SUPPLEMENTARY INFORMATION

Kynurenine is a novel endothelium-derived relaxing factor produced during inflammation

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Supplementary Fig. 1. Oxidative metabolism of Trp along the Kyn pathway
Supplementary Fig. 2. The Ido inhibitor 1–Me–Trp increases BP in vivo in unconscious mice. Mean arterial pressure (MAP) was determined in unconscious mice as described in Materials and Methods on day five post-PbA infection after infusion of 1–Me–Trp (1 mM) via the jugular vein. Results represent mean ± SEM, with the number of animals (n) used for each treatment indicated. *P < 0.05, versus uninfected control.

Supplementary Fig. 3. Contribution of NOS to BP in uninfected and PbA-infected mice. L–NAME-induced changes in mean arterial pressure (ΔMAP) in unconscious mice on day 5 post-PbA infection. Blood pressure was determined after infusion of L–NAME (estimated blood concentration 300 μM) via the jugular vein. Results represent mean ± SEM, with the number of animals (n) used for each treatment indicated. *P < 0.05 compared with PbA-infected wild type (Ido++).
**Supplementary Fig. 4.** Participation of Nos2 in the regulation of blood pressure during cerebral malaria infection. Systolic blood pressure (SBP) of wild type (WTGR, ○) and Nos2 knockout mice (●) during the course of PbA infection. Results represent mean ± SEM of n = 6 animals per group. *P < 0.05, wild type versus Nos2 knockout mice.

**Supplementary Fig. 5.** Ido contributes to the regulation of blood pressure in PbK-infected mice. (a,b) Expression of Ido in resistance vessels of kidney. Ido expression was assessed by immunohistochemistry in non-infected mice and in mice 8 days post-infection with PbK (bar = 40 μm). Ido was expressed strongly in endothelial cells of PbK-infected mice (a) but was absent in non-infected mice (b). (c) Plasma concentration of Trp (open columns) and Kyn (filled columns). *P < 0.05 compared with control. (d) SBP determined by the tail cuff method in conscious mice on day 8 after PbK-infection. *P < 0.05 compared with control and PbK + 1-Me-Trp.
**Supplementary Fig. 6.** LPS induces changes to plasma Trp and Kyn concentrations. Plasma concentrations of Trp (triangles) and Kyn (squares) in C57BL/6J mice at different times after LPS administration. Results shown represent mean ± SEM with n = 3–5 animal for each time point.

![Graph showing changes in Trp and Kyn concentrations](image)

**Supplementary Fig. 7.** Time-dependent induction of Ido mRNA in mice by LPS. Kidneys collected at 0, 2, 5, 10, 16 and 24 h after treatment of mice with LPS (7.5 mg kg⁻¹, i.p.), were subjected to qRT-PCR. Results shown represent mean ± SEM of three separate experiments.

![Graph showing induction of Ido mRNA](image)
Supplementary Fig. 8. Implantation of telemetry device causes inflammation associated with induction of Ido activity. (a) Plasma Kyn/Trp ratios determined in control mice and mice 24 h after sham operation or transmitter implantation. (b–c) Plasma was collected from control mice and animals at 3, 6, 12 and 24 h after transmitter implantation, and analyzed for (b) IL-6 and (c) MCP-1 using a commercially available kit. Results shown represent mean ± SEM of four animals per treatment group. *P < 0.05 versus other groups.
**Supplementary Fig. 9. Contribution of Ido to MAP in mice.** MAP was monitored by telemetry in wild type (filled columns) and Ido−/− mice (a) 7 and (b) 21 days after transmitter implantation. n, number of animals. *P < 0.05 versus wild type mice.

**Supplementary Fig 10. IFN-γ-mediated induction of Ido mRNA.** Porcine coronary arteries with intact endothelial cells (EC+) or denuded of endothelium (EC−) were incubated in Medium 199 in the absence or presence of 400 ng IFN-γ ml−1 for 24 h. Real time RT-PCR analysis of Ido and hypoxanthine-guanine phosphoribosyltransferase (Hprt, used as control) was performed on 100 ng of total RNA extracted from arteries. Results shown are representative of three separate experiments. The extent of induction of Ido mRNA increased with increasing IFN-γ concentration and time of incubation (not shown).
Supplementary Fig. 11. IFN-γ dose- and time-dependently induces active Ido protein in human endothelial cells. Human umbilical vein endothelial cells were incubated with IFN-γ (100 U ml⁻¹) for the indicated time (a), or with varying concentration, of IFN-γ for 48 h (b). L-Trp (200 µM) was included in the culture medium. Following incubation, cells were harvested and Ido expression analyzed by Western blotting (top panels). Ido activity (bottom panels) was assessed by HPLC determination of the Kyn accumulated in the cell culture supernatant. Results show mean ± SEM of three to four independent experiments.

Supplementary Fig. 12. Pre-treatment with Kyn decreases subsequent contraction of arteries. Rabbit aortic rings were incubated for 1 h at 37 °C in Krebs solution in the absence (–Kyn, control) or presence of 6 mM Kyn (+Kyn). Contraction was subsequently recorded in response to 25 mM K⁺. Results show mean ± SEM for six independent experiments. *P < 0.05 versus control.
Supplementary Fig. 13. Kyn relaxes pre-constricted rabbit aortic rings independent of an intact endothelium. Rabbit aortic rings with intact endothelium (▲) or denuded (●) of the endothelium were constricted with phenylephrine to half maximal contraction. Relaxation in response to increasing concentrations of Kyn (a) or bradykinin (b) was then determined. Note that denuded rings no longer relax in response to the endothelium-dependent relaxant bradykinin, whereas relaxation in response to Kyn was preserved. Results show mean ± SEM of three independent experiments.

Supplementary Fig. 14. Kynurenine pathway metabolites other than Kyn fail to relax constricted arteries. Porcine coronary arteries were contracted with U-46619. Relaxation in response to the indicated concentration of kynurenic acid (KA), 3-hydroxykynurenine (3-OH-Kyn), 3-hydroxyanthranilic acid (3-HAA), or quinolinic acid (QA) was then determined. Results show mean ± SEM of three independent experiments.
Supplementary Fig. 15. ODQ does not inhibit vessel relaxation induced by Kyn. Rabbit aortic rings incubated for 1 h in the absence (■, ●) or presence of ODQ (1 μM, ▲) and constricted with phenylephrine to half maximal contraction. Relaxation in response to increasing concentrations of Kyn (■, ▲) or vehicle (DMSO, ●) was then determined. Results show mean ± SEM of three independent experiments.

Supplementary Fig. 16. Inhibition of PKA has no effect on vessel relaxation in response to Kyn. Response of mouse aortas to 3 mM Kyn after pretreatment for 30 min with 10 μM KT 5720, an inhibitor of PKA. Results show mean ± SEM of five independent experiments.
Supplementary Methods

**Animals.** C57BL/6J (Animal Resources Centre, Perth, Australia), *Ido*−/− and *Ifng*−/− mice (Genentech), male New Zealand White rabbits (1.8–2.2 kg, Merunga Farm) were used to obtain abdominal aortic rings used for vessel function studies, as described². For porcine coronary arteries, porcine hearts were obtained freshly from a local abattoir, placed in Krebs buffer pH 7.0, and transported on ice to the laboratory. Coronary arteries (circumflex branch) were then immediately isolated and sectioned into 4–5 mm long segments. Where indicated, the endothelium was denuded by gently rubbing the luminal surface of the vessel with a pair of forceps.

**Physiology.** Mean arterial pressure (MAP) of unconscious mice with PbA infection, and SBP of conscious mice with PbK infection were recorded in the common carotid artery and by tail cuff, respectively, as described in the main Methods section. MAP was also monitored using implantable telemetry devices (Data Sciences International, Model TA11PAC10), principally as described previously³. Briefly, the catheter was inserted into the left carotid artery of anesthetized male C57BL/6J and *Ido*−/− mice at 12–16 weeks of age. The catheter was secured without use of adhesive and the transmitter then implanted subcutaneously along the right flank. Immediately following implantation of the transmitter, mice received anti-inflammatory (Carprofen, 4 mg kg⁻¹, s.c.) and antibiotic (Procaine Penicillin G 30 mg kg⁻¹, s.c.) drugs. Throughout the procedures, animals were kept warm and monitored closely until fully recovered. MAP was determined on days 7 and 21 after implantation of the devices.

For vascular reactivity, rabbit aortic rings were incubated in Krebs buffer and their viability confirmed by incremental constriction to phenylephrine (2.5-g load). After pre-constriction to 50% maximal response, rings were exposed to incremental doses of Kyn or bradykinin. For some experiments, rings were pre-incubated with Kyn (6 mM) or vehicle (DMSO) for 1
h, washed and then exposed to KCl. For other experiments, rings were pre-incubated with ODQ (1 µM) or vehicle (Krebs buffer) for 1 h before constriction to 50% maximal response with phenylephrine and relaxation in response to Kyn. For porcine coronary artery rings, viability was confirmed as described above for rabbit aortic rings. Rings were then pre-constricted to 50% maximal response using U46619, before relaxation in response to the indicated concentration of different Kyn pathway metabolites was determined.

**RT-PCR.** To determine the effect of IFN-γ on Ido mRNA expression, porcine coronary arteries were transferred to 12-well plates containing 1 mL serum-free Medium 199 with or without 400 ng porcine IFN-γ ml⁻¹, and incubated for up to 48 h at 37°C under 95% O₂ and 5% CO₂. Total RNA was extracted using Trizol reagent (Invitrogen) and qRT-PCR performed using MMLV (Life Technology) and Taq polymerase (Promega) according to the manufacturer’s instructions. Sense and anti-sense primers for Ido were 5’–GCTTCTTCTGGTCCTCCTATTGGTGA–3’ and 5’–TGCTTTGGCCTGCACTGCCCCCTG–3’, respectively. Ido mRNA was normalized to that of hypoxanthine-guanine phosphoribosyl transferase (Hprt), determined by RT-PCR as described previously⁴. RT-PCR products were separated by agarose gel electrophoresis and visualized by UV, with respective sizes for Ido and HPRT of 318 and 206 bp.

To determine LPS-induced expression of Ido mRNA, kidneys were removed at 0, 2, 5, 10, 16 and 24 h after injection of LPS (0111:B4, Sigma, 7.5 mg kg⁻¹), and stored overnight in RNALater before freezing. RNA was extracted using an RNAeasy kit and Qiacube (both from Qiagen). Two µg of RNA was reverse transcribed into cDNA using 0.1 µg random hexamers and MMLV-RT (both from Invitrogen). Expression of Ido and two reference genes (Hprt and Rpl13a) was quantified using a Rotorgene PCR machine (Corbett Research, Australia) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Amplification was performed in a two-step PCR with 40 cycles of 95 ºC for 15 s and 60 ºC for 45 s.
Specificity of amplification was determined by melting curve analysis and expression levels were measured via a standard curve. Idof expression was normalized to reference gene expression and graphed relative to Idof expression in control mice. Primers were AGATGAAGATGGGCTTTGCT and GGCAGATTTCTAGCCACAAGGA for Idof, CTTAGGCACTGCTCCTGTGGAT and GGTCGCTGTGCTAGCCTCTCTAAT for RPL13a, and CATCTAAGAGGTGGTCGTCAAGTGG and ACAGCCAACACTGCTGAAACAT for HPRT1.

Cell culture. Human umbilical vein endothelial cells were isolated from human umbilical cords and cultured in M199 medium as described previously. MEG-01 cells, kindly provided by Dr Beng H. Chong (St George Hospital, Sydney, Australia), were cultured in RPMI 1640 medium as described previously. Cell viability was assessed by Trypan blue exclusion and lactate dehydrogenase release assays.

Immunohistochemistry. Expression of Idof in mice 8 d post infection with PbK was assessed as described for PbA-infected animals in the Materials & Methods section.

Biochemical analyses. Mouse plasma was treated with 5% trichloroacetic acid to precipitate proteins prior to HPLC analysis of Trp and Kyn, as described in the Materials & Methods section. Plasma cytokines were assayed using a cytometric bead array (BD Biosciences) and flow cytometer (FC500, Beckman Coulter) according to the manufacturer’s instructions.

Idof protein expression and enzyme activity. Human umbilical vein endothelial cells were incubated with human IFN-γ (100 U ml⁻¹) for the indicated time or with varying concentration of IFN-γ for 48 h. Trp (200 µM) was included in the culture medium. Following incubation, cells were lysed and equal amounts of cell protein subjected to SDS-
PAGE Western for Ido expression as described previously\textsuperscript{8}. Protein content was determined using the bicinchoninic acid kit (BCA\textsuperscript{TM} kit, Sigma).

**Supplementary References**


