

## ORIGINAL RESEARCH ARTICLE

# Hyporesponsiveness to pathogen stimulation in CD56<sup>+</sup> natural killer cells and CD8<sup>+</sup> T cells from patients with primary sclerosing cholangitis

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## Abstract

Primary sclerosing cholangitis (PSC) is a rare cholestatic liver disease characterized by progressive inflammation of the intra- and extrahepatic bile ducts and a high association with inflammatory bowel disease. Since bacterial and viral stimuli are thought to contribute to the pathogenesis of autoimmune diseases, our study aims to investigate the effects of synthetic pathogenic agonists on the cytotoxic function of cluster of differentiation (CD)56<sup>+</sup> natural killer (NK) cells and CD8<sup>+</sup> T cells in PSC. A total of 17 PSC patients, 18 autoimmune hepatitis (AIH) patients, and 14 healthy controls (HCs) were included in this study. Using multicolor flow cytometry, we analyzed cytotoxic activity (CD107a assay) and secretion of interferon-gamma and tumor necrosis factor-alpha in peripheral CD56<sup>+</sup> NK-cell subsets and CD8<sup>+</sup> T cells after *in vitro* stimulation with synthetic bacterial (Pam3CSK, lipopolysaccharide [LPS], CpG-ODN-2216, and flagellin) and viral agonists (polyinosinic: polycytidylic acid [poly(I: C)], poly(I: C)-high molecular weight/LyoVec<sup>™</sup>, 5'ppp-dsRNA/LyoVec<sup>™</sup>, and R837). Compared with AIH patients and HCs, CD56<sup>+</sup> NK-cell subsets from PSC patients showed reduced cytotoxicity both at baseline and upon *in vitro* stimulation with synthetic bacterial/viral agonists. Notably, the percentage of unstimulated CD107a<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup> NK cells positively correlated with clinical serum parameters in PSC. In addition, the percentage of CD107a<sup>+</sup>CD8<sup>+</sup> cells was significantly reduced after synthetic bacterial/viral stimulation in PSC patients. Finally, in PSC patients, production of pro-inflammatory cytokines was markedly reduced in CD56<sup>high</sup>CD16<sup>-</sup> NK cells upon *in vitro* stimulation with Pam3CSK and LPS, respectively. This hyporesponsiveness of CD56<sup>+</sup> NK cells and CD8<sup>+</sup> T cells may contribute to the development of hepatic inflammation, representing a hallmark of PSC pathogenesis.

**Keywords:** Primary sclerosing cholangitis; Autoimmune liver disease; Synthetic bacterial/viral agonists; CD56<sup>+</sup> natural killer cells; CD8<sup>+</sup> T cells; Cytotoxicity; Cytokine production

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**Citation:** Kalthoff S, Dold L, Wolf J, Strassburg CP, Langhans B. Hyporesponsiveness to pathogen stimulation in CD56<sup>+</sup> natural killer cells and CD8<sup>+</sup> T cells from patients with primary sclerosing cholangitis. *Microbes & Immunity*. 2026;3(2):025360095. doi: 10.36922/MI025360095

**Received:** September 1, 2025

**Revised:** November 27, 2025

**Accepted:** December 15, 2025

**Published online:** January 6, 2026

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## 1. Introduction

Primary sclerosing cholangitis (PSC) is a rare multifactorial cholestatic liver disease with autoimmune features characterized by progressive inflammation of the intra- and

extrahepatic bile ducts, eventually leading to liver cirrhosis and end-stage liver disease. Currently, no medical treatment is available that effectively slows disease progression, and liver transplantation remains the only therapeutic option in severe cases. PSC is also an important risk factor for cholangiocellular carcinoma (CCA).<sup>1</sup> Moreover, given the markedly increased risk of CCA in PSC, recent advances in systemic therapies—notably targeted therapies and immunotherapeutic strategies—have broadened the therapeutic landscape beyond conventional chemotherapy and may, in the future, offer avenues for intervention in neoplastic transformation in PSC.<sup>2,3</sup> Moreover, up to 80% of patients with PSC present with concomitant chronic inflammatory bowel disease (IBD), such as ulcerative colitis (UC) or Crohn's disease.<sup>1,4</sup>

The etiology of PSC remains poorly understood, but it is widely believed to involve a complex interplay between genetic predisposition, dysregulated immune responses, and environmental factors.<sup>5</sup> The high prevalence of IBD in patients with PSC suggests a close correlation with microbial dysbiosis and an exaggerated or inappropriate immune response to pathogen-associated molecular patterns (PAMPs) derived from bacteria and viruses.<sup>6</sup> PAMPs are recognized by immune cells expressing pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and retinoic acid inducible gene I-like helicase receptors (RLRs). Membrane-bound TLR2, TLR4, TLR5, and TLR9 recognize bacterial ligands, whereas retinoic acid inducible gene I (*RIG-I*) and melanoma differentiation-associated protein 5 (MDA-5) respond to cytosolic viral RNA or DNA. Furthermore, TLR3 and TLR7 are stimulated using viral nucleic acids within the endosomal compartment.<sup>7</sup> Although PRRs are predominantly expressed on macrophages and dendritic cells, natural killer (NK) cells<sup>8</sup> and T cells<sup>9</sup> also express several TLRs and RLRs.

Both NK cells and cluster of differentiation (CD)8<sup>+</sup> T cells are key effector lymphocytes secreting inflammatory cytokines, such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha, as well as cytotoxic molecules essential for combating invading pathogens.<sup>10</sup> However, whether PAMP stimulation affects the functional activity of NK cells and CD8<sup>+</sup> T cells in PSC remains unclear. Although an increased percentage of activated CD8<sup>+</sup> T cell subsets has been reported in PSC patients,<sup>11</sup> no studies have directly evaluated the cytotoxic activity of peripheral CD8<sup>+</sup> T cells in these patients. Moreover, the contribution of NK cells to PSC pathophysiology remains incompletely defined. There is evidence suggesting decreased cytotoxic activity of liver-derived NK cells in PSC patients,<sup>12</sup> whereas others reported increased cytotoxicity in specific NK-cell subsets in peripheral blood.<sup>13,14</sup>

Despite accumulating evidence indicating altered NK cells and CD8<sup>+</sup> T cell functions in PSC, the role of PAMP/PRR signaling in PSC pathogenesis has not been systematically assessed. Given that bacterial and viral stimuli are suggested to contribute to the initiation and progression of autoimmune diseases,<sup>15,16</sup> defining how bacterial and viral agonists shape cytotoxic immune responses in PSC may offer important insights into disease mechanisms. Therefore, in this study, we investigated the cytotoxicity and cytokine secretion in CD56<sup>+</sup> NK-cell subsets and CD8<sup>+</sup> T cells isolated from the peripheral blood of PSC patients following *in vitro* stimulation with synthetic bacterial and viral agonists.

## 2. Methods

### 2.1. Patients

An *a priori* power analysis was performed to determine the required sample size. Effect sizes for CD56<sup>+</sup> NK cell and CD8<sup>+</sup> T cell functional readouts (e.g., CD107a degranulation and cytokine production) were derived from our previously published work that employed a comparable experimental design.<sup>17</sup> Power calculations were conducted using G\*Power (v3.1, Heinrich-Heine-Universität Düsseldorf, Germany), applying two-sample or paired *t*-tests as appropriate, with a significance level of  $\alpha = 0.05$  and a target power of  $1 - \beta = 0.80$ . Based on these calculations, we included 17 patients with PSC in this study. PSC diagnosis was based on the European Association for the Study of the Liver clinical practice guidelines on sclerosing cholangitis.<sup>18</sup> All PSC patients had been regularly monitored at the Department of Internal Medicine I at the University Hospital of Bonn. None of them had current bacterial cholangitis or biliary stent drainage at the time of blood sampling. Concomitant IBD was diagnosed according to established clinical criteria, and IBD phenotypes were classified as UC, Crohn's disease, or indeterminate colitis.<sup>19</sup> None of the PSC patients with IBD showed clinical signs of acute intestinal inflammation at the time of blood sampling. Data from PSC patients were compared with those of healthy controls (HCs;  $n = 14$ ) and patients with autoimmune hepatitis (AIH;  $n = 18$ ), who served as disease controls. All patients were recruited from our outpatient department, and HCs were recruited from the Bonn University blood banking service. Clinical characteristics and demographic data of the study groups are summarized in Table 1. Ursodeoxycholic acid (UDCA) therapy was administered to 17 patients with PSC and to 5 AIH patients. Immunosuppressive treatment (budesonide, prednisolone, and azathioprine) was given to three PSC patients and 18 AIH patients, respectively. Fourteen PSC patients received mesalazine due to underlying IBD.

**Table 1. Comparison of demographics and laboratory parameters in patients with primary sclerosing cholangitis, autoimmune hepatitis, and healthy controls**

Demographic and laboratory parameters	Healthy controls	PSC	AIH
Total number of individuals	14	17	18
Age (years)	43±20	39±12	58±17
Gender (male/female)	7/7	8/9	4/14
Clinical parameters			
IBD (%)	n.a.	70.6	0
Ulcerative colitis (% of IBD)	n.a.	91.7	0
Colitis indeterminata (% of IBD)	n.a.	8.3	0
PSC/AIH variant-phenotype (% of PSC)	n.a.	11.8	0
Laboratory parameters			
Amsterdam-Oxford Score	n.d.	1.51±0.43	n.a.
Model for end-stage liver disease score	n.d.	6.5±1.1	7.0±1.3
Transient elastography (kPa)	n.d.	9.01±6.40	6.66±2.09
Aspartate aminotransferase (U/L)	n.d.	53.6±49.9	40.6±50.7
Alanine aminotransferase (U/L)	n.d.	77.4±103.7	51.4±87.6
Alkaline phosphatase (U/L)	n.d.	171.4±139.2	96.1±86.9
Bilirubin (mg/dL)	n.d.	0.69±0.42	0.59±0.41
C-reactive protein (mg/L)	n.d.	3.9±4.5	1.3±0.7
Leukocytes (g/L)	n.d.	8.6±3.7	7.7±2.0
Platelets (Giga/L)	n.d.	303±127	260±71

Notes: Data are expressed as mean±standard deviation.

Abbreviations: AIH: Autoimmune hepatitis; IBD: Inflammatory bowel disease; n.a.: Not applicable; n.d.: Not done; PSC: Primary sclerosing cholangitis.

The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (number 128/23-EP) in accordance with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from all individuals included in this study.

## 2.2. Reagents

All PRR-activating reagents were purchased as synthetic agonists from *InvivoGen* (France), including triacylated lipoprotein Pam3CSK (P3C) for TLR1/2; ultrapure lipopolysaccharide from *Escherichia coli* LPS-B5 (LPS) for TLR4; Class A CpG-ODN-2216 (CpG) for TLR9; purified flagellin from *Pseudomonas aeruginosa* (FLA-PA) for TLR5; high-molecular-weight (HMW) polyinosinic: polycytidylic acid (Poly(I: C) [pIC]; HMW synthetic analog of double-stranded RNA [dsRNA]) for TLR3; Poly(I: C)-HMW/LyoVec™ (pIC-LV) for RIG-I/MDA-5; 5'ppp-dsRNA/LyoVec™ (dsRNA) for RIG-I; and R837 (small synthetic antiviral molecule) for TLR7.

## 2.3. Flow cytometric antibodies

The following antibodies from BioLegend (United Kingdom) were used for flow cytometric analyses: APC-Cy7-labeled anti-CD3 (clone ID: UCHT1), PerCp-labeled anti-CD4 (clone ID: SK3), APC-labeled anti-CD8 (clone ID: Leu2), Brilliant Violet 421-labeled anti-CD56 (clone ID: 5.1H11), PerCP-labeled anti-CD16 (clone ID: B73.1), and PE-labeled anti-IFN-gamma (clone ID: B27). The FITC-conjugated CD107a antibody (clone ID: H4A3) was purchased from BD Biosciences (Heidelberg, Germany).

## 2.4. Isolation of NK and T-cell subsets from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using density gradient centrifugation (Pancoll human; density: 1.077 g/mL, PAA Laboratories, Cölbe, Germany) and cryopreserved in liquid nitrogen until analysis. PBMCs were thawed immediately before use. Afterward, NK cells and T cells were isolated through immunomagnetic separation using commercial Magnetic-Activated Cell Sorting kits (NK Cells Isolation Kit [Catalog no. 130-092-657] and Pan T Cell Isolation Kit [Catalog no. 130-096-535], both from Miltenyi Biotec, Germany). Purities of CD56<sup>+</sup> NK cell and CD8<sup>+</sup> T cell preparations exceeded 95%, as confirmed using flow cytometry (BD FACSCanto™ II, BD Biosciences, Germany).

## 2.5. Functional analysis of immune cells after stimulation with synthetic pathogenic agonists

### 2.5.1. CD56<sup>+</sup> NK cells

Activation of purified NK cells was assessed by measuring induction of CD107a expression (a validated surrogate for cytotoxic degranulation<sup>20,21</sup>) as well as intracellular IFN-gamma and TNF-alpha production following stimulation with synthetic bacterial and viral agonists. Co-incubation of NK cells with major histocompatibility complex-deficient K562 target cells (#ACC 10, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany; ratio 1:2) served as a positive control.

NK cells were first pre-activated overnight with recombinant interleukin (IL)-2 (100 U/mL) and then stimulated for 16 h with P3C (1 µg/mL), LPS (50 ng/mL), CpG (2.5 µM), FLA (50 ng/mL), pIC (5 µg/mL), pIC-LV (500 ng/mL), dsRNA (1 µg/mL), or R837 (5 µg/mL). One hour after adding CD107a antibody, Golgi Stop (BD Biosciences, United States of America) and BFA (3 µg/mL; Enzo Life Sciences GmbH, Germany) were added for an additional 3 h to permit intracellular cytokine detection. Cells were stained with Zombie Aqua™ viability dye (BioLegend, USA) followed by surface staining with anti-CD3, anti-CD56, and anti-CD16. After fixation and

permeabilization, NK cells were stained intracellularly with anti-IFN-gamma and anti-TNF-alpha. The cell percentages in NK-cell subsets (overall CD56<sup>+</sup> NK cells, CD56<sup>dim</sup>CD16<sup>+</sup> NK cells, and CD56<sup>high</sup>CD16<sup>-</sup> NK cells, Figure S1) that expressed CD107a, IFN-gamma, or TNF-alpha were measured at baseline via flow cytometric analysis, after stimulation with synthetic agonists, as well as after co-culture with K562 target cells.

In preceding experiments, fluorescence-activated cell sorting antibodies had been titrated to detect optimal dilutions. Appropriate fluorescence minus one and/or isotype controls were included in every experiment to ensure consistent gating strategies and to exclude non-specific staining. Our gating strategy for identifying NK-cell subset percentage is shown in Figure S1. Gating boundaries to define the percentages of CD107a<sup>+</sup>, IFN-gamma<sup>+</sup>, and TNF-alpha<sup>+</sup> cells in NK-cell subsets are shown in Figure S2.

Due to the limited availability of PBMCs, not all analyses could be performed in all individuals. The final numbers included in each analysis are reported in the corresponding figure legends.

### 2.5.2. CD8<sup>+</sup> and CD4<sup>+</sup> T cells

Analogous to NK cells, activation of CD107a degranulation, IFN-gamma, and TNF-alpha induction through synthetic pathogenic agonists was measured in CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells using flow cytometry. As a positive control, we studied CD107a degranulation and cytokine production in the presence of concanavalin-A (2.5 mg/mL, *In vivo* Gen, France)-loaded P815 effector cells (#ACC 1, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany; ratio 1:2). Figures S1 and S2 illustrate gating strategies in CD8<sup>+</sup> T cells via flow cytometry.

### 2.6. Statistical analysis

Data were analyzed using GraphPad Prism (version 9.0, Dotmatics, United Kingdom). Data were tested for normality. Non-normally distributed data were analyzed using the Mann-Whitney test (unpaired) and the Wilcoxon matched-pairs signed-rank test (paired) as appropriate. Correlations between clinical parameters and experimental data were evaluated using Spearman's rank correlation. A  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Reduced CD107a degranulation in CD56<sup>+</sup> NK cell subsets upon stimulation with synthetic bacterial/viral agonists in PSC

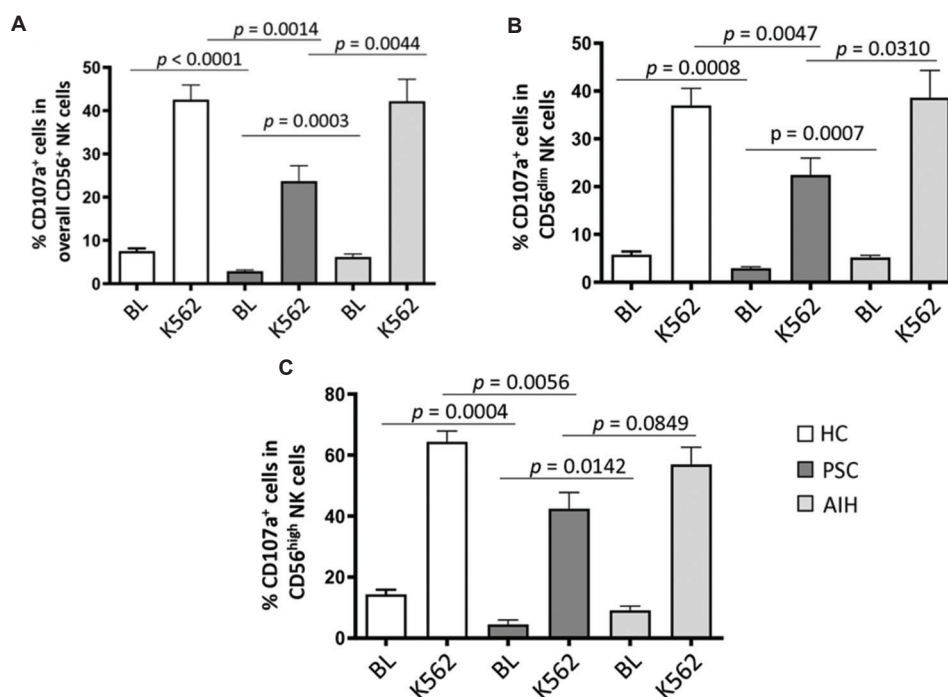
Human NK cells can be categorized into distinct subsets based on the relative surface expression of CD56 and CD16.

As important defenders against invading pathogens, both CD56<sup>dim</sup>CD16<sup>+</sup> and CD56<sup>high</sup>CD16<sup>-</sup> NK-cell subsets exert cytotoxic functions and produce inflammatory cytokines.<sup>22</sup> To assess functional characteristics of NK cells, we first assessed CD107a degranulation, a widely used and robust surrogate marker of cytotoxicity in NK cells and T cells.<sup>20,21</sup> After *in vitro* stimulation with various synthetic pathogenic agonists or K562 target cells (positive control), CD107a expression was quantified in overall CD56<sup>+</sup> NK cells (including both CD56<sup>dim</sup>CD16<sup>+</sup> and CD56<sup>high</sup>CD16<sup>-</sup> NK cells) as well as in the distinct subgroups CD56<sup>dim</sup>CD16<sup>+</sup> NKs and CD56<sup>high</sup>CD16<sup>-</sup> NK-cell subsets. The obtained data were compared across study groups. Compared with AIH patients and HCs, the percentage of CD107a<sup>+</sup> cells was significantly lower in PSC patients at baseline and after K562 stimulation in overall CD56<sup>+</sup> NK cells (Figure 1A), in CD56<sup>dim</sup>CD16<sup>+</sup> NK cells (Figure 1B), and in CD56<sup>high</sup>CD16<sup>-</sup> NK cells (Figure 1C).

To further evaluate functional inducibility, we next examined whether CD107a degranulation could be activated in each NK-cell subset using synthetic bacterial (P3C, LPS, CpG, and FLA) and viral agonists (pIC, pIC-LV, dsRNA, and R837) via *in vitro* stimulation and compared the results to baseline. Stimulation with either bacterial or viral agonists significantly increased CD107a expression in CD56<sup>overall/dim/high</sup> NK-cell subsets from HCs relative to baseline (Figure 2A-F). In contrast, CD56<sup>overall/dim/high</sup> NK-cell subsets from PSC patients showed minimal or absent inducibility of CD107a degranulation following bacterial stimulation, with CpG as the only agonist to elicit a detectable response (Figure 2A-C). Similarly, activation with viral agonists pIC-LV and dsRNA failed to induce CD107a expression in CD56<sup>overall/dim/high</sup> NK-cell subsets from PSC patients, whereas pIC and R837 led to markedly increased CD107a degranulation (Figure 2D-F).

When directly comparing across groups, PSC patients exhibited significantly lower percentages of CD107a<sup>+</sup> cells in the different CD56<sup>overall/dim/high</sup> NK-cell subsets following *in vitro* stimulation with P3C, LPS, and FLA compared with HCs (Figure 2A-C). Viral agonists pIC-LV and dsRNA likewise induced markedly less CD107a expression in PSC patients compared with healthy individuals (Figure 2D-F). Apart from a few exceptions, stimulation of NK cells with synthetic bacterial/viral agonists in AIH patients yielded results similar to those observed in HCs. Furthermore, we stratified our patients and reanalyzed the data regarding medication; however, medical treatment (UDCA, mesalazine, corticosteroids, and azathioprine) did not affect the results (data not shown). Taken together, these findings demonstrate a pronounced impairment in





**Figure 1.** Percentage of CD107a<sup>+</sup> cells in NK-cell subsets. Overall CD56<sup>+</sup> (A), CD56<sup>dim</sup> (B), and CD56<sup>high</sup> (C) NK cells induced with K562 target cells. Boxes and whiskers indicate mean and standard error of the mean, respectively.  $p < 0.05$  indicates statistically significant differences, assessed using the unpaired non-parametric Mann–Whitney test for independent comparisons and the Wilcoxon matched-pairs signed-rank test for paired comparisons, as indicated by the bars. Abbreviations: AIH: Autoimmune hepatitis; BL: Baseline; CD: Cluster of differentiation; HCs: Healthy controls; NK: Natural killer; PSC: Primary sclerosing cholangitis.

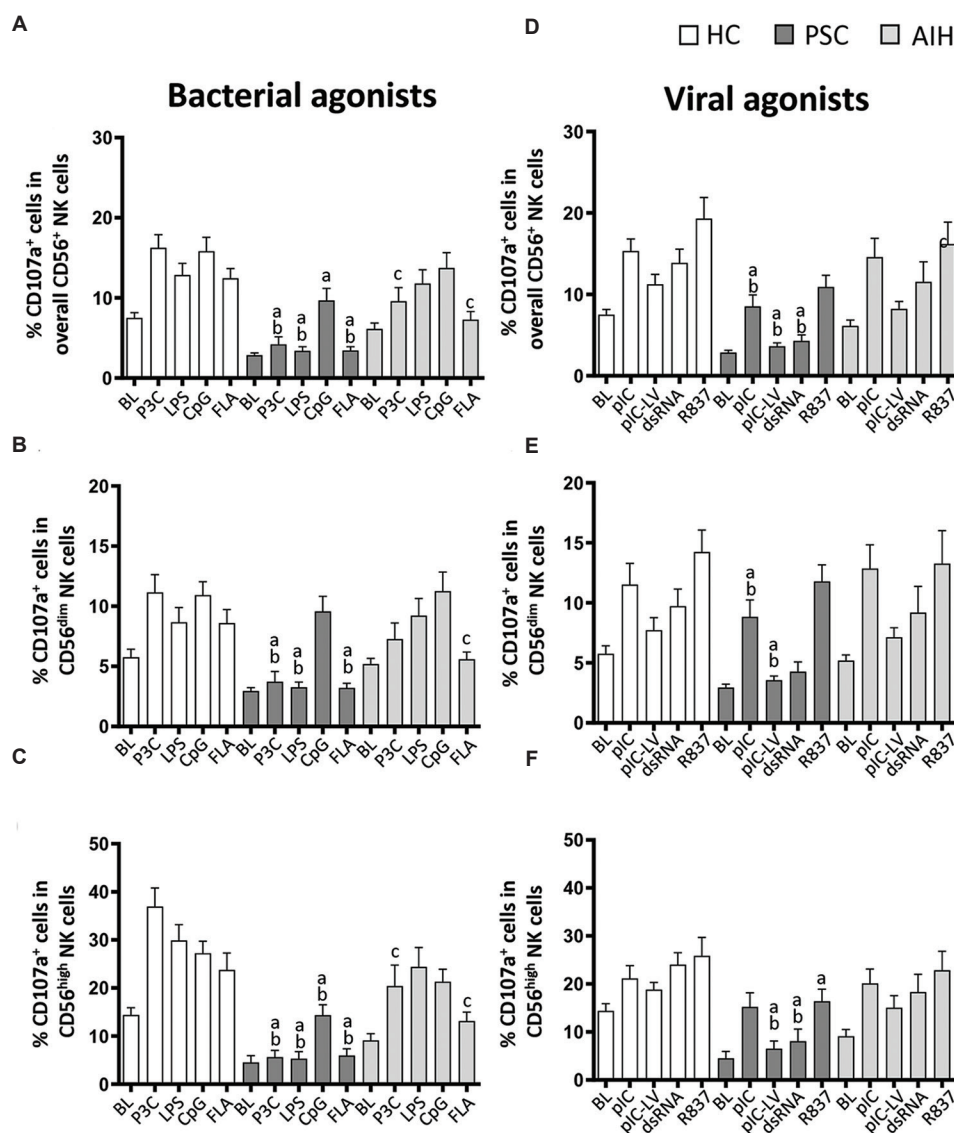
both basal and inducible cytotoxic degranulation of NK cells in PSC across all major NK-cell subsets.

### 3.2. Reduced CD107a degranulation in CD8<sup>+</sup> T cells upon stimulation with synthetic bacterial agonist CpG-ODN-2216 in PSC

Since human T cells were shown to express various PRRs/RLRs, we investigated CD107a degranulation after *in vitro* stimulation with bacterial/viral agonists and P815 effector cells (positive control) in CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells. In line with NK cell results, the percentage of CD107a<sup>+</sup> cells in CD8<sup>+</sup> T cells from PSC patients was significantly lower after stimulation with P815 cells than in AIH patients and HCs (Figure 3A). When analyzing inducibility relative to baseline, stimulation with CpG was the only agonist that elicited a significant increase in CD107a expression among CD8<sup>+</sup> T cells from PSC patients and HCs. All other bacterial and viral agonists failed to induce CD107a upregulation in CD8<sup>+</sup> T cells from PSC patients (Figure 3B and C). When directly comparing across groups, CpG-induced percentage of CD107a<sup>+</sup>CD8<sup>+</sup> T cells was significantly lower in PSC than in HCs. In contrast, CD4<sup>+</sup> T cells from patients with PSC exhibited no significant changes in CD107a degranulation (data not shown).

### 3.3. Reduced production of IFN-gamma and TNF-alpha in CD56<sup>high</sup>CD16<sup>-</sup> NK cells upon stimulation with Pam3CSK and lipopolysaccharide-B5 in PSC

In contrast to CD56<sup>dim</sup>CD16<sup>+</sup> NK cells, CD56<sup>high</sup>CD16<sup>-</sup> NK cells predominantly produce cytokines, such as IFN-gamma and TNF-alpha, in response to invading pathogens.<sup>22</sup> To further characterize potential functional defects in PSC, we analyzed intracellular production of IFN-gamma and TNF-alpha in CD56<sup>high</sup>CD16<sup>-</sup> NK cells after stimulation with synthetic bacterial and viral agonists. In CD56<sup>high</sup>CD16<sup>-</sup> NK cells from HCs, stimulation with K562 target cells, P3C, and LPS significantly induced both IFN-gamma (Figure 4A) and TNF-alpha (Figure 4B). However, CD56<sup>high</sup>CD16<sup>-</sup> NK cells from PSC patients displayed a distinct response pattern. While K562 cells induced both IFN-gamma (Figure 4A) and TNF-alpha (Figure 4B), only P3C stimulation led to a significant increase in the percentage of TNF-alpha-producing cells (Figure 4B). Notably, the P3C- and LPS-induced IFN-gamma (Figure 4A) and TNF-alpha production in CD56<sup>high</sup>CD16<sup>-</sup> NK cells was markedly reduced in PSC compared with HCs (Figure 4B). In AIH, stimulation of CD56<sup>high</sup>CD16<sup>-</sup> NK cells with P3C and LPS yielded results similar to those observed in HCs.



**Figure 2.** Percentage of CD107a<sup>+</sup> cells in NK-cell subsets upon stimulation with synthetic pathogenic agonists. (A–C) Overall CD56<sup>+</sup> (A), CD56<sup>dim</sup> (B), and CD56<sup>high</sup> (C) NK cells induced with bacterial agonists. (D–F) Overall, CD56<sup>+</sup> (D), CD56<sup>dim</sup> (E), and CD56<sup>high</sup> (F) NK cells were induced with viral agonists. Boxes and whiskers indicate mean and standard error of the mean, respectively.

Notes: <sup>a</sup>*p*<0.05 for comparisons between PSC and HC; <sup>b</sup>*p*<0.05 for comparisons between PSC and AIH; <sup>c</sup>*p*<0.05 for comparisons between AIH and HC. Abbreviations: AIH: Autoimmune hepatitis; BL: Baseline; CD: Cluster of differentiation; CpG: Class A CpG-ODN-2216; FLA: Flagellin from *Pseudomonas aeruginosa*; HCs: Healthy controls; LPS: Lipopolysaccharide from *Escherichia coli*; NK: Natural killer; P3C: Triacylated lipoprotein Pam3CSK; pIC: High molecular weight polyinosinic: polycytidylic acid; pIC-LV: High molecular weight polyinosinic: polycytidylic acid/LycoVec<sup>TM</sup>; PSC: Primary sclerosing cholangitis.

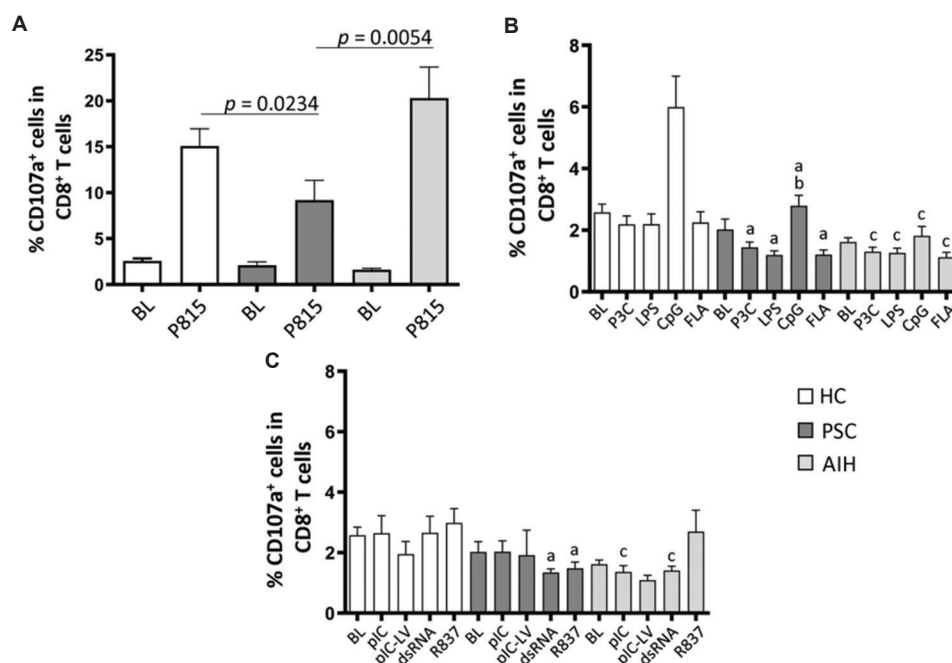
### 3.4. Correlation of CD107a<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup> NK cells with clinical serum parameters in PSC

To assess how NK-cell cytotoxic activity relates to clinical disease parameters in PSC, we performed correlation analyses with standard laboratory markers. These analyses revealed an inverse correlation between the percentage of unstimulated CD107a<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup> NK cells and serum levels of alanine aminotransferase (Figure 5A) and gamma-

glutamyl transferase (Figure 5B). A similar trend was observed for alkaline phosphatase, although the data were not statistically significant (Figure 5C). Moreover, albumin levels demonstrated a modest positive correlation with the percentage of CD107a<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup> NK cells (Figure 5D).

## 4. Discussion

The gut–liver axis has been shown to play an important role in the pathogenesis of PSC.<sup>1,23</sup> In this context, immune cells



**Figure 3.** Percentage of CD107a<sup>+</sup> in CD8<sup>+</sup> T cells. (A) Stimulation with P815 cells. (B) Stimulation with bacterial agonists. (C) Stimulation with viral agonists. Boxes and whiskers indicate mean and standard error of the mean, respectively.

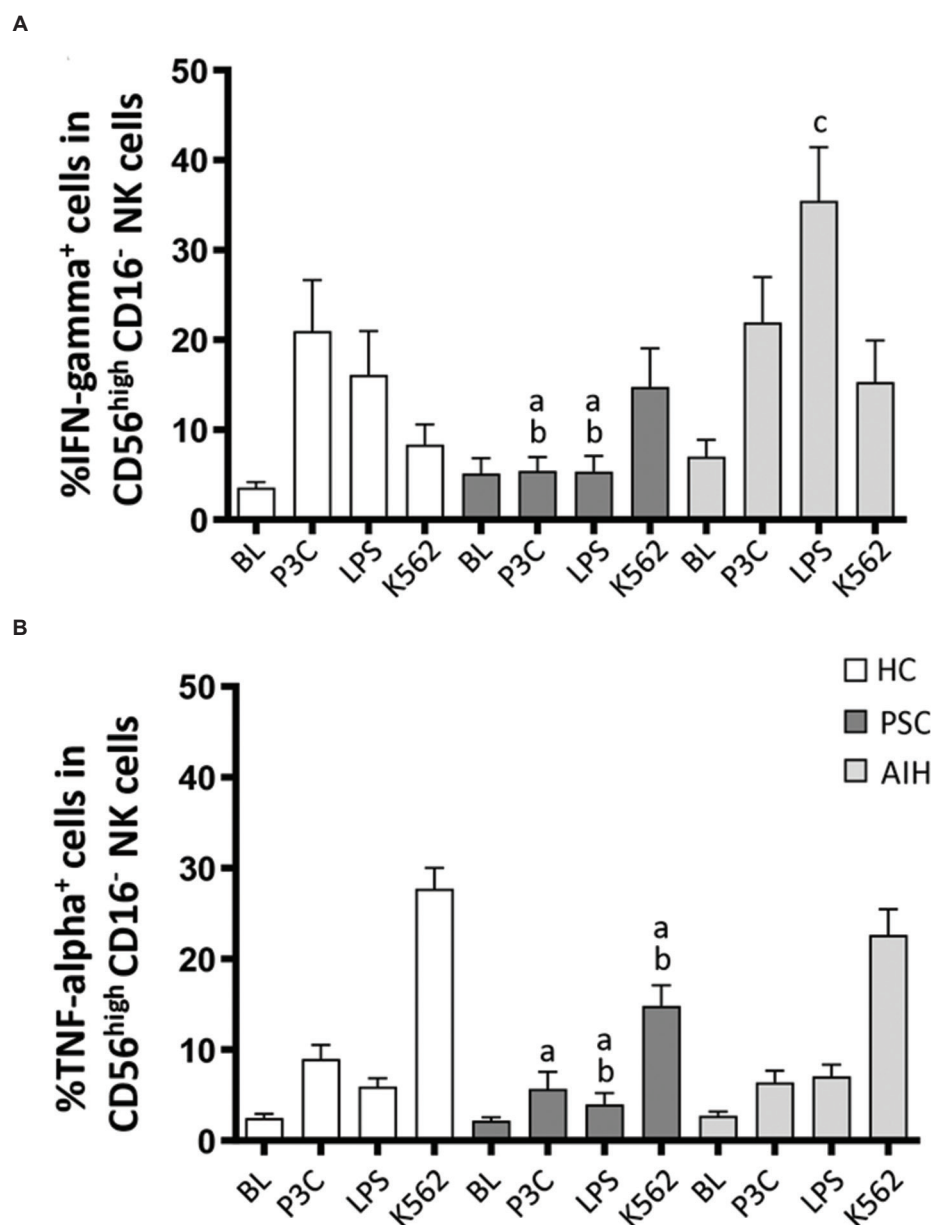
Notes: <sup>a</sup> $p < 0.05$  for comparisons between PSC and HC; <sup>b</sup> $p < 0.05$  for comparisons between PSC and AIH; <sup>c</sup> $p < 0.05$  for comparisons between AIH and HC. Abbreviations: AIH: Autoimmune hepatitis; BL: Baseline; CD: Cluster of differentiation; CpG: Class A CpG-ODN-2216; FLA: Flagellin from *Pseudomonas aeruginosa*; HCs: Healthy controls; LPS: Lipopolysaccharide from *Escherichia coli*; P3C: Triacylated lipoprotein Pam3CSK; pIC: High molecular weight polyinosinic: polycytidylic acid; pIC-LV: High molecular weight polyinosinic: polycytidylic acid/LycoVec<sup>TM</sup>; PSC: Primary sclerosing cholangitis.

can trigger inflammatory processes in the liver.<sup>24</sup> However, the impact of pathogenic agonists on cytotoxicity and on the functional properties of NK cells and CD8<sup>+</sup> T cells in PSC remains poorly understood.

To our knowledge, this study is the first to report reduced inducibility of CD107a degranulation in peripheral CD56<sup>+</sup> NK cells and CD8<sup>+</sup> T cells using synthetic bacterial and viral agonists, suggesting that pathogen-derived signals may influence cytotoxic immune responses in PSC. Furthermore, cytotoxic activity in CD56<sup>dim</sup> NK cells correlated with PSC-specific clinical serum markers, with lower NK-cell activity associated with increased disease severity. In addition, the production of pro-inflammatory IFN-gamma and TNF-alpha was markedly decreased in PSC-derived CD56<sup>high</sup>CD16<sup>-</sup> NK cells following *in vitro* stimulation with P3C and LPS, respectively.

As an immune-triggered disease, PSC is characterized by upregulated production of pro-inflammatory cytokines and elevated numbers of IFN-gamma-producing CD4<sup>+</sup> T helper type 1 cells and IL-17A-producing CD4<sup>+</sup> T helper type 17 cells.<sup>25-29</sup> A distinct IL-17-producing CD103<sup>+</sup>CD69<sup>+</sup>CD8<sup>+</sup> T-cell subset has also been reported to be enriched in the biliary tissue from patients with PSC.<sup>11</sup> In contrast, both the percentage and immunomodulatory

capacity of CD4<sup>+</sup> regulatory T cells were shown to be reduced in PSC.<sup>30-33</sup> However, the precise contribution of NK cells to PSC pathogenesis remains unclear. It is well known that NK cells play a pivotal role in defense against pathogens and in regulating inflammatory immune responses and autoimmunity.<sup>34,35</sup> Consistent with our findings in unstimulated NK cells, Bo *et al.*<sup>12</sup> reported significantly impaired cytotoxic activity of liver-derived CD56<sup>+</sup>CD16<sup>+</sup> NK cells in PSC. Langenecker *et al.*<sup>14</sup> identified a specific CCR7<sup>+</sup>CXCR3<sup>+</sup> NK-cell subset characterized by an increased CD107a degranulation in the blood and liver from patients with PSC. However, this analysis lacked comparison with HCs (only CCR7<sup>+</sup>CXCR3<sup>+</sup> NK cells vs. CCR7<sup>-</sup>CXCR3<sup>-</sup> NK cells in PSC patients), leaving it unclear whether this phenotype is specific to PSC. Similarly, Liu *et al.*<sup>13</sup> reported elevated cytotoxic activity in CD56<sup>dim</sup> NK cells from PSC patients compared to healthy individuals. However, their study used a small number of K562 target cells and focused on PBMCs rather than purified NK cells. PSC was reported to be associated with several genetic risk alleles, including the human leukocyte antigen (HLA) region.<sup>36</sup> A recent study identified HLA-DPA1\*02:01-DPB1\*01:01 as a risk haplotype for PSC and reported that an increased binding of activating NK-cell receptor NKp44 to HLA-DPA1\*02:01-DPB1\*01:01 molecules enhanced



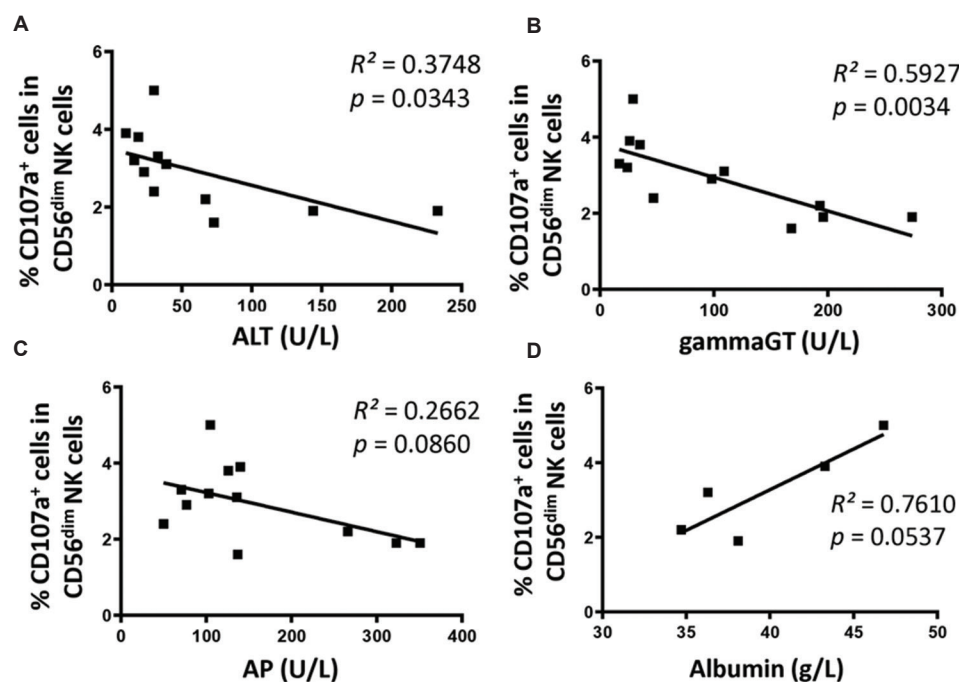
**Figure 4.** Percentage of IFN-gamma- and TNF-alpha-producing cells in CD56<sup>high</sup>CD16<sup>-</sup> NK cells. (A) IFN-gamma-producing cells. (B) TNF-alpha-producing cells. Boxes and whiskers indicate mean and standard error of the mean, respectively.

Notes: <sup>a</sup> $p < 0.05$  for comparisons between PSC and HC; <sup>b</sup> $p < 0.05$  for comparisons between PSC and AIH; <sup>c</sup> $p < 0.05$  for comparisons between AIH and HC. Abbreviations: HC: Healthy controls; IFN: Interferon; LPS: Lipopolysaccharide from *Escherichia coli*; NK: Natural killer; P3C: Triacylated lipoprotein Pam3CSK; PSC: Primary sclerosing cholangitis; TNF: Tumor necrosis factor.

cytotoxic activity of CD56<sup>+</sup> NK cells.<sup>37</sup> However, the low allelic percentage of this haplotype in PSC (0.095) suggests only a minor contribution to our observations. To link our findings of low CD56<sup>+</sup> NK and CD8<sup>+</sup> T cell reactivity to the pathogenesis of PSC, we propose the following mechanistic interpretation: The majority of patients with PSC also have IBD, characterized by continuous exposure to microbial products along the disrupted gut–liver axis

(leaky gut syndrome). Pathogen translocation can lead to chronic inflammation and hepatic stellate cell (HSC)-mediated fibrogenesis.<sup>38</sup> NK cells have been reported to kill activated HSCs, thereby preventing the development of liver fibrosis.<sup>39</sup> Thus, the reduced NK-cell cytotoxic activity observed in our cohort might contribute to the elevated levels of disease activity. The deficit in NK-cell activity may further lead to inadequate clearance of microbial





**Figure 5.** Correlation between cluster of differentiation (CD)107a<sup>+</sup>CD56<sup>dim</sup> natural killer (NK) cells and clinical serum parameters in primary sclerosing cholangitis patients. (A) Alanine aminotransferase (ALT). (B) Gamma-glutamyl transferase (GT). (C) Alkaline phosphatase (AP). (D) Albumin. The *p*-values and the correlation coefficients ( $R^2$ ) were calculated using Spearman's rank correlation analysis.

stimuli, perpetually driving ongoing immune activation and thereby fostering persistent inflammation and progressive fibrosis characteristic of PSC. NK cells play an important role in autoimmune liver diseases by dampening exaggerated immune responses.<sup>40</sup> Given that PSC exhibits autoimmune features, the dysregulated immune response might be explained by a lack of an appropriate NK-cell response.

Limitations of this study include the small number of patients and the lack of longitudinal analysis, which undermines the generalizability of the results. As the endoscopic retrograde cholangiography is not recommended for diagnosing PSC, we restricted our analyses to peripheral blood. Therefore, the hyporesponsiveness of NK and T cells to bacterial/viral agonists should be interpreted with caution, especially in the context of differences in the microenvironment or local inflammatory signaling between blood and tissues. Moreover, synthetic pathogenic agonists used in the study, although standardized, may not fully recapitulate the complexity of microbial recognition *in vivo*.

## 5. Conclusion

Our data demonstrate that CD56<sup>+</sup> NK cells and CD8<sup>+</sup> T cells in PSC exhibit a markedly reduced cytotoxic response to defined bacterial and viral agonists. This impaired inducibility suggests a state of hyporesponsiveness due to

chronic activation and provides a plausible immunological contributor to the persistent inflammatory and fibrotic process that defines PSC.

## Acknowledgments

We thank all patients and healthy individuals for their participation in this study and for donating blood samples.

## Funding

The study was funded by the German Center for Infection Research (DZIF; TI 07.003-MD program).

## Conflict of interest

The authors declare they have no competing interests.

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## Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (number 128/23-EP). Informed consent was obtained from all patients/healthy blood donors for being included in the study. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 2008.

## Consent for publication

All patients/healthy blood donors have given their written consent to participate in the study and to the publication of their data. The authors declare that they have made every effort to mask or conceal all identifying information about the patients/healthy blood donors that appears in writing.

## Availability of data

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

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