

Supplementary Information

PNA-based microRNA inhibitors elicit anti-inflammatory effects in microglia cells

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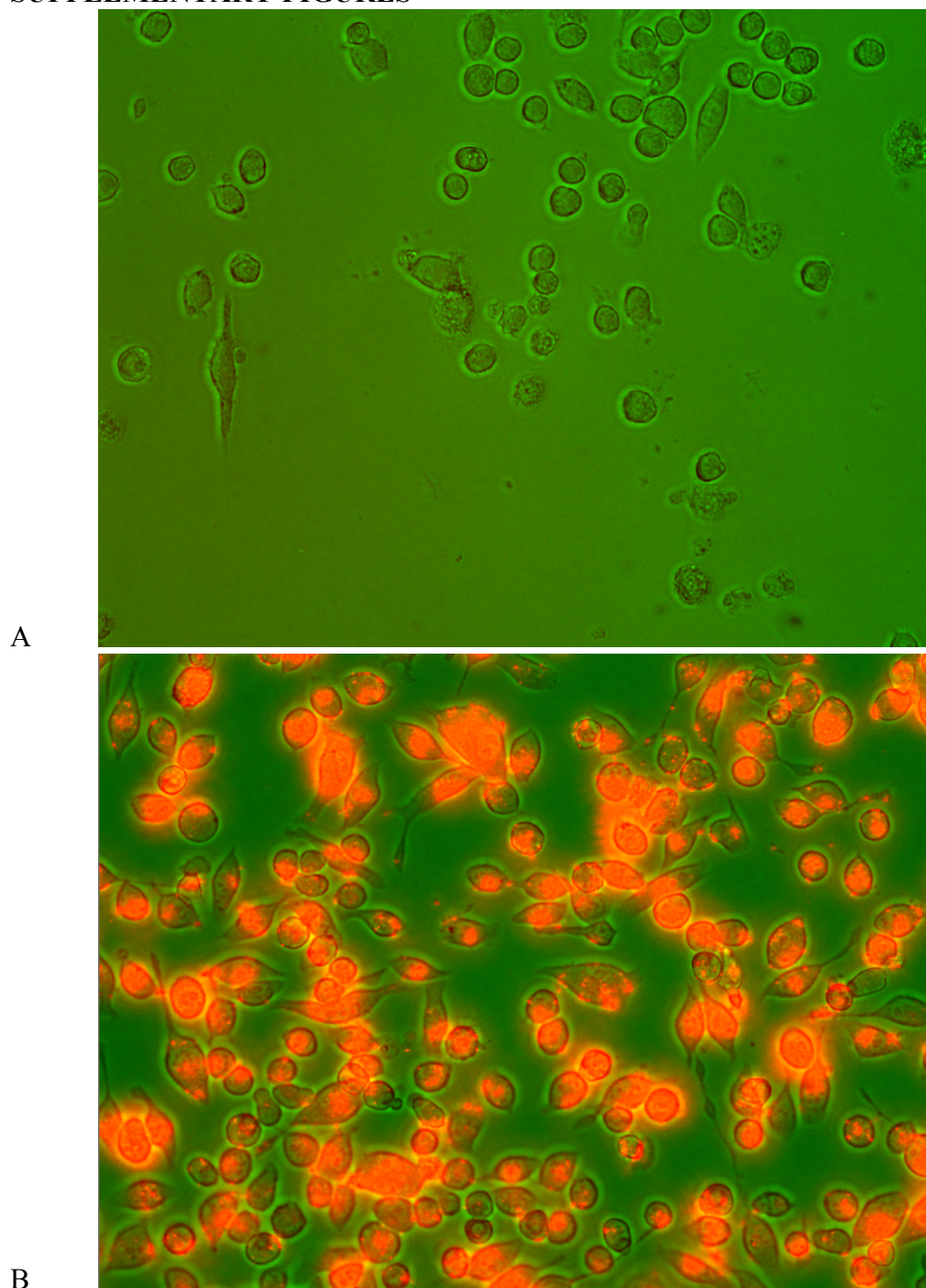


Fig. S1. Transfection of BV-2 microglia cells with high efficiency. Green denotes white light image and red represents the fluorescent signal of the transfectant. Both images were taken at the same time and both camera and image settings were unchanged. A. Untransfected cells showing negligible auto fluorescence of BV-2 cells. B. Transfected cells with fluorescent label showing high transfection efficiency.

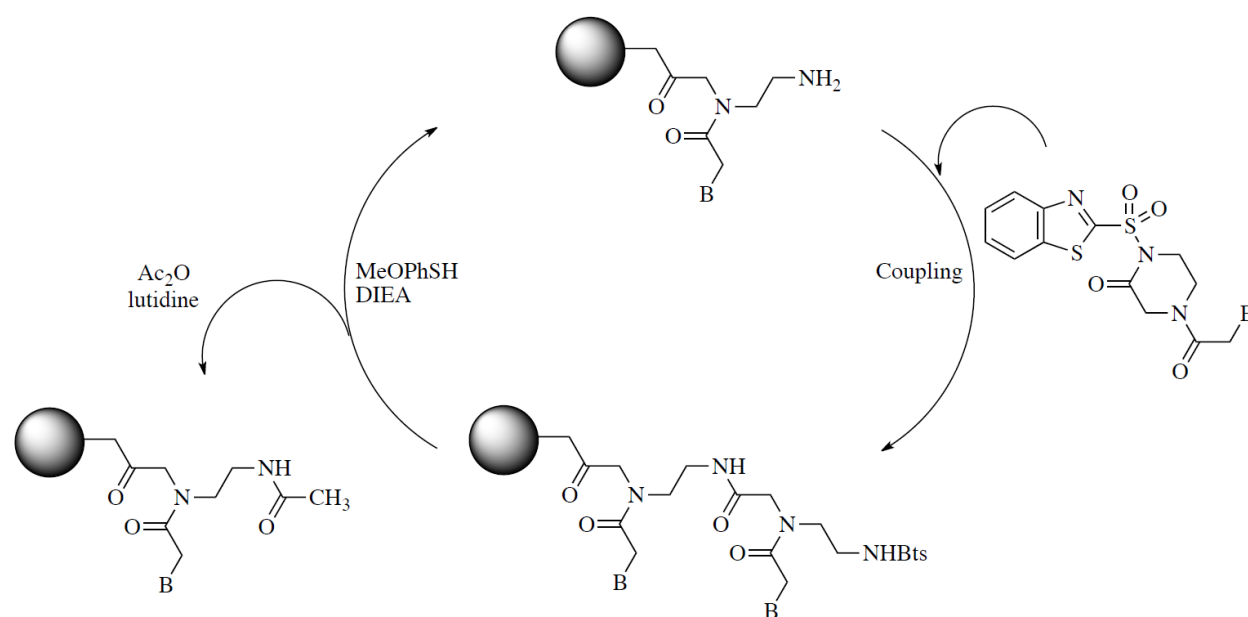


Fig. S2. Scheme for the production of the PNA polymer. The polymer is produced using an established solid state synthesis protocol. The Bts protected monomer results in an activated piperazinone which can be readily attacked by nucleophiles. This monomer readily reacts with the primary amine of the PNA polymer adding the next base. Unreacted polymers are capped with Acetic Anhydride and lutidine in order to take no further part in the polymerisation reaction. Subsequently, Reacted polymers are deprotected using 4-methoxybenzenethiol and N,N-diisopropylethylamine (DIEA) in dimethylformamide (DMF). This cycle can be repeated for the addition of the next PNA monomer.

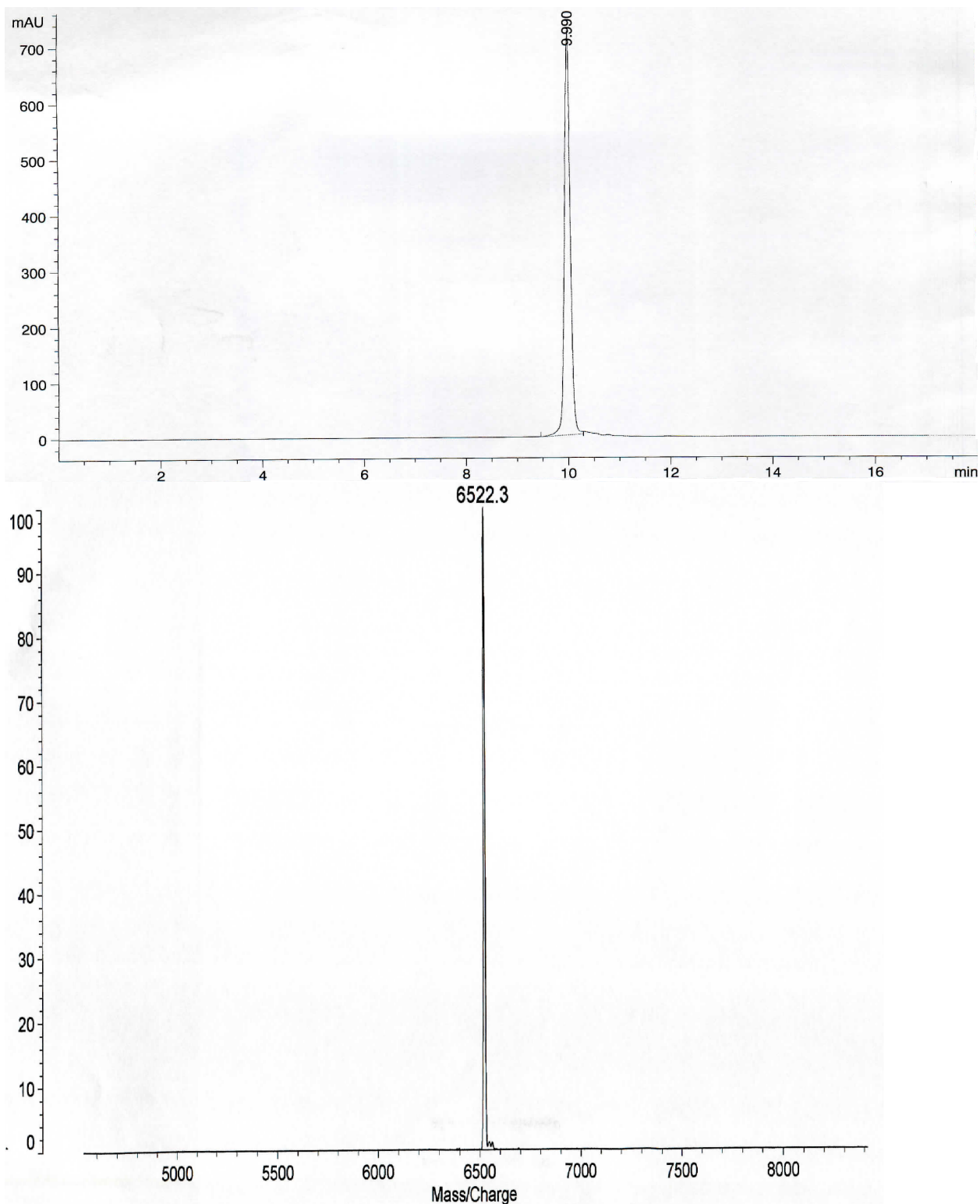


Fig. S3. Representative Purification by HPLC and characterisation by MALDI-MS of the PNA inhibitor. (Top) The HPLC purification spectrum of CU-PNA-221 at 60°C showing a >95% purity. (Bottom) The MALDI-MS spectrum of CU-PNA-221 demonstrating high purity at the predicted mass (calculated mass: 6521.2Da).

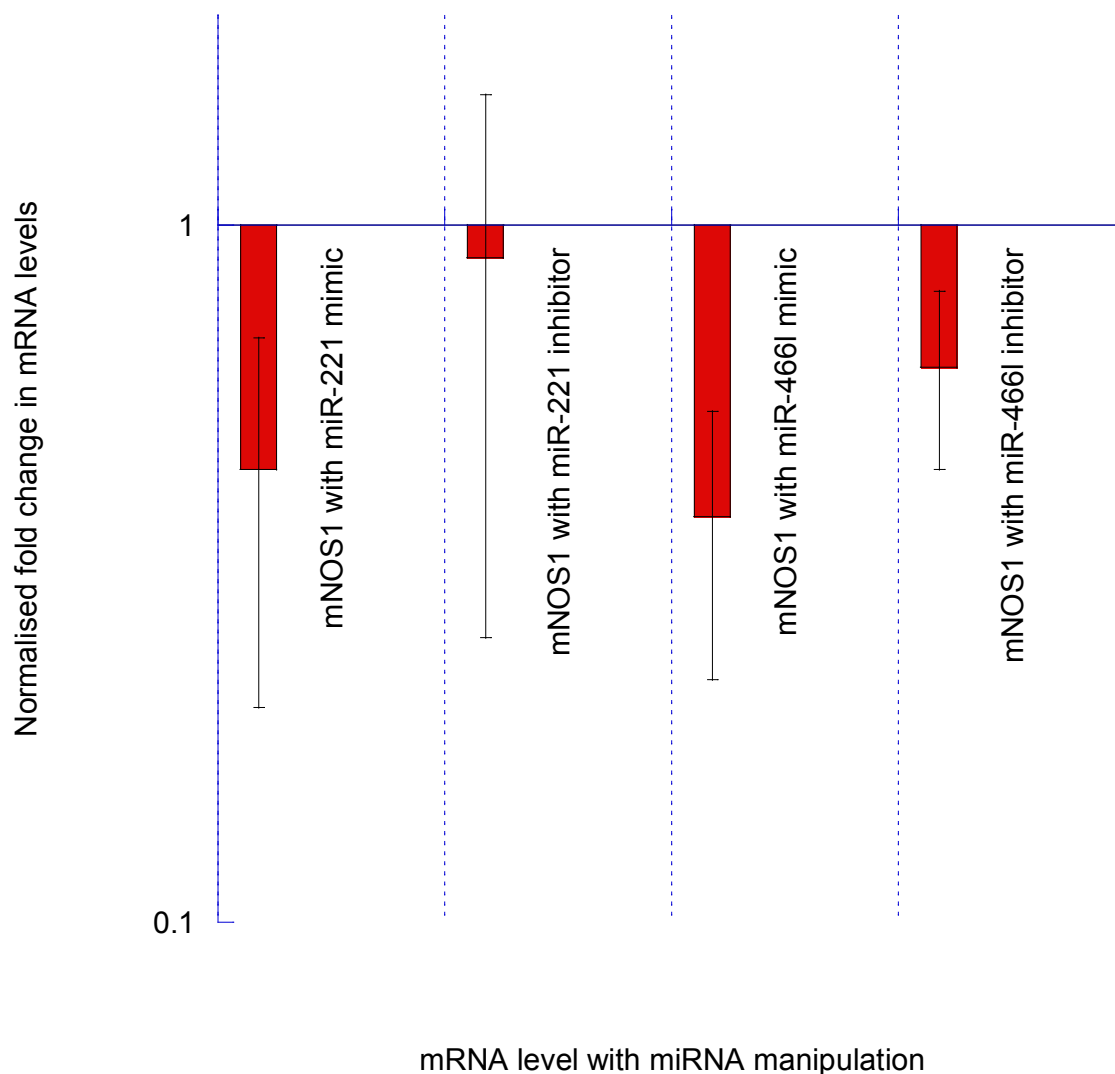


Fig. S4. Changes in mRNA levels of nNOS (NOS1) in BV-2 cells with modulation of miR-221-3p and miR-466l-3p upon LPS stimulation as determined by qPCR. The regulation of nNOS mRNA upon LPS stimulation in BV-2 cells transfected with miR-221-3p or miR-466l-3p mimics and inhibitors. These signals are normalised to the level at time 0 hours and show the signal for 2 hours post LPS stimulation. The levels of nNOS mRNA shows no statistically significant variation upon miRNA modulation.

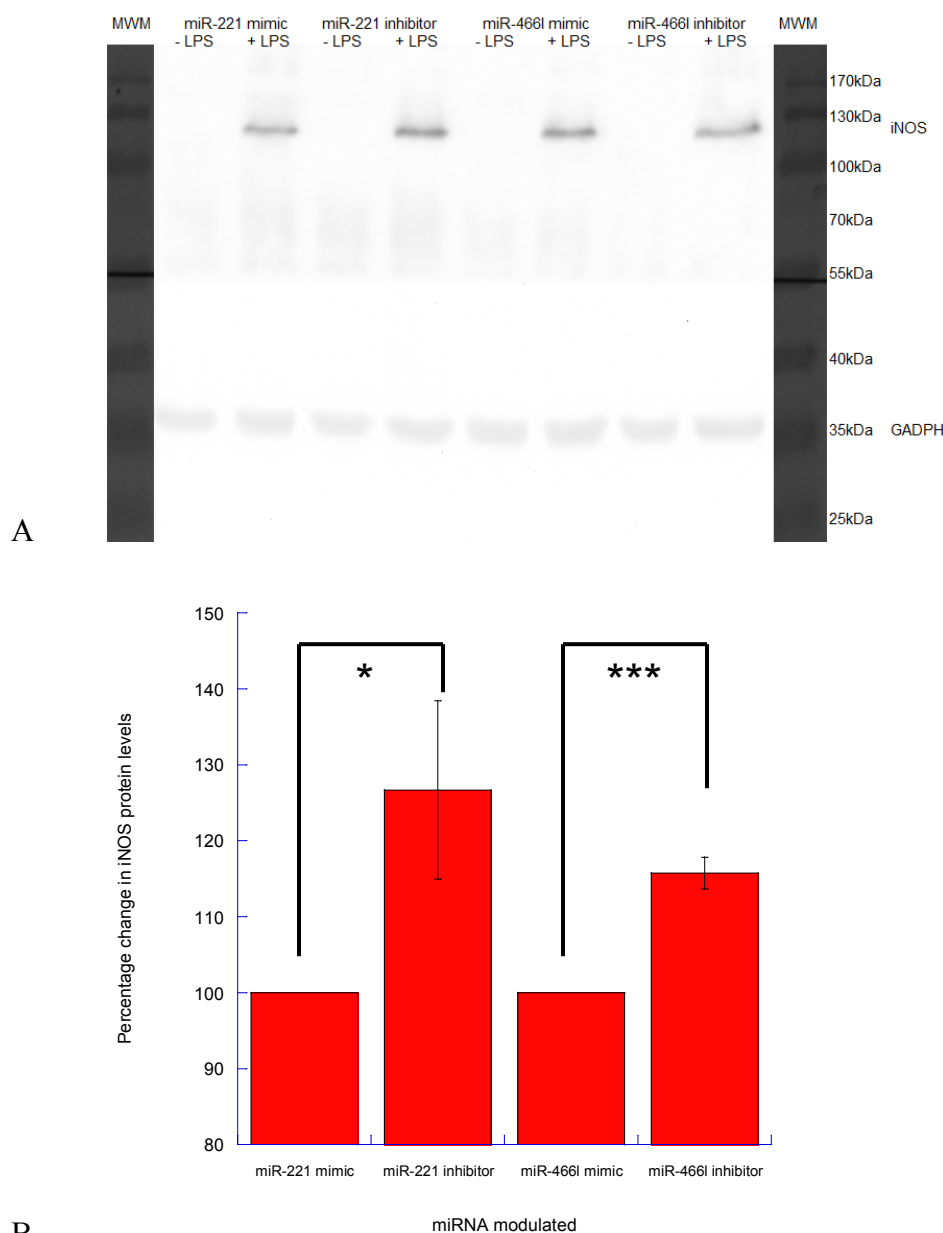


Fig. S5. Changes in iNOS protein expression levels upon miRNA modulation. The Stimulation of BV-2 cells with LPS (24 hours) results in the up regulation of iNOS. The increase in iNOS expression has been shown to be modified by miRNA modulation. A. Chemi-luminescent western blot of BV-2 cells probing for iNOS (top) and GADPH (bottom) flanked by white light image of molecular weight markers present on the nitrocellulose membrane. The lanes are shown with and without LPS stimulation showing iNOS only present upon LPS stimulation. GADPH loading control shows no significant variation. B. Quantification of iNOS levels of miRNA inhibitors normalised to values from miRNA mimics. These data demonstrate an elevated expression of iNOS upon inhibition of both miR-221-3p (p-value 0.017) and miR-466l-3p (p-value 0.0002).

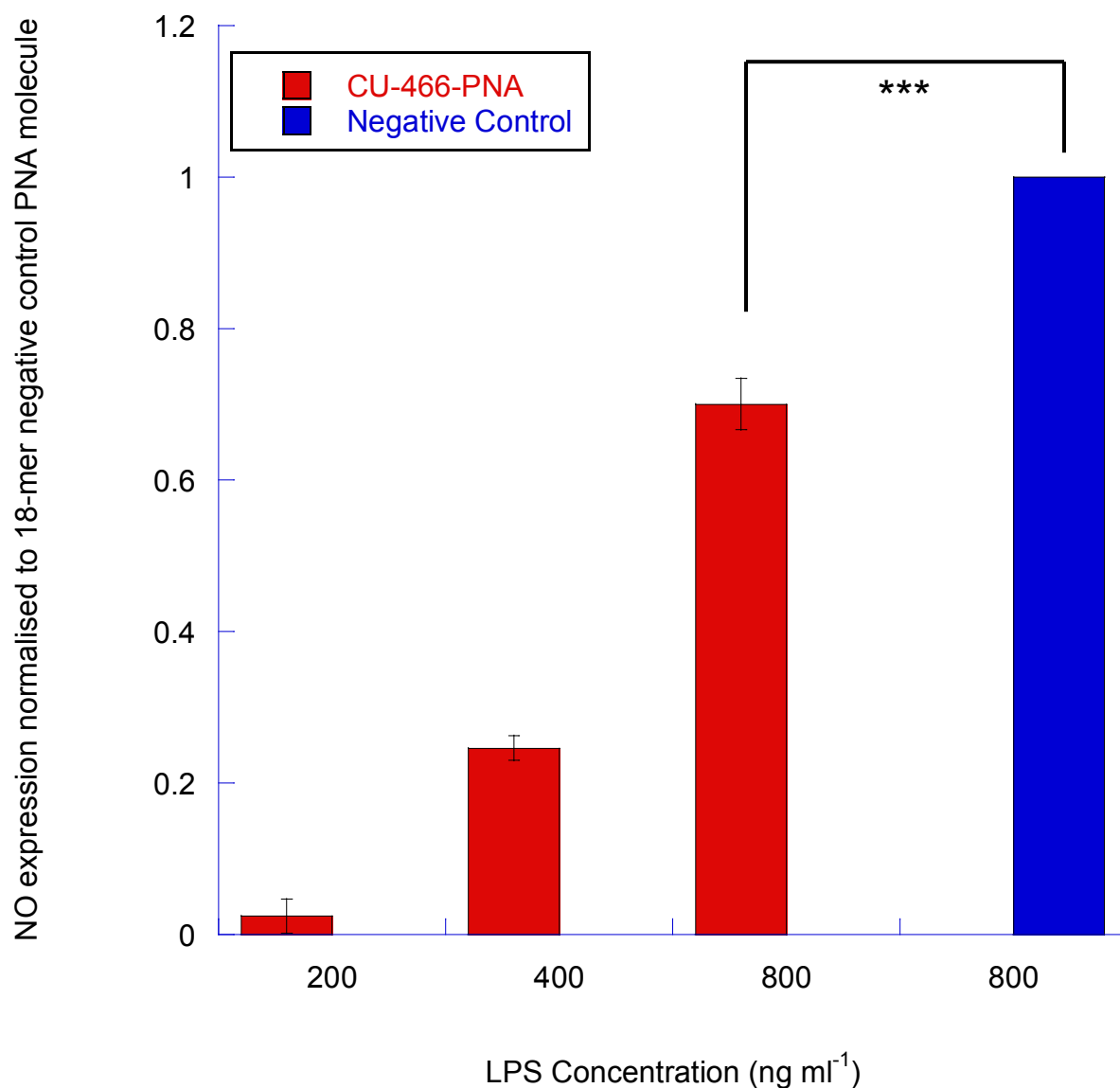


Fig. S6. NO release upon CU-466-PNA treatment compared to longer negative control sequence. The change in NO release levels from BV-2 cells under LPS stimulation (200, 400 and 800 ng ml⁻¹) with CU-466-PNA and NC3 (the longer negative control sequence). These signals have the signals from cells treated with no LPS deducted and normalised to the negative control after a 24 hour incubation. P-values represented as follows * <0.025 , ** <0.010 and, *** <0.005 .

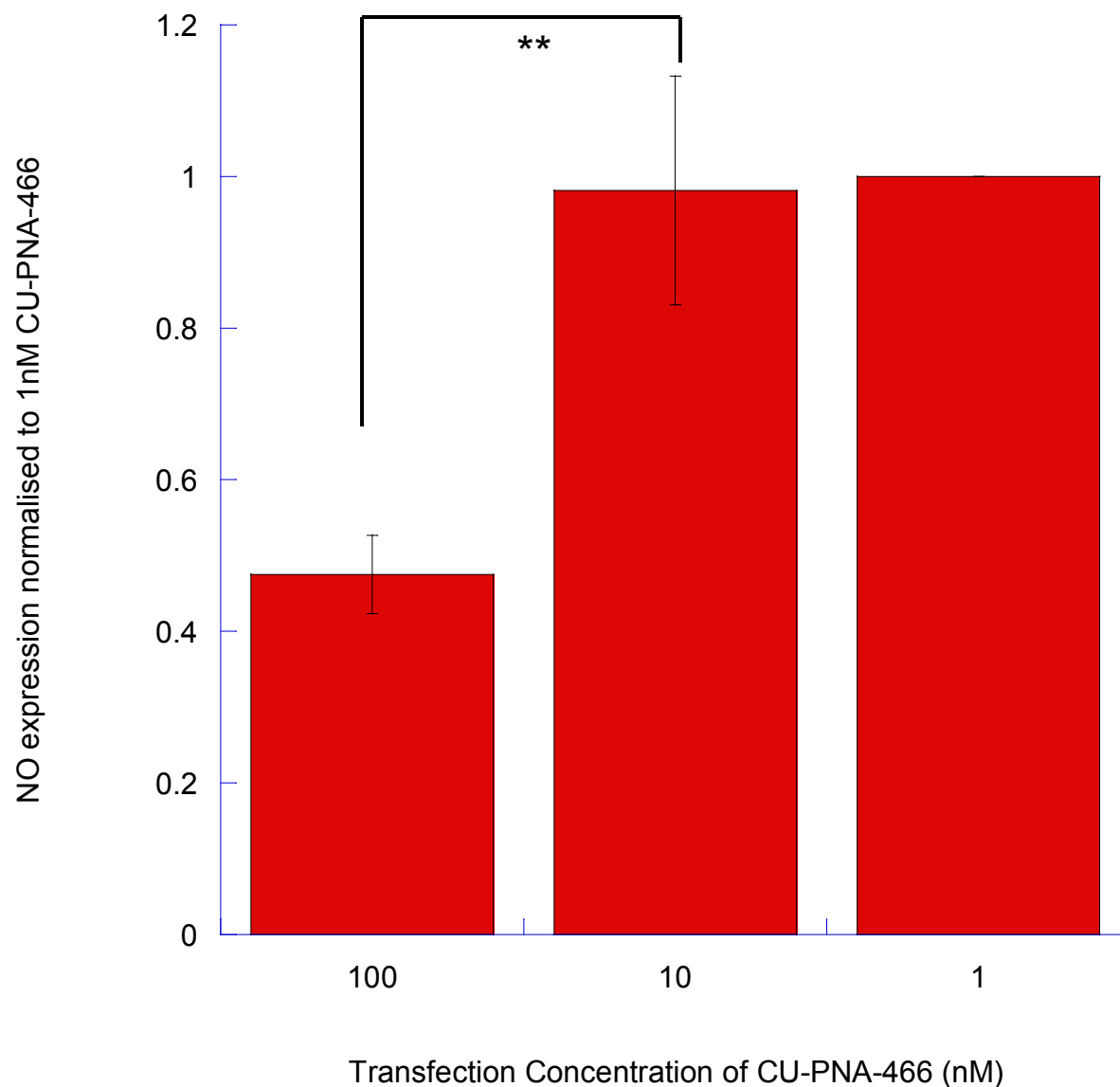
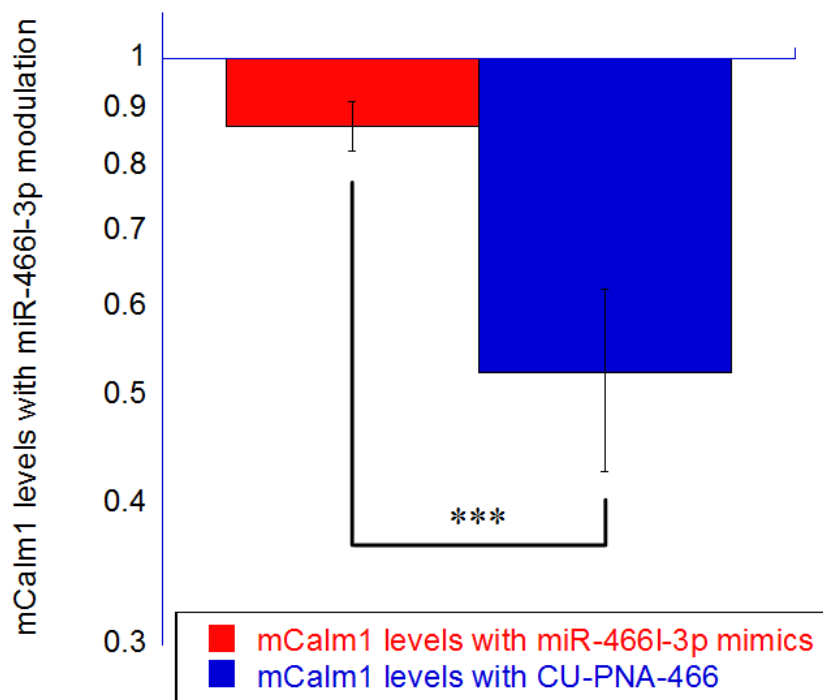
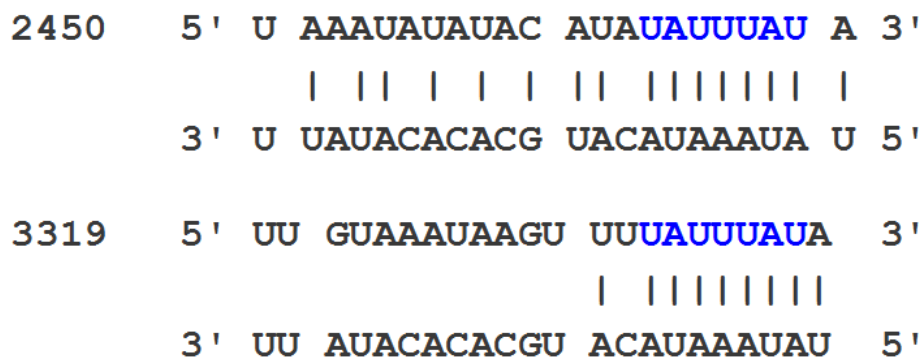


Fig. S7. The concentration dependence of CU-466-PNA upon NO expression. BV-2 cells transfected with 100, 10 or 1nM concentration of CU-466-PNA and treated with 800ng ml⁻¹ LPS for 24 hours. The NO expression was determined and the signals were normalised to values for 1nM CU-466-PNA. P-values represented as follows *<0.025, **<0.010 and, ***<0.005.

A miR-466l sites within calmodulin UTR



B

Fig. S8 Modulation of calmodulin in BV-2 cells by miR-466l-3p upon LPS stimulation. (A) Potential interaction sites for miR466l-3p within the 3'-UTR region of murine calmodulin. The seed sequences of miR-466l-3p in the 3'UTR region of calmodulin are shown in blue. (B) Real time qPCR analysis of calmodulin mRNA in BV-2 cells 12 hours after an LPS challenge. These signals are normalised to the level at time 0 hours and show values for miR-466l-3p mimics and inhibitors. P-value represented as follows ***<0.005.

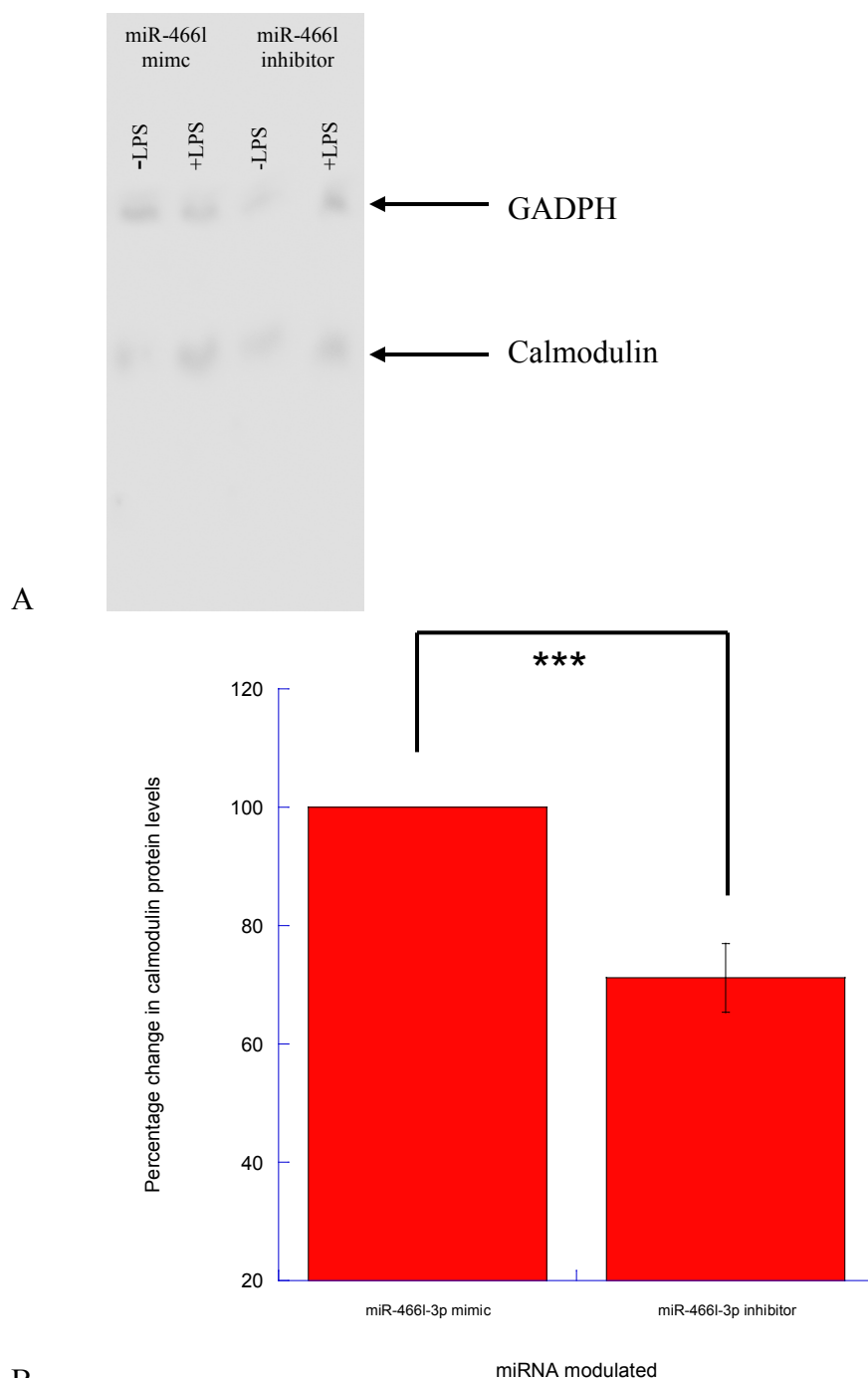


Fig. S9. Analysis of changes in calmodulin protein levels upon miRNA modulation. Cells were transfected miRNA mimics or inhibitors and treated with LPS for 24 hours. Signal for calmodulin protein was normalised to that of GADPH. A. Chemi-luminescent western blot of BV-2 cells probing for GADPH (top) and calmodulin (bottom). GADPH loading control shows some variation in loading level, therefore signals are normalised to GADPH. B. The calmodulin protein level showed an $\sim 28.9\% \pm 5.8\%$ decrease with 466l inhibitors compared to miR-466l mimics. Differences in protein levels are statistically significant (p-value 0.001).

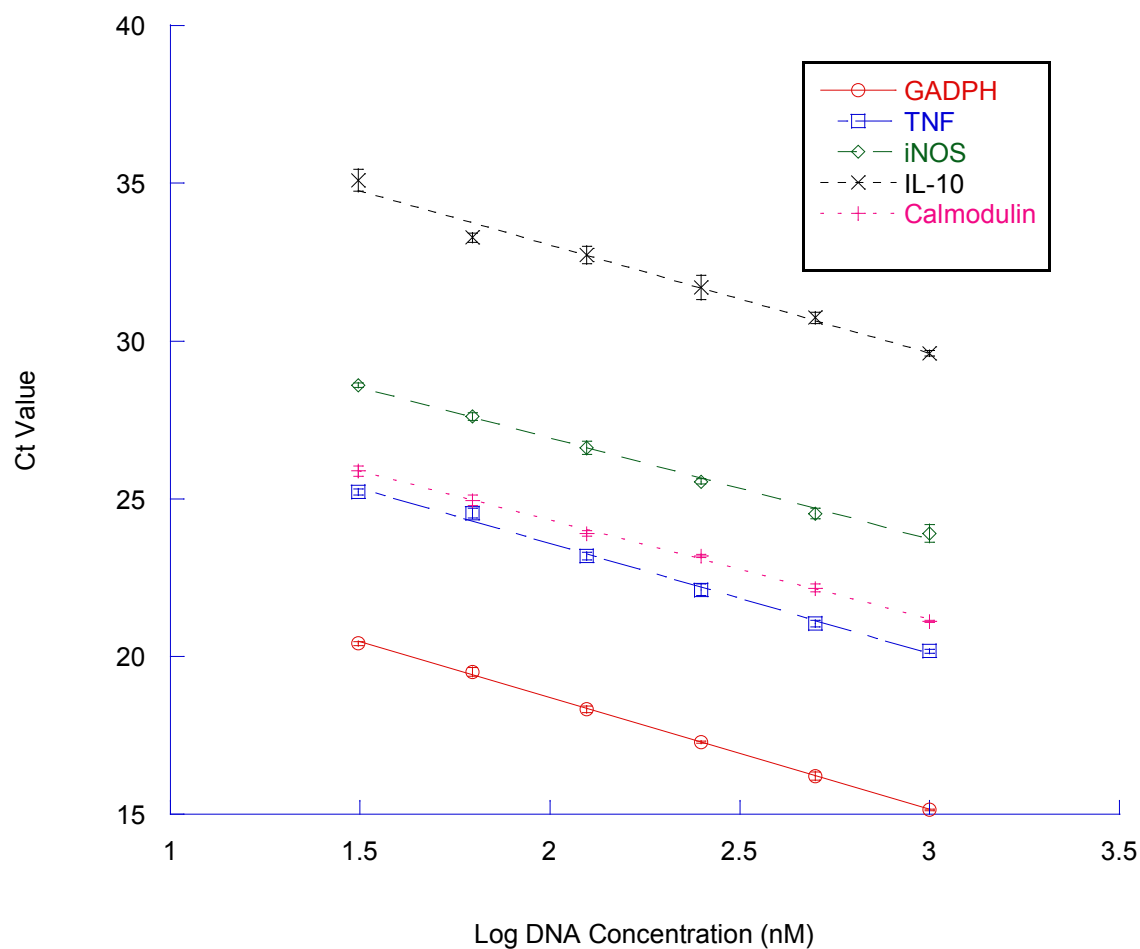


Fig. S10. Efficiencies of primers used for qPCR as determined by cDNA dilution series. Ct values of primers measured against a cDNA dilution series. The efficiencies of primers used to quantify mRNA levels of GADPH, TNF, iNOS, IL-10, and Calmodulin. All efficiencies were determined to be between 90 and 110% with an R-values greater than 0.99.

SUPPLEMENTARY METHODS

Cell handling and transfections - BV-2 cells were a kind gift from Linda R. Watkins lab and stored at 2×10^6 cells ml^{-1} at 143K. Cells were grown in 10cm Petri dishes in RPMI media (10% FBS, Pen-strep, and L-Glutamine). Transfection with PNA miRNA inhibitors (PANAgene) and miRIDIAN miRNA mimics (Dharmacon) were performed by the same method. PNA inhibitors were purified by HPLC and verified by MALDI mass spectrometry (for representative HPLC and MALDI spectra, see *Supplementary Fig. S3*). A longer negative control sequence was also used NC3 (AACAGTGTACGTACGAAC). Cells were grown to approximately 80% confluency and transfections were performed using DMR1E-C (Invitrogen) (100nM transefectant mixed in a 1:1 (w/w) ratio) suspended in unsupplemented opti-MEM serum free media overnight. Transfected cells were washed and re-plated and allowed to grow overnight in RPMI media (10% FBS, Pen-strep, and L-Glutamine).

mRNA quantification - Samples transfected with a single transfectant were grown in a 6-well plate format in triplicate and seeded at 1×10^6 cells well^{-1} . After 24 hours supplemented growth medium was removed and replaced with unsupplemented RPMI + 400ng ml^{-1} LPS (InvivoGen) and time-points were taken at 0, 2, 4, 6, 9, and 12 hours after addition. Total cellular RNA was extracted using the RNeasy mini kit (QIAGEN) using standard protocols with DNase treatment (QIAGEN). cDNA was immediately transcribed from extracted RNA using the iScript Reverse Transcription supermix (Bio-Rad) prepared with 500ng RNA per 20 μl reaction using standard conditions as determined by nano-drop quantification ND-1000 (Thermo Fisher). cDNA was used as quickly as possible once generated and real time qPCR analysis was performed using 1 μg of cDNA with SsoAdvanced SYBR Green Supermix (Bio-Rad), specific information is available from Bio-Rad. Primers for IL-10 (PPM03017B), TNF (PPM03113F), neuronal NOS (PPM05114E), inducible NOS (PPM02928B), GAPDH (PPM02946E), and Calm1 (PPM04591E) were used to determine relative levels of mRNA in the total RNA extract (SABiosciences) and have been shown to be specific and generate a single amplicon by melting curve analysis, specific information is available from SABiosciences. All real time qPCR analysis was performed in a 96 well plate (Bio-Rad) format on the CFX96 (Bio-Rad). After a 10minute 95°C melting step samples were run at 95°C melting step for 15seconds, 55°C annealing step for 40seconds, 70°C elongation step for 15seconds for 40 cycles. All amplified samples were analysed for primer specificity by melting curve analysis, with no significant amplicon contamination determined. Ct values were obtained using CFX manager 2.0 (Bio-Rad) and all fell within the limit of detection. Samples were confirmed as presenting non-detectable levels of DNA contamination using samples prepared with a no RT negative control (Bio-Rad). All primers used in this work demonstrated an efficiency between 90 and 110% with fitting R values greater than 0.99 (supplementary data, Fig. S10). Data was deconvoluted using the $\Delta\Delta\text{Ct}$ method with GAPDH as a housekeeping gene, normalised to time at 0 hours and errors derived from the standard deviation of three biologically separate replicates.

Protein analysis - BV-2 cells were transfected as above with miRNA mimics or inhibitors and seeded in a 6 well plate at 6×10^5 cells well^{-1} in supplemented RPMI and grown for 24 hours. Cells were treated with either 0 or 800 ng ml^{-1} LPS in unsupplemented RPMI. After 24hours stimulated cells were harvested and lysed in PBS (+1% Triton X-100 (Sigma), protease inhibitor tablet (Roche)) and run on a 10% SDS-PAGE gel. Immunoblotting was performed in standard

conditions (100mA for 1hour). Membrane was split at the 55kDa band and blocked in 5% non-fat milk (Safeway) for three hours then the upper half was probed for iNOS (Abcam ab49999) and the lower half probed for GADPH (Sigma G9545) in 1% milk for 2 hours. Membrane was washed extensively in TBST and incubated with secondary for 2hours, anti-mouse (ThermoFisher 31430) for iNOS, anti-rabbit (Abcam ab6112) for GADPH. Membranes were extensively washed and visualised by ECL with SuperSignal West pico (Thermo Fisher). Immunoblots for calmodulin were performed in the same manner with the following exceptions. the membrane was split at the 25kDa band and the upper section probed for GADPH as above. The lower half was probed for calmodulin (Abcam ab45689) as the primary and anti-rabbit (Abcam ab6112) as the secondary antibodies. Signals for calmodulin was normalised to those for GADPH.

Nitric Oxide assays - Samples transfected with a single transfection reagent were seeded onto a 96 well plate at 80k cells well⁻¹ in supplemented RPMI and grown for 24 hours. Growth medium was removed and replaced with unsupplemented RPMI with 0, 200, 400, and 800 ng ml⁻¹ LPS with four replicates on each plate. After 24 hours 100µl of growth medium was removed and analysed using the standard protocols for the nitric oxide assay using 2,3-diaminonaphthalene (36). Data was deconvoluted using Excel and signal recorded for cells with no LPS were deducted from treated samples. Final signals are the averages across the three separate plates with a total of 12 replicates and error bars are presented as standard deviation.

Statistical analysis - Data is presented as the mean average of three independent measurements with error represented as the standard deviation. Statistical significance was determined by students t-test, p-values are represented in the figures by * < 0.025, ** < 0.010, and *** < 0.005. All graphs presented in this work were generated using KaleidaGraph 4.0 (SynergySoftware).