Acid sphingomyelinase involvement in tumor necrosis factor α-regulated vascular and steroid disruption during luteolysis *in vivo*

Luiz E. Henkes*†, Brian T. Sullivan*, Maureen P. Lynch*†, Richard Kolesnick‡, Danielle Arsenault§, Mark Puder†§, John S. Davis¶, and Bo R. Rueda*†‖

*Vincent Center for Reproductive Biology, Massachusetts General Hospital, Boston, MA 02114; †Harvard Medical School, Boston, MA 02115; ¶Olson Center for Women's Health, University of Nebraska Medical Center, VA Medical Center, Omaha, NE 68198; ‡Laboratory of Signal Transduction, Memorial Sloan–Kettering Cancer Center, New York, NY 10021; and §Department of Surgery and the Vascular Biology Program, The Children's Hospital, Boston, MA 02115

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved March 27, 2008 (received for review December 28, 2007)

TNF is well known for its role in inflammation, including direct effects on the vasculature. TNF also is implicated in the regulation of reproduction by its actions to affect ovarian steroidogenic cells and to induce apoptosis of corpus luteum (CL)-derived endothelial cells *in vitro*. We hypothesized that the disruption of TNF signaling would postpone the regression of the highly vascularized CL *in vivo*, and this effect could be replicated in mutant mouse models lacking TNF receptor (TNFRI$^{-/-}$) and/or a critical enzyme of TNF signaling, acid sphingomyelinase (ASMase$^{-/-}$). In the current study, the treatment of pseudopregnant mice with the luteolytic mediator prostaglandin F2-α (PGF) significantly increased TNF in the ovaries when compared with saline-treated controls. Treatment with PGF also reduced serum progesterone (P4) concentrations and caused involution of the CL. However, pretreatment of pseudopregnant mice with Etanercept (ETA), a TNF-neutralizing antibody, inhibited the PGF-induced decrease in P4 and delayed luteal regression. A similar outcome was evident in pseudopregnant TNFRI$^{-/-}$ animals. Treatment of luteal microvascular endothelial cells (MVECs) with TNF provoked a significant increase in ASMase activity when compared with the corresponding controls. Furthermore, TNF-induced MVEC death was inhibited in the ASMase$^{-/-}$ mice. The ASMase$^{-/-}$ mice displayed no obvious evidence of luteal regression 24 h after treatment with PGF and were resistant to the PGF-induced decrease in P4. Together these data provide evidence that TNF plays an active role in luteolysis. Further studies are required to determine the deleterious effects of anti-inflammatory agents on basic ovarian processes.

corpus luteum | cytokines | endothelial cells | ovary | progesterone

Tumor necrosis factor α (TNF) is most well known for its role in pathogenic inflammatory disorders, including, but not limited to, asthma, septic shock, rheumatoid arthritis, inflammatory bowel disease, endometriosis, and cancer (1–5). Depending on the immediate microenvironment, TNF can stimulate cell proliferation or induce apoptosis. Aside from its role in inflammation, TNF has been implicated in ovarian follicular development and corpus luteum (CL) regression (6–8). TNFRSFI$^{-/-}$ mice have irregular estrous cycles and spend an inordinate amount of time in diestrus, suggesting a defect in luteal regression (9). However, despite the apparent physiological significance of TNF to fertility, the mechanisms by which TNF initiates and transmits intracellular signals to regulate ovarian function have received limited attention.

The CL, a transient endocrine organ, evolves from the remnants of the ovulating follicle and provides progesterone to induce uterine quiescence in preparation for embryo implantation. The development of the CL has been likened to the development of a tumor that is evidenced by the induction of angiogenesis (10–12). The CL consists of steroidogenic cells, endothelial cells, pericytes, and immune cells. By far the endothelial cells make up the greatest percentage of cells within the CL (13–16), and for a short tenure the CL becomes one of the most highly vascularized organs in the body (17). However, unlike a tumor, the CL undergoes regression in the absence of pregnancy. Luteal regression includes the combination of a decrease in progesterone (P4) synthesis and the involution of the luteal structure. Prostaglandin F2-α (PGF) is considered a primary luteolysin in most mammalian species (18). This role for PGF is further supported by studies of the PGF receptor KO mice, which fail to undergo luteal regression at the end of pregnancy and, as a result, fail to undergo parturition (19). These data suggest that PGF signaling is upstream of the angioregression and structural involution affiliated with luteolysis.

Although PGF is luteolytic *in vivo*, it is not capable of inducing apoptosis of microvascular endothelial cells (MVECs) or steroidogenic cells *in vitro*, suggesting that PGF regulates the expression or activity of other factors that contribute to the process of luteal regression *in vivo*. It is generally accepted that cytokines serve a significant role in PGF-induced luteal regression because they are capable of disrupting steroidogenesis (20–23) and stimulating apoptosis of MVECs (24, 25) and steroidogenic luteal cells (26, 27). Each of the cell types within the CL has the capacity to respond differently to various cytokines (18). *In vitro* experiments clearly show that TNF induces apoptosis in isolated luteal-derived endothelial cells (24), whereas both TNF and IFN-γ are required to induce apoptosis in luteal steroidogenic cells (25, 28, 29). In contrast, another member of the TNF superfamily, FasL, induces apoptosis in luteal steroidogenic cells, but has no effect in isolated luteal MVECs (24, 30).

Cytokines, including TNF, activate and/or signal via the sphingomyelin pathway. Acid sphingomyelinase (ASMase) is responsible for the conversion of sphingomyelin to ceramide, and both ASMase and ceramide are potent inducers of apoptosis in luteinized granulosa cells (26) and MVECs (30–34). Thus, it is possible that the disruption of TNF signaling directly or indirectly by inhibiting ASMase would disrupt luteal regression.

In view of our previous studies demonstrating that the MVECs of the CL are not directly responsive to PGF (35), we postulated that TNF might serve a more direct role in MVEC demise and the

Author contributions: R.K., M.P., J.S.D., and B.R.R. designed research; L.E.H., B.T.S., M.P.L., and D.A. performed research; R.K. and M.P. contributed new reagents/analytic tools; L.E.H., B.T.S., M.P.L., R.K., D.A., M.P., J.S.D., and B.R.R. analyzed data; and L.E.H., B.T.S., M.P.L., J.S.D., and B.R.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

‖To whom correspondence should be addressed. E-mail: brueda@partners.org.

This article contains supporting information online at www.pnas.org/cgi/content/full/0712260105/DCSupplemental.

luteolytic process *in vivo*. Within the vasculature, TNF is primarily known to increase vascular permeability and initiate vascular cell death (36, 37). To better define the physiological significance of TNF in the disruption of microvascular integrity and to confirm or refute the obligatory role of the sphingomyelin pathway in response to TNF, we evaluated its effects on the microvasculature of the CL. In the present study, we tested the hypothesis that the disruption of TNF signaling would postpone luteal regression *in vivo* and that this effect could be replicated in mutant mouse models that lack the TNF receptor (TNFRSFI$^{-/-}$) and/or a critical component of TNF signaling, ASMase$^{-/-}$. Furthermore, we tested whether the failed physiological process of luteal regression could be, at least in part, attributed to the protection of the microvascular component from TNF actions.

Results

Inhibition of TNF Signaling Ameliorates the Luteolytic Effects of PGF. In pseudopregnant mice, PGF treatment causes regression of the CL as evidenced by the loss of CL morphology and a reduction ($P < 0.01$) in P4 (control 16 ± 2.2 ng/ml, $n = 6$ vs. PGF treated 6.3 ± 1.3 ng/ml, $n = 6$). PGF also significantly elevated ($P < 0.05$) ovarian TNF levels (814 ± 68 pg/mg, $n = 5$) within 4 h over the nontreated controls (532 ± 0.11 pg/mg, $n = 5$). To test whether the actions of PGF on luteal regression are mediated by TNF, we treated pseudopregnant mice with Etanercept (ETA), a TNF-neutralizing antibody, before treatment with PGF. Pretreatment with ETA was sufficient to inhibit PGF-induced luteal regression as indicated by the maintenance of the morphology of the CL (Fig. 1 *a–d*) and maintenance of serum progesterone (Fig. 1*e*). To extend these results, we challenged pseudopregnant mice lacking the TNFR1 (TNFRSFI$^{-/-}$) with PGF. The TNFRSFI$^{-/-}$ mice showed no physical evidence of advanced luteal regression (i.e., involution of the structure) [see supporting information (SI) Fig. S1] 24 h after receiving a luteolytic dose of PGF when compared with the controls. Although the level of P4 observed in the TNFRSFI$^{-/-}$ mice was less than controls, it was significantly greater than observed in the PGF-treated WT mice (Fig. 1*f*).

TNF Induces Death in Microvascular Cells but Not the Steroidogenic Cells of the CL. Isolated mouse luteal MVECs treated with 5–50 ng/ml recombinant mouse TNF underwent a significant increase ($P < 0.002$) in apoptosis regardless of whether an inhibitor of protein synthesis, cycloheximide (CHX, 2 μg/ml), was present in the cultures (Fig. S2*a*). The mouse luteal MVECs responded differently than endothelial cells derived from the mouse aorta, whereby CHX was required for TNF-induced apoptosis (Fig. S2*b*). TNF-induced apoptosis of the mouse luteal MVECs was inhibited in cells pretreated with ETA (Fig. 2*a*).

In vivo, the luteal MVECs are immediate neighbors of the steroidogenic cells, which makes up a good portion (30–40%) of the CL (38). To verify that TNF does not directly induce the death of mouse luteal steroidogenic cells, we treated granulosa-luteal cells with TNF, C16 ceramide, dihydro ceramide (inactive form), C2 ceramide (cell-permeable ceramide analogue), or recombinant ASMase. TNF did not induce cell death ($P > 0.05$) in granulosa-luteal cells (Fig. 2*b*). With the exception of the inactive cell-permeable ceramide analogue, all ceramide analogues and recombinant ASMase significantly induced ($P < 0.05$) cell death in granulosa-luteal cells (Fig. 2*b*). Thus, although TNF was not sufficient to induce apoptosis, components of the ceramide-signaling pathway were able to induce death in granulosa-luteal cells.

TNF Binding Does Not Induce Capping but Does Result in Ceramide Accumulation Within Putative Lipid Domains. It has been shown previously that cytokine activation of FAS results in the "capping" or hyperoligomerization of the receptor within the plasma membrane of lymphocytes and hepatocytes (39, 40) and in steroidogenic luteal cells (41). Using the same strategy (41), we were unable to detect any evidence of capping in response to TNF treatment of luteal MVECs (Fig. S3).

TNF binding to TNFRI causes oligomerization of the receptor (42) and is required for TNF-induced death (43). Similarly, oligomerization of FAS and subsequent apoptosis can be inhibited by cholesterol-depleting agents that prevent formation of ceramide-rich platforms (44). In this regard, pretreatment of MVECs with filipin, which blocks intracellular ceramide accumulation and platform formation (40), inhibited TNF-induced

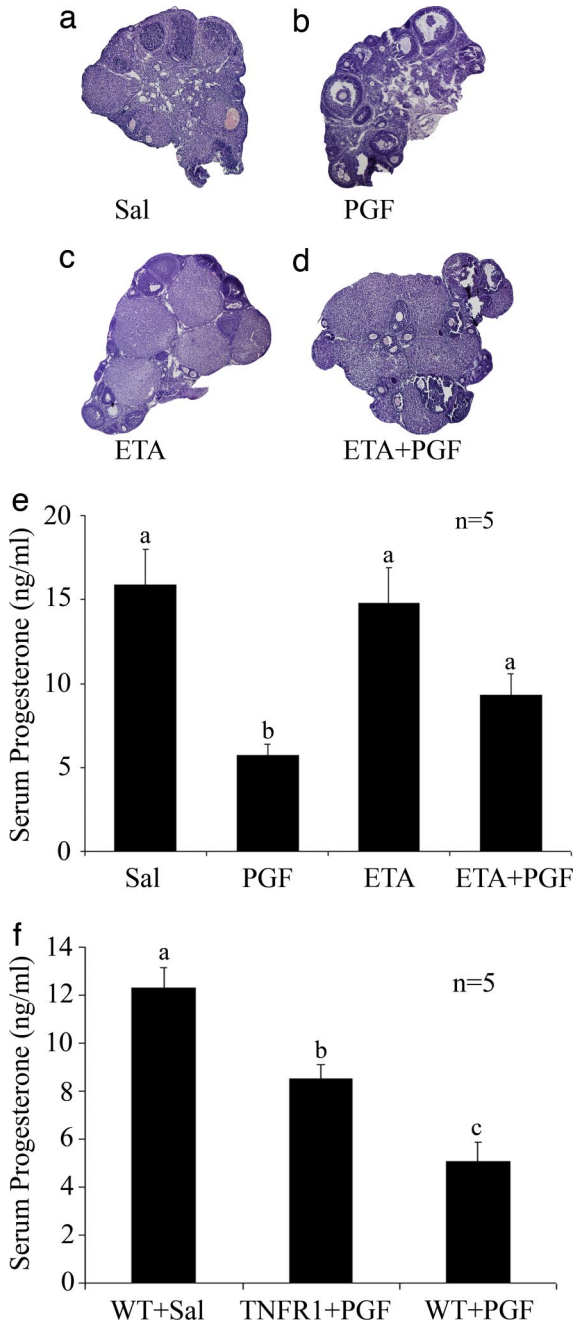

Fig. 1. Interruption of TNF activity impairs regression of the CL. (*a–d*) Morphology of pseudopregnant mouse ovaries 24 h after i.p. injection of saline (*a*), PGF (*b*), TNF-neutralizing antibody (ETA) (*c*), or PGF plus ETA (*d*). (*e* and *f*) Levels of circulating progesterone 24 h after treatment with saline or PGF either in the presence of absence of ETA (*e*) or in control mice (C567BL/6J) or mutant mice lacking the TNF type 1 receptor gene (*Tnfrsf1a*$^{-/-}$) (*f*).

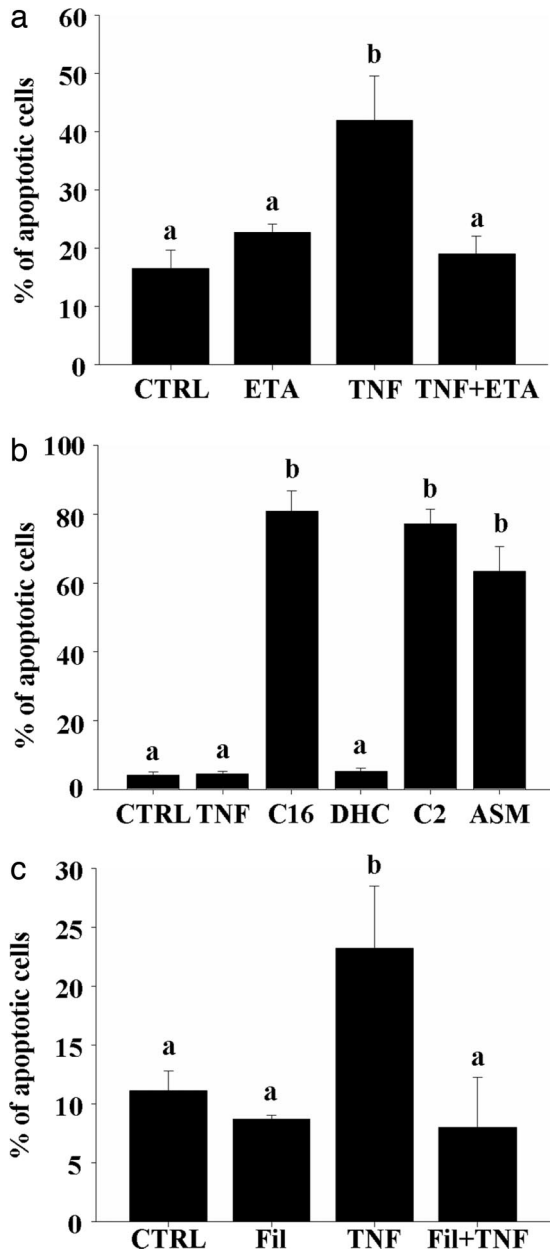

Fig. 2. TNF induces apoptosis of mouse CL MVECs. (*a*) Treatment with 50 ng/ml TNF induced apoptosis of isolated MVECs, which was prevented by treatment with 15 μg/ml ETA. (*b*) Treatment with 50 ng/ml TNF or an inactive ceramide dihydro ceramide (50 μM DHC) did not induce apoptosis of mouse luteinized granulosa cells, whereas treatment with active ceramide analogues (50 μM C2 and 200 nM C16) or 100 milliunits/ml rASMase increased apoptosis. (*c*) Treatment with 0.5 μg/ml filipin to block ceramide accumulation inhibited TNF-induced apoptosis of MVECs.

apoptosis in luteal MVECs (Fig. 2*c*). To test whether TNF increased ceramide in intact luteal MVECs, we used immunofluorescence to monitor ceramide distribution in the cell before and immediately after TNF treatment. Interestingly, treatment with TNF resulted in a punctate distribution of ceramide near the plasma membrane within 30 s of treatment. In contrast, those cells receiving vehicle alone showed no evidence of this phenotype (Fig. S3).

TNF-Induced ASMase Activity. Endothelial cells are a rich source of ASMase (45, 46), a common mediator of cytokine signaling. It has been suggested that ASMase is required to execute the cytokine-induced death response in hepatocytes (47). To further explore the mechanisms by which TNF induces luteal MVEC death, we measured ASMase activity in luteal MVECs treated with TNF. We observed a significant increase ($P < 0.05$) in ASMase activity after treatment of MVEC with TNF when compared with the corresponding controls (Fig. S4), whereas PGF did not increase ASMase in MVECs at any time (data not shown).

TNF Induction of Death in Luteal MVECs Is Inhibited in ASMase$^{-/-}$ Mice. Luteal MVECs were isolated from WT and ASMase$^{-/-}$ mice and then treated with vehicle or TNF 48 h before determination of MVEC death. The levels of apoptotic cells in the vehicle-treated WT and ASMase$^{-/-}$ MVEC and TNF-treated ASMase$^{-/-}$ MVECs were not different (10.7 ± 2.2, 7.4 ± 2.3, and 10.7 ± 1.6% mean ± SEM, $n = 3$, respectively), whereas the level of apoptotic cells in TNF-treated MVECs from WT mice was significantly increased (40.4 ± 2.0%; $P < 0.05$), suggesting that ASMase is required for TNF-induced MVEC death.

Similarly, luteal MVECs were isolated from ASMase$^{-/-}$ mice and treated with vehicle, recombinant ASMase, 50 ng/ml TNF, or a combination of the two and analyzed 48 h later. The percentage of cell death in vehicle, ASMase$^{-/-}$, and TNF-treated luteal MVECs were similar (14.4 ± 2.5%, 18.8 ± 5.8%, and 9.2 ± 2.0%, respectively). Replacement of ASMase, in combination with TNF, increased the percentage of cell death observed (30.3 ± 4.8; $P < 0.05$) when compared with the vehicle, recombinant ASMase, and TNF treatments alone. These data further support the argument that TNF-induced death of MVECs requires ASMase activity.

ASMase-Deficient Mice Are Resistant to the Luteolytic Effects of PGF. Because treatment of pseudopregnant mice with PGF resulted in elevated TNF and inhibition of TNF receptor (by Etanercept or in TNFRSFI$^{-/-}$ mice) results in resistance to PGF-induced luteal regression, we expected that inhibition of ASMase, a major mediator of TNF-induced cell death, also would result in inhibition or delay in luteal regression. Fig. 3 *a–d* illustrates that, similar to the TNFRSFI$^{-/-}$ mice, the mice lacking ASMase were protected from PGF-induced luteal regression. There was no gross evidence of PGF-induced disruption of the CL in the ASMase$^{-/-}$ mice, which was supported by the maintenance of P4 levels relative to the saline-treated controls (Fig. 3*e*). These results suggest that ASMase serves a pivotal role in PGF-induced luteal regression. These findings were further supported by the fact that the ASMase$^{-/-}$ mice have irregular estrous cycles (Fig. S5).

PGF Increases Ovarian TNF in WT but Not ASMase$^{-/-}$ Mice. We also determined the TNF content of ovaries 24 h after treatment of pseudopregnant ASMase$^{-/-}$ mice with PGF or saline. Unlike the increases in TNF we observed in the PGF-treated WT mice (≈60%, see above), the levels of TNF in the ovaries of ASMase$^{-/-}$ mice treated with PGF or vehicle were similar (459 ± 104 and 481 ± 103 pg/mg, respectively; $n = 3$ per group), suggesting that ASMase activity may contribute to the increase in ovarian TNF.

Pretreatment With the TNF Receptor Antagonist Inhibits PGF-Induced Disruption of the Microvascular Density (MVD). PGF has long been suspected to coordinate the reduction in vascular blood flow through the CL and ultimately initiate the disruption of the vascular integrity. If PGF-induced actions on the vascular component are mediated by TNF, its receptor antagonist should prevent PGF-induced changes in the luteal MVD. Pretreatment with ETA prevented the decline in progesterone and, importantly, it also inhibited the decrease in MVD (Fig. 4), suggesting

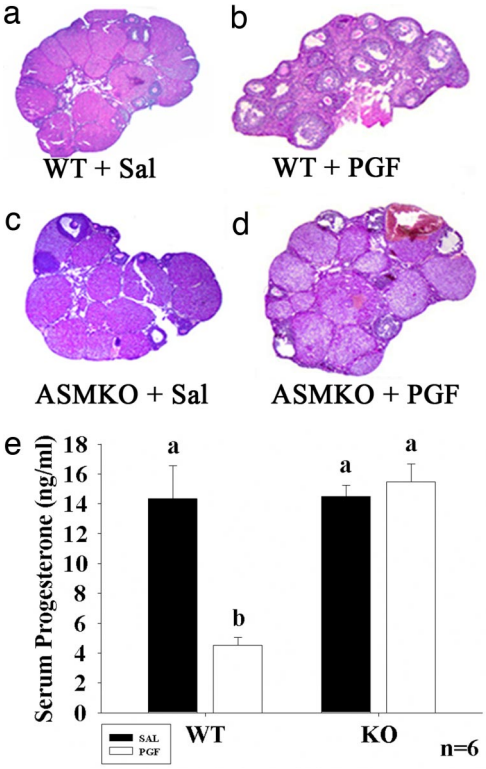

Fig. 3. Mice lacking the acid sphingomyelinase gene, *Smpd1* (ASMase$^{-/-}$), are resistant to PGF-induced CL regression. (*a–d*) Morphology of pseudopregnant mouse ovaries 24 h after injection of saline (*a*) or PGF (*b*) in WT mice or 24 h after injection of saline (*c*) or PGF (*d*) in ASMase$^{-/-}$ mice and corresponding serum progesterone levels (*e*) 24 h after treatment.

that TNF mediates the PGF-induced disruption of the luteal microvasculature.

Discussion

The present studies further define the physiological/biological significance of TNF in the disruption of the microvasculature of the CL and confirm the significance of the sphingomyelin pathway in the disruption of MVECs' integrity in the CL. Our results provide important evidence to support the concept that TNF is a critical mediator of the vascular integrity of the CL. Results of these studies demonstrate that disruption of TNF receptor signaling with TNFR blocking antibody (ETA) or via mice lacking TNFRI inhibits PGF-induced CL regression in mice.

It has been proposed that PGF induces vasoconstriction resulting in a hypoxic environment within the CL, ultimately resulting in apoptosis. Moreover, elegant histological studies describe an ordered and synchronous involution of the luteal vasculature and demonstrate that endothelial cells are among the first cells to show evidence of apoptosis (48–50) during luteal regression. These early studies provided indirect evidence that the vascular component is critical to the survival of the CL. The absence of direct cytotoxic effects of PGF on isolated luteal MVECs argues that luteal regression in response to PGF requires other entities (i.e., cytokines) to disrupt vascular integrity.

The present data suggest that TNF production in response to PGF is involved in the reduction of P4 and involution of the luteal tissue. Treatment with PGF causes a significant increase in TNF during CL regression. Although the mechanisms required for the increase in TNF are currently unknown, PGF was shown to influence TNF promoter activity (51) in HEK cells transfected with the PGF receptor. The PGF-induced increase in TNF preceded the decline in progesterone after PGF treatment. Importantly, we observed that treatment with ETA inhibited the PGF-induced decrease in P4 and delayed luteal regression. A reduction in progesterone can render steroidogenic cells more susceptible to apoptosis (52). Furthermore, TNF was directly cytotoxic to luteal MVEC. The significance of TNF as an important regulator of the luteal regression process was reinforced by studies using TNFRSFI$^{-/-}$ mice. Treatment of TNFRSFI$^{-/-}$ mice with PGF resulted in a moderate decrease in P4 compared with saline-treated WT controls, but the decline was not as steep as seen in PGF-treated WT mice. There also was no morphological evidence of CL regression. Roby *et al.* (9) established that TNFRSFI$^{-/-}$ mice have irregular estrus cycles, spending more time in diestrus than in estrus. Although these investigators did not analyze the CL specifically, CL were present based on the observations that some mice were fertile and the fact that the mice were locked into a diestrus phase, suggesting that there was a problem with luteal regression. These data allow for speculation as to whether this differential response to PGF in the WT and TNFRI$^{-/-}$ mice is due to activation of TNFRII or whether this incomplete blockage could be due to alternative pathways activated by PGF since it reduces P4 synthesis in luteal steroidogenic cells *in vitro*. Studies have shown that TNF can increase luteal production of PGF (53). Therefore, it is also possible that luteal production of PGF is reduced in TNFRI$^{-/-}$ mice, which may account for the partial reduction in P4 synthesis.

Previous studies demonstrated that PGF does not have a direct effect on the luteal MVECs of the CL *in vitro* as is observed with TNF (30, 35). It is of interest that luteal MVECs do not require inhibition of protein synthesis for TNF to induce death as seen in the isolated aortic endothelial cells. Whether the direct cytotoxic actions of TNF are specific to MVEC derived from the CL is not known. Despite its obvious ability to disrupt luteal MVEC, we show that TNF is not cytotoxic to mouse granulosa-luteal cells.

ASMase is responsible for the conversion of sphingomyelin to ceramide (54), and both ASMase and ceramide are potent inducers of apoptosis in luteinized granulosa cells. PGF-induced luteolysis can increase TNF, and, in turn, TNF can stimulate an increase in ASMase activity, which can increase the production of ceramide and can stimulate death of mouse luteal MVECs. Endothelial cells are a rich source of ASMase (45). Therefore,

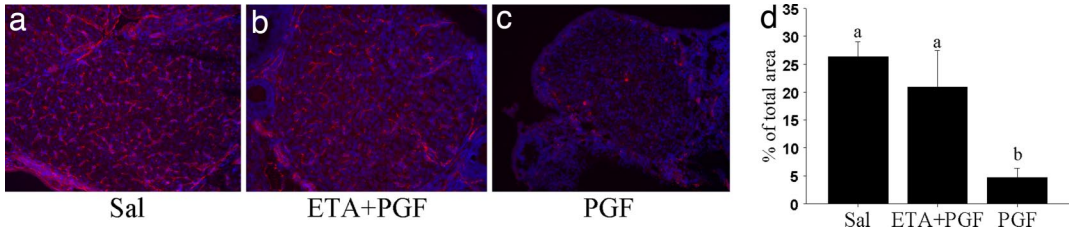

Fig. 4. MVD is disrupted during CL regression. MVD was assessed by staining with CD31 antibody. (*a–c*) Microvessel staining of pseudopregnant mouse ovaries 24 h after injection of saline (*a*) or PGF (*b*) in ETA-pretreated WT mice or 24 h after injection of PGF alone (*c*) and percentage of the MVD in total area counted (*d*).

the rich vascular entity of the CL may provide an ample source of extracellular ASMase during PGF-induced vasoconstriction associated with luteal regression. If true, the availability of ASMase could alter ceramide levels in the surrounding steroidogenic cells, resulting in alterations in membrane dynamics, including membrane fluidity and macrodomain formation.

In FAS-mediated cell death, intracellular accumulation of ceramide results in ceramide-rich platform formation and "capping" of FAS ligand on the cell surface (39). It has been previously proposed that alterations of the cell membrane allowing translocation of ASMase from an intracellular compartment to the cell surface may be critical to produce the ceramide needed for FAS-induced caspase 8 activation and apoptosis (40, 55). Additionally, inactivation of ASMase renders cells resistant to FAS clustering and apoptosis. Our studies showed that inhibition of the ceramide macrodomains with filipin (39, 40) rendered luteal MVEC resistant to TNF-induced death. The present studies also indicate that TNF induces a punctate distribution of ceramide near the plasma membrane within 30 s. We propose that normal luteal function and likely the vascular component are protected in the absence of ASMase because of failure to generate the ceramide-rich compartment necessary for transmembrane signaling of the TNF-mediated death response. This idea is further supported in our *in vitro* studies, whereby luteal MVECs collected from ASMase-deficient mice were resistant to TNF-induced death.

The significance of ASMase is not limited to TNF- or FAS-mediated signaling. LPS-induced death in disseminated endothelial cells requires ceramide generation. Lung, thymus, and fat endothelial cells of ASMase-deficient mice are protected from LPS-induced death (56), similar to what was seen in our present study.

This article emphasizes the complexity of the potential interactions between the vascular elements and the steroidogenic cells, and the prospective signaling pathways that maintain or disrupt luteal function. Collectively, these data support the importance of TNF and ASMase to the disruption of the microvasculature of the CL and its role in luteal regression.

Methods

Animals. WT ASMase$^{-/-}$-deficient female mice (congenic C57BL/6) were generated by mating heterozygous mice. Female mice were genotyped by PCR analysis of tail-snip genomic DNA using specific primers. Prepubertal TNFRI$^{-/-}$ female mice (Tnfrsf1a^{tm1Imx}) were obtained from The Jackson Laboratory. All animal protocols were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and were performed in strict accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

In Vivo* Treatments.** ***Superovulation and generation of pseudopregnant females. Superovulation was induced in all experiments at days 24–27 postpartum by i.p. injection of 10 units of eCG (Professional Compounding Centers of America) followed by 10 units of hCG (Serono Laboratories) 46 h later. Females were placed with vasectomized males after hCG injection, vaginal plugs were checked the next day, and pseudopregnancy was confirmed by vaginal cytology.

Induction of luteolysis. On day 4.5 after plug detection, the mice were treated with normal saline, 0.9% sodium chloride (100 μl per mouse i.p.; Abbot Laboratories), or PGF (10 μg per mouse i.p.; Lutalyse; Amersham Pharmacia-Upjohn).

In vivo *inhibition of TNF actions with ETA.* On days 2.5 and 3.5 after plug detection, animals were treated with or without 375 μg per mouse ETA i.p. (Enbrel; Amgen/Wyeth-Immunex) before injection with or without 10 μg per mouse PGF i.p. on day 4.5. Normal saline (100 μl per mouse i.p.) was used as vehicle control. Ovaries and blood were collected 24 h after treatment. Serum was separated and stored at −80°C until analysis for P4 concentrations. Ovaries were frozen at −80°C for TNF measurement and were fixed/embedded in 4% paraformaldehyde or OCT for histology.

Assessment of MVD in CL sections. To assess the density of the CL microvascular layer, a staining for the endothelial cell-specific cell-surface marker was performed by using Purified Rat Anti-Mouse CD31 (PECAM-1) Monoclonal Antibody (BD Pharmigen). Briefly, frozen sections were cut into 8-μm sections, mounted onto slides, fixed in cold acetone, and washed with 0.1% BSA/PBS. Incubation with CD31-specific antibody at a dilution of 1:50 was then performed overnight, followed by fluorescent secondary antibody (Alexa Fluor 594 chicken anti-rat IgG; Molecular Probes) at a dilution of 1:500 for 1 h. Sections were then washed three times 10 min in 0.1% BSA/PBS at 4°C in the dark, counterstained with Hoescht, mounted with Gel Mount Aqueous Mounting Medium (Sigma), covered with cover glasses, and examined under a fluorescent microscope (Nikon). Assessment of MVD was performed by computer-assisted image analysis as described previously (57) using Image-Pro Plus image analysis software (Media Cybernetics).

Serum progesterone. P4 was extracted from the thawed serum with diethyl ether and analyzed as described previously (58).

TNFα measurement. Ovaries were homogenized in 1 ml of PBS, and the suspension was centrifuged at 10,000 × *g* for 10 min. TNF was measured in the supernatant by using a mouse TNFα enzyme immunometric assay kit (Assay Designs) according to the manufacturer's instructions. The TNF concentration was normalized against the ovarian weight.

In Vitro* Analysis of MVECs.** ***MVEC isolation. Luteal MVECs were isolated from isolated CL of pseudopregnant WT and ASMase$^{-/-}$ through positive magnetic cell sorting by Dynabeads according to the manufacturer's instructions. Specific details are described in *SI Methods*.

Aortic endothelial cell isolation. Cells were isolated through positive magnetic cell sorting and plated as described above, details are provided in *SI Methods*.

In vitro *endothelial cell treatments.* Once 90% confluency was reached, the medium was replaced with serum-free medium for 24 h. At the end of the preincubation period, the MVECs were treated with or without 50 ng/ml recombinant mouse TNF (Cell Sciences) in the presence or absence of 2 μg /ml cycloheximide (CHX), 0.5 μg/ml filipin (Sigma), monoclonal antibody to 1:50 ceramide (Alexis Corporation), 100 milliunits/ml rASMase (Biomol), or 15 μg/ml Etanercept (ETA). Mouse aortic endothelial cells were treated with or without 50 ng/ml recombinant mouse TNF in the presence or absence of 2 μg/ml CHX.

In vitro *assessment of apoptosis.* To identify apoptosis-inducing agents in MVECs, culture medium was removed after treatments, and cells were washed one time with ice-cold PBS and fixed for 10 min with 4% PFA. The cells were rinsed one time with PBS and nuclei stained by placing 200 μl of glycerol (80% in 1× PBS) containing 2 μg/ml Hoechst 33258 in each well. The average number of apoptotic cells in 10 fields was determined and presented as the percentage of the total number of cells.

Statistical analysis. Data are displayed as mean ± SEM. Statistical analysis was calculated by a one-way ANOVA and the two-tailed paired *t* test using GraphPad Prism software.

ACKNOWLEDGMENTS. This work was supported in part by National Institutes of Health Grants HD35934 (to B.R.R.) and HD38813 (to J.S.D.), National Institutes of Health/National Cancer Institute Grant CA085704 (to R.K.), and the Department of Veterans' Affairs (J.S.D.).

1. Oettgen HF, *et al.* (1980) Endotoxin-induced tumor necrosis factor. *Recent Results Cancer Res* 75:207–212.
2. Weems CW, Reynolds LP, Huie JM, Hoyer GL, Behrman HR (1985) Effects of prostaglandin E1 or E2 (PGE1; PGE2) on luteal function and binding of luteinizing hormone in nonpregnant ewes. *Prostaglandins* 29:161–173.
3. Vassalli P (1992) The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411–452.
4. Sarzi-Puttini P, Atzeni F, Shoenfeld Y, Ferraccioli G (2005) TNF-alpha, rheumatoid arthritis, and heart failure: A rheumatological dilemma. *Autoimmun Rev* 4:153–161.
5. Mukhopadhyay S, Hoidal JR, Mukherjee TK (2006) Role of TNFalpha in pulmonary pathophysiology. *Respir Res* 7:125.
6. Terranova PF (1997) Potential roles of tumor necrosis factor-alpha in follicular development, ovulation, and the life span of the corpus luteum. *Domest Anim Endocrinol* 14:1–15.
7. Gadsby J, Rose L, Sriperumbudur R, Ge Z (2006) The role of intra-luteal factors in the control of the porcine corpus luteum. *Soc Reprod Fertil Suppl* 62:69–83.
8. Okuda K, Sakumoto R (2003) Multiple roles of TNF super family members in corpus luteum function. *Reprod Biol Endocrinol* 1:95.
9. Roby KF, Son DS, Terranova PF (1999) Alterations of events related to ovarian function in tumor necrosis factor receptor type I knockout mice. *Biol Reprod* 61:1616–1621.
10. Gaytan F, Bellido C, Morales C, Aguilar E, Sanchez-Criado JE (1997) Evidence for steroidogenic luteal cell hypertrophy and hyperplasia during pregnancy in the rat. *J Endocrinol* 154:211–217.

11. Folkman J (2000) *Tumor Angiogenesis* (Decker, Ontario, Canada).
12. Reynolds LP, Grazul-Bilska AT, Redmer DA (2000) Angiogenesis in the corpus luteum. *Endocrine* 12:1–9.
13. Davis JS, Rueda BR, Spanel-Borowski K (2003) Microvascular endothelial cells of the corpus luteum. *Reprod Biol Endocrinol* 1:89.
14. Wiltbank MC, Gallagher KP, Christensen AK, Brabec RK, Keyes PL (1990) Physiological and immunocytochemical evidence for a new concept of blood flow regulation in the corpus luteum. *Biol Reprod* 42:139–149.
15. Redmer DA, Reynolds LP (1996) Angiogenesis in the ovary. *Rev Reprod* 1:182–192.
16. Zheng J, Fricke PM, Reynolds LP, Redmer DA (1994) Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biol Reprod* 51:623–632.
17. Liptak AR, *et al*. (2005) Cooperative expression of monocyte chemoattractant protein 1 within the bovine corpus luteum: Evidence of immune cell–endothelial cell interactions in a co-culture system. *Biol Reprod* 72:1169–1176.
18. Davis JS, Rueda BR (2002) The corpus luteum: An ovarian structure with maternal instincts and suicidal tendencies. *Front Biosci* 7:d1949–d1978.
19. Sugimoto Y, *et al*. (1997) Failure of parturition in mice lacking the prostaglandin F receptor. *Science* 277:681–683.
20. Fairchild DL, Pate JL (1991) Modulation of bovine luteal cell synthetic capacity by interferon-gamma. *Biol Reprod* 44:357–363.
21. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW (2000) Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 80:1–29.
22. Pate JL (1995) Involvement of immune cells in regulation of ovarian function. *J Reprod Fertil Suppl* 49:365–377.
23. Pate JL, Landis Keyes P (2001) Immune cells in the corpus luteum: Friends or foes? *Reproduction* 122:665–676.
24. Friedman A, Weiss S, Levy N, Meidan R (2000) Role of tumor necrosis factor alpha and its type I receptor in luteal regression: Induction of programmed cell death in bovine corpus luteum-derived endothelial cells. *Biol Reprod* 63:1905–1912.
25. Pru JK, Lynch MP, Davis JS, Rueda BR (2003) Signaling mechanisms in tumor necrosis factor alpha-induced death of microvascular endothelial cells of the corpus luteum. *Reprod Biol Endocrinol* 1:17.
26. Pru JK, Hendry IR, Davis JS, Rueda BR (2002) Soluble Fas ligand activates the sphingomyelin pathway and induces apoptosis in luteal steroidogenic cells independently of stress-activated $p38^{MAPK}$. *Endocrinology* 143:4350–4357.
27. Quirk SM, Cowan RG, Joshi SG, Henrikson KP (1995) Fas antigen-mediated apoptosis in human granulosa/luteal cells. *Biol Reprod* 52:279–287.
28. Suter J, *et al*. (2001) Mediators of interferon gamma-initiated signaling in bovine luteal cells. *Biol Reprod* 64:1481–1486.
29. Benyo DF, Pate JL (1992) Tumor necrosis factor-alpha alters bovine luteal cell synthetic capacity and viability. *Endocrinology* 130:854–860.
30. Pru JK, Lynch MP, Davis JS, BR R (2002) Tumor necrosis factor is a potent inducer of luteal microvascular endothelial cell death. *J Soc Gynecol Investig* 9 (Supp 1):336A.
31. Paris F, *et al*. (2002) Sphingosine 1-phosphate preserves fertility in irradiated female mice without propagating genomic damage in offspring. *Nat Med* 8:901–902.
32. Pru JK, *et al*. (2001) Interferon-tau suppresses prostaglandin F2α secretion independently of the mitogen-activated protein kinase and nuclear factor kappa B pathways. *Biol Reprod* 64:965–973.
33. Morita Y, *et al*. (2000) Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. *Nat Med* 6:1109–1114.
34. Tilly JL, Kolesnick RN (1999) Sphingolipid signaling in gonadal development and function. *Chem Phys Lipids* 102:149–155.
35. Cavicchio VA, *et al*. (2002) Secretion of monocyte chemoattractant protein-1 (MCP-1) by endothelial cells of the bovine corpus luteum: Regulation by cytokines but not prostaglandin F2 alpha. *Endocrinology* 143:3582–3589.
36. Brett J, *et al*. (1989) Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J Exp Med* 169:1977–1991.
37. Royall JA, *et al*. (1989) Tumor necrosis factor and interleukin 1 alpha increase vascular endothelial permeability. *Am J Physiol* 257:L399–L410.
38. Wiltbank MC (1994) Cell types and hormonal mechanisms associated with mid-cycle corpus luteum function. *J Anim Sci* 72:1873–1883.
39. Cremesti A, *et al*. (2001) Ceramide enables fas to cap and kill. *J Biol Chem* 276:23954–23961.
40. Grassme H, *et al*. (2001) CD95 signaling via ceramide-rich membrane rafts. *J Biol Chem* 276:20589–20596.
41. Fanzo JC, *et al*. (2003) CD95 rapidly clusters in cells of diverse origins. *Cancer Biol Ther* 2:392–395.
42. Roulston A, Reinhard C, Amiri P, Williams LT (1998) Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J Biol Chem* 273:10232–10239.
43. van Loo G, *et al*. (2002) The role of mitochondrial factors in apoptosis: A Russian roulette with more than one bullet. *Cell Death Differ* 9:1031–1042.
44. Grassme H, *et al*. (2001) CD95 signaling via Ceramide-rich membrane rafts. *J Biol Chem* 276:20589–20596.
45. Marathe S, *et al*. (1998) Human vascular endothelial cells are a rich and regulatable source of secretory sphingomyelinase. Implications for early atherogenesis and ceramide-mediated cell signaling. *J Biol Chem* 273:4081–4088.
46. Andrieu-Abadie N, Levade T (2002) Sphingomyelin hydrolysis during apoptosis. *Biochim Biophys Acta Mol Cell Biol Lipids* 1585:126–134.
47. Garcia-Ruiz C, *et al*. (2003) Defective TNF-alpha-mediated hepatocellular apoptosis and liver damage in acidic sphingomyelinase knockout mice. *J Clin Invest* 111:197–208.
48. O'Shea JD, Nightingale MG, Chamley WA (1977) Changes in small blood vessels during cyclical luteal regression in sheep. *Biol Reprod* 17:162–177.
49. Azmi TI, O'Shea JD (1984) Mechanism of deletion of endothelial cells during regression of the corpus luteum. *Lab Invest* 51:206–217.
50. Sawyer HR, Niswender KD, Braden TD, Niswender GD (1990) Nuclear changes in ovine luteal cells in response to PGF2 alpha. *Domest Anim Endocrinol* 7:229–237.
51. Fujino H, Regan JW (2004) Prostaglandin F2α amplifies tumor necrosis factor-α promoter activity by the FPB prostanoid receptor. *Biochem Biophys Res Commun* 317:1114–1120.
52. Rueda BR, *et al*. (2000) Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. *Biol Reprod* 62:269–276.
53. Townson DH, Pate JL (1996) Mechanism of action of TNF-alpha-stimulated prostaglandin production in cultured bovine luteal cells. *Prostaglandins* 52:361–373.
54. Kolesnick R, Hannun YA (1999) Ceramide and apoptosis. *Trends Biochem Sci* 24:224–245, 227.
55. Grassme H, Cremesti A, Kolesnick R, Gulbins E (2003) Ceramide-mediated clustering is required for CD95-DISC formation. *Oncogene* 22:5457–5470.
56. Haimovitz-Friedman A, *et al*. (1997) Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med* 186:1831–1841.
57. King TW, Brey EM, Youssef AA, Johnston C, Patrick CW, Jr (2002) Quantification of vascular density using a semiautomated technique for immunostained specimens. *Anal Quant Cytol Histol* 24:39–48.
58. Carambula SF, *et al*. (2003) Prostaglandin F2alpha- and FAS-activating antibody-induced regression of the corpus luteum involves caspase-8 and is defective in caspase-3 deficient mice. *Reprod Biol Endocrinol* 1:15.