Research Article

Journal of Innate Immunity

J Innate Immun 2019;11:359–374
DOI: 10.1159/000495528

Received: February 27, 2018
Accepted after revision: November 1, 2018
Published online: January 17, 2019

# CD14 Counterregulates Lipopolysaccharide-Induced Tumor Necrosis Factor-α Production in a Macrophage Subset

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**Keywords**

Lipopolysaccharide · Macrophages · CD14 · Blocking antibody · Signal transduction

**Abstract**

In response to GM-CSF or M-CSF, macrophages (MΦ) can acquire pro- or anti-inflammatory properties, respectively. Given the importance of CD14 and Toll-like receptor (TLR) 4 in lipopolysaccharide (LPS)-induced signaling, we studied the effect of anti-CD14 antibody mediated CD14 blockade on LPS-induced cytokine production, signal transduction and on the expression levels of CD14 and TLR4 in GM-MΦ and M-MΦ. We found M-MΦ to express higher levels of both surface antigens and to produce more interferon (IFN)-β and interleukin-10, but less tumor necrosis factor (TNF)-α than GM-MΦ. Blockage of CD14 at high LPS concentrations increased the production of proinflammatory cytokines and decreased that of IFN-β in M-MΦ but not in GM-MΦ. We show that phosphorylation states of signaling molecules of the MyD88 (myeloid differentiation primary response 88), TRIF (TIR-domain-containing adapter-inducing IFN-β) and MAPK (mitogen-activated protein kinase) pathways are not altered in any way that would account for the cytokine overshoot reaction. However, CD14 blockage in M-MΦ decreased TLR4 and CD14 expression levels, regardless of the presence of LPS, indicating that the loss of the surface molecules prevented LPS from initiating TRIF signaling. As TNF-α synthesis was even upregulated under these experimental conditions, we suggest that TRIF is normally involved in restricting LPS-induced TNF-α overproduction. Thus, surface CD14 plays a decisive role in the biological response by determining LPS-induced signaling.

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## Introduction

Macrophages (MΦ) form a heterogeneous cell population displaying multiple functions. For the sake of simplicity they are generally categorized into two broad but distinct subsets termed M1-MΦ (classically activated) and M2-MΦ (alternatively activated) [1]. However, rather than being ontogenetically distinct, these classes represent the extremes of a continuum of functional phenotypes [2]. While M1-MΦ promoted by lipopolysaccha-

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ride (LPS) or interferon (IFN)-γ exhibit proinflammatory activities necessary for host defense, M2-MΦ generated in response to interleukin (IL)-4 and IL-13 are found to dampen inflammation, promote tissue remodeling and to support tumor progression. M1-MΦ are exquisitely sensitive to LPS, a prototypical activator of cells of the immune system. They respond in a robust manner by releasing numerous inflammatory mediators including the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-6 which support the eradication of pathogenic microbes. However, when uncontrolled, the same mediators can cause systemic inflammation, septic shock and death [3]. In contrast, M2-MΦ produce only low levels of the proinflammatory cytokines in response to LPS [4, 5] but secrete instead the anti-inflammatory cytokines IL-10 and IFN-β [6–8].

In search of MΦ that are functionally distinct, we differentiated monocytes in the presence of colony-stimulating factors (CSF), which reflects in vivo situations that have an impact on MΦ development [9]. Consistent with elevated levels of granulocyte-macrophage (GM)-CSF during inflammation or the constitutive production of macrophage (M)-CSF in the steady state, MΦ developed in the presence of GM-CSF or M-CSF also exhibit a predisposition for polarization towards an M1- and M2-like status, respectively [4, 10]. As the features of MΦ developed in the presence of CSFs do not exactly map those of M1- and M2-MΦ [7, 11], we will refer to the two subsets used here as GM-MΦ and M-MΦ, respectively. The first step of LPS-induced MΦ activation is the transfer and binding of LPS to the cell surface Toll-like receptor 4 (TLR4)-myeloid differentiation factor 2 (MD2) complex. This transfer is catalyzed by two accessory molecules: LPS-binding protein and cluster of differentiation 14 (CD14) [12]. CD14 is a 56-kDa glycosylphosphatidylinositol-anchored protein found on the surface of many TLR-expressing cells [13]. It contains no transmembrane or cytoplasmic domains and therefore cannot directly induce cell signaling. LPS-binding protein forms a high-affinity complex with LPS that is subsequently delivered to CD14, which in turn transfers monomeric LPS to TLR4-MD2 to initiate TLR4 dimerization and activation of two distinct pathways [12, 14, 15]. The first of these is the rapid myeloid differentiation primary response 88 (MyD88) pathway, which is activated directly from the plasma membrane leading to NF-κB activation and the production of proinflammatory cytokines [16–18]. The second (TIR-domain-containing adapter-inducing interferon-β/TRIF-related adapter molecule [TRIF/TRAM]) pathway requires the CD14-mediated internalization of TLR4/MD2 to endosomes and leads both to a delayed NF-κB response and to the activation of the transcription factor interferon regulatory factor 3 that results in IFN-β production. While TRAM/TRIF signaling has an absolute requirement for CD14 that cannot be overcome by simply increasing LPS concentrations, activation of the MyD88 pathway requires CD14 only when the LPS concentration is low [19].

These properties identify CD14 as a potential target for the suppression of pathological inflammation. However, while blocking CD14 has been shown to reduce the production of proinflammatory cytokines [20, 21] and to have promising clinical effects [22–24], inflammation still persists. It has recently been suggested that the combined inhibition of CD14 and complement may be more effective in this respect [25, 26].

In the present study we examine the role of CD14 in regulating TLR4 expression as well as the LPS-induced production of TNF-α, IL-6 and IL-10. We show that blockage of CD14 resulted in an overproduction of proinflammatory cytokines in M-MΦ but not GM-MΦ when exposed to high LPS concentrations. We exclude IL-10 and IFN-β as negative regulators of the overproduction and suggest that the increased cytokine release after blocking CD14 is mainly due to an interruption of the CD14-mediated crosstalk between TLR4 signaling pathways.

## Materials and Methods

*Reagents and Antibodies*

LPS from *Escherichia coli* (serotype 055:B5) was obtained from Sigma-Aldrich (Taufkirchen, Germany) and LPS from *Salmonella abortus equi* S-form (TLR grade) from Enzo Life Sciences (Farmingdale, NY, USA). Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine (OxPAPC) was from InvivoGen (San Diego, CA, USA). OxPAPC was dissolved in chloroform (1 mg/mL) and divided into aliquots. Thereafter, chloroform was removed by evaporation. The thin film of lipid on the tube wall was suspended in medium. As a control, one tube without OxPAPC but with chloroform was used (CF). Recombinant human IFN-β was purchased from R&D Systems (Minneapolis, MN, USA).

Anti-CD14 antibody (Ab) (MEM-18), mouse IgG1 isotype (MOPC-21) and mouse IgG2b isotype (MCP-11) were obtained from EXBIO Praha (Vestec, Czech Republic). Anti-CD14 Ab (My4, 322A-1) was from Beckman Coulter (Brea, CA, USA). Anti-CD14-APC Ab (M5E2), anti-CD14-PerCP/Cy5.5 Ab (HCD14), anti-CD18 Ab (TS1/18, LEAF), anti-TLR4 Ab (HTA125, LEAF), mouse IgG1 isotype (MOPC-21, LEAF), and mouse IgG2a isotype (MOPC-173, LEAF) were purchased from BioLegend (San Diego, CA, USA). Anti-TLR4-PE Ab (610015), anti-IFN-β Ab (polyclonal goat IgG), anti-IL-10 Ab (25209) and normal goat IgG isotype

(polyclonal) were from R&D Systems. Anti-MD2 (18H10) was from Hycultec, Beutelsbach, Germany. The FITC-labeled goat anti-mouse Ab was obtained from SIFIN (Berlin, Germany).

*Cell Separation and Cell Culture*

Human peripheral blood mononuclear cells from healthy donors were obtained by Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK) density centrifugation. After repeated washing in PBS containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-5.0 elutriation system (Beckman Coulter, Brea, CA, USA), as described previously [27]. The purity of the cell preparation was >90% as assessed by morphological screening and immunofluorescence staining with a monoclonal APC-labeled Ab against CD14.

Monocytes were suspended in RPMI 1640 medium (GE Healthcare) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich, Taufkirchen, Germany), 100 U/mL penicillin and 100 mg/mL streptomycin (Seromed Biochrom KG, Berlin, Germany) to a final amount of $5 \times 10^5$ cells/mL. Cells were supplemented with 500 U/mL GM-CSF (leukine, sargramostim) for GM-MΦ differentiation or 50 ng/mL M-CSF (Life Technologies, Darmstadt, Germany) for M-MΦ differentiation and incubated for 7 days at 37 °C, 5% $CO_2$ in Teflon bags (Zell-Kontakt, Nörte-Hardenberg, Germany; fluorinated ethylene propylene foil, 50 µm, hydrophobic). After harvesting, the MΦ ($1 \times 10^6$/mL) were incubated for 2 h in cell culture plates before analysis or stimulation.

*Detection of Cytokines in Culture Supernatants*

MΦ ($1 \times 10^6$/mL) were incubated for 15 min with the corresponding blocking antibodies or reagents prior to the stimulation with LPS. Culture supernatants were collected at the specified time points and tested for TNF-α, IL-6, IL-10 and IFN-β using a human TNF-α, IL-6 or IL-10 ELISA kit (PeproTech, Rocky Hill, NJ, USA) or an IFN-β-chemiluminescent ELISA kit (InvivoGen) according to the manufacturer's protocol.

*Flow Cytometry Analysis*

MΦ ($1 \times 10^6$/mL) seeded in cell culture plates were washed and incubated with PBS/EDTA for 15 min at 37 °C prior to rinsing off the detached cells. $2 \times 10^5$ cells were incubated at 4 °C with 10% human AB-serum (Institute of Transfusions Medicine, University Hospital Leipzig) to saturate Fc receptors and block unspecific bindings. After 15 min the cells were washed (PBS + 10% Emagel [Pirmal Healthcare, Morpeth, UK] + 0.1% $NaN_3$), and the direct dye-labeled Ab anti-CD14-APC (M5E2 or MEM-18), anti-TLR4-PE, the nonlabeled anti-CD18 and anti-MD2 Ab as well as the corresponding isotypes were added for 20 min at 4 °C. Cells were then washed and the nonlabeled probes further incubated for 15 min with a FITC-labeled goat anti-mouse Ab. After washing and fixation the cells were analyzed on a FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and median fluorescence intensity (MFI) values of the isotype were subtracted from those of the sample.

*Chip Cytometry Analysis*

Prior to sample loading, ZellSafe™ chips (Zellkraftwerk GmbH, Leipzig, Germany) were rinsed with 1,000 µL PBS. MΦ ($2 \times 10^5$/100 µL) were loaded onto the chip, incubated for 5 min to allow the cells to attach to the chip surface and fixed by rinsing with 1,000 µL 4% paraformaldehyde at 4 °C. After 45 min, loaded chips were rinsed with 1,000 µL PBS. Chip cytometry was performed as described previously [28] with anti-CD14-PerCP/Cy5.5 (HCD14) and TLR4-PE (610015) stained separately through iterative cycles of bleaching, staining and imaging on the ZellScanner ONE instrument (Zellkraftwerk). Image and data processing was performed automatically by Zellkraftwerk's ZellExplorer software. The fluorescence signal of each cell after staining was corrected for the background fluorescence, and MFI values were calculated.

*Western Blot Analysis*

Western blot analysis was carried out as described previously [29]. MΦ ($1 \times 10^6$/mL) were suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P 40, 0.5% deoxycholate, 0.1% SDS; pH 7.5) supplemented with cOmpleteTM EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and with phosphatase inhibitors (1 mM $Na_3VO_4$ and 50 mM NaF). After sonication the samples were centrifuged for 5 min at 15,000 *g* and 4 °C. The protein concentrations in the supernatants were determined using a DC Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Cell lysates (30–50 µg) boiled in 1× Laemmli sample buffer run on a 12% SDS-polyacrylamide gel (Protean II, Bio-Rad GmbH) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Munich, Germany). Membranes were probed with anti-phospho-IKKα/β Ab (Ser176/180, 16A6, 1:1,000, Cell Signaling), anti-phospho-RelA Ab (phospho-NF-κB p65, Ser536, 93H1, 1:1,000, Cell Signaling), anti-phospho-TBK1 Ab (Ser172, D52C2 XP®, 1:1,000, Cell Signaling), anti-phospho-extracellular signal-regulated kinase (p-ERK) 1/2 Ab (Thr202/Tyr204, E10, 1:2,000, Cell Signaling), anti-phospho-c-Jun amino-terminal kinase (JNK) Ab (Thr183/Tyr185, 81E11, 1:1,000, Cell Signaling), anti-phospho-p38 Ab (Thr180/Tyr182, 1:1,000, Cell Signaling) or anti-β-actin Ab (AC74, 1:2,000, Sigma-Aldrich). The following POD-conjugated secondary antibodies were used: goat anti-rabbit IgG Ab (1:20,000, Dianova, Hamburg, Germany) or goat anti-mouse IgG Ab (1:8,000, Sigma-Aldrich).

Chemiluminescent detection was achieved by using the ECL-A/ECL-B reagents (both from Sigma Aldrich) and a Luminescent Image Analyzer (LAS 1000, Fujifilm, Tokyo, Japan).

*RNA Isolation, Reverse Transcription and Real-Time PCR*

Total RNA was isolated from MΦ using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNase I treatment and reverse transcription were performed as previously described [29].

The reaction mixture for the quantitative PCR contained 7.5 µL SYBR Green PCR Mastermix (Bio-Rad), 250 nM forward and reverse primers (see below) and 1.5 µL of cDNA template in a final volume of 15 µL. The following primers were used for the PCR:

*GNB2L1* forward 5′-GAGTGTGGCCTTCTCCTCTG-3′
reverse 5′-GCTTGCAGTTAGCCAGGTTC-3′
*TNF-α* forward 5′-TCAGCCTCTTCTCCTTCCTG-3′
reverse 5′-GGCTACAGGCTTGTCACTCG-3′
*IL-6* forward 5′-GGATTCAATGAGGAGACTTGC-3′
reverse 5′-GTTGGGTCAGGGGTGGTTAT-3′
*IL-10* forward 5′-CGAGATGCCTTCAGCAGAGTG-3′
reverse 5′-TCATCTCAGAACAAGGCTTGGC-3′

The reactions were performed as previously described [29] using *GNB2L1* as the reference gene.

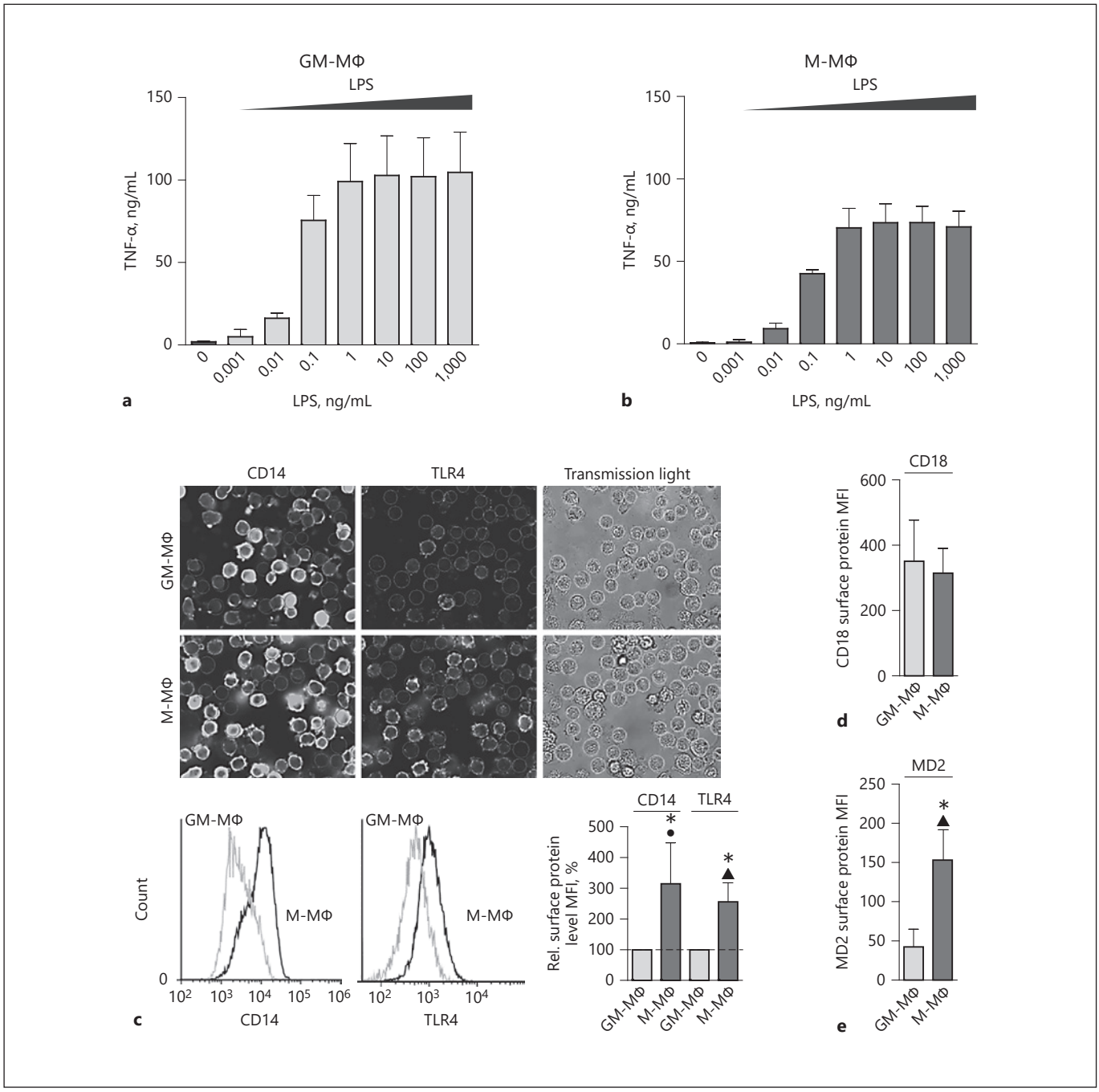


**Fig. 1.** LPS-induced TNF-α-release and expression of the LPS-receptor complex by GM-MΦ and M-MΦ. GM-MΦ ($1 \times 10^6$/mL) (**a**) and M-MΦ ($1 \times 10^6$/mL) (**b**) were incubated in the presence of different LPS concentrations (0.001–1,000 ng/mL). After 6 h, TNF-α concentrations in the supernatants were determined by ELISA. Data represent means ± SD ($n = 3$). **c** GM-MΦ and M-MΦ were immobilized on a ZellSafe™ chip, fixed and stained with fluorescence-labeled anti-CD14 (HCD14) ($n = 5$) and anti-TLR4 (610015) Ab ($n = 3$). Fluorescence intensity was measured by iterative chip-based cytometry (iCBC). One representative fluorescence light picture and the corresponding histogram of each cell surface antigen and one representative transmission light picture of each subtype are shown. Surface staining of CD18 (**d**) and MD2 (**e**) was determined by flow cytometry. Mean values of the median fluorescence intensity (MFI) ± SD are shown ($n = 4$ [CD18], $n = 3$ [MD2]). Significances were calculated using the unpaired one-tailed $t$ test (●) or the one-tailed Mann-Whitney U test (▲). * $p \leq 0.05$.

*Statistical Analysis*

All statistical analyses were done using GraphPad Prism software (GraphPad Inc., La Jolla, CA, USA). First, the values were tested for Gaussian distribution using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for $p$ value. If the values follow a Gaussian distribution, the unpaired $t$ test with Welch's correction and one-tailed or two-tailed calculation was used. If the values did not follow a normal distribution the Mann-Whitney U test was considered (● unpaired one-tailed $t$ test, ○ unpaired two-tailed $t$ test, ▲ one-tailed Mann-Whitney U test, △ two-tailed Mann-Whitney U test).

The statistical significance was classified as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

## Results

*Differential TNF-α Production and LPS-Receptor Complex Expression on GM-MΦ and M-MΦ*

Both GM-MΦ and M-MΦ produce TNF-α in response to LPS in a concentration-dependent manner, with a near-maximal response at 1 ng/mL (Fig. 1a, b). The levels of TNF-α produced by M-MΦ were lower than those produced by GM-MΦ under these conditions, consistent with previous observations [8, 29].

Given the critical role of the LPS receptor TLR4 and its coreceptor CD14 in mediating LPS-induced signaling pathways, we next determined the expression of these molecules on GM-MΦ and M-MΦ by chip cytometry [28]. By the use of this method we verified previous data [29, 30] generated by flow cytometry, showing that M-MΦ express higher levels of both TLR4 and CD14 (Fig. 1c). This pattern was further confirmed using a different CD14 Ab together with the corresponding isotype to control for nonspecific binding (online suppl. Fig. S1; www.karger.com/doi/10.1159/000495528 for all online suppl. material). MD2, another surface molecule involved in LPS signaling, displays an expression pattern similar to that of TLR4 and CD14 (Fig. 1e). MD2 forms a complex with TLR4 which upon binding of LPS results in a dimerization of two TLR4-MD2 complexes and activation of downstream signaling cascades [31]. The expression of CD18, which also participates in LPS recognition [32] and LPS signaling [33], hardly differed between the two subsets (Fig. 1d).

*CD14 and CD18 Limit TNF-α Production by M-MΦ in Response to LPS*

The unexpected finding that anti-inflammatory M-MΦ express higher levels of TLR4 and CD14 than do the proinflammatory GM-MΦ prompted us to assess the involvement of these molecules in LPS-induced cytokine production in this system. We tested the effects of an inhibitor of the TLR4 signal pathway (OxPAPC [34]), as well as antibodies blocking TLR4 (HTA125 [35]), CD14 (MEM-18 [36] and My4 [37]), CD18 (TS1/18 [38]) and MD2 (18H10 [39]) for their ability to affect TNF-α production induced by 1 ng/mL and 100 ng/mL LPS (Fig. 2a, b). The two LPS concentrations were chosen because of their differing requirements for CD14 in order to induce TNF-α production [40]. Blockage of the LPS receptor TLR4 by HTA125, of the TLR4 pathway by OxPAPC or of MD2 by 18H10 each led independently to a downregulation of TNF-α production by both MΦ subtypes, confirming that TLR4 and MD2 contribute to the TNF-α production at both low and high LPS concentrations. The effects of the other blocking reagents were highly cell type dependent: TNF-α release from LPS-stimulated proinflammatory GM-MΦ was suppressed by CD14 blockage, most markedly when the LPS concentration was low (Fig. 2a). However, these Ab hardly affected the TNF-α release from the anti-inflammatory M-MΦ under the same conditions and actually increased the amount of TNF-α released in response to the high LPS concentration (Fig. 2b). Ab-mediated blockage of CD18 had little or no effect on GM-MΦ (Fig. 2a) but strongly increased TNF-α production from M-MΦ (Fig. 2b) induced by either low or high concentrations of LPS. A combination of antibodies blocking both CD14 and CD18 in M-MΦ showed no additive or synergistic response (Fig. 2a, b).

These results suggest that CD14 normally acts to boost the TNF-α response of GM-MΦ to low concentrations of LPS but to limit TNF-α production from M-MΦ exposed to high concentrations of LPS, thus maintaining TNF-α production within both upper and lower limits. CD18 is required to prevent TNF-α overproduction in M-MΦ under both low and high LPS concentrations.

*CD14-Mediated Suppression of Cytokine Overproduction by M-MΦ Is Not Restricted to TNF-α*

Having described that TNF-α production is differentially dependent on CD14 in GM-MΦ and M-MΦ, we went on to examine effects on the production of the proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 under the same conditions. Here, too, cytokine release from GM-MΦ exposed to low levels of LPS was markedly reduced by the anti-CD14 Ab MEM-18, while the induction under high LPS concentrations was less susceptible to Ab inhibition and thus less reliant on CD14 (Fig. 3a). The pattern of IL-6 and IL-10 production in LPS-treated M-MΦ was also similar to that of TNF-α, CD14 blockage resulting in a suppression of IL-6 produc-

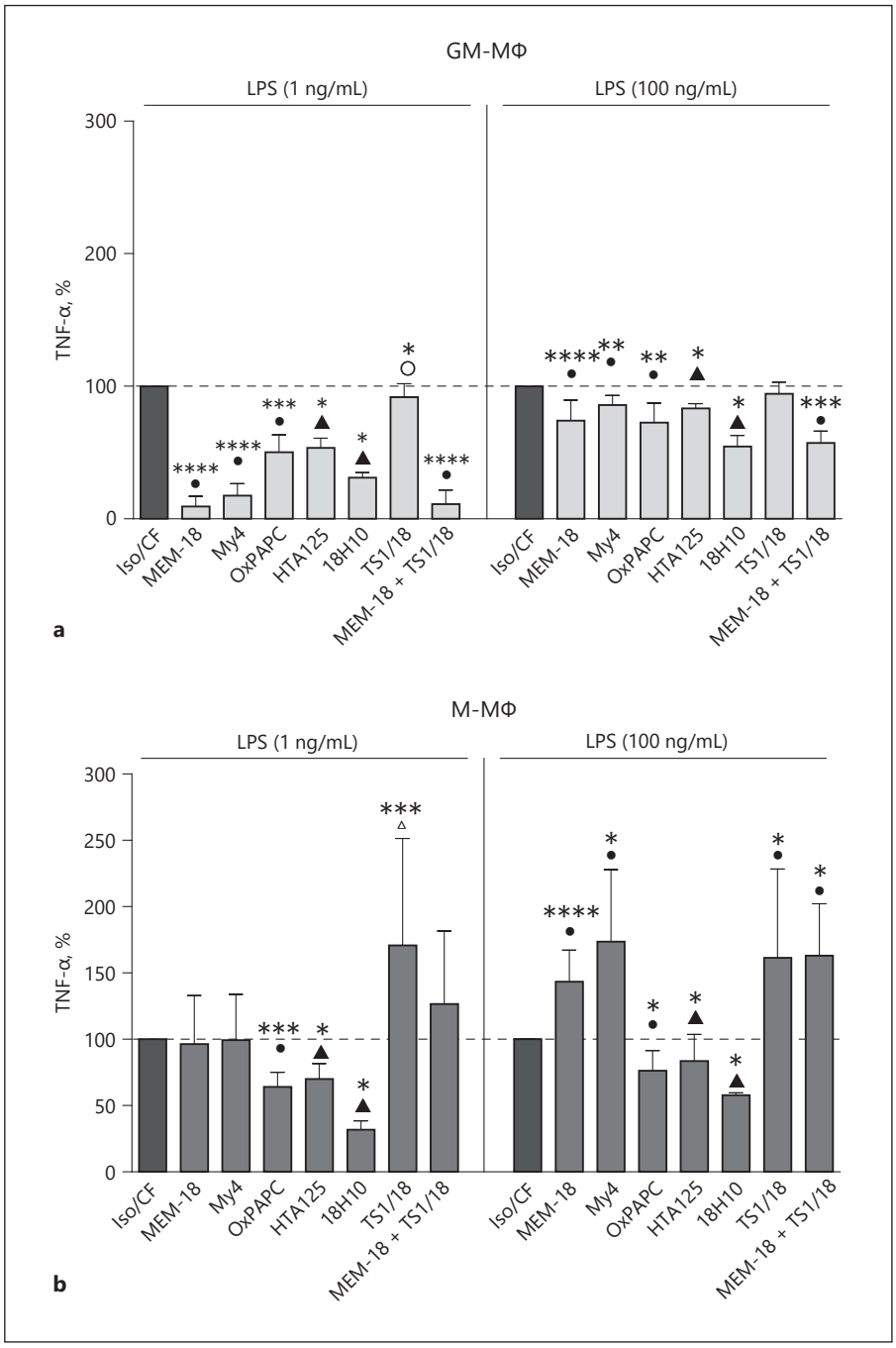


**Fig. 2.** CD14 and CD18 prevent overproduction of TNF-α in M-MΦ. GM-MΦ ($1 \times 10^6$/mL) (**a**) and M-MΦ ($1 \times 10^6$/mL) (**b**) were treated with anti-CD14 Ab (MEM-18, 10 µg/mL, $n = 30$ and My4, 10 µg/mL, $n = 6$ [GM-MΦ], $n = 7$ [M-MΦ]), TLR4 signal pathway inhibitor (OxPAPC, 20 µg/mL, $n = 5$), anti-TLR4 Ab (HTA125, 10 µg/mL, $n = 3$), anti-MD2 Ab (18H10, 10 µg/mL, $n = 3$), anti-CD18 Ab (TS1/18, 10 µg/mL, $n = 11$) or the respective isotype control (solvent CF for OxPAPC) for 15 min prior to the incubation with LPS (1 ng/mL or 100 ng/mL). After 6 h TNF-α concentrations in the supernatants were determined by ELISA. Data represent means ± SD. Significances were calculated using the unpaired one-tailed *t* test (●), unpaired two-tailed *t* test (○), one-tailed Mann-Whitney U test (▲) or two-tailed Mann-Whitney U test (△) to the respective isotype (or CF for OxPAPC) (isotype/CF = 100%). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

tion at low LPS concentration and in an increased production of both IL-6 and IL-10 in M-MΦ exposed to high concentrations of LPS (Fig. 3b).

To describe in more detail the differential involvement of CD14 in the LPS response of GM-MΦ and M-MΦ, we examined the time course of both secreted protein and cellular mRNA levels up to 6 h following LPS exposure (Fig. 4). Experiments were carried out with a concentration of 100 ng/mL LPS because at this concentration CD14 deficiency resulted in a cytokine overspill by M-MΦ but not GM-MΦ. In the GM-MΦ (Fig. 4a, c), the reduction in TNF-α and IL-6 protein levels in the presence of the blocking CD14 Ab MEM-18 was reflected by a reduction in levels of the corresponding mRNA, while the

slight increase in IL-10 production was not associated with changes at the mRNA level. Both the mRNA and protein responses in these cells were durable over 4–6 h.

Figure 4b shows that under CD14 blockade M-MΦ produce more TNF-α, IL-10 and, although delayed, also more IL-6 than do the controls. Furthermore, in the absence of LPS, these cells secreted minor amounts of IL-10 in response to MEM-18 probably due to a signaling event induced by the Ab. In M-MΦ (Fig. 4d) expression of these three RNAs followed different kinetics in response to MEM-18. Compared to controls TNF-α and IL-10 mRNA peaked at later time points, and IL-6 mRNA remained elevated for a prolonged time. IL-6 expression levels did not exceed those of controls until 4 h of incubation, consistent with the delayed IL-6 production. Comparing the mRNA and protein levels of GM-MΦ and M-MΦ it seems that the mRNA levels are not reflected in protein levels as normally assumed. This apparent discrepancy is due to the fact that the baseline mRNA levels of both GM-MΦ (Fig. 4c) and M-MΦ (Fig. 4d) were normalized to 1 at time zero, effectively obscuring the approximately 100-fold lower level of IL-6 mRNA and 3-fold lower level of TNF-α mRNA in M-MΦ compared to GM-MΦ (online suppl. Fig. S2a). As shown in online supplementary Figure S2b IL-6 mRNA levels of M-MΦ are always lower than those of GM-MΦ while TNF-α mRNA values reached after 30 min and 1 h are similar between the two subsets. At later time points M-MΦ express less TNF-α mRNA than GM-MΦ supporting a recent publication in which we show that the half-life of TNF-α mRNA in M-MΦ is shorter than in GM-MΦ [29].

Taken together these data demonstrate that treatment of GM-MΦ and M-MΦ with MEM-18 resulted in TNF-α, IL-6 and IL-10 mRNA expression levels which mirror the protein levels. However, the kinetics of mRNA expression under CD14 shortage differed between M-MΦ and GM-MΦ.

*IFN-β and IL-10 Are Not Involved in Preventing Overproduction of TNF-α*

In search of candidates that may be involved in preventing the TNF-α overshoot reaction in M-MΦ we concentrated on IFN-β and IL-10, each of which has been described to display anti-inflammatory properties and to be produced at higher levels by M-MΦ than by GM-MΦ in response to LPS (Fig. 5a, d) [6–8, 41]. LPS-induced IFN-β production depends on the activation of the TRAM/TRIF signaling pathway that requires CD14 to mediate internalization of TLR4 into endosomes, to which TRIF is recruited [16–18].

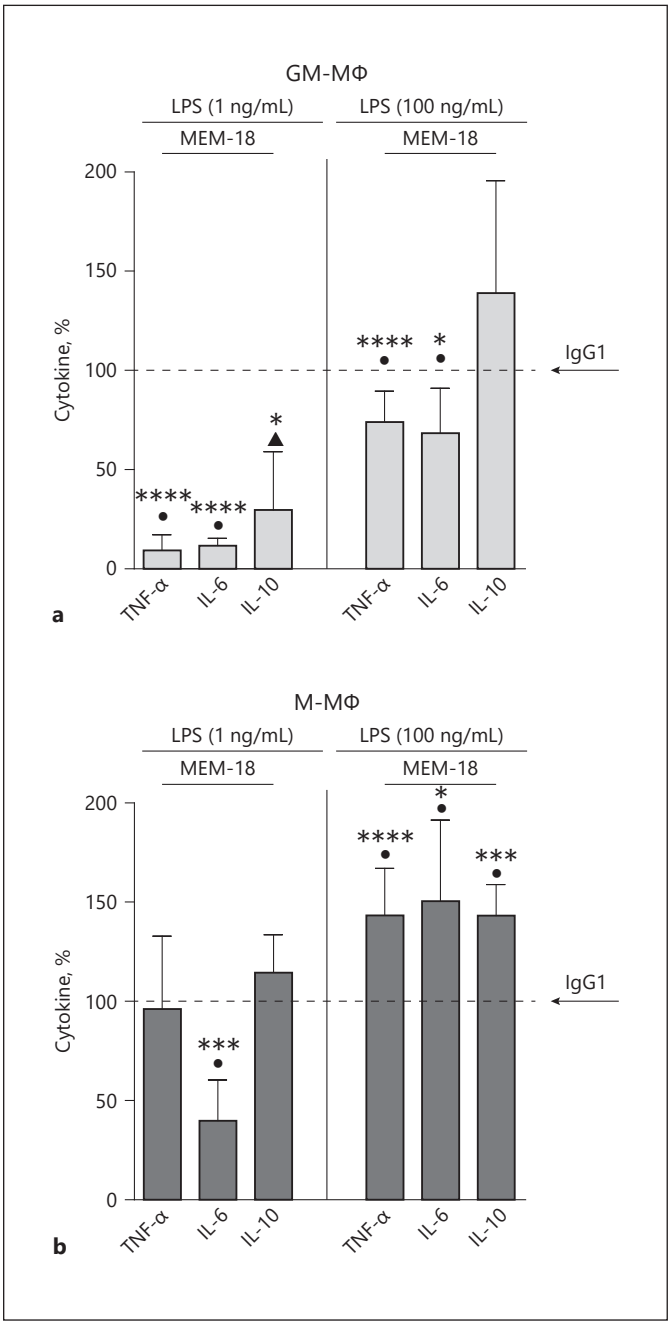


**Fig. 3.** CD14 prevents overproduction of cytokines other than TNF-α in M-MΦ. GM-MΦ ($1 \times 10^6$/mL) (**a**) and M-MΦ ($1 \times 10^6$/mL) (**b**) were treated with anti-CD14 Ab (MEM-18, 10 μg/mL) or the corresponding isotype (IgG1) for 15 min prior to the incubation with LPS (1 ng/mL and 100 ng/mL). After 6 h TNF-α ($n = 30$), IL-6 ($n = 5$) and IL-10 ($n = 4$ [GM-MΦ], $n = 6$ [M-MΦ]) concentrations in the supernatants were determined by ELISA. Data represent means ± SD. Significances were calculated using the unpaired one-tailed *t* test (●) or the one-tailed Mann-Whitney U test (▲) to IgG1 isotype control (IgG1 = 100%, dashed line). * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

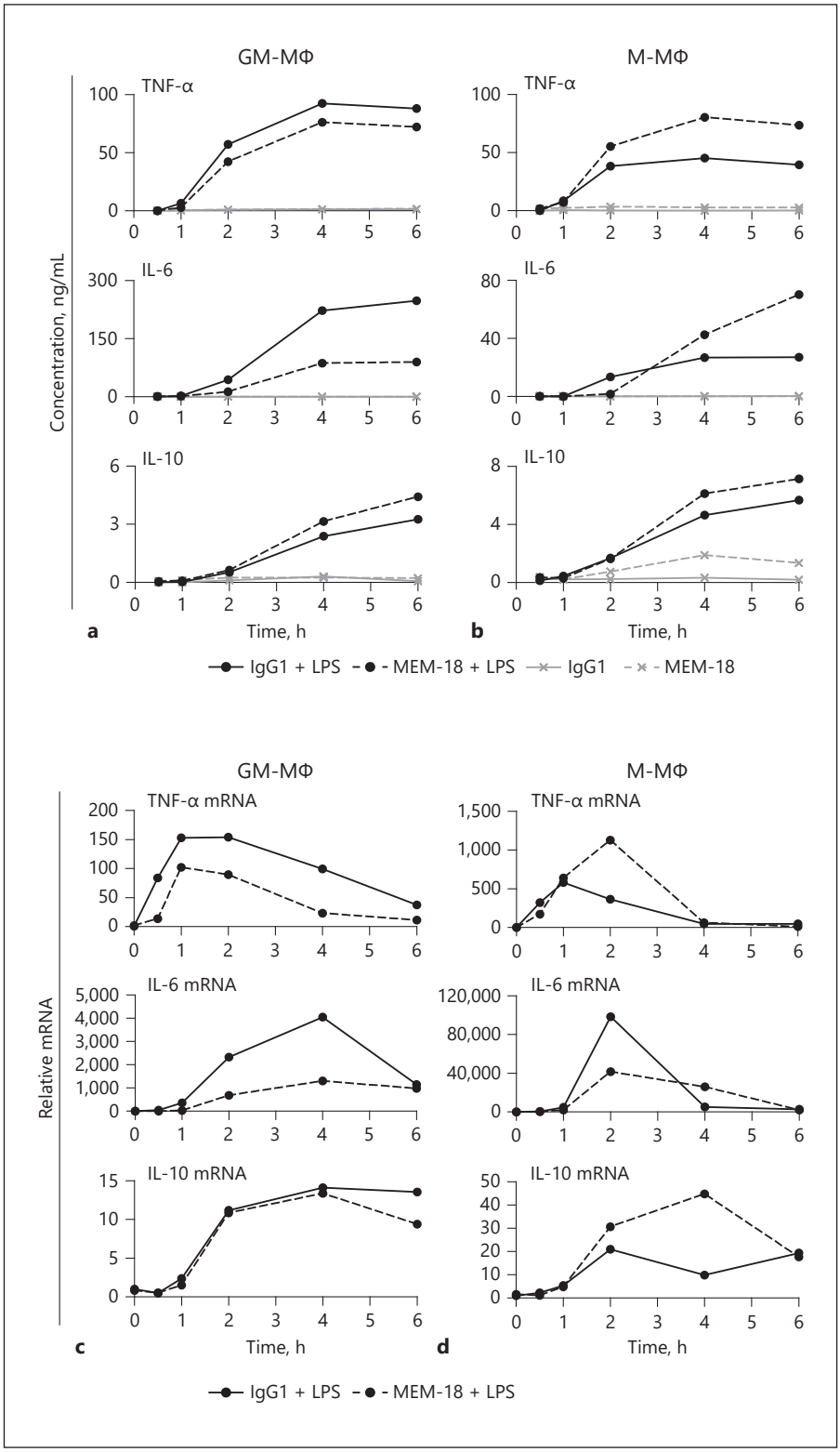


**Fig. 4.** Differential effect of anti-CD14 Ab on cytokine expression levels and kinetics of GM-MΦ and M-MΦ. GM-MΦ ($1 \times 10^6$/mL) (**a**, **c**) and M-MΦ ($1 \times 10^6$/mL) (**b**, **d**) were treated with anti-CD14 Ab (MEM-18, 10 µg/mL, dashed line) or the corresponding isotype control (IgG1, solid line) for 15 min prior to the incubation with 100 ng/mL LPS (black lines) or without LPS (gray lines, **a**, **b** only). After the indicated times the TNF-α, IL-6 and IL-10 protein concentrations in the supernatants were determined by ELISA, and the corresponding cellular mRNA levels were quantified by quantitative PCR (IgG1 + LPS, 0 h = 1). Data show one representative experiment out of three.

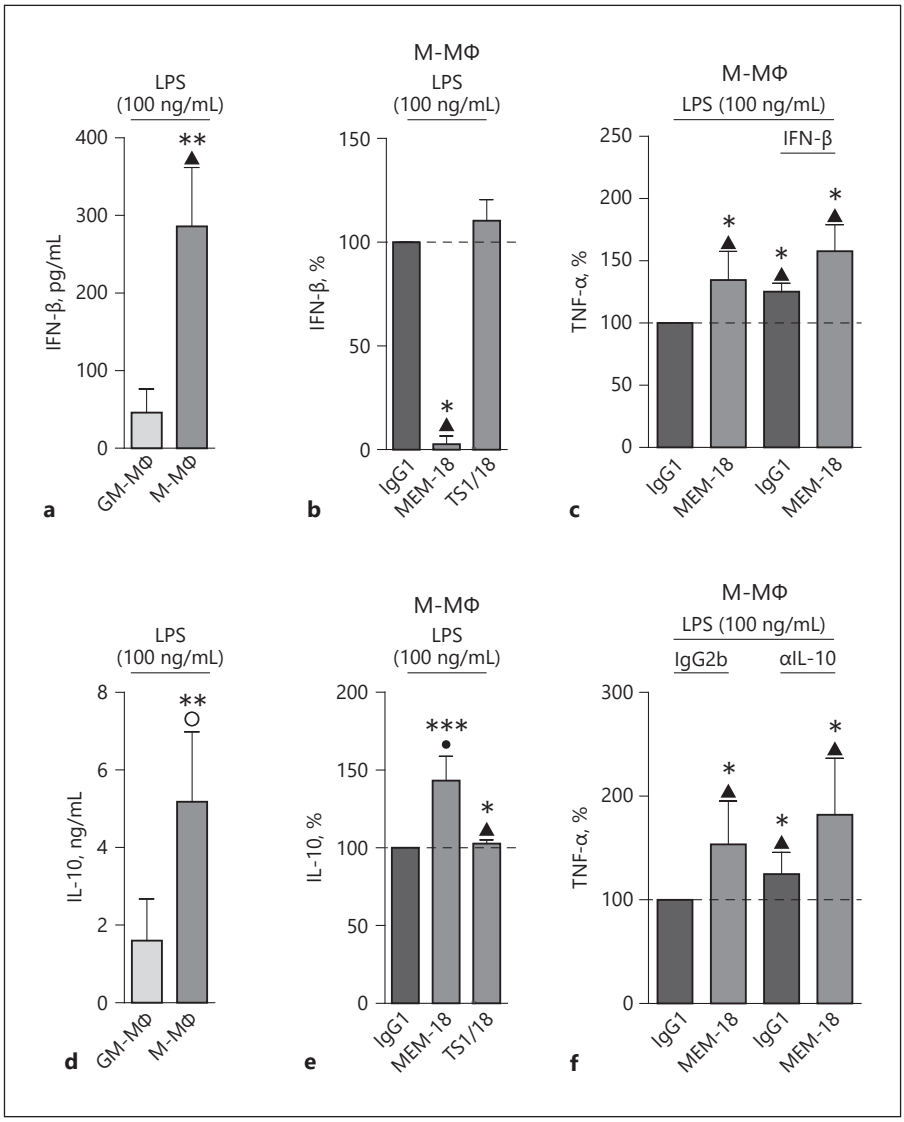


**Fig. 5.** IFN-β and IL-10 do not interfere with the LPS-induced TNF-α overproduction in M-MΦ. GM-MΦ ($1 \times 10^6$/mL) and M-MΦ ($1 \times 10^6$/mL) (**a**, **d**) were incubated with LPS (100 ng/mL). After 4 h IFN-β concentrations ($n = 4$ [GM-MΦ], $n = 5$ [M-MΦ]) (**a**) and after 6 h IL-10 concentrations ($n = 5$) (**d**) in the supernatants were determined by ELISA. Data represent means ± SD. Significances are calculated compared to GM-MΦ. M-MΦ ($1 \times 10^6$/mL) (**b**, **e**) were treated with anti-CD14 Ab (MEM-18, 10 μg/mL), anti-CD18 Ab (TS1/18, 10 μg/mL) or the isotype control (IgG1) for 15 min prior to the addition of LPS (100 ng/mL). After 4 h IFN-β concentrations ($n = 3$) (**b**) and after 6 h IL-10 concentrations ($n = 6$ [MEM-18], $n = 4$ [TS1/18]) (**e**) in the supernatants were determined by ELISA. Data represent means ± SD. Significances are calculated compared to IgG1 isotype control (IgG1 = 100%). M-MΦ ($1 \times 10^6$/mL) (**c**, **f**) were treated for 15 min with anti-CD14 Ab (MEM-18, 10 μg/mL) or the isotype control (IgG1) and IFN-β (200 pg/mL) ($n = 3$) (**c**) or anti-IL-10 Ab (100 ng/mL) and the corresponding isotype (IgG2b, 100 ng/mL) ($n = 3$) (**f**) prior to the addition of LPS (100 ng/mL). After 6 h, TNF-α-concentrations in the supernatants were determined by ELISA. Data represent means ± SD. Significances are calculated compared to the corresponding isotypes (IgG1or IgG1 + IgG2b = 100%). Statistical analysis was performed using the unpaired one-tailed *t* test (●), the unpaired two-tailed *t* test (○) or the one-tailed Mann-Whitney U test (▲). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

In line with a previous report, describing an absolute requirement for CD14 in LPS-induced TRAM/TRIF signaling [19], we found the IFN-β production to be drastically reduced in M-MΦ pretreated with the CD14-blocking Ab MEM-18 (Fig. 5b). Blockage of CD18 with TS1/18 had no inhibitory effect. We reasoned that if the absence of IFN-β was responsible for the LPS-induced overspill of TNF-α production in MEM-18-treated M-MΦ, then addition of IFN-β should reverse the effect. However, rather than leading to a decrease in TNF-α production, exogenous IFN-β actually increased the TNF-α release independently of functional CD14 (Fig. 5c). The upregulation of TNF-α was observed when IFN-β was applied either at 5 min before or 1 h or 2 h after stimulation with LPS (data not shown). Thus, IFN-β can be effectively excluded as a mediator of the CD14-dependent overshoot prevention.

IL-10 is also produced at far higher levels by M-MΦ than by GM-MΦ in response to LPS (Fig. 5d). In contrast to IFN-β, IL-10 production was actually increased by blocking CD14, while the blocking Ab to CD18 had no effect (Fig. 5e). This raised the question of whether IL-10 may actually contribute to the increased production of TNF-α in CD14-blocked M-MΦ. However, we were able to exclude this possibility by treatment with an IL-10-neutralizing Ab [42], which slightly increased, rather than decreased TNF-α production, both in the presence and absence of the CD14-blocking Ab MEM-18 (Fig. 5f). Thus, a role of IL-10 in preventing the TNF-α overshoot reaction can be excluded.

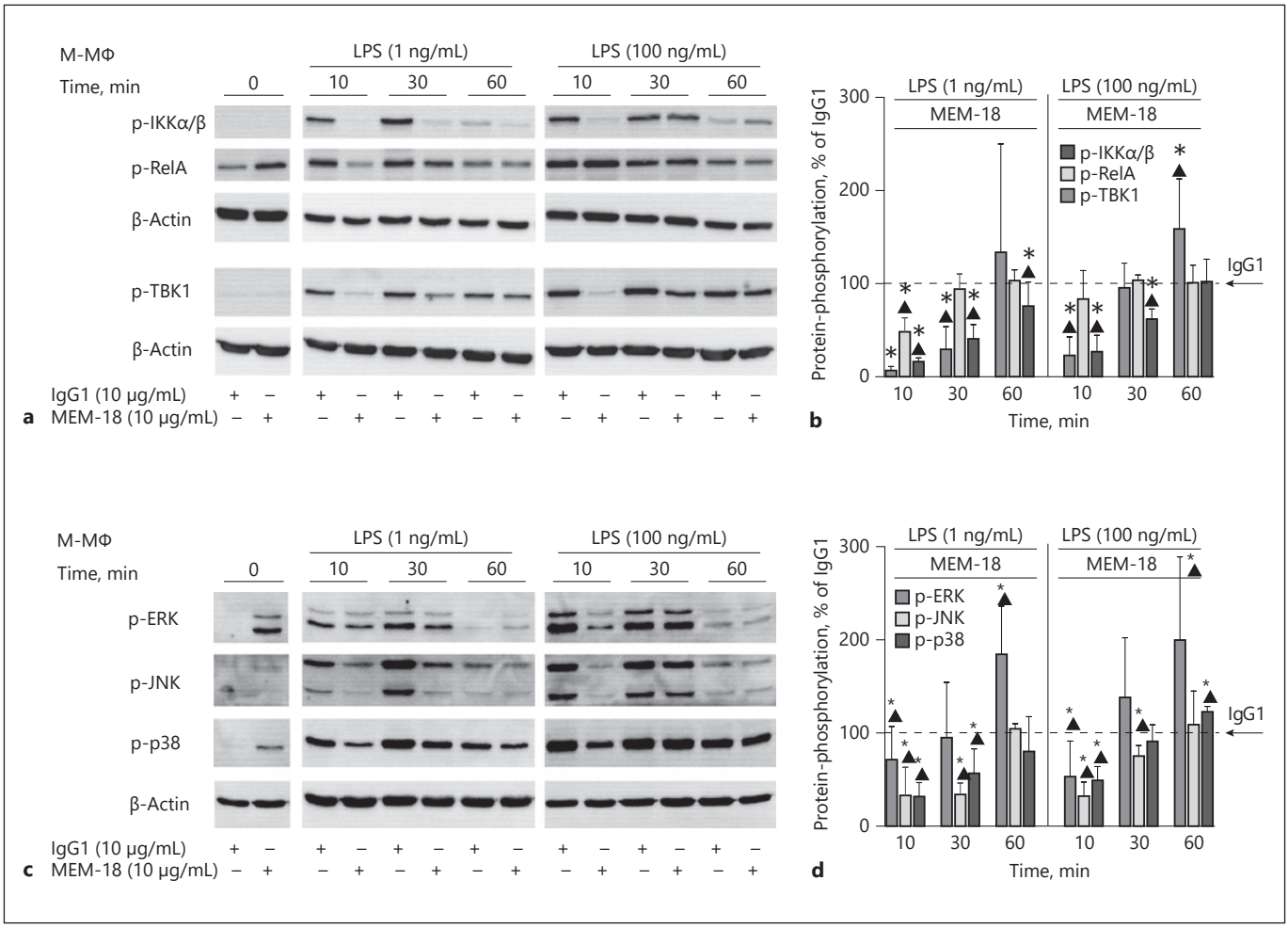


**Fig. 6.** Role of CD14 in LPS-induced signaling of M-MΦ. M-MΦ ($1 \times 10^6$/mL) were treated with anti-CD14 Ab (MEM-18, 10 μg/mL) or the corresponding isotype control (IgG1) for 15 min followed by LPS stimulation (1 ng/mL and 100 ng/mL) for various times. **a, c** Phosphorylated IKKα/β, RelA and TBK1 (**a**) and ERK, JNK and p38 (**c**) were determined using Western blot analysis. **b, d** Densitometry data of Western blots from **a** and **c** were normalized to the corresponding β-actin control. Data represent levels of phosphorylated proteins in anti-CD14 Ab-treated cells relative to the IgG1 isotype control (IgG1 = 100%, dashed line) ± SD ($n = 3$). The nonparametric one-tailed Mann-Whitney U test (▲) was calculated compared to IgG1 isotype control. * $p \leq 0.05$.

*CD14 Regulates LPS-Induced Signaling in M-MΦ*

Having shown that responses of M-MΦ to high LPS concentrations under CD14 blockade include an overproduction of TNF-α and a strong impairment of IFN-β production, we reasoned that CD14 blockade must affect the signal transduction pathways leading to the production of these cytokines.

We analyzed signaling molecules of the MyD88- (p-RelA, p-IKKα/β) and TRIF-dependent pathways (p-TANK-binding kinase1 [p-TBK1]) after stimulation with high and low LPS concentrations. With the exception of p-RelA, these molecules have been shown previously to be expressed at higher levels in M-MΦ than in GM-MΦ [29]. The primary target of the MyD88 canonical pathway is the RelA/p50 NF-κB complex, activation of which enhances transcription of proinflammatory cytokines. This effect is achieved by degradation of the NF-κB inhibitor IκB, following phosphorylation by the IκB kinase (IKK) complex, which is itself activated by phosphorylation. The IKK complex comprises two catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ [43]. TBK1 is an essential mediator of TRIF signaling: a signaling complex involving TBK1 and IKKε is responsible for the phosphorylation and consequent activation of interferon regulatory factor 3, an important transcription factor of the *IFN-β* gene [16].

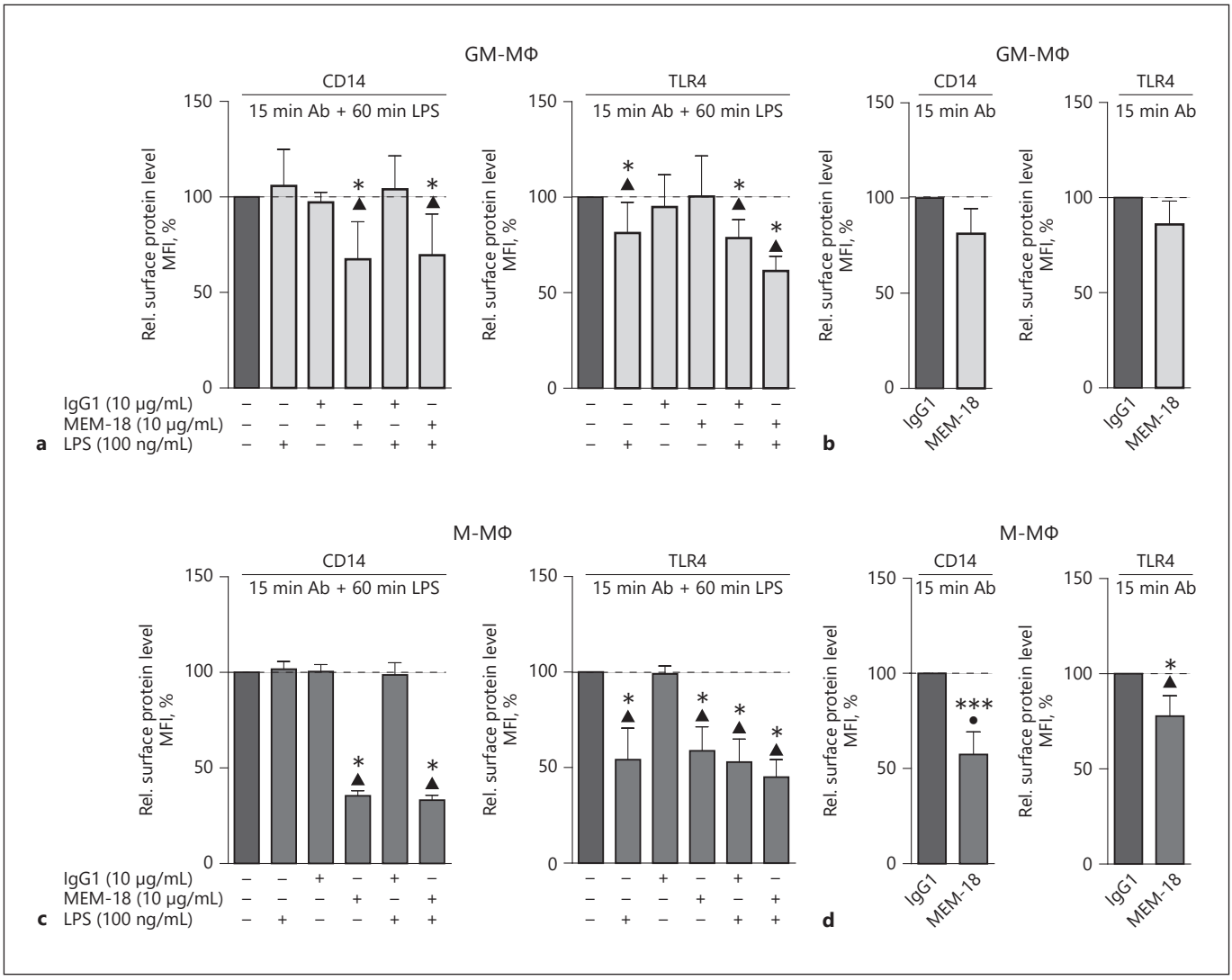


**Fig. 7.** Role of CD14 in regulating CD14 and TLR4 expression by GM-MΦ and M-MΦ. GM-MΦ ($1 \times 10^6$/mL) (**a**) and M-MΦ ($1 \times 10^6$/mL) (**c**) were treated with or without anti-CD14 Ab (MEM-18, 10 µg/mL) or the corresponding isotype control (IgG1) for 15 min prior to incubation with or without LPS (100 ng/mL) for 60 min. Surface levels of CD14 (anti-CD14-APC Ab [M5E2]) and TLR4 expression were measured by flow cytometry. Percent values of the median fluorescence intensity (MFI) ± SD are shown ($n = 3$). Significances are calculated compared to unstimulated macrophages (= 100%). **b**, **d** Effect of anti-CD14 Ab (MEM-18, 10 µg/mL) and the corresponding isotype control (IgG1) (15 min) on CD14 (anti-CD14-APC Ab [M5E2]) ($n = 6$) and TLR4 ($n = 3$) expression of GM-MΦ ($1 \times 10^6$/mL) (**b**) and M-MΦ ($1 \times 10^6$/mL) (**d**). Percent values of the median fluorescence intensity (MFI) ± SD are shown ($n = 3$). Significances are calculated compared to IgG1 isotype control (IgG1 = 100%). Statistical analysis was performed using the unpaired one-tailed *t* test (●) or the one-tailed Mann-Whitney U test (▲). * $p \leq 0.05$, *** $p \leq 0.001$.

M-MΦ preincubated for 15 min with MEM-18 were stimulated with LPS for 10 min, 30 min and 60 min, and the expression of p-IKKα/β, p-RelA and p-TBK1 was measured by Western blot analysis. As depicted in Figure 6a and b, LPS at both concentrations led to a rapid phosphorylation of both IKKα/β and TBK1 which peaked between 10 and 30 min and either declined (IKKα/β) or remained elevated up to 60 min (TBK1). In the presence of MEM-18, phosphorylation of IKKα/β and TBK1 was greatly diminished in response to 1 and 100 ng/mL LPS at 10 min. While after 30 min phosphorylation of both proteins induced by 1 ng/mL LPS was still below that of controls, the inhibitory effect of MEM-18 on IKKα/β almost vanished at 100 ng/mL LPS. The effect of MEM-18

on the phosphorylation of RelA was less prominent. In contrast to p-IKKα/β and p-TBK1, p-RelA was constitutively expressed in unstimulated cells. Addition of MEM-18 in the absence of LPS marginally enhanced phosphorylation. The only other effect of blocking CD14 was a reduction in RelA phosphorylation detected 10 min after stimulation with the low LPS concentration.

To examine the role of CD14 in activating the mitogen-activated protein kinase (MAPK) pathway, one of the pathways most relevant to LPS signaling [44, 45], M-MΦ were treated as described above in a second set of experiments and the expression of p-p38, p-ERK and p-JNK was determined (Fig. 6c, d).

Treatment with LPS at either low or high concentration resulted in an increased phosphorylation which was clearly detectable 10 min after exposure. Maximal phosphorylation was achieved after 30 min at the low LPS concentration and after 10 min at the high LPS concentration. By 60 min, phosphorylation of the three kinases declined, although p-p38 levels remained higher than those of p-ERK or p-JNK.

Densitometric analyses (Fig. 6d) demonstrate that MEM-18 reduces the p-JNK and p-p38 levels in response to 1 ng/mL LPS after 10 and 30 min of incubation. The inhibitory effect is also seen at high LPS concentrations but only during the first 10 min. It is noteworthy that, in the absence of LPS, MEM-18 leads to an increased phosphorylation of ERK and to a lesser extent of p38, indicating that these kinases can be activated by MEM-18. However, treatment with the My4 Ab does not lead to phosphorylation of ERK (online suppl. Fig. S3).

Together, these data show that the activation of signal transduction proteins of the different pathways is slightly greater in intensity in M-MΦ stimulated with 100 ng/mL LPS than in those stimulated with 1 ng/mL LPS. However, the level of phosphorylated proteins in response to MEM-18 and 100 ng/mL LPS does not identify these pathways as the cause of increased TNF-α production or impairment of IFN-β following CD14 blockade.

*CD14 Regulates CD14 and TLR4 Expression in M-MΦ*

Since CD14 is known to chaperone LPS molecules to the TLR4-MD2 complex and to mediate LPS-induced TLR4 endocytosis [16], we examined the effect of LPS on CD14 and TLR4 expression at the cell surface in the presence and absence of MEM-18. As shown in Figure 7a and c, LPS had no effect on the expression of CD14, but led to a decreased TLR4 expression on M-MΦ without affecting GM-MΦ. Assuming that the loss of membrane TLR4 is an indicator of endocytosis and the initiation of TRIF signaling, these data are consistent with the finding that large amounts of IFN-β are produced in response to LPS by M-MΦ but not by GM-MΦ.

In the presence of MEM-18 a slight reduction of CD14 expression was seen in GM-MΦ (Fig. 7a), independent of the activation state of the cells. This decrease was far more pronounced in M-MΦ (Fig. 7c) under the same experimental conditions, with expression levels already declining 15 min after preincubation with MEM-18 (Fig. 7d).

CD14 blockade by MEM-18 had no influence on TLR4 expression in GM-MΦ (Fig. 7a, b). It did, however, result in a downregulation in resting M-MΦ (Fig. 7c, d). The decrease was not affected further by the presence of LPS, which by itself led to a similarly diminished expression of TLR4. It should be noted that TLR4 expression levels are particularly low in GM-MΦ and that changes in MFI-values are very small, thus interpretation of the experiments should be handled with care.

Taken together our data show that treatment of resting M-MΦ with MEM-18 results in a reduction of cell surface TLR4 and CD14 expression, which then remains unaltered in the presence of LPS. Thus, assuming that the Ab initiates internalization of CD14 and TLR4, LPS added 15 min later is no longer capable of exerting an effect via TLR4 internalization and TRIF signaling. Although reduced, the expression level of CD14 and TLR4 seem to be sufficient to facilitate MyD88 signaling.

## Discussion

In the current study we examined the role of CD14 in regulating the LPS-induced cytokine response of GM-MΦ and M-MΦ. LPS initiates activation by binding to CD14 which chaperons LPS molecules to the TLR4-MD2-signaling complex.

Having confirmed that GM-MΦ produce far more TNF-α than M-MΦ but display lower levels of CD14 and TLR4, we examined the extent to which the cell surface molecules control TNF-α production. Consistent with previous work [40] we found that in GM-MΦ CD14 is required for MyD88-dependent TNF-α production at low but not high concentrations of LPS. This is likely to be explained by the ability of CD14 to transport LPS to MD2, when the concentration of LPS is limiting [46, 47], while at higher LPS doses LPS may bind to MD2 directly.

Unexpectedly, we found LPS (100 ng/mL) stimulated TNF-α production by M-MΦ to be higher when CD14 was blocked. This suggests that CD14 normally acts to

restrict TNF-α production by M-MΦ, keeping it below that produced by GM-MΦ. This effect was also seen when using LPS from *S. abortus equi* instead of LPS from *E. coli* (data not shown), indicating that the effect is not LPS strain specific.

The CD14 blockade also resulted in an overspill of IL-6 production [48] suggesting that functional CD14 limits multiple components of the proinflammatory response.

These results support previous findings that CD14 deficiency causes an overspill of proinflammatory cytokines in various contexts, including peritoneal MΦ [31], microglia [49] and bone-marrow-derived MΦ [19]. While most of the reports to date do not address the unusual inhibitory role of this cell surface antigen, we were able to relate the protective function of CD14 to a subset of human MΦ mainly involved in tissue repair, tumor progression and humoral immunity [50]. Thus, functional CD14 exerts suppressive effects on proinflammatory cytokine production specifically from M-MΦ, a finding that adds to our understanding of the anti-inflammatory nature of these cells. These data, together with those derived from well-established murine models of bacterial infection [48] showing exacerbated inflammation in $CD14^{-/-}$ mice, may be of importance when considering the therapeutic use of anti-CD14 antibodies to treat inflammatory diseases with inappropriate innate immune activation.

There is evidence that members of the leukocyte transmembrane CD11/CD18 family are also involved in LPS-induced signaling [33, 51–53]. CD18 is the common subunit of the four members of the integrin $\beta_2$-subfamily: $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1), $\alpha_X\beta_2$ (CD11c/CD18, p150, p95), and $\alpha_D\beta_2$ (CD11d/CD18) [54]. Of the $\beta_2$-integrins detectable on leukocytes, MΦ predominantly express CD11b/CD18. CD11b/CD18 has been postulated to interact with TLR4 and CD14 as part of the multimeric LPS-receptor complex to elicit optimal gene expression responses [55]. Perera et al. [55] found that each of the three receptors CD11b/CD18, CD14 and TLR4 was required for LPS-induced mRNA expression of cyclooxygenase-2, IL-12, and p53, whereas CD11b/CD18 was not necessary for the induction of TNF-α mRNA at low levels of LPS (0.5 ng/mL LPS).

We confirmed and extended these studies by showing that TNF-α production from GM-MΦ was CD18 independent at both low and high LPS concentrations and that in M-MΦ it even exceeded that of controls, indicating that CD18 exerted a suppressive effect in this population.

An increase in LPS-induced TNF-α release in response to treatment with anti-CD18 Ab has previously been demonstrated in monocytes [56]. Otterlei et al. [56] speculate that the upregulation of TNF-α production may be associated with an increase in CD14 expression seen after overnight incubation with LPS and anti-CD18 Ab. However, as the LPS-induced TNF-α production in both MΦ subsets peaks at earlier time points (between 2 and 4 h), we consider it unlikely that these two events are casually related. After 15 min of preincubation with anti-CD18 Ab we did not observe any changes in CD14 expression either on GM-MΦ or M-MΦ (data not shown).

The work of Han et al. [57] has paved the way to a better understanding of the role of CD11b in regulating TLR signaling. By using CD11b-deficient mice and MΦ treated with anti-CD11b/CD18 blocking Ab they found that CD11b negatively regulates TLR-triggered inflammatory responses by promoting degradation of the Toll-like receptor adapters MyD88 and TRIF. These findings help to explain why CD11b deficiency exacerbates dextran sodium sulfate-induced colitis [58] and renders mice more susceptible to endotoxin shock and *E. coli*-caused sepsis [57]. It seems likely that the overspill of TNF-α and IFN-β production in CD18-blocked M-MΦ described here is mediated by a similar molecular mechanism.

Having shown that both CD14 and CD18 restrict TNF-α production, we tested whether the absence of both surface molecules at the same time had an impact on the TNF-α response. Our data indicate that CD18 requires the presence of intact CD14 to function as a negative regulator of TNF-α production. Elevated TNF-α levels are not modulated by functional CD18 in the absence of CD14.

Having identified the MΦ subtype and the conditions under which CD14 negatively impacts TLR4 signaling, we reasoned that the activation state of components of TLR4 signaling pathways might determine the biological response. In addition to the MyD88- and TRIF-dependent pathways, we analyzed components of the MAPK pathway, which plays a significant role in LPS-induced signaling and cytokine production [59]. Comparing the kinetics and phosphorylation levels of the respective compounds in MEM-18-treated and untreated M-MΦ stimulated with either 1 or 100 ng/mL LPS, we were surprised that the phosphorylation patterns of the mediators were much alike, although they belong to different signal transduction pathways. We found the phosphorylation levels to be slightly increased and the MEM-18-mediated inhibitory effect less persistent in response to 100 ng/mL LPS. These data might explain differences in TNF-α production between the two LPS concentrations. However,

they do not necessarily account for the TNF-α overspill seen after stimulation with 100 ng/mL LPS.

Phosphorylation of ERK not only occurred in response to LPS, but also upon treatment with MEM-18 in the absence of LPS, indicating that the Ab by itself is able to activate the cells. These data are in line with previous reports [60, 61] showing that anti-CD14 Ab induces a rise in $[Ca^{2+}]_i$ in human monocytes, a process found to be caused by cross-linking monocytic antigen to an Fc receptor. Detailed analysis of Fc receptor cross-linking processes revealed the involvement of ERK signaling in downstream effects in stimulated cells [62]. However, blockage of CD14 by the My4 Ab led also to a TNF-α overspill without phosphorylation of ERK, indicating that p-ERK is not responsible for the TNF-α overproduction.

Considering the importance of CD14 and TLR4 in regulating LPS-induced signaling and the relatively high expression levels of the two molecules on M-MΦ, we addressed more specifically the role of the surface antigens in the MEM-18-mediated LPS-induced TNF-α overshoot reaction. Assuming that the loss of surface CD14 and TLR4 is due to internalization [63], we found that this process was greatly enhanced in response to MEM-18 independently of the presence of LPS. As CD14 is essential for LPS-induced TLR4-endocytosis and TRIF signaling, the loss of CD14 would explain the reduction in TLR4 internalization and IFN-β production in response to LPS. On the other hand, as downregulation of CD14 in the absence of the endocytic machinery resulted in an overproduction of TNF-α, one could speculate the failure of critical steps or interactions between signal transduction pathways that normally control or prevent extreme release activities. A potential candidate of such interactions, IFN-β, has been suggested as a negative regulator [49, 64, 65]. However, we did not find any evidence to support this notion. Instead, we suggest that a lack of CD14-dependent LPS-induced TLR4 internalization might provoke increased and prolonged signaling via MyD88. To unravel the precise molecular mechanism underlying the LPS-induced TNF-α overspill in M-MΦ will require further detailed investigations.

Taken together, our data show that CD14 contributes to the anti-inflammatory signature of M-MΦ by preventing excessive responses to high LPS challenges in this population. In response to CD14 shortage the cells shift toward a phenotype that in some aspects bears resemblance to GM-MΦ. Thus, expression levels of CD14 may determine the outcome of the biological response, by orchestrating LPS-induced signaling.


## Acknowledgment

We thank Dr. Michael Cross (Division of Hematology and Oncology, University of Leipzig, Leipzig, Germany) for critically reading the manuscript.


## Statement of Ethics

The manuscript contains studies using human monocyte-derived MΦ (ethics license 272-12-13082012).

## Disclosure Statement

N. Stanslowsky is currently employed by Zellkraftwerk Leipzig, Germany. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or material discussed in the paper apart from those disclosed.


## Funding Sources

This work was partially supported by the European Union (EFRE/SAB 100210189).


## Author Contributions

A.G., R.W. and S.H. conceived and planned the experiments. A.G., R.W., E.S. and N.S. performed the experiments. U.S. aided in interpreting the results. A.G. and S.H. wrote the manuscript with input from all authors.

## References


1 Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 2014 Mar;6:13.
2 Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010 Oct;11(10):889–96.
3 Dinarello CA. Cytokines as mediators in the pathogenesis of septic shock. Curr Top Microbiol Immunol. 1996;216:133–65.
4 Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J Immunol. 2007 Apr;178(8):5245–52.
5 Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage M1-M2 polarization balance. Front Immunol. 2014 Nov;5:614.

6 Fleetwood AJ, Dinh H, Cook AD, Hertzog PJ, Hamilton JA. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. J Leukoc Biol. 2009 Aug;86(2):411–21.
7 Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. J Leukoc Biol. 2006 Feb;79(2):285–93.
8 Weiss R, Schilling E, Grahnert A, Kölling V, Dorow J, Ceglarek U, et al. Nicotinamide: a vitamin able to shift macrophage differentiation toward macrophages with restricted inflammatory features. Innate Immun. 2015 Nov;21(8):813–26.
9 Lacey DC, Achuthan A, Fleetwood AJ, Dinh H, Roiniotis J, Scholz GM, et al. Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. J Immunol. 2012 Jun;188(11):5752–65.
10 Hamilton TA, Zhao C, Pavicic PG Jr, Datta S. Myeloid colony-stimulating factors as regulators of macrophage polarization. Front Immunol. 2014 Nov;5:554.
11 Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. Proc Natl Acad Sci USA. 2004 Mar;101(13):4560–5.
12 Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, et al. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc Natl Acad Sci USA. 2004 Mar;101(12):4186–91.
13 Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990 Sep;249(4975):1431–3.
14 O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol. 2007 May;7(5):353–64.
15 Barton GM, Medzhitov R. Toll-like receptor signaling pathways. Science. 2003 Jun;300(5625):1524–5.
16 Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004 Jul;4(7):499–511.
17 Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol. 2008 Apr;9(4):361–8.
18 Tanimura N, Saitoh S, Matsumoto F, Akashi-Takamura S, Miyake K. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. Biochem Biophys Res Commun. 2008 Mar;368(1):94–9.
19 Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, et al. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell. 2011 Nov;147(4):868–80.
20 Thorgersen EB, Hellerud BC, Nielsen EW, Barratt-Due A, Fure H, Lindstad JK, et al. CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses in Escherichia coli sepsis in pigs. FASEB J. 2010 Mar;24(3):712–22.
21 Thorgersen EB, Pischke SE, Barratt-Due A, Fure H, Lindstad JK, Pharo A, et al. Systemic CD14 inhibition attenuates organ inflammation in porcine Escherichia coli sepsis. Infect Immun. 2013 Sep;81(9):3173–81.
22 Olszyna DP, Verbon A, Pribble JP, Turner T, Axtelle T, van Deventer SJ, et al. Effect of IC14, an anti-CD14 antibody, on plasma and cell-associated chemokines during human endotoxemia. Eur Cytokine Netw. 2003 Jul-Sep;14(3):158–62.
23 Spek CA, Verbon A, Aberson H, Pribble JP, McElgunn CJ, Turner T, et al. Treatment with an anti-CD14 monoclonal antibody delays and inhibits lipopolysaccharide-induced gene expression in humans in vivo. J Clin Immunol. 2003 Mar;23(2):132–40.
24 Reinhart K, Glück T, Ligtenberg J, Tschaikowsky K, Bruining A, Bakker J, et al. CD14 receptor occupancy in severe sepsis: results of a phase I clinical trial with a recombinant chimeric CD14 monoclonal antibody (IC14). Crit Care Med. 2004 May;32(5):1100–8.
25 Barratt-Due A, Pischke SE, Nilsson PH, Espevik T, Mollnes TE. Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases-C3 or C5 emerge together with CD14 as promising targets. J Leukoc Biol. 2017 Jan;101(1):193–204.
26 Gustavsen A, Nymo S, Landsem A, Christiansen D, Ryan L, Husebye H, et al. Combined Inhibition of Complement and CD14 Attenuates Bacteria-Induced Inflammation in Human Whole Blood More Efficiently Than Antagonizing the Toll-like Receptor 4-MD2 Complex. J Infect Dis. 2016 Jul;214(1):140–50.
27 Grage-Griebenow E, Lorenzen D, Fetting R, Flad HD, Ernst M. Phenotypical and functional characterization of Fc gamma receptor I (CD64)-negative monocytes, a minor human monocyte subpopulation with high accessory and antiviral activity. Eur J Immunol. 1993 Dec;23(12):3126–35.
28 Hennig C, Adams N, Hansen G. A versatile platform for comprehensive chip-based explorative cytometry. Cytometry A. 2009 Apr;75(4):362–70.
29 Schilling E, Weiss R, Grahnert A, Bitar M, Sack U, Hauschildt S. Molecular mechanism of LPS-induced TNF-α biosynthesis in polarized human macrophages. Mol Immunol. 2018 Jan;93:206–15.
30 Sauer RS, Hackel D, Morschel L, Sahlbach H, Wang Y, Mousa SA, et al. Toll-like receptor (TLR)-4 as a regulator of peripheral endogenous opioid-mediated analgesia in inflammation. Mol Pain. 2014 Feb;10:10.
31 Rajaiah R, Perkins DJ, Ireland DD, Vogel SN. CD14 dependence of TLR4 endocytosis and TRIF signaling displays ligand specificity and is dissociable in endotoxin tolerance. Proc Natl Acad Sci USA. 2015 Jul;112(27):8391–6.
32 Wright SD, Jong MT. Adhesion-promoting receptors on human macrophages recognize Escherichia coli by binding to lipopolysaccharide. J Exp Med. 1986 Dec;164(6):1876–88.
33 Ingalls RR, Monks BG, Savedra R Jr, Christ WJ, Delude RL, Medvedev AE, et al. CD11/CD18 and CD14 share a common lipid A signaling pathway. J Immunol. 1998 Nov;161(10):5413–20.
34 von Schlieffen E, Oskolkova OV, Schabbauer G, Gruber F, Blüml S, Genest M, et al. Multihit inhibition of circulating and cell-associated components of the toll-like receptor 4 pathway by oxidized phospholipids. Arterioscler Thromb Vasc Biol. 2009 Mar;29(3):356–62.
35 Wang R, Stephens J, Lacy MJ. Characterization of monoclonal antibody HTA125 with specificity for human TLR4. Hybrid Hybridomics. 2003 Dec;22(6):357–65.
36 Plötz SG, Lentschat A, Behrendt H, Plötz W, Hamann L, Ring J, et al. The interaction of human peripheral blood eosinophils with bacterial lipopolysaccharide is CD14 dependent. Blood. 2001 Jan;97(1):235–41.
37 Viriyakosol S, Kirkland TN. A region of human CD14 required for lipopolysaccharide binding. J Biol Chem. 1995 Jan;270(1):361–8.
38 Van Epps DE, Potter J, Vachula M, Smith CW, Anderson DC. Suppression of human lymphocyte chemotaxis and transendothelial migration by anti-LFA-1 antibody. J Immunol. 1989 Nov;143(10):3207–10.
39 Elson G, Dunn-Siegrist I, Daubeuf B, Pugin J. Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. Blood. 2007 Feb;109(4):1574–83.
40 Perera PY, Vogel SN, Detore GR, Haziot A, Goyert SM. CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. J Immunol. 1997 May;158(9):4422–9.
41 Benveniste EN, Qin H. Type I interferons as anti-inflammatory mediators. Sci STKE. 2007 Dec;2007(416):pe70.
42 Fontaine M, Planel S, Peronnet E, Turrel-Davin F, Piriou V, Pachot A, et al. S100A8/A9 mRNA induction in an ex vivo model of endotoxin tolerance: roles of IL-10 and IFNγ. PLoS One. 2014 Jun;9(6):e100909.
43 Sun SC. Non-canonical NF-κB signaling pathway. Cell Res. 2011 Jan;21(1):71–85.
44 Cavalli V, Vilbois F, Corti M, Marcote MJ, Tamura K, Karin M, et al. The stress-induced MAP kinase p38 regulates endocytic trafficking via the GDI:Rab5 complex. Mol Cell. 2001 Feb;7(2):421–32.

45 Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol. 2002;20(1):55–72.
46 Akashi S, Saitoh S, Wakabayashi Y, Kikuchi T, Takamura N, Nagai Y, et al. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. J Exp Med. 2003 Oct; 198(7):1035–42.
47 Fitzgerald KA, Rowe DC, Golenbock DT. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. Microbes Infect. 2004 Dec;6(15):1361–7.
48 Roy S, Karmakar M, Pearlman E. CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and Pseudomonas aeruginosa killing in vivo. J Biol Chem. 2014 Jan;289(2): 1174–82.
49 Janova H, Böttcher C, Holtman IR, Regen T, van Rossum D, Götz A, et al. CD14 is a key organizer of microglial responses to CNS infection and injury. Glia. 2016 Apr;64(4):635–49.
50 Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. Annu Rev Immunol. 2010;28(1):157–83.
51 Ingalls RR, Golenbock DT. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J Exp Med. 1995 Apr;181(4): 1473–9.
52 Todd RF 3rd, Petty HR. β 2 (CD11/CD18) integrins can serve as signaling partners for other leukocyte receptors. J Lab Clin Med. 1997 May;129(5):492–8.
53 Petty HR, Todd RF 3rd. Integrins as promiscuous signal transduction devices. Immunol Today. 1996 May;17(5):209–12.
54 Aziz MH, Cui K, Das M, Brown KE, Ardell CL, Febbraio M, et al. The Upregulation of Integrin αDβ2 (CD11d/CD18) on Inflammatory Macrophages Promotes Macrophage Retention in Vascular Lesions and Development of Atherosclerosis. J Immunol. 2017 Jun; 198(12):4855–67.
55 Perera PY, Mayadas TN, Takeuchi O, Akira S, Zaks-Zilberman M, Goyert SM, et al. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J Immunol. 2001 Jan;166(1):574–81.
56 Otterlei M, Sundan A, Ryan L, Espevik T. Effects of anti-CD18 and LPS on CD14 expression on human monocytes. Scand J Immunol. 1995 Jun;41(6):583–92.
57 Han C, Jin J, Xu S, Liu H, Li N, Cao X. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. Nat Immunol. 2010 Aug;11(8): 734–42.
58 Abdelbaqi M, Chidlow JH, Matthews KM, Pavlick KP, Barlow SC, Linscott AJ, et al. Regulation of dextran sodium sulfate induced colitis by leukocyte beta 2 integrins. Lab Invest. 2006 Apr;86(4):380–90.
59 Rao KM. MAP kinase activation in macrophages. J Leukoc Biol. 2001 Jan;69(1):3–10.
60 MacIntyre EA, Roberts PJ, Jones M, Van der Schoot CE, Favalaro EJ, Tidman N, et al. Activation of human monocytes occurs on cross-linking monocytic antigens to an Fc receptor. J Immunol. 1989 Apr;142(7):2377–83.
61 Lund-Johansen F, Olweus J, Symington FW, Arli A, Thompson JS, Vilella R, et al. Activation of human monocytes and granulocytes by monoclonal antibodies to glycosylphosphatidylinositol-anchored antigens. Eur J Immunol. 1993 Nov;23(11):2782–91.
62 Luo Y, Pollard JW, Casadevall A. Fcgamma receptor cross-linking stimulates cell proliferation of macrophages via the ERK pathway. J Biol Chem. 2010 Feb;285(6):4232–42.
63 Kim D, Kim JY. Anti-CD14 antibody reduces LPS responsiveness via TLR4 internalization in human monocytes. Mol Immunol. 2014 Feb;57(2):210–5.
64 Kovarik P, Sauer I, Schaljo B. Molecular mechanisms of the anti-inflammatory functions of interferons. Immunobiology. 2007; 212(9-10):895–901.
65 Stockinger S, Kastner R, Kernbauer E, Pilz A, Westermayer S, Reutterer B, et al. Characterization of the interferon-producing cell in mice infected with Listeria monocytogenes. PLoS Pathog. 2009 Mar;5(3):e1000355.