Protein arginine methyltransferases as modulators of lipid metabolism and inflammation and the relevance for atherosclerosis

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Chapter

PRMT4

Chapter 3

PRMT4 inhibitor TP-064 inhibits the pro-inflammatory macrophage lipopolysaccharide response in vitro and ex vivo and induces peritonitis-associated neutrophilia in vivo

Biochim Biophys Acta Mol Basis Dis. 2021 Nov 1;1867(11):166212.

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ABSTRACT

Previous in vitro studies have shown that protein arginine N-methyltransferase 4 (PRMT4) is a co-activator for an array of cellular activities, including NF-κB -regulated pro-inflammatory responses. Here we investigated the effect of PRMT4 inhibitor TP-064 treatment on macrophage inflammation in vitro and in vivo.

Exposure of RAW 264.7 monocyte/macrophages to TP-064 was associated with a significant decrease in the production of pro-inflammatory cytokines upon a lipopolysaccharide challenge. Similarly, thioglycollate-elicited peritoneal cells isolated from wildtype mice treated with TP-064 showed lowered mRNA expression levels and cytokine production of pro-inflammatory mediators interleukin (IL)-1β, IL-6, IL-12p40, and tumor necrosis factor-α in response to lipopolysaccharide exposure. However, TP-064-treated mice exhibited an ongoing pro-inflammatory peritonitis after 5 days of thioglycollate exposure, as evident from a shift in the peritoneal macrophage polarization state from an anti-inflammatory $LY6C^{low}CD206^{hi}$ to a pro-inflammatory $LY6C^{hi}CD206^{low}$ phenotype. In addition, TP-064-treated mice accumulated (activated) neutrophils within the peritoneum as well as in the blood (7-fold higher; $P<0.001$) and major organs such as kidney and liver, without apparent tissue toxicity. °TP-064 treatment downregulated hepatic °mRNA° expression levels of the PRMT4 target genes °glucose-6-phosphatase catalytic subunit (-50%, °$P<0.05$) and the cyclin-dependent kinases°2 (-50%, $P<0.05$) °and°4 (-30%, $P<0.05$), suggesting a direct transcriptional effect of PRMT4 also in hepatocytes.

In conclusion, we have shown that the PRMT4 inhibitor TP-064 induces peritonitis-associated neutrophilia in vivo and inhibits the pro-inflammatory macrophage lipopolysaccharide response in vitro and ex vivo. Our findings suggest that TP-064 can possibly be applied as therapy in NF-κB -based inflammatory diseases.

Keywords: Gene expression, pro-inflammatory cytokines, thioglycollate-induced peritonitis, LPS, CARM1

1. INTRODUCTION

Macrophages and their monocyte progenitors play an important role in host defense and inflammation, as well as the resolution of inflammation and stimulation of repair, processes strongly influenced by their plasticity. Depending on environmental stimuli, such as the presence of lipopolysaccharides (LPS) from the outer membrane of gram-negative bacteria or the cytokines tumor necrosis factor α (TNFα) and interleukin-4 (IL-4), monocytes can switch their polarization state and differentiate into different types of macrophages. Upon exposure to LPS, M1 macrophages are formed that promote inflammation, amongst others by stimulating the production of vast amounts of pro-inflammatory cytokines like interleukin-6 (IL-6), TNFα, IL-12, and IL-1β [1, 2]. These cytokines play a central role in host defense, but are on the other hand also associated with chronic pathologies, including atherosclerosis, cancer, diabetes, and asthma [3-5].
Interestingly, Surdziel et al. have identified protein arginine N-methyltransferase 4 (PRMT4), also known as co-activator-associated arginine methyltransferase (CARM1), as co-activator for M1 macrophage polarization [6]. In line, augmented expression of PRMT4 in peripheral blood monocytes of patients with atherosclerosis-related cardiovascular disease correlated with higher levels of pro-inflammatory mediators, such as IL-8, IL-4, IL-7, IL-13, IL-17, IFN-γ, TNFα, interferon-inducible protein-10 (IP-10), and monocyte chemoattractant protein 1 (MCP-1) [1, 2]. Conversely, shRNA-mediated blockade of PRMT4 function in the human monocyte cell line THP1 downregulates expression levels of the pro-inflammatory genes TNFα and IP-10 [7]. Similarly, reduced expression levels of macrophage inflammatory protein 2 (MIP-2) and IP-10 protein were observed in PRMT4 deficient fibroblasts after LPS exposure [8]. Thus, inhibition of PRMT4 activity might be a promising strategy to reduce the

pro-inflammatory monocyte/macrophage response.

Recent studies have identified the small molecule TP-064 as a selective and potent inhibitor of PRMT4 function in vitro [9]. In the current study, we, for the first time, apply TP-064 as PRMT4 inhibitor in vivo with the overall aim to study the effects of TP-064 treatment on monocyte/macrophage-driven inflammation.

2. MATERIALS AND METHODS

2.1. Cell culture

RAW 264.7 murine monocyte-like cells (passage 10) were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% fetal bovine serum, 1% penicillin, and 1% L-glutamine at 37°C with 5% CO_2. The plating density for all cell cultures was 1 x 10^6 cells/ml. Cells were treated with 0.1% of DMSO dimethyl sulfoxide (DMSO) or 10 μM TP-064 (N-methyl-N-((2-(1-(2-(methylamino) ethyl) piperidin-4-yl) pyridin-4-yl) methyl)-3-phenoxybenzamide, Cat.No.6008, TOCRIS) in an equivalent concentration of DMSO for 24 hours. Subsequently, cells were stimulated with LPS (1 ng/ml; L9764-5MG, Sigma Aldrich, USA/Roche Diagnostics, Mannheim, Germany) for 0, 4, or 18 hours. After the LPS stimulation, cells and supernatants were collected and stored at -20°C until further gene expression or cytokine analysis.

2.2. Gene expression analysis

Total RNA from cells or tissues was isolated according to the standard protocol of Chomczynski and Sacchi [10]. In short, RNA was converted into cDNA using Maxima H Minus Reverse Transcriptase. After adding the SensiMix SYBR low-ROX mix, the PCR cycle threshold (Ct) values were measured using the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The average Ct values of the housekeeping genes acidic ribosomal phosphoprotein P0 (36B4), hypoxanthine guanine phosphoribosyl transferase (HPRT), and ribosomal protein L27 (RPL27) were used to calculate deltaCt values. Genes of interest were expressed relative compared to the housekeeping values using the 2^deltaCt. Primer sequences of the house keeping genes and the genes of interest can be found in Table 1.

Table 1: Nucleotide sequence of primers used for RT-PCR

Gene	GenBank Accession no.	Forward primer	Reverse primer
HPRT	NM_013556.2	5'GCTGGTGAAAAGGACCTCTCGAAGT	5'CAACTTGCGCTCATCTTAGGCTTTGT
RPL27	NM_011289.3	5'CGCCAAGCGATCCAAGATCAAGTCC	5'AGCTGGGTCCCTGAACACATCCTTG
36B4	NM_007475.5	5'CTGAGTACACCTTCCCACTTACTGA	5'CGACTCTTCCTTTGCTTCAGCTTT
TNFα	NM_013693.3	5'CAAAGGGATGAGAAGTTCCCAAATGGC	5'CACTCCAGCTGCTCCTCCACTTG
IL-1β	NM_008361.4	5'AACGACAAAATACCTGTGGCCTTG	5'CCGTTTTTCCATCTTCTTCTTTGGGT
IL-6	NM_031168.2	5'TGTATGAACAACGATGATGCACTTGCA	5'CTCTCTGAAGGACTCTGGCTTTGTCT
IL-12p40	NM_001303244.1	5'GCAAGTGGGCATGTGTTCCCTG	5'AAAGCCAACCAAGCAGAAGACAGC
CXCL1	NM_008176.3	5'TTGACCCTGAAGCTCCCTTG	5'AGGTGCCATCAGAGCAGTC
CXCL2 (MIP-2)	NM_009140.2	5'AACATCCAGAGCTTGAGTGTGACGC	5'CCAGGTCAGTTAGCCTTGCCTTTGT
CXCL10 (IP-10)	NM_021274.2	5'GTCATTTTCTGCCTCATCCTGCTG	5'CCTATGGCCCTCATTCTCACTGG
CCL2	NM_011333.3	5'CTGAAGCCAGCTCTCTCTTCCTC	5'GGTGAATGAGTAGCAGCAGGTGA
Ly6G	NM_001310438.1	5'GATGGATTTTGCGTTGCTCTGGA	5'GAGTAGTGGGGCAGATGGGAAG
MMP8	NM_008611.4	5'TGACCTCAATTTCATATCTCTGTTCTG	5'TCATAGCCACTTAGAGCCCAGTACT

2.3. Cytokine Enzyme-linked immunosorbent assay (ELISA)

The concentration of pro-inflammatory cytokines was analyzed in the medium of RAW macrophages and peritoneal macrophages using the ELISA protocol of BD Biosciences and Biolegend (San Jose and San Diego, CA, USA). Absorbances were measured at 450 nm and 570 nm.

2.4. Experimental animals

Male C57BL/6 wildtype (WT) mice were bred at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research (LACDR) in Leiden, The Netherlands. Mice were housed in groups at a 12h light/dark cycle and had ad libitum access to food and water. During the experiments, the mice were monitored for overall health every day and weighed twice during the 5 days of the

experiment. WT mice on chow diet were injected i.p. three times with 10 µl DMSO or 10 mg/kg TP-064 dissolved in a similar volume of DMSO. Together with the first i.p. injection, mice were treated with 3% thioglycollate to recruit macrophages to the peritoneum, in order to investigate the short-term effects of TP-064 treatment in macrophages in vivo. After 5 days of treatment, mice were anaesthetized at two hours after the last TP-064 injection with a s.c. administration of a mixture of 70 mg/kg xylazine, 1.8 mg/kg atropine, and 350 mg/kg ketamine. Under deep anesthesia, as monitored by absence of the leg withdrawal reflex, mice were killed by exsanguination. Blood was collected by tail vein bleeding in EDTA-coated tubes (Greiner Bio One, Kremsmünster, Austria). The number of leukocytes, specifically neutrophils, lymphocytes, and monocytes, were analyzed using an automated Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Etten-Leur, The Netherlands). The aspartate aminotransferase/alanine aminotransferase (ALT and AST) ratio was analyzed in plasma by using ALT (MAK052-1KT, Sigma) and AST (MAK055-1KT, Sigma) activity assay kits, in order to determine possible drug-induced liver injury [11, 12]. Peritoneal leukocytes were obtained by flushing the peritoneal cavity of the mice with 10 ml of ice-cold PBS. Subsequently, in preparation for harvesting the vital organs, the diaphragm was severed, and the organs were perfused with PBS via the left ventricle of the heart. Livers were harvested and fixed in 3.7% formalin (Formal-fix; Shandon Scientific Ltd, UK) for 24 hours and stored in 0.1% sodium azide in PBS until histological analysis. The remaining parts were snap-frozen in liquid nitrogen and stored at −20°C until gene expression analysis. Animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare, the directive 2010/63/EU of the European Union and the ARRIVE guidelines. The experimental protocol was approved by the Leiden University Animal Ethics Committee.

2.5. Flow cytometry (FACS)

Thioglycollated-elicited peritoneal cells isolated from the two treatment groups were washed and resuspended in 5 ml PBS. Approximately 100,000 cells were stained with appropriate antibodies, including F4/80, CD45, Ly6G, CD19, CD11b, Ly6C and CD206 (eBioscience) in PBS containing 2% fetal bovine serum. Flow cytometry analyses were performed on a Beckman Coulter Cytoflex S or BD Biosciences CantoII using FlowJo software.

2.6. Ex vivo thioglycollate-elicited peritoneal macrophage assay

Thioglycollate-elicited peritoneal cells, isolated from the two treatment groups, were plated in 12-well plates overnight at a density of 1 x 10^6 leukocytes/well in DMEM, containing 10% fetal bovine serum, 1% penicillin, and 1% L-glutamine (DMEM complete). Subsequently, cells were provided complete medium with or without LPS (1 ng/ml) for 18 hours. Then, cells and supernatants were collected for gene expression analysis or cytokine measurements, respectively.

2.7. Whole blood assay

A whole blood cytokine release assay was performed according to the protocol of De Groote et al [13]. After sacrifice of wildtype mice treated with TP-064 and controls, blood was isolated from tail vein into EDTA tubes, and directly diluted (1:5) in RPMI 1640 medium containing 1% penicillin and 1% L-glutamine without fetal bovine serum and incubated with 1 ng/ml LPS for 6 hours at 37^{o}C to stimulate monocyte TNFα production. Subsequently, supernatant was collected after centrifugation at 1500 rpm and the TNFα concentration in the supernatant was measured using ELISA (BD Bioscience, USA).

2.8. Liver histology and glycogen quantification

Formalin-fixed liver specimens were embedded in Tissue-Tek® O.C.T. Com-

pound (Sakura Finetek Europe, Alphen aan den Rijn, NL) and cryosections (8 µm) were prepared. Liver cryosections were stained with Eosin-Hematoxylin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Representative photomicrographs were taken for each group with 100× magnification. Liver glycogen contents from snap frozen liver lobes were quantified according to the standard protocol of Hassid and Abraham [14].

2.9. Statistical analysis

All statistical tests were performed using the GraphPad Prism 8.0 Software (2020). Outliers were detected using Grubb's outlier test. Data are presented as the mean with SEM. Dependent on the number of groups and variables in the data, unpaired Student's t-tests, one-way ANOVAs, or two-way ANOVAs with Bonferroni post hoc tests were performed. P values < 0.05 were considered significant.

3. RESULTS

3.1. TP-064 treatment lowers the LPS-induced pro-inflammatory cytokine response of RAW 264.7 cells in vitro

Previous studies in fibroblasts have suggested that genetic PRMT4 deficiency is associated with a reduced protein expression of the macrophage inflammatory protein 2 (MIP-2) and interferon-γ-induced protein 10 (IP-10) after LPS exposure [8]. To test whether TP-064 could reduce the expression of PRMT4 target genes in macrophages, RAW 264.7 cells were pretreated overnight with TP-064 or the solvent control DMSO prior to LPS stimulation. Different exposure times with LPS, i.e., 4 hours and 18 hours, were applied to identify the activation of both early and late response cytokines. See figure 1A for a detailed overview of the experimental in vitro setup. In accordance with the hypothesis that MIP-2 and IP-10 are PRMT4 target genes, both MIP-2 and IP-10 expression levels were lower after TP-064-mediated pharmacological inhibition of PRMT4 (Fig.1B). More specifically, in control cells MIP-2 levels were increased 106-fold and 188-fold ($P<0.05$) after 4 hours and 18 hours LPS treatment, respectively. TP-064-treated cells expressed >30% lower MIP-2 levels at both 4 and 18 hours of LPS exposure (two-way ANOVA, $P<0.05$ for treatment). IP-10 levels peaked at 4 hours after the addition of LPS. At this time point, IP-10 expression was induced 70-fold in TP-064–treated cells, as compared to 100-fold in control cells ($P<0.001$). At 18 hours, TP-10 expression had almost returned to baseline under both treatment conditions (Fig.1B). TP-064 treatment did not affect the LPS-induced TNFα and IL-1β response, neither on gene expression level nor on protein level (Fig.1C). In contrast, TP-064 did lower the mRNA expression of the late response pro-inflammatory cytokines IL-6 and IL-12p40 about 10 times after 18 hours of LPS exposure (two-way ANOVA: $P<0.01$ for treatment), also translating

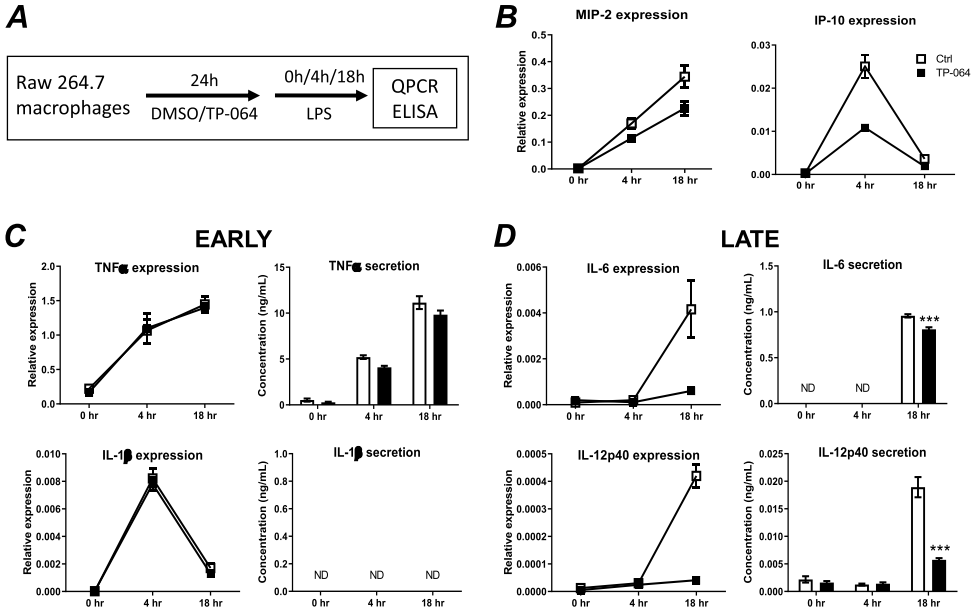

Figure 1: *TP-064 decreases the mRNA expression and production of pro-inflammatory cytokines in RAW264.7 murine macrophages upon LPS stimulation.*

*(A) Schematic overview of the in vitro experimental setup. Relative mRNA expression levels of PRMT4 target genes (B) and gene expression and protein secretion levels of early (C) and late (D) response pro-inflammatory cytokines in RAW macrophages that were treated with DMSO as control group (Ctrl; white) or 10 μM TP-064 (black) for 24 hours and subsequently treated with LPS (1 ng/ml) for 0, 4, or 18 hours. Data are expressed as means ± SEM (N=6 per group). ND, not detected. *** P<0.001 (Two-way ANOVA with Bonferroni post-test).*

into an attenuated secretion of these cytokines (medium concentrations 2.7-fold and 2.9-fold lower, respectively; two-way ANOVA: P<0.001 for treatment, Fig.1D).

In summary, TP-064 treatment of RAW 264.7 cells inhibited PRMT4-dependent upregulation of the mRNA expression and secretion of a selection of pro-inflammatory cytokines upon exposure to LPS.

3.2. TP-064 treatment was associated with a shift in macrophage phenotype in mice with thioglycollate-induced peritonitis

A previous study by Lastrucci et al. showed that thioglycolate-induced peri-

tonitis distinguishes 3 consecutive phases: 1) rapid influx of neutrophils, 2) recruitment of inflammatory monocytes that are transdifferentiate into macrophages, and 3) polarized into M2-like macrophages to resolve the inflammation [15]. In order to assess whether in vivo treatment with TP-064 could also reduce the LPS-induced inflammatory response of peritoneal macrophages ex vivo, thioglycollate was injected into the peritoneal cavity of wildtype (WT) C57BL/6 mice that were subsequently treated with either TP-064 (10 mg/kg i.p.) or the solvent control DMSO 3 times during the following 5 days until peritoneal leukocyte isolation [16, 17]. See figure 2A for a detailed overview of the in vivo / ex vivo experimental setup. As expected, in the control DMSO group about 75% of peritoneal leukocytes were identified as being $LY6G^{-}F4/80^{+}$ macrophages (Fig.2B & 2C). Within this macrophage population, 90% of the cells presented a $LY6C^{low}CD206^{high}$ M2-like (resolving) macrophage phenotype [18] (Fig.2D & 2E). Interestingly, we observed that only 20% of the total peritoneal leukocytes were $F4/80^{+}$ macrophages in the mice injected with TP-064 and that the fraction of $LY6C^{low}CD206^{high}$ resolving M2-like macrophages from TP-064 treated mice was 2-fold lower than that from control mice (Fig.2C & 2E). As can also be appreciated from Figure 2B, the relative decrease in macrophages numbers could be attributed to a higher peritoneal LY6G+ neutrophil count (absolute numbers: $54\pm17.5x10^{6}$ for TP-064 mice versus $12\pm2.5x10^{6}$ for controls; $P<0.05$). In contrast to the control group which had only 1.5% of $LY6C^{hi}CD206^{low}$ pro-inflammatory macrophages [19], 48% of the macrophages from TP-064 treated mice showed this phenotype (Fig.2D & 2E). In accordance, we also found that the mRNA expression level of pro-inflammatory mediators, like CXC and CC motif ligand 1 (CXCL1; 10-fold, $P<0.001$), CXC and CC motif ligand 2 (MIP-2; CXCL2; 7-fold, $P<0.05$), CXC and CC motif ligand 10 (IP-10; CXCL10; 5-fold, $P<0.01$), and C-C motif chemokine ligand 2 (CCL2; 5-fold, $P<0.001$) were all significantly increased in the thioglycollate-elicited peritoneal

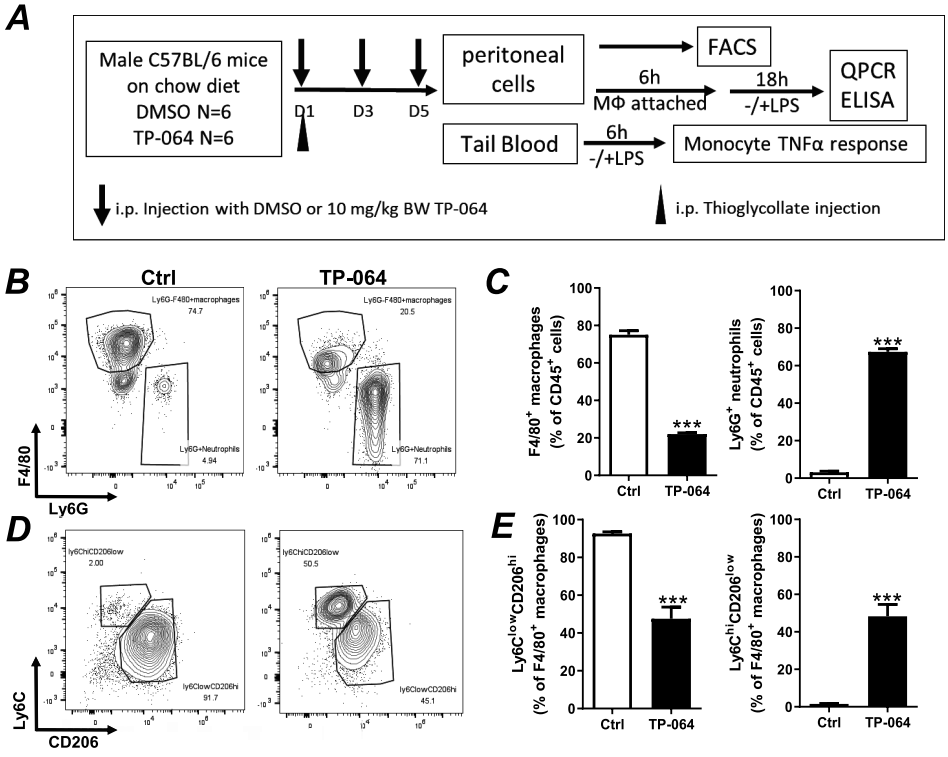

Figure 2: *TP-064 treatment alters macrophage phenotype in mice with thioglycollate-induced peritonitis*

*(A) Schematic overview of the in vivo / ex vivo experimental setup. (B) Representative flow cytometry plots showing peritoneal populations of F4/80+LY6G- macrophages and F4/80-LY6G+ neutrophils in thioglycollate-injected wildtype mice treated with either 10 mg/kg TP-064 or DMSO control (Ctrl). (C) Quantification of the relative numbers of peritoneal macrophages and neutrophils as percentage of live CD45+ leukocytes. (D) Representative flow cytometry plots showing peritoneal populations of anti-inflammatory LY6ClowCD206hi cells and pro-inflammatory LY6ChiCD206low macrophages. (E) Quantification of the relative numbers of the different macrophage subtypes as percentage of total F4/80+ cells. Data are expressed as means ± SEM (N=6 per group). *** P<0.001 (Unpaired t-test).*

macrophages isolated from TP-064-treated mice as compared to DMSO-treated controls (Fig.3A). Altogether, these findings suggest that TP-064 treatment, combined with thioglycollate stimulation, induced an on-going pro-inflammatory peritonitis.

3.3. TP-064 treatment of mice reduces the LPS-induced inflammatory response of thioglycollate-elicited peritoneal macrophages ex vivo

To verify that in vivo treatment with TP-064 was also able to block NF-κB -mediated cytokine secretion by macrophages, isolated peritoneal cells were plated in culture dishes and subsequently adherent macrophages were exposed to LPS. Baseline pro-inflammatory cytokine mRNA expression levels were not changed by TP-064 treatment in the thioglycollate-elicited peritoneal macrophages (Fig. 3B). However, after 18 hours LPS stimulation ex vivo, we found that macrophages from TP-064-treated mice showed an attenuated inflammatory response

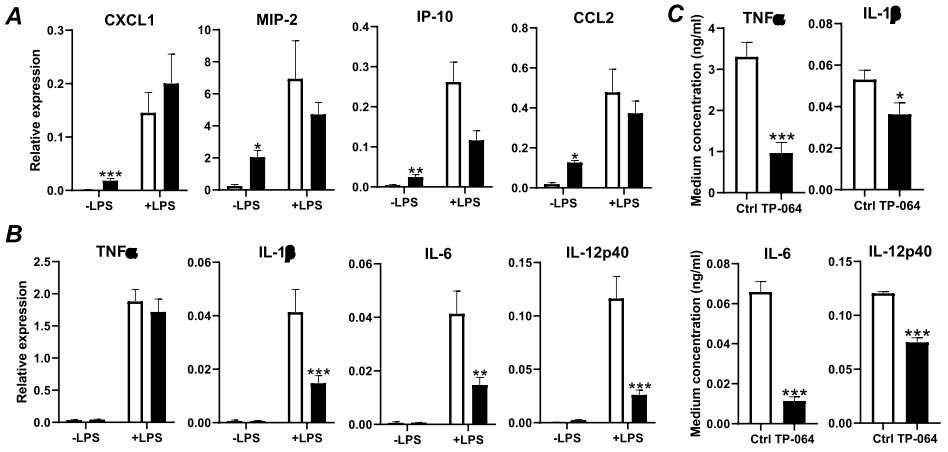

Figure 3: *In vivo TP-064 treatment decreases pro-inflammatory gene expression and cytokine production by thioglycollate-induced peritoneal macrophages upon ex vivo LPS stimulation.*

*Relative mRNA expression levels of pro-inflammatory trigger genes (A) and pro-inflammatory cytokine genes (B) in thioglycollate-elicited peritoneal macrophages isolated from wildtype mice treated with DMSO as control group (Ctrl; white) or 10 mg/kg TP-064 (black), that were treated with or without LPS (1 ng/ml) for 18 hours. (C) Concentrations of pro-inflammatory cytokines in culture medium collected from the LPS-treated peritoneal macrophages. Data are expressed as means ± SEM (N=6 per group). * P<0.05, ** P<0.01, *** P<0.001 (Unpaired t-test).*

(Fig.3B). More specifically, except for the early response inflammatory gene TNFα, the mRNA expression levels of the pro-inflammatory cytokines IL-

12p40 (8-fold, P<0.001), IL-6 (3-fold, P<0.01), and IL-1β (4.5-fold, P<0.001) were all significantly downregulated in cells from TP-064-treated mice as compared to controls (Fig.3B). Analysis of cytokine secretion by ELISA showed that TP-064 lowered LPS-induced TNFα secretion 3-fold (P<0.001, Fig.3C). Moreover, the secretion of IL-1β, IL-6, and IL-12p40 was decreased by 26% (P<0.05), 82% (P<0.001), and 31% (P<0.001), respectively in peritoneal macrophages isolated from TP-064-treated mice (Fig.3C). Thus, TP-064 treatment of mice reduces the inflammatory response of peritoneal macrophages upon ex vivo exposure to LPS.

3.4. TP-064 treatment lowers LPS-induced monocyte TNFα secretion in whole blood

Monocytes in blood, the precursors of tissue macrophages, play an important role in the elimination of LPS from the circulation and are considered early responders in the systemic inflammatory response after LPS exposure [20]. Upon exposure of whole blood to LPS, the secretion of TNFα by monocytes is stimulated in a time-dependent manner, with an optimal detection of TNFα between 2 and 8 hours [13, 21]. Using this whole blood assay, we also examined the effect of PRMT4 inhibitor treatment on the systemic monocyte activation after 6 hours of LPS treatment. As expected from our peritoneal macrophage findings, the TNFα production by monocytes in whole blood was significantly lower in the TP-064 treatment group (0.025±0.007 ng/ml) as compared to the control group (0.068±0.009 ng/ml) upon LPS stimulation (P<0.001; Fig.4A). To exclude that the lower responsiveness of whole blood from TP-064 treated mice was the result of altered monocyte counts, a full white blood cell differential and count was performed. TP-064 treatment did not change the number of circulating monocytes (t-test p>0.05, Fig.4B), indicating that the observed effects of TP-064 treatment on TNFα release in the whole blood assay was not the result

of lower monocyte counts but likely due to inhibition of the PRMT4-mediated inflammatory response in monocytes. Notably, we did observe a 30% decrease in the number of blood lymphocytes in the TP-064-treated group ($P<0.05$, unpaired t-test, Fig.4B). This is in line with previous findings of Jia et al. showing that PRMT4 deficient mice display an impaired thymocyte differentiation and defective lymphocyte development [22]. TP-064 treatment was also associated with a dramatic increase in neutrophil numbers (7-fold; $P<0.001$; Fig.4B), which is probably related to TP-064-induced on-going neutrophil-driven peritonitis.

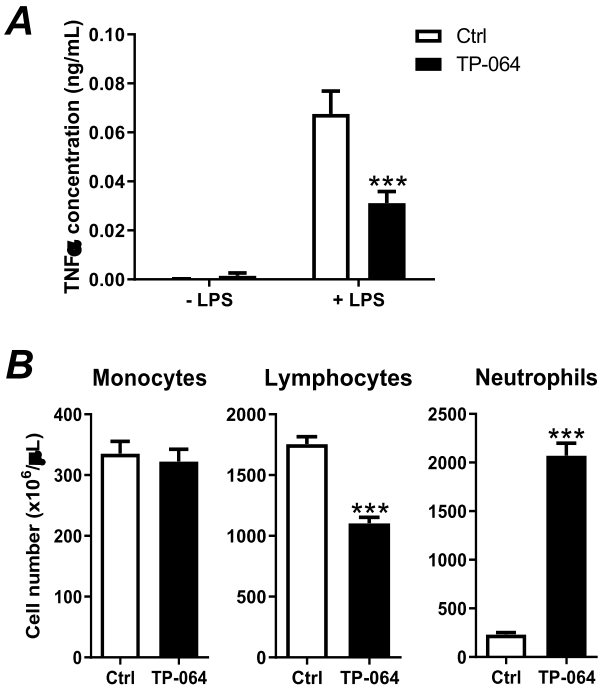

Figure 4: *TP-064 treatment lowers LPS-induced TNFα secretion by monocytes in the whole blood assay.*

*(A) TNFα production in cultured blood samples collected from tail veins of wildtype (WT) mice treated with DMSO solvent (White) or TP-064 (Black) that were treated with or without LPS stimulation (1 ng/ml) for 6 hours. (B) Blood monocyte, lymphocyte, and neutrophil counts. Data are expressed as means ± SEM (N=6 per group). *** P<0.001 (Two-way ANOVA with Bonferroni post-test or unpaired t-test).*

3.5. TP-064 treatment is associated with neutrophil accumulation in kidney and liver without inducing toxicity

High levels of neutrophils in blood can also affect major organs, such as kidney and liver [23, 24]. Therefore, to verify the infiltration of neutrophil into these tissues, we measured the mRNA expression levels of the neutrophil marker genes Ly6G (general) and matrix metalloproteinase-8 (MMP8) (activation) [25, 26]. In kidney from control mice, neither Ly6G or MMP8 were reliably detected (Ct>35) (Fig.5A). In contrast, kidney from TP-064 treated mice did express both LY6G (Ct=29±0.2; P<0.001) and MMP8 (Ct=30±0.2, P<0.001 Fig.5A). In liver, TP-064 did not significantly alter relative expression levels of Ly6G, but MMP8 expression was increased 5-fold (Fig.5B; P<0.001), as compared to control. Importantly, no changes were observed in plasma AST and ALT activities that are generally induced as a result of liver damage (Fig.5C), suggesting that the TP-064 induced-neutrophil infiltration did not cause liver injury [27].

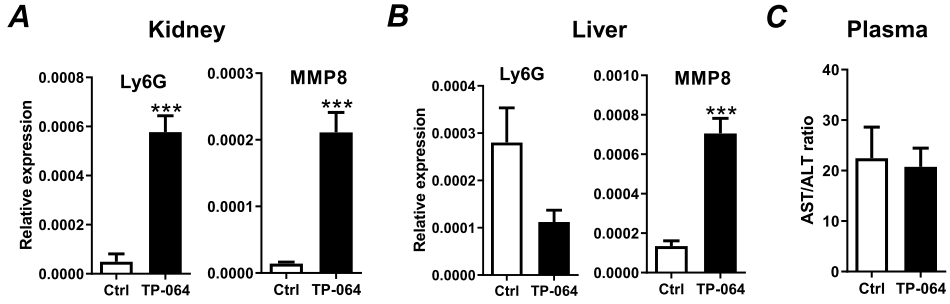

Figure 5: *TP-064 treatment is associated with neutrophil accumulation in kidney and liver without inducing toxicity*

*Relative mRNA expression levels of neutrophil markers LY6G (general) and MMP8 (activation) in kidney (A) and liver (B) from mice treated with DMSO solvent (Ctrl; white) or TP-064 (Black). (C) Plasma AST/ALT ratio. Data are expressed as means ± SEM (N=6 per group). *** P<0.001 (Unpaired t-test).*

3.6. TP-064 treatment lowers liver PRMT4 target gene expression

A previous study by Yeom et al. showed that PRMT4 plays an important role in

the upregulation of genes involved in hepatocyte proliferation and metabolism [28-30]. In line, the PRMT4 target genes cyclin-dependent kinase 2 (CDK2; -50%; P<0.05), CDK4 (-30%, P<0.05), and glucose-6-phosphatase catalytic subunit (G6PC; -50%; P<0.05) were all downregulated upon treatment with the PRMT4 inhibitor TP-064 (Fig.6A) Although G6PC is important for glycogen metabolism, no changes were observed in the liver glycogen stores and liver weight corrected for bodyweight, upon TP-064 treatment (t-test: P>0.05; Fig.6B and 6C). Histological sections of livers from TP-064-treated mice and controls were morphologically analyzed and showed no obvious differences Fig.6D). In summary, we showed that the PRMT4 inhibitor TP-064 efficiently lowers PRMT4 target genes in liver without any obvious negative hepatic side effects.

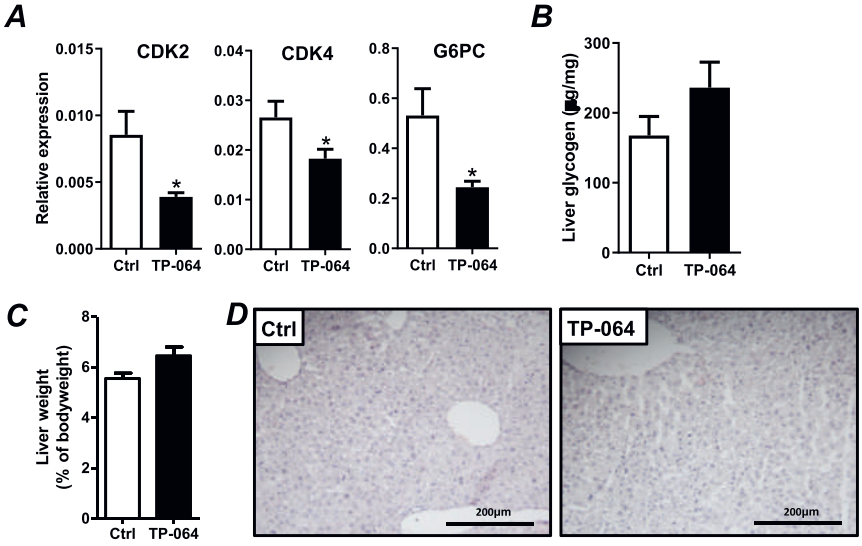

Figure 6: *TP-064 treatment inhibits liver PRMT4 target genes without any liver damages*

*(A) Relative mRNA expression levels of PRMT4 target genes CDK2, CDK4 and G6PC in liver from mice treated with DMSO solvent (Ctrl; white) or TP-064 (Black). (B) Hepatic glycogen levels expressed per mg of liver tissue. (C) Liver weights corrected for body weights. Data are expressed as means ± SEM (N=6 per group). * P<0.05 (Unpaired t-test). (D) Representative microphotographs of livers stained with Eosin & Hematoxylin (100x magnification). Scale bars represent 200µm length.*

4. DISCUSSION

Our study showed that TP-064 treatment is able to inhibit LPS-induced synthesis of IL-6, IL-12, IL-1β, and TNFα by macrophages in vitro and ex vivo. In addition, we found that TP-064 treated mice presented an on-going pro-inflammatory peritonitis after a thioglycollate challenge. Furthermore, TP-064 inhibited PRMT4-driven transcriptional activities in liver.

The finding that TP-064 reduces pro-inflammatory cytokine secretion is in line with the earlier observations that PRMT4 acts as a co-activator of NF-κB [7, 8]. Furthermore, it suggests that inhibition of PRMT4 function by TP-064 could be a potential novel therapeutic strategy for the treatment of NF-κB -driven pathologies [31]. NF-κB activation by extracellular inflammatory triggers is highly associated with metabolic diseases, such as obesity, insulin resistance, and type II diabetes [32-34]. Moreover, NF-κB-mediated pro-inflammatory signalling in endothelial cells and leukocytes induces the chronic inflammatory pathology atherosclerosis [31]. In agreement, drug-induced inhibition of NF-κB activation in pro-inflammatory monocytes/macrophages reduced atherosclerotic lesion development in apolipoprotein E deficient mice [34]. Moreover, siRNA-induced reduction of NF-κB signalling protects mice against high fat-diet induced insulin resistance and steatosis [35]. Therefore, it will be of interest to evaluate the effects of chronic TP-064 treatment on NF-κB -mediated macrophage function in disease models of diabetes and atherosclerosis.

An unexpected finding from our studies was that TP-064 treatment, combined with thioglycollate stimulation in peritoneal cavity, resulted in an on-going peritonitis and systemic neutrophil accumulation. The increase in (peritoneal) neutrophil numbers coincided with a higher basal expression level of neutrophil attraction mediators CXCL1, MIP-2, and IP-10 in peritoneal macrophages. In-

terestingly, studies by von Vietinghoff et al. have shown that genetic deficiency of NF-κB is also associated with elevated neutrophil numbers in blood after exposure to thioglycollate [36]. As such, it could be that TP-064 treatment not only disrupted the pro-inflammatory NF-κB -related function in macrophages, but also reduced NF-κB signalling in neutrophils leading to increased neutrophil mobilization in response to the thioglycollate challenge [36, 37]. Importantly, the higher activated neutrophil count can theoretically also underlie the shift in the basal macrophage phenotype observed in TP-064-treated mice.

Previous studies from Yeom et al. suggested that hepatocyte proliferation is directly upregulated by PRMT4 overexpression [28]. In further support, TP-064 treatment also restricts cell proliferation of NCI-H929 multiple myeloma cells [9]. In line with PRMT4's key role in liver cell proliferation, we showed that

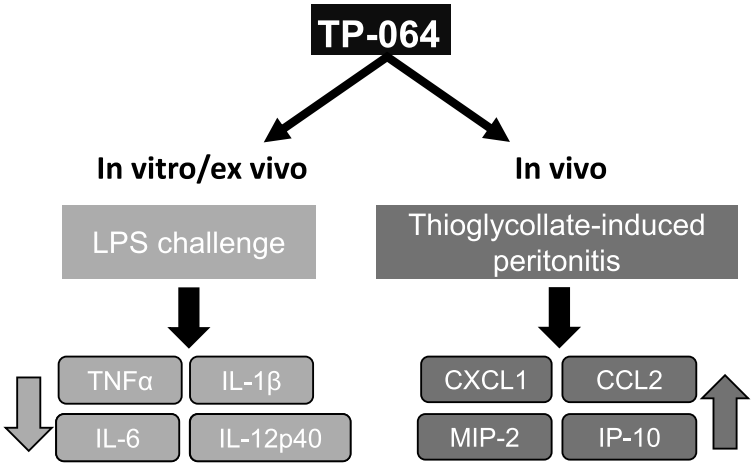

Figure 7: Schematic overview of the effects of TP-064 on monocytes / macrophages in vitro and in vivo.

TP-064 showed an anti-inflammatory effect in macrophages by reducing LPS-challenged pro-inflammatory TNFα, IL-1β, IL-6, and IL-12p40 responses both in vitro and ex vivo. In addition, TP-064 treatment in a thioglycollate-induced peritonitis mouse model was associated with a pro-inflammatory peritoneal macrophage phenotype differentiation instead of a resolving macrophage phenotype, as judged from upregulated mRNA expression levels of CXCL1, MIP-2, IP-10, and CCL2.

PRMT4 inhibition by TP-064 downregulates the expression of the cell cycle-related genes CDK2 and CDK4 in mouse liver [38]. Based on these combined findings, we anticipate that TP-064 may also be valuable for the treatment of hepatocellular carcinoma.

In conclusion, we have shown that PRMT4 inhibitor TP-064 inhibits the pro-inflammatory macrophage lipopolysaccharide response in vitro and ex vivo, but induces peritonitis-associated neutrophilia in vivo (Fig.7). Our findings suggest that TP-064 can possibly be applied as therapy in NF-κB -driven inflammatory diseases. However, in order to reach an optimal balance between the pro-inflammatory effects of neutrophilia and the anti-inflammatory effect of TP-064 though restricted NF-κB activation in macrophages, it would be worthwhile to evaluate the effects of different TP-064 dosages in specific disease settings.

ACKNOWLEDGMENTS

This study was supported by grants from the Netherlands Organization for Scientific Research (VICI 91813603) awarded to Miranda Van Eck. Miranda van Eck is head of the Cardiovascular and Metabolic Therapeutics group (https://www.universiteitleiden.nl/en/science/drug-research/biotherapeutics/cardiovascular-and-metabolic-therapeutics) and an Established Investigator of the Dutch Heart Foundation (2007T056). Yiheng Zhang is supported by the Chinese Scholarship Council (CSC).

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