TNF receptors regulate vascular homeostasis through a caspase-8, caspase-2 and P53 apoptotic program that bypasses caspase-3

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Short title: TNF receptors and vascular homeostasis.
Abstract

Although it is known that TNF receptor signaling plays a critical role in vascular integrity and homeostasis, the contribution of each receptor to these processes and the signaling pathway involved are still largely unknown. Here, we show that targeted gene knockdown of TNFRSF1B in zebrafish embryos results in the induction of a caspase-8, caspase-2 and P53-dependent apoptotic program in endothelial cells that bypasses caspase-3. Furthermore, the simultaneous depletion of TNFRSF1A or the activation of NF-κB rescue endothelial cell apoptosis, indicating that a signaling balance between both TNFRs is required for endothelial cell integrity and that, in endothelial cells, TNFRSF1A signals apoptosis through caspase-8, while TNFRSF1B signals survival via NF-κB. Similarly, TNFα promotes the apoptosis of human endothelial cells through TNFRSF1A and triggers caspase-2 and P53 activation. We have identified an evolutionary conserved apoptotic pathway involved in vascular homeostasis that provides new therapeutic targets for the control of inflammation and tumor driven angiogenesis.

Keywords: cytokines / inflammation / angiogenesis/ apoptosis/ cell signaling/ zebrafish.
Introduction

Tumor necrosis factor α (TNFα) is a powerful pro-inflammatory cytokine produced and released mainly by mononuclear phagocytes that regulates endothelial cell functions and strongly and specifically alters their gene expression profile (Miura et al., 2006). TNFα exerts its functions through interaction with two specific cell surface receptors (TNFRs): the 55 kDa TNFRSF1A/TNFRSF1A and the 75 kDa TNFRSF1B/TNFRSF1B (Shalaby et al., 1990). TNFRSF1A is expressed in most cell types, even in transformed cells, whereas TNFRSF1B function seems to be restricted to immune and endothelial cells (Aggarwal, 2003). Recent studies with deficient mice have shown that TNFRSF1A predominantly triggers apoptosis or inflammation, while TNFRSF1B promotes tissue repair and regeneration (Aggarwal, 2003). Neither TNFRSF1A nor TNFRSF1B has intrinsic enzymatic activity, so they both need to recruit accessory proteins for signal transduction. Three main types of proteins interact with the cytoplasmic domains of TNFRs: TNFR-associated factors (TRAFs), FAS-associated via death domains (FADDs) and TNFR-associated via death domains (TRADDs). TNFRSF1A promotes the recruitment of TRAF2 and TRADD which interact with several signaling proteins, such as the E3-ubiquitin ligases BIRC2 (cIAP1) and BIRC3 (cIAP2), to form a so called complex I that induces the proteasome-dependent degradation of the NF-κB inhibitor IkB and, hence, nuclear translocation of NF-κB and the transcription of pro-inflammatory and survival genes (Locksley et al., 2001; MacEwan, 2002). A complex II can also be generated from complex I upon release from TNFRSF1A and which then recruits FADD and caspase-8, resulting in caspase-8 activation and leading to cell death (Locksley et al., 2001; MacEwan, 2002). In contrast, TNFRSF1B triggers the recruitment of TRAF1, TRAF2, which interact with BIRC2 and BIRC3 (Rothe et al., 1995), leading to NF-κB activation.

Therefore, TNFα has been dubbed a “double-edged sword”, since it might initiate distinct or overlapping signal transduction pathways by binding to TNFRSF1A and/or
TNFRSF1B, resulting in a variety of cellular responses, such as survival, differentiation, proliferation and migration, or, on the other hand, cell death (Aggarwal, 2003). This pleiotropic activity links TNFα with a wide variety of human diseases, including inflammatory and autoimmune disorders, ischemia/reperfusion injury and cancer.

Using a forward genetic approach in the zebrafish (Danio rerio), Santoro et al. (Santoro et al., 2007), identified BIRC2 as an essential molecule involved in maintaining endothelial cell survival and vascular homeostasis. In the absence of BIRC2, a caspase-8- and caspase-3-dependent apoptotic program leads to vessel regression. Given that human BIRC2 plays a key role in the TNFRSF1B signaling pathway (Rothe et al., 1995) and endothelial cells have previously been reported a major target for TNFα in fish (Roca et al., 2008), we used gain and loss-of-function studies to analyze the role played by each TNFR in the development and maintenance of endothelial cells in this species. We found that targeted gene silencing of TNFRSF1B results in the induction of a caspase-8-dependent apoptotic program in endothelial cells that can be rescued by depletion of TNFRSF1A, indicating that an appropriate signaling balance between both TNFRs is required for endothelial cell integrity and vascular homeostasis. In addition, the data also showed that, in endothelial cells, TNFRSF1A signals apoptosis through complex II formation and caspase-8 activation, while TNFRSF1B signals survival via complex I and NF-κB activation. Furthermore, we were able to establish a TNFRSF1A apoptotic program that involved caspase-8, caspase-2 and P53, but bypassed caspase-3. The molecular mechanism proposed applies not only for the zebrafish, since activation of TNFRSF1A also promoted apoptosis and caspase-2 and P53 activation in human endothelial cells following TNFα treatment. This evolutionary conserved apoptotic pathway involved in vascular development and homeostasis places TNFRs and caspase-2 in the front line for inflammatory, angiogenesis and tumor drug development.
Results

*Genetic depletion of TNFRSF1B results in blood circulation disruption, blood pooling and vascular hemorrhages*

The zebrafish has single orthologues of mammalian TNFRSF1A and TNFRSF1B, which showed 38% amino acid similarity to their human counterparts and conserved TNFR superfamily and death domains (Grayfer and Belosevic, 2009). In addition, we confirmed by pull-down assays that TNFα was able to bind both TNFRs (Figure S1). Quantitative gene expression analysis by RT-qPCR showed that the mRNA of both TNFRs was present in zebrafish embryos at spawning, indicating that it was maternally transferred (Figure S2A). However, TNFR gene expression profiles were different; while the mRNA levels of TNFRSF1A drastically increased 24 hours post-fertilization (hpf) and reached a plateau, those of TNFRSF1B peaked at 4 hpf, strongly decreased at 7 hpf and then remained low. *In situ* hybridization (ISH) corroborated RT-qPCR analyses and, additionally, showed that both TNFRs were ubiquitously expressed in zebrafish embryos (Figure S2B). In addition, TNFα mRNA was first detected 24 hpf and increased after 72 hpf (Figure S2A), suggesting that other ligands beside TNFα might be signaling through these receptors early in development. Lymphotoxin α (LTα), which is able to bind both TNFRs in mammals (Schneider et al., 2004), would be a plausible candidate for signaling through TNFRs in early embryos since it was found to be expressed and peaked as early as 7 hpf (Figure S2A).

In zebrafish, one important intraembryonic primitive haematopoiesis site is conformed by the intermediate cell mass (ICM), equivalent to the mammalian yolk sac blood island and located in the trunk ventral to the notochord. Subsequently, the definitive haematopoietic stem cells emerge from the ventral wall of the dorsal aorta and then migrate to the posterior region in the tail called the caudal hematopoietic tissue (CHT). Hematopoiesis starts at around 18 hpf in the ICM, where cells within this site differentiate into the endothelial cells of the trunk.
vasculature and proerythroblasts, which begin to enter the circulation around 24 hpf (de Jong and Zon, 2005; Paik and Zon, 2010). Initially, one unique dorsal vessel is formed that will generate the dorsal aorta (DA)/caudal artery (CA). By sprouting of cells from the DA, intersegmental primary vessels (IPVs) appeared anterior, while posterior cardinal (PCV) and caudal vein (CV) appeared posterior (Isogai et al., 2001). During a second dorsal sprouting from the PCV/CV, the functional fate of the IPVs of the primary vascular network varies depending on whether or not a functional connection is made to a secondary sprout. Thus, approximately half of the IPVs eventually become part of intersegmental veins (ISVs), while the remainders give rise to intersegmental arteries (ISAs) (Isogai et al., 2001) (Figure 1A).

Depletion of TNFRSF1B by a morpholino antisense oligonucleotide (MO) targeting the intron 1-exon 2 boundary of pre-mRNA (Table S1, Figure S3) resulted in viable zebrafish for up to 7 days post-fertilization (dpf) with no obvious developmental delay (Table S2). However, less than 5% of fish in all experiments showed altered head development, delayed yolk reabsorption and pericardial edema at 54 and 72 hpf compared with fish injected with a standard control MO (Figs. 1B and 1C). Analysis of double transgenic \textit{fli1a:GFP/gata1:dsRed} fish to visualize \textit{in vivo} blood vessel formation (endothelial cells/green) and circulation (erythrocytes/red) revealed that TNFRSF1B depletion resulted in the absence of blood circulation and erythrocyte accumulation within the DA/CA at 54 hpf (Figure 1B) and the appearance of large hemorrhages throughout the body, blood pooling and aberrant CV formation at 72 hpf (Figs. 1C-1F). Although IPV formation was largely unaffected, abnormal, non-functional and bypassed ISVs and ISAs were observed (Figure 1E). These defects resulted in a caudal vessel net with or without blood circulation (Figure 1F). In addition, we found increased numbers of TUNEL positive endothelial cells in TNFRSF1B-deficient embryos, while there were no statistically significant differences in the numbers of TUNEL positive non-endothelial cells between control and TNFRSF1B-deficient
larvae (Figs. 1G and 1H). These results suggest that apoptosis of endothelial cells was largely responsible for the vascular defects triggered by TNFRSF1B depletion.

The vascular defects observed in TNFRSF1B-deficient larvae were also found with two additional MOs (Table S1), which targeted the exon 1-intron 1 boundary and the atg/5'UTR (Figure S4), and they were also rescued by the over-expression of wild type TNFRSF1B mRNA but not by antisense TNFRSF1B mRNA (Figure 2A). In addition, to further confirm the specificity of these MOs, we generated a dominant negative mutant of TNFRSF1B, DN TNFRSF1B, and expressed the mRNA in embryos. The DN TNFRSF1B is lacking the entire intracellular signaling domain, but is identical to full-length TNFRSF1B in its transmembrane and extracellular domains. Trimerization of DN TNFRSF1B with endogenous TNFRSF1B is expected to extinguish TNFRSF1B signaling (Fang et al., 2008). Hence, it was found that over-expression of the mRNA of DN TNFRSF1B resulted in similar vascular defects; although the phenotype was less penetrating and hemorrhages were less frequent (Figure S5). Strikingly, although TNFRSF1A knockdown (Figure S3) had no effect on vascular development, it was able to rescue the vascular defect observed in TNFRSF1B-deficient embryos (Figure 2B), further confirming the specificity of the MOs used. As pharmacological and genetic manipulation of NF-κB has shown that NF-κB signaling via TNFRs is involved in endothelial cell survival (Santoro et al., 2007), we over-expressed NEMO, which is the regulatory subunit of the IκB kinase and is required for NF-κB activation. NEMO was able to rescue in a dose-dependent manner the wild type vascular phenotype in TNFRSF1B-deficient fish (Figure 2C). Notably, TNFRSF1B deficiency also resulted in the induction of TNFRSF1A and TNFα gene expression, which was fully rescued by the depletion of TNFRSF1A (Figure 2D). These results suggest that the depletion of TNFRSF1B results in an imbalance between TNFRSF1A and TNFRSF1B signaling, which, in turn, results in the
activation of an apoptotic pathway through TNFRSF1A, leading to endothelial cell death, vasculature disruption and/or hemorrhages.

**TNFRSF1B depletion triggers a caspase-8 apoptotic pathway via TNFRSF1A in endothelial cells**

Since caspase-8 is the main initiator caspase involved in TNFR signaling, we analyzed caspase-8 activity in TNFRSF1B-deficient fish. Figure 3A shows that the depletion of TNFRSF1B not only increased caspase-8 activity, but also, that of caspase-9, an initiator caspase of the intrinsic apoptotic pathway that is usually activated following mitochondrion architecture demolition (Taylor et al., 2008). Genetic depletion of caspases-8 with specific MOs (Sidi et al., 2008) and the inhibition of caspase-8 by overexpression of the mRNA of the specific inhibitors CASP8 and FADD-like apoptosis regulator (CFLAR) and cytokine response modifier A (CRMA), zebrafish endogenous and cow poxvirus caspase-8 inhibitors, respectively, resulted in a partial rescue of the vascular defect promoted by TNFRSF1B deficiency (Figure 3B and 3C). However, caspase-9 depletion was unable to rescue the vascular defect of TNFRSF1B deficient embryos (Figure 3D). As the caspase-9 MO is not 100% efficient (Sidi et al., 2008), we cannot rule out an involvement of caspase-9 in the vascular defects observed in TNFRSF1B morphant. Collectively, these data suggest the activation of an apoptotic pathway initiated by caspase-8 via TNFRSF1A that promotes endothelial cell apoptosis in the absence of NF-κB-dependent survival signals provided by TNFRSF1B activation.
**P53 activation is indispensable for promoting caspase-8-dependent apoptosis of endothelial cell in TNFRSF1B deficient zebrafish embryos**

The activation of caspase-9 in TNFRSF1B-deficient embryos led us to look more closely at the contribution of the intrinsic apoptotic pathway in vascular development and maintenance. We observed the induction of P53 at both mRNA (Figure 4A) and protein (Figure 4B and 4C) levels in TNFRSF1B deficient zebrafish. Strikingly, P53 was mainly induced in endothelial cells (Figure 4C), which further suggests that TNFR signaling plays a critical role in vascular development. Furthermore, the induction of P53 observed in TNFRSF1B-deficient embryos was strongly reduced in double TNFRSF1A/TNFRSF1B-deficient embryos and it was caspase-8-dependent, since genetic depletion of caspase-8 largely attenuated P53 induction (Figure 4A). Finally, the activation of P53 was also confirmed by the sharp upregulation of the P53-dependent genes BAX and MDM2 (Figure 4D) and P21 (Figure 4E) in TNFRSF1B-deficient embryos.

To clarify the importance of P53 induction and activation in vascular integrity, we depleted P53 by using a specific MO (Langheinrich et al., 2002) and used the P53 zebrafish mutant line P53M214K (Berghmans et al., 2005). We found that the P53 MO almost completely rescued the vascular defects of TNFRSF1B-deficient embryos (Figure 4F) and abrogated the induction of caspase-8 gene expression (Figure 4G). Similarly, the P53M214K mutant embryos did not show significant vascular alteration (data not shown) and P21 and caspase-8 gene expression was unaltered (Figure 4H) following genetic depletion of TNFRSF1B. Strikingly, however, TNFRSF1B deficiency in this line resulted in caspase-8 activation, while caspase-9 activity was unaltered (Figure 3A). Collectively, these results suggest the re-amplification of TNFRSF1A-dependent apoptosis of endothelial cells by a crosstalk between extrinsic (caspase-8) and intrinsic (P53) apoptotic pathways (Figure 4I).
DNA fragmentation in endothelial cells triggers P53 activation in TNFRSF1B-deficient zebrafish

Since CAD is an endonuclease which is activated once caspases cleave its inhibitor (ICAD) and because TNFRSF1B depletion resulted in DNA fragmentation in endothelial cells, we overexpressed zebrafish ICAD in TNFRSF1B deficient embryos. We found that ICAD overexpression rescued the vascular defects of TNFRSF1B deficient embryos in a dose-dependent manner (Figure 5A) and reduced (about 25%) the induction of caspase-8 and P53 (data not shown). We next asked whether the checkpoint kinase CHK1 might be involved in the activation of P53 following CAD-mediated DNA damage. To inhibit CHK1, we used the indolocarbazole small molecule Gö6976 which has a high specificity for CHK1 and has successfully been used in whole zebrafish embryos (Sidi et al., 2008). Genetic depletion of P53 failed to rescue the vascular defects of TNFRSF1B deficient embryos in the presence of Gö6976 (Figure 5B), suggesting that CHK1 might be downstream of P53 in this apoptotic signaling pathway triggered by TNFRSF1A in endothelial cells.

The TNFRSF1A apoptotic signaling pathway is independent of caspase-3 but requires caspase-2

To identify the executor caspase linking TNFRSF1A/caspase-8 and CAD/P53 axis, we analyzed the activation of caspase-3, which is a hallmark of both extrinsic and intrinsic apoptotic pathways. Interestingly, although TNFRSF1B depletion resulted in increased numbers of apoptotic endothelial cells in the CHT (Figs. 1I and 1J), it failed to significantly increase active caspase-3 levels in endothelial cells compared with control embryos (injected with a standard MO) (Figs. 6A). Since CAD is involved in the TNFRSF1A apoptotic signaling pathway of zebrafish endothelial cells, we studied the involvement of caspase-2, which has been shown to process ICAD (Dahal et al., 2007) and is involved in an apoptotic
response to DNA damage that bypasses caspase-3 (Sidi et al., 2008). We found that caspase-2 is activated in TNFRSF1B deficient embryos and seems to be placed downstream of caspase-8, since genetic depletion of caspase-8 reduced caspase-2 activation in TNFRSF1B deficient animals (Figure 6B). To corroborate the relevance of caspase-2 activation in endothelial cell apoptosis, we ablated caspase-2 using a specific MO (Sidi et al., 2008). The results showed that caspase-2 deficiency was able to partially rescue the vascular defects of TNFRSF1B-deficient embryos (Figure 6C).

The caspase-2/P53 apoptotic signaling pathway triggered by TNFRSF1A is evolutionary conserved

We next wondered whether this new signaling pathway also operates in human endothelial cells. We first confirmed by RT-PCR that primary human umbilical vein endothelial cells (HUVECs) expressed both TNFRs. Treatment of primary HUVECs with recombinant TNFα resulted in the induction of apoptosis in a small proportion of cells (ranging from 10-20 %) (Figures 7A-C), while the neutralization of the TNFRSF1B with a specific antibody slightly increased the percentage of cells undergoing apoptosis in response to TNFα (Figure 7A). More importantly, the numbers of caspase-2 positive cells significantly increased (Figure 7B), while caspase-3 levels hardly increased following treatment of the cells with a TNFα mutein that specifically interact with TNFRSF1A (Figure 7D). In addition, P21 and P53 mRNA levels also increased following treatment with TNFα mutein (Figure 7E) and, notably, pharmacological inhibition of CHK1 with G66976 resulted in a further upregulation of P53 and P21 gene expression (Figure 7E) and accumulation of P53 protein (Figure 7F). These results suggest that this kinase is involved in a P53- and P21-dependent cell cycle arrest triggered by TNFRSF1A in endothelial cells.
Discussion

Tight molecular control of endothelial cell survival, integrity and apoptosis is essential for both embryonic and adult angiogenesis. Santoro and coworkers (Santoro et al., 2007) recently showed that the anti-apoptotic adaptor protein BIRC2 plays an essential role in regulating endothelial cell survival in vivo. However, since BIRC2 is known to be involved in the signaling pathways of several death receptors, including FAS (Rueemmele et al., 2002), CD40 (Bureau et al., 2002; Fotin-Mleczek et al., 2002), TNFRSF1A (Shu et al., 1996) and TNFRSF1B (Rothe et al., 1995), the nature of the receptor(s) involved in vascular development and integrity remains unclear. Using a gain- and loss-of-function approach, we found that the TNFRSF1A and TNFRSF1B signaling pathways must be balanced for endothelial cell development to be maintained in zebrafish and that, in endothelial cells, TNFRSF1A signals apoptosis through complex II and caspase-8, while TNFRSF1B signals survival via complex I and NF-κB. A similar crosstalk between TNFRSF1A and TNFRSF1B signaling pathways has also been observed in human aortic endothelial cells, whose susceptibility to TNFα treatment can be rescued by blocking TNFRSF1A or NF-κB (Okada et al., 2001). These results, together with the ability of TNFRSF1B to induce the depletion of TRAF2 and BIRC2 proteins and accelerate the TNFRSF1A-dependent activation of caspase-8 (Fotin-Mleczek et al., 2002), suggest that BIRC2 is required for TNFRSF1B complex I formation and NF-κB activation in endothelial cells, despite the high promiscuity of this adaptor molecule.

One of the most interesting observations made in our study is that the TNFRSF1A-dependent apoptotic pathway of endothelial cells is independent of caspase-3 and caspase-9, but requires caspase-2, an enigmatic caspase that possesses both initiator and executioner caspase characteristics (Krumschnabel et al., 2009). Our in vivo epistasis analysis led us to envisage a model whereby TNFRSF1A engagement leads to the following sequential events.
(Figure 8): (i) activation of caspase-8, (ii) activation of caspase-2, (iii) processing of ICAD by caspase-2 and translocation of CAD to the nucleus, (iv) activation of P53, and (v) P53-dependent induction of caspase-8 gene expression. As human caspase-2 has been shown to cleave ICAD in vitro (Dahal et al., 2007), we hypothesised that caspase-2 would replace caspase-3 as the main executor caspase and would mediate the cleavage of ICAD and the subsequent activation of P53. In fact, we observed that P53 was firmly involved in the TNFRSF1A-mediated apoptosis of endothelial cells. Furthermore, P53 might also be involved in the direct activation of caspase-2 in TNFRSF1B-deficient endothelial cells, since it was very recently found that caspase-2 is involved in a P53-positive feedback loop during DNA damage responses (Oliver et al., 2011). Thus, caspase-2 cleaves MDM2 promoting p53 stability, which leads to the transcription of PIDD, a P53 target gene product which activates caspase-2 in a complex called the caspase-2-PIDDosome. Regardless of the mechanism, the apoptotic program triggered by TNFRSF1A in endothelial cells involves a positive regulatory feedback between the intrinsic and extrinsic apoptotic pathways, which ends in the upregulation of caspase-8, further contributing to the apoptosis of endothelial cells. To the best of our knowledge, this observation has not previously been reported in vivo but it is not unexpected since P53 was also found to be able to upregulate caspase-8 gene transcription in human cancer cells treated with cytotoxic drugs (Ehrhardt et al., 2008).

The inhibition of the cell cycle checkpoint kinase CHK1, which is activated by ATR in response to replication stress (Cuadrado et al., 2006), impairs the ability of P53 knockdown to rescue the vascular defect promoted by TNFRSF1A in zebrafish embryos and further induces the expression of P21 in TNFα-treated HUVECs, indicating that CHK1 might be downstream P53 in the apoptotic signaling pathway triggered by TNFRSF1A in endothelial cells or, alternatively it might inhibit a P53-independent apoptotic pathways in these cells. This is not unexpected, since it has recently been described a role for CHK1 in the inhibition of an
ATM/ATR-caspase-2 apoptotic response to irradiation-induced DNA damage that bypasses P53 and caspase-3 (Sidi et al., 2008). Although the CHK1-dependent and P53-independent apoptotic pathway triggered by TNFRSF1A deserves further investigation, our results also suggest that CHK2 might also mediate activation of P53 in TNFRSF1B-deficient endothelial cells.

Although several systemic inhibitors of TNFα, such as soluble TNF receptors and anti-TNFα antibodies, have been approved for the treatment of human diseases where TNFα plays a pathogenic role, these drugs exhibit severe side effects and are expensive. Hence new active blockers of TNFα that are safe, efficacious and inexpensive are urgently needed (Sethi et al., 2009). We believe that the TNFRSF1A apoptotic axis described in this study reveals new molecular targets for the development of therapeutic drugs for human diseases where TNFα plays a major role, such as inflammatory and autoimmune disorders, ischemia/reperfusion injury and cancer. The TNFα-induced caspase-2/P53 apoptotic program in HUVECs, the vascular defects of TNFRSF1A deficient zebrafish and the recent observations in transgenic mice overexpressing TNFRSF1B within endothelial cells showing decreased death of these cells after ischemia/reperfusion and higher endothelial cells proliferation, neovascularization and vessel maturation after injury (Luo et al., 2010), strongly suggest that TNFRSF1A and TNFRSF1B, rather than TNFα, might be the better clinical targets.

In conclusion, we have identified an evolutionarily conserved apoptotic program in endothelial cells that is triggered by the imbalance between survival and death signals provided by TNFRs. This pathway involves crosstalk between intrinsic (caspase-8) and extrinsic (P53) apoptotic programs but, intriguingly, bypasses caspase-3. Caspase-2 replaces caspase-3 and would link both apoptotic programs probably due to its dual activity as initiator and executor caspase. This genetic pathway reveals new therapeutic targets for the control of inflammation- and tumor-induced angiogenesis.
Methods

Animals

Wild-type zebrafish (Danio rerio H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC) and maintained as described in the zebrafish handbook (Westerfield, 2000). The transgenic zebrafish line that expresses enhanced GFP (eGFP) driven by the endothelial cell-specific promoter \textit{flila} gene \textit{Tg(fli1a:egfp)y1} (Lawson and Weinstein, 2002) was obtained from ZIRC. The transgenic line with red fluorescent erythrocytes \textit{Tg(gata1:dsRed)sd2} (Traver et al., 2003) and the P53 mutant line \textit{zdf1} (P53^{M214K}) (Berghmans et al., 2005) were kindly provided by Prof. LI Zon. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the Bioethical Committee of the University of Murcia (approval number #333/2008) for the use of laboratory animals.

Embryos/larvae were anesthetized in Tricaine (200 \(\mu\)g/ml) and examined using a LEICA MZ16FA stereomicroscope. The vascular defects were scored semiquantitatively in blind samples at 48 and 72 hpf as wild type (no defects), mildly affected (erythrocyte accumulation in the CHT, partial blood circulation and hemorrhages) and severely affected (erythrocyte accumulation in the CHT and no blood circulation).

Morpholinos (MOs), mRNA microinjection and chemical treatment

Splice- or translation-blocking MOs were designed by and purchased from Gene Tools and solubilized in water (1-3 mM) (Table S1). All eggs were injected with the same amount of MOs. Full-length TNFRSF1A (BC068424), TNFRSF1B (ENSDARG00000070165) and ICAD (NM_001002631), and DN TNFRSF1B (amino acids 1-162) were subcloned into the pCS2+ or pBluescript II KS+ vectors. NEMO and CFLAR in PCS2 backbone were provided
by Dr. M. Santoro (Santoro et al., 2007). CMRA construct was obtained from BCCM/LMBP (http://bccm.belspo.be/about/lmbp.php).

mRNA was synthesized using the mMESSAGE MACHINE kit (Ambion) and polyadenylated using a polyadenylation kit (Ambion) according to the manufacturer's instructions. mRNA (0.5-1 ng/egg) and MOs (1-5 ng/egg) were mixed in microinjection buffer (0.5 x Tango buffer and 0.05 % phenol red solution) and microinjected (1-5 nl) into the yolk sac of one-cell-stage embryos using a Narishige IM300 microinjector. Knockdown efficiencies of TNFRSF1A and TNFRSF1B MOs are shown in Fig S2.

In some experiments, 24-hpf embryos were dechorionated and transferred to fresh E3 medium containing 1% DMSO with or without the CHK1 inhibitor Gö6976 (1 μM; Calbiochem) (Sidi et al., 2008).

Analysis of development

The effect of MOs on development was evaluated as previously reported (Kimmel et al., 1995). Briefly, it was recorded the side-to-side flexures at 22 hpf (25-26 somites stage); heartbeat, red blood cells on yolk and early pigmentation in retina and skin at 24 hpf (Prim 5 stage); head trunk angle (HTA), retina pigmented, early touch reflect and straight tail at 30 hpf (Prim 15 stage); and early motility and tail pigmentation at 36 hpf (Prim 25).

Determination of caspase activity

The activation of caspase-9, caspase-8 and caspase-2 in whole embryos was quantified using the luminescent assays Caspase-Glo 9, Caspase-Glo 8 and Caspase-Glo 2 (Promega), respectively, as described previously (Geiger et al., 2006). The substrate specificity and sensitivity to pharmacological inhibitors of zebrafish caspase-8 and caspase-9 has been found to be similar to their mammalian orthologues (Chen et al., 2001; Chiu et al., 2010; Friedrich et
al., 2004; Santoro et al., 2007; Stanton et al., 2006; Wrasidlo et al., 2008). Caspase-3 activation was determined by flow cytometry using an affinity-purified rabbit anti-human/mouse caspase-3 active (0.5 µg/ml, R&D Systems) (Sepulcre et al., 2011). As appropriate controls, 56 hpf larvae were treated for 16 h with 0.2 µM camptothecin (Sigma-Aldrich) in the presence or absence of the caspase-3 specific inhibitor Ac-DEVD-CHO (100 µM, Sigma-Aldrich).

**Analysis of gene expression**

Total RNA, extracted as indicated above, was treated with DNase I, Amplification grade (1 unit/µg RNA, Invitrogen) and the SuperScript III RNase H\(^{-}\) ReverseTranscriptase (Invitrogen) was used to synthesize the first strand of cDNA with an oligo-dT\(_{18}\) primer from 1 µg of total RNA at 50°C for 50 min.

Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For each mRNA, gene expression was normalized to the ribosomal protein S11 (rps11) content in each sample Pfaffl method (Pfaffl, 2001). In all cases, the PCR was performed with triplicate samples and repeated with at least two independent samples. The primers used are shown in Table S3.

**Whole-mount immunohistochemistry**

Embryos/larvae were fixed for 2 h in 4% paraformaldehyde (PFA) at room temperature (RT), dehydrated in methanol/PBS solutions (25, 50, 75 and 100 %, 5 min each) and stored in 100 % methanol at −20°C. For staining, they were rehydrated in 75, 50 and 25 % methanol/PBT (PBS and 0.1 % Tween-20) solutions (5 min each), washed three times for 5
min in dH$_2$O, permeabilized in cold acetone for 10 min at -20°C, washed again twice in dH$_2$O (5 min each) and treated with blocking solution (PDT=PBS containing 0.1 % Tween-20, 1% DMSO, 5% FBS and 2 mg/ml BSA) for 2 h at RT. After blocking, embryos were incubated overnight at 4°C with affinity-purified rabbit anti-human/mouse caspase-3 active (0.5 µg/ml, epitope CRGTELDCGIETD, #AF835, R&D Systems) or anti-zebrafish P53 (1:200, #55915, AnaSpec) diluted in PDT, washed six times in PDT (10 min each), incubated for 2 h at RT in PDT, incubated overnight at 4°C with a 1/1000 dilution in PDT of a phycoerythrin-conjugated secondary antibody (Invitrogen), washed five times in PBT (5 min each) and finally examined under a LEICA MZ16FA stereomicroscope.

**In situ hybridization**

Whole mount *in situ* hybridization was performed according to Schulte-Merker et al. (Schulte-Merker et al., 1994) with minor modifications, while in situ hybridization on paraffin sections was based on a protocol developed by Mallo et al. (Mallo et al., 2000). Similar results were obtained with three different riboprobes against TNFRSF1A (probe1: +64 to +572; probe 2: +666 to +1177; full length probe: +64 to +1177) and TNFRSF1B (probe1: +113 to +667; probe 2: +678 to +1189; full length probe: +113 to +1189), respectively. The primers used to amplify probes are shown in Table S3.

**Detection of apoptotic cell death by TUNEL labelling**

After overnight fixation in 4% PFA at 4°C embryos were washed in PBS containing 0.1% Tween 20 (PBT) for five minutes and dehydrated in graded Methanol series until reaching 100% Methanol. After storage at -20°C they were rehydrated gradually to PBT, washed twice for 10 minutes in PBT and digested in proteinase K (Roche) solution in PBT (10 µg/µl) at room temperature for 15 min. After two washes in PBT they were postfixed in
4% PFA for 20 min. Embryos were washed again twice in PBT for 10 min each and endogenous biotin was blocked using the Biotin blocking kit (Vector, Burlingame, CA, USA). Embryos were washed in PBT and put into equilibration buffer (Roche) for 1 hour. Embryos were subsequently incubated with the TdT reaction mix (Roche) overnight at 37°C. Reaction was stopped with washes in equilibration buffer during 3 hours at 37°C followed by three washes in PBT at RT. Streptavidine-Cy3 was used as a secondary antibody (Jackson laboratories) incubated during 1h at RT. For concomitant GFP detection, embryos were incubated with anti-GFP antibody (Clontech, Mountain View, CA, USA) followed by Alexa-488 secondary antibody (Invitrogen). Embryos were washed and mounted in Vectashield supplemented with DAPI (Vector).

Methanol-fixed embryos were also embedded in paraffin, sectioned at 5 µm and processed as previously described (Gonzalez-Rosa et al., 2011).

Immunoprecipitation and western blot assays

The physical interaction between zebrafish TNFα and TNFRs was analyzed by means of immunoprecipitation. Plasmid DNA was prepared using the Midi-Prep procedure (Qiagen) and transfected into HEK293 cells with LyoVec transfection reagent (Invivogen), according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed twice with PBS and lysed in 200 µL lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and a 1:20 dilution of the protease inhibitor cocktail P8340 (Sigma-Aldrich). Whole cell extracts were then mixed and incubated overnight at 4°C before being immunoprecipitated with the anti-V5 mAb (Invitrogen) and protein G sepharose (Sigma-Aldrich). After extensive washing with the lysis buffer, the resins were boiled in SDS sample buffer, and the bound proteins were resolved on 12% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). Blots were probed with specific
antibodies to V5 or Xpress (Invitrogen) and developed with enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer’s protocol.

Ten µg extracts from dechorionated and deyolked embryos obtained as indicated above for HEK293 cells were probed with a ½ dilution of the monoclonal antibody LLzp53-9E10, which is specific to zebrafish P53 (MacInnes et al., 2008). Membranes were then reprobed with a 1/5,000 dilution of a commercial rabbit antibody to histone 3 (#ab1791, Abcam), as an appropriate loading control.

**HUVEC culture and treatments**

Primary human endothelial cells (HUVECs) were cultured in appropriate media according to the manufacturer’s protocol (Lonza). Cells were stimulated for 16 h with 40 ng/ml recombinant TNFα (Sigma-Aldrich) alone or in the presence of 5 µg/ml TNFRSF1B neutralizing antibody (#MAB226) or an isotype control (#MAB003) (R&D Systems). Alternatively, a TNFα mutein specific for TNFRSF1A (GenScript) was also used (Loetscher et al., 1993). In some experiments, cells were also treated with 0.5 µM camptothecin, 1 µM Gö6976 or 0.25 µM staurosporine (Sigma-Aldrich) for 16 h in the presence or absence of 50 µM of the caspase-3 inhibitor Ac-DEVD-CHO. Cell viability was determined spectrophotometrically using an MTT-based test (Mosmann, 1983). Caspase-2 activation was determined by flow cytometry using the CaspGLOW™ Fluorescein Active Caspase-2 Staining Kit (Biovision) and the accompanying staining protocols. Caspase-3 activation was determined by immunofluorescence as indicated for zebrafish embryos. Phosphatidylserine exposure on the extracellular leaflet of the membrane was determined by flow cytometry using a phycoerythrin-Annexin V conjugate (ENZO Life Sciences) according to the manufacturer's instructions. Finally, P53 protein levels were evaluated by western blot using
monoclonal antibodies to human P53 (#P6749) and β-actin (#A5441) (both from Sigma-Aldrich).

**Statistical analysis**

All experiments were performed at least three times, unless otherwise indicated. The total number of animals used is indicated above each bar. Data were analyzed by Student’s *t* test, or ANOVA and a Tukey’s multiple range test to determine the differences among groups. Statistical significance was defined as *p* < 0.05. A Chi-square contingency test was used to determine the differences between vascular defects scores.

**Acknowledgements**

We thank Inma Fuentes, Pedro J. Martínez, Eduardo Díaz and Encarnación Sánchez for excellent technical assistance, Marina Peralta-López and Claudio Badía-Careaga for assistance with ISH and TUNEL, Prof. LI Zon for the *Tg(gata1:dsRed)sd2* and the P53 mutant line *zdf1*, Dr. M Santoro for the NEMO and CFLAR constructs, and Prof. JA Lees for the antibody to zebrafish P53.

**Funding**

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Author contribution


Conflict of interest

The authors declare no conflict of interest.
References


Translational Impact Box

Clinical Issue

Tumor necrosis factor α (TNFα) is a powerful pro-inflammatory cytokine produced and released mainly by mononuclear phagocytes that plays a prominent pathogenic role in several human diseases, including inflammatory and autoimmune disorders, ischemia/reperfusion injury and cancer. Although it is well known that TNFα signals via TNFRSF1A and TNFRSF1B, the contribution of each receptor to the development of these diseases and the signaling pathway involved are still largely unknown.

Results

Using the optically clear developing zebrafish, we found that genetic depletion of TNFRSF1B in zebrafish embryos results in the induction of a caspase-8-, caspase-2- and P53-dependent apoptotic program in endothelial cells that bypasses caspase-3. In addition, the simultaneous depletion of TNFRSF1A rescues endothelial cell apoptosis, indicating that a signaling balance between both TNFRs is required for endothelial cell integrity. Our in vivo epistasis analysis led us to envisage a model whereby TNFRSF1A engagement leads to the following sequential events: (i) activation of caspase-8, (ii) activation of caspase-2, (iii) processing of ICAD by caspase-2 and translocation of CAD to the nucleus, (iv) DNA breaks generated by CAD induce activation of P53, and (v) P53 induces caspase-8 gene expression. Importantly, this apoptotic pathway is evolutionary conserved, since TNFα promotes the apoptosis of human endothelial cells and triggers caspase-2 and P53 activation in these cells via TNFRSF1A.

Implications and Future Directions

Several systemic inhibitors of TNFα, such as soluble TNF receptors and anti-TNFα antibodies, have been approved for the treatment of human diseases where TNFα plays a pathogenic role, these drugs, however, exhibit severe side effects and are expensive. Hence new active blockers of TNFα that are safe, efficacious and inexpensive are urgently needed. The critical signaling balance between TNFRSF1A and TNFRSF1B identified in this study suggests that drugs specifically targeting TNFRs and their signaling adaptor molecules, rather than TNFα, should be developed to treat TNFα-driven human diseases.
Figure Legends

**Figure 1. Genetic depletion of TNFRSF1B results in endothelial cell apoptosis and blood circulation disruption.** (A) Scheme showing the main vessels of a 3 days-old zebrafish larvae (left) and sagittal sections of the formation of the caudal artery (CA) and the caudal vein (CV) from an unique initial vessel, the first dorsal and ventral sprouting from the CA to generate intersegmental primary vessels (IPV), and the second dorsal sprouting that results in the generation of intersegmental vessels (ISV). (B-G) Lateral view of 54 (B) and 72 hpf (C-F) and tranverse and sagittal sections (G, bottom panels) of double transgenic *flila:eGFP* and *gata1:dsRed* larvae microinjected at the one-cell stage with standard (STD) and TNFRSF1B MOs. (B) TNFRSF1B depletion results in impaired differentiation of the CA and CV during the first sprouting, leading to blood pooling (bp) inside a enlarged unique dorsal vessel (asterisk). (C-D) At 72 hpf, blood pooling can still be observed in the caudal body part. In addition, hemorrhages appear throughout the body (arrowheads). (E, F) Zoomed views of trunk vasculature of 72 hpf larvae. TNFRSF1B deficiency results in the alteration of the second sprouting leading to the formation of a net of vessels that replace the CA and CV (E, F) and altered development of ISV (E). Arrows: ISA without blood circulation. (G, H) Confocal Z-stack sections of whole larvae (G) and sections (H) of the CHT of 60 hpf Tg(*flila:eGFP*) injected with STD and TNFRSF1B MOs showing TUNEL positive cells (red) (arrowheads). Nuclei were counterstained with DAPI (blue). (I) Quantification of TUNEL positive non-endothelial cells (eGFP⁻) and endothelial cells (eGFP⁺) at 60 hpf from serial Z-stack sections. Each dot represents the number of TUNEL positive cells per single larvae. The mean ± S.E.M. of the TUNEL positive cells for each group of larvae is also shown. **P<0.001. Scale bars, 100 μm unless otherwise indicated. CA, caudal artery; CV, caudal vein; CHT, caudal haematopoietic tissue; DA, dorsal aorta; DLAV: dorsal longitudinal
anastomotic vessel; ISV, intersegmental vessels; PCV: posterior cardinal vein, SIV, subintestinal vessels.

Figure 2. A critical balance between TNFRSF1A and TNFRSF1B signaling is required for endothelial cell development and maintenance. Zebrafish embryos were microinjected at the one-cell stage with STD and TNFRSF1B MOs alone or in combination with the indicated mRNAs. At 72 hpf, the vascular defects were scored. Larvae revealing no defects were scored as wild type (white bar section), larvae showing erythrocyte accumulation in the CHT, partial blood circulation and hemorrhages were scored as mildly affected (grey bar section) and larvae displaying erythrocyte accumulation in the CHT and no blood circulation as severely affected (black bar section). (A) Effect of wild type and antisense TNFRSF1B mRNA overexpression in morphant embryos. Note that wild type, but not antisense TNFRSF1B mRNA partially rescues the TNFRSF1B morphant phenotype (B) Partial rescue of the vascular defect promoted by genetic depletion of TNFRSF1B by TNFRSF1A depletion. (C) NEMO mediated activation of NF-κB partially rescues the vascular defect promoted by genetic depletion of TNFRSF1B. The data shown in Figs. 2B and 2D are representative of two independent experiments. (D) mRNA quantification of the indicated genes were determined by real-time RT-PCR in 10 pooled larvae. The gene expression is normalized against rps11. Each bar represents the mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.0001, n.s.: not significant according to Chi-square contingency (A-C) or Student’s t tests (D).

Figure 3. TNFRSF1B depletion promotes a caspase-8 apoptotic pathway via TNFRSF1A in endothelial cells. Wild type and P53M214K zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1A, TNFRSF1B, P53, CASP8 and CASP9 MOs alone or in
combination with the mRNAs encoding the caspase-8 inhibitors CFLAR and CMRA. (A) The activation of caspase-8 and caspase-9 in whole embryos was quantified at 60 hpf using the luminescent assays Caspase-Glo 8 and Caspase-Glo 9. Represented are mean values ± S.E.M. (B-D) Vascular defects scored at 72 hpf as indicated in the legend to Figure 2. Different letters denote statistically significant differences among the groups according to a Tukey test. Note that inhibition of the caspase-8 but not caspase-9 pathway partially rescues the TNFRSF1B phenotype. *P<0.05, **P<0.01, ***P<0.0001. n.s.: not significant, according to a Chi-square contingency test (B-D)

Figure 4. P53 activation is indispensable for promoting caspase-8-dependent apoptosis of endothelial cell in TNFRSF1B deficient zebrafish. Wild type (A, B, D-G), transgenic fli1a:eGFP (C) and P53M214K (H) zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1A, TNFRSF1B and P53 MOs. At 72 hpf, the mRNA levels of the indicated genes were determined by real-time RT-PCR in 10 pooled larvae (A, D, E, G, H). The gene expression is normalized against rps11, each bar represents the mean ± S.E. M. Different letters denote statistically significant differences among the groups according to a Tukey test. *P<0.05, n.s.: not significant according to Chi-square contingency (F) or Student’s t tests (D, E and H). (A) Coinjection of TNFRSF1A- or CASP8-mo reduces TNFRSF1B-mo-mediated P53 upregulation. (B) P53 protein levels were assayed by western blot in dechorionated and deyolked embryos at 24 hpf. Note that P53 levels are upregulated in TNFRSF1B-deficient larvae. (C) Whole mount immunohistochemistry against P53 at 3 dpf in STD- and TNFRSF1B MO-injected Tg(fli1a:eGFP) larvae. Note a massive increase in P53 expression (red) in the vascular endothelium (green) of TNFRSF1B-deficient larvae. (D, E) TNFRSF1B silencing leads to upregulation of bax, mdm2 and p21. (F) The vascular defects in TNFRSF1B/P53-deficient larvae were scored as indicated in the legend to Figure 2 and are
representative of two independent experiments. Note that TNFRSF1BP53 deficiency partially rescues the vascular defects observed in TNFRSF1B-deficient fish. (G) Upregulation of caspase-8 by TNFRSF1B-deficiency is dependent on TNFRSF1A and P53. (H) \( p21 \) mRNA levels in P53 mutant larvae are not altered by TNFRSF1B silencing. (I) Schematic representation of the proposed amplification loop between extrinsic and intrinsic apoptotic pathways triggered by TNFRSF1A. CA, caudal artery. CV, caudal vein. ISV, intersegmental vessels. Scale bars, 25 \( \mu \)m.

**Figure 5. DNA fragmentation in endothelial cells triggers P53 activation in TNFRSF1B-deficient zebrafish.** Wild type zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1B, ATR and/or ATM MOs alone or in combination with the mRNAs encoding ICAD or the antisense mRNA for TNFRSF1B, as an appropriate control. Some larvae were treated at 48 hpf by bath immersion for 16 h with 1\( \mu \)M of the CHK1 specific inhibitor G\( \text{o6976} \) and the vascular defects were scored at 72 hpf as indicated in the legend to Figure 2. The data shown in Figs. 5B are representative of two independent experiments. *\( \text{P}<0.05 \), **\( \text{P}<0.01 \), ***\( \text{P}<0.0001 \), n.s.: not significant according to a Chi-square contingency test.

**Figure 6. The TNFRSF1A apoptotic signaling pathway of endothelial cells is independent of caspase-3 but requires caspase-2.** Wild type and transgenic \( \text{fli1a:eGFP} \) zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1B, CASP8 and/or CASP2 MOs. The activation of caspase-3 in embryos was quantified at 60 hpf by flow cytometry (A) using a specific antibody for active caspase-3, while caspase-2 levels were determined using the luminescent assays Caspase-Glo 2 (B). The vascular defects were also scored at 72 hpf as indicated in the legend to Figure 2 (C). Note that TNFRSF1B depletion...
resulted in the caspase-8 dependent activation of caspase-2 and that caspase-2 depletion rescued the vascular defects of TNR2-deficient fish. Some larvae were treated at 48 hpf by bath immersion for 16 h with 0.5 µM camptothecin (campt) or 0.2 µM staurosporine (stauros) in the presence or absence of 100 µM of the caspase-3 inhibitor Ac-DEVD-CHO, as appropriate controls for caspase activation. Different letters denote statistically significant differences among the groups according to a Tukey test. *P<0.05, ***P<0.0001. n.s.: not significant, according or Student’s t (A) and Chi-square contingency tests (C).

Figure 7. The caspase-2/P53 apoptotic signaling pathway triggered by TNFRSF1A is evolutionary conserved and its activation reduces in vivo the invasiveness of malignant cancer cells. HUVEC were treated for 16 h with 40 ng/ml human TNFα (hTNFα) alone or in combination with 5 µg/ml of a mouse IgG isotype control or a neutralizing antibody to hTNFRSF1B (αhTNFRSF1B) (A, E) or with a TNFα mutein specific for TNFRSF1A (B-D, F). Cell viability was determined by the MTT colorimetric assay (A), PS flip and caspase-2 activity were evaluated by flow cytometry and immunofluorescence using phycoerythrin-Annexin V conjugate and the CaspGLOW Fluorescein Active Caspase-2 Staining kit, respectively (B, C), caspase-3 activation was determined by immunofluorescence using a specific antibody to active caspase-3 (D). Some cultures were treated for 16 h with 0.5 µM camptothecin (campt), 1 µM Gö6976 or 0.5 µM staurosporine (stauros) in the presence or absence of 50 µM of the caspase-3 inhibitor Ac-DEVD-CHO. Note that annexin V+ cells showed membrane blebbing (arrowheads). (E) The mRNA levels of the indicated genes were determined by real-time RT-PCR, normalized against rps11, and shown relative to non-treated cells (mean ± S.E.M.). (F) P53 protein levels were assayed by western blot using monoclonal antibodies to human P53 (TP53) and β-actin (ACTB). (G-I) Wild type zebrafish embryos were microinjected at the one-cell stage with STD or TNFRSF1B MOs. The
vascular defects were scored at 72 hpf as indicated in the legend to Figure 2. Different letters denote statistically significant differences among the groups according to a Tukey test. The groups labeled with “a” or “A” in Figure 7E did not show statistically significant differences with non-treated cells. *P<0.05 according to a Student’s t test. ND: not determined.

Figure 8. Model of TNFR signaling in endothelial cells. The TNFRSF1A apoptotic and TNFRSF1B survival pathways, the main elements involved, and the crosstalk between extrinsic and intrinsic apoptotic programs. TNFRL, TNFR ligands (TNFα and LTα).
Figure 3

A

B

C

D

Disease Models & Mechanisms
DMM
Accepted manuscript

Vascular defect score (percentage)

Vascular defect score (percentage)

Vascular defect score (percentage)

Vascular defect score (percentage)

Disease Models & Mechanisms (DMM) is a peer-reviewed journal that publishes articles on all aspects of disease models, including their design, development, and application. The journal covers a wide range of topics, from basic research to translational studies, and is open to contributions from all fields of medicine and biology. It is a leading journal in the field of disease modeling and mechanistic approaches to understanding and treating human diseases. The journal is committed to publishing high-quality, innovative research that advances our understanding of disease and contributes to the development of new therapeutic strategies.
Figure 6

A

B

C

Disease Models & Mechanisms
Figure S1. Interaction of zebrafish TNFα with TNFRSF1A and TNFRSF1B. HEK293 cells were transfected with TNFα-Xpress, IL-1β-Xpress, TNFRSF1A-V5, TNFRSF1B-V5 or RP105-V5 expression constructs. Forty-eight hours after transfection, cells were washed twice with PBS and lysed for the pull down assay as described in Materials and Methods. Cell extracts from HEK293 cells expressing TNFα-Xpress or IL-1β-Xpress (control), were incubated overnight with those of cells expressing V5 tagged TNFRSF1A, TNFRSF1B or RP105 (control), immunoprecipitated with the anti-V5 mAb and protein G sepharose, and probed with anti-Xpress (A) or V5 (B) mAbs. Note that TNFα was able to interact with both TNFR but failed to bind to RP105, a decoy receptor of TLR signaling. Similarly, IL-1β failed to interact with TNFRs.
Figure S2. Expression of $tnfrsf1a$, $tnfrsf1b$ and their ligands $tnfa$ and $lta$ during zebrafish embryonic and larval development. (A) The mRNA levels of the gene encoding the TNFRs ($tnfrsf1a$ and $tnfrsf1b$) and their ligands ($tnfa$ and $lta$) were determined by real-time RT-PCR in 10-30 pooled larvae at the indicated times. The gene expression is normalized against $rps11$, each bar represents the mean ± S.E. of triplicate readings from pooled larvae and the data are representative of three independent experiments. ND: not detected. (B) $tnfrsf1a$ and $tnfrsf1b$ whole-mount in situ hybridization (ISH) (a-h) and ISH on sagittal sections (i-m). Embryonic stages and used antisense riboprobes were as indicated in each panel. Anterior is to the left, dorsal to the top in all panels except in i, where dorsal is to the left. (a,b and e,f). Note that in general, the expression pattern is rather ubiquitous. $tnfrsf1a$ expression did not appear to be restricted to the vascular endothelium (arrow in k marks intersegmental vessel). A stronger expression of $tnfrsf1b$ could be observed in the head mesenchyme (arrow in l). hpf, hours postfertilization; nt, notochord; s, somite. Scale bars, 100 µm.
Figure S3. Efficiency of splice-blocking MOs against zebrafish TNFRs. RT-PCR analysis of TNFRSF1A-mo- (A) and TNFRSF1B-mo- (B) induced altered splicing of the 
\textit{tnfrsf1a} transcripts at 1, 3, 5 and 7 dpf. (A) A 540 bp product containing a deletion of the last 16 bp of exon 6 of \textit{tnfrsf1a} transcript was observed in samples injected with TNFRSF1A-mo, while it was absent from STD-mo-injected fish. This deletion resulted in a predicted TNFRSF1A protein lacking the signaling domain. (B) A 611 bp product containing a deletion of whole exon 2 of \textit{tnfrsf1b} transcript was observed in samples injected with TNFRSF1B-mo, while it was absent from STD-mo-injected fish. This deletion resulted in a predicted TNFRSF1B protein lacking most extracellular domain and the whole signaling domain. Samples without template (cDNA-) and those obtained in the absence of reverse transcriptase (RT-) gave no amplifications. The annealing of MOs (dashed line) and the inframe premature stop codons (arrowheads) are indicated.
Figure S4. Validation and effects of two additional MOs targeting zebrafish TNFRSF1B. (A) RT-PCR analysis of TNFRSF1B-E1/I1-mo induced altered splicing of the \textit{infrs1b} transcript at 3 dpf. A dose-dependent decline of the wild type RNA was observed, suggesting the insertion of intron 1, which has 15,726 bp length. This insertion resulted in a predicted TNFRSF1B protein lacking both TNF$\alpha$ binding and signaling domains. Samples without template (-) gave no amplifications. The annealing of MO (dashed line) and the inframe premature stop codons (arrowheads) are indicated. (B) Embryos injected with 8 ng/egg (1 mM) TNFRSF1B-E1/I1-mo (middle panel) or TNFRSF1B-ATG-mo showed impaired differentiation of the caudal artery (CA) and caudal vein (CV) (asterisks), hemorrhages throughout the body (arrowheads) and altered blood circulation. A control larvae injected with STD-mo is shown on the left panel for comparison. Scale bars, 100 $\mu$m. (C) The vascular defects shown in (B) were scored at 72 hpf as indicated in the legend to Figure 2. ***P<0.0001 according to Chi-square contingency test.
Figure S5. Confirmation of the vascular defects promoted by genetic depletion of TNFRSF1B using a DN mutant form. (A) Representative embryo injected with 200 ng/egg DN TNFRSF1B (right panel) showed impaired differentiation of the caudal artery (CA) and caudal vein (CV). Note the interruption of the CA (arrow) and blood pooling in the altered CV (asterisks). A control larva injected with 200 ng/egg antisense mRNA is shown on the left panel for comparison. Scale bars, 500 µm. (C) The vascular defects shown in (A) were scored at 72 hpf as indicated in the legend to Figure 2. ***P<0.0001 according to Chi-square contingency test.
Table S1. Morpholinos used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

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Table S2. Effects of TNFRSF1B MO targeting the intron 1-exon 2 boundary of pre-mRNA in development.

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**HUMAN PRIMERS**

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