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Activation of μ-opioid receptor and Toll-like receptor 4 by plasma from morphine-treated mice.

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Running title: Plasma of morphine-treated mice activates TLR4

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ABSTRACT

In this study, we quantified the ability of opioids present in biological samples to activate the μ-opioid receptor and TLR4 using cell-based assays. Each assay was standardised, in the presence of plasma, using morphine, its μ receptor-active metabolite morphine-6 glucuronide (M6G) and its μ receptor-inactive, but TLR4-active metabolite morphine-3 glucuronide (M3G). Specificity was verified using antagonists. Morphine- and M6G-spiked plasma samples exhibited μ receptor activation, which M3G-spiked plasma lacked. In contrast, M3G showed moderate but consistent activation of TLR-4. Plasma samples were collected at a number of time points from mice administered morphine (1 or 10 mg/kg every 12 h for 3 days) or saline. Morphine administration led to intermittent μ receptor activation, reversed by μ receptor antagonists, and to TRL4 activation at time points where M3G is measured in plasma. Interestingly, this protocol of morphine administration also led to TLR4-independent NF-κB activation, at time points where M3G was not detected, presumably via elevation of circulating cytokines including, but not limited to, TNFα. Circulating TNFα was increased after three days of morphine administration, and TNFα mRNA elevated in the spleen of morphine-treated mice.

Keywords: morphine; TLR-4; μ-opioid receptor; TNFα

1. INTRODUCTION

Contemporary literature indicates that non-opioid receptors can be activated by opiates. The Toll-like receptor 4 (TLR4) has been recently examined in this context (Hutchinson et al., 2010b; Stevens et al., 2013). Opioids produce a slight but significant activation of TLR4 in a non-stereospecific fashion (i.e. the (+) isomer of morphine is active at TLR4 receptor but not at OR level) (Wang et al., 2012) while antagonising the effect of the natural ligand, LPS, in a non-competitive fashion (Stevens et al., 2013), presumably via binding to MD2 (Hutchinson et al., 2010b). The morphine metabolite, morphine-3-glucuronide (M3G), is able to activate TLR4 signalling (Hutchinson et al., 2010b). Activation of TLR4 by opioids is proposed to mediate some of the undesirable effects of opioids, such as neuro-inflammation through central innate immune and endothelial cells (Grace et al., 2014; Wang et al., 2012); contribution to the reinforcing/rewarding effects of opioids (Hutchinson et al., 2012) and to morphine-induced suppression of colon peristalsis (Farzi et al., 2015); but also to modulate peripheral endogenous opioid-mediated analgesia in the context of inflammation (Sauer et al., 2014).

In this study, we established new methods to quantify µ receptor and TLR-4 agonism in biological samples and have applied them to mouse plasma samples in order to demonstrate, for the first time, circulating receptor agonism in morphine-treated mice.

2. MATERIALS AND METHODS

2.1 Materials

DMEM, foetal bovine serum (FBS), trypsin-EDTA, penicillin/streptomycin and G418 sulfate solutions were obtained from Invitrogen (Life Technologies, VIC, Australia). DBL^{TM} morphine sulfate and naloxone were purchased from Hospira (Mulgrave, VIC, Australia). Morphine-6β-D-glucuronide (M6G), morphine-3β-D-glucuronide (M3G), morphine-D$_3$, morphine-6β-D-glucuronide-D$_3$ (M6G-D$_3$), morphine-3β-D-glucuronide (M3G-D$_3$) were purchased from Novachem (Collingwood, VIC, Australia). Methylnaltrexone (MNTX) was from Link Medical Products (Warriewood, NSW, Australia). Normocine™, HEK-Blue™ Selection, QUANTI-Blue™ and lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) were obtained from Jomar Life Research (Scoresby, VIC, Australia). 3-isobutyl-1-methylxanthine (IBMX) and forskolin (FSK) were supplied by Sapphire Bioscience (Redfern, NSW, Australia). AlamarBlue™ was purchased from Thermo Fisher Scientific (Life Technologies, VIC, Australia). The following reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, and Australia): Amitriptyline, lipopolysaccharide (LPS) from E. Coli, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Tween^{TM} 20. The mouse inflammation antibody array was purchased from Abcam (Melbourne, VIC, Australia). TNFα was from Lonza (Mount Waverley, VIC, Australia). Alphascreen^{TM} cAMP assay kit for the evaluation of opioid receptor activation was obtained from PerkinElmer (MA, USA).

2.2 Cell culture

HEK-MOP cells (human embryonic kidney 293 cells stably transfected with μ-opioid receptor) were cultured in DMEM supplemented with 10% (v/v) FBS, 500 μg/mL

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G418 sulfate, 100 U/mL penicillin and 100 µg/mL streptomycin. HEK-Blue™-hTLR4 cells (human embryonic kidney 293 cells co-transfected with the human TLR4, MD-2 and CD14 co-receptor genes) were cultured in DMEM supplemented with 10% (v/v) FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL Normocin™ and 1X HEK-Blue™ Selection reagent to protect the stability of the transgenes. Both cell lines were incubated at 37℃ in a 5% CO_2 atmosphere.

2.3 AlphaScreen^{TM} cAMP Assay

The µ receptor activation assay relies on measurements of cAMP by cells overexpressing the µ receptor and was performed according to the manufacturer's instructions. Cyclic AMP was measured in 96-well plates with suspension cells in a total volume of 25 µL. Five µL of HEK-MOP cells in suspension (~25,000 cells), acceptor beads (at a final concentration of 15 µg/mL), and stim buffer (1x HBSS, 1%BSA, 0.5 mM IBMX, 5 mM HEPES) mixture were added into each well previously filled with drugs or/and biological samples (5 µL in total volume) and incubated in the dark at room temperature for 30 min. Streptavidin donor beads and biotin cAMP in 15 µL lysis buffer (0.1% BSA, 0.3% Tween-20, 5 mM HEPES) were then added, and the plates were incubated and shaken in the dark at room temperature overnight before reading in the Perkin EnVision-Alpha Reader 2101 (PerkinElmer, MA, USA).

2.4 HEK-Blue™-hTLR4 assay (TLR4 signaling assay)

The determination of TLR4 activation employs cells engineered to express hTLR4 and MD-2/CD14 co-receptor genes, together with a secreted embryonic alkaline phosphatase (SEAP) reporter gene under NF-κB and AP-1 control. TLR4 activation results in NF-κB-dependent production of SEAP detected using a colorimetric

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substrate placed in the cell culture medium. These HEK-Blue™-hTLR4 cells were seeded in a 96-well plate (10,000 cells/well). After 48 h incubation, the medium was replaced with serum-free medium. The cells were then treated with drugs and/or biological samples, and incubated for 12 h at 37℃ in 5% CO_2. A second-step QUANTI-Blue™ assay reagent was introduced to measure the level of secreted embryonic alkaline phosphatase (SEAP). QUANTI-Blue™ substrate was pre-warmed at 37℃, then distributed in 180 μL aliquots per well to 96-well plates. 20 μL supernatant of treated cell culture media was then added and incubated for 4 h at 37℃ in 5% CO2. The plates were read at a wavelength of 655 nm with a spectrophotometer (Bio-Rad Laboratories Inc.).

2.5 AlamarBlue^{TM} Cell Viability Assay

Cells were seeded in a 96-well plate (10,000 cells/well). After 48 h incubation, the medium was replaced with serum-free medium. The cells were then added with drugs, and incubated for 6 h at 37℃ in 5% CO_2. Control samples included untreated cells and background (culture medium without cells). A volume of 10 μL AlamarBlueTM (AB) reagent was then added yielding a final concentration of 10% AB in 100 μL / well assay volume. The plates were then incubated for an additional 6 h and the absorbance was measured at a wavelength of 570 nm and 600 nm using a SPECTROstar Nano absorbance microplate reader. The calculation of the percentage of AB reduction (% AB reduction) was performed according to the manufacturer's protocol:

$$\% \text{ Reduction of AB Reagent} = \frac{(Eoxi600 \times A570) - (Eoxi570 \times A600)}{(Ered570 \times C600) - (Ered600 \times C570)} \times 100$$

In the formula, E_{oxi} and E_{red} are molar extinction coefficient (E) of oxidized AB reagent at 570 and 600 nm, respectively (E_{oxi} = 80586 and E_{red} = 117216). A570 and A600 represent absorbance of test wells at 570 and 600 nm, respectively. C570 and C600 represent absorbance of negative control at 570 and 600 nm, respectively.

2.6 Animals

Reporting of the animal studies follows the ARRIVE guidelines (McGrath and Lilley, 2015). Female Balb/c mice (6 weeks) from University of Queensland Biological Resources (The University of Queensland, QLD, Australia) were maintained on a 12 h light/dark cycle in temperature- and humidity-controlled conditions with water and food supplied ad libitum. The University of Queensland Animal Ethics Committee approved all procedures involving mice (AEC Approval Number: PHARM/381/13/ANZCA), and they are conducted according to the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised in 1985).

2.7 Morphine treatment and blood collection

Mice were given an intra-peritoneal injection of 1 or 10 mg/kg of morphine or an equivalent volume of saline at 12 h intervals for three days. The 10 mg/kg dose was chosen because it is analgesic and leads to circulating concentrations of morphine in mice similar to the dose range we use in our in vitro experiments (Afsharimani et al., 2014; Khabbazi et al., 2015), while the 1 mg/kg dose was selected to generate M3G concentrations that are lower and thus easier to compare to M3G concentrations in humans for TLR4 activation. To capture peak and trough concentrations of morphine,

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the mice were euthanised for blood collection at 10 min and 12 h after the first (day 1, D1) and last (day 3, D3) injection.

2.8 Quantitative LC–MS/MS determination of morphine and morphine metabolites in biological samples

Circulating concentrations of morphine, M6G and M3G were measured using an Agilent 6460 Triple Quadrupole system (Agilent Technologies, Santa Clara, CA, USA). The analytes were extracted from plasma samples (100 µL) using protein precipitation. Samples were extracted into 100% acetonitrile (Merck, Darmstadt, Germany), the acetonitrile dried down by nitrogen and the samples reconstituted in a mixture of 5% acetonitrile and water (Milli-Q system, Milford, USA) with 0.1% formic acid (Fisher Chemical, Geel, Belgium) prior to HPLC analysis. The analytes (M, M3G, M6G and the internal deuterated standards) were separated using reversed phase chromatography on a Poroshell 120 PFP (150 x 2.1 mm, 2.7 µm)-Agilent column (Agilent Technologies, Santa Clara, CA, USA) and eluted using 5% acetonitrile containing 0.1% formic acid as the mobile phase. The compounds were ionised using electrospray ionization, their m/z ratios were detected using tandem mass spectrometry running in the positive multiple reaction monitoring (MRM) mode, and their product ion transitions (M, 286.2-151.1 m/z; Morphine-D_3, 289.2-155.1 m/z; M3G & M6G, 462.2-286.1 m/z; M3G-D_3 & M6G-D_3, 465.2-289.1 m/z) monitored to provide unequivocal quantitative data. Results were analyzed using a Mass Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

2.9 Mouse inflammation antibody array

The mouse inflammation antibody array was purchased from Abcam (Melbourne, VIC, Australia). The assay was carried out following the manufacturer's protocol.

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Three membranes were incubated separately in blocking buffer for 30 min at room temperature before overnight incubation at 4^oC with an equal volume of combined serum collected from 3 individual mice undergoing the same treatment (D3 12 h, saline, morphine 1 mg/kg or morphine 10 mg/kg). The membranes were incubated in 1X Biotin-Conjugated Anti-Cytokine antibodies (1 mL/membrane) at 4^oC overnight. The following 2 h incubation step (at room temperature) for signal detection was carried out with 1X HPR-Conjugated Streptavidin (2 mL/membrane). Between each incubation, a 2 steps washing was performed to reduce background interference. The array assay was performed twice with samples collected from two separated experiments. Chemiluminescence was detected using the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories Inc.). Optical density was analyzed using Image J software.

2.10 **Quantitative RT-PCR**

The expression of TNFα transcripts was quantified by real-time reverse transcriptase polymerase chain reaction (Real time RT-PCR). Total RNA was isolated from mouse tissue and purified using a Pure link™ RNA Mini Kit (Life Technologies, VIC, Australia). Purified RNA concentration was determined by 260 nm absorbance in a nano-drop spectrophotometer (Thermo Fisher Scientific, VIC, Australia). RNA was reverse transcribed to complementary DNA using a High-Capacity cDNA reverse transcription kit (Life Technologies, VIC, Australia) and cDNA quantified using using TaqMan™ Fast Universal PCR Master Mix (Life Technologies, VIC, Australia) with AmpliTaq Gold™ DNA Polymerase and TaqMan™ Gene Expression Assay for human TNFα (Hs01113624-g1) in a StepOnePlus 7500 real time PCR system (Applied Biosystems, Carlsbad, CA, USA). The amount of target gene was normalized to 18S ribosomal RNA (18S rRNA) control gene and quantification was

performed using the comparative critical threshold (Ct) method (Schamittgen and Livak 2008).

2.11 ELISA quantification of TNFα

The mouse TNF single analyte ELISA kit was obtained from QIAGEN (Melbourne, VIC, Australia). The concentrations of TNFα in serum collected from saline or morphine-treated mice were determined according to the manufacturer's instructions. Mouse serum (50 µl/well) was incubated with assay buffer in 8-well ELISArray strips for 2 h, with detection antibody for 1 h and with avidin-horseradish peroxidase for 30 min. At least 3 washing steps were performed between each incubation. A substrate solution was added to each well for 15 min. The color development was stopped by addition of the stop solution and the absorbance at 450 nm was read in a spectrophotometer (Bio-Rad Laboratories Inc.). All incubation and washing steps were performed at room temperature. A standard curve was generated in a log-log graph for quantification. Protein amount of TNFα in experimental samples was determined based on the best straight-line curve fit through the points on the standard curve.

2.12 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (v. 7.00). A P-value of < 0.05 was considered significant. Data are presented as mean ± SEM of at least three replicates, independent experiments or individual mice as detailed in figure legends. To test three or more groups defined by one factor, data were analysed using One Way ANOVA with post hoc Dunnett's multiple comparison test. To compare individual groups of interest, data were analysed using Student's t test.

3. RESULTS

3.1 Quantification of μ receptor agonism in the presence of plasma.

In order to measure circulating μ receptor activity (resulting from the combination of exogenously administered and endogenously produced active factors), we have optimised the conditions to quantify μ receptor activation. The μ receptor activation determination is achieved via Alphascreen™ measurement of cAMP produced by μ receptor-overexpressing human embryonic kidney 293 cells (HEK-MOP cells). Cellular production of cAMP is measured by reference to a cAMP standard curve (Figure 1A). Forskolin (fsk)-induced cAMP production (Figure 1B) is decreased by Gi-coupled μ receptor; therefore, in this assay, decreased cAMP production reflects μ receptor activation. We have confirmed that morphine and its active metabolite M6G activate μ receptor (thus inhibiting fsk-induced cAMP production) while M3G does not reduce cAMP production (Figure 1C). Furthermore, naloxone and methylnaltrexone (MNTX) dose-dependently reverse the effect of morphine (Figure 1D). Serum interfered with the assay and caused artefactually high readings of cAMP concentration (data not shown). We determined that plasma could be used up to 0.5 μl (a concentration of 5% of the assay volume) without significant interference with AMP measurement, and would permit the detection of μ receptor activation by morphine (Figure 2A). Plasma spiked with morphine or M6G concentrations as low as 1 nM caused significant inhibition of fsk-induced cAMP production while plasma spiked with M3G up to 1 μM did not (Figure 2B). Lastly, naloxone and methylnaltrexone (MNTX) can be added to the assay to demonstrate μ receptor involvement in the detected activity (Figure 2C).

3.2 Quantification of TLR4 receptor activation in the presence of plasma.

We measured TLR4 activation by LPS in HEK-Blue™ cells and determined that plasma was a better option than serum to apply this assay to mouse samples (Figure 3A). TLR4 activation by LPS was reversed by the TLR4 antagonist LPS-RS (Figure 3B) and by amitriptyline (Hutchinson et al., 2010a) at concentrations that did not induce loss of HEK-Blue™ cell viability (Figure 3C). The µ receptor-inactive morphine metabolite M3G is a weak but consistent activator of the TLR4 in the absence or presence of 5% plasma, while in our experimental conditions, morphine (with a modest but significant increase at only one of the concentrations tested) and M6G are not (Figure 4A). TLR4 involvement in the observed activation was verified using the specific TLR4 antagonist, LPS-RS (Figure 4B) as well as by amitriptyline (Figure 4C) at concentrations that did not result in loss of HEK-Blue™ cell viability (Figure 4D and 4E, up to 30 µM amitriptyline).

3.3 Plasma from morphine-treated mice activates µ receptor

The ability of plasma from morphine treated mice to activate µ receptor was detected at some, but not all time points and treatment doses. When present, the activation was comparable to that obtained with 1-100 nM morphine, which, when accounting for the 5% dilution of plasma in the assay, would correspond to high nM, low µM morphine concentrations in the plasma (if all activity was due to non-metabolised morphine). Mu opioid receptor agonism was present in the plasma of morphine-treated mice 10 min after the first injection, but not 12 h after injection, indicating that the mice are subjected to intermittent µ receptor activation arising from the protocol that we employed (Figure 5A). Accordingly, no morphine was measured in plasma at the D1 12 h time point (Figure 5B). Activation of µ receptor was seen 10 min after the final injection even in mice treated with 1 mg/kg morphine (Figure 5A). Interestingly, at the D3 12h time point, there was significant µ receptor activation present (Figure

5A) but no morphine was detected (Figure 5B). We hypothesised that the activity was due to a μ receptor-active metabolite; accordingly, μ receptor activation detected in all samples was reversed by 1 μM naloxone (Figure 6 A-D). As expected (Milne et al., 1996), M6G was not detected in any of the samples by LC-MS/MS (Data not shown).

3.4 Plasma from morphine-treated mice activates TLR4

Our results show, for the first time, TLR4 activation by plasma of morphine-treated mice (Figure 7A). This was statistically significant at 10 min and 12 h after the last injection for both the 1 and 10 mg/kg doses. Mean ± SD concentrations of M3G of 4.9 ± 1.6 and 7.7 ± 2.2 μM were found in the plasma of 1 mg/kg morphine-treated mice 10 min after the first and last injection, respectively. Mean concentrations of M3G of 39.3 ± 11.8 and 68.9 ± 9.1 μM were found in the plasma of 10 mg/kg morphine-treated mice 10 min after the first and last injections, respectively. No M3G was detected 12 h after injections, indicating that exposure to this metabolite is also intermittent when using 12 h separated IP injections of morphine. Most intriguingly, the activity at the D3 12 h time point did not correlate with the presence / concentration of M3G in plasma (Figure 7B). The TLR4 assay relies on NF-κB activation in TLR4 over-expressing cells. To assess whether the activity detected at D3 12 h might be due to NF-KB activation independent of TLR4, we repeated the assay in the presence of the TLR4 antagonist LPS-RS (Figure 8). The antagonist reversed the activity of D1 10 min plasma, (compatible with M3G present in the sample activating TLR4), but only partially reversed the activity of D3 10 min plasma and did not alter the activity of the D3 12 h plasma. These results indicate that the mice subjected to the protocol of morphine administration that we employed: (i)

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exhibit circulating TLR4 activation correlating with the presence of M3G; and (ii) accumulate, within 3 days, circulating TLR4-independent, NF-κB activating factor(s).

3.5 HEK-Blue™ cells can increase secreted embryonic alkaline phosphatase (SEAP) production in response to TLR4-independent signals.

To test whether SEAP production in HEK-Blue™ cells can be triggered in a TLR4-independent fashion, we compared the activation induced by LPS (Figure 9A) and TNFα (Figure 9B) and showed that TNFα at concentrations above 1 ng/mL indeed elicited SEAP production. While the LPS-induced activation of SEAP production was reversed by the specific TLR4 antagonist LPS-RS (Figure 9C), the activation induced by TNFα was not reversed by LPS-RS (Figure 9D). This suggests that mice treated with intermittent IP injections of morphine for 3 days have increased circulating concentrations of TNFα and/or other cytokines and factors resulting in NF-κB-dependent activation of SEAP in the HEK-Blue™ cells. We verified whether this might be the case for TNFα by comparing the levels of TNFα and its soluble receptors sTNFR I and II in the serum of saline and morphine-treated mice (Figure 10A and 10B). We measured a slight but consistent increase in the circulating TNFα concentration in mice administered morphine compared to saline controls. In contrast, there was no change in the concentrations of circulating TNFR I or II. We confirmed the increase in circulating TNFα in morphine-treated mice using ELISA quantification (Figure 10C). The concentration of TNFα measured in serum was below the threshold required for *in vitro* activation of TLR4 in the reporter cell line (Figure 9B), indicating that TNFα is not the only NF-κB activating factor increased in the circulation of morphine-treated mice. In an attempt to identify the origin of the increased TNFα found in the circulation, organs were collected from mice treated with saline or 10 mg/kg morphine and the tissue expression of TNFα mRNA was

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determined. The mRNA was induced by a factor ~3 in the spleen of morphine-treated mice (Figure 10D), in favor of an effect of morphine intermittent administration on TNFα production by immune cells. These results suggest that other NF-κB-activating factors may be increased in the morphine-treated mice, which together with TNFα may explain the LPS-RS-unaltered increased production of SEAP in HEK-Blue™ at time points where M3G is not measured in the circulation.

3.6 Plasma from morphine-treated mice inhibits LPS-induced TLR4 activation

Opioids are reported to inhibit LPS-induced TLR4 activation (Stevens et al., 2013). We tested whether M3G and morphine altered the TLR4 activation induced by 1 ng/mL LPS across a wide range of concentrations, covering the range of concentrations of M3G and morphine found in the plasma of morphine treated mice (Figure 11A). Both M3G and morphine significantly decreased the TLR4 signal, with a maximum effect of ~50% inhibition. To examine whether this would be relevant *in vivo*, we tested the effect of plasma from morphine or saline-treated mice added at 0.5 μl (or 5% (v/v) of the reaction volume), on LPS-induced TLR4 activation (Figure 11B). At time points where M3G and morphine were detected in plasma, there was a statistically significant inhibition of TLR4 signal with a maximum inhibition of ~38% observed with plasma from 10 mg/kg morphine-treated mice collected 10 minutes after the final injection.

4. DISCUSSION

This study has demonstrated for the first time that the administration of morphine to mice results in circulating M3G concentrations and TLR4 activation, compatible with a clinically relevant effect on TLR4-expressing cells. Of note, and as previously reported in *in vitro* experiments (Hutchinson et al., 2010b) the activation of TLR4 by M3G is modest compared to that elicited by natural ligand LPS. Future experiments will have to examine whether TLR4 activation by M3G has functional consequences *in vivo*. In addition to modest activation of the TLR4, opioids have been shown to significantly decrease LPS-induced activation of TLR4, by a mechanism suggested to be non-competitive antagonism (Stevens et al., 2013). We confirm that morphine, and reveal that M3G, both alter LPS-induced TLR4 over a range of concentrations observed in the circulation of morphine-treated mice. Interestingly, our study discloses that plasma of morphine-treated mice can prevent LPS-induced TLR4 activation only at time points when morphine and M3G are present. This suggests that the net effect of morphine (and possibly other opioids) administration on the immune system may vary greatly depending on the presence of TLR4 activating factors such as endotoxin or danger-associated molecular patterns (DAMPs) and that opioid modulation of innate immunity and inflammation via TLR4 may have relevance in pathological contexts.

Our results also show that the administration of morphine to mice every 12 h results in discontinuous circulating μ receptor agonism. There is a paucity of information in the preclinical or clinical literature regarding the effect of intermittent *vs.* continuous morphine administration on immune parameters. Intermittent morphine (every 12 h for 4 days) is documented to be a stressor, activating the hypothalamo-pituitary-adrenal (HPA) axis, and promoting a stress response (Houshyar et al., 2003) in rats.

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It has been proposed that natural killer (NK) cell suppression in heroin (diacetylmorphine) addicts may result from multiple daily withdrawals, while neuroendocrine and immune functions normalise upon μ receptor exposure to the long-acting agonist methadone (Kreek, 1996). On the other hand, in rodents, morphine-induced NK cell suppression is subject to tolerance whether morphine administration is discontinuous (daily SC injections) (Shavit et al., 1986), or continuous (via mini-pumps) (West et al., 1998).

Our results serendipitously show that intermittent morphine administration results in the production, within 3 days, of circulating NF-κB-activating factor(s) that act independently of TLR4, including (but most likely not limited to) TNFα. Increased TNFα has been documented in association with dependence and withdrawal. In rats rendered morphine-dependent by escalating IP injections of morphine every 12 h (10 to 40 mg/kg) for 5 days, precipitated withdrawal by naloxone injection (4 mg/kg) resulted, within 1 h, in increased TNFα expression in the brain periaqueductal grey matter (Hao et al., 2011). C3HeB/FeJ female mice subjected to morphine withdrawal (implanted with a 75 morphine pellet for 48 h which was then surgically removed for 24 h) were shown to react to LPS administration by an increased (more than two-fold) TNFα concentration in serum collected 6 h after LPS injection, compared to placebo pellet-inserted mice (Rahim et al., 2004). Similar results were obtained with an experimental design where tolerance was induced over 96 h and withdrawal assessed 24 h following removal of the pellets (Feng et al., 2005). In this latter study, TNFα serum concentration and splenic mRNA expression were tested in morphine-withdrawn mice challenged with saline instead of LPS, and there was no detectable increase compared to placebo pellet-inserted mice, indicating that morphine withdrawal *per se* is insufficient to induce TNFα using this experimental protocol.

Taken together with our current results, this raises an interesting consideration of the choice of pharmacokinetic characteristics and mode of administration of opioid analgesia to patients.

One pathological context where our findings may have translational relevance is tumor growth and metastasis. Both μ receptors and TLR4 can be activated by opioids or their metabolites, are expressed on cancer cells and tumour-associated cells, and control signalling pathways important in cancer metastasis. Cancer cells and immune cells, such as macrophages in the tumour microenvironment, are able to respond to TLR4 ligands (Ikebe et al., 2009; Liao et al., 2012). The actions of opioids at TLR4 may contribute to their net effect, both promoting and preventing tumour growth and metastasis. TLR4 signalling in cancer cells can increase their ability to invade (Ikebe et al., 2009; Liao et al., 2012); TLR agonists are, however, also powerful immunostimulants, now becoming part of the anticancer therapeutic arsenal (Adams, 2009). Endogenous TLR4 agonists such as danger-associated molecular patterns (DAMPs), released by cancer cells upon chemotherapeutic treatment, activate dendritic cells and promote an anti-tumour T-cell response (Fang et al., 2014). Recently, perioperative treatment with a TLR4 agonist in rats and mice was documented to reduce cancer metastasis (Matzner et al., 2015), seemingly via an increased immune response towards the cancer cells. Preclinical research on the influence of opioids on cancer is abundant and discrepant, with morphine or other opioid agonists documented to promote, prevent or have no effect on tumour growth or metastasis. To the factors likely to explain the conflicting results, which we have previously reviewed (Afsharimani et al., 2015), our current study now adds TLR4 as a confounding factor. Firstly, mice differ greatly in their ability to respond to TRL4 agonists (Poltorak et al., 1998; Sultzer, 1968; Tsukamoto et al., 2013). If the action of

opioids on TLR4 contributes to the response of the host to tumours, the mouse strain employed must be taken into consideration. Similarly cancer cells derived from mice with deficient TLR4 signalling (e.g. 4T1 breast cancer cells originate from BALB/c mice) may respond differently to opioids or their metabolites. Another level of variability between mouse strains is their ability to biotransform opioids into metabolites with TLR4 activation ability such as M3G, which in our experiments is a more potent activator of the TLR4 than morphine (Figure 4A), and is found at high concentrations in morphine-treated mice (Figure 7B). Mouse species differences in UDP-glucuronosyl transferase (UGT) activities are documented (Shiratani et al., 2008). Lastly, we have previously identified the continuity of administration as a factor likely generating discrepancies in the outcomes of preclinical studies exploring the influence of morphine on tumour growth and metastasis (Afsharimani et al., 2015). This is now reinforced by the finding that TNFα is induced in mice subjected to discontinuous μ receptor activation, and could potentially be evaluated in mouse tumour model(s) comparing continuous osmotic mini-pump administration to IP injections separated by 12 h.

Our preclinical study shows that intermittent morphine administration results in increased TNFα production and suggests a novel non-opioid-mediated role for morphine administration, including weak activation of the TLR4 by M3G, but effective antagonism by both M3G and morphine in the presence of the pro-inflammatory TLR4 ligand, LPS. The assays we have developed can be applied to clinical samples to further elucidate the effect of morphine administration on the immune system.

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FIGURE LEGENDS

Figure 1. Determination of µ-opioid receptor activation by measurement of cAMP production. A) Standard curve of cAMP signal-concentration relationship. B) Concentration-dependent increase in cellular cAMP levels upon forskolin stimulation. C) Production cAMP in response to 10nM and 1µM of morphine, M6G or M3G. All samples were adjusted to contain the same volume of solvent, i.e. saline for morphine, water:methanol (80:20) for M6G, methanol with 0.05% sodium hydroxide (w/v) for M3G. Data are shown as mean ± SEM, n=3 replicates. D) Concentration-dependent reversal of the effect of morphine on FSK-induced cAMP production by µ receptor antagonists. Indicated concentrations of naloxone and methylnaltrexone were included in the reaction system which contained 1 µM of morphine, and receptor activation was determined by the Alphascreen™ cAMP assay. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, morphine ± naloxone or methylnaltrexone vs FSK, One way ANOVA with Dunnett's multiple comparison. Data are shown as mean ± SEM, n=3 replicates.

Figure 2. Optimisation of the µ-opioid receptor activation assay with plasma. A) Different volumes of plasma were tested in the reaction system. µ receptor activation by 10 nM morphine was determined by the Alphascreen™ cAMP assay. Values are expressed as the % of cAMP produced in the presence of forskolin alone. *, p<0.05, ****, p<0.0001, forskolin + 0.5 µl plasma versus forskolin + 0.5 µl plasma and morphine, unpaired Student's t test. Mean ± SEM is shown, n=3 replicates. B) Screening of 0.5µL plasma spiked with morphine, M6G or M3G at indicated concentrations. µ receptor activation was determined by the Alphascreen™ cAMP assay. *, p<0.05, ****, p<0.0001, plasma spiked with morphine or M6G vs 0.5 µL plasma alone (+FSK), One way ANOVA with Dunnett's multiple comparisons. Mean

± SEM is shown, n=6. C) Modulation of cAMP production in the presence of antagonists. Indicated concentrations of naloxone and methylnaltrexone were mixed with the reaction system which contained 0.5 µL of plasma and 1 µM morphine, and receptor activation was determined by the Alphascreen™ cAMP assay. *, p<0.05, **, p<0.01, ***, p<0.001, morphine, morphine + naloxone or morphine + methylnaltrexone vs plasma alone + FSK, One way ANOVA with Dunnet's multiple comparisons. Data are shown as mean ± SEM, n=3 replicates.

Figure 3. Optimization of the determination of TLR4 activation. A) HEK-Blue™ hTLR4 cells were treated with LPS at increasing concentrations (0-1000 ng/mL) in the absence or presence of 5% (v/v) plasma or serum for 12 h. 20 µL cell supernatant was added to 180 µL QUANTI-Blue™ substrate and incubated for an additional 4 h. The response ratio (absorbance at 655 nm for LPS-treated cells / absorbance at 655 nm for control cells) was calculated and plotted as a function of LPS concentration. Data are shown as mean ± SEM, n=3 replicates. B, C) Reversal of LPS-induced SEAP production by TLR4 inhibitors. Indicated concentrations of LPS-RS or amitriptyline were added to cells stimulated with 1 ng/mL LPS, and receptor activation was determined by the QUANTI-Blue™ assay. ***, p<0.001, ****, p<0.0001, LPS + LPS-RS or amitriptyline vs LPS alone, One way ANOVA with Dunnet's multiple comparisons. Data are shown as mean ± SEM, n=3 replicates. D, E) Determination of cell viability in the presence of LPS-RS and TLR4 inhibitors. HEK-Blue™ hTLR4 cells were exposed to 1 ng/ml LPS and various concentrations of LPS-RS (D) or amitriptyline (E) for 6 h. Cells were then added with AlamarBlue Reagent, incubated for a further 6 h, and the absorbance was measured at 570 nm / 600 nm. Cell viability is shown as the percentage of AlamarBlue™ reagent reduction.

****, p<0.0001, amitriptyline and LPS-RS vs LPS alone, one way ANOVA with Dunnett's multiple comparison test. Data are shown as mean ± SEM, n=3 replicates.

Figure 4. M3G activates TLR4. A) HEK-Blue™ hTLR4 cells were incubated in cell culture media and stimulated with indicated concentrations of M3G, morphine or M6G. After 12 h incubation, the levels of SEAP were measured with two-step QUANTI-Blue™. *, p<0.05, **, p<0.01, ****, p<0.0001, morphine or M3G treatment vs media control, One way ANOVA with Dunnet's multiple comparisons. Data are shown as mean ± SEM, n=6 replicates. B, C) Reversal of M3G-induced SEAP production by TLR4 inhibitors. Indicated concentrations of LPS-RS and amitriptyline were added to cells stimulated with M3G, and receptor activation was determined by the QUANTI-Blue™ assay. *, p<0.05, ***, p<0.001, ****, p<0.0001, M3G + LPS-RS or amitriptyline vs M3G alone, One way ANOVA with Dunnet's multiple comparisons. Data are shown as mean ± SEM, n=6 replicates (LPS-RS) and n=3 replicates (amitriptyline). D, E) Determination of cell viability in the presence of M3G and TLR4 inhibitors. HEK-Blue™ hTLR4 cells were exposed to 10 µM M3G and various concentrations of LPS-RS (D) or amitriptyline (E) for 6 h. Cells were then added with AlamarBlue Reagent, incubated for a further 6 h, and the absorbance was measured at 570 nm / 600 nm. Cell viability is shown as the percentage of AlamarBlue™ reagent reduction. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, LPS-RS or amitriptyline vs M3G alone, One way ANOVA with Dunnet's multiple comparisons. Data are shown as mean ± SEM, n=3 replicates.

Figure 5. A) Plasma of morphine-treated mice activates µ receptor. Mice were treated with morphine (1 or 10 mg/kg) every 12 h for up to 3 days and blood samples collected at indicated times. µ receptor activation was determined by the Alphascreen™ cAMP assay using 0.5 µL plasma into 100 µL reaction system. White

bars show a standard curve of morphine concentrations, *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, morphine vs no morphine, One way ANOVA with Dunnet's multiple comparisons. Mouse plasma samples were tested at 5% (v/v): *, p<0.05, **, p<0.01, unpaired Student's t test. Results are shown as mean ± SEM, n = 3 individual mice. B) Morphine concentration in mouse plasma. Morphine was quantified using LC–MS/MS. Data are shown as mean ± SEM, n=3 individual mice and expressed as µM morphine.

Figure 6. Effect of µ receptor antagonist naloxone on receptor activation by plasma of morphine-treated mice. Mouse plasma samples (0.5 µL) as well as 1 µM naloxone were added to the Alphascreen™ cAMP reaction system simultaneously, and cellular cAMP production was then measured. *, p<0.05, **, p<0.01, ***, p<0.001, unpaired Student's t test. Mean ± SEM is shown, n=3 individual mouse samples.

Figure 7. A) Plasma of morphine treated mice activates TLR4. Mice were treated with morphine (1 or 10 mg/kg) every 12 h for up to 3 days and blood samples collected at indicated times. HEK-Blue™ hTLR4 cells were stimulated with 5% (v/v) mouse plasma. After 12 h incubation, the levels of NF-kB-induced SEAP were determined using two-step QUANTI-Blue™. White bars show a standard curve of M3G concentrations, *, p<0.05, **, p<0.01, ****, p<0.0001, M3G vs no M3G, One way ANOVA with Dunnet's multiple comparisons. Mouse plasma samples were tested at 5% (v/v): *, p<0.05, **, p<0.01, unpaired Student's t test. Results are shown as mean ± SEM, n=3 individual mice. B) M3G concentration in mouse plasma. M3G was quantified using LC–MS/MS. Data are shown as mean ± SEM, n=3 individual mice and expressed as µM M3G.

Figure 8. Effect TLR4 antagonist on receptor activation by plasma from morphine-treated mice. HEK-Blue™ hTLR4 cells were co-treated with 1 µg/mL LPS-RS and 5% morphine-treated mouse plasma. After 12 h incubation, the levels of NF-kB-induced SEAP were determined with two-step QUANTI-Blue™ *, p<0.05, **, p<0.01, unpaired Student's t-test. Mean ± SEM is shown, n=3 individual mouse samples.

Figure 9. TNFα stimulates HEK-Blue™ hTLR4 cells SEAP production. A, B) HEK-Blue™ hTLR4 cells were treated with LPS (A) or TNFα (B) at indicated concentrations for 12 h. Cell supernatant (20 µL) was added to 180 µL QUANTI-Blue™ substrate and incubated for 4 h. The response ratio (absorbance at 655 nm for LPS-treated cells / absorbance at 655 nm for control cells) was calculated and plotted as a function of cytokine concentration. C, D) Indicated concentrations of LPS-RS were added to cells stimulated with LPS (C) or TNFα (D), and SEAP production was determined by the QUANTI-Blue™ assay. ****, p<0.0001 One way ANOVA with Dunnet's multiple comparisons (LPS 1ng/ml) and unpaired Student's t test (LPS 100 ng/ml). Mean ± SEM is shown, n=3 replicates.

Figure 10. Morphine treatment increases circulating TNFα. A) Serum from 3 morphine- (1 mg/kg, 10 mg/kg) or saline-treated mice were combined and added to mouse inflammation antibody array membranes. Chemiluminescence was detected using the ChemiDoc™ Touch Imaging System. B) Data were normalized to positive control according to the manufacturer's instructions, and presented as % of the signal intensities of saline control. Mean ± SEM is shown, n=4 determinations each performed with serum from 3 individual mice mixed in equal parts. C) ELISA quantification of TNFα in mouse serum samples. *, p<0.05, **, p<0.01, morphine-versus saline-treated mice, unpaired Student's t test. Mean ± SEM is shown, n=6 individual mice per group. D) Organs were collected from saline or morphine (10

28

mg/kg)-treated mice at Day 3 10 min and the levels of TNFα mRNA determined by qRT-PCR. Results are shown as mean ± SEM, n=6 individual mice per group. ****, p<0.0001, unpaired Student's t-test.

Figure 11. Effect of morphine or M3G on LPS-induced TLR4 activity. A) HEK-Blue™ hTLR4 cells were treated with indicated concentrations of morphine or M3G in combination with 1 ng/mL LPS. After 12h incubation, the activity of SEAP was determined by two-step QUANTI-Blue™ assay. **, p<0.01, ***, p<0.001, ****, p<0.0001. LPS + morphine or M3G vs LPS alone, One way ANOVA with Dunnet's multiple comparisons. Results are shown as mean ± SEM, n=3 replicates. B) Mice were treated with morphine (1 or 10 mg/kg) every 12 h for up to 3 days and blood samples collected at indicated times. HEK-Blue™ hTLR4 cells were treated with 1 ng/mL LPS and 5% (v/v) mouse plasma. After 12 h incubation, the levels of NF-kB-induced SEAP were determined using two-step QUANTI-Blue™.*, p<0.05, **, p<0.01, unpaired Student's t test. Results are shown as mean ± SEM, n= 6 individual mice.

Figure 1

A

cAMP standard

B

Forskolin optimisation

C

Morphine/M6G/M3G

D

Morphine + Naloxone/MNTX
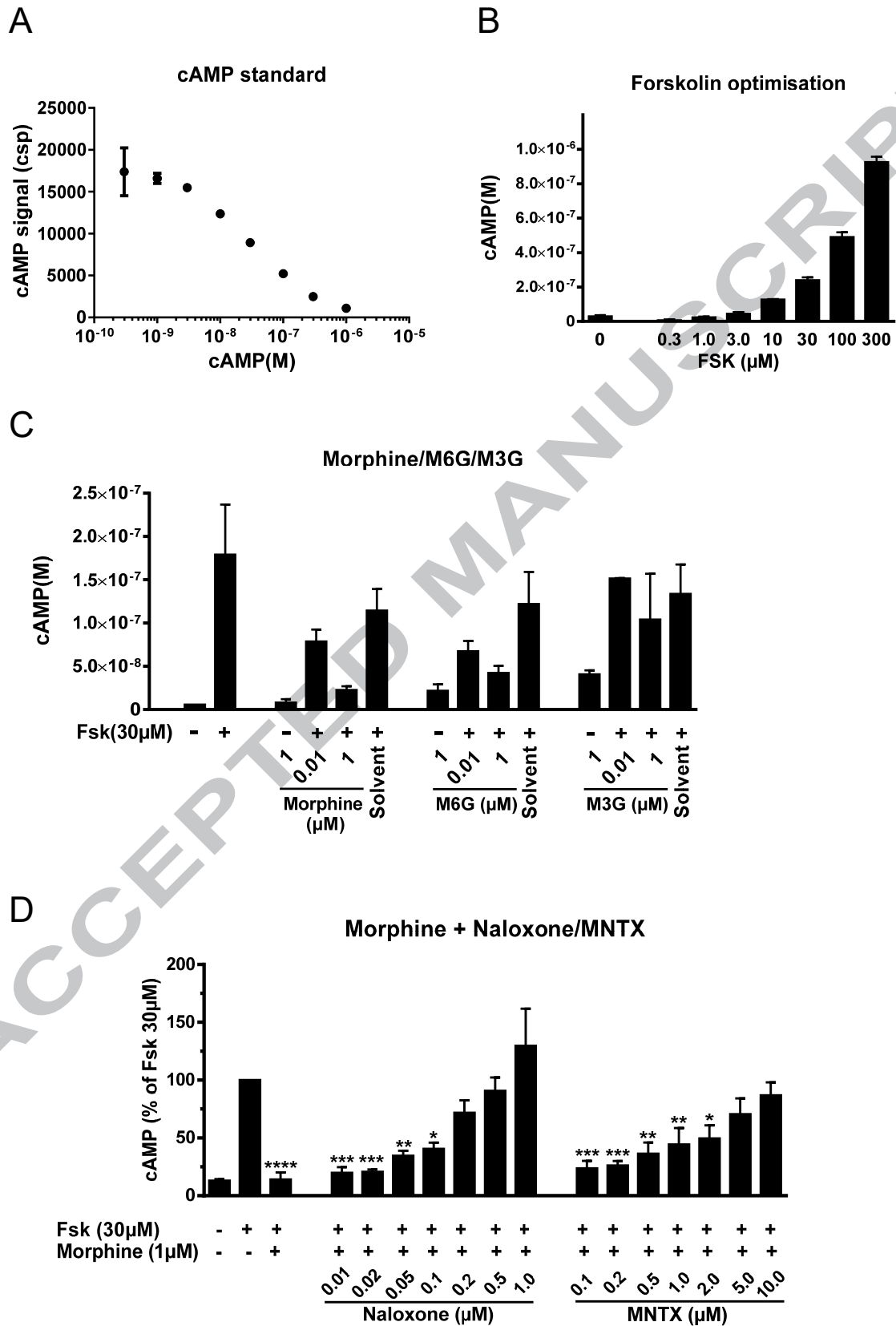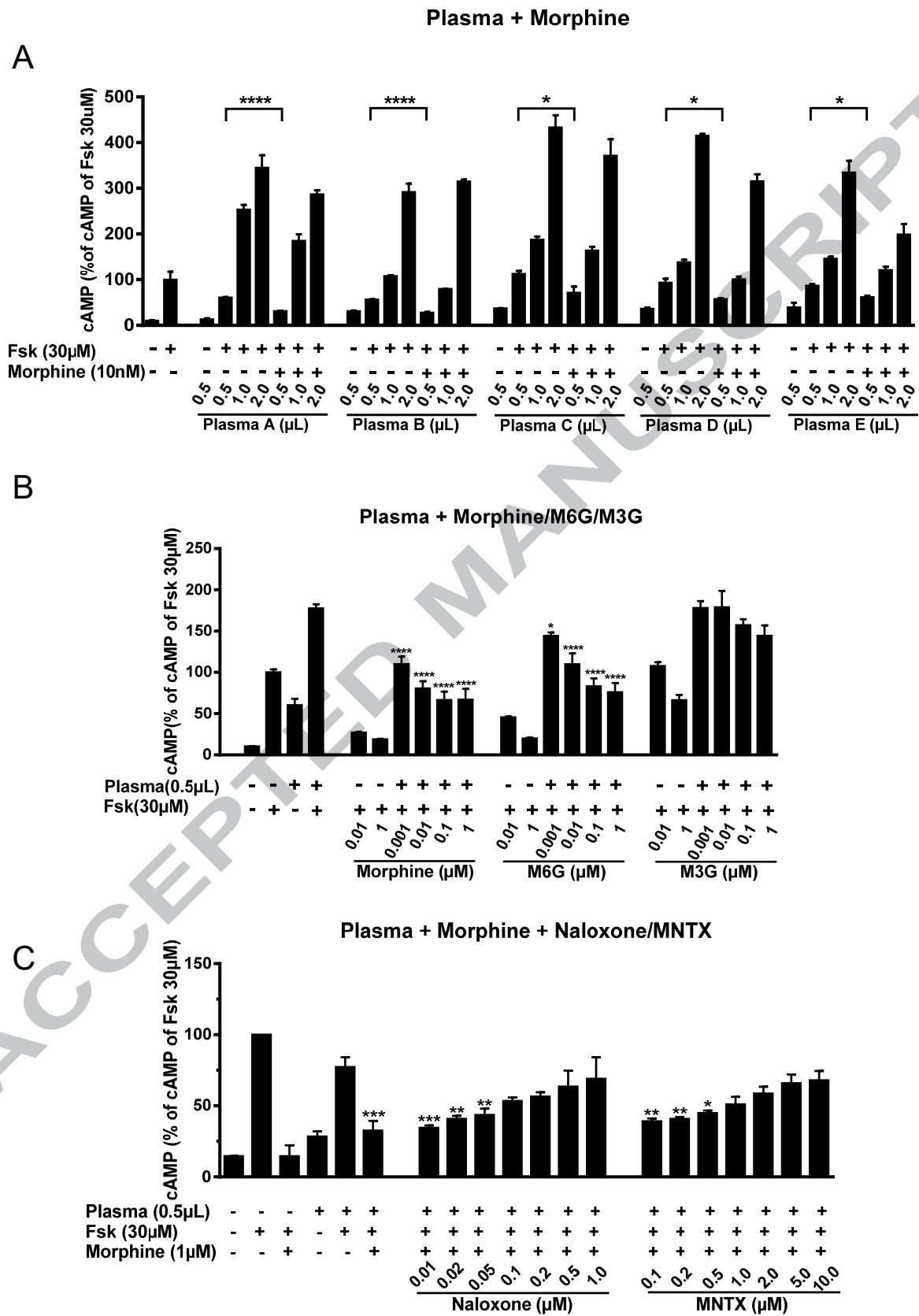
Figure 2

Figure 3

Figure 4

Figure 5

A

B

Figure 6

A

D1 10min

B

D1 12h

C

D3 10min

D

D3 12h
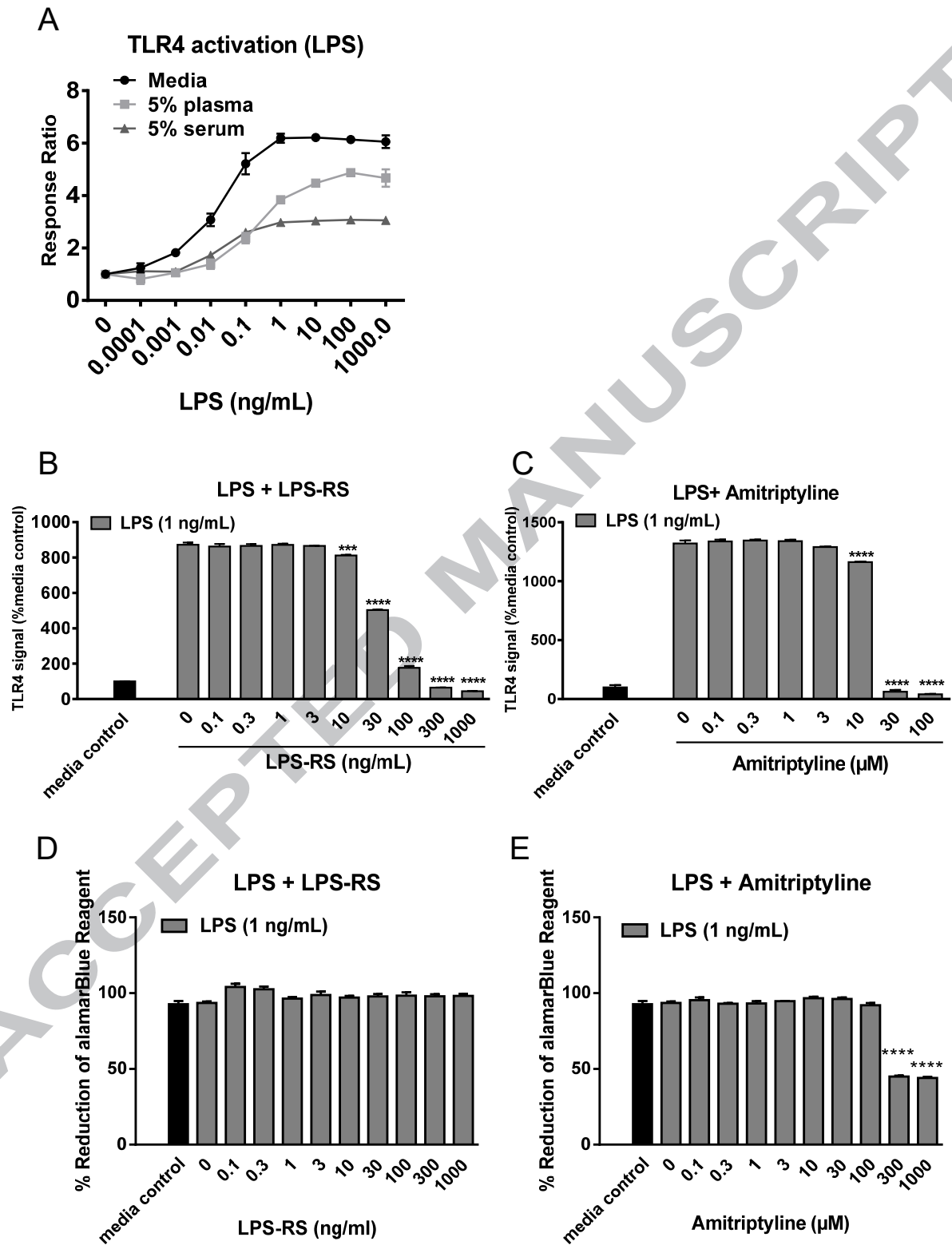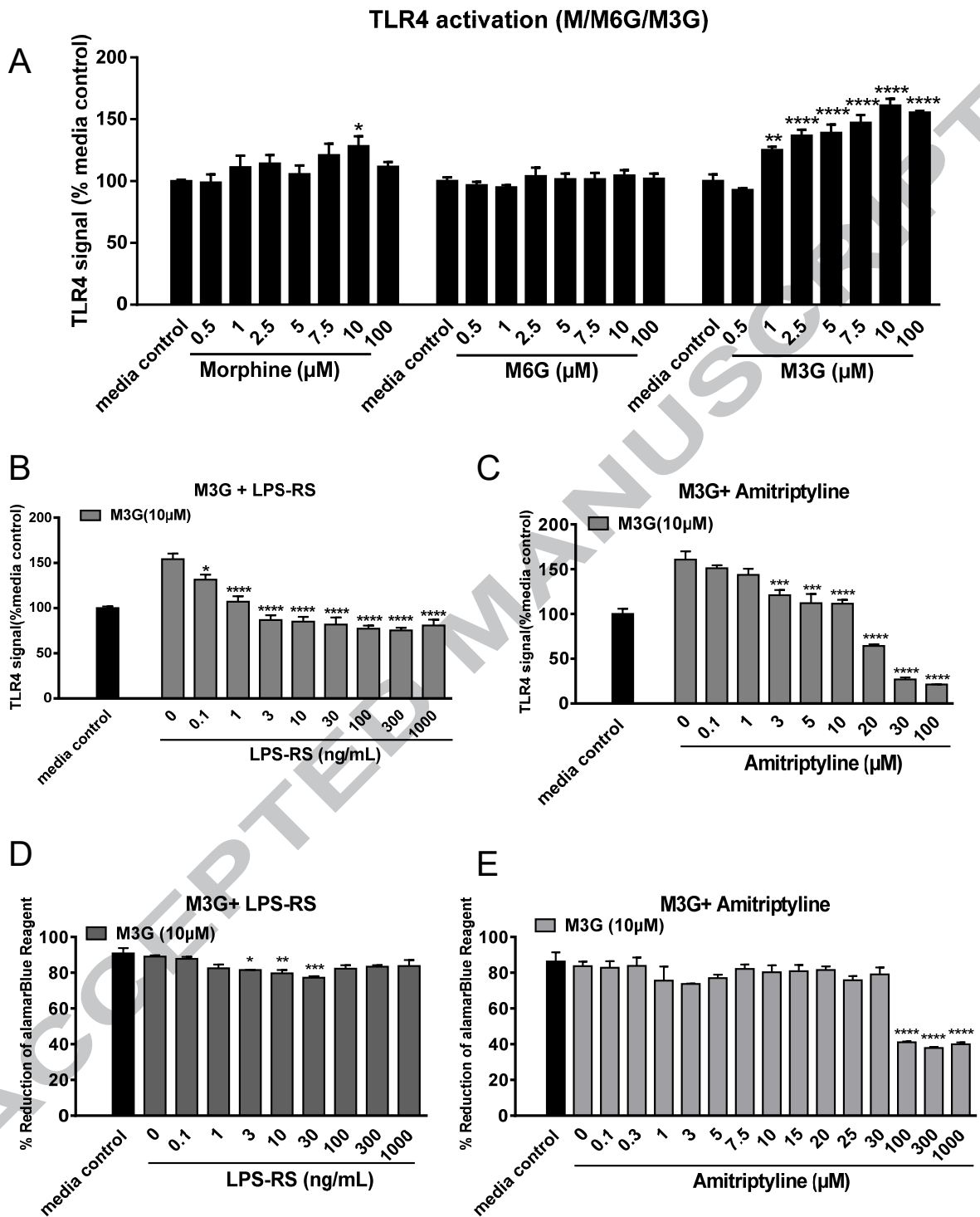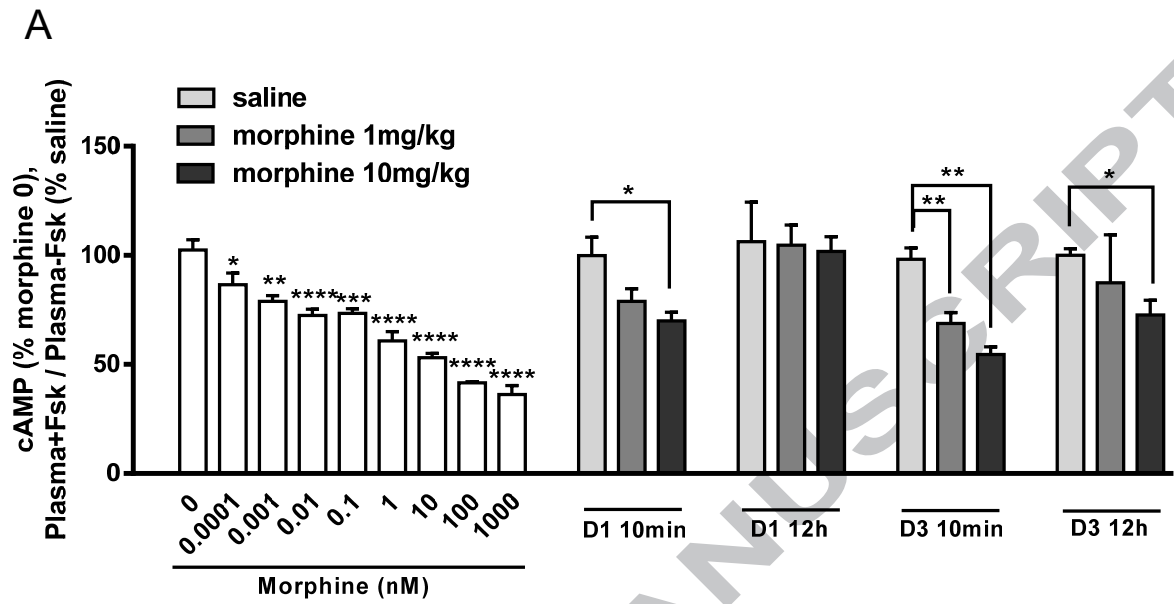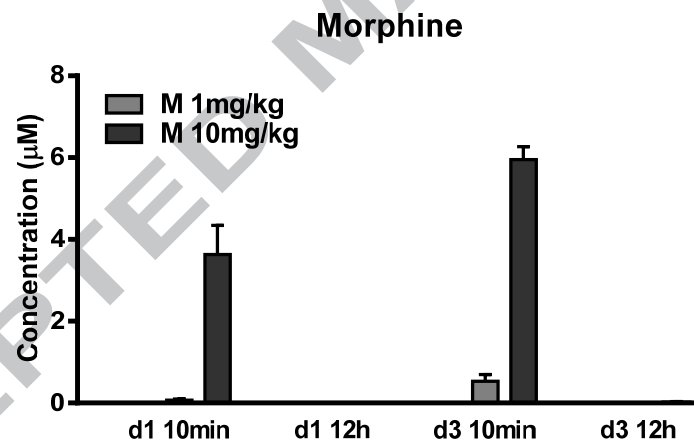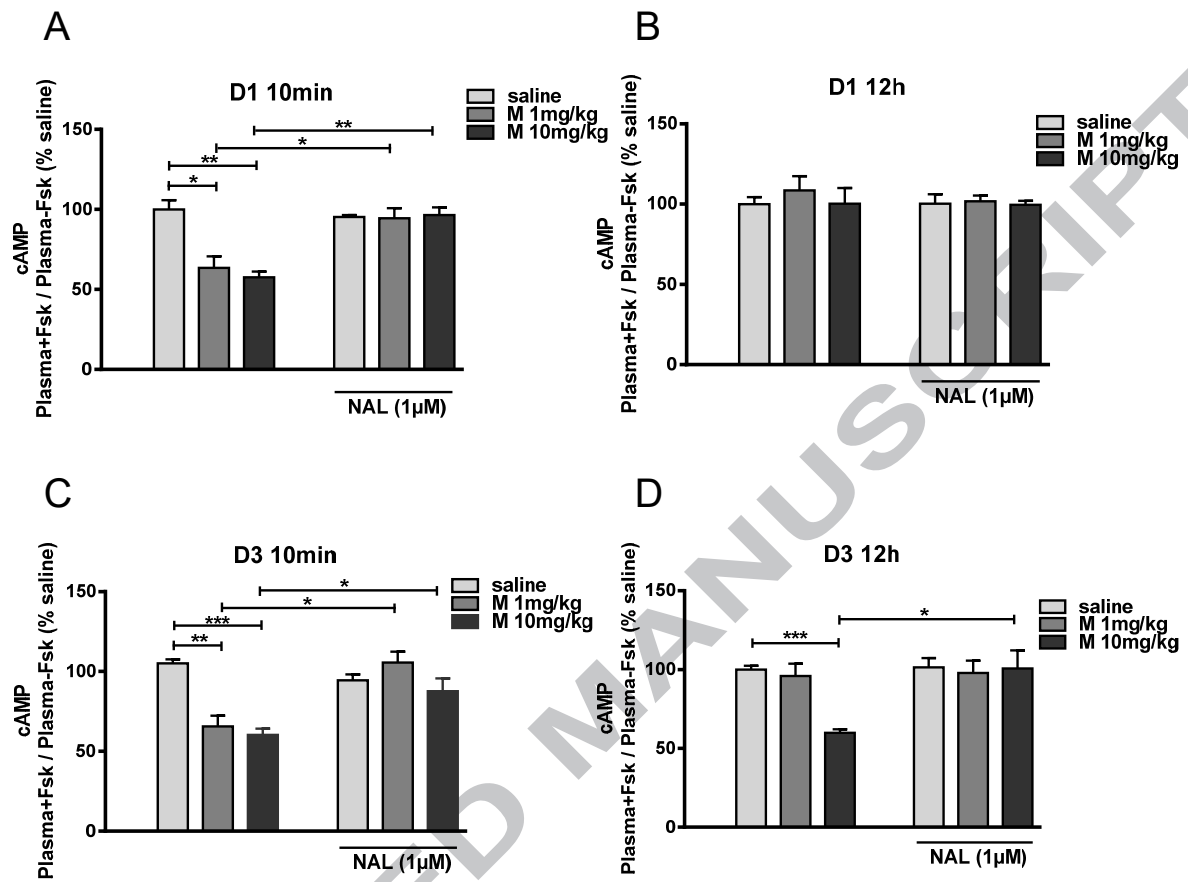
Figure 7

A

B

Figure 8

Figure 9

A

LPS

B

TNFα

C

LPS + LPS-RS

D

TNFα + LPS-RS

Figure 10

A

B

C

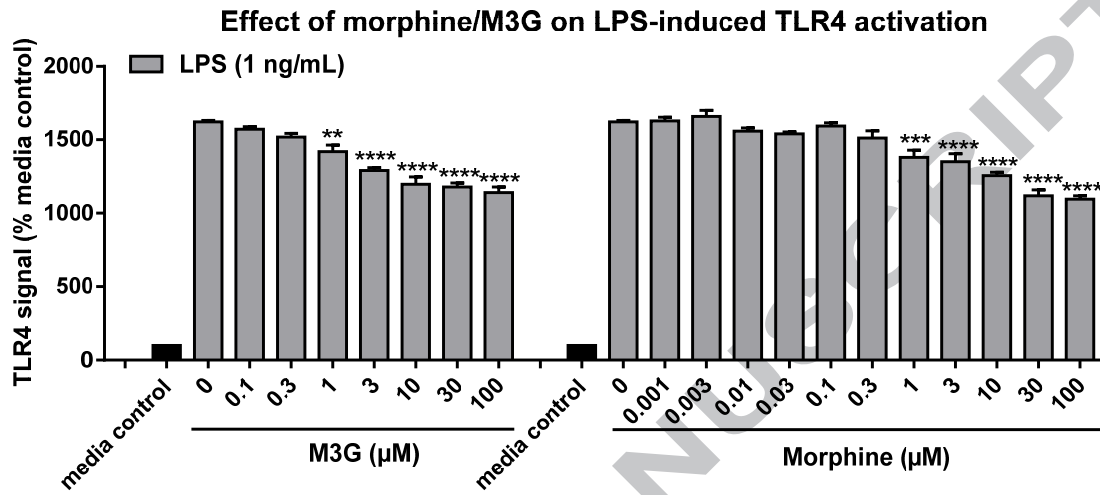
D
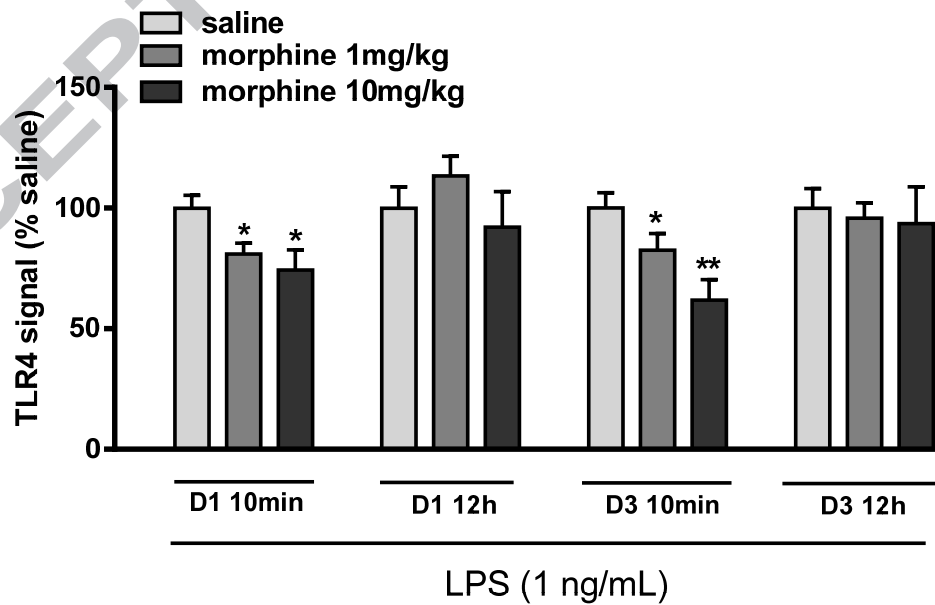
Figure 11

A

Effect of morphine/M3G on LPS-induced TLR4 activation
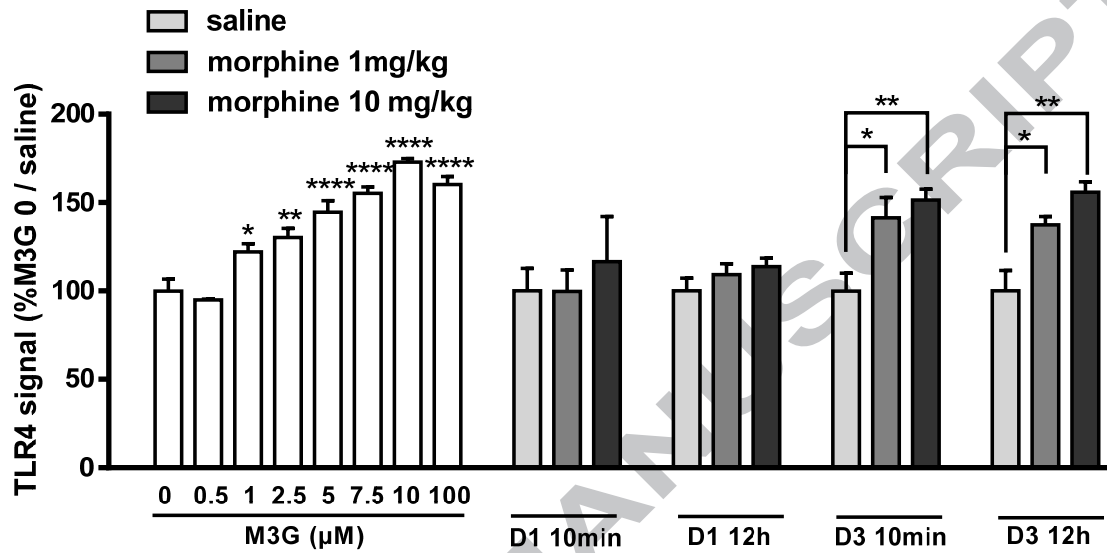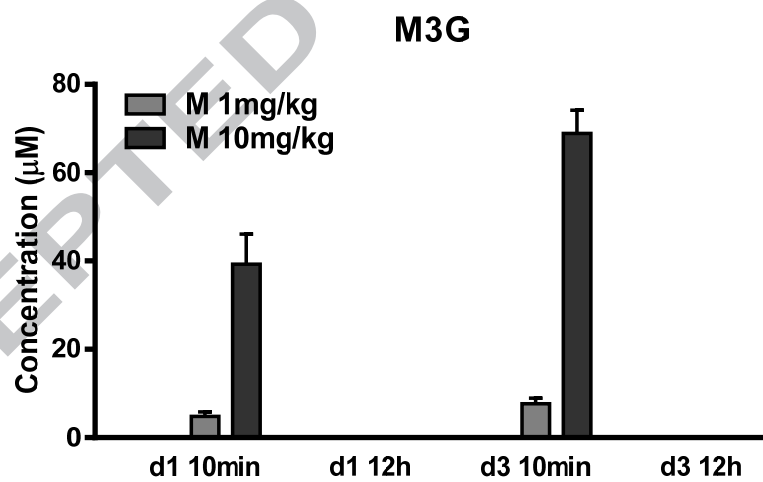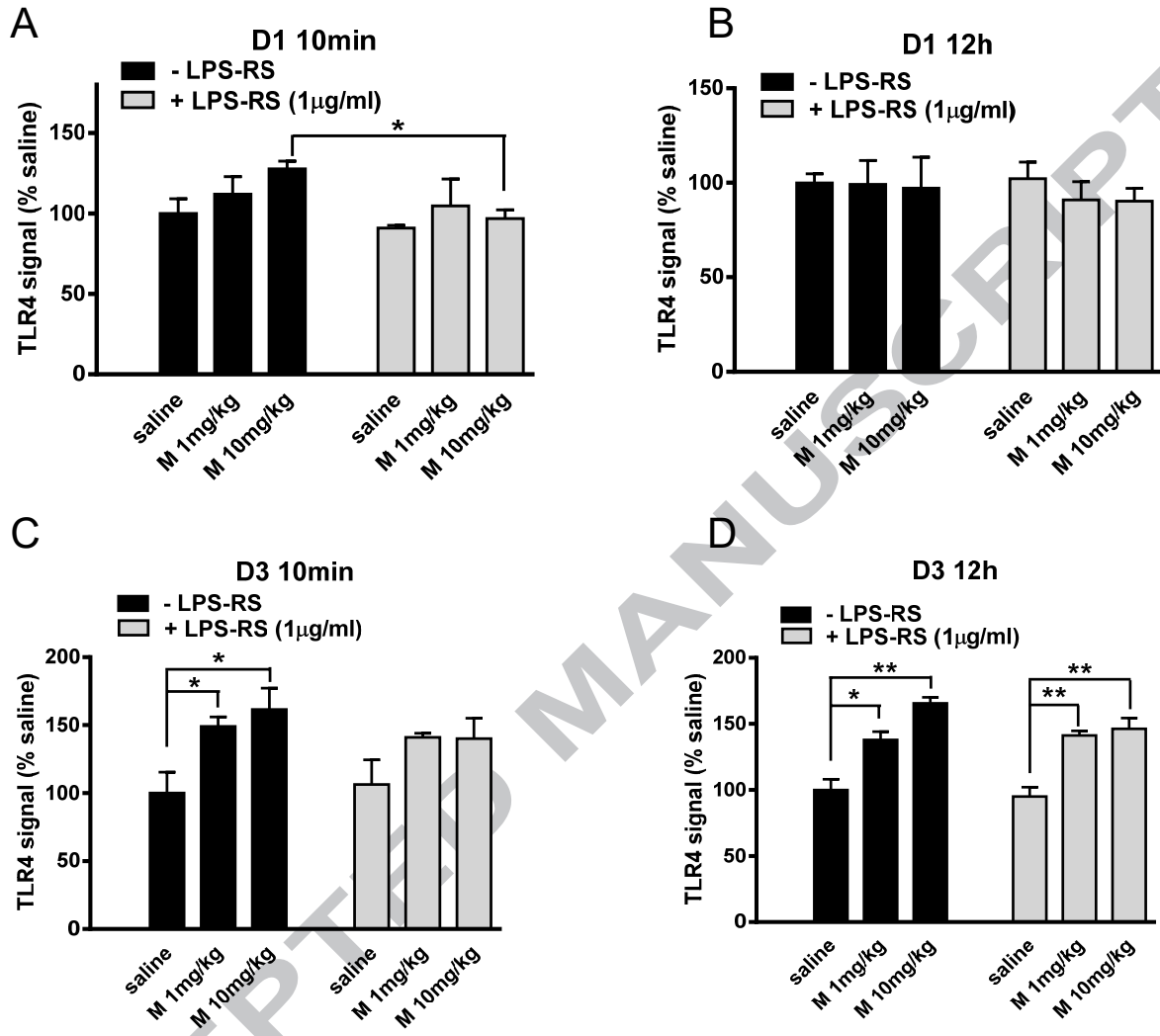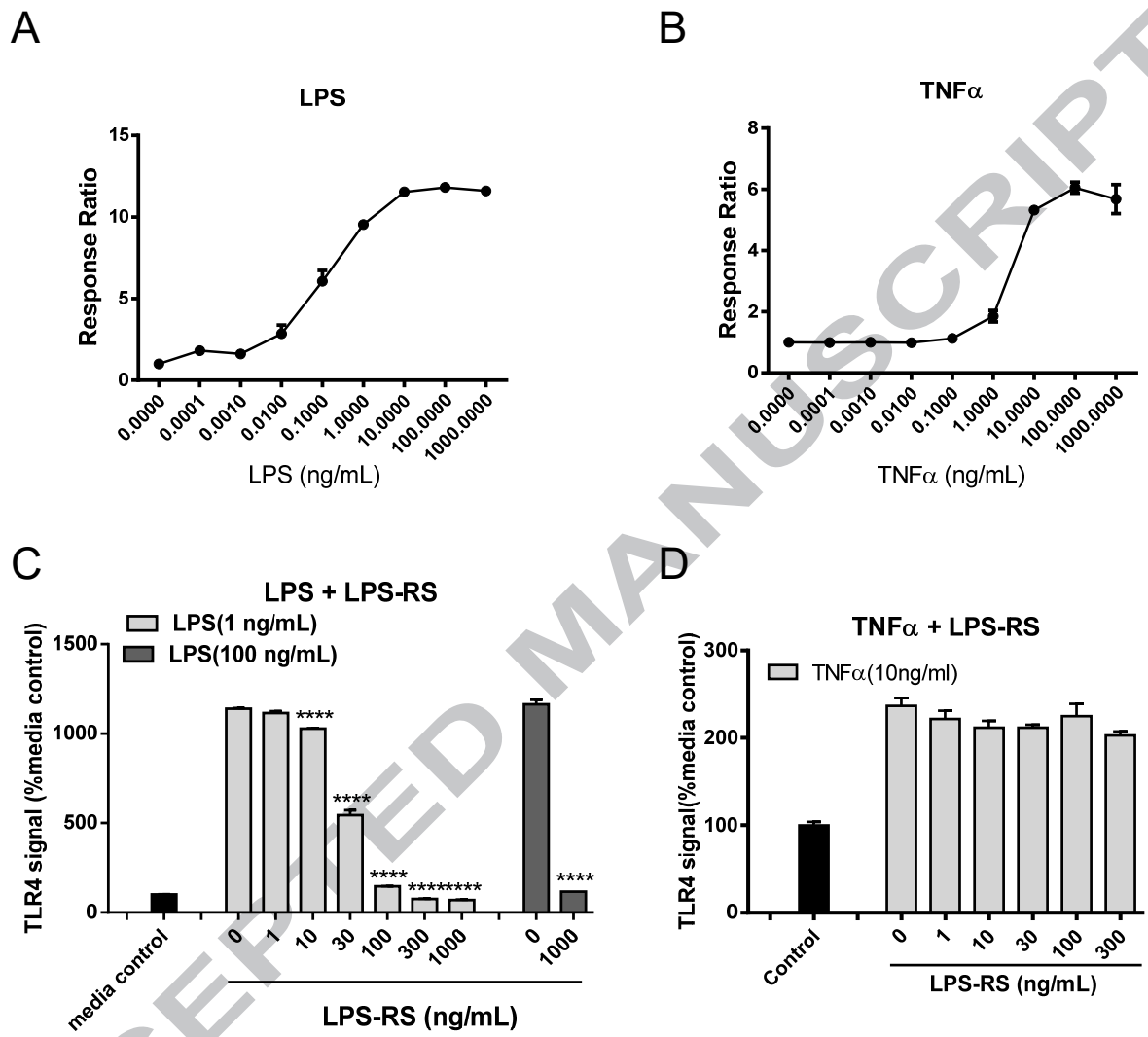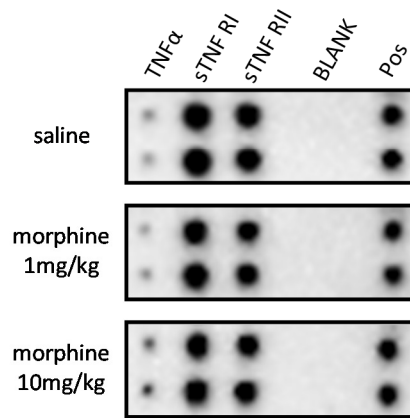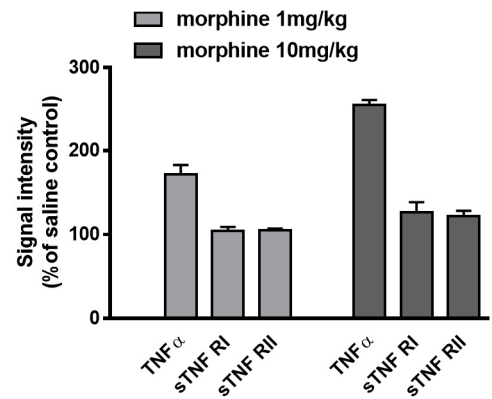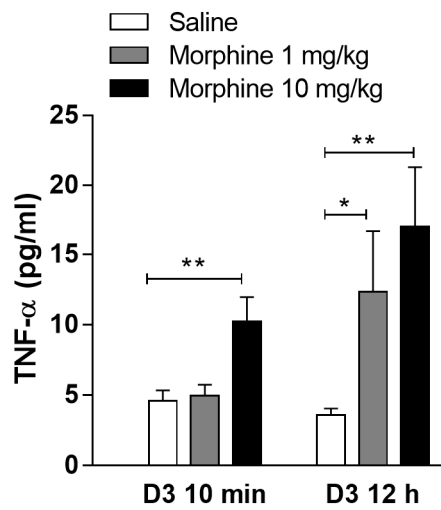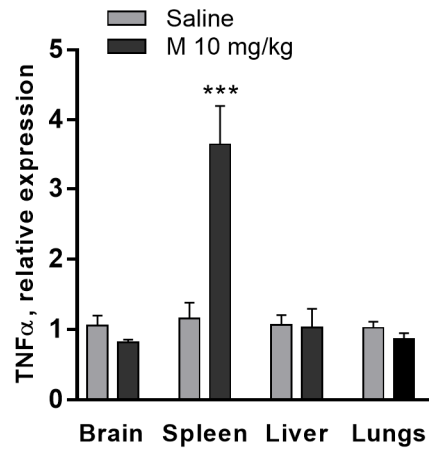
B

STUDY HIGHLIGHTS

• Both μ-opioid receptor and TLR4 can be activated by opioids or their metabolites

• Cell based assays can be used to quantify receptor agonism in biological samples

• Morphine administration results in circulating MOR and TLR4 agonism

• IP injections of morphine to mice every 12 h lead to intermittent MOR activation

• This mode of morphine administration results in TLR4-independent NF-κB activation