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CXCL4 is a driver of cytokine mRNA stability in monocyte-derived dendritic cells

Sandra C. Silva-Cardoso[a,b], Cornelis P.J. Bekker[a,b], Marianne Boes[a,c], Timothy R.D.J. Radstake[a,b,*,1], Chiara Angiolilli[a,b,1]

[a] Laboratory of Translational Immunology, University Medical Center Utrecht, the Netherlands
[b] Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, the Netherlands
[c] Department of Pediatrics, University Medical Center Utrecht, the Netherlands

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ABSTRACT

The chemokine CXCL4 has been implicated in several immune diseases. Exposure of monocyte-derived dendritic cells (moDCs) to CXCL4 potentiates the production of inflammatory cytokines in the presence of TLR3 or TLR7/8 agonists. Here we investigated the transcriptional and post-transcriptional events underlying the augmented inflammatory responses in CXCL4-moDCs. Our results indicate that CXCL4-moDCs display an increased expression and secretion of IL-12, IL-23, IL-6 and TNF upon TLR3 activation. Analysis of the cytokine transcripts for the presence of AU-rich elements (ARE), motifs necessary for ARE-mediated mRNA decay, revealed that all these cytokine transcripts are, at least *in silico*, possibly regulated at the level of mRNA stability. *In vitro* assays confirmed that mRNA stability of *IL6* and *TNF*, but not *IL12B* and *IL23A,* is increased in CXCL4-moDCs. We next screened the expression of ARE-binding proteins (ARE-BPs) and found that TLR stimulation of CXCL4-moDCs induced tristetraprolin (TTP or ZFP36). Increased TTP mRNA expression was found to be a consequence of TTP phospho-mediated inactivation, which over time causes the protein to degrade its own mRNA. Concomitantly with TTP inactivation, we observed increased MAPK p38 signalling, upstream of TTP, in stimulated CXCL4-moDCs. P38 inhibition restored TTP activation and subsequently reduced the production of inflammatory cytokines. Finally, TTP knockdown in moDCs resulted in an increased production of *IL6* and *TNF* after TLR stimulation. Overall, our study shows that the pro-inflammatory phenotype of CXCL4-moDCs relies in part on enhanced cytokine mRNA stability dictated by TTP inactivation.

1. Introduction

CXCL4 is a chemokine produced by activated platelets and immune cells involved in pathological conditions such as cancer (Aivado et al., 2007), infections (Schwartzkopff et al., 2009; Srivastava et al., 2008) and inflammatory diseases like systemic sclerosis (SSc), rheumatoid arthritis (RA) and psoriatic arthritis (PsA), among others (Affandi et al., 2018b; Ah Kioon et al., 2018; Patsouras et al., 2015; van Bon et al., 2014; Yeo et al., 2016). CXCL4 plays a determinant role in distinct physiological processes such as in hematopoiesis (Han et al., 1990), angiogenesis (Maione et al., 1990), coagulation (Dehmer et al., 1995) and modulation of immune responses. For instance, exposure of monocytes to CXCL4 prevents apoptosis, induces the production of TNF and reactive oxygen species (ROS) and promotes monocyte differentiation into a unique macrophage-like phenotype (Gleissner et al., 2010; Scheuerer et al., 2000). In addition, we and others have shown that CXCL4 also modulates T-cell activation and regulates both the phenotype and the TLR-mediated innate responses of dendritic cells (DCs) (Affandi et al., 2018b; Fleischer et al., 2002; Fricke et al., 2004; Gouwy et al., 2016; Silva-Cardoso et al., 2017; Xia and Kao, 2003).

DCs are professional antigen-presenting cells (APCs) playing a crucial role in the maintenance of peripheral tolerance and bridging innate and adaptive immune responses. External- and self-dangerous molecules activate DCs downstream inflammatory signalling pathways which, when unresolved, can result in cytokine storm and autoimmunity. Immune regulatory positive and negative feedback mechanisms are therefore crucial to control inflammatory responses, prevent tissue damage and restore immune homeostasis. In DCs, as well as

Abbreviations: moDC, monocyte-derived dendritic cells; polyI:C, poly inosinic-polycytidylic acid; ARE, AU-rich elements; ARE-BPs, ARE-binding proteins

* Corresponding author at: The Laboratory of Translational Immunology, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands.
E-mail addresses: t.r.d.j.radstake@umcutrecht.nl (T.R.D.J. Radstake), c.angiolilli@umcutrecht.nl (C. Angiolilli).

[1] Authors contributed equally.

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in other immune and non-immune cells, a tight control of inflammation starts at the transcriptional level. However, post-transcriptional regulation of cytokine mRNA stability is a determining factor for final cytokine abundance (Barreau et al., 2005; Stoecklin and Anderson, 2007). In fact, disturbances in mRNA regulatory processes are notoriously associated with pathological manifestations, such as chronic inflammation and cancer (Brooks et al., 2002; Huang et al., 2016; Khabar, 2010; Seko et al., 2006; Zhang et al., 2013).

mRNA decay mediated by adenosine uridine (AU)-rich elements (AREs) is one of the best described mechanisms of mRNA regulation in mammalian cells. ARE motifs are found in the three prime untranslated region (3'UTR) of many short-lived inflammatory and oncogenic mRNAs. AREs conventionally act as mRNA destabilizing factors, however the interaction of AREs with *trans*-acting ARE-binding proteins (ARE-BPs) ultimately determines mRNA degradation or stabilization (Barreau et al., 2005; Carpenter et al., 2014; Stoecklin et al., 2004). Tristetrapolin (TTP or *ZFP36*), TTP family members BRF1 (*ZFP36L1*) and BRF2 (*ZFP36L2*), AU-rich binding factor-1 (AUF1 or *HNRNPD*), KH-type splicing regulatory protein (*KHSRP*), Hu antigen R (HuR, or *ELAVL1*), T-Cell-Restricted Intracellular Antigen 1 (*TIA1*) and TIA1-related protein (TIAR or *TIAL1*) are examples of well characterized ARE-BPs. Among ARE-BPs, TTP is a critical regulator of DC maturation and DC-mediated activation of T-cell responses (Emmons et al., 2008) and it is implicated in the regulation of inflammatory cytokines, including TNF, IL-6, IL-12 and IL-23 (Brooks and Blackshear, 2013; Bros et al., 2010; Carballo et al., 1998; Molle et al., 2013; Tudor et al., 2009). TTP expression is influenced by transcriptional events, as well as by negative feedback loop mechanisms that lead to TTP mRNA degradation following the interaction of TTP protein with its own mRNA (Kratochvill et al., 2011; Ross et al., 2015; Tiedje et al., 2016). At the same time, TTP is tightly controlled by MAPK signalling pathways, which promote TTP phosphorylation and subsequent inactivation (Brooks and Blackshear, 2013; Kratochvill et al., 2011; Sandler and Stoecklin, 2008). Multiple studies have shown that deregulated expression and activity of TTP are associated with aberrant inflammatory conditions. Mice whose myeloid cells lack TTP manifest exacerbated inflammation in response to LPS exposure and septic shock (Kratochvill et al., 2011; Qiu et al., 2012). Moreover, constitutive TTP knockout mice spontaneously manifest a severe autoimmune syndrome characterized by the erosion of peripheral joints and TNF overproduction, features analogously observed in RA patients (Taylor et al., 1996). Conversely, genetic modifications in mice either enhancing TTP endogenous levels (Patial et al., 2016) or ensuring constitutive TTP activation (Ross et al., 2017) were shown beneficial in dampening inflammation and preventing arthritis progression. While drugs directly reverting TTP activation status are currently unavailable, understanding when and which mRNA stability mechanisms play a role in inflammatory and autoimmune settings is a recent challenge in biomedical research (Patial and Blackshear, 2016; Ross et al., 2017) and a necessary condition for the discovery of new targets aimed at therapeutic intervention.

Earlier studies, inclusive ours, have supported the established association of CXCL4 with inflammation and autoimmunity (Affandi et al., 2018b; Patsouras et al., 2015; van Bon et al., 2014; Yeo et al., 2016). Furthermore, we identified a role of CXCL4 in sensitizing monocyte-derived dendritic cells (moDCs) to aberrant TLR-mediated TNF and IL-12 production (Affandi et al., 2018b; Silva-Cardoso et al., 2017). Here, we investigated whether deregulation of TTP expression and cytokine mRNA stability could underlie the aberrant inflammatory phenotype of CXCL4-moDCs.

2. Materials and methods

2.1. Monocyte isolation and moDC differentiation

Monocyte isolation and moDC differentiation were performed as described previously (Silva-Cardoso et al., 2017). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers that was collected in accordance with institutional ethical approval. After the isolation of PBMCs by density-gradient centrifugation over FicollPaque™ Plus (GE Healthcare), monocytes were purified using anti-CD14 magnetic beads, based on positive isolation by autoMACS Pro Separator-assisted cell sorting (MiltenyiBiotec). For moDC differentiation, monocytes were cultured at a density of 1×10^6 cells/ml using the medium RPMI 1640 with GlutaMAX (Life Technologies), supplemented with 10% (v/v) heat-inactivated FCS (Biowest) and 1% (v/v) antibiotics (penicillin and streptomycin) (both form Life Technologies), for 6 days at 37 °C in the presence of 5% CO_2. Recombinant human IL-4 (500 U/ml; R &D) and GM-CSF (800 U/ml; R&D) were added to the medium on day 0 and day 3. Recombinant human CXCL4 (10 μg/ml; PeproTech) was added on day 0 and day 3 to differentiate CXCL4-moDCs.

2.2. moDC treatment and stimulation

After the differentiation of moDCs and CXCL4-moDCs, cells were left overnight in medium supplemented with FCS and antibiotics. When indicated, cells were pre-treated or not with 10 μM p38 inhibitor (SB202190; Sigma-Aldrich), MEK1/2-ERK inhibitor (U0126; Calbiochem) and JNK inhibitor (SP600125; Bio Connect) 30 min prior to stimulation with polyI:C (25 μg/ml; Invitrogen) for 2 or 8 h.

For mRNA stability analysis, moDCs and CXCL4-moDCs were stimulated with polyI:C for 2 h, followed by actinomycin D (ActD) (5 μg/ml; Sigma-Aldrich) treatment. Cells were harvested at 0, 2, 4 and 6 h after treatment.

2.3. RNA purification and real-time quantitative PCR

RNA was purified using the RNeasy Micro Kit (Qiagen) and reverse-transcribed with SuperScript® Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's protocols. Real-Time quantitative-PCRs (RT-qPCR) were performed on the QuantStudio 12k flex system using SYBR Select Master Mix (Life Technologies). For each time point analysed, the difference between the expression of a gene of interest and housekeeping gene (*RPL32*) was calculated by using either the $2^{-\Delta Ct}$ or the $2^{-\Delta\Delta Ct}$ methods. Sequences of the primers are listed in Table 1.

2.4. Primers design

Primers were designed with Primer-BLAST. FASTA sequences retrieved from GRCh38 Primary Assembly (TNF, NC_000006.12; IL6, NC_000007.14; IL12B NC_000005.10; IL23A, NC_000012.12; TTP, NC_000019.10) were used as template for the design of primers recognizing primary transcripts. FASTA sequences from protein-coding transcripts (IL6, NM_000600.5; TNF, NM_000594.4; IL12B, NM_002187.2; IL23A NM_016584.3; TTP, NM_003407.4) were used as template for the design of primers recognizing mature mRNA.

2.5. Cytokine quantification by Luminex

Supernatants were collected after 8 h stimulation with polyI:C and cytokine measurements were assessed by Luminex technology as described before (de Jager et al., 2005) at the MultiPlex Core Facility of the Laboratory of Translational Immunology in the University Medical Center of Utrecht.

2.6. Western blot

MoDCs and CXCL4-moDCs were left unstimulated or stimulated with polyI:C (25 μg/ml) for 15 min, 30 min, 1 or 2 h. When indicated, prior to stimulation, cells were pre-treated with the p38 inhibitor SB202190. Cells were lysed in Laemmli's buffer and protein concentration was quantified using a BCA Protein Assay Kit (Pierce, Thermo Scientific) according to the manufacture's protocol. Equal amounts of protein lysate were mixed with loading buffer and boiled at 95 °C for 5 min, and

Table 1
Sequences of primers used for qPCR analysis.

Gene	Forward primer	Reverse primer
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
IL12B	TGCCGTTCACAAGCTCAAGT	TGGGTCAGGTTTGATGATGTCC
PT IL12B	TCATCTGCCGCAAAAATGCC	TTTGAGGGCCTGCTCACCTA
IL23A	CAACAGTCAGTTCTGCTTGC	GAAGGCTCCCCTGTGAAAAT
PT IL23A	AGCCTTCTCTGCTCCCTGATA	ATCCTCCACGCCCCTACTT
TNF	CCCATGTTGTAGCAAACCCT	TGAGGTACAGGCCCTCTGAT
PT TNF	TCAGGATCATCTTCTCGAACC	GAGTCCTTCTCACATTGTCTC
IL6	GACAGCCACTCACCTCTTCA	CCTCTTTGCTGCTTTCACAC
PT IL6	ACATCCTCGACGGCATCTCAG	CCCAGCAAAGACCTCCTAATG
AUF1	TTTGTTGGTGGCCTTTCTCC	ATTCCACCTCACCAAAACCAC
BRF1	ATGCAAGGGTAACAAGATGCTC	CACTGCCTTTCTGTCCAGC
BRF2	TCCAGAAACATGTCGACCAC	AGGGATTTCTCTGTCTTGCAC
KHSRP	CTTACAAAGTGCAGCAAGCC	AGATCCGTACTCATTCCGGT
HuR	AAGCCTGTTCAGCAGCATTG	CCAAGCTGTGTCCTGCTACT
TIAL1	GGAGTAGATCAATCACCTTCTGCTG	ATCCGGCTTGGTTAGGAGGA
TIA1	GATGCCCGAGTGGTAAAAGAC	CCCATCTGTTGAATGGCGTTT
TTP	CTGCCATCTACGAGAGCCT	ACTCAGTCCCTCCATGGTC
PT TTP	GCCATCTACGAGGTGAGTCC	AGTTTGCGGCGCTAGAGAG

separated by electrophoresis on a 4–12% Bis-Tris SDS NuPAGE gels (Invitrogen). Alternatively, samples were ran on 10% SDS-PAGE gels for 5 h at constant 70 V for better separation of immune-reactive bands ranging between 26 and 55 kDa. Gels were transferred to a PVDF membrane (Millipore). The membranes were blocked with Tris-buffered saline (pH 8) containing 0.05% Tween-20 and 4% milk (Bio-Rad) for 1 h at room temperature (RT) and probed overnight at 4 °C with antibodies for total p38 and phospho-p38; ERK and phospho-ERK; TTP and histone 3 (H3) (all from Cell signaling) or tubulin (Sigma-Aldrich). After washing, membranes were incubated for 1 h at RT with the secondary anti-rabbit HRP-conjugated Ab (Dako). Protein detection was assessed using a ChemiDoc MP System (Bio-Rad). For protein visualization and densitometry analysis, the Image Lab software (version 5.1, Bio-Rad) was used. The ratio between the levels of the protein of interest and H3 or tubulin was calculated to determine relative expression.

2.7. Lambda phosphatase treatment

CXCL4-moDCs were lysed in non-denaturing lysis buffer (10 mM Tris HCl pH 8.0, 10 mM NaCl, 1% NP-40). Protein lysates were supplemented with 10X NEBuffer for Protein MetalloPhosphatases (PMP) and 10 mM MnCl2 (New England Biolabs). Cell lysates were incubated on ice for 30 min, after which supernatants were centrifuged at 4 °C for 10 min (10.000 *g*) and collected. Supernatants were incubated with 1000 units of lambda (λ) phosphatase (New England Biolabs) at 30 °C for 30 min, after which loading buffer was added to the protein lysate. Samples were boiled at 95 °C for 5 min, and further processed for immunoblotting as described above.

2.8. siRNA transfection

On day 6 of differentiation, moDCs were transfected for 4 h with 20 nM control non-targeting siRNA (siCtrl) or specific siRNA targeting TTP (siTTP), using DharmaFECT1 (all from Dharmacon). Transfection reagents were replaced with complete cell culture medium, and cells were left resting overnight. Cells were stimulated with polyI:C for 8 h for gene expression analysis.

2.9. Analysis of AU rich motifs

3'UTR sequences for *IL-12B* (ENST00000231228.2), *IL-23A* (ENST00000228534.5), *IL-6* (ENST00000404625.5) and *TNF* (ENST00000449264.2) were retrieved from UCSC Genome Browser. ARE sequences in the 3'UTR of the same transcripts were obtained from AREsite2 database (Fallmann et al., 2016) and mapped by making use of a customized C# application made with Unity3D which identifies specific ARE sequences in the 3'UTR and outputs an image assigning different colours to the ARE family motifs.

2.10. Statistical analysis

Data analyses and graphs were performed using GraphPad Prism software (version 7). Data are represented as mean ± SD. To compare two groups the paired *t* test was used or one-way analysis of variance (ANOVA) was applied when more than two groups were compared. The significance was defined as $p \leq 0.05$. Statistical significance indicated as * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; **** for $p < 0.00013$.

3. Results

3.1. Cytokines induced in CXCL4-moDCs display ARE sequences in their 3'UTR

Previously, we have shown that exposure of moDCs to CXCL4 (CXCL4-moDCs) during differentiation results in aberrant IL-12 and TNF production after TLR3 (polyI:C) and TLR7/8 (CL075 and R848) triggering (Silva-Cardoso et al., 2017). In this study we used the same cell culture model to differentiate moDCs in the presence of CXCL4 for 6 days, and further stimulated the cells for 8 h with polyI:C. We confirmed that stimulated CXCL4-moDCs secrete increased levels of IL-12 and TNF in comparison to moDCs, and additionally found that IL-6 and IL-23 are also strongly produced by CXCL4-moDCs (Fig. 1A). No differences in the expression of these inflammatory cytokines were found between unstimulated moDCs and CXCL4-moDCs (Supplementary Fig. S1). As these inflammatory cytokines have been reported to be regulated at the level of mRNA stability (Carballo et al., 1998; Molle et al., 2013; Tudor et al., 2009), we screened the 3'UTR regions of their transcripts for the presence of AU-rich motifs, which constitute necessary sites for ARE-mediated mRNA decay (Fig. 1B) (Fallmann et al., 2016). All cytokine transcripts displayed at least one AUUUA pentamer and one WWWUUUWWW nonamer motif, preferential binding sites for ARE-BPs (Fig. 1C–D) (Barreau et al., 2005; Kratochvill et al., 2011). Thus, based on the presence of ARE motifs, we hypothesized that the mRNA of these cytokines could be stabilized in stimulated CXCL4-moDCs.

3.2. Enhanced cytokine production by CXCL4-moDCs relies on both transcriptional and post-transcriptional regulation

Given the presence of ARE motifs in *IL6*, *TNF*, *IL12B* and *IL23A* transcripts, we next hypothesized that the protein induction observed in

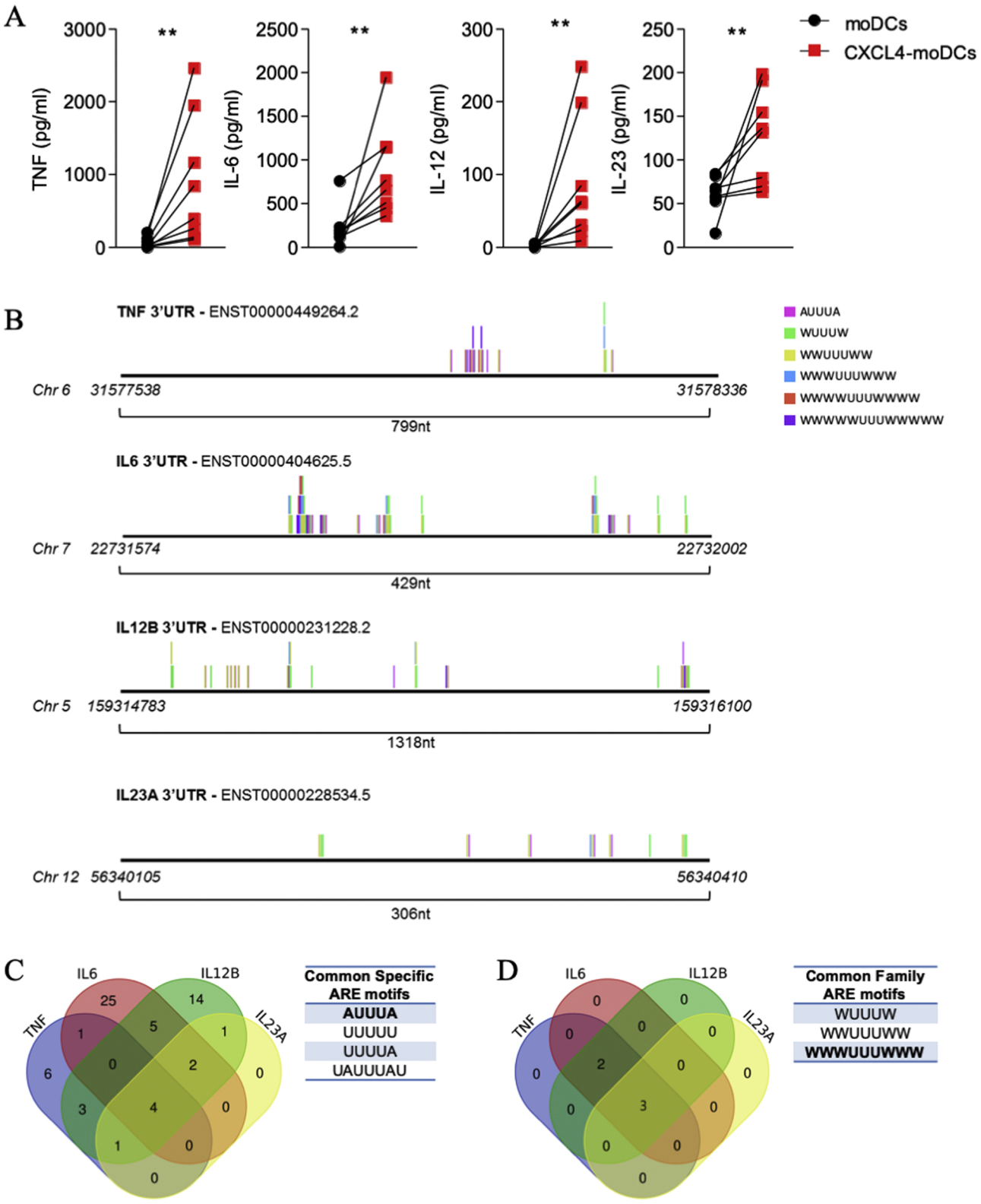

Fig. 1. Cytokines induced in CXCL4-moDCs display enriched ARE sequences. (A) Monocyte-derived dendritic cells (moDCs) were differentiated in the absence or presence of CXCL4 (CXCL4-moDCs) for 6 days. Cytokine production was measured by Luminex after 8 h stimulation with polyI:C. Lines connect individual donors. (N = 8); Wilcoxon matched-pairs signed rank test. (B) ARE sequences in *IL12B*, *IL23A*, *IL6* and *TNF* protein-coding transcripts were obtained from AREsite2 database and mapped in the 3'UTR of the respective transcripts. In the figure, specific ARE sequences are illustrated and coloured based on their belonging to defined ARE family motifs. W indicates either A (adenine) or U (uracil) nucleotide. (C–D) Specific AREs (C) and family ARE motifs (D) retrieved from (B) were overlapped in Venn-Diagram, and common sequences are shown in the table.

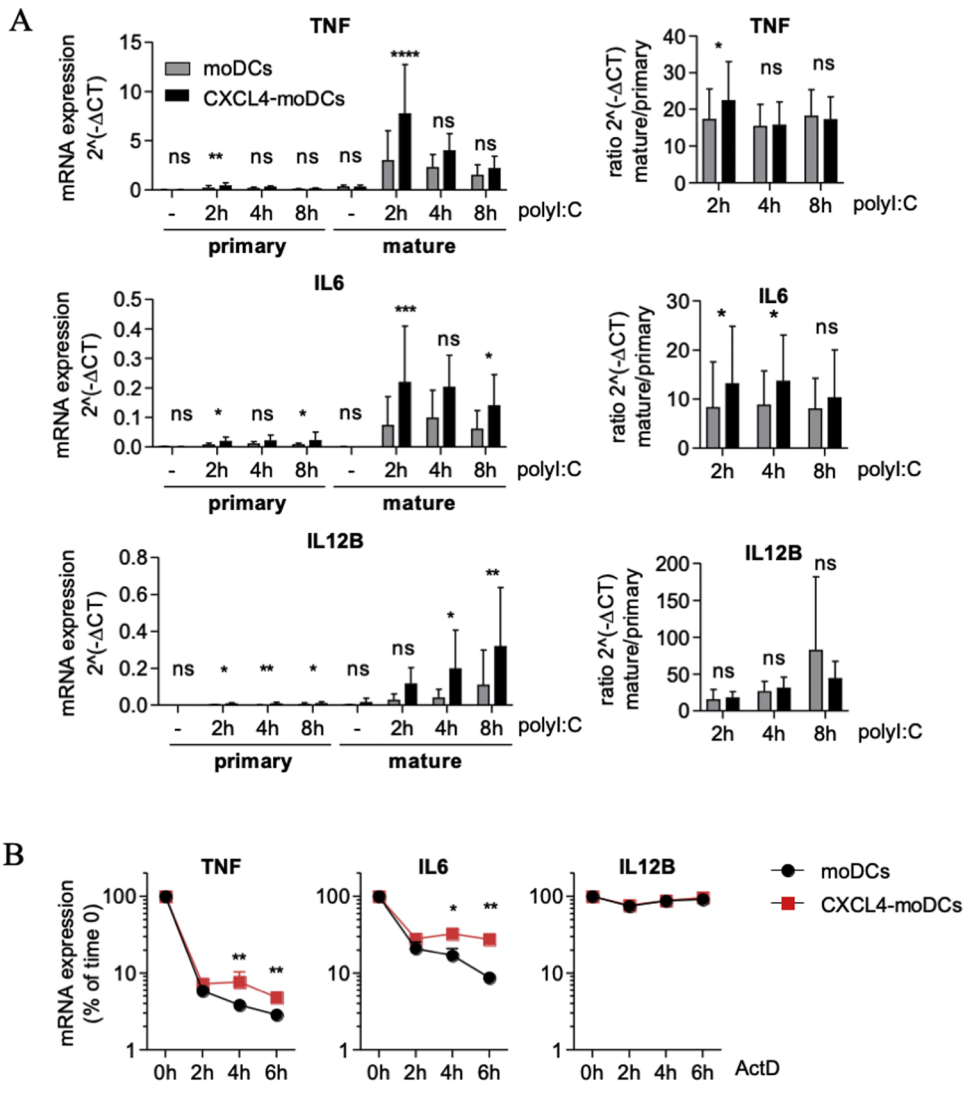

Fig. 2. Increased cytokine production by CXCL4-moDCs relies on both transcriptional and post-transcriptional regulation. (A) Primers for *IL6*, *TNF* and *IL12B* were designed in a way to amplify either intron-exon regions or exon-exon regions, allowing the detection of primary and mature transcripts, respectively. MoDCs and CXCL4-moDCs were left unstimulated (-) or stimulated for 2, 4 and 8 h with polyI:C. Gene expression of primary and mature transcripts of inflammatory cytokines is represented as relative expression levels for both primary and mature transcripts (2^-ΔCT), while ratios were calculated according to the formula: 2^-ΔCT mature transcript/2^-ΔCT primary transcript. (N = 8); One-way ANOVA followed by Fisher's LSD test. (B) MoDCs and CXCL4 moDCs were stimulated with polyI:C for 2 h, and further treated with actinomycin D (ActD) for the indicated time points, followed by gene expression analysis. (N = 10); Wilcoxon matched-pairs signed rank test.

stimulated CXCL4-moDCs would be a consequence of post-transcriptional, rather than transcriptional, regulation. To test our hypothesis, we designed primers specifically recognizing the unspliced 'primary' transcript or the spliced 'mature' transcript of all 4 cytokines (Fig. 2A and Supplementary Fig. S2). In fact, while primary transcript expression reflects transcriptional regulation, the expression of mature transcripts is sensitive to mRNA degradation (Scherrer, 2018). Analyses of the mature/primary ratios revealed that the mature transcript expression of *IL6* and *TNF*, but not *IL-12B* and *IL-23A,* was significantly higher in stimulated CXCL4-moDCs at early time points (Fig. 2A and Supplementary Fig. S3A), suggesting mRNA stabilization of *IL6* and *TNF* transcripts. In order to validate these results, we made use of an alternative assay using actinomycin D (ActD), a transcriptional inhibitor, to determine mRNA stability. In line with previous reports (Loupasakis et al., 2017; Paschoud et al., 2006), the mRNA of *IL-6* and *TNF* quickly decayed after 2 h ActD treatment. However, in CXCL4-moDCs, *IL6* and *TNF* transcripts retained higher stability at later time points (4 and 6 h ActD treatment) in comparison to conventional moDCs (Fig. 2B). The expression of *IL12B* and *IL23A* was intrinsically stable, in agreement with previous studies (O'Neil et al., 2017) and showed slow mRNA decay rates in both moDCs and CXCL4-moDCs (Fig. 2B and Supplementary Fig. S3B). These results indicate that stimulated CXCL4-moDCs display an enhanced production of IL-6 and TNF, which is explained by their post-transcriptional regulation (enhanced mRNA stability). On the contrary, the increased expression of IL-12 and IL-23 observed in these cells is dependent on transcriptional regulatory mechanisms.

3.3. TTP expression and activity are altered in CXCL4-moDCs

Several ARE-BPs have been shown to play a crucial role on mRNA stability regulation. Thus, we analysed the expression of well characterized ARE-BPs retaining either destabilizing or stabilizing properties (Carpenter et al., 2014). We found a strong induction of TTP mature transcript expression by CXCL4-moDCs after 2 h stimulation with polyI:C, in comparison to conventional moDCs (Fig. 3A). No differences were found for all the other analysed ARE-BPs (BRF1, BRF2, AUF1, KHSRP, TIA, TIAL1 and HuR) (Fig. 3A). We confirmed that TTP was induced at the protein level in polyI:C stimulated CXCL4-moDCs (Fig. 3B). However, given the mRNA-degrading properties of this ARE-BP, we hypothesized that the increased TTP expression observed by Western blot would be a consequence of TTP phosphorylation. In fact, MAPK-induced TTP phosphorylation, besides making TTP protein inactive, also prevents it from being processed for proteasomal degradation (Brooks and Blackshear, 2013; Deleault et al., 2008). Improved resolving for TTP protein by Western Blot allowed the identification of two immune-reactive bands of to 45 and 47 kDa (Angiolilli et al., 2018a). ActD treatment inhibited de novo TTP protein synthesis, leading to the reduction of the lower immune-reactive band, but it did not affect the higher phosphorylated band (Supplementary Fig. S4A). Furthermore, treatment of the protein lysates with lamda phosphatase (λ-phosphatase) reduced the expression of the higher, but not the lower, band (Supplementary Fig. S4B). In line with these results, and provided that active TTP destabilizes its own mRNA (Patial et al.,

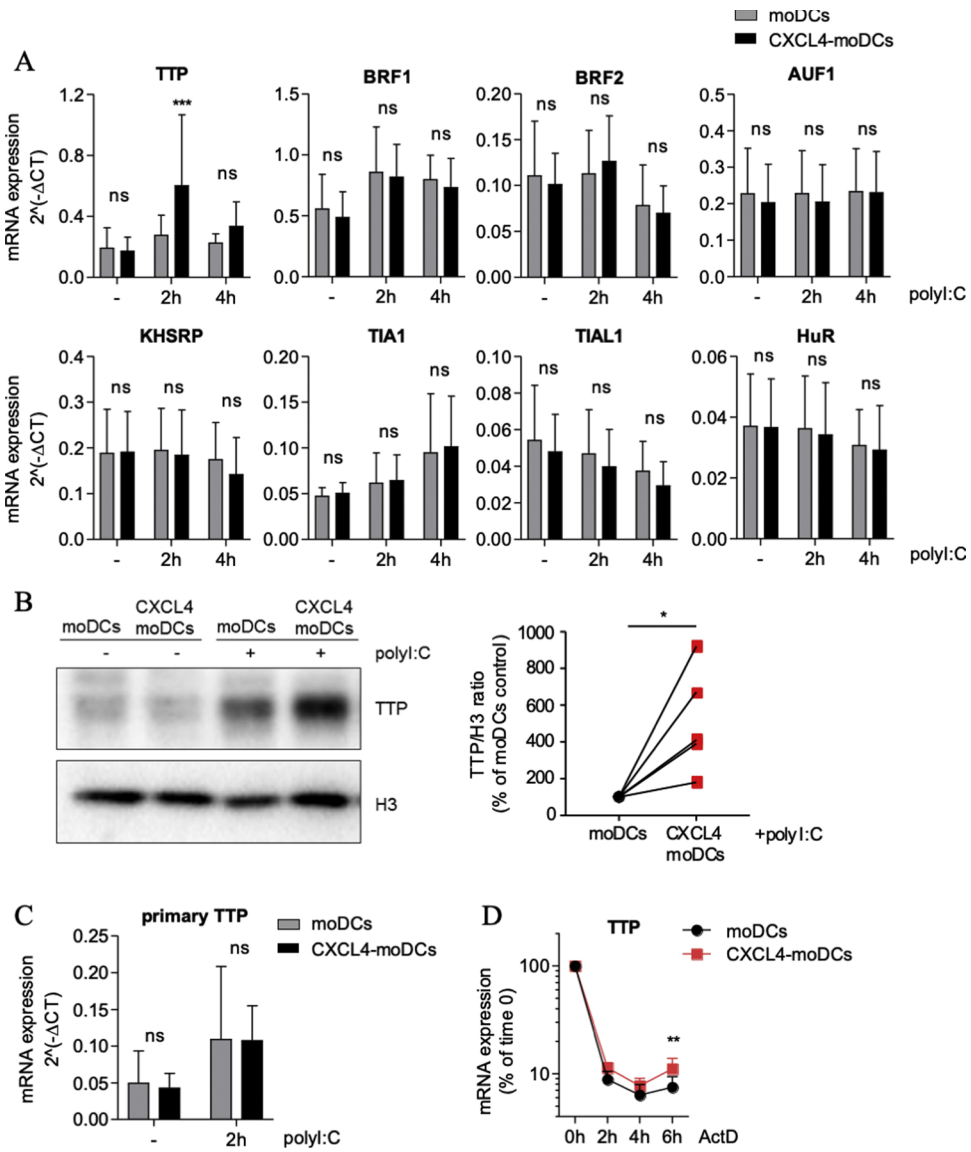

Fig. 3. TTP expression and activity are altered in CXCL4-moDCs. (A) MoDCs and CXCL4-moDCs were stimulated with polyI:C for the indicated time points and ARE-BPs expression was analysed by qPCR. Data are represented as relative expression levels. (N = 9); One-way ANOVA followed by Fisher's LSD test (B) TTP protein induction was analysed by Western blot after stimulating moDCs and CXCL4-moDCs for 2 h with polyI:C. Densitometry analysis indicates the signal intensity of TTP expression normalized to control Histone 3 (H3). Lines connect individual donors. (N = 5); Paired *t* test. (C) TTP primary transcript expression upon 2 h stimulation with polyI:C was analysed by qPCR. (N = 6); One-way ANOVA followed by Fisher's LSD test (D) MoDCs and CXCL4-moDCs were stimulated with polyI:C for 2 h. Cells were treated with ActD for the indicated time points and TTP mature transcript was analysed by qPCR. (N = 10); Wilcoxon matched-pairs signed rank test.

2016), we found that the induced TTP mRNA expression observed in stimulated CXCL4-moDCs was a consequence of increased TTP mRNA stability (Fig. 3D), but not transcription (Fig. 3C). Overall, our findings indicate that the increased mRNA expression of TTP in stimulated CXCL4-moDCs reflects TTP phospho-mediated inactivation.

3.4. Aberrant cytokine production by CXCL4-moDCs is dependent on MAPK p38 activation

TTP phosphorylation and consequent inactivation is a result of MAPK p38 activation upon inflammatory triggering (Carballo et al., 2001; Mahtani et al., 2001; Stoecklin et al., 2004; Tudor et al., 2009). Thus, we investigated whether stimulated CXCL4-moDCs would also display a perturbed MAPK signalling as compared to moDCs. We found that stimulation of CXCL4-moDCs with polyI:C for 30 and 60 min was followed by an increased phosphorylation of p38 (Fig. 4A). Treatment of CXCL4-moDCs with p38 inhibitor (p38i) SB202190 prior to polyI:C stimulation significantly reduced the protein expression of IL-6 and TNF, but also IL-12 and IL-23 (Fig. 4B). However, while affecting mature *IL6* and *TNF* expression, p38i effects on IL-12 and IL-23 production were mostly linked to the suppression of their primary transcripts, as the mature/primary ratio of *IL12B* and *IL23A* expression was not significantly altered by p38i treatment (Fig. 4C and Supplementary Fig. S5). Furthermore, while MEK/ERK, but not JNK, inhibition also resulted in a modest suppression of IL-6 and TNF production (Supplementary Fig. S6A-B), ERK signalling was not found potentiated in stimulated CXCL4-moDCs (Supplementary Fig. S6C). Overall, these results suggest that p38 is the predominant MAPK driving inflammatory activation in stimulated CXCL4-moDCs and that p38 inhibition uniformly reduces cytokine expression. However the mechanisms underlying p38i effects could be dependent on either control of cytokine mRNA stability (in case of *IL6* and *TNF*) or transcriptional regulation (in case of *IL12B* and *IL23A*).

3.5. Suppression of TTP expression or activity leads to increased cytokine production

To further assess the phosphorylation status of TTP in CXCL4-moDCs, we pre-treated cells with p38i before stimulation with polyI:C. In the presence of p38 inhibitor, the higher immune-reactive band corresponding to phosphorylated TTP was reduced in CXCL4-moDCs (Fig. 5A). As expected, treatment with p38i led to decreased TTP mature transcript after 2 h polyI:C stimulation (Fig. 5B), a possible consequence of the restored TTP activity which also causes TTP mRNA degradation. Conversely, neither ERK or JNK inhibition reduced TTP expression (Supplementary Fig. S7). These results suggest that, in CXCL4-moDCs, polyI:C stimulation mediates the aberrant activation of

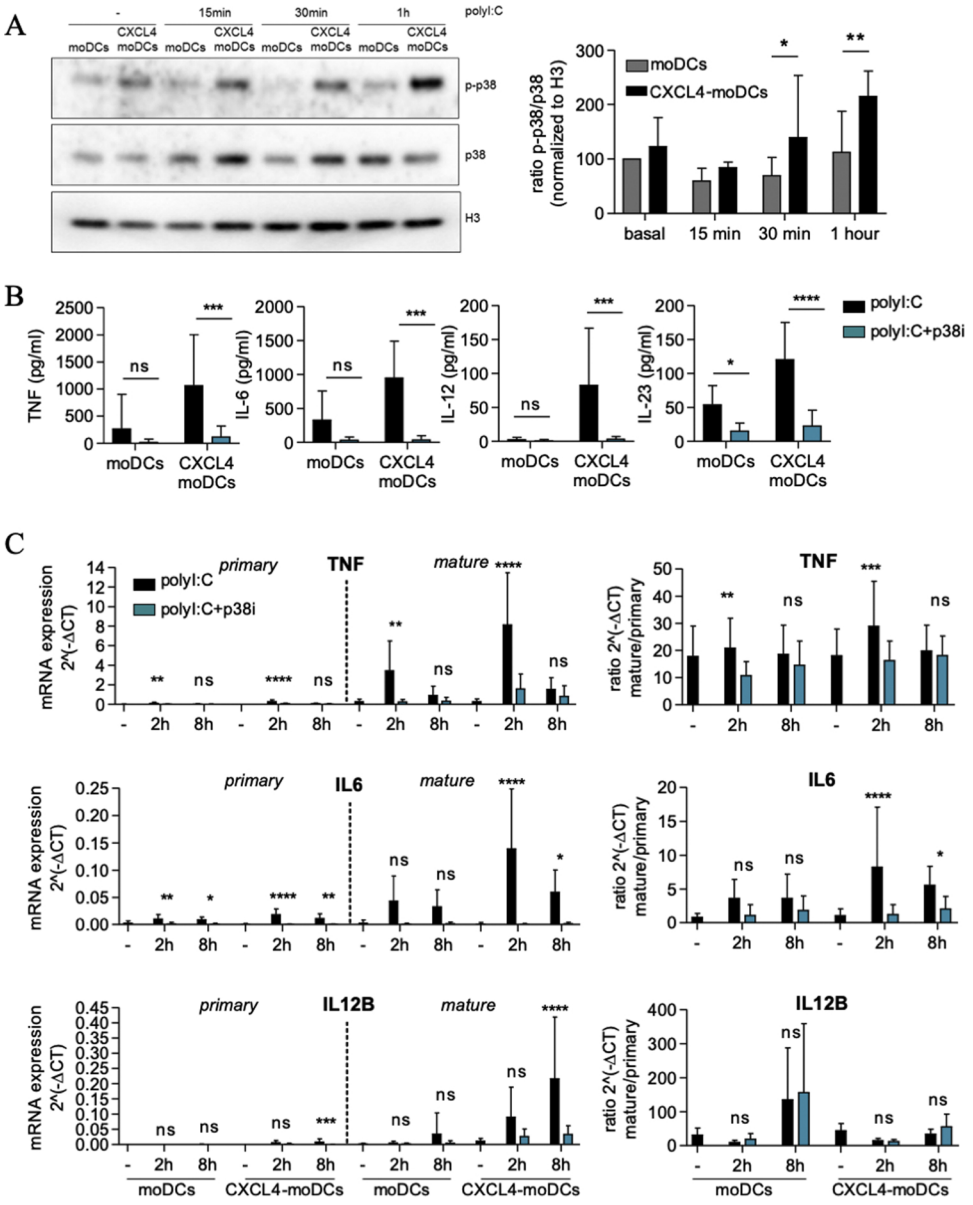

Fig. 4. Amplified activation of MAPK p38 in CXCL4-moDCs contributes to increased cytokine production. (A) MoDCs and CXCL4-moDCs were stimulated with polyI:C for the indicated time points. Phosphorylated p38 (p-p38), total p38 (p38) and H3 expression were measured by Western blot. Densitometry analysis indicates the signal intensity of p-p38/p38 ratio normalized to control H3. (N = 5); One-way ANOVA followed by Fisher's LSD test (B) Prior to polyI:C stimulation for 8 h, moDCs and CXCL4-moDCs were pre-treated with the p38 inhibitor SB202190 and cytokine production was measured by Luminex. (N = 9); One-way ANOVA followed by Fisher's LSD test (C) Cells were pre-treated as in (B), further stimulated for 2 and 8 h with polyI:C and processed for qPCR analysis. Gene expression of primary and mature transcripts of inflammatory cytokines is represented as relative expression levels for both primary and mature transcripts (2ˆ-ΔCT), while ratios were calculated according to the formula: 2ˆ-ΔCT mature transcript/2ˆ-ΔCT primary transcript. (N = 6); One-way ANOVA followed by Fisher's LSD test.

MAPK p38 signalling and the inactivation of TTP, the latter contributing to the increase mRNA stability of inflammatory cytokines. Interference of this pathway by the use of a p38i leads to restored activation of TTP function and consequent cytokine and TTP mRNA degradation. To further confirm the role of TTP on the regulation of cytokine production by moDCs, we performed TTP silencing by siRNA. TTP knockdown was confirmed at the mRNA (Fig. 5C) and protein level (Fig. 5D). While not reporting changes in cell viability after TTP silencing (Supplementary Fig. S8), we observed that *IL6* and *TNF* mRNA expression was boosted in TTP knockout moDCs upon polyI:C stimulation (Fig. 5E). In contrast, the expression of *IL-12B* and *IL-23A* was not significantly affected after TTP silencing. Altogether, our results indicate that *TNF* and *IL6* expression in CXCL4-moDCs is regulated at the post-transcriptional level and relies on TTP inactivation.

4. Discussion

CXCL4 is a chemokine which is massively released by activated platelets, as well as immune cells, under pathological conditions (Affandi et al., 2018a,b; Fox et al., 2018). Increased levels of CXCL4 were detected in individuals with chronic autoimmune diseases, both in circulation and at the site of inflammation (Affandi et al., 2018b; Patsouras et al., 2015; van Bon et al., 2014; Yeo et al., 2016). Furthermore, we and others have previously shown that during the differentiation of monocytes into DCs (moDCs) the prolonged exposure to CXCL4, a condition that mimics chronic inflammatory status, is able to significantly affect DCs phenotype and activation (Fricke et al., 2004; Silva-Cardoso et al., 2017; Xia and Kao, 2003). Specifically, we reported that CXCL4-moDCs produce higher levels of TNF and IL-12 cytokines after stimulation with polyI:C, a TLR3 agonist (Silva-Cardoso et al., 2017). Here we show that IL-6 and IL-23, two cytokines notoriously produced by DCs and playing a central role in autoimmune conditions (Abdel-Magied et al., 2016; Komura et al., 2008; Lee et al., 2004; Nakayama et al., 2017; Schmidt et al., 2005), are also upregulated in CXCL4-moDCs upon stimulation, in comparison to conventional moDCs. As these inflammatory cytokines have been reported to be regulated at the level of mRNA stability (Carballo et al., 1998; Molle et al., 2013; Tudor et al., 2009), and given that ARE sequences in the 3'UTR play a determinant role in mRNA regulation (Barreau et al., 2005; Carpenter et al., 2014; Stoecklin and Anderson, 2007), we hypothesized that these cytokines could all be subject to post-transcriptional events, explaining their higher production in stimulated CXCL4-

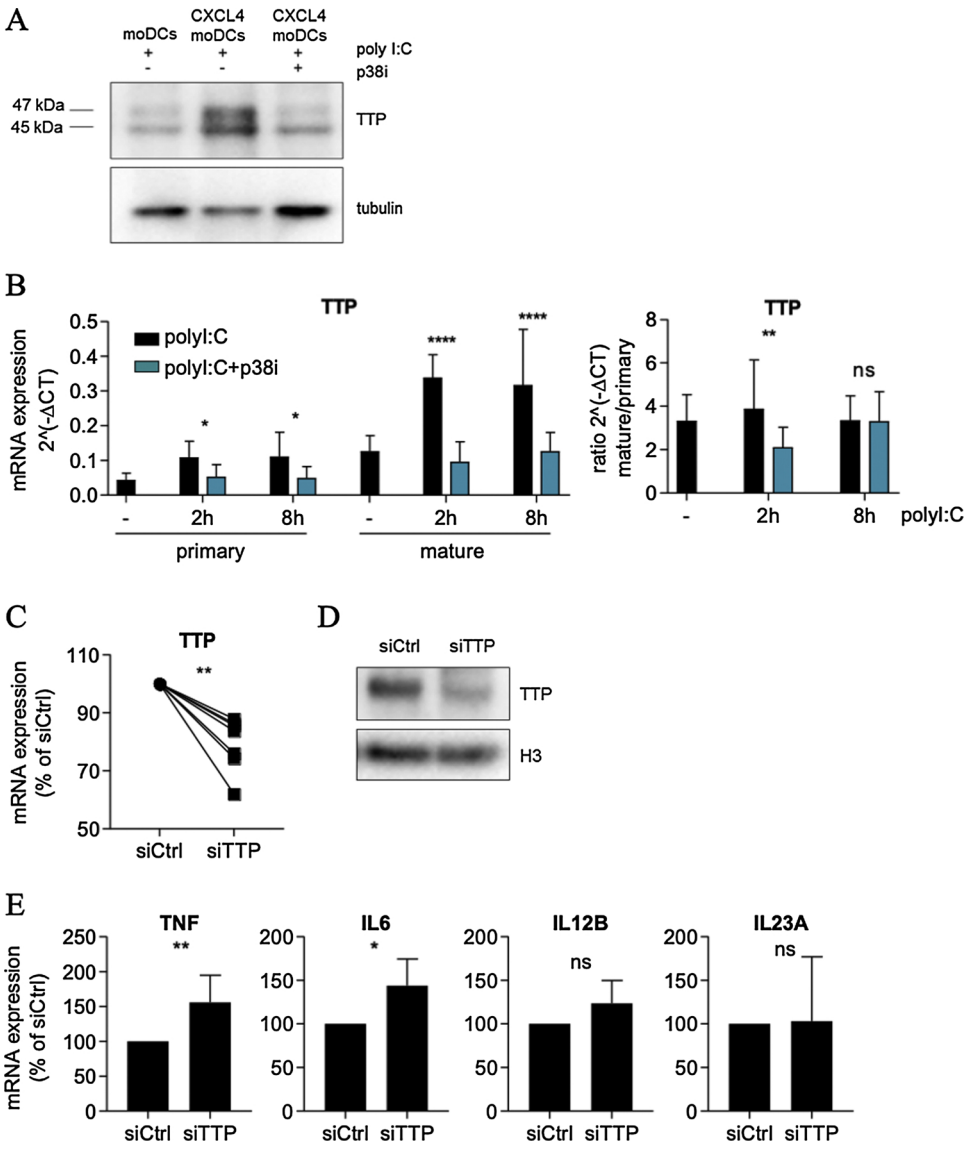

Fig. 5. Suppression of TTP activity or expression leads to increased cytokine production. (A) Prior to polyI:C stimulation for 2 h, CXCL4-moDCs were pre-treated or not with the p38i SB202190.TTP and Tubulin expression were measured by Western blot. (N = 3). (B) Cells were pre-treated as in (A) and stimulated for 2 and 8 h with polyI:C. TTP primary and mature transcripts were analysed by qPCR. Data are represented as relative expression levels for both primary and mature TTP transcripts (2ˆ-ΔCT), while ratio is calculated as 2ˆ-ΔCT mature transcript/ 2ˆ-ΔCT primary transcript. (N = 6); One-way ANOVA followed by Fisher's LSD test (C–E) moDCs were transfected with either control non-targeting siRNA (siCtrl) or with specific siRNA targeting TTP (siTTP). In (C), knockdown efficiency was determined by qPCR. Data are represented as fold change mRNA expression compared to siCtrl condition. (N = 7); Paired *t* test. In (D), transfected moDCs were stimulated with polyI:C for 2 h, and knockdown efficiency was confirmed by Western blot. (N = 4). In (E), transfected moDCs were stimulated with polyI:C for 8 h and cytokine expression was analysed by qPCR. (N = 6) Paired *t* test.

moDCs. However, our assays used to define mRNA stability (Angiolilli et al., 2018a; Smallie et al., 2015) indicated that *IL6* and *TNF*, but not *IL12B* and *IL23A*, transcripts are stabilized in TLR3-triggered CXCL4 moDCs. Although *IL12B* and *IL23A* mRNA can both be physically recognized by ARE-BPs (Molle et al., 2013; Qian et al., 2011; Sedlyarov et al., 2016), more studies indicated no effects of ARE-BPs on *IL12B* mRNA stability (Jalonen et al., 2006; Molle et al., 2013; O'Neil et al., 2017), and rather hinted for transcriptional mechanisms of regulation mediated by inhibition of NF-κB nuclear translocation (Gu et al., 2013). Additionally, interaction of ARE-BPs with target ARE-containing mRNAs is dictated by different parameters, such as cellular activation status, and different accessibility and adaptability of mRNA binding motifs (Garcia-Maurino et al., 2017; Ripin et al., 2019). Thus, it is plausible that in our experimental settings *IL23A* mRNA is not sufficiently affected by mRNA decay due to missing molecular counterparts or structural mRNA components that are instead present in other cells, or induced upon different stimulations. In line with this hypothesis, we also observed that TTP knockdown in moDCs failed to upregulate *IL12B* and *IL23A* mRNA expression upon poly:IC stimulation, while it potentiated *TNF* and *IL6* mRNA expression. Even though the use of primary cells in our study did not allow us to confirm a direct interaction between TTP and the target cytokines (data not shown), TTP binding to *TNF* and *IL6* has been previously confirmed in a variety of human and murine cells upon different experimental conditions (Patino et al., 2006; Shi et al., 2012; Tiedje et al., 2016; Zhao et al., 2011). Furthermore, ARE sequences in the 3'UTR region of *TNF* and *IL6* mRNAs are highly similar within mammalian species, suggesting a highly conserved mechanism of mRNA degradation for these cytokines (Carrick et al., 2004; Paschoud et al., 2006). Thus, in our experimental model, we could speculate that the mRNA stabilization of *TNF* and *IL6* mRNA in CXCL4-moDCs is likely a direct consequence of TTP inactivation, which results in the reduced binding of TTP to its target mRNAs.

Binding of ARE-BPs to AREs plays a determinant role in mRNA degradation. Among several ARE-BPs, TTP plays a pivotal role in cytokine regulation, by both guiding cytokine mRNA for degradation or preventing translation (Brooks and Blackshear, 2013). Carballo et al. had first described that LPS stimulation of macrophages derived from TTP knockout mice results in increased *TNF* mRNA and protein production (Carballo et al., 1997, 1998). Also, it has been shown that TTP on one hand regulates DC maturation and cytokine production in response to TLR agonists and on the other hand regulates DC-mediated activation of T-cell responses (Emmons et al., 2008; Molle et al., 2013). In this work, expression analysis of several ARE-BPs showed that TTP is significantly and selectively upregulated in stimulated CXCL4-moDCs, as compared to the other ARE-BPs. While it has been previously shown that TTP regulates the expression of ARE- and non-ARE- enriched genes

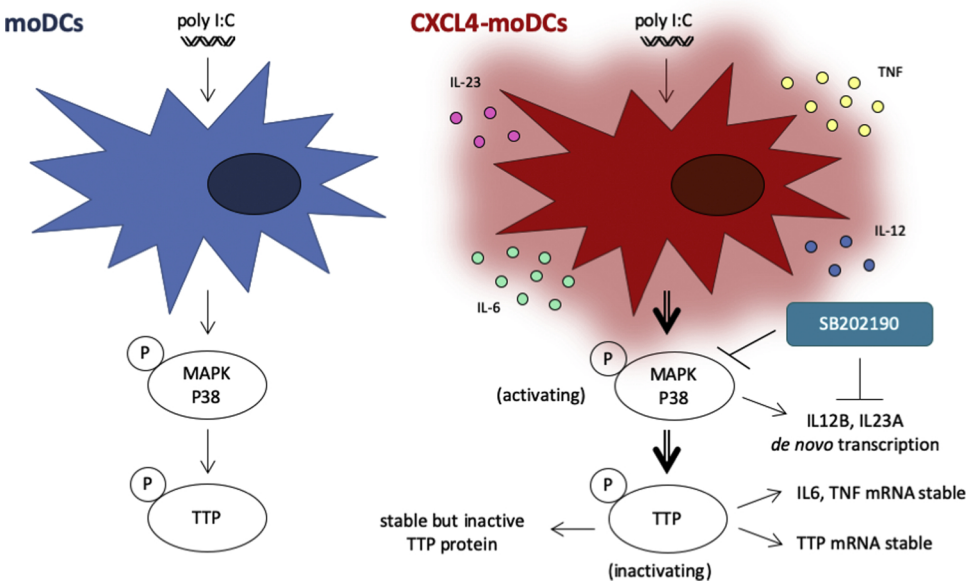

Fig. 6. Schematic overview of proposed mechanism. Conventional monocyte differentiation in the presence of IL-4 and GM-CSF generates monocyte-derived dendritic cells (moDCs). In our study we added CXCL4 during moDCs differentiation to generate CXCL4-moDCs. Compared to conventional moDCs, polyI:C stimulation of CXCL4-moDCs leads to abnormal phospho(P)-mediated activation of MAPK p38 signalling, which in turn mediates the phospho-mediated inactivation of TTP. When inactive, TTP does not degrade its target mRNAs, including its own mRNA. In our study we observe that the expression of *IL6* and *TNF* transcripts is increased in moDCs lacking TTP, a model mimicking TTP inactivation observed in CXCL4-moDCs. In line with this model, p38 inhibition suppresses not only IL-6 and TNF, but also IL-12 and IL-23, production possibly via mRNA stability-independent mechanisms.

with a critical function for DC maturation and activation (Emmons et al., 2008), it is worth mentioning that inflammatory triggers, besides enhancing cytokine production, typically induce TTP mRNA expression. Despite counterintuitive, given the mRNA degrading properties of this ARE-BP (Brooks and Blackshear, 2013), higher TTP levels are explained by the impaired activity of TTP protein upon inflammatory conditions, which also renders TTP less efficient in degrading its own mRNA (Patial et al., 2016). For instance, stimulation of RA FLS with IL-1β leads to increased phospho-inactive TTP protein levels, higher TTP mRNA expression and subsequent increased production of IL6 and CXCL8 (Angiolilli et al., 2018a). Similarly, in LPS-stimulated murine macrophages, induced expression of TTP is coupled to increased *IL23A* mRNA stability (Qian et al., 2011). High expression of TTP by macrophages and fibroblasts has also been found in the inflamed synovial tissue of RA patients, and reflected higher p38 activation in these sites (Ross et al., 2017). Overall our results indicate that induced, but phospho-inactive TTP in CXCL4-moDCs fails to degrade *TNF* and *IL6* mRNA. Additionally, our data indicate for the first time that inactivation of TTP function is one of the mechanisms by which CXCL4 controls cytokine production. In the longer term, this could explain the inflammatory and autoimmune features of the diseases where CXCL4 has been implicated.

MAPK play an important function in inflammation and tissue damage by regulating cytokines at the post-transcriptional level, promoting mRNA stability and increasing protein translation (Kratochvill et al., 2011; Mahtani et al., 2001; Ross et al., 2015; Stoecklin et al., 2004). Specifically, MAPK p38 and downstream kinase MAPK-activated protein kinase 2 (MK2) regulate the activity of several ARE-BPs, including TTP (Mahtani et al., 2001; Tiedje et al., 2016). MK2 mediates Ser-52 and Ser-178 phosphorylation of TTP, simultaneously inhibiting TTP activity and promoting its binding to 14-3-3 proteins, which prevent its degradation by the proteasome complex (Chrestensen et al., 2004; Stoecklin et al., 2004; Tiedje et al., 2016). In keeping with the need for therapeutic drugs able to prevent, or inhibit, inflammatory cytokine production, several immunomodulatory inhibitors have emerged in the last decades. Given the pleiotropic role of MAPK p38 in the regulation of transcriptional and mRNA-stabilizing events which control cytokine production (Arthur and Ley, 2013), compounds affecting p38 activity have been extensively investigated in experimental and clinical settings (Cohen et al., 2009; Salgado et al., 2014). Although p38 inhibitors have proven therapeutic potential in different experimental models of autoimmune diseases, including arthritis (Mihara et al., 2008), colitis (Li et al., 2013) and systemic sclerosis (Matsushita et al., 2017), their effects on the suppression of inflammatory parameters was shown to be only transient, possibly explaining their moderate effects in clinical trials. While 'the unexpected failure' of p38 inhibitors has not been completely understood, recent work elucidated how p38 acts as a pro- but also anti-inflammatory protein depending on the cell context and on the presence of co-stimulatory signalling (Jones et al., 2018; Raza et al., 2017). Thus, investigation of p38 inhibitors in combination therapies, and the development of second-generation inhibitors able to boost anti-inflammatory properties of p38, while preventing its pro-inflammatory effects, remains a current need.

Our study shows for the first time that TLR3-mediated activation of CXCL4-moDCs leads to transcriptional and post-transcriptional events that underlie the enhanced inflammatory cytokine production in these cells (Fig. 6). Future investigations aimed at systematically assessing CXCL4 contribution to immune processes will possibly help discerning the role of CXCL4 in health and disease.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.09.004.

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