AUS DER KLINIK UND POLIKLINIK FÜR ANÄSTHESIOLOGIE, INTENSIVMEDIZIN UND SCHMERZTHERAPIE

Direktor: Prof. Dr. med. Daniel A. Reuter

Analyse und Optimierung des Immune-Cell-Enhancement Systems – Ein praxisorientierter Ansatz in der Medizinproduktentwicklung

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Vorgelegt von: Dr. med. Gerd Klinkmann

Geb. am: 31.08.1985 in Teterow

Wohnhaft in: Am Wald 11, 18198 Kritzmow

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Gutachter der Habilitationsschrift:

1. Prof.Dr. D. A. Reuter (Universitätsmedizin Rostock)
2. Prof.Dr. A. Zarbock (Universitätsklinikum Münster)
3. Prof.Dr. C. Putensen (Universitätsklinikum Bonn)

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Inhaltsverzeichnis

Abkürzungsverzeichnis

Abb	Abbildung
EU MDR	Medizinprodukteverordnung 2017/745 der Europäischen Union
FFP	fresh frozen plasma
G-CSF	Granulocyte-Colony Stimulating Factor
HES	Hydroxyethylstärke
HLA-DR	humanen Leukozyten-Antigens DR
ICE	Immune-Cell-Enhancement
MDRL	Medical Device Readiness Levels
OA	Originalarbeit
PICS	Persistent Inflammation, Immunosuppression and Catabolism Syndrome
RCT	randomisierten kontrollierten multizentrischen Studie
RegNr	Registrierungsnummer
WBK	Leukozyten

Verwendete Originalarbeiten

1. Klinkmann G, Doss F, Goudeva L, Doss S, Blasczyk R, Milej M, Koch S, Mitzner S, Altrichter J. Prolonged storage of purified granulocyte concentrates: Introduction of a new purification method. Transfusion. 2022 Jan;62(1):194-204.

2. Klinkmann G, Doss F, Doss S, Schwarz A, Reichert S, Reuter DA, Selleng K, Thiele T, Mitzner S, Altrichter J. Purified Granulocyte Concentrates from Buffy Coats with Extended Storage Time. Transfus Med Hemother 2024

3. Klinkmann G, Wild T, Heskamp B, Doss F, Doss S, Milej M, Thiele LM, Goudeva L, Blasczyk R, Reuter DA, Altrichter J, Mitzner S. Extracorporeal therapy of sepsis by purified granulocyte concentrates: ex vivo circulation model. Artif Organs. 2023 Jul;47(7):1151-1162.

4. Klinkmann G, Wild T, Heskamp B, Doss F, Doss S, Arseniev L, Aleksandrova K, Sauer M, Reuter DA, Mitzner S, Altrichter J. Extracorporeal immune cell therapy of sepsis: ex vivo results. Intensive Care Med Exp. 2022 Jun 16;10(1):26.

5. Klinkmann G, Wild T, Heskamp B, Doss F, Doss S, Sauer M, Reuter DA, Mitzner S, Altrichter J. Purified Granulocytes in Extracorporeal Cell Therapy: A Multifaceted Approach to Combat Sepsis-Induced Immunoparalysis. Int J Artif Organs. 2024 Jul 23:3913988241262901.

1 Einleitung

1.1 Sepsis – Pathophysiologie, klinische Implikationen und Therapie

Sepsis ist definiert als eine lebensbedrohliche Organfunktionsstörung, die durch eine dysregulierte Reaktion des Wirts auf eine Infektion verursacht wird[1,2]. Gemäß dem Bericht zur globalen Krankheitslast sind weltweit nahezu 50 Millionen Fälle von Sepsis pro Jahr zu verzeichnen[3]. Im Jahr 2017 wurde die Sepsis für etwa 11 Millionen Todesfälle verantwortlich gemacht, was einem Anteil von nahezu 20% an allen Todesfällen entspricht. Die altersstandardisierte Sterblichkeit belief sich auf 148 pro 100.000 Einwohner[3]. Auf Intensivstationen überlebt einer von drei Patienten mit Sepsis die ersten 30 Tage nicht[4,5]. Die Analyse von Daten aus Krankenhäusern offenbart, dass Überlebende einer Sepsis einem erhöhten Risiko für eine erneute Hospitalisierung und Mortalität ausgesetzt sind[5–9]. In Anbetracht dessen ist die dringende Notwendigkeit hervorzuheben, neue Ansätze zur Therapie der Sepsis zu identifizieren und zu evaluieren.

Aktuelle Konzepte zur Darstellung der komplexen immunbiologischen Wirtsreaktion bei Patienten mit Sepsis propagieren die Theorie simultan beginnender Phasen der Hyperinflammation und Immunsuppression, an denen verschiedene Zellarten, Kompartimente und Organsysteme in unterschiedlichem Ausmaß beteiligt sein können[10–12]. Dabei wird der sogenannten „Sepsis-induzierten Immunparalyse“ ein signifikanter Anteil der Sepsis-assoziierten Mortalität zugeschrieben[12–18]. Die immunpathologischen Veränderungen der Immunparalyse stehen im Zusammenhang mit einer gesteigerten Anfälligkeit von Patienten mit Sepsis für Sekundärinfektionen, die v.a. durch opportunistische Erreger oder virale Reaktivierung verursacht werden[19–23]. Die Dysfunktion der Immunzellen stellt einen zentralen Aspekt der Sepsis-induzierten Immunparalyse dar[10,12,14,15,24–33]. Stellt sich die Immunhomöostase trotz initialer Stabilisierung nicht wieder ein, kann sich eine chronisch kritische Erkrankung manifestieren, die auch als „Persistent Inflammation, Immunosuppression and Catabolism Syndrome” (PICS) bezeichnet wird und ihrerseits zu einer Multiorgandysfunktion mit erhöhter Mortalität führen kann[15,18,34–40].

Die Standardtherapie der Sepsis basiert auf den drei therapeutischen Ansätzen einer kausalen Therapie (Fokussanierung und Antibiotikatherapie), einer supportiven Therapie (Stabilisierung der Hämodynamik, Optimierung des Volumen- und endokrinologischen Status und Vermeidung

bzw. Therapie von Organversagen) und einer adjuvanten Therapie (z.B. Immuntherapie)[41,42]. Die Immunmodulation wurde als adjuvante Therapie für die durch Sepsis verursachte Immundysfunktion bereits untersucht, aber es wurde kein signifikanter Überlebensvorteil gefunden[43]. Extrakorporale Blutreinigungsverfahren besitzen neben ihrer detoxifizierenden Wirkung auch das Potenzial die Immundysregulation zu modulieren und somit den klinischen Verlauf der Sepsis positiv zu beeinflussen[44–51]. Allerdings konnte auch hier kein eindeutiger Einfluss auf die Überlebensrate festgestellt werden, weshalb keine der verfügbaren und in der Klinik eingesetzten Methoden gegenwärtig in den internationalen Leitlinien für den routinemäßigen Einsatz in der klinischen Praxis empfohlen wird[2].

1.2 Konzept der extrakorporalen Immunzelltherapie

Mitzner et al. leisteten Pionierarbeit bei der Verwendung von Immunzellen, insbesondere von neutrophilen Granulozyten, in der extrakorporalen Sepsisbehandlung, was zur Entwicklung des „Immune-Cell-Enhancement“ (ICE) Systems führte[52–57]. Die zugrunde liegende Idee basiert auf der Erkenntnis, dass eine erhöhte Sterblichkeit in fortgeschrittenen Stadien der Sepsis mit einer beeinträchtigten zellulären Immunkompetenz einhergeht[10,12,14,15,24–33]. Das ICE-Konzept basiert auf der Nutzung der plasmamodifizierenden Fähigkeiten humaner Granulozyten, welche beispielsweise zur Entfernung antigenen Materials aus dem Blutkreislauf sowie zur Regulierung der Immunantwort durch Adsorption und Sekretion von Zytokinen eingesetzt werden können. Das ICE-System stellt eine Kombinationstechnologie für die Behandlung humanen Plasmas dar, welche auf der extrakorporalen Nutzung von Granulozytenkonzentraten basiert[52–57]. Dazu wird das Plasma kontinuierlich aus dem extrakorporalen Blutkreislauf des Patienten gefiltert und in einen geschlossenen „Zellkreislauf“ überführt. In diesem wird das Plasma des Patienten in direkten Kontakt mit therapeutisch wirksamen Immunzellen eines gesunden menschlichen Spenders (Granulozytenkonzentrat) gebracht. Die Immunzellen werden später im extrakorporalen Kreislauf zurückgehalten und nach Abschluss der Behandlung entsorgt. Dadurch lassen sich potenzielle unerwünschte Auswirkungen von Granulozytentransfusionen durch direkten Gewebekontakt vermeiden. So kann die systemische immunologische Funktion der Zellen des Granulozytenkonzentrates genutzt werden, während die Kontrolle über diese Zellen durch ihren Verbleib im extrakorporalen Kreislauf gewährleistet bleibt. In diesem Ansatz zeigte sich ein potentieller Nutzen für die Behandlung der Sepsis-induzierten Immunparalyse[53–57]. Die Evidenzlage zur Anwendung des ICE-Systems beruht auf empirisch gewonnenen Erkenntnissen,

die durch experimentelle Untersuchungen sowie klinische Pilotstudien gewonnen werden konnten[52–57].

Eine Pilotstudie mit zehn Patienten mit septischem Schock demonstrierte eine gute Verträglichkeit, eine verringerte Endotoxin-Konzentration, eine signifikante Reduktion der Noradrenalin-Dosierung bei gleichzeitig stabilem mittlerem arteriellem Druck. Zudem zeigten sich eine deutliche Reduktion des C-reaktiven Proteins, des Procalcitonins sowie des humanen Leukozyten-Antigens DR (HLA-DR)[53]. Eine erweiterte Studie mit zehn weiteren Probanden konnte die zuvor gewonnenen Ergebnisse bestätigen und unterstrich zudem die positiven Auswirkungen auf das Noradrenalin-Management sowie die Leberfunktion. Die beobachtete Sterblichkeitsrate betrug 40% nach 28 Tagen und 50% während des Krankenhausaufenthalts[54]. Beide Studien waren als unkontrollierte klinische Studien angelegt und konnten Nachweise zur Sicherheit und Verträglichkeit dieses Therapieansatzes erbringen[53,54].

1.3 Entwicklung von Medizinprodukten

In Übereinstimmung mit der Medizinprodukteverordnung 2017/745 der Europäischen Union (EU MDR) kann ein Medizinprodukt als jedes Instrument, jeder Apparat, jede Vorrichtung, jede Software, jedes Implantat, jedes Reagenz, jeder Stoff oder jeder andere Gegenstand definiert werden, der vom Hersteller dazu bestimmt ist, allein oder in Kombination für den Menschen zur Diagnose, Vorbeugung, Überwachung, Vorhersage, Prognose, Behandlung oder Linderung von Krankheiten verwendet zu werden[58]. Die Entwicklung von Medizinprodukten erfolgt in einem mehrstufigen Prozess, der von der Konzeptualisierung über das Entwickeln eines Prototyps bis hin zur präklinischen und klinischen Validierung reicht. In jeder Phase werden iterative Tests und Verbesserungen durchgeführt, um die Sicherheit der Patienten und die Wirksamkeit des Produkts zu gewährleisten[59–62]. Regulatorische Standards spielen hierbei eine entscheidende Rolle, wobei der Schwerpunkt zunächst auf der Sicherstellung von Sicherheit und klinischer Wirksamkeit liegt [63–73]. Ein Aspekt, der bei der Entwicklung von Medizinprodukten häufig vernachlässigt wird, ist die Orientierung an der Praxis[74]. Diese Dimension ist von besonderer Relevanz, um eine sinnvolle Integration von Medizinprodukten in den klinischen Kontext zu ermöglichen und die Grundlage für einen ordnungsgemäßen Betrieb zu schaffen. Eine einseitige Ausrichtung auf technische Aspekte birgt das Risiko, dass Medizinprodukte zwar grundlegende technische Anforderungen erfüllen, jedoch in der klinischen Anwendung mit erheblichen Schwierigkeiten konfrontiert werden[75–78].

Die Klassifikation mittels Medical Device Readiness Levels (MDRL) stellt ein spezifisches Rahmenwerk zur Einschätzung der Technologieeinsatzbereitschaft für die Entwicklung von Medizinprodukten dar[74]. Dieses Rahmenwerk adressiert spezifische medizinische Belange sowie die erforderliche enge Mensch-System-Interaktion. Das ICE-System kann als Kombinationsprodukt aus Medizinprodukt und Arzneimittel (Granulozytenkonzentrat) kategorisiert werden[79–81]. Es fällt in die Hochrisikogruppe der Sicherheits-Kategorie III und kann nach MDRL in seiner bisherigen Entwicklung auf Level sechs eingestuft werden (Pilotstudie zu Wirksamkeit und Verträglichkeit)[74,79]. Der weiterführende Entwicklungsprozess beinhaltet gemäß dem aktuellen wissenschaftlichen Standard die Durchführung klinischer Studien mit dem Goldstandard der randomisierten kontrollierten multizentrischen Studie (RCT)[82–88]. Die Wirksamkeit des Systems muss demnach patientenseitig nachgewiesen werden, damit ein routinemäßiger praktischer Einsatz in Erwägung gezogen werden kann. Um die Voraussetzung für diesen Entwicklungsschritt und damit die MDRL-Stufe sieben zu erreichen, soll im Rahmen der vorliegenden Arbeit die medizinisch-technische Entwicklung des ICE-Systems als Kombinationsprodukt praxisorientiert optimiert werden. Vor diesem Hintergrund wird die Hypothese aufgestellt, dass der Bezug zur sinnvollen klinischen Anwendbarkeit als multidisziplinärer Ansatz als Normativ und Regulativ des Entwicklungsprozesses eine Steigerung des MDRL des Systems schaffen kann.

2 Arbeitsprogramm

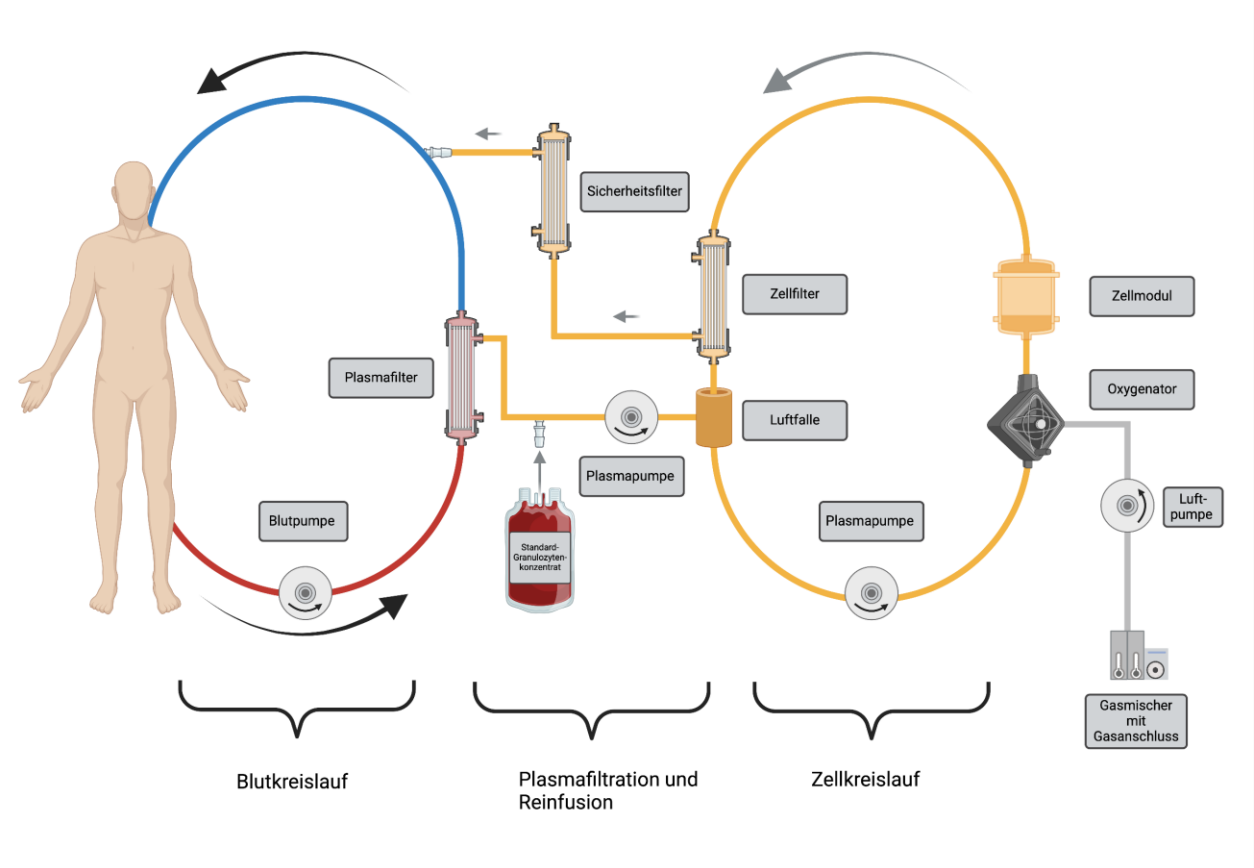
Im Rahmen der Initiierung des Entwicklungsprozesses ist zunächst die Identifizierung von Optimierungspotentialen der Systemkomponenten erforderlich, um eine Grundlage für die nachfolgenden Entwicklungsschritte zu schaffen.

2.1 Identifizierung von Optimierungspotenzialen im ICE-System

2.1.1 Komplexität des ICE-Systems

Als gravierender Nachteil des aktuellen Entwicklungsstands ist die hohe Materialbeanspruchung der aus zwei Kreisläufen bestehenden 1. Generation des ICE-Systems zu nennen (Abb.1). Die Plattform des Systems besteht aus einem Komplex mehrerer Apheresegeräte (BM25 (Baxter/Edwards (Unterschleißheim Deutschland/Irvin, USA)), MARS Monitor (Teraklin/Gambro, Rostock, Deutschland)), welcher aufwändige Hardware- sowie bislang ungelöste Software-Lösungen umfasst[53,54,57]. Insgesamt sind vier Rollerpumpen aufeinander abzustimmen. Darüber hinaus erfordert die Verbindung des gesamten Systems einen intensiven Einsatz von Verbrauchsmaterialien (Schlauchsystem, Konnektoren, Haltevorrichtung). Des Weiteren war die Applikation der angestrebten Granulozytenzahl von 3,5 x 10^{10} im Rahmen einer klinischen Pilotstudie mit der Integration eines mikroporösen Hohlfasermembran-Oxygenators verbunden, um den erhöhten Sauerstoffbedarf der Zellen zu bewältigen. Die Implementierung eines Oxygenators erwies sich jedoch als äußerst ressourcenintensiv und in der klinischen Umgebung als kaum praktikabel[57] (siehe Abb. 1). Die Größe der extrakorporalen Oberfläche eines Medizinprodukts im Kontakt mit Blut und Zellen hat zudem potenziell einen Einfluss auf dessen Biokompatibilität. Eine Vergrößerung dieser Oberfläche kann zu einer Reduktion der biologischen Verträglichkeit führen, wie z.B. die Aktivierung des Komplementsystems, eine Stimulierung humoraler und zellulärer Immunreaktionen sowie die Aktivierung des Gerinnungssystems[89–93]. Vor diesem Hintergrund ist das ICE-System im derzeitigen Format nur eingeschränkt für den praktischen klinischen Einsatz geeignet.

2.1.1.1 Abbildung 1



Schematische Darstellung der 1.Generation des ICE-Systems. Der Blutzugang erfolgte veno-venös über einen Shaldon-Katheter und die Plasmaseparation wurde mit einem Dialysemonitor (BM25, Edwards Lifesciences GmbH, Unterschleißheim, Deutschland) unter Verwendung eines Plasmafilters mit einer Porengröße von 0,5 µm (PF 1000 N, Gambro Hospal GmbH, Planegg-Martinsried, Deutschland) durchgeführt. Das Plasma wurde in ein kontinuierlich rezirkulierendes Spenderzellenkompartiment infundiert, das mit der Hämofiltrationslösung HF-BIC 35-410 (Fresenius Medical Care, Bad Homburg, Deutschland) vorgefüllt war und Standard-Granulozytenkonzentrate enthielt. Der Rückfluss des Plasmas zum Patienten erfolgte durch einen zweiten PF 1000 N Plasmafilter, um zu verhindern, dass die Spenderzellen in den Patienten infundiert werden. Das gesamte extrakorporale Volumen betrug fast 400 ml. Die Blutflussrate betrug 110-150 ml/min mit einer Plasmaseparationsrate von 16,7-33,3 ml Plasma/min unter Verwendung des BM 25 Monitors (Baxter/Edwards (Unterschleißheim Deutschland/Irvin, USA)). Der MARS-Monitor 1 TC (Gambro Rostock GmbH, Rostock, Deutschland) wurde für den rezirkulierenden Bioreaktorkreislauf mit einer Rate von 200 ml/min verwendet.

2.1.2 Standard-Granulozytenkonzentrate

Die Transfusion von Standard-Granulozytenkonzentraten stellt eine supportive Maßnahme für Patienten mit einer lebensbedrohlichen Neutropenie oder neutrophilen Dysfunktion dar. Die Anwendung dieser Präparate erfolgt bereits seit über 60 Jahren, obgleich die Evidenzlage bezüglich ihrer Wirksamkeit insgesamt unzureichend ist[94–97]. Die Herstellung eines Standard-Granulozytenkonzentrates kann auf zwei unterschiedliche Arten erfolgen: einerseits durch Granulozytapherese, andererseits durch Gewinnung des Konzentrats aus einem Pool von Buffy-Coats, der durch Vollblutspenden gewonnen wurde[96,98,99]. Ein Standard-Granulozytenkonzentrat besteht in überwiegendem Maße aus Erythrozyten, Thrombozyten sowie verschiedenen Leukozyten (WBK). Darunter fallen auch neutrophile Granulozyten[100]. Aufgrund uneinheitlicher Standards variiert der Vorbereitungsprozess für die Isolierung der Zellen zwischen den Transfusionsabteilungen hinsichtlich der Verwendung von Spenderstimulationsprotokollen, Sedimentationsmitteln, Maschinen und bedienerabhängigen Faktoren[37,101–104]. Die Anwendung unterschiedlicher Methoden zur Entnahme, Verarbeitung und Lagerung von Konserven resultiert in signifikanten Unterschieden in der Gesamtzahl der Granulozyten, anderer Leukozyten, Erythrozyten und Thrombozyten, was potenziell Auswirkungen auf deren Zellfunktion und Lebensdauer haben kann. In den Leitlinien zur Herstellung und Verwendung von Blutprodukten wird eine Mindestanzahl von 1×10^{10} Granulozyten pro Präparat empfohlen[100,104,105]. Probleme bereitet die Lebensfähigkeit von Granulozyten im menschlichen Körper. Sie reicht *in vivo* von einigen Stunden bis zu mehreren Tagen, wobei ihre Funktionalität und morphologische Integrität mit der Zeit abnehmen, was eine Herausforderung besonders für die *in vitro* Lagerung bedeutet[106,107]. Zudem ist die Verfügbarkeit von Standard-Granulozytenkonzentraten aufgrund des notwendigen AB0-Kompatibilitätsabgleichs eingeschränkt, was die routinemäßige Verwendung zusätzlich erschwert[100]. In Deutschland stellt derzeit die Gewinnung von Standard-Granulozytenkonzentraten durch Apherese den Standardansatz dar[104]. Die Gewinnung und Aufbereitung von Standard-Granulozytenkonzentraten durch Apherese hat jedoch mehrere Nachteile[108–110]. Die übliche präkonditionierende Gabe von G-CSF und Steroiden stellt für die Spender eine Belastung dar[109]. Zudem kann dieser Prozess die Bereitstellung von Standard-Granulozytenkonzentraten um mehrere Tage verzögern. Des Weiteren können Sedimentationsbeschleuniger wie Hydroxyethylstärke (HES), Dextran oder Gelatine beim Empfänger unerwünschte Arzneimittelwirkungen hervorrufen[111–125]. In europäischen und

amerikanischen Richtlinien wird empfohlen Standard-Granulozytenkonzentrate bei einer Temperatur von 20–24°C nicht länger als 24 Stunden zu lagern und vorzugsweise so schnell wie möglich nach der Spende zu transfundieren[104,105,126]. Für die in dieser Arbeit angestrebte Optimierung der praktischen Einsetzbarkeit von Granulozytenkonzentraten ist es erforderlich, die Lagerungsbedingungen so zu gestalten, dass die Funktionalität der Granulozyten über einen längeren Zeitraum erhalten bleibt.

2.2 Entwicklungsprozess des ICE-Systems

Die Analyse der Optimierungspotentiale der Systemkomponenten offenbart, dass das System zahlreichen technischen Einschränkungen unterliegt, welche sich nachteilig auf die klinische Anwendbarkeit auswirken. Im Rahmen der Untersuchungen zu **Originalarbeit (OA) 1** und **OA2** wurden daher zunächst Verfahren zur Optimierung der Lagerfähigkeit von Standard-Granulozytenkonzentraten entwickelt. Auf Grundlage der durchgeführten Untersuchungen konnte die Funktionalität der neu entwickelten, hochreinen Granulozytenpräparate im ICE-System *in vitro* erfolgreich demonstriert werden. Dabei wurden wesentliche Erkenntnisse zur technischen Vereinfachung des ICE-Systems gewonnen (**OA3**). Anschließend wurden die Auswirkungen der neu entwickelten Komponenten des vereinfachten ICE-Systems auf die Systemleistung untersucht (**OA4**). In der Folge wurde eine detaillierte Re-Evaluation des bisher verwendeten Dosisregimes durchgeführt, um eine optimierte Voraussetzung für den praxisorientierten Einsatz des ICE-Systems zu schaffen. (**OA5**).

3 Methodik und Ergebnisse der Originalarbeiten

3.1 Originalarbeit 1: Längere Lagerbarkeit von gereinigten Granulozytenkonzentraten aus Granulozytapherese

Hintergrund: Die Verwendung von Standard-Granulozytenkonzentraten ist aufgrund der kurzen Lagerbarkeit auf 24 Stunden begrenzt[104,105]. Dieses Phänomen kann auf den Einfluss der hohen Anzahl von enthaltenen Erythrozyten und Thrombozyten im Standard-Granulozytenkonzentrat zurückgeführt werden. Idealerweise sollten die Lagerungsbedingungen so gestaltet sein, dass sie den Stoffwechsel der Granulozyten aufrecht erhalten, deren Aktivierung verhindern und die Funktionalität der Zelle erhalten.

Zielstellung: Entwicklung eines geschlossenen Aufreinigungssystems mit dem Ziel die Lagerfähigkeit von Standard-Granulozytenkonzentraten aus Granulozytapherese durch Reduktion von Erythrozyten und Thrombozyten zu verbessern.

Methoden: Aus Granulozytapherese gewonnene Standard-Granulozytenkonzentrate wurden sedimentiert, zweimal mit 0,9% Natriumchlorid gewaschen und in blutgruppenidentischem, gefrorenem Frischplasma (FFP) resuspendiert. Die resultierenden gereinigten Granulozytenkonzentrate wurden dann in Thrombozytenkonzentratbeuteln bei einer Zellkonzentration von $5 \times 10^7 \pm 1{,}8 \times 10^7$ Leukozyten/ml ohne Bewegung bei Raumtemperatur bis zu 72 Stunden gelagert.

Ergebnisse: Die Sedimentation führte zu einer signifikanten Reduktion der Erythrozytenzahl um 98%, während die Waschvorgänge eine Reduktion der Thrombozytenzahl um 96% bewirkten. Die WBK konnten angereichert werden, wobei der Anteil der neutrophilen Granulozyten im gereinigten Granulozytenkonzentrat auf 96% der WBK erhöht wurde. Nach einer Lagerungszeit von 72 Stunden konnten noch über 90% der ursprünglichen Anzahl an Leukozyten in den gereinigten Granulozytenkonzentraten nachgewiesen werden, wobei eine hohe Vitalität (≥ 97%) gegeben war. Zudem wiesen die Granulozyten eine vergleichbare Phagozytose- und oxidative Burst-Funktionalität auf, die mit einem Standard-Granulozytenkonzentrat nach 24 Stunden vergleichbar war.

Schlussfolgerung: Die Entwicklung einer Methode zur Herstellung einer hochreinen, neutrophilenreichen Präparation aus Standard-Granulozytenkonzentraten war erfolgreich. Die Sedimentation und die anschließenden Waschschritte konnten in einem geschlossenen

Blutbeutelsystem durchgeführt werden. Die Methode führt zu einer verlängerten Lagerfähigkeit der Leukozyten von mindestens 72 Stunden bei erhaltener Zellfunktionalität und Vitalität.

3.2 Originalarbeit 2: Längere Lagerbarkeit von gereinigten Granulozytenkonzentraten aus gepoolten Buffy Coats

Hintergrund: Nachdem in **OA1** eine Aufreinigungsmethode von Standard-Granulozytenkonzentraten aus Granulozytapherese aufgezeigt wurde, soll im Rahmen dieser Studie eine Methode zur Aufreinigung von Granulozytenpräparaten aus gepoolten Buffy-Coats entwickelt werden. Dadurch soll eine weitere Quelle für die Isolierung von Granulozytenkonzentraten erschlossen werden, um die Verfügbarkeit von Granulozytenkonzentraten durch die Nutzung von Buffy-Coats zu erhöhen.

Zielstellung: Entwicklung eines geschlossenen Aufreinigungssystems mit dem Ziel die Lagerfähigkeit von Standard-Granulozytenkonzentraten aus gepoolten Buffy-Coats durch Reduktion der Zahl von Erythrozyten und Thrombozyten zu verbessern.

Methoden: Sechs AB0-Blutgruppen-identische Buffy-Coats wurden gepoolt und Erythrozyten durch Zugabe von HES sedimentiert. Der daraus resultierende mit Leukozyten angereicherte Überstand wurde zweimal mit Saline 0,9% gewaschen, um auch die Thrombozytenzahl zu reduzieren, und in AB0-identischem Spenderplasma resuspendiert. Das entstandene Leukozytenkonzentrat wurde in einen Thrombozyten-Lagerungsbeutel überführt und bis zu 72 Stunden bei 20-24°C ohne Bewegung gelagert.

Ergebnisse: Die Anzahl der Erythrozyten und der Thrombozyten reduzierte sich auf 0,4% bzw. 6,1% der Ausgangswerte. Die Wiederfindungsrate der WBK lag bei 49,8%. Im Verlauf der 72-stündigen Lagerung gab es keine signifikanten Veränderungen der Anzahl der WBK. Die Vitalität lag während des gesamten Zeitraums bei über 98%. Die Rate der Granulozyten, in denen Phagozytose und oxidativer Burst nachgewiesen wurde, blieb jederzeit über 95%.

Schlussfolgerung: Aus gepoolten Buffy-Coats hergestellte Granulozytenkonzentrate stellen eine Alternative zu Granulozytenpräparaten, die aus Granulozytapherese gewonnen werden, dar. Durch die Reduktion des Anteils an Erythrozyten und Thrombozyten konnte die maximale Lagerbarkeit der gereinigten Granulozytenkonzentrate von 24 Stunden auf 72 Stunden bei erhaltener metabolischer Aktivität, Zellfunktionalität und Vitalität verlängert werden.

3.3 Originalarbeit 3: Vergleich der funktionellen Eigenschaften von gereinigten Granulozytenpräparaten und Standard-Granulozytenkonzentraten im ICE-System

Hintergrund: Nachdem Daten zur Sicherheit und Verträglichkeit des ICE-Systems mit Standard-Granulozytenkonzentraten in klinischen Pilotstudien an insgesamt 20 Patienten erhoben wurden[53,57], soll geklärt werden, ob auch gereinigte Granulozytenkonzentrate im ICE-System angewendet werden können.

Zielstellung: *Ex vivo* Studie der funktionellen Eigenschaften von gereinigten Granulozytenkonzentraten (**OA1**) im ICE-System und Vergleich mit Standard-Granulozytenkonzentraten.

Methoden: Gereinigte Granulozytenkonzentrate wurden nach **OA1** hergestellt und 24 bzw. 72 Stunden gelagert. Als Patientenmodell diente in Szenario A ein Pool aus aufgetautem FFP gesunder Spender. In Szenario B wurde zudem Patientenplasma von Sepsispatienten, welches aus dem Austauschplasma einer therapeutischen Plasmaaustauschstudie (RegNr. II PV 12/2000) gewonnen wurde, untersucht. Die Plasmaseparation erfolgte mit einem manuell gesteuerten Mehrzweckdialysegerät unter Verwendung eines Plasmafilters mit einer nominalen Porengröße von 0,5 *µm*. Das gesamte „extrakorporale" Volumen betrug ca. 900 ml. Die Blutflussrate betrug 110-150 ml/min mit einer Plasmaseparationsrate von 33,3 ml/min. Alle Kreislaufexperimente wurden sechs Stunden ohne technische Probleme durchgeführt.

Ergebnisse: Die Zellen der gereinigten Granulozytenkonzentrate zeigten sowohl nach einer Lagerungsdauer von 24 Stunden als auch nach 72 Stunden vergleichbare Verläufe. Die Zellen waren während des gesamten Untersuchungszeitraums in beiden Szenarien vital, zeigten eine erhaltene metabolische Aktivität sowie eine erhaltene Phagozytose- und oxidative Burst-Funktionalität. Die Bestimmung der Anzahl zirkulierender WBK im ICE-System ergab signifikante Unterschiede ($p \leq 0{,}05$) zwischen gereinigten Granulozytenkonzentraten und Standard-Granulozytenkonzentraten während des gesamten Behandlungszeitraums.

Schlussfolgerung: Die WBK gereinigter Granulozytenkonzentrate zeigten in den sechs-stündigen extrakorporalen Kreisläufen auch nach 72 Stunden Lagerung sowohl in physiologischer als auch in pathologischer Umgebung eine erhaltene Funktionalität. Die signifikanten Unterschiede bzgl. der Anzahl der zirkulierenden WBK deuten darauf hin, dass eine kontinuierliche Zirkulation der WBK bei der Verwendung von gereinigten Granulozytenkonzentraten im ICE-System keine notwendige Voraussetzung darstellt.

3.4 Originalarbeit 4: Evaluation der funktionellen Eigenschaften von gereinigten Granulozytenpräparaten im ICE-System mittels Dead-End Plasmafiltration

Hintergrund: Basierend auf den vorangegangenen Erkenntnissen (**OA3**) deutet sich an, dass unter Verwendung von gereinigten Granulozytenkonzentraten (**OA1**) eine kontinuierliche Zirkulation der WBK im extrakorporalen System keine zwingende Voraussetzung für ihre funktionale zelluläre Integrität darstellt. Folglich erscheint es möglich, die Systemkomplexität des ICE-Systems zu reduzieren. Im Rahmen einer technischen Vereinfachung des ICE-Systems wurde der Zellkreislauf durch eine Dead-End-Plasmafiltration ersetzt (Abb. 2).

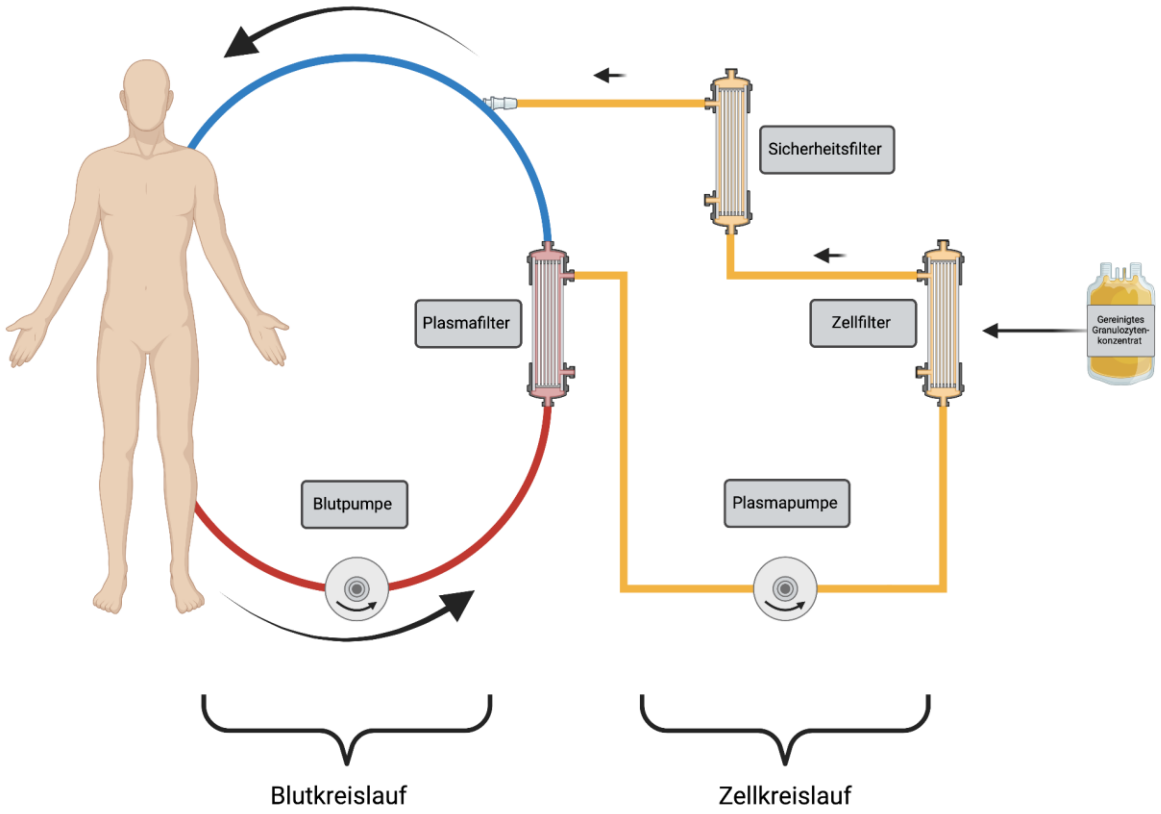
Zielstellung: *Ex vivo* Studie zur Bestimmung der funktionellen Eigenschaften der gereinigten Granulozytenkonzentrate in einer vereinfachten Dead-End Plasmafiltration.

Methoden: Gereinigte Granulozytenkonzentraten wurden nach **OA1** hergestellt und 24 bzw. 72 Stunden gelagert. Das extrakorporale Zelltherapiesystem wurde mit FFP vorgefüllt. Im folgenden Behandlungsmodus gelangt das Blut des Patienten (oder das Poolplasma im Simulationsexperiment) in den Plasmafilter (150 ml/min), wo das Plasma abgetrennt wird (33 ml/min). Dieses Plasma durchströmt den Zellfilter mit den darin enthaltenen Immunzellen im Dead-End-Filtrationsmodus. Anschließend fließt das Plasma durch einen weiteren Plasmafilter im Dead-End-Filtrationsmodus, der als Sicherheitsbarriere im Falle eines technischen Defektes im Zellfilter dient. Danach fließt das behandelte Plasma in die Venenkammer, wo es mit dem „Patientenblut“ gemischt und dem „Patienten“ wieder zugeführt wird (Abb. 2). Diese Simulation wurde kontinuierlich über sechs Stunden durchgeführt.

Ergebnisse: Die Zellen waren während des gesamten Untersuchungszeitraums sowohl nach vorangegangener Lagerung von 24 Stunden als auch nach 72 Stunden vital und wiesen eine erhaltene Funktionalität und Stoffwechselaktivität auf. Es konnte ein signifikanter Anstieg in der Freisetzung von Zytokinen im Verlauf des Beobachtungszeitraumes detektiert werden.

Schlussfolgerung: Die Erhaltung der Vitalität und Funktionalität der WBK konnte während der sechs-stündigen Simulation sowohl nach 24-stündiger als auch nach 72-stündiger Lagerung demonstriert werden. Die Resultate belegen die zelluläre Funktionsfähigkeit für ein vereinfachtes ICE-System. Die Analyse der Zytokinkonzentrationen im Verlauf lässt den Schluss zu, dass eine Verlängerung des Untersuchungszeitraums eine sinnvolle Option darstellen könnte.

3.4.1.1 Abbildung 2



Schematische Darstellung des neu entwickelten ICE-Systems: Das gereinigte Granulozytenkonzentrat wird in den Zellkreislauf eingefüllt und verbleibt während der Behandlung in den Hohlfasern des Zellfilters. Im Behandlungsmodus wird das Plasma im Plasmafilter abgetrennt und durchströmt den Zellfilter im Dead-End-Filtrationsmodus. Ein weiterer Plasmafilter dient als Sicherheitsbarriere bei Membranbrüchen. Danach fließt das behandelte Plasma in die Venenkammer, wird mit dem „Patientenblut“ vermischt und dem „Patienten“ zurückgegeben.

3.5 Originalarbeit 5: Evaluation der funktionellen Eigenschaften von gereinigten Granulozytenpräparaten im ICE-System mittels Dead-End Plasmafiltration mit prolongierter Therapiedauer

Hintergrund: Bislang wurden alle extrakorporalen Immunzellbehandlungen für jeweils sechs Stunden durchgeführt[53,57]. Die Erkenntnisse aus **OA4** deuten darauf hin, dass die Dynamik der Zytokinfreisetzung das Dosierungsregime beeinflussen kann. In diesem Zusammenhang stellt sich die Frage nach dem optimalen, klinischen Anwendungsschema für das ICE-System.

Zielstellung: *Ex vivo* Studie zur Untersuchung der technischen Durchführbarkeit und der zellulären Auswirkungen eines prolongierten Behandlungsintervalls von bis zu 24 Stunden.

Methoden: Gereinigte Granulozytenkonzentrate wurden nach **OA1** hergestellt, 72 Stunden gelagert und anschließend in das vereinfachte ICE-System (**OA4**) implementiert. Für die Untersuchung prolongierter Therapieregime wurden zwei Umgebungen definiert: Eine „10-Stunden-Umgebung“ (33 ml/min Plasmafiltration) und eine „24-Stunden-Umgebung“ (13,5 ml/min Plasmafiltration), welche sich sowohl in der Therapiedauer als auch in den jeweiligen Plasmafiltrationsraten unterschieden.

Ergebnisse: Nach einer Lagerung von 72 Stunden waren die WBK während des gesamten Studienzeitraums lebensfähig und zeigten eine erhaltene Funktionalität und effiziente Stoffwechselaktivität. Die Ergebnisse zeigen auch, dass die Zytokinausschüttung von WBK im extrakorporalen Kreislauf zeitabhängig ist, da die Zytokinausschüttung von IL-8 innerhalb von sechs Stunden ihren Höhepunkt erreichte, während MCP-1, IL-6, IL-1β und TNF-α erst nach 24 Stunden Zirkulation signifikant zunahmen.

Schlussfolgerung: Die Erkenntnisse früherer Arbeiten (**OA3, OA4**) zur metabolischen Aktivität und Funktionalität von WBK aus gereinigten Granulozytenkonzentraten im ICE-System konnten bestätigt und auf eine Therapiedauer von 10 bzw. 24 Stunden erweitert werden. Die dynamischen Veränderungen der Zytokinprofile deuten auf ein differenziertes Reaktionsmuster bei verlängerter Zirkulation hin. Ein weiterer Aspekt dieser Studie war die Untersuchung der Plasmaflussrate. Dabei zeigte sich, dass die Rate in der „24-Stunden“-Einstellung mit 13,5 ml/h deutlich niedriger war als in der „10-Stunden“-Einstellung mit 33 ml/h. Allerdings war der Anstieg der Zytokinkonzentrationen in den ersten 10 Stunden in beiden Einstellungen vergleichbar. Dies deutet darauf hin, dass der wesentliche Parameter nicht die Menge des behandelten Plasmas ist, sondern vielmehr die Kontaktzeit zwischen Plasma und Zellen im extrakorporalen Kompartiment.

4 Diskussion

Der Entwicklungsprozess eines jeden Medizinprodukts ist als ein komplexer, interaktiver Prozess zu verstehen, der sich in einem Spannungsfeld zwischen Design, Technik und medizinischen Spezifikationen bewegt[127]. Das Ziel muss immer die Gewährleistung von Sicherheit und Wirksamkeit sein[72,80,127]. Allerdings wird in diesem Prozess die für eine effektive Integration unerlässliche Praxisnähe oft vernachlässigt[58,59,68,71,73,74,78,127,128]. Das Beispiel des ICE-Systems zeigt auf, dass es gelingt wissenschaftliche Belege zur Sicherheit und Wirksamkeit durch klinische Pilotstudien zu erheben[53,57]. Eine umfassende Analyse der Systemkomponenten ergab, dass die Nutzung von Standard-Granulozytenkonzentraten sowie die Komplexität des ICE-Systems für den praktischen Einsatz Optimierungspotenzial aufweisen. Um den derzeitigen Entwicklungsstand des ICE-Systems gemäß geltenden wissenschaftlichen Standards weiterzuentwickeln und damit die MDRL-Stufe sieben zu erreichen, wurde die Hypothese aufgestellt, dass ein Bezug zur klinischen Anwendbarkeit als Normativ und Regulativ des Entwicklungsprozesses des ICE-Systems eine sinnvolle Zielvorgabe darstellen kann.

In einem ersten Schritt wurden Methoden zur Optimierung der Einsetzbarkeit von Standard-Granulozytenkonzentraten entwickelt. Neben den klinischen Herausforderungen, die es dabei zu bewältigen gilt, müssen auch die logistischen Aspekte berücksichtigt werden, die mit dem Einsatz von Granulozytenkonzentraten in der Praxis einhergehen. Die industrielle Produktion von Granulozytenkonzentraten kann zu Zellprodukten mit unterschiedlichen Eigenschaften führen, die sich von der Produktion in akademischen Zentren unterscheiden und sogar von einer Produktionsstätte zur anderen variieren[37]. Die Verfügbarkeit von Granulozytenkonzentraten ist, wie bei anderen Blutprodukten auch, stark von der Verfügbarkeit geeigneter Spender abhängig[103–105]. Da Sepsis und Sepsis-induzierte Immunparalyse zeitkritische medizinische Indikationen darstellen[2,129], könnte ein Reservevorrat an Granulozytenkonzentraten mit verlängerter Überlebenszeit sinnvoll sein. Im Rahmen von **OA1** konnte ein Reinigungsverfahren für Standard-Granulozytenkonzentraten aus Apherese etabliert werden. Neben der in Deutschland als Standardmethode geltenden Apherese stellt die Gewinnung von Granulozytenpräparaten aus gepoolten Buffy-Coats aus regulären Blutspenden eine weitere alternative Quelle dar. Die Verwendung von Buffy-Coat-Präparaten erfordert keine Vorkonditionierung der Spender und ermöglicht somit eine schnellere Produktion und

Einsetzbarkeit der Granulozytenkonzentrate[94–99,103–105]. Die hier vorgestellten neuen Reinigungsverfahren entfernen effektiv Erythrozyten, Thrombozyten sowie Verunreinigungen durch Sedimentationsmittel. Dies resultiert in einer Anreicherung von neutrophilen Granulozyten sowie einer Verlängerung der Lagerbarkeit auf bis zu 72 Stunden, unter Erhaltung von Zellfunktionalität und Vitalität. Die optimierten Lagerbedingungen sowie der reduzierte Erythrozytengehalt eröffnen die Möglichkeit einer Verwendung von gereinigten Granulozytenkonzentraten, die nicht mehr vom AB0-System abhängig ist und damit zur deutlichen Verbesserung der klinischen Anwendbarkeit von Granulozytenkonzentraten führt[100,130]. Ferner gestatten die Verfahren die exakte Anpassung der Zellzahlen, was eine präzise Dosierung im Vergleich zu Standard-Granulozytenkonzentraten ermöglicht. Um die Produktionsmöglichkeiten weiter zu optimieren, wurde die Entwicklung auf die Durchführbarkeit in geschlossenen Systemen mit konventionellen Blutbanktechnologien ausgelegt und standardisiert, wodurch potentiellen Varianzen zwischen verschiedenen Produktionsstätten vorgebeugt werden kann.

Im Rahmen von **OA3** wurden *ex vivo* Zirkulationsversuche mit Standard- und gereinigten Granulozytenkonzentraten in unstimulierter (FFP) und stimulierter Umgebung (Sepsis-Plasma) durchgeführt, um die optimierten Zellpräparate im extrakorporalen Setting gegen den bestehenden Entwicklungsstand zu vergleichen. Die Ergebnisse der Untersuchungen belegen, dass sowohl die Standard- als auch die gereinigten Präparationen eine vergleichbare Zellfunktionalität und Vitalität aufweisen. Dabei zeigte sich, dass die gereinigten Granulozytenkonzentrate über einen Zeitraum von sechs Stunden eine bessere Stoffwechselaktivität und einen stabileren pH-Wert beibehielten, selbst nach vorheriger Lagerung über 72 Stunden. Dies deutet auf einen klinisch bedeutsamen Vorteil im Sinne einer optimierten klinischen Anwendbarkeit hin. Eine weitere zentrale Erkenntnis ergab sich aus der Analyse der Zahl zirkulierender Zellen im ICE-System. Während die WBK der Standard-Granulozytenkonzentrate kontinuierlich zirkulierten, konnten die meisten WBK der gereinigten Granulozytenkonzentrate im Zellfilter nachgewiesen werden (Abb. 2). Dies lässt sich auf diverse Ursachen zurückführen. Zunächst könnten die adsorptiven Eigenschaften der MicroPES™ TF10 Kapillarmembran von Bedeutung sein, die aus Polyethersulfon in der hydrophilisierenden Legierung mit Polyvinylpyrrolidon besteht, welches pH-abhängig eine überwiegend positive Oberflächenladung besitz[131–134]. Die negative Oberflächenladung der Granulozyten fördert ihre

Adhäsion an diese Membran[135–137]. Darüber hinaus sind die Wechselwirkungen zwischen WBK und Erythrozyten, welche die Adhäsion der WBK verhindern, in gereinigten Granulozytenkonzentraten aufgrund der geringeren Erythrozytenzahl minimiert, sodass die Zellen vermehrt im Filter persistieren. Darüber hinaus können funktionelle Veränderungen bei WBK im extrakorporalen Kreislauf aufgrund der Interaktionen mit Thrombozyten zur Bildung von heterotypischen Ko-Aggregaten führen[138,139]. Da der Gehalt an Thrombozyten in gereinigten Granulozytenkonzentraten minimiert ist, spielen Interaktionen in diesem Setting eine untergeordnete Rolle. Aus der Erkenntnis, dass bei der Anwendung von gereinigten Granulozytenkonzentraten im ICE-System eine kontinuierliche Zirkulation der WBK für deren Funktionalität nicht erforderlich ist, lässt sich ableiten, dass WBK in einem plasmadurchlässigen Hohlfaserfilter funktionsfähig bleiben, sofern sie kontinuierlich mit Plasma gespült werden. Dies ermöglichte eine deutliche technische Vereinfachung des ICE-Systems. Ausgehend von dem ursprünglichen System, welches aus zwei Kreisläufen mit insgesamt vier Rollerpumpen bestand, konnte eine technisch weniger komplexe Lösung entwickelt werden. Dabei konnte der Plasmakreislauf durch eine Plasmaperfusion ersetzt werden. Die Realisierung dieser neuen Lösung erfordert, neben einer deutlich reduzierten extrakorporalen Oberfläche, lediglich zwei Rollerpumpen. Diese Entwicklung markiert einen bedeutenden Fortschritt hinsichtlich der Biokompatibilität von extrakorporalen Therapiesystemen. Die Reduktion der extrakorporalen Oberfläche sowie die Verringerung der Anzahl an Rollerpumpen, welche mit Hämolyse und den damit assoziierten klinischen Komplikationen in Verbindung gebracht werden, zielt direkt auf die zentralen Mechanismen der biologischen Interaktion der Geräte ab. Die Reduktion der Anzahl der Rollerpumpen bewirkt eine Verringerung der Scherbelastung der Blutbestandteile, was wiederum eine Reduktion der Hämolyse zur Folge hat. Des Weiteren wird durch die Minimierung der extrakorporalen Oberfläche die Adhäsion von Plasmaproteinen, Lipiden, Kalzium und weiteren Substanzen an der Oberfläche des Geräts sowie die Adhäsion von zellulären Elementen und die Adsorption ihrer Bestandteile reduziert. Andernfalls kann es zur Bildung einer Pseudointima oder Gewebekapsel auf der Geräteoberfläche kommen[89–92,140–142]. Die genannten biologischen Wechselwirkungen können eine Veränderung der mechanischen und funktionellen Eigenschaften des Geräts bewirken, was sich möglicherweise auf dessen Leistung und Langlebigkeit auswirkt. Unter Berücksichtigung der genannten Faktoren kann eine Verbesserung der Biokompatibilität sowie der allgemeinen Sicherheit von Medizinprodukten im klinischen Einsatz erreicht werden.

In der **OA4** wurden die gereinigten Granulozytenkonzentrate erstmalig in dem weiterentwickelten Plasmaperfusionssystem angewendet. In den *ex vivo* Zirkulationsexperimenten konnten eine erhaltene Zellfunktionalität und Vitalität bei einer stabilen metabolischen Aktivität über einen Zeitraum von sechs Stunden nachgewiesen werden. Die Analyse der Zytokinkonzentrationen im Verlauf deutet auf eine potentiell sinnvolle Verlängerung des Untersuchungszeitraumes hin. Diese potenziell zeitabhängige Wirksamkeit stellt eine Besonderheit des zellbasierten Therapieansatzes dar, da Erkenntnisse über membranbasierte und adsorptive Blutreinigungsverfahren zur Behandlung von Organinsuffizienzen auf eine erhöhte Wirksamkeit aufgrund des Gesamtvolumens der therapierten Flüssigkeit (Blut, Plasma) hinweisen[143–157]. In diesem Zusammenhang stellt sich die Frage, welches Anwendungsschema für das extrakorporale Immunzellplasma-Perfusionssystem für den klinischen Einsatz geeignet ist. Dies ist von besonderer Relevanz, da alle bisherigen extrakorporalen Immunzelltherapien über einen Zeitraum von sechs Stunden ohne vorherige Dosisfindungsstudien und damit ohne wissenschaftliche Evidenz durchgeführt wurden[53,57]. Diesbezüglich liefern die Ergebnisse aus **OA5** elementare Erkenntnisse über die Funktionalität und das Zytokinsekretionsprofil von gereinigten Granulozytenkonzentraten im *ex vivo* Zirkulationsmodell. Erstens blieben die Immunzellen im gereinigten Granulozytenkonzentrat auch nach drei Tagen Lagerung voll funktionsfähig, wobei sie im Behandlungsmodell zusätzlich 24 Stunden zirkulierten, was die Erhaltung der Funktionalität über längere Zeiträume unterstreicht. Zweitens wurden bei den *ex vivo* Zirkulationsexperimenten dynamische Veränderungen der Zytokinprofile beobachtet, was auf ein differenziertes Reaktionsmuster bei längerer Zirkulation hindeutet[137,158–168]. Schließlich konnte eine zeitabhängige Dynamik der Zytokinspiegel beobachtet werden, was eine Besonderheit der bioartifiziellen Therapie darstellt.

In der abschließenden Betrachtung lässt sich festhalten, dass durch die Einbeziehung der Dimension der klinischen Anwendbarkeit die medizinisch-technische Entwicklung des bioartifiziellen Plasmaperfusionssystems ICE von MDLR Stufe sechs weiterentwickelt und die Voraussetzung für die Durchführung einer RCT zum Nachweis der Wirksamkeit und damit dem Erreichen der MDLR Stufe sieben hergestellt werden konnte. Diese Entwicklung führte zur Initiierung einer RCT (NCT05442710), welche sich aktuell in der Rekrutierungsphase befindet.

In der modernen Medizin sind komplexe technische Therapieoptionen allgegenwärtig[127,128]. Die Ergebnisse der Studie stützen die These, dass ein multidisziplinärer Ansatz bei der Entwicklung

solcher Geräte unmittelbar den Entwicklungserfolg fördert, was letztlich auch zu einer Verbesserung der Therapieergebnisse führen kann. Die Integration praktischer klinischer Erkenntnisse in fortgeschrittene Phasen der Produktentwicklung von medizinischen Kombinationsprodukten kann einen signifikanten Einfluss auf den Entwicklungserfolg haben. Diese Arbeit hat gezeigt, dass die Ausrichtung auf einen praxisorientierten Ansatz die Verbesserung des ICE-Systems für den klinischen Einsatz erfolgreich geleitet und einen vielversprechenden Weg für zukünftige Innovationen in diesem Sektor geliefert hat.

5 Zusammenfassung

Die Behandlung komplexer Syndrome, wie beispielsweise der Sepsis-induzierten Immunparalyse, die auch heute noch hohe Mortalitätsraten aufweist, stellt eine große Herausforderung für das Gesundheitssystem und das Behandlungsteam dar. Extrakorporale Verfahren zur Sepsistherapie besitzen grundsätzlich das Potenzial die Prognose von Patienten mit Sepsis zu verbessern. Die Entwicklung des bioartifiziellen ICE-Systems hat bereits Belege zur Sicherheit und Verträglichkeit der Therapie der Sepsis-induzierten Immunparalyse erbracht. Die hohe technische Komplexität sowie die begrenzte Einsetzbarkeit von Standard- Granulozytenkonzentraten stellen jedoch wesentliche praktische Nachteile und Limitationen dar. Vor diesem Hintergrund wurden im Rahmen einer systematischen Analyse Optimierungspotenziale von Komponenten des ICE-Systems für gezielte Weiterentwicklungen identifiziert. Die dieser kumulativen Habilitationsschrift zugrunde liegenden Untersuchungen und Publikationen konnten demonstrieren, dass der Zellquelle als primärer Fokus der Entwicklung eine signifikante Bedeutung beizumessen ist. Die Verbesserung der Lagerbarkeit der Granulozytenkonzentrate durch die Entwicklung von speziellen Aufreinigungsverfahren eröffnet neben dem Einsatz im ICE-System auch das Potenzial den klinischen Einsatz in bereits bestehenden Indikationsfeldern zu optimieren. Die Anwendung der gereinigten Granulozytenkonzentrate im ICE-System hat gezeigt, dass neben der Aufrechterhaltung der Funktionalität und Integrität im System auch die kontinuierliche Bewegung von Immunzellen im Kreislauf keine unabdingbare Voraussetzung für die Aufrechterhaltung der Funktionalität der Zellen ist. Dies ermöglichte die technische Weiterentwicklung des Systems durch eine Reduktion der Komplexität zugunsten einer besseren klinischen Anwendbarkeit. Des Weiteren konnte das Dosisregime durch eine präzise Einstellbarkeit des Zellzahlbereichs mittels der Aufreinigungsverfahren optimiert werden. Zudem

gelang der Nachweis der zeitabhängigen Zytokinfreisetzung, welcher als spezifischer Aspekt der extrakorporalen Zelltherapie betrachtet werden kann. Insgesamt ist es durch die im Rahmen dieser Habilitationsschrift vorgestellten experimentellen Arbeiten gelungen einen Beitrag zur Optimierung der klinischen Anwendbarkeit zellbasierter extrakorporaler Therapieverfahren zu leisten.

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7 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich diese Arbeit selbstständig angefertigt, alle verwendeten Ergebnisse und Daten anderer vollständig aufgeführt und korrekt zitiert habe. Die Mitwirkung Dritter habe ich offengelegt.

Ich versichere weiterhin, dass diese Arbeit nicht zuvor und auch nicht bei einer anderen Fakultät zur Eröffnung eines Habilitationsverfahrens eingereicht wurde.

Ich erkläre hiermit ebenfalls, dass ich die deutsche Staatsbürgerschaft besitze und mir die Habilitationsordnung sowie alle zughörigen Bestimmungen bekannt sind.

Rostock, den 05.08.2024 Dr. med. Gerd Klinkmann

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9 Curriculum Vitae

Persönliche Daten

Name	Dr.med. Gerd Klinkmann
Anschrift	Am Wald 11, 18198 Kritzmow
Familienstand	verheiratet, 4 Kinder

Berufserfahrung

11/2024	Zusatzbezeichnung spezielle Intensivmedizin
Seit 03/2024	International Renal Research Institute of Vicenza (IRRIV), Research Fellow Direktor: Prof. Claudio Ronco
Seit 04/2023	Fraunhofer-Institut für Zelltherapie und Immunologie, Abteilung für extrakorporale Therapiesysteme, Rostock Direktor: Prof. Dr. med. S. Mitzner
Seit 01/2023	Clinician Scientist, Universitätsmedizin Rostock
11/2022	Facharzt für Anästhesiologie und Intensivmedizin
Seit 01/2022	Präsident, internationale Arbeitsgruppe „Albunet“ (Europäische Gesellschaft für künstliche Organe)
Seit 08/2019	Klinik für Anästhesiologie, Intensivmedizin und Schmerztherapie, Universitätsklinikum Rostock Direktor: Prof. Dr. med. D. Reuter
01/2017 - 07/2019	Klinik für Anästhesiologie und Intensivmedizin, Sana Hanse Klinikum Wismar, Direktor: Dr. med. A. Meyer
12/2016	Approbation

Bildung

05/2014 - 10/2015	Doktorand und studentische Hilfskraft, Klinik für Frauenheilkunde und Geburtshilfe, Medizinische Universität Greifswald, Greifswald
2010 - 2016	Studium der Humanmedizin an der Universität Szeged, der Universität Ulm und der Universität Greifswald
2006 - 2010	Studium der Mathematik und Sportwissenschaften an der Universität Rostock

Dissertation „Ovarialkarzinomzellen sekretieren das zytoprotektive Hitzeschockprotein HSP27 abhängig von der intrazellulären Proteinkonzentration jedoch unabhängig vom sekretorischen Stoffwechselweg des endoplasmatischen Retikulums"

Prädikat: „Magna cum laude“

10 Wissenschaftliche Aktivitäten

Mitgliedschaften

- Deutsche Gesellschaft für Anästhesiologie und Intensivmedizin (DGAI)
    - Wissenschaftlicher Arbeitskreis „Intensivmedizin“ (WAKI)
    - Wissenschaftlicher Arbeitskreis „Wissenschaftlicher Nachwuchs“ (WAKWiN)
    - Wissenschaftlicher Arbeitskreis „molekulare Medizin"
- Europäische Gesellschaft für künstliche Organe (ESAO)
    - Präsident der internationalen Arbeitsgruppe „Albunet"
- Europäische Gesellschaft für Intensivmedizin (ESICM)
- European Sepsis Care Study Group

Forschungsaktivitäten und wissenschaftliche Auszeichnungen

- Clinician Scientist Universitätsmedizin Rostock
    - "*Extrakorporale Zelltherapie bei Sepsis-induzierter Immunoparalyse*"
- Aufbau und Leitung der internationalen Arbeitsgruppe „Albunet“
- Dierichs-Stipendium der Deutschen Gesellschaft für Anästhesiologie und Intensivmedizin (DGAI), 2024
- 42nd Vicenza Course on AKI, CRRT & ECOS, 2024
    - Best Abstract Award: “*Purified Granulocytes in Extracorporeal Cell Therapy: A Multifaceted Approach to Combat Sepsis-Induced Immunoparalysis*”
- 41st Vicenza Course on AKI&CRRT, Vicenza, 2023
    - Poster Award : “*Characterization of albumin binding function in patients with acute kidney injury*”
- Fellowship am International Renal Research Institute of Vicenza, Italien
- Absolvierung des Mentoring-Programms des Wissenschaftlichen Arbeitskreises Wissenschaftlicher Nachwuchs in der Anästhesie (WAKWIN) der Deutschen Gesellschaft für Anästhesiologie und Intensivtherapie (DGAI)

Reviewer für internationale Fachzeitschriften

- Artificial Organs (seit 2020)
- Intensive Care Medicine Experimental (seit 2023)
- Blood Purification (seit 2024)
- BMJ open (seit 2022)
- iScience (seit 2023)
- Journal of Clinical Medicine (seit 2023)

11 Publikationsliste

Publikationen – Erstautor

1. **Klinkmann G**, Au B, Mitzner S, Kielstein J. Bridge over troubled water or road to nowhere? Adsorptive Verfahren bei kritisch kranken Patient:innen auf der Intensivstation. Die Nephrologie. 2024
2. **Klinkmann G**, Brabandt S, Möller M, Wild T, Heskamp B, Schewe JC, Sauer M, Altrichter J, Mitzner S. Purified Granulocytes in Extracorporeal Cell Therapy: A Multifaceted Approach to Combat Sepsis-Induced Immunoparalysis in Varied Environmental Conditions. International Journal of Artificial Organs. 2024
3. **Klinkmann G**, Waterstradt K, Klammt S, Schnurr K, Schewe JC, Wasserkort R, Mitzner S. Exploring Albumin Functionality Assays: A Pilot Study on Sepsis Evaluation in Intensive Care Medicine. International Journal of Molecular Sciences. 2023
4. **Klinkmann G**, Koball S, Reuter DA, Mitzner S. Hemoperfusion with CytoSorb®: Current Knowledge on Patient Selection, Timing, and Dosing. Contributions to Nephrology. 2023
5. **Klinkmann G**, Goudeva L, Blasczyk R, Mitzner S, Altrichter J. Granulocyte products: The saga continues. Transfusion
6. **Klinkmann G**, Wild T, Heskamp B, Doss F, Doss S, Milej M, Zielske D, Thiele LM, Goudeva L, Blasczyk R, Reuter DA, Altrichter J, Mitzner S. Extracorporeal therapy of sepsis by purified granulocyte concentrates - ex vivo circulation model. Artificial Organs. 2023
7. **Klinkmann G**, Wild T, Heskamp B, Doss F, Doss S, Arseniev L, Aleksandrova K, Sauer M, Reuter DA, Mitzner S, Altrichter J. Extracorporeal immune cell therapy of sepsis - ex vivo results. Intensive Care Medicine experimental. 2022
8. **Klinkmann G**, Altrichter J, Reuter DA, Mitzner S. Therapeutic apheresis in sepsis. Therapeutic Apheresis and Dialysis. 2022
9. **Klinkmann G**, Klammt S, Jäschke M, Henschel J, Gloger M, Reuter DA, Mitzner S. Impact of Albumin Binding Function on Pharmacokinetics and Pharmacodynamics of Furosemide. Medicina (Kaunas). 2022
10. **Klinkmann G**, Doss F, Goudeva L, Doss S, Blasczyk R, Milej M, Koch S, Mitzner S, Altrichter J. Prolonged storage of purified granulocyte concentrates: Introduction of a new purification method. Transfusion. 2021
11. **Klinkmann G**, Stope MB, Meyer A. Cytokine adsorption as a promising option for septic shock and multiple organ failure due to Candida infection and decompensated type 1 diabetes mellitus. Artificial Organs. 2020

Publikationen – Ko-Autor

1. Körtge A, Kamper C, **Klinkmann G**, Wasserkort R, Mitzner S. In vitro Assessment of Drug Adsorption Profiles During Hemoadsorption Therapy. Blood Purif. 2025 Mar 20:1-17.

2. Reis T, Ramírez-Guerrero G, Pecoits-Filho R, Lorenzin A, de Cal M, Corradi V, **Klinkmann G**, Ronco F, Neves FAR, Bellomo R, Ronco C. Iodinated Contrast Adsorption in Cartridges With Styrene-Divinylbenzene Sorbent. Artif Organs. 2025 Jan 24.
3. Ehler J, Klawitter F, von Möllendorff F, Zacharias M, Fischer DC, Danckert L, Bajorat R, Hackenberg J, Bertsche A, Loebermann M, Geerdes-Fenge H, Fleischmann R, **Klinkmann G**, Schramm P, Schober S, Petzold A, Perneczky R, Saller T. No substantial neurocognitive impact of COVID-19 across ages and disease severity: a multicenter biomarker study of SARS-CoV-2 positive and negative adult and pediatric patients with acute respiratory tract infections. Infection. 2024 Oct 1
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Übersichtsarbeiten

1. **Klinkmann G**, Koball S, Reuter DA, Mitzner S. Hemoperfusion with CytoSorb®: Current Knowledge on Patient Selection, Timing, and Dosing. Contributions to Nephrology. 2023
   Impact-Faktor: 1,9
2. **Klinkmann G**, Altrichter J, Reuter DA, Mitzner S. Therapeutic apheresis in sepsis. Therapeutic Apheresis and Dialysis. 2022
   Impact-Faktor: 1,8

Buchbeiträge

1. **Klinkmann G**. Peripartum Cardiomyopathy: Diagnostic and Therapeutic Challenge. Best 2022 Clinical Cases in Intensive Care Medicine, From the ESICM NEXT Committee Clinical Case Contest. 2023
2. **Klinkmann G**, Reuter DA, Haas S. Hemodynamic Monitoring and Optimization in Cardiac Surgery. Advanced Hemodynamic Monitoring: Basics and New Horizons. 2021.
3. **Klinkmann G**, Ehler J. Neurokognitives Defizit bei septischer Enzephalopathie. Intensivmedizin. ecomed MEDIZIN. 2021

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ORIGINAL RESEARCH

TRANSFUSION

Prolonged storage of purified granulocyte concentrates: Introduction of a new purification method

Gerd Klinkmann[1] | Fanny Doss[2] | Lilia Goudeva[3] | Sandra Doss[2,4] | Rainer Blasczyk[3] | Magdalena Milej[2] | Stephanie Koch[4] | Steffen Mitzner[2,4,5] | Jens Altrichter[2]

[1]Department of Anaesthesiology and Intensive Care Medicine, University of Rostock, Rostock, Germany

[2]Artcline GmbH, Rostock, Germany

[3]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Germany

[4]Department of Extracorporeal Immunomodulation, Fraunhofer Institute for Cell Therapy and Immunology, Rostock, Germany

[5]Department of Medicine, Division of Nephrology, Medical Faculty, University of Rostock, Rostock, Germany

Correspondence
Gerd Klinkmann, Department of Anaesthesiology and Intensive Care Medicine, University of Rostock, Schillingallee 35, 18055 Rostock, Germany.
Email: gerd.klinkmann@med.uni-rostock.de

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Abstract

Background: Use of donor granulocyte concentrate (GC) has been limited due to its short storage time of 6–24 h, which is partially due to residual red blood cells (RBCs) and platelets and the resulting lactate production leading to an acidotic milieu. To increase this storage time, we developed a closed system procedure compatible with standard blood bank technologies to remove RBC and platelets and to enrich the GC.

Methods: Standard GCs (sGCs) were sedimented, washed twice with 0.9% sodium chloride (NaCl), and resuspended in blood group-identical fresh frozen plasma. The resulting purified GCs (pGCs) were then stored in platelet bags at a cell concentration of about $5 \times 10^7 \pm 1.8 \times 10^7$ leukocytes/ml without agitation at room temperature for up to 72 h. Cell count and viability, pH, blood gases, phagocytosis, and oxidative burst were monitored daily.

Results: A significant reduction in RBC (98%) through sedimentation, and platelets (96%) by washing, purified the white blood cell (WBC) population and enriched the granulocytes to 96% of the WBC in the pGC. After 72 h of storage, over 90% of the initial WBC count of pGC remained, was viable (≥97%), and the granulocytes exhibited a high phagocytosis and oxidative burst functionality, comparable to sGC after 24 h.

Conclusion: Purification extends the maximum storage period of GC from 24 to 72 h and may therefore improve the availability of GC and its clinical use.

KEYWORDS

granulocyte, purification, transfusion medicine, transfusion

Abbreviations: AABB, association for the advancement of blood and biotherapies; AO, acridine orange; DAPI, 4′,6-diamidino-2-phenylindole; DHR, dihydrorhodamine; DR, G-CSF granulocyte-colony-stimulating factor; FITC, fluorescein isothiocyanate; GC, Granulocyte concentratet; GvH, graft versus hos; HES, hydroxyethyl starch; HLA-DR, human leucocyte antigen; iGC, intermediate granulocyte concentrate; LDH, lactate dehydrogenase; pGC, purified Granulocyte concentrate; RBC, red blodd cells; WBC, white blood cells.

Department of Anesthesiology and Intensive Care Medicine, University Medical Center Rostock, Schillingallee 35, 18057 Rostock, Germany.

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1 | INTRODUCTION

Sepsis is a common cause of morbidity and mortality worldwide with 48.9 million cases in 2017 and 11.0 million sepsis-related deaths, representing 19.7% of all deaths.[1]

Neutrophils are the body's main defense against bacterial and fungal infection, and a neutrophil count less than 1×10^9/L is directly linked to the risk of systemic infection.[2]

Despite being such important cells and having different functions in the control of invading microorganisms, neutrophils have been reported to be short-living cells. Neutrophil numbers are maintained by a fine balance between granulopoiesis, bone marrow storage and release, intravascular margination (prolonged transit through the spleen, liver, bone marrow, and lung, with the formation of discrete intravascular pools) and clearance and destruction.[3] However, lifespan is increased during inflammation by mechanisms delaying neutrophil apoptosis, for example, through the release of granulocyte-macrophage colony-stimulating factor.[4]

Intensive myelosuppressive chemotherapy regimens associated with neutropenia predispose to infections and sepsis. As a result, neutropenia is the most important cause of treatment-related morbidity and mortality and the most important dose-limiting toxicity.[2]

Furthermore, patients with sepsis and especially septic shock develop a state of immunoparalysis associated with increased mortality.[5,6] This comprises dysfunction of phagocytosis and bacterial clearance by granulocytes[7–11] and macrophages,[12] dysfunction of antigen presentation (e.g., low human leucocyte antigen DR [HLA-DR] expression on monocytes),[13,14] and dysfunction in T-cell activation as well as lymphopenia.[15,16]

Granulocyte concentrate (GC) transfusion is a supportive treatment for patients with life-threatening neutropenia or neutrophil dysfunction. It has been used for transfusion for over 60 years,[17,18] although its efficacy has not yet been well established.[2,19] Price et al. were unable to definitively answer this question in the largest prospective study to date, although secondary analyses provide suggestive evidence of potential efficacy when higher doses of granulocytes are actually administered to the patient.[20]

In addition to transfusion, GC has been used as an extracorporeal immune therapy for patients in septic shock with promising results.[21]

The majority of the cells in a standard GC (sGC) are red blood cells (RBCs) and platelets;[22] white blood cells (WBCs) often comprise less than 20% of the cells (own observation). Donor GC contains multiple immune cells, mainly granulocytes, which as phagocytes eliminate cell detritus and microbial remnants.[22] In addition, granulocytes modulate immune function by secreting and adsorbing immunologic messenger molecules (e.g., cytokines, chemokines, growth factors, prostaglandins).[23] Granulocytes can therefore influence and potentially correct the immune dysfunction observed in sepsis.

In the future, indications for GC treatment are likely to increase because of the increasing use of myelosuppressive therapy[24] and to address the high mortality and morbidity rates of sepsis.

Donor GC may be obtained by apheresis or from pooled buffy coats.[2] The preparation process varies between transfusion departments in the use of donor stimulation protocols, sedimentation agents, machines, and operator-dependent factors.

These different methods for collecting, processing, and storing GC lead to large differences in the total number of granulocytes, other WBC, RBC, and platelets in the GC and are likely to impact their survival and function after transfusion.

Guidelines for transfusion services in the USA and Germany recommend that 1 unit of GC should contain at least 1×10^{10} granulocytes.[22,25] However, higher numbers have shown a benefit.[26]

Storage of cellular blood products is challenging because whole blood deteriorates over time with lysis of RBC, loss of complement, change of pH,[27] and induction of WBC apoptosis, pointing to the crucial role of neutrophils.[28] Neutrophils can survive for several days in storage,[28] but the key question is how sustainably they remain active and functional. Apoptosis suppresses neutrophil activity, decreases neutrophil count, and results in morphologic changes.

We have therefore developed a method for solving the problem by purifying sGC in accordance with guidelines for the collection of blood and blood components,[22] and have analyzed the resulting purified GC (pGC) for vitality and function. Our aim was to increase storage time by substantially removing both RBC (>95% depletion) and platelets (>90% depletion), and enriching granulocytes.

Furthermore, we have designed the entire purification process within a closed blood bag system that can be operated by trained personnel in transfusion medicine facilities.

Delivery of pGC to patients with septic shock using an extracorporeal circuit opens up the potential for pGC

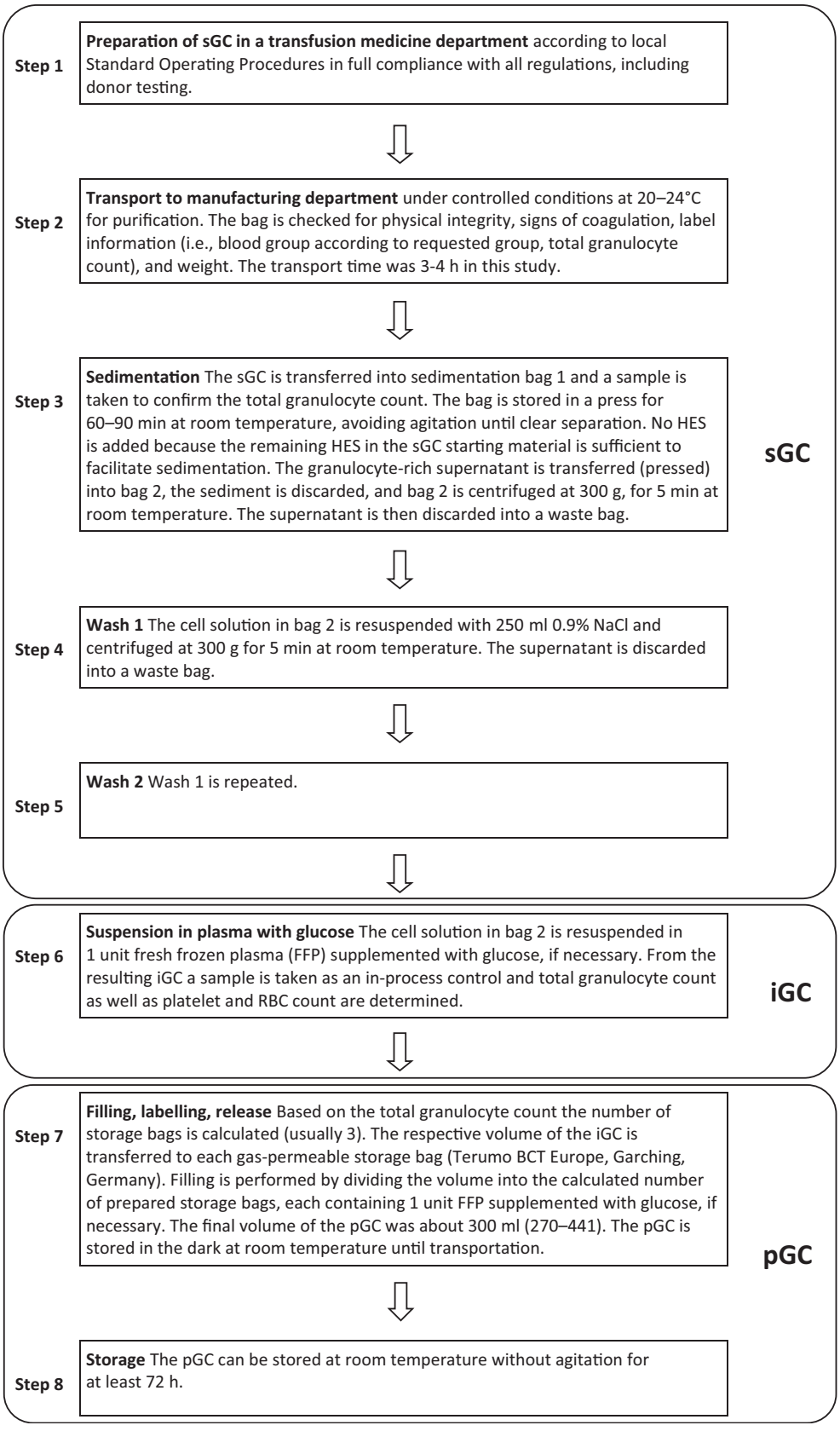

FIGURE 1 Flowchart of the purified granulocyte concentrate (pGC) manufacturing process

therapy in the management of sepsis. Side effects due to cellular components are avoided because cellular components do not come into contact with the patients, yet the absorbing and immunomodulatory benefits of the immune cells are retained to support regeneration of the patient's immune system.

2 | MATERIALS AND METHODS

2.1 | Donors

GC was obtained from healthy donors from the donor pools of the Institute of Transfusion Medicine and Transplant Engineering (Hannover Medical School, Germany). All donors met the screening and testing criteria for granulocytapheresis defined by "Guidelines for the preparation of blood and blood components and the use of blood products (Hemotherapy)," published by the German Medical Association (Bundesärztekammer).[29] Donors were stimulated with a subcutaneous injection of granulocyte-colony-stimulating factor (G-CSF) 6 μg/kg body weight (Lenogastrim, Kohlpharma, Merzig, Germany) and 8 mg oral dexamethasone approximately 16–18 h before granulocytapheresis.

2.2 | Granulocytapheresis

sGC was collected by continuous-flow apheresis with the COBE Spectra OPTIA™ Apheresis System (Terumo BCT Europe, Garching, Germany) using 6% hydroxyethyl starch (HES; Hespan, B.Braun, Irvine, CA) as the sedimentation agent supplemented with sodium citrate (SERACIT 1M, SERAG-Wiessner, Naila, Germany) for anticoagulation.

2.3 | New purification procedure for sGC

Figure 1 demonstrates the manufacturing process to purify sGC developed by ARTCLINE.

The process was performed under aseptic conditions, using single-use materials, as one continuous flow, without hold steps.

Twenty four sGCs were used to prepare a total of 42 pGC. The iGC is a concentrated intermediate used to determine the residual concentration of erythrocytes and platelets. About one unit of the resulting pGC was prepared for every 2×10^{10} granulocytes in the iGC and contained at least 1×10^{10} granulocytes. The pGC with its purified cells was supplemented with 40% or 5% glucose solution in ABO-compatible citrate-anticoagulated blood plasma to ensure an initial glucose concentration of at least 15 mmol/L.

The pGC was transferred to a gas-permeable storage bag, for example, platelet storage bag (Terumo BCT Europe, Garching, Germany). Both sGC and pGC were stored at room temperature (22 ± 2°C).

Samples were taken and analyzed to determine leukocyte recovery rate, viability, and functional capacity. Furthermore, the course of the leucocyte and platelet counts, pH, electrolyte, glucose, lactate, and lactate dehydrogenase (LDH) concentrations as well as oxygen and carbon dioxide partial pressure were determined.

2.4 | Measurement of blood cell counts and WBC viability

Blood cell content and WBC differentiation were evaluated automatically using a hematology analyzer (KX-21N, Sysmex, Norderstedt, Germany).

WBC viability was determined using the NucleoCounter NC-200 (ChemoMetec, Allerod, Denmark) according to the manufacturer's specifications. The Via1-Cassette™ is a specific device for cell sampling and staining for viability and cell counting applications with NucleoCounter® NC-200™. It incorporates the fluorophores acridine orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI) immobilized in the channels of the cassette. Using approximately 60 μl of a cell sample, the dyes AO and DAPI are added to the cassette, causing them to dissolve in the mixing channels. AO stains both live and dead nucleus-containing cells and provides a total count, whereas DAPI stains the dead cells only. The method does not discriminate between the different WBC subsets. However, in pGC, approximately 96% of WBCs were neutrophilic granulocytes. Thus, WBC viability provides a sufficiently accurate approximation of neutrophil viability.

2.5 | Measurement of electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures

Electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures were measured using an ABL90 Flex Plus blood gas analyzer (Radiometer, Krefeld, Germany) according to the manufacturer's specifications.

2.6 | Measurement of lactate, and LDH concentrations

Lactate and LDH concentrations were measured using a Cobas Mira Plus CQ (Roche, Ludwigsburg, Germany) according to the manufacturer's specifications.

2.7 | Evaluation of granulocyte morphology and function

Samples of all processed GC were collected before and after purification to evaluate neutrophil morphology. Blood smears were prepared, air-dried, methanol-fixed (99.9%, 10 min), stained using the Pappenheim method (May–Grünwald–Giemsa), and examined by light

microscopy using the DM IL LED (Leica Microsystems GmbH, Wetzlar, Germany).

Granulocyte function was analyzed in vitro with oxidative burst and phagocytosis assays using the commercial Phagoburst-Kit and Phagotest-Kit (Celonic, Heidelberg, Germany), respectively, by flow cytometry. Phagotest allows quantitative determination of leukocyte phagocytosis. It determines the percentage of phagocytes that take up fluorescein isothiocyanate (FITC)-labeled opsonized *E. coli* bacteria and their activity (mean fluorescence intensity that correlates with number of phagocytosed bacteria per cell). The Phagoburst test allows the quantitative determination of the oxidative burst of leukocytes. It detects the proportion of leukocytes that oxidize the fluorogenic substrate dihydrorhodamine (DHR) 123 to rhodamine 123 and their enzymatic activity (mean fluorescence intensity that correlates with amount of rhodamine 123 per cell). Both tests were used according to the manufacturer's instructions with one modification because the granulocyte concentration in GC is approximately 10 times higher than in whole blood. To achieve a concentration of 5000 granulocytes/μl and therefore the same ratio of granulocytes to the stimulus (ratio about 1:80) as with heparin-anticoagulated blood (4000–10,000 granulocytes/μl), the samples were diluted in heparin-anticoagulated plasma.

Cells are analyzed by flow cytometry using the blue-green excitation light (488 nm argon ion laser). Leukocytes were defined by fluorescence-2 (DNA staining), and the region containing leukocytes was analyzed by forward scatter versus side scatter to distinguish leukocyte subpopulations and identify neutrophils. Neutrophils from the control sample were then used to adjust the fluorescence-1 marker to detect <3% of positive events in the negative control.

2.8 | Statistical analysis

Statistical analysis of the data was performed using IBM SPSS Statistics (version 27, Chicago, IL, USA). The results are expressed as the median ± standard deviation (SD) and range. Box plots were used for graphics. The horizontal line within the boxes represents the median, whereas the upper part represents the 75th and the lower part the 25th percentiles. The whiskers represent the range of the values, whereas the circles and the asterisks show the outliers (extreme values that deviate from the rest of the sample). According to the distribution of data (using Shapiro–Wilk test), the Wilcoxon test was used for two dependent samples for continuous variables. The Friedman test was used when the data arose from more than two related samples. The Kruskal–Wallis test was used to test the difference between multiple independent samples with non-normal underlying population distribution, and appropriate post-hoc tests were applied if necessary. Statistical differences were considered significant at a p value <.05 and highly significant at $p < .01$.

3 | RESULTS

The sGC used to produce pGC were prepared according to clinical standard procedures by granulocytapheresis from healthy donors and met the quality criteria of the guidelines of the German Medical Association.[29]

3.1 | Lower RBC and platelet counts, and their effect on pH and lactate

The purification procedure of sedimentation and washing reduced the RBC and platelet counts in iGC, by approximately 98.08 ± 1.87% and 95.91 ± 5.1%, respectively, compared with sGC (Table 1, Figure 2A).

The higher purity and greatly diminished RBC count in the pGC resulted in its yellow color, in contrast to the red color of the sGC (Figure 2B).

Within 10 h after donation, the median pH of sGC was 6.89 ± 0.19 and decreased further within 24 h after donation to less than 6.75. In comparison, the pH in pGC during storage remained less acidic, with a median value above 6.90, even after 72 h of storage (Figure 3A). RBC and platelets produce huge amounts of lactate in sGC, leading to lactate concentrations of up to 19.15 mmol/L within the

TABLE 1 Red blood cell (RBC), white blood cell (WBC), and platelet counts in standard granulocyte concentrate (sGC) and intermediate granulocyte concentrate (iGC) preparations

	sGC	iGC	Recovery rate (%)
WBC ($\times 10^{10}$)	7.03 (4.01–11.30)	3.84 (2.10–6.21)	54.06 (41.37–78.95)
Lymphocytes ($\times 10^{9}$)	3.34 (1.36–5.63)	0.807 (0.484–1.98)	24.16 (15.38–42.71)
Monocytes ($\times 10^{9}$)	4.59 (1.82–7.64)	2.53 (0.882–4.59)	55.11 (34.99–66.67)
Granulocytes ($\times 10^{10}$)	6.24 (3.22–10.30)	3.58 (1.92–5.83)	57.37 (44.01–81.29)
Red blood cells ($\times 10^{11}$)	2.00 (1.09–2.92)	0.048 (0.017–0.102)	2.40 (0.82–7.99)
Platelets ($\times 10^{10}$)	7.79 (4.73–16.90)	0.313 (0.00–1.25)	4.01 (0.00–21.03)

Note: Data are reported as median (minimum–maximum) and percentage.

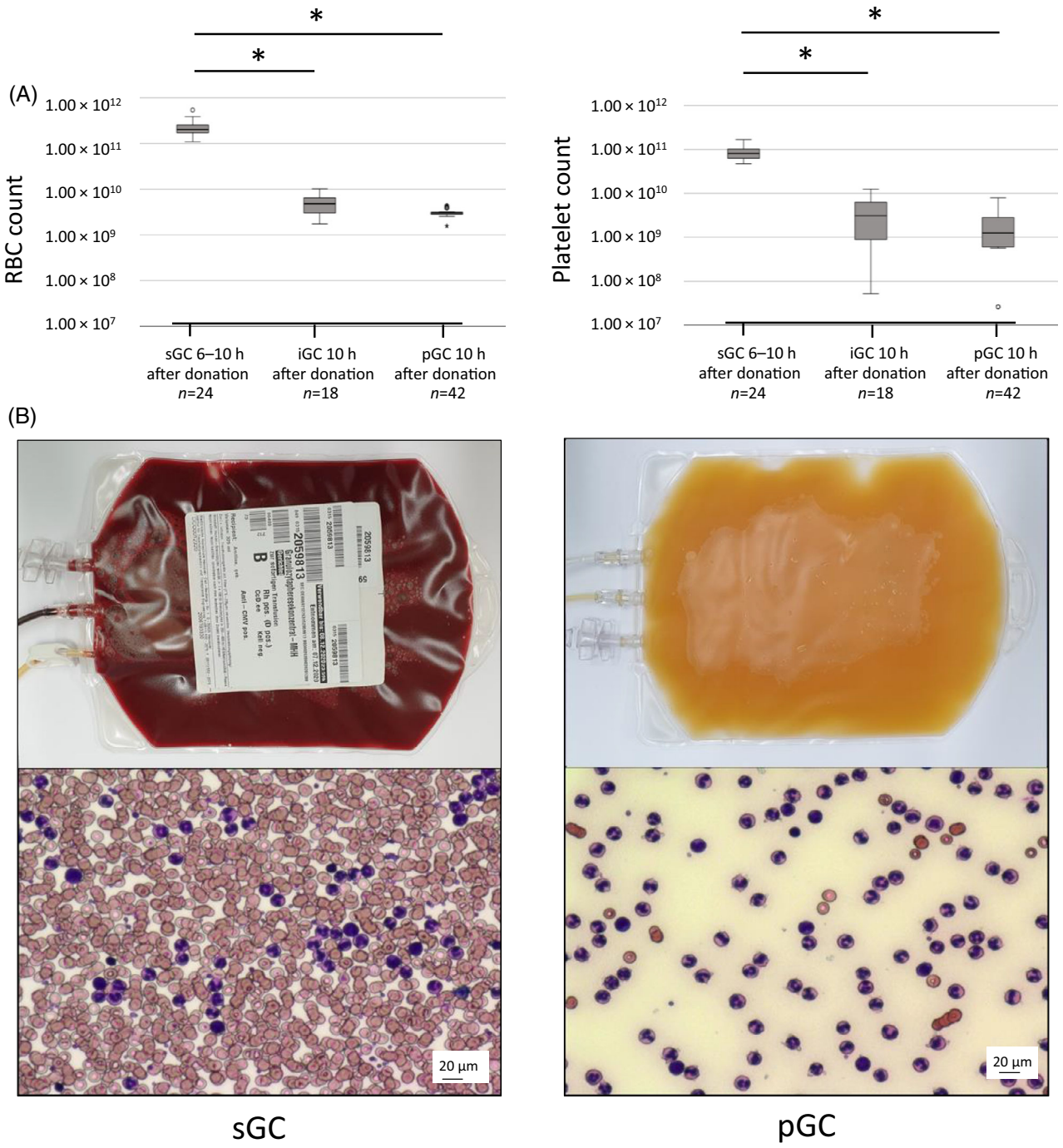

FIGURE 2 (A) Red blood cell (RBC) and platelet counts in standard granulocyte concentrate (sGC), intermediate granulocyte concentrate (iGC), and purified granulocyte concentrate (pGC). (B) Appearance and histology (400×) of sGC and pGC. The higher purity and greatly diminished RBC count in the pGC is evident from its yellow color resulting from the few remaining RBC and platelets. *p* values ≤.05 (+) and ≤.01 (*) were considered significant [Color figure can be viewed at wileyonlinelibrary.com]

first 10 h after donation (reference range 0.5 to 2.2 mmol/L)[30] (Figure 3B). Glucose levels in sGC were 1.2 mmol/L ± 1.88 6–10 h after donation and 0 mmol/L after 24 h. In contrast, in pGC, the glucose level was initially higher and although declining gradually over time remained above the minimal physiologic level (reference range 3.9 to 5.5 mmol/L),[30] even after 72 h of storage (Figure 3C).

3.2 | Granulocyte function and viability

Phagocytosis and oxidative burst assays demonstrated better preservation of granulocyte function in the pGC than in the sGC (Figure 4).

Oxidative burst activity of sGC over the storage time of 24 h showed a gradual decline down to 54.66%, whereas

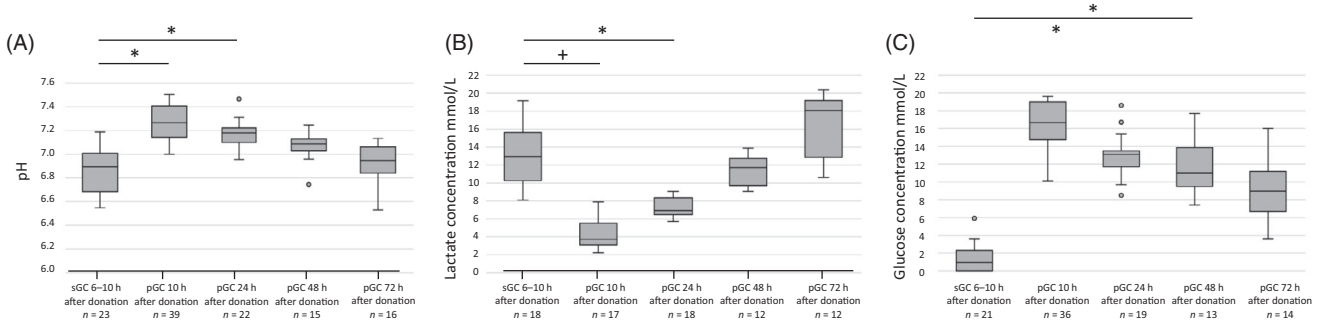

FIGURE 3 sGC and pGC analyses during storage. (A) pH. (B) Lactate concentration. (C) Glucose concentration. Already 6-10 h after donation the pH of sGC is often below 7 and worsens during the allowed storage of 24 h. In comparison, the pH in pGC is maintained much longer under less acidic conditions and after 72 h of storage is at the pH that sGC exhibits after 6–10 h of storage. This is mainly due to the reduced lactate production but also due to optimal storage bags releasing the CO_2 metabolically produced by the cells. Glucose in sGC is often below physiological concentration limiting cell functions. Glucose levels in sGC are around 1.5 mmol/L after 6–10 h. In contrast, in pGC, glucose is on a much higher level, and while declining gradually over time, still above minimal physiological level even after 72 h of storage. p values ≤.05 (+) and ≤.01 (*) were considered significant

oxidative burst activity of pGC remained consistently high over 72 h of storage (Figure 4A). Phagocytosis in the pGC remained robustly high at 90%–100% over the entire period of 72 h of storage comparable to that of the sGC (Figure 4B). Leukocyte viability was consistently high at over 90% in pGC across all time points, whereas it was more variable in sGC at 98.90 ± 4.20% (86.98%–99.80%) (Figure 4C).

3.3 | WBC recovery

Purification resulted in a median WBC recovery of 54.06 ± 12.21% (41.37%–78.95%). The median WBC count before and after purification was $7.03 \times 10^{10} \pm 2.30 \times 10^{10}$ (4.01–11.30) and $3.84 \times 10^{10} \pm 1.55 \times 10^{10}$ (2.10–6.21), respectively (Table 1). The median recovery rates of the granulocyte and monocyte populations were higher than those for lymphocytes. The recoveries of the WBC subpopulations are shown in Figure 5A. 57.37% ± 12.86% (44.01%–81.29%) of granulocytes, 55.11% ± 10.55% (34.99%–66.67%) of monocytes and 24.16% ± 9.32% (15.38%–42.71%) of the lymphocytes were detected after purification.

3.4 | Granulocyte count

The specifications for sGC usually only define a lower limit (e.g., $1x10^{10}$ granulocytes) but no upper limit, leading to a large variation in the total number of granulocytes in sGC. Preparation of pGC means that a much more reproducible and narrower range of granulocytes per unit can be achieved than with sGC, for example, between 1.2×10^{10} and 2.5×10^{10} granulocytes/pGC unit. In addition, the granulocyte count is largely maintained over 72 h of storage and does not fall below 1×10^{10} granulocytes/pGC unit (Figure 5B).

3.5 | Impact on LDH and blood gas dynamics

The LDH concentration was significantly reduced from 250 ± 200.15 U/L in the sGC to 152 ± 22.82 U/L by the purification process. It increased slightly during storage to 189 ± 42.95 U/L after 72 h, but this is well within the normal range. The partial pressures of the blood gases showed significant changes due to the purification process. The partial pressure of oxygen in the pGC was significantly increased from 51.7 ± 31.89 mmHg in the sGC to 149 ± 25.09 mmHg after purification and was constantly high (132.5 ± 19.83 mmHg after 72 h). The partial pressure of carbon dioxide was significantly reduced from 91.6 ± 21.88 mmHg in the sGC to 40.9 ± 12.92 mmHg in the pGC and stayed low (42.35 ± 9.24 mmHg after 72 h), probably due to the use of gas-permeable storage bags.

4 | DISCUSSION

Ideally, storage conditions should maintain granulocyte metabolism, prevent activation, and preserve neutrophil function. European and American guidelines recommend that GC should be stored at a temperature of 20–24°C for no longer than 24 h and preferentially transfused as soon as possible after donation.[22,26]

The search for an ABO-compatible donor and the subsequent preparation of a fresh GC considerably delay availability. Optimal collection yields require the use of a sedimentation agent, such as hydroxyethyl starch

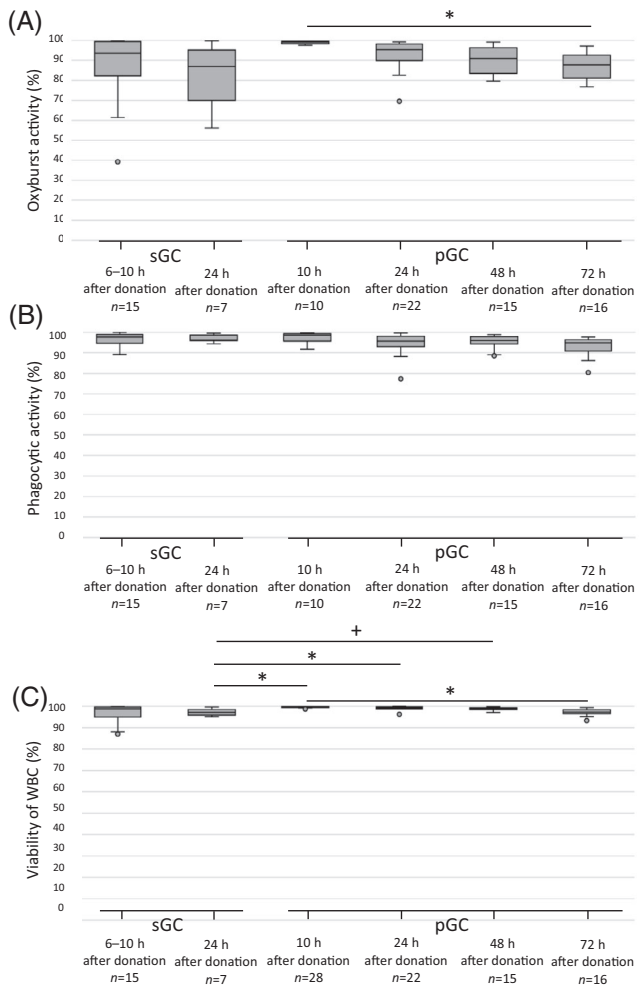

FIGURE 4 Functional granulocyte activity sGC and pGC during storage. (A) Oxidative burst activity of sGC has been evaluated over the permitted storage time of 24 h; the results are displayed in the left part of the figure showing a gradual decline. In contrast, oxidative burst activity of pGC remains robustly constant and high over 72 h of storage. (B) In the phagocytosis assay, a similar pattern can be observed: In the left part of (B), the activity of sGC over 24 h is shown. As can be seen in the right part, phagocytosis in pGC remains robustly high around 90%–100% over the entire period of the proposed 72 h of storage. (C). Viability of WBC was constantly high at above 95% in the pGC across all timepoints but was more variable in the sGC. p values ≤.05 (+) and ≤.01 (*) were considered significant

(HES), dextran, or modified fluid gelatin, which might lead to rare side effects.[31] Together with the limited storage time, these factors have restricted the routine use of GC.

We have developed a method for producing a high-purity, neutrophil-rich, WBC preparation from sGC. The sedimentation and subsequent washing steps can be carried out in a closed blood bag system and lead to an increased storage time of at least 3 days with high viability and functionality. Furthermore, the washing steps eliminate residual sedimentation agents, therefore avoiding potential side effects from these agents.

Compared with sGC, the purification method led to significantly higher removal of RBC ($p < .001$), significantly higher removal of platelets ($p < .05$), and recovery of 57.37% (44.01–81.29) of granulocytes. Granulocyte function was maintained and granulocyte viability and oxidative burst function were improved, thus optimizing quality.

The lower RBC and platelet count in the pGC result in much less lactate production during storage, and therefore, the pH is more stable than in the sGC, supporting longer storage ability. The pH stability also results from the use of optimal storage bags releasing carbon dioxide produced by the cells.

Furthermore, glucose in the sGC is below the physiologic concentration (reference range 3.9 to 5.5 mmol/L),[30] limiting cell functions. In contrast, in pGC, the glucose level is initially higher and, although declining gradually over time, remains above the minimal physiologic level, even after 72 h of storage. In line with this, Glasser et al. reported neutrophil functions to be adversely affected due to low glucose levels in the surrounding medium.[32]

The extended storage ability of RBC-reduced GC has also been reported for pooled GC from buffy coats.[33] However, although phagocytosis was well preserved, oxidative burst was reduced to 80% without, and 69% with, irradiation after 68 h; in contrast, these properties were well preserved for 72 h in our pGC preparations.

While the implementation of the new pGC protocol promotes neutrophil viability and essential cellular functions for up to 72 h, no comparative data are shown for sGCs for storage up to 72 h. However, the functional parameters of granulocytes in pGC were better preserved for 72 h than those in sGC after 24 h of storage. Conventional storage of sGC is not recommended for more than 24 h. Since the stimulation of donors with G-CSF ethically requires the genuine use of the GC, we applied all sGC either for the preparation of pGC or for other scientific objectives within 24 h. Hence, the storage of sGC beyond 24 h remained unexamined.

Granulocyte viability is of paramount importance during the development of this product. Viability assessment was performed using the NucleoCounter, an instrument that does not distinguish between the different subpopulations of the WBC. However, in the pGC, approximately 96% of WBCs were neutrophilic granulocytes. Thus, WBC viability provides a sufficiently accurate approximation of leukocyte viability.

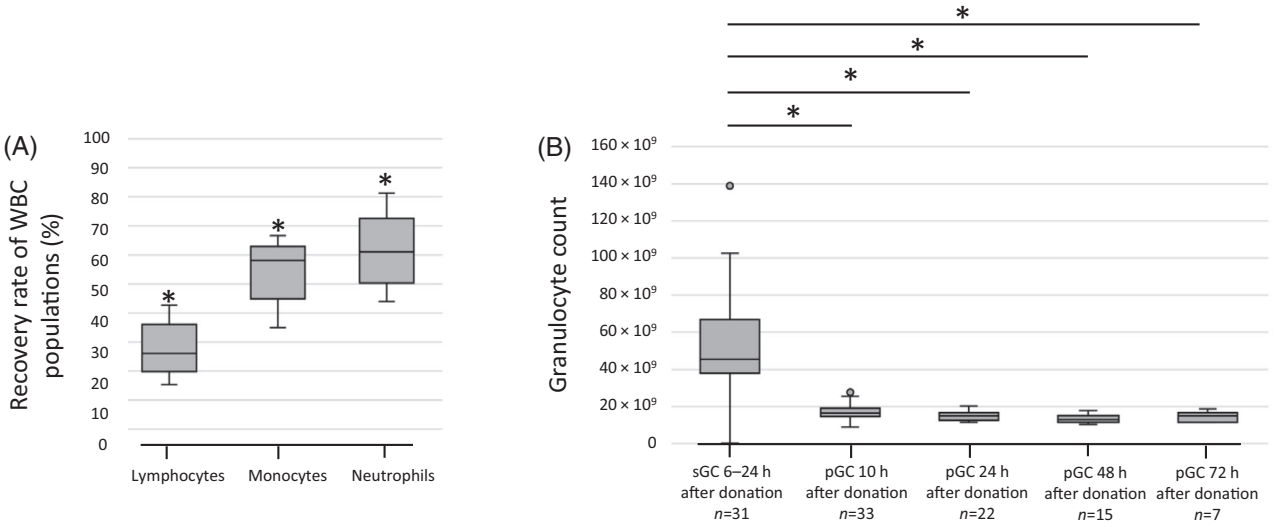

FIGURE 5 sGC and pGC white blood cell (WBC) recovery rates and granulocyte counts during storage. (A) WBC recovery rates: The purified granulocyte concentrates contained 57.37% (44.01–81.29) of the granulocytes and 55.11% (34.99–66.67) of the monocytes of the untreated concentrates. In contrast, only 24.16% (15.38–42.71) of the lymphocytes remained. (B) Granulocyte count: sGCs usually are not specified regarding a maximum granulocyte count. For the pGC, a tighter range in granulocyte count (e.g., between 1x and 2.5 x 10^{10} granulocytes per pGC unit) in order to better compare the individual treatments by more homogenous dosing. A comparatively reproducible and narrower range between 1× and 2.5 × 10^{10} granulocytes in pGC can be achieved in pGC. The granulocyte count is largely maintained over 72 h of storage and does not fall below 1 × 10^{10} cells. p values ≤.05 (+) and ≤.01 (*) were considered significant

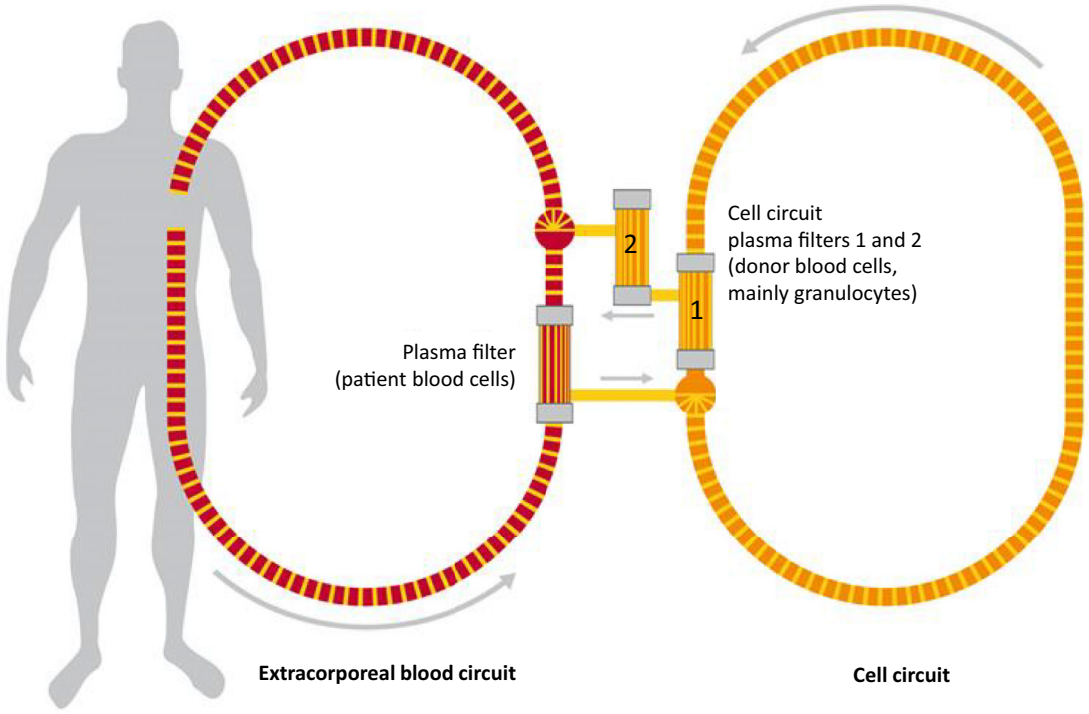

FIGURE 6 Diagram illustrating extracorporeal immune cell therapy using pGC. Plasma from patients with sepsis is perfused through the tubing and housing with the donor immune cells of the pGC and only the treated plasma is reinfused online into the patient. The donor immune cells are retained in the extracorporeal system and discarded after the treatment [Color figure can be viewed at wileyonlinelibrary.com]

The recovery rates were found to be highly variable. In fact, this could be based on the different initial cell counts of the sGC and as well as their composition with respect to the leukocyte subpopulation. Especially if the lymphocyte population differs, the purification result varies since mainly the lymphocytes are lost during purification.

The purification and storage procedure developed and presented here can be carried out with all necessary steps in a closed bag system with comparatively low time and cost expenditure.

Furthermore, the very low RBC content in pGC (less than 5×10^9, corresponding to less than 0.5 ml packed RBC) does not permit reliable ABO compatibility testing. AABB guidelines waive the ABO testing requirement, for example, thrombocyte concentrates when the total RBC volume is less than 2 ml.[22] This may allow ABO-incompatible transfusions, thus considerably expanding the pool of potential donors. This is consistent with the work of Bryant et al. who transfused similar ABO-incompatible preparations (sedimented but not washed and stored) with even higher RBC content with no transfusion reactions or signs of hemolysis after the transfusion of 66 ABO-incompatible GCs (RBC volume, 1.6–8.2 ml).[34]

GCs for transfusion are required to be irradiated with 30 Gy because viable lymphocytes can cause fatal transfusion-associated graft versus host (GvH) disease, particularly in severely immunocompromised patients.[22] Literature from the 1980s showed only minor reduction in granulocyte function, for example, in reduced chemotaxis, while massive reduction in lymphocyte proliferation leads to the compromise of radiation for GCs to avoid GvH reaction.[35] However, the negative effect on granulocytes is not negligible and has been even shown also for other granulocyte functions like oxidative burst.[36] We have used GC in a strictly extracorporeal mode, especially in patients with advanced stages of sepsis or septic shock.[21] This bedside immune cell perfusion therapy consists of a plasma-separating device and an extracorporeal circuit containing sGC/pGC (Figure 6). Because all immune cells are fully retained extracorporeally and not transfused into the patient, there is no need for irradiation to avoid GvH reactions. The avoidance of irradiation not only better preserves granulocyte function[37,38] but also results in the preservation of the immunomodulatory function of the lymphocytes in the extracorporeal application, further extending the potential use of GC.

In Conclusion, the inherent merits of this new purification method involve extending the maximum storage time of GC from 24 h to 3 days, thus improving GC availability. However, the clinical value of this procedure remains to be elucidated.

The main limitations for extensive use of GCs are the very short storage period combined with the donor-specific blood group compatible manufacturing and transfusion leading to a time lag of at least 1 day between decision and transfusion due to donor selection and donor stimulation. A combination of increased storage time, less cell number variation, and ABO independency has the potential to provide pGCs as off the shelf products allowing a precise adaptation to the individual requirements of the patient. Furthermore, the diminished variations in cell concentration are of great relevance for the use of the pGC in the context of extracorporeal treatment.

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CONFLICT OF INTEREST

Fanny Doss, Sandra Doss, Magdalena Milej, Steffen Mitzner, and Jens Altrichter are employees or shareholders of ARTCLINE GmbH. All others have no conflict of interest.

ORCID

Gerd Klinkmann https://orcid.org/0000-0002-8922-8700

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Purified Granulocyte Concentrates from Buffy Coats with Extended Storage Time

Gerd Klinkmann[a, b] Fanny Doss[c] Sandra Doss[b, c] Antje Schwarz[d, e] Susanne Reichert[d] Daniel A. Reuter[a] Kathleen Selleng[f] Thomas Thiele[g] Steffen Mitzner[d, b] Jens Altrichter[c]

[a]Department of Anesthesiology, Intensive Care Medicine and Pain Therapy, University Medical Center, Rostock, Germany; [b]Department of Extracorporeal Immunomodulation, Fraunhofer Institute for Cell Therapy and Immunology, Rostock, Germany; [c]Artcline GmbH, Rostock, Germany; [d]Department of Medicine, Division of Nephrology, University Medical Center, Rostock, Germany; [e]Institute of Transfusion Medicine, University Medicine Greifswald, Greifswald, Germany; [f]Institute for Transfusion Medicine, University Medical Center Rostock, Rostock, Germany; [g]Labormedizinisches Versorgungszentrum Westmecklenburg GmbH, Schwerin, Germany

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Keywords
Granulocyte concentrate · Buffy coat · Transfusion medicine · Transfusion

Abstract

Background: Granulocyte concentrates (GCs) are usually prepared by single-donor apheresis after G-CSF pretreatment and have to be transfused within 24 h after cell collection because of the rapid decrease in pH and cell survival due to high lactate production by red blood cell contamination. GCs pooled from buffy coats of whole blood donations could improve the availability of these products. Methods to reduce red blood cell and platelet contamination may improve storability. We developed a manufacturing process for pooled GCs and investigated cell viability and functionality over time. ***Methods:*** Six ABO blood group-identical buffy coats were pooled. Subsequently, the red blood cells spontaneously sedimented after the addition of hydroxyethyl starch. The resulting leukocyte-enriched supernatant was washed twice with saline to reduce platelets and was resuspended in ABO-identical donor plasma. The leukocyte concentrate was transferred to a platelet storage bag and stored up to 72 h at 20–24°C w/o agitation. Cell count and viability, pH, blood gases, phagocytosis, and oxidative burst activity were monitored. ***Results:*** The number of red blood cells and platelets was reduced to 0.4% and 6.1% of the baseline levels. About 50% of the original present leukocytes could be extracted (n = 76). In the course of 72 h of storage, there were no significant changes in white blood cell counts (p = 0.12). The viability exceeded 98% during the entire period. The rate of granulocytes performing phagocytosis and oxidative burst remained above 95% anytime. ***Conclusion:*** GCs prepared from pooled buffy coats provide a precious alternative to granulocytes obtained from apheresis. Reduction of red blood cells and platelets by more than 90% extends the maximum shelf life of GCs from 24 h to 72 h. For a therapeutic dose of at least 1×10^{10} granulocytes, 15–20 buffy coats are required.

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Introduction

Bacterial and viral infections, especially sepsis, account for 20% of all deaths and are thus among the major causes of mortality worldwide [1]. Neutrophil granulocytes play

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Correspondence to:
Gerd Klinkmann, gerd.klinkmann@uni-rostock.de

an important role in fighting bacterial and fungal infections, and diseases associated with decreased neutrophil counts increase the risk of systemic infections [2]. Patients suffering from sepsis, especially septic shock, develop a state of immune paralysis associated with increased mortality due to various cellular dysfunctions [3, 4] such as phagocytosis and clearance of bacteria by granulocytes [5–9] and macrophages [10], impaired antigen presentation, lymphopenia, and impaired T-cell activation [11, 12].

Transfusion of granulocyte concentrates (GCs) can adjunctively support the treatment of patients with sepsis, life-threatening neutropenia, or neutrophil dysfunction [13, 14]. However, although GCs have been transfused for several decades for this indication, their efficacy has not been well substantiated [2]. Furthermore, an extension of the range of indications for GCs to the area of intensive care medicine has already been approached [15]. In particular, a GC has been used in an extracorporeal therapy system for the treatment of sepsis-associated immune paralysis based on standard and purified GCs from apheresis. The extracorporeal circuit prevents cellular components from exposing the patient while harnessing the absorptive and immunomodulatory benefits of immune cells to regain immune homeostasis [16, 17].

GCs are produced by two approaches: (i) by apheresis or (ii) from a pool of buffy coats (BCs) from whole blood donations [18, 19]. Currently, in Germany, collection of GCs by apheresis is the standard approach. However, there are several disadvantages inherent to the collection and processing of GCs by apheresis [20–22]. First, donors require preconditioning with G-CSF and/or steroids before apheresis to mobilize a high number of granulocytes into the peripheral blood, which may cause adverse reactions [21, 23]. Further, preconditioning may impose a time delay of several days between an urgent request for GCs and their release in most cases. Finally, throughout granulocyte apheresis, hydroxyethyl starch (HES) (or gelatin) needs to be added directly into the cell collection system to increase the separation efficacy for granulocytes and decrease the number of red blood cells (RBCs) and platelets (PLTs) in the final product, which adversely affects the storage duration and functionality of the GCs. HES or gelatin is to some extent infused into the donor during apheresis and can induce adverse reactions in apheresis donors.

In contrast, BCs derived from whole blood donations can serve as an alternative source of granulocytes. BCs are available in every blood donation center and do not require preconditioning of donors, and GCs can be produced in large quantities, enabling rapid release in case of urgent demand [24]. In practice, however, BCs are predominantly used to prepare pooled PLT concentrates because they contain high amounts of PLTs [18], which could be a disadvantage for GC production.

Transfusion guidelines recommend that one unit of GC should contain at least 1×10^{10} granulocytes. However, higher numbers have been shown to be beneficial [25–27]. GC storage is crucial for this process. GCs have a limited shelf life and should be stored at a temperature of 20–24°C for no longer than 24 h and preferentially transfused as soon as possible after donation [26–28]. Throughout storage, apoptosis suppresses neutrophil activity, reduces neutrophil numbers, and leads to morphological changes, all of which are undesired.

Therefore, we designed a leukocyte preparation approach for GCs from pooled BCs in accordance with good manufacturing practice guidelines to enrich granulocytes and substantially remove RBCs and PLTs. The quality of these GCs was evaluated by investigation of cell counts, granulocyte viability, and cell functionality up to 72 h of storage.

Materials and Methods

Whole Blood Donation and Preparation

Whole blood donations were collected after informed consent was obtained from the donors. Each donor fulfilled the applicable donor suitability criteria of the national and European guidelines for hemotherapy [26, 28, 29]. Whole blood (450–500 mL, containing CPD as an anticoagulant) was processed according to the manufacturing license of the blood donation service (4,000 g, 10 min, 20–24°C). Plasma, BCs, and RBCs were separated by a plasma expresser (Macopharma, Langen, Germany). BCs (62–82 mL) were stored recumbently without agitation at room temperature until processing.

BC-Derived Granulocyte Purification Procedure

All bag and tubing connections were done by sterile techniques according to industrial standards (Sterile tubing welder TSCD-II, Terumo, Leuven, Belgium). Six BCs from ABO-identical donors were connected in a row.

After the bags were massaged gently to detach the adherent leukocytes from the inner wall of the bag, the thoroughly mixed contents of these BCs were pooled together in the last BC bag of the row (pool bag). The clamps between the BC bags were closed to prevent reflux.

HES (500 mL, Infukoll HES 6% 200/0.5 KS; Serumwerk, Bernburg, Germany) was filled into a transfer bag (Compoflex; Fresenius Kabi, Bad Homburg, Germany). The HES bag was welded to the first empty BC bag of the chain, and about 50 mL of HES was transferred to the BC bag, which was rinsed thoroughly by panning and manual massage.

This step was repeated with the following BC bags by opening the tube passage, filling the next bag with the 50-mL HES cell suspension, and then closing the tube connection with a clamp to prevent reflux. Finally, the HES solution was passed into the pool bag. This operation, called "washing of the BC-bags," was performed a second time. The pool bag was then welded from the BC chain and connected directly to the HES bag. The pool bag was filled with HES and all air bubbles were removed. Using this procedure, a total of 416 ± 13 mL BC was mixed with about 258 ± 30 mL HES.

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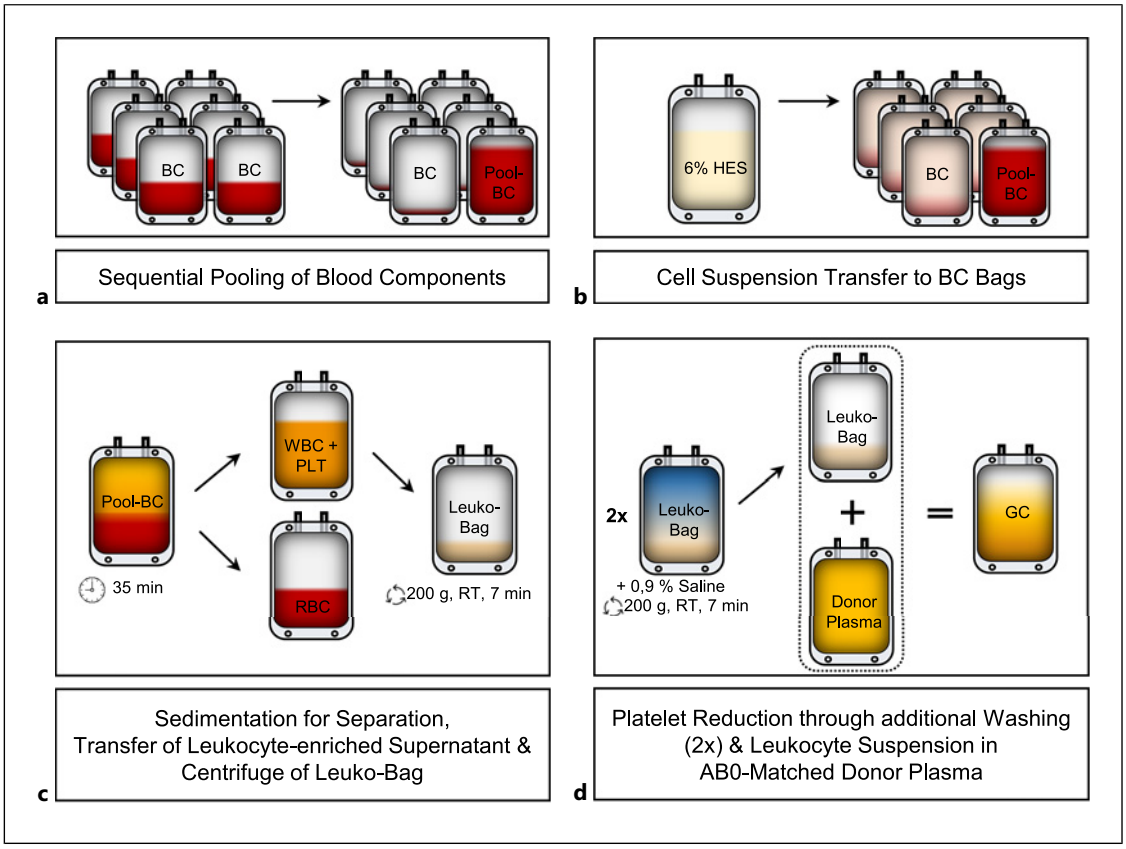

Fig. 1. a–d Manufacturing process. Schematic overview of the manufacturing process.

The pool bag was then connected to a transfer bag (Compoflex), keeping the connection between the bags closed initially. The unit was hung up with the connecting tube at the top and left undisturbed for 35 min at 22 ± 2°C, allowing the cells to sediment by gravity and to develop a sharp line of demarcation between the sedimented RBCs and the leukocyte-enriched supernatant. The pool bag was carefully placed in a manual plasma expresser (Medeiros, Rostock, Germany) to transfer the leukocyte-enriched supernatant to the empty transfer bag ("leuko-bag") after the tube connection was opened. RBC sediment was discarded.

The leuko-bag was connected to an empty bag (e.g., a bag of washed BC) while the connection between the bags remained closed. The bags were then centrifuged at 200 *g* at 22 ± 2°C for 7 min (Centrifuge Roto Silenta RS, Hettich, Tuttlingen, Germany), resulting in a sediment of leukocytes and a leukocyte-enriched supernatant containing plasma, HES, and most of the PLTs. Using a plasma expresser, the leukocyte-enriched supernatant was transferred to an empty bag, which was disconnected and discarded.

Subsequently, two washing steps were performed for PLT reduction. The leuko-bag was connected to a bag of 0.9% saline (1,000 mL Macoperf, Macopharma, Langen, Germany). 30 mL of saline was added to the leuko-bag to resuspend the cell sediment prior to filling the leuko-bag with further saline to a total weight of 350 g. Subsequently, the bag was centrifuged (200 *g*, 7 min, 22 ± 2°C) and the washing solution was pressed into an empty bag and discarded. This washing process was repeated once. Finally, the leukocytes were resuspended in 200 mL of ABO-matched donor plasma from one of the BC donors.

The resulting GC was transferred to a gas-permeable PLT storage bag (CompoStop F730; Fresenius Kabi, Bad Homburg, Germany), and the bubbles were removed. GCs were stored flat at RT in a gas-permeable bag without agitation for 72 h. Figure 1 provides a schematic overview of the manufacturing process.

Sampling

After 24, 48, and 72 h, the leukocyte concentrates have been mixed, and after discarding the first milliliter (content of the tube end) about 2 mL of the sample was taken for analysis.

Measurement of Blood Cell Counts and White Blood Cell Viability

Residual PLTs, RBCs, and white blood cells (WBCs), including their differentiation, were evaluated automatically using a hematology analyzer (KX-21N, Sysmex, Norderstedt, Germany). Viability of leukocytes was determined by trypan blue exclusion test.

Evaluation of Granulocyte Function

For functional analysis, the leukocyte concentration was adjusted to 5×10^6 leukocytes/mL by adding AB plasma. Granulocyte function was analyzed in vitro with OxyBURST and phagocytosis assays using commercial Phagoburst-Kit and Phagotest-Kit (Celonic, Heidelberg, Germany), respectively.

Both tests were designed for whole blood and were used according to the manufacturer's instructions after adaptation of the cell concentration because the granulocyte concentration in a GC is approximately 10 times higher than that in whole blood. To achieve a concentration of 5,000 granulocytes/μL and the same ratio of granulocytes to the stimulus (e.g., *Escherichia coli*) as in

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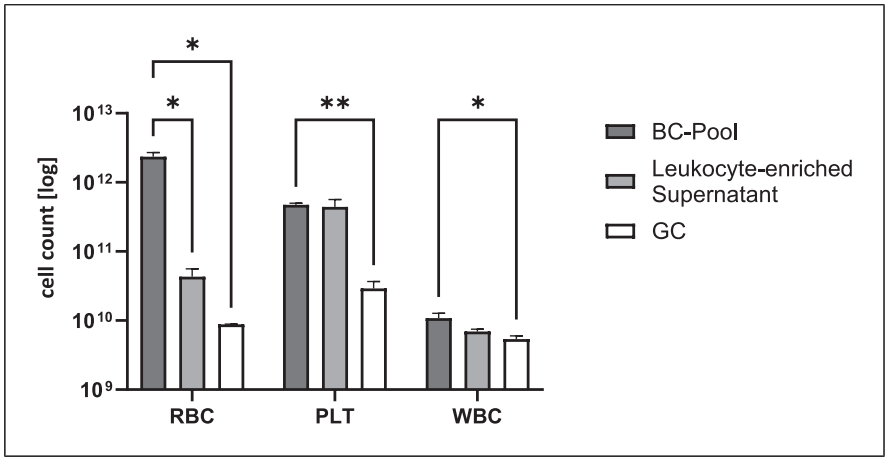

Fig. 2. Distribution of cell types during the production steps. Red blood cell (RBC), platelet (PLT), and white blood cell (WBC) counts in pooled buffy coats (BC pool), leukocyte-enriched supernatant, and granulocyte concentrate (GC) preparations. p values ≤0.05 (*) and ≤0.01 (**) were considered significant. N = 53.

heparin-anticoagulated whole blood (4,000–10,000 granulocytes/μL), the samples were diluted in heparin-anticoagulated AB plasma.

Measurement of Electrolytes, pH, Glucose, Oxygen, and Carbon Dioxide Partial Pressures

pH, glucose, oxygen, and carbon dioxide partial pressures were measured using an ABL77 blood gas analyzer (Radiometer, Krefeld, Germany).

Measurement of Lactate Concentration and Lactate Dehydrogenase Activity

Lactate concentration and lactate dehydrogenase (LDH) activity were measured using Cobas Mira Plus CC (Roche, Ludwigsburg, Germany).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (version 27, Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software Inc., San Diego, USA). Determination of the corresponding parameters was carried out in the BC pool, in the leukocyte-enriched supernatant, in the GC after preparation, and during storage up to 72 h. The results are expressed as mean ± standard deviation. The analytical evaluation was performed using univariate single-factor analysis of variance (ANOVA). If the F test was significant, the Fisher/least significant difference test was performed. According to the distribution of data (using the Shapiro-Wilk test), the Wilcoxon test was used for two dependent samples for continuous variables. The Kruskal-Wallis test was used to test the difference between multiple independent samples with the non-normal underlying population distribution, and appropriate post hoc tests were applied if necessary. The Friedman test was used when data arose from more than two related samples. Statistical significance was set at $p < 0.05$.

Results

Reduction of Contaminating Cells during Production Steps

A total of 76 BCs were used for all experiments. For each experiment, five to six BC bags from donors of the same blood type were combined, resulting in a total volume of 416 ± 13 mL for each BC pool. A total volume of 258 ± 30 mL HES 200 per BC pool was added sterilely. After 35 min sedimentation, in the leukocyte-enriched supernatant the number of RBCs was $4.31 \times 10^{10} \pm 1.29 \times 10^{10}$ compared to $2.36 \times 10^{12} \pm 0.33 \times 10^{12}$ in the BC pool, the number of PLTs $4.41 \times 10^{11} \pm 1.24 \times 10^{11}$ versus $4.73 \times 10^{11} \pm 0.25 \times 10^{11}$ in the BC pool, and the number of WBCs $6.97 \times 10^{9} \pm 0.53 \times 10^{9}$ versus $1.08 \times 10^{10} \pm 0.19 \times 10^{10}$ in the BC pool (Fig. 2). RBCs were reduced by more than 98% ($p = 0.012$), but PLTs only by 7% ($p = 0.89$). Two subsequent washing steps reduced the RBC content to 0.4% and the PLT content to 6.1% of the original RBC and PLT concentrations, respectively ($p = 0.012$ for RBC; $p = 0.001$ for PLT). However, the yield of WBCs was $5.38 \times 10^{9} \pm 0.62 \times 10^{9}$ cells/pool, which corresponds to 49.8% of the initial leukocyte count in the BC pool (Fig. 2). Finally, the leukocytes were resuspended in 200 mL of ABO-matched donor plasma, resulting in a total volume of 230 ± 25 mL per unit. During subsequent storage, no change in the total WBC content nor in one of the major WBC subsets was observed (Fig. 3).

Granulocyte Viability and Function

The percentage of granulocytes that performed phagocytosis of FITC-labeled *E. coli* accounted for 96.9 ± 2.1% in the BC pool and remained >90% at all time points (72 h: 97.3 ± 0.9%) (Fig. 4a). The percentage of granulocytes that performed *E. coli*-induced oxidative burst was 94.6 ± 2.7% in the BC and remained stable until 72 h of storage (95.1 ± 3.1%) (Fig. 4b). Viability was 99.8 ± 0.2% in the BC pool and showed a consistent trajectory for up to 72 h (98.7 ± 0.2%) for the whole observation period (Fig. 4c).

Glucose Consumption and Lactate Generation

Glucose levels in BCs as well as in the GC after preparation were measured: 19.1 ± 1.2 mmol/L and 20.8 ± 2.2 mmol/L, respectively. During storage, glucose levels decreased to 18.6 ± 1.1 mmol/L after 72 h ($p = 0.015$)

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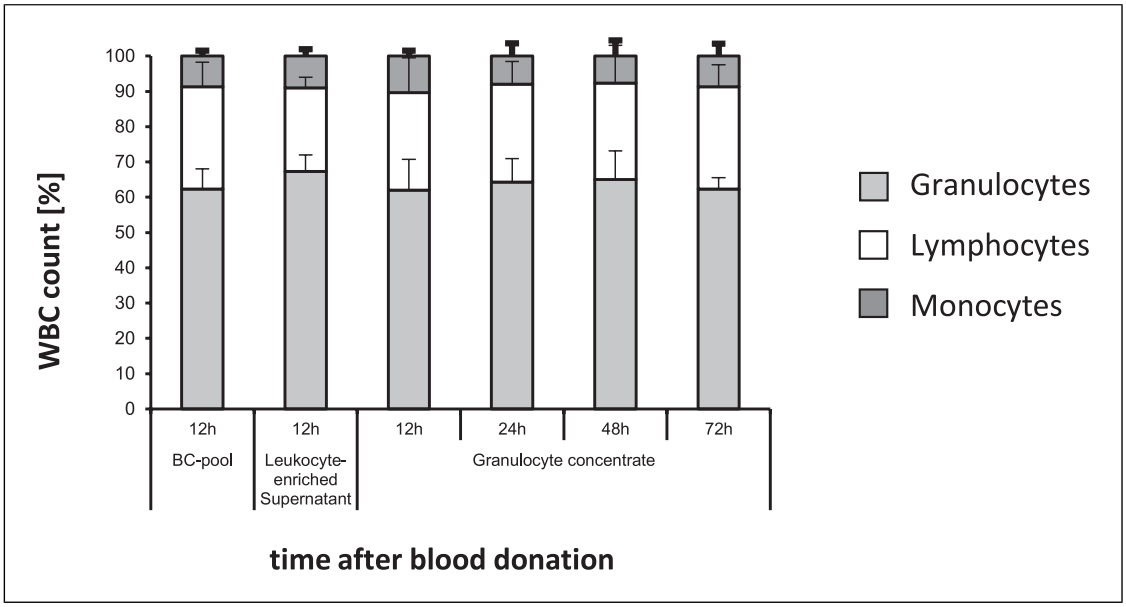

Fig. 3. Quantification of WBC subpopulations. Percentage distribution of WBC subpopulation contained in each preparation; Leukocyte concentrate during storage until 72 h at room temperature (N = 23); no difference between WBC populations.

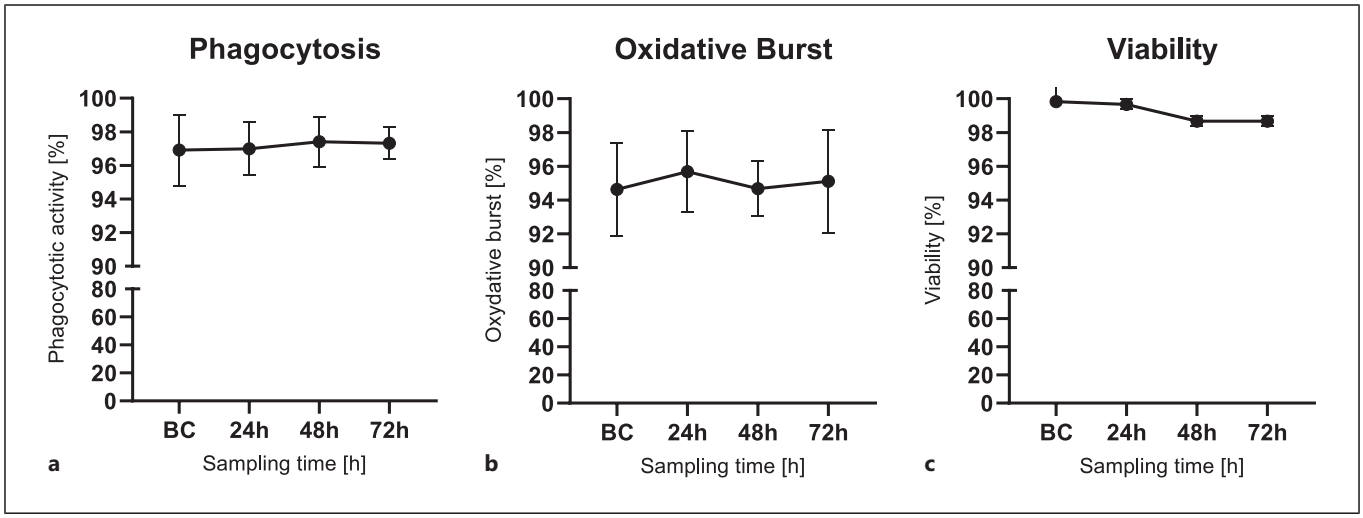

Fig. 4. Functional granulocyte activity of purified GC. Functional granulocyte activity of purified GC at defined observation time points. **a** Phagocytic activity. **b** Oxidative burst activity. **c** Viability. No statistically significant differences in cellular functionality were detected during the study period of 72 h of storage.

(Fig. 5a). The significant changes over the storage period are of minor clinical importance. In the BCs, lactate levels were 5.5 ± 1.5 mmol/L. In the fresh GC, lactate levels equaled 4.7 ± 1.1 mmol/L and increased during storage to 7.2 ± 0.7 mmol/L after 72 h (p = 0.010) (Fig. 5b).

pH Values

At baseline, the mean pH of the GC was 7.2 ± 0.07 immediately after manufacturing and showed a significant increase after 24 h up to 7.5 ± 0.06 and stable values up to 72 h of storage (7.5 ± 0.12; p = 0.009) (Fig. 5c).

Impact on LDH Activity

Figure 5d shows LDH activity as an indicator of cell damage, which in turn allowed conclusions to be drawn on the viability of the granulocytes. LDH values were within the physiological range (<225 U/L) and were stable during the experiment (Fig. 5d).

Discussion

This study describes a method for preparing GCs from whole blood donation-derived BCs. These GCs contain less than 1% of RBCs and less than 6% of PLTs

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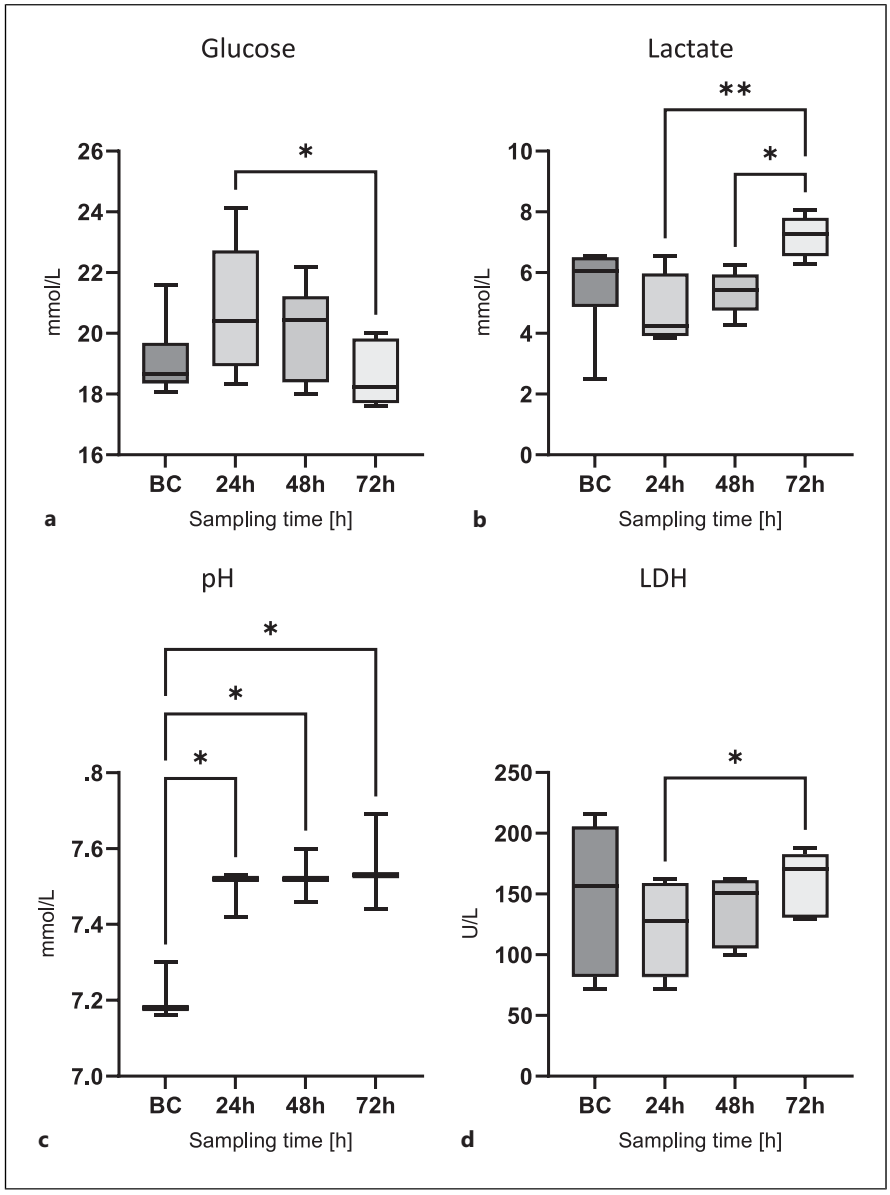

Fig. 5. Cellular metabolism: pH, glucose, lactate, and LDH activity. **a** Glucose concentration. **b** Lactate concentration. **c** pH value. **d** LDH activity was analyzed as an indication for cell damage. Compared to the reference value (<225 U/L), measured values are within the physiologic range and stable during the experiments. Therefore, no relevant cell damage was observed. *p* values ≤0.05 (*) and ≤0.01 (**) were considered significant.

compared to pooled BCs, while ~50% of leukocytes remain in the product. The final preparation contains approximately 3.4×10^9 granulocytes in 200 mL of ABO-compatible plasma, which can be stored at a temperature of 20–24°C for at least 72 h without significant loss of cellular function. In particular, the oxidative burst and phagocytosis ability remained above 90% of the baseline values of the fresh GC, even after 72 h of storage. The processing and storage procedure complies with the current GMP guidelines in a closed bag system with comparatively low time and cost expenditures. We provide a proof-of-principle concept that BCs could be used as an alternative to apheresis-derived GCs as a way to overcome potentially unacceptable risks for apheresis donors.

Regarding the capacity of the bags, an optimal yield could be achieved with pools of six BCs. Since a dose of at least 1×10^{10} granulocytes is required for clinically meaningful use, approximately 15–20 BCs would need to be pooled [29].

In Germany in 2021, about 3.7 million whole blood donations have been performed, of which about 1.1 million BCs are used for the preparation of 275,000 pooled PLT productions [30]. Approx. 1–2 million BCs are discarded each year in Germany and could be used for purified pooled GCs. However, we see the need for improvements in granulocyte purification to open up the perspective to produce purified pooled GCs by single blood donation centers. The quantitative dimensions of these results warrant a more detailed discussion. However, the optimal dosing of BC-derived GCs needs to be addressed in clinical studies. Further refinement of the described methodology and additional clinical research is imperative

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to advance our understanding and optimize the application of neutrophil transfusions in medical practice.

An important aspect to consider is the tendency of granulocytes to adhere to extraneous material [31–33]. This may explain why, after the initial sampling from the BC bags, the leukocytes in the leukocyte-enriched supernatant were dominated by the lymphocyte number. To prepare the samples, the granulocytes adhering to the bag surface were solubilized by rinsing the BC bags with the HES solution. Thus, a granulocyte content of approximately 70% in the leukocyte-enriched supernatant could be obtained. This will be a source of optimization to reduce granulocyte loss through this phenomenon.

In 2008, Bashir's method involved pooling 10 BCs, adding a PLT additive solution, centrifuging the mixture, and transferring the granulocyte-rich layer to a PLT storage bag with plasma addition. Our method applied in the present study resulted in a product with a lower hematocrit of 0.3% compared to Bashir's method, which obtained a hematocrit of 21%. In addition, our method resulted in a tenfold lower PLT content than Bashir's method. After 72 h of storage, our method resulted in a higher percentage of vital granulocytes, with 98.7% being viable in the final product compared to 84% for Bashir's method [18].

The number of neutrophils in the final product was higher in Bashir's method with 8.8×10^9 compared to 3.4×10^9 extracted from the leukocyte preparation in our method, whereas we used fewer BCs for the manufacturing process. Moreover, our method showed a higher rate of phagocytic activity by granulocytes, with a constant rate of 97%, whereas phagocytosis decreased from 94 to 92% in the Bashir method. In addition, our method showed a higher rate of reactive oxygen species production by granulocytes, with a constant rate of 95%, whereas oxidative burst activity decreased from 87 to 81% in the Bashir method [18].

Another innovation of the method presented here relates to the washing of leukocytes, which serves the purpose of PLT depletion, as well as HES removal. HES removal was intended to avoid additional stimulation of leukocytes and to minimize the risk of allergic reactions and other side effects of HES [34]. PLT depletion is necessary for better storage of leukocyte suspensions because PLTs should be stored with uniform agitation [28, 35], whereas resting storage is recommended for granulocytes [28]. Keating et al. [36] also reported the formation of PLT-leukocyte aggregates during storage of non-leukocyte-depleted RBC concentrates. These aggregates appear to be associated with increased leukocyte apoptosis and procoagulant activity. Although these processes are preceded by PLT activation, and HES 200 is thought to have a more inhibitory effect on PLT function [37], the risk of PLT-leukocyte aggregate formation should be circumvented by depleting PLTs. The very low RBC content in our purified preparations (less than 5×10^9, which corresponds to less than 0.5 mL of packed RBCs) precludes meaningful ABO compatibility testing. This was confirmed by the AABB guidelines. Herein, for example, ABO testing is waived for PLT concentrates if the total RBC volume is less than 2 mL [27]. This may allow ABO-independent transfusions and thus significantly expand the pool of potential donors. This is also consistent with the work of Bryant et al. who transfused 66 ABO-incompatible GCs (RBC volume 1.6–8.2 mL) with even higher RBC content, with no post-transfusion reactions or signs of hemolysis [38].

We show that 72 h of storage is feasible for BC-derived GCs. Schwanke et al. [39, 40] investigated granulocyte function after 72 h of storage in standard GC obtained by apheresis. These were tested either undiluted or diluted 1:4 in autologous plasma. In the undiluted preparation, no oxidative burst and only 15% phagocytic activity were detectable after 72 h. Similar to our results, 1:4 dilution with plasma showed only a slight decrease in oxidative burst and phagocytosis activities after 72 h. In another study by Schwanke et al. [39, 40], similar results were obtained for GCs diluted 1:8 with autologous plasma. Mochizuki et al. [41] investigated the influence of donor pretreatment on granulocyte function after 72 h of storage using the bag separation method. An untreated control sample, composed of a BC, was used for comparison. During storage (resting, RT), there was a successive loss of 10% of granulocytes, while in our observation, counts remained consistent, with similar viability (Mochizuki et al. [41]: 96 vs. Klinkmann et al. [15]: 99%). Oxidative burst was stable at a high level, similar to our findings, whereas our cells showed a significantly higher phagocytosis rate (Mochizuki et al. [41]: 83 vs. Klinkmann et al. [15]: 97%). Using another innovative purification concept in which standard GC was collected by granulocytapheresis, we succeeded in developing a closed system procedure compatible with standard blood bank technologies to remove 98% of RBCs and 96% of PLTs and enrich the GC. Purification extends the maximum storage time of purified GCs from 24 to 72 h with high viability and functionality [15].

The range of indications for GCs can be extended to the field of intensive care medicine, particularly extracorporeal therapy of sepsis-associated immune paralysis, using purified BCs. The use of leukocytes to treat sepsis in an extracorporeal setting was proposed by Mitzner et al. [42]. Regarding cellular immunocompetence, functional impairment of neutrophils and monocytes and lymphopenia have been linked to increased mortality in advanced stages of sepsis [4–12, 43, 44]. The use of donor granulocytes to treat critically ill sepsis patients in an

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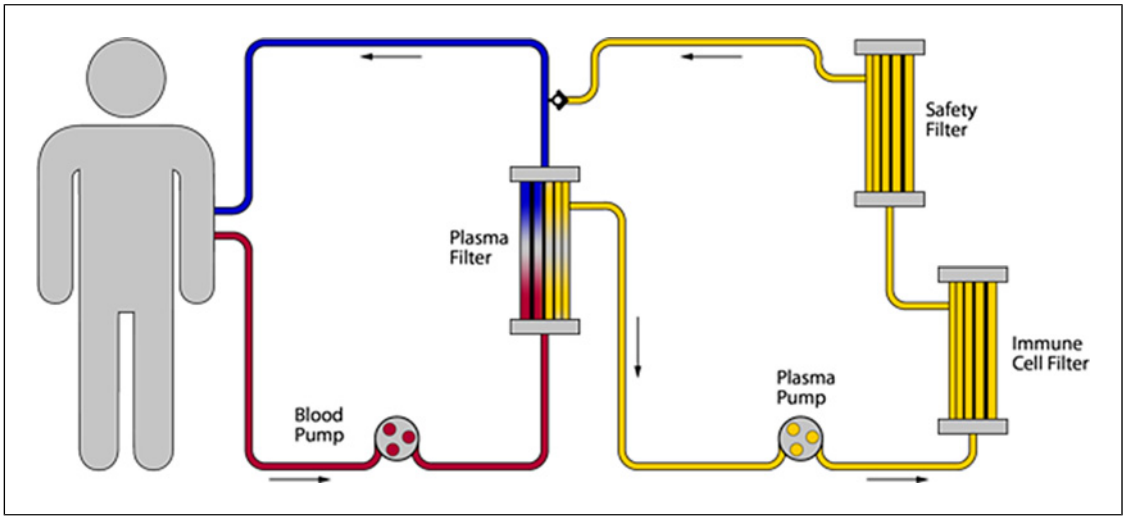

Fig. 6. Extracorporeal immune cell therapy. Extracorporeal immune cell therapy is a plasma treatment technology. Plasma is continuously filtered from the patient's extracorporeal bloodstream and transferred to a closed "cell circuit" where the patient's plasma is brought into direct contact with therapeutically active immune cells from a human donor (i.e., the GC). The schematic illustrates the optimized "one-way" immune cell perfusion method using purified GCs. The plasma filter CC2 serves as a redundant safety filter.

extracorporeal setting have been investigated both preclinically and in pilot clinical trials [45–49]. This therapeutic approach has already yielded promising results for its potential improvement of established sepsis therapies [47, 48]. As such, the immune cell perfusion therapy consists of a plasma separation unit and an extracorporeal circuit containing purified GCs (Fig. 6). Because all immune cells are fully retained extracorporeally and not transfused into the patient, irradiation is not required to prevent GvH reactions. Avoiding irradiation may better preserve granulocyte function [50, 51] and immunomodulatory function of lymphocytes in extracorporeal applications. However, Schwanke et al.'s [39] findings suggest the preservation of neutrophil function in irradiated plasma-diluted preparations stored for 72 h, prompting consideration for initial and late irradiation in cases where purified preparations are intended for direct transfusion. No radiation tests were carried out during the development of our method, so further studies are needed to evaluate the clinical use of the purified preparations.

The development of the extracorporeal therapy of sepsis-associated immune paralysis using purified BCs culminated in the initiation of an RCT (NCT05442710), which began in July 2022 and is currently actively enrolling patients. Purified GCs from granulocytapheresis [15] at a dose of $1.2–2.5 \times 10^{10}$ granulocytes per day are being used clinically in this extracorporeal immune cell plasma perfusion therapy.

This is a striking example demonstrating the potential for further clinical expansion of the indications for the use of GCs. To create infrastructural conditions for clinical use, it is essential to make the best possible use of the various sources, in addition to the quality of the preparations.

Conclusion

To date, the clinical applicability and usability of GCs have been limited owing to the rapid deterioration in quality caused by an autolytic process of the granulocytes and high levels of lactate production from large amounts of RBCs. This led to a low pH within the first 24 h. However, a new purification process has been developed to remove impurities, such as RBCs, thrombocytes, and sedimentation agents, resulting in better storage conditions for leukocytes and fewer side effects for recipients. This purification process increases the shelf life of GCs, while maintaining cell functionality, making it more usable in clinical practice. Additionally, the reduced RBC content could allow for the ABO-independent use of purified GCs, enabling the immediate use of GCs without relying on a specific recipient.

Overall, the development of a closed blood bag system and the production of GCs from pooled BCs have significant potential to solve the problem of storage of cellular blood products and could have important implications for the treatment of sepsis. The preceding quantitative investigation highlights the necessity for a thorough investigation in this domain. It is crucial to enhance our comprehension and refine the methodology, alongside continuous clinical research, to optimize the

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utilization of neutrophil transfusions. Subsequent research endeavors are warranted to substantiate the efficacy of GC therapy in diverse clinical settings.

Statement of Ethics

Whole blood donations were collected after informed consent was obtained from the donors. Each donor fulfilled the applicable donor suitability criteria of the national and European guidelines for hemotherapy. The study was approved by the Local Ethics Committee of the University of Greifswald (Reg. No.: BB014/14).

Conflict of Interest Statement

Fanny Doss, Sandra Doss, Steffen Mitzner, and Jens Altrichter are employees or shareholders of Artcline GmbH. Kathleen Selleng received personal fees from Aspen Germany, travel support from Sobi, and research funding from Immucor and Grifols, outside the submitted work. All others have no conflict of interest to declare.

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Author Contributions

Gerd Klinkmann, Fanny Doss, Jens Altrichter, and Steffen Mitzner: concept, data collection, data interpretation, and drafting and critical revision of this article. Antje Schwarz, Susanne Reichert, and Kathleen Selleng: GC preparation, performance of the in vitro experiments, data collection, data interpretation, and critical revision of this article. Sandra Doss, Daniel A. Reuter, and Thomas Thiele: data interpretation and critical revision of this article.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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MAIN TEXT

Extracorporeal therapy of sepsis by purified granulocyte concentrates—ex vivo circulation model

Gerd Klinkmann[1] | Thomas Wild[2] | Benjamin Heskamp[2] | Fanny Doss[2] | Sandra Doss[2,3] | Magdalena Milej[2] | Lea-Marie Thiele[4] | Lilia Goudeva[5] | Rainer Blasczyk[5] | Daniel A. Reuter[1] | Jens Altrichter[2] | Steffen Mitzner[3,4]

[1]Department of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University of Rostock, Rostock, Germany

[2]ARTCLINE GmbH, Rostock, Germany

[3]Department of Extracorporeal Immunomodulation, Fraunhofer Institute for Cell Therapy and Immunology, Rostock, Germany

[4]Department of Medicine, Division of Nephrology, Medical Faculty, University of Rostock, Rostock, Germany

[5]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Germany

Correspondence
Gerd Klinkmann, Department of Anesthesiology, Intensive Care Medicine and Pain Therapy, University Medical Center Rostock, Schillingallee 35, 18057 Rostock, Germany.
Email: gerd.klinkmann@uni-rostock.de

Abstract

Background: Immune cell dysfunction is a central part of immune paralysis in sepsis. Granulocyte concentrate (GC) transfusions can induce tissue damage via local effects of neutrophils. The hypothesis of an extracorporeal plasma treatment with granulocytes is to show beneficial effects with fewer side effects. Clinical trials with standard GC have supported this approach. This ex vivo study investigated the functional properties of purified granulocyte preparations during the extracorporeal plasma treatment.

Methods: Purified GC were stored for up to 3 days and compared with standard GC in an immune cell perfusion therapy model. The therapy consists of a plasma separation device and an extracorporeal circuit. Plasma is perfused through the tubing system with donor immune cells of the GC, and only the treated plasma is filtered for re-transfusion. The donor immune cells are retained in the extracorporeal system and discarded after treatment. Efficacy of granulocytes regarding phagocytosis, oxidative burst as well as cell viability and metabolic parameters were assessed.

Results: In pGC, the metabolic surrogate parameters of cell functionality showed comparable courses even after a storage period of 72 h. In particular, glucose and oxygen consumption were lower after extended storage. The course of lactate dehydrogenase concentration yields no indication of cell impairment in the extracorporeal circulation. The cells were viable throughout the entire study period and exhibited preserved phagocytosis and oxidative burst functionality.

Conclusion: The granulocytes demonstrated full functionality in the 6 h extracorporeal circuits after 3 days storage and in septic shock plasma. This is demonstrating the functionality of the system and encourages further clinical studies.

KEYWORDS

clinical use, extracorporeal therapy, granulocyte concentrate, sepsis

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1 | INTRODUCTION

1.1 | Background

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.[1] The hospital mortality rate of patients with sepsis according to the Sepsis 3 definition[1] is 35.3% and 52% for the respective septic shock population.[2] Sepsis treatment is based on causal therapy (antimicrobial therapy, focus rehabilitation) and supportive and adjunctive therapy.[3] Regarding the high incidence of sepsis in conjunction with a high mortality rate and high costs, efficient therapeutic procedures are required. Any favorable impact of attenuating resource consumption due to sepsis and septic shock may save critical intensive care resources in times of increased demand as in times of pandemic and mass casualty events.

A wide range of new treatment modalities has been evaluated for sepsis and septic shock. However, none of these treatments has currently been granted approval.[4]

Immune cell dysfunction is a central component of immune paralysis in sepsis. With regard to cellular immunocompetence, functional impairment of neutrophils and monocytes as well as lymphopenia is associated with increased mortality in advanced stages of sepsis.[5–7] Thus, transfusion of healthy donor immune cells would be a straightforward therapy. Nonetheless, transfusions of granulocyte concentrates, the only immune cell concentrates available, can provoke tissue damage via local effects of neutrophils.[8] Administration of granulocyte colony-stimulating factor (G-CSF) to increase neutrophil count during sepsis has also failed to improve clinical survival rates in several studies.[9] Use of immune cells to treat sepsis in an extracorporeal setting was previously propounded by Mitzner et al[10] and has been investigated both preclinically and in pilot clinical trials.[11–14] To date, still, only data on the functionality of standard granulocyte concentrates in an extracorporeal circuit is available.

The majority of cells in a standard GC are red blood cells (RBCs) and platelets.[15] White blood cells (WBCs) often comprise less than 20% of the cells (own observation). Donor GC contain a variety of immune cells, especially granulocytes, which act as phagocytes to eliminate cellular detritus and microbial debris.[15] In addition, granulocytes modulate immune function by secreting and adsorbing immunologic messenger molecules (e.g., cytokines, chemokines, growth factors, and prostaglandins).[16] Granulocytes can therefore influence and potentially correct the immune dysfunction observed in sepsis.[6]

Standard GC used in a clinical pilot trial contained approximately 1.5×10^{10} neutrophil granulocytes for a group of 10 septic shock patients and approximately 3.5×10^{10} neutrophil granulocytes for another 10 sepsis patients. Application of the higher neutrophil count in the second group required the integration of a microporous hollow fiber membrane oxygenator in order to support the increased oxygen consumption. Integration of the oxygenator leads to a beneficial effect on granulocyte function, whereas no oxygenator was required for the lower neutrophil counts.[17] Nevertheless, technical implementation of integrating the oxygenator turned out to be highly laborious and hardly practicable in the clinical setting. Consequently, further refinement of standard GC which entails an optimization of purity and cell count was encouraged. Furthermore, storage of cellular blood products is challenging for blood cell mixtures, since whole blood deteriorates over time due to red cell lysis, loss of complement, change in pH, and induction of cell apoptosis. To improve the storage stability of standard GC, our group has developed a purification procedure compatible with standard blood bank technologies to remove RBCs and platelets and enrich the neutrophils.[18] During the purification process, the absolute neutrophil count was adjusted to a level of about 1.5×10^{10} as used in the first 10 patients (range 1.0 to 2.5×10^{10}). The result of the purification is a significant optimization of storability. A significant enhancement of the storage duration was achieved from 24 h to 3 days while maintaining a consistently high level of cell functionality.

The aim of the present study was to investigate these purified granulocyte concentrates in the context of the extracorporeal circuit focusing on the functionality of the cells.

2 | MATERIALS AND METHODS

2.1 | Donors

Standard GC was obtained from volunteer healthy donors from the donor pool of the Institute of Transfusion Medicine and Transplant Engineering (Hannover Medical School). All donors met the screening and testing criteria for granulocytapheresis defined by the Guidelines for the preparation of blood and blood components and the use of blood products (Hemotherapy) of the German Medical Association (Bundesärztekammer).[19] Donors were stimulated by a single subcutaneous injection of G-CSF 6 µg/kg body weight (Lenogastrim, kohlpharma, Merzig, Germany) and 8 mg oral dexamethasone approximately 16–18 h before granulocytapheresis. Written informed consent was obtained from all donors.

2.2 | Granulocytapheresis

Standard GC was collected by continuous-flow apheresis with the Spectra OPTIA™ Apheresis System (Terumo

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BCT Europe, Garching, Germany). Samples collected from the standard GC were tested for blood cell counts, viability, and functionality.

2.3 | Purification

Development of the purification process and the successful extension of storage time to at least 3 days have been described recently.[18] In short, standard GC was sedimented to remove RBC, washed twice with 0.9% saline solution to remove platelets, split into units containing about 1.5×10^{10} granulocytes, and stored in ABO-compatible, citrate anticoagulated blood plasma with glucose in gas permeable storage bag. The purification procedure of sedimentation and washing reduced the RBC and platelet counts in purified GC, by approximately $98.08 \pm 1.87\%$ and $95.91 \pm 5.1\%$, respectively, compared with standard GC. One resulting purified GC unit contained at least 1×10^{10} granulocytes and maximal 2.5×10^{10}. Both standard GC and purified GC were stored at room temperature ($22 \pm 2°C$) without agitation.

2.4 | Patient model

The laboratory model of the extracorporeal immune cell therapy was essentially equivalent to the clinical application, except for the presence of a real patient. A standardized 1000 mL plasma pool from healthy donors or from sepsis patients was used as a patient model. As a model for healthy subjects, a pool of thawed fresh frozen plasma was used. Septic patient plasma was obtained from the discarded exchange plasma of a study on therapeutic plasma exchange (University of Rostock, RegNr. II PV 12/2000) in septic shock patients. Written informed consent was obtained from all participants or from the patients' representatives if direct consent could not be received. This exchange plasma was portioned in shares of 1000 mL each and frozen. 1000 mL of the frozen healthy or sepsis plasma was thawed in a water bath at 37°C on the day before the respective experiment, transferred into 2000 mL IMF infusion bags, and stored at 4°C overnight. During thawing, heparinization was performed with 25 I.U./mL. Citrate-containing plasma was re-calcified. The intended concentration of free Ca^{2+} in the extracorporeal circulation was >1.0 mmol/L. Since standard GC and purified GC cell preparations contain sodium citrate as anticoagulant and therefore almost no free Ca^{2+}, initial value in the plasma pool was set correspondingly higher to reach the goal of >1.0 mmol/L after equilibration. Therefore, the plasma pool was recalcified with respective amount of 1 M $CaCl_2$ solution to reach a free calcium concentration of about 1.6–2.0 mmol/L. To create a reservoir of oxygen in the "patient" pool bag, 1000 mL of sterile filtered ambient air was added to the plasma pool bag.

2.5 | Circuit description

Plasma separation was carried out by a manually controlled multipurpose dialysis machine (Infomed HF440, Infomed SA, Meinier, Switzerland) using a 0.5 μm pore-size plasma filter (Infomed LF-060 Plasma Filter—0.60 sqm Granopen, Infomed SA, Meinier, Switzerland). The filtered plasma was infused into a continuously recirculating donor cell compartment that was prefilled with therapeutically effective human immune cells (standard GC or purified GC), which are kept in circulating flow by the cell circuit pump. Plasma reflux to the patient model (plasma pool) was performed by filtration through two additional LF-060 plasma filters, which are referred to as plasma filters cell circuits 1 and 2 (PF CC1 and CC2), to prevent the donor cells from being infused into the "patient". Therefore, only the treated plasma is retransfused online into the (model) patient. Donor immune cells are retained in the extracorporeal system and discarded after treatment. The total "extracorporeal" volume was about 900 mL. Flow rate of the primary "blood" circuit was 110–150 mL/min with a plasma separation rate of 33.3 mL plasma/min. All fluids were heparinized: cell bags (10 IU/mL), rinsing solution (5 IU/mL), and plasma pool (20 IU/mL). A schematic depiction and a technical illustration of the circuit are presented in Figure 1.

2.6 | Sampling

Samples were collected at different sample ports in the circuits and time points. Sampling time points were before start, after 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h. Sampling ports were before and after cell circuit and within the cell circuit. To also obtain cells sticking in the plasma filter CC1 after 3 h, a sample was taken by backwashing plasma filter CC1 with 50 mL plasma from the circuit and the 6 h sample by backwashing with 2000 mL cold (4°C) NaCl solution.

Samples were taken and analyzed to determine leukocyte recovery rate, viability, and functional capacity. Furthermore, the course of pH, electrolytes, glucose, lactate, and lactate dehydrogenase (LDH) concentrations as well as oxygen and carbon dioxide partial pressure were determined.

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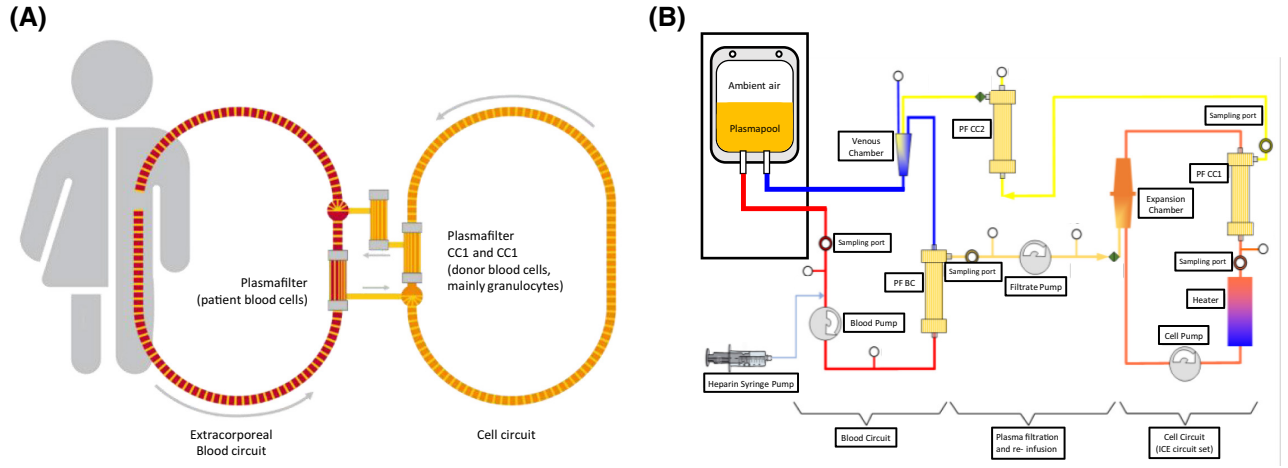

FIGURE 1 (A) The extracorporeal immune cell therapy is a plasma treatment technology. Plasma is continuously filtered from the patient's extracorporeal blood circuit and transferred into a closed-loop 'cell circuit', where the patient's plasma is brought into direct contact with therapeutically effective, human-donor immune cells (i.e. the granulocyte concentrate). (B) Schematic of the treatment simulation experiments shows a depiction of the components involved in the therapy system.

At each filter, pressure before and behind the plasma filter membrane was monitored during the whole treatment duration of six hours. For verification of donor cell retention, fluid behind the membrane of plasma filter CC1 was collected, centrifuged and concentrated and then examined.

2.7 | Measurement of blood cell counts and WBC viability

Blood cell content and WBC differentiation were evaluated automatically using a hematology analyzer (KX-21 N, Sysmex, Norderstedt, Germany).

WBC viability was determined using the Nucleo Counter® NC-200™ (ChemoMetec, Allerod, Denmark) according to the manufacturer's specifications.

2.8 | Measurement of granulocyte function

Function of the granulocytes was analyzed with oxidative burst and phagocytosis assays using commercial Phagoburst-Kit and Phagotest-Kit (Celonic, Heidelberg, Germany), respectively. Both tests were used according to manufacturers' instructions with one modification because granulocyte concentration in GC is approximately 10 times higher than in whole blood. To achieve a concentration of approximately 5000 granulocytes/μL and therefore the same ratio of granulocytes to the stimulus (e.g., *Escherichia coli*) like with heparin-anticoagulated blood (4000–10 000 granulocytes/μL), samples were diluted in heparin-anticoagulated blood group compatible plasma of healthy donors.

2.9 | Measurement of electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures

Electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures were measured using an ABL90 Flex Plus blood gas analyzers (Radiometer, Krefeld, Germany).

2.10 | Measurement of lactate and lactate dehydrogenase concentrations

Lactate and lactate dehydrogenase concentrations were measured using a Cobas Mira Plus CQ (Roche, Ludwigsburg, Germany).

2.11 | Statistical analysis

Statistical analysis of the data was performed using IBM SPSS Statistics (version 27, Chicago, IL, USA). Results are expressed as the median ± standard deviation (SD) and range. Box plots were used for graphics. The horizontal line within the boxes represents the median, whereas the upper part represents the 75th and the lower part the 25th percentiles. The whiskers represent the range of the values, whereas the circles and the asterisks show the outliers (extreme values that deviate from the rest of the sample). According to the distribution of data (using Shapiro–Wilk test), Mann–Whitney U-test was used for two independent samples for continuous variables. Kruskal–Wallis test was used to test the difference between multiple independent samples with non-normal underlying population distribution, and appropriate post hoc tests were applied if

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necessary. Statistical differences were considered significant at a p value <0.05.

3 | RESULTS

Standard GC used to produce purified GC was prepared according to clinical standard procedures by granulocytapheresis from healthy donors and met the quality criteria of the guidelines of the German Medical Association.[19] All circuit experiments were run for 6 h without technical problems. A total of 18 circuit experiments were performed. Results were statistically analyzed and are illustrated here.

3.1 | Standard GC versus purified GC in healthy donor plasma circuits

3.1.1 | Granulocyte function and viability

Viability in purified GC was consistently high at over 94% across all subgroups at 6 h of treatment (Figure 2A). Phagocytosis (Figure 2B) and oxydative burst assays (Figure 2C) indicated the preservation of granulocyte function of purified GC in the circuit. Phagocytosis remained robustly high even in the purified GC that were stored for 3 days prior to the circuit experiment (Figure 2B).

3.1.2 | pH values

At baseline, median pH of standard GC was about 6.99 ± 0.02 after storage, about 7.15 ± 0.03 after equilibration at t = 30 min, and decreased to 7.05 ± 0.01 during 6 h of treatment. In comparison, pH remained significantly ($p \leq 0.05$) less acidic in purified GC, with a mean value of 7.13 ± 0.05 after 6 h of treatment in both subgroups (24 h of storage and after 3 days) (Figure 3A).

3.1.3 | Glucose and lactate

Glucose levels of standard GC were initially in the physiological range of 3.5–5.5 mmol/L, showed an increase to 5.80 ± 0.36 mmol/L after equilibration at t = 30 min, and decreased to 4.70 ± 0.31 mmol/L over time. In contrast, glucose levels were initially higher in both 24 h stored and 3 days stored purified GC due to glucose addition prior to storage. Graphs show a similar course, with a slight decrease to values of 6.30 ± 0.21 mmol/L and 6.50 ± 0.65 mmol/L, respectively (Figure 3B). After equilibration of the system at t = 30 min, there were significant differences ($p \leq 0.5$) in glucose concentration of standard GC compared to all subgroups of purified GC.

High number of RBC and platelets in standard GC produce huge amounts of lactate, leading to lactate concentrations of up to 6.49 ± 0.65 mmol/L within the 6 h of treatment. In contrast, the lactate concentration of purified GC showed a low value at the onset and a notably lower increase, ranging from 4.67 ± 0.94 mmol/L to 5.59 ± 1.03 mmol/L after 6 h in the circuit (Figure 3C).

3.1.4 | Impact on LDH and blood gas dynamics

LDH concentrations were analyzed as an indication for cell damage. Compared to reference value (<225 U/L), measured values are within the physiologic range and stable during the experiments. Therefore, no relevant cell damage was observed. Furthermore, no significant differences were observed between the subgroups.

Although standard GC started with a much lower oxygen partial pressure than purified GC after equilibration both standard GC and purified GC showed a similar course over the entire observation period with only slight decrease. Likewise, oxygen partial pressure course in 3 days stored purified GC was rather similar (Figure 4A). Although course of carbon dioxide partial pressures during the treatment period showed considerably lower values in purified GC (6 h treatment: 69.90 ± 4.60 mmHg) than in standard GC (6 h treatment: 76.70 ± 7.65 mmHg), no significant difference could be determined (Figure 4B).

3.1.5 | WBC cell count

Regarding standard GC, of 1.88×10^{10} leukocytes introduced, the number of circulating leukocytes is initially 1.3×10^{10}, remains constantly high, and is 8.03×10^{9} cells after 6 h. Consequently, 1.08×10^{10} leukocytes adhered to the filter and tubing after 6 h of treatment, representing 57.35% of the initial WBC count. Concerning purified GC, significant differences ($p \leq 0.5$) in WBC count were detected throughout the entire treatment period compared to standard GC. 1.50×10^{10} were introduced in to the circuit. After an initial peak cell count, there was a rapid decrease in circulating cells due to adhesion to the plasma filter hollow fiber. After 6 h in the circuit, the range of circulating WBCs varied from 8×10^{7} to 2.5×10^{8}. Thus, an average of 98.78% of WBCs adhered to the filter (Figure 5).

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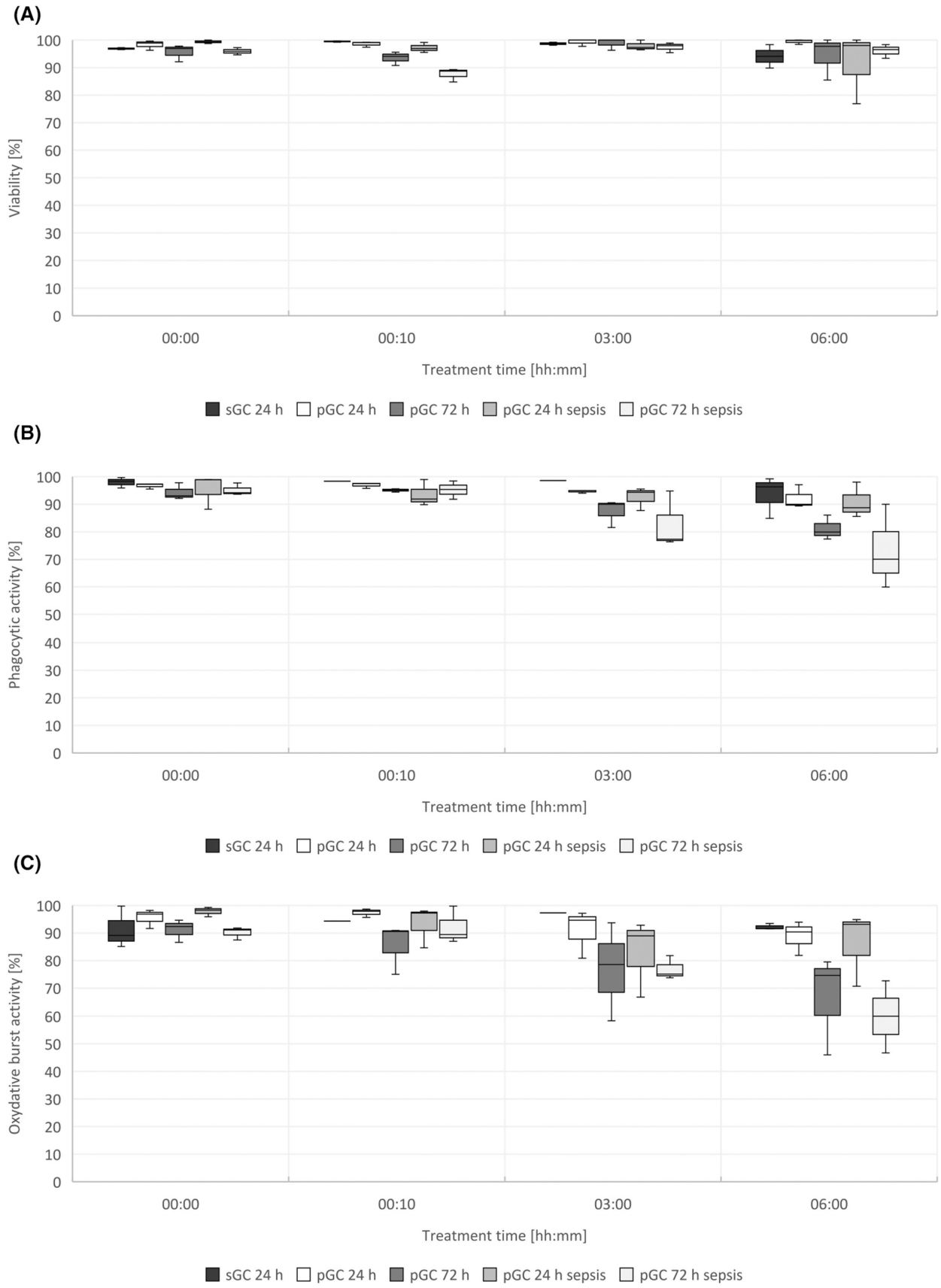

FIGURE 2 Functional granulocyte activity of standard granulocyte concentrate and purified granulocyte concentrate at defined observation time points. (A) Viability; (B) Phagocytic activity; (C) Oxydative burst activity. No statistically significant differences in cellular functionality were detected during the study period, while slight reductions were observed after 72 h of storage and after 6 h of circulation time.

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3.2 | Healthy donors versus sepsis plasma

Phagocytic activity showed a consistent high functional activity level even after 3 days of storage. Only a slight decrease at the end of the 6 h treatment could be observed in standard GC after 24 h and similarly in purified GC after 72 h but not in purified GC after 24 h. Concerning pH values, no significant differences were found between pH decrease slopes in the subgroups. Time trend of glucose consumption was parallel in both normal plasma and sepsis plasma. Glucose concentration in sepsis plasma group was 4.40 mmol/L and 4.80 mmol/L and that of normal plasma group was between 6.30 mmol/L and 6.50 mmol/L after 6 h of treatment. Lactate concentrations of all subgroups of purified GC showed a similar parallel increase in both normal plasma and septic environments, including purified GC stored for 72 h. However, although lactate concentrations were elevated in sepsis plasma pool, there were no significant differences between the subgroups. Blood gas analysis revealed equal courses during circulation time for all subgroups.

4 | DISCUSSION

The main finding of the present study is that granulocytes of both standard GC and purified GC show high viability and functionality in normal plasma as well as in sepsis plasma. Continuous glucose consumption and resulting lactate production occur, demonstrating a preserved metabolic activity throughout 6 h of treatment simulation, even for purified GC stored for 3 days prior to circuit experiments. Lactate production, and thus pH degradation, was found to be significantly worse using standard GC compared to all experiments performed with purified GC. The major reason for this is the significantly reduced erythrocyte and platelet concentration of purified GC due to the purification process.[18] In 3 days, stored purified GC and, especially in sepsis plasma circuits, lactate levels are elevated. However, these remain lower when compared to standard GC. Oxygen supply has been sufficient in all experiments.

In pilot clinical studies, efficacy of the bioreactor-based cellular sepsis therapy was investigated in terms of cell count dosage. In two groups of patients, 1.5×10^{10} cells or 3.5×10^{10} granulocytes were administered. High dose group showed a significantly higher oxygen consumption in the circuit. In order to counteract this process, an additional oxygenator was incorporated into the system. The oxygenator led to an unfavorable complexity of the system and thus significantly limits practical applicability. Hence, a dose of approximately 1.5×10^{10} cells being targeted and, on top of that, further development of GC was pursued in order to improve the storability and availability of preparations.[18] Use of purified GC in the circuit showed preservation of oxygen and carbon dioxide concentration without the need to implement an oxygenator as an additional device. Furthermore, purification of the standard GC resulted in lower carbon dioxide production thus, system design allowed further simplification for a more practical application on the patient.

Lower red blood cell and platelet count in purified GC results in lower lactate production and thus a less acidic pH compared to standard GC. Incorporation of special storage bags designed to release the carbon dioxide produced by the cells during storage further contributes to improved pH values of the purified GC supplied to the circuit.[18] In contrast, lactate concentration of septic plasma was elevated in our experiments. This is in correspondence to the disease-specific metabolic environment of sepsis patients.[20] Despite unfavorable environmental conditions for cells compared to normal plasma, their functionality in the circuit proved to be comparably high.

Standard GC-related glucose level declines during the course of storage.[18] In contrast, glucose level in purified GC initially is elevated and, although it gradually decreases over time, remains above minimum physiological level, even after 3 days of storage. Consistent with this, Glasser et al. reported impaired neutrophil functions due to low glucose levels in the surrounding medium.[21]

Granulocyte concentrates for transfusion require irradiation with 30 Gy since viable lymphocytes can cause fatal transfusion-associated graft-versus-host disease (GvH), especially in severely immunocompromised patients.[22] Evidence revealed only a minor impairment of granulocyte function, e.g., in the form of reduced chemotaxis, whereas lymphocyte proliferation was massively reduced, leading to the need to irradiate granulocyte concentrates to avoid a graft-versus-host reaction.[23] However, negative effects on granulocytes are not negligible and have even been demonstrated for other granulocyte functions such as oxidative burst.[24] We have used GC in a strictly extracorporeal mode, especially in patients with advanced sepsis or septic shock.[11] Introduction of a second plasma filter (PF CC2) as a redundant safety measure for the unlikely event of hollow fiber breach in PF CC1 secures that all immune cells are completely retained extracorporeally and not transfused to the patient. Therefore, unlike for transfusion of GC, irradiation is not required to avoid GvH reactions. Avoiding irradiation not only better preserves granulocyte function but also the immunomodulatory function of lymphocytes in extracorporeal use.[25,26]

A further important aspect relates to the analysis of cell counts, which indicated that many of the WBCs of standard GC kept moving constantly in the circuit throughout the

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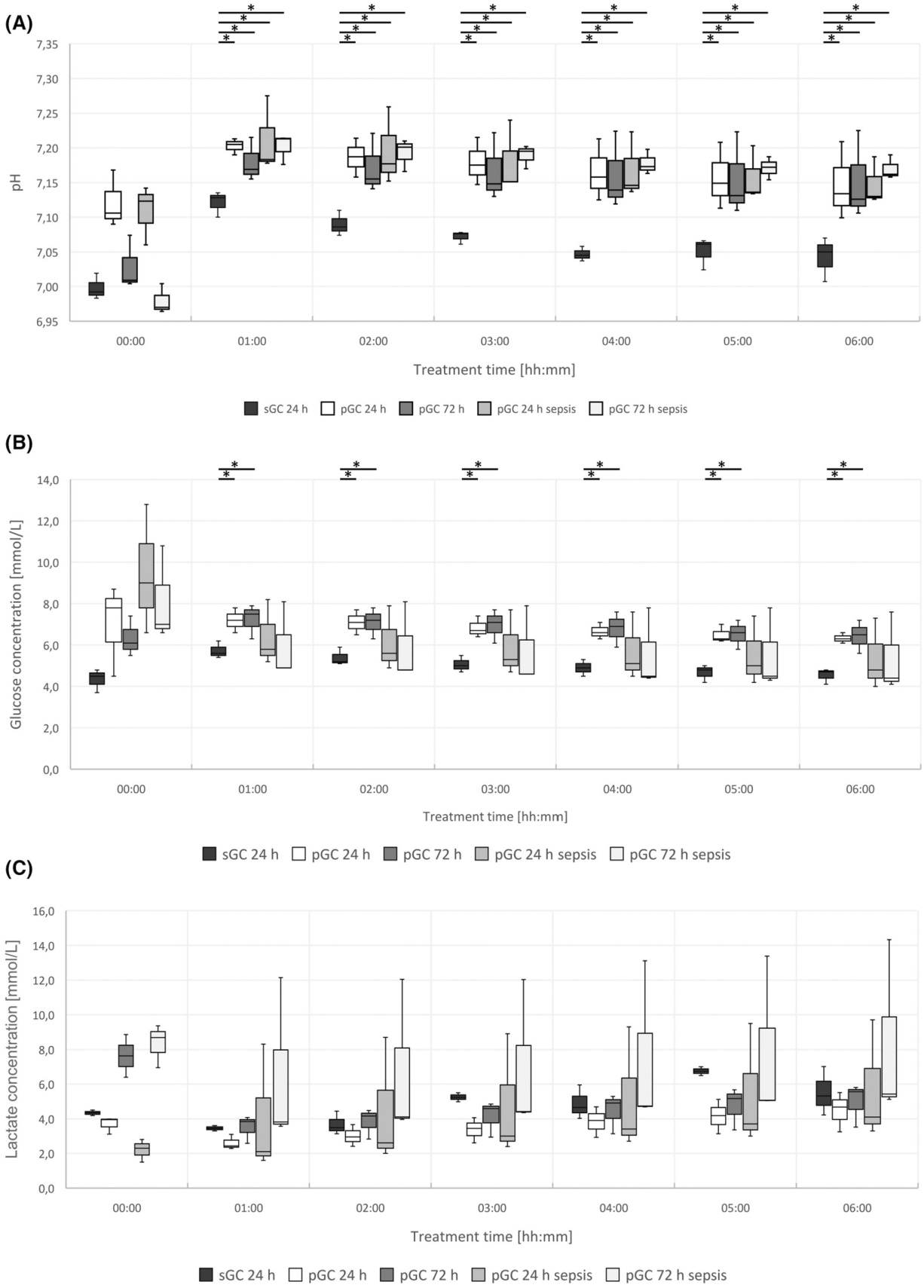

treatment cycle, whereas most of the WBC of purified GC settled in the filter. This phenomenon might be attributable to the interaction of WBCs and RBCs. These interactions may prevent the adhesion of WBCs in the filter. Given significantly reduced RBC cell count, these effects are minimized in the circulation of purified GC, leading resultatively to the

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FIGURE 3 Standard granulocyte concentrate and purified granulocyte concentrate analyses during treatment simulation at defined observation time points. *p* values ≤0.05 (*) were considered significant. (A) pH values. There are significant different pH values between sGC and pGC after equilibration at $t = 30$ min. There are no significant differences between the subgroups of pGC; (B) glucose concentration. There are significant differences between the pH values of sGC and pGC, starting at $t = 30$ min. No significant differences exist between the subgroups of pGC; (C) lactate concentration. There are no statistically significant differences, although a tendency toward less lactate concentrations in the pGC subgroups could be determined.

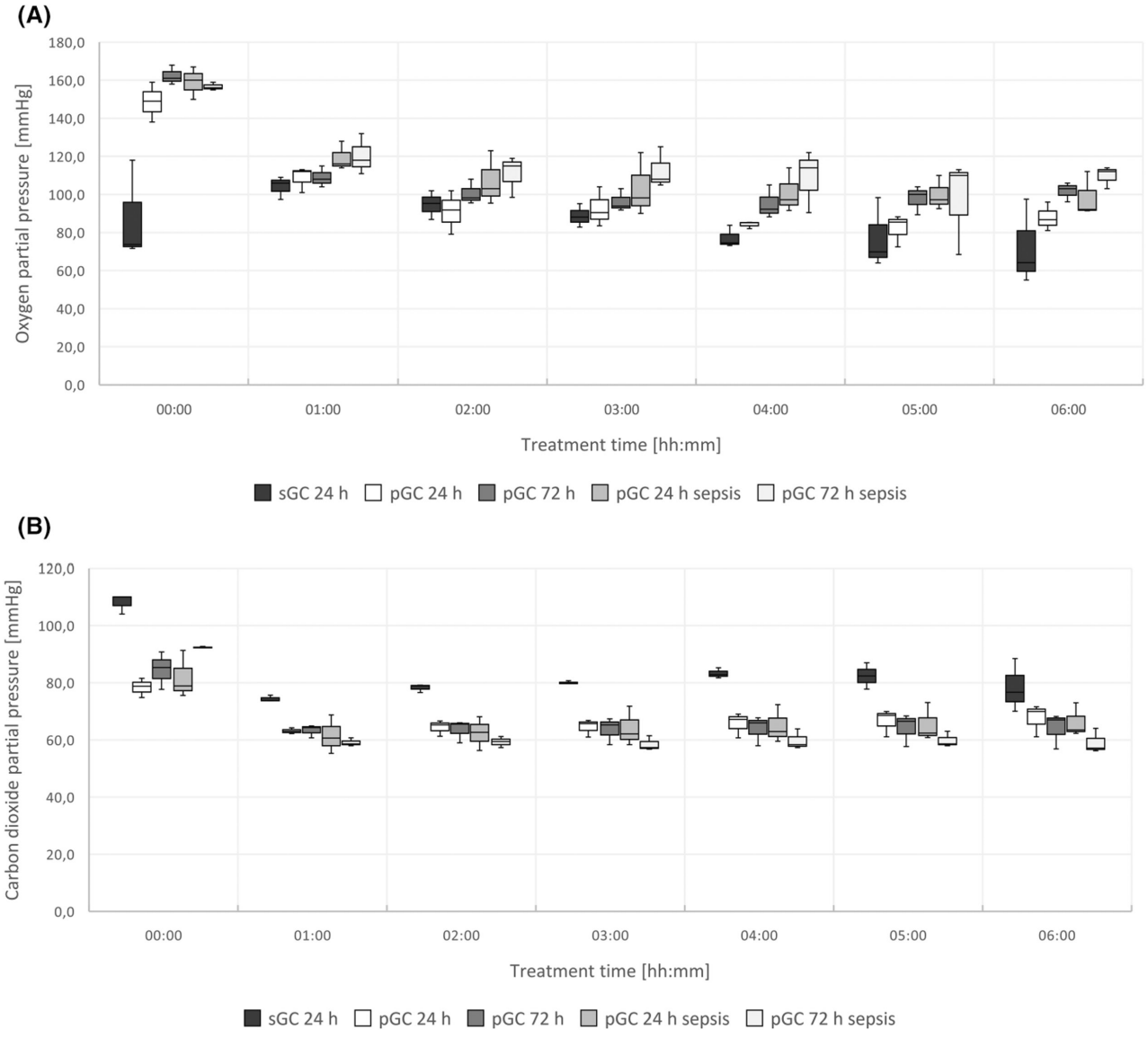

FIGURE 4 Blood gas dynamics of standard granulocyte concentrate and purified granulocyte concentrate at defined observation time points. *p* values ≤0.05 (*) were considered significant. (A) Oxygen partial pressure; (B) Carbon dioxide partial pressure.

persistence of cells in the filter. Furthermore, extracorporeal circuits may lead to the induction of adhesion molecules on granulocytes.[27] Moreover, Sbrana et al. demonstrated functional changes in neutrophil granulocytes and mononuclear cells during cardiopulmonary bypass. In particular, the interactions between leukocytes and platelets lead to the formation of heterotypic co-aggregates. The absence of platelet activation suggests that leukocyte membrane modifications play a major role in controlling the formation and stability of heterotypic leukocyte-platelet co-aggregates with extracorporeal circulation.[28] Since platelet fraction in purified GC is minimized, interactions play a minor role for this immune cell-based extracorporeal sepsis therapy. Based on these results, it was revealed that continuous movement of immune

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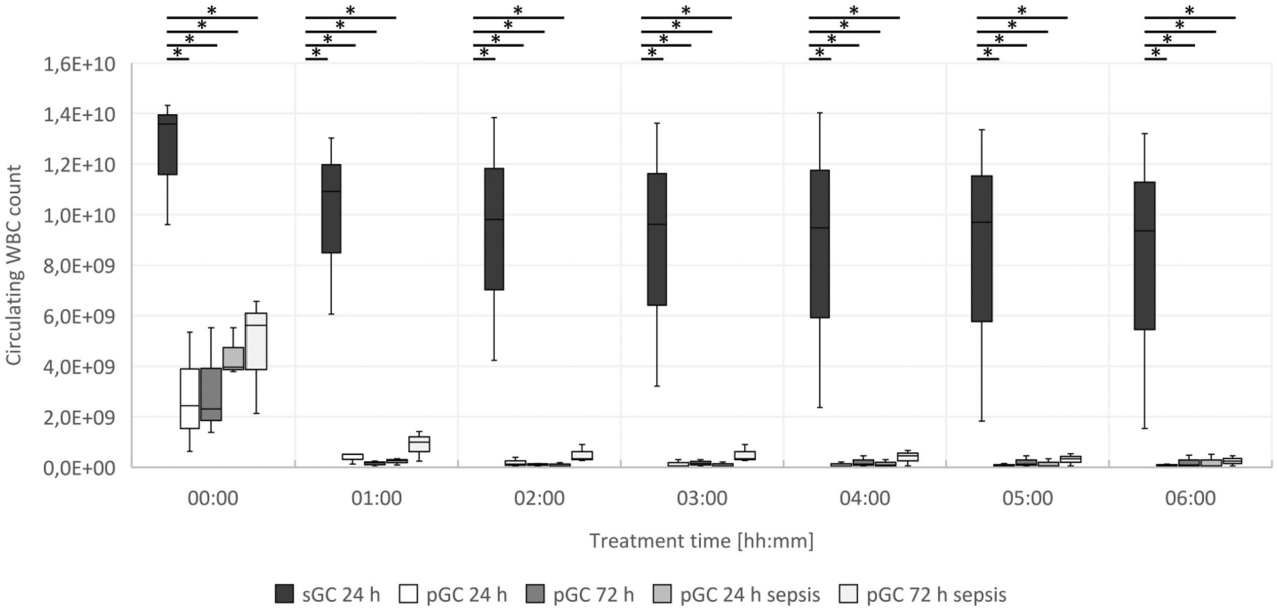

FIGURE 5 WBC count at defined observation time points. p values ≤0.05 (*) were considered significant. There is a significant difference in the amount of circulating WBC between sGC and pGC at all monitoring timestamps, but no significant differences between the subgroups of pGC.

cells in the circuit has apparently turned out not to be an indispensable prerequisite for maintaining the activity of the cells. In particular, it appears possible to provide immune cells in a plasma-permeable hollow fiber filter without compromising their functionality when continuously flushed with plasma. Further research might address this straightforward approach to store the cells in prefilled hollow fiber cartridges.

Several limitations apply to the validity of the data of this study. In vitro treatment simulation differs from clinical application in various parameters. No patient was connected to the system, but a plasma pool with a volume of 1000 mL was used. Hence, the solute distribution volume was smaller than in the clinical setting. A stationary mixture of water, electrolytes, and blood proteins constituted the plasma pool utilized. Therefore, the adsorption, resorption, and synthesis functions of a human body were not completely modeled. To avoid technical problems arising from clotting in the rather static plasma pool bag, anticoagulation was performed more intensively than in clinical applications. Oxygen supply to the cells was weaker than in a clinical situation where oxygen is continuously supplied by the patient. Since plasma filtration from whole blood is a standard technique and also for ethical reasons, the patient model was set up using human donor plasma instead of whole blood.

5 | CONCLUSION

In conclusion, high functionality of immune cells was adequately preserved even after 3 days of storage and also in extracorporeal circuits with sepsis plasma. This is a promising indicator of the efficacy of the system supporting the use of purified GC stored for up to 3 days in subsequent clinical trials.

AUTHOR CONTRIBUTIONS

Gerd Klinkmann, Jens Altrichter: Conceptualization, methodology, investigation, statistical analysis, data interpretation, writing – reviewing and editing, visualization. Steffen Mitzner: Supervision, conceptualization, methodology, data interpretation, critical revision of article. Thomas Wild, Benjamin Heskamp, Lea-Marie Thiele: Investigation, data interpretation, critical revision of article. Fanny Doss, Sandra Doss, Magdalena Milej, Daniel Reuter, Lilia Goudeva, Rainer Blasczyk: Data interpretation, critical revision of article.

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CONFLICT OF INTEREST STATEMENT

Fanny Doss, Sandra Doss, Magdalena Milej, Steffen Mitzner, and Jens Altrichter are employees or shareholders of ARTCLINE GmbH. All others have no conflict of interest.

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ORCID

Gerd Klinkmann https://orcid.org/0000-0002-8922-8700

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Extracorporeal immune cell therapy of sepsis: ex vivo results

Gerd Klinkmann[1,7*], Thomas Wild[2], Benjamin Heskamp[2], Fanny Doss[2], Sandra Doss[2,4], Lubomir Arseniev[5], Krasimira Aleksandrova[5], Martin Sauer[6], Daniel A. Reuter[1], Steffen Mitzner[3,4] and Jens Altrichter[2]

*Correspondence:
gerd.klinkmann@med.uni-rostock.de

[1] Department of Anaesthesiology and Intensive Care Medicine, University of Rostock, Schillingallee 35, 18055 Rostock, Germany
Full list of author information is available at the end of the article

Abstract

Background: Immune cell dysfunction plays a central role in sepsis-associated immune paralysis. The transfusion of healthy donor immune cells, i.e., granulocyte concentrates (GC) potentially induces tissue damage via local effects of neutrophils. Initial clinical trials using standard donor GC in a strictly extracorporeal bioreactor system for treatment of septic shock patients already provided evidence for beneficial effects with fewer side effects, by separating patient and donor immune cells using plasma filters. In this ex vivo study, we demonstrate the functional characteristics of a simplified extracorporeal therapy system using purified granulocyte preparations.

Methods: Purified GC were used in an immune cell perfusion model prefilled with human donor plasma simulating a 6-h treatment. The extracorporeal circuit consisted of a blood circuit and a plasma circuit with 3 plasma filters (PF). PF1 is separating the plasma from the patient's blood. Plasma is then perfused through PF2 containing donor immune cells and used in a dead-end mode. The filtrated plasma is finally retransfused to the blood circuit. PF3 is included in the plasma backflow as a redundant safety measure. The donor immune cells are retained in the extracorporeal system and discarded after treatment. Phagocytosis activity, oxidative burst and cell viability as well as cytokine release and metabolic parameters of purified GCs were assessed.

Results: Cells were viable throughout the study period and exhibited well-preserved functionality and efficient metabolic activity. Course of lactate dehydrogenase and free hemoglobin concentration yielded no indication of cell impairment. The capability of the cells to secret various cytokines was preserved. Of particular interest is equivalence in performance of the cells on day 1 and day 3, demonstrating the sustained shelf life and performance of the immune cells in the purified GCs.

Conclusion: Results demonstrate the suitability of a simplified extracorporeal system. Furthermore, granulocytes remain viable and highly active during a 6-h treatment even after storage for 3 days supporting the treatment of septic patients with this system in advanced clinical trials.

Keywords: Extracorporeal, Therapy, Sepsis, Clinical Use, Granulocyte, Concentrate

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Introduction

Background

Sepsis is defined as life-threatening organ dysfunction arising from a dysregulated host response to infection [1].

Immune dysfunction is a hallmark of sepsis and septic shock. This so-called "sepsis-induced immunoparalysis" is characterized by a phase of immunosuppression preceded or even paralleled by the early hyperinflammatory state [2]. Sepsis-induced immunoparalysis renders patients unable to clear their primary infection and exposes them to a higher risk of dying at a later stage from secondary or opportunistic infections in an immunosuppressed state [3–6]. Although its importance is increasingly recognized, there is still an absence of consensus on the relevance of this clinically emerging phenomenon [7, 8].

Despite our increasing understanding of the molecular and pathophysiologic processes underlying sepsis-related organ injury, all treatment options remain nonspecific with respect to host response [1]. In recent years, immunomodulation has been introduced as a complementary therapeutic strategy to overcome immune system dysfunction in sepsis [9]. The transfusion of granulocyte preparations failed to improve survival in sepsis and neutropenic patients [10, 11]. There is some indication that steroid- or G-CSF-stimulated high-yield granulocyte donations might result in better survival in severe infections associated with neutropenia and cancer. However, reported clinical benefits remain ambiguous [11, 12]

Similarly, a compelling biological rationale has emerged supporting the application of extracorporeal therapies based on our pathophysiologic understanding of sepsis. Extracorporeal therapies have been suggested to influence successfully immune imbalances and subsequently the clinical course of multiorgan failure and sepsis [13]. Some studies showed hemodynamic stabilization of patients during extracorporeal treatment of sepsis. However, no clear impact on survival was seen [14, 15]

Extracorporeal cell-based treatment is another promising approach emerging in this area. As such, cell perfusion therapies have been investigated for the treatment of liver failure and acute renal failure associated with sepsis using hepatocytes or renal tubule cells. Appropriate cell source selection appeared to be crucial in this regard. With regard to cellular immunocompetence, functional impairment of neutrophils and monocytes has been associated with increased mortality in advanced stages of sepsis [16–18]. Accordingly, the concept of using immune cells to provide extracorporeal treatment for sepsis-induced immunoparalysis arose from previous suggestions [19].

In order to deploy the beneficial features of neutrophils such as phagocytosis of cellular debris, antigenic material, or pathogens and at the same time to circumvent the possible damaging local effects of systemically transfused neutrophils, a bed-side cell perfusion therapy which uses immune cells (e.g., from granulocyte concentrates from healthy blood donors) in a strictly extracorporeal mode seems to be a promising therapeutic approach. The immune cells are retained in the extracorporeal system and discarded after the treatment, thus avoiding potential unwanted effects of granulocyte transfusions resulting from direct tissue contacts in the patient and his or her intravascular system and especially the endothelium.

The general rationale for such an approach is that on one hand the plasma-modifying capacity of human immune cells can be used (e.g., to remove antigenic material from the circulation, immune regulation via adsorption and secretion of cytokines) while on the other hand control over these cells can be maintained (e.g., retention of the cells in the extracorporeal circuit, preventing local tissue effects).

Granulocyte concentrates have already been investigated as cell perfusion therapy both preclinically and in pilot clinical trials, demonstrating safety and tolerability [20–22]. Recently, we reported a novel method to yield a high-purity, neutrophil-rich, functional leukocyte preparation from standard granulocyte concentrates. By developing this procedure, storage of GC was extended from 1 day to at least 3 days with preserved granulocyte viability and function. Furthermore, combination of longer storage time, lower cell count variation and ABO independence has the potential to offer purified GC as off-the-shelf immune cell preparations [23]. Thus, for the first time, a pure leukocyte population is recruitable for intensive care settings.

It is the aim of this ex vivo study to demonstrate the suitability of these purified GCs in a simplified extracorporeal treatment system in preparation for clinical trials in patients suffering from sepsis and septic shock.

Methods

Donors

Standard GCs were obtained from volunteer healthy donors from the donor pools of the Institute of Transfusion Medicine and Transplant Engineering (Hannover Medical School, Germany) according to national and local GMP guidelines for donation of granulocyte concentrates. All donors met the screening and testing criteria for granulocytapheresis defined by the Guidelines for the preparation of blood and blood components and the use of blood products of the German Medical Association [24]. Following informed consent, donors were stimulated as follows by a single subcutaneous injection of G-CSF 6 µg/kg body weight (Lenograstim, Kohlpharma, Merzig, Germany) and 8 mg oral dexamethasone approximately 16–18 h before granulocytapheresis.

Granulocyte apheresis, purification

Standard GC were collected by continuous-flow apheresis with the COBE Spectra OPTIA™ Apheresis System (Terumo BCT Europe, Garching, Germany). Samples collected from the standard GC were tested for blood cell counts, viability and functionality.

Development of the purification process and the successful extension of storage time to at least 3 days have been described recently. In short, standard GC were sedimented to remove red blood cells, washed to remove platelets and stored in ABO-compatible, citrate anticoagulated blood plasma with glucose in gas permeable storage bag. Each resulting purified GC unit contains at least 1×10^{10} granulocytes purified GC were stored at room temperature (22 ± 2 °C) without agitation [23]. Purification was performed according to national and local GMP guidelines at Cellular Therapy Centre (Hannover Medical School, Germany). Figure 1 provides a photograph of both the standard GC and the purified GC.

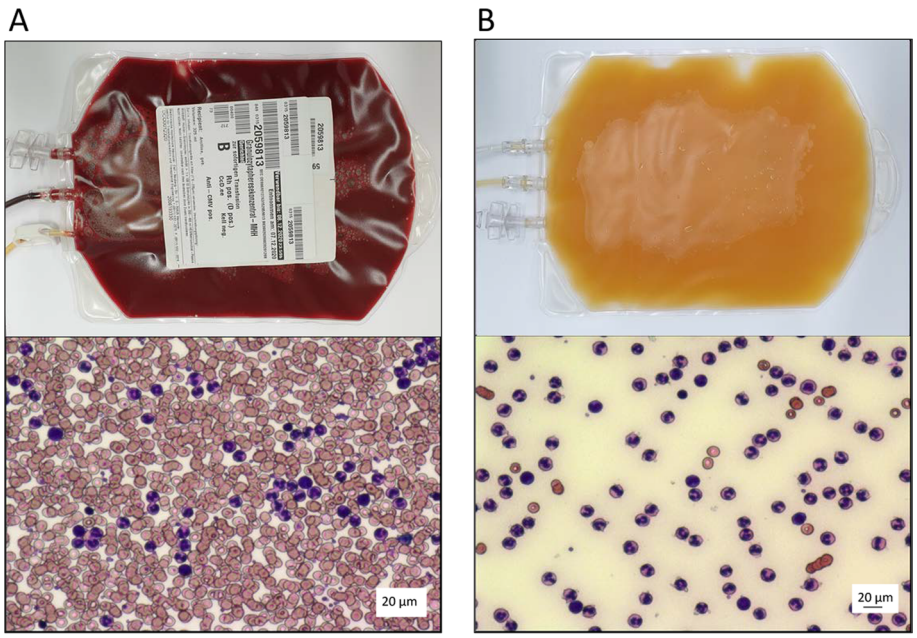

Fig. 1 Appearance and histology (×400) of **A** standard GC and **B** purified GC. The higher purity and greatly diminished RBC count in the purified GC is evident from its yellow color resulting from the few remaining RBC and platelets

In vitro plasma perfusion

The intended use of the purified GC is the therapeutic application in an extracorporeal treatment system to treat patients in septic shock. In essence, the laboratory model of extracorporeal immune cell therapy was similar to the clinical application, except for the presence of a genuine subject. A standardized 1000 mL plasma pool from donors was used as a model subject. Frozen plasma was thawed in a water bath at 37 °C until it was connected to the system.

The CE marked disposable tubing set (Meise GmbH, Schalksmühle, Germany) and plasma filters (Medica S.p.A., Medolla, Italy) were connected to form a closed, sterile system. This system was filled and rinsed with a hemofiltration fluid (MultiBic 4 mmol/L Kalium, Fresenius Medical Care, Bad Homburg, Germany). Figure 2 provides a schematic illustration of the therapeutic setup.

Hemofiltration solution used for rinsing had following contents:

K^+: 4.0 mmol/L	Cl^-: 111 mmol/L
Na^+: 140 mmol/L	HCO_3^-: 35 mmol/L
Ca^{2+}: 1.5 mmol/L	Glucose: 5.55 mmol/L
Mg^{2+}: 0.5 mmol/L	

Heparin was added as anticoagulant in a concentration of 5 IU/mL. After rinsing the assembled tubing and filter system, purified GC preparation was heparinized with 10 IU/mL and subsequently filled into the plasma part of the tubing system. The rinsing solution was displaced by the volume of the cell preparation (approx. 400–450 mL). The cells remained inside the hollow fibers of a plasma filter (PF CC1)

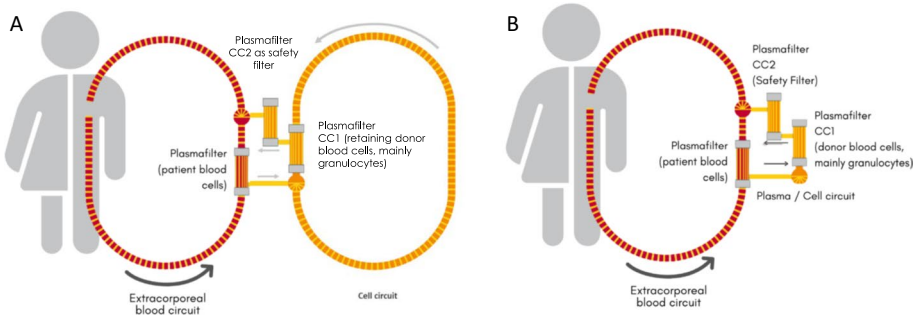

Fig. 2 The extracorporeal immune cell therapy is a plasma treatment technology. Plasma is continuously filtered from the patient´s extracorporeal blood circuit and transferred into a closed-loop 'cell circuit', where the patient's plasma is brought into direct contact with therapeutically effective, human-donor immune cells (i.e. the granulocyte concentrate). **A** Illustrates the procedure of separating plasma from an extracorporeal blood circuit through a plasma filter and feeding the plasma into a second circuit with circulating granulocytes as it has been used in clinical trials using standard granulocyte concentrates **B** illustrates the streamlined "one way" immune cell perfusion method using purified granulocyte concentrates. Plasma filter CC2 serves as a redundant safety filter

during the treatment. The system was then connected to a plasma pool, which was adjusted to 20 IU/mL heparin and 1.6–2.0 mmol/L free Ca^{2+} ions. The intended concentration of free Ca^{2+} ions in the extracorporeal circulation was > 1.0 mmol/L. Since purified GC cell preparations contain sodium citrate as anticoagulant and therefore almost no free Ca^{2+} ions, the initial value in the plasma pool was set correspondingly higher to reach a level of > 1.0 mmol/L after mixing. Glucose concentration was set to 3.5–7.0 mmol/L. Total fluid volume including the plasma pool was about 1850 mL. The different components mixed completely in the first 30 min of treatment simulation. In treatment mode, pool plasma enters the plasma filter PF BC (150 mL/min), where plasma is separated (33 mL/min). This plasma perfuses the plasma filter PF CC1 with the contained treatment cells inside in dead-end filtration mode. Subsequently, the plasma flows through another plasma filter PF CC2 in dead-end filtration mode, which acts as a safety barrier in case of a membrane rupture in PF CC1 to avoid donor cells entering the patient. After this, treated plasma flows into the venous chamber where it is mixed with the "patient´s blood" and returned to the "patient". This treatment is performed continuously for 6 h. Samples from plasma stream were taken before and after cell filter PF CC1 after every hour. After 3 and 6 h the cell filter was flushed back to obtain cells for analysis. 10 extracorporeal experiments were performed and analyzed. A schematic illustration of the circuit is presented in Fig. 3A.

Sampling

Samples were collected at different sample ports in the circuits and time points. Sampling time points were: before start, after 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h. Sampling ports were: before and after the cell filter PF CC1. To obtain cells sticking in the plasma filter PF CC1 after 3 h a sample was taken by backwashing plasma filter PF CC1 with 50 mL plasma and the 6 h sample by backwashing with 2000 mL cold (4 °C) NaCl solution at end of circulation.

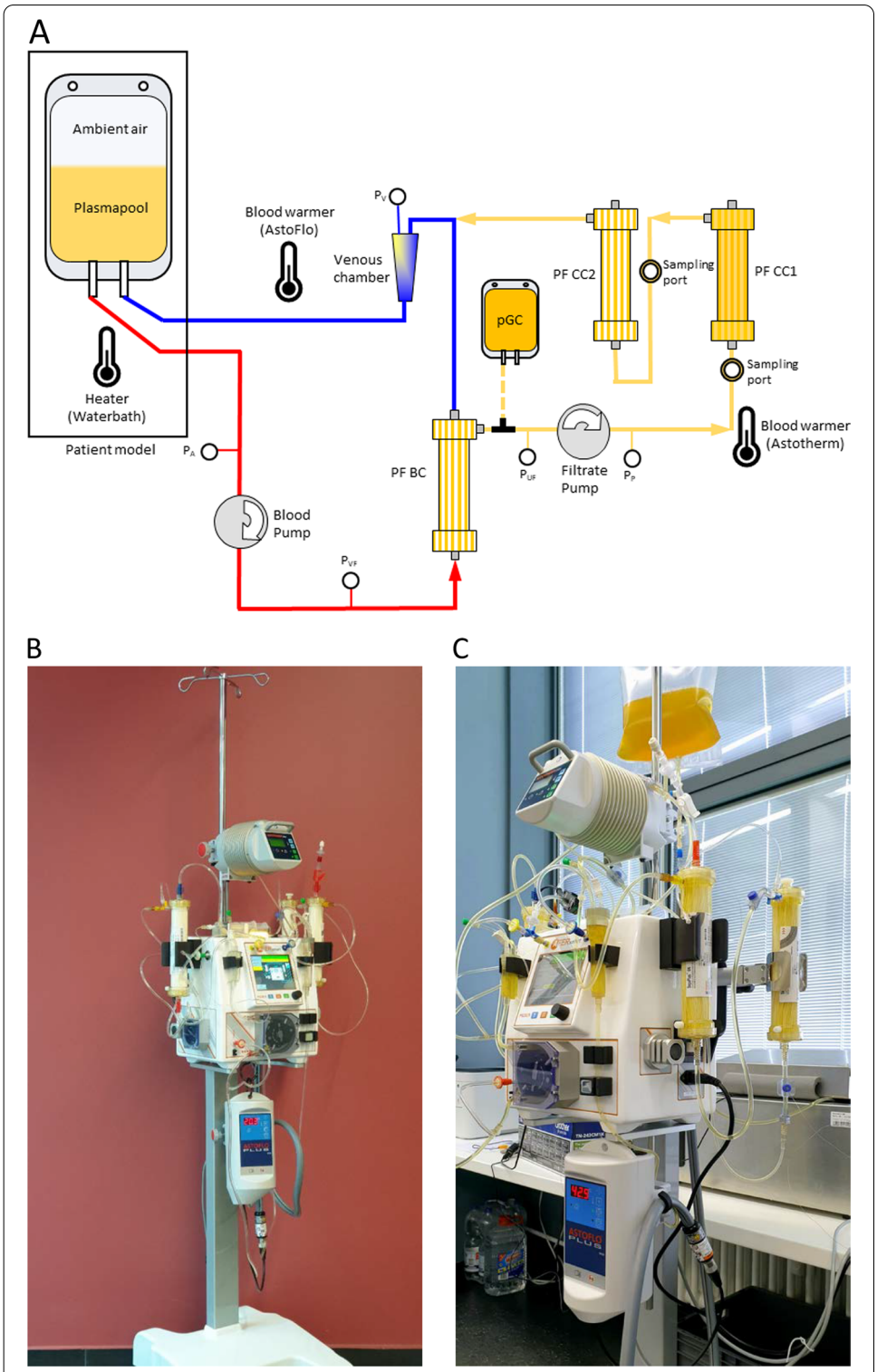

Fig. 3 **A** Schematic of the treatment simulation experiments depicts a graphical representation of the components involved in the immune cell enhancement single-pass therapy system. In vitro treatment simulation. **B** Apheresis machine AFERsmart with blood warmers and installed tubing set. **C** Simulation experiment using a purified GC preparation

Samples were taken and analyzed to determine leukocyte recovery rate, viability and functional capacity. Furthermore pH, electrolyte, glucose, lactate, lactate dehydrogenase and free hemoglobin concentrations as well as oxygen and carbon dioxide partial pressure were determined.

At each filter, the pressure before and behind the plasma filter membrane was monitored over the whole treatment duration of 6 h. For verification of donor cell retention, the fluid behind the membrane of plasma filter PF CC1 was collected, centrifuged and concentrated and then examined. Figure 3B and C provides photographs of the system.

Measurement of granulocyte function

Function of the granulocytes was analyzed with oxidative burst and phagocytosis assays using commercial Phagoburst-Kit and Phagotest-Kit (Celonic, Heidelberg, Germany), respectively. Both tests were used according to manufacturer's instructions with one modification, because granulocyte concentration in GC is approximately 10 times higher than in whole blood. To achieve a concentration of approximately 5000 granulocytes/μL and therefore the same ratio of granulocytes to the stimulus (e.g., Escherichia coli) like with heparin-anticoagulated blood (4000–10,000 granulocytes/μL), samples were diluted in heparin-anticoagulated blood group compatible plasma of healthy donors.

Measurement of cytokine concentration

Measurement of cytokine concentrations was performed by use of the LEGENDplex™ Human Essential Immune Response Panel (13-plex, BioLegend, Amsterdam, Netherlands). The panel is a bead-based multiplex assay panel that uses fluorescence-encoded beads suitable for use on various flow cytometers. Immunoassay was performed according to manufacturer's instructions. Quantitative analysis was carried out by a flow cytometer (MACS Quant 16, Miltenyi Biotec, Bergisch-Gladbach, Germany) and analysis was performed using LEGENDplex v8.0 software.

Measurement of blood cell counts and WBC viability

WBC viability was determined using NucleoCounter® NC-200™ (ChemoMetec, Allerod, Denmark) according to manufacturer's specifications.

Blood cell content and white blood cell differentiation were evaluated automatically using a hematology analyzer (KX-21 N, Sysmex, Norderstedt, Germany).

Values at time point 3 h are measured in samples that were taken by drawing back a volume of 50 mL from PF CC1. By this method, about 4% of the original cell count were retrieved, mixed, a sample was taken, and the rest of the cells was re-introduced to PF CC1. The values at time point 6.5 h are measured in sample that were taken at the end of the experiment by flushing back PF CC1 with a volume of 2000 mL cold sodium chloride solution. By this method, an average of 46% of the original cell count were retrieved, mixed, a sample was taken and analyzed. Therefore, cell-associated results at 3 h and 6 h do not necessarily represent total cell amount.

Measurement of electrolytes, pH, glucose, oxygen and carbon dioxide partial pressures

Electrolytes, pH, glucose, oxygen and carbon dioxide partial pressures were measured using an ABL90 Flex Plus blood gas analyzers (Radiometer, Krefeld, Germany).

Measurement of lactate, and lactate dehydrogenase and free hemoglobin concentrations

Concentrations of lactate, free hemoglobin (fHb) and lactate dehydrogenase (LDH) were determined using a Cobas Mira Plus CQ (Roche, Ludwigsburg, Germany) according to manufacturer's specifications. fHb concentration was measured using the 3-wavelength method (380/415/450 nm) according to the method of Harboe [25] on the spectral photometer Dr. Lange LS 500 (Type LPG 244) according to manufacturer's specifications.

Pressures

Transmembrane pressures (TMP) of plasma filters were calculated from respective pressure values at the inlet of the plasma filter and its outlet(s) as TMP = Pre-membrane mean pressure – post-membrane pressure. TMP of PF BC (plasma filter blood circuit) was measured and displayed by the apheresis machine AFERsmart (Medica, Medolla, Italy). TMPs at PF CC1 and PF CC2 were determined by additional pressure measurements in the tubing set.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 27, Chicago, IL, USA). Results are expressed as median ± standard deviation (SD) and range. According to the distribution of data (using Shapiro–Wilk test), Mann–Whitney test was used for two independent samples for continuous variables. Kruskal–Wallis test was used to test differences between multiple independent samples with non-normal underlying population distribution, and appropriate post hoc tests were applied if necessary. Statistical differences were considered significant at P value < 0.05.

Results

Standard GC used to produce purified GC were prepared according to clinical standard procedures by granulocytapheresis from healthy donors and met the quality criteria of the guidelines of the German Medical Association [24]. All 10 circuit experiments were run for 6 h without technical problems.

Granulocyte function and viability

Viability in purified GC was consistently high at over 94% across all subgroups during 6 h of treatment (Fig. 4A). Phagocytosis and oxidative burst assays demonstrated preservation of granulocyte function of purified GC in the circuit. Phagocytosis and oxidative burst remained preserved in the purified GC that were stored for 3 days prior to the circuit experiment (Fig. 4B).

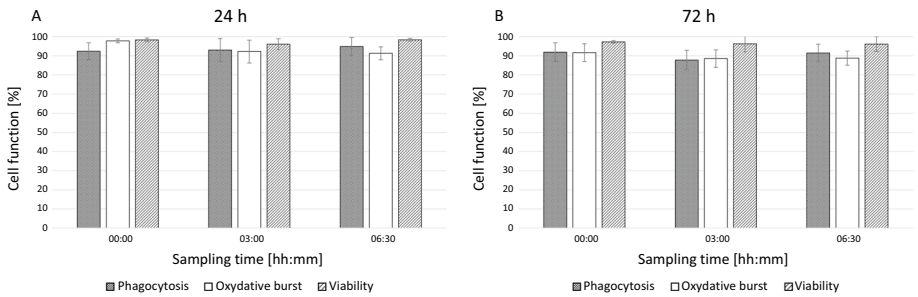

Fig. 4 Cell function during the extracorporeal treatment simulation—phagocytosis, oxidative burst rate and viability ($n = 10$). The charts show the function parameters phagocytosis rate and oxidative burst rate of the WBC inside the treatment system before, during and after the simulated treatment. No significant differences were found between specific time points

Glucose consumption and lactate generation

During preparation of the purified GC glucose was supplemented to support a longer storage time. After storage for 1 day glucose concentration in purified GC amounts to 15.42 ± 1.13 mmol/L. After equilibration at $t = 30$ min within the circuit, a value of 8.06 ± 0.35 mmol/L before PF CC1 and 7.76 ± 0.31 mmol/L after PF CC1 was observed. Subsequently, the concentration decreased steadily to 6.52 ± 0.57 mmol/L before PF CC1 and 6.30 ± 0.60 mmol/L after PF CC1 at the end of the 6-h analysis interval. A comparable course was detected in the circuits with purified GC that were stored for 3 days prior to use. In particular, no significant differences were observed between these two cell populations (Fig. 5A, B).

After storage for 1 day, lactate concentration of the purified GC was 6.05 ± 1.62 mmol/L. At 30 min after initiation of the simulation, equilibrated values in the circuit are 2.10 ± 0.49 mmol/L before PF CC1 and 2.97 ± 0.58 mmol/L after PF CC1. At 6 h, after a continuous increase, levels have reached 5.31 ± 1.26 mmol/L before PF CC1 and 5.82 ± 1.21 mmol/L after PF CC1. However, no significant differences between the test sites before and after the cell filter were determined. A comparable course is seen after storage for 3 days (Fig. 5A, B).

pH values

At baseline, mean pH of purified GC was 7.09 ± 0.09 after 1 day of storage, about 7.28 ± 0.07 after equilibration at $t = 30$ min and decreased to 7.12 ± 0.07 during 6 h of treatment. In comparison, after 3 days of storage pH in purified GC showed a mean value of 6.92 ± 0.23, about 7.23 ± 0.06 after equilibration at $t = 30$ min and decreased to 7.13 ± 0.07 during 6 h of treatment. Although a constant difference between the sample points before and after PF CC1 was detectable in all experiments with mean values of 0.06 after $t = 30$ min to 0.04 at $t = 6$ h no significance was reached (Fig. 5C, D).

Oxygen and carbon dioxide partial pressures

Commencing at 162.00 ± 3.52 mmHg, the course of oxygen partial pressure before PF CC1 shows a decrease over the entire observation period to a value of

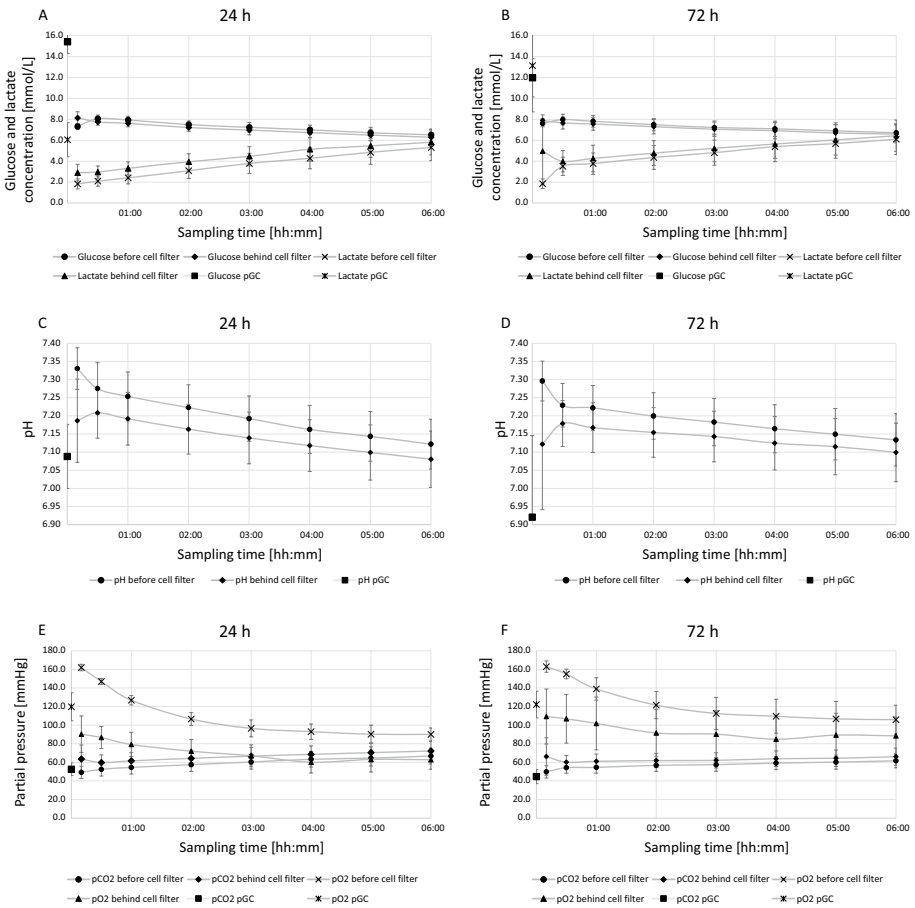

Fig. 5 Glucose consumption and lactate generation during the extracorporeal treatment simulation ($n = 10$). Glucose is consumed continuously. Likewise, lactate is generated as a product of cell metabolism. Also, there is a difference in concentrations before and after cell filter CC1 until the end of the treatment, showing that the cells are still active. pH values pressures in the plasma before and after cell filter CC1 during the extracorporeal treatment simulation ($n = 10$). Purified GC have a low pH, especially on day 3. After mixing with the physiologically conditioned plasma pool, the resulting pH is about 7.2 and decreases continuously by the metabolic activity of the cells. Oxygen and carbon dioxide partial pressures in the plasma before and after cell filter CC1 during the extracorporeal treatment simulation ($n = 10$). Oxygen is consumed, and carbon dioxide is generated as a product of cell metabolism

90.06 ± 7.00 mmHg after 6 h in the circuits after 1 day of storage. Oxygen partial pressures after PF CC1 exhibit considerably but non-significant lower values. Carbon dioxide partial pressures maintain a constant level during the entire circuit duration. After 3 days of storage, comparable courses of pO_2 and pCO_2 were detectable albeit at a slightly higher level (Fig. 5E, F).

Impact on LDH and fHB dynamics

Figure 6A and B shows free hemoglobin concentrations as an indication for erythrocyte damage which in turn allows conclusions on viability of the granulocytes. In view of the reference value (< 10 mg/dL), all values were low and rather decreasing during the experiment. Similarly, Fig. 5C and D shows LDH concentrations as an indication

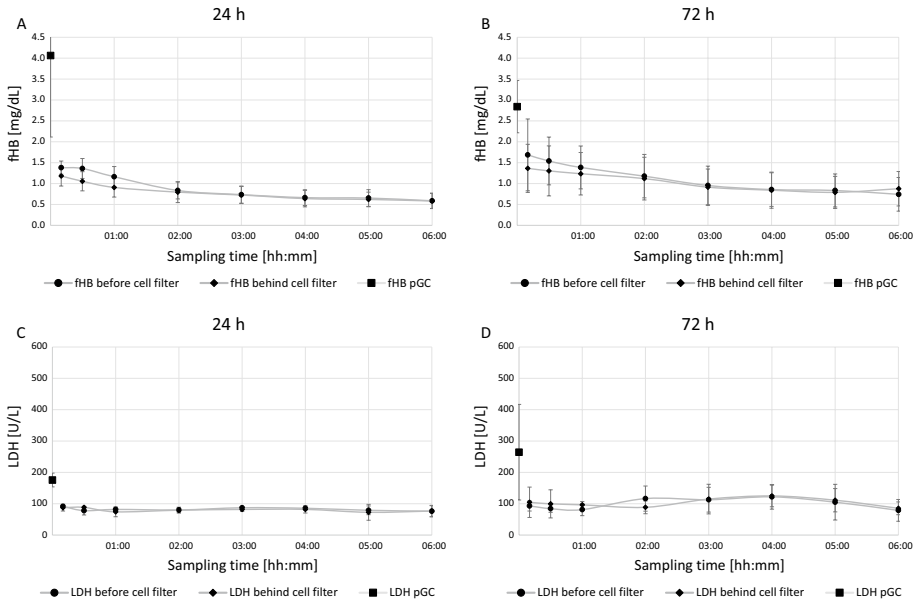

Fig. 6 Free haemoglobin concentrations as an indication for erythrocyte damage ($n = 10$). Reference value is < 10 mg/dL. All values are very low and rather decreasing during the experiments. LDH activity as an indication for cell damage. Reference value is < 225 U/L ($n = 10$). All values are within the physiologic range and stable during the experiments

for cell damage. Compared to the reference value (< 225 U/L), measured values were within the physiologic range and stable during the experiment

Cytokine expression

Interleukin-8 (IL-8) is a chemotactic factor that attracts neutrophils, basophils, and T-cells during the inflammatory process [26]. Monocyte chemoattractant protein-1 (MCP-1) is a potent monocyte-attracting chemokine [27]. IL-8 concentration of purified GC prior to initiation of the circuit simulation amounted to 14.78 ± 13.21 pg/mL (1 days of storage) and 115.39 ± 186.00 pg/mL (3 days of storage). During investigation, there was a significant increase up to values of 4648.67 ± 2637.27 pg/mL (1 day of storage) and 6744.53 ± 4547.30 pg/mL (3 days of storage) after 6 h ($P < 0.05$) (Fig. 7A, B). MCP-1 concentration of purified GC remained below 40 pg/mL for both cell populations before onset of the circuit simulation. During the experiment, there was a significant increase up to values of 887.63 ± 526.05 pg/mL (1 day of storage) and 587.01 ± 292.83 pg/mL (3 days of storage) after 6 h of treatment. Of particular interest is the fact that the cells performed the same on day 1 and day 3 supporting the extended storability of purified GC (Fig. 7C, D).

Discussion

The main findings of this ex vivo investigation demonstrate that purified GC were for the first time successfully used in an extracorporeal immune cell plasma perfusion system. The preparations are thereby capable of being used at least until the end of the third day after donation and purification. Its potential therapeutic effect is sustained

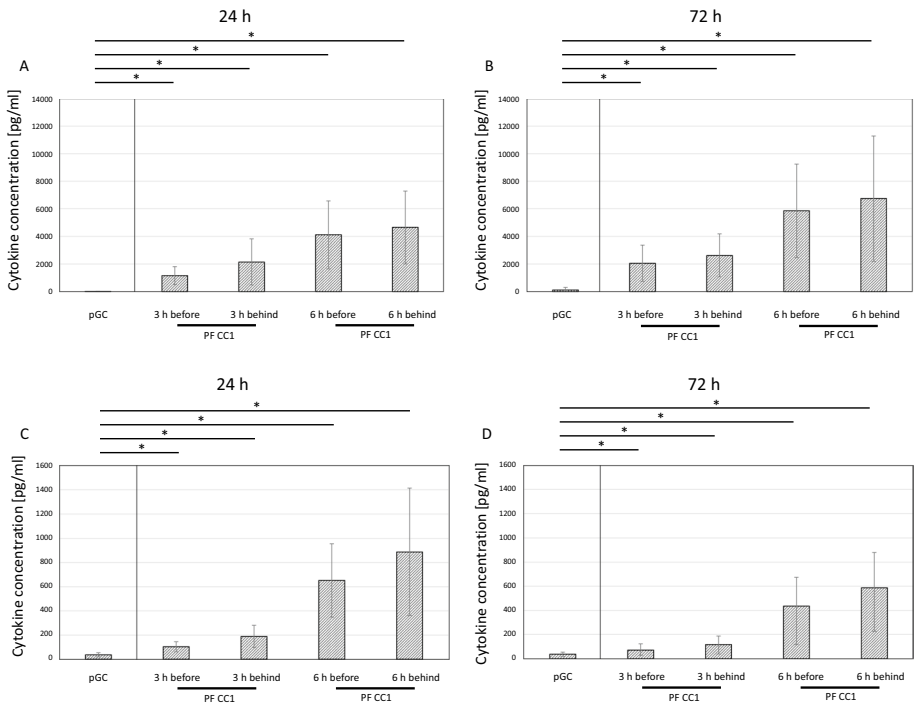

Fig. 7 Interleukin-8 concentration pressures in the plasma before and after cell filter CC1 during the extracorporeal treatment simulation ($n = 10$). IL-8 level is low in the pGC and secreted during the extracorporeal treatment simulation both on day 1 and day 3. *P* values ≤ 0.05 (*) were considered significant. MCP-1 concentration pressures in the plasma before and after cell filter CC1 during the extracorporeal treatment simulation ($n = 10$). MCP-1 is low in the pGC and actively secreted during the extracorporeal treatment simulation both on day 1 and day 3. *P* values ≤ 0.05 (*) were considered significant

for a minimum of 6 h of extracorporeal plasma perfusion. Cytokine release dynamics may even argue for a longer treatment period.

While therapeutic plasma exchange has shown some positive signals in treatment of septic shock other extracorporeal blood purification techniques targeting the early hyperimmune "cytokine storm" phase have not yet been able to provide convincing evidence for routine clinical practice [13, 28, 29]. Indeed, this is reflected in the recommendation issued by the surviving sepsis campaign [1]. It is noteworthy that recently several clinical trials addressing early septic shock indicate a potential mortality risk for patients. For example, the COMPACT-2 trial, which investigated the use of high-volume coupled plasma filtration and adsorption in patients in septic shock, was terminated prematurely. Mortality was found to be 54% in the treatment group and 29% in the control group [30]. Likewise, hemoadsorption using the CytoSorb adsorber failed to provide positive results in patients in refractory septic shock [31]. Another clinical investigation examined COVID-19 patients suffering from severe acute respiratory distress syndrome who underwent extracorporeal membrane oxygenation. Additive cytokine adsorption increased mortality by 58% as a secondary outcome parameter [32]. A commonality of these trials is the therapeutic approach focusing on the early proinflammatory phase during sepsis. As an underlying principle of action, circulating mediators are removed from the patient's blood.

However, current intensive care supports septic patients through this early sepsis phase into a state referred to as immunoparalysis and characterized by, e.g., lymphopenia, low human leukocyte antigen DR (HLA-DR) on monocytes and impaired granulocyte function [9, 33]. Consequently, sepsis patients are at higher risk of mortality due to secondary infections and multiorgan failure. Therefore, a therapeutic principle that provides restorative support to the patient's dysregulated immune system represents one approach in solving this challenge [19, 34].

Different extracorporeal approaches have been proposed to address this issue and to successfully influence the immune system imbalance, thereby affecting the clinical course and outcome of multiorgan failure and sepsis. Specifically, David et al. reported the results of a randomized controlled trial evaluating additive therapeutic plasma exchange in patients with severe refractory septic shock and observed rapid hemodynamic improvement after this intervention confirming earlier results of Busund et al [28, 29]. Moreover, encouraging results have been obtained from pilot clinical trials evaluating the extracorporeal immune cell therapy as an additive cell-based approach in patients with severe refractory septic shock [20, 21]. There are two main concepts underlying this approach: on the one hand, the plasma-modifying ability of human immune cells is exploitable (e.g., removal of antigenic material from the circulation, immune regulation by adsorption and secretion of cytokines), and on the other hand, control over these cells can be maintained (e.g., retention of cells in the extracorporeal circulation, prevention of local tissue effects) [19]

Granulocyte concentrates were studied as extracorporeal cell perfusion therapy in a pilot phase I trial with ten septic shock patients and showed safety and compatibility of this complex therapy. The pilot trial was conducted as a prospective uncontrolled clinical phase I/II study with 28-day follow-up. The subjects were treated twice for 6 h within 3 days with about 1.5×10^{10} granulocytes from healthy donors. On average, about 10 L separated plasma were treated by the therapeutic donor cells. During treatments, bacterial endotoxin concentration showed significant reduction. Furthermore, noradrenaline dosage could be significantly reduced while mean arterial pressure was stable. Also, C-reactive protein, procalcitonin, and HLA-DR showed significant improvement. Four subjects died in hospital, six subjects could be discharged [20].

An extension of that trial including 9 subjects with septic shock and 1 subject with severe sepsis using about double the amount of granulocytes focused on the dosage of norepinephrine in subjects and influence on dynamic and cell-based liver tests during extracorporeal therapies. Extracorporeal treatment with donor granulocytes showed promising effects on dosage of norepinephrine in subjects, liver cell function, and viability in a cell- based biosensor [21]

A drawback of these trials was that a two-loop system requires substantial material input, complex apheresis equipment and personnel. Besides, such a process is demanding in handling. Further, immune cells are subjected to high stress due to constant movement of the cells through the tubes. Therefore, a streamlined "one-way" plasma perfusion system was developed compatible with rather simple apheresis equipment providing additional advantages like ease of use and less space requirement on ICU.

Foremost, the use of purified GC provides a significantly reproducible and narrower range of granulocytes per unit than obtained by standard GC. This enables precise therapy control in everyday clinical practice. Another aspect involves the performance of the cells in the circuit. A previous investigation comparing standard GC and purified GC in a recirculating system revealed WBCs of purified GC to settle in the filter after a short period of time, whereas many of the WBCs of standard GC moved continuously in the recirculating system throughout the entire treatment cycle [20]. This phenomenon appeared to be related to the interaction between WBCs and erythrocytes. These interactions potentially prevented adhesion of the WBCs in the filter. Considering the significantly lower amount of erythrocytes, these effects are minimized in the circuit of purified GC, which consequently leads to the persistence of cells in the filter. However, granulocytic activity including phagocytosis and oxidative burst were preserved completely. Consequently, continuous movement of cells in the circuit system is superfluous in the presence of purified GC. This could be confirmed in the present study using a streamlined "one-way" plasma perfusion system, which demonstrated high viability, phagocytosis, and oxidative burst throughout the entire treatment cycle. This observation is also consistent with IL-8 and MCP-1 concentration being low in baseline purified GC and increasing significantly during extracorporeal treatment simulation on both day 1 and day 3.

Lower erythrocyte and platelet counts in purified GC lead to lower lactate production and thus a more balanced pH in the circuit compared to standard GC in earlier studies. Use of special storage bags that release carbon dioxide produced by the cells during storage also contributes to better pH values of purified GC supplied to the circuit. Glucose is continuously consumed. Likewise, lactate is generated as a product of cell metabolism. Moreover, concentrations before and after the cell filter PF CC1 differ until the end of the treatment, indicating the cells remain active.

There are several limitations to the validity of these data. Since plasma filtration from whole blood is a standard technique and also for ethical reasons, the patient model was designed using human donor plasma rather than whole blood. Therefore, an ex vivo simulation of the treatment differs from clinical application in several parameters. While no patient was connected to the system, a plasma pool with a volume of 1000 mL was applied. Hence, the solute distribution volume was inferior to that in a clinical environment. A stationary mixture of water, electrolytes and blood proteins constituted the used plasma pool. In consequence, adsorption, resorption, and synthesis functions of a human body remained incompletely modeled. Additionally, in order to avoid technical problems with clotting in the rather static plasma pool bag, anticoagulation was performed with greater intent than in clinical applications. Oxygen supply to the cells was attenuated compared to a clinical situation with oxygen continuously supplied by the patient.

Conclusion

The objective of the current study was to deploy purified GC in a streamlined extracorporeal plasma circuit designed for the treatment of septic patient. In conclusion, these results demonstrate that the streamlined "one-way" design entirely resembles previous results of the immune cell plasma perfusion system involving circulating

immune cells with regard to immune cell functions including phagocytosis, oxidative burst, cytokine secretion, viability, and metabolism. Furthermore, fully preserved functionality of purified granulocyte concentrates stored for 3 days could be confirmed. Therefore, subsequent clinical trials with the extracorporeal immune cell therapy can be applied by the streamlined "one way" system with the purified granulocyte concentrates stored for at least up to 3 days prior to extracorporeal use. In 2022, a multicenter randomized controlled trial with the one-way system will be started in septic shock patients.

Abbreviations
BC: Blood circuit; CC: Cell circuit; CE: Conformité européenne; GC: Granulocyte concentrates; G-CSF: Granulocyte-colony stimulating factor; GMP: Good Manufacturing Practice; NaCl: Sodium chloride; PF: Plasma filters.

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Author contributions
GK, SM, JA: supervision, conceptualization, methodology, investigation, statistical analysis, data interpretation, writing—reviewing and editing, visualization. LA, TW, BH, KA: investigation, data interpretation, critical revision of article. FD, SD, MS, DR: data interpretation, critical revision of article. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The ethical basis of preparation and utilization of granulocyte concentrates in the Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Germany including scientific purposes is of general validity and in accordance with informed consent of the donors that covers the procedure of this experimental investigation and for scientific publication.

Consent for publication
All coauthors provided consent.

Competing interests
FD, SD, TW, BH, SM, JA are employees or shareholders of ARTCLINE GmbH.
All other authors declare that they have no competing interests.

Author details
[1]Department of Anaesthesiology and Intensive Care Medicine, University of Rostock, Schillingallee 35, 18055 Rostock, Germany. [2]ARTCLINE GmbH, Schillingallee 68, 18057 Rostock, Germany. [3]Division of Nephrology, Department of Medicine, Medical Faculty, University of Rostock, Ernst-Heydemann-Str. 6, 18057 Rostock, Germany. [4] Department of Extracorporeal Therapy Systems, Fraunhofer Institute for Cell Therapy and Immunology, Schillingallee 68, 18057 Rostock, Germany. [5]Cellular Therapy Centre (CTC), Medizinische Hochschule Hannover, Feodor-Lynen-Str. 21, 30625 Hannover, Germany. [6]Center for Anesthesiology and Intensive Care Medicine, Hospital of Magdeburg, Birkenallee 34, 39130 Magdeburg, Germany. [7]Department of Anesthesiology and Intensive Care Medicine, University Medical Center Rostock, Schillingallee 35, 18057 Rostock, Germany.

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Purified granulocytes in extracorporeal cell therapy: A multifaceted approach to combat sepsis-induced immunoparalysis

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Gerd Klinkmann[1,2,3], Sophie Brabandt[1], Marlene Möller[1], Thomas Wild[4], Benjamin Heskamp[4], Jens-Christian Schewe[1], Martin Sauer[5,1], Jens Altrichter[2] and Steffen Mitzner[2,6]

Abstract

Background: Immune cell dysfunction plays a central role in sepsis-induced immunoparalysis. Targeted treatment using healthy donor immune cell transfusions, particularly granulocyte concentrates (GC) potentially induces tissue damage. Initial trials using GC in an extracorporeal immune cell perfusion system provided evidence for beneficial effects with fewer side effects, by separating patient and donor immune cell compartments. A multicenter clinical trial is exploring feasibility and effects of a 6-h treatment (NCT06143137). This ex vivo study examines technical feasibility and cellular effects of an extended treatment interval up to 24 h.

Methods: Standard GC were purified to increase the potential storage time and subsequently implemented in the extracorporeal immune cell perfusion system. Parameters assessed included cell viability, phagocytosis activity, oxidative burst, cytokine release, and metabolic parameters of purified. GC during an extended circulation time of up to 24 h.

Results: After storage of 72 h granulocytes were viable throughout the study period and exhibited preserved functionality and metabolic activity. The findings highlight a time-dependent nature of cytokine release by neutrophils in the extracorporeal circuit, as cytokine secretion patterns showed IL-8 peaking within 6 h, while MCP-1, IL-6, IL-1β, and TNF-α increased after 24 h of circulation.

Conclusion: Purified GC remain functional after 72 h of storage and additional 24 h in the circulating treatment model. Cytokine secretion patterns revealed a significant increase, especially between 10 and 24 h of treatment. Extending treatment time holds promise for enhancing immune response against sepsis-induced immunoparalysis. These findings provide valuable insights for optimizing immune-targeted therapeutic interventions.

Keywords

Extracorporeal therapy, sepsis, immunoparalysis, cytokine release, granulocyte concentrate

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Introduction

Septic conditions represent major challenges for health-care worldwide, as it places a significant burden on health-care systems and affects millions of patients every year. The disease results from a dysregulated host immune response to infection, leading to life-threatening organ dysfunction.[1] The early phase of sepsis is characterized by a hyperinflammatory state in which the immune system responds vigorously to fight the invading pathogen. However, a critical turning point is the immunoparalysis induced under septic conditions, which leads to a phase of immunosuppression.[2]

[1]Department of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University of Rostock, Rostock, Germany
[2]Department of Extracorporeal Therapy Systems, Fraunhofer Institute for Cell Therapy and Immunology, Rostock, Germany
[3]International Renal Research Institute of Vicenza, Vicenza, Italy
[4]Artcline GmbH, Rostock, Germany
[5]Center for Anesthesiology and Intensive Care Medicine, Hospital of Magdeburg, Magdeburg, Germany
[6]Department of Medicine, Division of Nephrology, Medical Faculty, University of Rostock, Rostock, Germany

Corresponding author:
Gerd Klinkmann, Department of Anaesthesiology, Intensive Care Medicine and Pain Therapy, University of Rostock, Schillingallee 35, Rostock, Mecklenburg-Vorpommern 18055, Germany.
Email: gerd.klinkmann@uni-rostock.de

Sepsis-induced immunoparalysis represents a complex and critical pathophysiologic state that has a significant impact on patient outcomes. During this phase, the immune system is severely impaired and key components such as neutrophils, monocytes, and other immune cells are functionally deficient.[3–6] This impaired immune response significantly reduces the patient's ability to clear the primary infection and makes them susceptible to secondary or opportunistic infections.[4] The clinical impact of sepsis-induced immunoparalysis is profound, as it significantly affects patient management and complicates the treatment of septic patients. The impaired immune response not only impedes recovery from the primary infection, but also makes it difficult to recognize and treat secondary infections, which can lead to sepsis-related complications and death.[5] In addition, the presence of immunosuppression can mask the typical clinical signs of infection, making early diagnosis and intervention difficult and further contributing to the negative consequences of sepsis.

While the importance of sepsis-induced immunoparalysis is well recognized, consensus on its clinical relevance and management remains elusive, and effective therapeutic principles capable of positively influencing immunoparalysis are currently lacking, resulting in guideline-based sepsis therapy primarily relying on supportive measures.[1,7,8] Thus, innovative and targeted therapeutic approaches are needed to effectively combat this complex immunosuppressive condition. One possible approach that is gaining increased attention is immunomodulation, which aims to restore the balance and function of the immune system in septic patients.[9]

Extracorporeal therapies have also emerged as a potential way to treat the immune system imbalance and multiorgan failure associated with sepsis.[10] The use of immune cells such as granulocytes in extracorporeal treatment promises to counteract sepsis-induced immunoparalysis and has so far shown promising results both experimental and clinically.[11–15] However, to realize the full potential of this therapeutic strategy, it is essential to rigorously evaluate the performance of granulocytes under realistic conditions that simulate the challenges they may encounter in clinical application. So far, all extracorporeal immune cell treatments were performed for 6 h each and up to 6 of these treatments per patient.[16,17] Here, we present *ex vivo* data regarding extended treatment times up to 24 h.

By studying the effects of this extended circulation time on cellular performance and in particular cytokine release, we aim to obtain valuable insights that can contribute to optimize immune cell based extracorporeal therapy strategies. Such advances promise to improve patient outcomes in critical care medicine to ultimately address the unmet clinical needs and challenges of sepsis-induced immunoparalysis. We hypothesize that an extended circulation time will result in altered cytokine release profiles without compromising cellular functionality.

Methods

Donors

Standard granulocyte concentrates (GC) were procured from healthy volunteers participating in the donor pools of the Institute of Transfusion Medicine and Transplant Engineering at Hannover Medical School, Germany. The donation process adhered strictly to the national and local Good Manufacturing Practice (GMP) guidelines for granulocyte concentrate donation.[18] To ensure the eligibility of donors for granulocytapheresis, the screening and testing criteria as specified by the Guidelines for the preparation of blood and blood components and the use of blood products of the German Medical Association were carefully met.[18]

Before undergoing granulocytapheresis, all donors provided written informed consent. Subsequently, 16–18 h before the granulocytapheresis procedure, they were stimulated by means of a single subcutaneous injection of granulocyte-colony stimulating factor (G-CSF) at a dosage of 6 µg/kg body weight. The specific G-CSF product used was Lenogastrim from Kohlpharma, Merzig, Germany. In parallel to the G-CSF, the donors were administered 8 mg of oral dexamethasone.

Granulocyte apheresis and purification

The procurement of standard GC was carried out using the continuous-flow apheresis method, employing the COBE Spectra OPTIA™ Apheresis System (Terumo BCT Europe, Garching, Germany).

Use of donor granulocyte concentrate (GC) has been limited due to its short storage time of 6–24 h, which is partially due to residual red blood cells (RBC) and platelets and the resulting lactate production leading to an acidotic milieu. To increase this storage time, our group developed a closed system procedure compatible with standard blood bank technologies to remove RBC and platelets and to enrich the GC. These efforts in the advancement of the purification process have been reported, leading to the successful extension of the storage duration to a minimum of 3 days.[16,17,19] To summarize, the standard GC underwent sedimentation (60–90 min) and centrifugation (300 *g* for 5 min at room temperature) to eliminate red blood cells, followed by a washing step to remove platelets. Thereafter, the purified GC were stored in gas-permeable storage bags, with ABO-compatible, citrate-anticoagulated blood plasma (final volume: 300 mL (range: 270–441 mL)). Each unit of the resulting purified GC was confirmed to contain at least 1×10^{10} granulocytes. Glucose concentration was set to 3.5–10.0 mmol/l. Storage conditions involved maintaining the purified GC at room temperature (22 ± 2°C), with no agitation.[19]

The purification procedure adhered strictly to the national and local Good Manufacturing Practice (GMP)

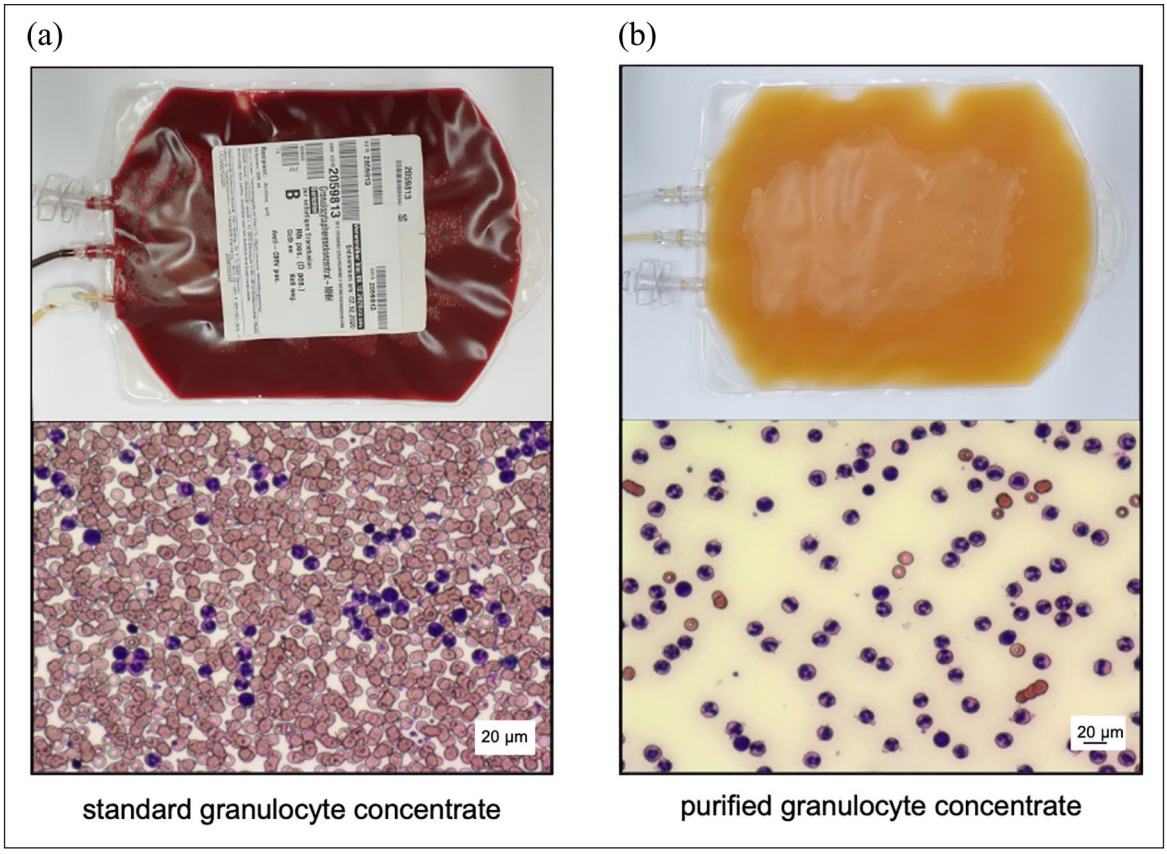


Figure 1. (a) Appearance and histology (400×) of A. standard GC and (b) purified GC. The higher purity and greatly diminished RBC count in the purified GC is evident from its yellow color resulting from the few remaining RBC and platelets.

guidelines and was conducted at the Cellular Therapy Centre, Hannover Medical School, Germany. Figure 1 provides a photograph of both the standard GC and the purified GC.

In vitro *plasma perfusion*

The purified GC is intended for therapeutic application in an extracorporeal treatment system designed to treat patients suffering from septic-induced immunoparalysis. Our laboratory *in vitro* model of extracorporeal immune cell therapy closely resembled the clinical application, with the exception of the absence of a human being. Instead, a standardized 1000 mL plasma pool obtained from donors was used as a representative model. Prior to its connection to the system, frozen plasma was thawed in a water bath set at 37°C.

To create a closed and sterile system, a CE marked disposable tubing set manufactured by Meise GmbH (Schalksmühle, Germany) and plasma filters (Medica SepaPlas, Surface area 0.6 m^2, max. Pore size 0.5 µm, Medolla, Italy) were interconnected. The resulting circuit was then filled and rinsed with a hemofiltration fluid consisting of MultiBic, supplied by Fresenius Medical Care (Bad Homburg, Germany) (Table 1). For a visual representation of the therapeutic setup, please refer to Figure 2.

Table 1. Composition of the rinsing solution.

K^+: 4.0 mmol/L	Cl^-: 111 mmol/L
Na^+: 140 mmol/L	HCO_3^-: 35 mmol/L
Ca^{2+}: 1.5 mmol/L	Glucose: 5.55 mmol/L
Mg^{2+}: 0.5 mmol/L	

In this study, heparin was utilized as an anticoagulant at a concentration of 5 IU/mL. To prime the system, 5 IU/mL of heparin were added to the rinsing solution. Following the assembly of the tubing and filter system, a purified GC was heparinized with 10 IU/mL and subsequently introduced into the plasma section of the tubing system, to avoid initiation of the coagulation cascade. This process effectively displaced the rinsing solution with an approximate volume of 400-450 mL of the cell preparation. Throughout the treatment, the GC remained contained within the hollow fibers of a plasma filter known as PF CC1.[16,17]

Subsequently, the system was connected to pooled human plasma, in which the concentrations of heparin and Ca^{2+} ions were adjusted to 20 IU/mL and 1.6-2.0 mmol/L,

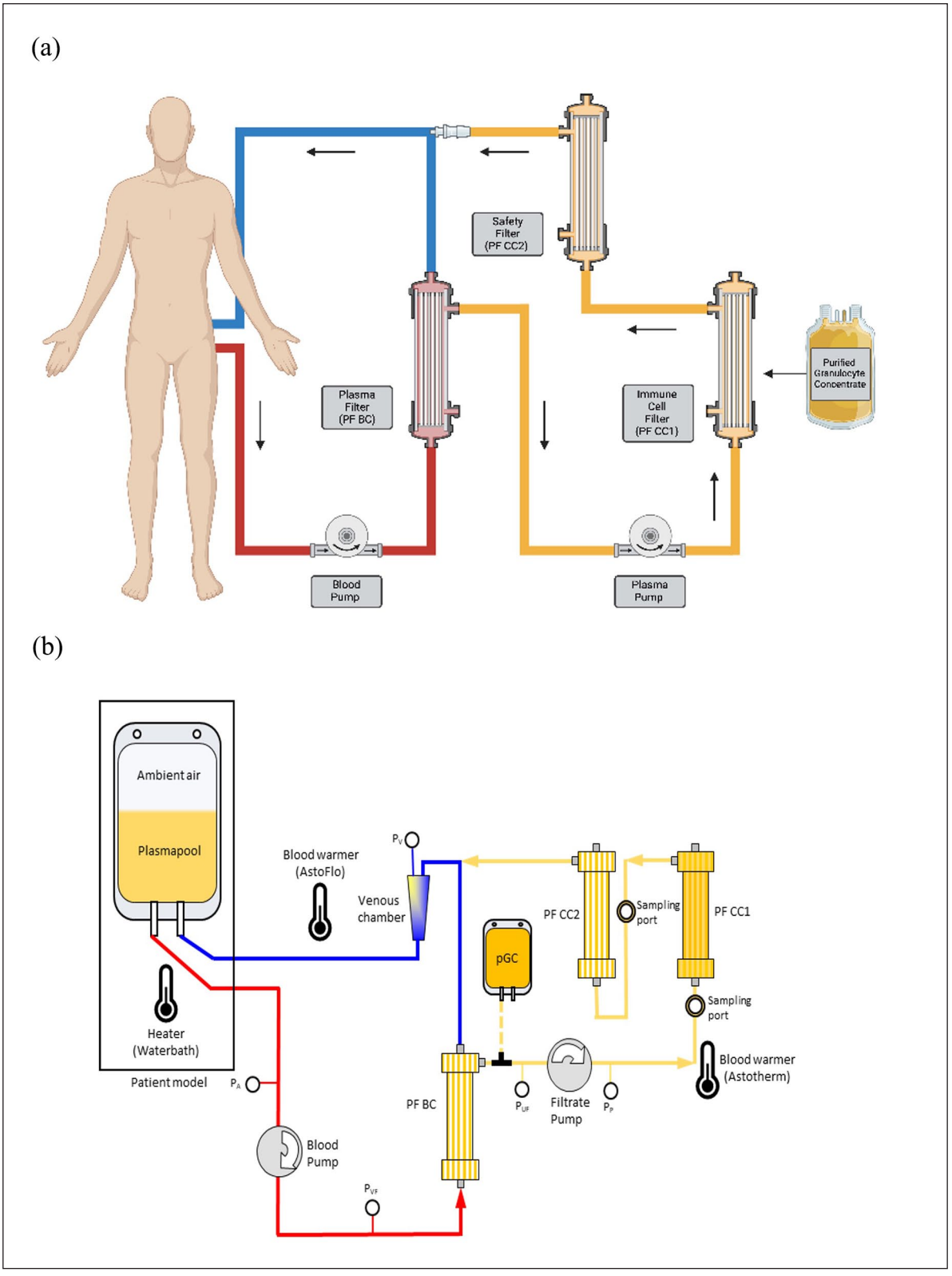


Figure 2. (a) The extracorporeal immune cell therapy is a plasma treatment technology. Plasma is continuously filtered from the patient's extracorporeal blood circuit and transferred into a closed-loop "cell circuit," where the patient's plasma is brought into direct contact with therapeutically effective, human-donor immune cells (i.e. the granulocyte concentrate). (b) Schematic of the treatment simulation experiments depicts a graphical representation of the components involved in the immune cell enhancement single-pass therapy system.

respectively. The targeted concentration of Ca^{2+} ions in the extracorporeal circuit was set to be above 1.0 mmol/L. Since purified GC cell preparations inherently contain sodium citrate as an anticoagulant, leading to minimal free Ca^{2+} ions, the initial Ca^{2+} ion concentration in the plasma pool was deliberately set higher to attain a level of >1.0 mmol/L after thorough mixing.

The total fluid volume, inclusive of the plasma pool, approximated 1850 mL. It was observed that the various components of the experimental setup homogeneously mixed within the first 30 min of the treatment simulation.

Settings for in vivo *application*

During the treatment phase, the pool plasma was introduced into the plasma filter PF BC at a rate of 150 mL/min with two different plasma filtration rates. A "10-h setting" (33 mL/min plasma filtration) and a "24-h setting" (13.5 mL/min) was defined, differing both in therapy duration and varying plasma filtration rates.

Subsequently, this separated plasma perfused the plasma filter PF CC1, wherein the treatment cells were housed, employing a dead-end filtration mode. Following this step, the plasma flowed through another plasma filter, PF CC2, also functioning in dead-end filtration mode. PF CC2 was designed to act as a safety barrier, specifically in the event of a membrane rupture in PF CC1, preventing donor cells from entering the patient's system. The treated plasma was then directed into the venous chamber, where it was thoroughly mixed with the "patient's blood" before being returned to the patient (Figure 2). This continuous treatment process spanned a duration of 10 or 24 h respectively.

Sampling

Plasma samples were obtained from the stream at regular intervals, upstream and downstream of the PF CC1 cell filter, ensuring hourly assessments. Furthermore, after 3, 6, 10, and 24 h of treatment, the immune cell containing filter PF CC1 was flushed back to obtain cells for subsequent analysis. A total of 13 extracorporeal experiments were conducted and analyzed. The experimental configuration of the circuit is illustrated in Figure 2(b).

Samples were collected at different sample ports in the circuits: upstream and downstream of cell filter PF CC1. To obtain cells sticking in the plasma filter PF CC1 after 3 and 6 h a sample was taken by backwashing plasma filter PF CC1 with 50 mL plasma and at the end of the circulations by backwashing with 2000 mL cold (4°C) NaCl solution.

Samples were taken and analyzed to determine viability and functional capacity. Furthermore pH, electrolyte, glucose, lactate, and lactate dehydrogenase activity.

At each filter, the pressure upstream and downstream the plasma filter membrane was monitored over the entire treatment duration.

Measurement of granulocyte function

Granulocyte function was analyzed in vitro with oxidative burst and phagocytosis assays using the commercial Phagoburst-Kit and Phagotest-Kit (Celonic, Heidelberg, Germany), respectively, by flow cytometry. Phagotest allows quantitative determination of leukocyte phagocytosis. It determines the percentage of phagocytes that take up fluorescein isothiocyanate (FITC)-labeled opsonized *E. coli* bacteria and their activity (mean fluorescence intensity that correlates with number of phagocytosed bacteria per cell). The Phagoburst test allows the quantitative determination of the oxidative burst of leukocytes. It detects the proportion of leukocytes that oxidize the fluorogenic substrate dihydrorhodamine (DHR) 123 to rhodamine 123 and their enzymatic activity (mean fluorescence intensity that correlates with amount of rhodamine 123 per cell). Both tests were used according to the manufacturer's instructions with one modification because the granulocyte concentration in GC is approximately 10 times higher than in whole blood. To achieve a concentration of 5000 granulocytes/μL and therefore the same ratio of granulocytes to the stimulus (ratio about 1:80; 20 μL of stabilized and opsonized FITC-labeled E.coli suspension containing approximately 4×107 bacteria were added) as with heparin-anticoagulated blood (4000–10,000 granulocytes/μL), the samples were diluted in heparin-anticoagulated plasma.

Cells are analyzed by flow cytometry using the blue-green excitation light (488 nm argon ion laser). Leukocytes were defined by fluorescence-2 (DNA staining), and the region containing leukocytes was analyzed by forward scatter versus side scatter to distinguish leukocyte subpopulations and identify neutrophils. Neutrophils from the control sample were then used to adjust the fluorescence-1 marker to detect $<3\%$ of positive events in the negative control.

Measurement of WBC viability

Blood cell content and white blood cell differentiation were evaluated automatically using a hematology analyzer (KX-21N, Sysmex, Norderstedt, Germany).

WBC viability was determined using the NucleoCounter NC-200 (ChemoMetec, Allerod, Denmark) according to the manufacturer's specifications. The Via1-Cassette™ is a specific device for cell sampling and staining for viability and cell counting applications with NucleoCounter® NC-200™. It incorporates the fluorophores acridine orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI) immobilized in the channels of the cassette. Using approximately 60 μL of a cell sample, the dyes AO and DAPI are

added to the cassette, causing them to dissolve in the mixing channels. AO stains both live and dead nucleus-containing cells and provides a total count, whereas DAPI stains the dead cells only. The method does not discriminate between the different WBC subsets. However, in pGC, approximately 96% of WBCs were neutrophilic granulocytes. Thus, WBC viability provides a sufficiently accurate approximation of neutrophil viability.

Measurement of electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures

Electrolytes, pH, glucose, oxygen, and carbon dioxide partial pressures were measured using an ABL90 Flex blood gas analyzer (Radiometer, Krefeld, Germany).

Evaluation of cell integrity by measurement of lactate concentration and lactate dehydrogenase activity

Concentrations of lactate and lactate dehydrogenase (LDH) activity were determined using a Cobas Mira Plus CQ (Roche, Ludwigsburg, Germany) according to manufacturer's specifications.

Pressures

Transmembrane pressures (TMP) of plasma filters were calculated from respective pressure values at the inlet of the plasma filter and its outlet(s) as TMP = Pre-membrane mean pressure − post-membrane pressure. TMP of PF BC (plasma filter blood circuit) was measured and displayed by the apheresis machine AFERsmart (Medica, Medolla, Italy). TMPs at PF CC1 and PF CC2 were determined by additional pressure measurements in the tubing set.

Measurement of cytokine concentration

Quantification of cytokine concentrations was conducted utilizing the LEGENDplex™ Human Essential Immune Response Panel (13-plex, BioLegend, Amsterdam, Netherlands). This panel constitutes a bead-based multiplex assay that employs fluorescence-encoded beads, rendering it compatible with a variety of flow cytometers. The immunoassay procedure was performed in strict accordance with the manufacturer's recommended instructions.

To achieve quantitative analysis, a flow cytometer (MACS Quant 16, Miltenyi Biotec, Bergisch-Gladbach, Germany) was employed, and the resulting data were subjected to analysis using the LEGENDplex v8.0 software.

Statistical analysis

Statistical analysis and data visualization was performed using IBM SPSS Statistics (version 27, Chicago, IL, USA) and GraphPad Prism 10 (GraphPad Software Inc., San Diego, USA). Results are expressed as median ± 95% confidence interval. According to the distribution of data (using Shapiro–Wilk test), Mann-Whitney *U* test was used for two independent samples for continuous variables. Kruskal-Wallis test was used to test differences between multiple independent samples with non-normal underlying population distribution, and appropriate post hoc tests were applied if necessary. A two-way mixed analysis of variance (ANOVA) was conducted to assess the change in time and its interaction with treatment mode. Statistical differences were considered significant at p value < 0.05.

Results

Distinct *in vitro* settings were examined to explore varied treatment strategies regarding treatment durations and cellular effects. A "10-h setting" and a "24-h setting" were defined, differing both in therapy duration and plasma filtration rates (33 mL/min vs. 13.5 mL/min).

Prior to extracorporeal application standard GC used to produce purified GC were prepared according to clinical standard procedures by granulocytapheresis from healthy donors and met the quality criteria of the guidelines of the German Medical Association.[18] The purified GC were stored for 72 h prior to use in the circuit experiments and still demonstrated high viability and functionality as described earlier.[19] All circuit experiments were run without technical problems.

Granulocyte function and viability

At predefined time points cell samples were taken by backflushing the cell containing filters. Viability in these samples was consistently high at over 90 % across all experiments during 10 h of treatment (Figure 3(a and b)). After 24 h viability decreased to a median of 84 ± 2.4%.

The percentage of granulocytes that performed phagocytosis of FITC labeled *E.coli* demonstrated preservation of granulocyte function in the circuit in both settings up to 24 h (Figure 3(c and d). The percentage of granulocytes that performed *E.coli* induced oxidative burst exhibited a trend toward improved cell performance within the "24-h setting" (Figure 3(e and f)).

Glucose consumption and lactate generation

During preparation of the purified GC glucose was supplemented to support a longer storage time. Therefore, after storage and prior to the circuit experiments the mean glucose concentration in purified GC amounts to 10.35 ± 1.08 mmol/L. Regarding the "10-h setting," after equilibration at $t = 30$ min within the circuit, a glucose concentration of 8.97 ± 0.81 mmol/L upstream of PF CC1 and 8.68 ± 0.82 mmol/L downstream of PF CC1 was observed.

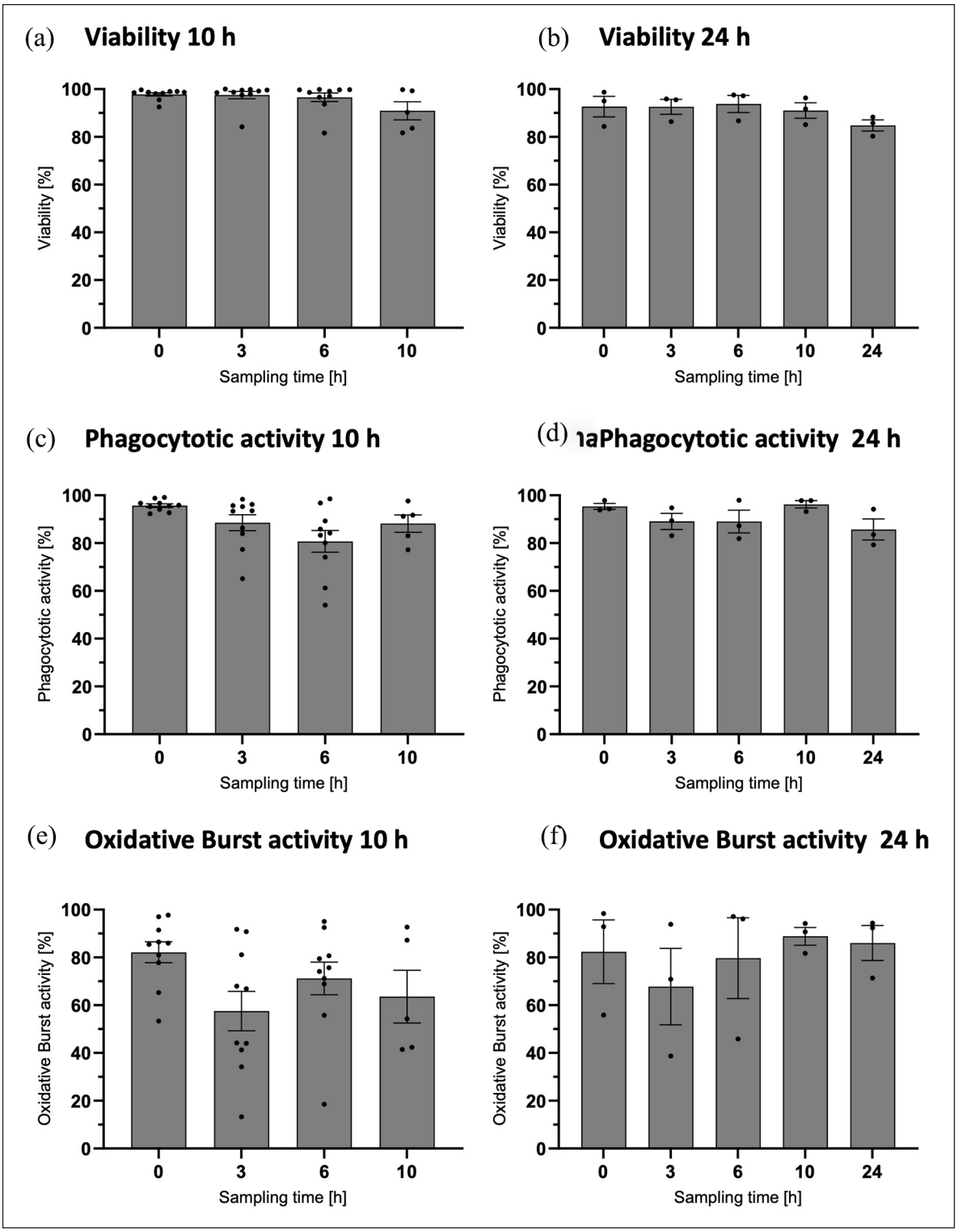


Figure 3. Functional granulocyte activity of purified granulocyte concentrate at defined observation time points. Viability (a and b); Phagocytic activity measured as the percentage of granulocytes that performed phagocytosis of FITC labeled E.coli (c and f); Oxidative burst activity measured as the percentage of granulocytes that performed E.coli induced oxidative burst (e and f). No statistically significant differences in cellular functionality were detected during the study period up to 24 h.

Subsequently, the mean concentration decreased steadily to 6.64 ± 1.22 mmol/L upstream of PF CC1 and 6.52 ± 1.22 mmol/L downstream of PF CC1 at the end of the 10-h analysis interval. A comparable pattern was observed in the "24-h setting." No significant differences were observed (Figure 4(a and b)).

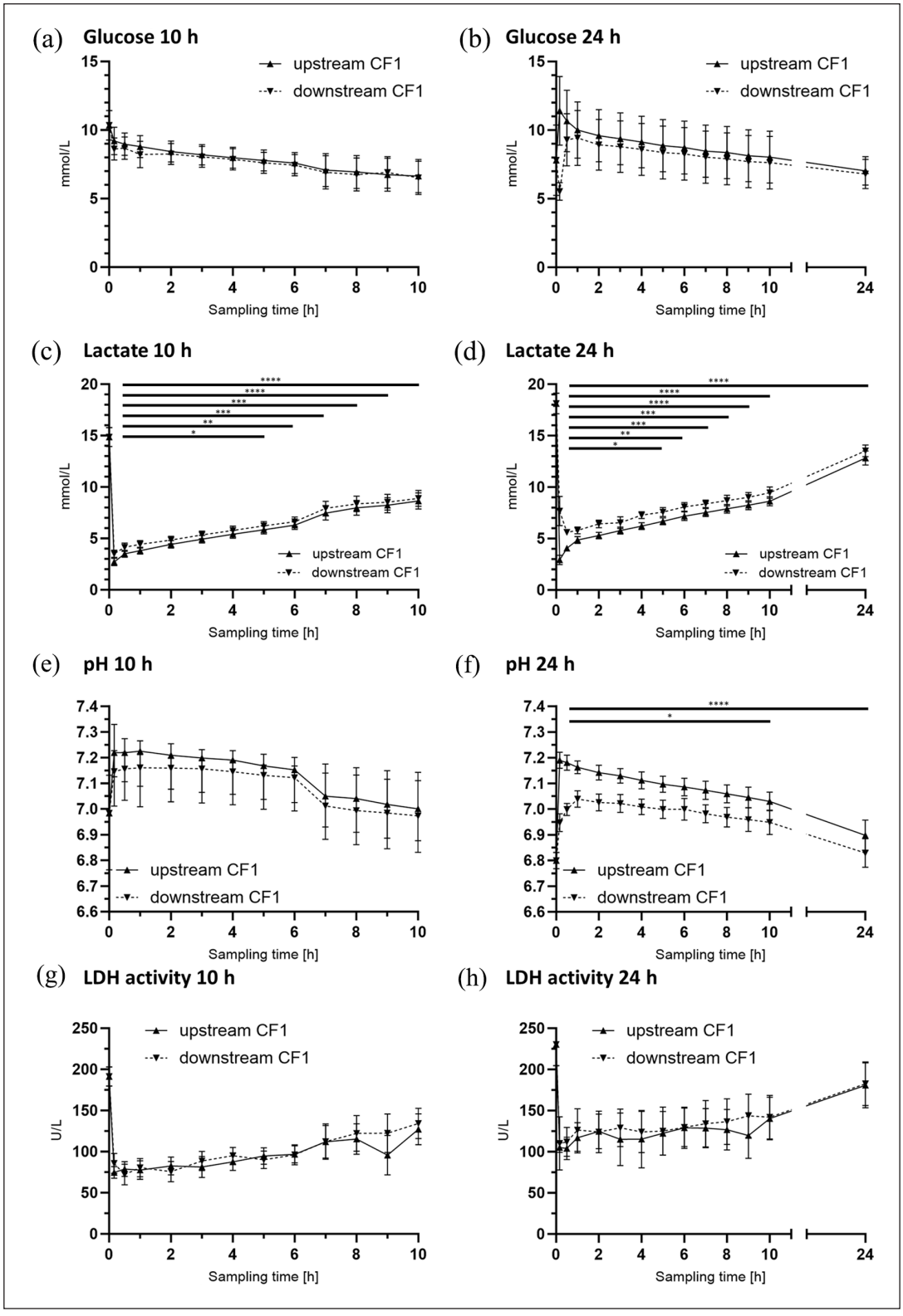


Figure 4. Cellular Metabolism: pH, Glucose, Lactate, and LDH Activity. Glucose is consumed continuously. Likewise, lactate is generated as a product of cell metabolism. Also, there is a difference in concentrations before and after cell filter PF CC1 until the end of the treatment, showing that the cells are still active (a–d). pH values in the plasma before and after cell filter CC1 during the extracorporeal treatment simulation (e and f). LDH activity was analyzed as an indication for cell damage. Compared to reference value (<225 U/L), measured values are within the physiologic range and stable during the experiments. Therefore, no relevant cell damage was observed (g and h). *$p \leq 0.05$, **$p \leq 0.01$,***$p \leq 0.001$, and ****$p \leq 0.0001$ were considered significant.

After storage, the mean lactate concentration of the purified GC was 14.86 ± 0.93 mmol/L. Regarding the "10-h setting," at 30 min after initiation of the simulation, equilibrated values in the circuit are 3.51 ± 0.34 mmol/L upstream of PF CC1 and 4.13 ± 0.41 mmol/L downstream of PF CC1. At 10 h, after a continuous increase, levels have 8.64 ± 0.78 mmol/L upstream of PF CC1 and 8.89 ± 0.77 mmol/L downstream of PF CC1. A comparable pattern was observed in the "24-h setting." Both the "10-h setting" and the "24-h setting" conditions exhibited a significant increase in lactate concentration after equilibration of the system until the end of each study period (Figure 4(c and d)).

PH values

After storage, the mean pH of the purified GC was 6.97 ± 0.05. Within the "10-h setting," after 30 min of equilibration, the pH values in the circuit were 7.22 ± 0.03 mmol/L upstream of PF CC1 and 7.16 ± 0.04 mmol/L downstream of PF CC1. At 10 h, after a continuous decrease, pH levels were 7.02 ± 0.05 mmol/L upstream of PF CC1 and 6.99 ± 0.05 mmol/L downstream of PF CC1. In comparison, the mean pH within the "24-h setting" showed a value of 7.23 ± 0.06 after equilibration at $t = 30$ min and decreased to 7.13 ± 0.07 during 10 h and to 6.89 ± 0.06 after 24 h of treatment. There was a significant difference in the "24-h setting" after 10 h ($p = 0.046$) and after 24 h ($p < 0.0001$) of treatment simulation (Figure 4(e and f))

Impact on LDH activity

Figure 4(g) and (h) show LDH activity as an indication for potential cell damage. Compared to the reference value (<225 U/L), measured values were within the physiologic range during the experiment.

Cytokine expression

Since there are two main concepts underlying the immune cell-based extracorporeal approach, namely, the plasma-modifying ability of human immune cells is exploitable (e.g. removal of antigenic material from the circulation, immune regulation by adsorption, and secretion of cytokines), and the maintenance of control over these cells (e.g. retention of cells in the extracorporeal circulation and prevention of local tissue effects),[20] we aimed to characterize the cytokines secreted by the cells during the treatment simulation.

For IL-8 the main secretion occurs until about 6 h of circulation. At 1 and 3 h the massive secretion of IL-8 is demonstrated both by the 100fold increase of the plasma concentration as well as in the higher concentrations downstream of PF CC1 compared to upstream of PF CC1.

IL-8 concentration of purified GC prior to initiation of the circuit simulation amounted to 135.39 ± 51.49 pg/mL. Both during the "10-h setting" investigation as well as in "24-h setting," the peak concentration was reached after 6 h (Figure 5(a and b)) with 13,511.39 ± 2463.1 pg/mL and 16,086.21 ± 1649.37 pg/mL respectively, followed by a plateau-like phase until the end of the investigations.

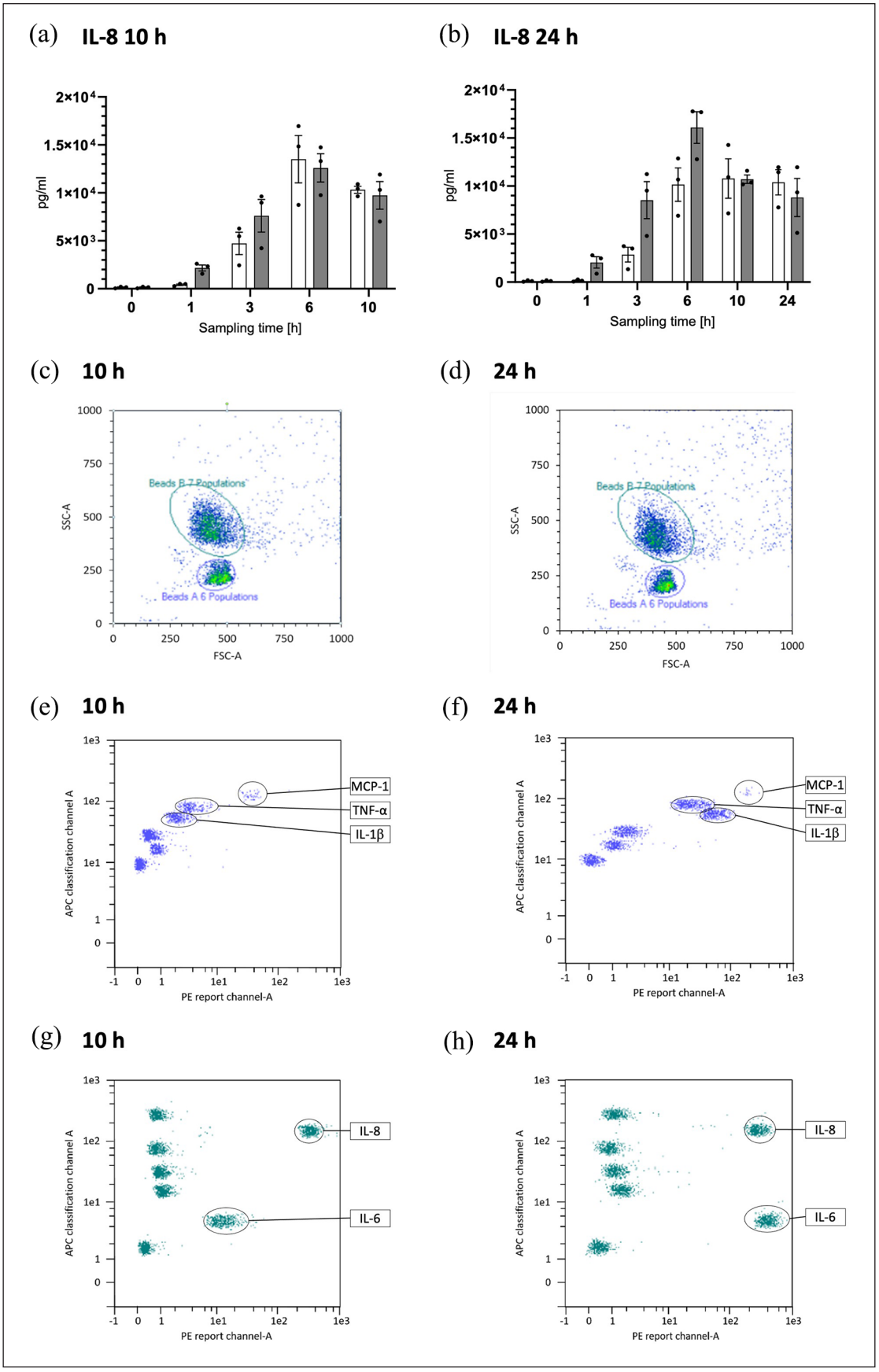
A different time pattern was observed with MCP-1 IL-6, IL-1β, and TNF-α. In the "10-h setting," the Monocyte chemoattractant protein-1 (MCP-1) concentration exhibited a moderate increase after 10 h. However, in the "24-h setting," a significant increase becomes apparent after a duration of 24 h ($p < 0.0001$; Figure 6).

The mean levels of the cytokines MCP-1, IL-6, IL-1β, and TNF-α followed comparable trajectories with main secretion post 10 h. After storage and prior to initiation of the circuit simulation cytokine levels remained low. Notably, only small changes were observed during the whole treatment simulation in the "10-h setting" (Figure 6 and Table 2). In contrast, a significant increase was observed in "24-h setting," which became apparent after a period of 24 h ($p < 0.0001$; Figure 6 and Table 3).

Discussion

We have performed this *ex vivo* investigation to study the effects of extended circulation time after prolonged storage of 72 h on the cellular performance of immune cells in the extracorporeal plasma perfusion system. The main findings indicate that the immune cells demonstrate preserved viability and functionality for the entire 24 h treatment period investigated here, even after 3 days of storage. Therefore, one can conclude, that the current 6-h clinical treatment regime can be extended up to 24 h. Furthermore, the incorporation of purified GC within the system elicits a time-dependent modulation of an active cytokine release.

The use of immune cells to treat sepsis-induced immunoparalysis in an extracorporeal setting was propounded by Mitzner et al. and has been generating beneficial effects both preclinically and in pilot clinical trials using standard GC and purified GC.[11–15,20] To date, data on functionality and optimal cell count of GC in the extracorporeal circuit is available.[12–15] The optimized purification process enables precise standardization of the cell count in the preparations, aligning with current specifications ranging between 1.2 and 2.5×10^{10} cells, a dosage recommended by Estcourt et al. for effectively mitigating infection risk.[19,21] Purified GC previously have been shown to maintain their effectiveness for at least 6 h during extracorporeal plasma perfusion. Specific evidence was provided by the preservation of various cell functions such as phagocytosis, oxidative burst, viability, and preserved cell metabolism. Moreover, purified GC retained their functional integrity even after being stored for 3 days prior to application in the extracorporeal circuit. In addition, initial *ex vivo*


(a) IL-8 10 h
pg/ml
Sampling time [h]
(b) IL-8 24 h
pg/ml
Sampling time [h]
(c) 10 h
Beads B 7 Populations
Beads A 6 Populations
SSC-A
FSC-A
(d) 24 h
Beads B 7 Populations
Beads A 6 Populations
SSC-A
FSC-A
(e) 10 h
MCP-1
TNF-α
IL-1β
APC classification channel A
PE report channel-A
(f) 24 h
MCP-1
TNF-α
IL-1β
APC classification channel A
PE report channel-A
(g) 10 h
IL-8
IL-6
APC classification channel A
PE report channel-A
(h) 24 h
IL-8
IL-6
APC classification channel A
PE report channel-A

(Continued)

Figure 5. Interleukin-8 concentration in the plasma upstream and downstream of cell filter PF CC1 during the extracorporeal treatment simulation (a and b). Representative illustration of cytokine determination using the LEGENDplex™ HU Essential Immune Response Panel Kit from Biolegend® (MACS Quant 16, Miltenyi Biotec, Bergisch-Gladbach, Germany). Cytokines bind to beads, which are divided into 2 populations in the forward/sidescatter dot plot: Beads A: (lower circle) contain the beads for the following cytokines: IL-4, IL-2; CXCL10 (P-10), IL-1β, TNF-α, and CCL2 (MCP-1). Beads B: (upper circle) include IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, CXCL8 (IL8), and Free Active TGF-ß1 (c and d). Plot of fluorescence intensity in the Allophycocyanin (APC) channel against fluorescence intensity in the Phycoerythrin (PE) channel (the more Streptavidin-Phycoerythrin (SA-PE) has bound, the higher the cytokine concentration ⩾right shift in the dot-plot diagram); Cloud populations of distinct cytokines are highlighted: ILIL-4, IL-2; CXCL10 (P-10), IL-1β, TNF-α, and CCL2 (MCP-1). (e and f) IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, CXCL8 (IL8), and Free Active TGF-ß1 (g and h).

findings indicated that cytokine release dynamics were in favor of an extended treatment protocol.[16,17]

This is a special feature of the cell-based therapeutic approach, as current findings on adsorptive blood purification procedures for the treatment of sepsis indicate increased efficacy due to dose dependency.[22] Hence, the question still arises as to which application scheme should most suitable for the extracorporeal immune cell plasma perfusion system.

The question of optimal dosing of extracorporeal therapy in critically ill patients is a key problem, even in areas that have been established for decades, such as renal replacement therapy (RRT). In clinical practice, determining the optimal dosage regime as well as the selection among different modalities involves a thorough consideration of primary treatment goals and associated risks. Tailoring the administered dose is linked to technical specifications such as membrane characteristics, pore sizes, and blood flow rates, in conjunction with an understanding of the pathophysiological basis of substance removal. While intensive dosing has been advocated in the past, a more moderate approach has recently been adopted due to conflicting results regarding efficacy.

In particular, high volume hemofiltration (HVHF) and very high volume hemofiltration (VHVHF) provide a high clearance and thus offer a reduced toxic potential and have been studied for immunomodulation in acute kidney injury (AKI) and sepsis, with small studies and the IVOIRE trial providing no conclusive evidence of increased mortality or hemodynamic benefits compared to standard volume hemofiltration (SVHF) at 35 mL/kg/h, leading to HVHF no longer being recommended in septic shock with AKI.[23–25]

Similarly, the current recommendations on hemoadsorption in sepsis indicate an intensive dosage regimen focused on the amount of blood treated may be beneficial to patients.[26,27] Schultz et al. reported the observed mortality rates to be reduced when processed blood purification volumes were higher than 6 L/kg. These results suggest that hemoadsorption may improve survival in critically ill patients, assuming that the applied dosage is sufficiently high.[28]

These nuanced approaches underscore the significance of dose regimen selection in established therapies, balancing therapeutic principles with clinical outcomes. Therefore, a personalized therapeutic strategy is even more pivotal, particularly within the complex domain of bioartificial therapies. Hence, an examination was conducted on the time course of cytokine release in different scenarios within the extracorporeal cell perfusion system.

In order to gain a differentiated understanding of this phenomenon, it is crucial to consider that alongside monocytes and macrophages, neutrophil granulocytes, a subgroup of granulocytes, form the primary line of defense against microbial threats.[29] In the past, neutrophils were exclusively considered to be functional effector cells that release antimicrobial and cytotoxic proteins stored in granules. However, according to recent evidence, neutrophils are also considered a major sources of secreted cytokines that influence the inflammatory response.[30] Indeed, neutrophils have been shown to produce newly synthesized cytokines by gene induction, beside the ability to constitutively express cytokines from pre-formed reservoirs. Cytokines, both preformed and newly synthesized, are released through regulated exocytosis upon ligand-receptor signaling, and via constitutive exocytosis following transport through recycling endosomes, while variations of these secretion pathways have also been demonstrated.

Indeed, it is known that the interaction of neutrophils with a specific agonist elicits a distinct, time-dependent response owing to the various transport pathways.[29]

Our findings clearly illustrate that the release of cytokines by purified GC that mainly contain neutrophils in the extracorporeal circuit is time-dependent. This was demonstrated by the significant differences in cytokine concentrations during the time course in comparison of the "24-h" setting to the "10-h" setting.

Since cytokines are released in a hierarchical seque nce,[29,31–35] relevant cytokines secreted by neutrophils and other immune cells, described to contribute to the development of pro- and anti-inflammatory process were selected, in order to investigate their release in the cell perfusion system.

IL8, a key cytokine involved in inflammatory responses, is secreted in a complex manner. It can be released into the extracellular milieu either from intracellular stores or by *de novo* synthesis via the classical secretory pathway.[36,37] This secretion process is closely associated with various cellular compartments, including secretory vesicles, which are readily mobilized organelles.[38] In addition, IL8 is

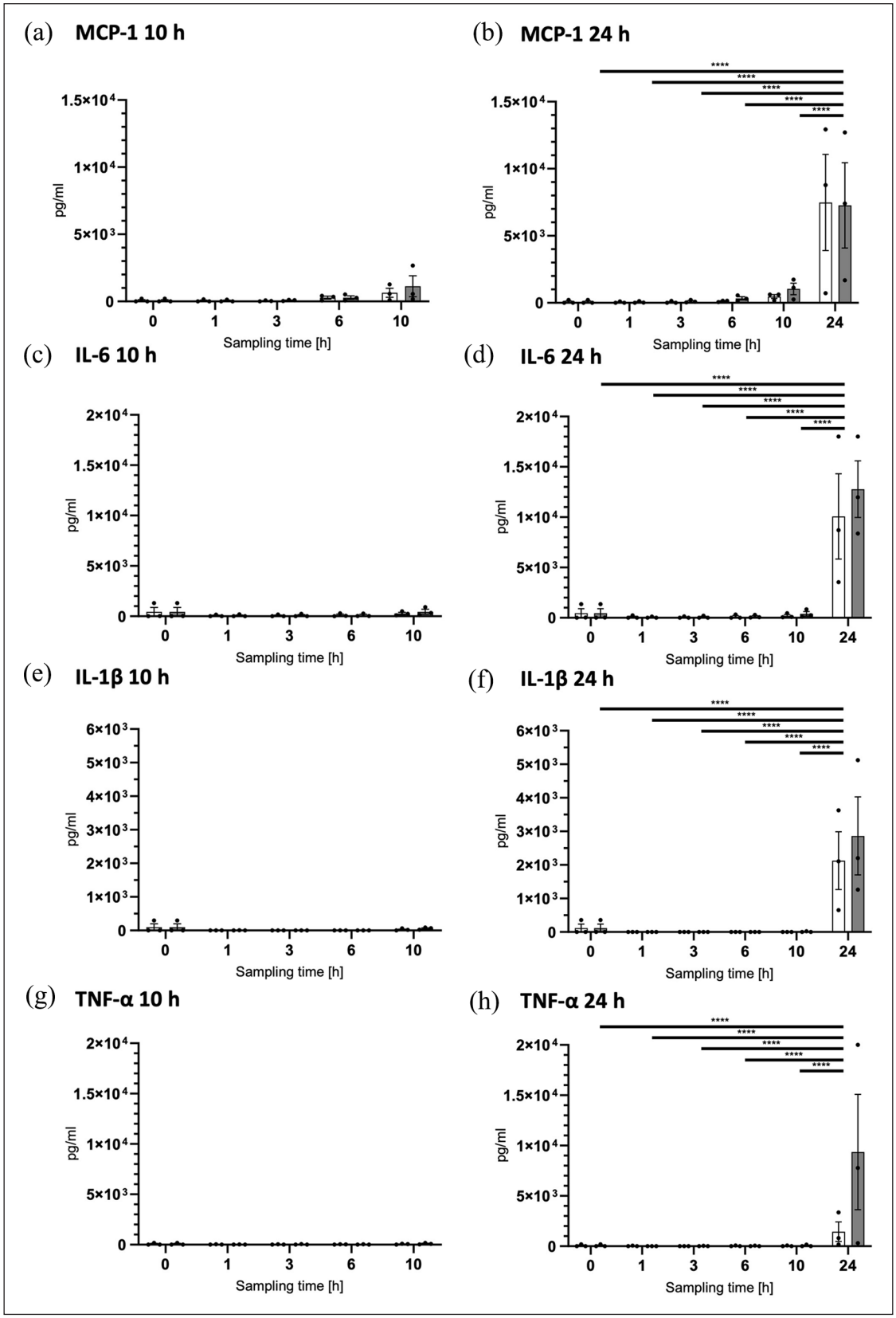


Figure 6. Interleukin concentrations in the plasma upstream and downstream of cell filter PF CC1 during the extracorporeal treatment simulation. Mean levels of the cytokines MCP-1, IL-6, IL-1β, and TNF-α follow comparable trajectories. Within the "10-h setting," concentrations show a slight increase after 10 h. Within the "24-h setting," a significant increase becomes apparent only after a duration of 24 h ($p < 0.0001$). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ were considered significant.

Table 2. Cytokine concentrations [pg/mL] "10-h setting."

Cytokine	Hours	Upstream PF CC1		Downstream PF CC1	
		Mean	SEM	Mean	SEM
IL-8	0:00:00	135.43	51.50	135.43	51.50
	1:00:00	423.01	73.66	2168.84	314.48
	3:00:00	4727.75	1165.35	7611.18	1699.69
	6:00:00	13511.39	2463.51	12599.71	1482.84
	10:00:00	10325.35	370.67	9736.08	1441.55
MCP-1	0:00:00	68.26	68.26	68.26	68.26
	1:00:00	50.78	50.78	49.19	44.03
	3:00:00	37.63	22.63	81.13	24.37
	6:00:00	278.44	109.39	284.04	131.84
	10:00:00	645.97	335.36	1133.44	777.73
IL-6	0:00:00	447.33	430.83	447.33	430.83
	1:00:00	50.18	50.18	62.49	62.49
	3:00:00	62.35	59.18	80.95	76.78
	6:00:00	118.58	86.60	111.17	85.81
	10:00:00	271.47	117.43	449.61	237.98
IL-1β	0:00:00	99.05	99.05	99.05	99.05
	1:00:00	0.00	0.00	2.81	2.81
	3:00:00	0.00	0.00	0.00	0.00
	6:00:00	0.00	0.00	0.00	0.00
	10:00:00	25.99	18.25	57.95	19.39
TNF-α	0:00:00	60.92	60.92	60.92	60.92
	1:00:00	11.47	11.47	9.49	7.20
	3:00:00	15.51	14.13	23.92	18.03
	6:00:00	26.31	11.79	21.07	10.20
	10:00:00	52.93	20.62	90.28	46.19

stored in cytoplasmic granules within neutrophils, allowing for rapid mobilization and release upon cellular activation.[37,38] Furthermore, the presence of IL8 in different types of granules suggests a mechanism for sustained secretion over time. IL8 secretion occurs in two distinct phases: an early secretory phase directly induced by stimulation and a late secretory phase triggered by the release of other pro-inflammatory mediators such as TNFα and IL-1β.[36] These data align with our observations, indicating that following the rapid increase and peak concentration, a plateau phase occurs after 6 h indicating no further secretion beyond 6 h. This phenomenon corresponds to the interactions between the signal pathways and is reflected by the late concentration increases of TNFα and IL-1β.[39]

TNF-α serves as a robust stimulus for neutrophils, enhancing phagocytosis, respiratory burst activity, and degranulation. Neutrophil's ability to release TNF-α in response to various stimuli suggests broader roles in host defense beyond microbial killing. This hints at neutrophils activating themselves through autocrine/paracrine mechanisms to enhance immune functions.[29,30] This finding also provides evidence of a time-dependent effect profile of the cell-based extracorporeal therapy.

The cellular components of the purified GC include monocytes as well as lymphocytes and platelets beside the majority of granulocytes. This composition is evident in the observed increases in Monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) concentrations after 24 h. MCP-1 is recognized for its role as a potent chemokine, specifically responsible for attracting monocytes. Conversely, the presence of IL-6 serves as an indication of potential monocyte presence. IL-6, acting as a proinflammatory cytokine, not only induces the expression but also facilitates the secretion of MCP-1, thereby amplifying its involvement in immune responses and inflammation.[40] These dynamics highlight the significance of time-dependent interactions within the treatment simulation, reflecting the complex interplay at the mediator and cytokine levels.

Our study furthermore provides valuable insights into the pattern of action of immune cells in an extracorporeal environment, as it incorporates an approach that considers both cell functionality and the time course of cytokine secretion. Cell activity demonstrated preserved viability, phagocytosis and oxidative burst throughout the entire treatment cycle. Moreover, cell functionality inside the cell containing filters might be even higher, as only a portion of the cells could be flushed out of the filters.[16,17] It is possible that the most active cells exhibited stronger adhesion to the filter, making them inaccessible for analysis.

Table 3. Cytokine concentrations [pg/mL] "24-h setting."

Cytokine	Hours	Upstream PF CC1		Downstream PF CC1	
		Mean	SEM	Mean	SEM
IL-8	0:00:00	117.93	42.80	117.93	42.80
	1:00:00	158.72	59.86	2044.01	593.67
	3:00:00	2856.62	761.06	8528.74	1922.40
	6:00:00	10153.22	1742.49	16086.21	1649.36
	10:00:00	10786.62	2055.57	10712.10	444.91
	24:00:00	10393.79	1319.44	8808.81	1990.59
MCP-1	0:00:00	73.84	73.84	73.84	73.84
	1:00:00	33.79	33.79	47.61	40.70
	3:00:00	46.50	46.50	108.03	56.60
	6:00:00	120.83	30.95	337.44	119.38
	10:00:00	467.97	152.26	1040.46	430.72
	24:00:00	7476.92	3587.93	7263.85	3184.27
IL-6	0:00:00	463.54	449.56	463.54	449.56
	1:00:00	82.24	82.24	43.93	43.93
	3:00:00	45.00	45.00	70.91	70.91
	6:00:00	115.95	101.81	125.34	87.43
	10:00:00	206.08	124.29	399.43	224.02
	24:00:00	10081.27	4231.80	12780.39	2809.72
IL-1β	0:00:00	118.63	118.63	118.63	118.63
	1:00:00	0.00	0.00	0.00	0.00
	3:00:00	0.00	0.00	0.00	0.00
	6:00:00	0.00	0.00	0.00	0.00
	10:00:00	0.00	0.00	7.35	7.35
	24:00:00	2129.28	860.09	2863.12	1162.76
TNF-α	0:00:00	65.75	65.75	65.75	65.75
	1:00:00	16.91	16.91	0.00	0.00
	3:00:00	0.00	0.00	14.61	12.93
	6:00:00	23.13	23.13	17.03	17.03
	10:00:00	28.16	24.94	62.70	49.84
	24:00:00	1444.65	970.24	9358.67	5740.37

Glucose is continuously consumed. Likewise, lactate is generated as a product of cell metabolism. Moreover, concentrations upstream and downstream of cell filter PF CC1 differ until the end of the treatment, indicating the cells remain active.

Another aspect of this study was, that the plasma flow rate of 13.5 mL/h in the "24-h" setting was markedly slower than in the "10-h" setting with 33 mL/h. However, the increase of the cytokine concentrations was rather similar during the first 10 h in both settings. This indicates that the amount of treated plasma itself does not constitute the essential parameter, but rather the contact time between plasma and cells in the extracorporeal compartment.

There are several limitations to the validity of these data. This study provides valuable information on the functionality of extracorporeal immune cell therapy, which is intended for the treatment of sepsis-induced immunoparalysis. In the *ex vivo* simulation presented here, the immune cells are exposed to healthy but foreign donor plasma. The adaptive immune response needs to be demonstrated in clinical studies in patients. Since plasma filtration from whole blood is a standard technique and also for ethical reasons, the patient model was designed using human donor plasma rather than whole blood. Therefore, an *ex vivo* simulation of the treatment differs from clinical application in several parameters. While no patient was connected to the system, a plasma pool with a volume of 1000 mL was applied. Hence, the solute distribution volume was inferior to that in a clinical environment. A stationary mixture of water, electrolytes, and blood proteins constituted the used plasma pool. In consequence, adsorption, resorption, and synthesis functions of a human body remained incompletely modeled. Additionally, in order to avoid technical problems with clotting in the rather static plasma pool bag, anticoagulation was performed with greater intent than in clinical applications. Oxygen supply to the cells was attenuated

compared to a clinical situation with oxygen continuously supplied by the patient.

Conclusion

In conclusion, significant findings emerged regarding the functionality and cytokine secretion profile of purified granulocyte concentrates in *ex vivo* circulation models. Firstly, the immune cells within the purified granulocyte concentrate remained fully functional even after 3 days of storage resulting from the purification process, with an additional 24 h of circulation in the treatment model, emphasizing their stability over extended periods. Secondly, dynamic changes in cytokine profiles were observed during *ex vivo* circuit experiments. Notably, IL-8 secretion peaked within the initial 6 h, while MCP1, IL-6, IL-1β, and TNF-α were predominantly secreted after 24 h, indicating a nuanced response pattern with prolonged circulation. Lastly, extending treatment time holds promise for enhancing combat against immunoparalysis, suggesting potential for further optimization of immune response mechanisms. These results offer valuable insights into the functional integrity and dynamic behavior of immune cells, informing future strategies for therapeutic interventions targeting immune dysfunction.

List of abbreviations

AKI acute kidney injury
BC blood circuit
CC cell circuit
CE conformité européenne
GC granulocyte concentrates
G-CSF Granulocyte-Colony Stimulating Factor
GMP Good Manufacturing Practice
HVHF High volume hemofiltration
NaCl sodium chlorid
PF plasma filters
SEM Standard of the mean
SVHF standard volume hemofiltration
VHVHF very high volume hemofiltration

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Author contributions

GK, SM, and JA: Supervision, Conceptualization, Methodology, Investigation, Statistical analysis, Data interpretation, Writing—Reviewing and Editing, and Visualization. TW, BH, SB, and MM: Investigation, Data interpretation, and Critical revision of article. JCS and MS: Data interpretation and Critical revision of article. All authors read and approved the final manuscript.

Declaration of conflicting interests

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Ethical approval and consent to participate

The ethical basis of preparation and utilization of granulocyte concentrates in the Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Germany including scientific purposes is of general validity and in accordance with informed consent of the donors that covers the procedure of this experimental investigation and for scientific publication.

Consent for publication

All coauthors provided consent.

ORCID iDs

Gerd Klinkmann https://orcid.org/0000-0002-8922-8700
Thomas Wild https://orcid.org/0009-0009-4024-8530

Availability of data and material

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

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