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RESEARCH PAPER

Role and interactions of annexin A1 and oestrogens in the manifestation of sexual dimorphisms in cerebral and systemic inflammation

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BACKGROUND AND PURPOSE
Gender differences in inflammation are well described, with females often showing more robust, oestrogen-associated responses. Here, we investigated the influence of gender, oestrogen and the anti-inflammatory protein annexin A1 (AnxA1) on lipopolysaccharide (LPS)-induced leukocyte–endothelial cell interactions in murine cerebral and mesenteric microvascular beds.

EXPERIMENTAL APPROACH
Intravital microscopy was used to visualize and quantify the effects of LPS (10 μg·per mouse i.p.) on leukocyte–endothelial interactions in male and female wild-type (WT) mice. The effects of ovariectomy ± oestrogen replacement were examined in WT and AnxA1-null (AnxA1$^{-/-}$) female mice.

KEY RESULTS
LPS increased leukocyte adherence in the cerebral and mesenteric beds of both male and female WT mice; females showed exacerbated responses in the brain versus males, but not the mesentery. Ovariectomy further enhanced LPS-induced adhesion in the brain but not the mesentery; its effects were reversed by oestrogen treatment. OVX AnxA1$^{-/-}$ mice also showed exaggerated adhesive responses to LPS in the brain. However, these were unresponsive to ovariectomy and, paradoxically, responded to oestrogen with a pronounced increase in basal and LPS-induced leukocyte adhesion in the cerebrovasculature.

CONCLUSIONS AND IMPLICATIONS
Our data confirm the fundamental role of AnxA1 in limiting the inflammatory response in the central and peripheral microvasculature. They also (i) show that oestrogen acts via an AnxA1-dependent mechanism to protect the cerebral, but not the mesenteric, vasculature from the damaging effects of LPS and (ii) reveal a paradoxical and potentially toxic effect of the steroid in potentiating the central response to LPS in the absence of AnxA1.

Abbreviations
AnxA1, annexin A1; BBB, blood–brain barrier; E2, 17β-oestradiol; FPR, formylpeptide receptor; IVM, intravital microscopy; LPS, lipopolysaccharide; OVX, ovariectomized; V_{WBC}, leukocyte rolling velocity

Introduction

Leukocytes are the first line of defence against bacteria and other infections, and a complex orchestration of events is required to prevent pathogen invasion. When this fails, severe sepsis occurs. Worldwide, sepsis kills well over 1400 people every day, with approximately 37 000 people dying each year in the UK alone. Current treatments are inadequate and further complicated by the development of antibiotic resistance and the emergence of multi-drug resistance Gram-negative bacilli and viruses (e.g. SARS) (Phillipson and Kubes, 2011). Thus, a greater understanding of how the host defence system responds to pathogens is required in order to understand the disease process and develop effective new drugs.

When activated, leukocytes undergo a sequential pattern of interaction with vascular endothelial cells, characterized by rolling, adhesion and emigration into inflamed/infected tissue. Leukocyte–endothelial cell interactions are induced in the peripheral and cerebral microvasculature within hours of inflammatory stimulus in animal models of sepsis (Vachharajani *et al.*, 2005; 2006; McAvoy *et al.*, 2011). One agent capable of modulating leukocyte–endothelial cell interactions is an endogenous protein annexin A1 (AnxA1; previously termed lipocortin 1). AnxA1-null ($AnxA1^{-/-}$) mice exhibit prolonged and exacerbated responses to inflammatory stimuli in the systemic circulation, which are reversed by administration of AnxA1 and AnxA1 peptides (Hannon *et al.*, 2003; Chatterjee *et al.*, 2005; Damazo *et al.*, 2005, p. 005). Exogenous AnxA1 is also neuroprotective in rodent brains following induction of stroke or fever (Relton *et al.*, 1991; Strijbos *et al.*, 1992; Gavins *et al.*, 2003). In addition, *AnxA1* gene deletion exacerbates inflammation in an animal model of stroke (Gavins *et al.*, 2007), while *de novo* synthesis of AnxA1 defends against toxic brain injury (Young *et al.*, 1999), suggesting that endogenous AnxA1 has an anti-inflammatory role in the brain.

The female hormone, oestrogen, may play a key role in affording protection against sepsis. Certainly, females of child-bearing age have a lower incidence of, and mortality from, sepsis (Erikoğlu *et al.*, 2005; Coyle *et al.*, 2006) and several other diseases, for example stroke, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Cutolo and Wilder, 2000; Czlonkowska *et al.*, 2005; Członkowska *et al.*, 2006). In particular, oestrogens exert neuroprotective effects in models of neuroinflammatory disease, partly through actions at the level of the blood–brain barrier (BBB) (Johnson *et al.*, 2006). Further evidence for a protective role of oestrogens is provided by *in vitro* (Deshpande *et al.*, 1997; Simoncini *et al.*, 2000), *in vivo* (Nathan *et al.*, 1999) and clinical studies (Koh *et al.*, 2001). Oestrogens also up-regulate the expression and function of AnxA1 (Nadkarni *et al.*, 2011; Davies *et al.*, 2007) and sexual dimorphisms have been noticed in the $AnxA1^{-/-}$ mouse (Hannon *et al.*, 2003; Morris *et al.*, 2006), some of which are oestrogen sensitive (Cover *et al.*, 2009), suggesting that interactions between AnxA1 and oestrogen may contribute to the aetiology of sexual dimorphisms in the host inflammatory response. The impact of such interactions on leukocyte–endothelial cell interactions in models of sepsis has not yet been explored.

Peripheral inflammogens cause a systemic inflammatory response that is well characterized in a number of different animal models (Allcock *et al.*, 2001; Buras *et al.*, 2005; Andreasen *et al.*, 2008) and is akin to that observed in sepsis. The inflammatory response induced in the cerebral circulation by peripheral inflammatory insults has been less well studied and characterized. Our study is the first (i) to compare the inflammatory responses initiated by i.p. injection of LPS in two distinct vascular beds: the mesentery and brain – and (ii) to explore the effects of gender, *AnxA1* gene deletion, ovariectomy and oestrogen replacement therapy. Specifically we hypothesized that the two vascular beds would respond differently to LPS (i.p. injection) and that oestrogen would afford protection via (in part) AnxA1. Our results support the premise that endogenous AnxA1 plays an important role in protecting the cerebral and peripheral vasculature from inflammatory insults. They also show for the first time that oestrogen exerts protective AnxA1-dependent actions on vessels in the brain but not the periphery and, paradoxically, reveal that in the absence of AnxA1, oestrogen exerts potentially harmful pro-inflammatory effects on the brain.

Methods

Drugs and reagents

17β-Oestradiol 3-benzoate (E2), lipopolysaccharide (LPS), rhodamine 6G and fluorescein isothiocyanate (FITC)-labelled albumin. E2 was dissolved directly in peanut oil for *in vivo* s.c. injection. LPS (from *Escherichia coli* 0111:B4; specific activity >500 000 EU·mg^{-1}) was dissolved in saline for i.p. injection. Rhodamine 6G and FITC-albumin were dissolved in de-ionized water for i.v. injection. All reagents were obtained from Sigma-Aldrich (Poole, UK).

In vivo *protocols*

Animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All animal care and experimental procedures were performed under licence and complied with the UK Animals (Scientific Procedures) Act, 1986. Animals were maintained on a standard chow pellet diet with tap water *ad libitum* using a 12 h light/dark cycle, in which temperature was maintained at 21–23°C. Experiments were performed on age-matched male (n = 6) and both intact pro-oestrous (intact; n = 6) and ovariectomized (OVX; n = 4–5) female wild-type (WT; C57BL/6) and $AnxA1^{-/-}$ mice (5–8 weeks). WT mice were supplied by Charles River Laboratories UK and allowed 72 h to acclimatize upon arriving in Imperial College. $AnxA1^{-/-}$ mice on a C57BL/6 background were generated as described in Hannon *et al.* (2003) and bred in-house.

Oestrous cycle determination by vaginal cytology. Vaginal cell smears were taken from female mice daily between 0800 and 1000 h, collected on glass slides, stained with methylene blue and examined under a light microscope. The cycle stage was determined according to cell morphology. Only regularly cycling pro-oestrous animals were used in the experiments.

Ovariectomy. Regularly cycling female mice were anaesthetized with isoflurane (Abbott Animal Health, Kent, UK) and

given buprenorphine (Alstoe Animal Health, York, UK) for analgesia at 0.1 mg·kg^{-1}, s.c., immediately before surgery. Bilateral ovariectomy was carried out with additional analgesia and fluid replacement administered as necessary. Animals were treated daily for 8 days postoperatively with E2 (40 ng per mouse, s.c.; Sigma; group termed 'E2') or a corresponding volume of vehicle (peanut oil; 50 µL·per mouse, s.c.; Sigma, UK; group termed 'OVX'). Sham animals underwent surgery without removal of the ovaries and were handled to the same schedule postoperatively, but not injected, as it is the former rather than the latter that is the major stressor in female mice (Drude *et al.*, 2011).

Intravital microscopy (IVM). Due to the requirements of the models used, separate groups of mice were used for IVM of the mesenteric and cerebral microcirculations (as discussed in Gavins and Chatterjee, 2004; Hughes and Gavins, 2010).

Surgery. Animals were injected with LPS (10 µg·per mouse, i.p.; Sigma) or saline vehicle (50 µL, i.p.). Anaesthesia was induced 2 h later by i.p. injection of ketamine (150 mg·kg^{-1}; Ketaset, Fort Dodge Animal Health, Southampton, UK) and xylazine (7.5 mg·kg^{-1}; Rompun, Bayer Healthcare, Newbury, UK) for imaging. Thereafter, the pedal reflex was tested every 15 min and further anaesthetic was administered as required. The jugular vein was exposed and cannulated with polyethylene tubing (PE10) for drug administration.

Visualization of the mesenteric microcirculation. A midline incision along the abdominal region was made; the mesenteric vascular bed exteriorized; gently laid across a Plexiglass viewing stage; and mounted on an Olympus 'BW61WI' microscope with a water-immersion objective lens (magnification of 40×; LUMPlan, FI/IR, Olympus, Japan). The tissue preparation was transilluminated with a 12-V, 100W halogen light source. Real-time videos were acquired with a black-and-white camera (model CoolSNAP HQ^2, Photometrics, Tucson, AZ) coupled to a Windows XP-based computer for recording by Slidebook 4.2 (Intelligent Imaging Innovations, Inc., Denver, CO). Mesenteries were superfused with a bicarbonate-buffered solution (gL^{-1}: NaCl, 7.71; KCl, 0.25; $MgSO_4$, 0.14; $NaHCO_3$, 1.51; and $CaCl_2$, 0.22; pH 7.4).

One to five randomly selected post-capillary venules (20–40 µm wide and 100 µm long) were observed per mouse. Cell flux was quantified as the number of leukocytes passing a fixed point in the vessel in a given time, expressed as cells·min^{-1}. Leukocyte rolling velocity (V_{WBC}) was measured and calculated in µm·s^{-1}. Leukocyte adhesion was quantified as cells that remained stationary for ≥30 s within 100 µm. Leukocyte emigration from the microcirculation into the tissue was quantified as the number of cells 50 µm outside the vessel wall on either side of the 100 µm. To determine post-capillary venule leakage, FITC-albumin was injected i.v. (0.25 mg g^{-1} body weight) 5–10 min before recording. A snapshot of vessel fluorescence was taken using block filter (excitation at 450–490 nm, emission at 535–620 nm). Albumin leakage was quantified by measuring mean fluorescence intensity using ImageJ64 (National Institute of Health, USA). Average fluorescence intensity in three areas of equal size was measured: inside the vessel (Fl_{in}), 10 µm outside the vessel (Fl_{out}) and background fluorescence in an area with no obvious leakage (bk). Albumin leakage was then determined as: $[(Fl_{out} \times bk)/(Fl_{in} \times bk)] \times 100\%$ (Gavins *et al.*, 2003).

Visualization of the cerebral microcirculation. The head of each mouse was fixed in a frame in a sphinx position. The left parietal bone was exposed and a cranial window drilled (diameter 2.5 mm, 1 mm posterior to bregma and 4 mm lateral to the midline). The dura mater was left intact. A circular 12 mm glass coverslip was placed over the window, and the space between the glass and the dura mater was filled with artificial CSF (gL^{-1}: $NaHCO_3$, 20.70; NaCl, 7.71; KCl, 0.22; $MgCl_2$, 0.047; $CaCl_2$, 0.14; urea, 0.402; and dextrose, 0.665; pH 7.4). Rhodamine 6G (100 µL of 0.02%) was administered i.v. to fluorescently labelled leukocytes. An Olympus 'BW61WI' microscope with a water-immersion objective lens (magnification of 40×; LUMPlan, FI/IR, Olympus) was used to visualise pial post-capillary venules in the cerebral cortex. Fluorescence was visualized using block filter (excitation at 450–490 nm, emission at 535–620 nm) and real-time videos were acquired using a camera (model CoolSNAP HQ^2, Photometrics) coupled to a Windows XP-based computer for recording by Slidebook 4.2 (Intelligent Imaging Innovations, Inc.). One to five randomly selected vessel segments, 30–70 µm in diameter and 100 µm long, were observed in each mouse. Rolling leukocyte flux was measured as number of leukocytes passing a fixed point in the vessel in a given time, as cells·min^{-1}. Leukocyte rolling velocity (V_{WBC}) was measured and calculated in µm·s^{-1}. Leukocyte adhesion was quantified as cells that remained stationary for ≥30 s within 100 µm. These parameters are expressed as the number of cells per mm^2 of the vessel surface. (Quantification of leukocyte emigration could not be determined using our intravital microscope, due to insufficient depth of penetration into the thick brain tissue.)

Collection and analysis of blood samples

Differential leukocyte counts. Blood was taken by cardiac puncture from terminally anaesthetized mice, collected into heparin-containing tubes and diluted 1:10 in Turk's solution (0.01% crystal violet in 3% acetic acid). Differential cell counting was performed using a Neubauer chamber under a light microscope (Nikon Eclipse E400; ×40 objective; Nikon, Tokyo, Japan).

Determination of plasma cytokine levels. Blood was taken by cardiac puncture from terminally anaesthetized mice, collected into heparin-containing tubes and centrifuged at 400× *g* at 4°C, for 10 min. The plasma was stored at –80°C until required. Plasma concentrations of the cytokines TNF-α, IL-6 and IL-10 were assayed using standard ELISA kits (R&D Systems, Abingdon, UK) with samples from 4 mice per group.

Statistical analysis

All values are expressed as mean ± SEM; $n = 6$ animals per group unless otherwise stated. Statistical analysis was conducted either by Student's *t*-test for two groups (used in experiments involving intact animals) or a two-way ANOVA followed by Bonferroni's *post hoc* test for more than two groups (GraphPad Software Inc., San Diego, CA). In all cases, a probability value of $P < 0.05$ was considered significant.

Results

LPS induced inflammatory responses in the mesenteric and cerebral microcirculation of male and female mice

LPS elicited inflammation in mesenteric and cerebral microcirculations of male and female mice, as determined by intravital microscopy (Figure 1 and Tables 1 and 2). A sharp increase in leukocyte adherence (Figure 1D and G) and emigration (Figure 1E) was quantified in both vascular beds, with no significant differences between sexes. By contrast, while LPS reduced rolling by approximately 50% in the mesenteric vessels of male and female mice (Figure 1A), it increased rolling in the cerebrovasculature with pronounced effects in the female over 60 times that in the male ($P < 0.001$, Figure 1F). Resting leukocyte cell flux was lower in the brain than the mesentery, with no apparent differences between sexes (Tables 1 and 2). In the male, LPS increased cell flux in both beds studied ($P < 0.05$). LPS also increased leukocyte flux in the female brain but reduced cell flux in the mesentery ($P < 0.05$).

Another cardinal sign of an inflammatory response, plasma protein extravasation (PPE), was measured in the mesentery. LPS increased PPE in both male (from 20.5 ± 1.5% to 36.1 ± 4.1%, $P < 0.01$) and female (20.5 ± 1.5% to 36.1 ± 4.1%, $P < 0.01$) mice, in comparison with saline treatment, with no significant differences between the sexes (data not shown).

Figure 2 shows the plasma concentrations of pivotal pro- and anti-inflammatory cytokines (TNF-α, IL-6 and IL-10) in mice 2 h after an injection of LPS or saline. Concentrations of all three cytokines were increased following LPS treatment versus saline. TNF-α was undetectable in the plasma of saline-treated mice but rose to 151.6 ± 11.7 pg·mL^{-1} in males and 692.7 ± 130.3 pg·mL^{-1} in females following treatment with LPS ($P < 0.0001$ vs. control, Figure 1A). The increase in the female was 4.5 times the effect of that seen in males ($P < 0.01$), suggesting the initiation of a more aggressive inflammatory response in the female. IL-6 levels were also increased versus controls in both male and female mice treated with LPS, but with no significant sex difference (Figure 2B). Similarly, the plasma concentrations of the anti-inflammatory cytokine IL-10 (Figure 2C) were also increased following LPS treatment; as with TNF-α, the response was approximately eight times higher in the female (522.3 ± 39.3 pg·mL^{-1}) than the male (70.5 ± 1.3 pg·mL^{-1}, $P < 0.001$). The blood polymorphonuclear (PMN) cell count in saline-treated mice was higher in females than males. LPS treatment reduced PMN cell numbers in both male and female

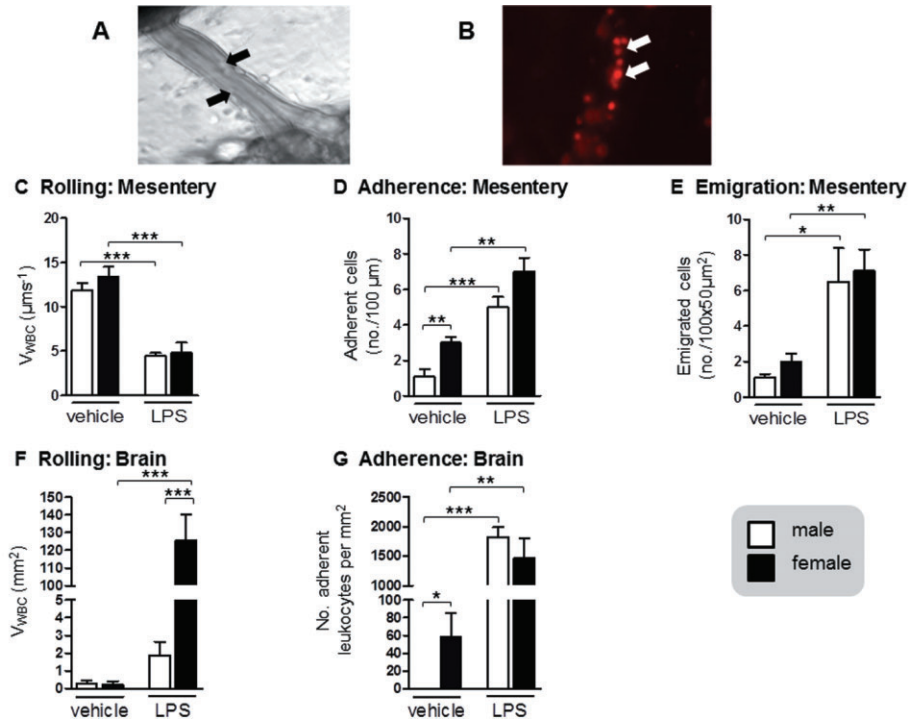

Figure 1

Effect of LPS on leukocyte–endothelial cell interactions in mesenteric and cerebral vascular beds. Mice were treated with LPS (10 μg·per mouse) i.p. or saline vehicle (100 μL). After 2 h, the (A) mesentery or (B) brain was visualized using intravital microscopy. Leukocytes can be clearly seen in vessels of both tissues. Leukocyte–endothelial cell interactions in mesenteric venules were quantified in terms of (C) leukocyte rolling velocity (expressed as V_{WBC}), (D) number of adherent leukocytes per 100 μm vessel length and (E) number of emigrated leukocytes per 100 × 50 μm^2. The leukocyte–endothelial cell interactions in cerebral venules were quantified in terms of (F) leukocyte rolling velocity (expressed as V_{WBC}) and (G) number of adherent leukocytes per 100 μm vessel length; results in (F) and (G) are expressed as the number of cells per mm^2 of the vessel surface. Data are mean ± SEM. $n = 6$ mice per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ using ANOVA with Bonferroni's *post hoc* test.

Table 1

Interactions of oestrogen status and AnxA1 on LPS-induced cell flux in mesenteric venules

	Cell flux (cells·min^{-1})							
	WT (intact)		WT			AnxA1$^{-/-}$		
	Male	Female	Sham	OVX	E2	Sham	OVX	E2
Saline	109.7 ± 14.1	76.9 ± 8.3	10.3 ± 6.2	61.4 ± 16.2[c]	36.2 ± 11.4	18.5 ± 12.8	113.1 ± 38.2[c]	19.3 ± 4.8[d]
LPS	254.0 ± 38.8[a]	29.7 ± 2.0[a,b]	5.8 ± 2.1	14.3 ± 3.3[a]	21.8 ± 5.8	17.8 ± 7.4	32.3 ± 17.6[a]	24.9 ± 4.1

The following mice were used: male and pro-oestrous female C57BL/6 (termed WT intact); female C57BL/6 (WT) and AnxA1$^{-/-}$ mice, some of which were ovariectomized and treated daily for 8 days with 40 ng 17β-oestradiol per mouse; s.c. (termed E2) or vehicle (termed OVX); control mice were subjected to sham operation only (termed sham). All groups were injected with LPS (10 μg per mouse) or saline vehicle i.p. (100 μL), and after 2 h, the leukocyte–endothelial cell interactions in mesenteric venules were quantified by intravital microscopy in terms of rolling leukocyte flux (expressed as cells·min^{-1}). Data are expressed as mean ± SEM. $n = 4$–6 mice per group.
[a]$P < 0.05$ versus saline vehicle-treated counterparts.
[b]$P < 0.05$ versus male counterparts.
[c]$P < 0.05$ versus sham counterparts.
[d]$P < 0.05$ versus OVX counterparts using ANOVA followed by Bonferroni's *post hoc* test.

Table 2

Interactions of oestrogen status and AnxA1 on LPS-induced cell flux in cerebral venules

	Cell flux (cells·min^{-1})							
	WT (intact)		WT			AnxA1$^{-/-}$		
	Male	Female	Sham	OVX	E2	Sham	OVX	E2
Saline	0.2 ± 0.2	0.3 ± 0.3	2.0 ± 1.2	5.5 ± 1.0[b]	4.8 ± 0.8[b]	2.5 ± 2.5	11.3 ± 4.2[b]	5.0 ± 2.4[c]
LPS	2.3 ± 0.8[a]	2.7 ± 0.7[a]	13.8 ± 6.3[a]	28.8 ± 5.5[a,b]	12.0 ± 1.6[a,c]	16.5 ± 5.7[a]	25.5 ± 2.1[a]	18.3 ± 1.2[a]

The following mice were used: male and pro-oestrous female C57BL/6 (termed WT intact); female C57BL/6 (WT) and AnxA1$^{-/-}$ mice, some of which were ovariectomized and treated daily for 8 d with 40 ng 17β-oestradiol per mouse; s.c. (termed E2) or vehicle (termed OVX); controls were subjected to sham operation only (termed sham). All groups were injected with LPS (10 μg per mouse) or saline vehicle (100 μL) i.p., and after 2 h, the leukocyte–endothelial cell interactions in cerebral venules were quantified by intravital microscopy in terms of rolling leukocyte flux (expressed as cells·min^{-1}). Data are expressed as mean ± SEM. $n = 4$–6 mice per group.
[a]$P < 0.05$ versus saline vehicle-treated counterparts.
[b]$P < 0.05$ versus sham counterparts.
[c]$P < 0.05$ versus OVX counterparts using ANOVA followed by Bonferroni's *post hoc* test.

animals but its effects were more pronounced in the male (Figure 2D).

Effects of OVX and E2 replacement on mesenteric and cerebral vascular inflammation

In order to investigate the potential role of ovarian hormones, in particular oestrogens, in causing sex differences in LPS-induced vascular inflammation, mice were ovariectomized (OVX) or subjected to a similar sham operation; OVX mice were given either E2 replacement (E2) or its peanut oil vehicle daily for 8 days post surgery.

Mesenteric vessels. Table 1 shows the effects of OVX and E2 replacement on cell flux in the mesentery of saline- and LPS-treated mice. OVX increased cell flux in saline-treated mice versus sham-operated controls ($P < 0.05$); its effects were partially reversed by E2 replacement therapy. LPS treatment reduced cell flux in all groups; its effects were exaggerated by OVX and attenuated by E2 (Table 1). By contrast neither OVX nor E2 influenced V_{WBC} in the mesenteric vessels of vehicle- or LPS-treated mice (Figure 3A). However, OVX enhanced both resting and LPS-induced adhesion but E2 replacement was without effect (Figure 3B). OVX also caused an E2-reversible increase in emigration in vehicle-treated mice but paradoxically reduced the increase in emigration caused by LPS via an E2-independent mechanism (Figure 3C).

Cerebral vessels. LPS increased cell flux in the cerebral vessels of sham-operated, OVX and E2-treated mice, as it did in intact controls; the responses to LPS were enhanced by OVX and reversed by E2 replacement (Table 2). OVX also increased rolling ($P < 0.001$) and adhesion ($P < 0.05$) in saline-treated mice; its effects on rolling ($P < 0.05$) but not adhesion were enhanced by E2 replacement (Figure 3D and E). The increase

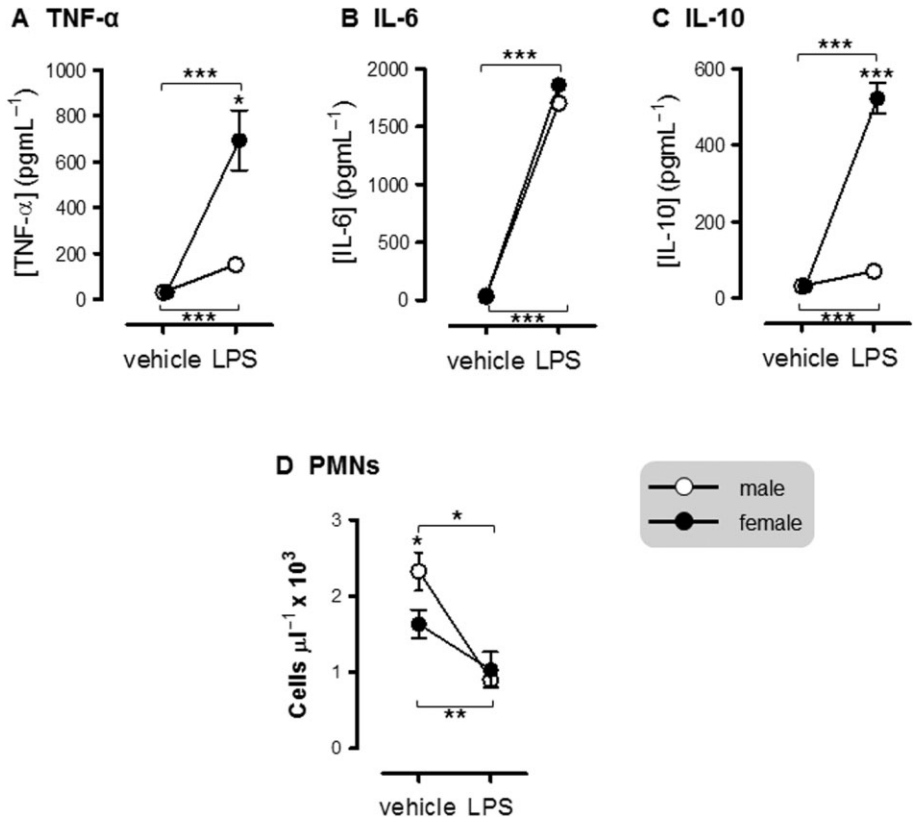

Figure 2

Sexual dimorphisms in the inflammatory responses of intact male and female C57BL/6 mice following LPS. LPS (10 µg·per mouse) or the saline vehicle (100 µL) was administered i.p. to intact male and pro-oestrous female C57BL/6 mice. After 2 h, blood was taken by cardiac puncture and plasma assayed by ELISA for (A) TNF-α, (B) IL-6 and (C) IL-10; $n = 4$ mice per group. (D) Whole blood was diluted 1:10 in Turk's solution, and PMNLs were counted by means of a Neubauer chamber; $n = 6$ mice per group. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ using a Student's *t*-test for two groups or a two-way ANOVA with Bonferroni's *post hoc* test.

in rolling induced by LPS was similar in sham-operated animals ($P < 0.05$ vs. saline) and OVX mice ± E2 replacement (Figure 3D). By contrast, the increase in adhesion induced by LPS in sham-operated controls ($P < 0.05$ vs. saline-treated control) was exaggerated by OVX ($P < 0.05$ vs. sham-operated) and corrected by E2 replacement ($P < 0.001$ vs. OVX, Figure 3E).

Plasma cytokines. Table 3 demonstrates the effects of OVX and E2 replacement on the plasma cytokine responses to LPS. TNF-α and IL-6 were undetectable in the plasma of saline-treated sham-operated, OVX and E2 mice. IL-10 was also not detected in the blood of saline-treated sham-operated mice but was evident in the E2-treated OVX group. LPS increased the concentrations of all three cytokines in sham-operated mice ($P < 0.001$); its effects were attenuated by OVX (TNF-α: 2177.0 ± 853.1 vs. 220.6 ± 128.5; IL-6: 4259.0 ± 72.4 vs. 2086.0 ± 254.2; IL-10: 1004.0 ± 501.9 vs. undetectable, $P < 0.05$). E2 replacement did not affect the inhibition of the TNF-α and IL-6 responses to LPS induced by OVX ($P > 0.05$), but it partially reversed the IL-10 response ($P < 0.05$).

Effects of AnxA1 gene deletion on the immunomodulatory effects of ovariectomy and oestrogen replacement

AnxA1$^{-/-}$ mice were used to examine the interactions of AnxA1, ovariectomy and oestrogen in modulating the leukocyte–endothelial cell interactions induced by LPS.

Mesenteric vessels. The increase in cell flux induced by OVX in the mesenteric vessels of saline-treated WT mice was potentiated by *AnxA1* gene deletion (61.4 ± 16.2 vs. 113.1 ± 38.2); this effect of OVX was attenuated by E2 replacement in the KO, but not the WT mice. By contrast, no strain differences in the increment in cell flux caused by LPS treatment in any of the three groups studied (sham, OVX or E2). V_{WBC} was ~3 higher times in saline-treated, sham-operated AnxA1$^{-/-}$ mice than in the corresponding WT controls (Figure 4Ai and ii; $P < 0.001$), with no further differences in V_{WBC} being observed in AnxA1$^{-/-}$ or WT mice after LPS treatment and/or OVX. However, E2 replacement reduced the increases in resting V_{WBC} induced by *AnxA1* gene deletion (Figure 4A; $P < 0.01$). No differences in adherence were observed between sham-operated WT and AnxA1$^{-/-}$ treated with saline or LPS (Figure 4B). OVX caused a marked increase in adhesion in

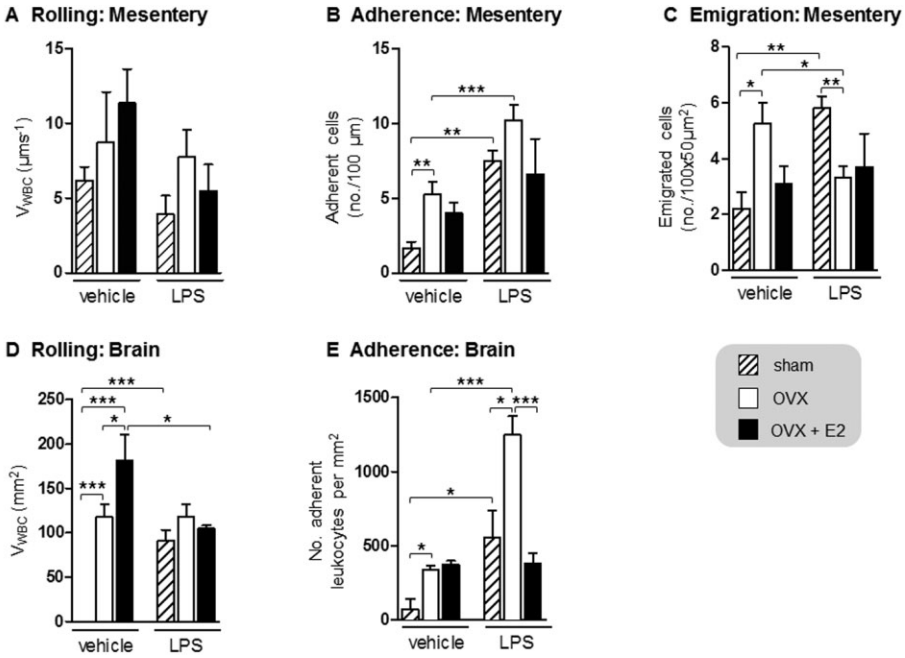

Figure 3

Effects of LPS on mesenteric and cerebral leukocyte–endothelium interactions in intact and ovariectomized female C57BL/6 mice. Animals were either taken whilst in pro-oestrous, as determined by vaginal cytology, or ovariectomized, followed by daily oestrogen replacement (40 ng 17β-oestradiol per mouse, s.c.; E2) or peanut oil vehicle (OVX) for 8 days. On day 8, LPS (10 μg per mouse) i.p. or saline vehicle (100 μL) was administered, and 2 h after this, the leukocyte–endothelium interactions in mesenteric venules were quantified in terms of (A) leukocyte rolling velocity (expressed as V_{WBC}), (B) number of adherent leukocytes per 100 μm vessel length and (C) number of emigrated leukocytes per 100 × 50 μm^2. The leukocyte–endothelium interactions in cerebral venules were quantified in terms of (D) leukocyte rolling velocity (expressed as V_{WBC}) and (E) number of adherent leukocytes per 100 μm vessel length; results in (D) and (E) are expressed as the number of cells per mm^2 of the vessel surface. Data are mean ± SEM. n = 5–6 mice per group *P < 0.05, **P < 0.01, ***P < 0.001 using a Student's t-test for two groups or a two-way ANOVA with Bonferroni's *post hoc* test.

Table 3

LPS-induced changes in plasma cytokine concentrations in ovariectomized female WT and AnxA1$^{-/-}$ mice

		[TNF-α] (pg·mL^{-1})		[IL-6] (pg·mL^{-1})		[IL-10] (pg·mL^{-1})	
		Vehicle	LPS	Vehicle	LPS	Vehicle	LPS
WT	Sham	0	2177.0 ± 853.1[a]	0	4259.0 ± 72.4[a]	0	1004.0 ± 501.9[a]
	OVX	0	220.6 ± 128.5[a,b]	0	2086.0 ± 254.2[a,b]	0	0[b]
	E2	0	176.3 ± 54.2[a,b]	0	2022.0 ± 170.3[a,b]	352.2 ± 130.7[b,c]	47.9 ± 16.7[a,b]
AnxA1$^{-/-}$	Sham	0	4542.0 ± 2477.0[a]	0	4230.0 ± 47.2[a]	0	1232.0 ± 306.7[a]
	OVX	0	0[b,d]	0	1854.0 ± 272.8[a,b]	0	58.3 ± 13.8[b]
	E2	34.6 ± 3.4	205.5 ± 53.1[a,b,c]	0	2215.0 ± 70.7[a,b]	35.8 ± 5.51[d]	76.9 ± 23.7[b]

Saline vehicle (100 μL) or LPS (10 μg per mouse) was administered i.p. to ovariectomized C57BL/6 (WT) and AnxA1$^{-/-}$ mice receiving daily oestrogen replacement therapy (E2) or peanut oil vehicle (OVX). Blood was taken 2 h later by cardiac puncture and plasma assayed by ELISA for TNF-α, IL-6 and IL-10. A value of 0 indicates that cytokine concentrations were below the detectable limits of the kits (31.25 pg·mL^{-1} for TNF-α and IL-10; 15.63 pg·mL^{-1} for IL-6). Data are mean ± SEM. n = 3–4 mice per group.

[a]P < 0.05 versus vehicle-treated counterparts.
[b]P < 0.05 versus sham-operated counterparts.
[c]P < 0.05 versus OVX counterparts.
[d]P < 0.05 versus WT counterparts using ANOVA with Bonferroni's *post hoc* test.

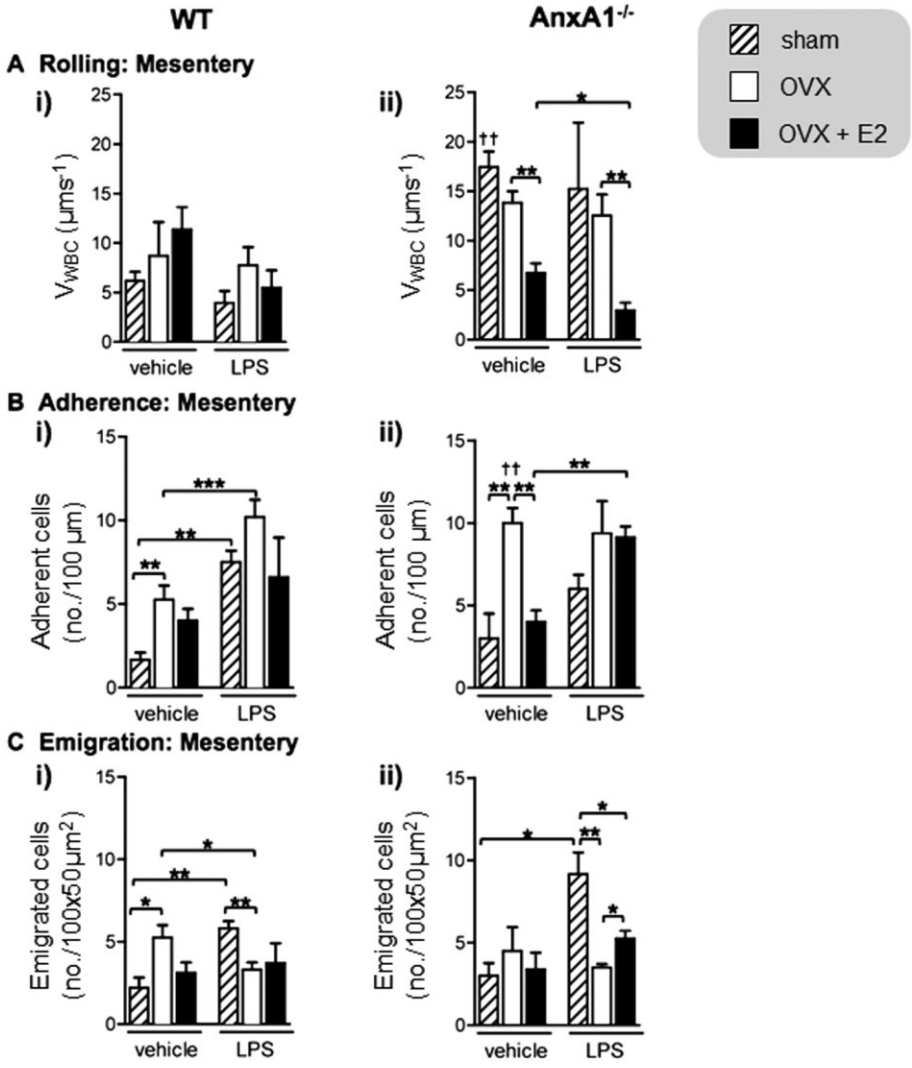

Figure 4

Effects of LPS on mesenteric leukocyte–endothelium interactions in ovariectomized female WT and AnxA1$^{-/-}$ mice. Animals were ovariectomized, and treated daily for 8 days with 17β-oestradiol (40 ng per mouse, s.c.; E2) or peanut oil vehicle (OVX). On day 8, LPS (10 μg·per mouse) i.p. or saline vehicle (100 μL) was administered. After 2 h, the leukocyte–endothelium interactions in mesenteric venules were quantified in terms of (A) leukocyte rolling velocity (expressed as V_{WBC}), (B) number of adherent leukocytes per 100 μm vessel length and (C) number of emigrated leukocytes per 100 × 50 μm^2. Data are mean ± SEM. n = 6 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 versus indicated groups; $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ < 0.01 and $^{\dagger\dagger\dagger}P$ < 0.001 versus wild-type counterparts using Student's t-test for two groups or a two-way ANOVA with Bonferroni's *post hoc* test.

AnxA1$^{-/-}$ mice versus WT mice (P < 0.01); this response, which was sufficient to mask the normal adhesive response to LPS was reversed by E2 (P < 0.01). LPS-induced emigration was exaggerated in AnxA1$^{-/-}$ mice versus WT mice (P < 0.01) and abolished in both strains by OVX. However, while E2 failed to reverse the effects of OVX in the WT mice, it caused a partial reduction in the KO mice (P < 0.05, Figure 4C).

Cerebral vessels. OVX caused a small increase in cell flux (P < 0.02) in WT mice, which was exaggerated by *AnxA1* gene deletion (P < 0.05); its effects were inhibited by E2 in KO (P < 0.05) but not in WT mice (Table 3). By contrast, *AnxA1* gene deletion did not affect LPS-induced changes in cell flux in any of the three groups examined (sham, OVX, E2). The increments in V_{WBC} induced by OVX were similar in WT and AnxA1$^{-/-}$ mice (Figure 4A). However, while the effects of OVX were exacerbated by E2 treatment in WT mice (P < 0.05, Figure 4Ai), they were reversed in KO mice (P < 0.05, Figure 4Aii). As with cell flux, *AnxA1* gene deletion did not affect the V_{WBC} response to LPS in sham, OVX or E2-replace mice. With regard to adherence (Figure 4B), OVX produced similar increases in baseline values in the WT and KO mice. However, in WT mice, the number of adherent leukocytes was unaffected by E2 (Figure 4Bi), but in KO mice, it was doubled by hormone replacement (P < 0.01, Figure 4Bii). Furthermore, while LPS caused a modest increase in adhesion in the WT, which was increased by OVX (P < 0.05) and reduced by E2 replacement (P < 0.01), in the KO mice LPS caused a

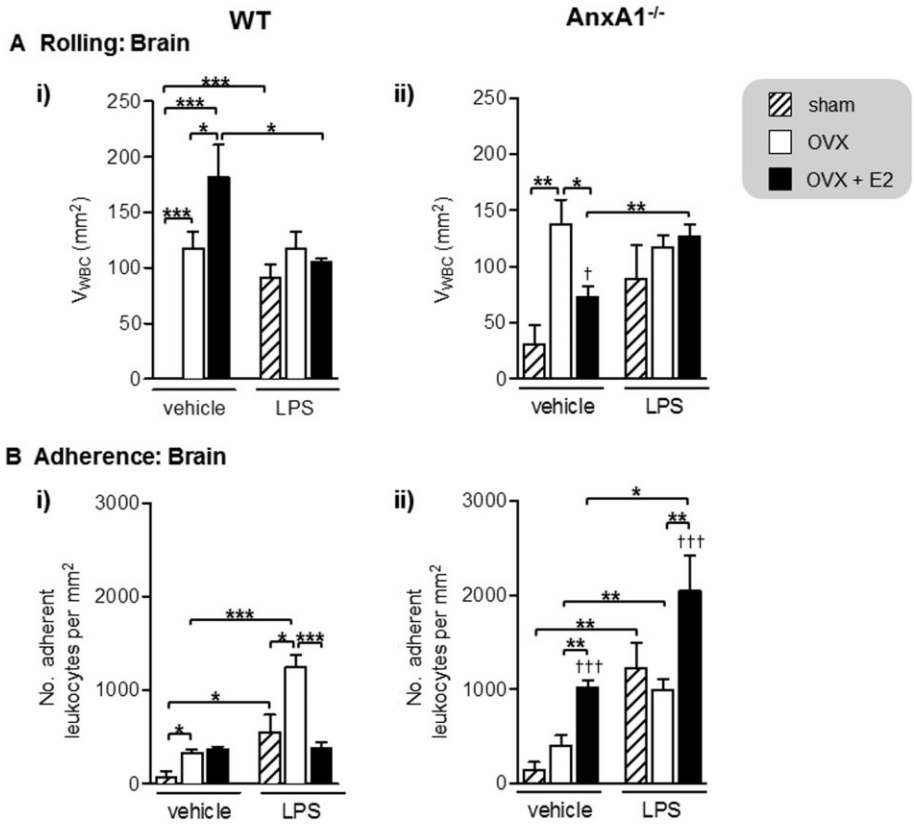

Figure 5

Effects of LPS on cerebral leukocyte–endothelium interactions in ovariectomized female WT and AnxA1$^{-/-}$ mice. Animals were ovariectomized and treated daily for 8 days with 17β-oestradiol oestrogen replacement (40 ng·per mouse, s.c.; E2) or peanut oil vehicle (OVX). On day 8, LPS (10 μg·per mouse) i.p or saline vehicle (100 μL) was administered; leukocyte–endothelium interactions in cerebral venules were quantified 2 h later in terms of (A) leukocyte rolling velocity (expressed as V_{WBC}) and (B) number of adherent leukocytes per 100 μm vessel length; results are expressed as the number of cells per mm^2 of the vessel surface. Data are mean ± SEM. $n = 6$ mice per group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus indicated groups; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ versus wild-type counterparts using Student's *t*-test for two groups or a two-way ANOVA with Bonferroni's *post hoc* test.

much larger increase in adhesion (~3-fold; $P > 0.01$), which was unaffected by OVX but potentiated by E2 ($P < 0.01$, Figure 4Bii). By contrast, *AnxA1* gene deletion did not affect leukocyte emigration, irrespective of the treatment.

Plasma cytokines. *AnxA1* gene deletion caused a two-fold increase in the amount of TNF-α released in response to LPS; furthermore, while OVX reduced the TNF-α response to LPS in WT mice, it completely abolished the response in the KO mice (Table 3) (Figure 5). By contrast the increases in IL-6 and IL-10 induced by LPS were similar in WT and KO mice irrespective of whether they had been subjected to a sham operation, OVX or E2 replacement therapy (Table 3).

Discussion

In this study, we compared the vascular inflammatory response to LPS in male and female mice, focusing on two distinct vascular beds, the brain and the mesentery, which have previously been shown to exhibit differential sensitivity to LPS (Mitchell *et al.*, 1993). In addition, we examined the potential roles and interactions of the ovary, the principal female hormone, 17β-oestradiol (E2), and the anti-inflammatory protein, AnxA1, in modifying the responses to LPS. Our data confirm reports that (i) LPS elicits an inflammatory response characterized by increased leukocyte–endothelial cell interactions, increased plasma extravasation and a shift towards a pro-inflammatory cytokine *milieu* (Zhou *et al.*, 2009; Kwan *et al.*, 2010) and (ii) the profiles of the responses differ in the mesenteric and cerebrovascular beds (Mitchell *et al.*, 1993). For the first time, we have demonstrated that gender is not a major factor in determining the intravascular leukocyte responses to LPS, although the toxin-induced increases in circulating TNF-α and IL-10 were exaggerated in the female, as also was the increase in leukocyte rolling observed in cerebral but not mesenteric vessels, thus supporting the premise of a heightened inflammatory response in the female (Cutolo and Wilder, 2000; Straub, 2007). Not withstanding these findings, our data demonstrate a significant role for the ovary in quelling inflammation, with removal of the ovary precipitating an inflammatory state characterized by increased leukocyte rolling, adhesion and

emigration in mesenteric vessels and by increased rolling and adhesion in cerebral vessels. As replacement with E2 proved protective only in the cerebral microcirculation our findings point to roles for ovarian factors other than E2 in exerting a tonic, protective inhibitory effect on the host defence system. We have also shown that the presence of AnxA1 limits the pro-inflammatory actions of E2, particularly in the brain and thus elucidates mechanisms by which females of child-bearing potential are afforded neuroprotection during inflammatory disease.

The dose of LPS used in our study was relatively low, chosen deliberately to activate the vasculature without causing either mortality or a drastic decrease in microvascular perfusion, as is seen at higher doses and which would complicate the inflammatory response (Zanetti *et al.*, 1992; Damazo *et al.*, 2005). Previous studies in our laboratory have revealed that leukocyte–endothelial cell interactions persist for at least 24 h after LPS injection in the same model, whilst cytokine concentrations peak early on (Hughes, Buckingham and Gavins, unpubl. data). Leukocyte–endothelial cell interactions are supported by expression of adhesion molecules on both leukocytes and endothelial cells (Ley *et al.*, 2007), which are up-regulated in both brain and mesentery in response to LPS (Eppihimer *et al.*, 1996; Henninger *et al.*, 1997). *In vitro* and *in vivo*, oestrogen inhibits systemic endothelial induction of selectins (which mediate rolling) and VCAM-1 and ICAM-1 (which mediate adhesion) as well as induction of ICAM-1 in cerebral endothelia (Caulin-Glaser *et al.*, 1996; Squadrito *et al.*, 1997; Cuzzocrea *et al.*, 2001). Reduced expression of adhesion molecules by E2 supports our observations that fewer leukocyte–endothelial cell interactions occurred both in intact female mice compared to males, and in WT E2 mice. However, a generalized reduction in adhesion molecule expression would not explain the clear differences between mesenteric and cerebral vascular beds observed here. It is known that local and temporal variations in adhesion molecule expression, such as E- and P-selectin, occur in response to LPS (Eppihimer *et al.*, 1996; Eppihimer *et al.*, 1999). Furthermore, constitutive and LPS-induced expression of PECAM-1, ICAM-1 and VCAM-1 are much lower in the brain than the mesentery, and the kinetics and relative up-regulation of these adhesion molecules varies between the tissues (Henninger *et al.*, 1997). Therefore, differences in intravascular leukocyte responses in the present study are most likely due to local variations in the endothelia.

The sexually dimorphic response to LPS was more evident in terms of cytokine production. The detection of IL-10 at the same time as TNF-α in both sexes indicates that the anti-inflammatory circuits (whose eventual purpose is to switch off the pro-inflammatory response) are already active at 2 h post LPS challenge. However, the fact that levels of both cytokines are significantly higher in females suggests that the female mounts a more aggressive pro-inflammatory response. These data together with the raised sensitivity of the intact female mice to sterile inflammation so far fit with the hypothesis that oestrogen renders females more immunoreactive (Straub, 2007).

OVX exerted pro-inflammatory effects in the brain, by promoting leukocyte rolling and adhesion both at rest and in response to LPS; these effects were fully reversed by E2 treatment. Since the effects of E2 are so complex and varied, it is difficult to draw many comparisons with the literature due to differences in treatment protocols, doses and models. The dose used in this study (40 ng E2·day^{-1} s.c.) has previously been reported to mimic pro-oestrous serum hormone concentrations of 150 pg·mL^{-1} (Jablonka-Shariff *et al.*, 1999; Thanky *et al.*, 2002). Straub (2007) discusses at length the way in which both dose and timing can affect numerous aspects of the inflammatory response: in relation to innate immune activity, the dose-response is biphasic, with both very low and very high doses acting in a pro-inflammatory capacity, whilst E2 concentrations pertaining to the physiological levels seen during pro-oestrus are protective. This is confirmed with respect to AnxA1 by Nadkarni *et al.* (2011), who showed that a particular anti-inflammatory PMN phenotype (AnxA1^{hi} CD62L^{lo} CD11b^{lo}) can only be induced if E2 administration precedes the inflammogen. In addition to regulation by E2 of inflammatory components of leukocytes such as PMN expression of CD11b and AnxA1 (Nadkarni *et al.*, 2011), the hormone is also able to potently up-regulate synthesis, release and activity of protective endothelial mediators and down-regulate pathological ones (Villar *et al.*, 2008).

Differential actions of E2 on the vasculature (attributed to the presence of ERα and β; Mendelsohn and Karas, 2005) would account for inherent differences in leukocyte recruitment presented here. It is known that background activation of cerebral ERs occurs, whilst in other organs such as the intestine it does not (Lemmen *et al.*, 2004). Whilst leukocyte recruitment in the brain was E2-sensitive, this was not the case in the peripheral circulation. Although some complex effects were observed in the mesentery in terms of LPS-induced leukocyte recruitment, wherein LPS-induced emigration was increased in sham-operated animals and reduced in OVX mice, the overall effect of OVX on mesenteric leukocyte recruitment was pro-inflammatory. These pro-inflammatory effects were not fully reversed by E2, suggesting ovarian hormones other than E2 govern the processes guiding LPS-induced emigration in the periphery (such as generation of chemokines and adhesion molecule up-regulation, which is supported by a recent study into sex differences of murine resident peritoneal leukocytes; Scotland *et al.*, 2011).

LPS-elicited production of TNF-α, IL-6 and IL-10 was diminished by OVX and sensitivity to E2 was variable, although replacement of the hormone failed to fully restore any of the three cytokines to levels of the sham animals and further supports the idea that another ovarian hormone is required to mount a proper inflammatory response. The abolition of the anti-inflammatory IL-10 further shows that OVX induces a baseline pro-inflammatory state, whilst its particular sensitivity to E2 (coupled with our observations that intact female mice produce more IL-10 than males) suggests that E2 is capable of producing anti-inflammation via IL-10. Other studies of E2 on cytokine production are contradictory, but long-term oestrogen treatments can enhance production of pro-inflammatory cytokines in response to LPS (Calippe *et al.*, 2008), supporting our observations that females simultaneously produce higher levels of pro- and anti-inflammatory cytokines. Therefore, our data imply firstly that ovarian hormones other than E2 are required to promote a proper inflammatory response to LPS; and secondly, a lack of these hormones seems to produce a pro-inflammatory state at rest, which holds implications for the health of women who may

lack these hormones due to, for example, reproductive dysfunction.

Sex differences in other endocrine axes potentially explain the divergence in the inflammatory response seen here. The output of glucocorticoid hormones by the hypothalamo–pituitary–adrenal (HPA) axis in response to inflammation or infection (including LPS) results in potent anti-inflammatory effects (Berczi, 1998). Males and females exhibit differential HPA axis reactivity, output and duration thereof, with oestrogens thought to be a key sensitizer of the axis via modulation of glucocorticoid and mineralocorticoid receptors and upregulation of CRH gene expression (Kudielka and Kirschbaum, 2005). However, this difference in HPA axis functioning is unlikely to fully explain the sex differences in our model, because a greater HPA axis output would be expected to limit rather than enhance cytokine production and leukocyte recruitment. Furthermore, the rodent mesenteric and cerebral vascular beds are both known to express the glucocorticoid receptor (Sousa *et al.*, 1989; DeLano and Schmid-Schönbein, 2004). Thus, it is unlikely that the E2-dependent modulation of glucocorticoid sensitivity can explain the differences observed between the vascular beds.

Bilateral ovariectomy eliminates hormones other than E2 such as the steroid progesterone, which modulates components of the TLR4 signalling pathways and offers protection via cytokine modulation in models including focal ischaemic stroke, traumatic brain injury and LPS-induced inflammation (Ghezzi *et al.*, 2000; Ishrat *et al.*, 2009; Hua *et al.*, 2011). Peptide ovarian hormones are also capable of regulating inflammation: activin A is present in CSF of rabbits with meningitis, and may regulate TNF-α and IL-1β (Jones *et al.*, 2004), whilst relaxin can inhibit neutrophil activity and limit adhesion molecule and chemokine expression by endothelial cells (Masini *et al.*, 2004; Brecht *et al.*, 2011), all of which could impact on leukocyte recruitment and explain the pro-inflammatory effects of OVX we have observed.

OVX also disrupts endocrine axes sensitive to E2. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are pituitary-derived hormones negatively regulated by E2, and therefore would both be raised in OVX animals. FSH and LH have been positively correlated with AnxA1 expression (Mulla *et al.*, 2005), so E2 could act synergistically with these hormones to exert anti-inflammatory actions via AnxA1, thus explaining the partial sensitivity of WT mice to E2 in this study. Prolactin, also pituitary-derived, is up-regulated by E2 and partly modulated by AnxA1 (Cover *et al.*, 2009). Prolactin potentiates the response of peripheral immune cell IL-8 and TNF-α production in response to LPS (Brand *et al.*, 2004; D'Isanto *et al.*, 2004); therefore, low prolactin levels in OVX mice might also explain the reduced cytokine production following LPS treatment.

In addition to direct removal or disruption of reproductive hormones by OVX, there is also cross-talk between the ovarian system with that of the natriuretic peptides, which can (amongst other effects) alter both inflammation and blood pressure (which can impact on leukocyte–endothelial interactions via alterations in local haemodynamic factors such as shear stress). Up-regulation of atrial natriuretic peptide (ANP; produced by cardiac myocytes) is associated with elevated oestrogen (Hong *et al.*, 1992; Karjalainen *et al.*, 2004) and inhibits expression of L-selectin, E-selectin and ICAM-1 (Biselli *et al.*, 1996; Kiemer *et al.*, 2002; 2005). C-type natriuretic peptide (CNP; produced by the pituitary gland) has similar effects to ANP on adhesion molecule expression and modulates vascular tone in a NO-dependent fashion (Qian *et al.*, 2002), as well as influencing prolactin (Brand *et al.*, 2004). These findings go towards explaining the non-E2-dependent effects of OVX in our study, particularly at baseline. Therefore, it is likely that the absence of multiple ovarian hormones in OVX mice affects the ability to sense and mount a response to LPS, suggesting that the ovarian system as a whole – rather than oestrogen alone – is important in sexual dimorphisms during inflammation.

To understand the role of AnxA1 in mediating sexually dimorphic effects of the inflammatory response to LPS and its interaction with E2, we also tested the model in female AnxA1$^{-/-}$ mice. This is the first time that such an investigation has been conducted; therefore, relevant data in the literature are limited.

The contrasting effects of OVX on rolling and adhesion in mesenteries of null mice further imply that female hormones differentially regulate adhesion molecule expression in the absence of AnxA1. Whilst constitutive up-regulation of selectins is indicated by elevated baseline rolling in these mice, this is independent of AnxA1 as it was also observed in the WT. However, the reversal by E2 of OVX-induced adherence in the AnxA1$^{-/-}$ mice only indicates that E2 specifically prevents up-regulation of those adhesion molecules regulating adhesion, but only in the absence of AnxA1. Furthermore, it appears that a female hormone has pro-emigratory effects on leukocytes, as OVX limited the emigration response to LPS in the periphery in wild-type mice. In the AnxA1$^{-/-}$ mice only, this effect was partially reversed by E2 replacement, which may indicate a synergistic action of E2 with other female hormones such as FSH and LH in regulating AnxA1 activity as discussed above. This differential E2-sensitive aspect of leukocyte recruitment was not seen in the WT mice, which implies that the AnxA1$^{-/-}$ mice are more sensitive to the effects of E2 than the WT mice, perhaps because AnxA1 masks or blocks some of the hormone's effects.

The interaction of AnxA1 with adhesion molecules has not been widely studied, although it is known that the N-terminus peptide AnxA1$_{Ac2\text{-}25}$ can specifically prevent monocyte adhesion *in vitro* by blocking the α4 integrin (Solito *et al.*, 2000), and that the protein's receptor FPR/ALX regulates the β2 integrin-dependent adhesion and transmigration of PMNs (Fiore and Serhan, 1995), a process that could be disrupted in the absence of AnxA1. The protein also modulates localization and expression of ICAM-1 and CD11b (the latter in an E2-sensitive manner) (Chatterjee *et al.*, 2005; Williams *et al.*, 2010; Nadkarni *et al.*, 2011). AnxA1 therefore seems to particularly interact with the integrins, and so its deletion would alter leukocyte adhesion in an E2-sensitive manner as seen here in the AnxA1$^{-/-}$ mice at baseline, an effect that is particularly potent in the brain.

Given the propensity of AnxA1$^{-/-}$ mice to suffer excessive inflammation (Hannon *et al.*, 2003; Chatterjee *et al.*, 2005; Damazo *et al.*, 2005), it was surprising to observe relatively few changes in mesenteric leukocyte–endothelial cell interactions in OVX AnxA1$^{-/-}$ mice. There is a small amount of

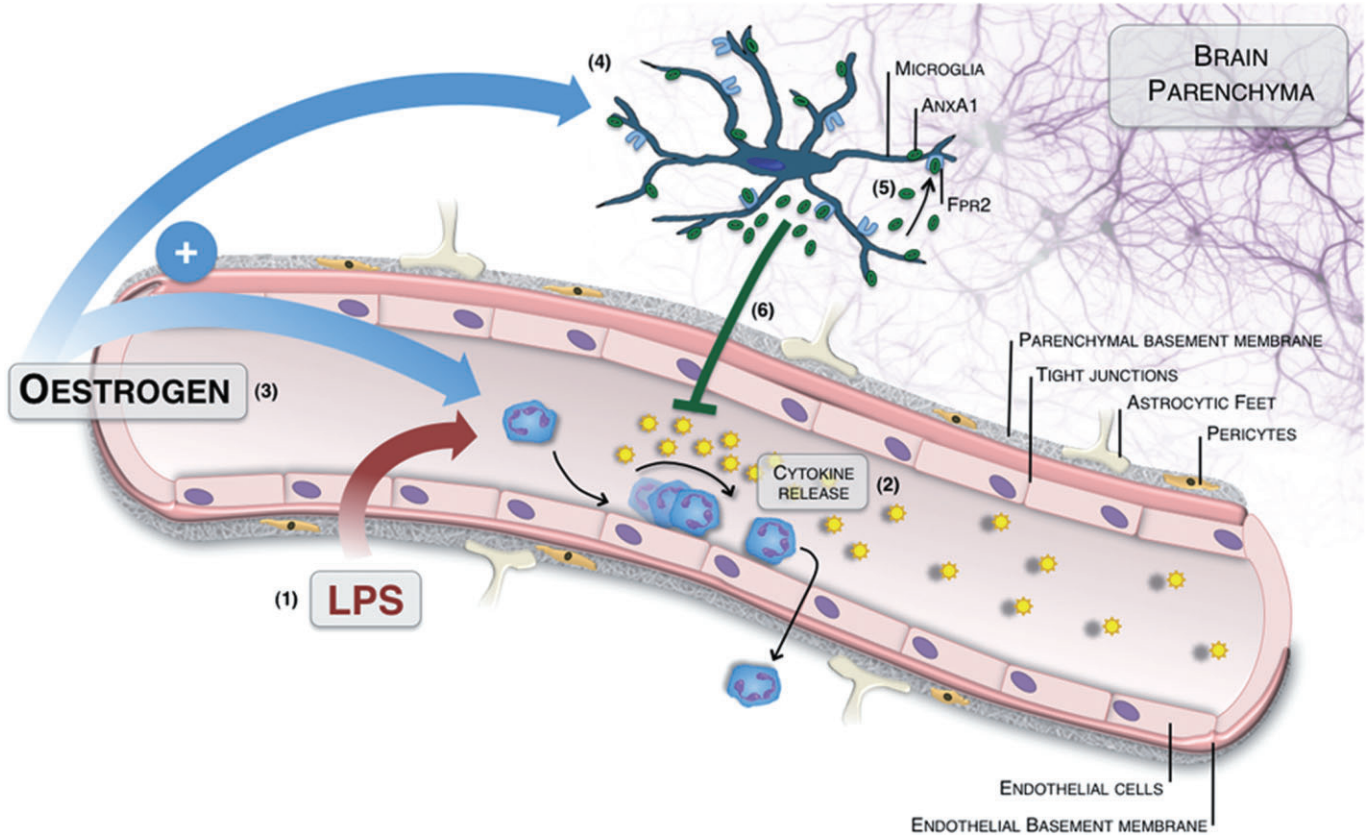

Figure 6

Summary diagram of the hypothesized mechanism by which oestrogen modulates cerebral inflammation. (1) Peripheral LPS challenge activates the inflammatory cascade in pial venules. It causes up-regulation of leukocyte–endothelial interactions in discrete stages that include rolling, adhesion and emigration. (2) Secretion of pro-inflammatory mediators such as the cytokines TNF-α and IL-6, from both leukocytes and endothelial cells, is also initiated. (3) Treatment with oestrogen directs the inflammatory cascade to become swifter and more aggressive, in order to better deal with pathogens. However, overblown inflammation within the confines of the skull can be injurious; therefore, (4) oestrogen also promotes expression of the anti-inflammatory protein AnxA1 on certain cell types within the brain, where (5) it can interact with its receptor, FPR2. (6) AnxA1 then acts to quench inflammation within the cerebral vasculature, possibly via inhibition of iNOS and PGE_2 production, thereby protecting the brain.

evidence to show that the inflammatory responses in mesenteric vessels of WT and AnxA1$^{-/-}$ mice do not differ following a mild inflammatory stimulus of IL-1β (Nadkarni *et al.*, 2011) – furthermore, oestrogen treatment did not exacerbate the response, correlating with our data here. However, in the brain, the story was very different, and we found that E2 treatment of AnxA1$^{-/-}$ mice severely potentiated LPS-induced leukocyte adhesion. This further implies that differences exist in the vascular beds rather than in the leukocytes. The presence of AnxA1 at the BBB (Eberhard *et al.*, 1994; Solito *et al.*, 2008) appears to play a protective role via promotion of endothelial anti-inflammatory eicosanoids (Herbert *et al.*, 2007); stimulation of IL-10 production (Souza *et al.*, 2007); and via anti-inflammatory paracrine signalling through locally expressed FPR2. This fits with the more subtle effects of E2 and AnxA1 deletion observed in the mesentery and in TNF-α production, where the presence of AnxA1 appears protective. However, the protection afforded by AnxA1 seems more potent in the brain.

Solito *et al.* (2008) postulate that AnxA1 tightly controls central immunity and prevents an excessive inflammatory response within the confines of the skull, which has devastating consequences. Oestrogen is a requisite for a proper immune response in the female brain: Soucy *et al.* (2005) found that OVX mice could only mount a cerebral response to i.c.v. or systemic LPS challenge if oestrogen replacement therapy had been administered, and that the response was ERα-dependent. If, therefore, oestrogen is required to mount a proper inflammatory response in the brain, and the presence of AnxA1 is required to limit the magnitude of the response, then the potentiation by E2 of LPS-induced leukocyte–endothelial cell interactions in cerebral microvessels of AnxA1$^{-/-}$ mice can be explained, as can the lack of TNF-α response in the OVX AnxA1$^{-/-}$ group.

In summary, specifically, we hypothesized that the two vascular beds would respond differently to LPS (i.p. injection) and that oestrogen would afford protection via (in part) AnxA1 (Figure 6). Indeed, our data show that the ovary as a whole appears to be just as – if not more – important than E2 in modulating both leukocyte recruitment and plasma cytokine levels, although the specific contributing factor(s) remain to be determined. The potentiating effects of OVX on

baseline leukocyte–endothelial cell interactions is important because it suggests that these animals exist in a chronic low-level pro-inflammatory state, which is a risk factor for cardiovascular and cerebrovascular accident (Roifman *et al.*, 2011). We have demonstrated a novel protective role for AnxA1 in the cerebral circulation, as indicated by the absence of AnxA1, which sensitizes mice to the pro-inflammatory effects of E2 in the brain, elucidating mechanisms by which the hormone affords neuroprotection during inflammation. Thus, our novel data are of relevance for the health of post-menopausal women and those with ovarian dysfunction such as polycystic ovary syndrome, wherein patients have been found to exist in a low-grade chronic inflammatory state with elevated cytokines such as IL-6, raised E-selectin and VCAM indicating chronic endothelial activation and raised leukocyte counts (Diamanti-Kandarakis *et al.*, 2006).

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Conflict of interest

The authors state no conflict of interest.

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