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BRIEF REPORT

TRANSFUSION

Detection of SARS-CoV-2-independent immunoregulatory activity of COVID-19 convalescent plasma

Regine Brox | **Susanne Achenbach** | **Holger Hackstein**

Department of Transfusion Medicine and Hemostaseology, University Hospital Erlangen, Erlangen, Germany

Correspondence
Regine Brox, Department of Transfusion Medicine and Hemostaseology, University Hospital Erlangen,
Erlangen 91054, Germany.
Email: regine.brox@uk-erlangen.de

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Abstract

Background: Convalescent plasma has emerged as a potential specific treatment for coronavirus disease 2019 (COVID-19), since it contains severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies. Several studies are currently investigating the efficacy of convalescent plasma for treatment of COVID-19, with a focus on neutralizing antibodies. However, there is little information on whether convalescent plasma may contain additional immunoregulatory constituents produced by the blood donor during convalescence. Therefore, using a standardized whole blood assay employing synthetic toll-like receptor (TLR) ligands, we have investigated the immunoregulatory capacity of convalescent plasma in direct comparison to ABO-matched allogeneic control plasma.

Study design and methods: Whole blood samples from healthy blood donors were collected, and autologous plasma was replaced by convalescent plasma or ABO-matched control plasma. Standardized innate immune triggering and monitoring was performed by adding different TLR ligands (Pam3CsK4 [TLR1/2], HKLM [TLR2], LPS [TLR4], flagellin [TLR5], ssRNA40 [TLR8], imiquimod [TLR7], and FSL-1 [TLR2/6]) and subsequent quantitative analysis of pro- and anti-inflammatory cytokines (IP-10, IL-1β, TNF-α, MCP-1, IL-6, IL-10, and IFN-γ) by cytometric bead array. Negative controls included unstimulated samples as well as samples spiked with autologous plasma.

Results: COVID-19 convalescent plasma (CCP) significantly decreased pro-inflammatory cytokines production triggered by different TLR ligands in healthy donors as compared with healthy control plasma. IL-6, MCP-1, and IFN-γ represented the cytokines that are most frequently downregulated by convalescent plasma.

Conclusion: Our experiments reveal a potential novel, SARS-CoV-2-independent immunomodulatory activity of CCP, which may be beneficial for COVID-19 patients.

Abbreviations: CCP, COVID-19 convalescent plasma; COVID-19, coronavirus disease 2019; IgG, Immunoglobulin G; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Th2, Type 2 helper T cells; TLR, toll-like receptor; TPE, therapeutic plasma exchange.

KEYWORDS
convalescent plasma, COVID-19

1 | INTRODUCTION

Owing to the current coronavirus disease 2019 (COVID-19) pandemic, which accounts for more than 150 million infections and 3 million deaths worldwide, there is a high demand for treatment options.[1] COVID-19 convalescent plasma (CCP), plasma collected from patients who have recovered from COVID-19, offers a potential therapy for critically ill patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[2] Historical evidence suggests the potential of convalescent plasma transfusions to improve the clinical course of different viral infectious diseases, such as influenza,[3] ebola,[4] SARS,[5] and Middle East respiratory syndrome (MERS)[6] with varying degrees of success. Current studies indicate that convalescent plasma collected from patients who have recovered from COVID-19 contains antibodies to SARS-CoV-2 that can be passively transferred to the plasma recipient and might protect against a severe course of the disease.[7,8] However, evidence supporting the use of convalescent plasma as a therapeutic strategy against COVID-19 remains inconclusive due to a high variability in selection criteria for donors and best-suited recipients, effective titers of neutralizing antibodies, and optimal timing of administration.[9] Randomized and controlled clinical trials evaluating the effectiveness and safety of CCP are ongoing.[10]

While more attention has been focused on direct antiviral activity of SARS-CoV-2 antibodies in convalescent plasma, plasma contains a large variety of immunomodulatory components including cytokines, clotting factors, hormones, natural antibodies, and other proteins, which may interfere with the antiviral and anti-inflammatory immune response.[11,12] We hypothesized that during convalescence from COVID-19 infection, the production of potential immunomodulatory plasma factors may have been enhanced and could contribute to its clinical effectiveness.

Therefore, we used an in vitro whole blood assay employing synthetic toll-like receptor (TLR) ligands and healthy donor WBCs to address the question of whether CCP has SARS-CoV-2-independent immunomodulatory effects.

2 | MATERIALS AND METHODS

To investigate the immunomodulatory effects of CCP, we used a standardized whole blood assay as a simple model for infection-induced inflammation. Stimulation of various TLRs, a family of pattern recognition receptors, serves as an ideal experimental model for infections since the first line of defense against pathogens is simulated, thereby bridging innate and adaptive immunity by the activation of antigen-presenting cells and the release of inflammatory cytokines.[13] The diversity of TLR ligands, which are derived from bacteria, viruses, or synthetic origin, offers the possibility of examining CCP in different immunological contexts. Before TLR stimulation, plasma from healthy donors was replaced: (1) by their own plasma (autologous plasma control), (2) by plasma from other healthy donors (healthy plasma control), or by CCP from fully recovered donors.

2.1 | Blood samples

After obtaining written informed consent, freshly drawn peripheral blood from healthy donors aged 18–60 years was anticoagulated using tri-sodium citrate monovettes (S.Monovette, Sarstedt). All CCP donors were healthy and donated plasma according to the Paul Ehrlich Institute (Federal Institute for Vaccines and Biomedicine, Germany) recommendations as early as 4 weeks after complete recovery from COVID-19 or 2 weeks after their last negative SARS-CoV-2 PCR diagnosis. SARS-CoV-2 immunoglobulin G (IgG) antibodies in the convalescent plasma and healthy plasma controls were measured by a semi-quantitative enzyme-linked immunosorbent assay (LIAISON® SARS-CoV-2 S1/S2 IgG, DiaSorin, Germany) with a defined cutoff of 15 AU/ml. The study was approved by the local ethics committee of University Hospital Erlangen (346_18B, 343_18B, 357_19B). Blood samples were kept at room temperature for no longer than 2 h before processing.

2.2 | Plasma replacement

The plasma was obtained from whole blood of healthy donors and CCP donors by centrifugation for 10 min at 1000 × g and the supernatant was stored at −20°C until use. To investigate the immunomodulatory effect of CCP in healthy donors, plasma from the whole blood of healthy donors was replaced after centrifugation with either the same amount of CCP or ABO-matched control plasma from other healthy donors

(healthy plasma control). The plasma used for replacement was ABO compatible, and replacement with the autologous plasma (autologous plasma control) served as a reference.

2.3 | Stimulation of whole blood

Monitoring of immunomodulatory response was performed adapting an in vitro whole blood assay.[14] After plasma replacement, the whole blood diluted 1:2 with RPMI 1640 (Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Gibco) were distributed in 96-well round bottom plates (total volume 200 µl/well). Samples were stimulated for 18 h in 5% CO_2 at 37°C with 20 µl TLR ligands from InvivoGen including 500 ng/ml Pam3CsK4 (TLR1/2), 10^8 cells/ml HKLM (TLR2), 10 ng/ml LPS E. *coli* K12 (TLR4), 1 µg/ml flagellin-ST (TLR5), 100 ng/ml FSL-1 (TLR6/2), 5 µg/ml imiquimod (TLR7), 2.5 µg/ml ssRNA40/LyoVec (TLR8), or vehicle. The supernatant was collected and frozen at −20°C until analysis.

2.4 | Measurement of cytokine production

Cytokines including interferon-induced protein (IP)-10, interleukin (IL)-1β, tumor necrotic factor (TNF)-α, monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-10, and interferon gamma (IFN-γ) were quantified by flow cytometry bead-based immunoassay (LEGENDplexTM human essential immune response panel, BioLegend) according to the manufacturer's protocol and analyzed using LEGENDplex version 7.0 software (Vigene Tech).

2.5 | Statistical analysis

Data were reported as mean ± SD unless otherwise stated. Statistical analysis was performed with GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, California USA). Statistical significance between groups was evaluated by Mann–Whitney U test. p value <.05 was considered statistically significant.

3 | RESULTS

The average SARS-CoV-2 IgG antibody level in convalescent plasma was 67 ± 29 AU/ml, and all 12 CCP donors had recovered from asymptomatic COVID-19 infection. In contrast, none of the healthy control plasma were positive for SARS-CoV-2 IgG antibodies with values <3.80 AU/ml.

An essential immune response panel of seven cytokines (IP-10, IL-1β, TNF-α, MCP-1, IL-6, IL-10, and IFN-γ) was evaluated, and the results revealed a distinct cytokine profile triggered by the TLR ligands in the autologous plasma control group (Table 1). The unstimulated CCP and healthy plasma control samples showed no measurable cytokine levels (Table S1).

To elucidate the immunomodulatory effect of CCP in comparison with the healthy plasma control, the cytokine release of these two groups was normalized to the respective autologous plasma control group (Figure 1). This analysis showed that replacement by CCP in healthy donors significantly downregulated a variety of cytokines following the TLR stimulation, as compared with the healthy plasma control. IL-1β, IL-6, and IL-10 levels were 1.5–2-fold lower in the CCP group than in the healthy plasma control group following stimulation with Pam3CsK4 (Figure 1A). Replacement by CCP induced a significant decrease of IL-10 and IFN-γ in HKLM-stimulated healthy donor cells compared with the plasma control group (Figure 1B). Following stimulation with LPS, several cytokines, including IL-1β (1.4-fold), TNF-α (2-fold), MCP-1 (1.6-fold), IL-6 (1.3-fold), and IFN-γ (2.3-fold), were reduced by CCP (Figure 1C). Compared with the healthy control plasma, we observed a decline in the release of IL-1β, TNF-α, and IFN-γ following stimulation with Flagellin after replacement by CCP (Figure 1D), whereas for ssRNA40, the amount of MCP-1, IL-6, and IFN-γ was markedly decreased (Figure 1E). Approximately 1.5-fold less MCP-1 and IL-6 were detected between the CCP group and the healthy control plasma group in imiquimod-stimulated whole blood (Figure 1F) and also lower levels of MCP-1 were induced by CCP following stimulation with FSL-1 (Figure 1G). Interestingly, MCP-1, IFN-γ, and IL-6 were most consistently inhibited by CCP.

To investigate the influence of the severity of COVID-19 disease on TLR-stimulated cytokine release, CCP donors were divided into moderate and severe disease group based on whether they were hospitalized or not (Figure 2). We found no association between the severity of COVID-19 infection and suppression of TLR-stimulated cytokine release.

4 | DISCUSSION

Since convalescent plasma is currently used to treat severely ill COVID-19 patients, our results highlight that CCP may have an immunomodulatory effect that is SARS-CoV-2 antibody independent. Herein, we report

TABLE 1 Cytokine release of autologous plasma control following different toll-like receptor stimulation ($n = 3$, ±SD)

TLR ligand	IP-10 (pg/ml)	IL-1β (pg/ml)	TNF-α (pg/ml)	MCP-1 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)
Pam3CsK4	176.3 ± 87.8	273.0 ± 146.0	15.1 ± 10.6	15,533 ± 6501	13,350 ± 7447	154.2 ± 90.2	17.1 ± 12.1
HKLM	1796 ± 361	8752 ± 1607	1408 ± 397	35,103 ± 5569	74,033 ± 8396	769.4 ± 179.7	920.3 ± 147.0
LPS	3647 ± 823	3227 ± 606	353.1 ± 70.6	11,320 ± 3555	40,244 ± 6609	112.0 ± 24.6	409.6 ± 86.5
Flagellin	1828 ± 443	5970 ± 616	481.0 ± 96.5	8294 ± 2986	74,532 ± 6667	493.3 ± 95.7	700.1 ± 274.6
ssRNA40	9194 ± 552	8683 ± 2444	1768 ± 416	6454 ± 1055	17,093 ± 4774	1389 ± 388	10,966 ± 3802
Imiquimod	5506 ± 610	135.4 ± 45.2	46.7 ± 33.6	9170 ± 2521	4001 ± 784	158.1 ± 76.9	81.4 ± 64.1
FSL-1	6.4 ± 2.3	42.3 ± 22.6	13.9 ± 12.4	9601 ± 1682	1460 ± 130	62.8 ± 9.7	16.5 ± 11.4

Abbreviations: IFN-γ, interferon gamma; IL, interleukin; IP, interferon-induced protein; MCP-1, monocyte chemoattractant protein-1; TLR, toll-like receptor; TNF, tumor necrotic factor.

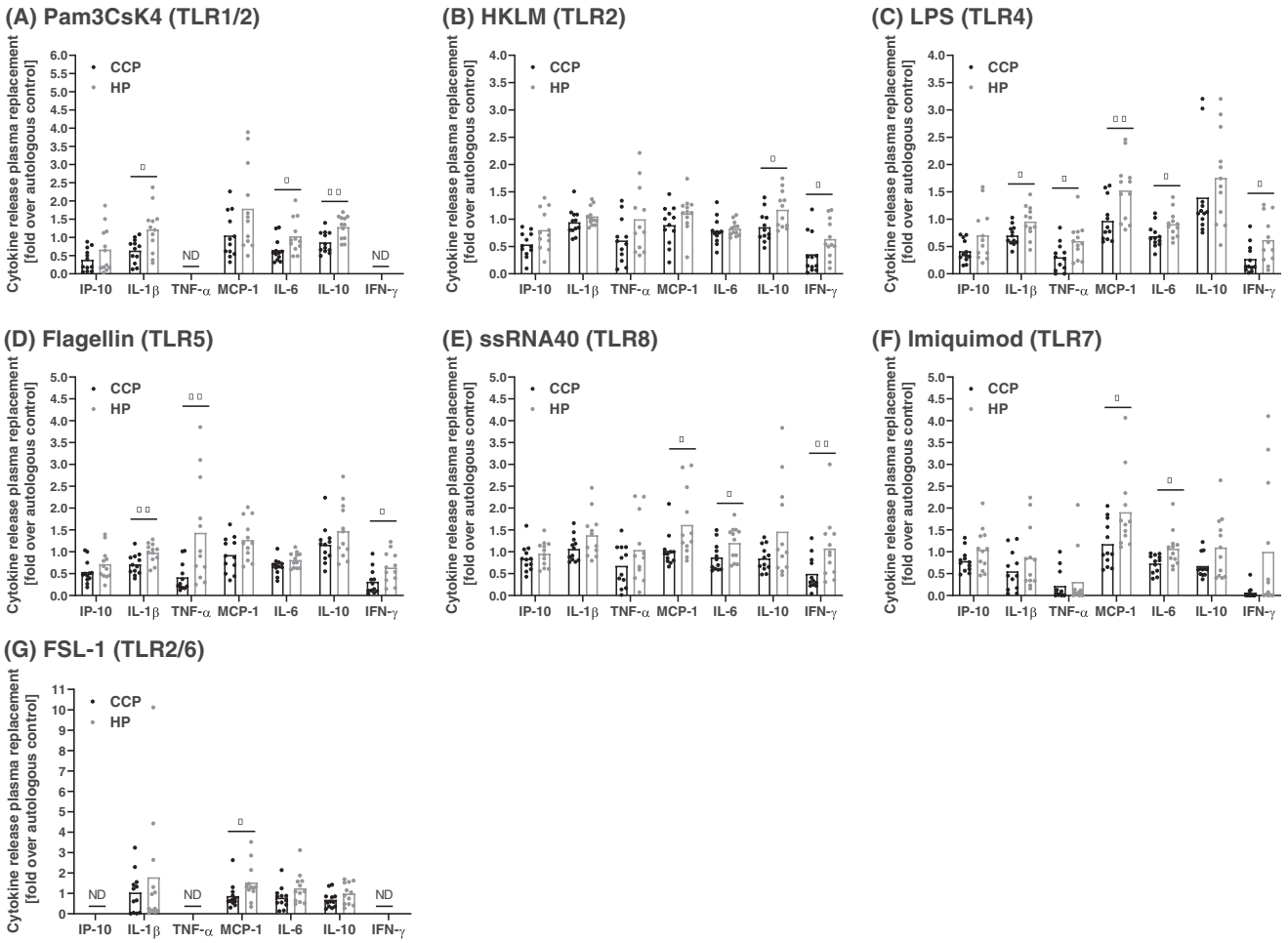

FIGURE 1 COVID-19 convalescent plasma (CCP) shows immunomodulatory effects on toll-like receptor (TLR)-triggered cytokine release. Cytokine release after plasma replacement by CCP (black) and healthy plasma control (HP; gray) following different TLR stimulation are represented as fold change of autologous plasma control levels, with $n = 12$ convalescent plasma, each performed in duplicates (A–G). Comparisons were made using the Mann–Whitney U test and differences were significant at $p < .05$ (*) and .01 (**). ND = not determined due to low cytokine levels

that CCP significantly suppresses the release of various cytokines in healthy donors, and this inhibitory effect can be demonstrated across different TLR receptors and linked signaling pathways. Based on the pattern of our results, we identified IL-6, MCP-1, and IFN-γ as the most frequently downregulated cytokines, following stimulation with different TLR ligands. IL-6 is an important pleiotropic cytokine whose production is related to

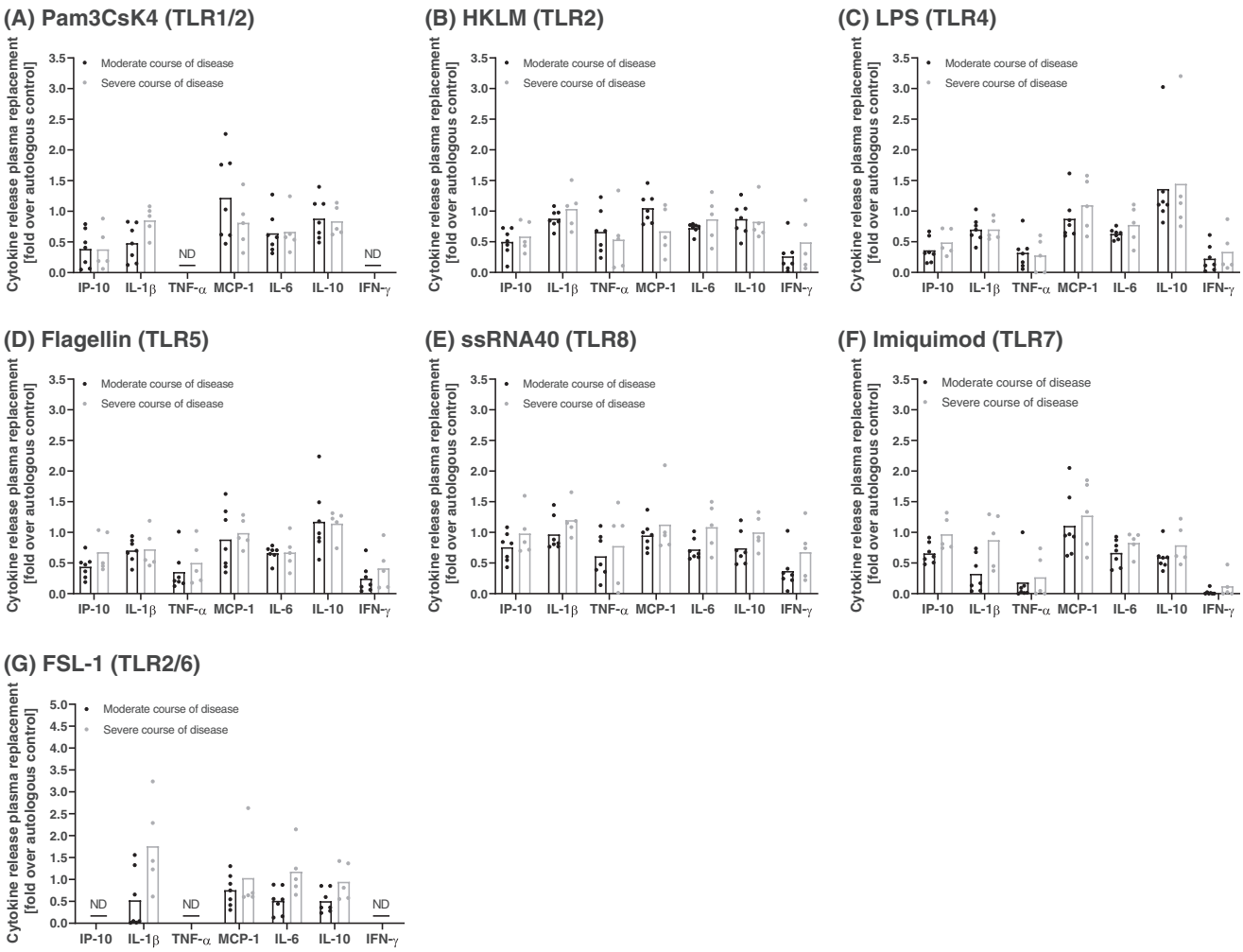

FIGURE 2 Comparison of TLR-stimulated cytokine release based on the severity of COVID-19 infection in the CCP group. The results of cytokine release after plasma replacement by CCP were grouped into moderate (black) and severe course of disease (gray) following different TLR stimulation, with n = 12 convalescent plasma, each performed in duplicates (A-G). Comparisons were made using the Mann–Whitney U test and differences were significant at $p < .05$ (*) and .01 (**). ND = not determined due to low cytokine levels

bacterial and viral infections, since it controls the differentiation of monocytes, increases B-cell IgG production, and promotes Th2 response.[15,16] MCP-1 is a potent monocyte-attracting chemokine that orchestrates the migration of myeloid and lymphoid cells during immune defense.[17] IFN-γ, which is normally produced during infection, is one of the most characteristic cytokines that are suggested to be essential in the fine-tuning and control of the extent of inflammatory conditions.[18] Interestingly, recent studies have also shown that increased levels of IL-6, MCP-1, and IFN-γ, among other cytokines, have been detected in COVID-19 patients under intensive care and found to correlate with clinical parameters and severity of the disease.[19-21] Early detection of cytokine storm and immediate initiation of treatments to reduce severity are both essential for the treatment of severe COVID-19. Thus, great efforts are being made to find an appropriate anti-inflammatory therapy. The application of therapeutic plasma exchange (TPE) and the use of convalescent plasma have both been suggested to reinforce immunity and provide the chance of overcoming sepsis-induced cytokine storm.[22] In a case report that described the beneficial effects of TPE on COVID-19, after plasma exchange, the patient showed clinical improvement with reduced inflammatory markers including IL-6.[23] Although a randomized clinical trial, evaluating the immunomodulatory effect of CCP on COVID-19-related cytokine storm, found no significant improvement of mortality rate and length of in-hospital stay, convalescent plasma therapy significantly decreased the mean levels of IL-6, TNF-α, and IFN-γ.[24] In addition, it is supposed that plasma from healthy donors provides immunomodulatory effects via the infusion of anti-inflammatory cytokines and antibodies that blockade complement, inflammatory cytokines, and auto-antibodies.[25]

While our finding regarding the decreased cytokine production following the replacement by CCP in our in vitro model for different pathogen-induced inflammation is notable, it remains unclear whether these effects are relevant enough to translate to a clinically improved outcome. Synthetic TLR ligands can serve as in vitro tool to investigate different immunological signaling pathways by stimulating whole blood in a reproducible and standardized manner and may help to design appropriate clinical trials that are necessary to determine the clinical relevance. However, a recent two-phase controlled study demonstrated that CCP attenuated the exhausted phenotype and increased memory T- and B-lymphocytes together with a reduction of IL-6/IFN-γ and IL-6/IL-10 ratios compared with those who received standard therapy alone.[21] Although our whole blood assay is optimal to monitor immunomodulatory effects by incorporating all immune cells and the microenvironment, further studies using peripheral blood mononuclear cells or TLR-expressing cell lines could be useful to unravel the molecular mechanism and signal transduction events that lead to the observed downregulation of cytokines. To gain more information about the immunomodulatory factor in the CCP, further experiments can be performed to investigate, for example, whether it is a protein or a lipid or whether it is heat stable. Additional immunological readouts to measure innate immune cell function, including phagocytosis, chemotaxis, and expression of surface molecules, may further reveal the immunomodulatory effects. Herein, we focused on cytokine production because it is also used as a readout in clinical trials for adverse outcomes in critically ill patients.[21] In conclusion, using a standardized whole blood assay and appropriate negative controls, we demonstrated that CCP has an inhibitory effect on cytokine release triggered by different TLR ligands in healthy donors as compared with healthy control plasma. In addition to the effects of neutralizing antibodies, CCP may therefore exhibit additional immunoregulatory properties. Due to the pleiotropic nature of cytokines, the use of CCP may either be beneficial in states of hyperinflammation, which is being manifested in a cytokine storm, or detrimental in states of immunodeficiency.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

ORCID

Regine Brox https://orcid.org/0000-0003-3104-4869
Holger Hackstein https://orcid.org/0000-0001-9722-8180

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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