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Molecular mechanism of LPS-induced TNF-α biosynthesis in polarized human macrophages

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ABSTRACT

In response to environmental stimuli such as granulocyte-macrophage or macrophage colony stimulating factor (GM-CSF/M-CSF), macrophages (MΦ) can acquire distinct functional phenotypes that control inflammatory processes on the one hand and contribute to a broad spectrum of pathologies on the other. Potential intervention strategies will require an understanding of the signalling processes that are associated with macrophage polarization.

In the present study, we show that M-MΦ produce more IFN-β and IL-10 and a lot less TNF-α than do GM-MΦ in response to LPS. To define the molecular mechanisms that underlie the biosynthesis of TNF-α we carried out a detailed investigation of the LPS-induced activation of the canonical and non-canonical myeloid differentiation primary response 88 (MyD88)-dependent signal transduction pathways as well as the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway. Our results show that all three pathways are activated in both cell types and that the activation is more pronounced in M-MΦ. While IL-10 was found to interfere with TNF-α production in M-MΦ, we exclude a decisive role for IFN-β in this respect. Furthermore, we demonstrate that TNF-α mRNA is markedly destabilized in M-MΦ and that expression of the mRNA destabilizing protein tristetraprolin is greatly enhanced in these cells.

Collectively, our study suggests that differential effects of LPS on TNF-α mRNA turnover and on signal transduction pathways influence the amount of TNF-α finally produced by GM-MΦ and M-MΦ.

1. Introduction

Macrophages are crucial for mediating and coordinating immunity and can adopt various activation states depending on the prevailing environmental signals. When undergoing “classical” activation in response to IFN-γ either alone or in combination with LPS, they assume the M1 phenotype, characterised by high microbicidal activity involving the production of pro-inflammatory mediators and reactive oxygen species (Martinez and Gordon, 2014). In contrast, “alternative” activation promoted by IL-4 and IL-13 generates M2 macrophages, which are mainly involved in tissue repair, tumour progression and humoral immunity (Martinez and Gordon, 2014).

The use of colony stimulating factors (CSF) to differentiate monocytes into macrophages reflects *in-vivo* situations that have an impact on macrophage development (Lacey et al., 2012). Consistent with elevated levels of GM-CSF at sites of inflammation GM-CSF gives rise to macrophages of a pro-inflammatory (“M1-like”) state. In contrast, macrophages normally exposed to the constitutive production of M-CSF in the absence of inflammation are likely to acquire an anti-inflammatory “M2-like” status, secreting relatively small amounts of pro-inflammatory mediators (Fleetwood et al., 2007; Hamilton, 2008).

As the features of cells derived by CSF treatment do not correspond exactly to those of M1 and M2 macrophages (Verreck et al., 2004; Verreck et al., 2006), we refer here to the two subsets as GM-MΦ and M-MΦ, respectively.

The distinct cytokine/chemokine profile of the two macrophage populations has recently been correlated with the usage of distinct LPS-induced signalling pathways (Fleetwood et al., 2007; Fleetwood et al.,

Abbreviations: TTP, tristetraprolin; TRIF, TIR-domain-containing adapter-inducing interferon-β; TRAM, TRIF-related adapter molecule; IRF3, interferon regulatory factor 3; IFNAR, interferon-α/β receptor; ActD, actinomycin D; TIRAP, TIR-domain-containing adapter protein; MΦ, macrophage; MyD88, myeloid differentiation primary response 88; TLR4, Toll-like receptor 4; CSF, colony stimulating factor

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2009; Wang et al., 2014; Zhou et al., 2014). Two main pathways have been described. The first is activated from the plasma membrane after TLR4 encounters LPS. Signal transduction requires the presence of the adaptors TIR-domain-containing adapter protein (TIRAP) and myeloid differentiation primary response 88 (MyD88), and leads ultimately to the activation of NF-κB and AP-1, two important transcription factors involved in the expression of many pro-inflammatory cytokines (Akira and Takeda, 2004; Kagan and Medzhitov, 2006). It has also been suggested that LPS can activate NF-κB via the non-canonical pathway (Bhattacharyya et al., 2010; Sun, 2011), by processing p100 to p52. Following association of p52 with RelB, the heterodimer moves into the nucleus and activates the transcription of NF-κB target genes (Sun, 2011).

The second pathway requires the internalization of TLR4 into the endosomal network and is triggered through the adaptors TRIF (TIR-domain-containing adapter-inducing interferon-β) and TRAM (TRIF-related adapter molecule). This leads to the activation of the transcription factor interferon regulatory factor 3 (IRF3) and expression of type I IFN as well as delayed NF-κB activation (Akira and Takeda, 2004; Kagan et al., 2008; Tanimura et al., 2008). Importantly, activation of both the MyD88- and TRIF-dependent pathways is necessary for induction of inflammatory cytokines via TLR4 signalling (Akira et al., 2006).

Among the pro-inflammatory cytokines secreted by macrophages, TNF-α plays a central role in the host response to injury and inflammation. While a rapid and transient pulse is primarily protective, overproduction of TNF-α can cause pathological conditions such as septic shock and rheumatoid arthritis (Feldmann et al., 1996; Tracey and Cerami, 1994). Strict regulation of TNF-α is therefore particularly important.

Control of TNF-α production can theoretically be achieved at a number of levels between transcription and secretion. As TNF-α is greatly enhanced in activated monocytes/macrophages (Beutler et al., 1986) and since polarized GM-MΦ/M-MΦ differ in their ability to generate this cytokine (Verreck et al., 2006; Weiss et al., 2015), we have investigated the subtype-specific molecular mechanisms that underlie the differential levels of TNF-α synthesis and that may therefore play an important role in regulating the immune response.

To this end, we compared the influence of LPS on the expression of distinct components of the MyD88- and TRIF-dependent pathways in GM-MΦ and M-MΦ and also assessed the involvement of post-transcriptional mechanisms in the control of TNF-α production. Given that M-MΦ are capable of higher IFN-β (Fleetwood et al., 2009) and IL-10 (Verreck et al., 2006; Weiss et al., 2015) production than are GM-MΦ when exposed to LPS, we also determined the contribution of these cytokines to the TNF-α response.

Our studies show that the lower level of TNF-α production by M-MΦ compared to GM-MΦ is not associated with a diminished MyD88-dependent NF-κB activation. We actually found the non-canonical MyD88- and TRIF-dependent pathways to be enhanced in M-MΦ. In addition, IL-10 but not IFN-β appears to be involved in TNF-α biosynthesis. Finally, we demonstrate that a diminished TNF-α mRNA stability combined with an increased expression of tristetraprolin appears to play an important role in controlling TNF-α biosynthesis in M-MΦ.

2. Material and methods

2.1. Reagents

Unless otherwise indicated, materials used in this study were from the following manufacturers: Sigma-Aldrich (Taufkirchen, Germany): fetal calf serum (FCS), LPS from *E. coli* (serotype 055:B5); Seromed Biochrom KG (Berlin, Germany): penicillin, streptomycin; GE Healthcare (Little Chalfont, Buckinghamshire, UK): RPMI 1640 (with L-glutamine, 25 mM HEPES and phenol red), Ficoll-Paque Plus.

2.2. Cell separation and cell culture

Buffy coats from healthy donors were acquired from the blood service (Institute of Transfusion Medicine, University Hospital Leipzig; ethics license 272-12-13082012). Human peripheral blood mononuclear cells were obtained from buffy coats by Ficoll-Paque Plus centrifugation (Ulmer et al., 1984). After washing three times with PBS containing 0.3 mM EDTA (Sigma-Aldrich), monocytes were isolated by counter-flow elutriation using the JE-5.0 elutriation system (Beckman Coulter, Brea, CA, USA), as described previously (Grage-Griebenow et al., 1993). The cell purity was > 90%, as assessed by immunofluorescence staining with a-CD14 (BL-M/G14, DiaMak, Leipzig, Germany) or a-CD14-APC Ab (M5E2, BioLegend, San Diego, CA, USA) and by morphological examination. Monocytes (5×10^5 cells/ml) suspended in RPMI 1640 medium supplemented with 10% (v/v) FCS, 100 U/ml penicillin and 100 mg/ml streptomycin were differentiated with 500 U/ml GM-CSF (Leukine, sargramostim, Genzyme, Neu-Isenburg, Germany) to GM-MΦ or with 50 ng/ml M-CSF (R&D Systems, Minneapolis, MO, USA) to M-MΦ at 37 °C and 5% CO_2 in teflon bags (Zell-Kontakt, Nörte-Hardenberg, Germany; Fluorinated ethylene propylene (FEP) foil, 50 µm, hydrophob). After 7 days, macrophages (1×10^6 cells/ml) were suspended in RPMI 1640 medium supplemented with 10% (v/v) FCS, 100 U/ml penicillin and 100 mg/ml streptomycin and incubated for 2-4 h in cell culture plates before analysis or stimulation with 100 ng/ml LPS.

Antibodies, corresponding isotypes, IFN-β (200 pg/ml) (R&D Systems) or IL-10 (10 ng/ml) (PeproTech, Rocky Hill, NJ, USA) were added 15 min prior to the stimulation with 100 ng/ml LPS. The following antibodies were used: blocking mouse anti human IFNAR Ab MMHAR (a-IFNAR, 1 µg/ml) (Moll et al., 2008) (Merck Millipore, Billerica, MA, USA), blocking anti-human IL-10 receptor Ab 3F9 (a-IL-10R, 5 µg/ml) (Liu et al., 1997) (BioLegend), neutralizing polyclonal goat anti-human IFN-β Ab (a-IFN-β, 0.2 µg/ml) (Clemens, 1987) and neutralizing anti-human IL-10 Ab 25209 (a-IL-10, 100 ng/ml) (Fontaine et al., 2014) (both Ab from R&D Systems, Minneapolis, MO, USA).

2.3. Detection of TNF-α, IL-10 and IFN-β in culture supernatants

Macrophages (1×10^6/ml) were stimulated with LPS (100 ng/ml) for various times. TNF-α, IL-10 and IFN-β were determined in culture supernatants using a human TNF-α or IL-10 ELISA kit (PeproTech) or an interferon-β bioluminescent ELISA kit (InvivoGen, Toulouse, France), respectively according to the manufacturer's protocol.

2.4. Cell surface receptor flow cytometry analysis

Macrophages (1×10^6/ml) seeded in cell culture plates were washed and incubated with PBS/EDTA for 10 min at 37 °C prior to rinsing off the detached cells. 2×10^5 cells were blocked with 10% AB-serum (Institute of Transfusion Medicine, University Hospital Leipzig) for 15 min at 4 °C and washed with wash buffer (PBS + 2% Emagel (Pirmal Healthcare, Morpeth, Nortumberland, UK) + 0.1% NaN_3). The direct dye labelled antibodies a-CD14-APC (M5E2, BioLegend) and a-TLR4-PE (610015, R&D systems) and the non-labelled a-IFNAR Ab (MMHAR-2) were added for 20 min at 4 °C. After washing with wash buffer the direct labelled probes were fixed with 1% formaldehyde. The non-labelled probes were incubated for 15 min at 4 °C with a FITC-labelled goat-anti-mouse Ab (SIFIN, Berlin, Germany) prior to fixation. Appropriate isotype-specific controls were used accordingly. Cells were analysed 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.

2.5. Intracellular flow cytometry (p-STAT-1) analysis

Macrophages (1×10^6/ml) were incubated with or without a-