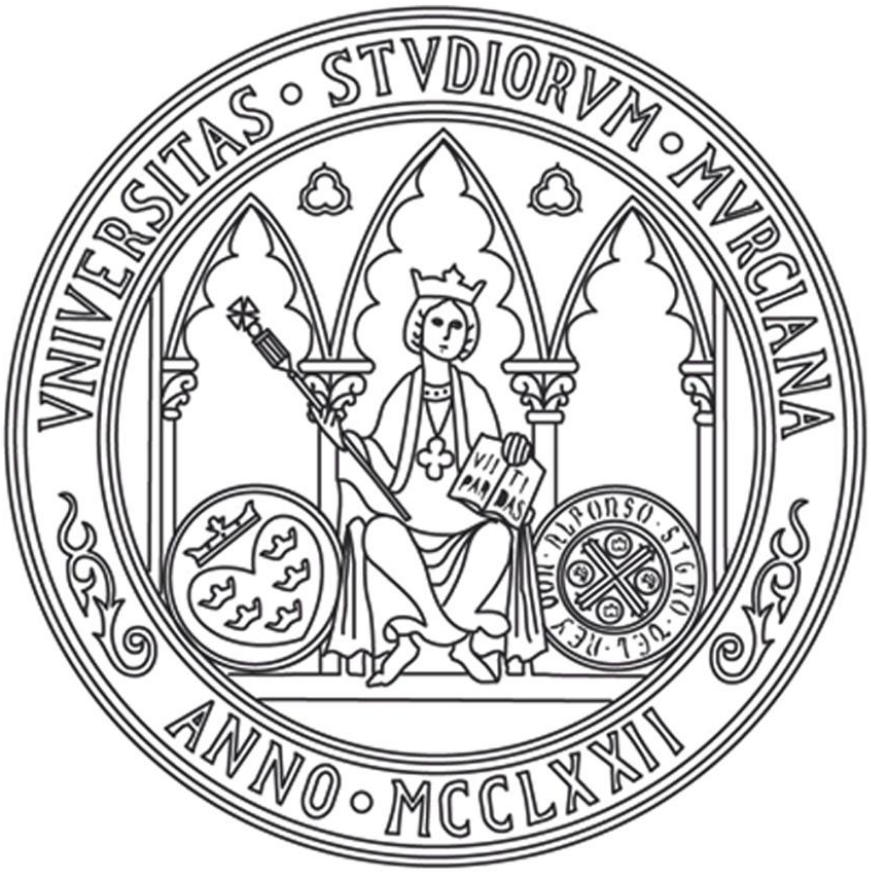

UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Functional Characterization of
TNF Receptors in Zebrafish

Caracterización Funcional de los
Receptores de TNF de Pez Cebra

Dña. Raquel Espín Palazón

2013

A todos aquellos que me han acompañado

en mi tesis doctoral y, en especial,

a mis padres y mi hermana.

INDEX

ABBREVIATIONS

AA	Aortic arches
AGM	Aorta-gonad-mesonephros
ALM	Anterior lateral plate mesoderm
AP-1	Activator protein-1
APRIL	Proliferation-inducing ligand
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BA	Branchial arches
BAFF	B-cell activating factor
CA	Caudal artery
CAD	Caspase activated DNase
CASP	Caspase
CCV	Common cardinal vein
CD27L	Cluster of differentiation 27 ligand
CD40L	Cluster of differentiation 40 ligand
CFLAR	FADD-like apoptosis regulator
CHK1	Checkpoint kinase
CHT	Caudal hematopoiestic tissue
CMP	Common myeloid progenitor
COX-2	Cyclooxygenase-2
CRDs	Cystein-rich domains
CRMA	Cytokine response modifier A
CV	Caudal vein
DA	Dorsal aorta
DC	Duct of Cuvier
DD	Death domains
dH_2O	Distilled water
DLAV	Dorsal longitudinal anastomotic vessel
DNA	Deoxyribonucleic acid

DMSO	Dimethyl sulfoxide
DN TNFRSF1B	Dominant negative mutant of TNFRSF1B
Dpf	Days post-fertilization
ECL	Enhanced chemiluminescence
EDA-A	Anhidrotic ectodermal displasia A
EMPs	Erythromyeloid progenitors
eph	Ephrin
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
FASL	Fibroblast-associated ligand
flk1	Fetal liver kinase-1
GFP	Green fluorescent protein
H	Heart
HEK293 cells	Human embryonic kidney 293 cells
Hpf	Hours post-fertilization
HSCs	Hematopoietic stem cells
HTA	Head trunk angle
HUVECs	Human umbilical vein endothelial cells
ICAD	Inhibitor of caspase activated DNase
ICM	Intermediate cell mass
IκB	NF-κB inhibitor
IKK	IκB kinase complex
IL-6	Interleukin 6
IL-18	Interleukin 18
iNOS	Inducible nitric oxide synthase
iPS	Induced pluripotent stem
ISVs	Intersegmental vessels
JNK	c-Jun N-terminal kinase
LDA	Lateral dorsal aorta

5-LOX	5-lipoxygenase
LT	Lymphotoxin
mem-TNFα	Membrane-anchored TNFα form
MKK	MAP kinase kinase
MO	Morpholino
N.D.	Not detected
NF-κB	Nuclear Factor κB
NK	Natural killer
Ns	Not significant
OX40L	OX40 ligand
PBI	Posterior blood island
PBS	Phosphate buffered saline
PBT	Phosphate buffered solution with 0.1 % Tween-20
PCV	Posterior cardinal vein
PFA	Paraformaldehyde
PHS	Primary head sinus
PLM	Posterior lateral plate mesoderm
p38MAPK	p38 mitogen-activated protein kinase
RIP	Receptor interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
SA	Segmental artery
Scl	Stem cell leukemia
SDS-PAGE	Sodium dodecyl sulfate polyacrilamide gel electrophoresis
S.E.M.	Standard error of the mean
STD-mo	Standard morpholino
sTNFα	Soluble TNFα

SV	Segmental vein
TACE	TNFα converting enzyme
TAK	TGF-β-activated kinase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFSF	Tumor necrosis dactor superfamily
TNFRSF	Tumor necrosis factor receptor superfamily member
TRADD	TNFR associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-induced ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTP	Uridine-5'-triphosphate
vegfr2	Vascular endothelial growth factor receptor 2
VEGI	Vascular endothelial cell-growth inhibitor
ZIRC	Zebrafish International Resource Center
ZFNs	Zinc finger nucleases

SUMMARY

During this Doctoral Thesis, we have characterized the role of Tumor necrosis factor alpha (TNFα) and TNF receptors (TNFRs) during endothelial and hematopoiesis development in the embryo using zebrafish (*Danio rerio*) as a vertebrate model.

Targeted gene knockdown of Tnfr2 (Tumor necrosis factor receptor 2) 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 Tnfr1 (Tumor necrosis factor receptor 1) or the activation of NF-κB (nuclear factor-κB) rescue endothelial cell apoptosis, indicating that a signaling balance between both TNFRs is required for endothelial cell integrity and that, in endothelial cells, Tnfr1 signals apoptosis through caspase-8 while Tnfr2 signals survival via NF-κB. Similarly, Tnfa promotes the apoptosis of human endothelial cells through Tnfr1 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.

On the other hand, we aimed to characterize the role of TNFα receptors and their ligands (TNFα and LTα, lymphotoxin alpha) in the embryonic development of the hematopoietic system. Our results indicate that Tnfa signaling through Tnfr2 is dispensable for the specification of HSCs, but is intrinsically required for their subsequent maintenance and expansion. The genetic inhibition of Tnfa or Tnfr2, but not Lta or Tnfr1, results in the apoptosis of HSCs soon after their emergence. These results are the first to describe the role of TNFR signaling in HSCs, and highlight a novel role for a signaling pathway predominantly associated with the regulation of inflammation and immunity.

INTRODUCTION

1. TUMOR NECROSIS FACTOR ALPHA (TNFα)

1.1 TUMOR NECROSIS FACTOR SUPERFAMILY (TNFSF)

Tumor necrosis factor alpha (TNFα) is a powerful proinflammatory cytokine which exerts its function by binding two specific receptors: TNFR1 and TNFR2. Since TNFα was cloned in 1984, many other proteins have been showed to have high sequence homology with this cytokine, including TNFα receptors. Thus, Tumor necrosis factor superfamily (TNFSF) was created to include all these sequence-related proteins. The TNFSF is nowadays composed by 19 ligands (TNF superfamily ligands: TNFSFs) and 29 receptors (TNF superfamily receptors: TNFSFRs) [1], and most of them are produced by immune cells such as natural killer (NK), T and B cells, macrophages, dendritic cells, mast cells, neutrophils and monocytes. However, other types of cells like hematopoietic cells, endothelial cells, skin cells and smooth muscle cells have been also reported to produce some of this proteins belonging to the TNFSF. Among TNFSF proteins, the following members can be emphasised: tumor necrosis factor beta, TNFβ (also called LTα); lymphotoxin beta, LTβ; fibroblast-associated ligand, FASL; LIGHT; LIGHTR; TNF-related apoptosis-induced ligand, TRAIL; proliferation-inducing ligand, APRIL; and vascular endothelial cell-growth inhibitor, VEGI.

All members of the TNF superfamily have been shown to have pro-inflammatory activity, yet some of them also exhibit proliferative activity on hematopoietic cells and play a role in morphogenetic changes and differentiation. Therefore, TNFSF members play roles as contradictory as cell apoptosis and survival, proliferation or differentiation (Figure 1).

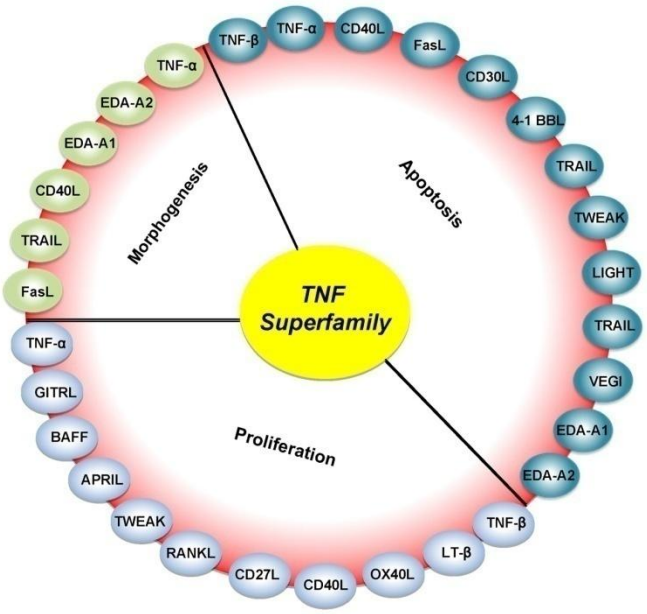

Figure 1. Roles of some members of TNFSF in apoptosis, morphogenesis and proliferation. All members of the TNF superfamily exhibit pro-inflammatory activity, in part through activation of the transcription factor NF-κB (full red circle); OX40L, CD40L, CD27L, APRIL, and BAFF exhibit proliferative activity in part through activation of various mitogen-activated kinases (sky blue); TNFα, TNFβ, FasL, and TRAIL control apoptosis (bluish-green); and EDA-A1, EDA-A2, TNFα, FasL, and TRAIL regulate morphogenesis (green). Adapted from Aggarwal [1].

1.2 TNFα ESTRUCTURE, NOMENCLATURE AND FUNCTION

TNFα presents an extracellular C- and an intracellular N- terminus when this cytokine is anchored to the plasma membrane (named pro-TNFα or membrane TNF-α, mem-TNFα) and it is composed of three identical TNFα units. Pro-TNFα can be processed by a TNFα converting enzyme (TACE), transforming the membrane precursor in a soluble C-terminal form (sTNFα), which exerts its biological actions as a 51 kDa soluble homotrimer [2] (Figure 2).

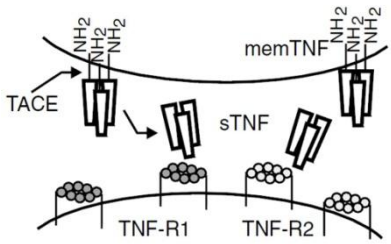

Figure 2. Mem-TNFα can be processed by TACE, releasing the extracellular C-terminal of pro-TNFα. Adapted from Wajant [2].

TNFα nomenclature can be confusing since it has changed along the years. In 1998, the name for TNFα was changed upon recommendation by the TNF Congress to TNF. However, the term TNFα is still widely used and it is used in this thesis as a term which refers to both, pro-TNFα and mem-TNFα.

TNFα is mainly produced by macrophages, but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, T and B cells, NK cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons. TNFα is known to affect the growth, differentiation, survival and physiological functions of a variety of different cells, including cells outside of the immune system. Thus, TNFα is a true pleiotropic factor which plays an important role in the immune response as well as in other physiological processes, such as metabolism and reproduction. Perhaps one of the best known actions of TNFα is in macrophage-mediated cytotoxicity due to the proapoptotic effects of TNFα. However, TNFα is increasingly recognized as a key regulator of lipid metabolism in adipose tissue and protein catabolism in muscle.

Also, TNFα has been shown to be linked to an array of pathophysiologies, including cancer, neurologic diseases, cardiovascular diseases, pulmonary diseases, autoimmune diseases and metabolic diseases [1] (Figure 3).

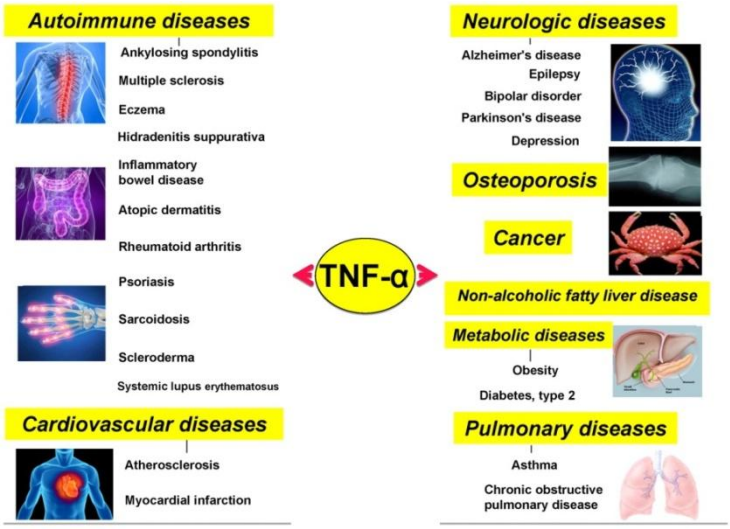

Figure 3. Various diseases that have been closely linked to TNFα. Adapted from Aggarwal [1].

2. TNFα RECEPTORS: TNFR1 AND TNFR2

Both forms, soluble and membrane-bound TNFα, can bind two transmembrane receptors: TNFR1 (also called TNFRSF1A, TNF receptor type 1, p55/60, CD120a) and TNFR2 (TNFRSF1B, TNF receptor type 2, p75/80, CD120b) (Figure 4). Both receptors belong to the TNFSFR family, sharing with all their members the peculiarity of having the named cystein-rich domains (CRDs) in their extracellular domain, which is involved in the ligand binding. Even though TNFR1 is activated by both TNFα forms, TNFR2 is mainly activated by the membrane TNFα [3].

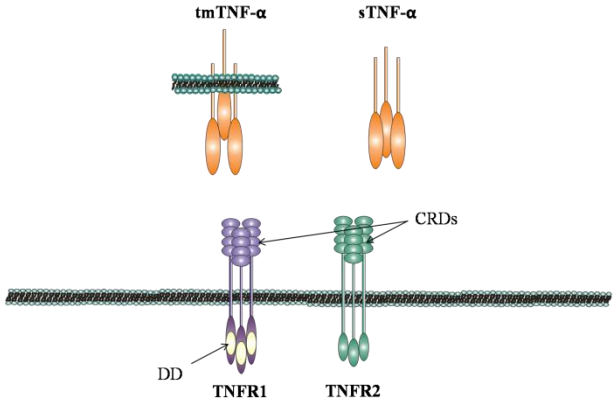

Figure 4. Schematic representation of the pro-TNFα and sTNFα and their receptors TNFR1 and TNFR2. DD: Death domains; CRDs: Cystein-rich domains.

2.1. ESTRUCTURE AND FUNCTION

TNFα receptors have been reported to have marked differences in structure, expression patterns, signalling and function (Table1). While TNFR1 has been found in all cell types, TNFR2 seems to have a more restricted expression to certain cells such as CD4 and CD8 T lymphocytes, thymocytes, endothelial cells, microglia, oligodendrocytes, neuron subtypes and human mesenchymal stem cells [4]. This means that each cell types bearing TNFR2 also have TNFR1. The ratio of expression of TNFR1 to TNFR2 and the signalling behind these receptors typically fluctuates in

relation to the cell type and its functional roles. Moreover, for immune cells, the state of prior activation of the cell is also a key variable.

Table 1. TNFR1 and TNFR2 expression, signalling and function. FADD, Fas-associated death domain; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; NF-κB, nuclear factor-κB; TNFR, tumour necrosis factor receptor; TRADD, TNFR1-associated death domain; TRAF, TNF receptor-associated factor. Adapted from [4].

STRUCTURE	EXPRESSION	SIGNALLING	FUNCTION
TNFR1			
-Extracellular domain: cystein-rich and similar to TNFR2 -Intracellular domain: death domain; no resemblance to TNFR2	Nearly all cells of the body	Adapter protein FADD and TRADD, which engage caspases	-Pro-apoptosis -Some pro-survival functions, depending on crosstalk with TNFR2, cell type, activation state, age and other factors.
TNFR2			
-Extracellular domain: cystein-rich and similar to TNFR1 -Intracellular domain: no death domain; no resemblance to TNFR2	Limited expression: CD4 and CD8 T lymphocytes, endothelial cells, microglia, oligodendrocytes, neuron subtypes, cardiac myocytes, thymocytes and human mesenchimal stem cells	One pathway involves adapter proteins TRAF1 and TRAF2 and the eventual cleavage of transcription factor NF-κB from its inhibitor molecule in the cytoplasm IκB	-Pro-survival functions in immunity to infection, injury, cancer and autoimmune disease via proliferation of T cell differentiation and recruitment of naive immune cells. -Some pro-apoptotic functions to limit immune response after injury or inflammation resolves. -Other pro-apoptotic functions depend on crosstalk with TNFR1, cell type, activation state, age and other factors

Both receptors comprise four domains: a hydrophobic signal peptide, an intracellular domain, a transmembrane segment and a soluble extracellular cysteine-rich domain which functions as the receptor locus [5]. The soluble extracellular domains of TNFR1 and TNFR2 have been purified, sequenced, and cloned [6] and they exhibit 28% of sequence homology [7], being capable of binding either TNFα or TNFβ. However, TNFR1 alone possesses a cytoplasmic death domain, an 80 amino-acid sequence that rapidly engages the apoptotic signalling pathway of the cells. The cytoplasmic domain of TNFR2 bears no structural or functional resemblance to that of TNFR1 [8, 9].

The DD of TNFR1 is capable of rapidly triggering apoptosis in the cell. Thus, TNFR1 has been described to mainly produce apoptosis, whereas proliferation is associated to TNFR2. TNFR2 can also induce apoptosis in certain circumstances, having been described that there is crosstalk between both receptors [3]. Therefore, both TNFR1 and TNFR2 can mediate proliferation, differentiation and inflammation [4].

2.2. SIGNALLING PATHWAYS

TNFα signalling through TNFR1 and TNFR2 receptors is complex; mainly due to that neither TNFR1 nor TNFR2 possesses enzymatic activity *per sé*, and different adaptor proteins need to be recruited. Moreover, some of these adaptor proteins are able to participate in both TNFR1 and TNFR2 pathways, making difficult to discriminate the receptor which is signalling.

TNFα induces at least 5 different types of signals that include activation of NF-κB, apoptosis pathways, extracellular signal regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK), and c-Jun N-terminal kinase (JNK) (Figure 5). When TNFα binds to TNFR1, it recruits a protein called TNFR associated death domain (TRADD) through its DD [9]. TRADD then recruits a protein called Fas-associated protein with death domain (FADD), which then sequentially activates caspase-8 and caspase-3, leading to apoptosis [10]. Alternatively, TNFα can activate mitochondria to sequentially release reactive oxygen species (ROS), cytochrome C, and Bax, leading to activation of caspase-9 and caspase-3 and thus apoptosis. Paradoxically, TNFα has also been shown to activate NF-κB, which in turn regulates the expression of proteins associated with cell survival and proliferation [11]. NF-κB activation by TNFα is mediated through sequential recruitment of TNFR1, TRADD, TNFR-associated factor 2 (TRAF2/TRAF5) and receptor interacting protein (RIP), resulting in activation of TGF-β-activated kinase 1 (TAK1) and IκB kinase (IKK) complex, the phosphorylation, ubiquitination, and degradation of inhibitor of nuclear factor-κBα (IκBα), and, finally, the nuclear translocation of p50 and p65 NF-κB subunits and DNA binding [12]. The proinflammatory effect of TNF is mediated through NFκB–induced transcriptions of genes encoding IL-6, IL-18, chemokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX), all major mediators of inflammation. Indeed, TNFα can induce expression of TNFα itself through activation of NF-κB [13]. TNFα can also activate cellular proliferation through activation of another transcription factor, activator protein-1 (AP-1) [14], which is activated by TNFα through sequential recruitment of TNFR1, TRADD, TRAF2, MAP/ERK kinase kinase 1 (MEKK1), MAP kinase kinase 7 (MKK7), and JNK. The activation of p38MAPK by TNFα is mediated through TRADD-TRAF2- MKK3. How TNFR2, which lacks a DD, activates cell signalling is much less clear than how TNFR1 activates cell signalling. Because TNFR2 can directly bind to TRAF2, it can activate both NF-κB and MAPK signalling quite well. Interestingly, TRADD has been reported recently to mediate cell signalling by TOLL-like receptors 3 and 4 [15].

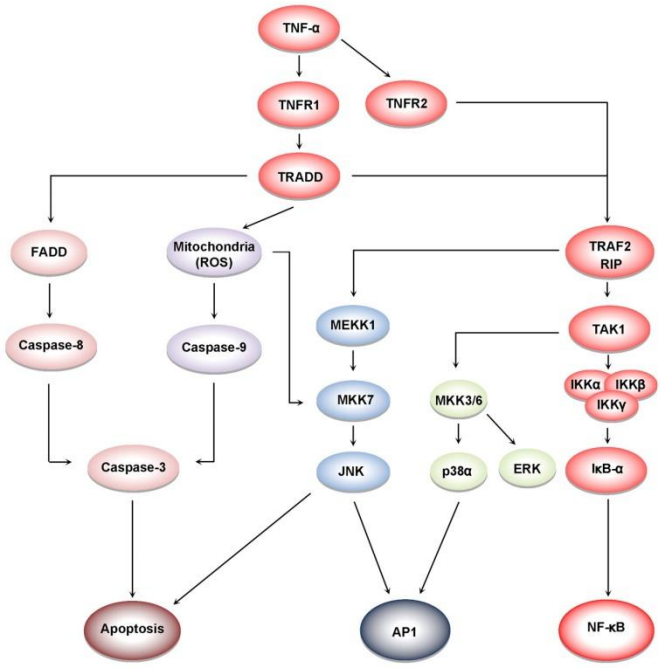

Figure 5. **Cell signalling pathways activated by TNFα**. TNFR1 activation leads to recruitment of intracellular adaptor proteins (TRADD, FADD, TRAF, and RIP), which activate multiple signal transduction pathways. TNFR sequentially recruits TRADD, TRAF2, RIP, TAK1, and IKK, leading to the activation of NF-κB; and the recruitment of TRADD, FADD, and caspase-8, leads to the activation of caspase-3, which in turn induces apoptosis. JNK is activated through the sequential recruitment of TRAF2, RIP, MEKK1, and MKK7. Exposure of cells to TNFα in most cases results in the generation of ROS, leading to activation of MKK7 and JNK. The activation of ERK and p38MAPK is via TRADD, TRAF2, RIP, TAK1, and MKK3/6. Adapted from Aggarwal [1].

3. ZEBRAFISH AS A VERTEBRATE MODEL

Zebrafish, *Danio rerio* (Figure 6), is a tropical freshwater fish belonging to the Cyprinidae family and order Cypriniformes. Since it was first used in a scientific laboratory 30 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models. Therefore, nowadays, zebrafish is a very popular model for the study of both, basic and applied science, being extensively used for large-scale screenings, developmental and genetic studies *in vivo* as well as a model of human pathologies.

Figure 6. Adult zebrafish. Adapted from Yong 2011.

Among the multiple advantages that make zebrafish an excellent research model, can be emphasized:

- As a vertebrate, zebrafish shares considerable genetic sequence similarity with humans.
- Low maintenance cost and small space needed.
- High resistance to pathogens.

- High fecundity and large production of embryos (around 200 eggs/female/week) makes phenotype-based forward genetics doable.
- Externally development following fertilization, making them easily accessible to embryonic manipulation and imaging.
- Transparency of zebrafish embryos, together with the large availability of transgenic lines, let *in vivo* tracking of cells easy (Figure 7).
- Fast zebrafish development from embryo to larva in just three days.
- Easy to transfer among different labs by transporting their eggs.
- It is relatively easy to knockdown specific genes by using morpholinos and overexpressing proteins by mRNA or plasmids.
- Use of reverse genetics approaches using zinc finger nucleases (ZFNs) [16] and a transposon strategy [17] for generating transgenic zebrafish, which help in analyzing new roles of additional genes in larval and adult zebrafish.

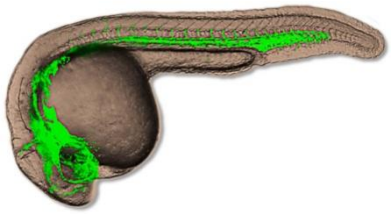

Figure 7. Transgenic *fli1a:eGFP* embryo at 24 hpf in which the development of the vasculature can be easily tracked *in vivo*.

All these advantages have lead to the increased interest of scientists using zebrafish as an animal model in the last years, and nowadays, zebrafish has been proposed as an excellent vertebrate model for the study of the immune system [18], hematopoiesis [19], vascular development Isogai [20-22], neurogenesis [23] and cancer research [24], among others.

4. VASCULATURE DEVELOPMET

4.1. ZEBRAFISH AS AN *IN VIVO* MODEL FOR VASCULAR DEVELOPMENT.

Adding to the general advantages that zebrafish has as a vertebrate model, it has been proposed specifically as an excellent model for vascular development research due to:

- As in mammals, zebrafish has a closed circulatory system.

- The anatomical form of the developing vasculature, the processes used to assemble vessels and the molecular mechanisms underlying vessel formation are highly similar to those in humans and other higher vertebrates (Figure 8).

- Zebrafish are amenable to large-scale forward-genetic analysis, making them very useful for genetic screens for identifying vascular-specific mutations in novel genes regulating vascular development.

- The small size of zebrafish embryos also allows them to receive sufficient oxygen for the first few days of development by passive diffusion alone, allowing other organs and tissue to continue to develop normally for several days in the absence of a functional cardiovascular system and greatly facilitating analysis of the specificity of vascular phenotypes caused by mutations or experimental manipulation.

- The availability of stable transgenic lines with different promoter-driven fluorescent proteins in which endothelial cells are marked, such as *fli1a:eGFP* [25] and *flk1:Dsred* [26].

Together, all of these characteristics make zebrafish an ideal system to study the development of the blood vascular system *in vivo*.

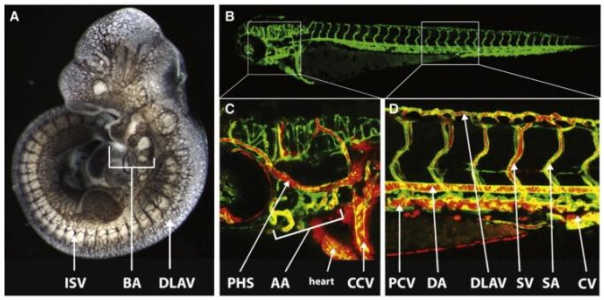

Figure 8. The basic anatomy of the initial embryonic circulatory system is quite similar among vertebrates. The embryonic vascular system in a day 10 mouse (A) and 3-day-old zebrafish embryos (B). BA: branchial arches (1st and 2nd); ISV: intersegmental vessel; DLAV: dorsal longitudinal anastomotic vessel; AA: aortic arches (1–6); CV: caudal vein; CCV: common cardinal vein; DA: dorsal aorta; PCV: posterior cardinal vein; PHS: primary head sinus; SA: segmental artery; SV segmental vein. Adapted from Ellertsdottir [27].

4.2. VASCULATURE DEVELOPMENT IN THE ZEBRAFISH EMBRYO.

In vertebrates, the cardiovascular system constitutes a highly ramified network of tubes that transports gas, nutrients, hormones and metabolites throughout the body. It also has important roles in the regulation of homeostasis and wound healing and is involved in the pathology of numerous diseases including cancer and inflammation [28].

The cardiovascular system emerges as one of the first organs during embryonic development and retains morphogenetic plasticity in adult life. There are two different mechanisms for blood vessels formation in vertebrates called vasculogenesis and angiogenesis. In the first one, vessels are form *de novo* by *in situ* aggregation of angioblasts [29-32], in contrast, in angiogenesis, vessels are formed from preexisting ones [33]. Both processes, vasculogenesis and angiogenesis, occur during zebrafish vasculature development.

The first embryonic vessels are formed by vasculogenesis (Figures 9 and 10), in which early mesoderm derived precursor endothelial cells or angioblasts coalesce to form the primary vasculature including the dorsal aorta (DA), posterior cardinal vein (PCV), and primitive cranial vasculature [34]. As these first vessels assemble, they concomitantly acquire arterial and venous identity. The vasculature is fundamentally divided into two types of blood vessels, arteries and veins, and the acquisition of arterial-venous identity and the assembly of distinct networks of arterial and venous vessels are critical for the proper functioning of the circulatory system.

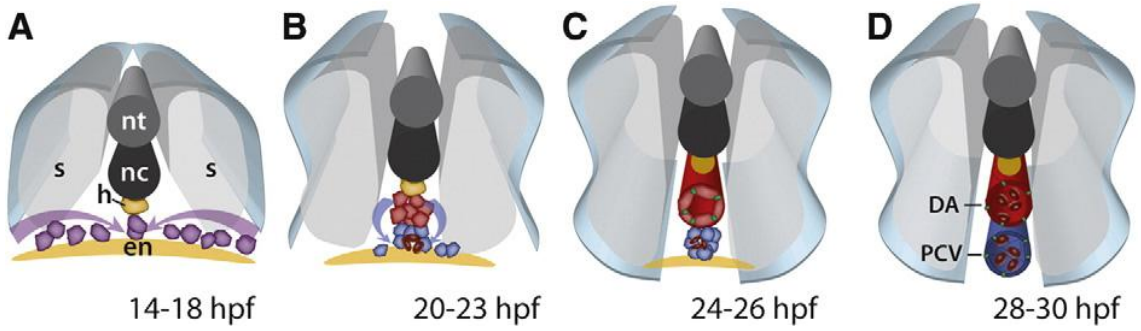

Figure 9. Phases of vasculogenesis in the zebrafish embryo. Schematic cross sections of the trunk region at representative stages of development. (A) Medial migration. From 14 hours post-fertilization (hpf) onward, angioblasts (purple) that originate in the lateral plate mesoderm migrate over the endoderm towards the midline just below the hypochord, where they aggregate to form a vascular cord (B). (B) Arterio-venous segregation and ventral sprouting. At around 17 hpf, angioblasts start to express markers of arterio-venous differentiation, such as ephrin-b2a (eph-b2a) in arterial cells (marked in red). These cells are located in the dorsal portion of the vascular rod and will give rise to the DA, whereas ephrin-b4a (ephb4a) expressing cells are located more ventrally and will contribute to the PCV and CV. At 21 hpf, angioblasts located in the ventral part of the vascular cord start migrating ventrally and accumulate below the forming DA (B, C). (C) Lumen formation. The DA forms and lumenizes prior to the PCV and CV in the absence of blood cells (brown) by cord hollowing. Venous angioblasts aggregate and coalesce around the blood cells to ultimately form a tube. (D) Functional Vasculature. At 30 hpf, both vessels are fully formed and carry blood flow. Endothelial cell junctions are indicated in green. Adapted from Ellertsdottir [27].

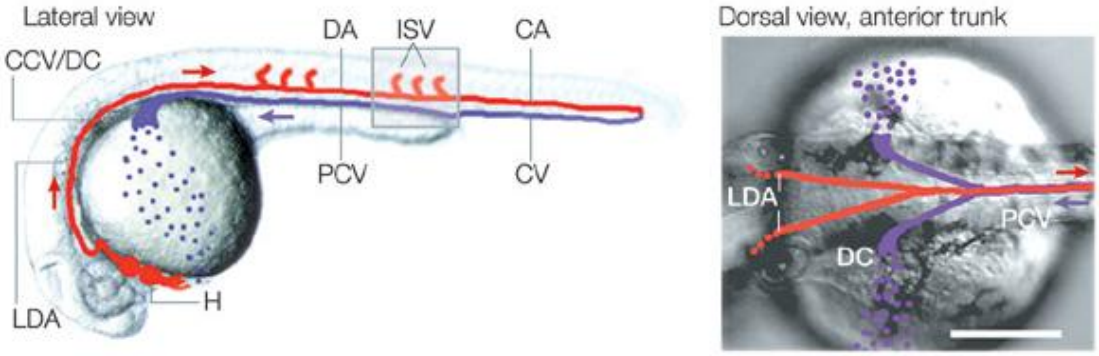

Figure 10. The zebrafish embryo vasculature. The circulatory system of a zebrafish embryo at 30 hpf (lateral and dorsal views). Blood leaves the heart (H) and enters the lateral dorsal aorta (LDA), which converge into the dorsal aorta (DA). The circulation progresses into the caudal artery (CA), where blood empties into the caudal vein (CV) and returns rostrally through the common cardinal vein (CCV), which is also referred to as the Duct of Cuvier (DC), before returning to the heart. At this time, intersegmental vessels are sprouting from the dorsal aorta, but do not yet carry circulation. ISV, intersegmental vessel. Adapted from Lawson [25].

Following initial formation of the primitive vasculature by vasculogenesis, most subsequent vessel formation during development takes place via angiogenesis. During this process, the formation of new vessels is by budding growth from or remodeling of preexisting vessels. The intersegmental vessels (ISVs) of the trunk are among the first angiogenic vessels to form in all vertebrates (Figure 10). A two-step process for intersegmental vessel formation has been documented by time-lapse imaging of vessel development in transgenic zebrafish [35]. Following formation of the DA and PCV, a set of new sprouts emerges from the dorsal side of the DA and grows dorsally along vertical somite boundaries. The migration of these sprouts is very rapid and dynamic,

with numerous filopodia-like cellular processes extending from the tips of the growing sprouts in all directions. As they reach the dorsal-lateral surface of the neural tube, the growing vascular segments branch rostrally and caudally, and then interconnect to form the dorsal longitudinal anastomotic vessel (DLAV). At the early stages the identity of all of these vessels is not defined, but as the vessels become interconnected and flow begins arterial-venous fate becomes established, likely in response to this flow [36].

5. ONTOGENY OF HEMATOPOIESIS

Hematopoiesis is a vital process for invertebrate and vertebrate organisms. The ontogeny of blood cells from multiple hematopoietic organs appears to be a feature common to all organisms with multiple hematopoietic lineages [37, 38]. In the adulthood, all blood lineages come originally from the hematopoietic stem cells (HSCs), defined by their self-renewal and differentiation into all blood cell types in transplanted recipients previously irradiated. It has been supported by lineage tracing experiments in zebrafish, that HSCs are specified during embryonic development, not arising *de novo* in the adulthood [39].

5.1. ZEBRAFISH AS AN *IN VIVO* MODEL FOR HEMATOPOIESIS

The zebrafish has contributed a lot to the field of developmental hematopoiesis in the past decade [40, 41]. This contribution is due to different aspects that make zebrafish an excellent model for the hematopoiesis research:

- The isolation of more than 50 mutant lines with diverse and interesting hematopoietic defects using multiple genetic screens [42-44] (Figure 11).

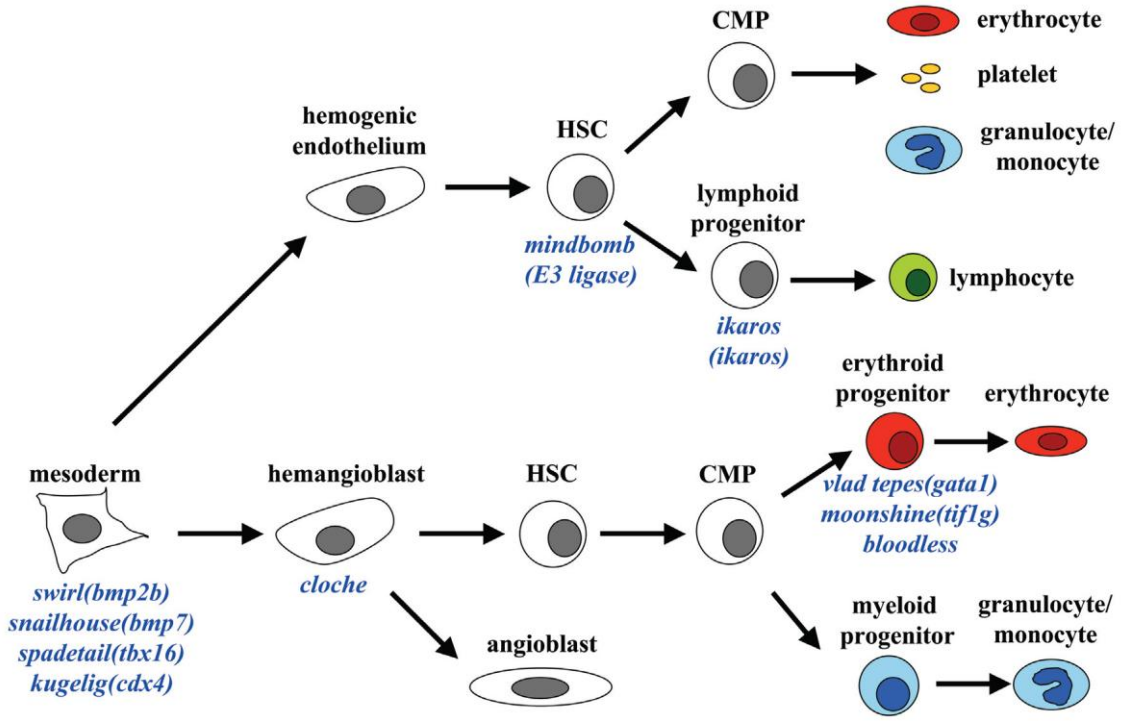

Figure 11. Overview of zebrafish mutants corresponding to the hematopoietic stage of development. HSC=hematopoietic stem cell; CMP= common myeloid progenitor. Adapted from Paik [44].

- The molecular basis of hematopoiesis is highly conserved between teleosts and higher vertebrates, including humans [45].

- As in all vertebrate embryos, zebrafish has different waves of hematopoiesis (Figure 12).

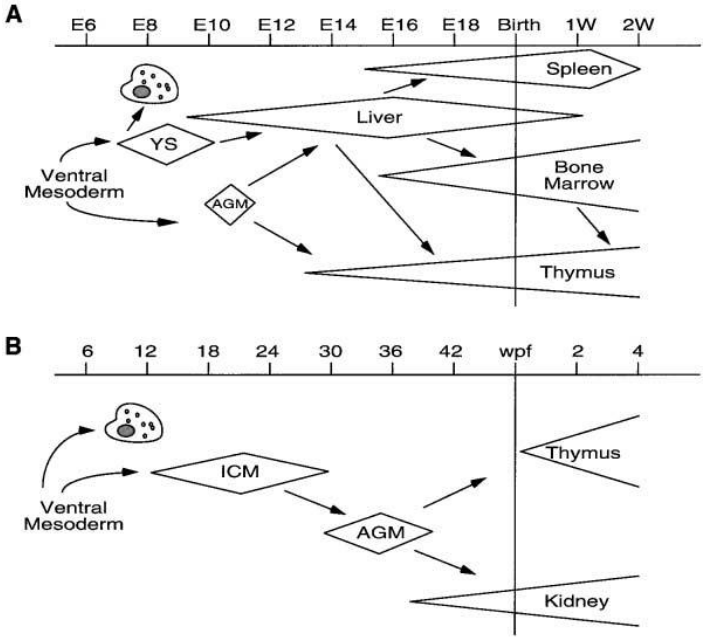

Figure 12. Sequential sites of hematopoiesis during development hematopoietic ontogeny in mice (A) and zebrafish (B). Adapted from Traver [46].

- The generation of a handful of transgenic lines has facilitated the *in vivo* analysis, in real time, of developing hematopoietic cells [45].

- Development of specific assays to examine zebrafish hematopoiesis such as irradiation recovery and kidney marrow transplantation [47, 48].

- Most of the critical hematopoietic transcription factor genes identified in mammals have orthologues in zebrafish.

5.2. ENDOTHELIAL AND HEMATOPOIETIC CELLS: A COMMON ORIGEN

As in other vertebrates, endothelial and hematopoietic cells in zebrafish embryos arise in close association with one to another, and are thought to be derived from a common precursor. In zebrafish embryos, the two cell lineages develop in a spatially distinct manner from other vertebrates but are thought to rely on common genetic programs [49]. In avian and mammalian embryos, endothelial and hematopoietic cells develop in extraembryonic yolk sac blood islands, whereas in zebrafish embryos they develop in the intermediate cell mass (ICM) derived from the ventral mesoderm [49]. However, early hematopoietic and endothelial cells in zebrafish and mammals express a common set of genes. Early expression of stem cell leukemia (*scl*) and fetal liver kinase-1/vascular endothelial growth factor receptor 2 (*flk1/vegfr2*) is required for endothelial and hematopoietic lineage formation in both mouse and zebrafish [50].

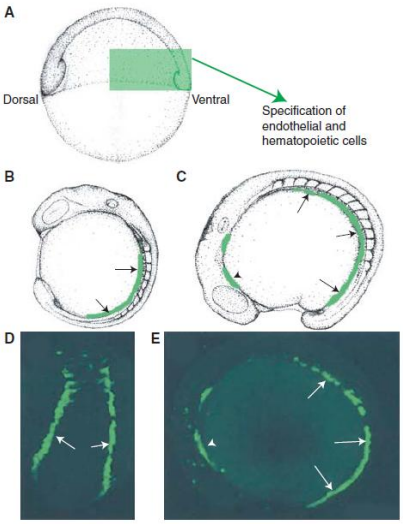

Figure 13. Endothelial cells and hematopoietic cells are closely specified during early embryogenesis. (A) Schematic diagram of an early stage zebrafish embryo, with green boxed area indicating the region of ventral mesoderm in which endothelial precursors are specified. (B,C) Schematic diagrams of 14 somite (B) and 16 somite (C) stage embryos shown in lateral view, with migrating angioblasts shown in green. (D, E) Fluorescence micrographs of live embryos of comparable ages to B and C, with green fluorescent protein expressed under the control of an endothelial-specific promoter. Arrows in B–E show migrating caudal angioblasts in the posterior lateral plate mesoderm. Arrowheads in C and E show the position of rostral angioblasts in the anterior lateral mesoderm. Adapted from [22].

5.3. HEMATOPOIESIS IN THE ZEBRAFISH EMBRYO

As in all vertebrates, zebrafish also experience different waves of hematopoiesis during embryogenesis: the primitive and definitive waves of hematopoiesis [45, 51]. These waves have been named differently regarding to the ability of the hematopoietic cells not to have or have self-renewal properties, respectively.

5.3.1. THE PRIMITIVE WAVES

The first wave, primitive hematopoiesis, takes place in two distinct areas of the embryo: the anterior lateral plate mesoderm (ALM) and the posterior lateral plate mesoderm (PLM) (Figure 14). This ventral mesoderm in zebrafish is equivalent to the yolk sac in mammals. At early somitogenesis stages, the ALM gives rise to a transient hemangioblast population that differentiates into myeloid and endothelial cells. In its turn, the PLM gives rise to a second population of hemangioblasts that differentiate into erythroid and endothelial cells. Red blood cells do not differentiate in the PLM, instead the erythroid derivatives of the PLM migrate to the midline to differentiate in the intermediate cell mass (ICM).

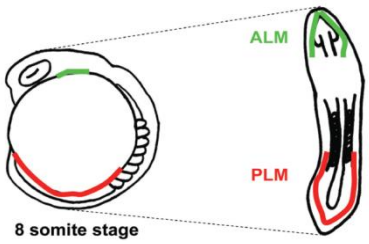

Figure 14. Areas of primitive hematopoiesis in the zebrafish embryo. Adapted from [44].

5.3.2. THE DEFINITIVE WAVES

The first wave of definitive hematopoiesis gives rise in the posterior blood island (PBI) of the zebrafish embryo between 24 and 48 hpf [52]. These transient cells are named erythromyeloid progenitors (EMPs), and they have erythroid and myeloid differentiation potential but lack lymphoid potential.

During the second wave of definitive hematopoiesis, HSCs are produced in the floor of the DA (Figure 15), and these cells seed the caudal hematopoiestic tissue (CHT) to expand [53]. Subsequently, HSCs seed the thymus and kidney, which are the definitive hematopoietic organs in the adult zebrafish. The floor of the DA in zebrafish counterparts with the amniotes AGM (aorta-gonad-mesonephros). This temporal site of hematopoiesis, the CHT, in the zebrafish embryo has a similar function to that of the mammalian fetal liver.

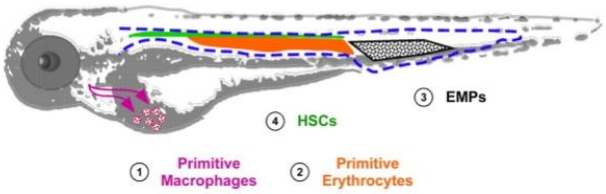

Figure 15. Waves of hematopoiesis in the zebrafish embryo. Hematopoietic development in the zebrafish occurs in four independent waves, each through precursors that arise in different anatomical regions. Adapted from [52].

OBJECTIVES

The specific objectives of the present work are:

1. Characterization of Tnfa receptors (Tnfr1 and Tnfr2) in vascular homeostasis during the zebrafish embryo development.

2. Characterization of the Tnfr1 and Tnfr2 signaling pathways involved in endothelial cell development and homeostasis.

3. Characterization of the role played by Tnfrs and their ligands (Tnfa and Lta) in the primitive wave of hematopoiesis in the zebrafish embryo.

4. Characterization of Tnfrs and their ligands (Tnfa and Lta) in HSCs specification and maintenance in the zebrafish embryo.

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

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.

1. INTRODUCTION

Tumor necrosis factor α (TNF α) 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 [54]. TNFα exerts its functions through interaction with two specific cell surface receptors (TNFRs): the 55 kDa TNFRSF1A/TNFRSF1A and the 75 kDa TNFRSF1B/TNFRSF1B [55]. TNFRSF1A is expressed in most cell types, even in transformed cells, whereas TNFRSF1B function seems to be restricted to immune and endothelial cells [13]. Recent studies with deficient mice have shown that TNFRSF1A predominantly triggers apoptosis or inflammation, while TNFRSF1B promotes tissue repair and regeneration [13]. 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 IκB and, hence, nuclear translocation of NF-κB and the transcription of pro-inflammatory and survival genes [56, 57]. 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 [56, 57]. In contrast, TNFRSF1B triggers the recruitment of TRAF1, TRAF2, which interact with BIRC2 and BIRC3 [58], 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 [13]. 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. [59], 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 [58] and endothelial cells have previously been reported a major target for TNFα in fish [60], 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.

2. MATERIALS AND METHODS

2.1. 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 [61]. The transgenic zebrafish line that expresses enhanced GFP (eGFP) driven by the endothelial cell-specific promoter *fli1a* gene *Tg(fli1a:egfp)y1* [25] was obtained from ZIRC. The transgenic line with red fluorescent erythrocytes Tg(gata1:dsRed)sd2 [47] and the P53 mutant line *zdf1* (P53M214K) [62] 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 μg/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).

2.2. 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 1). 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 [59]. CMRA construct was obtained from BCCM/LMBP (http://bccm.belspo.be/about/lmbp.php).

Gene	acc. # or ensembl ID	MO target	Sequence (5'→3')	wk. conc. (mM)	Reference
casp2	DQ812119	e4/i4	CGCTGAAACCCTGTTGTACCTGTGG	0.14	Sidi et al., 2008
casp8	BC081583	e5/i5	ACAGGGTTTTAACTCACAGTAGATC	0.30	Sidi et al., 2008
casp9	BC097103	e3/i3	GATGGAAAAACACACTTACGGACTG	0.6	Sidi et al., 2008
tp53	NM_131327	atg/5'UTR	GCGCCATTGCTTTGCAAGAATTG	0.25	Langheinrich et al., 2002
tnfrsf1a	BC068424	e6/i6	CTGCATTGTGACTTACTTATCGCAC	0.65	This work
tnfrsf1b	ENSDARG00000070165	i1/e2	GGAATCTGTGAACACAAAGGGACAA	0.25	This work
		e1/i1	AAAGCTGTTAGTTACCTTTCCTTCC	1.0	This work
		atg/5'UTR	CGCCATGAGCCACACCACCGTCATA	1.0	This work

Table 1. Morpholinos used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

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 Figure 4.

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) [63].

2.3. Analysis of development

The effect of MOs on development was evaluated as previously reported [64]. 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).

2.4. 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 [65]. 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 [59, 66-70]. 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) [71]. 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).

2.5. 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-dT18 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 [72]. 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 3.

2.6. 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 –20ºC. 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 dH2O, permeabilized in cold acetone for 10 min at -20ºC, washed again twice in dH2O (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 4ºC 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.

2.7. In situ hybridization

Whole mount in situ hybridization was performed according to Schulte-Merker et al. [73] with minor modifications, while in situ hybridization on paraffin sections was based on a protocol developed by Mallo et al. [74]. 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 (probe 1: +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 3.

Table 3. Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html) and the Human Genome Organisation (HUGO) Gene Nomenclature Committee (http://www.genenames.org/).

Gene	Accesion	Name	Nucleotide sequence (5'→3')	Use
ZEBRAFISH PRIMERS				
Lta	AB183467	F2	AAGCCAAACGAAGGTCA	RT-PCR
		R2	AACCCATTTCAGCGATTGTC	
rps11	NM_213377	F1	GGCGTCAACGTGTCAGAGTA	
		R1	GCCTCTTCTCAAAACGGTTG	
Tnfa	NM_212859	F2	GCGCTTTTCTGAATCCTACG	
		R2	TGCCCAGTCTGTCTCCTTCT	
tnfrsf1a	NM_213190	F	AAAGGATCCTGGATGAGGATATGTCAACTGACC	Morpholino efficiency
		R4	TGCTGGTTTTGCATAGGTGA	
		F5	AGCATTCCCCCAGTCTTTTT	RT-PCR
		R5	GCAGGTGACGATGACTGAGA	
		TNFR1-F1	TCAGGACTTGGTGGAAACAA	ISH
		TNFR1-R1T7	TAATACGACTCACTATAGGGAAAAAGACTGGGGGAATGCT	
		TNFR1-F2	TATGGCATACGAAGGCATCA	
		TNFR1-R2T7	TAATACGACTCACTATAGGGCGAAACGCTTGTGTTCTGTG	
tnfrsf1b	NM_001089510	F5	GCGGGTCTCTGAAGGATCAT	Morpholino efficiency
		F10	AAAGGATCCTGGATGACGGTGGTGTGGCTCTTGGCG	
		R13	AAAGGGCCCGGGTCAGGCGGAGGGTTGCTTCGGATC	
		R13b	CATTGCACAGACAAGCGAGT	
		F14	CACACAAGAGATCCGAAGCA	RT-PCR
		R14	GGCATCTGTGATGGGAACTT	
		TNFR2-F1	GCTGCAGTAGATGCAAACCA	ISH
		TNFR2-R1T7	TAATACGACTCACTATAGGGTTGACGTCGCTGCTCAATAC	
		TNFR2-F2	CACACAAGAGATCCGAAGCA	
		TNFR2-R2T7	TAATACGACTCACTATAGGGCCTCTTGTTGGCAGGATGAT	
tp53	NM_000546	F	GATGGTGAAGGACGAAGGAA	RT-PCR
		R	AAATGACCCCTGTGACAAGC	
casp8	BC081583	F	AACTCTTCGGCTGGCACA	
		R	TGCCTGTTCGTTTGAGCA	
Bax	NM_131562	F	GGAGATGAGCTGGATGGAAA	
		R	GAAAAGCGCCACAACTCTTC	
mdm2	NM_131364	F1	AACTCCCAACACAACCTTCG	
		R1	GGCTGTGATGATGTGGTTTG	
p21/cdkn1a	NM_000389	F	AACGCTGCTACGAGACGAAT	
		R	CGCAAACAGACCAACATCAC	
HUMAN PRIMERS				
ACTB	NM_001101	F3	GGCACCACACCTTCTACAATG	RT-PCR
		R3	GTGGTGGTGAAGCTGTAGCC	
TP53	NM_000546	F	CCGCAGTCAGATCCTAGCG	
		R	AATCATCCATTGCTTGGGACG	
P21/CDKN1A	NM_000389	F	ATGTGGACCTGTCACTGTCTTG	
		R	GGCGTTTGGAGTGGTAGAAA	
TNFRSF1A	NM_001065	F2	GGGCTGAGCGACCACGAGATC	
		R	AAAGGATCCTCTGAGAAGACTGGGCGCGGG	
TNFRSF1B	NM_001066	F2	GTTGGACTGATTGTGGGTGTG	
		R	AAAGGATCCACTGGGCTTCATCCCAGCATC	

2.8. Detection of apoptotic cell death by TUNEL labelling

After overnight fixation in 4% PFA at 4ºC 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 -20ºC 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 37ºC. Reaction was stopped with washes in equilibration buffer during 3 hours at 37ºC followed by three washes in PBT at RT. Strepavidine-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 [75].

2.9. Immunoprecipitation and western blot assays

The physical interaction between zebrafish TNFα and TNFRs was analyzed by means of immunuprecipitation. 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 [76]. 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.

2.10. 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 [77]. 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 [78]. Caspase-2 activation was determined by flow cytometry using the CaspGLOWTM 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).

2.11. 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.

3. RESULTS

3.1. 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 [79]. In addition, we confirmed by pull-down assays that TNFα was able to bind both TNFRs (Figure 1).

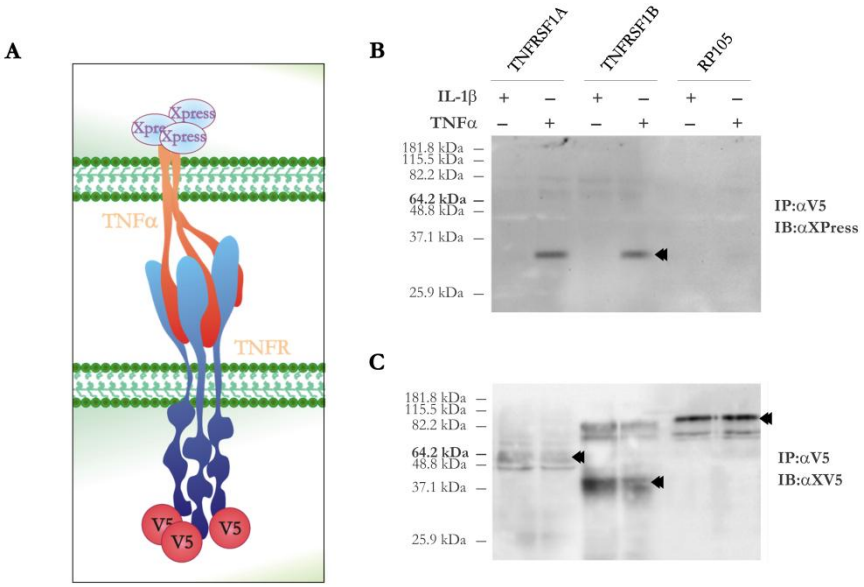

Figure 1. Interaction of zebrafish TNFα with TNFRSF1A and TNFRSF1B. HEK293 cells were transfected with TNFα-Xpress, IL-1β-Xpress, TNFRSF1A-V5, TNFRSF1B-V5 or RP105-V5 expression constructs. Forty-eight hours after transfection, cells were washed twice with PBS and lysed for the pull down assay as described 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.

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 2A). 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 2B). In addition, TNFα mRNA was first detected 24 hpf and increased after 72 hpf (Figure 2A), 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 [80], 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 2A).

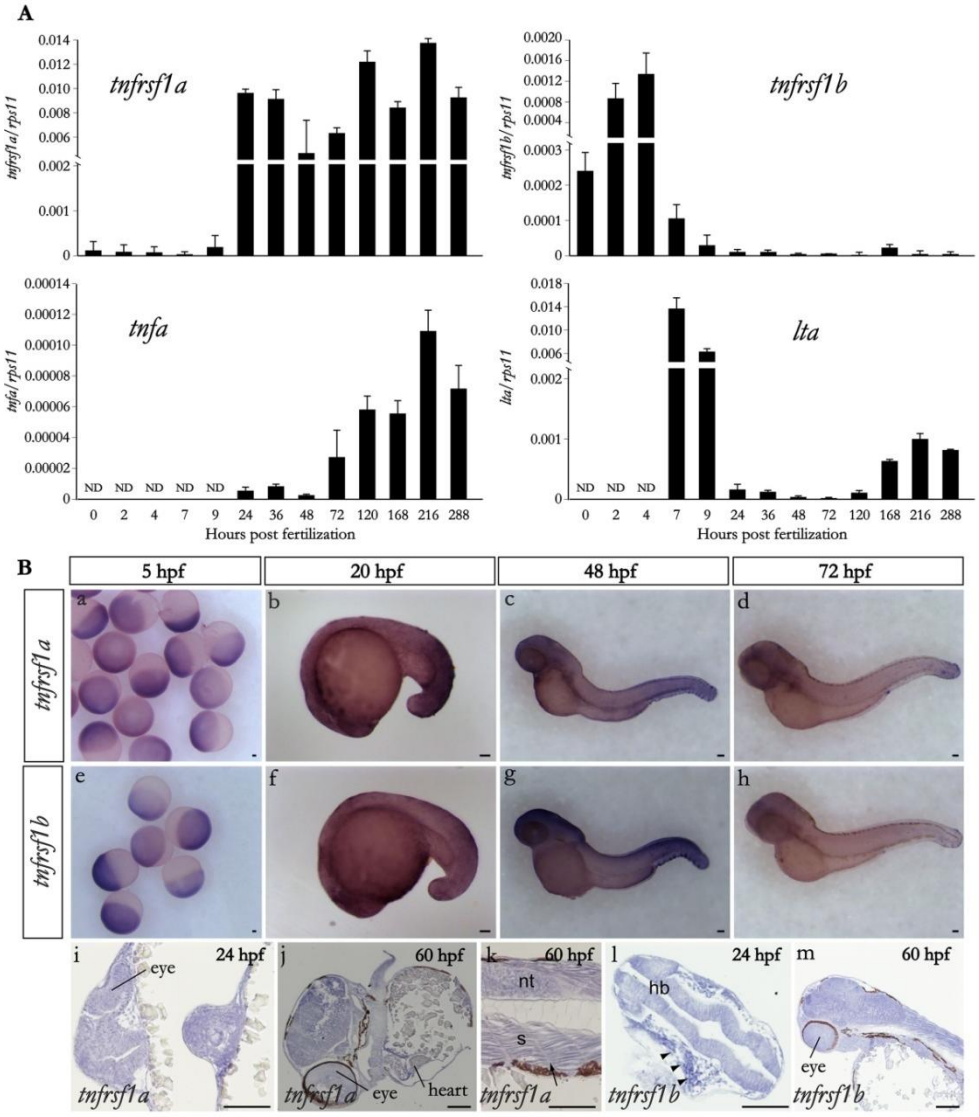

Figure 2. Expression of TNFRSF1A (tnfrsf1a), TNFRSF1B (tnfrsf1b) and their ligands tnfa and lta during zebrafish embryonic and larval development. (A) The mRNA levels of the gene encoding the TNFRs (*tnfrsf1a* and *tnfrsf1b*) and their ligands (*tnfa* and *lta*) were determined by real-time RT-PCR in 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.

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 [44, 45]. 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 [35]. 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) [35] (Figure 3A).

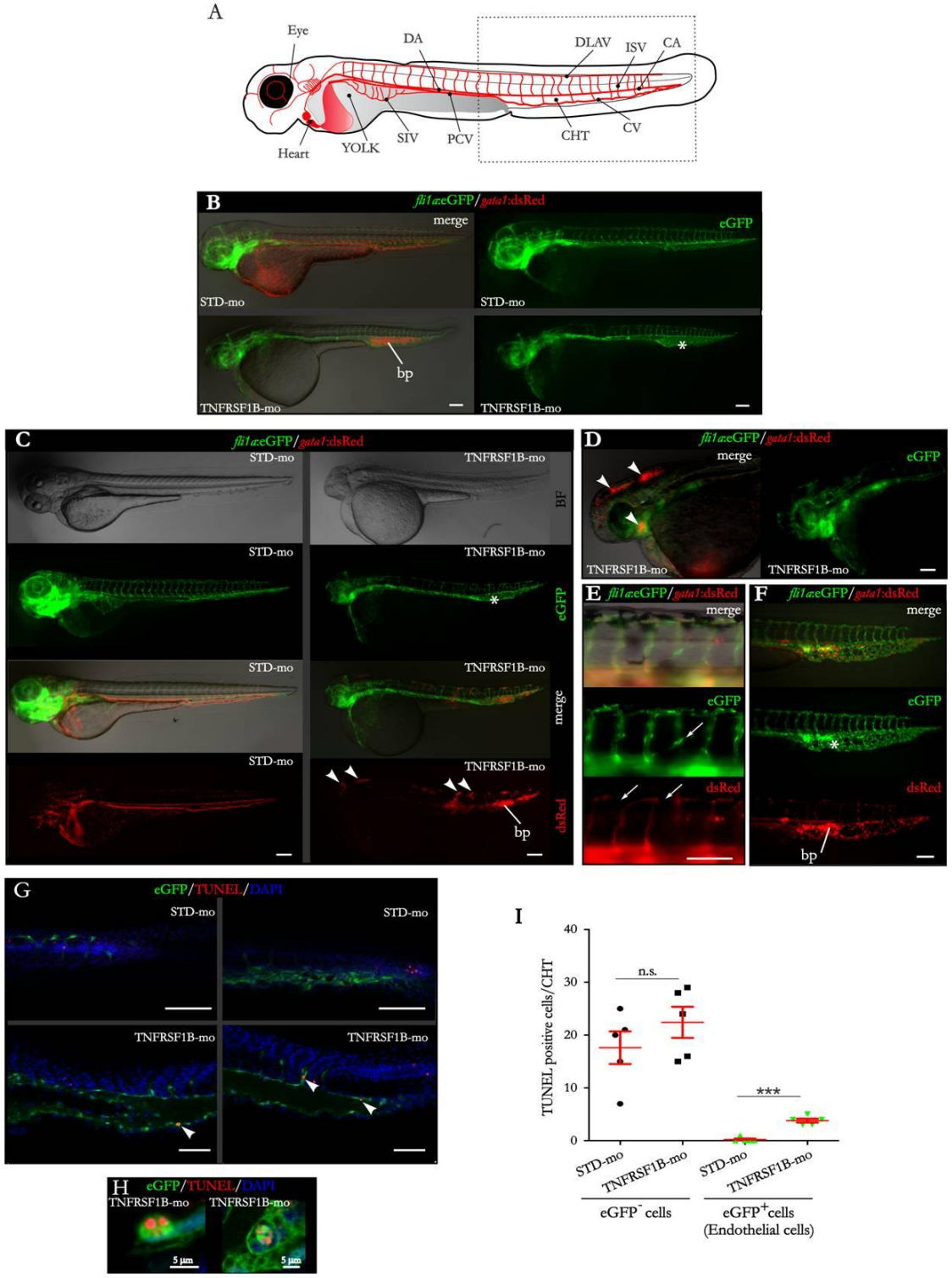

Figure 3. Genetic depletion of TNFRSF1B results in endothelial cell apoptosis and blood circulation disruption. (A) Scheme showing the main vessels of a 3 days-old zebrafish larvae (left) and sagittal sections of the formation of the caudal artery (CA) and the caudal vein (CV) from an unique initial 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 fli1a: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(fli1a: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.

Depletion of TNFRSF1B by a morpholino antisense oligonucleotide (MO) targeting the intron 1-exon 2 boundary of pre-mRNA (Table 1, Figure 4) resulted in viable zebrafish for up to 7 days post-fertilization (dpf) with no obvious developmental delay (Table 2). 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 (Figures 3B and 3C). Analysis of double transgenic *fli1a:eGFP/gata1:dsRed* fish to visualize 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 3B) and the appearance of large hemorrhages throughout the body, blood pooling and aberrant CV formation at 72 hpf (Figures 3C-3F). Although IPV formation was largely unaffected, abnormal, non-functional and bypassed ISVs and ISAs were observed (Figure 3E). These defects resulted in a caudal vessel net with or without blood circulation (Figure 3F). 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 (Figures 3G and 3H). These results suggest that apoptosis of endothelial cells was largely responsible for the vascular defects triggered by TNFRSF1B depletion.

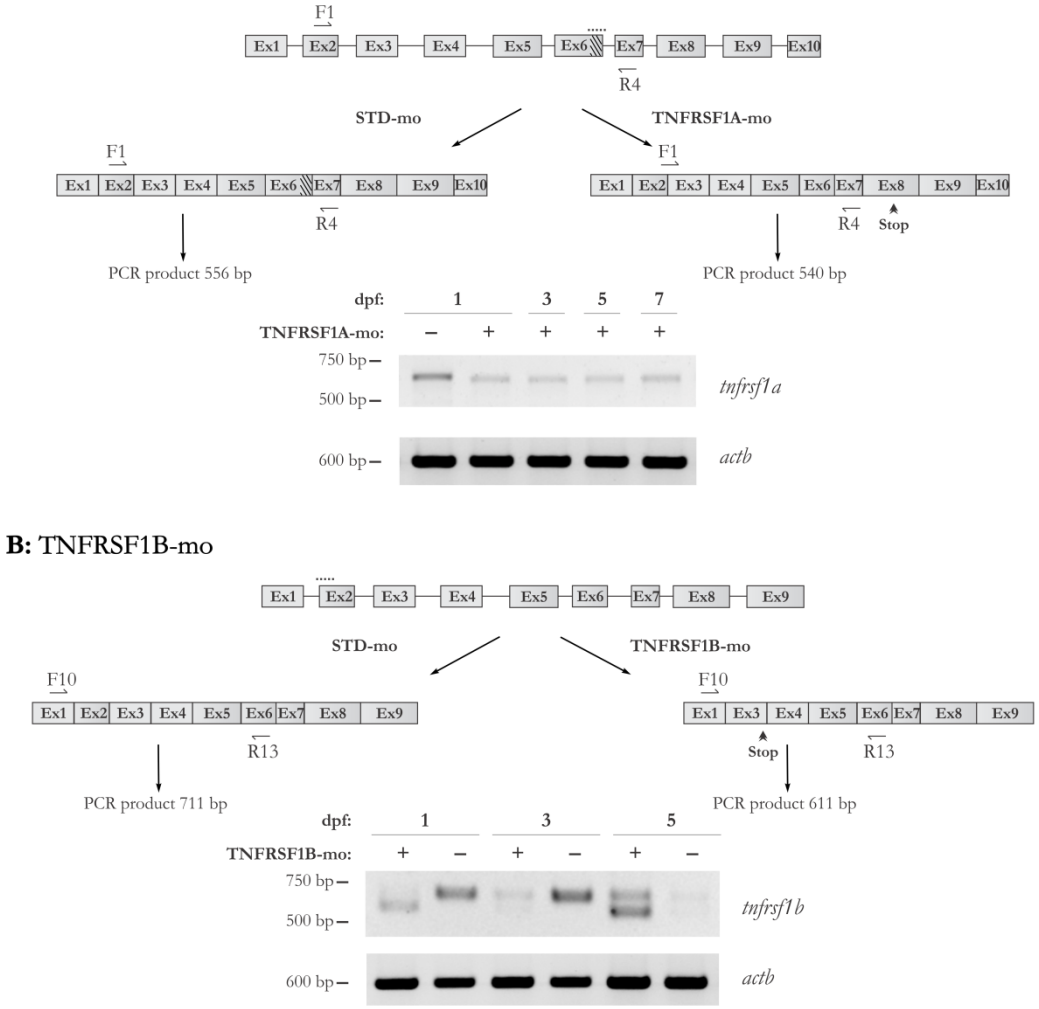

Figure 4. Efficiency of splice-blocking MOs against zebrafish TNFRs. RT-PCR analysis of TNFRSF1A-mo- (A) and TNFRSF1B-mo- (B) induced altered splicing of the tnfrsf1 transcripts at 1, 3, 5 and 7 dpf. (A) A 540 bp product containing a deletion of the last 16 bp of exon 6 of tnfrsf1a transcript was observed in samples injected withTNFRSF1A-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 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.

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

Developmental stages	Features	Morphants	
		STD (n=18)	**TNFRSF1B** (n=24)
25-26 somites (22 hpf)	Side-to-side flexures	Yes	Yes
Prim 5 (24 hpf)	Heartbeat	Yes	Yes
	Red blood cells on yolk	Yes	Yes
	Early pigmentation of retina/skin	Yes	Weak/Absent
Prim 15 (30 hpf)	HTA	95°	95°
	Early touch reflect	Yes	Yes
	Straight tail	Yes	Yes
	Retina pigmented	Yes	Weak
Prim 30 (36 hpf)	Early motility	Yes	Yes
	Tail pigmentation	Yes	Yes

The vascular defects observed in TNFRSF1B-deficient larvae were also found with two additional MOs (Table 1), which targeted the exon 1-intron 1 boundary and the atg/5'UTR (Figure 5), and they were also rescued by the over-expression of wild type TNFRSF1B mRNA but not by antisense TNFRSF1B mRNA (Figure 6). 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 7). Strikingly, although TNFRSF1A knockdown had no effect on vascular development, it was able to rescue the vascular defect observed in TNFRSF1B-deficient embryos (Figure 6B), 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 6C). Notably, TNFRSF1B deficiency also resulted in the induction of TNFRSF1A and TNFα gene expression, which was fully rescued by the depletion of TNFRSF1A (Figure 6D). 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.

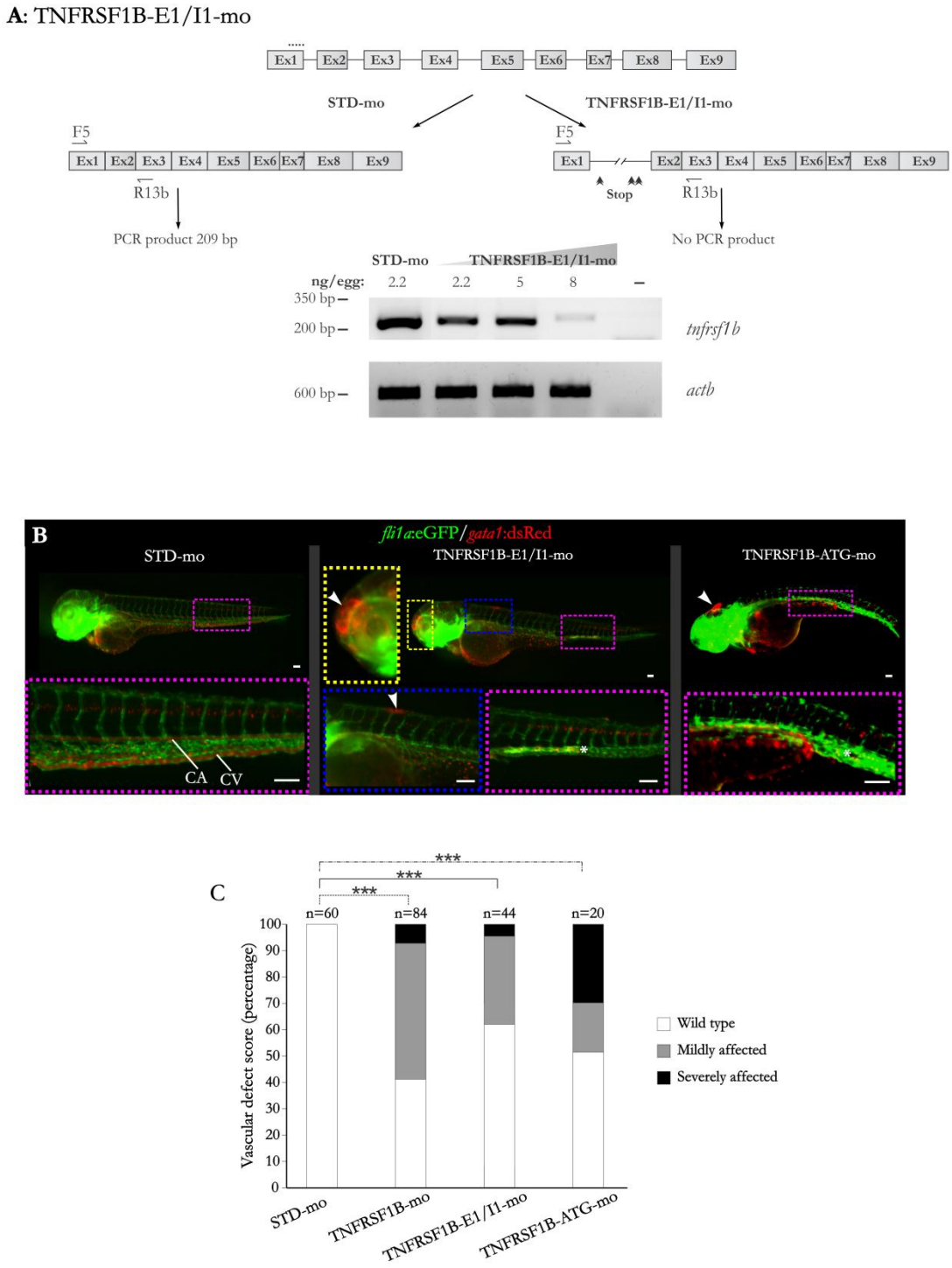

Figure 5. Validation and effects of two additional MOs targeting zebrafish TNFRSF1B. (A) RT-PCR analysis of TNFRSF1B-E1/I1-mo induced altered splicing of the tnfrsf1b transcript at 3 dpf. A dose-dependent decline of the wild type RNA was observed, suggesting the insertion of intron 1, which has 15,726 bp length. This insertion resulted in a predicted TNFRSF1B protein lacking both TNFα 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 µm. (C) The vascular defects shown in (B) were scored at 72 hpf as indicated in the legend to Figure 6. ***P<0.0001.

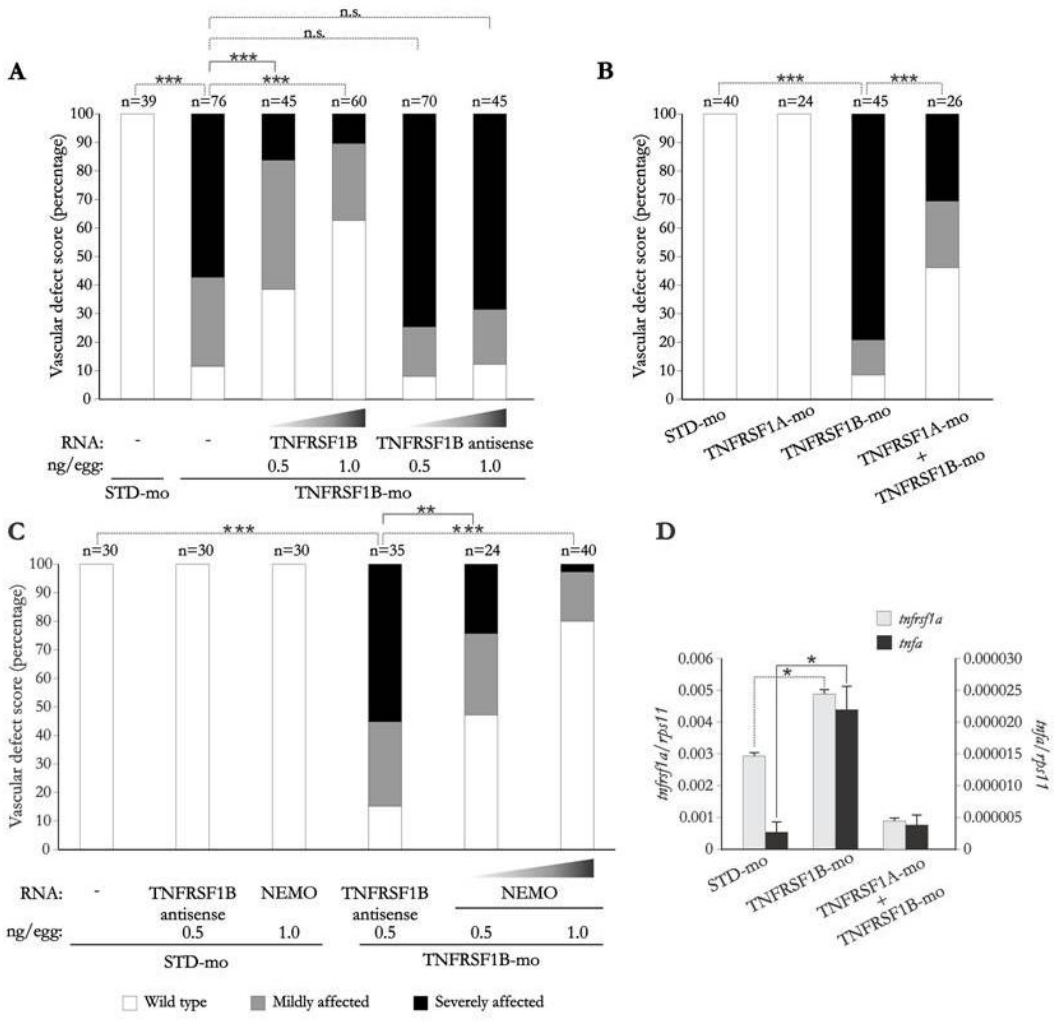

Figure 6. A critical balance between TNFRSF1A and TNFRSF1B signaling is required for endothelial cell development and maintenance. Zebrafish embryos were microinjected at the one-cell stage with STD and TNFRSF1B MOs alone or in combination with the indicated mRNAs. At 72 hpf, the vascular defects were 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 Figures 6B and 6D 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).

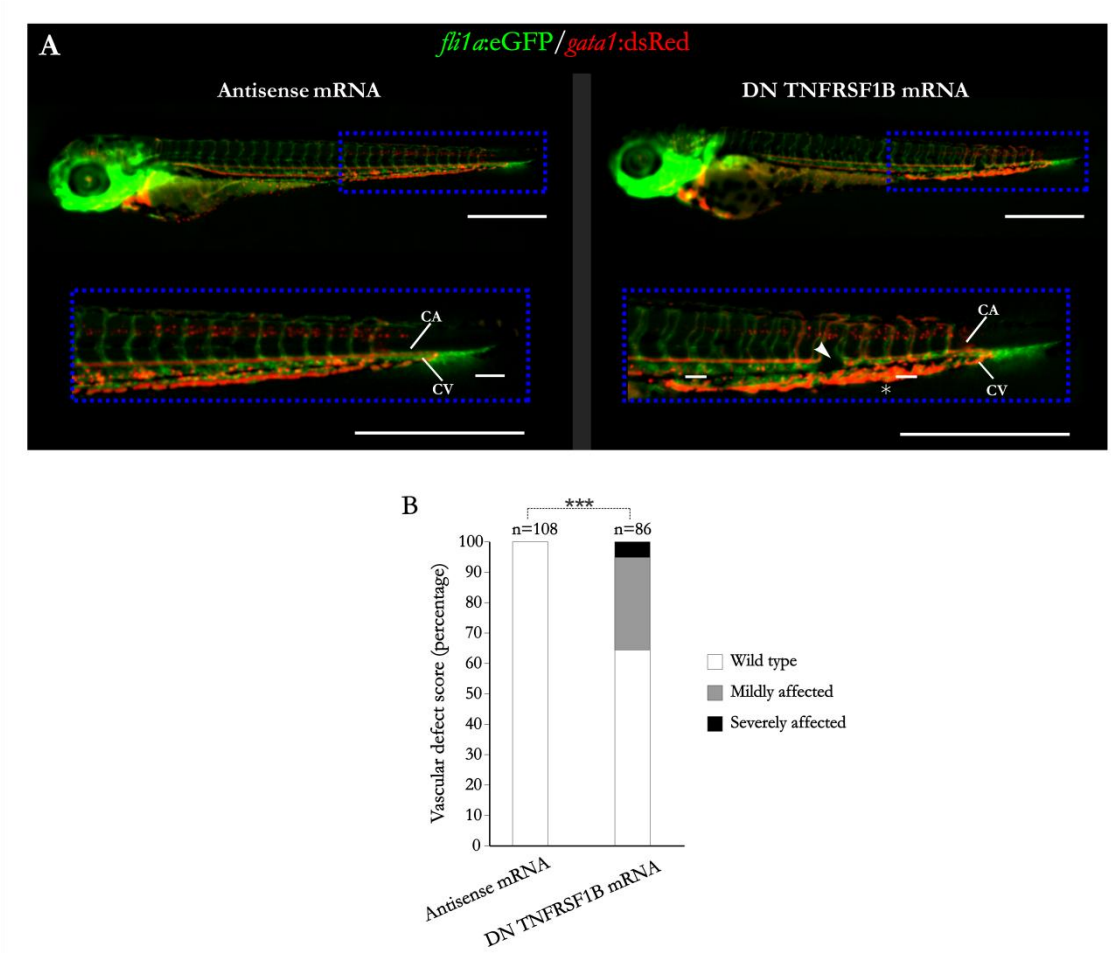

Figure 7. Confirmation of the vascular defects promoted by genetic depletion of TNFRSF1B using a DN mutant form. (A) Representative embryo injected with 200 ng/egg DN TNFRSF1B (right panel) showed impaired differentiation of the caudal artery (CA) and caudal vein (CV). Note the interruption of the CA (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 6. ***P<0.0001.

3.2. 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 8A 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 [81]. Genetic depletion of caspases-8 with specific MOs [63] 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 8B and 8C). However, caspase-9 depletion was unable to rescue the vascular defect of TNFRSF1B deficient embryos (Figure 8D). As the caspase-9 MO is not 100% efficient [63], 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.

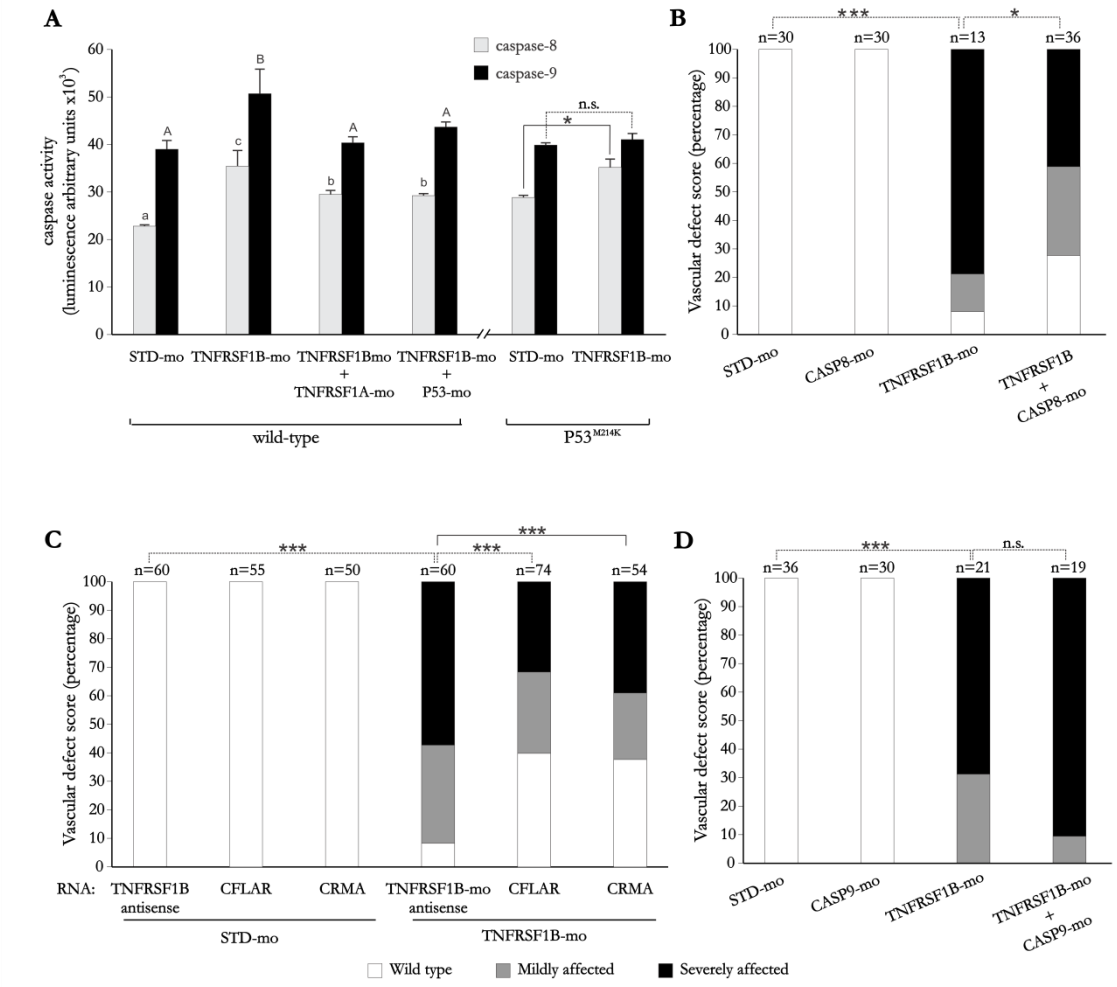

Figure 8. TNFRSF1B depletion promotes a caspase-8 apoptotic pathway via TNFRSF1A in endothelial cells. Wild type and P53M214K zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1A, TNFRSF1B, P53, CASP8 and CASP9 MOs alone or in combination with the mRNAs encoding the caspase-8 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 6. 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)

3.3. 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 9A) and protein (Figure 9B and 9C) levels in TNFRSF1B deficient zebrafish. Strikingly, P53 was mainly induced in endothelial cells (Figure 9C), 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 9A). Finally, the activation of P53 was also confirmed by the sharp upregulation of the P53-dependent genes BAX and MDM2 (Figure 9D) and P21 (Figure 9E) in TNFRSF1B-deficient embryos.

To clarify the importance of P53 induction and activation in vascular integrity, we depleted P53 by using a specific MO [82] and used the P53 zebrafish mutant line P53M214K [62]. We found that the P53 MO almost completely rescued the vascular defects of TNFRSF1B deficient embryos (Figure 9F) and abrogated the induction of caspase-8 gene expression (Figure 9G). Similarly, the P53M214K mutant embryos did not show significant vascular alteration (data not shown) and P21 and caspase-8 gene expression was unaltered (Figure 9H) following genetic depletion of TNFRSF1B. Strikingly, however, TNFRSF1B deficiency in this line resulted in caspase-8 activation, while caspase-9 activity was unaltered (Figure 8A). 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 9I).

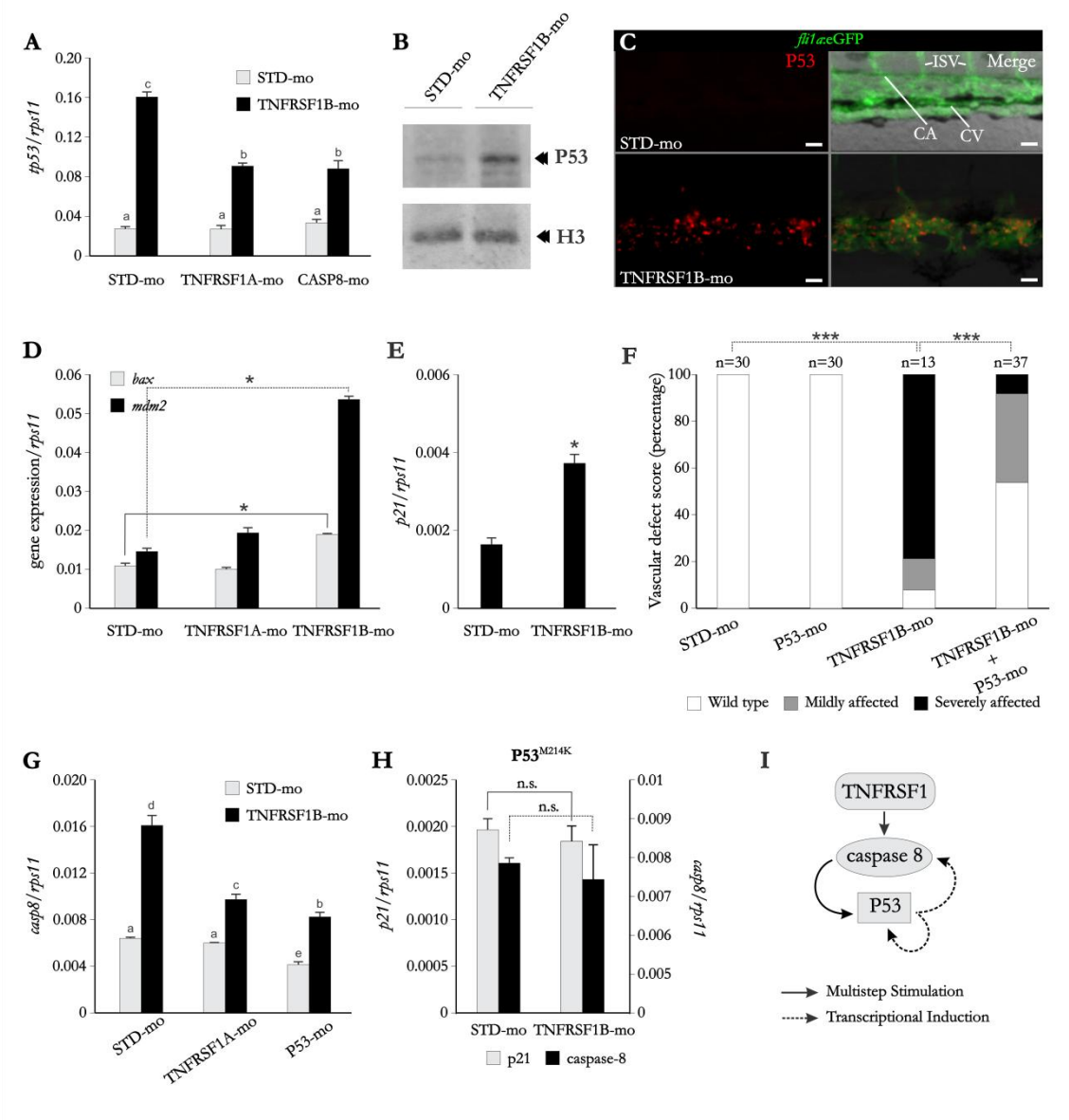

Figure 9. P53 activation is indispensable for promoting caspase-8-dependent apoptosis of endothelial cell in TNFRSF1B deficient zebrafish. Wild type (A, B, D-G), transgenic fli1a:eGFP (C) and P53M214K (H) zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1A, TNFRSF1B and P53 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 6 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 µm.

3.4. 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 10A) 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 [63]. Genetic depletion of P53 failed to rescue the vascular defects of TNFRSF1B deficient embryos in the presence of Gö6976 (Figure 10B), suggesting that CHK1 might be downstream of P53 in this apoptotic signaling pathway triggered by TNFRSF1A in endothelial cells.

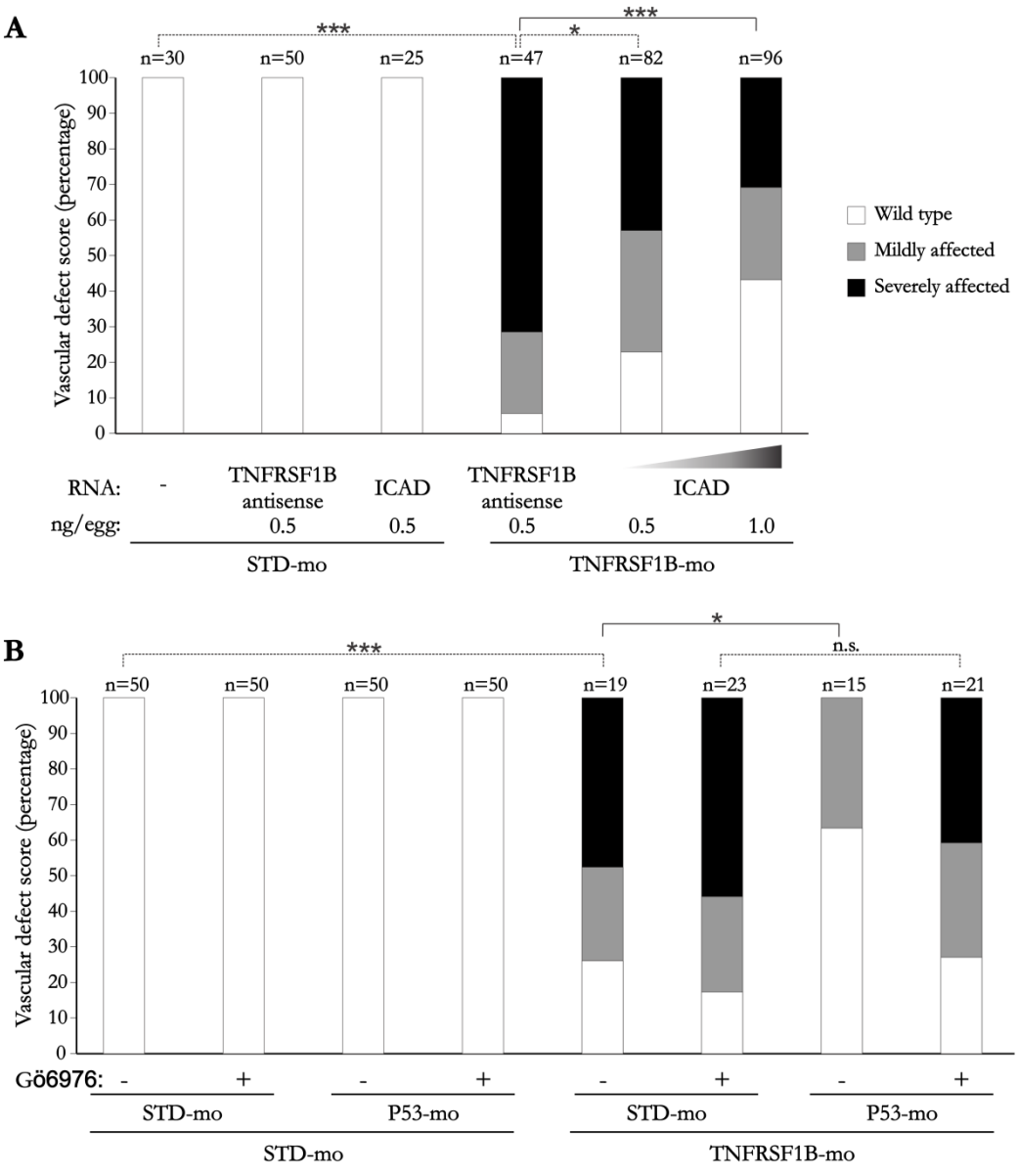

Figure 10. DNA fragmentation in endothelial cells triggers P53 activation in TNFRSF1B-deficient zebrafish. Wild type zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1B, ATR and/or ATM MOs alone or in combination with the mRNAs encoding ICAD or the antisense mRNA for TNFRSF1B, as an appropriate control. Some larvae were treated at 48 hpf by bath immersion for 16 h with 1µM of the CHK1 specific inhibitor Gö6976 and the vascular defects were scored at 72 hpf as indicated in the legend to Figure 6. The data shown in Figures 10B are representative of two independent experiments. *P<0.05, **P<0.01, ***P<0.0001, n.s.: not significant according to a Chi-square contingency test.

3.5. 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 (Figures 3I and 3J), it failed to significantly increase active caspase-3 levels in endothelial cells compared with control embryos (injected with a standard MO) (Figure 11A). 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 [83] and is involved in an apoptotic response to DNA damage that bypasses caspase-3 [63]. 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 11B). To corroborate the relevance of caspase-2 activation in endothelial cell apoptosis, we ablated caspase-2 using a specific MO [63]. The results showed that caspase-2 deficiency was able to partially rescue the vascular defects of TNFRSF1B-deficient embryos (Figure 11C).

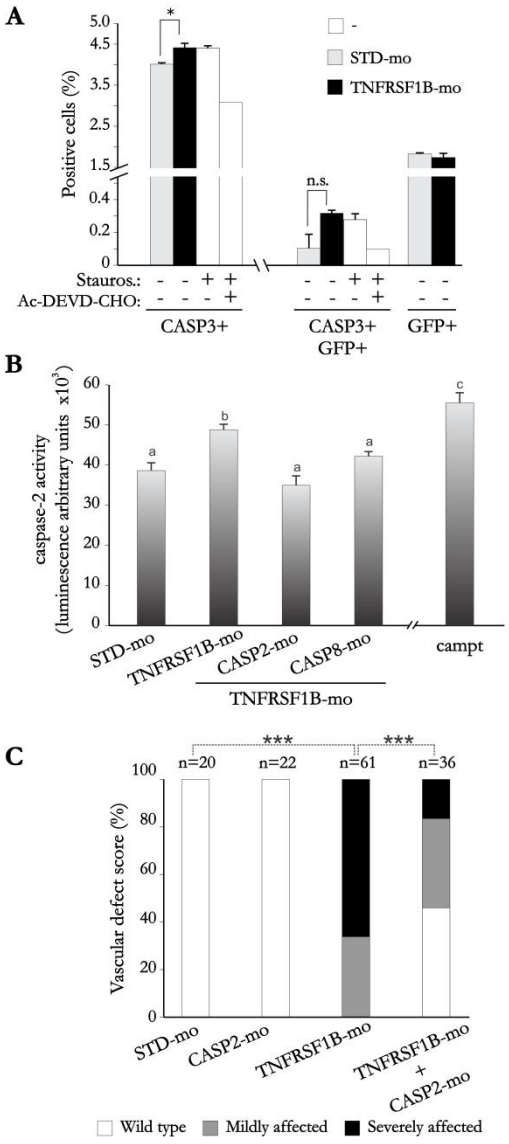

Figure 11. The TNFRSF1A apoptotic signaling pathway of endothelial cells is independent of caspase-3 but requires caspase-2. Wild type and transgenic *fli1a:eGFP* zebrafish embryos were microinjected at the one-cell stage with STD, TNFRSF1B, CASP8 and/or CASP2 MOs. The activation of caspase-3 in embryos 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 4 (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)

3.6. 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 12A-C), while the neutralization of the TNFRSF1B with a specific antibody slightly increased the percentage of cells undergoing apoptosis in response to TNFα (Figure 12A). More importantly, the numbers of caspase-2 positive cells significantly increased (Figure 12B), while caspase-3 levels hardly increased following treatment of the cells with a TNFα mutein that specifically interact with TNFRSF1A (Figure 12D). In addition, P21 and P53 mRNA levels also increased following treatment with TNFα mutein (Figure 12E) and, notably, pharmacological inhibition of CHK1 with Gö6976 resulted in a further upregulation of P53 and P21 gene expression (Figure 12E) and accumulation of P53 protein (Figure

12F). These results suggest that this kinase is involved in a P53- and P21-dependent cell cycle arrest triggered by TNFRSF1A in endothelial cells.

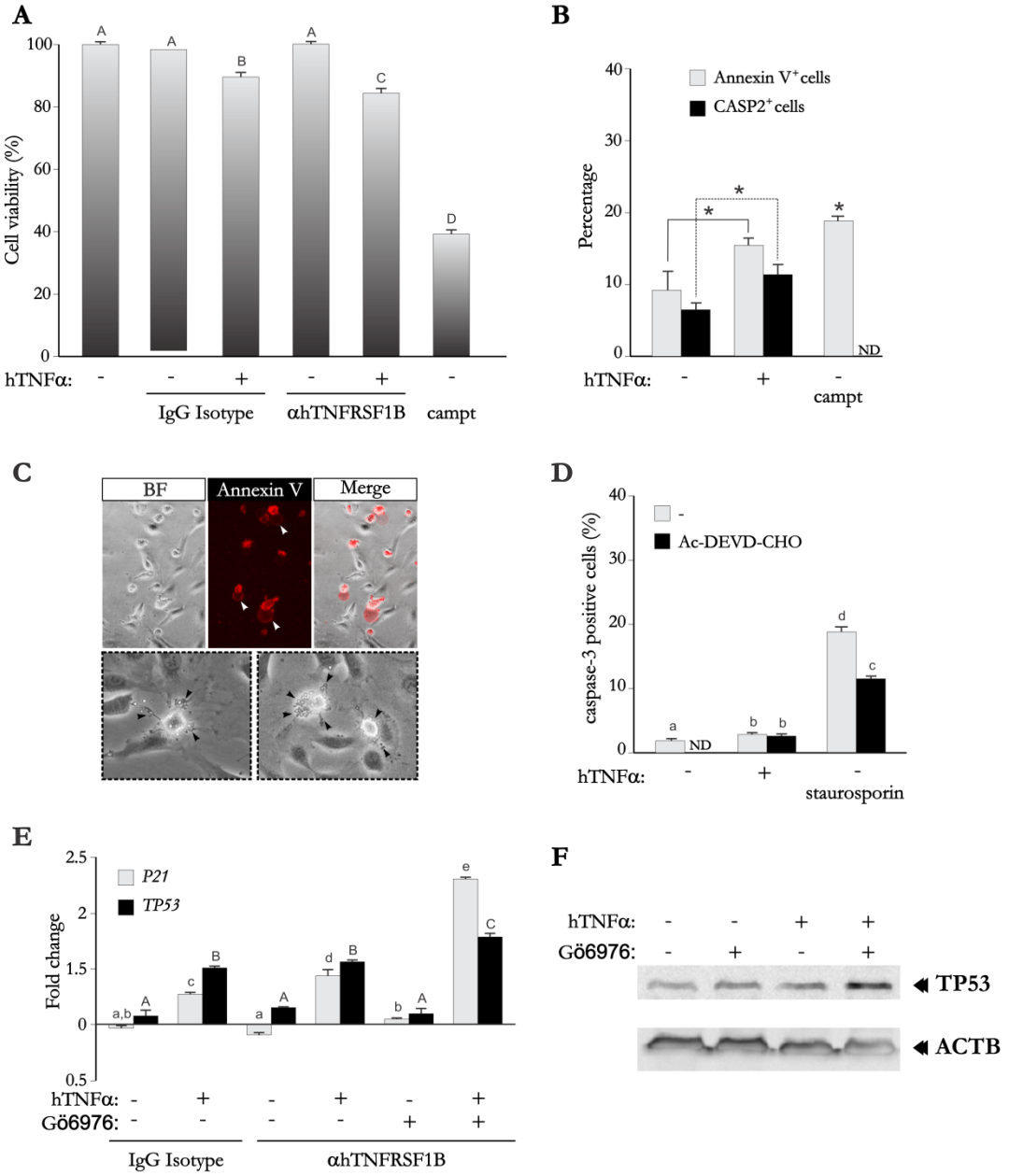

Figure 12. The caspase-2/P53 apoptotic signaling pathway triggered by TNFRSF1A is evolutionary conserved and its activation reduces *in vivo* the invasiveness of malignant cancer cells. HUVEC were treated for 16 h with 40 ng/ml human TNFα (hTNFα) alone or in combination with 5 µg/ml of a mouse IgG isotype 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). Different letters denote

statistically significant differences among the groups according to a Tukey test. The groups labeled with "a" or "A" in Figure 12E did not shown statistically significant differences with non-treated cells. *P<0.05 according to a Student's t test. ND: not determined.

4. DISCUSSION

Tight molecular control of endothelial cell survival, integrity and apoptosis is essential for both embryonic and adult angiogenesis. Santoro and coworkers [59] 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 [84], CD40 [85, 86], TNFRSF1A [87] and TNFRSF1B [58], 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 [88]. 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 [86], 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 [89]. Our *in vivo* epistasis analysis led us to envisage a model whereby TNFRSF1A engagement leads to the following sequential events (Figure 13): (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* [83], 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 [90]. 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 [91].

The inhibition of the cell cycle checkpoint kinase CHK1, which is activated by ATR in response to replication stress [92], 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 [63]. 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 [93]. 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 [94], 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.

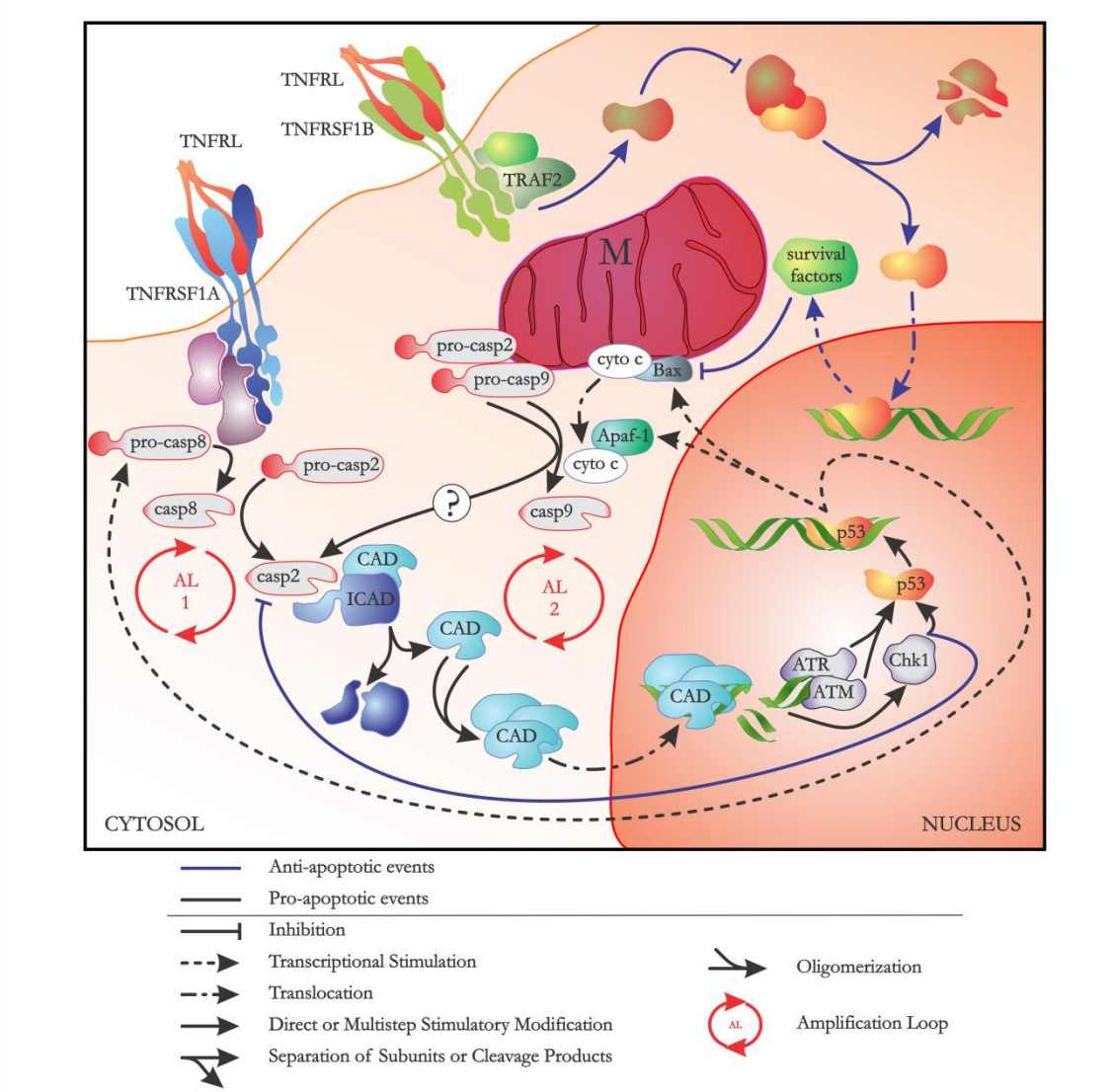

Figure 13. Model of TNFR signaling in endothelial cells. The TNFRSF1A apoptotic and TNFRSF1B survival pathways, the main elements involved, and the crosstalk between extrinsic and intrinsic apoptotic programs. TNFRL, TNFR ligands (TNFα and LTα).

CHAPTER II: TNF Signaling is Critical for Hematopoietic Stem Cell Survival and Expansion in the Developing Embryo.

ABSTRACT

While bone marrow failure syndromes are associated with tumor necrosis factor α (TNFα) overexpression, the role of TNFα signaling in the regulation of hematopoiesis is unclear. Here, we have utilized the unique advantages of the zebrafish to study the impact of Tnf receptor (Tnfr) signaling in the emergence and maintenance of hematopoietic stem cells (HSCs). Our results indicate that Tnfa signaling through Tnfr2 is dispensable for the specification of HSCs, but is intrinsically required for their subsequent maintenance and expansion. The genetic inhibition of Tnfa or Tnfr2, but not of lymphotoxin α (Lta) or Tnfr1, results in the apoptosis of HSCs soon after their emergence. These results are the first to describe the role of TNFR signaling in HSCs, and highlight a novel role for a signaling pathway predominantly associated with the regulation of inflammation and immunity.

1. INTRODUCTION

Tumor necrosis factor α (TNFα) is a powerful pro-inflammatory cytokine that plays a pivotal role in the regulation of inflammation and immunity. TNFα exerts its functions via engagement of two specific cell surface receptors (TNFRs), namely the 55 kDa TNFR1 (also known as TNFRSF1A) and the 75 kDa TNFR2 (also known as TNFRSF1B) [55]. TNFR1 is expressed in most cell types, whereas TNFR2 is restricted to immune and endothelial cells [13]. Several studies have suggested that signaling through TNFR1 predominantly triggers apoptosis or inflammation, while signaling through TNFR2 promotes tissue repair and regeneration [13]. However, receptor crosstalk has been reported that varies depending upon the cell type and activation state [95], complicating interpretation of these data. This pleiotropic activity links TNFα signaling to a wide variety of human diseases, including inflammatory and autoimmune disorders, ischemia followed by reperfusion injury, and cancer.

Like many other acute and chronic inflammatory diseases, bone marrow failure syndromes such as Fanconi anemia are associated with TNFα overexpression [96, 97]. However, the role of TNFα in the regulation of hematopoiesis, specifically in HSCs, has remained unclear. Although earlier studies reported that TNFα negatively affected HSC growth and maintenance *in vitro* [98, 99], the *in vivo* relevance of TNFα in these processes is enigmatic. While one study indicated that TNFα signaling through TNFR1, and to some extent through TNFR2, restricted the activity of HSCs in mice [100], another showed that HSCs lacking TNFR1 have impaired proliferative and self-renewal abilities [101]. Membrane-anchored TNFα produced by precursor plasmacytoid dendritic cells was also reported to significantly enhance the engraftment of purified HSCs in allogeneic and syngeneic recipients [102].

TNFα and TNFRs are conserved in all vertebrate organisms. Recent studies in the zebrafish (*Danio rerio*) indicate that zebrafish Tnfa functions as a pro-inflammatory cytokine mainly through activating endothelial cells, while it has only modest effects on macrophage activation [60]. In addition, the genetic inhibition of Tnfrs identified the important role of Tnfa signaling in the development and maintenance of endothelial cells [103]. We have taken advantage of the external development and optical transparency of zebrafish embryonic development to examine the role of Tnfr signaling in the emergence, maintenance, and expansion of HSCs. Our results indicate that Tnfa signaling through Tnfr2 is critical for the survival and expansion of HSCs. Genetic inhibition of Tnfa or Tnfr2, but not of Tnfr1, resulted in HSC apoptosis soon after their emergence from the floor of the dorsal aorta.

2. MATERIALS AND METHODS

2.1. Animals

Zebrafish (*Danio rerio*) were obtained from the Zebrafish International Resource Center and mated, staged, raised and processed as described [61]. The tg(*gata1:DsRed*) [47], tg(*cmyb:eGFP*) [104] and tg(*kdrl:DsRed2*) [105] lines were previously described. The tg(*cd41:eGFP*) [106] and tg(*lck:eGFP*) [107] lines were kindly provided by Drs. RI Handin and AT Look, respectively. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and IACUC. Experiments and procedures were performed as approved by the Bioethical Committee of the University of Murcia (Spain).

2.2. DNA constructs

The *runx1* promoter was kindly provided by Dr. O. Tamplin as a 5' entry clone for the Tol2kit [108] and the DN-Tnfr2 was described previously [103]. The runx1:DN-Tnfr2-Cherry, runx1:Cherry, and *runx1:Tnfr2* were generated by MultiSite Gateway assemblies using LR Clonase II Plus (Life Technologies) according to standard protocols and using Tol2kit vectors described previously [108]. The destination vector used in all constructs was pDestTol2CG2 (#395). This vector has a cardiac marker (*cmlc2:eGFP*), which allows the assessment of Tol2-mediated integration and expression.

2.3. Morpholino, DNA/RNA injection, and chemical treatments

Specific morpholinos (Gene Tools) were resuspended in nuclease-free water to 1mM (Table 1). *In vitro*-transcribed RNA was obtained following manufacturer's instructions (mMESSAGE mMACHINE kit, Ambion). Morpholinos, DNA (100 ng/µl) and/or Tol2 RNA (50 ng/µl) were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into the yolk ball of one- to eight-cell-stage embryos using a microinjector (Narishige) (0.5-1 nl per embryo). The same amount of MOs, DNA, and/or RNA were used in all experimental groups.

In some experiments, 24 hpf embryos were dechorionated and transferred to fresh E3 medium containing 1% DMSO with or without the pan-caspase inhibitor Q-VD-OPh (100 µM; Sigma-Aldrich) [109, 110].

Table 1. Morpholinos used in this study.

Gene	ENA or Ensembl ID	Target	Sequence (5'>3')	Concentration (mM)	Reference
tnfr1	ENSDARG00000018569	e6/i6	CTGCATTGTGACTTACTTATCGCAC	0.65	Espín et al., 2013
tnfr2	ENSDARG00000070165	i1/e2	GGAATCTGTGAACACAAAGGGACAA	0.2	Espín et al., 2013
tnfa	ENSDARG00000009511	e1/i1	GCAGGATTTTCACCTTATGGAGCGT	0.5	López-Muñoz et al., 2011
lta	ENSDARG00000013598	e1/i1	AATTTCAGTCTTACCATCACATGCC	1	This study

2.4. Flow cytometry

Approximately 100 to 200 embryos were dechorionated with pronase (Roche), anesthetized in tricaine, minced with a razor blade, incubated at 28°C for 30 min with 0.077 mg/ml Liberase (Roche) and the resulting cell suspension passed through a 40 µm

cell strainer. Sytox Red (Life Technologies) was used as a vital dye to exclude dead cells. Flow cytometric acquisitions were performed on a FACS LSRII (Becton Dickinson) or FACSCalibur (BD Biosciences), and cell sorting was performed on a FACS ARIA1 (BD). Analyses were performed using FlowJo software (Treestar).

2.5. Whole-mount RNA in situ hybridization (WISH)

WISH was carried out as described [111]. *gata1a, csfr1ra, kdrl, cmyb, runx1, foxn1,* and *rag1* RNA probes were generated using the DIG RNA Labeling Kit (Roche Applied Science) from linearized plasmids. Embryos were imaged using a Leica S8APO stereomicroscope equipped with a digital camera (EC3, Leica).

2.6. Detection of apoptotic cell death by TUNEL labeling

The TUNEL assay was performed as previously described [103] with slight modifications. After overnight fixation in 4% PFA at 4ºC, 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 -20ºC they were rehydrated gradually to PBT, washed twice for 10 minutes in PBT and permeabilizated in PBS containing 0.5% Triton and 1% DMSO at room temperature for 3 hours. Embryos were washed again twice in PBT for 10 min each and endogenous biotin was blocked using the Biotin blocking kit (Vector). 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 37ºC. Reaction was stopped by six washes in PBT at RT. Strepavidin-Alexa647 was used as a secondary antibody (Jackson Laboratories) incubated during 1h at RT. For concomitant GFP and DsRed detection, embryos were incubated with anti-GFP and anti-DsRed (Clontech Laboratories) antibodies followed by Alexa488 and Alexa555 secondary antibodies (Life Technologies), respectively. Embryos were washed and mounted in Vectashield.

2.7. Confocal imaging and analysis

Embryos were imaged using a Leica SP5 inverted confocal microscope (Leica, Germany). Stacked images were captured using 3.7 µm increments and deconvolved using Huygens Essential Confocal software (v 4.1 0p6b) by Scientific Volume Imaging. Three-dimendional reconstruction and maximum intensity projections of the deconvolved images were accomplished using the Imaris package 7.4.0 (Bitplane AG).

2.8. Analysis of gene expression

Total RNA was extracted from whole embryos/larvae or sorted cell suspensions with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/µg RNA; Invitrogen). SuperScript III RNase H□ Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with 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 *ef1a* content in each sample using the Pfaffl method [72]. The primers used are shown in Table 2. In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples.

Table 2. Primers used in this study.

Gene	ENA ID	Name	Nucleotide sequence (5'>3')	Use
tnfr1	NM_213190	F5	AGCATTCCCCCAGTCTTTTT	RT-qPCR
		R5	GCAGGTGACGATGACTGAGA	
tnfr2	NM_001089510	F14	CACACAAGAGATCCGAAGCA	
		R14	GGCATCTGTGATGGGAACTT	
tnfa	NM_212859	F2	GCGCTTTTCTGAATCCTACG	
		R2	TGCCCAGTCTGTCTCCTTCT	
lta	AB183467	F2	AAGCCAAACGAAGGTCA	
		R2	AACCCATTTCAGCGATTGTC	
		F	AAAGGATCCTGGATGGTGAGA	Morpholino efficiency
		R2	ACCCATTTCAGCGATTGTC	
ef1a	NM_131263	FP	GAGAAGTTCGAGAAGGAAGC	RT-qPCR
		RP	CGTAGTATTTGCTGGTCTCG	
acta1a	NM_001017750	F3	GGCACCACACCTTCTACAATG	RT-PCR
		R3	GTGGTGGTGAAGCTGTAGCC	

2.9. Statistical analysis

Data were analyzed by analysis of variance (ANOVA). The differences between two samples were analyzed by the Student t-test.

3. RESULTS

3.1. Tnfr signaling is dispensable for primitive hematopoiesis

As in all vertebrates, zebrafish possess multiple waves of hematopoiesis during embryogenesis [45]. We first assessed if Tnfr signaling was required for the first waves of hematopoiesis, commonly referred to as "primitive" due to the transience of these cells and lack of upstream multipotent progenitors. In zebrafish, primitive hematopoiesis generates macrophages, neutrophils, and erythrocytes. The expression of *csf1ra*, a specific marker of macrophages [112], was unaffected in Tnfa-, Tnfr1- and Tnfr2- deficient embryos at 24 hpf (30 somites) (Figure 1). In addition, primitive neutrophils were also unaffected at 30 hpf, as assayed using transgenic *mpx:eGFP* fish (data not shown). Similarly, primitive erythropoiesis, assessed by expression of the erythroid-specific transcription factor *gata1a*, was not affected in these embryos (Figure 1). We also analyzed the effect of lymphotoxin α (Lta, also known as Tnfb) depletion on primitive hematopoiesis, since Lta also signals through both Tnfrs in mammals [80]. However, Lta-deficient embryos also had unaltered *csf1ra* and *gata1a* expression (Figures 1 and 2). Collectively, these results indicate that Tnfr signaling is dispensable for primitive hematopoiesis in the zebrafish.

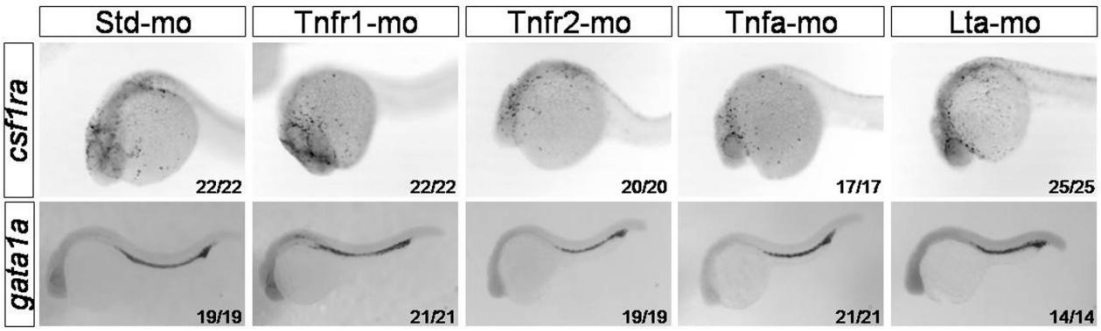

Figure 1. Tnfr Signaling is Dispensable for Primitive Hematopoiesis. Zebrafish one-cell embryos were microinjected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Lta morpholinos (mo) and the expression of *csf1ra* and gata1a within the anterior part and the intermediate cell mass (ICM) of the

zebrafish embryo was analyzed by WISH at 24 hpf (30 somites). All views, anterior to left. Numbers represent the frequency of embryos with the shown phenotype.

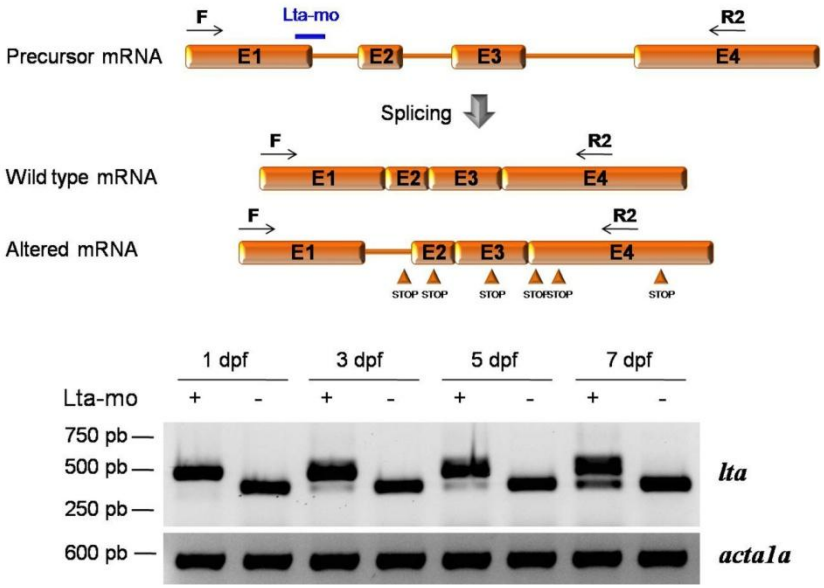

Figure 2. Efficiency of the Splice-blocking Morpholino Against Lymphotoxin α (Lta). RT-PCR analysis of Lta-mo induced altered splicing of lta transcripts at 1, 3, 5 and 7 dpf. The 348 bp wild type product is not (1 dpf), or vaguely detected (3, 5 and 7 dpf) in embryos/larvae injected with Lta-mo, while a 435 bp is detected instead. This aberrant mRNA is generated by retention of intron 1 in the mature *lta* mRNA and results in the presence of premature STOP codons (arrowheads), which would lead to the production of truncated Lta protein. RT-PCR of the housekeeping gene acta1a for mRNA quality checking on the same samples is also shown.

3.2. Tnfa signaling via Tnfr2 is required for the maintenance of HSCs, but dispensable for their emergence

In the zebrafish embryo, HSCs arise directly from hemogenic endothelium lining the ventral wall of the dorsal aorta [39, 113, 114]. To visualize this process and to isolate these cells for further study, we utilized double transgenic animals in which upstream regulatory sequences (URS) of the *cmyb* gene, a marker of emerging definitive hematopoietic stem and progenitor cells [115], and the *kdrl* gene (also known as *flk1* and *vegfr2*), a marker of vascular cells [105], were utilized to drive fluorescent protein expression. To evaluate if Tnf receptors and their ligands were specifically expressed in endothelial cells and HSCs, *kdrl*$^{+}$; *cmyb*$^{-}$ endothelial cells and *kdrl*$^{+}$; *cmyb*$^{+}$ HSCs were isolated from 48hpf *kdrl:mCherry; cmyb:eGFP* embryos by fluorescence-activated cell sorting (FACS). RT-qPCR analysis of these highly purified cell fractions showed that while *tnfr1* and *tnfa* mRNA levels were similar in HSCs and endothelial cells, those of *tnfr2* were significantly higher in HSCs (Figure 3A). While *lta* transcripts were not detected in endothelial cells, *lta* was highly expressed in HSCs, at levels comparable to whole kidney, the main site of hematopoiesis in adult zebrafish (Figure 3A). These results prompted us to examine the impact of Tnfr signaling in HSCs.

In the zebrafish embryo, HSCs can be observed between the dorsal aorta and posterior cardinal vein by *cmyb* expression using whole-mount *in situ* hybridization (WISH) [115]. The number of *cmyb*$^{+}$ cells in or near the floor of the dorsal aorta was reduced in Tnfa- and Tnfr2-deficient embryos compared with their wild type (wt) siblings (Figures 3B and 3C). However, Tnfr1 and Lta depletion showed no effect on these cells (Figures 3B and 3C). This result was verified by fluorescence microscopy of

cd41:eGFP HSCs [116]; *cd41:eGFP*$^{+}$ cells were significantly decreased in Tnfr2- and Tnfa- deficient fish at 3 days post fertilization (dpf) (Figure 3D). Notably, Tnfr1 deficiency failed to rescue the reduced number of *cmyb*+ HSCs in Tnfr2-deficient embryos (Figures 3B and 3C), despite the critical signaling balance that exists between Tnfr1 and Tnfr2 in the maintenance of endothelial cell integrity [103].

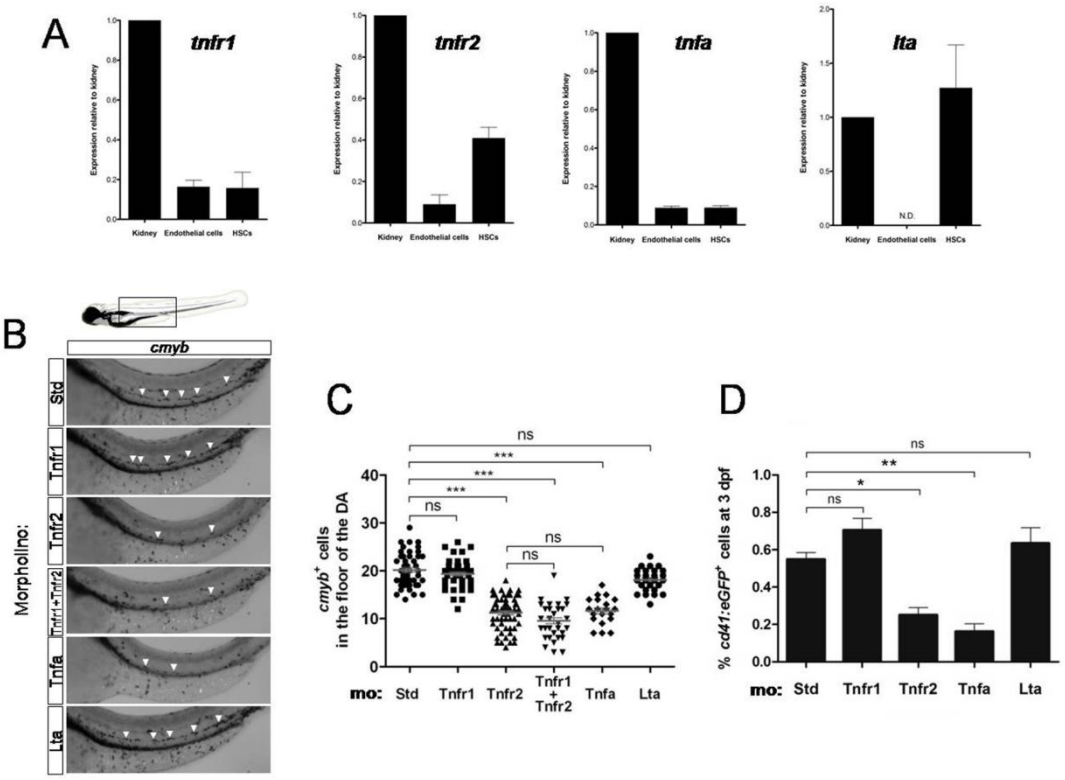

Figure 3. Tnfr2 or Tnfa Depletion Leads to Decreased Number of cmyb+ and cd41+ HSCs. (A) RT-qPCR analysis of the expression of *tnfr1*, *tnfr2*, *tnfa* and *lta* in FACS-sorted endothelial cells (*cmyb*$^{-}$; *kdrl*$^{+}$) and HSCs (*cmyb*$^{+}$; *kdrl*$^{+}$) from *cmyb:eGFP; kdrl:memCherry* transgenic embryos at 48 hpf. Units on y-axis represent *tnfr1*, *tnfr2*, *tnfa* and *lta* mRNA levels relative to adult kidney after being normalized with the *ef1a* content in each sample. Data are shown as the mean ± S.E.M. of triplicate samples and are representative of two independent experiments. (B-D) Zebrafish one-cell embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa and/or Lta morpholinos (mo) and the expression of *cmyb* in the floor of the DA of the zebrafish embryo was analyzed by WISH at 48 hpf. Representative images (B) and quantitation of the number of *cmyb*$^{+}$ cells in the floor of the DA. White arrowheads mark *cmyb*-expressing cells in the floor of the dorsal aorta. Each dot represents the number of *cmyb*$^{+}$ cells per single embryo. The mean ± S.E.M. of the *cmyb*$^{+}$ cells for each group of embryos is also shown. (D) Zebrafish one-cell *cd41:eGFP* transgenic embryos were injected with the above morpholinos and the number of GFP^{+} cells was determined by flow cytometry at 3 dpf. Data are the mean ± S.E.M of 3-7 independent samples (each with 5 pooled embryos). ns., not significant; N.D., not detected.*p<0.05; **p<0.01; ***p<0.001.

To further confirm the reduction of HSC numbers in Tnfr2- and Tnfa-deficient embryos, we directly visualized emerging HSCs from the floor of the dorsal aorta in *kdrl:mCherry; cmyb:eGFP* double transgenic embryos at 48 hours post fertilization (hpf) by confocal microscopy (Figure 4A). Consistent with our previous results, the number of *kdrl*$^{+}$; *cmyb*$^{+}$ HSCs in the floor of the dorsal aorta was reduced 50% when compared to control embryos (Figures 4A and 4B). Additionally, WISH for the nascent HSC marker runx1 at 28 hpf in Tnfr2- and Tnfa- deficient embryos also showed significantly reduced HSC numbers in the floor of the dorsal aorta (Figures 5A and 5B).

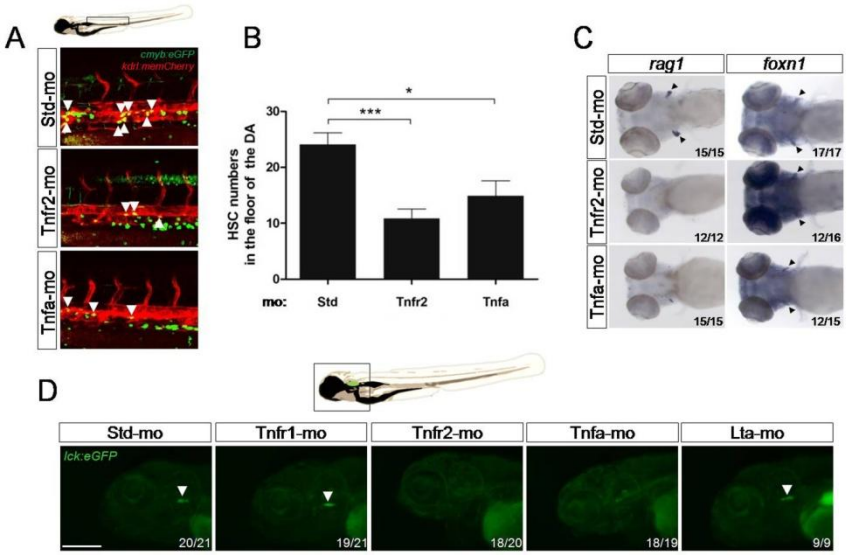

Figure 4. Tnfr2 and Tnfa Depletion Leads to Decreased Number of *cmyb*$^{+}$; *kdrl*$^{+}$ HSCs and Impaired T Lymphocyte Development. Zebrafish one-cell embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Lta morpholinos (mo). (A) Maximum projections of representative confocal microscopy stacks of *cmyb:eGFP; kdrl:memCherry* double transgenic embryos in the region of the DA at 48 hpf. Arrowheads denote *cmyb*$^{+}$; *kdrl*$^{+}$ HSCs (yellow). All views, anterior to left. (B) The number of HSC from (A) in Std (n=13), Tnfr2 (n=13) and Tnfa (n=8) morphant embryos. (C) WISH for the T lymphocyte and thymic epithelium markers *rag1* and *foxn1* (black arrowheads), respectively, in Tnfr2 and Tnfa morphants compared with Std controls at 4 dpf. All views, anterior to left within ventral views of the larvae heads. Numbers represent frequency of embryos with displayed phenotype (D) Representative images showing the thymus in *lck:eGFP* transgenic embryos at 4 dpf deficient for Tnr1, Tnfr2, Tnfa and Lta compared with Std controls. Arrowhead marks GFP^{+} cells in Std, Tnfr1 and Lta morphants, being obviously missing in their Tnfr2 and Tnfa morphant siblings. All views anterior to left within lateral views of the head. Numbers represent the frequency of larvae with shown phenotype. Scale bar, 250 μm. ns., not significant.*p<0.05; **p<0.01; ***p<0.001.

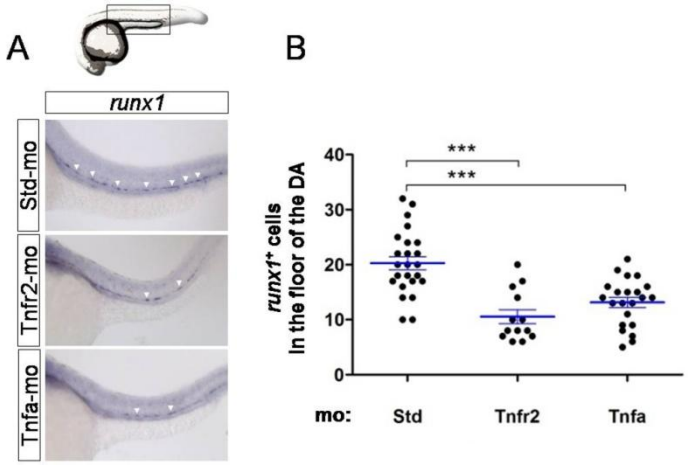

Figure 5. Tnfr2 or Tnfa Depletion Leads to Decreased Number of *runx1*$^{+}$ HSCs. (A) Zebrafish one-cell embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (mo) and WISH for *runx1* was performed at 28 hpf. All views anterior to left, lateral views. (B) Quantitation of *runx1*-expressing cells in the floor of the DA from (A). Each dot represents the number of *runx1*$^{+}$ cells in the floor of the DA per single embryo. The mean ± S.E.M. of the *runx1*$^{+}$ cells for each group of embryos are also shown. *p<0.05; **p<0.01; ***p<0.001.

We next examined the expression of *rag1* and *lck*, two genes expressed in developing thymic T cells [107], since they are exclusively derived from HSCs [116, 117]. Although *rag1* and *lck* expression was unaffected by Tnfr1 depletion, it was

undetectable in Tnfr2- and Tnfa-deficient animals at 4 dpf (Figure 4C). However, the thymic anlage developed normally in all morphant animals, assessed by the expression of the thymic epithelial marker *foxn1* (Figure 4C). These results were further verified in *lck:eGFP* transgenic fish; Tnfr2- and Tnfa- deficient larvae thymi were not visible at 4 dpf, while Tnfr1- and Lta-deficient siblings showed normal T cell development (Figure 4D). Together, these results indicate that Tnfa signals via Tnfr2, and that this signaling pathway is required for HSC emergence and/or maintenance in the zebrafish embryo, while Tnfr1 and Lta are dispensable.

3.3. Tnfr2- and Tnfa-deficient embryos show no vascular or circulatory defects

Since blood circulation is required for the transit of nascent HSCs to the caudal hematopoietic tissue (CHT), a site of HSC expansion, [53], we assessed whether the reduction of HSCs in Tnfr2- and Tnfa-deficient embryos was a consequence of vasculature abnormalities. However, no vasculature abnormalities were observed in Tnfr1-, Tnfr2-, Tnfa- or Lta-deficient embryos at 24 hpf (30 somite stage) at the morpholino doses used in these studies (Table 1) when assayed by WISH for the endothelial marker *kdrl* (Figure 6A). These results were confirmed using double transgenic *cd41:eGFP*; *gata1:Dsred* embryos, which allowed the visualization of circulating *gata1:DsRed*$^{+}$ red blood cells [47], *cd41:eGFP*$^{+}$ HSCs in the CHT, and circulating *cd41:eGFP*$^{+}$ thrombocytes [106, 118]. We observed that Tnfr2- and Tnfa-deficient embryos had normal circulation, but had a reduced number of HSCs and thrombocytes at 3 dpf (Figure 6B), indicating that Tnfr2 and Tnfa are involved specifically in HSC specification and/or maintenance.

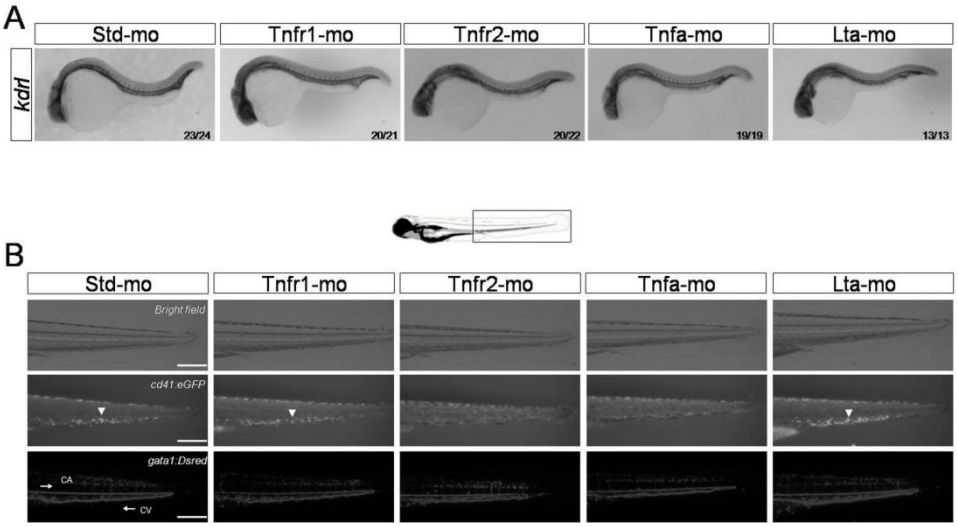

Figure 6. Tnfr2- and Tnfa-deficient Embryos Show Normal Vascular Development and Blood Circulation. Zebrafish one-cell embryos were injected with standard control (Std), Tnfr1, Tnfr2, Tnfa or Lta morpholinos (mo). (A) The expression of the endothelial marker *kdrl* in Std, Tnfr1, Tnfr2, Tnfa and Lta morphants was analyzed by WISH at 24 hpf. All views, anterior to left within lateral views of the embryos. Numbers represent the larvae with the shown phenotype. (B) Representative images of *cd41:eGFP; gata1a:Dsred* double transgenic larvae at 3 dpf. All views, anterior to left within lateral views of the larvae. Arrowheads indicate GFP^{+} cells in the caudal hematopoietic tissue (CHT) located between the caudal artery (CA) and the caudal vein (CV). Note that *cd41*$^{+}$ cells are hardly observed in Tnfr2- and Tnfa-deficient larvae, while Tnfr1- and Lta-deficient siblings show similar number of *cd41*$^{+}$ cells than control animals. Arrows indicate blood direction.

3.4. Tnfa and Tnfr2 deficiency leads to early apoptosis of HSCs

As our results suggested that HSC emergence from hemogenic endothelium was unaffected while HSC expansion was impaired, we analyzed the number of *cmyb*$^{+}$; *kdrl*$^{+}$ HSCs at 36 and 48 hpf in individual embryos by confocal microscopy. Whereas wt animals expanded their HSC numbers between 36 to 46 hpf, Tnfr2- and Tnfa-deficient siblings had the same number of HSCs at both timepoints (Figures 7A and 7B). Additionally, while Tnr2- and Tnfa-deficient embryos had similar numbers of HSCs at 36 hpf, they had approximately 50% less HSCs than their wt siblings at 48 hpf (Figure 7B), suggesting that Tnfa signaling through Tnfr2 was required in HSC maintenance soon after their emergence from the aortic endothelium. To determine if deficient HSCs were undergoing apoptosis, we performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in whole *cmyb:eGFP; kdrl:DsRed2* embryos. In Tnfa- and Tnfr2-deficient animals, numerous HSC budding events initiated, with *kdrl*$^{+}$; *cmyb*$^{-}$ cells transitioning to *kdrl*$^{+}$; *cmyb*$^{+}$ cells, although these cells rapidly become apoptotic before losing their contact with the endothelium (Figure 7C). Strikingly, 50% of the HSCs detected in Tnfr2- and Tnfa-deficient embryos were TUNEL^{+}, compared with almost no TUNEL^{+} cells found in controls (Figure 7D).

To test if the early apoptosis of Tnfa- and Tnfr2-deficient HSCs was responsible for their inability to expand, we treated deficient embryos with Q-VD-OPh, a pan-caspase inhibitor that blocks apoptosis in zebrafish [109, 110]. Q-VD-OPh treatment was able to partially rescue the number of *cmyb*$^{+}$ HSCs in Tnfr2-deficient animals (Figure 7E). Thus, the Tnfa/Tnfr2 signaling axis is required for HSC maintenance following their emergence from the hemogenic endothelium. These results further support a critical role for Tnfa/Tnfr2 signaling in HSC survival.

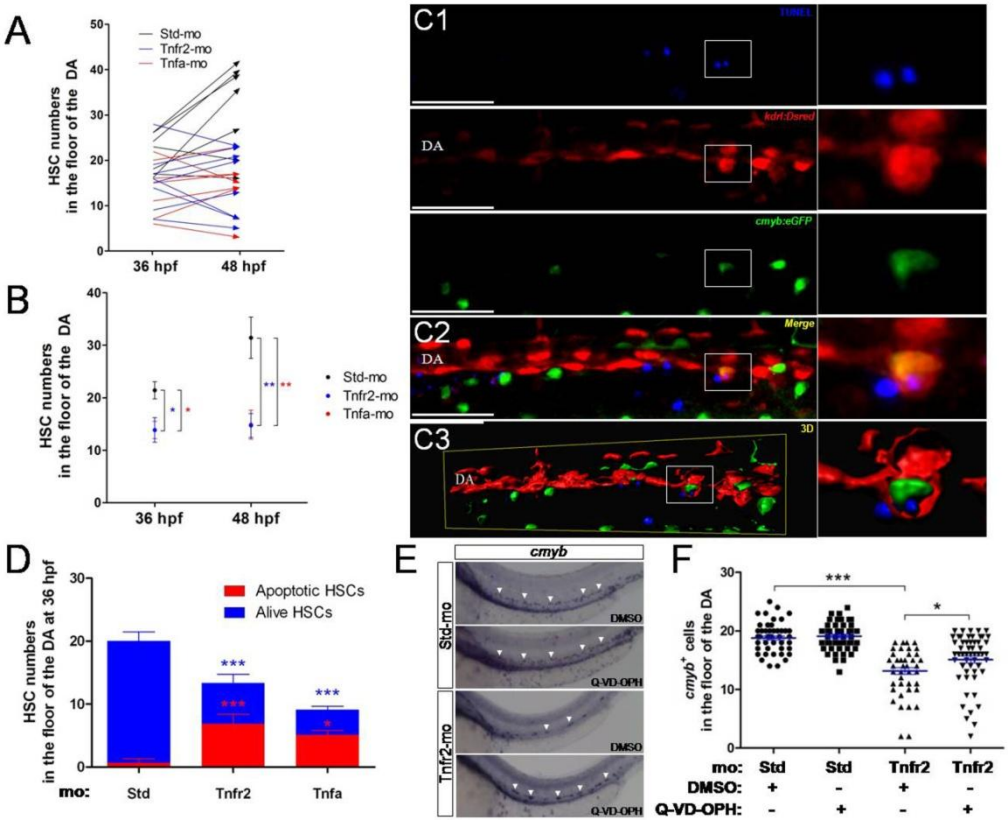

Figure 7. Tnfa and Tnfr2 Deficiency Leads to Early Apoptosis of HSCs. Zebrafish one-cell embryos were injected with standard control (Std), Tnfr2 or Tnfa morpholinos (mo). (A) Confocal tracking of HSC numbers in the floor of the DA from individual *cmyb:eGFP; kdrl:DsRed2* transgenic animals at 36 and 48 hpf following depletion of Tnfr2 or Tnfa compared with standard controls. (B) The mean ± S.E.M. of the *cmyb*$^{+}$ cells for each group of embryos at 36 and 48 hpf are also shown. (C) Representive confocal microscopy image of the TUNEL assay showing an apoptotic HSC (TUNEL^{+}; *cmyb*$^{+}$;*kdrl*$^{+}$) budding from the hemogenic endothelium in a Tnfr2-deficient embryo. A higher magnification of the apoptotic cell (squares) is also shown (right pictures). All views, anterior to left. (C1) One z-stack of the three channels showing a TUNEL^{+} HSC. (C2) Maximum projection. (C3) 3D reconstruction from (C2) in which cut has been performed to show that the apoptotic HSC is budding from the endothelium. The reconstruction has been flip towards the right side to facilitate the apoptotic HSC visualization. (D) Quantitation of the number of apoptotic (TUNEL^{+}) and alive (TUNEL^{-}) HSCs in the floor of the DA of Tnfr2 and Tnfa morphants compared with Std morphants at 36 hpf (n=6, 9 and 3 embryos, respectively). (E, F) The number of *cmyb*$^{+}$ cells in the floor of the DA was analyzed by WISH in Std and Tnfa morphants at 3 dpf after being treated with DMSO (vehicle control) or the pan-caspase inhibitor Q-VD-OPH from 24 hpf onward. Each dot represents the number of *cmyb*$^{+}$ cells per single embryo. The mean ± S.E.M. of the *cmyb*$^{+}$ cells for each group of embryos are also shown. Scale bar, 50 μm in (C). *p<0.05; **p<0.01; ***p<0.001.

3.5. Tnfr2 is intrinsically required for HSC maintenance

To evaluate if Tnfr2 was intrinsically required for HSC maintenance, we designed a truncated form of Tnfr2 lacking the intracellular signaling domain of the receptor. This truncated Tnfr2 (DN-Tnfr2) functions as a dominant negative form of the receptor [103], presumably by forming trimers with endogenous Tnfr2 receptors and preventing their signal transduction. DN-Tnfr2 was expressed in HSCs by injecting *runx1:DN-Tnfr2-Cherry* into wt embryos at the one-cell stage of development. WISH analysis of *runx1*$^{+}$ HSCs in the floor of the dorsal aorta revealed a significant reduction in HSCs in embryos expressing the DN-Tnfr2 compared to control embryos (Figures 8A and 8B), suggesting that Tnfr2 is intrinsically required for HSCs maintenance.

To further verify that Tnfr2 signaling was required for HSC maintenance, we rescued Tnfr2-deficient embryos by expressing wt Tnfr2 under the control of the *runx1* URS. Interestingly, the decreased number of *runx1*$^{+}$ cells in the floor of the dorsal aorta at 36 hpf in Tnfr2-deficient embryos was fully rescued by the restricted expression of Tnfr2 in HSCs (Figures 8C and 8D). These results strongly support that Tnfa/Tnfr2 signaling is intrinsically required for HSC survival after they emerge from the hemogenic endothelium.

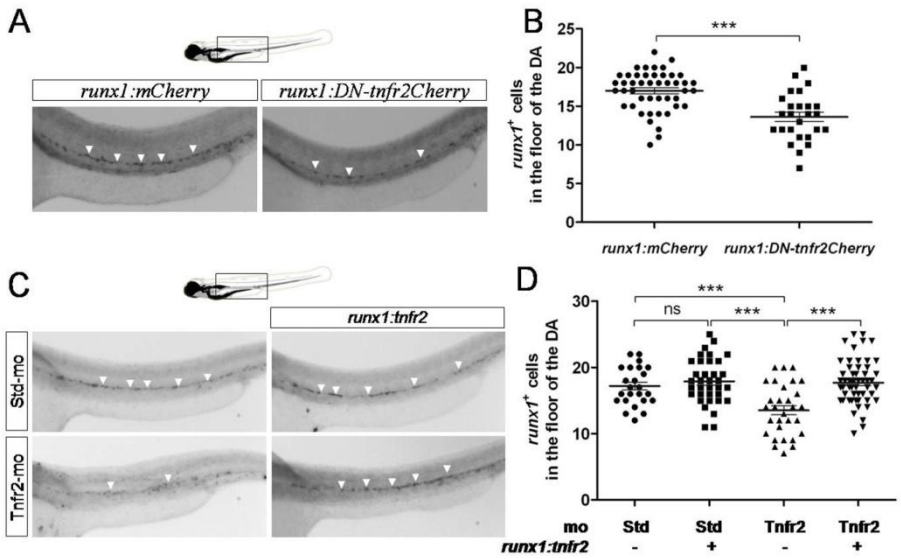

Figure 8. Tnfr2 is Intrinsically Required for HSC Maintenance. (A, B) Zebrafish one-cell embryos were injected with 100 ng/egg of *runx1:mCherry* or *runx1:DN-tnfr2Cherry* plasmid constructs plus 50 ng/egg of the Tol2 transposase RNA. The embryos that integrated the construct into their genome were selected with the cardiac marker *cmlc2:eGFP* and the number of *runx1*$^{+}$ cells in the floor of the dorsal aorta (DA) was then analyzed by WISH at 36 hpf. (A) Representative images of WISH. (B) Each dot represents the number of *runx1*$^{+}$ cells per single embryo. The mean ± S.E.M. of the *runx1*$^{+}$ cells for each group of embryos is also shown. (C, D) Zebrafish one-cell embryos were injected with standard control (Std) or Tnfr2 morpholinos (mo) and 100 ng/egg of the *runx1:tnfr2* construct plus 50ng/egg of the Tol2 transposase RNA and the number of *runx1*$^{+}$ cells in the floor of the DA of embryos expressing (+) or not (-) the wild type tnfr2 driving by the promoter *runx1a* were selected using the cardiac marker present in the construct (see Experimental Procedures for details). (C) Representative images of WISH. (B) Each dot represents the number of *runx1*$^{+}$ cells per single embryo. The mean ± S.E.M. of the *runx1*$^{+}$ cells for each group of embryos is also shown. All views, anterior to left. ns., not significant. *p<0.05; **p<0.01; ***p<0.001.

4. DISCUSSION

The exacerbated production of TNFα during acute and chronic inflammatory diseases has been implicated in bone marrow failure typically associated with inflammatory disorders [119]. Several TNFα inhibitors, such as etanercept, infliximab, and adalimumab, have proven efficacy in the treatment of human inflammatory diseases. However, multiple reports describing non-malignant hematological abnormalities associated with anti-TNFα therapy indicate a strong correlation between the use of TNFα inhibitors and neutropenia and thrombocytopenia (reviewed by [120]). Therefore, the role of TNFα in the regulation of hematopoiesis and HSCs needs to be clarified. We have utilized the unique advantages of the zebrafish embryo to deplete Tnfa or Tnfr2, causing apoptosis of HSCs just after their emergence from the ventral wall of the dorsal aorta (Figure 9). This result helps to explain non-malignant hematological abnormalities associated with anti-TNFα therapy [120], and supports the idea that specific inhibition of the TNFα/TNFR1 signaling axis while leaving TNF/TNFR2 signaling untouched would inhibit the pathological effects of TNFα and potentially reduce side effects associated with this treatment modality [103, 121].

In vivo imaging of whole embryos by confocal microscopy allowed us to demonstrate that although the endothelial-to-hematopoietic transition events were initiated at both morphological (cells began to bud) and molecular (cells transitioned from *kdrl*$^{+}$*; cmyb*$^{-}$ to *kdrl*$^{+}$*; cmyb*$^{+}$) levels in Tnfa- and Tnfr2-deficient embryos, nascent HSCs rapidly became apoptotic before losing their contact with the endothelium. In contrast, Lta and Tnfr1 were both dispensable for HSC emergence and expansion. In addition, Tnfr1 was unable to rescue the early apoptosis of Tnfr2-deficient HSCs. The reduced number of HSCs in embryos deficient in the ligand for both receptors (Tnfa) further supports this conclusion. Therefore, these results suggest that a balance between Tnfr1 and Tnfr2 signaling is not required for the maintenance and expansion of HSCs, in contrast to endothelial cells [103]. It will be of interest to further elucidate the role of Tnfr1 signaling in HSCs that occurs in acute and chronic inflammation, since exacerbated Tnfr1 signaling might result in HSC apoptosis, explaining the bone marrow failure associated with inflammatory diseases [119].

The transient knockdown of Tnfr signaling by antisense morpholinos indicates that there is a critical threshold for Tnfr2 signaling that exists in different cell types, since a lower dose of morpholino against Tnfr2 promotes the apoptosis of HSCs, while higher doses trigger the apoptosis of endothelial cells by unbalancing survival (via Tnfr2) and apoptotic (via Tnfr1) signals [103]. This observation should be taken into account for increasing the efficiency and reducing the side effects of anti-TNFα therapies in different human inflammatory disorders. The ability to control the timing and level of Tnfr knockdown is a benefit of using the zebrafish for these studies, and will likely be helpful for further investigating the role of Tnfrs in a multitude of hematopoietic diseases.

The *in vivo* role of TNFα and the contribution of each TNF receptor in the regulation of the HSC compartment have not been thoroughly investigated with *in vivo* loss-of-function studies. One study indicated that TNFα signaling through TNFR1, and to some extent through TNFR2, restricted HSC activity in mice, as determined in competitive transplantation assays [100]. Similarly, TNFR1-deficient mice have increased numbers of HSCs without a marked change in bone marrow cellularity [122]. However, another study indicated that TNFR1-deficient HSCs display impaired

proliferative and self-renewal abilities [101]. In addition, membrane-anchored TNFα produced by precursor plasmacytoid dendritic cells significantly enhanced engraftment of purified HSCs in allogeneic and syngeneic recipients [102]. Although it is difficult to reconcile these contradictory results, our data indicate that these findings are likely due to the fact that these previous studies were performed in mice deficient for either TNFR, allowing signaling through the remaining, unaffected wt receptor. Therefore, the increased number of HSCs found in TNFR1-deficient mice [122] might be explained by a potentiated signaling of TNFα via TNFR2, rather than by the negative regulation of the HSC compartment by TNFR1. Therefore, we propose that experiments utilizing single TNFR-deficient animals should be performed in parallel with their double deficient siblings to fully understand their role in these processes.

The signaling pathways downstream of TNFRs are very complex [56-58], and receptor crosstalk has been reported depending on the cell type and activation state [95]. Similarly, it was recently reported that signaling through both TNFRs must be balanced for endothelial cell development and maintenance in zebrafish [103]. Tnfr2 deficiency triggers a caspase-8-, caspase-2-, and p53-dependent apoptotic program in endothelial cells that bypasses caspase-3 [103]. Interestingly, this apoptotic program is evolutionary conserved, since TNFα promotes the apoptosis of human endothelial cells through TNFR1, triggering caspase-2 and p53 activation [103]. Similarly, our results indicate that the Tnfa/Tnfr2 axis provides an intrinsic survival signal that is critical for the maintenance and expansion of HSCs, preventing their early apoptosis. Nevertheless, the contribution of Tnfr1 to HSC emergence, early expansion, and differentiation seems to be dispensable, suggesting that the Tnfa/Tnfr2 axis is required for the induction of critical signals required for HSC specification, rather than counteracting the apoptotic signals induced by Tnfr1. The high expression of Tnfr2 by emerging HSCs further supports the intrinsic nature of the survival signal provided by Tnfr2. It is of interest that *lta* was also expressed at very high levels in emerging HSCs, but that the depletion of Lta had no effect on HSC emergence or survival. Further studies are likely required to elucidate the full role of Tnfr1 and Lta in HSC emergence, specification, and differentiation.

The results from our study are also relevant for developing methods to stimulate *in vitro* production of HSCs. HSCs are specified during early embryonic development, and persist for the lifetime of the organism [39]. Therefore, signaling events that occur during this critical time window of hematopoietic ontogeny are responsible for the only *de novo* production of HSCs. Our study indicates that signaling through the TNFα/TNFR2 axis is important for the efficient generation of HSCs from hemogenic endothelium, suggesting that it is also likely important in the generation of HSCs from induced pluripotent stem (iPS) cells. This is important, as the generation of hematopoietic cells from iPS cells needs to be significantly improved before cellular therapy can be considered an effective clinical treatment modality [123, 124].

In conclusion, Tnfr signaling is not required for primitive hematopoiesis, but Tnfa signaling through Tnfr2 is intrinsically required for the survival of HSCs after their emergence from the hemogenic endothelium (Figure 9). In addition, Lta and Tnfr1 are dispensable for HSC emergence and early expansion. These studies are novel, as they conclusively show that TNFα, once thought to only play a role in inflammation and immune regulation, also plays an essential role in the maintenance and expansion of HSCs, the keystone cells of the hematopoietic system. Not only do these studies shed light on the development and maintenance of HSCs, they also highlight the utility of

developing targeted therapies that specifically inhibit the TNFα/TNFR1 axis, and not TNFα/TNFR2 to reduce the side effects caused by current anti-TNFα therapies. Finally, our studies suggest that manipulation of the TNFα/TNFR2 axis may aid current efforts to generate HSCs *de novo* from iPS cells, an important yet daunting task.

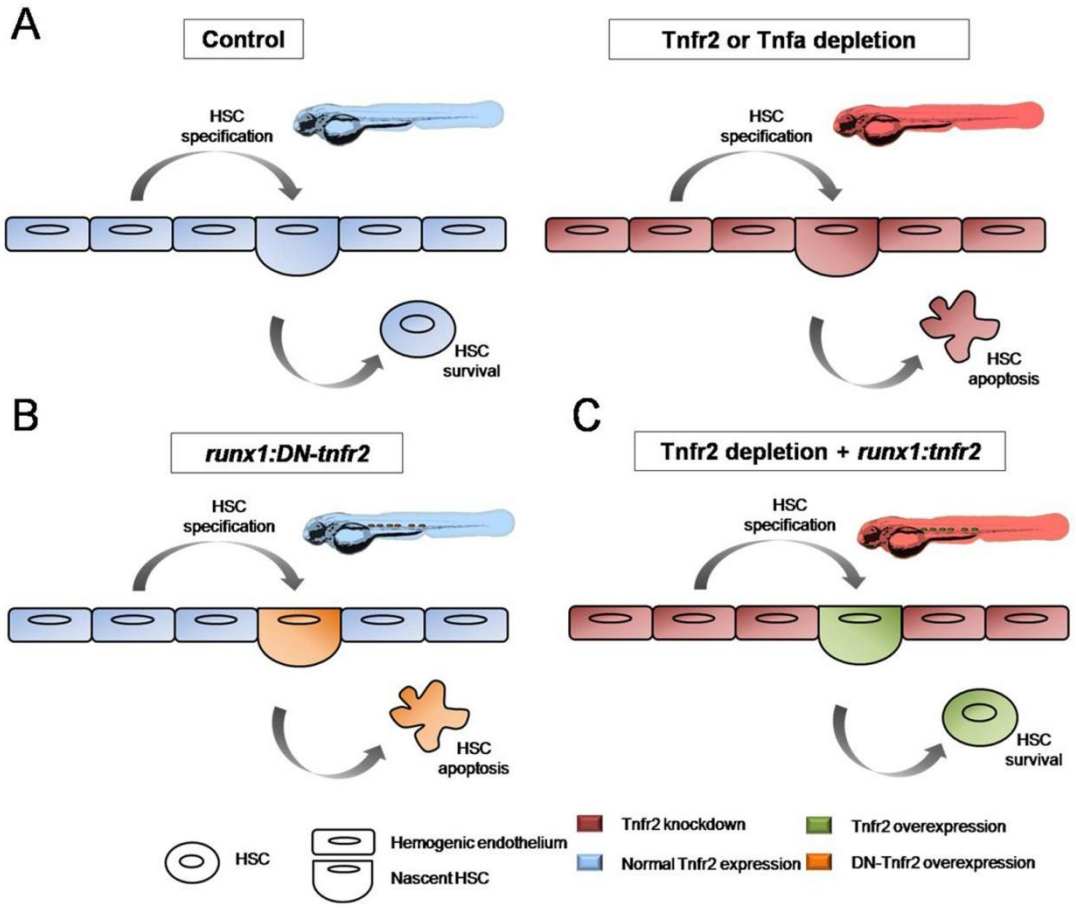

Figure 9. Proposed model illustrating the role of Tnfa signaling viaTnfr2 in HSC survival. (A) Tnfa or Tnfr2 depletion results in the apoptosis of HSCs soon after their emergence from the hemogenic endothelium. (B) Expression of dominant negative tnfr2 (DN-tnfr2) in HSCs using the *runx1* promoter leads to the early apoptosis of HSCs. Collectively, these results indicate that Tnfa signaling via Tnfr2 is intrinsically required for HSCs survival in the zebrafish embryo. (C) Restricted expression of *tnr2* in HSCs using the *runx1* promoter rescues the early apoptosis of HSC in Tnfr2-deficient embryos.

CONCLUSIONS

The results obtained in this work lead to the following conclusions:

1. Target gene silencing of Tnfr2 results in the induction of a caspase-8-dependent apoptotic program in endothelial cells. This apoptosis can be rescued by depletion of Tnfr1, indicating that an appropriate signaling balance between both Tnfrs is required for endothelial cell integrity and vascular homeostasis.

2. In endothelial cells, Tnfr1 signals apoptosis through complex II formation and caspase-8 and caspase-2 activation, while Tnfr2 signals survival via complex I and NF-κB activation.

3. The apoptotic program induced by Tnfr1, which involves caspase-8, caspase-2 and P53, is evolutionary conserved in human endothelial cells.

4. Tnfrs signaling is dispensable for primitive hematopoiesis in the zebrafish embryo.

5. Genetic inhibition of Tnfa or Tnfr2, but not of Tnfr1 or Lta, results in HSC apoptosis soon after their emergence from the floor of the dorsal aorta.

6. Tnfa/Tnfr2 signaling is intrinsically required for HSC survival after their emergence from the hemogenic endothelium.

PUBLICATIONS

Espín R., Roca F. J., Candel S., Sepulcre M. P., González-Rosa J. M., Alcaraz-Pérez F., Meseguer J., Cayuela M. L., Mercader N., Mulero V. Tumour necrosis factor receptors regulate endothelial cell survival and vascular homeostasis through a caspase-8, caspase-2 and P53 apoptotic programme that bypasses caspase-3. *Disease Models and Mechanisms*. 6, 383-396 (2013) doi:10.1242/dmm.010249.

Espín-Palazón R, Stachura D.L., García-Moreno D., Campbell C.A., Candel S., Meseguer J., Traver D., Mulero V. TNF Signaling is Critical for Hematopoietic Stem Cell Survival and Expansion in the Developing Embryo. Under review.

RESUMEN EN CASTELLANO

INTRODUCCIÓN

INTRODUCCIÓN

El Factor de Necrosis Tumoral α (TNFα) es una citoquina que ha sido muy bien caracterizada por su función en la inflamación. Sin embargo, el TNFα se caracteriza por tener un papel pleiotrópico, y se le ha atribuido un papel en procesos biológicos tan dispares como desarrollo embrionario, diferenciación, supervivencia celular, cáncer, hematopoyesis, metabolismo lipídico y reproducción.

El TNFα se produce principalmente por células del sistema inmunitario tales como macrófagos, linfocitos T y B, células linfoides y células NK. Sin embargo, otros tipos celulares también son capaces de producir TNFα, entre los que podemos destacar células endoteliales, miocitos, tejido adiposo, células hematopoyéticas, fibroblastos y neuronas. La producción de TNFα por esta gran variedad de tipos celulares es un indicativo de los múltiples procesos en los que esta citoquina está involucrada. Además, el efecto pleiotrópico del TNFα relaciona esta citoquina con una gran variedad de enfermedades humanas, tales como desórdenes inflamatorios y autoinmunes (psoriasis, artritis reumatoide y enfermedad inflamatoria intestinal), isquemia seguida de daño por reperfusión y cáncer [1].

El TNFα ejerce su acción a través de la unión a dos receptores de membrana denominados TNFR1, también llamado TNFRSF1A, y TNFR2 o TNFRSF1B (Shalaby et al., 1990). Mientras que TNFR1 está presente en la mayoría de células, TNFR2 tiene una expresión más restrictiva, encontrándose fundamentalmente en células del sistema inmunitario y células endoteliales [13]. En cuanto al efecto que desencadena cada receptor de TNFα, se ha demostrado que TNFR1 provoca apoptosis o inflamación, pero TNFR2 promueve la reparación tisular y regeneración [13]. Sin embargo, hay cierto solapamiento en sus funciones, ya que los receptores de TNFα no poseen actividad enzimática per sé, y es a través del reclutamiento de diferentes proteínas adaptadoras cómo ejercen su función, pudiendo compartirlas [95].

Son tres tipos de proteínas las que interaccionan con los dominios citoplasmáticos de los TNFRs: TAFs, factores asociados a TNFR; FADDs: dominios de muerte asociados a FAS y TRADDs, dominios de muerte asociados a TNFR. La unión de TNFα a TNFR1 provoca el reclutamiento de TRAF2 y TRADD, los cuales interaccionan con otras proteínas señalizadoras tales como BIRC2 (cIAP1) y BIRC3 (cIAP2). De esta forma se forma lo que se denomina complejo I que induce la degradación de IκB, que es el inhibidor de NF-κB. De esta forma, NF-κB se trasloca al núcleo, desencadenando la transcripción de genes pro-inflamatorios y de supervivencia [56, 57]. A partir de este complejo I se puede formar el complejo II. Este complejo II recluta FADD y caspasa-8, lo que resulta en la activación de ésta última que desencadenará muerte celular [56, 57]. Por el contrario, la unión de TNFα a TNFR2 desencadena el reclutamiento de TRAF1 y TRAF2, que interaccionan con BIRC2 y BIRC3 [58], llevando a la activación de NF-κB.

Mediante el uso de una aproximación genética directa en pez cebra, Santoro y colaboradores [59], identificaron BIRC2 como una proteína esencial en la supervivencia y homeostasis vascular. En ausencia de BIRC2, ocurre una regresión de los vasos debida a la aparición de un programa apoptótico en estas células dependiente de caspasa-8 y caspasa-3. Dado que BIRC2 está implicado en la ruta de señalización de TNFR2 [58] y que previamente hemos demostrado en nuestro grupo que las células endoteliales son una de las principales dianas de TNFα en peces [60], junto con el hecho de que TNFα y TNFRs se encuentran conservados a lo largo de los vertebrados [125],

decidimos analizar el papel que juega cada receptor de TNF en el desarrollo y mantenimiento de las células endoteliales durante el desarrollo embrionario de pez cebra y comprobar si esta función se encuentra conservada en humanos.

Por otro lado, la sobreexpresión de TNFα se ha relacionado con fallos en la médula ósea tales como la anemia de Fanconi [96, 97]. Sin embargo, la contribución del TNFα en la regulación de la hematopoyesis y, en concreto, en el desarrollo y mantenimiento de la células madre hematopoyéticas (HSCs) no está clara. Hay estudios que muestran que el TNFα afecta negativamente a la especificación y mantenimiento de las HSCs *in vitro* [98], pero los pocos estudios *in vivo* que hay hasta la fecha son contradictorios: el TNFα afecta positiva y negativamente a la especificación y mantenimiento de las HSCs [100-102]. Por ello sería de gran importancia aclarar el papel que, tanto el TNFα como sus receptores, tienen en la especificación y mantenimiento de las HSCs.

OBJETIVOS

En el presente trabajo se proponen los siguientes objetivos concretos:

1. Caracterización de los receptores de Tnf (Tnfr1 y Tnfr2) en la homeostasis vascular durante el desarrollo en pez cebra.

2. Caracterización de las rutas de señalización de Tnfr1 y Tnfr2 involucradas en el desarrollo de las células endoteliales y su homeostasis.

3. Caracterización del papel que juegan Tnfrs y sus ligandos (Tnfa y Lta) en la ola primitiva de hematopoyesis en el pez cebra.

4. Caracterización de los Tnfrs y sus ligandos (Tnfa y Lta) en la especificación y mantenimiento de las HSCs en el embrión de pez cebra.

RESULTADOS Y DISCUSIÓN

1. LOS RECEPTORES DE TNF REGULAN LA HOMEOSTASIS VASCULAR A TRAVÉS DE UN PROGRAMA APOPTÓTICO QUE IMPLICA CASPASA-8, CASPASA-2 Y P-53, PERO NO CASPASA-3

El proceso de angiogénesis tanto en el embrión como en el adulto necesita de un fino control a nivel molecular de la supervivencia e integridad en células endoteliales. Santoro y colaboradores [59] demostraron que la proteína antiapoptótica BIRC2 es muy importante en la regulación de la supervivencia de las células endoteliales *in vivo*. Sin embargo, BIRC2 es una proteína adaptadora que participa en diferentes rutas de señalización, entre las que destacan FAS [84], CD40 [85, 86], TNFRSF1A [87] and TNFRSF1B [58], pero se desconoce cuál es el receptor necesario para el desarrollo vascular y su integridad.

Mediante experimentos de pérdida y ganancia de función, hemos descubierto que debe haber un equilibrio en la señalización a través de los receptores TNFRSF1A y TNFRSF1B en las células endoteliales para que éstas se desarrollen de forma normal durante la embriogénesis de pez cebra. De esta forma, TNFRSF1 produce apoptosis a través del complejo II y activación de caspasa-8 en este tipo celular, mientras que TNFRSF2 provoca supervivencia a través del complejo I y NF-κB. Además, hemos demostrado que estas rutas se encuentran conservadas en células endoteliales humanas mediante el uso células primarias HUVEC, en la que se ha visto que la susceptibilidad al tratamiento con TNFα se rescata al bloquear el receptor TNFRSF1A o NF-κB [88]. Estos resultados, junto con la capacidad de TNFRSF1B de inducir TRAF2 y BIRC2 y que la deficiencia en TNFRSF1B conlleva acelerar la activación dependiente de caspasa-8 dependiente de TNFRSF1B [86], sugiere que BIRC2 se requiere para la formación del complejo I y activación de NF-κB por TNFRSF1B en células endoteliales, a pesar de que BIRC2 es una proteína adaptadora promiscua.

Una de las observaciones más importantes realizadas en nuestro estudio es que la vía apoptótica dependiente de TNFRSF1A en células endoteliales es independiente de caspasa-3 y caspasa-9, pero requiere caspasa-2. Caspasa-2 es una caspasa misteriosa que posee características de caspasa iniciadora y ejecutadora [89]. Nuestro estudio epistático ha dado lugar a un modelo en el que la unión de TNFα a TNFRSF1A conlleva la sucesión de los siguientes eventos: (i) activación de caspasa-8, (ii) activación de caspasa-2, (iii) procesamiento de ICAD mediante caspasa-2 y translocación de CAD al núcleo, (iv) activación de P53 y (v) inducción de la expresión del gen de caspasa-8 dependiente de P53. Como se ha demostrado que la caspasa-2 humana es capaz de procesar ICAD *in vitro* [83], hipotetizamos que quizás caspasa-2 sería capaz de reemplazar caspasa-3 como principal caspasa ejecutora, mediando el procesamiento de ICAD y la consiguiente activación de P53. De hecho, pudimos observar que P53 está involucrado en la apoptosis de células endoteliales mediado por TNFRSF1A. Además, P53 podría estar involucrado en la activación directa de caspasa-2 en las células deficientes en TNFRSF1B, ya que se ha demostrado recientemente que caspasa-2 participa en un bucle positivo con P53 en respuesta a daño de DNA [90]. De esta manera, caspasa-2 procesa MDM2, lo que lleva a un aumento en la estabilidad de p53 y a la transcripción de PIDD, que es un gen diana de P53 que activa caspasa-2 en un complejo denominado caspasa-2PIDDosoma.

El programa apoptótico desencadenado por TNFRSF1A en células endoteliales implica una regulación positiva entre las rutas intrínsicas y extrínsecas, que termina en la regulación positiva de caspasa-8, contribuyendo así a la apoptosis de las células

endoteliales. Esta observación no había sido descrita previamente *in vivo*, pero no es extraño, ya que se ha visto que P53 puede regular positivamente la transcripción del gen de caspasa-8 en células cancerígenas humanas tratadas con drogas citotóxicas [91].

La inhibición de la quinasa CHK1 del punto de control de ciclo celular, la cual es activada por ATR en respuesta al estrés replicativo [92], impide el que la inactivación de P53 rescate de los defectos vasculares promovidos por TNFRSF1A en embriones de pez cebra y la inducción de la expresión de P21 en células HUVEC tratadas con TNFα, indicando así que CHK1 podría estar aguas debajo de P53 en esta ruta apoptótica de señalización. De forma alternativa, también podría inhibir otras rutas apoptóticas independientes de P53. Esto no es extraño, ya que se ha descrito recientemente un papel de CHK1 en la inhibición de la respuesta apoptótica a través de ATM/ATR-caspasa-2 inducida por daño al DNA por irradiación, que no implica P53 ni caspasa-3 [63]. Aunque la ruta apoptótica dependiente de CHK1 e independiente de P53 desencadenada por TNFRSF1A necesita de una investigación más en detalle, nuestros resultados sugieren que CHK2 podría también mediar una activación de P53 en células endoteliales deficientes en TNFRSF1B.

Aunque varios inhibidores sistémicos de TNFα tales como receptores solubles de TNF y anticuerpos anti TNFα, se han aprobado para el tratamiento de enfermedades humanas en las que el TNFα juega un papel patológico, estas drogas producen severos efectos secundarios y son caras. De esta forma, se necesitan nuevas drogas anti TNFα que sean seguras, eficaces y baratas [93]. Creemos que la ruta apoptótica de TNFRSF1A descrita en este estudio revela nuevas dianas moleculares para el desarrollo de drogas terapéuticas para enfermedades humanas donde el TNFα juega un importante papel tales como desórdenes autoinmunes e inflamatorios, daño por reperfusión después de isquemia, y cáncer. El programa apoptótico caspasa-2/P53 inducido por TNFα en células HUVECs, los defectos vasculares de peces cebra deficientes en TNFRSF1A y las observaciones recientes en ratones transgénicos que sobreexpresan TNFRSF1B en células endoteliales que demuestran una disminución de la muerte de estas células después de la isquemia/reperfusión y un incremento en la proliferación de células endoteliales, neovascularización y maduración de los vasos después del daño [94], sugiere que TNFRSF1A y TNFRSF1B, el lugar de TNFα, podría tener ventajas para la búsqueda de dianas terapéuticas.

Como conclusión, en este trabajo hemos identificado un programa apoptótico evolutivamente conservado en células endoteliales debido a un desbalance entre las señales de supervivencia y muerte generadas por los receptores de TNFα. Esta ruta implica un diálogo entre los programas apoptóticos intrínsecos (caspasa-8) y extrínsecos (P53) y que de forma llamativa, no necesitan caspasa-3. Caspasa-2 reemplaza a la caspasa-3 y uniría ambos programas apoptóticos debido, probablemente, a su actividad dual como una caspasa iniciadora y ejecutora. Esta ruta genética revela nuevas dianas terapéuticas para el control de la inflamación y de la angiogénesis inducida por tumores.

2. LA SEÑALIZACIÓN A TRAVÉS DE TNFR2 ES IMPRESCINDIBLE PARA LA SUPERVIVENCIA Y EXPANSIÓN DE LAS CÉLULAS MADRE HEMATOPOYÉTICAS DURANTE EL DESARROLLO EMBRIONARIO.

La producción excesiva de TNFα en enfermedades inflamatorias crónicas y agudas se ha relacionado con el fallo en la médula ósea que ocurre en ciertos desórdenes

inflamatorios [119]. Diferentes inhibidores de TNFα, tales como etanercept, infliximab y adalimumab se usan eficazmente para el tratamiento de ciertas enfermedades inflamatorias en humanos. Sin embargo, hay ciertos estudios en los que se ha descrito ciertas anormalidades hematológicas no malignas como neutropenia y trombocitopenia [120] asociadas a la terapia anti-TNFα, pero la causa se desconoce completamente. De esta forma, la función que tiene el TNFα en la regulación de la hematopoyesis necesita ser estudiado.

Utilizando las ventajas incuestionables del modelo de pez cebra, hemos eliminado genéticamente Tnfa o Tnfr2 en embriones de pez cebra, provocando apoptosis en las HSCs justo después de su formación en la pared ventral de la aorta dorsal. Este resultado ayuda a explicar las anormalidades hematológicas no malignas que se han descrito en estrecha asociación con pacientes tratados con fármacos anti TNFα [120], apoyando la idea de que la inhibición específica de la señalización vía TNFα/TNFR1 dejando intacta la ruta TNFα/TNFR2 podría reducir los efectos secundarios asociados a este tipo de tratamientos [103, 121].

El estudio *in vivo* de embriones usando microscopía confocal nos ha permitido demostrar que aunque la transición de células endoteliales a hematopoyéticas se inicia tanto morfológica (las células son capaces de gemar) como molecularmente (expresión transicional de $kdrl^{+}; cmyb^{-}$ a $kdrl^{+}; cmyb^{+}$) en embriones deficientes en Tnfa o Tnfr2, las HSCs emergentes entran rápidamente en apoptosis, incluso antes de perder el contacto con el endotelio. Sin embargo, tanto Lta como Tnfr1 son dispensables para la emergencia y expansión de HSCs. Además, Tnfr1 es incapaz de rescatar la temprana apoptosis en las HSCs carentes de Tnfr2. El número reducido de HSCs en los embriones deficientes en el ligando para ambos receptores (Tnfa) apoya esta conclusión. De esta forma, estos resultados sugieren que no se necesita un balance entre Tnfr1 y Tnfr2 para el mantenimiento y expansión de las HSCs, al contrario de lo que ocurre en las células endoteliales [103]. Sería importante dilucidar el papel de la señalización de Tnfr1 en las HSCs que ocurre en la inflamación aguda y crónica, ya que la activación excesiva de la ruta desencadenada por el eje Tnfa/Tnfr1 podría resultar en apoptosis de las HSCs, explicando así el fallo en la médula ósea que ocurre en pacientes que padecen enfermedades inflamatorias crónicas [119].

El bloqueo temporal de la señalización a través de Tnfr mediante el uso de morfolinos indica que hay un límite crítico para la señalización de Tnfr2 que difiere según el tipo celular, ya que una dosis menor del morfolino que bloquea el Tnfr2 conlleva apoptosis de HSCs, mientras que una mayor dosis desencadena apoptosis en células endoteliales debido a un desbalance entre señales de supervivencia (Tnfr2) y apoptosis (Tnfr1) [103]. Es importante tener en cuenta esta observación para incrementar la eficacia y reducir los efectos secundarios en las terapias anti-TNFα en los diferentes desórdenes inflamatorios en los que TNFα está implicado. La habilidad para controlar el tiempo y nivel del bloqueo de los receptores de Tnf es un beneficio del uso del pez cebra para estos estudios, y será útil para investigar el papel de los Tnfrs en una multitud de enfermedades hematopoyéticas.

El papel *in vivo* del TNFα y la contribución de cada uno de sus receptores en la regulación del compartimento de las HSCs no ha sido investigado lo suficiente con estudios de pérdida de función. Un estudio de trasplante competitivo indica que la señalización de TNFα a través del receptor TNFR1 restringe la actividad de las HSCs en ratón [100]. De forma similar, ratones deficientes en TNFR1 tienen un mayor número

de HSCs [122]. Sin embargo, otro estudio indica que HSCs sin TNFR1 muestran incapacidad proliferativa y de auto renovación [101]. Además, el TNFα anclado a membrana producido por el precursor de células dendríticas mejora el injerto de HSCs purificadas en receptores alogénicos y singénicos [102]. Aunque es difícil conciliar estos resultados contradictorios, nuestros datos indican que estos descubrimientos probablemente son debidos al hecho de que los estudios previos se llevaron a cabo en ratones deficientes en uno de los receptors, permitiendo la señalización a través del otro receptor. De esta forma, el incremento en el número de HSCs encontrado en los ratones deficientes en TNFR1 [122] se podría explicar por una potenciación en la señalización de TNFα via TNFR2, más que por la regulación negativa de las HSCs por TNFR1. De esta manera, proponemos que en este tipo de experimentos, además de usar animales deficientes en uno de los receptores, se debería estudiar en paralelo el efecto que tiene en el doble mutante para poder comprender completamente el papel de cada receptor de TNFα en estos procesos.

Las rutas de señalización aguas debajo de los TNFRs son muy complejas [56-58]. De forma similar, hemos demostrado en esta tesis que la señalización a través de ambos receptores de TNFα debe estar equilibrada para el correcto desarrollo y mantenimiento de las células endoteliales en pez cebra [103]. La deficiencia en Tnfr2 conlleva un programa apoptótico dependiente de caspasa-8, caspasa-2 y P53, pero que no incluye caspasa-3 [103]. De forma similar, nuestros resultados indican que el eje Tnfa/Tnfr2 proporciona una señal de supervivencia intrínseca que es crucial para el mantenimiento y expansión de las HSCs. Sin embargo, la contribución de Tnfr1 a la emergencia de las HSCs, expansión y diferenciación parece ser dispensable, sugiriendo que el eje Tnfa/Tnfr2 se requiere para la inducción de señales críticas requeridas para elmantenimiento de las HSCs, más que para contrarrestar las señales apoptóticas producidas por Tnfr1. El aumento en la expresión de *tnfr2* cuando las HSCs emergen del endotelio hemogénico respalda la naturaleza intrínseca de la señal de supervivencia producida por Tnfr2. Es interesante que *lta* es expresada a altos niveles en las HSCs emergente, pero que los embriones a los que se les ha bloqueado Lta no afecte a la emergencia o supervivencia de las HSCs. Estudios más en detalle deberían llevarse a cabo para elucidar el papel de Tnfr1 y Lta en la emergencia, especificación y diferenciación de las HSCs.

Los resultados obtenidos en nuestro estudio son también importantes para el desarrollo de métodos para la producción *in vitro* de HSCs. Las HSCs se especifican durante el desarrollo embrionario temprano, y persisten durante toda la vida del organismo [39]. De esta forma, las señales que ocurren durante esta crítica ventana de tiempo en la ontogenia de la hematopoyesis es responsable del único momento en la vida del organismo en el que las HSCs son producidas *de novo*. Nuestros estudios indican que la señalización a través del eje TNFα/TNFR2 es importante para la formación eficaz de HSCs desde el endotelio hemogénico, sugiriendo que es también probablemente importante en la generación de HSCs *in vitro* a partir de células madre pluripotentes inducidas (iPS). Esto tiene gran importancia, ya que la generación de células hematopoyéticas a partir de células iPS necesita mejorar antes de considerar esta terapia celular un tratamiento clínico efectivo [123, 124].

Como conclusión, la señalización de Tnfr no se requiere para la hematopoyesis primitiva, pero la señal de Tnfa a través de Tnfr2 es necesaria por las HSCs para su supervivencia después de su especificación desde el endotelio hemogénico. Además, Lta y Tnfr1 son dispensables para la emergencia y expansión de las HSCs. Estos

estudios son novedosos, ya que demuestran que TNFα, al que se le atribuyó originalmente un papel en la regulación del sistema inmunitario e inflamación, también juega un papel esencial en el mantenimiento y expansión de las HSCs, células clave del sistema hematopoyético. No sólo nuestro estudio arroja luz en el campo del desarrollo y mantenimiento de las HSCs, sino que sugiere la utilidad de desarrollar terapias que específicamente inhiban el eje TNFα/TNFR1 y no TNFα/TNFR2 para reducir los efectos secundarios causados por las actuales terapias anti-TNFα. Por último, nuestros estudios sugieren que la manipulación del eje TNFα/TNFR2 podría ayudar a los esfuerzos actuales para generar HSCs *de novo* a partir de células iPS, un tema que actualmente carece de respuesta.

CONCLUSIONES

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. El silenciamiento génico dirigido contra Tnfr2 resulta en la inducción de un programa apoptótico dependiente de caspasa-8 en células endoteliales. Esta apoptosis se rescata mediante la eliminación de Tnfr1, lo que indica que se requiere un balance adecuado entre los Tnfrs para la integridad de las células endoteliales y la homeostasis vascular.

2. En las células endoteliales, el Tnfr1 señaliza apoptosis a través de la formación del complejo II y la consecuente activación de caspasa-8 y caspasa-2, mientras que Tnfr2 señaliza supervivencia mediante el comple I y la activación de NF-κB.

3. El programa apoptótico inducido por Tnfr1, en el cual participa caspasa-8, caspasa-2 y P53, se encuentra conservado evolutivamente en células endoteliales humanas.

4. La señalización de Tnfrs es dispensable para la hematopoyesis primitiva en el embrión de pez cebra.

5. La inhibición génica de Tnfa o Tnfr2, pero no Tnfr1 o Lta, resulta en la apoptosis de las HSCs cuando éstas emergen de la aorta dorsal.

6. La señalización Tnfa/Tnfr2 se requiere de forma intrínseca para la supervivencia de las HSCs a partir del endotelio hemogénico.

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