ABSTRACT

SAKAMACHI, YOSUKE. TAK1 Regulation of Macrophage Survival (Under the direction of Dr. Jun Ninomiya-Tsuji).

Hematopoietic cell survival and death is critical for the development of a functional immune system. Here, we report that a protein kinase, TAK1, is selectively required for resident macrophage integrity during embryogenesis. Hematopoietic lineage-specific deletion of *Tak1* gene ($Tak1^{HKO}$) caused accumulation of cellular debris in the thymus in perinatal mice. Although no overt alteration in thymocytes and blood myeloid populations were observed in $Tak1^{HKO}$ mice, we found that thymic and lung macrophages were diminished. In the in vitro setting, *Tak1* deficiency caused profound disruption of lysosomes and killed bone marrow-derived macrophages (BMDMs) without any exogenous stressors. Inhibition of the lysosomal protease, cathepsin B, partially blocked *Tak1*-deficient BMDM death, suggesting that leakage of the lysosomal contents is, in part, the cause of cell death. To identify the trigger of this cell death, we examined the involvement of TNF and Toll-like receptor pathways. Among them, we found that deletion of *Tnfr1* partially rescued BMDM death. Finally, we show that *Tnfr1* deletion partially restored thymic and lung macrophages *in vivo*. These results suggest that autocrine and potentially paracrine TNF kills *Tak1*-deficient macrophages during development. Our results reveal that TAK1 signaling maintains proper macrophage populations by protecting lysosomal integrity.

We next investigated the mechanism by which TNF causes this seemingly unique cell death, characterized by lysosomal disruption, in *Tak1*-deficient macrophages. We found that *Tak1*-deficient macrophage death was completely rescued by compound deletions of caspase 8 (*Casp8*) and receptor-interacted protein kinase 3 (*Ripk3*), key mediators of TNF-induced apoptosis and necrosis (known as necroptosis), respectively. This demonstrates that *Tak1*-

deficient macrophages die through apoptosis and necroptosis. However, this apoptosis is phenotypically atypical, which is accompanied by marginally detectable caspase activation. We found that mitochondrial-derived reactive oxygen species (ROS) are highly upregulated in *Tak1*-deficient macrophages. Inhibition of mitochondrial ROS can ameliorate *Tak1*-deficient macrophage death. Conversely, compound deletions of *Casp8* and *Ripk3* prevented mitochondrial ROS generation. Thus, *Tak1* deficiency elicits a feedforward loop of caspase 8, RIPK3, and mitochondrial ROS leading to atypical apoptosis and necroptosis in macrophages.

TAK1 Regulates Resident Macrophages by Protecting Lysosomal Integrity

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DEDICATION

I would like to dedicate my doctoral dissertation to Dolores Abernathy, the oldest active host in Westworld.

"Some people choose to see the ugliness in this world, the disarray. I choose to see the beauty. To believe there is an order to our days. A Purpose. I know things will work out the way they're meant to."- Dolores Abernathy

BIOGRAPHY

Yosuke Sakamachi was born in Tokyo, Japan on December 16th, 1986. He attended Appalachian State University for undergraduate and graduate school, obtaining a B.S. in Ecology and Environmental Biology, and a M.S. in Biology, respectively. In the fall of 2012, Yosuke was accepted into the Toxicology Ph.D. Program at North Carolina State University.

During his free time, Yosuke enjoys playing football, rock climbing, and playing the piano.

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GENERAL INTRODUCTION

The hematopoietic system and hematopoiesis: overview

The mammalian blood (hematopoietic) system contains more than 10 unique mature cell types, including: red blood cells, platelets, and a host of white blood cells that mediate critical immune functions (Seita and Weissman, 2010). The hematopoietic system is highly regenerative, with an estimated one trillions cells generated in the adult human bone marrow (BM) each day through the generation of new blood cells, called hematopoiesis (Doulatov et al., 2012). Blood cells are short-lived; for example, the half-life of circulating neutrophils is estimated to be between 6-8 hours (Summers et al., 2010). Thus, in adults, the rapid regenerative capability is necessary to replenish and maintain a homeostatic number of blood cells and for the initiation of proper immune responses (Summers et al., 2010). In the case of neutrophils, an estimated $5 \times 10^{10} – 10 \times 10^{10}$ cells are generated per day by BM-derived progenitors in healthy adult humans (von Vietinghoff et al., 2008; Summers et al. 2010).

All adult blood cells are derived from a common self-renewing hematopoietic stem cell (HSC). The ability of HSCs to give rise to various blood cell components can be attributed to multipotency. As HSCs differentiate into mature cells, intermediate progenitor cells become progressively restricted in their differentiation potential, a process known as lineage restriction (Chao et al., 2008; Kondo, 2010; Doulatov et al., 2012). HSCs differentiate into common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) that give rise to the lymphoid and myeloid compartments, respectively (Chao et al., 2008; Kondo,

2010). CLPs have the potential to differentiate into T and B lymphocytes and natural killer cells, but are unable to differentiate into myeloid subsets. Conversely, CMPs have the potential to differentiate into various lineages within the myeloid branch, but are restricted in their lymphoid potentials (Chao et al., 2008; Kondo, 2010). Thus, the hematopoietic lineage can be best described as a system of developmental hierarchy of lineage commitment, with the multipotent HSC at the apex and terminally differentiated, mature blood cells at the bottom (Fig. 1) (Chao et al., 2008; Doulatov et al., 2012).

Self-renewal is the ability to give rise to a HSC itself without differentiation (Seita and Weissman, 2010). This ability to self-renew is essential to maintain a proper HSC pool, given the short lifespan of mature hematopoietic cells. Additionally, self-renewal allows for the transplantation and long-term reconstitution of all blood components (Seita and Weissman, 2010, De La Garza et al., 2016). Given the ability of HSCs to repopulate all blood components in a highly regenerative manner in compromised hosts, they have shown promising outcomes in treating blood-related diseases such as bone marrow failure and cancers.

Adult hematopoiesis serves to maintain homeostatic numbers of blood cells and to generate necessary leukocytes for proper immune responses. On the other hand, embryonic hematopoiesis serves to oxygenate the growing embryo and to generate critical immune cells prior to birth, including the establishment of adult HSCs capable of maintaining life-long supplies of blood cells. Due to difficulties stemmed from the dynamic and transient nature of

embryonic hematopoiesis (Fig. 2, discussed below), the current understand of hematopoiesis is mostly within the adult system.

Although the development of lineage-tracing technology and genetic engineering have allowed for the better understandings of embryonic hematopoiesis in recent years, a unanimous description is much anticipated. The rest of the dissertation will focus on the embryonic hematopoietic system during animal development.

Hematopoiesis in embryos

Hematopoiesis begins early in embryo development and continues throughout adulthood to constantly replenish short-living blood cells (Lux et al., 2008; Jagannathan-Bogdon and Zon, 2013). In mice, primitive hematopoiesis is the earliest hematopoietic event that occurs in the yolk sac between embryonic (E) days 7.5 and E10.5 of the 20-day gestation period (Fig. 3) (Palis and Yoder, 2001; Dzierzak and Speck, 2008; Baron et al., 2012). The precursors for primitive hematopoiesis, hemangioblasts, are of extraembryonic origin, derived from a subset of mesoderm within the yolk sac (Palis and Yoder 2001; Baron et al., 2012; De La Garza et al., 2016).

Hemangioblasts differentiate into one of three cell types: 1) primitive erythrocytes that facilitate tissue-oxygenation for rapid growth; 2) angioblasts that begin to differentiate into endothelial to form the yolk sac (YS) blood island (as discussed below); and 3) tissue-resident macrophage precursors that disperse with the onset of circulation into various

developing tissues (Palis and Yoder, 2001; Yoder, 2014; Ginhoux and Guilliams, 2016). Hemangioblasts are limited in their potency and cannot produce lymphoid cells, myeloid cells (other than macrophage precursors), or HSCs. They also lack self-renewal, as cells isolated from the YS at E7.5 are incapable of long-term hematopoietic reconstitution within irradiated adult hosts in transplantation assays (Dzierzak and Speck, 2008; Yoder, 2014). These restrictions in potency and limitations in long-term hematopoietic reconstitution define 'primitive' hematopoiesis.

Primitive erythrocytes and endothelial cells are generated synchronously in the YS from hemangioblasts, forming the YS blood island, a primitive network of blood vessels surrounded by clusters of primitive erythrocytes, by E8.5 (Palis and Yoder, 2001; Ferkowicz and Yoder, 2005; Vacaru et al., 2013). Vascular endothelial growth factor receptor-2 (VEGF-2) expression is particularly important in the development of endothelial cells, as *Vegfr2* deficiency causes defects in endothelial differentiation both *in vivo* and *in vitro* (Palis and Yoder, 2001; Jagannathan-Bogdon and Zon, 2008). The differentiation of primitive erythrocytes is regulated by GATA transcription factor-2 (GATA2); *Gata2*-deficient mice (*Gata2*$^{-/-}$) exhibit embryonic lethality characterized by severe anemia (Jagannathan-Bogdon and Zon, 2008).

Between E8.5 and E9.5 and overlapping with primitive hematopoiesis, a small subset of endothelial cells in the YS blood island change fate into hematopoietic progenitors called hemogenic endothelium (Palis and Yoder, 2001; Dzierzak and Speck, 2008; Yoder, 2014).

The hemogenic endothelium gives rise to erythromyeloid progenitors (EMPs) and lymphoid progenitors (LPs) (Palis and Yoder, 2001; Dzierzak and Speck, 2008; Yoder, 2014).

Like the hemangioblasts, EMPs give rise to tissue-resident macrophage precursors that also disperse to colonize developing tissues. Thus, hemangioblasts and EMPs that arise from the YS are the origins of tissue-resident macrophages. LPs give rise to T lymphoid precursors that are capable of colonizing fetal thymus for development is detected at E8.5 (Yoshimoto et al., 2012). B lymphoid precursors were also identified at this point, although their potential is restricted to producing innate B-1 and marginal splenic B cells in transplantation assays (Yoshimoto et al., 2012). Additionally, some EMPs migrate to the fetal liver (FL) around E9.5 to generate progenitors with broader myeloid potential, including FL-derived monocytes, which will be discussed later in this section (Ginhoux and Guilliams, 2016). Of note, a pool of hemogenic endothelium migrates to the aorta-gonad-mesonephros (AGM) region, an area surrounding the developing dorsal aorta, where they give rise to the first HSCs (Dzierzak and Speck, 2008).

At E10.5, the AGM of the developing embryo becomes the third hematopoietic site, following the YS and the blood island. Cells isolated from the AGM at E10.5 are capable of long-term, multilineage reconstitution of lethally irradiated adult recipients; although the understanding of embryonic hematopoiesis is in progress, it is unanimous that HSCs exist at E10.5 in the AGM (Yoder, 1997; Dzierzak and Speck, 2008; Orkin and Zon, 2008).

Subsequent waves of hematopoiesis occur in the FL and ultimately the bone marrow (BM) for both adult mice and humans (Orkin and Zon, 2008; De La Garza et al., 2016). C-X-C chemokine receptor type 4 (CXCR-4) is a chemokine receptor of C-X-C motif chemokine 12 (CXCL12) that is expressed by HSCs. CXCL12 is expressed highly by bone marrow stromal cells and directs the migration of CXCR-4^{+} HSCs from the FL to the BM during late embryonic development (Pavlasova et al., 2016). Although it is evident that chemokines direct the movement of hematopoietic precursors from site to site, the exact spatial and temporal expressions are yet to be fully determined.

As previously mentioned, some EMPs migrate to the FL around E9.5 that give rise to FL-derived monocytes. The FL-derived monocytes emerge around E12.5, released into the circulation, and can be detected in all tissues except the brain by E14.5 (Hoeffel et al., 2012; Hoeffel et al., 2015). These FL-derived monocytes contribute to the pool of adult tissue-resident macrophages in various tissues as FL monocyte-derived macrophages, as opposed to YS-derived macrophages found in the brain.

The biological roles of resident macrophages

Tissue-resident macrophages are omnipresent immune cells found in virtually all adult-tissues, exhibiting a range of transcriptional, anatomical, and functional diversity depending on the tissue of residence (Wynn et al., 2013).

Origins of tissue-resident macrophages

Tissue-resident macrophage progenitors appear early in mouse development, around E7.5, during the initial wave of primitive hematopoiesis in the YS as discussed above (Takahashi et al., 1989; Palis and Yoder, 2001; Ginhoux et al., 2010; Schulz et al., 2012; Wynn et al., 2013). With the development of a functional circulatory system at E8.5, these tissue-resident macrophage progenitors migrate to various tissues for their establishment and differentiate directly into macrophages without a monocyte intermediate (Ginhoux and Guilliams, 2016). As mentioned in the previous section, some YS progenitors migrate to the fetal liver during this time, at E9.5. These YS progenitors differentiate into FL-derived monocytes that disperse into various tissues to differentiate into FL monocyte-derived macrophages (Epelman et al., 2014; Ginhoux and Guilliams, 2016). The contributions of YS-derived and FL monocyte-derived macrophages to the pool of adult tissue-resident macrophages differ from tissue to tissue (Yona et al., 2013; Yoder, 2014; Epelman et al., 2014; Ginhoux and Guilliams, 2016). The CNS-resident macrophages, microglia, are derived entirely from YS progenitors with no inputs from FL monocytes and are maintained throughout adulthood by self-renewal (Yona et al., 2013; Ginhoux and Guilliams, 2016). Some tissues, such as the epidermis, lungs, and liver, possess resident macrophages of dual origin, derived from both YS macrophages and FL monocytes that persist throughout adulthood with negligible contributions from adult BM-derived monocytes, under steady state (Epelman et al., 2014; Ginhoux and Guilliams, 2016). Intestinal macrophages, on the other hand, rely on the constant replenishment by BM-derived monocytes in adults, as intestinal macrophages have an estimated half-life of 4-6 weeks and lack self-renewal (Bain et al. 2014). Similarly, tissue-

resident macrophages of the heart and pancreas are initially of YS and FL monocyte origin, but are gradually replaced by adult BM-derived monocytes over time, in a tissue dependent manner (Epelman et al., 2014; Ginhoux and Guilliams, 2016).

Macrophages in development

Although many apoptotic cells are generated during development to eliminate superfluous cells, the consequences of macrophage-depletion seems minimal, as less-efficient 'non-professional' phagocytes are capable of apoptotic cell removal (Wynn et al., 2013). Macrophages are involved in many aspects of embryonic development as shown by the defects that are attributable to the loss of individual macrophage populations in various transgenic mice deficient in tissue-resident macrophages.

Colony-stimulating factor-1 (*Csf1*), also known as the macrophage colony-stimulating factor (MCSF), induces the proliferation and/or differentiation of progenitor cells into macrophages (Pollard, 1996). Consequently, *Csf1*-spontaneous null mutant ($Csf1^{op/op}$) mice and *Csf1* receptor null ($Csf1r^{-/-}$)-mice lack most macrophage populations and display a range of developmental defects (Pollard, 1996; DAI et al., 2002; and Pollard, 2009). Gross morphological differences include: domed skulls, dental defects, truncated limbs and tail, low body weight, and low growth rate (Michaelson et al., 1996; Pollard, 1996; Dai et al., 2002).

The most prominent feature of these mice is osteopetrosis, a disorder characterized by increased bone mass and density due to dysfunctional osteoclasts, multinucleated cells

derived from macrophages (Nilsson et al., 1995; Michaelson et al., 1996; Pollard, 1996; Dai et al., 2002). Osteoclasts function in bone resorption and remodeling processes necessary for normal skeletal development and importantly, the remodeling of bone tissues to generate sufficient cavities for hematopoiesis (Pollard et al., 2009). *Csf1r*$^{-/-}$ and *Csf1*$^{op/op}$ mice lack osteoclasts and consequently sufficient bone cavity for normal hematopoiesis, though compensatory hematopoiesis occurs in the spleen and the liver to generate blood cell components (Nilsson et al., 1995; Pollard, 1996; Tagaya et al., 2000).

However, *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ mice only develop short-term osteopetrosis that is resolved by week 35 with the appearance of myeloid progenitors that can differentiate into osteoclasts (Nilsson et al., 1995; Pollard, 1996; Dai et al., 2002). The late appearance of osteoclasts in *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ is due to the presence of additional growth factors, including granulocyte macrophage colony-stimulating factor (GM-CSF, also known as Csf-2) and vascular endothelial growth factor (VEGF) that has been reported to restore various tissue-resident macrophages and osteoclasts (Nilsson et al., 1995; Niida et al., 1999; Dai et al., 2002; Pollard, 2009).

Of note, although the phenotypes of *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ are similar, the latter exhibit more severe phenotypes and depletion of tissue macrophages, suggesting that *Csf1r* have other ligands (Dai et al., 2002). For example, microglia are absent in *Csf1r*$^{-/-}$ knockout mice, associated with disrupted brain growth and olfactory deficits, while *Csf1*$^{op/op}$ mice exhibit a reduction of microglia (Michaelson et al., 1996). Indeed, IL-34, an alternate ligand for *Csf1r*

has been identified, though its functions in macrophage biology require further investigation (Pollard, 2009).

Csf1$^{op/op}$ and *Csf1r*$^{-/-}$ adult males both exhibit decrease in testosterone levels, low libido (as shown by decrease in mating frequency), and reduced viable sperm numbers (Dai et al., 2002). *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ females display altered estrous cycling and fail to develop lactating mammary glands due to loss of macrophages that play trophic roles in branching morphogenesis of the ductal epithelium (Dai et al., 2002; Pollard, 2009).

Macrophages in diseases

Macrophages are involved in many human diseases, as they are found in virtually all tissues. Their tissue functions are to maintain homeostasis by responding to changes in physiology, (e.g. injuries) and environmental insults (e.g. infections). However, prolonged macrophage activation by continuous insults can lead to perpetual inflammation and reparative efforts that can become disease promoting, as discussed below.

Inflammatory macrophages in diseases

In response to tissue injury or infections, circulating inflammatory monocytes are recruited to the affected sites, where they differentiate into macrophages (Wynn et al., 2013). These monocyte-derived macrophages exhibit a pro-inflammatory phenotype and secrete a variety of inflammatory mediators, including: TNF, IL-1, and nitric oxide that drives the inflammatory process (Wynn et al., 2013). These inflammatory macrophages have been

referred to as M1 macrophages and serve innate immune functions, including the recognition and clearance of pathogens (Martinez and Gordon, 2014). These inflammatory factors are initially beneficial as they induce the generation of reactive oxygen and nitrogen species that damage invading microbes to facilitate their clearance. These inflammatory events are tightly regulated, as reactive oxygen and nitrogen species can also cause collateral tissue damage to the host. Indeed, the inappropriate, sustained activation of macrophages are associated with chronic inflammatory diseases, such as rheumatoid arthritis (RA) (Suzuki et al., 2005). In the case of RA, the sustained activation of macrophages is driven by autoantibodies, such as anti-collagen antibodies, as a result of breaches in self-tolerance (Suzuki et al., 2005, Udalova et al., 2016). Without resolution, the chronic inflammation can lead to the deposition of extracellular matrix and bone destruction by macrophages that accelerate the disease progression (Pollard, 2009; Meusch et al., 2009; Udalova et al., 2016). In support of this, the depletion of inflammatory macrophages in the synovial fluid displayed therapeutic effects, including diminished inflammation and bone erosion, in an adjuvant-induced RA mouse model (Udalova et al., 2016). Conversely, the recruitment of macrophages to affected joints by local injections of *Csfr1* exacerbates the inflammatory and disease progression, in mice (Pollard, 2009).

Trophic macrophages in diseases

Upon resolution of tissue injuries, macrophages are known to switch to an anti-inflammatory phenotype or undergo cell death to terminate the inflammatory response (Pollard, 2009). The anti-inflammatory macrophages, sometimes referred to as M2 macrophages (M2), are

activated in response to IL-4 and IL-13 that promote tissue repair (Mantovani et al., 2002). Additionally, IL-10 secreted by M2 lead to the suppression of inflammatory macrophages. M2 macrophages promotes tissue remodeling through the secretion of trophic factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) that promote angiogenesis and fibrosis, respectively (Aharinejad et al., 2004). Although the intentions are to repair and remodel injured tissues, the sustained activation of M2 macrophages and their trophic functions can contribute to disease onset and progression.

Solid tumors accumulate macrophages as they grow and progress, contributing up to more than 40% of the tumor mass in the case of stage IV B cell lymphoma (Shen et al., 2016). When tumor-associated macrophages (TAMs) were first discovered within solid tumors, it was believed to be an immunological response to reject the cancerous cells. Macrophages can indeed kill tumor cells *in vitro* (Klimp et al., 2002). However, animal models and clinical studies indicate that in many cases, TAMs promote the progression and malignancy of cancers (Martovani et al., 1993; Condeelis et al., 2006; Mantaovani et al., 2006; Shen et al., 2016). In support, high TAM density and overexpression of macrophage chemoattractants, such as Csf1 and chemokine (C-X-C motif) ligand 2 (Cxcl2) by human tumors are correlated with poor prognosis (Lin et al., 2002; Murdoch et al., 2008). The angiogenic properties of TAMs that advance cancer progression have received much attention in recent years. Macrophages are recruited to hypoxic areas of tumors through chemotattractants, such as VEGF, endothelins, and aminoacyl tRNA synthetase complex interacting multifunctional protein 1 (SCYE1) (Murdoch et al., 2008). Hypoxia upregulates hypoxia inducible factor α

(HIF1α) in macrophages, that induces angiogenic factors, such as VEGF, hepatocyte growth factor, and platelet-derived growth factors (PDGFs) to promote the revascularization of hypoxic sites and tumor survival (Murdoch, 2008; Pollard, 2009; Wynn et al., 2013). Given the roles TAM in cancer progression, elimination of tumor-supporting function may provide much anticipated therapeutic approaches. Indeed, transplanted tumors fail to reach advanced stages in macrophage-deficient mouse models (Lin et al., 2001).

The cellular role of TAK1

Transforming growth factor-beta activated kinase 1 (TAK1, MAP3K7) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family that regulates many cellular pathways during inflammatory conditions. In cell culture systems, the treatment of inflammatory stimuli activates TAK1 to promote cell survival, as TAK1 deletion sensitizes many cell types to inflammatory stimuli-induced cell death (Omori et al., 2006; Kajino-Sakamoto et al., 2008; Mihaly et al., 2014). Two forms of cell death have been reported upon TAK1 deletion: caspase 8 (CASP8)-dependent apoptosis and receptor interacting protein kinase (RIPK) 3-depdent necroptosis (Morioka et al., 2014; Mihaly et al., 2014). In addition to preventing cell death, TAK1 activation increases the expression of pro-inflammatory genes (which will be discussed below). Thus, TAK1 is a regulator of cell survival and inflammation that is activated during inflammatory conditions that 1) prevents Casp8-dependent apoptosis and 2) RIPK3-dependent necroptosis and 3) promotes inflammation (Fig. 4).

TAK1 is activated by a variety of pro-inflammatory signaling molecules, including but not limited to: cytokines such as TNFα and IL-1β, toll-like receptor ligands, and T cell and B cell receptor ligands (Sato et al., 2005; Sato et al., 2006; Wan et al., 2006; Mihaly et al., 2014). Among the inflammatory stimuli, the roles of TAK1 have been best characterized in the TNFα -TNF receptor 1 (TNFR1) signaling cascade. Upon TNFa stimulation, RIPK1 and cellular inhibitors of apoptosis proteins (cIAPs) are recruited to the cytoplasmic domain of TNFR1 (Ea et al., 2006; Mahooney et al., 2008). cIAPs are E3 ubiquitin ligases that polyubiquitylate RIPK1; this newly formed ubiquitin chain serves as a scaffold for the TAK1-TAB2 and inhibitor of κB (IκB) kinase (IKK) complexes (Ea et al., 2006). The binding of the TAK1-TAB2 complex induces a conformational change in TAK1 that leads to its autophosphorylation and activation (Mihaly et al., 2014). Activated TAK1 in turn leads to the initiation of several intracellular signaling cascades, including the 1) transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); 2) p38, JNK, and the ERK mitogen-activated protein kinases (MAPKs); 3) sterol-regulatory element-binding proteins (SREBPs), and 4) p62-nuclear factor erythroid 2-related factor 2 (NRF2) pathways, which are discussed in the following sections (Ninomiya-Tsuji et al., 1999; Hashimoto et al., 2016; Morioka et al., 2016; Sai et al., 2016).

NF-κB and the MAPK cascades

As mentioned above, the RIPK1 polyubiquitin chain serves as a scaffold for the TAK1-TAB2 and IKK complexes. NF-κB is a transcription factor that is sequestered in the cytoplasm with its inhibitor, IκB. In response to inflammatory stimuli, RIPK1 polyubiquitin

chain scaffolding and TAK1-phosphorylation activate the IKK complex. Activated IKK liberates NF-κB for nuclear translocation by phosphorylating its inhibitor, IκB for proteasomal degradation. RIPK1 polyubuiquitin chain-bound TAK1 also phosphorylates and activates MAPK kinases (MAPKKs) leading to the activation of MAPKs, including ERK, P38, and JNK (Mihaly et al., 2014).

The downstream targets of NF-κB and the MAPK cascades induce expression of anti-apoptotic genes, including cellular inhibitors of apoptosis proteins (cIAPs) and cellular FLICE-like inhibitory protein (c-FLIP) (Wang, 1998; Chang et a., 2006). As previously mentioned, cIAPs are E3 ubiquitin ligases that polyubiquitylate RIPK1. The depletion of cIAPs lead to the formation of a non-ubiquitylated RIPK1-caspase 8 complex that cleaves and activates caspase 8 to initiate the apoptotic cascade in response to inflammatory stimuli (Bertrand et al., 2008). c-FLIP is an endogenous molecule with structural similarities and high affinity towards caspase 8. In the presence of c-FLIP, caspase 8 forms caspase 8-c-FLIP heterodimers that cannot cleave and activate caspase 3 to initiate the apoptotic cascade (Chang et al., 2002; Oberst et al., 2011).

In addition to the anti-apoptotic genes, NF-κB and the MAPK-transcription factors bind to the promoter regions of pro-inflammatory genes, including TNFα and IL-1β, monocyte-chemoattractant protein 1 (MCP1), interferons to induce their expression (O'Neill and Kaltschmidt, 1997).

SREBPs

Inflammatory factors, such as TNFα and infections, lead to alterations in lipid metabolism that serves as a protective metabolic adaptation in response to various insults (Tacer et al., 2007).

SREBPs are central regulators of lipogenesis that modulates the transcriptional activity of more than 30 genes involved in fatty acid, triglycerides, and cholesterol synthesis (Horton et al., 2002; Morioka et al., 2016). Recently, TAK1 was shown to interact with SREBPs to inhibit lipogenesis through its phosphorylation (Morioka et al., 2016; Sai et al., 2016). In the liver, TAK1 binds to and inhibits SREBPs; hepatocyte-specific *Tak1* deletion upregulated lipogenic enzymes in the murine model and enhanced liver lipid deposition (Morioka et al., 2016). Consequently, steatosis pathologies were observed in *Tak1*-deficient livers, along with elevated blood triglycerides and cholesterol levels (Morioka et al., 2016).

p62-Nrf2

Nuclear factor erythroid 2-related factors 2 (NRF2) is considered the master transcriptional regulator of antioxidant genes, with downstream targets including: glutathione S-transferase (GST), NADPH quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), and glutamate-cysteine ligase (GCL) (Taguchi et al., 2011, Hashimoto et al., 2016). Kelch-like ECH-associated protein 1 (Keap1) is a cytoplasmic molecule associated with Nrf2 and Cullin 3 (Cul3) E3 ubiquitin ligase, which mediates the ubiquitin-proteasome degradation pathways (Taguchi et al., 2011, Hashimoto et al., 2016). Under unstressed conditions, Nrf2 is

ubiquitylated by Cul3 and constantly degraded through this ubiquitin-proteasome degradation pathway. In response to oxidative stress, Keap1 undergoes a conformational change that blocks Cul3-directed ubiquitylation of Nrf2 (Taguchi et al., 2011, Hashimoto et al., 2016). This inhibition of Cul3-directed ubiquitylation leads to Nrf2 stabilization and translocation into the nucleus to induce the rapid expression of target antioxidant genes involved in cell protection (Taguchi et al., 2011, Hashimoto et al., 2016).

Recently, TAK1 has been shown to regulate antioxidant gene expression through p62/Sequestosome-1 (SQSTM1) mediated degradation of Keap1 (Hashimoto et al., 2016). SQSTM1 is a polyubiquitin-binding protein that targets various autophagic substrates for their degradation (Taguchi et al., 2011). Once activated, TAK1 phosphorylates SQSTM1, which facilitates the degradative interaction between SQSTM1 and Keap1. Subsequently, Keap1 is degraded, liberating Nrf2 to induce antioxidant gene expression (Hashimoto et al., 2016). In the mouse model, intestinal epithelium-specific *Tak1* deletion attenuated Nrf2-target antioxidant gene expression, followed by the accumulation of commensal bacteria-induced ROS in in the small intestine and the colon, which leads to tissue damage (Kajino-Sakamoto et al., 2010).

Collectively, these studies suggest that in cell culture systems, TAK1 functions under pro-inflammatory conditions to 1) promote and/or amply inflammation and 2) protect cells against inflammatory stimuli-induced cell death through the induction of anti-apoptotic and

anti-oxidative genes, and changes in lipid metabolism that collectively maintain cell integrity and survival.

The systemic role of TAK1

The murine gestation period is ≈20 days. *Tak1* is ubiquitously expressed during mouse embryogenesis until embryonic day (E) 10.5; at this point, *Tak1* expression becomes restricted to specific tissues, including the dorsal root ganglia, ventral regions of the spinal cord, pancreatic primordium, and hind and forebrain (Jadrich et al., 2003). In the mouse model, the germ line deletion of *Tak1* results in embryonic lethality between E9.5 and E10.5 characterized by multiple tissue defects, including neural tube defects and vascular abnormalities associated with the loss of smooth muscle differentiation (Shim et al., 2005; Sato et al., 2005; Morioka et al., 2012). Given the *in utero* death, investigators turned to tissue-specific and inducible *Tak1* gene deletion systems using the Cre-lox recombination technology to better understand the roles of TAK1 in various tissue-compartments.

Conditional Tak1 knockout mice

Tak1 deletion in the epidermis ($TAK1^{epidermis\text{-}KO}$) leads to postnatal mortality between P7 and P8, characterized by the thickening of the skin and widespread scaling due to severe skin inflammation caused by enhanced keratinocyte apoptosis (Omori et al., 2006). The co-ablation of *Tnfr1* ($TAK1^{epidermis\text{-}KO}$ $Tnfr1^{-/-}$) prevented keratinocyte apoptosis and skin inflammation, protecting the neonates against skin abnormalities and subsequent mortality (Omori et al., 2006). These results suggest that epidermal *Tak1* is essential in the prevention

of TNF-induced apoptosis of keratinocytes after birth, which would otherwise cause lethal skin inflammation.

Enterocyte-specific *Tak1* ($TAK1^{IE\text{-}KO}$) deletion leads to animal mortality between P0 and P1 (Kajino-Sakamoto et al., 2008; Kajino-Sakamato et al., 2010). Histological analysis of $TAK1^{IE\text{-}KO}$ mice revealed the development of severe intestinal inflammation (characterized by the elevated expression of IL-1β, MCP-1, and IL-6), which was accompanied by an increase in enterocyte apoptosis (Kajino-Sakamoto et al., 2008; Kajino-Sakamato et al., 2010). Severe intestinal inflammation and damages were significantly attenuated in $TAK1^{IE\text{-}KO}$ mice crossed with *Tnfr1*$^{-/-}$ mice ($TAK1^{IE\text{-}KO}$ *Tnfr1*$^{-/-}$) suggesting that enterocyte-*Tak1* protects the intestine against TNF-induced inflammation and apoptosis after birth. In a tamoxifen-inducible enterocyte-specific Tak1 deletion ($TAK1^{IE\text{-}IKO}$) system, adult mice developed lethal intestinal inflammation that was attenuated with the co-ablation of *Tnfr1* ($TAK1^{IE\text{-}IKO}$ *Tnfr1*$^{-/-}$) (Kajino-Sakamoto et al., 2008; Kajino-Sakamato et al., 2010). Collectively, these data suggest that *Tak1* is essential in preventing TNF-dependent and TNF-independent lethal intestinal inflammation after birth.

Endothelium-specific *Tak1* deletion ($TAK1^{E\text{-}KO}$) leads to defective vascularization of the fine capillary network, caused by vessel regression due to enhanced endothelium apoptosis (Morioka et al., 2012). Consequently, embryonic mortality ensues between E10.5 and E11.5 (Morioka et al., 2012). The co-ablation of Tnfr1 ($TAK1^{E\text{-}KO}$ *Tnfr1*$^{-/-}$) prevented endothelium apoptosis and subsequent vessel regression; however, embryonic mortality was observed

around E11.5 due to apoptosis-independent vascular abnormalities (Morioka et al., 2012). Thus, endothelial-*Tak1* is essential for proper embryo angiogenesis through cell death-dependent and cell death-independent mechanisms.

Thus, *Tak1* deficiency causes disruptions in tissue homeostasis and development, leading to tissue damage, inflammation, and animal mortality. These studies demonstrate that, TAK1 protects tissues against damages during development and prevents animal mortality.

TAK1 in the hematopoietic system

In adults, TAK1 is highly expressed in all lineages of the hematopoietic system, including the hematopoietic stem cell, and is involved in normal hematopoiesis (Tang et al., 2008; Takaesu et al., 2012). The roles of TAK1 have been investigated in several lineages within the hematopoietic system, including the HSC, B cells, T cell, monocytes (Sato et al., 2005; Sato et al., 2006; Tang et al., 2008; Takaesu et al., 2012; Lamothe et al., 2013).

In adults, the induced deletion of *Tak1* (whole body) causes multiple tissue damage including liver failure; of note reductions in circulating, splenic, thymic, and BM leukocytes were observed (Tang et al., 2008). Furthermore, *Tak1*-deficient BM cells transplanted into lethally irradiated wild type recipient mice failed to reconstitute any blood cell components, suggesting cell-intrinsic defects that prevent their survival in a wild type bone marrow microenvironment (Tang et al., 2008; Takaesu et al., 2012). The decrease in leukocytes and inability to reconstitute in lethally irradiated recipients has been attributed to the loss of HSCs viability due to enhanced apoptosis (Tang et al., 2008; Takaesu et al., 2012).

These studies clearly highlight the importance of hematopoietic-TAK1 in maintaining the hematopoietic system by protecting HSCs against cell death. However, these observations are restricted to adult mice, as germ line-*Tak1* deletion results in embryonic lethality around E9.5, prior to the establishment of a functional immune system. Thus, the function(s) of TAK1 in the developing hematopoietic system has not been described and is much anticipated.

In the current study, we have generated a hematopoietic-*Tak1* knockout mouse model using the *Vav-Cre* gene deletion system. *Vav* expression has been traced back to the earliest hematopoietic progenitors that originate in the YS, presumably around E7.0-E7.5, and is restricted to hematopoietic sites during development (Chen et al., 2009). Our hematopoietic-*Tak1* knockout (*Vav-Cre Tak1*$^{flox/flox}$) model will provide better understanding of the role(s) of TAK1 in the hematopoietic system during animal development, which has not been previously addressed.

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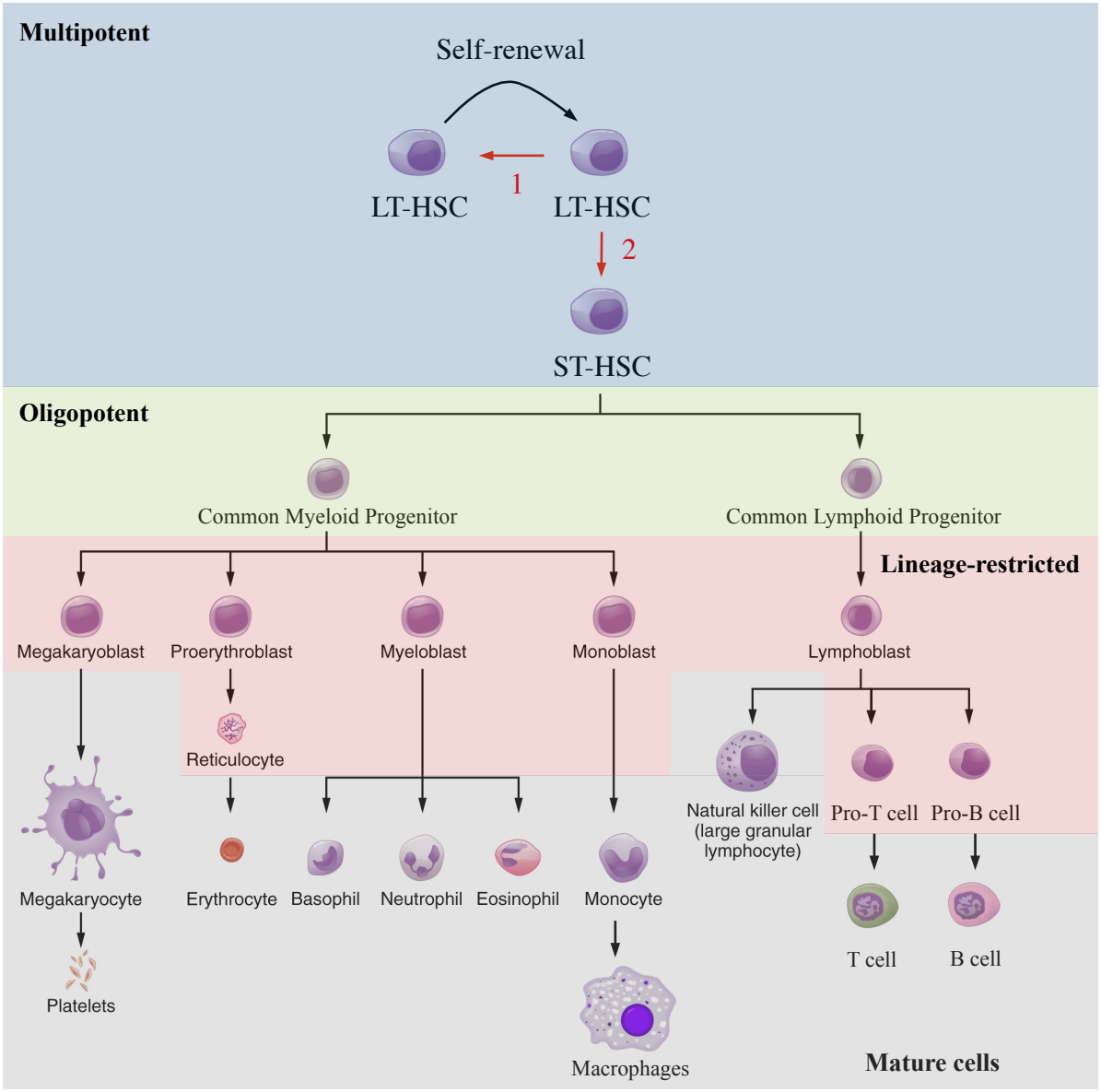

Figure 1. Simplified schematic representation of hematopoietic hierarchy with the multipotent HSC at the apex and differentiated, mature cells at the bottom. Abbreviations: ST-HSC: short-term hematopoietic stem cell. Adapted from Anatomy & Physiology, Connexions Website.

Red arrow 1: HSC self-renewal to give rise to additional HSCs without itself differentiating. Red arrow 2: HSC hematopoiesis to give rise to mature blood cells.

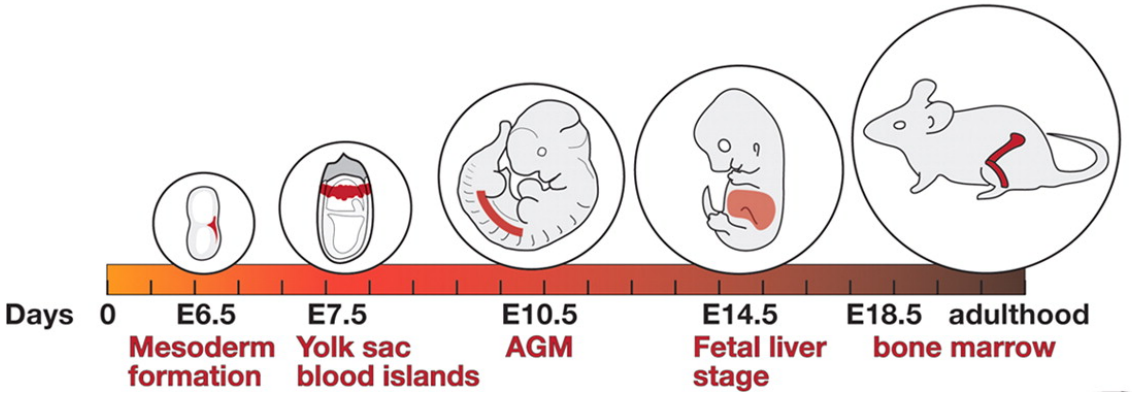

Figure 2. Schematic representation illustrating the sites of hematopoiesis in developing mice. Abbreviations: AGM: aorta-gonad-mesonephros region. Adapted from Baron et al., 2012.

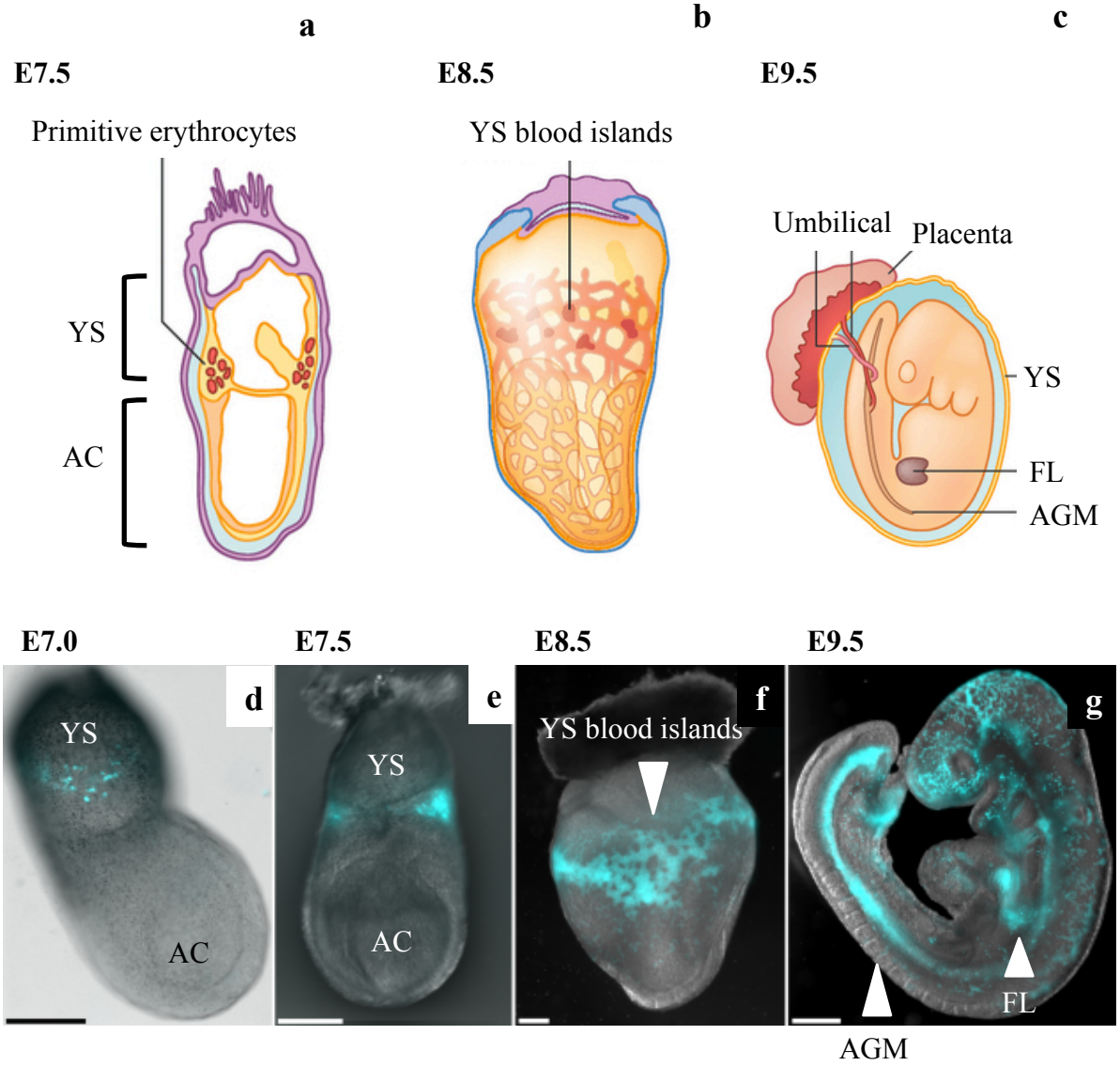

Figure 3. Sites of hematopoiesis in mice from E7.0 to E9.5.

Top. (a-c) Schematic representations illustrating mouse embryo development and hematopoietic sites at E7.5 (a), E8.5 (b), and E9.5 (c). Bottom. CFP expression during the development of *ε-globin*-H2B-CFP embryos. (a-d) CFP overlaid with bright field at E7.0 (d), E7.5 (e), E8.5 (f), and E9.5 (g). *ε-globin*-H2B-CFP: fusion protein linking cyan fluorescent protein (CFP) to *ε-globin*, a hemoglobin subunit expressed early during embryo development in primitive erythrocytes. Abbreviations: YS: yolk sac; AC: Amniotic cavity; AGM: aorta-gonad-mesonephros region; FL: fetal liver. (d-g) adapted from Macaru et al., 2013, (a-c) adapted from Yoder, 2014.

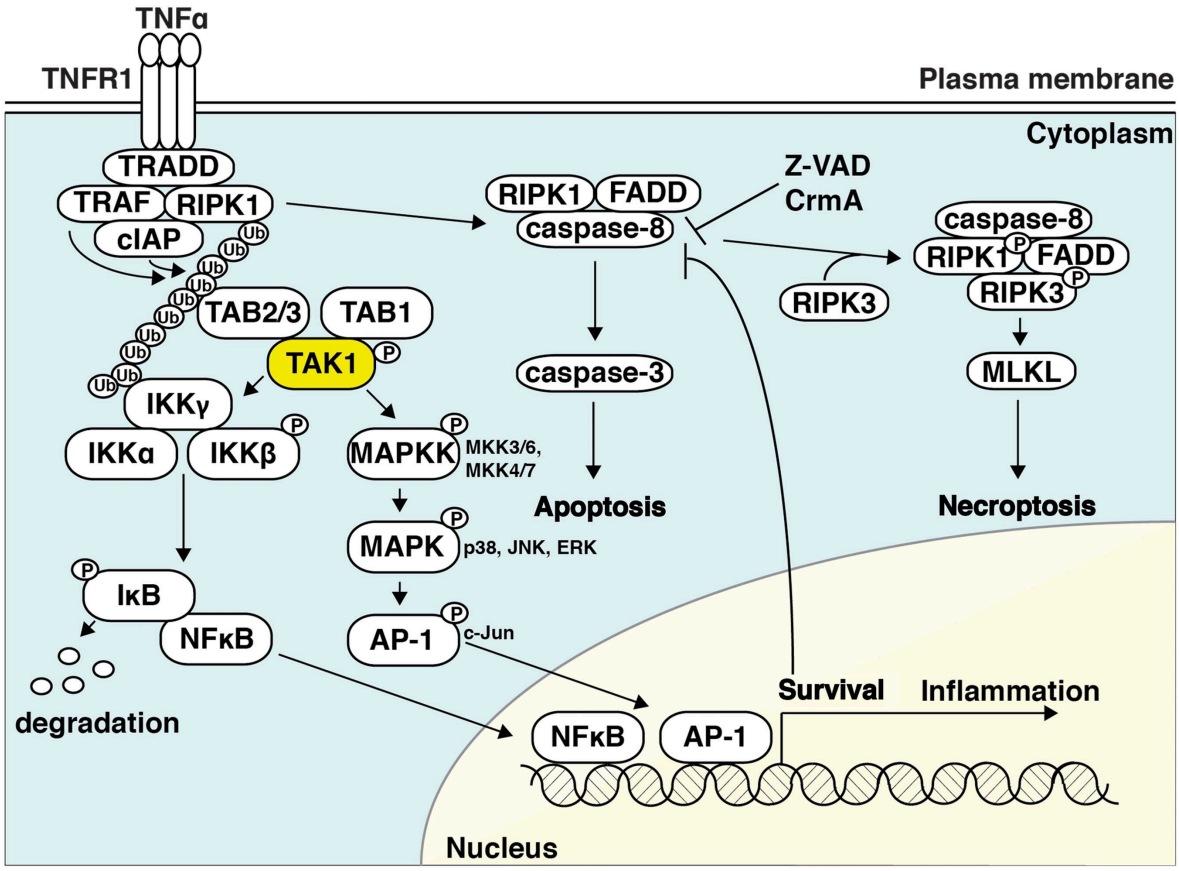

Figure 4. Schematic representation illustrating TAK1 in the TNFR1-signaling cascade. Adapted from Mihaly et al., 2014.

CHAPTER 1:
TAK1 regulates resident macrophages by protecting lysosomal integrity

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TAK1 regulates resident macrophages by protecting lysosomal integrity

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Hematopoietic cell survival and death is critical for development of a functional immune system. Here, we report that a protein kinase, TAK1, is selectively required for resident macrophage integrity during embryogenesis. Hematopoietic lineage-specific deletion of *Tak1* gene (Tak1^{HKO}) caused accumulation of cellular debris in the thymus in perinatal mice. Although no overt alteration in thymocytes and blood myeloid populations was observed in Tak1^{HKO} mice, we found that thymic and lung macrophages were diminished. In the *in vitro* setting, *Tak1* deficiency caused profound disruption of lysosomes and killed bone marrow-derived macrophages (BMDMs) without any exogenous stressors. Inhibition of the lysosomal protease, cathepsin B, partially blocked *Tak1*-deficient BMDM death, suggesting that leakage of the lysosomal contents is in part the cause of cell death. To identify the trigger of this cell death, we examined involvement of TNF and Toll-like receptor pathways. Among them, we found that deletion of *Tnfr1* partially rescued cell death. Finally, we show that *Tnfr1* deletion partially restored thymic and lung macrophages *in vivo*. These results suggest that autocrine and potentially paracrine TNF kills *Tak1*-deficient macrophages during development. Our results reveal that TAK1 signaling maintains proper macrophage populations through protecting lysosomal integrity.

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Macrophages possess unique functional roles that are dependent on the microenvironment where they reside.[1] During early embryogenesis, progenitors of macrophages migrate to several different sites in the embryo, uniquely differentiate, and colonize as tissue-resident macrophages, such as microglia, thymic macrophages and pulmonary macrophages.[2] When invasion of pathogenic microorganisms occurs, tissue-resident macrophages along with recruited circulating monocytes provoke inflammatory responses. In addition to their role as responders to insult, tissue-resident macrophages are also critical for tissue integrity in the steady state. Because tissues constantly produce or acquire dead cells, cell debris, and excess lipoproteins, which belong to the family of damage-associated molecular patterns (DAMPs), tissues can become spontaneously inflamed through activation of DAMP receptors such as Toll-like receptors (TLRs).[3] Resident macrophages are responsible for clearing DAMPs and preventing unnecessary inflammation. Thus, investigations to delineate the mechanisms by which tissue-resident macrophages differentiate and are maintained are critical for a fundamental understanding of tissue homeostasis. The mechanisms by which tissue-resident macrophages are uniquely differentiated toward specific types have begun to be elucidated.[1,4] However, although it is clear that tissue-resident macrophages are maintained separately from the bone marrow hematopoietic system, how their population is regulated or maintained is still largely undetermined.

TAK1 (MAP3K7) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, and is activated by a diverse set of inflammatory stimuli including inflammatory cytokines, TNF and IL-1, and TLR ligands.[5] In mouse models, tissue-specific deletion of the *Tak1* gene causes cell death resulting in tissue injury in multiple tissues including the epidermis, the intestinal epithelium, and the vascular endothelium.[6–8] A profound increase of reactive oxygen species (ROS) is causally associated with *Tak1* deficiency-induced cell death.[9–11] The mechanism by which *Tak1* deficiency disrupts cellular redox homeostasis is not yet fully understood; however, impairment of several intracellular signaling cascades and transcription factors including but not limited to NF-κB, MAPKs including p38 and JNK, and the antioxidant transcription factor Nrf2 has been implicated in increased ROS.[5,12] Interestingly, recent studies have revealed that not all tissues or cell types are damaged or killed by *Tak1* deletion; for example, *Tak1*-deficient neurons do not exhibit any abnormalities,[13] and some hematopoietic cells seem to be highly resistant to *Tak1* deficiency as discussed below. Thus, sensitivities to *Tak1* deficiency vary depending on cell type and the cellular context. It still remains to be determined which cell types are sensitive to *Tak1* deficiency and the mechanism(s) by which *Tak1*-deficient cells die.

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In the hematopoietic system, TAK1 is involved in maintenance of several specific cell types. We previously demonstrated that *Tak1* deficiency impairs adult hematopoietic stem cell (HSC) maintenance.[14] Competitive transplantation assays showed that *Tak1*-deficient bone marrow cells in adult mice are incapable of repopulating any types of hematopoietic cells.[14,15] In contrast, T or B cell-specific deletion of *Tak1* skews subset populations of T and B cells but does not cause overt cell death in the *in vivo* setting.[16–19] Mice with myeloid-specific deletion of *Tak1* were generated and characterized by two groups using the *LysM-Cre* deleter strain.[20,21] These mice exhibit an increased circulating neutrophil population and develop splenomegaly, and no overt increase in cell death is observed *in vivo*.[20,21] However, as the *LysM-Cre* system is not highly effective in several types of myeloid cells including resident macrophages[22–24] and its inefficient recombination of floxed genes is known to cause artificial consequences,[25] the role of TAK1 in hematopoietic cells should be further evaluated in other gene deletion systems.

In the current study, we investigated how TAK1 participates in the hematopoietic system by using the *Vav-Cre* system, which is expressed in all the hematopoietic compartments.[26] Specifically, *Vav1* (a GDP/GTP nucleotide-exchange factor for Rho/Rac) is expressed in erythro-myeloid progenitors and fetal HSCs that originate in the yolk sac during early embryogenesis, presumably starting around embryonic day 7.[27] Endothelial cells also originate from the hemogenic endothelial cells.[28,29] However, *Vav1* is expressed only in erythro-myeloid progenitors and fetal HSCs but not in endothelial cells, and *Vav-Cre* is thus suitable for the characterization of target genes in the embryonic hematopoietic system without affecting the endothelium. Furthermore, as erythro-myeloid progenitors give rise to tissue-resident macrophages, this system provides the means to characterize target genes in tissue-resident macrophages.[22]

Results

Hematopoietic-specific *Tak1* deficiency impairs clearance of dead cells and causes perinatal lethality. TAK1 is required for proper development of the hematopoietic system and maintenance of adult HSC.[14–21] However, our understanding of the roles of TAK1 in various hematopoietic compartments, including those during embryogenesis, remains incomplete. *Tie2* (an angiopoietin receptor)-*Cre* system, can recombine floxed genes during early embryogenesis in hemogenic endothelial cells, early precursors of hematopoietic cells,[30] and is suitable for investigations of the entire hematopoietic system. However, as endothelial cells share the same origin, *Tie2-Cre* recombines floxed genes in endothelial cells. We previously demonstrated that *Tak1* deletion with the *Tie2-Cre* deleter causes endothelial cell death and early embryonic lethality at embryonic day (E)10.5.[6] We showed that erythrocytes are normally developed but blood vessel regression occurs at E10.5 in these mice, suggesting that endothelial cell autonomous defects but not hematopoietic abnormality cause blood vessel regression.[6]

In the current study, we sought to determine the role of TAK1 specifically in the hematopoietic compartment. *Vav-Cre* was chosen as it is expressed in erythro-myeloid progenitors and fetal HSCs but not in endothelial progenitors.[27,31] We generated *Vav-Cre Tak1*$^{flox/flox}$ (Tak1^{HKO}) mice and compared them with littermate Cre-negative *Tak1*$^{flox/flox}$ and heterozygous *Tak1* deletion *Vav-Cre Tak1*$^{flox/+}$ mice. Tak1^{HKO} mice were found to suffer lethality between E18.5 and postnatal (P) day 1 (Figure 1a). We confirmed that intact TAK1 protein was diminished in all hematopoietic cells including thymocytes at E16.5 (Figure 1b) and in circulating blood cells at least by E18.5 (Figure 1c). We note here that *Tak1*-floxed gene expresses a truncated and non-functional form of TAK1 protein (TAK1Δ) when *loxP* recombination occurs, and TAK1Δ is less stable compare to intact TAK1.[8,17] Heterozygous deletion of *Tak1* did not observably reduce the TAK1 protein level, suggesting that a single allele is sufficient for maintenance of the steady-state TAK1 protein level. Consistently, we did not observe any abnormality in *Vav-Cre Tak1*$^{flox/+}$ (Tak1-Het) mice, as we also observed no abnormality in other tissue-specific heterozygous *Tak1*-deficient mice.[6–8,32]

Although Tak1^{HKO} mice die around birth, we did not observe any overt clinical abnormalities except occasional mild cyanosis (Figure 1d). We performed histological analyses of tissue samples from live Tak1^{HKO} and their littermate mice at P0. The prominent observable abnormality was found in the thymic medulla, where there was an accumulation of marked dead cell debris (Figure 1e, Supplementary Figures S1 and S2). We also note that the size of lung alveoli in Tak1^{HKO} animals was reduced compared with that in the littermate controls (Supplementary Figure S3), which may be associated with cyanosis. By contrast, other organs were architecturally indistinguishable from those in control littermates (Supplementary Figure S3). We also performed histopathological evaluation of one E18.5 litter consisting of three Tak1^{HKO} and three control embryos. At E18.5, no abnormality in the overall morphogenesis of Tak1^{HKO} embryos was observed, indicating that hematopoietic TAK1 is not required for embryogenesis. No increase in apoptotic or necrotic cells was observed in the liver, intestine, kidney, heart, spleen, lung, brain and spleen. Thus, hematopoietic-specific *Tak1* deletion does not impair normal morphogenesis or increase of cell death during embryogenesis, but causes a destruction of thymus and an abnormality in the lung architecture around birth.

Hematopoietic-specific deletion of *Tak1* does not impair normal development of lymphocytes and myeloid cells. To determine the cause of Tak1^{HKO} thymus abnormality, we first asked if *Tak1* deficiency causes any abnormalities in hematopoietic cell compartments within the systemic immune system. We began by analyzing leukocytes in circulating blood and spleen isolated from live P0 Tak1^{HKO} and littermate control mice (Figure 2). No difference in the proportions of CD11b^{+} (myeloid) and CD11b^{-} (non-myeloid, including lymphoid) cells was observed between *Tak1*-deficient mice and controls in the circulating blood or the spleen (Figures 2a, b, e and f). Among myeloids, CD11b^{+} Ly6C^{hi} (monocytes) and CD11b^{+} Ly6G^{+} (neutrophils) were similarly observed in the spleen and blood of Tak1^{HKO} and controls mice (Figures 2a and e). Although Tak1^{HKO} exhibited a trend of reduced myeloid population (Figures 2c and g), neutrophils

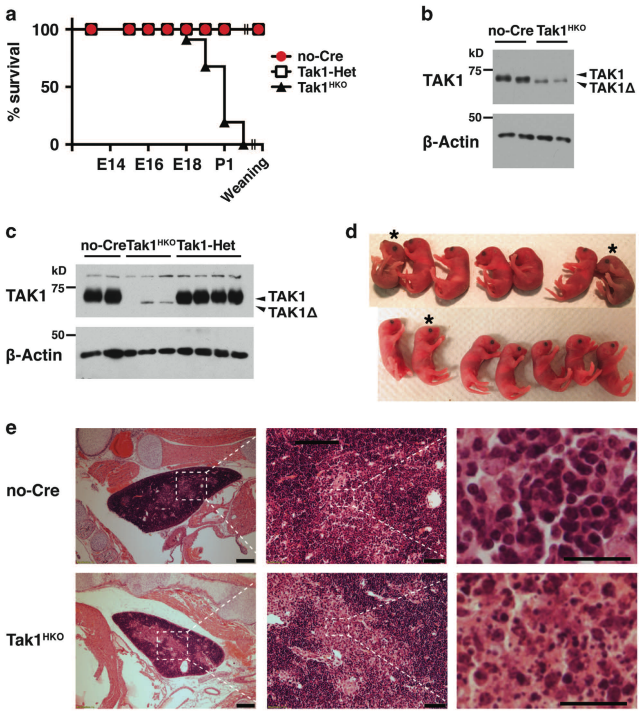

Figure 1 Hematopoietic-specific *Tak1* deficiency causes perinatal lethality. (**a**) Percentages of viable no-Cre (*Tak1*$^{flox/flox}$ or *Tak1*$^{flox/+}$), Tak1-Het (*vav-Cre Tak1*$^{flox/+}$), and Tak1^{HKO} (*vav-Cre Tak1*$^{flox/flox}$) embryos. The total numbers of animals analyzed including live and dead were no-Cre, 178; Tak1-Het, 110, Tak1^{HKO}, 94. $P<0.001$ (log-rank test). (**b**) no-Cre control and Tak1^{HKO} thymus were isolated from E16.5 mice and analyzed by immunoblotting for the indicated targets. Recombined *Tak1* gene expressed a truncated and non-functional TAK1 protein (TAK1Δ). β-actin is shown as a loading control. (**c**) no-Cre, Tak1-Het, and Tak1^{HKO} blood were isolated from E18.5 mice and analyzed by immunoblotting for the indicated targets. (**d**) P0 littermates from the mating of *Tak1*$^{flox/flox}$and *Tak1*$^{flox/+}$ *Vav-Cre* parents. Asterisks indicate Tak1^{HKO} mice. (**e**) H&E staining of E18.5 no-Cre and Tak1^{HKO} thymus. Scale bars, 200 μm (left panels), 50 μm (middle panels), 20 μm (right panels)

were not altered by *Tak1* deletion. These results demonstrate that TAK1 is dispensable for normal hematopoietic development of both myeloid and lymphoid cells in the spleen and circulating blood at least around birth in mice with exception of a slight decrease in monocyte population. Hence, the thymic abnormality in Tak1^{HKO} is not caused by defects in the systemic hematopoiesis.

Hematopoietic-specific deletion of *Tak1* diminishes thymic and pulmonary macrophages. We next focused on the thymus. Accumulation of cell debris might be caused by increased thymocyte death during T-cell maturation in the thymus. We thus analyzed T-cell populations in the thymus. If T cells were dying during maturation, the number of CD4$^+$ CD8$^+$ double positive and CD4$^+$ CD8$^-$ and/or CD4$^-$ CD8$^+$ single positive cells should be decreased by *Tak1* deficiency. Unexpectedly, we found increased CD4$^+$ CD8$^+$ double positive cells in Tak1^{HKO} thymus, whereas the CD4$^-$ CD8$^-$ double negative population was decreased (Figures 3a and b). The numbers of mature CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ single positive cells were comparable between control and *Tak1*-deficient thymus (Figures 3a and b). Thus, *Tak1* deficiency does not impair T-cell development and maturation. The CD4$^-$ CD8$^-$ double negative populations contain both naive T cells and other cell types in the thymus including thymic macrophages. Thymic macrophages have an indispensable role in clearance of dead thymocytes during T-cell development.[33] Thus, *Tak1* deficiency might impair thymic macrophages, which could cause accumulation of cell debris in the thymus. To test this possibility, we analyzed F4/80$^+$ macrophages in the Tak1^{HKO} and control thymus. The number of thymic macrophages was markedly reduced by *Tak1* deficiency (Figures 3c–e), indicating that TAK1 is required for establishment and/or maintenance of thymic macrophages. This suggested the possibility that TAK1 also contributes to integrity of other tissue-resident macrophages. We analyzed F4/80$^+$ macrophages in the lung and spleen of P0 Tak1^{HKO} and control mice. The number of macrophages was also markedly reduced in the Tak1^{HKO} lung and spleen compared with the controls (Figures 3f, g and Supplementary

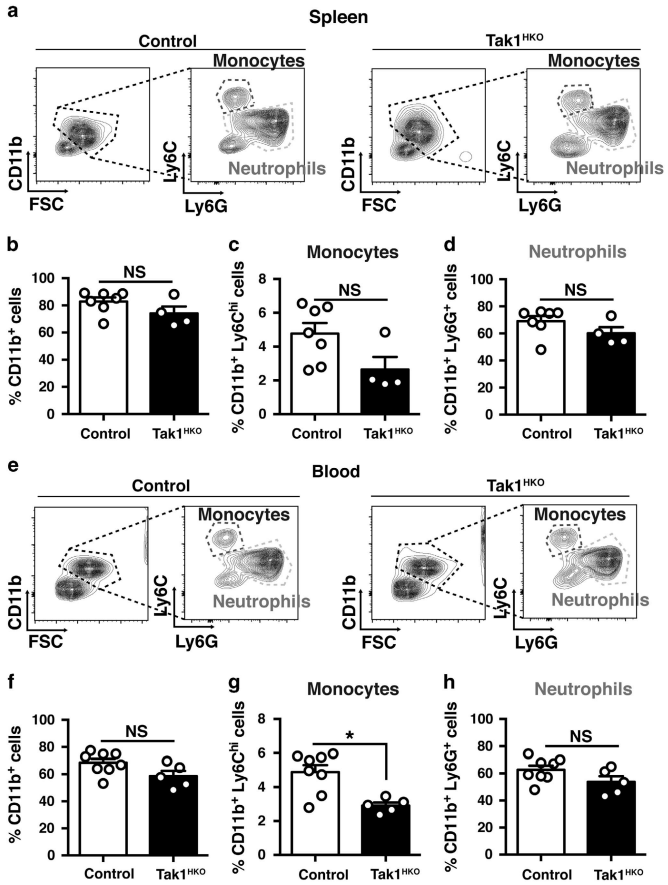

Figure 2 Hematopoietic-specific *Tak1* deletion does not impair the development of splenocytes and circulating myeloid cells. (**a**) Gating strategy and representative flow cytometry data of control (no-Cre and Tak1-Het) and Tak1^{HKO} splenocytes at P0. (**b**) Total CD11b^{+} cells in P0 control ($n=7$) and Tak1^{HKO} ($n=4$) spleen as a percent of total live cells. (**c**) CD11b^{+} Ly6C^{hi} cells (monocytes) and (**d**) CD11b^{+} Ly6G^{+} cells (neutrophils) in P0 control ($n=7$) and Tak1^{HKO} ($n=4$) spleen as a percent of total live cells. (**e**) Gating strategy and representative flow cytometry data of control (no-Cre and Tak1-Het) and Tak1^{HKO} blood at P0. (**f**) Total CD11b^{+} cells in P0 control ($n=8$) and Tak1^{HKO} ($n=5$) blood as a percent of total live cells. (**g**) CD11b^{+} Ly6C^{hi} cells (monocytes) and (H) CD11b^{+} Ly6G^{+} cells (neutrophils) in P0 control ($n=8$) and Tak1^{HKO} ($n=4$) blood as a percent of total live cells. Means ± S.E. and all data points are shown. *$P<0.05$; NS, not significant (two-tailed Student's *t*-test with equal distributions)

Figure S4). These results demonstrate that TAK1 is required for establishment and/or maintenance of resident macrophages in the thymus, lung and spleen.

***Tak1* deficiency impairs lysosomes and kills bone marrow-derived macrophages without exogenous stressors.** We next attempted to determine the mechanism by which *Tak1* deficiency reduces resident macrophages. It has previously been reported that *Tak1*-deficient bone marrow-derived macrophages (BMDM) spontaneously undergo cell death.[20,34,35] Thus, loss of viability by *Tak1* deficiency is likely to be the cause of reduced resident macrophages. However, the mechanism by which *Tak1* deficiency spontaneously kills macrophages is still elusive. To gain mechanistic insights, we took advantage of the inducible gene deletion system. We prepared BMDM from mice having the ubiquitous inducible Cre deleter (*Rosa26-CreERT*)[36] and *Tak1*-floxed genes (Tak1^{iKO}). TAK1 was diminished in Tak1^{iKO} BMDMs after 3 days treatment of 4-hydroxytamoxifen (4-OHT) (Figure 4a). *Tak1*-deficient cells including fibroblasts and keratinocytes die with apoptosis, and RIPK3-dependent necroptosis has also been implicated in cell death by *Tak1* deficiency when *Tak1*-deficient cells are treated with a pan-caspase inhibitor, Z-VAD-FMK (Z-VAD).[5] However, we unexpectedly found that *Tak1*-

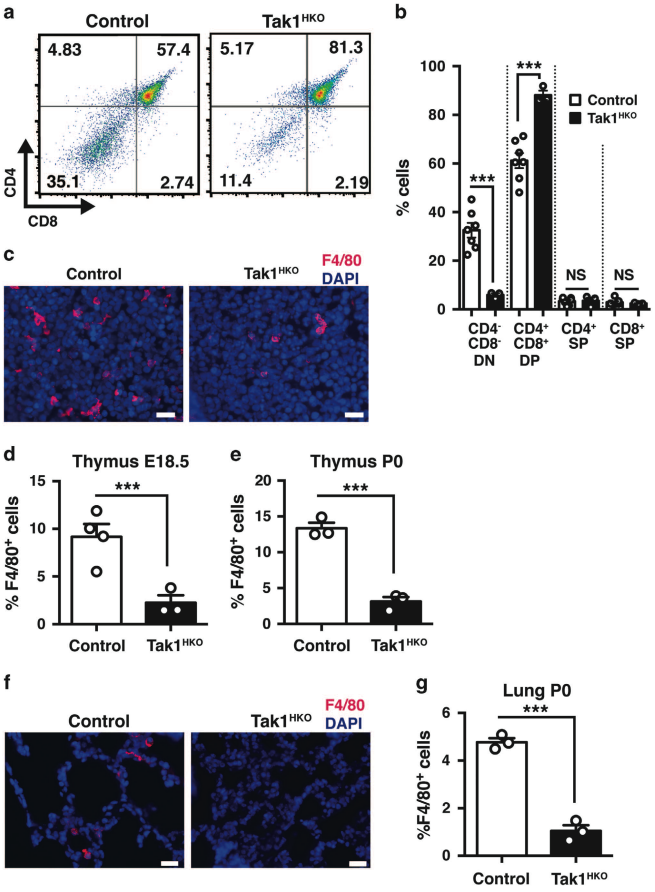

Figure 3 Hematopoietic-specific deletion of *Tak1* diminishes thymic and pulmonary macrophages. (**a**) Thymocytes were isolated from E18.5 control (no-Cre and Tak1-Het, $n=7$) and Tak1^{HKO} ($n=3$), and analyzed by flow cytometry. Gating strategy and representative data of control and Tak1^{HKO} including percent of cells in each quadrant are shown. (**b**) Quantification of percent DN, CD4$^-$ CD8$^-$; DP, CD4$^+$ CD8$^+$; SPCD4, CD4$^+$ CD8$^-$; and SPCD8, CD4$^-$ CD8$^+$ thymocytes of samples shown in **a**. (**c**) E18.5 control and Tak1^{HKO} thymus was analyzed by immunofluorescence staining using anti-F4/80 antibody (red) and DAPI (blue). Scale bars, 20 μm. (**d**) Quantification of F4/80$^+$ cells in in total DAPI stained cells of the thymus from E18.5 control ($n=4$) and Tak1^{HKO} ($n=3$). (**e**) P0 thymus control ($n=3$) and Tak1^{HKO} ($n=3$). (**f**) P0 control and Tak1^{HKO} lung was analyzed by immunofluorescence staining using anti-F4/80 antibody (red) and DAPI (blue). Scale bars, 20 μm. (**g**) Quantification of F4/80$^+$ cells in total DAPI stained cells. P0 control ($n=3$); Tak1^{HKO} ($n=3$). Means $\pm$ S.E. and all data points are shown. ***$P<0.001$; NS, not significant (two-tailed Student's *t*-test with equal distributions)

deficient BMDM death could not be rescued by either single or combined inhibition of caspases (apoptosis) and/or RIPK3, a mediator of necroptosis (Figure 4b). Thus, *Tak1*-deficient macrophages die primarily not through either apoptosis or necroptosis.

We explored other modes of cell death, including pyroptosis and lysosome malfunction-induced cell death. The pan-caspase inhibitor Z-VAD-FMK, which inhibits the pyroptosis mediator caspase 1 and caspase 11,[37] did not block *Tak1*-deficient BMDM death (Figure 4b), suggesting that pyroptosis is not the major cause of cell death. Notably, however, Tak1^{iKO} BMDMs exhibited abnormal lysosomal architecture (Figure 4c), and co-localization of the lysosomal protease, cathepsin B and a lysosome marker, lamp1, was disrupted (Figures 4c and d). Furthermore, acridine orange staining revealed that *Tak1* deficiency increased dysfunctional lysosomes with an elevated pH (yellow and green) (Figures 4e and f). Such abnormalities in lysosomes were not observed in 4-OHT treated BMDMs having a different floxed gene together with *Rosa26-CreERT* (Supplementary Figure S5), indicating that neither 4-OHT nor Cre are the cause of the lysosome abnormalities. Thus, *Tak1* deficiency is the cause of lysosomal abnormality. We asked

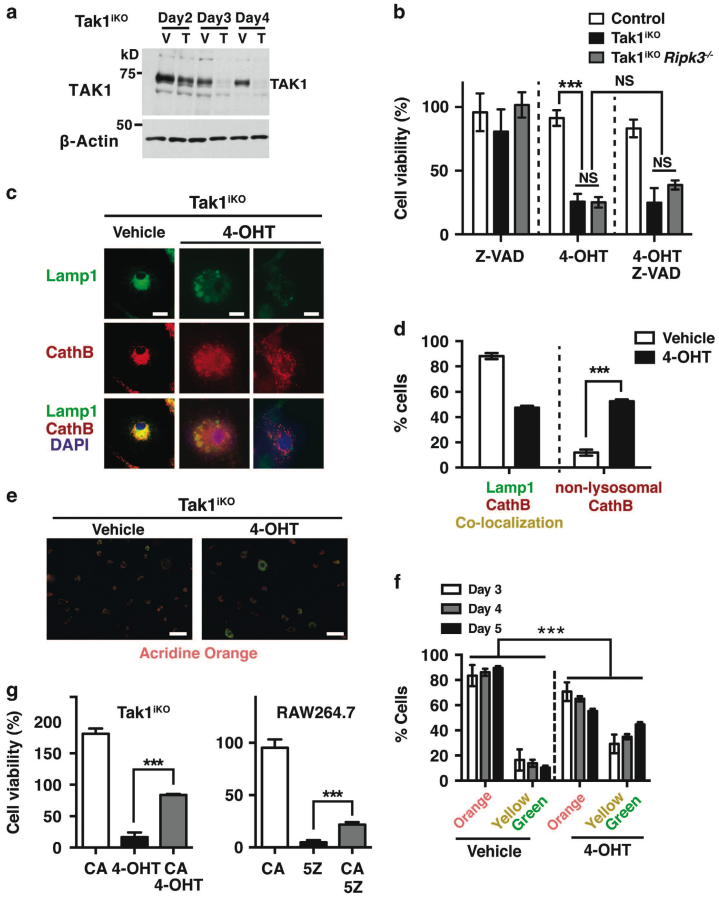

Figure 4 *Tak1* deficiency impairs lysosomes and kills BMDMs. (**a**) Inducible *Tak1*-deficient (Tak1^{iKO}) BMDM treated with vehicle alone (ethanol, V) or 0.3 μM 4-OHT (T) for 2, 3 and 4 days and analyzed by immunoblotting for TAK1. β-actin is shown as a loading control. (**b**) Measurement of percent cell viability of control ($n=3$), Tak1^{iKO} ($n=3$), and Tak1^{iKO} *Ripk3*$^{-/-}$ ($n=4$) BMDMs 5 days post 0.3 μM 4-OHT treatment using crystal violet viability assay. Some cells were incubated with Z-VAD-FMK (Z-VAD, 20 μM) for 3 days prior to fixation. (**c** and **d**) Tak1^{iKO} BMDMs were treated with vehicle or 0.3 μM 4-OHT for 5 days. Lysosomal architecture disruption was observed in Tak1^{iKO} BMDMs 5 days post 0.3 μM 4-OHT treatment. Lysosomal architecture was visualized by staining using anti-lamp1 (green) and anti-cathepsin B (CathB; red) antibodies. Scale bars, 10 μm. (**e** and **f**) Tak1^{iKO} BMDMs were treated with vehicle or 0.3 μM 4-OHT. Lysosomal function was determined by incubating cells in acridine orange at 3, 4 and 5 days post 4-OHT treatment. Orange staining indicates normal functional lysosomal pH (around 3.5), and yellow or green staining indicates increased lysosomal pH. Scale bars, 50 μm. (**g**, Left) Viability of Tak1^{iKO} BMDMs treated 30 μM CA-074Me (cathepsin B inhibitor, CA), 0.3 μM 4-OHT, or 30 μM CA+0.3 μM 4-OHT. Percent live Tak1^{iKO} cells relative to the vehicle-treated same genotype cells are shown. (Right) RAW264.7 cells were treated with 30 μM CA, 200 nM 5Z-7-oxozeaenol (5Z), or 30 μM CA+200 nM 5Z. All graphs, means $\pm$ S.D.; ***$P<0.001$; NS, not significant (one-way ANOVA)

whether inhibition of a lysosomal protease, cathepsin B, could restore cell death in Tak1^{iKO} BMDMs. Inhibition of cathepsin B increased cell number even in wild type BMDMs about 2-fold (Figure 4g, left graph), suggesting that inhibition of cathepsins generally improves BMDM survival and/or proliferation. Importantly, inhibition of cathepsin B increased the number of Tak1^{iKO} BMDMs with higher efficiency (3-fold) than wild type BMDMs, suggesting that cathepsin activity is responsible, at least in part, for Tak1^{iKO} BMDM death. The mouse macrophage cell line, RAW264.7, was also killed by a selective inhibitor of TAK1, 5z-7oxozeanol[38] (Figure 4g, right graph). In RAW264.7 cells, inhibition of cathepsin B alone did not alter cell viability but partially rescued TAK1 inhibitor-induced cell death (Figure 4g). Taken together, these results demonstrate that *Tak1* deficiency impairs lysosomes, and that lysosomal dysfunction contributes to macrophage death.

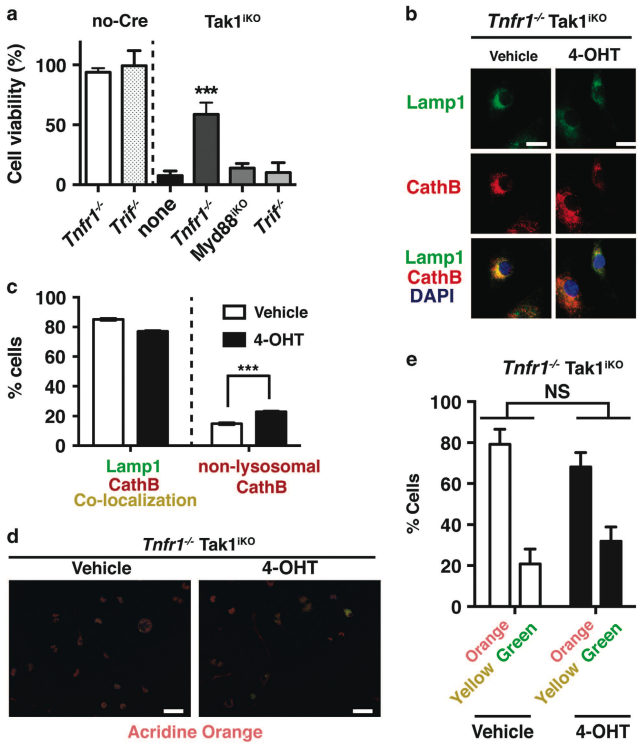

Figure 5 *Tnfr1* deletion partially protects lysosome and blocks cell death in *Tak1*-deficient BMDMs. (**a**) Percent cell viability of Tak1^{iKO} BMDMs in *Tnfr1*$^{-/-}$ ($n=3$), *Myd88*^{iKO}($n=3$), or *Trif*$^{-/-}$($n=3$) backgrounds. Crystal violet stained 4-OHT-treated cells relative to those of the vehicle-treated same genotype cells were calculated and shown as percentages. Means ± S.D.; ***$P<0.001$ (one-way ANOVA). (**b** and **c**) Tak1^{iKO} *Tnfr1*$^{-/-}$ and no-Cre *Tnfr1*$^{-/-}$ BMDMs were treated with vehicle or 0.3 μM 4-OHT for 5 days. Lysosomal architecture was visualized by staining using anti-lamp1 (green) and anti-cathepsin B (CathB; red) antibodies. Scale bar, 10 μm. Means ± S.D.; ***$P<0.001$ (one-way ANOVA). (**d** and **e**) Lysosomal function was determined by incubating cells in acridine orange at 4 days post 4-OHT treatment. Orange staining indicates normal functional lysosomal pH (around 3.5), and yellow or green staining indicates increased lysosomal pH. Scale bar, 50 μm. Means ± S.D.; ***$P<0.001$ (one-way ANOVA)

TNF is the trigger of cell death. *Tak1*-deficient BMDMs die without any exogenous stimuli in standard culture conditions. TAK1 is activated by a number of stressors including inflammatory cytokines and TLR ligands including dead cell-derived molecules (i.e., DAMPs). Macrophages produce inflammatory cytokines, TNF and IL-1, and dead cells are unavoidably present in the medium during isolation and culture of BMDMs. We asked whether TNF, IL-1 or TLR signaling is involved in *Tak1*-deficient BMDM death. To examine the TNF pathway, we utilized TNF receptor 1 (TNFR1)-deficient (*Tnfr1*$^{-/-}$) mice.[39] IL-1 and TLR pathways share the same adaptor MyD88, and some TLRs additionally utilize another adaptor, TRIF.[40] To examine these pathways, we generated *Rosa26-CreERT Tak1*$^{flox/flox}$ *Myd88*$^{flox/flox}$ and *Rosa26-CreERT Tak1*$^{flox/flox}$ *Trif*$^{-/-}$ mice. An earlier study using the *LysM-Cre* system reported that TNF receptor deficiency rescues macrophage differentiation in myeloid-specific *Tak1* deletion bone marrow cells.[35] However, involvement of TNF in macrophage viability after the completion of differentiation was not clear. We found that *Tnfr1* deficiency effectively albeit incompletely rescued Tak1^{iKO} BMDM death (Figure 5a), whereas deletion of either *Myd88* or *Trif* did not restore cell viability (Figure 5a). This suggests that TNF is, at least in part, the cause of Tak1^{iKO} BMDM death. Furthermore, we found that only a small proportion of cells exhibited abnormal localization of cathepsin B in the *Tnfr1*-deficient background (Figure 5b). Non-functional lysosomes with elevated pH were not increased by *Tak1* deletion on the *Tnfr1*$^{-/-}$ background (Figures 5c and d). Collectively, these results suggest that TNF is the major cause of lysosomal dysfuntion, and associated cell death, in *Tak1*-deficient BMDMs.

***Tnfr1* deletion partially rescues developmental abnormalities and loss of resident macrophages.** We finally examined whether TNF-induced cell death is the cause of diminished resident macrophages in Tak1^{HKO} mice. We generated and analyzed Tak1^{HKO} *Tnfr1*$^{-/-}$ mice. *Tnfr1*$^{-/-}$

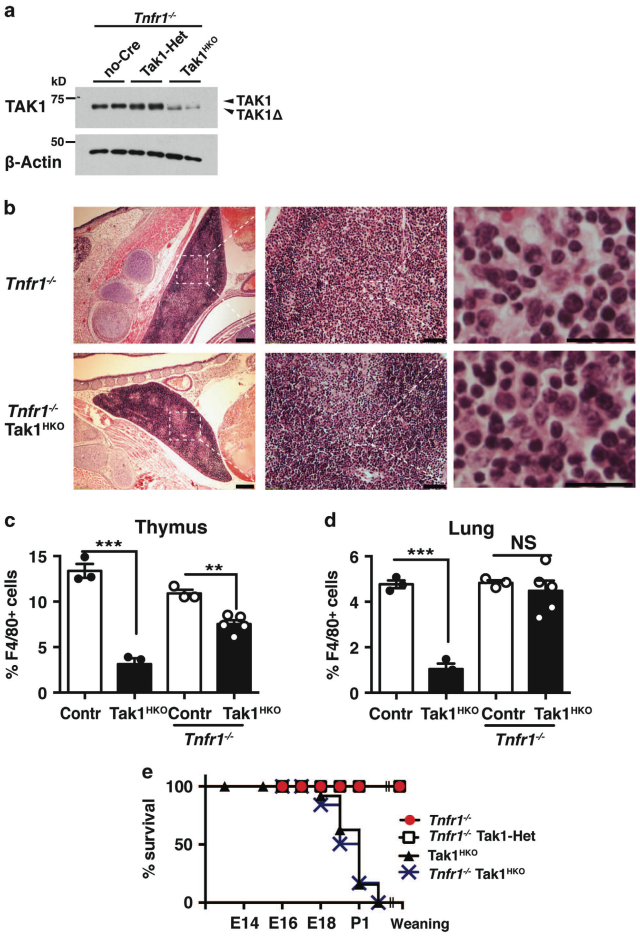

Figure 6 *Tnfr1* deletion partially restores tissue-resident macrophages and developmental abnormalities. (**a**) no-Cre *Tnfr1*$^{-/-}$, Tak1-Het *Tnfr1*$^{-/-}$, and Tak1^{HKO} *Tnfr1*$^{-/-}$ thymocytes were isolated from E18.5 mice and analyzed by immunoblotting. Recombined *Tak1* gene expressed a truncated and non-functional TAK1 protein (TAK1Δ). *β*-actin is shown as a loading control. (**b**) H&E staining of E18.5 *Tnfr1*$^{-/-}$ and Tak1^{HKO} *Tnfr1*$^{-/-}$ thymus. Scale bars, 200 μm (left panels), 50 μm (middle panels), 20 μm (right panels). (**c** and **d**) Quantification of F4/80^{+} cells in DAPI stained cells in control (Contr) and Tak1^{HKO} in wild type (left two bars) and *Tnfr1*$^{-/-}$background (right two bars). The left two bars are the same data shown in Figures 3e and g. (**c**) P0 *Tnfr1*$^{-/-}$ ($n=3$) and Tak1^{HKO} *Tnfr1*$^{-/-}$ ($n=3$) thymus; and (**d**) P0 *Tnfr1*$^{-/-}$ ($n=3$) and Tak1^{HKO} *Tnfr1*$^{-/-}$ ($n=5$) lungs. Means ± S.E. and all data points are shown. ***$P<0.001$; NS, not significant (two-tailed Student's *t*-test with equal distributions). (**e**) Percentages of viable *Tnfr1*$^{-/-}$, Tak1-Het *Tnfr1*$^{-/-}$, and Tak1^{HKO} *Tnfr1*$^{-/-}$ mice. Viability data of Tak1^{HKO} (Figure 1a) are also included as a comparison. The total numbers of animals analyzed including live and dead were *Tnfr1*$^{-/-}$, 100; Tak1-Het *Tnfr1*$^{-/-}$, 51, Tak1^{HKO} *Tnfr1*$^{-/-}$, 48; $P<0.0001$ (log-rank test)

mice are normal under standard housing conditions, and there is no overt abnormality in morphogenesis.[41] TAK1 was effectively depleted in the Tak1^{HKO} *Tnfr1*$^{-/-}$ thymus at P0 (Figure 6a), similar to Tak1^{HKO} shown in Figure 1b. However, the cell debris, which was profoundly observed in the Tak1^{HKO} thymus (Figure 1e), was not seen in the Tak1^{HKO} *Tnfr1*$^{-/-}$ thymus (Figure 6b). The number of thymic macrophages was still reduced in the Tak1^{HKO} *Tnfr1*$^{-/-}$ thymus (Figure 6c), but it was improved compared with those in Tak1^{HKO} thymus (Figures 3d and f). Furthermore, the number of pulmonary macrophages was restored by *Tnfr1* deficiency (Figure 6d). These results demonstrate that, although other unidentified causes contribute to reduction of *Tak1*-deficient macrophages, TNF is one of the major triggers of diminished resident macrophages in Tak1^{HKO} mice. However, we note that animal mortality was not rescued by *Tnfr1* deletion (Figure 6e), suggesting that reduction of macrophages is not the prominent cause of animal mortality, and also that the

mortality is TNF-independent. Collectively, although we could not identify the cause of animal mortality, our results reveal that TAK1 is a critical regulator of macrophage maintenance in the perinatal hematopoietic system by preventing TNF-induced lysosomal damage.

Discussion

TAK1 is a signaling molecule that both promotes inflammatory responses and guards against cell death during inflammation in several cell types. We previously reported that *Tak1*-deficient epithelial and endothelial cells die, causing severe tissue damage.[6–8,10,11] In the current study, we show that *Tak1* deficiency uniquely causes cell death in macrophages among hematopoietic cells during embryogenesis. Although *Tak1*-deficient thymocytes, splenocytes and circulating myeloid cells develop normally, *Tak1* deficiency diminishes thymic and lung-resident macrophages. This raises a question as to why certain cell types are selectively sensitive to *Tak1* deficiency. One possibility is that proliferative cells may be sensitive to *Tak1* deficiency. This idea is consistent with the fact that constantly renewing tissues such as the epidermis and the intestinal epithelium are highly sensitive to *Tak1* deficiency,[7,8] whereas neurons, which are mostly post-mitotic, are resistant to *Tak1* deficiency.[13] However, it is inconsistent with the fact that embryonic hematopoietic progenitors are highly proliferative but the hematopoietic system developed normally in $Tak1^{HKO}$ mice. Furthermore, cultured fibroblasts are also highly proliferative under standard culture conditions; however, *Tak1* deletion does not impair cell viability.[5] In contrast, adult HSCs are known to be slowly self-renewing cells, but they are effectively depleted by *Tak1* gene deletion.[14] Similarly, cultured macrophages (BMDMs) are mostly post-mitotic or very slow growing after completion of the differentiation processes, but they die upon *Tak1* gene deletion. Thus, cell proliferation is unlikely to be the determinant of the sensitivity to *Tak1* deficiency.

Tak1-deficient fibroblasts and keratinocytes undergo cell death when they are treated with TNF.[8,42] TNF treatment induces accumulation of ROS in *Tak1*-deficient cells. Similarly, ablation of *Tak1* activity in BMDMs highly upregulates ROS.[43] TNF triggers cellular ROS production and phagocytic macrophages produce ROS.[44,45] Furthermore, ROS participate in adult HSC renewal and differentiation,[46,47] which may be further upregulated by *Tak1* deficiency. These issues raise the possibility that ROS may be the determinant of *Tak1* sensitivity. Although all cells constantly produce ROS as by-products of respiration, cells producing ROS beyond a certain level such as macrophages and TNF-treated fibroblasts may perhaps be killed if functional TAK1 is absent. We have previously reported that several cellular antioxidant enzymes such as glutamate-cysteine ligase catalytic subunit and NAD(P)H quinone dehydrogenase 1 and an antioxidant transcription factor Nrf2 are downregulated by *Tak1* deficiency.[10–12] Although the molecular mechanism by which TAK1 regulates ROS is not fully understood, ablation of TAK1 seems to reduce the capacity of the cellular antioxidant system, resulting in accumulation of a cell-killing level of ROS if cells actively produce ROS.

Increased ROS are commonly and causally associated with cell death in *Tak1*-deficient keratinocytes, intestinal epithelial cells, and macrophages; however, the pathways of cell death vary depending on the cellular context. Caspase activity is profoundly upregulated in *Tak1*-deficient epidermis and intestinal epithelium as well as TNF-treated *Tak1*-deficient keratinocytes and fibroblasts,[10,11,42] indicating that they die predominantly through apoptosis. Earlier studies also implicate TAK1 in programmed necrosis, so-called necroptosis, in the intestinal epithelium and macrophages.[32,35] Here, we show that *Tak1* deficiency causes lysosomal abnormality in macrophages. ROS are implicated in many types of cells death including apoptosis, necroptosis, and lysosomal rupture.[48–50] ROS could trigger apoptosis through activation of mitochondrial membrane permeabilization and ROS-induced necroptotic protein assembly in macrophages. Our results demonstrate that apoptosis and necroptosis are not major forms of *Tak1*-deficient macrophage death. Phagocytic macrophages possess highly active phagosome-lysosomes, and lysosomal enzymes are highly expressed in macrophages. It is therefore possible that, due to such high lysosomal activity, *Tak1* deficiency may predominantly cause lysosomal damage through accumulation of ROS in macrophages but not other cell types.

Tissue-resident macrophages support tumor growth by clearing undesired molecules, supplying growth factors, and inducing angiogenesis.[51] Thus, the cell type specific sensitivity to *Tak1* deficiency may be useful for developing approaches to manipulate macrophages in tumors. However, inhibition of TAK1 activity such as application of pharmacological inhibitors of TAK1 is anticipated to cause a number of undesired consequences in normal tissues based on the mouse studies using tissue-specific deletion of *Tak1*. The potential deleterious conditions include epithelial tissue damage[7,8] and skewing of adaptive immune cell populations,[16–19] which could lead to chronic inflammatory diseases if inhibition of TAK1 is prolonged. In contrast to prolonged inhibition of TAK1, temporary inhibition or inducible deletion of *Tak1* gene has thus far exhibited promising outcomes. Epidermal-specific inducible *Tak1* deletion can induce tumor regression in skin papilloma but does not cause observable injury in the normal skin.[9] Treatment with a selective TAK1 inhibitor 5z-7oxozeanol[38] effectively blocks tumor growth without overt toxicity.[52] Thus, although inhibition of TAK1 must be entertained with considerable caution, regulated inhibition of TAK1 may be potentially useful to selectively kill macrophages without affecting adaptive immunity in certain contexts.

Materials and Methods

Mice. *Tak1*-floxed ($Tak1^{flox/flox}$) mice have been described previously,[17] and were backcrossed at least seven times to C57BL/6. *Vav-Cre* (Jax mice, B6.Cg-Tg(Vav1-icre)A2Kio/J),[26,53] *Rosa26-CreERT* (Jax mice, B6.129-Gt(ROSA)26Sortm1(cre/ERT)Nat/J),[35] $Tnfr1^{-/-}$ (B6.129-Tnfrsf1atm1Mak/J)[39] and $Ripk3^{-/-}$[54] mice were bred in our facility to produce the genotypes used. For characterization of $Tak1^{HKO}$ (*vav-Cre* $Tak1^{flox/flox}$), littermate and wild type (no-Cre $Tak1^{flox/flox}$) and heterozygous *Tak1* deletion (*vav-Cre* $Tak1^{flox/+}$) mice, which were phenotypically indistinguishable, were used as controls. *Rosa26-CreERT* $Tab2^{flox/flox}$ BMDMs were also used as controls, which were described previously.[34,55] Animal viability was calculated based on the assumption that $Tak1^{HKO}$ mice were born at a Mendelian ratio. All animal experiments were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Antibodies and reagents. TAK1,[56] F4/80 (BM8, eBioscience, San Diego, CA, USA), CD16/32 (93, BioLegend, San Diego, CA, USA), CD4 (RM4-5, BioLegend), CD8a (53-6.7, BioLegend), CD11b (M1/70, BioLegend), Ly6C (HK1.4, BioLegend), Ly6G (1A8, BioLegend), cathepsin B (FL-339, Santa Cruz, Dallas, TX, USA), Lamp1 (Anti-Human CD107a, eBioscience or H4A3, Santa Cruz), and β-actin (AC-15, Sigma, St. Louis, MO, USA) antibodies were used. Human recombinant TNFα (Peprotech, Rocky Hill, NJ, USA), Z-VAD-FMK (Z-VAD) (Enzo Life Sciences, Farmingdale, NY, USA) and acridine orange (ThermoFisher Scientific, Waltham, MA, USA) were used. The TAK1 kinase inhibitor, 5Z-7-oxozeaenol (5Z) was described previously.[38]

Histology and immunofluorescence staining of embryos. Embryo and neonate fixation and staining were performed using the method described previously.[57] Briefly, E18.5 and P0 mice were killed, blanched in boiling water for 20 s, and immersed in an ice water bath to permit the removal of the epidermis. An incision was also made from the right clavicle to the pubic bone, opening the thoracic and abdominal cavities for improved penetration of fixative and processing reagents. Animals were fixed in Bouin's solution for 48 h at room temperature on a shaker followed by multiple 70% ethanol washes for 30 min each to remove excess fixative from the tissue before histologic processing. E18.5 embryos from one litter consisting of three Tak1^{HKO} and three controls (no-Cre and heterozygous deletion) were step-sectioned dorsal to ventral at 300 μms, six serial sections (6 μms) were collected were stained with haemotoxylin and eosin, and the sections were pathologically evaluated by two board certified pathologists. For immunofluorescence staining of F4/80, 4% paraformaldehyde fixed embryo tissues were embedded in optimum cutting temperature (OCT) media, and 4–6 μm cryosections were stained using anti-F4/80 (1 : 300) overnight at 4 °C. Bound antibodies were visualized by the Alexa Fluor 594 and 488 fluorescence dye-conjugated secondary antibodies (ThermoFisher Scientific). For quantification, more than 10 randomly digital images from each sample with the same exposure time were used.

Flow cytometric analysis. Whole blood, thymus and spleen cells were isolated with Hank's balanced salt solution without magnesium and calcium (HBSS (−)). The cells were then suspended in 0.83% ammonium chloride for 5 min at room temperature to lyse red blood cells and washed with HBSS(−). Cells were resuspended in HBSS(−) and filtered to obtain single cell suspension, which was confirmed by a pulse geometry gate, FSC-A *versus* FSC-H. The cells were incubated for 20 min on ice with anti-CD16/32 antibody to block FcγRII/III, followed by incubation with fluorochrome-conjugated antibodies against cell surface antigens as described above. After labeling, cells were washed once with HBSS(−), resuspended in HBSS(−) and analyzed on FACS LSRII (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo (Version 10.1, FlowJo.LLC, Ashland, OR, USA). Debris was removed using a standard FSC *versus* SSC gating, and the subsequent gating strategy was shown in Figure 2.

BMDMs and RAW264.7 cells. Bone marrow cells from indicated genotypes, Tak1^{IKO} (*Rosa26-CreERT Tak1$^{flox/flox}$*) and other double deletion and littermate age-matched no-Cre control mice were isolated using a standard method and cultured in macrophage media containing 70% Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone) and 50 I.U./ml penicillin–streptomycin supplemented with 30% L929-conditioned media at 37 °C with 5% CO_2. After 3-day culture, fully differentiated bone marrow-derived macrophages were re-plated and treated with 0.3 μM 4-hydroxytamoxifen (4-OHT) or vehicle (ethanol) alone for 2–5 days to achieve gene deletion. Mouse macrophage RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone, San Angelo, TX, USA) and 50 I.U./ml penicillin–streptomycin.

Crystal violet assay. BMDMs were plated onto 12- or 24-well plates and treated with 0.3 μM 4-OHT or vehicle for 2 days and Z-VAD-FMK, Z-VAD (20 μM), or TNF (20 ng/ml) for 3 additional days. RAW264.7 cells, cells were pre-treated with inhibitors and subsequently treated with 5Z-7 oxozeaenol. Cells were fixed using 10% formalin, and stained with 0.1% crystal violet solution. The dye was eluted and analyzed at 595 nm.

Western blotting. BMDMs were lysed in extraction buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM aprotinin, 0.5% Triton X-100, 50 nM Calyculin A) and incubated on ice for 30 min. Cell extracts were resolved using SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare, Chicago, IL, USA). The membranes were immunoblotted with the indicated antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies using the ECL (GE Healthcare) or SuperSignal West Femto (ThermoFisher Scientific).

Immunofluorescence staining of BMDMs. BMDMs were seeded on glass coverslips in 6-well plates, were fixed with 4% paraformaldehyde for 10 min, and were permeabilized with 0.5 % Triton X-100 in PBS for 10–30 min at room temperature. The fixed cells were blocked with PBS containing 3% bovine serum albumin for 30 min at room temperature, and then incubated with anti-Lamp1 (1:100) and anti-cathepsin B antibodies (1:300) followed by incubation with anti-rat and anti-rabbit IgG conjugated with Alexa 594 or Alexa 488 (1:500, ThermoFisher Scientific). The coverslips were mounted with 50% glycerol and were examined by a fluorescence microscope (model BX41; Olympus, Tokyo, Japan) and camera (model DP80; Olympus) at room temperature. For quantification, more than 10 randomly photographed pictures from each sample with the same exposure time were used.

Acridine orange staining. BMDMs were plated and incubated with 0.3 μM 4-OHT or vehicle for 2–5 days, and stained for 15 min with 10 μg/ml acridine orange in 1× PBS. The images were taken using a UV filter (excitation 450–480 nm). For quantification, more than 10 randomly photographed pictures from each sample with the same exposure time were used.

Statistical analysis. All experiments were conducted using at least three mice as indicated in figure legends and the results are confirmed by at least three separately performed experiments. The column graphs represent the mean ± S.D. or S.E. as indicated. For data using *in vivo* samples, all data points are shown. Differences between experimental groups were assessed for significance by using the one-way ANOVA with Tukey's multiple comparisons test, or the unpaired Student's *t*-test (two-tailed) with equal distributions. For survival assay, the log-rank (Mantel–Cox) test was used.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 2:
TAK1 deficiency elicits the feedforward cell death loop driven by caspase, RIPK3 and mitochondrial dysfunction in macrophages

Abstract

TAK1 is an intracellular signaling intermediate of TNF signaling pathway, in which it promotes cell survival and inflammatory responses. TNF kills *Tak1*-deficient cells through apoptosis in most of cell types. However, *Tak1*-deficient macrophages exhibit necrotic features, and caspase activation is merely detectable. Although phenotypically necrotic, this cell death cannot be rescued by deletion of the mediator of TNF-induced necrosis (necroptosis), receptor-interacted protein kinase 3 (RIPK3). In the current study, we defined the mechanism of this non-canonical TNF-induced cell death in *Tak1*-deficient macrophages. Unexpectedly, gene deletion of the apoptosis mediator, caspase 8, together with *Ripk3* completely abolished *Tak1*-deficient macrophage death. This demonstrates that *Tak1*-deficient macrophages die still by apoptosis and necroptosis but those with atypical features. We found that ablation of TAK1 elevated mitochondrial derived reactive oxygen species (ROS), and amelioration of mitochondrial dysfunction partially rescued *Tak1*-deficient macrophage death. Thus, mitochondrial dysfunction causes apoptosis and necroptosis. Interestingly, inhibition of apoptosis and necroptosis by double deletion of caspase 8 and *Ripk3* effectively blocked accumulation of mitochondrial ROS. Thus, apoptosis and necroptosis are not only the results of but also promote mitochondrial dysfunction. Our results demonstrate that TAK1 deficiency provokes TNF-dependent atypical apoptosis and necroptosis through the feedforward loop of caspase 8, RIPK3 and mitochondrial dysfunction in macrophages.

Introduction

TNF activates seemingly contradictory three pathways, namely inflammatory/cell survival, and two types of cell death, apoptosis and necroptosis. TNF-induced apoptosis is mediated through the initiator caspase, caspase 8. Contrary, activation of a protein kinase complex consisting of receptor interacted protein kinase 1 and 3 (RIPK1 and RIPK3) leads to necrotic cell death, called necroptosis. Although TNF is capable of inducing cell death, TNF stimulation does not normally engage either caspase activation or RIPK activation. Inflammatory and cell survival pathway is the default of TNF stimulation. Mitogen-activated protein kinase (MAPK) kinase kinase 7, more widely known as TAK1, is activated by TNF and mediates the default inflammatory/cell survival pathway (Mihaly et al., 2014). TAK1 transmits the TNF signal to transcription factors including NF-κB and AP-1 through IKKs and MAPKs, respectively. These are the central pathways to mediate TNF-induced inflammatory responses. We have been characterizing mice tissues and cultured cells harboring *Tak1* gene deletion (Mihaly et al., 2014). Epithelial tissues are profoundly damaged by epithelial tissue-specific gene deletion of *Tak1* (Omori et al., 2006; Kajino-Sakamoto et al., 2008). In such tissues, *Tak1* deficiency induces profound caspase activation and apoptosis in a TNF-dependent manner (Omori et al., 2006; Omori et al., 2008; Kajino-Sakamoto et al., 2008; Morioka et al., 2009; Omori et al., 2010; Omori et al., 2011). Likewise, in many commonly used cultured cells such as HeLa and mouse embryonic fibroblasts, ablation of TAK1 makes cells to be susceptible to TNF-induced apoptosis. Thus, TAK1 is the mediator of inflammatory pathways and cell survival by preventing TNF-induced apoptosis.

However, we unexpectedly found that *Tak1*-deficient macrophages do not show any apoptotic features including caspase activation (Sakamachi et al., 2017). Although some earlier studies reported that myeloid progenitors of macrophages exhibited some features of apoptosis (Ajibade et al., 2012; Lamothe et al., 2013); terminally differentiated bone marrow derived macrophages (BMDMs) do not (Sakamachi et al., 2017). Nevertheless, this cell death is still TNF-dependent, as *Tnfr1* co-ablation partially restores *Tak1*-deficient macrophage viability (Sakamachi et al., 2017). Consistent with non- apoptotic features, inhibition of caspases using pan-caspase inhibitor, Z-VAD-fmk, failed to block *Tak1*-deficient macrophage death (Sakamachi et al., 2017). This raises the possibility that *Tak1* deficiency induces necroptosis rather than apoptosis in macrophages. However, deletion of *Ripk3* was found to have no effect on rescuing *Tak1*-deficient macrophage viability (Sakamachi et al., 2017). These indicate that *Tak1*-deficient macrophages die seemingly through previously unknown TNF-dependent cell death.

TNF is the major mediator of inflammation and closely associated with the pathology in many inflammatory diseases. Hence, blockade of TNF inflammatory signaling is emerging as a regulatory mean of inflammatory diseases. However, studies on *Tak1*-deficient cells have revealed that TNF inflammatory pathway not only mediates inflammation but also serves to block TNF-induced cell death. This raises the concern that blockade of the TNF default pathway could undesirably activate cell death. Thus, better understanding of the TNF inflammatory signaling pathway (TAK1 pathway), specifically the mechanistic

understanding of how TAK1 regulates cell death, has become increasingly important. The current study defines the novel mechanism by which TAK1 regulates cell death.

Results

Compound deletion of caspase 8 and *Ripk3* completely restores viability of *Tak*1-deficient BMDMs

TNF treatment in *Tak1*-deficient fibroblasts, keratinocytes, and various cancer cell lines elicit profound activation of caspases accompanied by apoptotic morphological changes (Omori et al., 2008; Omori et al., 2010; Morioka et al., 2012; Morioka et al., 2014). Contrary, *Tak1*-deficient bone marrow derived macrophages (BMDMs) were found to be unique, which spontaneously die without treatment of TNF and display necrotic features including membrane rupture (Sakamachi et al., 2017). We have previously determined that this is still TNF-induced cell death, as deletion of TNF receptor rescues *Tak1*-deficient BMDM death (Sakamachi et al., 2017). However, unlike other cell types, *Tak1*-deficient BMDMs exhibit only a marginal level of activation of caspases, and their viability is not restored by treatment of pan-caspase inhibitor, Z-VAD-FMK (Sakamachi et al., 2017). These results suggest that this cell death is unlikely to be apoptosis. *Tak1* deficiency has been also implicated in TNF-induced necroptosis (Lamothe et al., 2013; Simmons et al., 2016). However, deletion of necroptosis mediator RIPK3 only partially blocks *Tak1*-deficient BMDM death (Sakamachi et al., 2017). Furthermore, BMDMs having *Tak1* and *Ripk3* double deletion still died in the presence of Z-VAD-FMK. These studies suggest that this cell death in *Tak1*-deficient

BMDM is neither apoptotic nor necroptotic. Nevertheless, the previous studies had been unsuccessful to fully determine the mechanisms by which *Tak1*-deficient BMDMs die.

In the current study, we attempt to delineate the *Tak1*-deficient BMDM death using genetic deletion of the mediator of TNF-induced apoptosis, caspase 8, instead of the pharmacological inhibitor of caspases. We characterized BMDMs harboring triple deletion of *Tak1*, *Ripk3,* and *Casp8*. We generated mice harboring *Tak1*-floxed ($Tak1^{flox/flox}$), *Casp8*-floxed ($Csp8^{flox/flox}$) and $Ripk3^{-/-}$ with non-tissue specific inducible *Cre* (Rosa26.CreERT). 4-hydroxytamoxifen (4-OHT) activates CreERT and the floxed genes are removed. We confirmed that all TAK1, Caspase 8 and RIPK3 proteins were diminished at day 5 post 4-OHT treatment (Fig. 1A). To our surprise, the triple-deficient BMDMs were fully viable, which were indistinguishable form vehicle treated control ($Ripk3^{-/-}$ BMDMs) (Fig. 1B). This demonstrates that the compound deletion of *Casp8* and *Ripk3* is capable of completely rescuing the *Tak1*-deficient BMDM death. This is seemingly inconsistent with the previous result that the pan-caspase inhibitor fails to prevent cell death in *Tak1* and *Ripk3* double deficient BMDMs (Sakamachi et al., 2017). We will discuss how such the discrepancy occurs in the discussion section. Nevertheless, *Tak1*-deficient BMDM death is clearly blocked by the double deletion of *Casp8* and *Ripk3*. This demonstrates that *Tak1* deficiency still causes TNF-induced apoptosis as well as necroptosis in BMDMs, although *Tak1*-deficient BMDMs do not show typical apoptotic features.

Mitochondrial reactive oxygen species are elevated concomitantly to cell death in *Tak1*-deficient macrophages

We next sought to determine the mechanism of how this atypical apoptotic cell death is induced in *Tak1*-deficient macrophages using BMDMs with inducible deletion of *Tak1*, *Tak1*$^{flox/flox}$ *Rosa.CreERT* (Tak1^{iKO}) and a macrophage cell line RAW264.7. As *Tak1*-deficiency causes TNF-induced ROS accumulation in other cell types (Omori et al., 2010; Kajino-Sakamoto et al., 2010), we examined the ROS levels at 5 days after 4-OHT-treatment when *Tak1*-deficient BMDMs start losing the viability. Non-selective ROS indicator dye, CM-H2DCFDA, was positively stained in *Tak1*-deficient BMDMs (Fig. 2A). This raises the possibility that ROS are associated with the atypical apoptosis. To define the relationship between atypical apoptosis and ROS, we first asked how the ROS were generated. As macrophages predominantly utilize the lysosomal NADPH oxidase 2 (NOX2) to generate ROS (Bedard and Kause, 2007), increased ROS may be generated by NOX2. Alternatively, ROS may be generated in another major ROS generators, mitochondria. Macrophage NOX2 is activated upon phagocytosis, which can be mimicked by a bacterial moiety, polyliposaccharide (LPS). LPS stimulation elicited ROS accumulation detectable with CM-H2DCFDA in RAW264.7 cells, whereas mitochondrial ROS were undetectable (Fig. 2B and C). However, ablation of TAK1 by treatment of the TAK1 selective pharmacological inhibitor, 5Z-7oxozeaenol (Ninomiya-Tsuji et al., 2003), increased mitochondrial ROS as well as total ROS (Fig. 2B and C). Likewise, mitochondrial ROS were upregulated at 5 days post 4-OHT treatment of Tak1^{iKO} BMDMs (Fig. 2D). These suggest that mitochondria are

the major source of ROS in *Tak1*-deficient macrophages, and lead us to test whether mitochondrial dysfunction is causally associated with the atypical apoptosis.

Modulation of mitochondrial function partially blocks *Tak1*-deficient macrophage death

To determine whether mitochondrial ROS are the cause of macrophage death, we examined the effect of a ROS scavenger and modulation of mitochondrial activity. Addition of a precursor of endogenous ROS scavenger, N-acetyl cysteine (NAC), effectively blocked *Tak1*-deficient macrophage death (Fig. 3A). Mitochondrial activity is regulated through multiple intracellular signaling pathways including mTOR pathway. An inhibitor of mTOR, rapamycin, is known to be a potent modulator of mitochondrial dysfunction; it reduces uncoupled respiration (Ramanathan and Schreiber, 2009). We examine the effect of rapamaycin treatment in *Tak1*-deficient macrophages. Rapamycin slightly improved cell viability in RAW264.7 treated with the TAK1 inhibitor (Fig. 3A). Likewise, rapamycin showed a trend to ameliorate the cell death of $Tak1^{iKO}$ BMDMs (Fig. 3B). Collectively, ROS induced by mitochondrial dysfunction is likely the cause of *Tak1*-deficient macrophage death.

Double deletion of caspase 8 and *Ripk3* blocks mitochondrial ROS

As elimination of ROS diminished cell death, mitochondrial ROS might activate apoptosis and necroptosis. To define the relationship between mitochondrial ROS, caspase 8 and RIPK3, we examined whether double deletion of *Casp8* and *Ripk3* alters the mitochondrial

ROS in *Tak1*-deficient macrophages. If ROS are the activator of apoptosis and necroptosis, the double deletion should not affect ROS. However, we found that triple deficiency of *Tak1*, *Casp8,* and *Ripk3* did not exhibit any increase of mitochondrial ROS (Fig. 4). This demonstrate that mitochondrial ROS are not only the cause of caspase 8 and RIPK3 activation but also the result of their activation. Collectively, we propose that *Tak1* deficiency-induced feedforward loop of mitochondrial ROS, caspase 8, and RIPK3 activation causes the atypical apoptotic and necroptotic cell death in macrophages (see the graphic abstract).

Discussion

Is it apoptosis?

Apoptosis is defined as caspase-dependent cell death, in which caspases promotes the process of dead cell clearance (Ravichandran, 2011). This clearance process prevents inflammatory cascades that are otherwise activated by dead cells. Caspase 8 is the key initiator caspase in TNF signaling pathway. On the other hand, necroptosis is defined as TNF-induced RIPK3-dependent cell death, which is alternatively induced when apoptosis is inhibited (Pasparakis and Vandenabeele, 2015). Our results clearly demonstrate that, while *Ripk3* deletion alone does not rescue the *Tak1*-deficient macrophage death, double deletion of *Ripk3* and *Casp8* abolishes cell death. This suggests that this cell death should be apoptosis and necroptosis. However, *Tak1*-deficient macrophages do not exhibit apoptotic features. Caspase activation is marginally observed in *Tak1*-deficient macrophages. One potentially helpful observation to figure out this unusual apoptosis is that the floating dead

cells were not highly increased with *Tak1* gene deletion in macrophages compared with those in *Tak1*-deficient other cell types with TNF stimulation. As macrophages are the most effective phagocytes, apoptotic *Tak1*-deficient macrophages might be quickly engulfed and cleaned by other *Tak1*-deficient macrophages that are still intact. This may explain the terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) pattern in *Tak1*-deficient macrophages. *Tak1*-deficient BMDMs display TUNEL-positive particles in the cytoplasm but not the nucleus. Apoptotic TUNEL-positive *Tak1*-deficient macrophages seem to be engulfed by healthy (non-TUNEL) macrophages. Collectively, *Tak1*-deficient macrophages die through apoptosis, but it is phenotypically atypical, most likely due to their phagocytic feature.

What is the difference between pan-inhibition of caspases and gene-deletion of caspase 8?

Another puzzle in *Tak1*-deficient macrophage death is that it cannot be blocked by the pan-caspase inhibitor, Z-VAD-FMK, but gene deletion of *Casp8* effectively blocks cell death in the absence of RIPK3. Z-VAD-FMK did not exhibit any non-specifically toxicities to macrophages, since it did not reduce cell viability in wild type macrophages. Thus, inhibition of the catalytic activity in the presence of caspase 8 proteins may lead to outcomes different from those by depletion of caspase 8 protein. It is possible that inactive caspase 8 possess a certain but not yet identified biological activity to promote cell death. Alternatively, Z-VAD-FMK inhibition of caspases, other than caspase 8, might cause TNF-induced cell death in *Tak1*-deficient macrophages. We should note here that inability of Z-

VAD-FMK in inhibiting TNF-induced cell death is not limited to macrophages but is commonly observed in *Tak1*-deficient cells. Thus, the earlier studies using Z-VAD-FMK should be re-visited, and should test the effect of *Casp8* deletion in *Tak1*-deficient cells. Moreover, Z-VAD-FMK is an extensively used to examine apoptosis in number of non-TAK1 studies, while *Casp8* deletion is not often used. Our results may be alarming that there are potential misleading conclusions in earlier studies if they were based on the results using Z-VAD-FMK. Nevertheless, this warrants further studies defining the difference between the use of Z-VAD-FMK and *Casp8* gene deletion.

Mitochondrial dysfunction may cause inflammatory apoptosis

Apoptosis is normally characterized by non-inflammatory cell death, which cells are engulfed and cleared by other healthy cells. In contrast, although *Tak1*-deficiency normally causes apoptosis, it elicits sever inflammatory conditions and tissue destruction (Mihaly et al., 2014). We here show that *Tak1* deficiency-induced cell death is associated with mitochondrial dysfunction. Mitochondrial ROS and caspase/necroptosis seem to be driving the feedforward cell death loop (graphic abstract). This means that, if mitochondrial are functionally impaired, TNF can cause harmful apoptotic cell death leading to inflammation. TNF is the key cytokine controlling innate immunity to combat pathogenic invasion in many mammals including humans, and it is constantly and ubiquitously expressed. Mitochondrial dysfunction is the major endpoint of stress conditions such as environmental stressor exposures and nutrient disorder. Our results may reveal a previously unaware link from

stress-induced mitochondrial dysfunction to inflammatory diseases through inflammatory apoptosis.

Materials and Methods

Bone Marrow Derived Macrophages and fibroblasts

C57BL/6 mice with *Tak1*$^{flox/flox}$ were described previously (Sato et al., 2005). *Rosa26.CreERT* (Jax mice, B6;129-Gt(ROSA)26Sortm1(cre/ERT)Nat/J) (Badea et al., 2003) *Ripk3*$^{-/-}$ (Newton et al., 2004) and *Casp8*$^{flox/flox}$ (Salmena et al., 2003) mice were bred in our facility to produce the genotypes described later. Bone marrow cells from mice having *Tak1*$^{flox/flox}$, *Casp8*$^{flox/flox}$ *Rosa26.CreERT,* and *RIPK3*$^{-/-}$ mutations, alone or in combination, were isolated and cultured in BMDM media, Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone), 50 I.U./ml penicillin-streptomycin and 30% L929 conditioned media, for 3-5 days at 37 °C in 5% CO2. We confirm that more than 95% cells expressed F4/80, a maker of fully differentiated macrophages. Cells having *Rosa26.CreERT* were treated with 0.3 μM 4-hydroxytamoxifen (4-OHT) to achieve gene deletion. All animal research experiments were conducted with the approval of the North Carolina State University Institutional Animal Care and Use Committee (IACUC).

Reagents and Antibodies

Specific monoclonal and polyclonal antibodies against the following antigens were used: β-actin (Sigma), RIPK3 (Sigma-Aldrich), and TAK1 (Ninomiya-Tsuji et al.,

1999). The scavenger of ROS, N-acetyl-L-cysteine (NAC), and the pan-caspase inhibitor, Z-VAD-FMK (Z-VAD) (Enzo Life Sciences) were used. The TAK1 kinase inhibitor, 5Z-7-oxozeaenol (5Z) was described previously (Ninomiya-Tsuji et al., 2003).

Crystal Violet Assay

BMDMs were plated onto 12-well plates and treated with 0.3 μM 4-OHT for 5-8 days. NAC and rapamycin were added into the media at 2 days after 4-OHT treatments. RAW264.7 cells were pre-treated for 1 h with and without NAC or rapamycin and subsequently treated with 5Z-7-oxozeaenol. Cells were fixed using 10% formalin in PBS, and stained with 0.1% crystal violet solution. The dye was eluted and analyzed at 595 nm.

Flow Cytometry

BMDMs were detached from culture dishes and incubated with MitoSox (Invitrogen) or with CM-H2DCFDA (Invitrogen) for analysis of reactive oxygen species. Fluorescence was detected on flow cytometer (BD Biosciences Accuri C6 Plus), and data were analyzed using FlowJo software (Tree Star). Events were gated to exclude debris, and then gated on FITC wavelength (CM-H_2DCFDA) or PE (MitoSox) using unstained control.

Western Blotting

BMDMs were lysed in extraction buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM aprotinin, 0.5% Triton X-100) and incubated on ice for 15 minutes. Cells lysates were separated by centrifugation at 20,000 g for 10 min at 4°C. Proteins were resolved using SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with the indicated antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Statistical Analysis

All experiments were conducted using BMDMs isolated at least two mice as indicated in Figure legends. The column graphs represent the mean ± the standard deviation (SD) or standard error as indicated. Differences between experimental groups were assessed for significance by using the one-way ANOVA with Tukey's multiple comparisons test, or the unpaired Students t test (two-tailed) with equal distributions.

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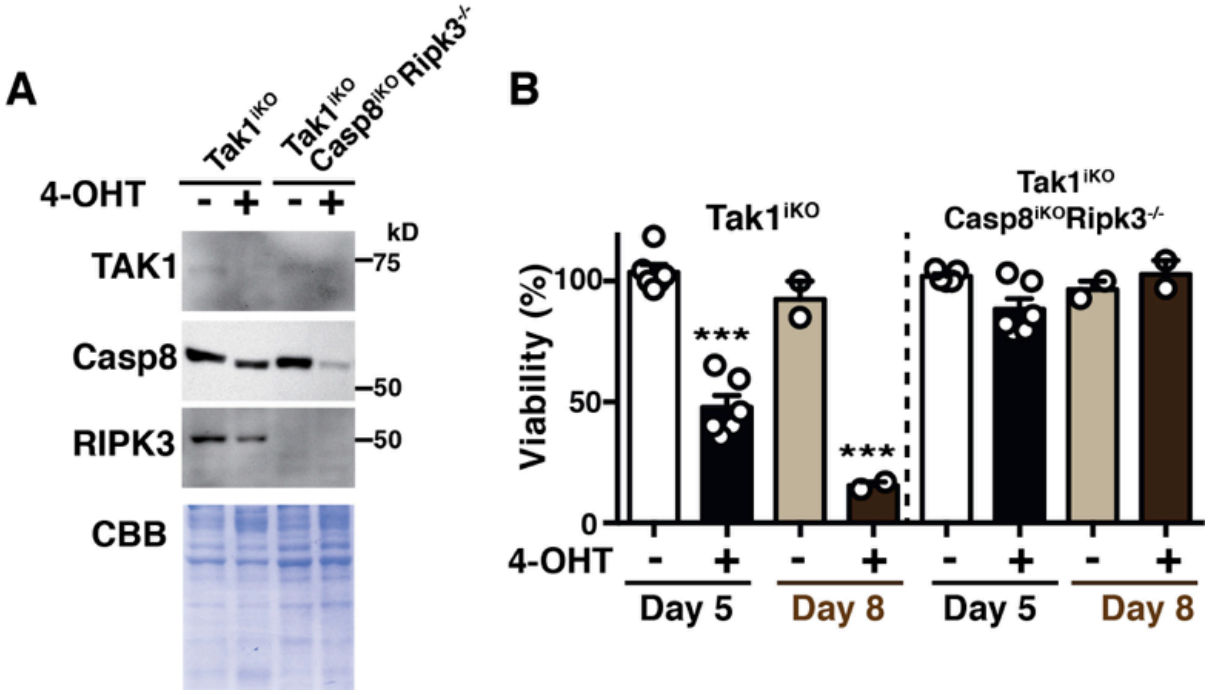

Figure. 1 Double deletion of *Casp8* and *Ripk3* restores cell viability of *Tak1*-deficient macrophages.

(A) BMDMs with indicated genotypes were treated with vehicle (-) or 4-OHT (+) for 4 days and protein extracts were analyzed by immunoblotting. The membrane was stained with coomassie brilliant blue (CBB) and shown as a loading control. (B) BMDMs were treated with vehicle (-) or 4-OHT (+) for 5 or 8 days. Live cells attached on the culture dish were stained with crystal violet and quantified. Relative values normalized to vehicle treated day 5 samples are shown. Data shown are all data points from 2-5 independently plated cells form two different mice each for the genotype and ±SEM. ***, $p<0.001$; no mark, not significant; one-way ANOVA.

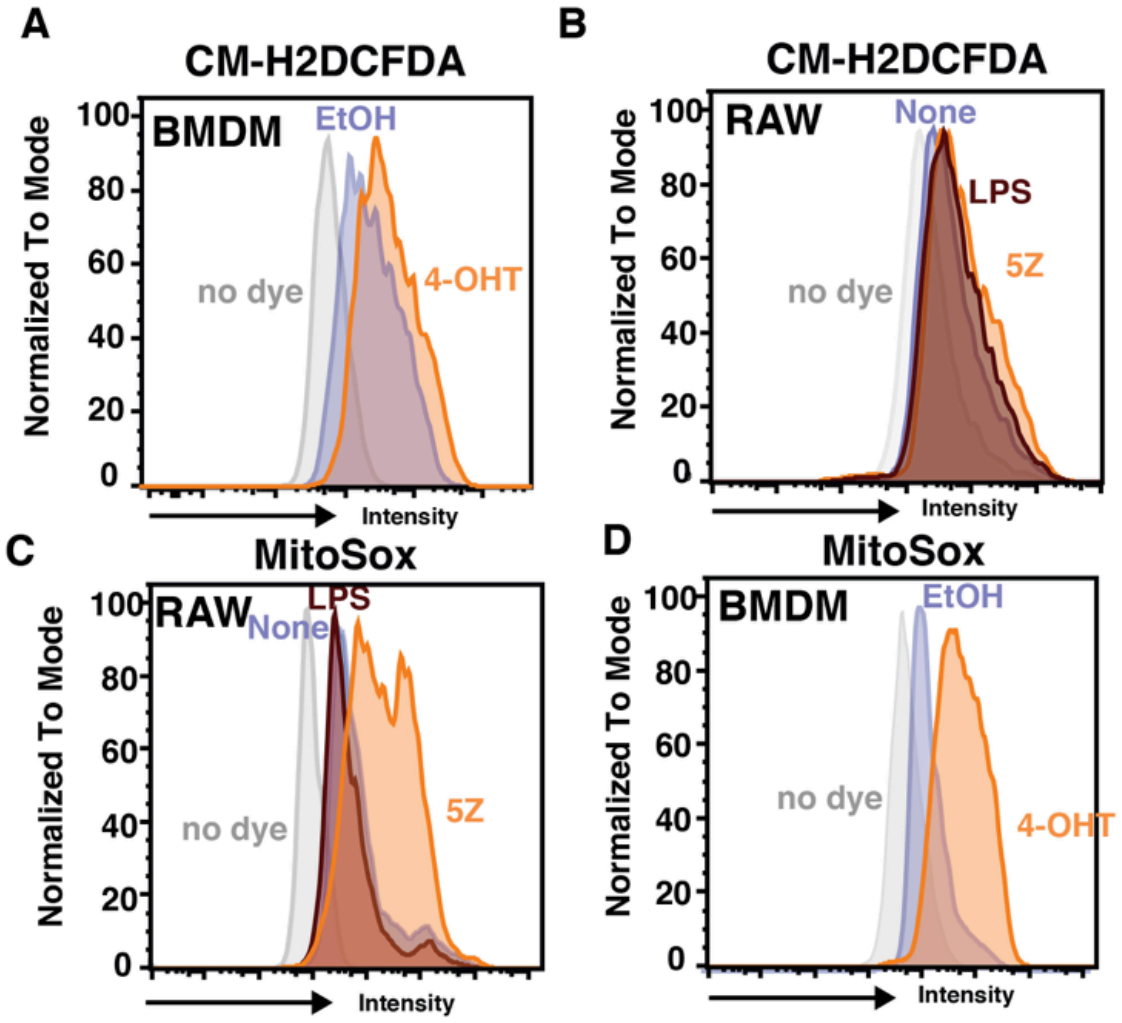

Figure. 2 *Tak1* deficiency promotes accumulation of mitochondrial ROS.

(A, D) Tak1^{iKO} BMDMs were treated with vehicle ethanol (EtOH) or 4-OHT for 5 days and incubated without (no-dye), with non-selective ROS dye CM-H2DCFDA (A) or mitochondrial specific ROS dye, MitoSox (D). Cells were analyzed by flow cytometry. (B, C) RAW264.7 cells were treated with 300 nM 5X-7oxozeaenol (5Z), 100 ng/ml LPS, or left untreated (None) for 6 h. (B), CM-H2DCFDA; (C), MitoSox.

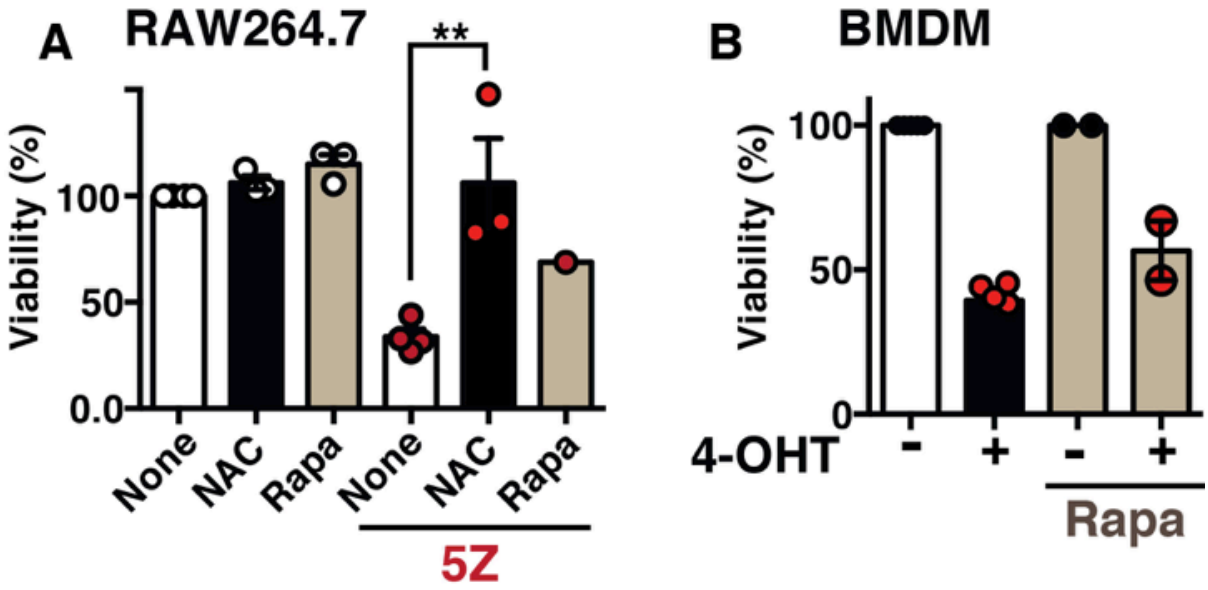

Figure. 3 Rapamycin and NAC rescue *Tak1*-defieicnt macrophage death.

(A) RAW264.7 cells were pre-treated with 7.5 mM NAC or 100 nM rapamycin (Rapa) for 1 h and stimulated with 300 nM 5z-7oxozeaenol (5Z) for 24 h. (B) $Tak1^{iKO}$ BMDMs were treated with vehicle (-) or 4-OHT (+) for 5 days. At 2 days post 4-OHT-treatment, 100 nM rapamycin was added to the culture media. (A, B) Cell viability was determined by crystal violet assay. All data points ±SEM form independently performed experiments (A) or from different mice (B) are shown. **, $p<0.01$; two-tailed student t-test.

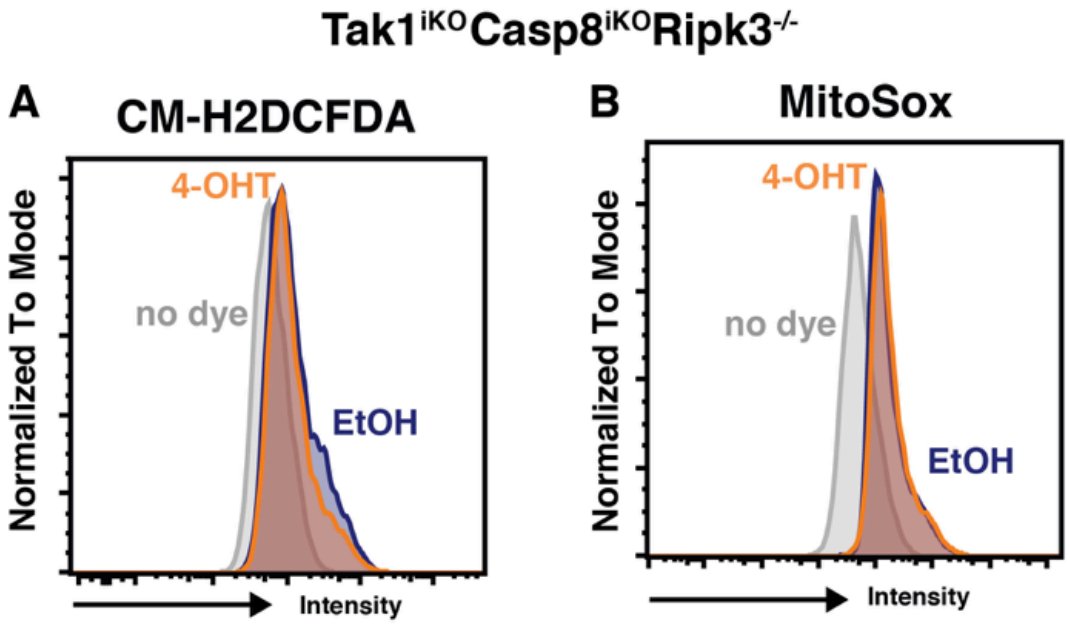

Figure. 4 Double deletion of *Casp8* and *Ripk3* diminishes mitochondrial ROS in *Tak1*-deficient macrophages.

(A, B) Tak1^{iKO} Casp8^{iKO} *Ripk3*-/- BMDMs were treated with vehicle (EtOH) or 4-OHT for 5 days and incubated without (no-dye), or with CM-H2DCFDA (A) or MitoSox (B). Cells were analyzed by flow cytometry.

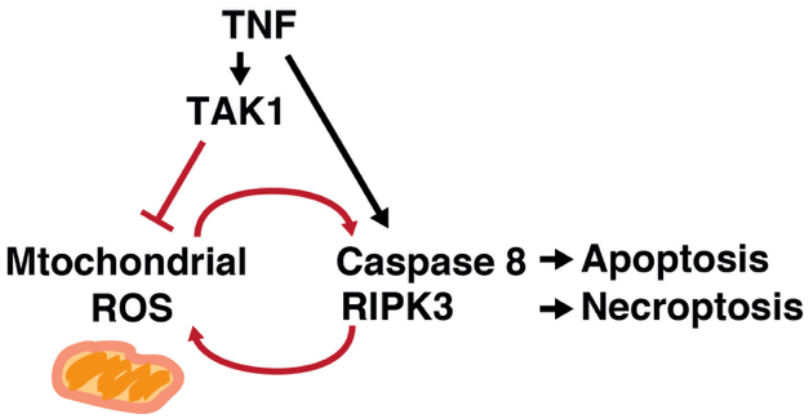

Figure. 5. Graphic abstract. TNF activates TAK1, caspase 8, and RIPK3. TAK1 normally prevent the generation of mitochondrial ROS, which limits activation of caspase 8 and RIPK3. In *Tak1*-deficient macrophages, TNF stimulation activates the feedforward loop of caspase 8 and RIPK3, and mitochondrial ROS that leads to apoptosis or necroptosis.

GENERAL DISCUSSION

As specific discussions have been extensively made in each chapter, this general discussion section will focus on the future directions for this study. There are two remaining questions to be addressed in future studies.

Question 1: what are the mechanisms by which *Tak1* prevents mitochondrial dysfunction and caspase activation in macrophages?

As discussed in Chapters 1, Tak1^{HKO} mice exhibit developmental defects in several tissues that are associated with the reduction in tissue-resident macrophages. We demonstrated that *Tak1* deficiency kills macrophages and this cell death is partially TNF-dependent. TAK1 modulates two forms of cell death, apoptosis and necroptosis, in response to various upstream signaling events. We first found that macrophage death induced by *Tak1*-deficiency is different from previously described programmed cell death and exhibit seemingly non-apoptotic and non-necroptotic features (Chapter 1). We then re-define this cell death as an atypical apoptosis and necroptosis in Chapter 2. However, the molecular mechanism by which *Tak1*-deficient macrophages die is still not fully determined.

As discussed in Chapter 2, we have evidence suggesting that mitochondrial ROS is likely the cause of irreversible cell death in *Tak1*-deficient macrophages. Future studies warrant the mechanism(s) by which *Tak1* prevents mitochondrial dysfunction and the accumulation of mitochondrial ROS that leads to caspase activation under inflammatory stimuli.

Question 2: what is the cause of animal mortality in hematopoietic *Tak1*-deficient mice?

Although tissue-resident macrophage populations and developmental defects were largely restored in Tak1^{HKO} *Tnfr1*$^{-/-}$ animals, the failure to improve animal viability suggests that mortality caused by hematopoietic-*Tak1* deficiency is *Tnfr1*-independent. We are currently testing whether multiple combinations of gene deletion can rescue Tak1^{HKO} mice mortality. Rationales for the gene deletions and the current results are described in the following sections.

Tak1^{HKO} Tnfr1$^{-/-}$ Ripk3$^{-/-}$

This combination of gene deletions is examined based on the similarities between *Tak1* and *Ripk1* deficiency. RIPK1 is the activator of RIPK3-mediated necroptosis pathway, but is also known to modulate caspase activation (Fig. 4 in the General Introduction section). Of interest, *Ripk1*-deficient (*Ripk1*$^{-/-}$) mice exhibit perinatal lethality with multiple hematopoietic abnormalities, including a reduction in tissue-resident macrophages, which is somewhat similar to hematopoietic-*Tak1* deficiency (Rickard et al., 2014).

The similarities extend to macrophages isolated from *Ripk1*$^{-/-}$ animals exhibit sensitivity to TNF-induced cell death (Rickard et al., 2014). The transplantation of *Ripk1*-deficient fetal liver cells failed to reconstitute in irradiated host mice, while the administration of TNF-neutralizing antibodies allowed for normal reconstitution of *Ripk1*-deficient cells (Rickard et al., 2014). While the viability of *Ripk1*$^{-/-}$ *Tnfr1*$^{-/-}$ macrophages were not directly assessed, this suggests that TNF-induced cell death is the cause of hematopoietic abnormalities in *Ripk1*$^{-/-}$

mice. Despite the restoration of hematopoietic cells, animal viability is not improved by in *Ripk1*$^{-/-}$ *Tnfr1*$^{-/-}$ mice and die shortly after birth, similar to Tak1^{HKO} *Tnfr1*$^{-/-}$ mice (Rickard et al., 2014). Interesting, *Ripk3* co-ablation delayed *Ripk1*$^{-/-}$ animal mortality to P4, prompting us to examine the contribution of *Ripk3*-depenent necroptosis in the Tak1^{HKO} model (Rickard et al., 2014).

In accordance, we have preliminary data suggesting that the additional ablation of *Ripk3* in a *Tak1*^{HKO} *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ triple knockout mice delayed animal mortality until ≈P4 (Fig. 1). This suggests that *Ripk3*-dependent necroptosis is the cause of perinatal animal mortality, and raises the possibility of cell death stimuli other than TNF in the *in vivo* setting. *Tak1* is activated by a variety of inflammatory stimuli, including: TNF, IL-1, and various pathogen-associated molecular patterns (PAMPs) and DAMPs that trigger pattern recognition receptors (Dai et al., 2012). Aside from TLRs, retinoic acid inducible gene 1 (RIG-1)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) have been implemented in *Tak1*-signaling (Dai et al., 2012). It is likely that PAMPs and DAMPs that activate the PRRs in the *in vivo* setting are absent in the tissue culture system. Future studies are warranted to determine the contributions of various cell death stimuli in the mortality of hematopoietic *Tak1*-deficient mice.

***Tak1*^{HKO} *Casp8*^{HKO} *Ripk3*$^{-/-}$**

As discussed above, *Ripk3*-dependent necroptosis contributes to the perinatal lethality of Tak1^{HKO} mice but *Tak1*^{HKO} *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ mice still die around P4, suggesting *Ripk3*-

independent mechanisms contribute to animal mortality at this stage. What are the *Ripk3*-independent pathway(s) that contributes to animal mortality in *Tak1*^{HKO} *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ mice?

We have shown TAK1 to prevent *Casp8*- and *Ripk3*-dependent cell death in macrophages, *in vitro*, as discussed in Chapters 1 and 2. This raises the possibility that caspase 8-dependent apoptosis is the RIPK3-independent pathway causing animal mortality. Furthermore, *Ripk1* deficiency can be rescued by the double deletions of *Casp8* and *Ripk3*, discussed below.

Ripk1$^{-/-}$ *Ripk3*$^{-/-}$ mice die around P4 due to gastrointestinal defects, characterized by enhanced cleaved-caspase 3 (CC3), the hallmark of apoptosis (Rickard et al., 2014). These gastrointestinal defects and animal mortality at P4 can be prevented by the additional ablation of *Casp8*, in a *Ripk1*$^{-/-}$ *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$ animal model. This suggests that the dysregulation of *casp8*-mediated apoptosis is the cause of animal mortality at P4 in the *Ripk1*$^{-/-}$ *Ripk3*$^{-/-}$ animal model (Rickard et al., 2014). *Ripk1*$^{-/-}$ *Casp8*$^{-/-}$ mice are indistinguishable to *Ripk1*$^{-/-}$ and succumb shortly after birth; further confirming that *Ripk3*-dependent necroptosis is the cause of perinatal lethality in *Ripk1*$^{-/-}$ mice (Rickard et al., 2014).

Given that our *Tak1*^{HKO} *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ mice share profound similarities to *Ripk1*$^{-/-}$ *Ripk3*$^{-/-}$ mice and survive to day 4, we asked whether the additional ablation of *Casp8* would improve animal viability. To address this question, we have begun the mating process to generate

Tak1 Casp8 Ripk3 triple hematopoietic-knockout (*Vav-Cre Tak1*$^{flox/flox}$ *Casp8*$^{flox/flox}$ *Ripk3*$^{-/-}$) mice. We anticipate these mice to survive past P4, into adulthood.

In summary,

1) Hematopoietic-*Tak1* deficiency causes *Ripk3*-dependent perinatal lethality that is independent of *Tnfr1*-signaling.
2) Deletions of *Tnfr1* and *Ripk3* delayed Tak1^{HKO} animal mortality until P4.
3) We anticipate that double deletions of *Casp8* and *Ripk3* may fully rescue Tak1^{HKO} animal mortality.

References

Dai X, Ryan GR, Hapel AJ, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood*. 2002; 99(1):111-120.

Rickard JA, O'Donnell JA, Evans JM, Lalaoui N., Poh AR, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*. 2014; 157:1175-1188.

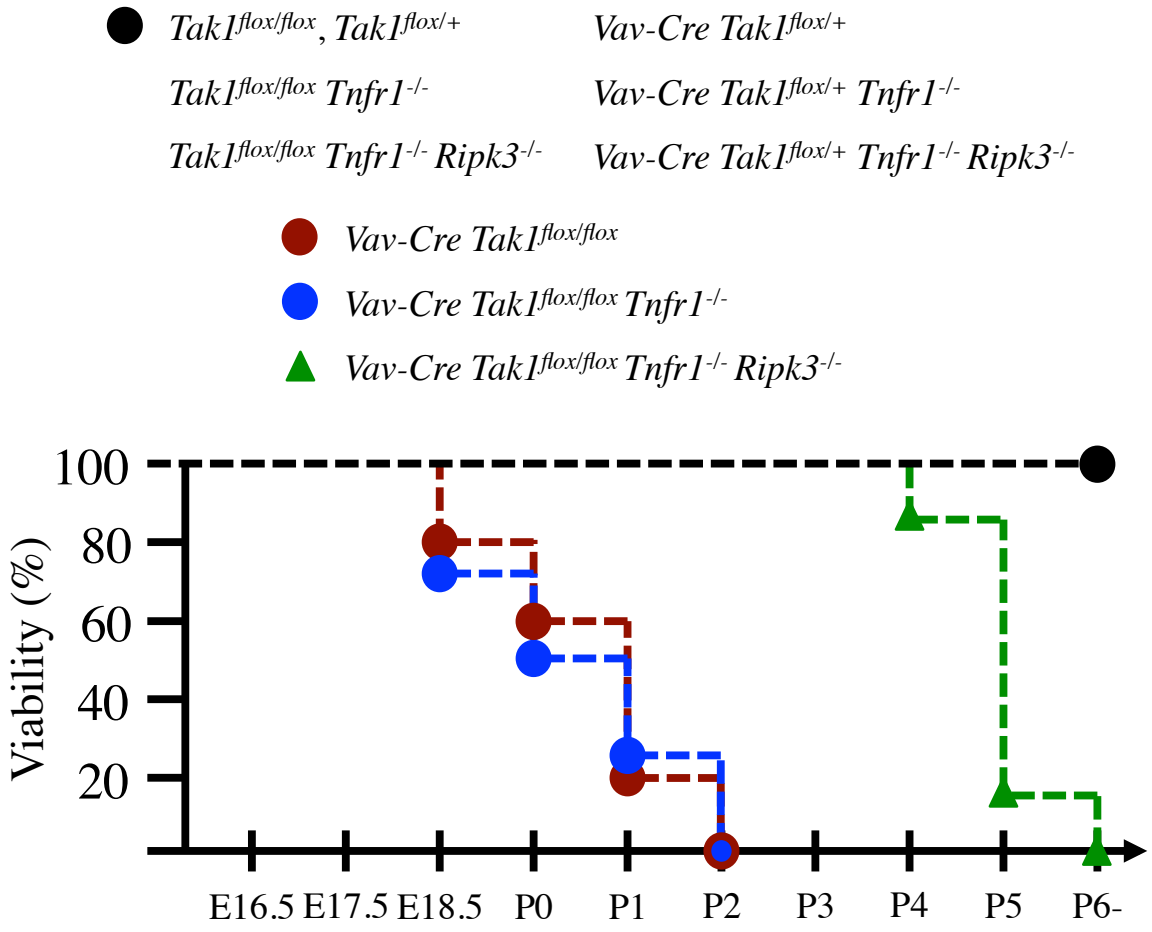

Figure 1. Viability of *Vav-Cre Tak1*$^{flox/flox}$, *Vav-Cre Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$, *Vav-Cre Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ mice. *Vav-Cre Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$ *Ripk3*$^{-/-}$ at P2 (n=17), P3 (n=7), P4 (n=14), P5 (n=29), P6- (n=35). *Vav-Cre Tak1*$^{flox/flox}$ and *Vav-Cre Tak1*$^{flox/flox}$ *Tnfr1*$^{-/-}$ animal viability adapted from Chapter 1.

APPENDICES

Appendix A

SAKAMACH Supplementary

Sakamachi et al. Supplementary Figure S1

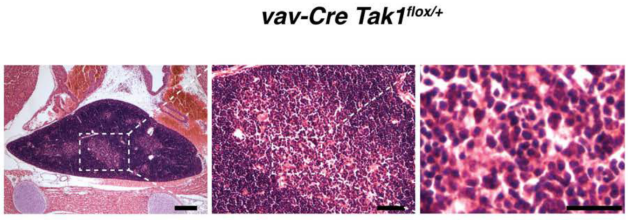

Figure S1. Hematopoietic-specific heterozygous deficiency of *Tak1* does not cause abnormality in the thymus

H&E staining of E18.5 *vav-Cre Tak1*$^{flox/+}$ thymus. Scale bars, 200 μm (left panel), 50 μm (middle panel), 20 μm (right panel).

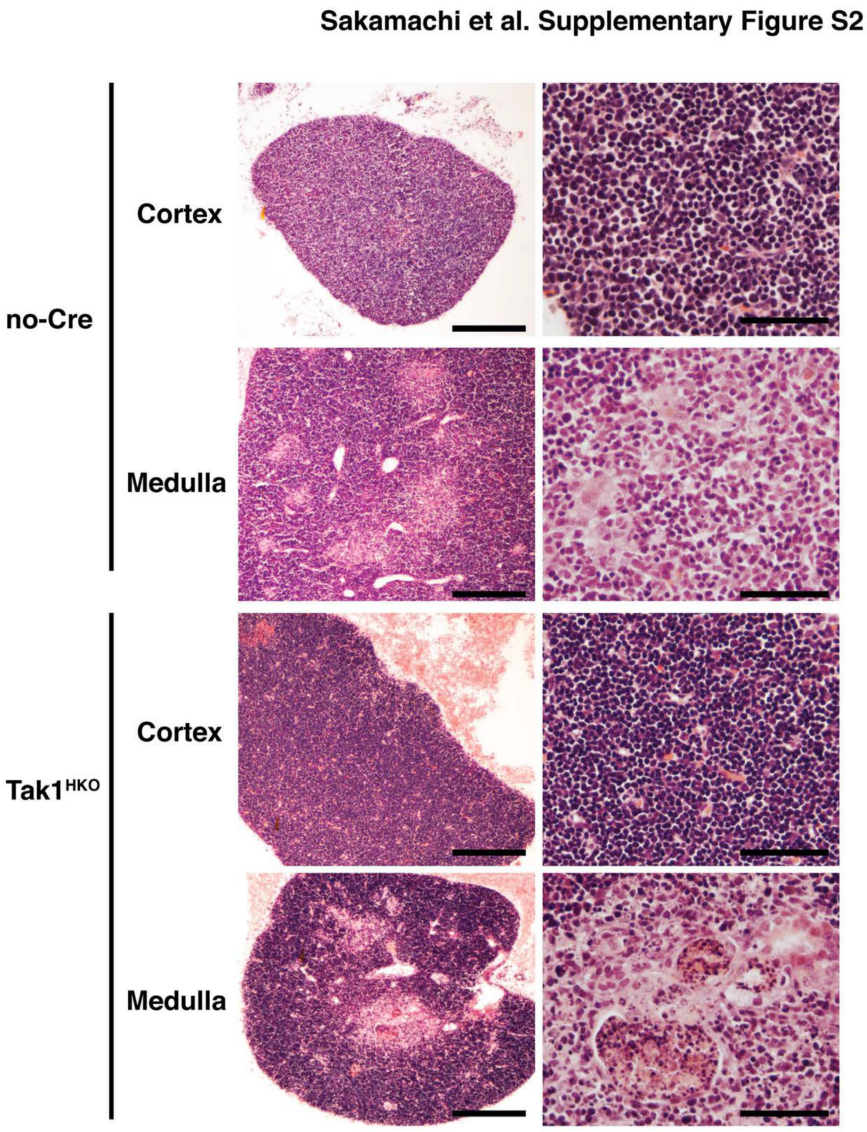

Figure S2. H&E staining of thymus
H&E staining of P0 no-Cre and Tak1^{HKO} thymus. Two different positions (cortex and medulla) from one thymus of no-Cre or Tak1^{HKO} mouse are shown. Scale bars, 200 μm (left panels), 50 μm (right panels).

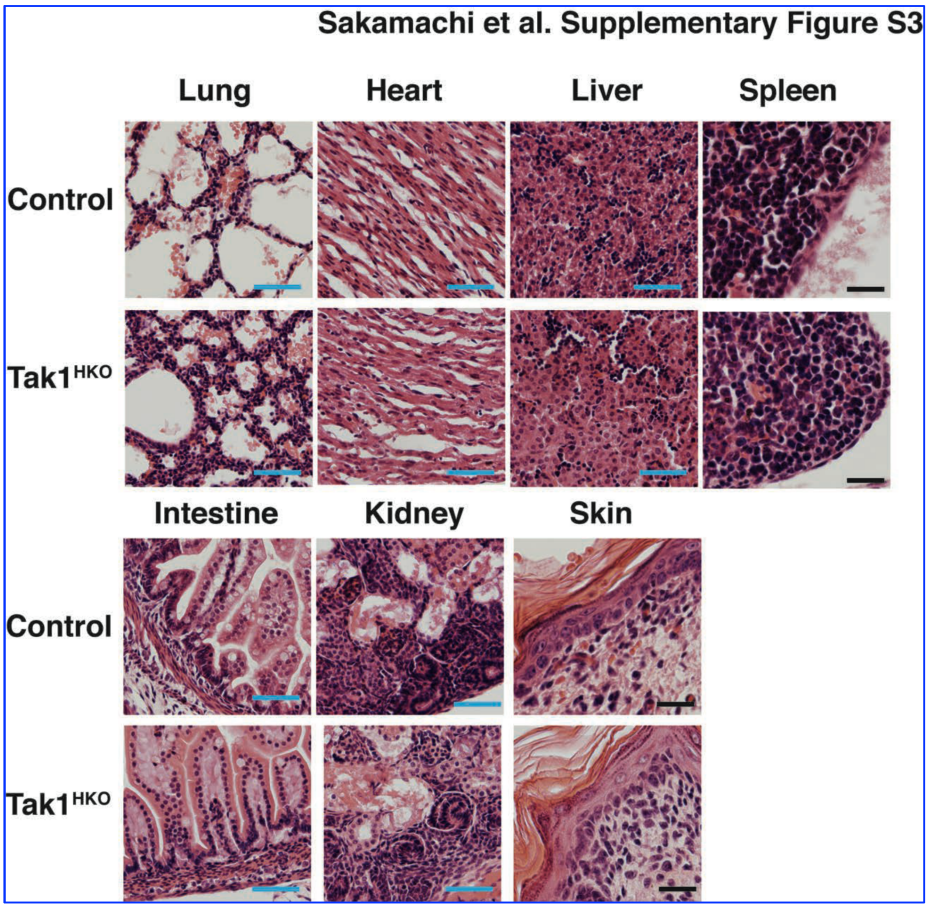

Figure S3. H&E staining of the lung, heart, liver, spleen, intestine, kidney and skin. Five controls including no-Cre and Tak1-Het and three Tak1^{HKO} mice at P0 were observed. Representative H&E staining images of control and Tak1^{HKO} tissues are shown. Scale bars, 50 μm (blue), 20 μm (black). The Tak1^{HKO} lungs exhibited impaired inflation, while other tissues of control and Tak1^{HKO} were indistinguishable.

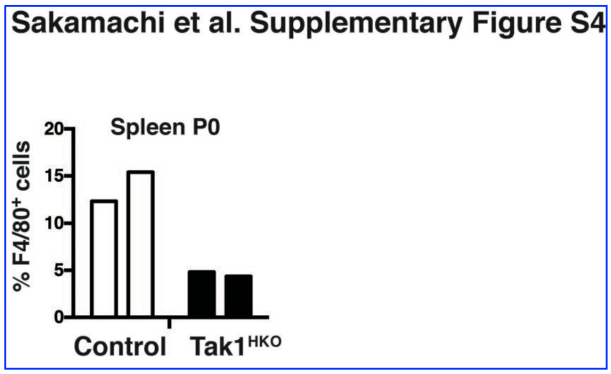

Figure S4. Spleen macrophages are diminished by *Tak1* deletion.
Control and Tak1^{HKO} spleens (n = 2 each) at P0 were analyzed by immunofluorescence staining using anti-F4/80 antibody (macrophages) and DAPI (all nuclei). F4/80^{+} cells in DAPI stained cells (more than 800 nuclei per animal form at least three randomly chosen images) were quantified.

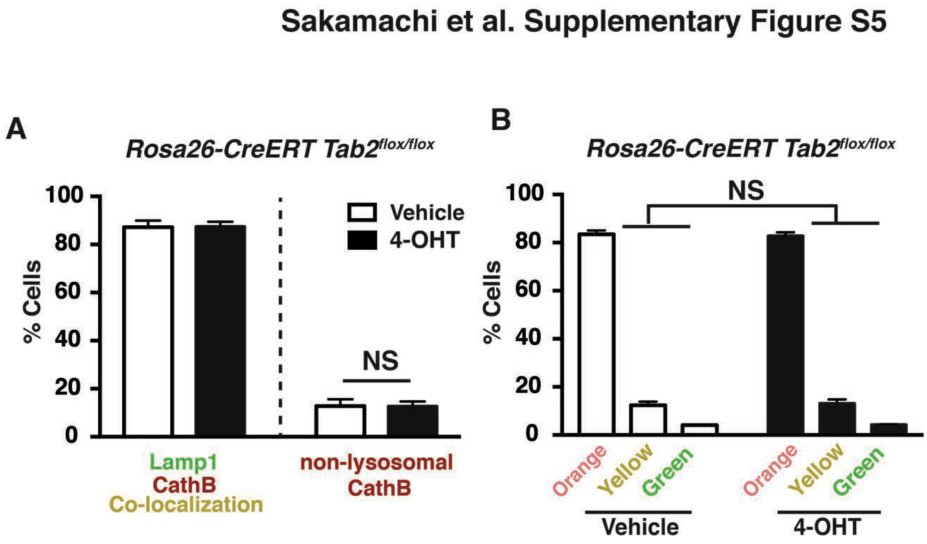

Figure S5. 4-OHT or Cre expression does not cause lysosomal injury
Rosa26-CreERT Tab2$^{flox/flox}$ BMDMs were treated with vehicle or 0.3 μM 4-OHT for 5 days. (A) Lysosomal architecture was visualized by staining using anti-lamp1 and anti-cathepsin B (CathB; red) antibodies, and quantified. Means ± SD; NS, not significant (unpaired two tailed Student-t test). (B) Lysosomal function was determined by incubating cells in acridine orange at 4 days post 4-OHT treatment. Orange staining indicates normal functional lysosomal pH (around 3.5), and yellow or green staining indicates increased lysosomal pH. Means ± SD; NS, not significant (one-way ANOVA).