Introduction
Portal hypertension is an important and potentially fatal complication of liver disease whereby cellular and fibrotic alterations manifest to increase portal venous pressure [1]. Increased portal venous pressure promotes the redirection of portal venous blood flow towards esophageal and gastric veins that dilate and hemorrhage [2]. This process is exacerbated by the formation of a hyper dynamic circulation that increases venous blood flow to the mesentery and liver [3]. Reduction of this increased forward flow is very important in reducing portal venous flow and preventing bleeding and death [4]. Numerous reports have identified the over expression of vasodilators as a key factor in the development of PHT associated hyper dynamic circulation, in particular, endothelial nitric oxide synthase (eNOS) catalysis of nitric oxide (NO) production and cyclooxygenase enzyme biosynthesis of prostaglandin I2 (PGI2) [5-7]. The cytokine tumor necrosis factor-a (TNFa) is reported to be important in the induction of eNOS and thus the development of PHT [8].

Brief Communication
Tumor necrosis factor alpha signaling in the development of experimental murine pre-hepatic portal hypertension

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Abstract: The cytokine tumor necrosis factor alpha (TNFa) has previously been identified in the development of portal hypertension (PHT) by facilitating portal venous and systemic hyperemia. TNFa is reported to contribute to hyperemia via endothelial nitric oxide synthase (eNOS) induction and nitric oxide (NO) production. This study examines this hypothesis by utilizing TNFa receptor knockout mice and a murine model of pre-hepatic PHT. Plasma TNFa and NOx and tissue TNFa mRNA levels were determined in wild-type mice 0-7d post induction of pre-hepatic PHT by partial portal vein ligation (PVL). TNFa receptor knockout mice also received PVL or sham surgery and splenic pulp pressure, abdominal aortic flow and portal-systemic shunting were recorded 7d following. Portal pressure and systemic hyperemia developed rapidly following PVL. Plasma NOx was increased temporarily 2-3 days following PVL and returned to baseline by day 7. Circulating TNFa was below detectable limits of the ELISA used, as such no increase was observed. Hepatic and vascular TNFa mRNA levels were transiently changed after PVL otherwise there was no significant change. TNFa receptor targeted gene deletion did not ameliorate plasma NOx following PVL and had no effect on the development of PHT. TNFa receptor signaling plays no detectable role in the development of systemic hyperemia in the murine model of pre-hepatic PHT. Consequently, increased TNFa observed in intra-hepatic inflammatory models (CCl4) and in patients is probably related to inflammation associated with intra-hepatic pathology. Alternatively, TNFa may be signaling via a TNFa receptor independent mechanism.

Keywords: Knockout, eNOS, nitric oxide, TNF alpha, shunting, hyperemia, splenic pulp pressure, abdominal aortic flow
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Evidence suggests that TNFRI is most important for circulating TNFα, while membrane-bound TNFα associates with TNFRII [14]. It is also suggested that TNFRI binds TNFα and transfers it to TNFRI, which then is activated and initiates a physiological response [15]. At 37 °C, soluble TNF binds to TNFRI with a Kd of 20 pM, and to TNFRII with a Kd of 300 pM. Normal levels of circulating TNFα are reported to be in the 10-80 pg/mL range [16]. Consequently, TNFα signaling will normally be mediated through TNFRI. TNFRI is a 55 kDa, 455 amino acid residue transmembrane glycoprotein that is expressed by virtually all nucleated mammalian cells [17, 18]. Activation can trigger apoptosis and NF-κB expression, although the mechanism determining the choice of pathways is not clear [14, 19]. Among the numerous cells known to express TNFRI are hepatocytes and endothelial cells [16, 20]. TNFRII is a 75 kDa, 461 amino acid residue transmembrane glycoprotein [21] and is expressed on endothelial cells [22]. Targeting TNFα may have therapeutic benefits. Anti TNFα antibody infusion reduces hyper-dynamic circulation [23] and in cirrhotic patients produces a highly significant, early, and sustained reduction in portal pressure [24, 25]. However, the precise mechanism by which TNFα participates in PHT pathology is not known. In this study we examine the role of TNFα in the development of PHT by utilizing double TNF receptor knockout mice (B6;129S-Tnfrsf1atmImxTnfrsf1btm1Imx/j) [26] and a murine model of pre-hepatic PHT. We report that TNFα is not induced significantly and known TNFα receptors do not play a role in the pre-hepatic non-inflammatory portal vein ligation model of PHT in mice.

Materials and methods

All studies were approved by the Indiana University institutional animal care and use committee and adhered to AAALC and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolate cages on a 12-hour light/dark cycle and were allowed access to food and water ad libitum. Unless otherwise stated all products were purchased from Sigma, MO.

Pre-hepatic PHT model; Partial portal vein ligation: Mice were anesthetized using halothane inhalation. A midline laparotomy was performed and the portal vein was exposed. A blunt-ended 27-gauge needle was placed alongside the portal vein and a 4-0 silk suture was tied around the vein and needle, after which the needle was withdrawn, producing a standardized stenosis. In sham animals the procedure consisted of dissection and visual inspection of the portal vein without ligature. The abdomen was closed and the animals were allowed to recover under a heat lamp. TNFα double receptor knockout mice (TNFr1r2-/-) and wild-type mice (B6;129p2) were purchased from Taconic (Germantown, NY).

Physiological measurements: Physiological measurements were performed as previously described. [6] At the indicated times animals were anesthetized and subjected to laparotomy to allow physiological measurements to be taken. Splenic pulse pressure (SPP) was measured as an index of portal venous pressure. To measure SPP, a micro-tip pressure transducer (#SPR-839 Millar Instruments, CA) was inserted into the spleen pulp. Abdominal aortic flow (Qao) was measured by placing an ultrasonic Doppler flow probe (Transonic #11RB) around the abdominal aorta between the diaphragm and celiac artery. Flow rates were obtained with a Transonic T201 Blood Flow Meter (Transonic Instruments, Ithaca, NY). Aortic blood flows were recorded and standardized per gram of body weight.

TNFα and NOx: Serum was obtained by cardiac puncture and withdrawn into a heparinized syringe. Serum NOx and TNFα levels were quantified using commercially available kits (Oxford Biomedical Research MA, Thermo-Clinical PA) as per manufacturer’s instructions.

Urinary 2,3-dinor-6-keto prostaglandin F1α: 2,3-Dinor-6-keto prostaglandin F1α (2,3-dinor-6-keto-PGF1α) is a stable β-oxidation metabolite of 6-keto-PGF1α and levels have previously been used to quantify systemic PG12 biosynthesis in mice and humans [27, 28]. TNF receptor null PVL and sham mice were placed in metabolic cages and 24hr urine was collected. Urine was stored at 4°C prior to analysis. 2,3-dinor-6-keto-PGF1α was extracted as previously described [5] and quantified using a competitive ELISA for 2,3-dinor-6-keto-PGF1α and 6-keto-PGF1α (Assay Designs, Ann Arbor, MI) as per manufacturer’s instructions.

TNFα RT-PCR: Hepatic and vascular tissues were snap frozen immediately following dissec-
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Tissues were homogenized in guanidine-isothiocyanate. RNA was extracted by phase separation following the addition of phenol and chloroform. cDNA was synthesized from RNA using invitrogen RT kit as per manufacturers instructions. Murine TNFa gene-specific primers were used to detect TNFa (CTCTTCAAGGGACAAGGCTG, CGGACTCCGCAAAGTCTAAG) β-actin gene specific primers (ATCCTGGTCTGGACCTGGCT, CTTGCTGATCCACATCTGCTG) were also used as a housekeeping gene to correct for mRNA levels. PCR conditions were 30 cycles for TNFa and 25 cycles for β-actin of 1 minute each of 94°C, 60°C, and 74°C. PCR products were separated on 1% agarose/TBE/ethidium bromide gels and observed under UV illumination.

Statistics: The data shown are means±S.E., with 4-7 animals per experimental group. Statistical significance was estimated using one-way ANOVA statistical analysis. A value of p<0.05 was considered significant.

Results

Circulating TNFa and TNFa expression in liver and vascular tissue: To examine changes in TNFa expression and TNFa circulation following PVL wild type mice received sham-operations or portal vein-ligated; Plasma, aorta and liver were collected 0-24hr, 2, 5 days later. TNFa levels were measured using a commercially available ELISA kit. Circulating TNF levels in untreated mice were below the detection limit of the assay; at no time following PVL was TNFa detectable. As a positive control, serum was collected from mice that had been given an intraperitoneal injection of LPS. We found that LPS caused TNF levels to rise to 100 pmoles/ml 60 min after LPS injection. TNFa mRNA levels were
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quantified in liver, portal vein, inferior vena cava and aorta cDNA by RT-PCR. Levels were normalized to b-actin, which was amplified in parallel. The acute response to PVL (0-24hr) involved a transient reduction in TNFa expression 1-3hr in both aortic and hepatic tissues. (Figure 1A-B) Chronically TNFa mRNA was transiently increased in the liver 2d following PVL (Figure 1C) but there was no increase in aortic levels. (Figure 1D) There was no significant change in TNFa expression following PVL in either the portal vein or the IVC. We conclude that if there is a response to PVL that includes elevation of TNFa levels, it is hepatic minor, transient, and does not lead to detectable levels of circulating TNFa.

Development of portal hypertension in TNFa receptor deficient mice: To determine the role of TNFa receptor signaling in pre-hepatic portal hypertension wild type and TNFa receptor null mice received either PVL or sham surgery. Seven days following PVL or sham surgery splenic pulp pressure, abdominal aortic flow and portal systemic shunting were measured. Abdominal flow was initially decreased (1d) following PVL after which levels increased rapidly and were significantly greater then sham controls. Splenic pulp pressure and portal systemic shunting increased steadily following PVL and was significantly increased at all time points when compared to shams. TNFa exerts its effects by binding to one of two types of cell surface receptor. Mice with targeted mutations in the genes for both receptors were also subjected to portal vein-ligation. Physiologically, mice deficient in both TNF receptors (TNFr1r2/−)

Figure 2. Splenic pulp pressure, aortic blood flow, serum NOx and urine 2,3-d-6-keto PGF1α following sham or PVL surgery in TNF receptor null mice. Pre-hepatic portal hypertension was induced in wild type and TNFa receptor null mice by partial ligation of the portal vein. (A-B) 7d post ligation aortic blood flow (A) and splenic pulp pressure (B) were measured. Both markers of PHT were increased following PVL when compared to shams. There was no significant difference between TNFa receptor null mice and wild type controls. (C-D). Serum NOx is a marker of NO production via nitric oxide synthase activity. Urine 2,3-dinor-6-keto PGF1α level is a marker of systemic PGI2 levels and cyclooxygenase activity. NOx and 2,3-dinor-6-keto PGF1α were increased in TNFa receptor null mice following PVL. Data represents mean±S.E from 5 mice.
responded to PVL by an increase in splenic pulp pressure, aortic flow and portal systemic shunting in a manner indistinguishable from that of wild-type mice (Figure 2A,B). From this data we conclude that TNF receptor signaling is not essential for altered hemodynamics in the PVL pre-hepatic model.

NOx and 2,3-dinor-6-k-PGF1α levels following PVL in TNFa receptor deficient mice: To examine the effect of TNFa receptor deletion on NOS and COX activity plasma and 24hr urine were collected 0-7d following PVL or sham surgery on TNF receptor null mice. Previous studies have shown that plasma NOx and urine 2,3-donor-6-k-PG1α (markers of NOS and COX activity respectively) are increased following PVL [5, 6]. NOx: Similar to results seen in wild types serum NOx increased in TNFa receptor null mice 5d following PVL (86.3±20.3mM) after which levels were not significantly different to sham operated controls (41±4.6mM). (Figure 2C) However, the increase was later then that typically seen in wild types (2-3d). 2,3-donor-6-k-PG1α: seven-day urine 2,3-donor-6-k-PG1α levels were significantly greater in PVL TNF receptor null mice (118%, P=0.04) when compared to shams. (Figure 2D) This increase was lower then that seen in wild type mice (336%). These data suggests that eNOS and COX activation in PHT is partly TNFa receptor mediated.

Discussion

TNFa is a potent cytokine and is reported to be important in numerous pathological events [29]. This manuscript reports studies designed to investigate TNFa signaling in PHT associated vascular hyper-dynamic circulation. Previous studies have identified TNFa as a key mediator in the development in PHT [30]. Using a murine model of pre-hepatic PHT we were unable to show an increase in TNFa in the absence of liver disease. Moreover, TNFa receptor null mice developed PHT in a similar manner to wild type controls.

This data conflicts with previous reports that have shown the potential of targeting TNFa to improve PHT and non-PHT vasculopathies [25, 31]. Administration of anti TNFa polyclonal antibodies to PVL rats has been shown to reduce portal venous flow, but because portal systemic shunt was also reduced the portal pressure was not reduced [25]. Portal pressure is dependent upon hepatic resistance and portal venous flow (similar to Ohm’s law V=IR). In the PVL model hepatic resistance is constant due to the mechanical stenosis but is countered by the development of portal systemic shunts that decompress the portal system. Consequently, the reduction in portal systemic shunt negates any reduction in portal venous flow. Our data suggests that any role for TNFa in pre-hepatic portal hypertension is not mediated through known TNFa receptors. However, TNFa may still be involved via a non-TNFa receptor signaling mechanism. The success of anti TNFa antibodies in reducing portal flow is evidence of this. TNFa may have efficacy for additional receptors that have, to date, not been identified. Moreover, TNFa is known to signal through peroxisome proliferator-activated receptorγ (PPARγ) [32]. Currently there is no information regarding the role of PPARg and the development of PHT. PPARg antagonists have been synthesized [33] but there use clinically would probably be limited. PPARs are a ubiquitous group of nuclear receptor proteins that function as transcription factors regulating the expression of genes involved in regulation of cellular differentiation, development, and metabolism [34]. Attempts to generate PPARg knock mice showed that homozygote knockouts were not viable but that heterozygotes were and had a significantly altered phenotype [35]. More recently, cell specific PPARg gene knockout mice have been successful generated [36, 37]. These mice may help investigate the mechanism by which TNFa participates in the pathophysiology of PHT.

The data presented in this manuscript also conflicts with the work reported by Wang et al., whom describe increased plasma TNFa in PVL treated rats [38]. This is probably species related. Studies in other tissues have shown significantly stronger induction of TNFa and iNOS expression in rats when compared to mice. [39]. Moreover, data generated using gene-deleted mice should always be taken with caution. As with all gene deletions there is a potential for adaptive effects such that observed effects are not directly related to an absence of TNFa receptor.

In conclusion, the development of systemic hyperemia and portal hypertension in the PVL pre-hepatic model of PHT is TNFa receptor inde-
pendent. Moreover, we found no evidence of increased TNFα expression in the absence of hepatic injury or inflammation. However, we cannot discount that TNFα levels may have been over-expressed on a micro-localized level. TNFα naturalizing antibodies argue in favor of TNFα but the data presented in this manuscript suggests that any role would not be via the two main TNFα receptors. Further research is required to better understand this interesting pathway in order to provide alternative options and improve clinical outcomes. At present the mainstay prophylactic treatments for patients with PHT, and are at risk of variceal formation and hemorrhage, are β-blockers and statins. Only 50% of patients respond normally. [4] Reduction of portal systemic shunting by the use of neutralizing TNFα antibody highlights TNFα synthesis and signaling as a fruitful target. Positive clinical outcomes following TNFα blockade [24] in combination with the emergence of infliximab and etanercept (fusion proteins consisting of the human TNFα receptor protein and human IgG1), supports direct intervention. However, our understanding for the role of TNFα in PHT is not complete. At present we do not know by what pathway TNFα signals and influences the development of PHT. Delineation of this pathway may provide selective and improved targets then direct blockade.

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