Supporting Information

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SI Text

MVEC Isolation. Ovaries were removed aseptically on day 5 of pseudopregnancy, rinsed in PBS (GIBCO), and digested in collagenase A (1 mg/ml, Sigma–Aldrich) for 45 min. Corpora lutea were mechanically dispersed and the cellular suspension was filtered through sterile 75-μm nylon mesh, centrifuged at 400 $\times$ *g* for 10 min, washed twice, and ressuspended in PBS. Cells were counted on a hemocytometer, and cell viability was determined by trypan blue dye exclusion. Luteal mouse microvascular endothelial cells were isolated from pseudopregnant WT and ASMase$^{-/-}$ through positive magnetic cell sorting by Dynabeads according to the manufacturer's instructions. Dynabeads M-450 Sheep anti-Rat IgG (Dynal) were precoated with purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen) for 30 min at 4°C (1 μg of rat IgG per 1 $\times$ 10^7 Dynabeads). Precoated Dynabeads were added to cells (four Dynabeads per cell) and incubated at 4°C for 30 min. Isolated mouse microvascular endothelial cells were then plated and cultured in EGM-2MV, as recommended by the supplier with progesterone (250 ng/ml) and 3% FBS added. The positive selection of microvascular endothelial cells was confirmed by Factor VIII immunoflorescence using rabbit anti-human von Willebrand Factor (factor VIII) IgG fraction of antiserum (Sigma) and Goat anti-rabbit IgG labeled with fluorescein isothiocyanate-FITC (Sigma).

Aortic Endothelial Cell Isolation. The thoracic aorta was dissected away from the fascia, removed, washed in ice-cold PBS and incubated in collagenase A (1 mg/ml, Sigma–Aldrich) for 45 min. The arterial tissue was mechanically dispersed and the cellular suspension was filtered through sterile 100-μm nylon mesh, centrifuged at 400 $\times$ *g* for 10 min, and washed twice and resuspended in PBS. Cells were counted on a hemocytometer, and cell viability was determined by trypan blue dye exclusion. Aortic endothelial cells were isolated through positive magnetic cell sorting and plated as described above.

ICC for Ceramide Accumulation After Activation of TNFRI. Isolated luteal microvascular endothelial cells were seeded onto four-chamber glass slides (Nalge Nunc) coated with Type II collagen (Sigma) and cultured in EGM-2 (Cambrex) at 37°C and 5% CO_2 in air. After 48 h of culture, the medium was removed and replaced with serum-free Dubelcco's MEM (Mediatech). After 24 h under serum-free conditions, the cells were cooled to 4°C, and then incubated for 1 h at 4°C with 50 ng of murine TNF (Cell Sciences) in MEM. Activation of the TNF receptor was induced by warming the cells to 37°C on a slide warmer for the indicated times. Activity was terminated by vacuum removal of treatment media, followed by immediate addition of cold 2% paraformaldehyde in PBS (pH 7.4). The cells were fixed for 20 min at 4°C, and then washed in PBS + 2% FBS and 0.1% Tween-20 (HyClone and Sigma). Nonspecific binding was blocked by incubation for 20 min in PBS + 2% FBS at 4°C. Mouse anti Ceramide (MID15B4; Alexis Biochemicals) was diluted 1:50 in wash buffer and incubated for 45 min at 4°C. Ceramide staining was detected with fluorescein-labeled anti mouse IgM (Roche) diluted 1:100 in wash buffer and incubated for 45 min at 4°C. Nuclei were counterstained with a Propidium Iodide/RNase A solution (Phoenix Flow Systems) for 15 min, and the slides were mounted by using Gel Mount (Sigma). Staining intensity was evaluated on a Radiance 2000 confocal microscope (Carl Zeiss) using either $\times$40 or $\times$60 oil-immersion objective lenses.

Acid Sphingomyelinase Activity. Once 90% confluency was reached, the medium was changed, and the cultures were maintained in serum, phenol red, and growth factor-free conditions (Minimum Essential Medium Alpha Medium; GIBCO/BRL) for 24 h. Medium was then removed once again, and fresh serum, phenol red and growth factor-free medium were applied. After 5-h treatment with or without recombinant Mouse TNFα (50 ng/ml; Cell Sciences) medium was removed, and the cells were lysed in 300 μl of buffer containing 50 mM sodium acetate (pH 5.0), 1% Triton X-100, 1 μg/ml aprotinin, 1 mM EDTA, and 100 μg/ml phenylmethylsulfonyl fluoride for 60 min on ice (or frozen immediately at −80°C until processing). The supernatant fraction was collected after centrifugation at 17,000 $\times$ *g* for 10 min at 4°C. Cell membrane-free supernatant fractions (adjusted to pH 5.0) were assayed for ASMase activity in a two-step reaction system using Amplex Red Sphingomyelinase Assay kit (cat no. A12220; Molecular Probes) according to the manufacturer's instructions.

Evaluation of the Estrous Cycle Length of WT and ASMase$^{-/-}$ Mice. Daily vaginal swabs were collected from WT and ASMase$^{-/-}$ mice for 21 days, stained with methylene blue and subjected to cytological analysis for assessment of estrous cycle phase.

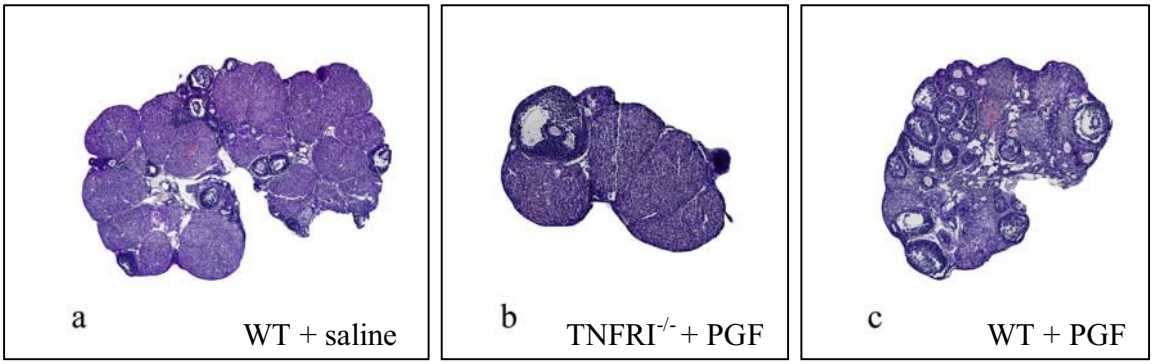

Fig. S1. Mice lacking the TNF Receptor 1 gene (TNFRI$^{-/-}$) are resistant to PGF2α-induced corpus luteum regression. TNFRI$^{-/-}$ mice showed no physical evidence of advanced luteal regression 24 h after PGF injection when compared with the controls. (*a*) WT mice treated with normal saline. (*b*) TNFRI$^{-/-}$ mice treated with PGF. (*c*) WT mice treated with PGF. The ovary of the saline-treated and PGF-treated TNFRI$^{-/-}$ had numerous intact CL, whereas the CL of the WT mice treated with PGF were much smaller in size, and there was evidence of numerous developing follicles, which corresponds with luteal regression.

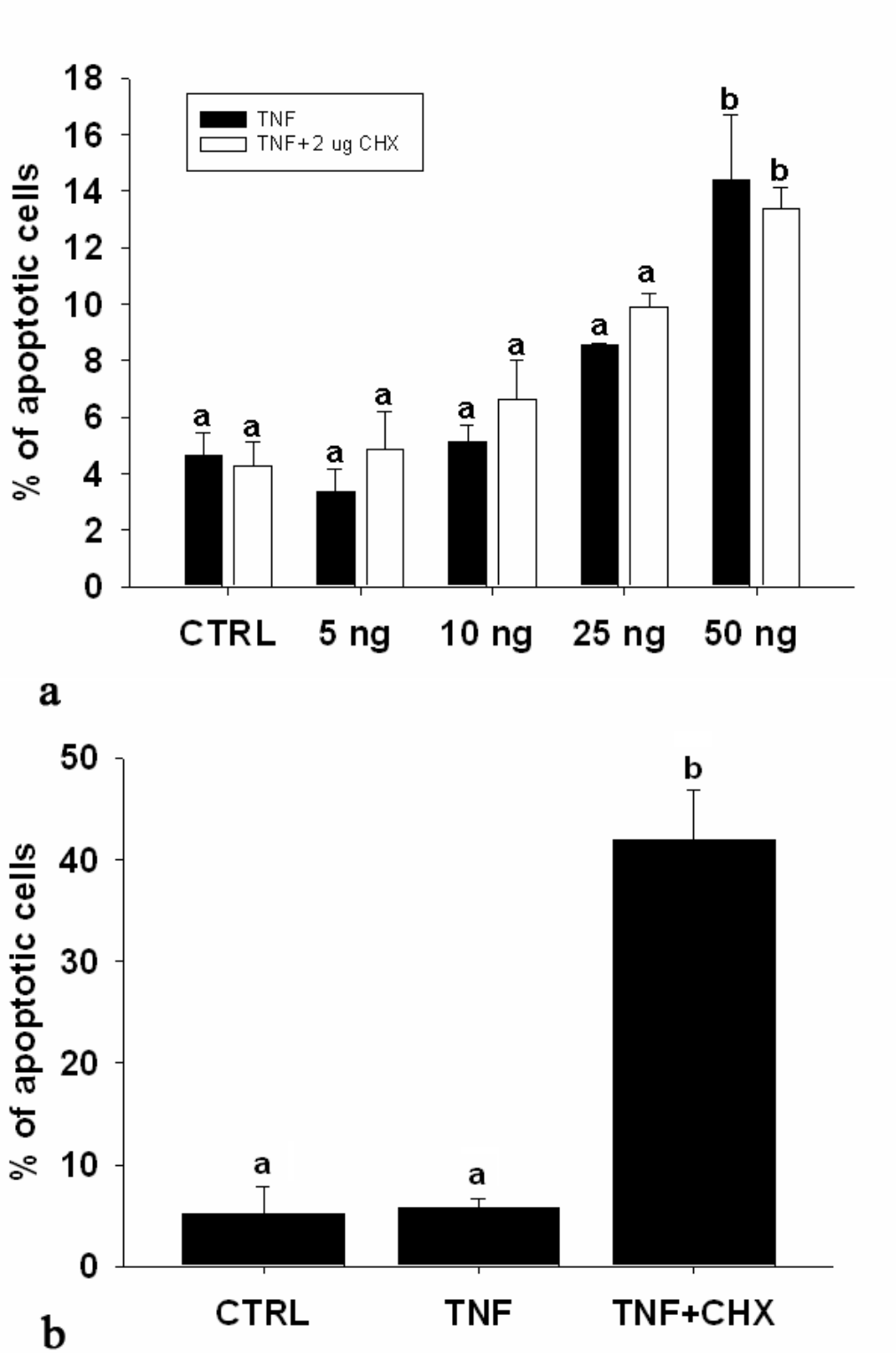

Fig. S2. Isolated mouse luteal MVECs treated with recombinant mouse TNF (10–50 ng/ml) underwent a significant increase ($P < 0.002$) in apoptosis whether or not an inhibitor of protein synthesis, cycloheximide (CHX, 2 μg/ml), was present in the cultures. In contrast, endothelial cells derived from the mouse thoracic aorta required CHX for TNF-induced apoptosis. (*a*) Dose–response to TNF and TNF + CHX in mouse luteal MVEC 48 h after treatment. (*b*) Apoptosis of mouse thoracic aorta endothelial cells 48 h after treatment with 50 ng of TNF or 50 ng of TNF + 2 μg of CHX.

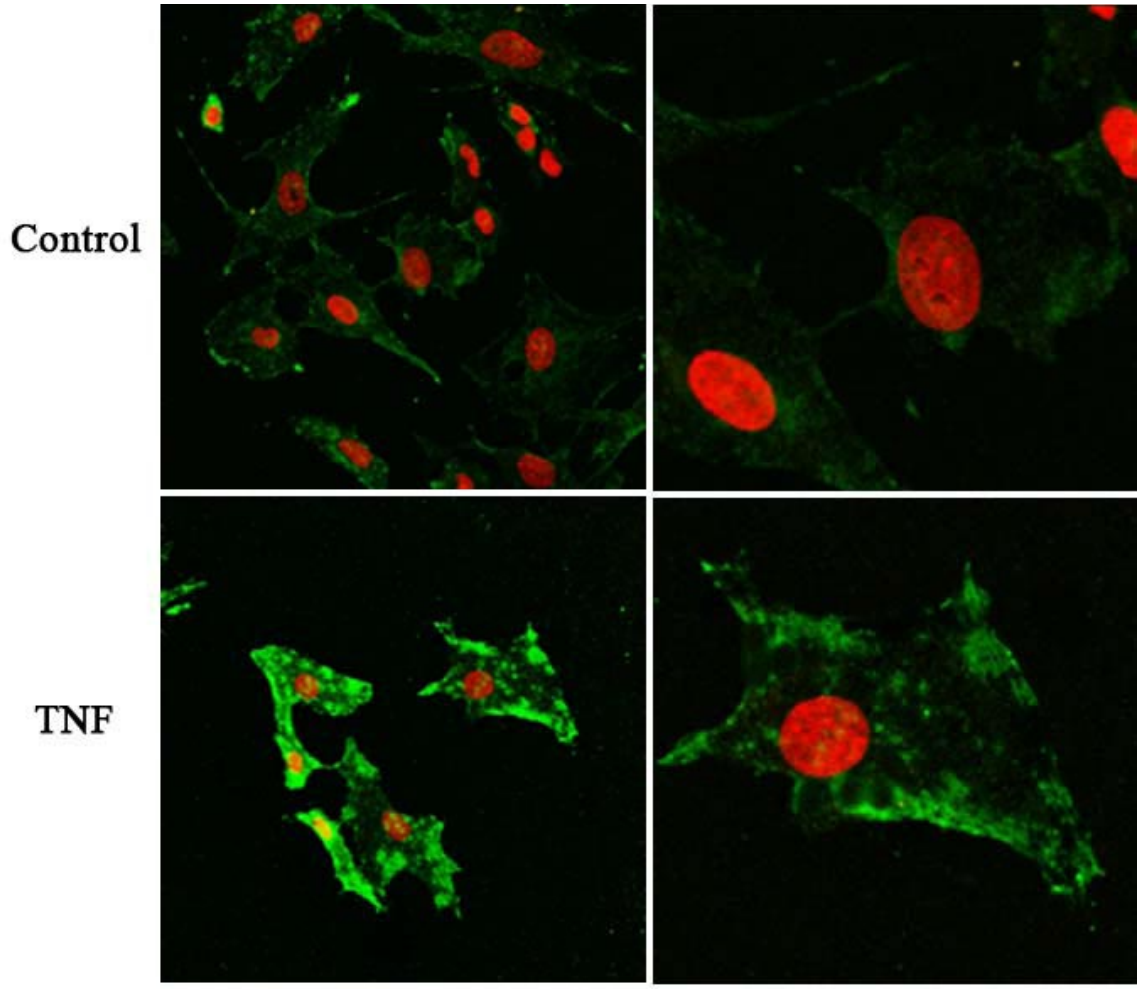

Fig. S3. TNF treatment promotes ceramide accumulation in mouse luteal MVEC. Treatment with TNF resulted in a punctate distribution of ceramide near the plasma membrane of mouse luteal MVEC within 30 s of treatment. In contrast, cells receiving vehicle alone had no evidence of this phenotype.

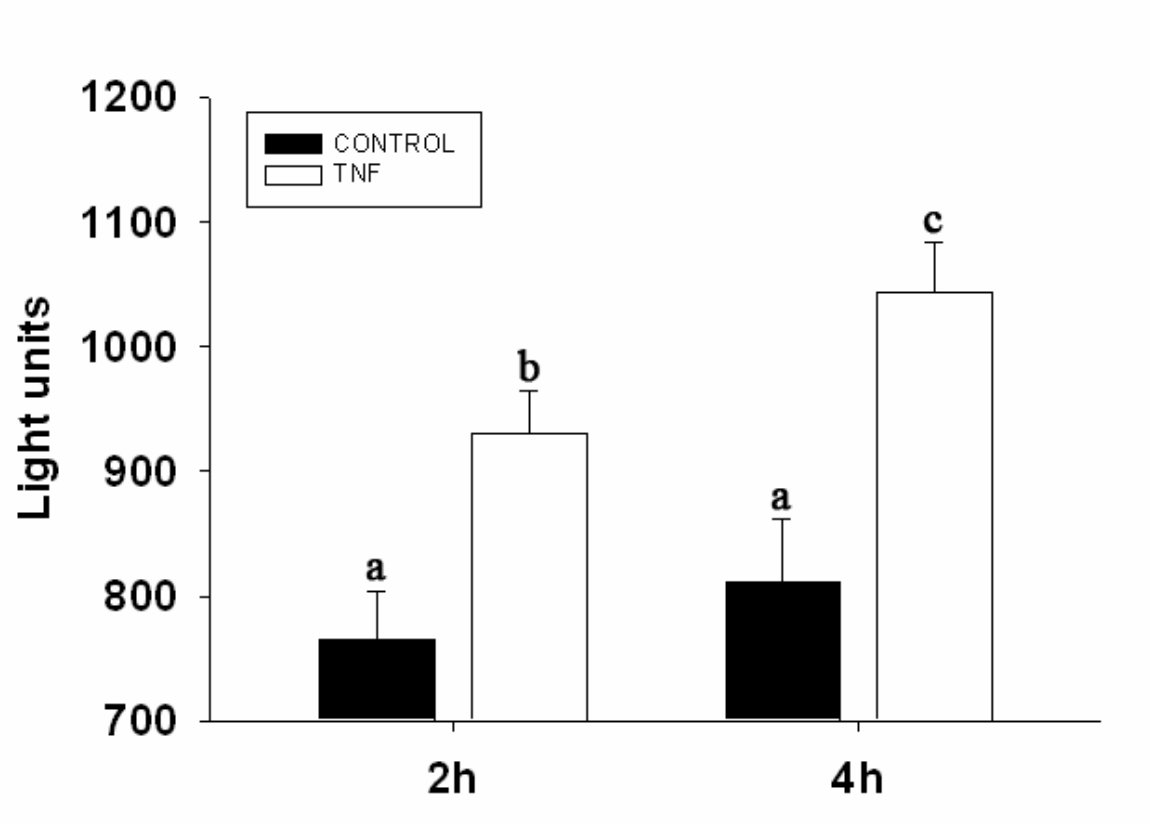

Fig. S4. TNF treatment causes an increase in ASMase activity in mouse MVECs. Mouse luteal MVECs show a significant increase ($P < 0.05$) in ASMase activity at 2 and 4 h after treatment with TNF when compared with the corresponding controls

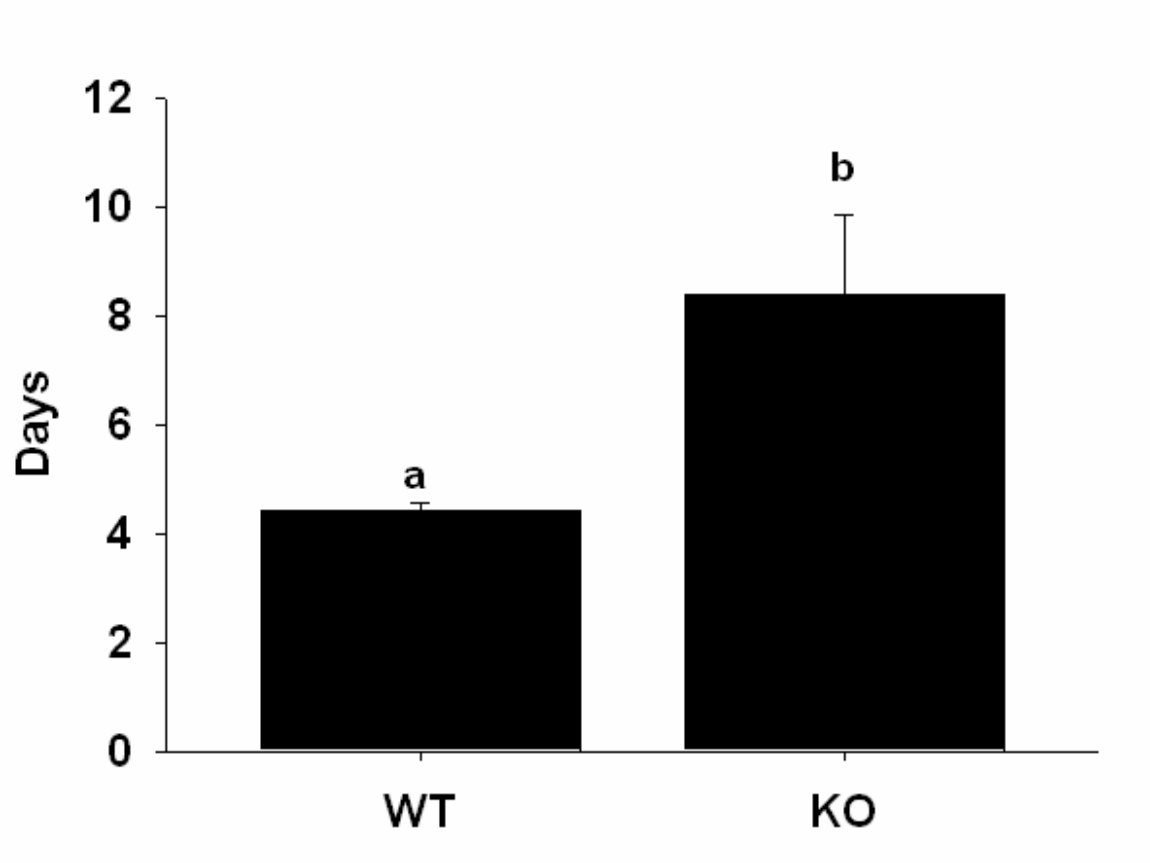

Fig. S5. ASMase$^{-/-}$ mice exhibited irregular estrous cycles. The mean estrous cycle length in ASMase$^{-/-}$ mice is significantly longer than their WT littermates.