Supplementary Methods

Animals

Studies were performed on male and female 8-10 week old *Cox2* luciferase knock-in reporter mice ($Cox2^{fLuc/+}$) (1). In these mice, luciferase activity indicates transcriptional activity from the endogenous *Cox2* promoter, which is a surrogate for *Cox2* gene expression. However, for the purpose of simplification this is referred to as 'COX-2 expression' within the body of the manuscript. Studies were also performed on transgenic luciferase reporter mice for activity of NFAT (NFAT-luc) (2) or NFκB (NFκB-RE-luc) (3) in which luciferase activity reflects activation of NFAT or NFκB response elements, as well as mice with germline knockout of COX-1 ($Cox1^{-/-}$) (4) or wild-type C57Bl/6 mice (Charles River, UK / Taconic, USA) raised under normal or germ-free conditions (5) (Farncombe Gnotobiotic Unit, McMaster University, Canada). Procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were subject to local ethical review and approval by the UCLA Animal Research Committee (Protocol No: 1999-066-32; $Cox2^{fLuc/+}$ experiments), Animal Care Committee of the Faculty of Health Sciences at McMaster University (NFκB-RE-luc and germ-free experiments), Malmö/Lund Animal Care and Use Committee (Permit Number: M29-12; NFAT-luc experiments) or the Imperial College Ethical Review Panel (PPL 70/7013; all other experiments).

Where indicated mice were treated with cyclosporin A (20mg/kg; once daily s.c.; Novartis, Switzerland; vehicle: 1:2:27 cremaphor-EL:ethanol:saline), BMS-345541 (10mg/kg; once daily i.v.; Sigma, UK; vehicle: 1:20 DMSO:saline), dexamethasone (3mg/kg; once daily i.p.; Organon, UK; vehicle: saline) or appropriate vehicle for 4 days. In some cases, an inflammatory response was induced by administration of LPS (10mg/kg; from E. coli serotype 055:B5; Sigma, UK; vehicle: saline), 4 hours prior to euthanasia and tissue collection. At the end of each experiment, animals were euthanized by an overdose of isoflurane. Where tissue was collected for gene expression or activity measurements the kidney was sub-divided into 'renal cortex'– defined as the outer 2mm layer of the kidney and 'renal medulla' – defined as the region within this comprising medulla and pelvis.

Bioluminescent imaging

Bioluminescent imaging of luciferase activity in tissue from luciferase reporter strains was performed essentially as we have previously described (6). Immediately post-mortem, tissues were rapidly dissected, cleaned of adherent fat/connective tissue and arranged on

petri dishes. Tissues were bathed in D-luciferin substrate solution (15mg/ml in saline; PerkinElmer, UK) and bioluminescent emission recorded over 30 secs (NFAT-luc, NFκB-RE-luc) or 3 mins ($Cox2^{fLuc/+}$) using an IVIS imaging system (Xenogen, USA). Image data was analyzed using Living Image software (Xenogen, USA) and quantified as the peak photon emission/pixel detected from each tissue.

Quantitative PCR

Tissue gene expression was measured by quantitative reverse transcriptase PCR. Snap frozen tissue segments were homogenized using a ceramic bead homogenizer (Precellys, UK) in RLT buffer (Qiagen, UK) with 1:50 β-mercaptoethanol (Sigma, UK) and RNA extracted using a magnetic silica bead-based kit (Life Technologies, UK). RNA was converted to cDNA using SuperScript II reverse transcriptase (Life Technologies, UK) with oligo(dT) primers. In most cases, gene expression was measured with TaqMan gene expression assays (Life Technologies, UK) for Ptgs2 (COX-2 gene; probe ID: Mm00478374_m1), Nos2 (iNOS gene; probe ID: Mm00440502_m1), Ptgs1 (COX-1 gene; probe ID: xxx); 18S rRNA (probe ID: Mm03928990_g1) and Gapdh (probe ID: Mm99999915_g1) using qPCR master mix with ROX (Thermo Scientific, UK) and a 7500 Fast real-time PCR system (Applied Biosystems, USA). For other target genes, expression was measured using SYBR®Green master mix (Bio-Rad, UK) and primer pairs recognizing Tnc (Tenascin C; fwd: ACGGCTACCACAGAAGCTG, rev: ATGGCTGTTGTTGCTATGGCA), Nfatc1 (fwd: GACCCGGAGTTCGACTTCG, rev: TGACACTAGGGGACACATAACTG), Nfatc2 (fwd: TCATCCAACAACAGACTGCCC, rev: GGGAGGGAGGTCCTGAAAACT), Nfatc3 (fwd: GCTCGACTTCAAACTCGTCTT, rev: GATGTGGTAAGCCAAGGGATG), Nfatc4 (fwd: GAGCTGGAATTTAAGCTGGTGT, rev: CATGGAGGGGTATCCTCTGAG) or Nfat5 (fwd: CAGCGCCCAATAGTTGGCA, rev: TGCTGGTGAAAAATTGACTGGT). Data was analyzed using the comparative Ct method, with reference to housekeeping genes (18S and Gapdh).

PGE_2 synthesis bioassay

COX-2-dependent prostaglandin synthesis was measured, as we have done before (6), in tissue from $Cox1^{-/-}$ mice, such that all prostaglandin production is through the COX-2 isoform. Immediately post-mortem, tissues of interest were removed and divided into 2x2x2mm segments. Segments were placed in wells of a 96 well microtitre plate containing DMEM media (Sigma, UK) with A23187 calcium ionophore (50μM; Sigma; UK) and incubated for 30 mins at 37°C. Media was then removed of PGE_2 concentration measured by immunoassay (Cisbio, France). PGE_2 release was normalized to tissue wet weight.

Plasma cytokine and creatinine measurement

Blood was collected from the inferior vena cava into heparin (10U/ml final; Leo Laboratories, UK) from mice euthanized with isoflurane, and plasma separated by centrifugation (10000g, 2 mins). Levels of CXCL10 (R&D Systems, UK), interferon-γ and TNF-α (Meso Scale Discovery, USA) were measured by immunoassay. Levels of creatinine were measured by a commercial veterinary biochemistry service (IDEXX Laboratories, UK).

Statistics and data analysis

Data were analyzed using Prism 6.0 software (GraphPad software, USA) and are presented as mean ± standard error for 'n' number of animals. Data were compared by Student's unpaired t-test or one-way ANOVA with Dunnett's post-test as indicated in figure legends and differences considered significant where $p<0.05$.

Supplementary Figures

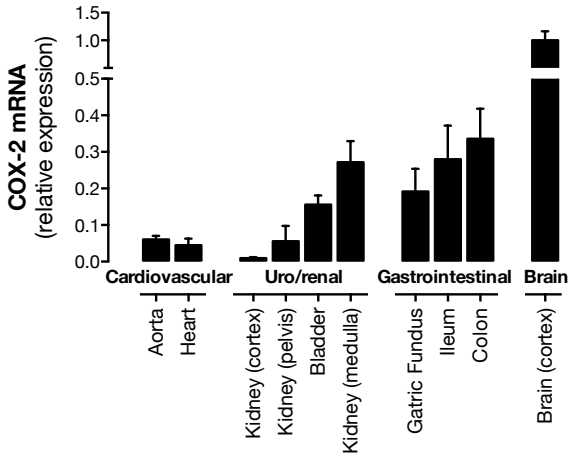

Figure S1: Tissue distribution of Cox2 mRNA expression. Quantification of basal expression of COX-2 mRNA in wild-type mice, measured by qPCR, demonstrates high expression in the renal medulla, bladder, gastrointestinal tract and brain but lower levels in the heart and aorta . Data are means ± SEM. n=5-18.

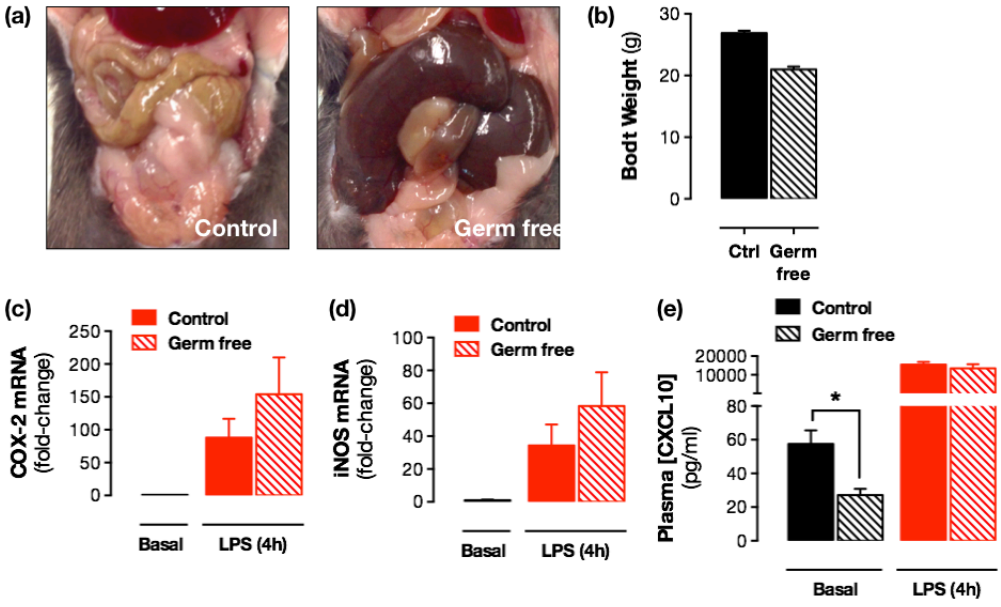

Figure S2: Phenotype of germ-free mice. Mice raised under germ-free conditions exhibited gastrointestinal abnormalities (a) and reduced body weight (b). Germ-free mice exhibited normal spleen cyclo-oxygenase (COX-2) (c) and iNOS (Nos2) induction (d) responses to LPS (10mg/kg; 4 hours; i.p.). Basal plasma levels of CXCL10 were reduced in germ-free mice (e), but when challenged with LPS, plasma CXCL10 levels were increased similarly in germ-free and to control animals. Data are means ± SEM. n=4-10. *, $p<0.05$ by unpaired t-test.

Tissue	Control	Germ Free	p-value
	COX-2 expression (fold change)		
Spleen	1.0 ± 0.2	5.4 ± 1.5	* 0.0086
Renal medulla	1.0 ± 0.3	0.8 ± 0.1	0.46
Large intestine	1.0 ± 0.3	1.3 ± 0.2	0.43
Brain	1.0 ± 0.2	2.6 ± 0.8	0.059
Small intestine	1.0 ± 0.2	3.9 ± 2.3	0.23
Thymus	1.0 ± 0.1	1.9 ± 0.2	* 0.0021
Stomach	1.0 ± 0.2	1.6 ± 0.4	0.19

Table S1: Constitutive and LPS-induced cyclo-oxygenase (COX)-2 mRNA expression in control and germ-free mice. In mice raised under germ-free conditions, constitutive COX-2 expression was detected by qPCR in all studied tissues. COX-2 expression levels were increased in the thymus and spleen of germ-free mice. Data are means ± SEM. n=12. *, $p<0.05$ by unpaired t-test.

Tissue	Control	BMS	CsA	BMS	CsA
	COX-2 luciferase activity (x10^5 photons/sec)			p-value (vs. control)	
Aorta	1.1 ± 0.1	0.9 ± 0.1	2.1 ± 0.4	0.88	0.052
Bladder	1.4 ± 0.3	1.5 ± 0.4	1.6 ± 0.5	0.99	0.96
Cerebral Cortex	5.8 ± 0.6	6.3 ± 0.5	9.2 ± 2.3	0.95	0.21
Heart	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.3	0.84	0.86
Cerebellum	0.8 ± 0.4	1.5 ± 1.0	0.6 ± 0.1	0.67	0.97
Hippocampus	3.0 ± 0.03	2.7 ± 0.8	3.4 ± 0.5	0.94	0.85
Large Intestine	14.9 ± 3.2	14.8 ± 3.2	13.1 ± 3.0	1.00	0.89
Liver	0.3 ± 0.0	0.3 ± 0.1	0.7 ± 0.3	1.00	0.23
Lung	3.5 ± 1.0	7.9 ± 4.2	7.2 ± 2.9	0.50	0.61
Renal cortex	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.92	0.14
Renal medulla	3.1 ± 0.6	2.3 ± 0.1	1.1 ± 0.3	0.31	* 0.011
Renal pelvis	4.6 ± 0.8	3.0 ± 0.6	1.9 ± 0.7	0.23	* 0.045
Skin	1.9 ± 0.3	2.4 ± 0.6	1.9 ± 0.5	0.66	1.00
Small Intestine	10.2 ± 1.4	9.8 ± 0.9	12.0 ± 1.6	0.97	0.52
Spleen	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.59	0.61
Stomach	11.5 ± 3.2	8.0 ± 1.3	11.2 ± 8.0	0.65	1.00
Thymus	2.6 ± 0.6	1.9 ± 0.6	5.7 ± 4.0	0.97	0.57

Table S2: Effect of NFAT and NFkB pathway inhibitors on luciferase activity driven from the endogenous *Cox2* gene. Constitutive COX-2 expression, measured using bioluminescent imaging of tissue from $Cox2^{fLuc/+}$ mice, was reduced by cyclosporin A (CsA; 4 days; 20mg/kg/day; s.c.), an inhibitor of calcineurin-dependent NFAT activation, in the renal medulla/pelvis but not other studied tissues. BMS-345541 (BMS; 4 days; 10mg/kg/day; i.v.), an inhibitor of IκB kinase-2-dependent NFκB activation had no effect in any studied tissue. Data are means ± SEM. n=4. *, $p<0.05$ by one-way ANOVA with Dunnett's post-test.

Tissue	Control	CsA	p-value
	[PGE_2], pg/ml		
Renal medulla	151.3 ± 52.4	26.7 ± 16.3	* 0.046
Renal cortex	10.2 ± 3.3	4.9 ± 3.3	0.28
Bladder	66.6 ± 29.9	80.9 ± 46.1	0.79
Cerebral cortex	39.6 ± 15.0	59.5 ± 28.7	0.55
Thymus	218.3 ± 25.9	273.0 ± 114.8	0.65
Colon	41.0 ± 5.2	43.7 ± 14.1	0.85

Table S3: Effect of cyclosporin A on constitutive cyclo-oxygenase (COX)-2-dependent PGE_2 formation. Constitutive COX-2 activity was measured as PGE_2 formation in A23187 calcium ionophore-stimulated (50μM; 30 mins) tissue segments from COX-1-deficient mice (where all prostaglandin formation is driven by COX-2). Treatment of mice with cyclosporin A (CsA; 4 days; 20mg/kg/day; s.c.) reduced COX-2-dependent PGE_2 formation in isolated renal medulla but not other studied tissues. Data are means ± SEM. n=6. *, $p<0.05$ by unpaired t-test.

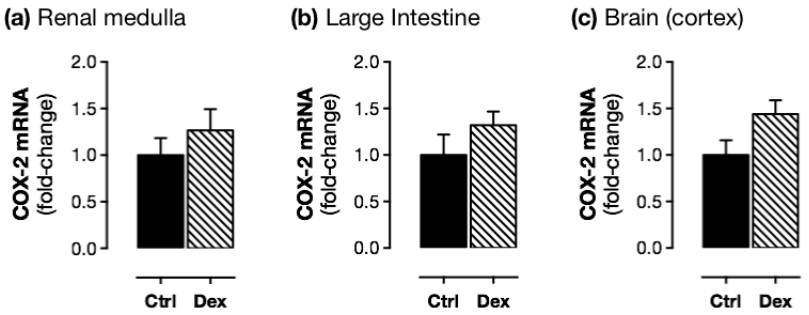

Figure S3: Effect of dexamethasone on regional constitutive cyclo-oxygenase (COX)-2 mRNA expression. Treatment of wild-type mice with dexamethasone (Dex; 4 days; 3mg/kg/day; i.p.) did not alter constitutive COX-2 expression, measured by qPCR, in the renal medulla (a), large intestine (b) or brain (c). Data are means ± SEM. n=6. All $p>0.05$ by unpaired t-test.

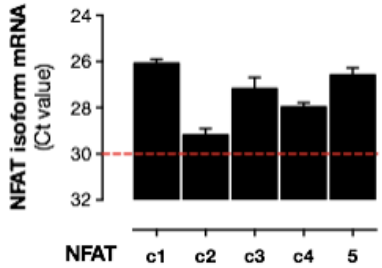

Figure S4: Relative expression NFAT isoforms in the renal medulla. qPCR analysis of NFAT isoform gene expression in the renal medulla of wild-type mice showed that all NFAT isoforms are expressed at the mRNA level (Ct <30 considered as level of detection, marked by red line). Data are means ± SEM. n=12.

Tissue	Control	LPS	LPS + CsA	p-value (LPS vs. LPS+CsA)
	COX-2 luciferase activity (x10^5 photons/sec)			
Aorta	1.0 ± 0.1	8.8 ± 2.6	4.4 ± 1.9	0.24
Bladder	1.4 ± 0.3	30.9 ± 11.0	14.0 ± 4.7	0.22
Cerebral Cortex	5.8 ± 0.6	17.1 ± 4.7	15.5 ± 3.6	0.80
Heart	0.9 ± 0.2	49.6 ± 31.6	20.6 ± 8.8	0.43
Hind brain	0.8 ± 0.4	12.5 ± 5.2	6.3 ± 1.9	0.32
Hippocampus	3.0 ± 0.3	11.4 ± 4.0	6.8 ± 1.4	0.34
Large Intestine	14.9 ± 3.2	105.5 ± 80.3	21.5 ± 8.4	0.36
Liver	0.3 ± 0.0	3.2 ± 1.4	1.8 ± 0.6	0.40
Lung	3.5 ± 1.0	65.7 ± 28.6	33.0 ± 13.3	0.36
Renal cortex	0.3 ± 0.0	3.5 ± 0.9	3.7 ± 2.0	0.93
Renal medulla	3.1 ± 0.6	30.9 ± 5.8	38.9 ± 18.6	0.70
Renal pelvis	4.6 ± 0.8	50.5 ± 10.3	55.5 ± 24.7	0.86
Skin	1.9 ± 0.3	9.6 ± 3.1	3.6 ± 1.3	0.16
Small Intestine	10.2 ± 1.4	90.2 ± 39.1	36.4 ± 11.8	0.26
Spleen	0.5 ± 0.1	34.3 ± 17.5	30.8 ± 13.6	0.88
Stomach	11.6 ± 3.2	47.9 ± 9.2	22.3 ± 7.5	0.10
Thymus	2.6 ± 0.6	20.0 ± 10.6	5.9 ± 1.7	0.26

Table S4: Effect of cyclosporin A on luciferase activity driven from the endogenous *Cox2* gene in LPS-treated mice. LPS treatment (10mg/kg, 4 hours) induced COX-2 expression in all tissues, as determined by bioluminescent imaging of tissue from $Cox2^{fLuc/+}$ mice. This response was not altered by cyclosporin A treatment (CsA; 4 days; 20mg/kg/day; s.c.) in any studied tissue. Data are means ± SEM. n=3-4. For all panels, effect of CsA $p>0.05$ by unpaired t-test.

References

1. Ishikawa TO, Jain NK, Taketo MM, & Herschman HR (2006) Imaging cyclooxygenase-2 (Cox-2) gene expression in living animals with a luciferase knock-in reporter gene. *Mol Imaging Biol* 8(3):171-187.
2. Wilkins BJ*, et al.* (2004) Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res* 94(1):110-118.
3. Carlsen H, Moskaug JO, Fromm SH, & Blomhoff R (2002) In vivo imaging of NF-kappa B activity. *J Immunol* 168(3):1441-1446.
4. Langenbach R*, et al.* (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* 83(3):483-492.
5. Slack E*, et al.* (2009) Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science* 325(5940):617-620.
6. Kirkby NS*, et al.* (2013) LC-MS/MS confirms that COX-1 drives vascular prostacyclin whilst gene expression pattern reveals non-vascular sites of COX-2 expression. *PLoS One* 8(7):e69524.