

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 (TNF α) has previously been identified in the development of portal hypertension (PHT) by facilitating portal venous and systemic hyperemia. TNF α 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 TNF α receptor knockout mice and a murine model of pre-hepatic PHT. Plasma TNF α and NOx and tissue TNF α mRNA levels were determined in wild-type mice 0-7d post induction of pre-hepatic PHT by partial portal vein ligation (PVL). TNF α 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 TNF α was below detectable limits of the ELISA used, as such no increase was observed. Hepatic and vascular TNF α mRNA levels were transiently changed after PVL otherwise there was no significant change. TNF α receptor targeted gene deletion did not ameliorate plasma NOx following PVL and had no effect on the development of PHT. TNF α receptor signaling plays no detectable role in the development of systemic hyperemia in the murine model of pre-hepatic PHT. Consequently, increased TNF α observed in intra-hepatic inflammatory models (CCl $_4$) and in patients is probably related to inflammation associated with intra-hepatic pathology. Alternatively, TNF α may be signaling via a TNF α receptor independent mechanism.

Keywords: Knockout, eNOS, nitric oxide, TNF alpha, shunting, hyperemia, splenic pulp pressure, abdominal aortic flow

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 I $_2$ (PGI $_2$) [5-7]. The cytokine tumor necrosis factor- α (TNF α) is reported to be important in the induction of eNOS and thus the development of PHT [8].

Human TNF α is a 233 amino acid, non-glycosylated polypeptide that exists as either a transmembrane or soluble protein [9]. The soluble protein is created by a proteolytic cleavage via TNF α converting enzyme [10]. While both membrane-bound and soluble TNF α are biologically active, soluble TNF α is reported to be more potent [11]. TNF α expression has been documented in many cells; include hepatocytes and endothelial cells [12, 13]. Two receptors for TNF α have been identified. TNFR1 and TNFR2 with a shared sequence of approximately 20%.

Evidence suggests that TNFR1 is most important for circulating TNF α , while membrane-bound TNF α associates with TNFR2 [14]. It is also suggested that TNFR2 binds TNF α and transfers it to TNFR1, which then is activated and initiates a physiological response [15]. At 37 °C, soluble TNF binds to TNFR1 with a K_d of 20 pM, and to TNFR2 with a K_d of 300 pM. Normal levels of circulating TNF α are reported to be in the 10-80 pg/mL range [16]. Consequently, TNF α signaling will normally be mediated through TNFR1. TNFR1 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- κ B expression, although the mechanism determining the choice of pathways is not clear [14, 19]. Among the numerous cells known to express TNFR1 are hepatocytes and endothelial cells [16, 20]. TNFR2 is a 75 kDa, 461 amino acid residue transmembrane glycoprotein [21] and is expressed on endothelial cells [22]. Targeting TNF α may have therapeutic benefits. Anti TNF α 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 TNF α participates in PHT pathology is not known. In this study we examine the role of TNF α in the development of PHT by utilizing double TNF receptor knockout mice (B6;129S-Tnfrsf1a^{tm1mx}Tnfrsf1b^{tm1lmx}/j) [26] and a murine model of pre-hepatic PHT. We report that TNF α is not induced significantly and known TNF α 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 por-

tal 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 ligation. The abdomen was closed and the animals were allowed to recover under a heat lamp. TNF α 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 pulp 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 in to 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.

TNF α and NO $_x$: Serum was obtained by cardiac puncture and withdrawn into a heparinized syringe. Serum NO $_x$ and TNF α 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 F1a: 2,3-Dinor-6-keto-prostaglandin F1a (2,3-dinor-6-keto-PGF1a) is a stable b-oxidation metabolite of 6-keto-PGF1a and levels have previously been used to quantify systemic PGI $_2$ 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-PGF1a was extracted as previously described [5] and quantified using a competitive ELISA for 2,3-dinor-6-keto-PGF1a and 6-keto-PGF1a (Assay Designs, Ann Arbor, MI) as per manufacturer's instructions.

TNF α RT-PCR: Hepatic and vascular tissues were snap frozen immediately following dissec-

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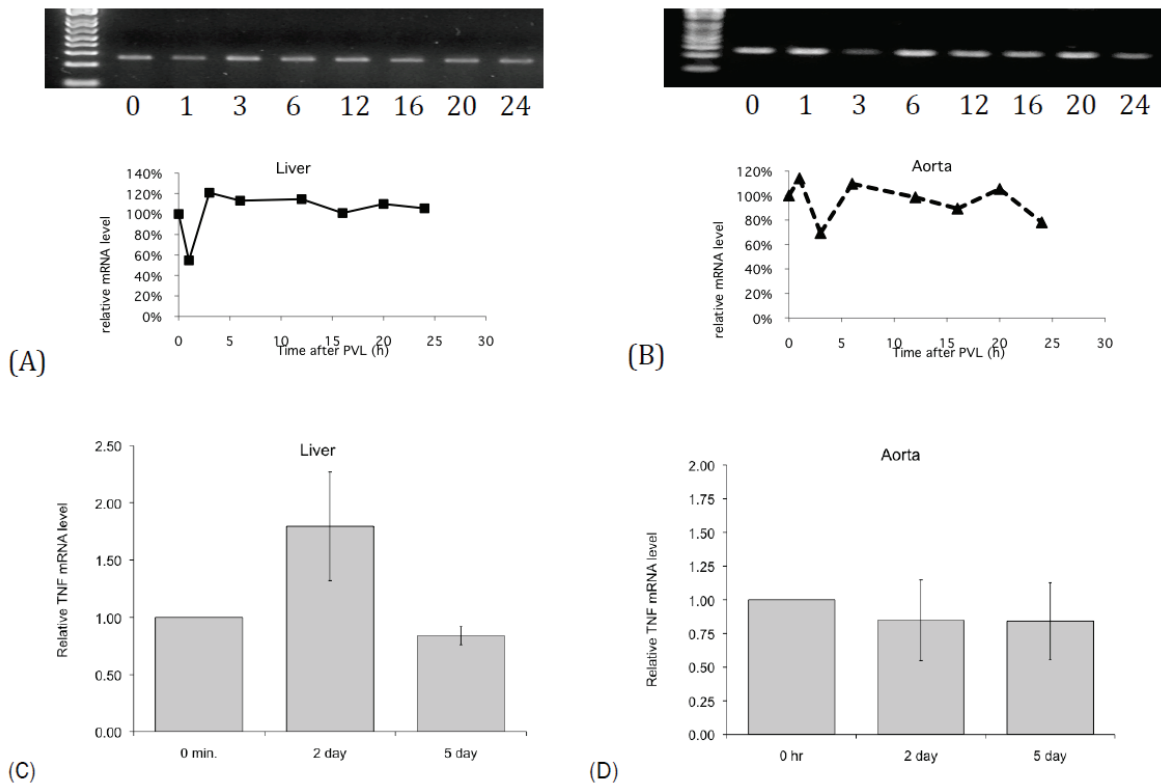


Figure 1. Portal vein ligation does not increase hepatic or aortic TNF α mRNA expression. Pre-hepatic portal hypertension was induced in wild type mice by partial ligation of the portal vein. 0-24hr and 0-5d post ligation livers (A,C) and aortas (B,D) were harvested, RNA isolated, cDNA generated and TNF mRNA quantified by RTPCR and volume densitometry. (A-B) TNF α mRNA levels were not significantly changed within livers and TA 0-24hr following PVL. (C-D) Chronic response to PVL (0-5d) included an increase in hepatic TNF α mRNA 2d following PVL (C) but no change in aorta (D). Data is representative of three experiments (mean \pm S.E. n=5 mice).

tion. 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 TNF α gene-specific primers were used to detect TNF α (CTCTCAAGGGACA AGGCTG, CGGACTCCGCAAAGTCTAAG) β -actin gene specific primers (ATCCTGGTCTGGACCT GGCT, CTTGCTGATCCACATCTGCTG) were also used as a housekeeping gene to correct for mRNA levels. PCR conditions were 30 cycles for TNF α 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 \pm 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 TNF α and TNF α expression in liver and vascular tissue: To examine changes in TNF α expression and TNF α 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. TNF α 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 TNF α 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. TNF α mRNA levels were

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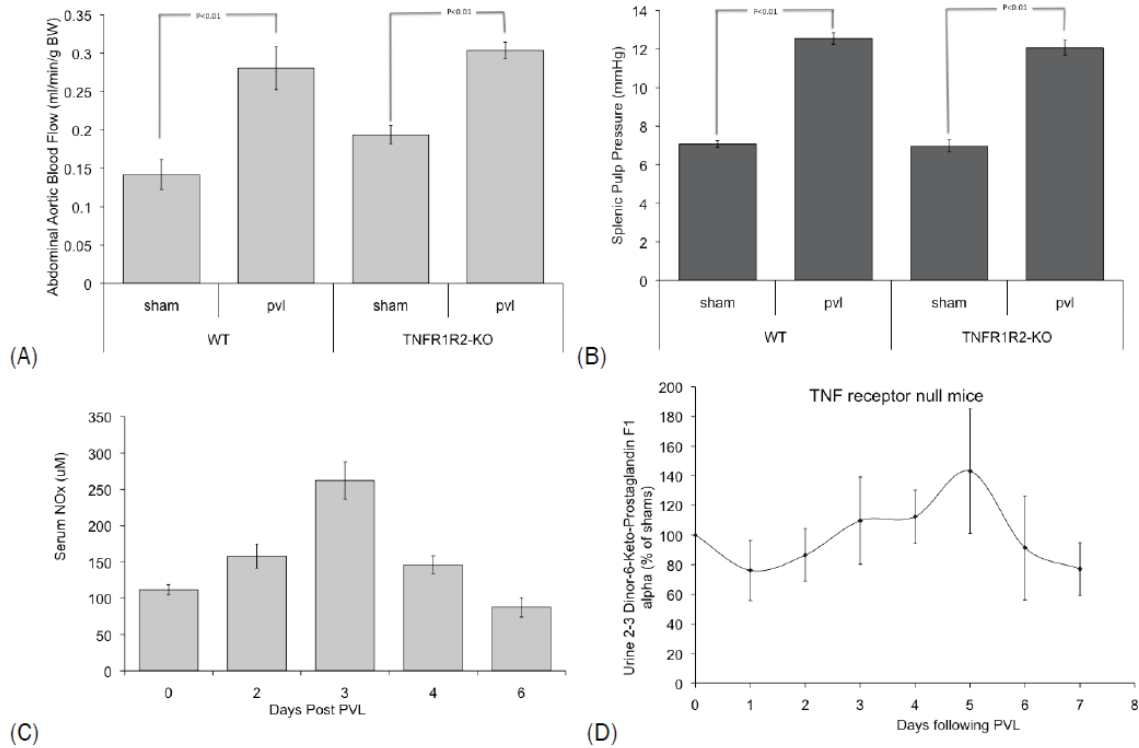


Figure 2. Splenic pulp pressure, aortic blood flow, serum NOx and urine 2,3-d-6-keto PGF1a following sham or PVL surgery in TNF receptor null mice. Pre-hepatic portal hypertension was induced in wild type and TNF α receptor null mice by partial ligation of the portal vein. (A-B) 7d post ligation aortic 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 TNF α 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 PGF1a level is a marker of systemic PGI₂ levels and cyclooxygenase activity. NOx and 2,3-dinor-6-keto PGF1a were increased in TNF α receptor null mice following PVL. Data represents mean \pm S.E from 5 mice.

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 TNF α expression 1-3hr in both aortic and hepatic tissues. (**Figure 1A-B**) Chronically TNF α 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 TNF α 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 TNF α levels, it is hepatic minor, transient, and does not lead to detectable levels of circulating TNF α .

Development of portal hypertension in TNF α receptor deficient mice: To determine the role of

TNF α receptor signaling in pre-hepatic portal hypertension wild type and TNF α 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 than 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. TNF α 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^{-/-})

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-PGF_{1a} levels following PVL in TNF α receptor deficient mice: To examine the effect of TNF α 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-dinor-6-k-PGF_{1a} (markers of NOS of COX activity respectively) are increased following PVL [5, 6]. NOx: Similar to results seen in wild types serum NOx increased in TNF α receptor null mice 5d following PVL (86.3 \pm 20.3mM) after which levels were not significantly different to sham operated controls (41 \pm 4.6mM). (**Figure 2C**) However, the increase was later than that typically seen in wild types (2-3d). 2,3-dinor-6-k-PGF_{1a}: seven-day urine 2,3-dinor-6-k-PGF_{1a} levels were significantly greater in PVL TNF receptor null mice (118%, P=0.04) when compared to shams. (**Figure 2D**) This increase was lower than that seen in wild type mice (336%). These data suggest that eNOS and COX activation in PHT is partly TNF α receptor mediated.

Discussion

TNF α is a potent cytokine and is reported to be important in numerous pathological events [29]. This manuscript reports studies designed to investigate TNF α signaling in PHT associated vascular hyper-dynamic circulation. Previous studies have identified TNF α 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 TNF α in the absence of liver disease. Moreover, TNF α 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 TNF α to improve PHT and non-PHT vasculopathies [25, 31]. Administration of anti TNF α 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 Ohms 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 TNF α in pre-hepatic portal hypertension is not mediated through known TNF α receptors. However, TNF α may still be involved via a non-TNF α receptor signaling mechanism. The success of anti TNF α antibodies in reducing portal flow is evidence of this. TNF α may have efficacy for additional receptors that have, to date, not been identified. Moreover, TNF α is known to signal through peroxisome proliferator-activated receptor- γ (PPAR γ) [32]. Currently there is no information regarding the role of PPAR γ and the development of PHT. PPAR γ antagonists have been synthesized [33] but their 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 PPAR γ knock mice showed that homozygote knockouts were not viable but that heterozygotes were and had a significantly altered phenotype [35]. More recently, cell specific PPAR γ gene knockout mice have been successfully generated [36, 37]. These mice may help investigate the mechanism by which TNF α 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 TNF α in PVL treated rats [38]. This is probably species related. Studies in other tissues have shown significantly stronger induction of TNF α 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 alternative protein expression to counteract gene deletion and provide an alternative phenotype. TNF α receptor null mice may manifest adaptive effects such that observed effects are not directly related to an absence of TNF α receptor.

In conclusion, the development of systemic hyperemia and portal hypertension in the PVL pre-hepatic model of PHT is TNF α receptor inde-

pendent. Moreover, we found no evidence of increased TNF α expression in the absence of hepatic injury or inflammation. However, we cannot discount that TNF α levels may have been over-expressed on a micro-localized level. TNF α naturalizing antibodies argue in favor of TNF α but the data presented in this manuscript suggests that any role would not be via the two main TNF α 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 TNF α antibody highlights TNF α synthesis and signaling as a fruitful target. Positive clinical outcomes following TNF α blockade [24] in combination with the emergence of infliximab and etanercept (fusion proteins consisting of the human TNF α receptor protein and human IgG1), supports direct intervention. However, our understanding for the role of TNF α in PHT is not complete. At present we do not know by what pathway TNF α signals and influences the development of PHT. Delineation of this pathway may provide selective and improved targets then direct blockade.

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