


The novel polyclonal Ab preparation trimodulin attenuates *ex vivo* endotoxin-induced immune reactions in early hyperinflammation

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Abstract

Sepsis is a syndrome associated with excessive inflammation. Since mortality from sepsis remains high, more laboratory research is needed to provide insight into more effective ways to use novel, potentially more beneficial agents in sepsis. We investigated the *ex vivo* immunomodulatory effect of a novel polyclonal Ab preparation, trimodulin, containing IgM (~23%), IgA (~21%) and IgG (~56%). Using whole blood and purified PBMCs from healthy volunteers and patients with sepsis, various *ex vivo* investigations upon endotoxin challenge and pre- and post-trimodulin treatment were performed. Endotoxin-induced TNF- α secretion was noticeably lower with than without trimodulin, implying attenuation of the hyper-responsive state. Trimodulin also lowered TLR2, TLR4, CD11b and CD64 detection on LPS/lipoteichoic acid-stimulated monocytes. These responses were observed in cells from healthy volunteers only shortly after *ex vivo* endotoxin stimulation and in whole blood from patients with early-stage sepsis. Furthermore, trimodulin markedly reduced lymphocyte proliferation and release of pro- and anti-inflammatory cytokines, but did not affect phagocytosis or oxidative-burst activities of endotoxin-stimulated cells. Thus, trimodulin mitigated monocyte and lymphocyte hyperinflammatory responses early after endotoxin exposure. Determining whether early *in vivo* administration of trimodulin will elicit similar positive immunomodulatory effects and offer a clinical benefit warrants investigation.

Keywords

Cytokines, Ig, inflammatory response, monocytes, sepsis

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Introduction

Sepsis and septic shock are life-threatening conditions caused by a dysfunctional host immune response to infection, which may lead to system-wide tissue damage, organ injuries and death.^{1,2} In sepsis, there are two recognised opposing responses:^{3–8} a dominant systemic hyper-inflammatory response, which is characterised by leukocyte activation, cytokine storm and tissue-wide damage; and counter-regulatory mechanisms, which start simultaneously. These mechanisms in turn may lead to down-regulation of effector-cell functions, including T-cell exhaustion, defective Ag presentation and immunosuppression. The resulting massive immune suppression is paralleled by high

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mortality due to unresolved septic foci and secondary infections.^{3–8}

Both monocytes and macrophages play a major role in orchestrating these host immune responses, detecting and phagocytosing pathogens opsonised with complement and/or Ig.⁹ Monocytes and macrophages also activate T cells by presenting bacterial Ags on major histocompatibility complex class II molecules. In addition, monocytes contribute to the cytokine storm by producing pro-inflammatory ILs and TNF- α , mainly via bacterial endotoxin-induced TLR4 signalling. Prolonged and repeated pathogen stimulation triggers endotoxin tolerance in the monocyte lineage, finally releasing anti-inflammatory factors and adapting the response to an immunosuppressive phenotype.¹⁰ Blood lymphocyte dysfunction during sepsis has also been reported with significant lymphopenia, including decreased T lymphocytes and NK cells.¹¹

Sepsis represents a major health-care problem worldwide, and novel adjunctive therapies are much needed. Ig preparations comprising polyvalent IgG molecules alone (IVIg) have not shown clear benefits.¹² However, a preparation containing pentameric IgM, Pentaglobin[®] (~12% IgM, ~12% IgA and ~76% IgG), reduced mortality amongst adults with sepsis.^{12,13} These findings may be related to the observed hypogammaglobulinemia and reduced IgM levels in early-stage sepsis and severe infections.^{14–16} Relevant mechanisms of action that may be deficient in sepsis, due to reduced IgM concentrations in severely infected patients, include agglutination and opsonisation activity against bacteria contributing to pathogen recognition and clearance.^{17–19} Furthermore, IgM has a higher neutralisation capacity for bacterial endotoxins and influences the complement activity cascade more efficiently than IgG alone.^{17,20} The pentameric nature of IgM renders it very efficient in these processes.²¹

A novel polyclonal Ab preparation, trimodulin, containing ~23% IgM, ~21% IgA and ~56% IgG, is being developed. Safety and efficacy of trimodulin has already been investigated in a randomised placebo-controlled trial as an add-on therapy in patients with severe community-acquired pneumonia requiring invasive mechanical ventilation.²² Although no significant difference was found in ventilator-free days and mortality between the trimodulin and the placebo group (albumin), *post hoc* analyses supported improved outcome regarding mortality with trimodulin in subsets of patients with elevated C-reactive protein, reduced IgM concentrations or both. C-reactive protein is a marker of inflammation and increases in response to bacterial infections. Improved outcomes of trimodulin-treated patients with reduced IgM concentrations at baseline support the important functions of this Ig preparation.

In this study, we investigated the immunomodulatory activity of trimodulin to gain insight into the potential utility of this preparation as a novel therapeutic agent in severe infections. *Ex vivo* experiments on whole blood from patients with sepsis and PBMCs from healthy donors were performed, including the endotoxaemia second-hit model. The model was used to reflect the onset, early phases and late phases of sepsis. It is based on the knowledge that the first endotoxin hit leads to a strong secretion of TNF- α and, additionally, in monocytes and macrophages, to an increased production of the TLR4-negative regulator, IL-1-receptor-associated-kinase M (IRAK-M). IRAK-M inhibits the release of IRAK/IRAK-4 from the TLR4/MD2 complex, blocking downstream signalling in monocytes via NF- κ B.²³ Despite a second endotoxin challenge, blockage of this intracellular signalling pathway prevents the new induction of TNF- α , which is a key event in immune suppression.²⁴

Here we show that early intervention with trimodulin impedes on pro- and anti-inflammatory functions of monocytes and lymphocytes induced by endotoxins and provide insight into the nature of the immunomodulating activity of this unique preparation.

Materials and methods

Ethics statement

The study was approved by the local institutional Ethics Committee, Medical University of Vienna, Vienna, Austria (approval number: EK Nr. 581/2008), and written informed consent was obtained from all patients or their next of kin. The study was conducted according to the ethical principles for medical research involving human patients described in the Declaration of Helsinki.

Reagents

The novel polyclonal Ig preparation, trimodulin (~23% IgM, ~21% IgA and ~56% IgG), was kindly provided by Biotest AG (Dreieich, Germany). Purified *Escherichia coli* LPS 0111:B4, *Staphylococcus aureus*-derived lipoteichoic acid (LTA), ConA and recombinant IL-2 were obtained from Sigma-Aldrich (St. Louis, MO).

Fluorescently labelled Abs for flow cytometry were purchased from the following manufacturers: anti-human leukocyte Ag-DR isotype (HLA-DR G46-6-PE) and anti-TNF- α (mAB11) from BD Biosciences (San Jose, CA); anti-TLR2 (CD282-PE) and anti-TLR4 (HTA125-PE) from eBioscience (San Diego, CA); anti-cluster of differentiation 16 (CD16; 3G8-PE), anti-CD64 (22-PC5), anti-CD11b

(Bear1-PC5), anti-CD11c (BU15-PC5), anti-CD3 (UCHT1-PC7), anti-CD4 (13B8.2-PC5) and anti-CD8 (SFCI21Thy2D3-ECD) from Beckman Coulter (Fullerton, CA); and anti-CD14 (My4; FITC, PC5 or PC7 conjugated) from Beckman Coulter (Indianapolis, IN).

Collection and cultivation of blood samples from patients with sepsis

Heparinised whole blood was collected from eligible adult patients with sepsis admitted to the intensive-care unit of the Departments of Surgery ($n = 10$) and Internal Medicine ($n = 5$), General Hospital of Vienna, Austria (Table 1). Sepsis was defined according to the International Sepsis Definitions Conference criteria.^{25,26} Blood samples were obtained within 24 h (early) of, or ≥ 5 d (late) after, a diagnosis of sepsis. Eligible patients had not received chemotherapy or immunotherapy 4 wk prior to blood sample collection. Heparinised whole blood was cultured undiluted in 2 ml pyrogen-free tubes (Eppendorf AG, Hamburg, Germany).

Purification and cultivation of PBMCs from healthy donors and patients with sepsis

Fresh blood was collected from apparently healthy adult donors into lithium-heparin-coated tubes (VACUETTE®; Greiner Bio-One International GmbH, Kremsmünster, Austria). Whole blood was either directly cultivated or PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) according to standard protocols. Approximately 2×10^6 PBMCs (or 1×10^6 for proliferation assays) were seeded onto 24-well plates and

cultured in RPMI 1640 medium plus GlutaMAX™-I (GIBCO®; Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Linaris GmbH, Wertheim, Germany). Toxins and trimodulin were added as described for individual investigations below. The medium was carefully exchanged before the second addition of endotoxins.²⁴ Generally, experiments (e.g. ELISA) were performed in duplicate per donor, or according to specifications in the manuals provided with the different assays (see below).

Ex vivo endotoxaemia second-hit model

The endotoxaemia LPS/LTA second-hit model (endotoxin-tolerance model) was based on methods published previously for LPS and was conducted as depicted in Figure 1.²⁷ The model reflects the onset, early phases (first hit) and late phases of sepsis (second hit), based on the knowledge that the first endotoxin hit leads to a strong secretion of TNF- α , which is inhibited via blockage of NF- κ B signalling and impedes response after a second toxin challenge.

The LPS concentrations inducing TNF- α responsiveness in PBMCs were determined in checkerboard experiments, and LTA concentrations were derived from literature.²⁸ The trimodulin concentration used in these investigations did not exceed concentrations measured in patients upon administration.²² After treatment of PBMCs or whole blood, the supernatants or plasma, respectively, were collected and stored at -80°C until analysis.

Quantitative PCR analysis

PBMCs were treated according to the second-hit model, and RNA was isolated using the RNeasy Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. A total of 400 ng RNA was reverse transcribed to cDNA using the ThermoScript RT-PCR System (Thermo Fisher Scientific, Waltham, MA). Quantitative (real-time) PCR of target cDNA was performed with the 7500 Fast Real-time PCR System (Thermo Fisher Scientific). All reagents and devices used for real-time PCR were obtained from Invitrogen. All primers were obtained from VBC Biotech (Vienna, Austria). Amplification of TLR2, TLR4 and TNF- α cDNA was normalised to amplification of 18S rRNA. Changes in transcript levels ($\Delta\Delta\text{Ct}$) were calculated by subtracting $\Delta\text{Ct}_{\text{treated control}}$ values (5 h LPS with or without trimodulin) from $\Delta\text{Ct}_{\text{untreated sample}}$ values (no LPS or trimodulin) and fold change with $2^{\Delta\Delta\text{Ct}}$ compared to non-treated cells.

Table 1. Blood samples collected from patients with sepsis upon admission to the intensive-care unit.

Parameter	Characteristics	
Sample	Early (≤ 24 h; $n = 7$)	Late (≥ 5 d; $n = 8$)
Age (yr) ^a	56.4 \pm 14.3	62.3 \pm 13.7
Male/female	5/2	4/4
Leukocytes ($\times 10^9/\text{L}$) ^{a,b}	18.3 \pm 2.9	17.2 \pm 1.5
Diagnosis at admission		
Septic shock	1	1
Peritonitis	0	3
Pneumonia	2	2
Mediastinitis	1	1
Septic-wound infection	2	1
Unknown	1	0

^aMean \pm SD.

^bLeukocyte counts upon admission to the intensive-care unit.

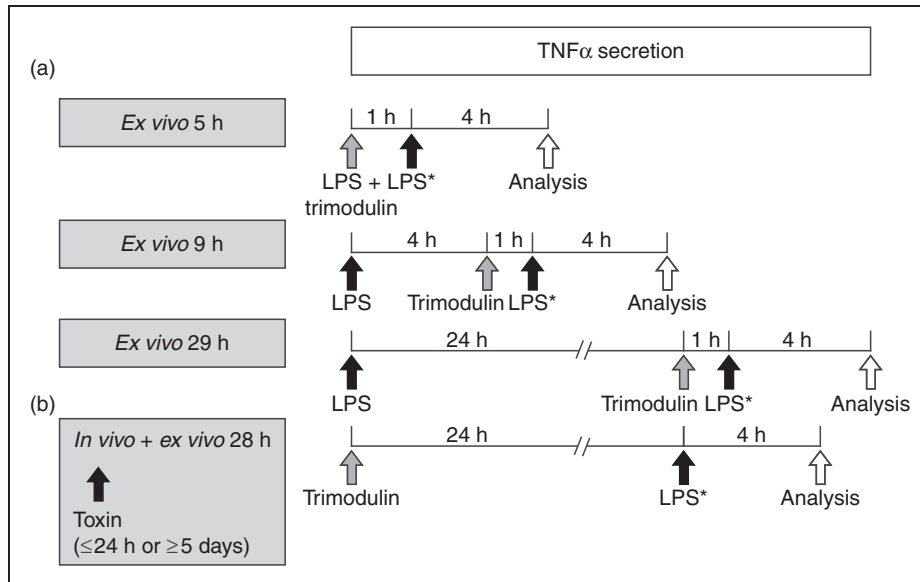


Figure 1. The ex vivo endotoxaemia second hit model. (a) PBMCs or whole blood from healthy donors were primed with endotoxins (LPS: either 0.05 or 0.5 ng/ml LPS, or 1 or 10 µg/ml lipoteichoic acid (LTA) for 1, 4 or 24 h). Different trimodulin concentrations (0, 0.5 or 5 mg/ml) were added for 1 h either simultaneously or after 4 or 24 h of incubation with LPS/LTA. Medium was carefully removed, and cells were challenged with a second high endotoxin dose (LPS*: either 0.5 ng/ml LPS or 10 µg/ml LTA) for exactly 4 h prior to analysis of TNF- α in supernatants. (b) Blood cells from patients with sepsis were considered primed *in vivo* with bacterial endotoxins for either maximally 24 h (early) or minimally 5 d (late). Whole blood was obtained and treated directly with 0, 0.5 or 5 mg/ml trimodulin for 24 h before *ex vivo* challenge with a second high LPS* dose for exactly 4 h prior to analysis of TNF- α in plasma. Total endotoxin exposure time is indicated in the grey boxes.

Flow cytometry analysis

Prior to flow cytometric analysis of the cells, purified PBMCs from healthy donors were treated according to the one-hit protocol, as described in detail for this specific investigation below. After treatment, cells were harvested, and Ab incubation was performed on ice in the dark for 25 min. All cells were co-stained with anti-CD14 and washed twice with Dulbecco's PBS without calcium and magnesium (DPBS^{-/-}) containing 0.1% NaN₃ and 1% FCS. To analyse various surface markers on monocytes, single samples with a total of 2500 CD14⁺ cells were analysed by flow cytometry (Beckman Coulter FC500 equipped with CXP software).

Determination of phagocytic activity

Whole blood from healthy donors and patients was treated according to the respective protocol, as shown below (phagocytosis experiments). Here, the second hit was not performed with purified LPS, but with fluorescently labelled *E. coli* cells. After pre-treatment with LPS (first hit), the isolated PBMCs from healthy donors were harvested, washed and incubated with non-opsonised FITC-labelled *E. coli* (second hit). Whole blood from patients was incubated directly with non-opsonised FITC-labelled *E. coli* cells (PHAGOTEST[®]; ORPEGEN Pharma, Heidelberg,

Germany) or with endocytosis/acidification-sensitive pHrodo-conjugated *E. coli* bioparticles (pHrodo[™] Red *E. coli* BioParticles[™] Conjugate for Phagocytosis; Life Technologies, Carlsbad, CA).²⁹ The percentage of phagocytic monocytes and phagocytic activity per cell were analysed by flow cytometry as recommended by the manufacturer.

Determination of oxidative-burst activity

Heparinised whole blood from healthy donors was treated for 4 h with LPS and subsequently stimulated for 1 h with various concentrations of trimodulin. Following the addition of non-opsonised, non-labelled *E. coli* (second hit), the percentage of phagocytic cells (granulocytes and monocytes) that produced reactive-oxygen metabolites and their mean fluorescence intensity were measured by flow cytometry (PHAGOBURST[™]; BD Biosciences, Heidelberg, Germany).

Proliferation assay

PBMCs from healthy donors and patients with sepsis were treated as described in detail for this specific investigation below. Cells were stained with 1 µM 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE; Sigma-Aldrich, Vienna, Austria) for 10 min at room

temperature and washed with RPMI 1640 prior to 2 h stimulation with LPS (one hit, with healthy PBMCs only) and 2 h treatment with trimodulin. To induce proliferation of T lymphocytes *in vitro*, the cells were treated with ConA (10 µg/ml) and/or IL-2 (100 ng/ml).

To analyse the number of completed T-lymphocyte divisions after 72 h (according to the CFSE instructions), cells were co-stained with anti-CD3, anti-CD4 and anti-CD8. PBMCs were washed with DPBS^{-/-} containing 0.1% NaN₃ and 1% FCS and then analysed by flow cytometry. Completed cycles of division were defined by the progressive halving of the CFSE fluorescence intensity after each cell division and were gated manually on lymphocyte subpopulations for quantification.

Cytokine analysis

To explore the influence of trimodulin on TNF- α expression, PBMCs or whole blood from healthy donors or whole blood samples from patients with sepsis were treated according to the second-hit model, and were analysed for TNF- α by colorimetric sandwich ELISA (Human TNF- α Quantikine ELISA kit; R&D Systems, Minneapolis, MN). In further experiments, PBMCs from healthy donors and patients with early-stage sepsis were treated according to the one-hit protocol used to measure proliferation. Cell culture supernatants from these cultures were collected and stored at -80°C. Cytokines (IFN- γ , IL-10, IL-17A and TNF- α) were measured in supernatants using a commercially available cytometric bead array (Human Th1/Th2/Th17 Kit; BD Biosciences, Heidelberg, Germany).

Statistical analysis

Normal distribution was assumed and confirmed by analyses of PBMCs or blood samples from more than five donors using the Kolmogorov-Smirnov test.³⁰ To compare treated and untreated donor PBMCs, a paired parametric *t*-test was applied if $n > 3$. Associations between trimodulin concentrations and cytokine secretion or surface-marker expression were analysed using paired one-way ANOVA with Greenhouse-Geisser correction.³¹ Pearson's chi-square test was used to determine if the observed number of lymphocyte generations after trimodulin treatment was significantly different from the untreated control. Associations between cytokine concentration and trimodulin were graphically represented by a scatterplot and assessed by Spearman's correlation coefficient (r_s). Statistical analysis was performed by MS Excel (Microsoft Corp., Redmond, WA), SPSS Statistics for Windows v17.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA). All calculated *P*-values are two-sided, and significance was set at $P < 0.05$.

Results

Trimodulin attenuates TNF- α secretion by blood cells from healthy donors after repeated endotoxin doses at the early stage

To explore the influence of trimodulin on expression of the pro-inflammatory cytokine TNF- α upon bacterial endotoxin stimulation, the *ex vivo* endotoxaemia second-hit model was used as depicted and described in detail in Figure 1. ELISA analysis of TNF- α secreted by PBMCs from healthy donors showed that its detection was dependent on LPS/LTA dose and incubation time ($P \leq 0.01$, ANOVA; Figure 2a and b). The highest TNF- α levels (hyper-inflammatory stage) were observed after 5 h, and levels had clearly lowered after 29 h of endotoxin challenge (hypo-inflammatory stage). Trimodulin attenuated TNF- α levels ($P \leq 0.05$, *t*-test) dose dependently and early during the first 5–9 h of endotoxin challenge ($P \leq 0.05$, ANOVA). The effect on TNF- α secretion was not elicited by serum protein albumin if used in equimolar protein concentrations (Figure 2c). Albumin was used as a placebo control in the trimodulin clinical trial.²²

Similar results to those seen with purified PBMCs were obtained using whole blood samples from healthy donors (Figure 2d). This was important, since experiments with patients samples (below) could only be done with whole blood due to volume limitations.

RT-PCR analysis revealed that the regulation of TNF- α , by LPS and increasing concentrations of trimodulin, occurs on the transcriptional level (Figure 2e), confirming the observed reduction in the secretion of TNF- α (Figure 2a).

Trimodulin attenuates TNF- α secretion by blood cells from patients with early-stage sepsis after repeated endotoxin doses

The impact of trimodulin on TNF- α secretion by blood samples from healthy donors (Figure 2d) was compared to the reactivity of *in vivo* endotoxin-primed samples from patients with early- and late-stage sepsis. Whole blood was collected from eligible patients with sepsis. Baseline characteristics of patients with early (≤ 24 h; $n = 7$) and late (≥ 5 d; $n = 8$) sepsis are shown in Table 1.

The TNF- α level secreted by *in vivo* and *ex vivo* endotoxin-stimulated cells obtained during early-stage sepsis was attenuated ($P = 0.0570$, *t*-test) with 5 mg/ml trimodulin compared to cells not treated with trimodulin (Figure 2f). Cells from patients with sepsis obtained within 24 h after diagnosis were responsive to endotoxin and trimodulin.

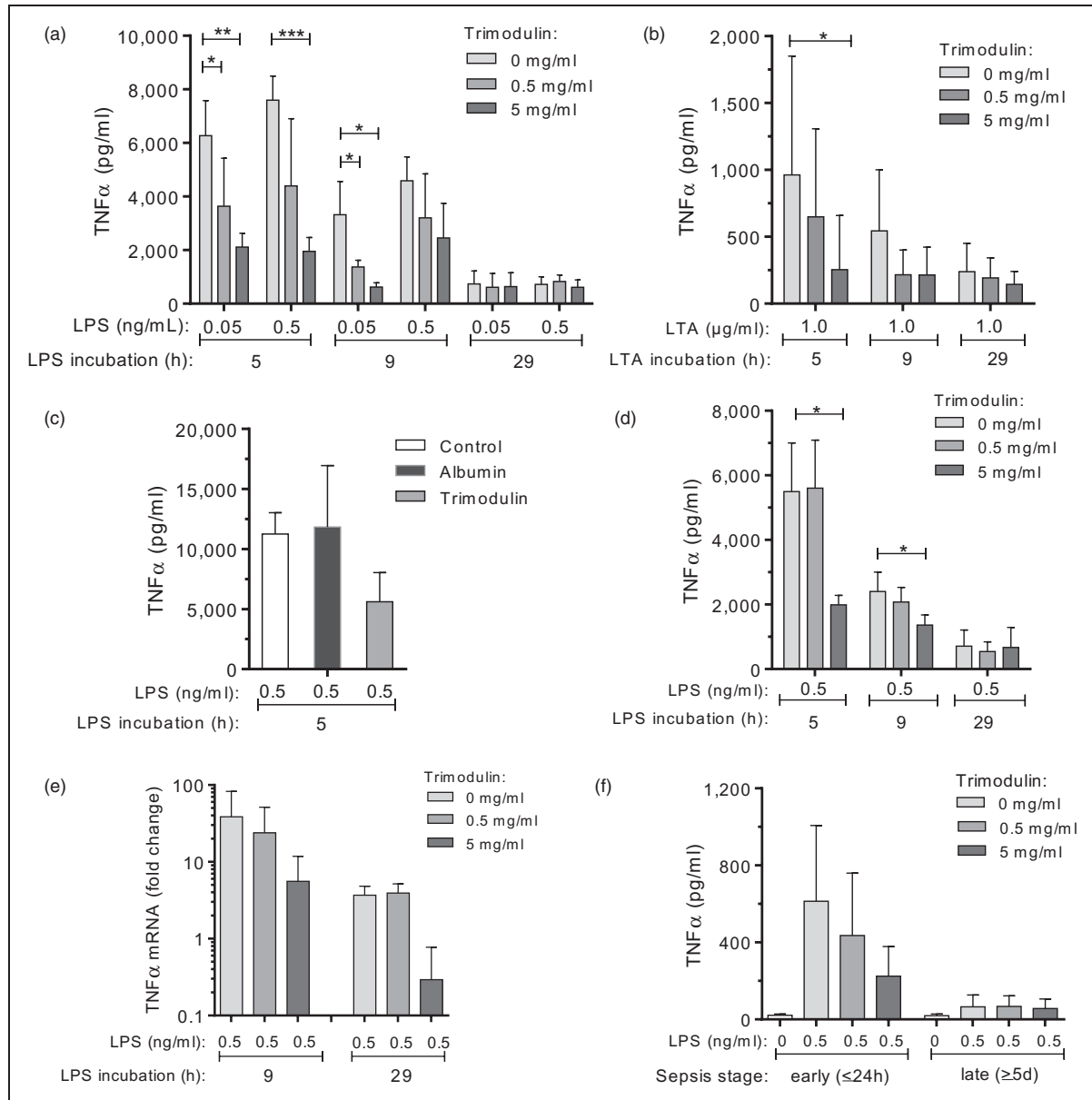


Figure 2. TNF- α secretion upon repeated endotoxin stimulation and trimodulin treatment. Purified PBMCs (a, $n = 5$; b, $n = 7$; c, $n = 3$) or whole blood (d, $n = 5$) from healthy donors were challenged with LPS (a, c and d) or LTA (b) and treated with trimodulin according to the second hit protocol shown in Figure 1(a). (c) Control experiment performed by simultaneous incubation of PBMCs ($n = 3$) with LPS, without (control) or with 5 mg/ml trimodulin, or with an equimolar concentration of albumin for 1 h, followed by a second LPS* hit for a total LPS incubation time of 5 h. (e) RT-PCR analysis showing fold change in TNF- α transcript levels in PBMCs ($n = 3$) from healthy donors upon treatment compared to non-treated cells (no LPS, no trimodulin). (f) Whole blood obtained from patients with sepsis in the early ($n = 7$) or late ($n = 8$) phase was treated with trimodulin and with 0.5 ng/ml LPS* or without endotoxins (control) as shown in Figure 1b. (a–d and f) The TNF- α concentration in PBMC supernatants or in plasma from whole blood was analysed by ELISA. Bars represent mean \pm SD. Differences versus control (0 mg/ml trimodulin) are indicated with asterisks (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.005$ using a parametric paired t -test). Paired one-way ANOVA showed strong correlations (a, $P \leq 0.001$; b, $P \leq 0.05$) between trimodulin dose (5 and 9 h) and TNF- α detection.

During late-stage sepsis, TNF- α levels (16–154 pg/ml) were not altered by trimodulin treatment (16–132 pg/ml) and were more than eightfold lower when compared to early-stage sepsis (134–1238 pg/ml). During late-stage

sepsis, cells were thus non-responsive to LPS and trimodulin. Furthermore, the TNF- α level with *ex vivo* repeatedly stimulated healthy PBMCs (Figure 2a and b) or whole blood (Figure 2d) was, after 29 h, similar to the

TNF- α levels observed in whole blood samples from patients with late-stage sepsis (Figure 2f, late), supporting the underlying mechanism of the endotoxaemia model.

Trimodulin down-regulates mRNA expression of TLRs in monocytes after repeated LPS doses

The second-hit experiments were further extended to gain more insight into the receptor-dependent regulation of TNF- α secretion by LPS and trimodulin treatment. RT-PCR analysis of TLR4 (LPS receptor, Figure 3a) and TLR2 (LTA receptor, Figure 3b)

showed that 9 or 29 h of LPS stimulation alone caused a mean 32- and 3.6-fold increase in TLR4-mRNA and a 40- and 34-fold increase in TLR2-mRNA, respectively, compared to untreated cells. The LPS-induced up-regulation of TLR4-mRNA after 9 or 29 h was clearly less pronounced after trimodulin treatment (7.6- and 1.3-fold, respectively) and supports the endotoxin neutralisation activity of trimodulin. Down-regulation of TLR2 (at 29 h) and TLR4 revealed through RT-PCR was confirmed by flow cytometry; a decrease in cell-surface TLR2 and TLR4 was seen on monocytes in this second-hit model (Figure 3c).

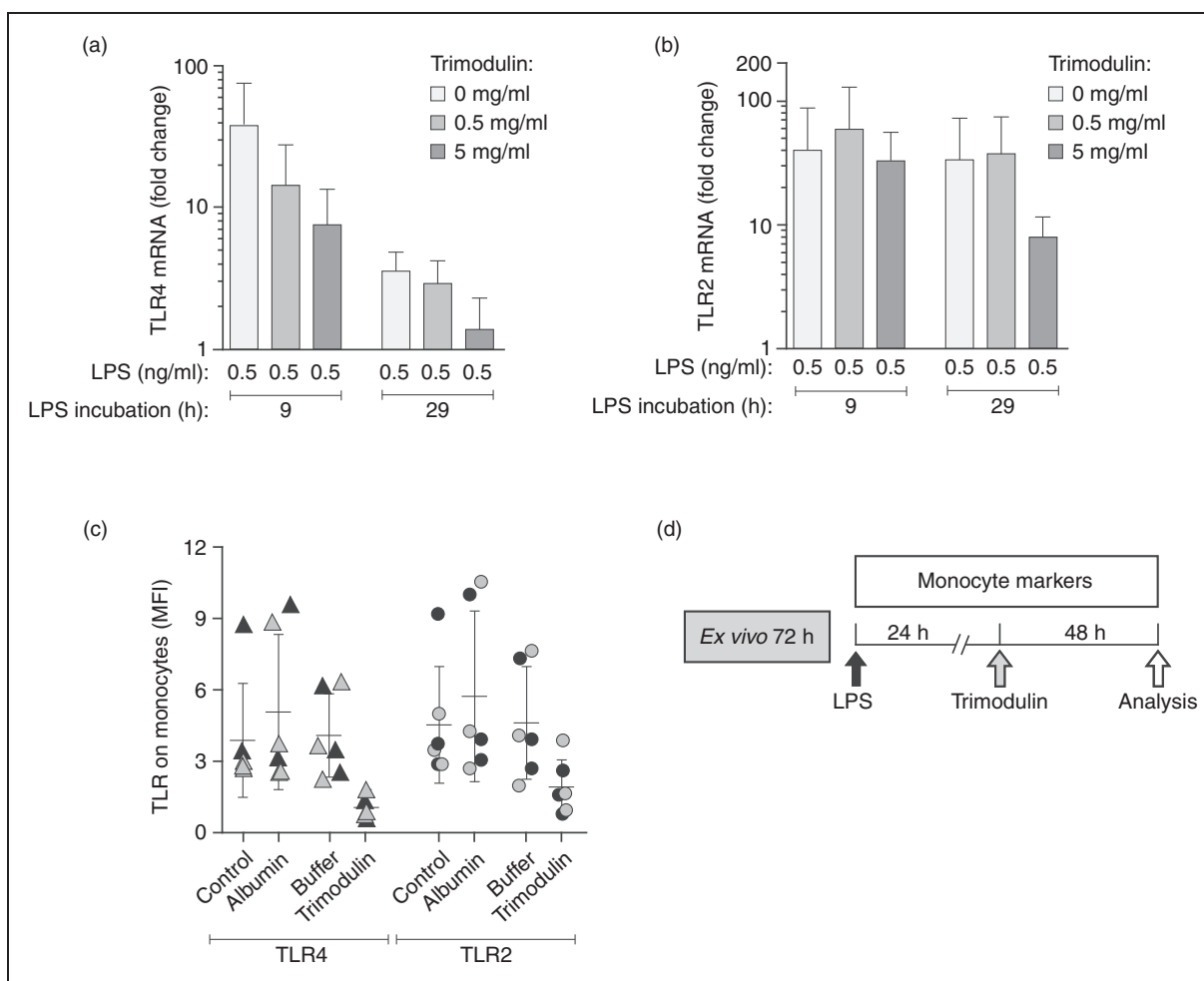


Figure 3. Regulation of TLR expression in monocytes. (a–c) PBMCs from healthy donors ($n = 3$) were treated according to the second-hit model depicted in Figure 1a for 9 or 29 h without endotoxin (control) or with 0.5 ng/ml LPS and LPS*, and with or without trimodulin. (a) RT-PCR analysis showing fold change in TLR4 mRNA and (b) TLR2 mRNA compared to untreated cells (no LPS, no trimodulin) and analysed after the second LPS* hit. (c) Detection of TLR2 and TLR4 on monocytes after 9 h (black symbols) or 29 h LPS/LPS* stimulation (grey symbols) including either 5 h albumin, formulation buffer or trimodulin treatment, or without further additives (control). (d) Single endotoxin dose (one hit) model representing treatment of early inflammation (24 h) with trimodulin. This model was used for experiments presented in Tables 2 and 3. PBMCs from healthy donors were stimulated with 0, 0.05 or 0.5 ng/ml LPS, or 0, 1 or 10 μ g/ml LTA. After 24 h of LPS/LTA, different trimodulin concentrations (0, 0.5 or 5 mg/ml) were added for 48 h prior to analysis of cells by flow cytometry (total incubation time: 72 h). Bars in a–c represent mean ($n = 3 \pm$ SD).

Trimodulin attenuates TLR4 on monocytes if applied early after a single endotoxin dose

The effect of trimodulin on the monocyte phenotype, if applied in the early endotoxin-induced hyper-inflammatory phase, was investigated in more detail (Figure 3d). PBMCs from healthy donors were stimulated with or without a single endotoxin dose, followed by early treatment at 24 h with or without trimodulin for an extended period of 48 h. The expression of TLR2 and TLR4 on CD14⁺ monocytes upon LPS or LTA stimulation was assessed by flow cytometry (Tables 2 and 3). Upon endotoxin stimulation alone, surface expression of these TLRs was largely unchanged. However, TLR2 and TLR4 decreased markedly and dose dependently upon the addition of trimodulin. This effect was most evident with LPS stimulation. These data are in agreement with the TLR4- and TLR2-mRNA results shown in Figure 3a and b. Reduced TLR4 expression was not observed after treatment with the control protein, albumin (data not shown).

Trimodulin modulates surface markers on monocytes if applied early after a single endotoxin dose

Fcγ-RI–III expressed on monocytes mediate Ab-dependent cellular cytotoxicity and phagocytosis activities. Their surface expression was investigated in the early inflammatory model according to Figure 3d. The level of expression of CD64 (Fcγ-RI, a high-affinity receptor for IgG1 and IgG3) on PBMCs from healthy donors markedly increased five- to sixfold ($P \leq 0.01$) upon strong LPS/LTA stimulation alone (0.5 ng/ml LPS or 10 μg/ml LTA) or trimodulin treatment alone. Interestingly, only high expression levels of CD64 on monocytes due to strong endotoxin induction

on monocytes were counterbalanced by trimodulin treatment (LPS; $P \leq 0.01$; Tables 2 and 3). Similarly, the increase in expression of CD16 (Fcγ-RIII) by LTA was reduced markedly by trimodulin ($P \leq 0.01$).

Expression of complement receptors on endotoxin-activated monocytes was analysed. Levels of both CD11b and CD11c were decreased upon endotoxin stimulation alone, which was further reduced by trimodulin ($P \leq 0.05$; Tables 2 and 3).

Trimodulin markedly reduced HLA-DR expression on CD14⁺ monocytes in a dose-dependent manner in the absence of endotoxins ($P \leq 0.05$; Tables 2 and 3). No additive down-regulation was observed with trimodulin over that seen with LPS/LTA activation. Monocytes from patients with early and late stage sepsis showed a slight dose-dependent increase in HLA-DR expression upon LPS treatment, which was not significant (data not shown). However, more detailed experiments are required to verify these results further.

No trimodulin-mediated alterations in expression levels of CD32b, CD35 and CD71 were observed (data not shown).

Antibacterial functions of endotoxin-activated monocytes and granulocytes are not affected by trimodulin

The effect of trimodulin on the phagocytic activity of monocytes before and after LPS/LTA stimulation was assessed. *In vitro* phagocytosis occurs quickly after opsonisation, and the treatment protocol was adjusted accordingly (Figure 4a). Whole blood from healthy donors was treated 24 h *ex vivo* with LPS or LTA, followed by 1 h of treatment with trimodulin (early inflammation model; Table 4). Whole blood samples

Table 2. Detection of surface markers on CD14⁺ monocytes after LPS and trimodulin treatment.

LPS ^a (ng/ml)	Trimodulin ^b (mg/ml)	TLR2 ^c	TLR4 ^c	CD64 ^c	CD11b ^c	HLA-DR ^c
0	0	5.0 ± 0.6	3.5 ± 1.6	7.5 ± 1.5	63.3 ± 8.3	204.4 ± 59.2
	0.5	2.8 ± 0.3***	1.6 ± 0.3*	5.9 ± 2.8	58.2 ± 7.7	170.2 ± 40.3
	5	1.8 ± 0.2***	1.5 ± 0.6*	13.2 ± 5.5	50.3 ± 14.3	137.6 ± 22.4*
0.05	0	8.5 ± 3.8	5.1 ± 3.3	10.2 ± 4.2	70.2 ± 18.3	115.0 ± 43.6
	0.5	4.4 ± 2.0	1.6 ± 0.6*	5.6 ± 1.6	71.5 ± 18.3	128.5 ± 32.6
	5	2.8 ± 1.0*	0.9 ± 0.3*	11.1 ± 2.4	55.5 ± 14.8	102.6 ± 38.6
0.5	0	8.2 ± 0.3	3.3 ± 1.6	46.4 ± 9.0	57.9 ± 12.7	42.5 ± 15.8
	0.5	5.0 ± 0.7***	1.4 ± 0.6*	25.7 ± 3.1**	58.5 ± 12.5	42.2 ± 16.6
	5	3.4 ± 1.1***	0.9 ± 0.5*	30.1 ± 4.0**	38.0 ± 11.0*	42.9 ± 15.5

^aTotal endotoxin treatment time (72 h) on healthy donor PBMCs.

^bTreatment 24 h after initiation of LPS stimulation.

^cMean ± SD values were obtained from five donors (values represent mean channel fluorescence intensity).

* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ showing the differences between trimodulin treated and untreated control (two-tailed t-test).

Bold numbers: increase or decrease is different with $P \leq 0.05$ for LPS stimulation alone compared to unstimulated cells (two-tailed t-test).

Table 3. Detection of surface markers on CD14⁺ monocytes after LTA and trimodulin treatment.

LTA ^a (μ g/ml)	Trimodulin ^b (mg/ml)	TLR2 (n = 6) ^c	TLR4 (n = 4) ^c	CD16 (n = 6) ^c	CD64 (n = 6) ^c	CD11b (n = 5) ^c	CD11c (n = 4) ^c	HLA-DR (n = 5) ^c
0	0	4.9 \pm 2.4	3.2 \pm 2.0	50.0 \pm 21.8	19.6 \pm 4.7	142.5 \pm 31.6	126.5 \pm 18.6	193.6 \pm 85.5
	0.5	3.3 \pm 0.8	1.8 \pm 0.5	57.8 \pm 34.4	29.7 \pm 12.2	136.1 \pm 24.9	138.9 \pm 8.3	142.4 \pm 73.3
	5	2.6 \pm 1.2	1.9 \pm 0.5	40.2 \pm 15.6	46.1 \pm 23.8*	94.9 \pm 20.6*	123.5 \pm 5.3	107.1 \pm 44.0
1	0	4.7 \pm 2.0	2.5 \pm 0.5	67.4 \pm 30.5	19.7 \pm 7.4	127.9 \pm 27.3	130.5 \pm 17.4	143.8 \pm 80.5
	0.5	3.1 \pm 1.3	2.2 \pm 0.9	53.8 \pm 22.2	15.6 \pm 6.1	115.6 \pm 19.3	131.5 \pm 3.8	130.4 \pm 64.6
	5	2.2 \pm 1.0*	1.4 \pm 0.6*	45.6 \pm 5.5	32.2 \pm 16.5	85.7 \pm 10.0*	104.4 \pm 12.0*	117.9 \pm 44.2
10	0	7.1 \pm 3.4	2.6 \pm 1.6	128.9 \pm 54.1	93.8 \pm 41.8	55.3 \pm 31.7	59.4 \pm 5.3	24.6 \pm 16.7
	0.5	4.6 \pm 1.6	1.1 \pm 0.5	81.6 \pm 27.2	66.7 \pm 29.7	67.4 \pm 21.9	63.2 \pm 8.9	26.7 \pm 13.6
	5	3.5 \pm 1.3*	0.9 \pm 0.3	36.0 \pm 5.2**	54.2 \pm 32.6	35.8 \pm 12.9	49.4 \pm 6.5	30.3 \pm 17.6

^aTotal endotoxin treatment time (72 h) on healthy donor PBMCs.

^bTreatment 24 h after initiation of LTA stimulation.

^cMean \pm SD values were obtained from four to six donors (values represent mean cell fluorescence).

* $P \leq 0.05$ and ** $P \leq 0.01$ statistically different compared to trimodulin untreated control (two-tailed t-test).

Bold numbers: increase or decrease is different with $P \leq 0.05$ for LTA stimulation alone compared to unstimulated cells (two-tailed t-test).

LTA: lipoteichoic acid.

from patients with sepsis were also treated with trimodulin for 1 h, but without additional *ex vivo* LPS or LTA pre-stimulation (Figure 4b and d). LPS, LTA and trimodulin did not have an overall reducing effect on the phagocytic activity of monocytes obtained from healthy donors or patients with sepsis (Figure 4d). Phagocytosis was, for most donors and patients, largely unchanged (Figure 4d).

To study oxidative-burst activity in monocytes and granulocytes, whole blood of healthy donors was treated as shown in Figure 4c. A slight, but not significant, increase in oxidative activity upon trimodulin treatment alone was observed for both monocytes and granulocytes (Table 4). The results imply that the antibacterial functions of the monocytes and granulocytes were not negatively influenced by endotoxins and trimodulin treatment.

Trimodulin impedes LPS-driven T lymphocyte proliferation

During sepsis high endotoxin concentrations induce a cytokine storm, which leads to accelerated polyclonal T cell proliferation in the acute phase and to T-cell exhaustion and immune suppression at the late stage.⁶ The influence of trimodulin on expansion of CFSE-stained T lymphocytes after a single LPS hit from healthy donors and patients was investigated (Figure 5a and b). After 72 h in culture, trimodulin dose dependently reduced the number of cell divisions completed by LPS-stimulated CD3⁺ T lymphocytes from healthy donors ($P \leq 0.01$). The percentage of CD3⁺-resting T lymphocytes in cultures pre-stimulated with LPS

increased from 44% to 61% and 94% upon the addition of 0.5 and 5 mg/ml trimodulin, respectively (Figure 5c). Upon LPS/ConA/IL-2 stimulation, about 10% of CD3⁺ T lymphocytes completed up to four cell divisions in 72 h; by contrast, >95% of trimodulin-treated cells did not divide more than once. Similar results were obtained with the CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocyte subpopulations.

Interestingly, upon treatment with an increasing trimodulin dose (0, 0.5 or 5 mg/ml), similar *ex vivo* enrichments in the percentages of CD3⁺-resting T lymphocytes were observed with PBMCs from patients in the early ($n = 3$; from 12% to 27% and 61%; Figure 5d) and late ($n = 3$; from 27% to 59% and 84%) phases of sepsis (Figure 5e). Untreated cells from late-stage sepsis showed a twofold increase in resting cells compared to cells from patients with early-stage sepsis, which might in part reflect exhaustion occurring during late-stage sepsis.

Trimodulin dampens endotoxin-induced cytokine secretion

The early phase of sepsis is characterised by an initial intense inflammatory response involving both pro- and anti-inflammatory cytokines (cytokine storm) secreted by activated monocytes and expanding T lymphocytes. Since trimodulin modulates monocyte and lymphocyte functions, cytokine patterns were analysed in the cell-culture supernatants of PBMCs cultivated according to Figure 5a and b.

In PBMCs from healthy donors pre-stimulated with LPS *ex vivo*, a noteworthy negative correlation was

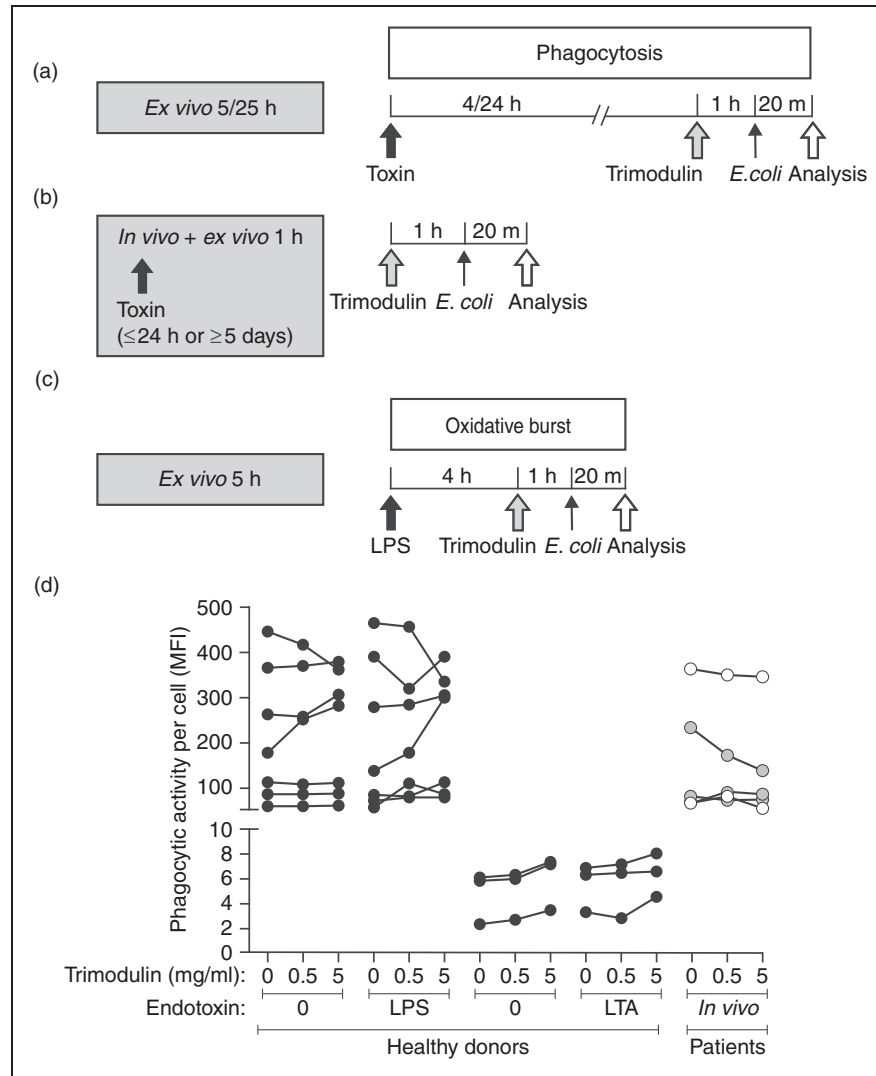


Figure 4. Phagocytosis and oxidative-burst activity in monocytes and granulocytes. (a) Whole blood of healthy donors was treated with 0, 0.05 or 0.5 ng/ml LPS or 0, 1 or 10 μ g/ml LTA. After 4 or 24 h of endotoxin stimulation, different trimodulin concentrations (0, 0.5 or 5 mg/ml) were added for 1 h. (b) Whole blood of patients was treated with trimodulin without *ex vivo* LPS/LTA stimulation. (a and b) Cells were harvested and incubated with 20 μ l FITC-(LPS) or pHrodo-conjugated *E. coli* cells (LTA) for 20 min before analysis in flow cytometry. (c) Whole blood of healthy donors was stimulated with 0, 0.05 or 0.5 ng/ml LPS for 4 h, and then treated with or without trimodulin (5.0 mg/ml) for 1 h before the addition of 20 μ l non-labelled *E. coli*. After 20 min of staining, cells were washed and analysed by flow cytometry. (d) Before–after plot of phagocytic activity measured in monocytes from seven or three healthy donors (black circles), three patients with early stage sepsis (grey circles) and two patients with late-stage sepsis (open circles) without or with trimodulin treatment. Results are shown for FITC-labelled *E. coli* with or without 0.5 ng/ml LPS for 24 h, or for pHrodo-conjugated *E. coli* without or with 10 μ g/ml LTA for 4 h. A paired parametric *t*-test revealed no difference between untreated and 0.5 mg/ml trimodulin-treated cells from healthy donors without LPS ($P=0.6527$) or with LPS ($P=0.6205$), nor for cells from patients with sepsis ($P=0.3167$).

observed between trimodulin dose and levels of the pro-inflammatory cytokines IL-17a ($r_s=-0.843$, $P=0.004$) and IFN- γ ($r_s=-0.685$, $P=0.042$), and also in this experimental setting for TNF- α ($r_s=-0.843$, $P=0.004$; Figure 6a–c). Additionally, a clear (72 h; $r_s=-0.632$, $P=0.068$) and relevant (120 h; $r_s=-0.791$, $P=0.011$; data not shown) reduction was detected in the anti-inflammatory cytokine, IL-10, with trimodulin

treatment (Figure 6d), in contrast to IVIG, which was shown to increase IL-10.³² Levels for IL-4 and IL-6 were below and above detection thresholds, respectively (data not shown).

Similarly, *ex vivo* cytokine release of PBMCs from patients with early-stage sepsis showed a negative correlation between trimodulin dose and IL-17a ($r_s=-0.896$, $P=0.001$), TNF- α ($r_s=-0.798$,

Table 4. Phagocytosis and oxidative-burst activity of immune cells after endotoxin and trimodulin treatment.

Trimodulin (mg/ml)	Phagocytosis ^{a,b}				Oxidative burst ^{a,c}			
	24 h LTA+1 h trimodulin (pHrodo-conjugated <i>E. coli</i>)		24 h LPS+1 h trimodulin (FITC-labelled <i>E. coli</i>)		Reactive oxidant-positive monocytes		Enzymatic activity	
	LTA ($\mu\text{g/ml}$)	% of cells ($n=3$) ^d	LPS (ng/ml)	% of cells ($n=7$)	LPS (ng/ml)	% of cells ($n=5$)	Monocytes ($n=5$)	Granulocytes ($n=5$)
0	0	7.7 \pm 2.1	0	92.0 \pm 4.8	0	45.9 \pm 14.6	2.3 \pm 1.4	3.8 \pm 1.5
0.5	0	7.5 \pm 2.3	0	91.6 \pm 5.4	0	n.d.	n.d.	n.d.
5	0	6.5 \pm 3.0	0	90.8 \pm 4.6	0	65.0 \pm 11.6	3.1 \pm 1.2	7.3 \pm 1.7*
0	1	7.0 \pm 2.0	0.05	88.5 \pm 6.2	0.05	56.2 \pm 9.4	2.6 \pm 1.3	5.3 \pm 0.7
0.5	1	7.3 \pm 2.2	0.05	89.8 \pm 7.5	0.05	n.d.	n.d.	n.d.
5	1	6.6 \pm 2.9	0.05	88.0 \pm 7.4	0.05	60.2 \pm 10.5	3.5 \pm 1.1	6.6 \pm 0.7
0	10	7.6 \pm 1.2	0.5	91.0 \pm 9.3	0.5	63.6 \pm 12.4	2.8 \pm 1.3	5.4 \pm 1.1
0.5	10	7.7 \pm 1.1	0.5	91.3 \pm 9.5	0.5	n.d.	n.d.	n.d.
5	10	7.0 \pm 1.8	0.5	91.6 \pm 6.8	0.5	70.8 \pm 10.6	3.8 \pm 1.7	7.5 \pm 2.1

^aActivities measured in whole blood samples.

^bPhagocytosis measured according to Figure 4a.

^cOxidative burst after 24 h of LPS stimulation of whole blood and 1 h of trimodulin treatment (Figure 4c).

^dMean and SD values obtained from a number (n) of donors as indicated.

*One condition comparison between untreated and trimodulin-treated cells resulted in $P \leq 0.05$ (t-test). All data are mean (SD) unless otherwise specified.

n.d.: not determined.

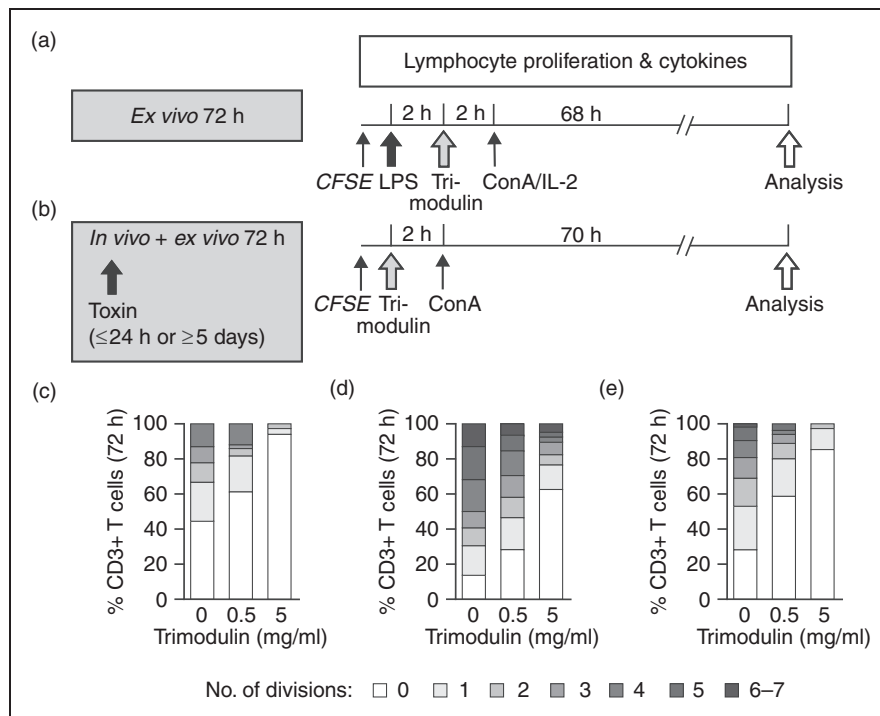


Figure 5. Proliferation analysis of T lymphocytes. (a) PBMCs from healthy donors ($n = 3$) were stained with CFSE before stimulation with 0.5 ng/ml LPS for 2 h and treatment with 0, 0.5 or 5 mg/ml trimodulin for 2 h. Subsequently, IL-2 (100 ng/ml) and/or ConA (10 $\mu\text{g/ml}$) were added to induce proliferation of T cells. (b) PBMCs from patients with early ($n = 3$) or late ($n = 3$) sepsis were treated only with trimodulin and ConA at the same concentrations as in (a). (c–e) After a total ex vivo incubation time of 72 h, (c) healthy donor PBMCs or PBMCs from patients with (d) early or (e) late-stage sepsis were analysed by flow cytometry to determine the percentage of CD3⁺ T lymphocytes that had divided up to seven times. Chi-square test, $P < 0.0001$.

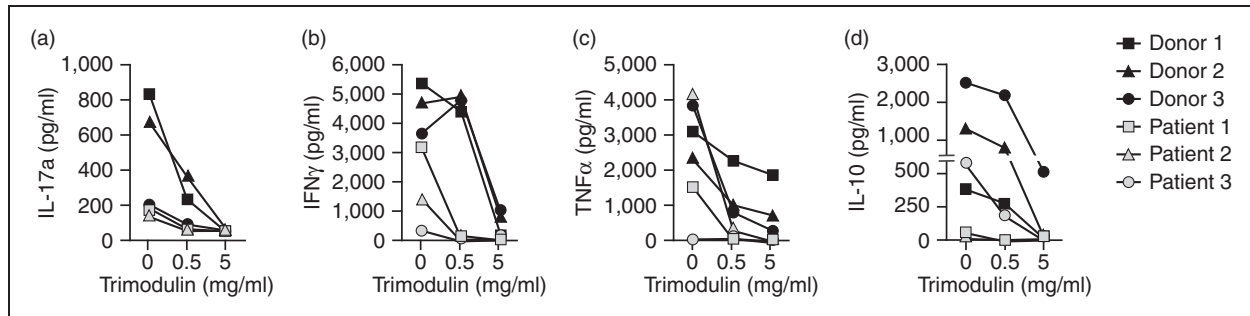


Figure 6. Cytokines expressed by PBMCs. Plots showing bead-array results of cytokine levels detected in supernatants of PBMC cultures from healthy donors and patients with early-stage sepsis. PBMCs were treated as shown in Figure 5a and b with 0.5 ng/ml LPS and increasing trimodulin concentrations (grey symbols: healthy donors; $n = 3$) or after trimodulin treatment alone (black symbols: patients with early stage sepsis; $n = 3$).

$P = 0.010$), IFN- γ ($r_s = -0.908$, $P = 0.001$) and IL-10 ($r_s = -0.751$, $P = 0.020$). The levels of the cytokines analysed were lower in PBMCs from patients with sepsis than in PBMCs from healthy donors. Furthermore, one patient did not show the cytokine storm, possibly because they were in an early septic stage (low LPS blood level) or already in an immune-suppressed stage.

Discussion

In this study, we present new insights into the underlying immunomodulatory mechanisms of trimodulin on the reactivity of cells in purified PBMCs and whole blood samples, with reference to sepsis. A translational approach, such as that employed here, is essential for the careful evaluation of biological plausibility and mode-of-action elucidation for this investigational immunomodulatory therapy. In addition, results from such studies may help to identify strategies for determining which patients are most likely to benefit from trimodulin.

Our results provide insights into the mechanism of action of trimodulin. First, trimodulin was shown to down-regulate endotoxin-induced secretion of pro- and anti-inflammatory cytokines, including TNF- α , IL-17a, IFN- γ and IL-10 (Figures 2 and 5). In contrast to IVIG, IL-10 was down-regulated by trimodulin.³³ Second, down-regulation of the complement receptors, CD11b (LPS and LTA) and CD11c (LTA), by high endotoxin concentrations was further enhanced by trimodulin (Tables 2 and 3). CD11b and CD11c mediate inflammation by regulating leukocyte adhesion and migration, phagocytosis, cytotoxicity and chemotaxis, and are able to bind the inactivated complement component, iC3b. Early trimodulin treatment thus seems to attenuate such pro-inflammatory responses. As shown previously, the IgM containing Ig preparation Pentaglobin[®] prevented complement deposition more effectively compared with IVIG.³⁴ For other sepsis

studies, early intervention with Pentaglobin[®] was also important for improved efficacy.^{35–37}

Furthermore, cell-surface markers on monocytes, including CD64, CD16, TLR2 and TLR4, were induced with high endotoxin concentrations, but were counterbalanced by trimodulin (Tables 2 and 3). Our analysis showed that the regulation of TLRs by endotoxins and trimodulin occurred at the mRNA level, resulting in up- and down-regulation, respectively, of TLR2 and TLR4 proteins on the monocyte surface (Figure 3).

For CD64 (and CD16), up-regulation was observed upon LPS and LTA treatment alone, and the same was true for monocytes treated with trimodulin alone (Tables 2 and 3). Although trimodulin treatment alone caused up-regulation of CD64, and oxidative burst was nearly doubled, the combination of endotoxin and trimodulin did not result in an additive effect, but resulted in counterbalancing the expression level of CD16 and CD64. The phagocytic and oxidative-burst activities of the monocytes were not related to CD16/CD64 expression. The activities were largely unchanged upon endotoxin and trimodulin treatment, indicating that these important functions are not negatively influenced by trimodulin (Table 4 and Figure 4d). This finding is in agreement with an *in vivo* study showing no change in oxidative-burst activity of polymorphonuclear leukocytes after trimodulin treatment.³⁸ Trimodulin appears to reduce the disproportionate expression of the CD64 receptor, which might prevent excessive release of pro-inflammatory cytokines induced by this receptor (Figures 2 and 5).³⁹ Thus, our *ex vivo* experiments demonstrated that early intervention with trimodulin impedes various important pro-inflammatory functions of monocytes and lymphocytes induced by endotoxins.

It was postulated that extended LPS/LTA stimulation leads to an increase in IRAK-M inhibitory protein, resulting in the steady suppression of TNF- α

during the late stages of sepsis.^{40,41} Trimodulin might prevent such immune suppression, since it prevents the initial hyperstimulation by endotoxins. IgG-based IVIG and IgM-enriched Pentaglobin® contain inhibitory or neutralising Abs against super Ag exotoxins released by *Staphylococci* and *Streptococci*.^{15,42–47} In contrast to IVIG, the IgM-enriched preparation Pentaglobin® contains superior concentrations of neutralising Abs against *E. coli*, *Klebsiella* species and *Pseudomonas aeruginosa* LPS.^{46,48,49} In accordance, recent publications have reported a benefit with Pentaglobin® in endotoxin-mediated diseases such as toxic shock syndrome and in severe infections with multidrug-resistant pathogens.^{18,50–52}

In addition to its effects on monocytes, trimodulin was found to impede T-cell proliferation, which may counteract the sepsis-induced phenomenon of immune anergy *in vivo* (Figure 5). This impediment may be caused indirectly by the decrease in the cytokine storm induced by endotoxin-activated monocytes (TNF- α) and lymphocytes (IL-17a and IFN- γ) in the presence of trimodulin (Figure 6). Upon infection in the early phase, IFN- γ and IL-6 are produced by naïve T cells activating the differentiation and proliferation of Th1 and Th17 cells while inhibiting Th2 proliferation. The following counter-regulatory, anti-inflammatory state, characterised by a reduction in IFN- γ and IL-17a, might be co-responsible for the observed shift from Th1/Th17 to the Th2/T_{reg} phenotype associated with poor prognosis in patients with sepsis. Trimodulin, if applied early, may prevent this cytokine storm and possibly the shift in T-cell phenotypes. Similarly for IVIG, it has been postulated that the modulation of T-cell populations and functions observed in treated patients was the indirect consequence of a direct effect of IVIG on accessory cells.^{53–55} Again, this suggests a window of opportunity for trimodulin during early-stage sepsis. Importantly, if trimodulin was added to cells during late-stage sepsis, suppression of proliferation was even stronger than observed during early sepsis. The administration of trimodulin at a time point that is too late may be immunosuppressive and may not support patient cure. Previous variable timing of therapy thus may also explain the inconsistent efficacy results of IVIG and Pentaglobin® in sepsis studies.¹²

In addition to modulating endotoxin-induced responses due to endotoxin-neutralising Abs, this study shows that trimodulin has additional immunomodulatory activity. A direct anti-inflammatory effect of trimodulin was observed by a dose-dependent reduction in cytokines (TNF- α , IL-17a and IFN- γ ; data not shown) and by the down-regulation of TLR2/4, HLA-DR and CD64 on monocytes in the absence of endotoxins (Tables 2 and 3). This direct anti-inflammatory

effect of Ig preparations is in accordance with previous studies with IVIG showing the dampening effect on TNF- α , for example in rheumatoid arthritis and Kawasaki disease.⁵⁶

There are limitations of this model and study data in that the potential of trimodulin to modulate cellular processes *ex vivo* is not evidence of *in vivo* activity in patients. Nonetheless, these results are supported by clinical findings showing that the early application of IgM-enriched Ig preparation was associated with a survival benefit.^{36,57–59} The present study has revealed a possible rationale for this observation in that the immunoregulatory effect seen with trimodulin is determined by the strength and duration of endotoxin stimulation: strong, short-term stimulation, either *ex vivo* or *in vivo*, resulted in a greater dampening effect by trimodulin but, importantly, without completely blocking the immune response. After extended and repeated endotoxin challenge or in the late phase of sepsis, cytokine secretion by hyporeactive monocytes was not affected by trimodulin.

We hypothesise that targeting ‘upstream’ mediators preventing early induction of the hyper-inflammatory stage may reduce the risk of driving the suppression of ‘downstream’ mediators in the following phase, resulting in immune suppression. Our findings offer a mechanistic rationale for Berlot’s thesis that we should not miss ‘the window of opportunity in the first days that follow sepsis diagnosis’.^{35,36,57}

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