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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 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

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 (H_2O_2), 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 Tsinti 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

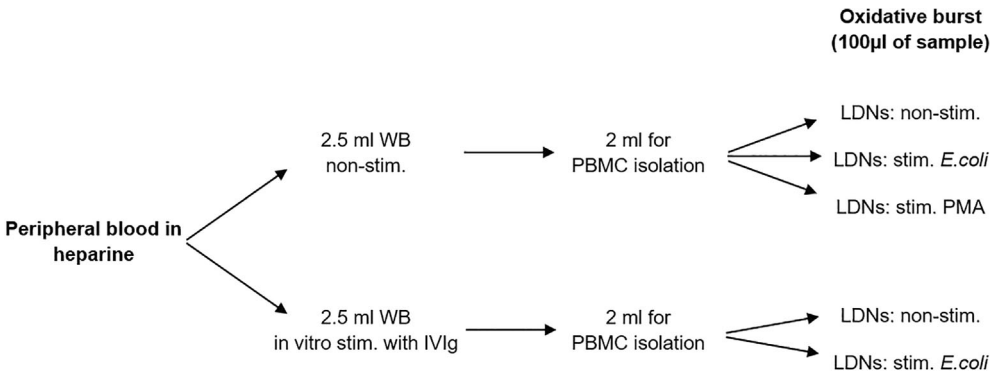

FIGURE 1 Experimental conditions used for quantifying oxidative burst. WB was non-stimulated or stimulated in vitro with IVIg. Part of the WB was further used for PBMC isolation. LDNs were stimulated with *E. coli* or PMA for the detection of oxidative burst.

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

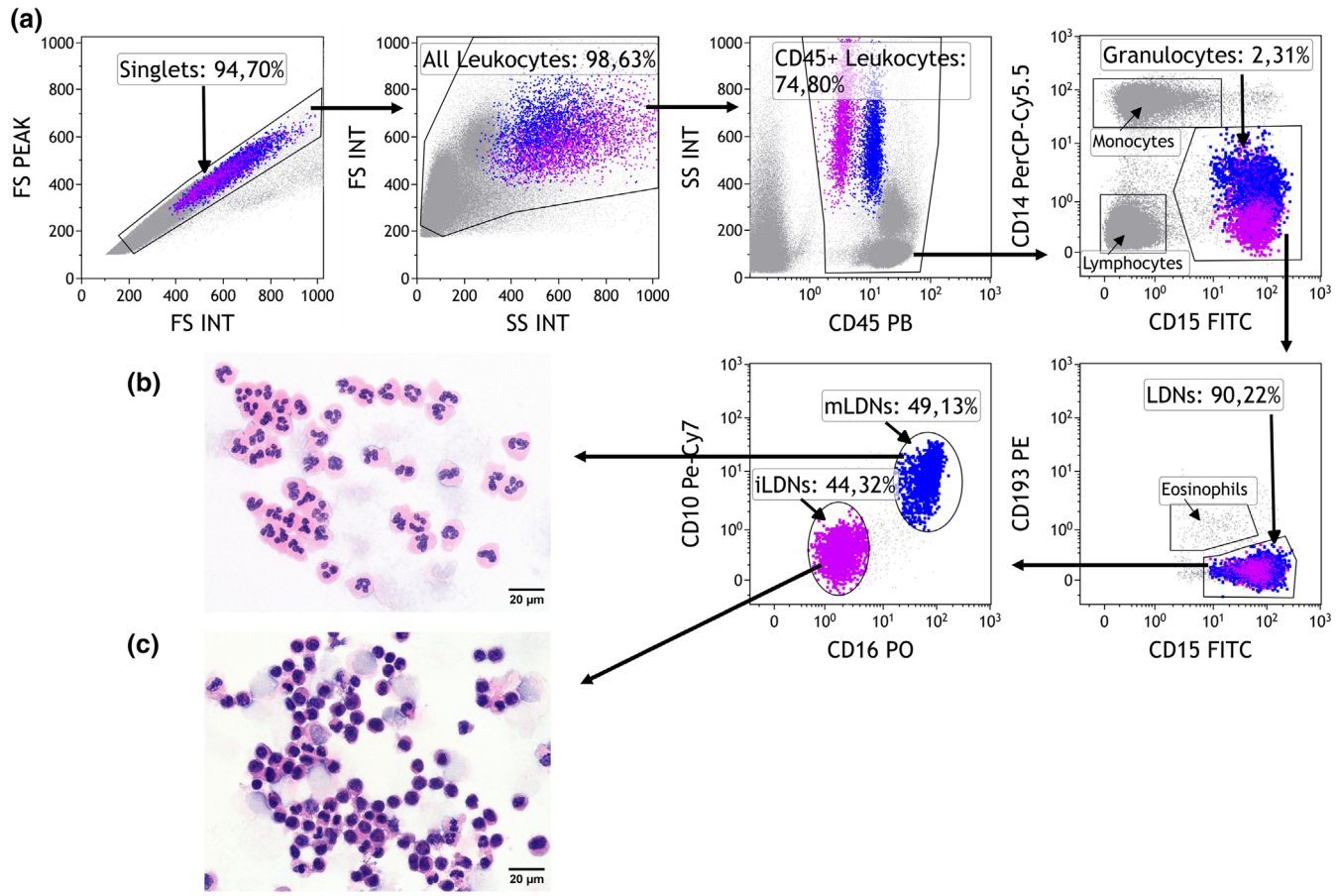

FIGURE 2 Definition of LDNs and LDN subtypes: immature and mature LDNs. (a) Plots showing the gating and sorting strategy of PBMC suspension purified by gradient centrifugation. First gate was set on FSC-A and FSC-H to eliminate doublets, then physical parameters, that on $CD45^+$ to eliminate debris 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.

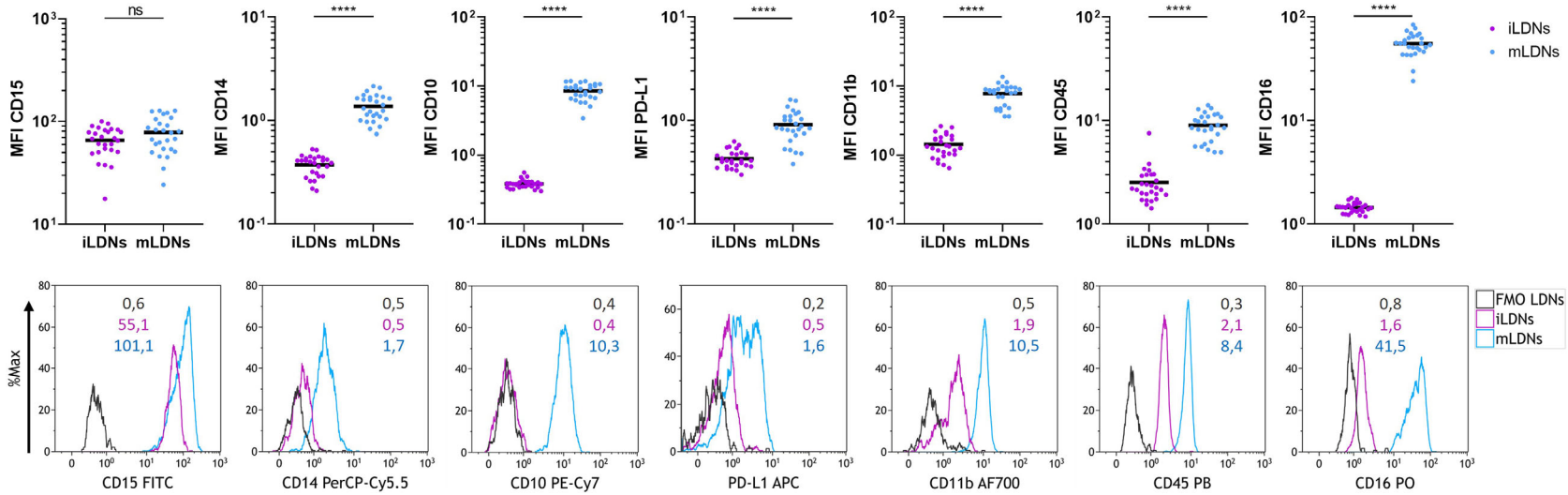

FIGURE 3 Comparison of phenotypes of LDNs from HD. (a) Surface expression of markers CD15, CD14, CD10, PD-L1, CD11b, CD45, and CD16 was determined by the flow cytometry measurement and compared between iLDNs and mLDNs from HD ($n = 27$, combined data from 12 independent experiments, each performed with 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]

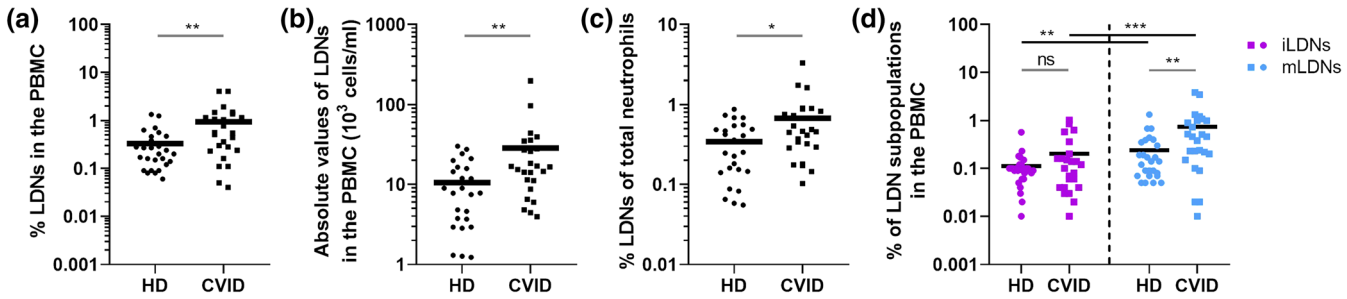

FIGURE 4 CVID patients show an increased frequency of predominantly mature LDNs. (a) The proportion and (b) absolute numbers of LDNs in the PBMC fraction and (c) the proportion of LDNs in the total neutrophils purified from non-stimulated WB samples by density gradient centrifugation was determined using 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		
		Median (%)	Range (%)	Author
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 squamos 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	Rheumathoid 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, evere 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

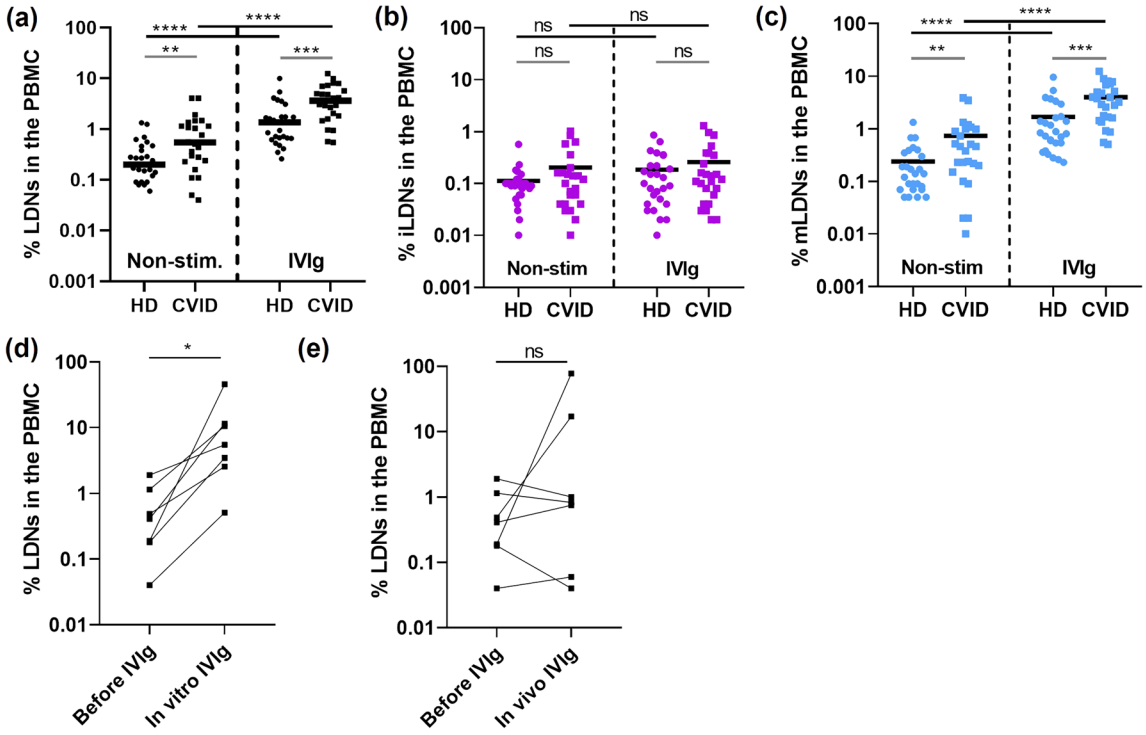

FIGURE 5 WB stimulation with IVIg caused increase of mLDNs. The proportion of (a) LDNs, (b) iLDNs, and (c) mLDNs in the PBMC purified from WB samples of HD and CVID patients stimulated with IVIg. Combined data of 27 HD and 25 CVID patients from 24 independent experiments, each performed with 2–4 samples, 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, *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; and ****$p \le 0.0001$). [Color figure can be viewed at wileyonlinelibrary.com]

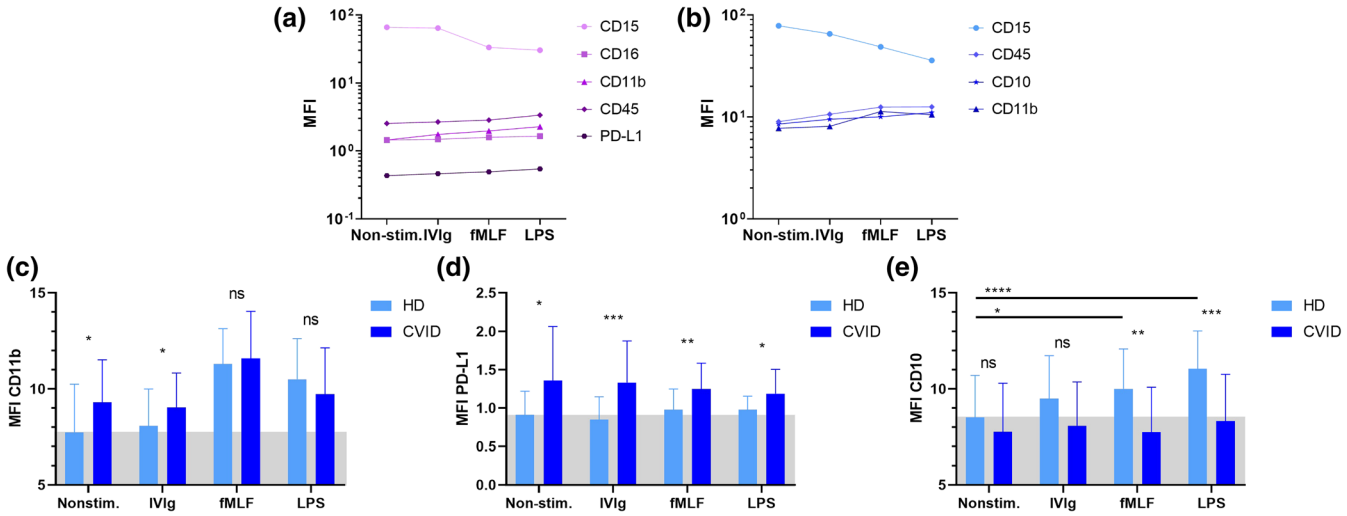

FIGURE 6 Changes in the surface expression of markers of LDN subpopulations after WB stimulation. The WB of 27 HD was either non-stimulated or stimulated with 5.7 mg/mL IVIg for 2 h, 2 μg/mL fMLF, and 2 μg/mL LPS for 1 h. Statistically significant changes in the expression levels of surface markers after 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, *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; and ****$p \le 0.0001$). [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

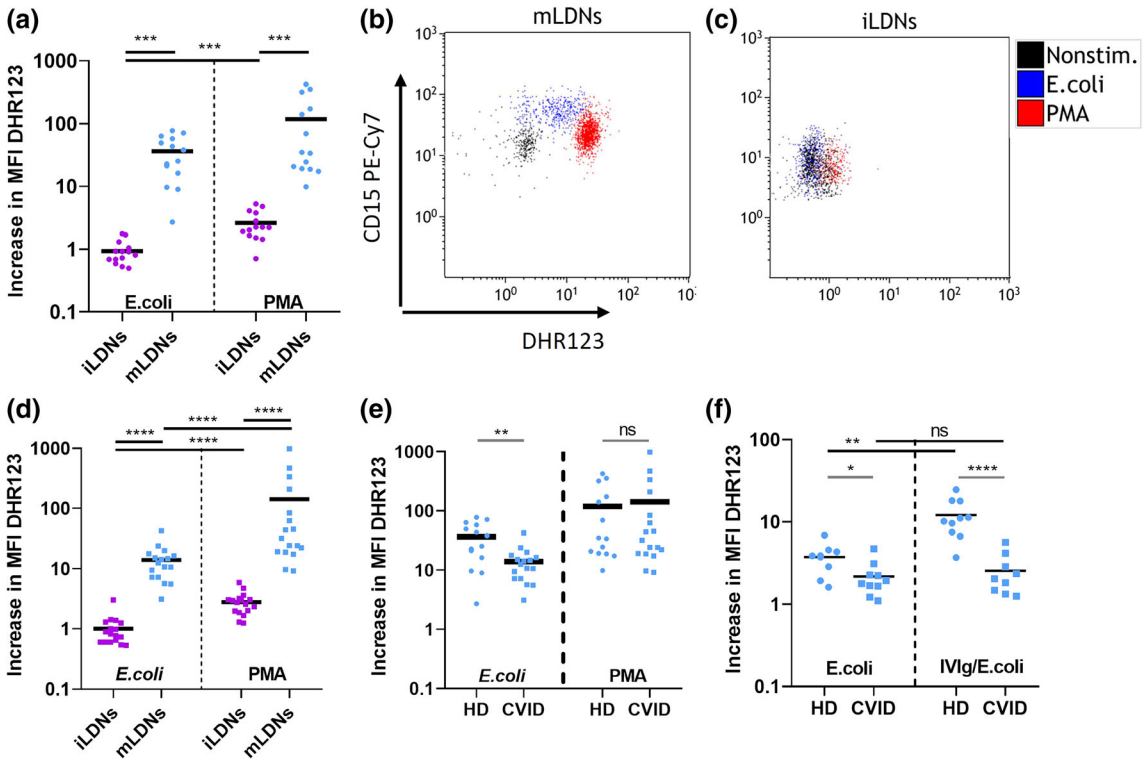

FIGURE 7 Oxidative burst of LDN subpopulations after *E. coli* or PMA stimulation and the effect of IVIg pre-stimulation. PBMC from (a) HD ($n = 14$) and (d) CVID patients ($n = 17$) were stimulated with opsonized *E. coli* or PMA to determine oxidative burst of LDNs using the flow cytometry measurement 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 or 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, $^{*}p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$; and $^{****}p \le 0.0001$). [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 preparates. Consistently, IgG aggregates are absent in the majority of IVIG preparates, 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 Ca^{2+} 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 (Rodriguez-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 FcγRIIa (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 Staniczkova 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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REFERENCES

Barbosa, R. R., Silva, S. P., Silva, S. L., Tendeiro, R., Melo, A. C., Pedro, E., Barbosa, M. P., Santos, M. C. P., Victorino, R. M. M., & Sousa, A. E. (2012). Monocyte activation is a feature of common variable immunodeficiency irrespective of plasma lipopolysaccharide levels. *Clinical and Experimental Immunology*, *169*, 263–272.

Blanco-Camarillo, C., Alemán, O. R., & Rosales, C. (2021). Low-density neutrophils in healthy individuals display a mature primed phenotype. *Frontiers in Immunology*, *12*, 672520.

Bonilla, F. A., Barlan, I., Chapel, H., Costa-Carvalho, B. T., Cunningham-Rundles, C., de la Morena, M. T., de la Morena, M. T., Espinosa-Rosales, F. J., Hammarström, L., Nonoyama, S., Quinti, I., Routes, J. M., Tang, M. L. K., & Warnatz, K. (2016). International consensus document (ICON): Common variable immunodeficiency disorders. *The Journal of Allergy and Clinical Immunology in Practice*, *4*, 38–59.

Borregaard, N., & Cowland, J. B. (1997). Granules of the human neutrophilic Polymorphonuclear leukocyte. *Blood*, *89*, 3503–3521.

Bouma, G., Ancliff, P. J., Thrasher, A. J., & Burns, S. O. (2010). Recent advances in the understanding of genetic defects of neutrophil number and function. *British Journal of Haematology*, *151*, 312–326.

Bowers, N. L., Helton, E. S., Huijbregts, R. P. H., Goepfert, P. A., Heath, S. L., & Hel, Z. (2014). Immune suppression by neutrophils in HIV-1 infection: Role of PD-L1/PD-1 pathway. *PLoS Pathogens*, *10*, 1.

Brandau, S., Trellakis, S., Bruderek, K., Schmaltz, D., Steller, G., Elian, M., Suttmann, H., Schenck, M., Welling, J., Zabel, P., & Lang, S. (2011). Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *Journal of Leukocyte Biology*, *89*, 311–317.

Carmona-Rivera, C., & Kaplan, M. J. (2013). Low density granulocytes: A distinct class of neutrophils in systemic autoimmunity. *Seminars in Immunopathology*, *35*, 455–463.

Casulli, S., Coignard-Biehler, H., Amazzough, K., Shoai-Tehrani, M., Bayry, J., Mahlaoui, N., Elbim, C., & Kaveri, S. V. (2014). Defective functions of polymorphonuclear neutrophils in patients with common variable immunodeficiency. *Immunologic Research*, *60*, 69–76.

Casulli, S., Topçu, S., Fattoum, L., von Gunten, S., Simon, H. U., Teillaud, J. L., Bayry, J., Kaveri, S. V., & Elbim, C. (2011). A differential concentration-dependent effect of IVIg on neutrophil functions: Relevance for anti-microbial and anti-inflammatory mechanisms. *PLoS One*, *6*, e26469.

Chandra, A., Zhang, F., Gilmour, K. C., Webster, D., Plagnol, V., Kumararatne, D. S., Burns, S. O., Nejentsev, S., & Thrasher, A. J. (2016). Common variable immunodeficiency and natural killer cell lymphopenia caused by Ets-binding site mutation in the IL-2 receptor γ (IL2RG) gene promoter. *Journal of Allergy and Clinical Immunology*, *137*, 940–942.e4.

Chapel, H., & Cunningham-Rundles, C. (2009). Update in understanding common variable immunodeficiency disorders (CVIDs) and the

management of patients with these conditions. *British Journal of Haematology*, *145*, 709–727.

Chen, Y., & Junger, W. G. (2012). Measurement of oxidative burst in neutrophils. *Methods in Molecular Biology*, *844*, 115–124.

Coxon, A., Cullere, X., Knight, S., Sethi, S., Wakelin, M. W., Stavrakis, G., Luscinskas, F. W., & Mayadas, T. N. (2001). Fc gamma RIII mediates neutrophil recruitment to immune complexes: A mechanism for neutrophil accumulation in immune-mediated inflammation. *Immunity*, *14*, 693–704.

Darcy, C. J., Minigo, G., Piera, K. A., Davis, J. S., McNeil, Y. R., Chen, Y., Volkheimer, A. D., Weinberg, J., Anstey, N. M., & Woodberry, T. (2014). Neutrophils with myeloid derived suppressor function deplete arginine and constrain T cell function in septic shock patients. *Critical Care*, *18*, R163.

Dolcino, M., Patuzzo, G., Barbieri, A., Tinazzi, E., Rizzi, M., Beri, R., Argentino, G., Ottria, A., Lunardi, C., & Puccetti, A. (2014). Gene expression profiling in peripheral blood mononuclear cells of patients with common variable immunodeficiency: Modulation of adaptive immune response following intravenous immunoglobulin therapy. *PLoS One*, *9*, e97571.

Dolman, C., Thorpe, S. J., & Thorpe, R. (2001). Enhanced efficacy of anti-D immunoglobulin for treating ITP is not explained by higher immunoglobulin polymer content. *Biologicals*, *29*, 75–79.

Dominguez-Soto, A., Simon-Fuentes, M., de las Casas-Engel, M., Cuevas, V. D., Lopez-Bravo, M., Dominguez-Andres, J., et al. (2018). IVIg promote cross-tolerance against inflammatory stimuli in vitro and in vivo. *Journal of Immunology*, *201*, 41–52.

Eichelberger, K. R., & Goldman, W. E. (2020). Manipulating neutrophil degranulation as a bacterial virulence strategy. *PLoS Pathogens*, *16*, e1009054.

Fernandes, M. J., Lachance, G., Paré, G., Rollet-Labelle, E., & Naccache, P. H. (2005). Signaling through CD16b in human neutrophils involves the Tec family of tyrosine kinases. *Journal of Leukocyte Biology*, *78*, 524–532.

Hardisty, G. R., Llanwarne, F., Minns, D., Gillan, J. L., Davidson, D. J., Findlay, E. G., et al. (2021). High purity isolation of low density neutrophils casts doubt on their exceptionality in health and disease. *Frontiers in Immunology*, *12*, 625922.

Hel, Z., Huijbregts, R. P. H., Xu, J., Nechvatalova, J., Vlkova, M., & Litzman, J. (2014). Altered serum cytokine signature in common variable immunodeficiency. *Journal of Clinical Immunology*, *34*, 971–978.

Kaneko, T., Stearns-Kurosawa, D. J., Taylor, F., Jr., Twigg, M., Osaki, K., Kinasewitz, G. T., et al. (2003). Reduced neutrophil CD10 expression in nonhuman primates and humans after in vivo challenge with *E. coli* or lipopolysaccharide. *Shock (Augusta, GA)*, *20*, 130–137.

Keir, M. E., Butte, M. J., Freeman, G. J., & Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. *Annual Review of Immunology*, *26*, 677–704.

Kessel, A., Peri, R., Haj, T., Snir, A., Slobodin, G., Sabo, E., Rosner, I., Shoenfeld, Y., & Toubi, E. (2011). IVIg attenuates TLR-9 activation in B cells from SLE patients. *Journal of Clinical Immunology*, *31*, 30–38.

Kozicky, L. K., Menzies, S. C., Zhao, Z. Y., Vira, T., Harnden, K., Safari, K., del Bel, K. L., Turvey, S. E., & Sly, L. M. (2018). IVIg and LPS Co-stimulation induces IL-10 production by human monocytes, which is compromised by a FcγRIIA disease-associated gene variant. *Frontiers in Immunology N.*, *9*, 2676.

Li, X.-K., Lu, Q.-B., Chen, W.-W., Xu, W., Liu, R., Zhang, S.-F., du, J., Li, H., Yao, K., Zhai, D., Zhang, P. H., Xing, B., Cui, N., Yang, Z. D., Yuan, C., Zhang, X. A., Xu, Z., Cao, W. C., Hu, Z., & Liu, W. (2018). Arginine deficiency is involved in thrombocytopenia and immunosuppression in severe fever with thrombocytopenia syndrome. *Science Translational Medicine*, *10*, eaat4162.

Liew, P. X., & Kubes, P. (2019). The Neutrophil's role during health and disease. *Physiological Reviews*, *99*, 1223–1248.

Litzman, J., Chovancová, Z., Bejdák, P., Litzman, M., Hel, Z., & Vlková, M. (2019). Common variable immunodeficiency patients display elevated plasma levels of granulocyte activation markers elastase and myeloperoxidase. *International Journal of Immunopathology and Pharmacology*, *33*, 2058738419843381.

Marini, O., Costa, S., Bevilacqua, D., Calzetti, F., Tamassia, N., Spina, C., de Sabata, D., Tinazzi, E., Lunardi, C., Scupoli, M. T., Cavallini, C., Zoratti, E., Tinazzi, I., Marchetta, A., Vassanelli, A., Cantini, M., Gandini, G., Ruzzenente, A., Guglielmi, A., … Scapini, P. (2017). Mature CD10+ and immature CD10− neutrophils present in G-CSF–treated donors display opposite effects on T cells. *Blood*, *129*, 1343–1356.

Marini, O., Spina, C., Mimiola, E., Cassaro, A., Malerba, G., Todeschini, G., Perbellini, O., Scupoli, M., Carli, G., Facchinelli, D., Cassatella, M., Scapini, P., & Tecchio, C. (2016). Identification of granulocytic myeloid-derived suppressor cells (G-MDSCs) in the peripheral blood of Hodgkin and non-Hodgkin lymphoma patients. *Oncotarget*, *7*, 27676–27688.

Martens, A., Eppink, G. J., Woittiez, A. J., Eidhof, H., & de Leij, L. F. (1999). Neutrophil function capacity to express CD10 is decreased in patients with septic shock. *Critical Care Medicine*, *27*, 549–553.

Matthews, N. C., Burton, C. S., & Alfred, A. (2021). Low-density neutrophils in chronic graft versus host disease (cGVHD) are primarily immature CD10- and enhance T cell activation. *Clinical and Experimental Immunology*, *205*, 257–273.

Mulay, S. R., & Anders, H.-J. (2020). Neutrophils and neutrophil extracellular traps regulate immune responses in health and disease. *Cells*, *9*, 2130.

Nagelkerke, S. Q., & Kuijpers, T. W. (2015). Immunomodulation by IVIg and the role of fc-gamma receptors: Classic mechanisms of action after all? *Frontiers in Immunology*, *5*, 674.

Nguyen, G. T., Green, E. R., & Mecsas, J. (2017). Neutrophils to the ROS-cue: Mechanisms of NADPH oxidase activation and bacterial resistance. *Frontiers in Cellular and Infection Microbiology*, *7*, 373.

Rocha, B. C., Marques, P. E., Leoratti, F. M. d. S., Junqueira, C., Pereira, D. B., Antonelli, L. R. d. V., et al. (2015). Type I interferon transcriptional signature in neutrophils and high frequency of low-density granulocytes are associated with tissue damage in malaria. *Cell Reports*, *13*, 2829–2841.

Rodriguez-Rosales, Y. A., Langereis, J. D., Gorris, M. A. J., van den Reek, J. M. P. A., Fasse, E., Netea, M. G., et al. (2021). Immunomodulatory aged neutrophils are augmented in blood and skin of psoriasis patients. *Journal of Allergy and Clinical Immunology*, *148*, 1030–1040.

Rosales, C. (2020). Neutrophils at the crossroads of innate and adaptive immunity. *Journal of Leukocyte Biology*, *108*, 377–396.

Scapini, P., Marini, O., Tecchio, C., & Cassatella, M. A. (2016). Human neutrophils in the saga of cellular heterogeneity: Insights and open questions. *Immunological Reviews*, *273*, 48–60.

Sharifi, L., Tavakolinia, N., Kiaee, F., Rezaei, N., Mohsenzadegan, M., Azizi, G., et al. (2017). A review on defects of dendritic cells in common variable immunodeficiency. *Endocrine, Metabolic & Immune Disorders – Drug Targets (Formerly Current Drug Targets – Immune, Endocrine & Metabolic Disorders)*, *17*, 100–113.

Shipp, M., Stefano, G., Switzer, S., Griffin, J., & Reinherz, E. (1991). CD10 (CALLA)/neutral endopeptidase 24.11 modulates inflammatory peptide- induced changes in neutrophil morphology, migration, and adhesion proteins and is itself regulated by neutrophil activation. *Blood*, *78*, 1834–1841.

Siedlar, M., Strach, M., Bukowska-Strakova, K., Lenart, M., Szaflarska, A., Węglarczyk, K., Rutkowska, M., Baj-Krzyworzeka, M., Pituch-Noworolska, A., Kowalczyk, D., Grodzicki, T., Ziegler-Heitbrock, L., & Zembala, M. (2011). Preparations of intravenous immunoglobulins diminish the number and proinflammatory response of CD14 +CD16 ++ monocytes in common variable immunodeficiency (CVID) patients. *Clinical Immunology*, *139*, 122–132.

Sun, R., Huang, J., Yang, Y., Liu, L., Shao, Y., Li, L., & Sun, B. (2022). Dysfunction of low-density neutrophils in peripheral circulation in patients with sepsis. *Scientific Reports*, *12*, 685.

Tay, S. H., Celhar, T., & Fairhurst, A.-M. (2020). Low-density neutrophils in systemic lupus erythematosus. *Arthritis & Rheumatology*, *72*, 1587–1595.

Trépanier, P., Chabot, D., & Bazin, R. (2014). Intravenous immunoglobulin modulates the expansion and cytotoxicity of CD8+ T cells. *Immunology*, *141*, 233–241.

Tsinti, G., Makris, D., Germenis, A. E., & Speletas, M. (2020). Persistent activation of innate immunity in patients with primary antibody deficiencies. *Journal of Immunology Research*, *2020*, 8317671.

Ui Mhaonaigh, A., Coughlan, A. M., Dwivedi, A., Hartnett, J., Cabral, J., Moran, B., Brennan, K., Doyle, S. L., Hughes, K., Lucey, R., Floudas, A., Fearon, U., McGrath, S., Cormican, S., de Bhailis, A., Molloy, E. J., Brady, G., & Little, M. A. (2019). Low density granulocytes in ANCA Vasculitis are Heterogenous and hypo-responsive to anti-myeloperoxidase antibodies. *Frontiers in Immunology*, *10*, 2603.

Uribe-Querol, E., & Rosales, C. (2020). Phagocytosis: Our current understanding of a universal biological process. *Frontiers in Immunology*, *11*, 1066.

van der Heijden, J., Nagelkerke, S., Zhao, X., Geissler, J., Rispens, T., van den Berg, T. K., et al. (2014). Haplotypes of FcγRIIa and FcγRIIIb polymorphic variants influence IgG-mediated responses in neutrophils. *Journal of Immunology*, *192*, 2715–2721.

Vlkova, M., Chovancova, Z., Nechvatalova, J., Connelly, A. N., Davis, M. D., Slanina, P., Travnickova, L., Litzman, M., Grymova, T., Soucek, P., Freiberger, T., Litzman, J., & Hel, Z. (2019). Neutrophil and granulocytic myeloid-derived suppressor cell–mediated T cell suppression significantly contributes to immune dysregulation in common variable immunodeficiency disorders. *The Journal of Immunology*, *202*, 93–104.

Walker, B. A., Hagenlocker, B. E., Stubbs, E. B., Jr., Sandborg, R. R., Agranoff, B. W., & Ward, P. A. (1991). Signal transduction events and fc γ R engagement in human neutrophils stimulated with immune complexes. *Journal of Immunology*, *146*, 735–741.

Wright, H. L., Makki, F. A., Moots, R. J., & Edwards, S. W. (2017). Low-density granulocytes: Functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signaling. *Journal of Leukocyte Biology*, *101*, 599–611.

SUPPORTING INFORMATION

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

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