnováhy mezi efektorovými a regulačními mechanismy, do nichž se zapojují i složky vrozené imunity.⁶⁵

Pacienti s CVID vykazují známky chronické imunitní aktivace, která není výhradně spojena s přítomností infekce nebo endotoxemie.^{66,67} Studie zaměřené na sérový cytokinový profil prokázaly výrazné změny v cirkulujících pro- i protizánětlivých mediátorech, včetně zvýšených hladin IL-6, TNF- α a dalších cytokinů spojených s aktivací monocytů a T-lymfocytů.⁶⁸ Zároveň byly u těchto pacientů opakovaně zjištěny zvýšené koncentrace solubilních aktivačních markerů, jako jsou sCD14 a sCD25, které odrážejí aktivaci vrozené (sCD14), i adaptivní imunity (sCD25) těchto pacientů.⁶⁹ Tyto nálezy poukazují na přítomnost systémového zánětlivého prostředí, které přetrvává i mimo období akutních infekcí. CVID je rovněž spojen se zvýšenými sérovými hladinami G-CSF, CXCL-10/IP-10, antagonisty IL-1R, TNF- α , IL-10, IL-12 (p40), CCL-2/MCP-1 a eotaxinu,⁶⁸ což ukazuje na probíhající aktivaci buněk monocytární a granulocytární linie. Rovněž koncentrace G-CSF, klíčového regulátoru granulopoézy a centrálního mediátoru nouzové granulopoézy,⁷⁰ byla u pacientů s CVID zvýšena ve srovnání se zdravými dárči.⁶⁸ Vzhledem k tomu, že neutrofily představují klíčovou složku vrozené imunity a významně se podílejí na regulaci zánětlivých reakcí, nabízí se otázka, zda právě jejich aktivace, fenotypové změny či funkční poruchy nehrají úlohu v pozorované imunitní dysregulaci u CVID. Výzkum na našem pracovišti se proto zaměřil na úlohu neutrofilních granulocytů v patogenezi CVID, přičemž ukázal, že neutrofily těchto pacientů nejsou pouze aktivované, ale vykazují znaky imunosupresivních buněk, poruchy efektorových funkcí a mohou tak přispívat k rozvoji imunitní dysregulace.

Ve studii „Neutrophil and Granulocytic Myeloid-Derived Suppressor Cell-Mediated T Cell Suppression in CVID“ jsme pozorovali 30% nárůst absolutního počtu neutrofilů u pacientů s CVID ve srovnání s kontrolními osobami, což může být v důsledku nouzové granulopoézy.⁷¹ Jedná se o proces rychlé tvorby neutrofilů prostřednictvím zvýšené proliferace myeloidních progenitorových buněk v kostní dřeni v důsledku reakce chronické mikrobiální translokace, čili proces přenosu celých bakterií a mikrobiálních produktů ze střevního lumenu do systémového oběhu.⁷² K detekci patogenů imunitním systémem dochází prostřednictvím signalizace TLR buňkami v postižených tkáních, avšak tyto patogeny mohou být rovněž detekovány hematopoetickými kmenovými a progenitorovými buňkami, což by mohlo přispívat k celkové granulopoetické odpovědi.⁷⁰ Nedávné studie ukázaly, že nouzová granulopoéza je řízena právě G-CSF produkovaným endotelovými buňkami v reakci na LPS signalizaci prostřednictvím dráhy TLR4/MyD88.^{70,73–75} Neutrofily indukované G-CSF procházejí alternativní diferenciační cestou z běžných myeloidních progenitorů, produkují ROS a vykazují silné imunosupresivní vlastnosti.⁷⁶ V naší práci jsme popsali u pacientů s CVID zvýšený výskyt granulocytárních myeloidně odvozených supresorových buněk (gMDSC). Tyto buňky byly fenotypicky charakterizovány jako CD15⁺CD16^{med}CD62L^{low}CD11b^{high}PD-L1^{high} granulocyty, které vykazovaly vysokou produkci reaktivních forem kyslíku (ROS) a v in vitro podmínkách byly schopny potlačit aktivaci a produkci IFN- γ CD8⁺ T buňkami prostřednictvím produkce ROS a částečně také prostřednictvím regulační dráhy PD-L1/PD-1. Tento nálezk podpořil hypotézu, že poruchy u CVID zahrnují nejen

nedostatečnou produkci imunoglobulinů B-lymfocyty spolu se zvýšenou aktivací T-lymfocytů, ale i patologickou aktivací a supresivní funkcí vrozených imunitních buněk. O zvýšené degranulaci neutrofilů svědčí i nalezené zvýšené koncentrace NGAL v séru pacientů s CVID.

Low density neutrophils

V rámci studie zaměřené na výše popsané supresorové vlastnosti neutrofilů, jsme také popsali zvýšený počet tzv. nízkodenzitních neutrofilů (LDNs). Jedná se o neutrofile, které jsou přítomny ve frakci PBMC po izolaci na Ficolu, tedy ve frakci mononukleárních buněk. Tyto neutrofile s nízkou densitou, původně popsané zejména u autoimunitních a nádorových onemocnění^{77,78} se v kontextu CVID poprvé objevují jako buňky se supresivním potenciálem, neboť jejich počet koreloval se sníženou produkcí IFN- γ produkovaného CD8⁺ T-lymfocyty. To nás vedlo k jejich hlubší analýze v navazujícím výzkumu.

Studie „Phenotype and oxidative burst of low-density neutrophil subpopulations are altered in common variable immunodeficiency patients“, zaměřená na detailní charakterizaci LDN u zdravých dárců ukázala, že populace LDN u zdravých dárců i CVID pacientů zahrnuje subpopulaci zralých (mature LDN - mLDN) i nezralých (immature LDN - iLDN) neutrofilů, které se liší svými funkčními vlastnostmi. Počet zralých neutrofilů v LDN může být zvýšen in vitro, a to stimulací plné heparinizované krve pomocí IVIg, fMLF nebo LPS. Naše výsledky ukázaly, že ve srovnání s mLDN mají iLDN výrazně sníženou schopnost oxidačního vzplanutí u obou vyšetřovaných skupin. Navíc, u pacientů s CVID mají nejen iLDN, ale také mLDN po stimulaci PMA omezenou kapacitu oxidačního vzplanutí. Tato snížená schopnost oxidačního vzplanutí představuje zásadní defekt antimikrobiální funkce LDN u pacientů s CVID. U pacientů s CVID mají LDN zvýšenou expresi supresorové molekuly PD-L1 a jejich mLDN dále nejsou schopny po stimulaci IVIg, fMLF nebo LPS zvyšovat expresi CD10. CD10 je receptor, objevující se na povrchu zralých neutrofilů, při stimulaci se jeho exprese zvyšuje a se stárnutím neutrofilní populace exprese CD10 klesá.^{79,80} Snížená schopnost zvyšovat expresi CD10 u mLDN může být v důsledku jejich nezralosti nebo také předčasné senescence mLDN pacientů s CVID.

Zvýšená přítomnost LDN u pacientů s CVID může odrážet epizody akutního zánětu a při dlouhodobém sledování u jednotlivých pacientů sloužit jako indikátor chronického zánětu.⁸¹

Biomarkery aktivace neutrofilů: elastáza a MPO

Doplněním výše uvedených fenotypových a funkčních nálezů neutrofilů u pacientů s CVID je studie „Common variable immunodeficiency patients display elevated plasma levels of granulocyte activation markers elastase and myeloperoxidase“, v níž byly na našem pracovišti zjištěny zvýšené plazmatické hladiny neutrofilní elastázy a myeloperoxidázy (MPO) u pacientů s CVID ve srovnání s kontrolní populací. Zvýšené hodnoty byly detekovány i mimo infekční epizody pacientů s CVID a korelovaly s výskytem autoimunity a lymfoproliferace. Tento náález dokládá, že neutrofile u CVID pacientů jsou trvale aktivovány i v nepřítomnosti akutní infekce a že hladiny elastázy a MPO lze vnímat jako potenciální biomarkery chronické subklinické aktivace,^{82,83} reflektující systémové zánětlivé prostředí typické pro imunodysregulační formy CVID.

Výzkum na našem pracovišti prokázal, že neutrofile u pacientů s CVID získávají regulační roli, která přesahuje jejich klasickou efektorovou funkci, vykazují supresivní účinky na adaptivní imunitní odpověď prostřednictvím ROS a interakce mezi PD-1 a PD-L1, současně však jejich klíčová antimikrobiální vlastnost, oxidační vzplanutí může být snížena. Navíc trvalá aktivace neutrofilů přispívá k chronickému zánětlivému prostředí. Tyto nálezy zásadně rozšiřují pohled na CVID jako poruchu postihující nejen adaptivní, ale i vrozenou složku imunitního systému.

Studium neutrofilních granulocytů v kontextu COVID zároveň otevřelo cestu k analýze obdobných mechanismů i u dalších stavů spojených s imunitní dysregulací – především u pacientů po těžkém traumatu, kterým se věnuje následující kapitola.

Následuje plný text článků:

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Neutrophil and Granulocytic Myeloid-Derived Suppressor Cell-Mediated T Cell Suppression Significantly Contributes to Immune Dysregulation in Common Variable Immunodeficiency Disorders

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Common variable immunodeficiency disorders (CVID) represent a group of primary immunodeficiency diseases characterized by hypogammaglobulinemia and impaired specific Ab response, resulting in recurrent infections due to dysfunctional immune response. The specific mechanisms mediating immune deficiency in CVID remain to be determined. Previous studies indicated that immune dysregulation in CVID patients is associated with chronic microbial translocation, systemic immune activation, and altered homeostasis of lymphocytic and myeloid lineages. A detailed phenotypic, functional characterization of plasma markers and immune cell populations was performed in 46 CVID patients and 44 healthy donors. CVID patients displayed significantly elevated plasma levels of a marker of neutrophil activation neutrophil gelatinase-associated lipocalin. Neutrophils from CVID patients exhibited elevated surface levels of CD11b and PD-L1 and decreased levels of CD62L, CD16, and CD80, consistent with a phenotype of activated neutrophils with suppressive properties. Neutrophils from CVID patients actively suppressed T cell activation and release of IFN- γ via the production of reactive oxygen species. Furthermore, CVID was associated with an increased frequency of low-density neutrophils (LDNs)/granulocytic myeloid-derived suppressor cells. LDN/granulocytic myeloid-derived suppressor cell frequency in CVID patients correlated with reduced T cell responsiveness. Exogenous stimulation of whole blood with bacterial LPS emulated some but not all of the phenotypic changes observed on neutrophils from CVID patients and induced neutrophil population with LDN phenotype. The presented data demonstrate that neutrophils in the blood of CVID patients acquire an activated phenotype and exert potent T cell suppressive activity. Specific targeting of myeloid cell-derived suppressor activity represents a novel potential therapeutic strategy for CVID. *The Journal of Immunology*, 2019, 202: 93–104.

Common variable immunodeficiency disorders (CVID) are a heterogeneous group of defects representing the most frequent form of primary symptomatic hypogammaglobulinemia (1). CVID patients suffer from clinically significant immunodeficiency that manifests by frequent and severe respiratory tract infections, diarrhea, and autoimmune disorders. Recurrent bacterial infections in CVID patients are predominantly caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* (2, 3). In ~2–10% of patients with CVID, specific mutations in more than 20 genes were described,

including genes coding for LPS-responsive beige-like anchor protein (LRBA), CTLA-4, CD19, CD20, CD21, CD81, ICOS, BAFF-R, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and others (2, 3). Previous studies have demonstrated a number of lymphocytic abnormalities in CVID patients, including chronic T cell activation, reduced T cell functionality, and decreased absolute numbers of CD4⁺ T cells and NK cells in the systemic circulation (4–10). The specific mechanisms underlying systemic immune dysregulation in CVID remain unclear. Studies by others and us demonstrated

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Abbreviations used in this article: CAT, catalase; CVID, common variable immunodeficiency disorder; G-MDSC, granulocytic myeloid-derived suppressor cell; iNOS, inducible NO synthase; LDN, low-density neutrophil; L-NHA, *N*⁶-hydroxy-L-arginine monoacetate; L-NMMA, *N*^ω-monomethyl-L-arginine acetate salt; NGAL, neutrophil gelatinase-associated lipocalin; ROS, reactive oxygen species; sCD14, soluble CD14; SOD, superoxide dismutase; SSC, side light scatter.

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that immune dysregulation in CVID is associated with chronic microbial translocation, systemic activation of myeloid cells, and altered phenotype of myeloid cell-derived cytokines, including increased production of G-CSF and other factors involved in granulopoiesis and regulation of neutrophil recruitment and activation (10–13).

Neutrophils represent the most abundant nucleated leukocyte population (50–70% of all WBCs) that is specifically geared for sensitive detection of bacterial products (14–16). Any significant functional alteration of this population is likely to exert a profound effect on the host. Systemic bacterial or viral infection induces a hematopoietic response referred to as emergency granulopoiesis, with up to 1×10^{11} granulocytes generated each day (17, 18). Recent elegant studies have demonstrated that emergency granulopoiesis is driven by G-CSF produced by endothelial cells in response to LPS signaling via TLR4/MyD88 pathway (18–20). G-CSF-induced neutrophils were shown to arise from alternative differentiation pathway from common myeloid progenitors and granulocyte/macrophage progenitors, produce reactive oxygen species (ROS), and display potent immunosuppressive properties (19–21).

A new appreciation of the significance of neutrophils for the host has emerged in light of recent findings on their heterogeneity and plasticity (16, 22), their ability to survive long term, and their ability to shape adaptive arm immune responses (14–16, 23, 24). Neutrophils regulate immune hyper and hypoactivation via a release of inflammatory and toxic mediators and by curbing excessive immune responses (16, 25). The altered functionality of neutrophils can lead to severe inflammatory complications and sepsis (26). Neutrophils colocalize and actively communicate with T cells in draining lymph nodes and other organized lymphoid tissues in which they are involved in negative regulation of T cell function via production of ROS and arginase-1 (15, 27–32). We have recently described a presence of immunosuppressive neutrophils in the blood of HIV-1-infected patients that are likely induced by microbial translocation and suppress T cell function via the production of ROS and programmed death PD-1/PD-L1 interaction (33). Recent studies have identified an immunosuppressive population of CD16⁺CD62L^{low} neutrophils that is induced in human volunteers following injection of a low dose of bacterial LPS and inhibits T cell function by local release of hydrogen peroxide (31) and via PD-L1/PD1 axis.

In sepsis, trauma, and various chronic inflammatory conditions, a population of low-density neutrophils (LDNs) with immunosuppressive properties has been detected (23, 30, 33, 34). In autoimmune diseases, LDNs have been associated with enhanced NETosis and extensive endothelial damage (35). A similar population of granulocytic myeloid-derived suppressor cells (G-MDSCs) has been reported by multiple studies, primarily in patients with cancer (30, 34, 36, 37). Both LDNs and G-MDSCs exert low-density phenotype and cosegregate in the PBMC fraction on a density gradient. It is unclear at present whether LDN and G-MDSC populations are identical or differ in their phenotype, origin, and functional properties (30). Furthermore, it remains to be established whether LDNs/G-MDSCs originate by granulopoiesis from dedicated suppressive progenitors in the bone marrow or, alternatively, they represent a functional subset of neutrophils that is acquired the immunosuppressive phenotype in response to specific signals in the periphery (30). Both LDNs and G-MDSCs display a remarkable ability to suppress T cell-mediated immune responses by multiple mechanisms, including production of ROS, the release of arginase-1 resulting in a depletion of arginine and downregulation of TCR ζ chain, production of regulatory cytokines, and induction of regulatory T cells

(30, 32, 34). LDNs/G-MDSCs are believed to serve as a negative feedback mechanism preventing extensive damage to the host in sepsis and inflammation (32).

The goal of the current study was to determine the phenotype of granulocytes in CVID patients with an emphasis on possible regulatory effect on T cell function. We present evidence that neutrophils in CVID patients acquire a specific activated phenotype and exert potent T cell suppressive activity, a previously unrecognized mechanism of immune suppression in CVID patients.

Materials and Methods

Patients and healthy donors

All participants in the study were white and from South Moravian region of Czech Republic. The study was approved by the Medical Ethics Committee of St Anne's University Hospital, Brno, Czech Republic. Informed consent was obtained from all volunteers prior to the inclusion in the study. A total of 46 CVID patients (25 females, 21 males; median age 45, range 22–82 y) and 44 healthy donors (23 females, 21 males; median age 41, range 19–78 y) were recruited. All patients fulfilled the International Consensus Document diagnostic criteria for CVID (38). Clinical characteristics of the study population are described in Table I. CVID patients were treated with regular i.v. (IVIG) ($n = 25$) or s.c. (SCIG) ($n = 21$) substitution. None of the patients suffered from opportunistic infections typical for late-onset combined immunodeficiency (39). One patient recently had splenectomy for splenomegaly and hypersplenism. One patient underwent partial gastrectomy for gastric cancer; no other malignancies were detected in any of the participants. Three patients were on long-term steroid treatment (owing to granulomatous disease, lung fibrosis, and demyelinating disease). One patient was treated with combined immunosuppressives (steroids and leflunomide) owing to lung fibrosis, arthritis, neurologic involvement, and diarrhea.

Cell isolation

Blood samples for isolation of PBMCs and T cells of patients on IVIG treatment were collected before the IVIG infusion. PBMCs and neutrophils were isolated from the blood harvested in EDTA-containing vacutainers by density gradient centrifugation ($400 \times g$, 30 min) using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and washed twice with PBS supplemented with 5% BSA (Sigma-Aldrich, St. Louis, MO). Following centrifugation, the upper mononuclear cell layer was removed, and the lower granulocyte layer was collected and resuspended. Erythrocytes were lysed using isotonic NH_4Cl erythrocyte lysis buffer (170 mM NH_4Cl , 10 mM KHCO_3 , 20 mM EDTA, pH 7.3) at room temperature. Cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (HyClone, South Logan, UT), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (HyClone), further referred to as complete RPMI medium. T cells were isolated from peripheral blood using RoboSep Human CD3 Positive Selection Whole Blood kit (STEMCELL Technologies, Rockford, IL). Separation was performed on the instrument RoboSep-S (STEMCELL Technologies) according to the manufacturer's instructions.

ELISA for the detection of neutrophil activation markers and cytokines

To prevent neutrophil degranulation and release of enzymes, EDTA blood was collected on ice. Within 20 min after the blood draw, the plasma was separated by centrifugation ($1500 \times g$, 4°C, 15 min). Plasma was removed and transferred to fresh polypropylene tube without disturbing white cells in the buffy coat. Harvested plasma was centrifuged second time under the same conditions to separate any remaining WBCs. Samples were cryopreserved at -80°C . ELISA assays for the determination of neutrophil gelatinase-associated lipocalin (NGAL; Hycult Biotech, Plymouth Meeting) and IFN- γ (BioLegend, San Diego, CA) were performed according to the manufacturers' protocols.

Cell culture

PBMCs and T cells were stimulated with $1 \mu\text{g/ml}$ purified plate-bound anti-CD3 mAb (CD3 mAb; clone Hit-3a; BioLegend) and with added $0.3 \mu\text{g/ml}$ anti-CD28 mAb (clone CD28.2; eBioscience) for 18 h in complete RPMI medium in 96-well flat-bottom plates with a starting concentration of 2×10^5 cells per well, (1×10^6 per ml) of PBMCs or 10^5 cells per well (1×10^6 ml) of T cells. For detection of cytokines by intracellular staining, Brefeldin A ($10 \mu\text{g/ml}$; Sigma-Aldrich) was added for the last 4 h. Cell

proliferation was determined by the incorporation of thymidine analog BrdU (PerkinElmer, Waltham, MA) into newly synthesized DNA and detection with anti-BrdU Ab. For proliferation, PBMCs and T cells were stimulated with 1 μ g/ml purified plate-bound anti-CD3 mAb and added to 0.3 μ g/ml anti-CD28 mAb for 48 h in complete RPMI medium at a starting concentration of 2×10^5 cells per well (1×10^6 per ml) for PBMCs and 1×10^5 cells per well for T cells (1×10^6 per ml) in 96 flat-bottom cells. BrdU was added for the last 12 h. Proliferating cells were processed using the DELFIA Cell proliferation kit (PerkinElmer) following the manufacturer's protocol. Briefly, cells are incubated with the nonradioactive pyrimidine analog BrdU to allow its incorporation into newly synthesized DNA. Subsequently, europium-labeled anti-BrdU Abs are used to detect the level of BrdU incorporation via time-resolved fluorescence, a measurement of the Eu-fluorescence in a time-resolved fluorometer EnSight (PerkinElmer). For the neutrophil/T cell suppression assays, purified T cells or PBMCs were stimulated with plate-bound anti-CD3 (1 μ g/ml) and soluble anti-CD28 (0.3 μ g/ml) Abs for 18 h for production of cytokines and 48 h for proliferation test. Neutrophils were cocultured with CD3⁺ T cells or PBMCs at 1:3 T cell/neutrophil ratio (10^5 cells; 3×10^5 neutrophils per well). To address the mechanism of neutrophil-mediated suppression of T cells, catalase (CAT; 1000 U/ml) and superoxide dismutase (SOD; 200 U/ml; Sigma-Aldrich) were added to media to neutralize ROS, *N*^ω-hydroxy-L-arginine monoacetate (L-NHA; 2.5 μ g/ml) was added to inhibit arginase-1, and *N*^ω-monomethyl-L-arginine acetate salt (L-NMMA; 2.5 μ g/ml; Sigma-Aldrich) was added to inhibit the function of inducible NO synthase (iNOS). Anti-PD-L1 (clone 29E.2A3) (5 μ g/ml) and anti-PD-1 (clone EH12.2H7) (5 μ g/ml, BioLegend) Abs were added to inhibit PD-L1/PD-1 signaling pathway.

Intracellular cytokine staining

Cells were washed, fixed, permeabilized using Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA), and stained with IFN- γ -BV421 (clone 4S.B3) (BioLegend). Dead cell exclusion was performed using aqua-fluorescent reactive dye LIVE/DEAD Fixable Stain Kit (Invitrogen, CA). T cell staining was performed using CD4-PE-Cy7 (clone SFCH1274D11), CD8-allophycocyanin A750 (clone B9.11) (Beckman Coulter, Miami, FL), CD3-allophycocyanin (clone SK7) (BD Biosciences, San Jose, CA), and CD25-PerCP-Cy5.5 (clone BC96) (BioLegend). Gating strategy for the analysis of the expression of CD25 and intracellular production of IFN- γ is depicted in Supplemental Fig. 1.

Flow cytometry characterization of neutrophils and LDNs

For the determination of activation markers on the surface of neutrophils, mAbs were used in the following combinations: 1) CD15-FITC (clone H198), CD80-PE (clone 2D10), CD14-PerCP-Cy5.5 (clone M5E2), CD274-allophycocyanin (PD-L1) (clone 29E.2A3), CD62L-BV421 (clone DREG-56) (BioLegend) and CD16-allophycocyanin A750 (clone 3G8), CD45-Krome Orange (clone J33) (Beckman Coulter); 2) CD15-FITC, CD87-PE (clone VIM5), CD14-PerCP-Cy5.5, CD11a-PE-Cy7 (clone H1111), HLA-DR-BV421 (clone L243), CD11b-BV 510 (clone ICRF44), (BioLegend), and CD45-allophycocyanin-H700 (clone 2D1) (BD Biosciences); 3) CD15-FITC, CCR3-PE (clone 5E8), CD14-PerCP-Cy5.5, CD64 PC7 (clone 10.1) CXCR4-allophycocyanin (clone 12G5), CD62L-BV421, CD11b-BV 510, CD16-allophycocyanin A750; 4) CD15-FITC, CCR3-PE, CD14-PerCP-Cy5.5, CD64 PE-Cy7, CD54-allophycocyanin (clone HA58), CD62L-BV421, CD11b-BV 510, CD16-allophycocyanin A750; 5) CD15-FITC, CCR3-PE, CD14-PerCP-Cy5.5, CD10-allophycocyanin (clone H110a), CD62L-BV421, CD11b-BV 510, and CD16-allophycocyanin A750. Briefly, fresh blood samples were incubated for 30 min at 4°C in the dark with mAbs. The erythrocytes were lysed by Multi-Q-Prep Lysing Workstation (Beckman Coulter). Samples were acquired using a Navios flow cytometer (10 colors, three lasers; Beckman Coulter), and cytometry data (LMD files) were analyzed using Kaluza software (Beckman Coulter). The data are presented as the median fluorescence intensity for each cell subset. Gating strategy for the analysis of neutrophils is depicted in Supplemental Fig. 2A.

For the characterization of LDNs, freshly isolated PBMCs were incubated for 30 min in the dark with the following mAbs: CD15-FITC, CCR3-PE, CD14-PerCP-Cy5.5, CD33-PE-Cy7 (clone D3HL60.251), CD10-allophycocyanin or CXCR4-allophycocyanin or CD54-allophycocyanin, CD62L-BV510 (BioLegend), CD16 allophycocyanin A750, and CD45-Krome Orange (Beckman Coulter). Gating strategy for the analysis of LDNs is depicted in Supplemental Fig. 2B.

Ex vivo stimulation of blood with bacterial products

Whole blood freshly obtained from healthy donors was either not stimulated or stimulated with fMLF (10 μ M; Sigma-Aldrich) or LPS (1 μ g/ml,

Escherichia coli 0111:B4; Sigma-Aldrich) at 37°C for the indicated time and immediately stained with CD14-allophycocyanin/Fire750 (clone 63D3), CD274-PE-Cy7 (PD-L1; 29E.2A3), CD11b-PerCP-Cy5.5 (ICRF44; BioLegend), CD62L-BV711 (DREG-56), CD193-BV510 (CCR3; 5E8; BD Biosciences), CD15-eFluor450 (H198), and CD16-allophycocyanin (eBioCB16; eBioscience) for 30 min at 4°C. Samples were washed with cold wash Dulbecco's PBS buffer with EDTA (1 mM EDTA in DPBS) and centrifuged at $200 \times g$ for 5 min to remove unbound Abs. Samples were lysed with 1 ml of 1-step Fix/lyse buffer (Invitrogen/Thermo Fisher Scientific) in the dark at room temperature for 15 min, washed, and resuspended in equal parts of 2% FBS in DPBS and intracellular Fixation buffer (Thermo Fisher Scientific) prior to the analysis on the Attune NxT flow cytometer (Thermo Fisher Scientific). Cytometry data were analyzed by FlowJo (FlowJo, Ashland, OR). For the analysis of induction of LDNs in whole blood ex vivo, whole blood from healthy donors was either not stimulated or stimulated with LPS (1 μ g/ml) or fMLF (10 μ M) at 37°C for the indicated time. PBMCs were purified by gradient centrifugation, and LDNs were characterized as described above.

Statistics

Intergroup differences were analyzed using the nonparametric Mann-Whitney *U* test or Wilcoxon signed-rank test as appropriate. Correlations were assessed using the Spearman rank correlation test. Fisher exact test was used for the analysis of categorical data. The *p* values <0.05 (two-tailed) were considered statistically significant. Data were analyzed using GraphPad Prism 5 (GraphPad Software, CA).

Results

CVID patients display elevated levels of NGAL in plasma

Granulocytes have been previously shown to contribute to several autoimmune and inflammatory complications in CVID patients, including granuloma formation and chronic lung, intestinal, and other organ inflammation (38). We have previously reported that CVID patients display increased levels of myeloid cell-derived cytokines and chemokines in plasma, including factors involved in granulopoiesis and neutrophil activation (10). To further address the potential role of granulocytes in CVID pathogenesis, we analyzed immune cell populations and plasma markers of granulocytic activation in 46 CVID patients and 44 healthy donors (clinical parameters of the study population are described in Table I). CVID patients displayed increased absolute number of neutrophils compared with controls (3.45×10^9 per l versus 2.71×10^9 per l; *p* = 0.014; Mann-Whitney *U* test) (Fig. 1) whereas the absolute number of lymphocytes was decreased (1.43×10^9 per l versus 1.97×10^9 per l; *p* < 0.0001), consistent with a previous study (5). Interestingly, the plasma concentration of NGAL, a marker of neutrophil activation and degranulation, was significantly increased in CVID patients compared with healthy controls (*p* < 0.0001; Mann-Whitney *U* test) (Fig. 2). Because the elevated plasma level of NGAL could be caused by the increase in the absolute number of circulating neutrophils, normalization to absolute neutrophil count was performed. Following normalization, the statistical significance of intergroup differences was preserved (*p* < 0.0001) (Fig. 2). The intergroup differences remained significant after exclusion of CVID patients with C-reactive protein (CRP) >10 mg/l (*n* = 7; *p* < 0.0001), indicating that the increase in neutrophil activation and degranulation marker could not be attributed solely to the level of systemic immune activation. No correlations between the concentration of NGAL and other laboratory and clinical parameters were observed. No statistically significant difference in plasma levels of arginase-1 between CVID patients and healthy donors was observed (Supplemental Fig. 3).

Neutrophils from CVID patients exhibit an activated phenotype

To further address the potential role of granulocytes in CVID pathogenesis, we evaluated the levels of neutrophil surface markers of activation and potential suppressor activity in fresh blood of

Table I. Clinical characteristics of the study population

	IVIG (n = 27)	SCIG (n = 19)	Total (n = 46)
F/M	14/13	11/8	25/21
Age, mean (range)	43.5 (23–77)	46.9 (21–83)	45.5 (21–83)
Length of Ig treatment, mean (range)	11.52 (0.17–25)	9.89 (1–21)	10.85 (0.17–25)
Bronchiectasis, n (%)	14/25 ^a (56)	3/19 (16)	17/44 ^a (39)
Autoimmune diseases, n (%)	4/27 (15)	7/19 (37)	11/46 (24)
Granuloma, n (%)	4/25 ^a (16)	3/19 (16)	7/44 ^a (16)
Chronic diarrhea, n (%)	4/27 (15)	5/19 (26)	9/46 (20)

^aData concerning bronchiectasis and granulomas were not available in two patients.
F, female; M, male.

CVID patients and healthy donors. Whole blood neutrophils were gated as side light scatter (SSC)^{high} CD45⁺ CD15⁺ population, and the surface levels of CD11a, CD11b, CD16, CD62L, CD80, CD87, PD-L1, and HLA-DR were examined. A significant increase of median fluorescence intensity of CD11b ($p < 0.01$) and PD-L1 ($p < 0.002$), and decreases of CD16 ($p < 0.01$), CD62L ($p < 0.0005$), and CD80 ($p = 0.0002$, Mann-Whitney U tests) were observed on neutrophils from CVID patients compared with neutrophils from healthy donors (Fig. 3). CD11a, CD87, HLA-DR, CD10, CXCR4, and CD54 levels were not significantly different between the experimental groups. Surface levels of CD10 and CXCR4 are depicted in Supplemental Fig. 4. Phenotypic analysis of T cells of CVID patients revealed an increased expression of PD-1 on both CD4⁺ and CD8⁺ T cells compared with controls, consistent with previous reports (9, 10). However, no significant correlations were found between the levels of PD-L1 on neutrophils and PD-1 on T cells in CVID patients ($p = 0.2$ and $p = 0.4$ for PD-1 on CD4⁺ and CD8⁺ T cells, respectively; Spearman correlation test).

Neutrophils from CVID patients suppress T cell activation and production of IFN- γ

CD11b⁺CD16⁺CD62L^{dim} neutrophils were previously described as a unique circulating population mediating the suppression of T cell activation and cytokine production (31, 32). We have, therefore, assessed the ability of neutrophils from CVID patients to suppress the T cell activation upon CD3/CD28 stimulation *ex vivo*. Surface CD25 expression was higher on CD8⁺ T cells of CVID patients compared with controls ($p < 0.02$) but was significantly reduced on

T cells following cocubation with autologous neutrophils ($p = 0.003$; Wilcoxon test) (Fig. 4A). In contrast, neutrophils suppressed CD4⁺ T cell activation in healthy controls ($p = 0.01$) but not CVID patients ($p = 0.1$) (Fig. 4B). Intracellular production of IFN- γ by CD8⁺ T cells following CD3/CD28 stimulation in the absence of neutrophils was higher in CVID patients ($p < 0.0001$ (Fig. 4C) and correlated with the frequency of effector memory CCR7[−]CD45RO⁺ CD8⁺ T cells in CVID patients ($r = 0.4$; $p = 0.003$), but not in healthy donors ($r = 0.3$; $p = 0.09$; Spearman test). Importantly, the production of IFN- γ by CD8⁺ T cells of CVID patients upon CD3/CD28 stimulation was suppressed following cocubation with autologous neutrophils ($p = 0.005$); however, no effect was observed in healthy donors ($p = 0.6$; Wilcoxon test). Intracellular production of IFN- γ by CD4⁺ T cells following CD3/CD28 stimulation in the absence of neutrophils was higher in CVID patients ($p = 0.004$) (Fig. 4D) and correlated with the frequency of effector memory CCR7[−]CD45RO⁺ CD4⁺ T cells in CVID patients ($r = 0.3$; $p = 0.03$), but not in healthy donors ($r = 0.2$; $p = 0.27$; Spearman test). No significant difference was found in the production of IFN- γ in CD4⁺ T cells after coculture with neutrophils of CVID patients or healthy controls. IFN- γ protein accumulation in the medium upon CD3/CD28 stimulation was lower in cocultures of T cells with neutrophils from CVID patients ($p = 0.02$) as well as healthy controls ($p = 0.04$; Wilcoxon test) (Fig. 4E). The production of IFN- γ in the medium upon CD3/CD28 stimulation was higher in the culture of T cells in CVID patients $p < 0.005$; Mann-Whitney U test). Suppressive activity of CVID neutrophils was not significantly associated with clinical symptoms, including bronchiectasis, splenomegaly, chronic diarrhea, granuloma, or autoimmunity (tested using Fischer exact test), duration of Ig treatment, or the time from the first manifestation of ID symptoms (tested using Mann-Whitney U test).

Neutrophil-mediated immune suppression of IFN- γ production by activated T cells in CVID patients is dependent on ROS

To address the mechanisms of neutrophil-mediated suppression of T cell function, T cells were cocultured with neutrophils in media supplemented with CAT and SOD to neutralize ROS, iNOS inhibitor L-NMMA, arginase inhibitor L-NHA, or anti-PD-L1 and anti-PD-1 Abs to inhibit the PD-L1/PD1 pathway. The production of IFN- γ by CD3⁺CD8⁺ T cells following stimulation with CD3/CD28 in coculture with neutrophils from CVID patients was restored in the presence of CAT and SOD (Fig. 5A). Inhibition of PD-L1/PD-1 signaling resulted in partial restoration of neutrophil-mediated suppression of the production of IFN- γ by CD3⁺CD8⁺ T cells in CVID patients (Fig. 5A). In healthy donors, the production of IFN- γ by stimulated CD3⁺CD8⁺ T cells cocultured with neutrophils was restored only in the presence of ROS inhibitors ($p < 0.0001$; Wilcoxon test). No significant alteration of the production of IFN- γ by CD4⁺ T cells stimulated in the presence of inhibitors was observed in CVID patients or healthy controls. Full restoration of neutrophil-mediated suppression of extracellular

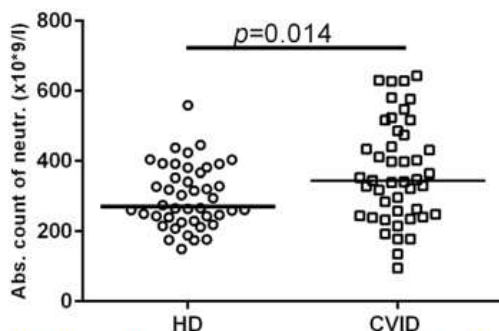
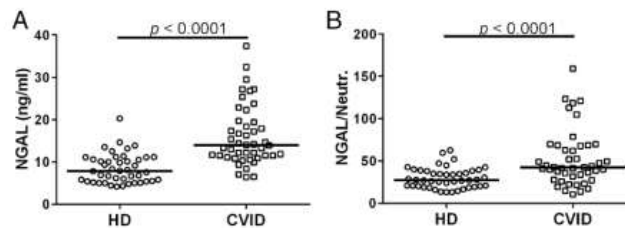


FIGURE 1. CVID patients display increased absolute neutrophil blood count. Total leukocyte counts were determined in whole blood. The neutrophils were gated as CD45⁺CD15⁺ CD16⁺SSC^{high} cells. The absolute number of circulating neutrophils was determined based on the flow cytometry analysis of population frequency and absolute leukocyte count. Horizontal bars represent medians; data were analyzed using the Mann-Whitney U test (CVID: $n = 46$; HD: $n = 44$). HD, healthy donors.

FIGURE 2. CVID patients display elevated plasma levels of NGAL. Plasma NGAL concentrations were determined using ELISA. The absolute concentration of NGAL (**A**) and concentration of NGAL normalized to absolute neutrophil count (**B**) are depicted. Horizontal bars represent medians; data were analyzed using the Mann–Whitney *U* test (CVID: *n* = 46; HD: *n* = 44). HD, healthy donors.



production of IFN- γ by stimulated T cells was observed in the presence of ROS inhibitors in CVID patients (Fig. 5B) as well as in healthy donors ($p < 0.0001$; tested by Wilcoxon test). A partial restoration was observed in the presence of the inhibitor of arginase-1 or blockade of the PD-L1/PD-1 pathway in CVID patients (Fig. 5B) as well as in healthy donors ($p = 0.0002$ for both L-NHA and PD-L1/PD-L1 inhibition; tested by Wilcoxon test).

Neutrophils from CVID patients suppress the proliferation of autologous T cells

To evaluate the suppressive effect of CVID neutrophils on autologous T cells, T cells were stimulated with CD3/CD28 in the presence or absence of neutrophils and proliferation was analyzed

after 48 h. The proliferation of T cells was significantly reduced following coincubation with autologous neutrophils from CVID patients ($p = 0.03$; Wilcoxon test) (Fig. 6A). No significant effect was detected following coculture of T cells of healthy donors with autologous neutrophils (Fig. 6A). To address the mechanism underlying neutrophil-mediated suppression of T cell proliferation in CVID patients, the experiments were performed in the presence of inhibitors of ROS, iNOS, arginase-1, and PD-L1/PD-1 pathway. No statistically significant effect of inhibitors of any of the regulatory pathways was observed (Fig. 6B). Therefore, the mechanism of neutrophil-mediated inhibition of T cell proliferation in CVID patients remains unclear. Importantly, a negative correlation was observed between the expression of CD62L on fresh blood

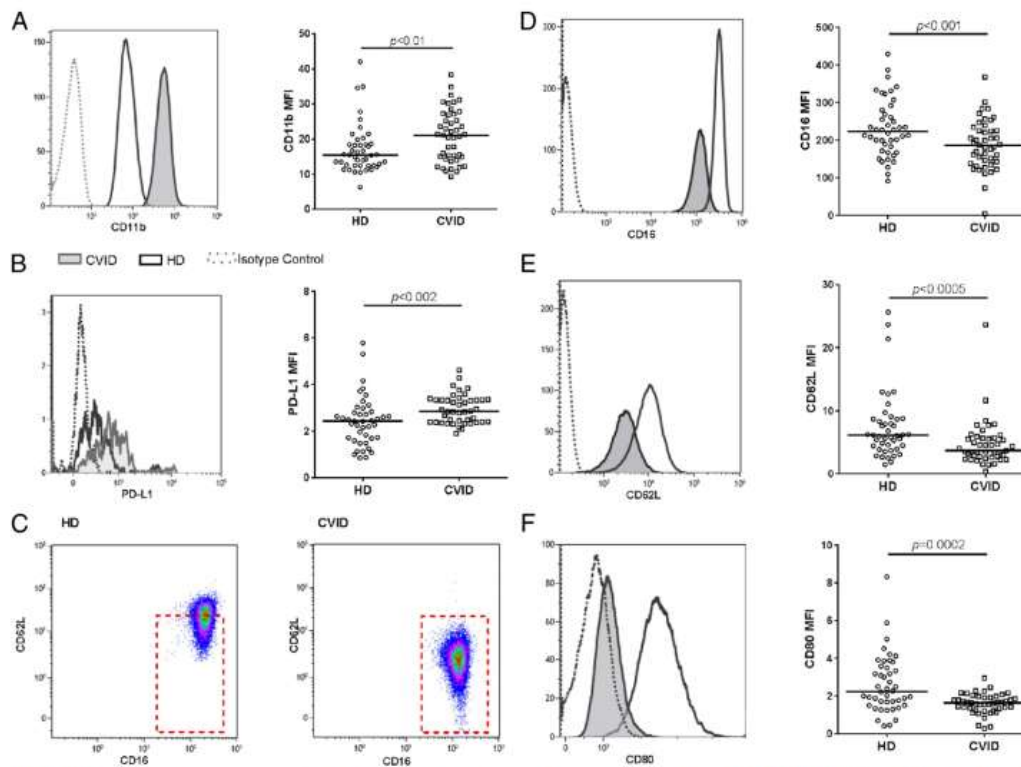
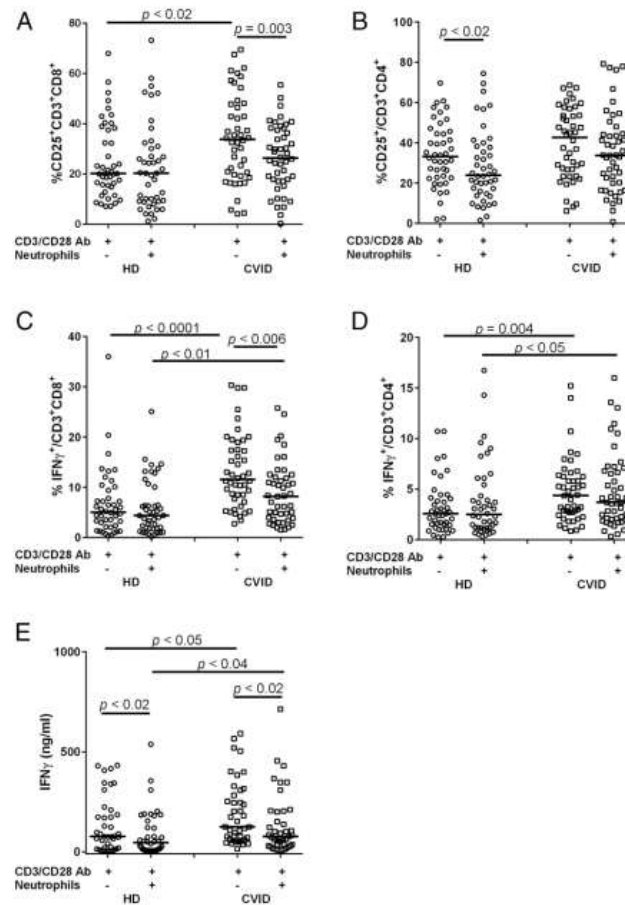


FIGURE 3. Neutrophils from CVID patients exhibit an activated phenotype. Neutrophils were gated as CD45⁺CD15⁺CD16⁺ SSC^{high} cells. (**A** and **B**) Increased expression of CD11b (**A**) and PD-L1 (**B**) on neutrophils in fresh blood of CVID patients; representative examples. (**C**) Decreased levels of CD16 and CD62L on neutrophils in fresh blood of CVID patients; representative examples. (**D–F**) Decreased expression of CD16 (**D**), CD62L (**E**), and CD80 (**F**) on neutrophils in fresh blood of CVID patients; cumulative data. Horizontal bars represent medians; data were analyzed using the Mann–Whitney *U* test (CVID: *n* = 46; HD: *n* = 44). HD, healthy donors.

FIGURE 4. Neutrophils from COVID patients suppress T cell activation and production of IFN- γ . Purified CD3 $^{+}$ T cells were isolated from healthy donors and COVID patients and stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (0.3 μ g/ml) Abs in the absence or presence of autologous neutrophils at 1:3 T cell/neutrophil ratio (1×10^5 T cells; 3×10^5 neutrophils per well). After 18 h of coculture, the frequencies of CD25 $^{+}$ and IFN- γ $^{+}$ CD4 $^{+}$ and CD8 $^{+}$ T cells were determined by flow cytometry. IFN- γ was determined by intracellular staining. **(A and B)** Percentages of CD25 $^{+}$ T cells of total CD3 $^{+}$ CD8 $^{+}$ or CD3 $^{+}$ CD4 $^{+}$ T cells. **(C and D)** Percentages of IFN- γ $^{+}$ T cells of total CD3 $^{+}$ CD8 $^{+}$ or CD3 $^{+}$ CD4 $^{+}$ T cells. **(E)** Cocubation of T cells with autologous neutrophils results in reduced accumulation of IFN- γ in culture supernatants in response to the stimulation with CD3/CD28. Horizontal bars represent medians, which were analyzed using Wilcoxon or Mann-Whitney U test as appropriate (COVID: $n = 46$; HD: $n = 44$). HD, healthy donors.



neutrophils and their capacity to suppress autologous T cell proliferation *ex vivo* ($r = -0.5$; $p = 0.002$; Spearman test) (Fig. 6C), consistent with the reported suppressive activity of CD62L dim neutrophils (31, 32). In contrast, no significant correlation was observed in healthy donors (Fig. 6C).

COVID patients display an increased frequency of LDNs/G-MDSCs

LDNs and G-MDSCs have been previously described as specific populations of suppressive granulocytes in cancer, trauma, and other inflammatory conditions (33, 34). Although it remains to be determined whether LDNs and G-MDSCs represent an identical cell population, they have been shown to share many key properties, most notably the low-density phenotype resulting in their retention in the PBMC layer on density gradients. We have addressed whether the frequency LDNs/G-MDSCs, characterized as SSC high CD33 $^{+}$ CD14 $^{+}$ CD15 $^{+}$ cells in fresh PBMCs, is increased in the blood of COVID patients. As shown in Fig. 7A and 7B, COVID patients exhibited a significantly increased frequency of LDNs in PBMCs compared with healthy donors ($p = 0.02$). A positive correlation was observed between CD11b expression on neutrophils from fresh blood and the frequency of LDNs in COVID patients ($r = 0.4$; $p = 0.007$) and

healthy donors ($r = 0.3$; $p = 0.04$; Spearman test). Next, we examined whether the presence of LDNs is associated with reduced production of IFN- γ by CD3/CD28-activated T cells *ex vivo*. A negative correlation was observed between the frequency of LDNs in PBMCs and intracellular production of IFN- γ by CD8 $^{+}$ T cells in PBMCs of COVID patients ($r = -0.4$; $p = 0.007$) but not healthy donors ($r = -0.05$; $p = 0.8$; Spearman test). CD10, CXCR4, and CD54 levels were not significantly different on LDNs between the experimental groups. No statistically significant correlation between the frequency of LDNs and administration of IVIG or any other laboratory and clinical parameters was observed.

Ex vivo stimulation of blood with bacterial products reduces neutrophil surface levels of CD62L, increases CD11b, and induces LDNs

Others and we have previously described evidence of microbial translocation in COVID patients (10–12). Circulating products of microbial translocation may be responsible for the activated neutrophil phenotype and altered profile of myeloid cell-derived cytokines and chemokines in COVID patients (10, 11). To address this possibility, fresh blood of healthy donors was stimulated with bacterial products fMLF and LPS. As shown in Fig. 8, stimulation

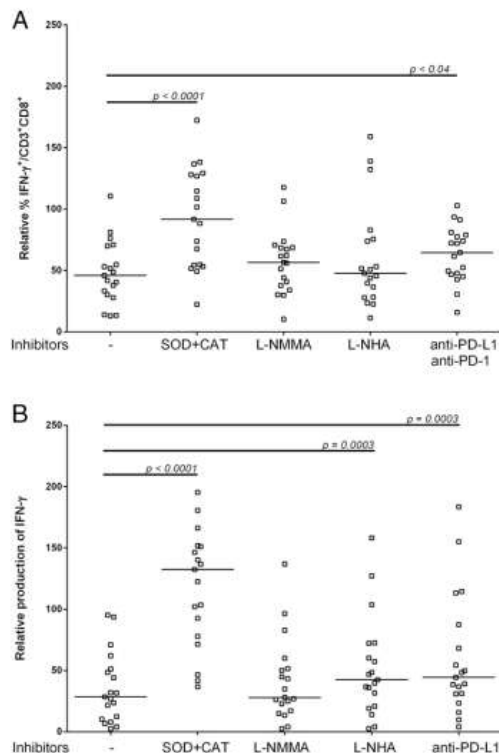


FIGURE 5. Mechanisms of neutrophil-mediated suppression of IFN- γ production by activated T cells in CVID patients. Purified CD3⁺ T cells were isolated from CVID patients and stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (0.3 μ g/ml) Abs in the presence of autologous neutrophils (10^5 T cells; 3×10^5 neutrophils per well) in the presence or absence SOD (200 U/ml) and CAT (1000 U/ml), L-NMMA (2.5 μ g/ml), L-NHA (2.5 μ g/ml), or anti-PD-1 (5 μ g/ml) and anti-PD-L1 (5 μ g/ml) Abs. After 18 h of coculture, the relative frequency of IFN- γ ⁺ of CD3⁺CD8⁺ cells compared with T cells stimulated in the absence of neutrophils is depicted (A). (B) IFN- γ concentration was determined in culture supernatants from cell cultures described in (A). Relative production of IFN- γ compared with T cells stimulated in the absence of neutrophils is depicted. Analyzed using Wilcoxon test (CVID: $n = 19$).

with LPS and fMLF lead to a rapid increase of surface levels of a degranulation marker CD11b and a decrease in the levels of CD62L. In contrast, the levels of CD16 were not significantly affected by either stimulation. The surface level of suppressor molecule PD-L1 was increased; however, the change did not reach statistical significance. Importantly, stimulation of whole blood from healthy donors ($n = 4$) with bacterial products fMLF or LPS increased the frequency of LDNs in the PBMC layer following density gradient centrifugation by 27-fold ($p = 0.02$) and 18-fold ($p = 0.02$), respectively (Fig. 9).

Discussion

In this study, we show that neutrophils from CVID patients exhibit significantly altered phenotype characterized by reduced surface levels of CD16, CD62L, and CD80 and elevated levels of CD11b and PD-L1. In addition, the presented data indicate that CVID

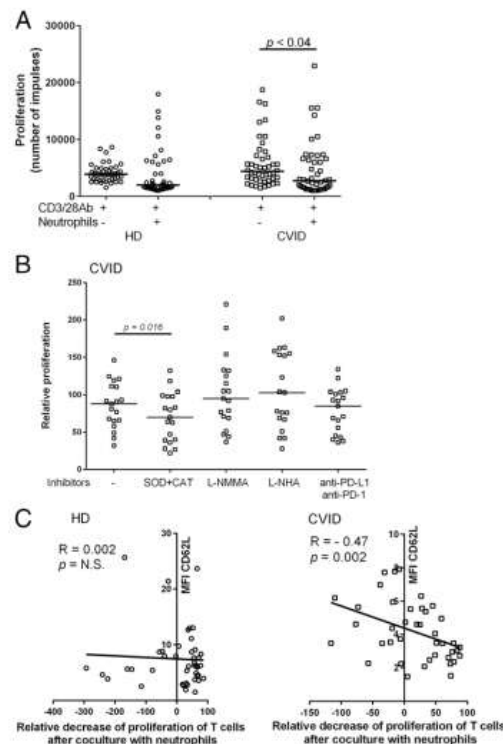


FIGURE 6. CVID neutrophils suppress the proliferation of autologous T cells. (A) Purified CD3⁺ T cells from healthy donors and CVID patients were stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (0.3 μ g/ml) Abs in the absence or presence of autologous neutrophils at 1:3 T cell/neutrophil ratio (1×10^5 T cells; 3×10^5 neutrophils per well). After 48 h of coculture, cell proliferation was determined by the incorporation of BrdU into newly synthesized DNA. Analyzed using the Mann-Whitney U test (CVID: $n = 46$; HD: $n = 44$). (B) Mechanism of neutrophil-mediated suppression of T cell proliferation. Purified CD3⁺ T cells were isolated from CVID patients and stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (0.3 μ g/ml) Abs in the presence of autologous neutrophils and in the presence or absence SOD (200 U/ml) and CAT (1000 U/ml), L-NMMA (2.5 μ g/ml), L-NHA (2.5 μ g/ml), or anti-PD-1 (5 μ g/ml) and anti-PD-L1 (5 μ g/ml) Abs. After 48 h of coculture, cell proliferation was determined by the incorporation of BrdU. Relative proliferation compared with T cells stimulated in the absence of neutrophils is depicted. Analyzed using Wilcoxon test (CVID: $n = 19$). (C) Correlation between the level of CD62L on fresh blood neutrophils and the relative decrease of T cell proliferation following coincubation with autologous neutrophils from healthy donors and CVID patients. Analyzed using Spearman rank correlation test; r and p values are indicated; lines represent linear regression analysis (CVID: $n = 46$; HD: $n = 44$). HD, healthy donors.

neutrophils actively suppress the activation and IFN- γ production by CD8⁺ T cells via the production of ROS and partially via PD-L1/PD-1 regulatory pathway. This observation may be of high potential importance as it provides a qualitatively new view on the mechanisms of immune suppression in CVID and potentially other immunodeficiencies and inflammatory conditions.

The mechanisms causing altered neutrophil phenotype in CVID are unclear. It is tempting to speculate that changes in neutrophil

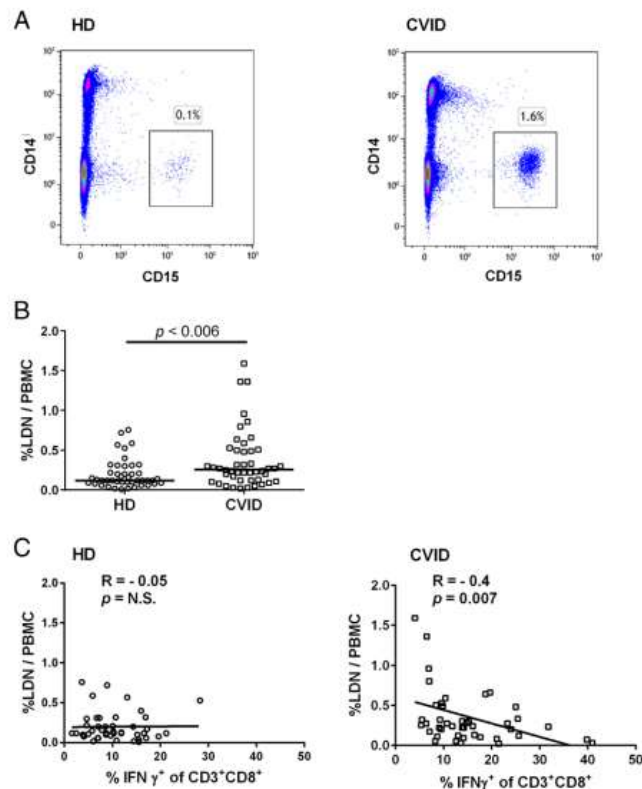


FIGURE 7. The frequency of LDNs is increased in the blood of COVID patients. LDNs were detected as CD45⁺CD15⁺CCR3⁺CD16⁺ cells in the PBMC fraction following density gradient centrifugation. **(A)** Representative flow cytometry plots of LDNs detected in PBMCs obtained from a healthy donor and COVID patient. **(B)** Cumulative frequencies of LDNs in PBMCs of healthy donors and COVID patients. Analyzed using the Mann–Whitney *U* test. **(C)** Correlation between the frequency of LDNs and the percentage of IFN- γ ⁺ CD3⁺CD8⁺ T cells following ex vivo stimulation with CD3/CD28. Analyzed using the Spearman rank correlation test; lines represent linear regression (COVID: $n = 46$; HD: $n = 44$). HD, healthy donors.

population are driven by chronic microbial translocation, a process of transfer of whole bacteria and microbial products from the intestinal lumen into the systemic circulation. Low level of microbial translocation occurs in healthy individuals; however, its extent dramatically increases in various pathological conditions including inflammatory bowel disease, celiac disease, visceral leishmaniasis, dengue virus infection, HIV infection, hepatic cirrhosis caused by alcohol abuse, or hepatitis B and C infections (40, 41). Translocation of bacterial and fungal products results in activation of both innate and acquired immune response mechanisms (10). Others and we have previously presented evidence of chronic microbial translocation in COVID patients (10–13, 42). Complex interactions between LPS, CD14, and TLR 4 results in the secretion of soluble CD14 (sCD14) from myeloid cells. Serum levels of sCD14 represent a marker of bacterial translocation and endotoxemia (43–45). We have previously demonstrated that COVID patients display elevated concentration of plasma sCD14 and other factors consistent with microbial translocation (10). One study showed that, in addition to elevated sCD14, COVID patients display chronic monocytic activation (12). Other investigators showed increased LPS levels together with increased sCD25 in COVID patients (13). We demonstrated that COVID patients display an altered profile of cytokine production, namely reduced serum levels of cytokines produced by CD4⁺ Th1 cells (IFN- γ , IL-2), Th2 (IL-9, IL-13), and Th17 (IL-17). In contrast, COVID is associated with elevated serum levels of G-CSF, CXCL-10/IP-10, IL-1R antagonist, TNF- α , IL-10, IL-12 (p40), CCL-2/MCP-1,

and eotaxin (10). This cytokine signature is consistent with an ongoing activation of cells of monocytic and granulocytic lineages. The concentration of G-CSF, a key granulopoietic regulator and a central mediator of emergency granulopoiesis (18), was increased by over 2-fold in COVID patients compared with healthy donors ($p = 0.0001$) (10).

Emergency granulopoiesis is a process of rapid generation of neutrophils via increased myeloid progenitor cell proliferation in the bone marrow in response to systemically disseminated bacterial products or invading pathogens. Pathogen sensing occurs mainly in nonhematopoietic cells through TLR signaling. However, sensing by hematopoietic stem and progenitor cells have also been suggested and might contribute to the overall granulopoietic response (17, 18). Recent studies showed that emergency granulopoiesis is driven by G-CSF produced by endothelial cells in response to LPS signaling via the TLR4/MyD88 pathway (18–20, 46). G-CSF-induced neutrophils undergo an alternative differentiation pathway from common myeloid progenitor and granulocyte/macrophage progenitors, produce ROS, and display potent immunosuppressive properties (19–21). In this study, we observed a 30% increase in the absolute numbers of neutrophils in COVID patients ($p = 0.014$; Fig. 1), consistent with enhanced granulopoiesis. However, the rate of granulopoiesis cannot be easily determined from absolute cell counts due to the contribution of complex processes of cell recruitment, trafficking, survival, and removal (47).

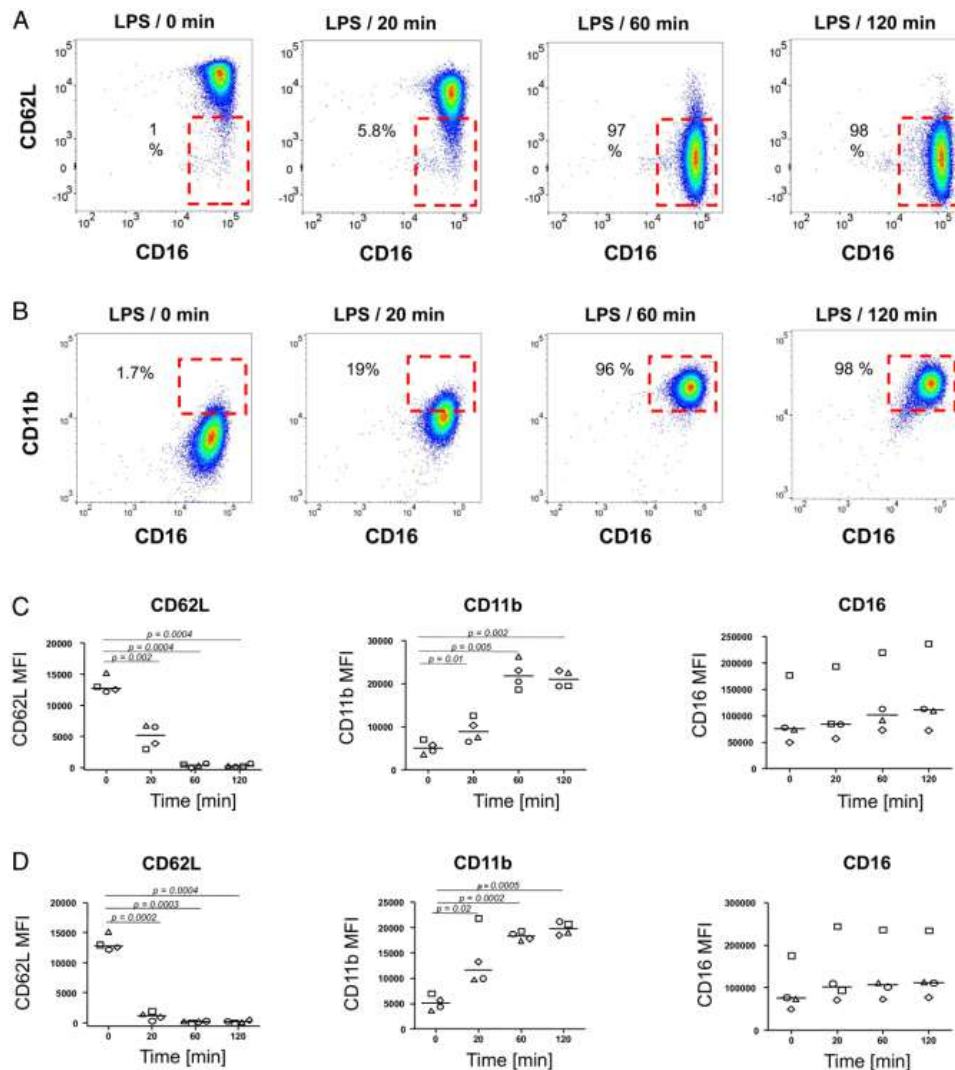


FIGURE 8. Stimulation of whole blood with bacterial products results in a rapid reduction of the neutrophil surface levels of CD62L and increased levels of CD11b. Whole blood from healthy donors was stimulated with bacterial LPS (1 μ g/ml) and fMLF (10 μ M) for the indicated time. The levels of surface markers were analyzed on SSC^{high} CCR3⁺ CD14⁺ CD15⁺ cells. (**A** and **B**) Stimulation of blood with LPS results in a rapid decrease of levels of CD62L and an increase of surface levels of CD11b on neutrophils. (**C** and **D**) Cumulative data depicting the changes in surface marker expression on neutrophils following the stimulation of whole blood with LPS (**C**) or fMLF (**D**). Data obtained from four independent healthy donors and analyzed using Mann-Whitney *U* test.

We show that CVID neutrophils exhibit low surface level of CD16 (Fig. 3). The level of CD16 (Fc γ receptor IIIb; CD16b) is the form of CD16 expressed on human neutrophils increases with neutrophil maturation and serves as a marker of neutrophil differentiation from myelocytes to metamyelocytes, banded neutrophils, and mature segmented neutrophils (48). Low level of neutrophil CD16 is observed in severe bacterial sepsis, infections, and inflammatory states and marks a population of “toxic” or “left-shift” neutrophils with elevated myeloperoxidase activity and

compromised phagocytic and bactericidal activity (49, 50). The level of CD16 is low on neutrophils induced in healthy volunteers following administration of G-CSF (51). The phenotype of neutrophils in CVID patients described in this article is consistent with the phenotype of human G-MDSCs found in the peripheral blood of patients with advanced renal cell carcinoma and pancreatic cancer characterized by low levels of CD16 and CD62L and high levels of CD11b (36, 52). CD16^{low} neutrophils expand in the blood of terminal cancer patients and strongly suppress T cell

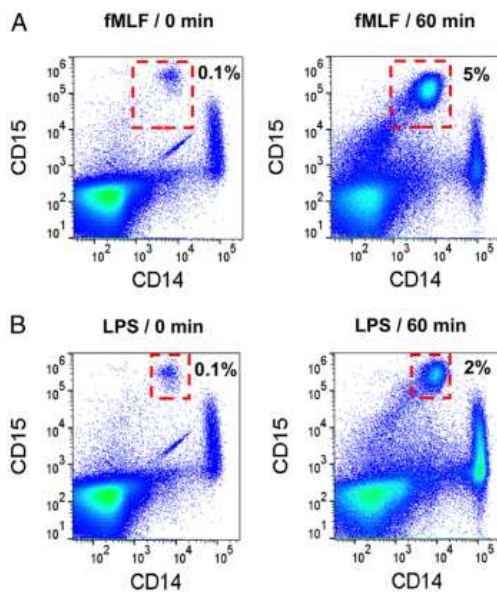


FIGURE 9. Stimulation of whole blood with fMLF or LPS results in an induction of LDNs. Fresh whole blood was either not stimulated or stimulated with fMLF (10 μ M) (A) or LPS (1 μ g/ml) (B) for 60 min. PBMCs were purified by gradient centrifugation, and the frequency of SSC^{high} CCR3⁺ CD14⁺ CD15⁺ LDNs was determined by flow cytometry. Representative data from one of four healthy donors.

proliferation (53). Elegant studies using pulse-chase labeling with 6,6-²H₂-glucose recently demonstrated that CD16^{low} neutrophils represent an immature neutrophil population that is released from the bone marrow several days earlier than mature segmented neutrophils (54). Early mobilization is believed to be a compensatory mechanism allowing the release of substantial numbers of neutrophils, although with lower maturation status and reduced capacity for pathogen clearance (54, 55). The levels of CD16 on CVID neutrophils described in our study closely correlate with the low levels of another myeloid maturation marker CD80 ($r = 0.9$; $p = 0.0008$; Spearman correlation test), consistent with the low maturation status of CD16^{low} cells. Based on these observations, we propose that microbial translocation in CVID patients drives accelerated granulopoiesis and recruitment of alternatively differentiated CD16^{low} neutrophils with potent T cell suppressive activity (19–21, 32).

We demonstrate that CVID is associated with lower neutrophil levels of CD62L (Fig. 2) and that CD62L expression inversely correlates with the level of suppression of T cell proliferation *ex vivo* (Fig. 4). CD62L (L-selectin) mediates the initial tethering and rolling of neutrophils on the endothelial surface. Reduction of CD62L levels can be explained by several distinct mechanisms. CD62L on neutrophils is reduced following administration of G-CSF *in vivo*, and it is shed from the cell surface upon activation with various stimuli, including various bacterial products (56). In this context, we demonstrate that incubation of whole blood with bacterial products fMLF and LPS results in a rapid downmodulation of CD62L on neutrophil surface (Fig. 6). It is, therefore, feasible that CD62L reduction *in vivo* is induced by contact with translocated microbial products. However, an alternative mechanism of the

observed downmodulation of neutrophil CD62L in CVID patients can be postulated. An immune suppressive population of CD62L^{low} neutrophils has been identified following injection of a low dose of LPS in human volunteers (31). This population inhibits T cell function via PD-L1/PD-1 interaction and local release of hydrogen peroxide into the immunological synapse between the neutrophil and T cell (31, 57). Using pulse-chase analysis, authors (54) demonstrated that CD62L^{low} neutrophils with hypersegmented phenotype represent a separate cell population distinct from the banded neutrophils and mature segmented neutrophils. Based on proteome analysis, CD62L^{low} neutrophils are closer to the banded neutrophils than to mature segmented neutrophil population (54). The authors suggest that CD62L^{low} neutrophils represent a distinct subset that enters the bloodstream in response to inflammation. CD62L^{low} neutrophils are not likely to be the reverse-transmigrated neutrophils returning from the tissue back into the bloodstream due to the lack of expression of CD54 or reduced expression of CXCR1 and CXCR2 as determined by flow cytometry (31) and proteomics (54). Low expression of CD62L was also observed in the aged neutrophils subset in mice (46). This subset displayed upregulated CXCR4 receptor. In this study, we have not observed elevated expression of CXCR4 on the CD62L^{low} subset of neutrophils or LDNs in CVID patients. It is feasible that the CD62L^{low} phenotype described here results from a combination of the above-described mechanisms. Recently, a subset of neutrophils in spleen (N_{BH}) with CD62L^{low} CD11b^{hi} phenotype and a tendency to produce NETs has been described (58). N_{BH} display higher expression of CD27, CD40L, CD86, CD95, and HLA-DR and activate Ig production by marginal zone B cells via BAFF, APRIL, and IL-21 (57–62). In addition, several recent reports have indicated the induction of neutrophil–dendritic cell hybrids expressing CD80 and HLA-DR and presenting Ags to CD4⁺ T cells in a variety of models and diseases (63–66). In this study, we have not observed elevated expression of CD80 and HLA-DR on neutrophils from CVID patients. Whether the alteration of neutrophil phenotype directly contributes to the dysregulation of Ig production in CVID patients remains unclear. Future studies should focus on more detailed characterization of a CD62L^{low} population of neutrophils in CVID patients to discern between the alternative mechanisms of downmodulation of CD62L.

CD11b is a marker of degranulation that can be rapidly upregulated on stimulated cells by stimulation with bacterial products, including LPS and fMLF (Fig. 6). In addition, anti-CD3-activated CD8⁺ T cells modulate neutrophil levels of CD11b by IFN- γ production (67). CD11b (complement receptor 3 [CR3], MAC-1) is an integrin that forms complexes with CD18 and is stored in secretory, gelatinase, and specific granules (49). Our observation of increased CD11b on CVID neutrophils (Fig. 2) is inconsistent with a previous study (68). The reason for the difference between studies is unclear but can be related to differences in cell processing and staining conditions that may result in different levels of surface CD11b and other markers of degranulation.

We show that CVID patients exhibit an increased frequency of LDNs (Fig. 5). Multiple studies have reported the elevated frequency of LDNs with potent T cell suppressive activity in various inflammatory conditions (30, 34, 36, 37). Although we have not directly addressed the immunosuppressive activity of LDNs in the current study, we report a negative correlation between the frequency of LDNs in PBMCs and intracellular production of IFN- γ by CD8⁺ T cells of CVID patients *ex vivo* ($r = -0.4$; $p = 0.007$). The mechanism of induction of LDNs is currently unclear. As shown in Fig. 9, stimulation of whole

blood with bacterial products fMLF and LPS results in rapid induction of LDNs in the PBMC layer following gradient centrifugation. Thus, it is feasible that LDNs arise from the contact of neutrophils with translocated circulating bacterial products in vivo (11, 12). However, it is unclear whether LDNs induced by ex vivo stimulation are phenotypically identical to those observed in the fresh blood of CVID patients. Overall, the accumulated evidence suggests that low-density phenotype is a cellular property rather than a marker of a specific cell population.

A major mechanism of neutrophil suppression of T cells is the production of ROS (30, 32). Production of ROS and release of arginase can result in downregulation of TCR ζ on T cells, thereby arresting the cells in the G0–G1 phase (32). Our observation that the suppression of T cell activation and production of IFN- γ is ROS-dependent is consistent with previous studies (33, 69). Rapid production of ROS by neutrophils can result in immediate suppression of T cell activation and IFN production. Another potential mechanism of T cell suppression is the upregulation of PD-L1 (Fig. 2) associated with IFN-dependent PD-1-mediated T cell apoptosis (32, 68, 70). The increase of expression of PD-1 on T cells in CVID patients was previously described (9, 10). Although it is feasible that PD-L1/PD-1 interaction contributes to T cell suppression in CVID, we did not observe a correlation between expression of PD-1 on T cells, and enhanced expression of PD-L1 on neutrophils and T cell proliferation was not affected by blocking the PD-L1/PD-1 interaction.

In conclusion, the study presented in this article suggests that neutrophils in CVID patients acquire an altered phenotype and exert potent T cell suppressive activity. The presented results have several limitations, including limited sample size of a cross-sectional study design. Although the data need to be confirmed in future detailed studies employing larger populations, the observations presented in this article provide a qualitatively new view on potential mechanisms of immune suppression in CVID and open new avenues for therapeutic targeting of CVID and other inflammatory disorders.

Disclosures

The authors have no financial conflicts of interest.

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Phenotype and oxidative burst of low-density neutrophil subpopulations are altered in common variable immunodeficiency patients

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Abstract

Common variable immunodeficiency disorder (CVID) is the most common form of primary antibody immunodeficiency. Due to low antibody levels, CVID patients receive intravenous or subcutaneous immunoglobulin replacement therapy as treatment. CVID is associated with the chronic activation of granulocytes, including an increased percentage of low-density neutrophils (LDNs). In this study, we examined changes in the percentage of LDNs and the expression of their surface markers in 25 patients with CVID and 27 healthy donors (HD) after in vitro stimulation of whole blood using IVIg. An oxidative burst assay was used to assess the functionality of LDNs. CVID patients had increased both relative and absolute LDN counts with a higher proportion of mLDNs compared to iLDNs, distinguished based on the expression of CD10 and CD16. Immature LDNs in the CVID and HD groups had significantly reduced oxidative burst capacity compared to mature LDNs. Interestingly we observed reduced oxidative burst capacity, reduced expression of CD10 after stimulation of WB, and higher expression of PD-L1 in mature LDNs in CVID patients compared to HD cells. Our data indicate that the functional characteristics of LDNs are closely linked to their developmental stage. The observed reduction in oxidative burst capacity in mLDNs in CVID patients could contribute to an increased susceptibility to recurrent bacterial infections among CVID patients.

KEYWORDS

common variable immunodeficiency, intravenous immunoglobulins, low-density neutrophils, oxidative burst, suppression

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1 | INTRODUCTION

Neutrophils represent the largest subpopulation of leukocytes in blood circulation (Bouma et al., 2010), and being key players in innate immunity, they are also the most predominant leukocytes at the site of inflammation (Liew & Kubes, 2019). Recent evidence suggests that neutrophils play an active role in the coordination of innate and adaptive immunity (Rosales, 2020). When triggered by pathogens, they activate multiple antimicrobial functions, such as phagocytosis (Uribe-Querol & Rosales, 2020), degranulation (Eichelberger & Goldman, 2020), and the formation of extracellular traps (NETs) (Mulay & Anders, 2020). Density gradient centrifugation of peripheral blood collected from patients with acute or chronic inflammatory diseases has revealed a heterogeneous group of neutrophils trapped in the peripheral blood mononuclear cell (PBMC) layer, the so-called low-density neutrophils (LDNs) (Scapini et al., 2016). Increased counts of LDNs were found in the periphery of patients with cancer (Brandau et al., 2011), infections including human immunodeficiency virus (Bowers et al., 2014) and sepsis (Darcy et al., 2014), as well as autoimmune diseases (Carmona-Rivera & Kaplan, 2013; Darcy et al., 2014; Ui Mhaonaigh et al., 2019; Wright et al., 2017). Phenotypically, LDNs in the PBMC layer are distinguished by the presence of common neutrophil lineage markers CD15 and CD66b. A detailed subclassification of LDNs was first published by Marini et al. (2017), who used flow cytometry to detect the expression of markers CD10 and CD16 on the cell surface. Based on their observations, LDNs are subdivided into CD16⁺CD10⁻ immature (iLDNs) and CD16⁺CD10⁺ mature (mLDNs).

As part of phagocytosis, the process of oxidative burst is activated, in which molecular oxygen is reduced due to the influence of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This process leads to the subsequent formation of the superoxide radical and reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]), and hypochlorous acid (HClO). ROS are main components of neutrophil killings in response to microbial invasion, serving as messengers in normal cell transduction and the cell cycle. Inside phagocytes, the formed hydrogen peroxide and hypochlorite ions strongly oxidize dihydrorhodamine 123 (DHR123) to fluorescent rhodamine 123, which is detectable by flow cytometry (Chen & Junger, 2012). While the presence of mature and immature LDNs has also been reported by other studies (Brandau et al., 2011; Matthews et al., 2021; Tay et al., 2020), none have performed a characterization of these cells during oxidative burst.

Common variable immunodeficiency (CVID) is the most frequent form of primary hypogammaglobulinemia characterized by decreased serum levels of immunoglobulin (Ig)G and (Ig)A in the presence of varying levels of (Ig)M (Chapel & Cunningham-Rundles, 2009). CVID represents a heterogeneous group of defects that clinically manifest themselves as frequent and complicated respiratory tract infections, diarrhea, and autoimmune disorders (Bonilla et al., 2016). Defects in the number and function of B, T, or NK cells have been previously reported (Chandra et al., 2016). Recent studies have also shown increased activation of innate immunity, altered expression of surface

markers, and changes in the number of dendritic cells (Sharifi et al., 2017) and monocytes (Barbosa et al., 2012), as well as defects in their function. Our previous studies showed that chronic neutrophil activation was associated with immune dysregulation in patients with CVID (Litzman et al., 2019; Vlkova et al., 2019). Furthermore, we previously observed a systemic activation of myeloid cells and an increased production of cytokines involved in granulopoiesis and the regulation of neutrophil recruitment and activation (Hel et al., 2014).

Intravenous immunoglobulin (IVIg) treatment is the standard therapy for antibody immunodeficiencies. IgG used in IVIg therapy binds to various immunoglobulin receptors on immune cells, which may lead to either the stimulation or inhibition of the immune system (Nagelkerke & Kuijpers, 2015). Recent evidence has shown that IVIg alleviates the state of chronic immune activation (Dominguez-Soto et al., 2018; Kessel et al., 2011; Kozicky et al., 2018; Trépanier et al., 2014). Through increased expression of CD64, flow cytometry measurements by Tsintzi et al. (2020) further confirmed the persistent activation of polymorphonuclear leukocytes (PMNs) and demonstrated that immunoglobulin treatment increases the activation capacity of neutrophils after exposure to lipopolysaccharide (LPS).

Recent research has revealed that LDNs manifest in numerous diseases, exhibiting a significant positive correlation with the severity of these conditions (Bowers et al., 2014; Rocha et al., 2015). In addition to increased activation of neutrophils and elastase serum levels, we have previously reported increased proportion of LDNs in the peripheral blood of patients with CVID after IVIg administration (Litzman et al., 2019; Vlkova et al., 2019). Although neutrophils have been extensively studied in patients with CVID, there seems to be limited emphasis on investigating the occurrence of LDNs. Taken together, this latest evidence raises new questions regarding more in-depth analyses of LDNs, which would examine their heterogeneity, oxidative burst capacity and characterize the effects of IVIg on the fraction of LDNs. New knowledge about LDNs could contribute to a better understanding of immune dysregulation in patients with CVID.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy donors

The study was reviewed and approved by the Medical Ethics Committee of St. Anne's University Hospital (Brno, Czech Republic), ethics approval number: 6G/2015. Written informed consent was obtained from all participants prior to inclusion in the study. A total of 25 CVID patients (15 females, 10 males; median age: 51, range: 23–86 years) were recruited. All patients fulfilled the International Consensus Document (ICON) diagnostic criteria for CVID (Bonilla et al., 2016). The control group consisted of 27 HD (16 females, 11 males; median age: 48, range: 24–73 years). CVID patients were treated with regular IVIg substitution, and peripheral blood samples were collected into S-Monovette heparin-containing vacutainers (Sarstedt, Nümbrecht, Germany) before the infusion. No participants suffered from acute health problems.

2.2 | Cell isolation

PBMCs were isolated from heparinized peripheral blood with density gradient centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). WB was layered on Ficoll and centrifuged for 25 min at 400 g without brake. The fraction of PBMCs was collected from the plasma-lymphocyte separation medium interface. Aside from monocytes and lymphocytes, PBMCs also contained LDNs. PBMC suspension was transferred to phosphate-buffered saline (PBS) and washed twice. Next, the cells were re-suspended in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and a combination of penicillin, streptomycin, and L-glutamine (Sigma, St. Louis, MO), referred to as complete RPMI medium. The number of cells was quantified using the Vicell XR cell counter (Beckman Coulter, Brea, CA, USA).

2.3 | Stimulation of the WB for LDN induction

Samples were stored at 22°C, and all procedures began at the latest 1 hour after blood collection. Samples were divided into 4 aliquots (1 non-stimulated aliquot and 3 stimulated aliquots), using 2 µg/mL formyl methionyl-leucyl-phenylalanine (fMLF; Sigma, St. Louis, MO), 2 µg/mL LPS (*E. coli* 0111:B4; Sigma) (Vlkova et al., 2019), and IVIg (Privigen, CSL Behring GmbH, Marburg, Germany) at a dose of 5.7 mg/mL of heparinized blood, calculated based on a therapeutically relevant dose for infusion (i.e., 400 mg IgG/kg). Samples treated with fMLF and LPS were incubated at 37°C for 60 minutes, while samples treated with IVIg were incubated for 120 min. Non-stimulated and stimulated WB was further processed for PBMC isolation according to the cell isolation protocol described above. LDNs were analyzed using flow cytometry.

2.4 | Flow cytometry characterization of LDNs

LDNs were characterized by identifying combination of markers expressed on their surface. A mixture of fluorescent-labeled human monoclonal antibodies (Exbio Praha, Vestec, Czech Republic) was

used: CD15 FITC (Fluorescein Isothiocyanate, clone MEM-158), CD193 PE (Phycoerythrin, clone 5E8), CD14 PerCP Cy5.5 (Peridinin-chlorophyll Cyanin 5.5, clone MEM-15), CD10 PC7 (R-Phycoerythrin Cyanin 7, clone MEM-78), CD274 APC (Allophycocyanin, clone 29E.2A3), CD11b AF 700 (Alexa Fluor 700, clone ICRF44), CD45 PB (Pacific Blue, clone 2D1), and CD16 PO (Pacific Orange, clone 3G8). Samples were incubated with monoclonal antibodies in the dark at 4°C for 20 min, followed by washing with PBS. Analysis was performed using the flow cytometer Navios (10 colors, 3 lasers, Beckman Coulter, Inc. Miami, FL, USA), and the results were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

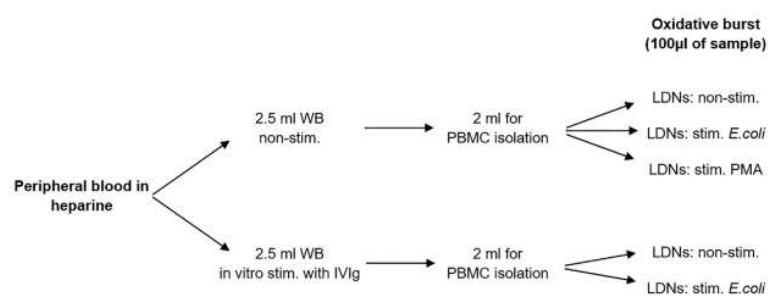
2.5 | Cell sorting and light microscopy

PBMCs were prepared according to the cell isolation protocol described above. Staining of LDN subsets in the fraction of PBMCs was performed with the mixture of antibodies used for the characterization of LDNs by flow cytometry, as described above. LDN subpopulations were sorted with the MOFLO Astrios cell sorter (Beckman Coulter, Brea, CA, USA). Sorted subpopulations were stained according to the standard Mayer's hematoxylin and eosin staining protocol. Stained cells were observed using a standard light microscope with 400x magnification.

2.6 | Oxidative burst of LDNs

The oxidative burst of LDNs was quantified as the mean fluorescence intensity of rhodamine 123 assessed by flow cytometry. Firstly, part of the heparinized WB was pre-stimulated with IVIg for 2 h at 37°C. PBMCs were isolated from the non-stimulated WB and WB pre-stimulated with IVIg according to the cell isolation protocol described earlier. Next, 1 aliquot from each condition served as a non-stimulated control for the oxidative burst, and 1 aliquot was stimulated with opsonized *E. coli* (Department of Biology, Faculty of Medicine, MU, Brno, Czech Republic). One aliquot of PBMCs from the non-stimulated WB was stimulated with 2 µg/mL Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). Experimental conditions used for quantifying the oxidative burst are shown in Figure 1. Tubes were

FIGURE 1 Experimental conditions used for quantifying oxidative burst. WB was non-stimulated or stimulated in vitro with IVIg. Part of the WB was further used for PBMC isolation. LDNs were stimulated with *E. coli* or PMA for the detection of oxidative burst.



incubated for 15 min at 37°C. After 15 min of incubation, dihydroxyrhodamine 123 (Sigma-Aldrich, Saint Louis, MO, USA) was added to the samples, which were then incubated for 15 minutes. Subsequently, the samples were washed with PBS and further incubated in the dark at 4°C for 30 min with the mixture of monoclonal antibodies: CD15 PC7 (R-Phycoerythrin-Cyanin 7, clone W6D3, Sony), CD10 APC (Allophycocyanin, clone HI10a, Sony), CD16 APC-Cy7 (Allophycocyanin-Cyanin 7, clone 3G8, Exbio), CD11b PB (Pacific Blue, clone ICRF44, Exbio), and CD45 PO (Pacific Orange, clone HI30, Exbio). Samples with PBMCs were washed with PBS. Analysis was performed on the flow cytometer Navios (10 colors, 3 lasers, Beckman Coulter, Inc. Miami, FL, USA), and the results were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

2.7 | Assessment of changes in LDNs

Peripheral blood of CVID patients was collected into heparin tubes before intravenous administration of IVIg. Part of the WB was processed immediately according to the PBMC isolation procedure. The rest of the WB was stimulated *in vitro* at 37°C for 2 h with IVIg preparation in a therapeutically relevant dose for infusion (i.e., 400 mg IgG/kg), followed by PBMC isolation. Two hours after *in vivo* IVIg administration, peripheral blood collection from CVID patients was again performed, and the WB was processed according to the PBMC cell isolation procedure. PBMC samples were further processed for the flow cytometry characterization as described earlier.

2.8 | Statistics

Differences between CVID patients and HD were explored using the non-parametric Mann-Whitney U test or Wilcoxon signed-rank test, as appropriate. Correlations were determined using Spearman's rank-order correlation. The *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed in GraphPad Prism (Version 5; GraphPad Software Inc., CA, USA).

3 | RESULTS

3.1 | Characterization of LDN subtypes

A specific surface marker capable of differentiating LDNs from peripheral PMNs has not yet been identified, and it remains necessary to use density centrifugation and characterize LDNs as a fraction of PBMCs based on the expression of surface markers characteristic of PMNs. In our experiments, we used combination of flow cytometry measurements and light microscopy for exact determination of LDNs. First, granulocytes were gated from PBMCs as CD45⁺CD14^{dim}CD15⁺ cells. Using the marker CD193, we removed eosinophils from selected granulocytes, thus we obtained a population of LDNs. Size (FSC) and granularity (SSC) of gated LDNs correspond with the PMNs. Next, we

used a combination of surface markers CD10 and CD16 to distinguish CD45⁺CD15⁺CD16⁺CD10⁺ immature (iLDNs) and CD45⁺CD15⁺CD16⁺CD10⁺ mature (mLDNs) LDNs (Figure 2a) (Marini et al., 2017). The accuracy of the gating strategy was confirmed by visualizing iLDNs and mLDNs in a light microscope, where iLDNs and mLDNs were obtained using the fluorescence-activated cell sorting method. mLDNs typically had segmented nuclei with abundant pale pink cytoplasm (Figure 2b), whereas iLDNs had round, less segmented nuclei with a higher nucleus/cytoplasm ratio (Figure 2c).

3.2 | Phenotype of LDNs in healthy donors

To better characterize mLDNs and iLDNs, we used flow cytometry to analyze the expression of surface markers on LDNs obtained from HD. In addition to the low expression of CD10 and CD16, iLDNs also displayed low expression of adhesion molecules CD62L (data not shown) and integrin CD11b, as well as reduced expression of CD45 and CD66b (data not shown). Thus, iLDNs showed a phenotype described as CD45^{dim}CD16⁺CD10⁺CD11b⁺CD62L⁺CD66b^{dim} compared to mLDNs that showed an overall phenotype described as CD45⁺CD16⁺CD10⁺CD11b⁺CD62L⁺CD66b⁺ (Figure 3a,b).

3.3 | PBMCs contain an increased proportion of mLDNs in CVID patients

To further examine the potential role of LDNs in CVID pathogenesis, we evaluated the percentage of LDNs in PBMCs isolated from fresh blood. CVID patients compared to HD displayed higher percentage (Figure 4a) and absolute numbers (Figure 4b) of LDNs in the PBMC and higher relative proportion (Figure 4c) of LDNs within the total neutrophils, which was consistent with our previous study (Vlkova et al., 2019). The frequencies of LDNs in various diseases are summarized in Table 1. In both CVID and HD groups, isolated PBMCs from the non-stimulated WB contained a significantly higher percentage of mLDNs relative to iLDNs (Figure 4d). Moreover, an increased proportion of all LDNs in CVID patients suggests that they had a significantly higher percentage of mLDNs compared to HD, whereas there was no significant difference in the percentage of iLDNs between the two groups (Figure 4d).

3.4 | LDN production is increased after *in vitro* stimulation with IVIg

A possible explanation behind the increased percentage of LDNs in CVID patients is the treatment with IVIg, as we have previously reported that CVID patients display increased levels of granulocyte activation markers (Litzman et al., 2019). To better understand the properties and sources of LDNs in CVID patients and HD, we examined changes in the percentage of LDNs after WB stimulation with IVIg. A significant increase in the percentage of LDNs was observed in

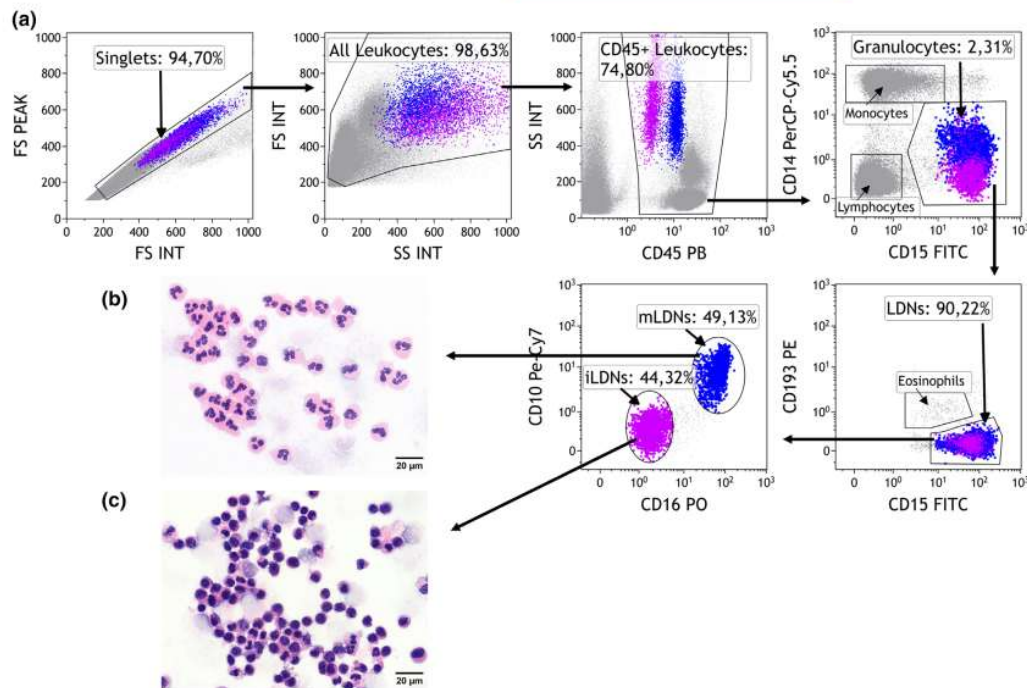


FIGURE 2 Definition of LDNs and LDN subtypes: immature and mature LDNs. (a) Plots showing the gating and sorting strategy of PBMC suspension purified by gradient centrifugation. First gate was set on FSC-A and FSC-H to eliminate doublets, then physical parameters, that on CD45⁺ to eliminate debris and LDNs were defined as CD45⁺CD14⁺CD15⁺CD193⁺ cells and further subdivided into CD15⁺CD10⁺CD16⁺ iLDNs and CD15⁺CD10⁺CD16⁺ mLDNs based on the surface expression of CD10 and CD16. Representative data from one CVID patient were analyzed in Kaluza. The maturity of sorted LDN subpopulations was controlled using light microscopy. (b) mLDNs had a typical morphology of mature segmented neutrophils with pale pink cytoplasm and condensed chromatin separated by thin filaments into 2–5 nuclear lobes, whereas (c) iLDNs had less abundant cytoplasm (higher nucleus/cytoplasm ratio) and unsegmented nuclei with less condensed chromatin. Representative Mayer's hematoxylin and eosin staining images of mLDNs and iLDNs (out of two independent experiments) were acquired with a 400x objective. Scale bar represents 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

CVID patients as well as in HD after WB stimulation with IVIg (Figure 5a). In both groups, an increase in LDNs after WB stimulation with IVIg was not dependent on the initial proportion of LDNs in the WB without stimulation (data not shown). WB stimulation with IVIg did not cause significant changes in the percentage of iLDNs (Figure 5b) in both groups, whereas there was significant increase in the percentage of mLDNs (Figure 5c). CVID patients, in comparison to HD, showed a significantly elevated proportion of mLDNs after WB stimulation with IVIg (Figure 5c).

3.5 | In vivo stimulation of LDNs after IVIg administration

Next, we aimed to determine the possible formation of LDNs in the blood of CVID patients after intravenous administration of IVIg and compare the results to those of in vitro experiments. The formation of LDNs was measured in the PBMC samples obtained from the

peripheral blood of 7 CVID patients collected prior to IVIg administration, 2 h after IVIg administration, and 2 hours after in vitro incubation of peripheral blood with IVIg. In vitro stimulation of the WB with IVIg caused an increase in the proportion of mLDNs in the fraction of PBMCs (Figure 5e). However, these results were not confirmed in the samples after IVIg application in vivo. In 4 CVID patients, the percentage of LDNs increased, but 3 patients responded with a reduced number of LDNs (Figure 5f).

3.6 | WB stimulation induces changes in the expression of LDN surface markers of HD

Our next goal was to detect changes in the expression of surface markers on iLDNs and mLDNs of HD after WB stimulation. WB was stimulated as described earlier, and surface expression of CD10, CD11b, CD15, CD16, CD45 and PD-L1 was measured on the surface of obtained LDNs.

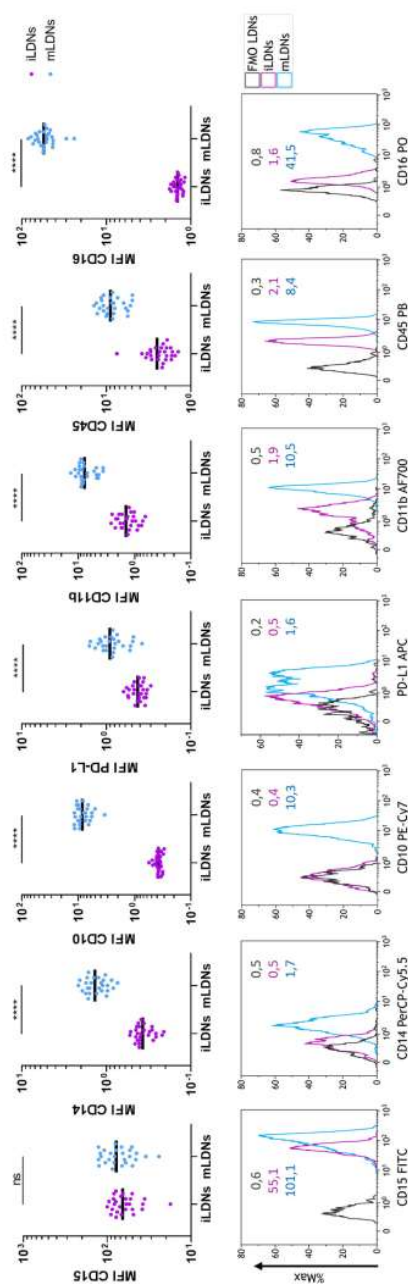


FIGURE 3 Comparison of phenotypes of LDNs from HD. (a) Surface expression of markers CD15, CD14, CD10, PD-L1, CD11b, CD45, and CD16 was determined by the flow cytometry measurement and compared between iLDNs and mLDNs from HD ($n = 27$, combined data from 12 independent experiments, each performed with 2–4 samples). iLDNs showed decreased expression of all measured markers compared to mLDNs, except for CD15. Horizontal bars represent the median. Wilcoxon signed-rank test was used for the statistical analysis (ns = non-significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$). (b) Illustrative overlay histograms showed expression of surface markers by LDN subpopulations from one HD (FMO control- gray line, iLDNs- purple line, mLDNs- blue line). MFI values of LDN subpopulations and FMO controls are illustrated in the plots. [Color figure can be viewed at wileyonlinelibrary.com]

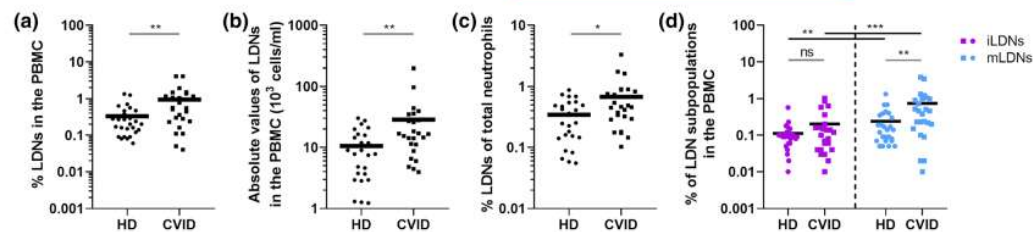


FIGURE 4 CVID patients show an increased frequency of predominantly mature LDNs. (a) The proportion and (b) absolute numbers of LDNs in the PBMC fraction and (c) the proportion of LDNs in the total neutrophils purified from non-stimulated WB samples by density gradient centrifugation was determined using the flow cytometry measurement. (d) The percentage of iLDNs and mLDNs was recalculated according to the total number of LDNs in the sample. Combined data of 27 HD and 25 CVID patients from 24 independent experiments, each performed with 2–4 samples, are shown. Horizontal bars represent the median. Data were analyzed using the Wilcoxon signed-rank test (black line) or Mann–Whitney U test (gray line), as appropriate (ns = non-significant, * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Overview of LDN frequency in various diseases.

		LDNs		Author
		Median (%)	Range (%)	
Immunodeficiency	CVID	0.55	0.05–4.06	
Sepsis	Sepsis with shock	19.2	4.4–29.5	Darcy et al. (2014)
	Sepsis no shock	2.7	1.5–6.1	Darcy et al. (2014)
	Sepsis	37.3	n.s.	Sun et al. (2022)
Cancer	Head and neck squamous cell carcinoma	5.5	2–34	Brandau et al. (2011)
	Hodgkin and non-Hodgkin lymphoma	2.2	0.02–70.92	Marini et al. (2017)
Autoimmunity	Rheumatoid arthritis	>1	1–24.2	Wright et al. (2017)
	ANCA vasculitis	17.4	11.2–40.7	Ui Mhaonaigh et al. (2019)
Infectious diseases	SFTS	46.8	n.s.	Li et al. (2018)

Note: LDN frequency for certain diseases is displayed as the median, and the range shows the minimum and maximum LDN percentage values. Abbreviations: CVID, common variable immunodeficiency; n.s. – not specified; SFTS, severe fever with thrombocytopenia syndrome.

Significant increase was detected in the expression of CD16 and PD-L1 on iLDNs of HD after WB stimulation with fMLF and LPS. All used stimulants caused increase in the relative numbers of PD-L1⁺ iLDNs (Supplementary Figure 2c). Moreover, CD11b and CD45 were also significantly increased after WB stimulation with IVIg. Conversely, significant decrease was detected in the expression of CD15 on iLDNs of HD after WB stimulation with fMLF and LPS (Figure 6a). No changes were detected in the expression of CD10 on iLDNs of HD after all used stimulants (data not shown).

WB stimulation with fMLF and LPS caused significant increase in the expression of CD11b and CD10 on mLDNs of HD and for CD45 also after WB stimulation with IVIg. Conversely, there was significant decrease in the expression of CD15 on mLDNs of HD after WB stimulation with fMLF and LPS. (Figure 6b) No changes were detected in the expression of PD-L1 on mLDNs of HD after all used stimulants (data not shown), however, there was increase in the relative numbers of PD-L1⁺ mLDNs after stimulation of WB with fMLF and LPS (Supplementary Figure 2d).

3.7 | Differences in the expression of LDN surface markers between CVID patients and HD

To provide more comprehensive characterization of LDNs of CVID patients, we compared expression of their surface markers with the LDNs of HD in the PBMC from non-stimulated WB and from WB stimulated with IVIg, fMLF, and LPS.

iLDNs from non-stimulated WB of CVID patients showed higher expression of CD15, CD10, and CD11b compared to iLDNs of HD (Supplementary Figure 1). No between-group differences were detected in the expression of CD16, CD45 and PD-L1 on iLDNs from non-stimulated WB (data not shown). iLDNs of CVID patients and HD had a comparable expression of the measured markers after WB stimulation with IVIg, fMLF, and LPS (data not shown). No significant difference was detected in the relative numbers of PD-L1⁺ iLDNs between CVID patients and HD (Supplementary Figure 2c).

mLDNs from non-stimulated WB of CVID patients had higher expression of CD11b and PD-L1 compared to mLDNs of HD

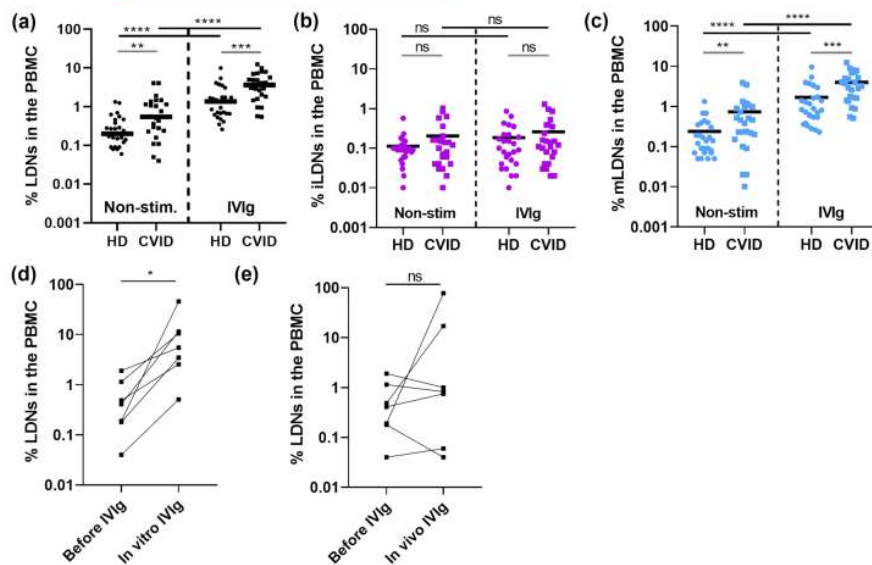


FIGURE 5 WB stimulation with IVIg caused increase of mLDNs. The proportion of (a) LDNs, (b) iLDNs, and (c) mLDNs in the PBMC purified from WB samples of HD and CVID patients stimulated with IVIg. Combined data of 27 HD and 25 CVID patients from 24 independent experiments, each performed with 2–4 samples, are shown. Horizontal bars represent the median. The proportion of LDNs in the PBMC fraction was compared in CVID patient samples ($n = 7$, combined data from 7 independent experiments) before and (e) 2 hours after in vivo IVIg administration or (d) after 2 h of in vitro IVIg stimulation of the WB collected prior to IVIg administration (in vitro IVIg). (d) In vitro stimulation of the WB with IVIg induced the formation of LDNs, whereas (e) in vivo administration of IVIg did not validate the results of the in vitro experiments. Data were analyzed using the Wilcoxon signed-rank test (black line) or Mann–Whitney U test (gray line), as appropriate (ns = non-significant, $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; and $****p \leq 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

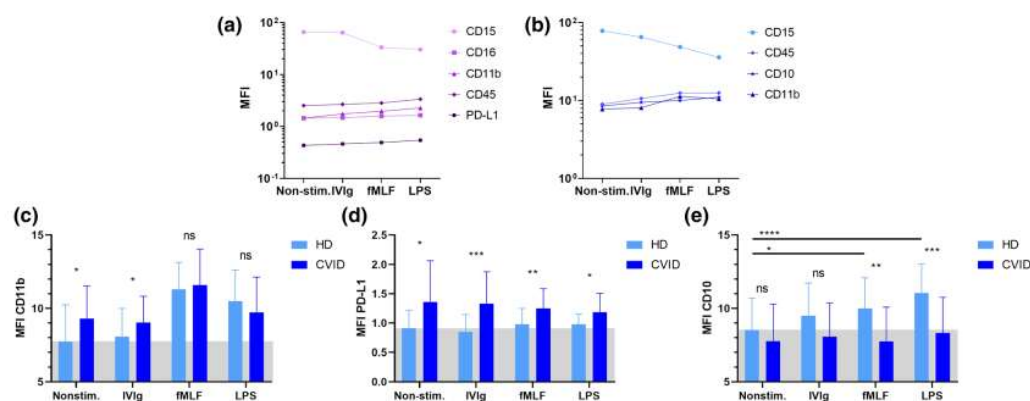


FIGURE 6 Changes in the surface expression of markers of LDN subpopulations after WB stimulation. The WB of 27 HD was either non-stimulated or stimulated with 5.7 mg/mL IVIg for 2 h, 2 μ g/mL fMLF, and 2 μ g/mL LPS for 1 h. Statistically significant changes in the expression levels of surface markers after stimulation on (a) iLDNs and (b) mLDNs are depicted in the graphs. Each point represents median of MFI values from 27 HD. Combined data from 24 independent experiments, each performed with 2–4 samples. The WB of 27 HD and 25 CVID patients were non-stimulated or stimulated as mentioned above. Statistically significant differences in the expression of (c) CD11b (d) PD-L1 and (e) CD10 in mLDNs of CVID patients compared with HD after stimulation of WB are shown. Combined data from 24 independent experiments, each performed with 2–4 samples are shown as mean + standard deviation. Data were analyzed using the Wilcoxon signed-rank test or Mann–Whitney U test, as appropriate (ns = non-significant, $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; and $****p \leq 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 6c,d). Moreover, CVID patients had higher proportion of PD-L1+ mLDNs in the PBMC from non-stimulated WB compared to HD (Supplementary Figure 2d). No between-group differences were detected in the surface expression of others measured markers on mLDNs from non-stimulated WB. mLDNs of CVID patients compared to HD showed higher expression of CD11b after WB stimulation with IVIg (Figure 6c) and higher expression of PD-L1 and higher relative numbers of PD-L1+ cells after all used stimulants (Figure 6d, Supplementary Figure 2d). Unlike in HD, IVIg, fMLF, and LPS did not increase CD10 expression in mLDNs of CVID patients (Figure 6e).

3.8 | iLDNs have limited oxidative burst capacity

Oxidative burst is one of the fundamental functions of neutrophils in antimicrobial host defense and inflammation. Therefore, we assessed whether iLDNs and mLDNs had comparable oxidative burst capacity. To detect the oxidative burst in HD, a DHR assay was performed

using stimulation with opsonized *E. coli* or PMA. A significant increase in oxidative burst was observed in mLDNs after both stimulations (Figure 7a,b). Our results showed that oxidative burst was markedly reduced in iLDNs than mLDNs regardless of the used stimulants (Figure 7a). iLDNs had a very limited oxidative burst capacity after being stimulated with opsonized *E. coli* or PMA (Figure 7a,c).

3.9 | mLDNs show a reduced oxidative burst capacity in CVID patients

To assess functional properties of LDNs in CVID patients and HD, we evaluated measurements of the oxidative burst using flow cytometry. When comparing the oxidative burst of iLDNs, we observed similar results in CVID patients as in HD: iLDNs were not capable of the oxidative burst after in vitro stimulation with opsonized *E. coli* or PMA, relative to mLDNs (Figure 7d). Our measurements showed that mLDNs of CVID patients had a reduced oxidative burst capacity than

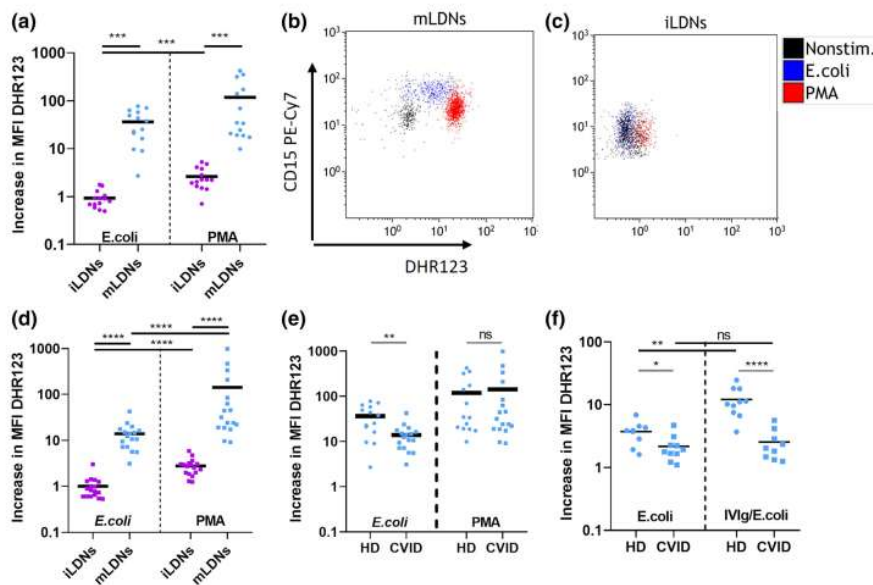


FIGURE 7 Oxidative burst of LDN subpopulations after *E. coli* or PMA stimulation and the effect of IVIg pre-stimulation. PBMC from (a) HD ($n = 14$) and (d) CVID patients ($n = 17$) were stimulated with opsonized *E. coli* or PMA to determine oxidative burst of LDNs using the flow cytometry measurement of DHR123 fluorescence. Comparison of the oxidative burst of LDNs between CVID patients ($n = 17$) and HD ($n = 14$) after stimulation is shown in graph (e). The increase in MFI DHR123 (Y-axis) expresses the ratio between MFI DHR123 of the non-stimulated sample and MFI DHR123 of the sample stimulated with opsonized *E. coli* or PMA. Horizontal bars represent the median. Combined data from 16 independent experiments, each performed with 2–4 samples, are shown. Representative dot plots from one HD comparing DHR123 expression on (b) mLDNs and (c) iLDNs from non-stimulated PBMC after stimulation with *E. coli* or PMA. (f) Comparison of the oxidative burst of LDNs between CVID patients ($n = 10$) and HD ($n = 10$) from 6 independent experiments, each performed with 2–4 samples, after combined 2-h pre-stimulation with IVIg and subsequent stimulation with *E. coli*. The increase in MFI DHR123 (Y-axis) expresses the ratio between the increase in MFI DHR123 after combined IVIg and *E. coli* stimulation and in MFI DHR123 after *E. coli* stimulation. Horizontal bars represent the median. Data were analyzed using the Wilcoxon signed-rank test (black line) or Mann–Whitney U test (gray line), as appropriate (ns = non-significant, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

mLDNs of HD after stimulation with opsonized *E. coli* (Figure 7e) and no difference was detected between the two groups after PMA stimulation (Figure 7e).

3.10 | Oxidative burst of LDNs after in vitro WB stimulation with IVIg

Our results demonstrated that IVIg stimulates neutrophils and may induce the formation of additional LDNs. Next, we wanted to explore whether in vitro stimulation with IVIg would positively affect the oxidative burst of iLDNs and mLDNs in patients with CVID. To determine the effects of IVIg on the oxidative burst of LDNs, the WB was first stimulated for 2 hours with the addition of IVIg. Afterward, LDNs were stimulated by opsonized *E. coli* to determine their oxidative burst capacity, later referred to as combined IVIg and *E. coli* stimulation. Our results showed that iLDNs of CVID patients and HD were not influenced by combined IVIg and *E. coli* stimulation and were not capable of oxidative burst (data not shown).

In HD, combined IVIg and *E. coli* stimulation caused a significant increase in the oxidative burst of mLDNs compared to stimulation with *E. coli* without IVIg pre-stimulation (Figure 7f). At the same time, the oxidative burst of mLDNs in CVID patients did not significantly change after combined IVIg and *E. coli* stimulation (Figure 7f). mLDNs of CVID patients did not have as high of an oxidative burst as mLDNs of HD after combined IVIg and *E. coli* stimulation. (Figure 7f).

4 | DISCUSSION/CONCLUSION

In this study, we showed that LDNs represent a small but highly specialized population of neutrophils that includes immature and mature cells. They differ in their functional properties: compared to mature neutrophils, immature neutrophils have a significantly reduced oxidative burst capacity. The number of mature neutrophils in LDNs can be increased in vitro by stimulation with IVIg, fMLF, or LPS.

LDNs are generally characterized as PMNs based on the expression of various surface antigens, including CD33⁺/CD66b⁺/CD15⁺/CD14⁺. Nowadays, several publications subdivide LDNs into mature and immature based on the surface expression of CD10, CD11b, or CD16. CD16⁺CD11b⁺ iLDNs and CD16⁺CD11b⁺ mLDNs have been previously described in patients with urological cancer or head and neck cancer (HNC) (Brandau et al., 2011), as well as Hodgkin and non-Hodgkin lymphoma (Marini et al., 2016). In patients with sepsis, LDNs have been reported to be formed during various stages of neutrophil maturation (Sun et al., 2022).

Our flow cytometry results showed that 60% of LDNs in the PBMC samples of HD were, on average, composed of mLDNs with a high expression of myeloid markers CD10, CD11b, CD15, CD16, CD45, CD62L, and CD66b. In comparison, 40% of LDNs were phenotypically immature based on the low expression of the measured myeloid markers. In contrast, recent publications describe LDNs in HD as mature cells with the equivalent expression of surface markers and

standard morphology of mature neutrophils (Blanco-Camarillo et al., 2021; Gareth R. Hardisty et al., 2021). We speculate that the variability in the results may be explained by differences in the protocols used to isolate LDNs or gating strategies for the flow cytometry measurements. Moreover, the frequency of LDNs in the PBMC samples of HD was around 0.2%, and immature neutrophils comprised only 0.08% of the total LDNs. Probably, iLDNs have not been analyzed in previous studies due to their very low quantity.

Previously published results, as well as our data, indicate that LDN composition is variable and dependent on the disease type, as shown in Table 1. The current study confirmed our previous findings: compared to HD, CVID patients produce more LDNs, mLDNs in particular, however it remains unknown why patients with CVID display an increase in the number of LDNs.

IVIg replacement, together with the subcutaneous immunoglobulin replacement route, is the first-line treatment for CVID patients. Our current study showed that in vitro stimulation of the WB with IVIg caused a significant increase in the proportion of mLDNs in the PBMC fraction of both CVID patients and HD. The increment of LDNs was significantly higher in CVID patients compared to HD. The mechanism leading to the formation of LDN after in vitro stimulation remains unknown. It could be potentially attributed to the presence of a dimeric form of IgG in therapeutic IVIg preparations. Consistently, IgG aggregates are absent in the majority of IVIg preparations, however up to 1%–10% of IgG in dimeric form can be found in most IVIGs depending on the manufacturer and a batch (Dolman et al., 2001). According to the study of Coxon et al. (2001), immune complexes, which are accessible for neutrophils, lead to their activation and migration into tissues. Multivalent cross-linking of CD16b elicits downstream signals including the release of Ca²⁺ from intracellular stores (Walker et al., 1991). Additional effector functions observed after CD16b cross-linking include degranulation and activation of respiratory burst (Fernandes et al., 2005). Degranulation and increased expression of integrins on the neutrophil surface could cause interaction with monocytes, lymphocytes or platelets, which may account the increased number of LDNs in PBMC fraction. Vlkova et al. (2019) described, that patients with CVID had activated neutrophils compared to HD, therefore, it could be possible that IVIG could activate neutrophils of CVID patients more significantly, which could eventually lead to increased LDNs frequency in PBMCs.

To examine the possibility of IVIg treatment inducing the formation of LDNs, we compared the effect of in vitro stimulation of the WB and in vivo stimulation after IVIg administration on the percentage of LDNs in 7 CVID patients. In vitro stimulation caused a substantial increase in the percentage of LDNs, while after in vivo stimulation, there was a decrease in the percentage of LDNs in some patients but an increase in others. While in vivo LDNs could have been produced from a pool of neutrophils in the bone marrow, and many factors could have influenced their production and maturity, these mechanisms are typically very limited in vitro. Based on the obtained results, we were unable to identify common mechanisms or pathways that could explain the differences in LDNs between in vitro and in vivo stimulation. It is possible that in vivo activation of neutrophils by IVIg

or microbial translocation may be different across individual patients and more data need to be collected.

CD10 is expressed on the surface of mature neutrophils. This antigen also has enzymatic activity, and its main function is to regulate the activation of neutrophils by degrading of inflammatory peptides and influencing the morphology and migration of neutrophils (Shipp et al., 1991). Kaneko et al. (Kaneko et al., 2003) further described a homogenous increase in CD10 expression after in vitro stimulation of neutrophils with LPS. Our data confirmed that stimulation of mLDNs induces elevated CD10 expression in HDs, with a tendency to even higher expression after IVIg, fMLF, and LPS administration, respectively. mLDNs of CVID patients did not display increased CD10 expression after administering IVIg or stronger stimulants, such as fMLF or LPS. Decreased capacity of neutrophils to express CD10 has been reported in patients with septic shock, indicating that secretory vesicles containing CD10 were fully mobilized in vivo and that neutrophils were unable to further upregulate CD10 after in vitro stimulation (Martens et al., 1999). Recently, “aged neutrophils” have been found in patients with psoriasis, with reduced expression of CD10, CD11b, and CD16. That subpopulation of neutrophils also had a reduced oxidative burst capacity compared to mature neutrophils (Rodríguez-Rosales et al., 2021). However, decreased CD10 expression has not been described in patients with chronic diseases yet, which is relevant to CVID patients. On the other hand, our study did not reveal a decrease in CD11b or CD16 expression on the mLDNs in CVID patients as published in the case of patients with psoriasis.

It remains uncertain based on our observations whether the diminished CD10 expression on mLDNs of CVID patients is a result of the mild immaturity of these neutrophils or if it is indicative of neutrophils showing signs of aging. Both of these populations showed changes in the expression of other markers such as CD11b, CD16 and a reduced capacity for oxidative burst. Therefore additional research is required to elucidate the function and mechanistic consequences of decreased CD10 expression after stimulation in mature neutrophils from patients with CVID.

PD-L1 is a molecule with an immunomodulatory function that is expressed in various cells, including myeloid cells. PD-L1 interaction with the programmed cell death protein 1 (PD-1) receptor triggers inhibitory signals that regulate T cell responses (Keir et al., 2008). mLDNs of CVID patients obtained from the non-stimulated WB expressed higher PD-L1 than HD, which could be related to the suppression of specific immune reactions and, as a consequence, chronic inflammation. It could be a characteristic feature of mLDNs in CVID patients, with other features including CD10 expression.

Oxidative burst plays an essential role in the response of neutrophils to a variety of pathogens. Using fMLF cell stimulation, Hardisty et al. (2021) reported that healthy LDNs had a production of ROS comparable to that of normal neutrophils. Moreover, Blanco-Camarillo et al. (2021) described that LDNs, compared to normal neutrophils, had enhanced ROS production after stimulation with PMA. Our tests of the oxidative burst performed on distinguished iLDNs and mLDNs showed that iLDNs were incapable of the oxidative burst after being stimulated with opsonized *E. coli* or PMA. Stimulation of neutrophils

via Fc receptors FcγRIIIa (CD32) and FcγRIIIb (CD16) is necessary for the oxidative burst that is mediated by the IgG immune complex (van der Heijden et al., 2014). Oxidative burst is also induced by the activation of β2 integrins (Nguyen et al., 2017). The phenotype of immature neutrophils with low expression of CD11b, CD16, or CD32 reflects incomplete maturation of granules, especially specific vesicles that have a membrane on which these receptors are expressed and from which they reach the plasma membrane upon activation (Borregaard & Cowland, 1997) and indicates a possible reduction in their oxidative burst capacity.

The varying results may be attributed to a different gating strategy than that described in Blanco-Camarillo et al. (Blanco-Camarillo et al., 2021), where the authors did not consider CD16^{low} neutrophils (i.e., iLDNs) in the analyses, or to the LDN isolation protocol itself presented in Hardisty et al. (Gareth R. Hardisty et al., 2021), where iLDNs may not have been captured.

Compared to mLDNs of HD, we observed a reduced capacity of mLDNs for the oxidative burst in CVID patients after stimulating LDNs with opsonized *E. coli*. In addition, combined pre-stimulation of the WB with IVIg and subsequent stimulation of LDNs with opsonized *E. coli* did not increase the oxidative burst of LDNs in CVID patients. On the other hand, no difference was detected in the oxidative burst of mLDNs between the two groups after PMA stimulation. IVIg has been shown to modulate the functions of various cells in both innate and adaptive immunity (Dolcino et al., 2014; Siedlar et al., 2011). Activation and increased oxidative burst were observed in peripheral blood neutrophils in vitro after low doses of IVIg (Casulli et al., 2011). In this study, the authors also observed increased CD11b expression and greater ROS production in neutrophils after IVIg (cIVIg = 5 mg/mL) + LPS stimulation. These data are consistent with our observed increased oxidative burst in HD after IVIg pre-activation and subsequent *E. coli* stimulation. However, we did not observe an increase in CD11b expression after IVIg stimulation in either HD or CVID patients on mLDN. Nevertheless, CD11b expression was increased on mLDN in comparison with neutrophils in whole blood in both investigated groups (data not shown). On the other hand, we also observed reduced oxidative burst capacity in mLDNs from CVID patients after *E. coli* stimulation, similar to Casulli et al., where peripheral blood neutrophils from CVID patients showed decreased degranulation, phagocytosis, and reactive oxygen species production (Casulli et al., 2014). These observations indicate that neutrophils from CVID patients including mLDN might show dysregulation in their functional properties.

In conclusion, CVID patients have defects in cells of specific and non-specific immunity, including neutrophils, in particular LDNs (Barbosa et al., 2012; Sharifi et al., 2017; Vlkova et al., 2019). We showed that LDNs are composed of immature and mature LDNs, which differ between CVID patients and HD. iLDNs in HD as well as in CVID patients are not capable of an oxidative burst. mLDNs in CVID patients have limited oxidative burst capacity, and expression of the PD-L1 suppressor molecule is enhanced in LDNs of CVID patients. Our study provides valuable and novel data by defining the phenotype and characterizing the oxidative burst capacity of LDNs in

both CVID patients and HD. We propose that LDNs represent the most activated state of neutrophils in peripheral blood. The increased presence of LDNs in CVID patients may serve as an indicator of chronic inflammation or potentially reflect episodes of acute inflammation.

The observed reduction in oxidative burst capacity in mLDNs is likely linked to the decreased production of ROS by neutrophils in CVID patients, as demonstrated in the study by Casulli et al. (2014). This reduction may contribute to an increased susceptibility to recurrent bacterial infections among CVID patients. However, we do not believe that the functionality of LDNs, specifically their oxidative burst capability, directly contributes to the pathogenesis of CVID. Instead, it likely indicates a potential dysregulation of the immune response in these patients. Nonetheless, further research is necessary to elucidate the relationship between activated neutrophils and mLDNs in CVID patients and to understand the implications of their limited functional capacity.

AUTHOR CONTRIBUTIONS

Peter Slanina performed the flow cytometry experiments and analyzed data. Julie Stichova and Terezie Plucarova helped in performing the experiments. Zita Chovancova recruited patients and obtained blood samples. Veronika Bosakova performed sorting of cells. Iva Stanickova Zambo evaluated and captured the light microscopy images. Marcela Hortova Kohoutkova and Petra Laznickova prepared cells for microscopy. Marcela Vlkova supervised the study. Peter Slanina, Jiri Litzman, Jan Fric, and Marcela Vlkova wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated and analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of St Anne's University Hospital, Brno. The patients/participants provided their written informed consent to participate in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Abstract

Common variable immunodeficiency disorders (CVIDs) represent a group of primary immunodeficiency diseases characterized by hypogammaglobulinemia and dysfunctional immune response to invading pathogens. Previous studies have indicated that CVID is associated with microbial translocation and systemic myeloid cell activation.

The goal of this study was to determine whether patients with CVID display elevated systemic levels of markers of granulocyte activation and whether the levels are further influenced by intravenous immunoglobulin (IVIg) infusions. The plasma levels of granulocyte activation markers elastase and myeloperoxidase were determined using enzyme-linked immunosorbent assay (ELISA) in 46 CVID patients and 44 healthy controls. All CVID patients were in a stable state with no apparent acute infection. In addition, granulocyte activation markers' plasma levels in 24 CVID patients were determined prior to and 1 h following IVIg administration. Neutrophil elastase and myeloperoxidase plasma levels were significantly higher in CVID patients than in healthy controls. Systemic elastase levels were further increased following IVIg administration. In vitro stimulation of 13 CVID patients' whole blood using IVIg in a therapeutically relevant dose for 2 h resulted in a significant increase in plasma elastase levels compared to unstimulated blood. The data presented here indicate that CVID is associated with chronic granulocytic activation which is further exacerbated by administering IVIg. Increased myeloperoxidase and elastase levels may contribute to associated comorbidities in CVID patients.

Keywords

CVID, elastase, immunoglobulin treatment, myeloperoxidase, neutrophil activation

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Introduction

Common variable immunodeficiency disorders (CVIDs) comprise the most frequent symptomatic primary hypogammaglobulinemia characterized by decreased immunoglobulin G (IgG) levels accompanied by decreased immunoglobulin A (IgA) and/or immunoglobulin M (IgM) serum levels and disturbed response to antigenic stimuli.¹ Clinically significant immunodeficiency in CVID patients is manifested by severe respiratory tract infections, diarrhea, and autoimmune disorders.¹

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Neutrophil elastase and myeloperoxidase are stored in large quantities in the neutrophils' azurophilic granules and are released into extracellular space following neutrophil activation. The release of elastase, a serine proteinase with broad substrate specificity, results in damage to host tissue in a wide range of inflammatory conditions.² Neutrophil elastase plasma levels were shown to be increased in patients with pneumonia,^{3,4} inflammatory bowel disease,⁵ and preeclampsia.⁶ Systemic levels of myeloperoxidase are elevated in patients with acute coronary syndrome,⁷ pelvic inflammatory disease,⁸ and rheumatic arthritis.⁹

CVID was shown to be associated with an altered neutrophil phenotype characterized by decreased expression of surface markers CD15, CD11b, and CD16.¹⁰ CVID patients display elevated systemic cytokine levels associated with granulocyte-macrophage lineage activation.¹¹ Decreased reactive oxygen species (ROS) production of granulocytes pretreated with toll-like receptor (TLR) 1/2 and TLR 4 agonists after stimulating with N-formylmethionyl-leucyl-phenylalanine (fMLP) was documented, suggesting disturbed granulocytic function in CVID patients.¹⁰ However, no significant difference in granulocytic oxidative burst activity following stimulation with opsonized *Escherichia coli* or phorbol-myristate-acetate (PMA) was observed in CVID patients compared to healthy donors.^{12–14}

The goal of this study was to determine whether the levels of granulocyte activation markers released from azurophilic granules are altered in patients with CVID and whether they are affected by the infusion of intravenous immunoglobulin (IVIg).

Materials and methods

Study participants

The study was approved by the Medical Ethics Committee of St. Anne's University Hospital. Informed consent was obtained from all participants prior to inclusion in the study. A total of 46 CVID patients (25 females, 21 males; median age: 45, range: 22–82 years) were recruited. All patients fulfilled the International Consensus Document (ICON) diagnostic criteria for CVID.¹ The control group consisted of 44 healthy donors (23 females, 21 males; median age: 41, range: 19–78 years). The control persons were recruited mainly from the hospital employees and their relatives, not suffering from a known immunodeficiency or autoimmune disease.

Splenomegaly was defined as a spleen length > 11 cm in ultrasonography. Bronchiectasis was determined by high-resolution computed tomography (HRCT). The clinical phenotypes were defined as described in Chapel et al.¹⁵ B-cell phenotypes were determined according to Wehr et al.¹⁶ (EUROclass).

Plasma neutrophil elastase and myeloperoxidase determination

The blood was collected in an acute infection-free period. In 24 patients on IVIg treatment (aged 22–82 years), elastase and myeloperoxidase plasma levels were determined prior to and 1 h after the completion of IVIg infusion.¹⁴

Human elastase and myeloperoxidase plasma levels were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Hycult Biotech, Plymouth Meeting, PA, USA). The samples were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes (S-Monovette; Sarstedt, Nümbrecht, Germany) and placed on ice. Within 20 min, the plasma was separated by centrifugation for 15 min at 1500 g at 4°C. Without disturbing the buffy coat, the plasma was transferred into a polypropylene tube and centrifuged again for 15 min at 1500 g at 4°C. All plasma samples were stored at –20°C.

In vitro stimulation of blood with IVIg

A total of 13 CVID patients' whole blood was collected into three heparinized tubes (S-Monovette) at room temperature. Plasma from the first tube was collected as described above. The second tube was incubated in a water bath at 37°C with gentle mixing every 30 min; after 2 h, plasma was collected. In the third tube, 10% IVIg at a dose of 5.7 mg/mL of heparinized blood (IVIg concentration adequate to the concentration in blood after infusion of 400 mg IgG/kg) was added. The subsequent procedure was identical to the one in tube number 2. Plasma elastase levels were determined as described above.

Statistical analysis

The results were analyzed using the Mann–Whitney rank-sum test, Wilcoxon signed-rank test, and analysis of variance (ANOVA) test as appropriate using Stata and GraphPad Prism 5 statistical

packages. The results are presented as median (5th–95th centile).

Results

Plasma elastase and myeloperoxidase levels

Basic clinical and laboratory characteristics of the CVID patients are shown in Table 1. The absolute number of neutrophils was higher in CVID patients than that in the healthy controls ($3.4 \times 10^9/L$ (1.8–6.8) vs $2.7 \times 10^9/L$ (1.8–4.4); $P=0.02$). The CVID patients displayed significantly higher neutrophil elastase (100.5 $\mu g/L$; (55.8–184.0) vs 66.9 $\mu g/L$ (42.2–99.6); $P<0.001$) and myeloperoxidase (28.2 $\mu g/L$ (15.2–56.6) vs 14.1 $\mu g/L$ (8.4–19.7); $P<0.001$; analyzed by Mann–Whitney test) plasma levels than the healthy controls (Figure 1). The difference remained significant after the CVID patients with C-reactive protein (CRP) higher than 10 mg/L ($n=7$) were excluded from the analysis (elastase: 89.2 $\mu g/L$ (54.8–162.1); $P=0.04$; myeloperoxidase: 27.5 $\mu g/L$ (12.1–55.9); $P<0.001$). No significant differences were observed between patients treated with IVIg ($n=25$) versus subcutaneous immunoglobulin (SCIg; $n=21$) ($P=0.2$ for elastase and $P=0.1$ for myeloperoxidase). Compared to healthy controls, the levels of granulocytic markers were significantly higher in both IVIg (elastase: 110.4 $\mu g/L$ (55.8–162.1); myeloperoxidase: 26.5 $\mu g/L$ (12.1–44.7)) and SCIg (elastase: 83.1 $\mu g/L$ (56.5–191.9); myeloperoxidase: 29.5 $\mu g/L$ (17.1–62.5)) recipients (all $P<0.001$; analyzed by Mann–Whitney test).

Significantly elevated neutrophil elastase and myeloperoxidase plasma levels were observed in patients with splenomegaly ($n=21$) than patients without splenomegaly ($n=24$) (elastase: 114.9 $\mu g/L$ (54.8–162.1) vs 76.4 $\mu g/L$ (56.5–156.9); $P=0.005$; myeloperoxidase: 35.2 $\mu g/L$ (12.1–56.6) vs 26.5 $\mu g/L$ (15.8–55); $P=0.02$). No significant differences were observed between patients with ($n=19$) and without ($n=26$) bronchiectasis.

Using clinical phenotype classification,¹⁵ patients classified as “no disease-related complications” ($n=28$) displayed a lower plasma myeloperoxidase concentration (26.9 $\mu g/L$ (15.2–55.9)) than patients with other complications of CVID ($n=18$) (40.1 $\mu g/L$ (12.1–71.7); $P=0.007$). Patients with enteropathy ($n=8$) had higher myeloperoxidase levels (42.9 $\mu g/L$ (12.1–71.7)) than patients without this complication ($n=38$) (27.3 $\mu g/L$ (15.2–55.9); $P=0.006$). No significant

differences were observed between patients with ($n=7$) and without ($n=39$) lymphocytic infiltration, and with ($n=9$) and without ($n=37$) autoimmune disorders. No patient suffered from lymphoid malignancy. No significant differences were observed in plasma elastase levels.

The ANOVA test did not show any significant difference in elastase ($P=0.35$) and myeloperoxidase ($P=0.06$) plasma levels comparing patient groups defined according to EUROclass.¹⁶

Effect of administering IVIg on plasma neutrophil elastase and myeloperoxidase levels

Administering IVIg resulted in a significant increase in neutrophil elastase plasma levels 1 h after treatment ($P=0.001$; Wilcoxon signed-rank test; Figure 2). A trend toward increased myeloperoxidase levels was observed; however, the effect did not reach statistical significance ($P=0.17$). No adverse reaction to infusion was observed in any of the participants.

The in vitro effect of IVIg on elastase release

To evaluate the potential activation effect of IVIg on CVID neutrophils, whole heparinized blood was stimulated with IVIg. As shown in Figure 3, 2-h cultivation of patients' blood at 37°C leads to an increase in the elastase concentration in plasma by 74.5% (37.2%–273.3%). Stimulating whole blood from CVID patients with IVIg leads to an increase in plasma elastase levels by 104.0% (56.2%–301.3%). The difference between the increase with and without IVIg was statistically significant ($P=0.017$; Wilcoxon signed-rank test; Figure 3).

Discussion

The presented data, together with previously published observations, are indicative of an ongoing chronic granulocytic activation in CVID patients. The cause of activation of granulocytes in CVID remains unclear. However, the data are consistent with a potential role of translocation of microbial products from the intestinal lumen into the systemic circulation. Microbial translocation is observed in various pathological conditions including HIV-1 infection, inflammatory bowel disease, and hepatitis B and C infections.^{17,18} Bacterial and fungal products' translocation results in systemic

Table 1. Clinical and laboratory characteristics of the patients at the time of the investigation.

Patient No.	Age	Sex	Treatment	CRP (mg/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	B-cell phenotype	Splenomegaly	Bronchiectasis	Clinical phenotype	Immunosuppressive treatment
1	31	F	SClg	28.80	6.48	<0.02	0.33	smB-2l low	No	Yes	A,E	Prednisone, leflunomide
2	39	M	IVlg	4.49	4.90	<0.02	0.09	smB+2l norm	No	No	N	
3	82	F	IVlg	5.60	6.42	<0.02	0.05	smB+2l low	No	Yes	E	
4	25	M	SClg	1.47	4.55	<0.02	0.13	smB+2l low	No	No	N	
5	66	F	IVlg	6.10	7.37	<0.02	0.06	smB-2l low	Yes	Yes	L	
6	52	F	SClg	4.51	7.59	<0.02	0.09	smB+2l norm	Yes	No	N	
7	42	F	SClg	1.70	5.90	0.02	0.04	smB-2l norm	No	No	A	
8	46	F	SClg	4.56	8.01	<0.02	0.06	B-	No	No	A	Prednisone
9	76	F	IVlg	8.26	7.68	<0.02	0.06	smB+2l norm	Yes	Yes	N	
10	58	M	IVlg	5.44	5.97	<0.02	0.04	B-	No	No	N	
11	62	M	IVlg	1.71	8.62	<0.02	0.27	smB+2l low	No	Yes	N	
12	30	M	IVlg	2.59	7.47	<0.02	0.06	smB-2l norm	Yes	No	N	
13	22	M	SClg	23.40	10.20	<0.02	0.05	B-	No	Yes	L,E	
14	38	M	IVlg	22.80	6.87	<0.02	0.07	smB-2l norm	Yes	Yes	N	
15	34	M	SClg	1.35	12.80	0.37	0.05	smB+2l low	No	No	N	
16	63	M	IVlg	7.25	5.87	<0.02	0.06	smB+2l low	Yes	Yes	N	
17	28	F	IVlg	8.24	6.40	<0.02	0.21	smB+2l norm	No	No	A	
18	20	M	IVlg	3.03	5.89	<0.02	0.18	smB+2l low	Yes	No	A	
19	76	M	SClg	8.87	7.93	0.10	0.11	smB-2l norm	No	Yes	L	
20	35	F	IVlg	1.27	7.07	<0.02	0.15	smB+2l low	ND	ND	N	
21	49	F	SClg	3.00	6.82	0.04	0.07	smB-2l norm	Yes	Yes	L	
22	29	F	SClg	2.43	5.52	0.04	1.41	smB+2l low	No	No	N	
23	45	F	SClg	6.92	6.72	0.02	0.04	smB-2l low Tr+	No	No	L,A	
24	48	M	SClg	12.40	7.37	<0.02	0.06	smB-2l low Tr+	Yes	No	E	
25	33	F	IVlg	8.02	8.86	<0.02	0.10	smB-2l norm	No	No	N	

Table 1. (Continued)

Patient No.	Age	Sex	Treatment	CRP (mg/L)	IgG (g/L)	IgA (g/L)	IgM (g/L)	B-cell phenotype	Splenomegaly	Bronchiectasis	Clinical phenotype	Immunosuppressive treatment
26	37	F	SClg	9.16	5.15	<0.02	0.04	B-	No	No	E,A	Methylprednisone
27	52	F	IVIg	25.40	4.00	<0.02	0.05	smB-2llow	No	No	N	
28	45	M	SClg	3.30	6.60	0.03	0.08	smB-2lnorm	Yes	No	N	
29	38	F	SClg	5.59	4.66	<0.02	0.08	smB-2lnorm	No	No	N	
30	48	F	SClg	3.27	8.65	<0.02	0.13	smB-2llow	Yes	No	N	
31	65	M	SClg	6.65	7.44	0.06	0.06	smB-2llow	Yes	Yes	E,A	
32	46	F	IVIg	2.91	9.22	<0.02	0.07	smB-2llow	Yes	Yes	N	
33	51	F	IVIg	4.75	8.06	<0.02	0.06	smB-2llow	No	Yes	N	
34	22	M	SClg	23.10	5.56	<0.02	2.29	smB-2llow	Yes	Yes	A	
35	31	M	IVIg	10.80	4.78	0.04	0.68	smB-2llow	Yes	No	LE	Prednisone
36	48	F	SClg	1.67	7.84	<0.02	0.06	smB-2lnorm	No	No	N	
37	30	M	SClg	12.20	6.40	<0.02	0.10	smB+2llow	No	No	N	
38	30	M	SClg	8.23	6.11	0.04	0.08	smB-2llow	Yes	Yes	N	
39	58	M	IVIg	7.80	6.24	0.29	0.04	smB+2llow	No	Yes	N	
40	52	F	IVIg	4.63	5.32	<0.02	0.06	smB-2lnorm	Yes	No	L	
41	63	M	IVIg	8.35	7.85	<0.02	0.29	smB-2llow	Yes	Yes	N	
42	30	F	IVIg	7.35	6.01	0.08	0.97	smB+2lnorm	Yes	No	N	
43	58	M	IVIg	8.38	9.88	<0.02	0.07	smB+2lnorm	Yes	No	N	
44	52	M	IVIg	25.50	9.46	<0.02	0.11	smB-2lnorm	No	Yes	N	
45	36	F	IVIg	2.26	8.72	<0.02	0.05	smB-2llow	No	Yes	N	
46	29	M	IVIg	1.81	5.95	<0.02	0.07	smB-2llow	Yes	No	E	

IgG: immunoglobulin G; IgA: immunoglobulin A; IgM: immunoglobulin M; F: female; M: male; IVIg: intravenous immunoglobulin; SClg: subcutaneous immunoglobulin; CRP: C-reactive protein; N: no disease-related complications; E: unexplained enteropathy; L: polyclonal lymphocytic infiltration; A: autoimmune; ND: not detected.
 Serum immunoglobulin levels were determined before IVIg infusion or during routine follow-up in SClg-treated patients.
 B-cell phenotypes were determined according to EUROclass;¹⁶ Clinical phenotypes were defined according to Chapel et al.¹⁵

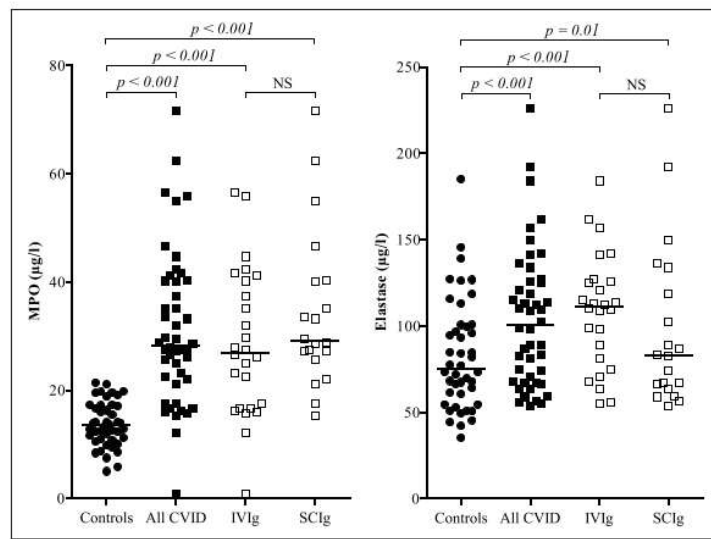


Figure 1. Plasma myeloperoxidase (MPO) and elastase levels in all CVID patients, patients on intravenous immunoglobulin (IVIg) and subcutaneous immunoglobulin (SCIg) treatments and healthy control donors.

NS: non-significant.

Horizontal lines indicate median values; Mann-Whitney rank-sum test was used for statistical evaluation.

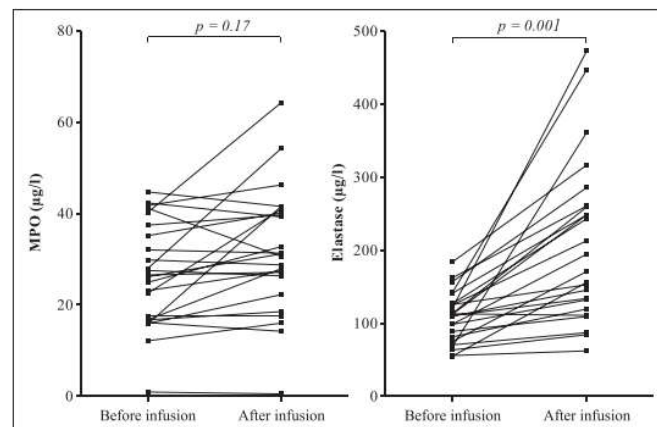


Figure 2. Plasma myeloperoxidase (MPO) and elastase levels before and 1 h after intravenous immunoglobulin infusion in CVID patients.

Wilcoxon signed-rank test was used for statistical evaluation.

immune activation.¹⁷ We and others have previously presented evidence of chronic microbial translocation in CVID patients.^{19–21} Furthermore, CVID patients display an altered cytokine signature profile consistent with an ongoing activation of cells of monocytic and granulocytic lineages.¹¹

In our study, we observed increased serum elastase and myeloperoxidase levels in patients with splenomegaly compared to patients without splenomegaly. Except for the observation showing higher serum myeloperoxidase levels in patients with splenomegaly and liver cirrhosis compared to

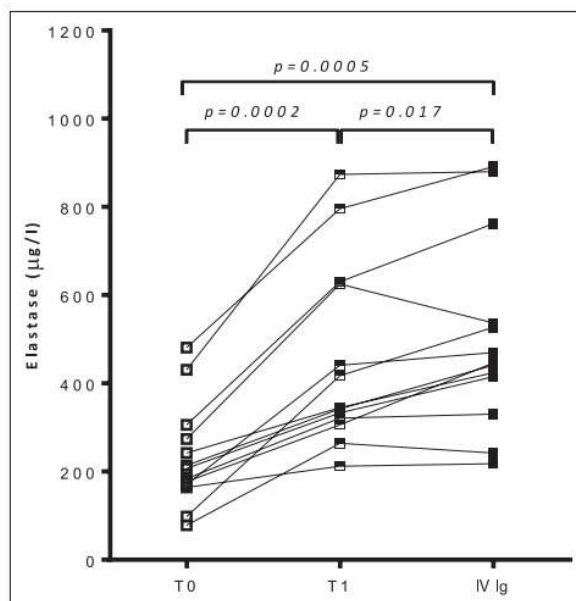


Figure 3. Plasma elastase concentration levels of 13 CVID patients after in vitro stimulation with IVIg.

Elastase concentration was determined in three plasma samples from each patient: T0—the plasma was obtained after the collection of heparinized blood from a patient; T1—heparinized blood was incubated in water bath at 37°C. After 1-h incubation plasma was collected; IVIg—heparinized blood was incubated in water bath at 37°C and stimulated with 10% intravenous immunoglobulin. After 2 h of incubation plasma was collected. All samples of plasma were separated by two consecutive centrifugations, and human elastase plasma levels were determined by ELISA, according to the manufacturer's protocol. Wilcoxon signed-rank test was used for statistical evaluation.

patients without splenomegaly,²² to our knowledge, no association of splenomegaly with myeloperoxidase and elastase levels has been previously reported. However, splenomegaly in CVID is frequently associated with other complications, for example, cytopenias, hepatomegaly, granuloma, enteropathy, autoimmunity, or bronchiectasis.^{15,23} Although we did not observe elevated levels of both granulocyte activation plasma markers in subjects with bronchiectasis, autoimmunity, or lymphatic infiltration in our cohort, the myeloperoxidase levels were lower in patients in the “no disease-related conditions” group¹⁵ than the group with disease-related conditions and increased in patients with enteropathy.

Elevated systemic elastase and myeloperoxidase levels may significantly contribute to the pathogenesis of CVID complications. Neutrophil elastase is involved in destroying the extra-cellular matrix in chronic obstructive pulmonary disease²⁴ and plays a pathogenic role in forming bronchiectasis.²⁵ Neutrophil-derived myeloperoxidase is involved in inducing oxidative stress

and producing proinflammatory cytokines including interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α .²⁴ Therapeutic approaches targeting granulocytic enzymes or curbing their proinflammatory properties may be beneficial to CVID patients.

Here, we report that the administration of IVIg resulted in elevated systemic concentrations of neutrophil elastase and a trend toward an increased concentration of myeloperoxidase at 1 h following the treatment. Limited data are available regarding the effect of IVIg on polymorphonuclear neutrophil (PMN) degranulation upon IVIg stimulation in vitro. Van Mirre et al.²⁶ observed decreased elastase release in isolated granulocytes stimulated by aggregated IgG by adding monomeric IgG (obtained from IVIg). This effect was not observed when PMNs were activated by fMLP/cytochalasin B; monomeric IgG alone did not stimulate PMN, suggesting that monomeric IgG acts as a low-affinity Fc γ R antagonist. On the other hand, in the whole blood, under the in vitro conditions more similar to our experiments, Teeling et al.²⁷ showed that in vitro exposure of whole fresh blood from

healthy donors to commercially available IVIg preparations at therapeutically relevant levels resulted in neutrophil degranulation and a release of elastase and lactoferrin. This effect was mainly mediated by the dimeric and polymeric fractions of the IVIg derivate and was dependent on FcγRII.²⁷ Currently available commercial preparations of IVIg contain less than 3% of polymeric IgG.²⁸ Whether or not neutrophil activation by IVIg depends on the presence of polymeric IgG warrants further investigation.

Other studies support potential activation of granulocytes by IVIg. Higurashi et al.²⁹ showed that IVIg administration increased the production of ROS from TNF-α-primed PMN in a process dependent on both the Fab and Fc domains of IgG and interaction with FcγRIII. Similarly, using whole-blood conditions, Casulli et al.³⁰ showed that IVIg at low concentrations induced PMN activation indicated by decreased CD62L and increased CD11b surface expression, enhancement of oxidative burst, and prolonged cell survival. In contrast, at higher concentrations, IVIg inhibited lipopolysaccharide (LPS)-induced CD11b degranulation and priming of oxidative burst.³⁰

Limited information is available regarding the effect of IVIg administration on neutrophils *in vivo*. A significantly reduced capacity for PMA-induced ROS production by PMN obtained from CVID patients after administering IVIg was observed.¹⁴ No effect on CD11b, CD16, and sialic acid-binding immunoglobulin-like lectin (Sialic) 9 expression on PMNs or ROS production induced by opsonized *E. coli* was documented in this study.¹⁴

Compared to our report, Prezzo et al.¹³ recently observed decreased serum elastase levels in patients with CVID treated by IVIg compared to healthy controls; the levels decreased 1 h after IVIg infusion. The results are not consistent with our study. The differences may be influenced by various factors, including the general clinical health of the investigated patients, the immunoglobulin brand used (stabilizers present in different brands), and others. A probable explanation may also be the variances in the handling of patients' plasma. Compared to our study, Prezzo et al.¹³ used heparinized plasma. Significant differences in sampling conditions when determining the plasma elastase levels were previously demonstrated.³¹ Our observation of increased plasma elastase levels after IVIg treatment is supported by the fact

that previous studies showed an increase in proinflammatory cytokines after IVIg infusions, including neutrophil-activating cytokines TNF-α, IL-6, and IL-8.^{32,33} The observation of an increase in plasma elastase levels following IVIg infusion is supported by other reports of *in vitro* elastase release²⁷ and general granulocyte activation^{29,30} after *in vitro* stimulation of whole blood by IVIg.

In summary, the data presented here indicate chronic granulocyte activation in CVID patients, independent of concurrent infection and other forms of disease exacerbation. Administering IVIg further enhances granulocytic degranulation in CVID patients. Pharmacologically targeting granulocytic activation and degranulation may represent a novel approach to CVID treatment.

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10 Přejchod k výzkumu granulocytů u imunokompromitovaných pacientů

Po více než dekádě výzkumu zaměřeného převážně na T- a B-lymfocytární subpopulace u pacientů s primárními imunodeficiencemi se odborné zaměření našeho pracoviště rozšířilo na studium funkčních vlastností granulocytů, zejména neutrofilů, u pacientů s imunokompromitovaným statusem – především po těžkém traumatu, u pacientů v intenzivní péči nebo s podezřením na sepsi.

Víceparametrová průtoková cytometrie nám umožnila detailně sledovat analyzovat fenotyp a funkci jednotlivých subpopulací neutrofilů. To zahrnovalo hodnocení jejich funkční kapacity (fagocytóza, oxidační vzplanutí, mitochondriální potenciál) a korelaci těchto parametrů s klinickým stavem pacientů (SIRS, CARS, septický šok, TRISS a ISS skóre). Cílem těchto studií bylo zjistit, jak těžký úraz remodeluje vzájemné poměry v zastoupení neutrofilních subpopulací a zda lze tyto změny využít pro časnou predikci komplikací nebo k individualizaci terapie pacienty.

Využili jsme kombinaci následujících markerů: CD16 (FcγRIII) jako marker maturace a aktivace, CD62L (L-selectin) jako adhezní molekulu, jejíž exprese klesá při aktivaci, a CD66b a CD11b jako markery degranulace a adheze. Na základě exprese CD16 a CD62L lze definovat hlavní subpopulace neutrofilů: (1) zralé neutrofile (CD16⁺CD62L^{hi}) (2) banded neutrofile (neutrofilní tyče) (CD16⁺CD62L^{low}), a CD16^{low}CD62L⁺ hypersegmentované supresorové neutrofile⁸⁴ a „exhausted“ neutrofile (CD16^{low}CD62L^{low}).⁸⁵

U pacientů po traumatu jsme pozorovali dynamické změny v zastoupení těchto subpopulací. V časně fázi po úrazu (do 24 hodin) dominovaly nezralé formy (neutrofilní tyče), zatímco ve fázi systémové zánětlivé odpovědi (TP2, 5.–7.den) výrazně přibývalo zralých, hypersegmentovaných a CD16^{low}CD62L^{low} neutrofilů. Tento posun byl doprovázen změnami v aktivačním profilu buněk, a to, poklesem oxidační kapacity. Tyto fenotypové a funkční změny jsme dále analyzovali z hlediska jejich souvislosti s klinickými parametry – zejména s vývojem SIRS, hladinami laktátu a skóre závažnosti poranění (TRISS, ISS).

U pacientů s komplikovaným průběhem, zejména s rozvojem SIRS, jsme zaznamenali sníženou fagocytární aktivitu „exhausted“ neutrofilů, sníženou schopnost generovat ROS po stimulaci *Staphylococcus aureus* a sníženou schopnost po aktivaci produkovat mitochondriální superoxid. Funkční poruchy exhausted neutrofilů a jejich zvýšený počet, který se typicky vyskytoval v pozdější fázi zánětlivé odpovědi, se pravděpodobně shoduje s poklesem celkové zánětlivé aktivity a přechodem do fáze kompenzační antiinflamatorní odpovědi u traumatizovaného pacienta.

Ukazujeme, že pacienti s neinfekčním SIRS vykazují zvýšené hladiny laktátu v séru ve srovnání s pacienty bez SIRS. Kromě toho jsme pozorovali, že hladiny laktátu v séru při přijetí negativně korelují s procentem neutrofilních tyčí. Zvýšený laktát, marker metabolického stresu a tkáňové hypoperfuze, odráží závažnost buněčného poškození a hypoxie a je široce uznáván jako prediktor závažnosti traumatu a časného klinického zhoršení.^{86,87} Podobně zvýšené hladiny kreatinikínázy (CK), indikátoru poškození svalů, korelují s procentem neutrofilních tyčí, zejména u pacientů s traumatickým SIRS. Hladiny CK jsou také spojeny s komplikacemi, jako je akutní poškození ledvin, zejména v případech rabdomyolýzy, což je stav spojený se zvýšenou úmrtností.⁸⁸ Pozorované korelace mezi neutrofilními tyčemi a hladinami laktátu nebo CK naznačují, že metabolický stres a svalové poranění vedou ke zvýšené poptávce po nezralých neutrofilech, doprovázené snížením počtu zralých neutrofilů. Tento jev

odráží nouzovou granulopoézu, ke které dochází v reakci na trauma.⁸⁹ Zajímavé je, že naše zjištění podporují hypotézu, že hladiny laktátu hrají klíčovou roli v řízení mobilizace neutrofilů z kostní dřeně.⁹⁰ Společná interpretace zastoupení neutrofilních populací s výsledky koncentrací laktátu a ktreatinázy v séru poukazují na roli metabolického a tkáňového stresu při formování reakce neutrofilů na trauma.

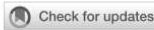
Podíl „exhausted“ neutrofilů naopak pozitivně koreloval s hladinami laktátu, kreatinázy a skóre závažnosti poranění (TRISS, ISS). Naše data podporují hypotézu, že neutrofilní dysfunkce představuje klíčový prvek sekundární imunosuprese u pacientů s polytraumatem.

Naše výsledky dále ukazují významné korelace mezi procentem neutrofilních tyčí a skórem závažnosti zranění (ISS), nebo se skórem závažnosti traumatu a zranění (TRISS). Zatímco ISS odráží anatomickou závažnost zranění, TRISS kombinuje závažnost poranění s fyziologickými parametry a demografickými faktory k predikci výsledků přežití. Pozoruhodné je, že skóre TRISS také korelovalo s procentuálním zastoupením neutrofilních tyčí v čase TP2, tedy přibližně čtyři dny po přijetí, což naznačuje souvislost mezi dynamikou změn v zastoupení neutrofilních populací a závažností traumatu.

Jak zánět postupuje, regulační mechanismy jsou zásadní pro obnovení homeostázy po odstranění poškozených tkání. Hypersegmentované CD16^{high}CD62L^{low} neutrofilie mohou přispívat k tomuto procesu regulací prozánětlivé aktivity T-lymfocytů prostřednictvím receptorů Mac-1, jak popsali Pillay et al.⁸⁴ V naší studii jsme pozorovali zvýšení hypersegmentovaných neutrofilů u pacientů bez SIRS na TP1 a u pacientů se SIRS na TP2. Negativní korelace mezi hypersegmentovanými neutrofilie a hladinami laktátu spolu s jejich nižším výskytem u pacientů se SIRS na TP1 naznačuje zhoršenou regulaci imunitní odpovědi u těchto jedinců. U TP2, zejména u pacientů se SIRS, jsme zaznamenali nárůst hypersegmentovaných neutrofilů doprovázený poklesem neutrofilních tyčí. Tento posun může znamenat nástup úspěšného návratu k homeostáze. K potvrzení této hypotézy a úplnému objasnění role hypersegmentovaných neutrofilů v imunitní regulaci po traumatu a neinfekčním SIRS jsou však zapotřebí další studie.

Získané výsledky ukazují, že fenotypová a funkční charakterizace neutrofilních subpopulací pomocí vícebarevné průtokové cytometrie může poskytnout cenné informace o aktuálním imunologickém stavu pacienta, prognóze vývoje zánětlivých a infekčních komplikací a eventuální potřebě cílené podpůrné nebo imunomodulační terapie.

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Pro-inflammatory role of neutrophils populations in trauma patients: monitoring neutrophil populations

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Background: Trauma is a leading global cause of mortality, and systemic inflammatory response syndrome (SIRS) remains a significant complication, contributing to adverse outcomes. Neutrophils, as first responders to tissue injury, undergo substantial phenotypic and functional changes following trauma. This study investigates neutrophil subpopulations defined by CD16 and CD62L expression in trauma patients, focusing on their correlation with clinical biomarkers, trauma severity, and functional properties.

Methods: We included 50 non-infectious trauma patients, categorized into SIRS and Non-SIRS groups, and 43 elective surgery patients as controls. Neutrophil subsets were analyzed at two time points (TP1 and TP2) using flow cytometry. Functional assays evaluated phagocytosis, oxidative burst, mitochondrial function, and degranulation. Correlations between neutrophil subpopulations and clinical markers, including lactate, creatine kinase, Injury Severity Score, and Trauma and Injury Severity Score, were examined.

Results: Patients with SIRS exhibited higher proportions of banded neutrophils and CD16^{low}CD62L^{low} neutrophils at TP1, alongside reduced levels of mature neutrophils. Elevated lactate and creatine kinase levels positively correlated with banded neutrophils and CD16^{low}CD62L^{low} neutrophils, while negatively correlating with

mature neutrophils CD16^{high}CD62L^{high} and hypersegmented neutrophils CD16^{high}CD62L^{low}. Hypersegmented neutrophils were more prevalent in Non-SIRS patients at TP1 and in SIRS patients at TP2. Banded neutrophils showed a positive correlation with Injury Severity Score and an inverse correlation with Trauma and Injury Severity Score (TRISS), whereas hypersegmented neutrophils were negatively associated with ISS and positively correlated with TRISS. These correlations likely reflect the pro-inflammatory role of banded neutrophils and the inflammation-resolving function of hypersegmented neutrophils. CD16^{low}CD62L^{low} neutrophils displayed impaired phagocytosis, oxidative burst, and degranulation capacity, indicating functional deficiencies.

Conclusion: This study highlights the dynamic changes in neutrophil subpopulations in trauma and their association with systemic inflammation and clinical severity. Increased banded neutrophils correlate with SIRS and metabolic stress, whereas hypersegmented neutrophils may contribute to resolving inflammation. CD16^{low}CD62L^{low} neutrophils exhibit functional impairments, warranting further investigation. Monitoring neutrophil subpopulations could aid in identifying trauma patients at risk for non-infectious SIRS and guide therapeutic interventions.

KEYWORDS

trauma, neutrophils, SIRS, lactate, creatine kinase, TRISS, ISS

Introduction

Trauma ranks among the ten leading causes of death worldwide and remains a significant public health challenge. While advancements in medical care have reduced trauma-related mortality (1, 2) caused by blood loss and coagulopathy, secondary complications such as systemic inflammatory response syndrome (SIRS) continue to pose considerable risks to patient outcomes (3). SIRS, characterized by a dysregulated cascade of pro-inflammatory cytokines and chemokines, is often accompanied by marked alterations in neutrophil phenotype and function (4–6).

Neutrophils, which constitute 50–70% of circulating white blood cells, are essential first responders to tissue injury. Their primary functions include the elimination of necrotic tissue and pathogens through mechanisms such as phagocytosis, degranulation, and the production of reactive oxygen species (ROS). Beyond their direct antimicrobial role, neutrophils actively participate in modulating immune responses by interacting with other immune cells and shaping the resolution or progression of inflammation (7).

Abbreviations: BN, banded neutrophils; CK, creatine kinase; DHR, Dihydrorhodamine 123; GCS, Glasgow Coma Scale; HSN, hypersegmented neutrophils; ICU, Intensive Care Unit; IL-8, Interleukin 8; ISS, Injury Severity Score; Mac-1, Macrophage-1 antigen; MFI, mean fluorescence intensity; MN, mature neutrophils; MTG, MitoTracker MTR Green; MTR, MitoTracker™ Red CMXRos; MTS, MitoSOX Red; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SA, *Staphylococcus aureus*; SIRS, systemic inflammatory response syndrome; TP, time point; TRISS, Trauma and Injury Severity Score.

Following trauma, emergency granulopoiesis results in the rapid release of heterogeneous neutrophil subsets from the bone marrow into circulation. These subsets include mature segmented neutrophils (CD16^{high}CD62L^{high}), immature banded neutrophils (CD16^{low}CD62L^{high}), and hypersegmented neutrophils (CD16^{high}CD62L^{low}) (8, 9). Additionally, a population of CD16^{low}CD62L^{low} neutrophils has been identified as neutrophil progenitors in infants with bacterial and viral infections (10). While these cells are believed to represent immature neutrophil forms, their functional roles in trauma-related immune responses remain to be elucidated.

Neutrophil dysfunction in trauma has significant clinical implications. Dysregulated neutrophil activation and recruitment can exacerbate tissue damage, prolong inflammation, and contribute to the development of severe complications such as sepsis and multiorgan failure (11–13). Specific subpopulations, such as hypersegmented neutrophils have been associated with immunoregulatory roles, including the ability to regulate pro-inflammatory T-lymphocyte activity through Mac-1 receptors (3). Furthermore, the early mobilization of banded neutrophils has been proposed as a potential biomarker for trauma severity and immune response dynamics (9).

Understanding the heterogeneity of neutrophil subsets and their functional properties is crucial for advancing our knowledge of trauma-related immune dysregulation. In trauma patients, the expression of neutrophil activation markers such as CD10, CD11b, the degranulation marker CD66b, and chemotaxis markers CD181 and CD182 is frequently discussed (3, 7). While recent years have seen

the classification of neutrophils into subsets based on CD16 and CD62L expression, the baseline expression of these markers in these subsets, as well as how their levels change upon activation, has not yet been clearly defined. In this study, we present for the first time the correlations between neutrophil subsets, clinical biomarkers such as lactate and creatine kinase, and trauma severity scores, including the Trauma and Injury Severity Score (TRISS) and Injury Severity Score (ISS). Subsequently, we focused on the functional properties of CD16^{low}CD62L^{low} neutrophils in trauma patients, analyzing their phagocytic capacity, oxidative burst activity, changes in activation markers (CD10, CD11b), the degranulation marker CD66b, and chemotaxis markers CD181 and CD182. Additionally, we examined the mitochondrial content, membrane potential, and mitochondrial ROS production on defined neutrophil populations. Through this integrative approach, we aim to elucidate the role of neutrophils in trauma-related inflammation and their potential impact on clinical outcomes.

Methods

Study participants

All participants in this study were patients suffering from combined injury or blunt polytrauma admitted to either high threshold Emergency of the Department of Anesthesiology and Intensive Care of St. Anne's University Hospital in Brno (n=38) or the Clinic of Accident Surgery of the University Hospital Brno (n=12), Czech Republic. Fifty trauma patients (22 females, 28 males; median age 43, range 19–78 years) were enrolled. At the time of admission to the Intensive Care Unit (ICU), none of the patients showed signs of infection. Based on the clinical and laboratory evaluation during the first 24 hours of hospitalization, patients were categorized into two groups: the SIRS group (Systemic Inflammatory Response Syndrome; n=16) and the Non-SIRS group (n=34). Patients in the SIRS group exhibited at least two of the following circumstances: tachycardia, hypotension, need for oxygen therapy, lactate elevation, or oliguria. Detailed clinical characteristics of the study population are presented in Table 1.

Blood samples were collected from all patients at two specific time points. The first sample (TP1) was obtained shortly after ICU admission, with a mean interval of 20.8 hours (SD ± 9.6 hours). The second sample (TP2) was collected on average 4 days later (SD ± 1 day). All blood samples were processed within 1 hour of collection. The control group consisted of 43 patients scheduled for hip or knee joint replacement surgery (27 females, 16 males; median age 71, range 43–91 years).

Flow cytometry characterization of neutrophils

Neutrophils were characterized using a combination of fluorescently labeled human monoclonal antibodies: CD15 FITC (clone MEM-158), CD193 PE (clone 5E8), CD45 PE-DY647 (clone

MEM-28), CD14 PerCP Cy5.5 (clone MEM-15), CD62L PC7 (clone LT-TD180), CD10 APC (clone MEM-78), CD11b PB (clone ICRF44), and CD16 PO (clone 3G8) (all antibodies from Exbio Praha, Vestec, Czech Republic). Additional antibodies included CD181 APC (clone 8F1/CXCR1), CD182 APC (clone 5E8/CXCR2), and CD66b Alexa Fluor 700 (clone 10F5; Sony Biotechnology, San Jose, CA, USA). Fresh whole blood samples were incubated with monoclonal antibodies for 30 minutes at 4°C in the dark. Erythrocytes were lysed using formic acid, followed by the addition of a stop solution. The samples were washed with phosphate-buffered saline (PBS) before analysis. Data acquisition was performed on a Navios flow cytometer (10 colors, 3 lasers; Beckman Coulter, Miami, FL, USA), and data were analyzed using the Kaluza software (Beckman Coulter, Brea, CA, USA).

Oxidative burst of neutrophils

To assess the oxidative burst of neutrophils, mean fluorescence intensity (MFI) of rhodamine 123 was measured using flow cytometry. Two tubes of whole blood were prepared per patient: one unstimulated and one stimulated with opsonized 1×10^7 *Staphylococcus aureus* (Wood strain without protein A) BioParticles™ (Invitrogen™, Life Technologies Corporation, Oregon, USA). Each tube contained 100 µl of blood and 10 µl of diluted Dihydrorhodamine 123 (final concentration 375 ng/ml; Sigma-Aldrich, St. Louis, MO, USA). Samples were incubated for 30 minutes at 37°C, washed with PBS, and incubated for another 30 minutes at 4°C with monoclonal antibodies: CD14 PerCP Cy5.5, CD66b Alexa Fluor 700, CD11b PB, CD10 PC7 (clone MEM-78), CD62L APC (clone LT-TF180), CD16 APC-Cy7 (clone 3G8) and CD45 PO (clone HI30), (all antibodies from Exbio Praha). Erythrocytes were lysed by formic acid as mentioned above and samples were measured using a Navios flow cytometer.

Phagocytosis assay

Phagocytic activity of neutrophils was assessed using FITC-labeled *Escherichia coli* strain K12. (IngoFlowEx kit; Exbio Praha). Whole blood samples were divided into two tubes: one unstimulated and one stimulated with 10 µl of FITC-labeled *E. coli*. Following a 30 minute incubation at 37°C, samples were washed with PBS, labeled with monoclonal antibodies, (CD14 PerCP Cy5.5, CD66b Alexa Fluor 700, CD11b PB, CD10 PC7, CD62L APC, CD16 APC-Cy7 and CD45 PO) lysed, and analyzed using a Navios flow cytometer.

Evaluation of mitochondrial functionality

Mitochondrial functionality was assessed using specific fluorescent probes: MitoTracker™ Green FM (MTG) for mitochondrial content and MitoTracker™ Red CMXRos (MTR)

for mitochondrial membrane potential (all probe, Invitrogen™) (14). Blood samples (100 µl) were incubated with these probes along with monoclonal antibodies (CD193 PE, CD62L PC7, CD64 APC, CD11b PB, CD16 PO, CD66b Alexa Fluor 700). Mitochondrial ROS production was evaluated using MitoSOX Red (MTS) in pre-lysed blood samples incubated at either 4°C (unstimulated) or 37°C (stimulated with *Staphylococcus aureus*). Following an incubation, samples were washed with PBS, labeled with monoclonal antibodies and lysed. Data were acquired on a Navios flow cytometer and analyzed with the Kaluza software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., California, USA). Data normality was assessed using the Shapiro-Wilk test. Non-parametric tests, including the Mann-Whitney U test, Wilcoxon signed-rank test, and Kruskal-Wallis test with Dunn's correction for multiple comparisons, were employed as appropriate. Correlations were analyzed using Spearman's rank correlation test. Results were considered statistically significant at $p < 0.05$.

TABLE 1 Clinical and laboratory characteristics of patients.

Demographics and Nature of trauma (N)		Non-SIRS	Standard deviation	SIRS	Standard deviation	p (Non-SIRS/SIRS)
Number of patients		38		12		
Male/Female (N)		22/12		12/2		
Age (years)		52,3	17,8	49,6	15,7	ns
BMI		26	4,1	27,2	4,5	ns
Motor-vehicle accident		32		6		
Fall from a height		6		5		
Other blunt injury		0		1		
Mortality due to MODS		1		1		
Laboratory parameters	Normal range	Median	Min - Max	Median	Min - Max	p (Non-SIRS/SIRS)
Leukocytes (*10 ⁹ /l)	4,0 - 10,0	11,05	7,02 - 26,6	15,1	11,21 - 31,14	0.0336
Immature granulocytes (%)	0,0 - 0,6	0,3	0,2 - 1,5	0,5	0,4 - 0,8	ns
C-reactive protein CRP (mg/l)	0,0 - 5,0	1,15	0,6 - 39,0	1,5	0,6 - 13,7	ns
Lactate (mmol/l)	0,5 - 2,2	1,4	0,6 - 4,2	2,1	1,4 - 5,9	0.0162
Thrombocytes (*10 ⁹ /l)	150 - 400	234	90 - 674	256,5	150 - 355	ns
Hemoglobin (g/l)	120 - 175	128,0	84 - 164	134,5	98 - 169	ns
Creatine kinase (ukat/l)	0,0 - 3,2	4,04	1,6 - 21,4	7,1	3,9 - 43,3	0.0008
Myoglobin (ug/l)	25,0 - 72,0	231,1	31,6 - 2864	713,0	279,5 - 1466	0.0491
Alcohol (‰)	0	0	0 - 2,5	0,0	0 - 1,8	ns
Injury Severity Score (ISS 0-75)		19,5	4 - 57	36,0	17 - 66	0.0037
Glasgow Coma Scale (GCS 3-15)		15	14 - 15	15,0	3 - 15	0.0443
TRISS Score - % of survival		96,75	53,45 - 99,61	86,7	2,11 - 98,43	0.0003

Demographic overview of the group of 50 trauma patients, including the cause of trauma and the evaluation of measured laboratory parameters upon hospital admission. The percentage of immature neutrophils represents the percentage of immature neutrophils measured using a hematology analyzer. The table also includes an assessment of scoring systems for both the SIRS and Non-SIRS patient groups. Statistically significant results are highlighted in bold, with a significance level of $p < 0.05$. Data were tested for normal distribution; for normally distributed data, a Student's t-test was applied, while non-normally distributed data were analyzed using the Mann-Whitney U test.

Results

A total of 50 non-infectious trauma patients were included in the study. Based on their clinical and laboratory presentation, 16 patients were categorized as trauma with SIRS (TRA with SIRS), while the remaining 34 were designated as TRA without SIRS (Non-SIRS). None of the patients displayed signs of infection upon admission. The clinical characteristics of the study population are summarized in Table 1. Initial laboratory assessments revealed that SIRS patients exhibited significantly higher leukocyte counts, as well as elevated serum lactate and creatine kinase (CK) levels, compared to Non-SIRS patients. Interestingly, despite the lower ISS observed in the SIRS group, these patients demonstrated lower Glasgow Coma Scale (GCS) scores and Trauma and Injury Severity Scores (TRISS) compared to Non-SIRS patients (Table 1).

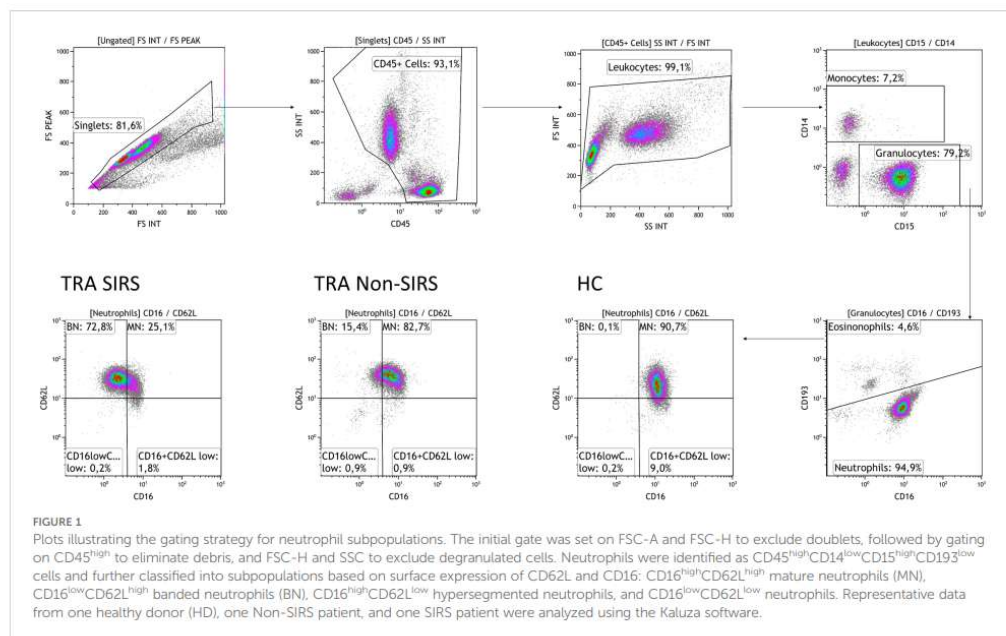
Blood samples were collected from trauma patients at two distinct time points (TP1 and TP2) to assess dynamic changes in neutrophil subpopulations and other parameters, allowing for the monitoring of immediate and evolving immune response following trauma. The control group consisted of 43 individuals undergoing elective hip or knee joint replacement surgery, who were used as a reference for baseline neutrophil characteristics.

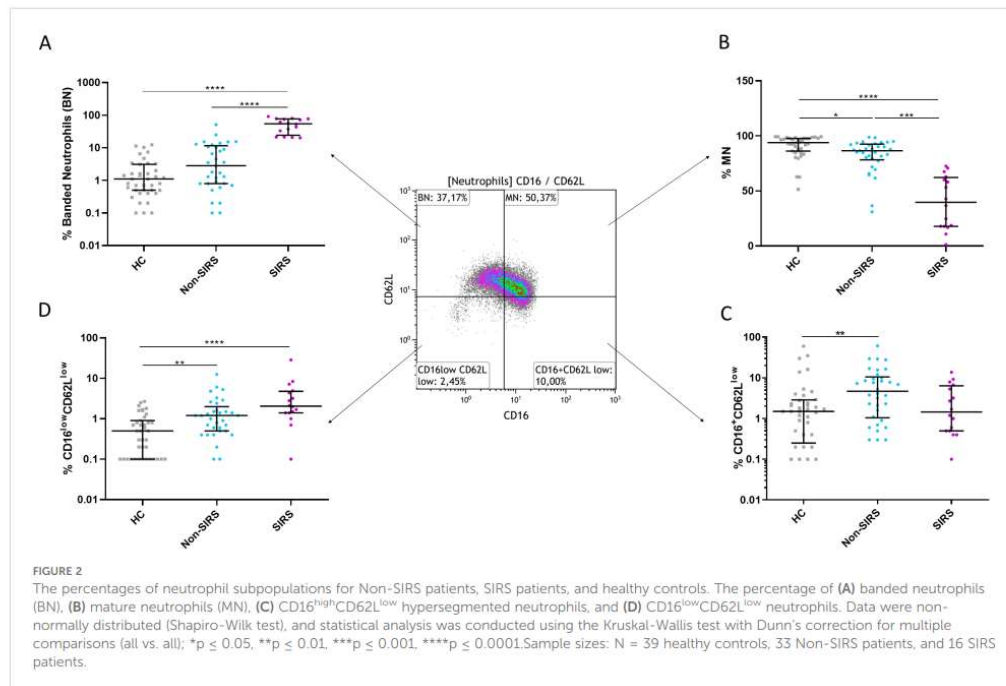
Neutrophil subpopulations in trauma patients

To identify and characterize neutrophil subpopulations, we utilized the markers CD16 and CD62L. Neutrophils were gated

based on their granularity while excluding eosinophils (gating strategy shown in Figure 1). Subpopulations included $CD16^{low}CD62L^{high}$ (banded neutrophils, BN), $CD16^{high}CD62L^{high}$ (mature neutrophils, MN), $CD16^{high}CD62L^{low}$ (hypersegmented neutrophils HSN) and $CD16^{low}CD62L^{low}$ neutrophils (Pillay J et al., 2012). Analysis of data collected at TP1 revealed a significant reduction in mature neutrophils (MN) in both trauma groups, with a more pronounced decrease in SIRS patients. Conversely, an significantly increase in BN was observed, particularly in SIRS patients (Figures 2A, B). The decline in MN among SIRS patients was primarily attributed to a rise in $CD16^{low}CD62L^{low}$ neutrophils, while in Non-SIRS patients, the reduction also involved HSN neutrophils (Figures 2C, D).

Although trauma patients exhibited elevated absolute neutrophil counts compared to controls, no significant differences were observed between SIRS and Non-SIRS patients in total leukocyte or neutrophil counts (Supplementary Figures 1A–C). The analysis of neutrophil subpopulations at TP2 revealed a normalization of BN and an increase in mature neutrophils MN in SIRS patients, while Non-SIRS patients showed an increase in both HSN and $CD16^{low}CD62L^{low}$ neutrophils (Figures 3A–D). In the overall study cohort, a significant correlation was found between patient age and the percentage of mature neutrophils, ($R = -0.2858$, $p = 0.0443$). This association was also confirmed in non-SIRS patients, ($R = -0.3614$, $p = 0.0358$), but did not reach statistical significance in the SIRS subgroup ($R = -0.496$, $p = 0.0526$, Spearman test). No significant correlations with age or associations with sex were identified for the remaining neutrophil populations.





Correlation of neutrophil subpopulations with creatine kinase and lactate levels

In SIRS patients, elevated levels of CK and lactate were observed at the time of hospital admission (Table 1). Therefore, we investigated whether these markers were associated with the proportional representation of specific neutrophil subpopulations. Serum CK levels, a marker of muscle injury, were negatively correlated with the percentages of MN and HSN at TP1. In contrast, a positive correlation was observed between CK levels and the percentage of BN at TP1, as well as CD16^{low}CD62L^{low} neutrophils at TP2 (Table 2).

Next, we analyzed the association between serum lactate concentrations, indicative of metabolic stress, anaerobic metabolism and tissue hypoxia and the distribution of neutrophil subpopulations. Higher lactate concentrations correlated positively with the percentages of immature neutrophils and CD16^{low}CD62L^{low} subsets at both TP1 and TP2. In contrast, MN and CD16^{high}CD62L^{low} subsets were negatively correlated with lactate levels at TP1 (Table 2).

Neutrophil subpopulations and clinical severity scores

Patients with SIRS exhibited a higher ISS and lower TRISS scores compared to Non-SIRS patients, reflecting increased trauma

severity and reduced survival probability (Table 1). To further address the potential role of neutrophil subpopulations in SIRS, we analyzed their associations between Injury Severity Score and Trauma and Injury Severity Score. The percentage of BN at TP1 was inversely correlated with the TRISS score and positively associated with ISS (Table 3). CD16^{low}CD62L^{low} neutrophils at TP1 showed a weaker inverse correlation with the TRISS score and no significant association with ISS. At TP2, BN maintained a weaker inverse correlation with TRISS and a positive association with ISS (Table 3).

Functional analysis of neutrophil subsets

Phenotypic analysis of neutrophil subpopulations, defined based on CD16 and CD62L expression, demonstrated that BN exhibit reduced expression of CD10, CD11b, and CD16, along with increased expression of CD181. CD16^{low}CD62L^{low} neutrophils exhibited low expression of neutrophil activation markers CD10, CD11b, CD181, CD182 suggesting potential functional impairments (Figure 4; Supplementary Figure 2). Therefore, we assessed their phagocytic capacity using FITC-labeled *E. coli* particles and oxidative burst activity. The total phagocytic capacity of neutrophils revealed no significant differences between trauma patients and controls. (Figure 5A). However, CD16^{low}CD62L^{low} neutrophils displayed reduced phagocytic activity in comparison to MN (Figures 5B, C). ROS production by neutrophils upon stimulation

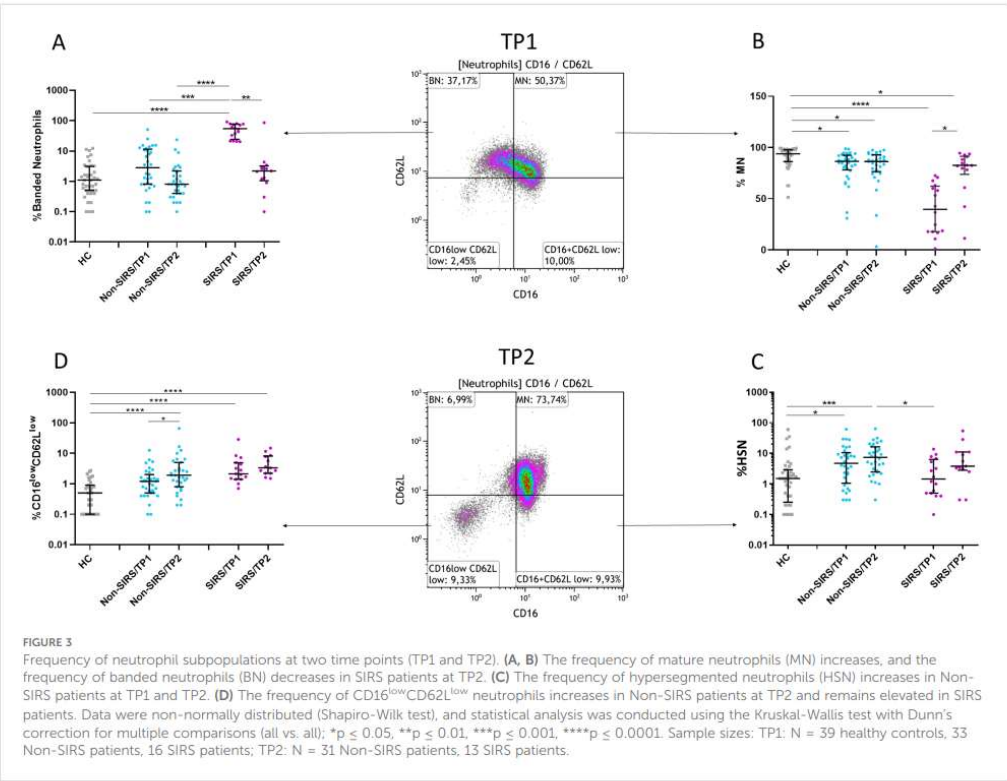


TABLE 2 Correlation of neutrophil subpopulations with creatine kinase and lactate levels.

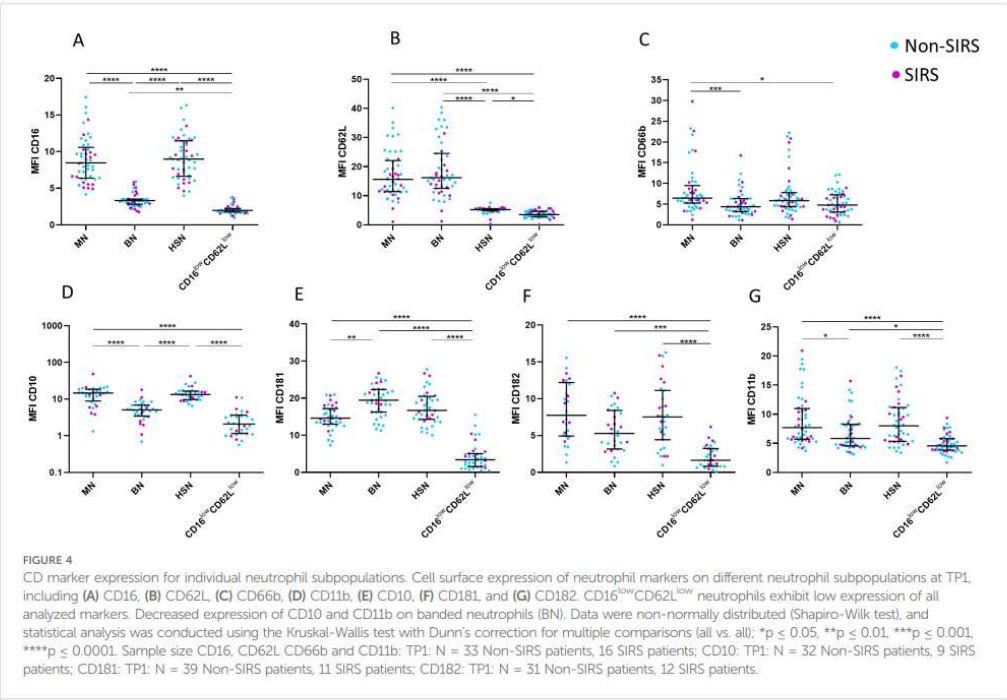
Correlation coefficient and probability	Creatine kinase (ukat/l)		Lactate (mmol/l)		Lactate (mmol/l)	
	ICU admission		ICU admission		TP1	
	r	p	r	p	r	p
% MN/TP1	-0,332	0.0416	-0,269	ns	-0,792	0.0019
% BN/TP1	0,473	0.0027	0,427	0.0028	0,823	0.0009
%CD16+CD62Llow /TP1	-0,378	0.0195	-0,265	ns	-0,638	0.0215
CD16lowCD62Llow/TP1	0,066	ns	0,377	0.009	0,131	ns
% MN/TP2	-0,122	ns	-0,177	ns	-0,360	ns
% BN/TP2	0,146	ns	0,005	ns	0,657	0.0318
%CD16+CD62Llow /TP2	-0,276	ns	0,010	ns	-0,242	ns
CD16lowCD62Llow/TP2	0,359	0.0401	0,337	0.0292	0,055	ns

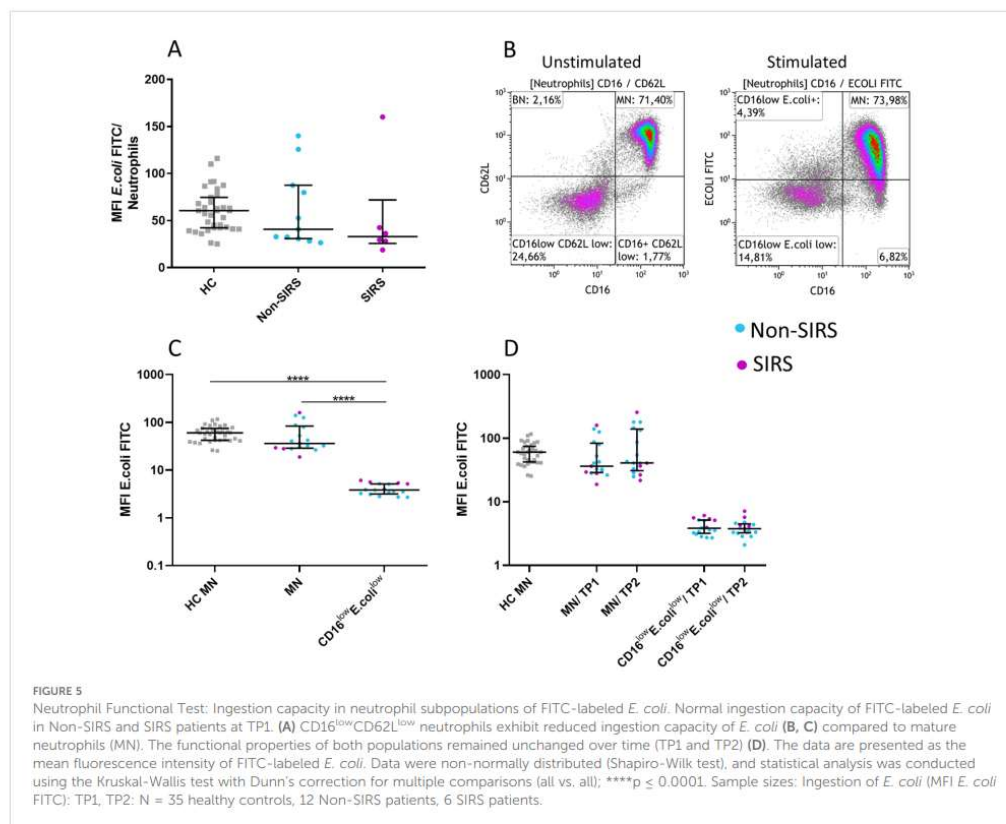
The table presents Spearman correlation coefficients for non-parametric correlations between creatine kinase concentrations and specific neutrophil populations at TP1 and TP2, as well as between lactate concentrations and neutrophil populations at TP1 and TP2. Statistically significant results are highlighted in bold. Sample sizes: Creatine kinase: N = 37 TRA patients; Lactate: N=47 TRA patients. r (Spearman correlation coefficients); ns (not significant).

TABLE 3 Neutrophil subpopulations and clinical severity score.

Correlation coefficient and probability	TRISS score		Injury severity score	
	r	p	r	p
c lactate (mmol/l)	-0,252	ns	0,279	ns
c kreatin kinase (μkat/l)	0,1975	ns	0,401	0.0126
% MN/TP1	0,455	0.0009	-0,284	0.0457
% BN/TP1	-0,541	<0.0001	0,541	<0.0001
% CD16+CD62L low/TP1	0,092	ns	-0,459	0.0008
% CD16low CD62L low/TP1	-0,357	0.0109	0,262	ns
% MN/TP2	0,005	ns	0,044	ns
% BN/TP2	-0,323	0.0306	0,005	ns
% CD16+CD62L low/TP2	0,183	ns	0,114	ns
% CD16low CD62L low/TP2	-0,124	ns	0,221	ns

The table presents Spearman correlation coefficients for non-parametric correlations between Trauma and injury severity score (TRISS) and specific neutrophil populations at TP1 and TP2, as well as between Injury severity score (ISS) and specific neutrophil populations at TP1 and TP2. Statistically significant results are highlighted in bold. Sample sizes: Both TRISS and ISS: N = 50 TRA patients. r (Spearman correlation coefficients); ns (not significant).





with *Staphylococcus aureus* (SA) was diminished in SIRS patients compared to Non-SIRS patients and controls (Figure 6A). Similarly, CD16^{low}CD62L^{low} neutrophils exhibited a significantly lower capacity for ROS production compared to MN a pattern observed consistently at both TP1 and TP2 (Figures 6B, C).

Functional analysis of degranulation and activation markers in neutrophil subpopulations

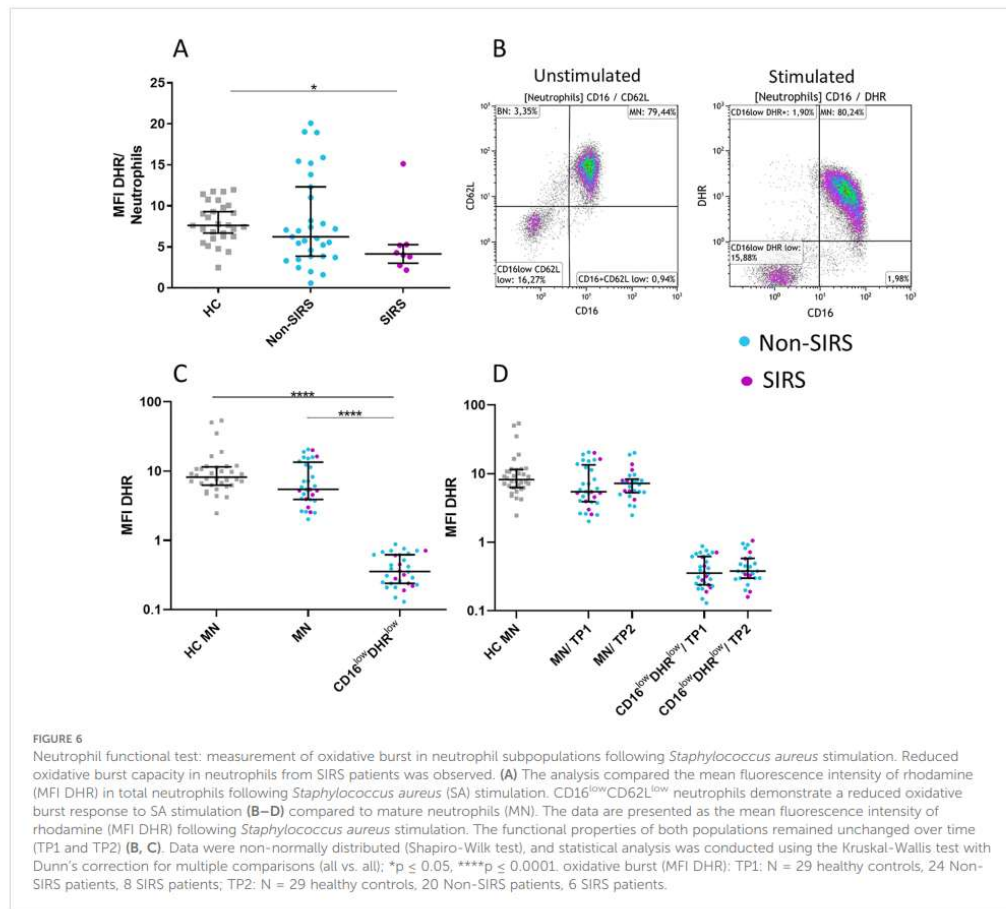
Given that CD16^{low}CD62L^{low} neutrophils demonstrated a significantly reduced capacity for both oxidative burst and phagocytosis, we subsequently evaluated whether this subpopulation also exhibits alterations in activation and degranulation. Functional assays were performed to evaluate the degranulation capacity of mature neutrophils and CD16^{low}CD62L^{low} neutrophils by measuring changes in CD66b expression following stimulation with SA. Upon stimulation, MN exhibited a significant increase in CD66b expression, while CD16^{low}CD62L^{low} neutrophils did not exhibit an increase in CD66b expression (Figure 7A). Activation levels were further assessed by

analyzing CD11b expression, which typically upregulates on the neutrophil surface following stimulation. MN from trauma patients displayed a CD11b expression increase comparable to that observed in healthy controls, whereas CD16^{low}CD62L^{low} neutrophils failed to show any significant upregulation (Figure 7B).

The expression of CD10, another activation marker, was also examined. Similar to the trend observed with CD66b, MN exhibited an increase in CD10 expression upon stimulation with SA, whereas CD16^{low}CD62L^{low} neutrophils remained unresponsive. Notably, MN from trauma patients demonstrated a lower upregulation of CD10 compared to MN from healthy controls (Figure 7C). Lastly, CD16 expression was analyzed, revealing consistent levels across both neutrophil populations, with no significant differences compared to MN from healthy controls, following stimulation with SA. (Figure 7D).

Mitochondrial functionality in neutrophil subpopulations

Given the low CD16 expression and the reduced functional capacity for phagocytosis and ROS production observed in



CD16^{low}CD62L^{low} neutrophils, we investigated whether this population retained mitochondrial functionality using mitochondrial functional assays. Mitochondrial content was assessed using the Mitotracker Green probe, mitochondrial membrane potential with the Mitotracker Red probe, and mitochondrial superoxide production with the MitoSOX probe (14).

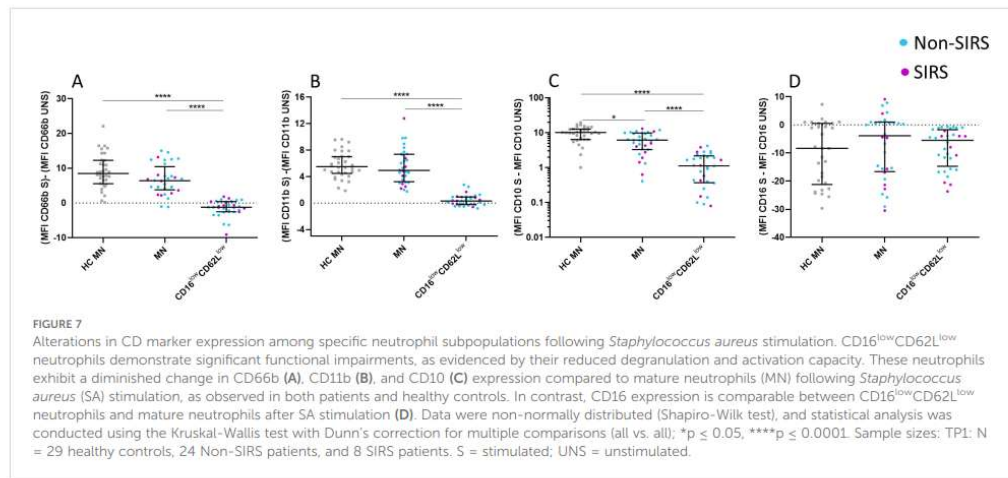
Compared to MN, CD16^{low}CD62L^{low} neutrophils exhibited a comparable mitochondrial content and preserved mitochondrial membrane potential, both clearly distinct from dead cells (Figures 8D, E). However, upon stimulation with SA, CD16^{low}CD62L^{low} neutrophils demonstrated a significantly reduced capacity for mitochondrial superoxide production (Figures 8A–C).

Discussion

In this study, we demonstrate that neutrophils from trauma patients exhibit significantly altered counts and proportions of

neutrophil subpopulations, with an increased representation of banded neutrophils, a trend that is further accentuated in patients who developed SIRS. The mechanisms driving this shift in neutrophil subsets remain unclear, but it is plausible that severe tissue injury and systemic inflammation contribute to the observed changes.

Trauma triggers the activation and recruitment of neutrophils from the bone marrow, leading to their accumulation both at the site of inflammation and in peripheral blood (6). This phenomenon is reflected in elevated neutrophil counts observed in trauma patients, which align with our clinical observations (15). Notably, the increase involves not only mature neutrophils but predominantly banded neutrophils (Figure 2). The early presence of banded, segmented, and hypersegmented neutrophils—characterized by CD16/CD62L expression and associated with nuclear segmentation—has been linked to late-onset infectious complications and progression to multi-organ failure during hospitalization in major trauma patients (8, 9). Our study confirmed a significant increase in the percentage and absolute



counts of BN in trauma patients, with the increase being more pronounced in those with non-infectious SIRS (Figure 2, Supplementary Figure 1). While standard hematology analyzers often fail to detect these immature neutrophils, they can be identified using microscopy or flow cytometry, where they exhibit a CD16^{low}CD62L^{high} phenotype as described by Hellebrekers et al. (9). Nevertheless, consistent with previous studies, our findings using hematology analyzers did not show a significant rise in immature neutrophils in SIRS patients compared to those without SIRS. The immaturity of CD16^{low}CD62L^{high} neutrophils we confirmed not only by their reduced expression of CD16 but also by lower expression of CD10 and CD11b (Figure 4), as is typically described for immature neutrophils (16).

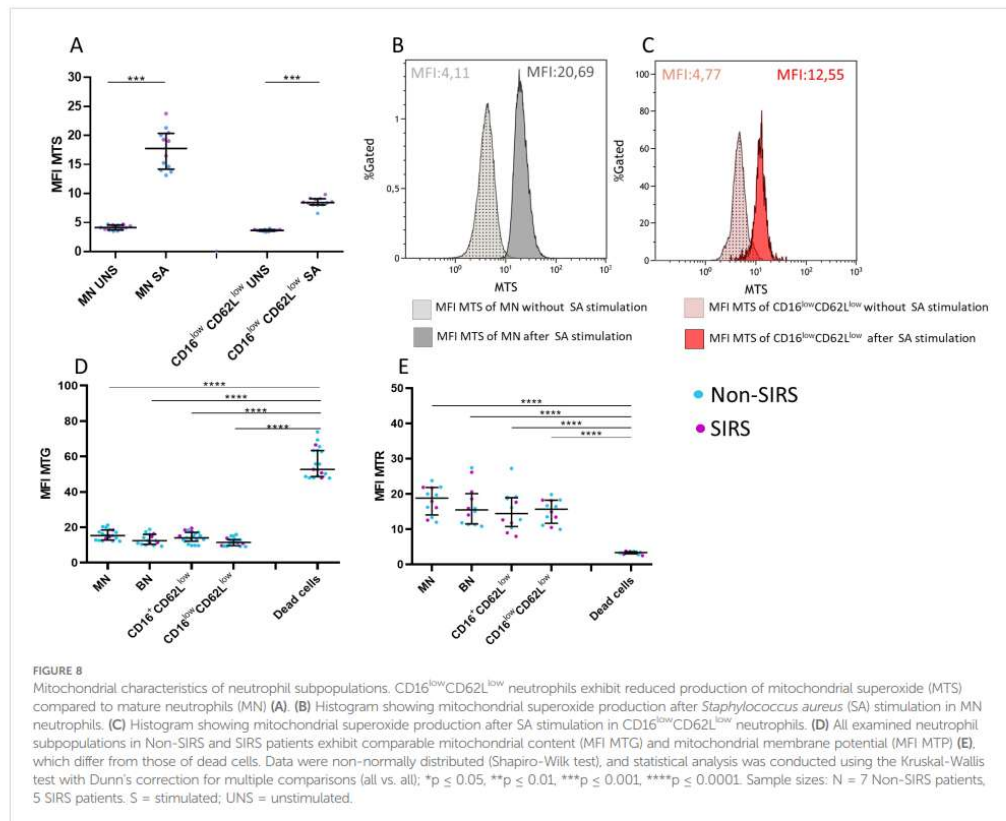
We show that patients with non-infectious SIRS exhibit increased serum lactate levels compared to Non-SIRS patients (Table 1). Furthermore, our data highlight that serum lactate levels at admission have the strong association with the proportion of banded neutrophils (Table 2). Elevated lactate, a marker of metabolic stress and tissue hypoperfusion, reflects the severity of cellular injury and hypoxia and has been widely recognized as a predictor of trauma severity and early clinical deterioration (17, 18). Similarly, elevated CK levels, an indicator of muscle damage, correlate with the percentage of banded neutrophils, particularly in patients with traumatic SIRS (Table 2). CK levels are also associated with complications such as acute kidney injury, particularly in cases of rhabdomyolysis, a condition linked to increased mortality (19). The observed correlations between banded neutrophils and lactate or CK levels suggest that metabolic stress and muscle injury drive an increased demand for immature neutrophils, accompanied by a corresponding reduction in mature neutrophils. This phenomenon reflects the emergency granulopoiesis that occurs in response to trauma, as previously described (6). Interestingly, our findings support the hypothesis that lactate levels play a pivotal role in driving neutrophil mobilization from the bone marrow. This mechanism has been previously described, with lactate shown to

influence emergency granulopoiesis and neutrophil release (20). These results point to the role of metabolic and tissue stress in shaping the neutrophil response to trauma.

Importantly, our data further reveal significant correlations between banded neutrophil levels, Injury Severity Scores, and Trauma and Injury Severity Scores (Table 3). While ISS reflects the anatomical severity of injuries, TRISS combines injury severity with physiological parameters and demographic factors to predict survival outcomes. Notably, TRISS scores also correlated with banded neutrophil proportions at TP2, approximately four days after admission, indicating a sustained association between neutrophil dynamics and trauma severity.

As inflammation progresses, regulatory mechanisms are crucial to restore homeostasis after the clearance of damaged tissues. Hypersegmented CD16^{high}CD62L^{low} neutrophils may contribute to this process by regulating pro-inflammatory T-lymphocyte activity through Mac-1 receptors, as described by Pillay et al. (8). In our study, we observed an increase in hypersegmented neutrophils in Non-SIRS patients at TP1 and in SIRS patients at TP2 (Figure 2; Supplementary Figure 1). The negative correlation between hypersegmented neutrophils and lactate levels, (Table 2), along with their lower abundance in SIRS patients at TP1, suggests an impaired immune response regulation in these individuals. By TP2, particularly in SIRS patients, we noted a rise in hypersegmented neutrophils accompanied by a decline in banded neutrophils (BN). This shift may indicate the onset of a successful return to homeostasis. However, further studies are needed to confirm this hypothesis and fully elucidate the role of hypersegmented neutrophils in immune regulation following trauma and non-infection SIRS.

The role of CD16^{low}CD62L^{low} neutrophils remains unclear. These cells display impaired functional properties, including reduced phagocytosis and oxidative burst (Figures 5, 6), likely reflecting reduced expression of the markers CD10, CD11b, CD181, and CD182 (Figure 4). Reduced expression of CD181 and CD182, key IL-



8 receptors, indicates reduced chemotactic capacity (21). In line with this, Cortjens et al. (10) reported that CD16^{low}CD62L^{low} neutrophils were restricted to peripheral blood but not found in bronchoalveolar lavage fluid, suggesting impaired migration. This population was further characterized by low expression of CD11b, which plays a crucial role in neutrophil migration and phagocytosis and T-cell regulation (8). Importantly, despite the loss of CD16, a characteristic commonly associated with apoptotic neutrophils (22), our mitochondrial assays confirmed that these cells were viable and non-apoptotic. Apoptosis is typically initiated by outer mitochondrial membrane permeabilization, which is closely linked to a loss of mitochondrial membrane potential (23). CD16^{low}CD62L^{low} population demonstrated a mitochondrial membrane potential comparable to that of mature neutrophils (Figure 8).

CD16^{low}CD62L^{low} neutrophils have previously been identified in infants with severe viral or bacterial infections, where they have been shown to include myelocytes and metamyelocytes (10). This population was characterized by decreased expression of CD11b and CD54 and increased expression of CD63 and CD66b. In infected infants, the number of neutrophil progenitors gradually increased between the third and sixth day of infection, a trend similar to what we observed in our

cohort of trauma patients. However, unlike the pediatric group, we did not observe elevated CD66b expression in trauma patients, which may be attributed to differences in age and the nature of the underlying conditions in the two studied populations.

In addition to their surface expression patterns, the functional roles of CD16 and CD62L provide further insight into the significance of the CD16^{low}CD62L^{low} neutrophil subset in trauma. CD62L (L-selectin) is a key adhesion molecule that facilitates neutrophil attachment and rolling along the endothelium during the early stages of migration into inflamed tissues. Beyond adhesion, CD62L also participates in outside-in and inside-out signaling pathways that regulate neutrophil activation, arrest, and transmigration (24, 25). Upon ligation, it triggers intracellular signaling cascades essential for neutrophil function, including calcium influx, tyrosine phosphorylation, MAPK (ERK, JNK) activation, and production of reactive oxygen species that collectively promote gene expression, cytoskeletal reorganization, and microbial killing (26). These responses also enhance the activity of integrins, particularly $\beta 1$, and support Rac-mediated actin rearrangements that are critical for firm adhesion and transmigration (26). The downregulation of CD62L in the

CD16^{low}CD62L^{low} subset suggests disruption of the aforementioned key signaling and adhesion processes, implying a reduced capacity for endothelial interaction and tissue infiltration. Similarly, decreased CD16 expression may contribute to impaired phagocytosis and oxidative burst (27), consistent with the functional deficits observed in this population.

In our cohort, a significant increase in the percentage of CD16^{low}CD62L^{low} neutrophils was observed around four days post-trauma, possibly indicating a subsequent phase of the inflammatory response in trauma patients. Despite their impaired effector functions, the expansion of this subset may reflect a regulatory mechanism within trauma-induced systemic inflammatory response syndrome (SIRS). Their accumulation in the circulation, combined with limited oxidative activity, reduced phagocytosis, and poor tissue migratory capacity, may serve to dilute the pool of highly reactive neutrophils, thereby moderating the overall oxidative and proteolytic burden characteristic of SIRS (28).

Our study demonstrates that all four neutrophil populations, defined by the expression of CD16 and CD62L, exhibit stable and characteristic surface marker expression of CD10, CD11b, and CD66b (Supplementary Figure 2). This expression remains unchanged over time and is comparable between patients with SIRS and those without SIRS. The stability of these surface markers suggests that the fundamental biological activity of these neutrophil populations is not influenced by the presence or absence of an acute inflammatory condition such as SIRS. Furthermore, the relative proportions of banded neutrophils, mature neutrophils, hypersegmented neutrophils and CD16^{low}CD62L^{low} neutrophils in trauma and SIRS may indicate their role as consistent indicators of a patient's current pro-inflammatory state.

However, this study has certain limitations. Due to the loss of CD62L expression upon neutrophil activation, we were unable to assess the functional capacity of banded and hypersegmented neutrophils following stimulation with *Staphylococcus aureus* or *E. coli* (Figures 5, 6). Future studies should focus on identifying alternative activation markers that remain stable under short-term stimulation conditions, thereby facilitating functional analysis of neutrophil subpopulations. Additionally, we lack morphological confirmation of the CD16^{low}CD62L^{low} population; however, its phenotypic similarity and temporal occurrence align with previous descriptions of progenitor neutrophils.

Conclusion

In conclusion, analysis of neutrophil subpopulations defined by CD16 and CD62L expression provides a comprehensive understanding of neutrophil activation and function in trauma patients. Increased proportion of banded neutrophils correlates with systemic inflammation and SIRS, whereas hypersegmented neutrophils may contribute to the resolution of inflammation. Monitoring of these subpopulations could help identify trauma patients at risk for developing non-infectious SIRS. Furthermore, we have shown that CD16^{low}CD62L^{low} neutrophils exhibit reduced functional properties, including impaired phagocytosis and oxidative burst. Neutrophil

activation and recruitment during trauma can exacerbate tissue damage and prolong inflammation. However, the presence of CD16^{low}CD62L^{low} neutrophils, characterized by their reduced capacity for ROS and associated NOS production, may serve as an anti-inflammatory factor. This hypothesis, however, requires further investigation in future studies.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of St. Anne's University Hospital Brno (11G/2021) and the Medical Ethics Committee of University Hospital Brno (04-070623). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MV: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. JŠ: Data curation, Methodology, Writing – original draft. KS: Data curation, Methodology, Writing – review & editing. KD: Data curation, Methodology, Writing – review & editing. VK: Data curation, Methodology, Writing – review & editing. IP: Data curation, Methodology, Writing – review & editing. GB: Data curation, Methodology, Writing – review & editing. ZT: Data curation, Methodology, Writing – review & editing. KB: Data curation, Methodology, Writing – review & editing. LD: Data curation, Methodology, Writing – review & editing. AM: Data curation, Methodology, Writing – review & editing. JŽ: Data curation, Methodology, Writing – review & editing. JK: Data curation, Methodology, Writing – review & editing. MK: Data curation, Methodology, Writing – review & editing. TT: Data curation, Methodology, Writing – review & editing. VŠ: Supervision, Writing – review & editing. MH: Data curation, Methodology, Writing – review & editing. AK: Data curation, Methodology, Writing – review & editing. JF: Supervision, Validation, Writing – original draft. MH: Supervision, Validation, Writing – original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1565606/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Frequency of leukocyte and neutrophil subsets at TP1 and TP2. Frequency of leukocytes (A), frequency of neutrophils (B), and absolute neutrophil counts (C) in healthy controls, Non-SIRS patients, and SIRS patients at two time points: TP1 and TP2. Absolute counts of neutrophil subsets in healthy controls and both patient groups at TP1 and TP2: (D) mature neutrophils (MN), (E) banded neutrophils (BN), (F) CD16^{high}CD62L^{low} neutrophils, and (G) CD16^{low}CD62L^{low} neutrophils. Data were non-normally distributed (Shapiro-Wilk test), and statistical analysis was conducted using the Kruskal-Wallis test with Dunn's correction for multiple comparisons (all vs. all); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Sample sizes: TP1: N = 39 healthy controls, 33 Non-SIRS patients, 16 SIRS patients; TP2: N = 31 healthy controls, 31 Non-SIRS patients, 13 SIRS patients.

SUPPLEMENTARY FIGURE 2

Phenotypic analysis of neutrophil subpopulations. Decreased expression of CD10 and CD11b on banded neutrophils (BN) at TP1. Cell surface expression of neutrophil markers in different neutrophil populations at TP1 and TP2, including (A) CD16, (B) CD62L, (C) CD66b, (D) CD11b, (E) CD10, CD16^{low}CD62L^{low} neutrophils consistently exhibit low expression of all analyzed markers at both time points. Data were non-normally distributed (Shapiro-Wilk test), and statistical analysis was conducted using the Kruskal-Wallis test with Dunn's correction for multiple comparisons (all vs. all); *p ≤ 0.05, ***p ≤ 0.001. Sample sizes: CD16, (B) CD62L, (C) CD66b, (D) CD11b, TP1: N = 39 healthy controls, 33 Non-SIRS patients, 16 SIRS patients; TP2: N = 31 healthy controls, 31 Non-SIRS patients, 13 SIRS patients; CD10, (E) TP1: N = 39 healthy controls, 23 Non-SIRS patients, 9 SIRS patients; TP2: N = 21 Non-SIRS patients, 7 SIRS patients.

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11 Závěr

V této habilitační práci jsou nejprve shrnuty základní poznatky o běžném variabilním imunodeficitu a selektivním deficitu IgA s důrazem na vývoj a diferenciaci B-lymfocytů. Následuje přehled dostupných klasifikačních systémů CVID pacientů, z nichž některé vycházejí z výsledků průtokové cytometrie a fenotypizace B buněk. Poté jsou představeny vlastní publikace autorky, které se zaměřují zejména na imunofenotypizaci T- a B-lymfocytů u pacientů s primárními protilátkovými deficity. Tyto práce přispěly k pochopení věkových změn a vzájemných vztahů mezi jednotlivými lymfocytárními odchylkami. V rámci mezinárodní studie EUROclass se autorka podílela na hodnocení pacientů našeho pracoviště podle jednotného protokolu a následně se spoluautorsky zapojila do publikace, která se stala základem jedné z klasifikací CVID pacientů. Na tuto část navázaly studie hodnotící vývojová stadia a funkční parametry B buněk pomocí pokročilé polychromatické cytometrie.

Další výsledky se týkaly T-lymfocytů, především změn v zastoupení naivních a paměťových subpopulací a zvýšeného výskytu terminálně diferencovaných buněk u CVID a IgA deficiencie. Důležitým přínosem bylo také zapojení myeloidních buněk, konkrétně neutrofilů a granulocytární supresorové buňky odvozené od myeloidů (granulocytic Myeloid-Derived Suppressor Cells, gMDSC), jejichž schopnost regulovat imunitní odpověď se ukázala jako významná pro porozumění imunopatogenezi CVID.

V posledním období se výzkum rozšířil o analýzu neutrofilních subpopulací u pacientů s těžkým traumatem, která ukázala jejich zásadní vliv na rozvoj zánětu a oslabení imunity. Další vědecké směřování autorky proto bude zaměřeno právě na tuto oblast.

Z dnešního pohledu již multiparametrová průtoková cytometrie překročila roli základního fenotypizačního nástroje a stala se nedílnou součástí diagnostiky a monitorace vrozených poruch imunity. Dokáže vyhodnotit specifické buněčné populace a subpopulace, proteiny na buněčném povrchu, intracelulární a intranukleární proteiny a určité funkční imunitní charakteristiky, přičemž každá z nich může být pro diagnostiku a hodnocení vrozených poruch imunity užitečná.

Závěrem lze shrnout, že multiparametrová průtoková cytometrie přináší zásadní přínos pro diagnostickou a výzkumnou praxi nejen v oblasti vrozených poruch imunity. Její schopnost rozlišovat komplexní buněčné fenotypy, doplněná o automatizované nástroje a standardizační schémata, přispívá k robustnější, rychlejší a přesnější identifikaci imunitních poruch a jejich klinických implikací. Její další integrace s molekulárními metodami a strojovým učením otevírá cestu k personalizované medicíně a další evoluci imunodiagnostiky.

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