NEW INSIGHTS INTO TUMOR NECROSIS FACTOR-ALPHA IN CANCER: DISTINCT ISOFORMS EXERT OPPOSING EFFECTS ON TUMOR ASSOCIATED MYELOID CELLS AND TUMORIGENESIS

By

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To my family

And

To all those whose right to higher education has been denied

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"Science is but a perversion of itself unless it has as its ultimate goal the betterment of humanity."

— Nikola Tesla

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ADAM-17	A disintegrin and metalloprotease domain
A-SMase	Acid sphingomyelinases
BM	Bone marrow
Btk	Bruton's tyrosine kinase
CAPK	Ceramide-activated protein kinase
CFSE	5-(and -6)-carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
cIAP	Cellular inhibitor of apoptosis
CM	Conditioned media
CYLD	Cylindromatosis
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DD	Death domain
DISC	Death inducing signaling complex
DMAP	4-Dimethylaminopyridine
DPI	Diphenyleneiodonium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMA	Enriched membrane antigen
Etk	Endothelial/epithelial tyrosine kinase
FADD	FAS-associated protein with death domain
FAN	Factor associated with N-SMase activation
GLUD1	Glutamate dehydrogenase 1
GLUL	Glutamine synthase
HUVECs	Human umbilical vein endothelial cells
JNK	c-Jun NH2-terminal kinase
LDH	Lactate dehydrogenase
LLC	Lewis lung carcinoma
MDSC	Myeloid derived suppressor cells
MMP	Matrix metalloproteinases
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor-κB
NK	Natural Killer
NOX	NADPH oxidase
NSCLC	Non-small-cell lung carcinoma
NSD	Neutral sphingomyelinase domain
N-SMase	Neutral SMase
PECAM-1	Platelet/endothelial cell adhesion molecule-1

PLAD	Preligand binding assembly domains
PYGL	Glycogen phosphorylase
RIP-1	Receptor-Interacting Protein-1
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SMase	Sphinglomylinase
TAB	Transforming growth factor-β-activated kinas binding protein
TACE	Tumor necrosis factor-alpha converting enzyme
TAK-1	Transforming growth factor-β-activated kinase
TAM	Tumor associated macrophages
TEM	Tie2 expressing monocytes
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TNFR	TNF receptor
TNFR-DKO	TNF receptor double knockout
TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRAF	TNF receptor associated factors
TRAIL-R1/2	TNF-related apoptosis-inducing ligand receptor-1 and -2
TTFA	Thenoyltrifluoroacetone
VEGF	Vascular endothelial growth factor
TAM	Tumor associated macrohpahges
MAPK	Mitogen-activated protein kinases

CHAPTER I

INTRODUCTION

Tumor Microenvironment

Tumor cells actively interact with each other and the surrounding interstitial tissue through cell-to-cell contacts and by both releasing and responding to soluble factors present in the tumor microenvironment. This interaction results in the generation of a population of non-malignant cells, collectively known as the “tumor stroma”, that are uniquely primed to support growth and metastasis of the malignant population (Goldoni and Iozzo, 2008; Whiteside, 2008). This “tumor-educated” stroma thus exhibits a unique phenotype that promotes the growth, invasion and metastasis of the malignant population (Joyce and Pollard, 2009; Pollard, 2008).

In recent years increasing attention has been focused on the role of the host inflammatory cells in the stroma (Balkwill et al., 2005). The first connection between inflammation and cancer was made in 1863 by Rudolf Virchow, who noted the presence of leukocytes in neoplastic tissues (Virchow, 1963). Decades of extensive *in vitro* and *in vivo* studies have led to a better understanding of the importance of inflammatory cells in tumor formation and progression, and have highlighted the significance of Virchow’s discovery. Both lymphocytes and leukocytes have been shown to contribute to tumor inflammation. Myeloid cells however, are the major component of the inflammatory infiltrate frequently seen

in primary tumors (Balkwill and Coussens, 2004; Balkwill and Mantovani, 2001; Murdoch et al., 2008).

Myeloid/macrophage cells play a key role in the immune response by presenting antigens to lymphocytes to start the recognition and immune inductive processes or by releasing regulatory molecules to stimulate lymphocyte functions (Mosser and Edwards, 2008). As a component of the innate immune response, myeloid cells activate dendritic cells and natural killer (NK) cells that can initiate an anti-tumor response. Consistent with this role, in some mouse experimental models, syngeneic macrophages from tumor bearing mice inhibited more melanoma growth in nude mice than control macrophages (Adelman et al., 1983). However, often in both mouse models and in patients, the myeloid population can instead drive tumor progression by promoting neovascularization, metastasis, and immunosuppression (Balkwill and Coussens, 2004; Murdoch et al., 2008; Pollard, 2008). This is achieved through various myeloid subpopulations, including tumor associated macrophages (TAM, M1/M2 subtypes), myeloid derived suppressor cells (MDSC), Tie2 expressing monocytes (TEM), vascular leukocytes and dendritic cells (Figure 1). Identifying signals that contribute to the generation of these protumorigenic myeloid populations and their link to cancer progression has been the subject of many studies. Based on *in vitro* studies it has been hypothesized that a complex network of pro-inflammatory mediators is probably involved (Balkwill, 2004a; Coussens and Werb, 2002). Tumor cells depend on these cytokines for both proliferation and reprogramming cells present in the tumor

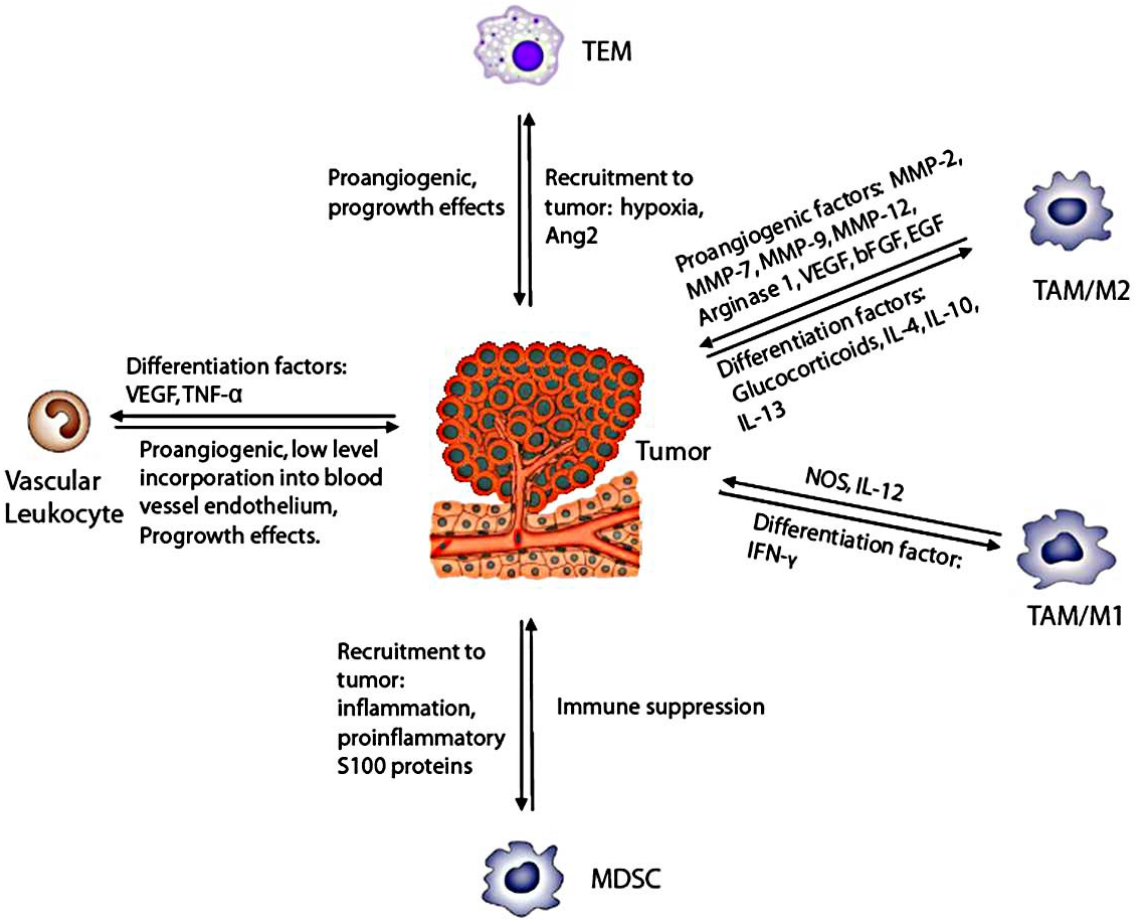

Figure 1. Schematic of interactions between tumor and bone marrow derived myeloid subtypes. Recruitment of diverse bone marrow-derived cell populations to the tumor microenvironment and their effects on tumor progression. Tumor and stromal cells mobilize various subpopulations of tumor promoting bone marrow-derived cells to the peripheral blood through secretion of cytokines and chemokines. Diverse chemoattractant factors promote the recruitment and infiltration of these cells to the tumor microenvironment where they suppress the antitumor immunity or promote tumor angiogenesis and vasculogenesis. In addition cytokines produced in the tumor microenvironment can give rise to macrophages with distinct physiologies. This population includes tumor associated macrophages-M1 (TAM/M1) and TAM/M2, Tie2 expressing monocytes (TEMs), myeloid derived suppressor cells (MDSCs) and myeloid/endothelial biphenotypic vascular leukocytes.

microenvironment toward a protumorigenic phenotype. The Nuclear factor-κB (NF-κB) pathway has emerged as a key regulator of the release of these pro-inflammatory cytokines, and is an important mediator of tumor proliferation and survival (Balkwill and Coussens, 2004). It has been proposed that NF-κB activates signaling pathways in both cancer cells and tumor-associated inflammatory cells, and regulates the pro-inflammatory mediator TNF-α and other pro-inflammatory cytokines, thus promoting malignancy (Pikarsky et al., 2004).

In the following section, we will provide an overview of the signaling molecules, cytokines and growth factors, which modulate the recruitment and differentiation of inflammatory cells. Furthermore, we will discuss the role of TNF-α, an interesting example of one of the cytokines present in tumor microenvironment that allows tumor invasion of the host inflammatory response, to promote its survival and progression.

Myeloid Cell Recruitment and Programming

Circulating blood monocytes are recruited to the tumor by a range of growth factors and chemokines, often produced by the tumors themselves (Murdoch et al., 2004). Other inflammatory cells, such as B lymphocytes, have been implicated in producing trophic factors that promote macrophage recruitment (de Visser et al., 2005). Following recruitment, it is widely accepted that the tumor/myeloid cell crosstalk drive recruited myeloid cells towards a tumor-promoting phenotype(s) (Balkwill et al., 2005; Pollard, 2004). Understanding the mechanisms by which pro-tumorigenic, myeloid

immunophenotypes are generated either in circulation or within the tumor milieu can be used to develop novel anti-tumor therapy. Hence, this area of study is at the forefront of cancer research. While a number of factors have been identified that recruit myeloid cells to tumor sites (Schmid and Varner, 2007), much less is known about tumor-derived signals that modulate the generation of unique myeloid subtypes during cancer growth.

Multiple reports have shown that tumor-derived factors contribute to bone marrow-derived myeloid recruitment and differentiation. A number of monocytes, chemoattractants and cytokines initiate recruitment of these cells to the tumor. Once recruited, additional tumor-derived factors, differentiate myeloid cells into tumor promoting cells. These tumor-associated myeloid derived cells promote cancer progression through several mechanisms, including promoting angiogenesis, inducing tumor growth and enhancing tumor cell migration and invasion. Several studies have demonstrated the association between increased tumor vascularity and macrophage infiltration in several human cancers (Conejo-Garcia et al., 2005; Li et al., 2009). Macrophage infiltration has been shown to correlate with vessel density in endometrial, ovarian, breast and central nervous system malignancies. Other unique myeloid populations, such as TEMs and MDSCs, are thought to be present in the circulation and are recruited to the tumor site (Bray et al., 1993; De Palma et al., 2005; Serafini et al., 2006).

Multiple studies have described a role for TNF-α in modulating the interactions between tumor cells and macrophages that result, in both increased invasive capacity of malignant cells, and the switch of macrophages to a tumor

promoting phenotype (Li et al., 2009; Pollard, 2004). TNF-α is not normally detected in the plasma or serum of healthy individuals but has been detected in the malignant and/or stromal cells in human ovarian, breast, prostate, bladder, and colorectal cancer, often in association with IL-1, IL-6 and macrophage colony stimulating factor (Bozcuk et al., 2004; Burke et al., 1996; Ferrajoli et al., 2002; Michalaki et al., 2004; Naylor et al., 1993; Pfitzenmaier et al., 2003). In epithelial ovarian cancer, TNF-α mRNA is found in epithelial tumor islands and is positively correlated with tumor grade (Naylor et al., 1993). There is substantial evidence that TNF-α is involved in the promotion and progression of cancer by regulating pathways that lead to cell proliferation, survival and angiogenesis (Balkwill, 2006). High doses of TNF-α can cause hemorrhagic necrosis through the destruction of tumor blood vessel and the generation of T-cell anti-tumor immunity (Balkwill, 2009). However, when chronically produced this cytokine may act as an endogenous tumor promoter, contributing to the tissue remodeling and stromal development that is necessary for tumor growth and progression. An example of this is TNF-α's role in the generation of vascular leukocytes.

Vascular leukocytes are a subpopulation of tumor-associated myeloid cells that express both endothelial and myeloid markers (Rehman et al., 2003; Romagnani et al., 2005; Sharpe et al., 2006). Although rare in the peripheral blood, vascular leukocytes are primarily associated with tumors where they enhance tumor growth and angiogenesis and decrease tumor necrosis. These myeloid/endothelial biphenotypic populations result from the endothelial

differentiation of myeloid progenitors, a process regulated by low levels of TNF-α expression in tumor cells.

In the following sections our focus will turn to TNF-α as one of the proinflammatory cytokines present in tumor environment. The role of this tumor-derived cytokine, which can destroy blood vessels and induced cell death at high concentration but can also induce angiogenic factors and tumor survival, will be discussed in the context of tumor biology.

Tumor Necrosis Factor-Alpha

The discovery of TNF-α

TNF-α was discovered in 1892 when William Coley's mixed bacterial toxin treatment cured a patient with sarcoma (Coley, 1991). Coley noted that when cancer patients developed certain bacterial infections, the tumors become necrotic. In the hope of finding a cure for cancer, Coley began to inject cancer patients with supernatants derived from various bacterial cultures. These cultured supernatants, called "Coley's toxins", induced hemorrhagic necrosis in tumors but also had undesirable side effects. This observation led to the conclusion that there is a factor derived from bacterial toxin that causes tumor necrosis, henceforth known as "tumor necrosis factor". Later on, it was discovered that tumor necrosis factor is found in the serum of infected endotoxin-treated animals and also caused tumor regression. In 1975, Carswell et al. reported that the active component of Coley's toxin was a lipopolysaccharide (endotoxin)

component of the bacterial cell wall. It was concluded that the necrotic factor was made by host cells in response to endotoxin and was not directly derived from bacteria. They identified macrophages as the source of tumor necrosis factor (Carswell et al., 1975).

TNF-α signal transduction

The TNF-α super family is a group of cytokines with important functions in immunity, inflammation, differentiation, control of cell proliferation and apoptosis. TNF family members exert their biological effects through the TNF (TNFR) superfamily of cell surface receptors that share a stretch of ~ 80 amino acids within their cytoplasmic region and the death domain (DD) that is critical for recruiting the death machinery. TNF-α is the founding member of the 19 different proteins that have so far been identified within this family. TNF-α is a 26 kDa type II transmembrane protein with an intracellular amino terminus. It can be cleaved by TNF-α-converting enzyme (TACE) into a 17 kDa soluble cytokine that is released into the extracellular space (McGowan et al., 2008) (Figure 2). TNF-α signals through two receptors: TNF receptor type 1 (TNFR-1), which is found in most cells in the body, and TNF receptor type 2 (TNFR-2), which is mainly expressed on hematopoietic cells (Grell, 1995). TNF-α can activate pathways leading to three different cellular responses: cell survival and proliferation, transcription of pro-inflammatory genes, and cell death (Waters et al., 2013b).

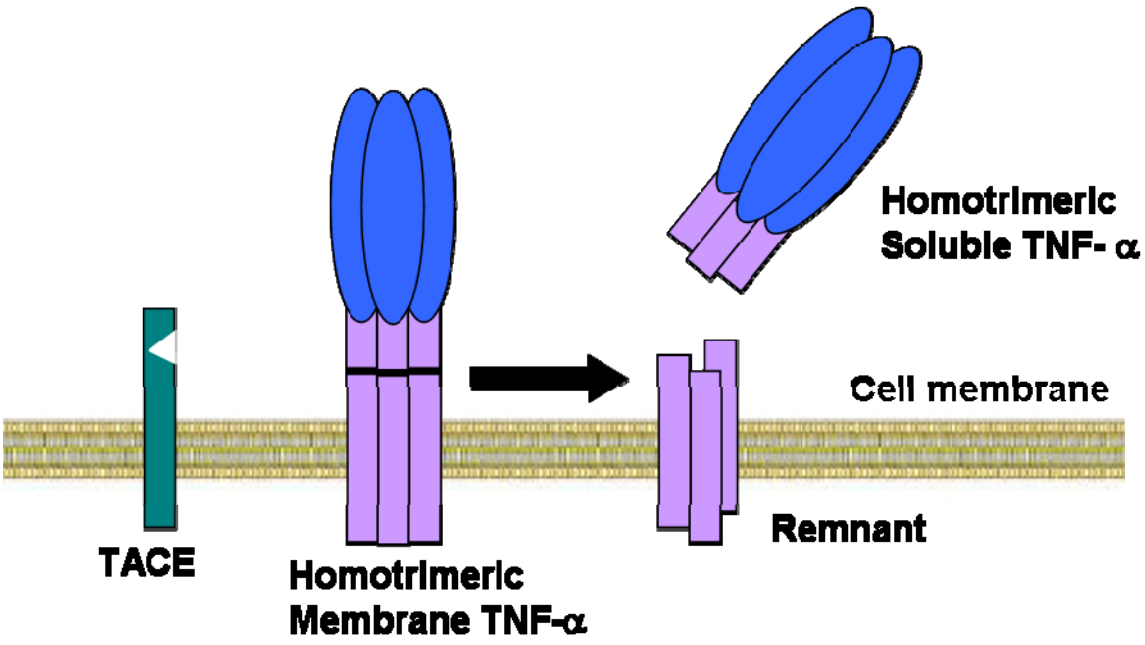

Figure 2. Schematic representation of membrane TNF-α cleavage by TACE into soluble TNF-α. TNF-α is produced as a 26 kDa membrane-associated protein, which is cleaved and released as a soluble 17 kDa protein by specific metalloproteinases, such as TNF-α-converting enzyme (TACE). TACE the major convertase for precursor TNF-α, processes the 78-kd homotrimeric membrane bound TNF-α to generate the 51-kd homotrimeric soluble TNF-α.

Both TNF receptors are capable of binding intracellular adaptor proteins that activate complex intracellular signaling processes and mediate the pleiotropic effects of TNF-α. Downstream signaling targets of TNFR-1 are activation of pro-survival/differentiation pathways (NF-κB or MAPK) or activation of apoptotic pathways (caspases-8,2,1). TNFR-2 lacks the death domain but can affect NF-κB and JNK signaling (Baud and Karin, 2001). *In vitro* studies suggest that TNF-α preferentially binds to the ubiquitously expressed TNFR-1 whereas mTNF-α is the primary activating ligand for TNFR-2, expressed mostly in endothelial and hematopoietic cells (including myeloid cells)(Grell, 1995).

Upon activation, TNFR-1 undergoes a conformational change in its cytoplasmic portion allowing it to interact with the death domain (DD) region of receptor interacting protein (RIP)-1, TNFR-associated death domain (TRADD), and TNFR-associated factor (TRAF)-2 and -5. They in turn recruit the cellular inhibitor of apoptosis (cIAP) forming the complex I. cIAP inhibits caspase-3 activation and allows ubiquitinylation of RIP-1. Next, transforming growth factor-β-activated kinase (TAK)-1/TAK-1 binding protein (TAB)-2/TAB-3 forms a complex that binds to ubiquitin residues on RIP-1 and activates NF-κB. Deubiquitinylation of RIP-1 by the enzyme cylindromatosis (CYLD), favors the transformation of complex I to complex II binding to the internalized death-inducing-signaling-complex (DISC, formed by FAS-associated protein with death domain (FADD) and procaspase-8 (Pro-C8)) and RIP-3 (Complex II) that can trigger cell death through apoptosis (Micheau and Tschopp, 2003). RIP-1 activates caspase-8 (C8) which cleaves RIP-1 and RIP-3 and induces apoptosis

via intrinsic (release of cytochrome C from mitochondrial) or extrinsic pathways resulting in caspase-3 activation. Preservation of RIP-1 kinase activity and autophosphorylation between RIP-1 with RIP-3 leads to the formation of necrosome. Necrosome induces reactive oxygen species (ROS) production via activation of NOX-1 at the cellular membrane or exerts direct effects in the mitochondria (Figure 3). The signaling pathways initiated by TNFR-2, which may be the preferential receptor for transmembrane TNF-α, are less characterized compared to those of TNF-R1. However, TNFR-2 shares some pathways similar to TNF-R1. In some cell types, TNFR-2 can initiate phosphorylation of IKK leading to nuclear translocation of NF-κB through a pathway similar to TNFR-1. Distinct TNFR-2 signal transduction pathways have also been described (Wajant et al., 2003; Waters et al., 2013a). Specifically TNFR-2 can activate endothelial/epithelial tyrosine kinase (Etk), a member of the Bruton's tyrosine kinase (Btk) non-receptor tyrosine kinase family implicated in cell adhesion, migration, proliferation, and survival (Pan et al., 2002). It has also been observed that TNFR-2 can potentiate the apoptotic response to TNF-α (Declercq et al., 1998; Haridas et al., 1998; Vandenabeele et al., 1995). This phenomenon has been explained by the "ligand-passing" model which suggests that TNFR-2 merely serves as a membrane-bound high-affinity trap of TNF-α that delivers the ligand to TNFR-1 (Weiss et al., 1997).

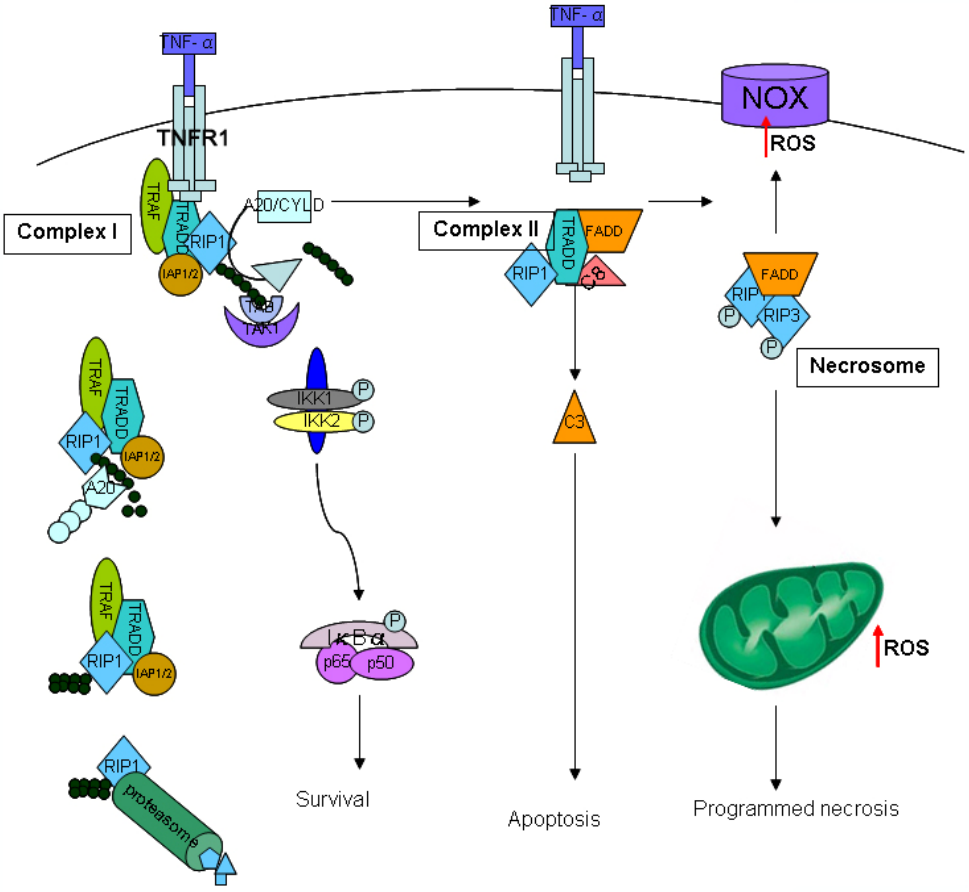

Figure 3. A schematic overview of TNFR-1 activation and downstream signaling events. Following TNF receptor activation by TNF-α, TNFR undergoes a conformational modification of its cytoplasmic portion allowing the interaction with receptor interacting protein (RIP)-1 with the death domain (DD), TNFR-associated death domain (TRADD), and TNFR-associated factor (TRAF). They in turn recruit the cellular inhibitor of apoptosis (cIAP) forming the complex I. cIAP inhibits caspase-3 activation and allows ubiquitylation of RIP-1. Next, transforming growth factor-β-activated kinase (TAK)-1/TAK-1 binding protein (TAB)-2/TAB-3 form a complex that binds to ubiquitin residues on RIP-1 and activates nuclear factor-κB (NFκB). This may occur via a p38 mitogen-activated-protein-kinase-(p38-MAPK-) dependent pathway. Deubiquitylation of RIP-1 by the enzyme cylindromatosis (CYLD) favors the transformation of complex I to complex II binding to the internalized death-inducing-signaling-complex (DISC, formed by FAS-associated protein with death domain (FADD) and procaspase-8 (Pro-C8)) and RIP-3 (Complex II). If energy is only partially insufficient, receptor interacting protein-1 (RIP-1) activates caspase-8 (C8) signaling for classical apoptosis. In this setting, caspase-8 cleaves RIP-1 and RIP-3 preserving signal for apoptosis; however, if caspase activity declines or prohibited autophosphorylation between RIP-1 with RIP-3 result in formation of the necrosome. Necrosome induces reactive oxygen species (ROS) production via activation of NOX 1 at the cellular membrane or direct effects in the mitochondria.

Biological activities of soluble TNF-α

Soluble TNF-α (sTNF-α) was originally described as a circulating factor that can induce hemorrhagic necrosis of tumors. Subsequently sTNF-α was found to mediate widespread systemic effects. It is well known for its role as a major proinflammatory mediator responsible for activating the immune system after an infection. Bacterial pathogens and many other stimuli induce TNF-α production through Toll-like receptors (TLRs) and NFκ-B signaling (Locksley et al., 2001; Tracey et al., 2008). Following the activation of a complex biological cascade involving chemokines, cytokines and endothelial adhesions, TNF-α along with other proinflammatory factors recruits and activates neutrophils, macrophages and lymphocytes at the sites of damage and infection (Sethi et al., 2008).

It is crucial that TNF-α is produced in the right place, at the right time and in the appropriate context. Unregulated production of TNF-α can cause chronic inflammation, septic shock and autoimmune diseases such as Crohn's disease (van Deventer, 1999), psoriasis (Mease et al., 2000), severe chronic asthma (Berry et al., 2006) and psoriatic arthritis (Tracey et al., 2008).

Biological activities of membrane TNF-α

The role of membrane TNF-α is less understood. Upon its discovery, it was thought that only the soluble form of TNF-α is biologically active. However, subsequent studies found that the membrane form of TNF-α also possessed biological activities (Perez et al., 1990; Xin et al., 2006; Yang Lin, 2007).

Membrane TNF-activated TNFR on target cells exerts various biological functions that contribute to the physiological as well as pathological responses in health and diseases. In endothelial cells, mTNF-α induces production of pro-coagulant agents, adhesion molecules and pro-inflammatory cytokines. Human umbilical vein endothelial cells (HUVECs) co-cultured with transmembrane TNF-α-expressing Chinese hamster ovary (CHO) cells expressed tissue factors with synergistic actions of both TNFR-1 and -2 in an adhesion molecule (E-selectin/ICAM-1)-dependent manner (Grell et al., 1995; Schmid et al., 1995). Membrane TNF-α is an important mediator for crosstalk between NK cells and dendritic cells (DC) (Xu et al., 2007). In mouse, proliferation and cytotoxic activity of NK cells were enhanced by membrane TNF-α on DCs through NK cell-surface TNFR-2. Expression of membrane TNF-α on adipocytes resulted in inhibition of differentiation by selectively activating TNFR-1 (Xu et al., 2007). In addition, patients with HIV infection and acute respiratory distress syndrome have functional and cytotoxic membrane TNF-α expression in the alveolar macrophages (Agostini et al., 1995; Armstrong et al., 2000), which is believed to be a mechanism for TNF-α-mediated lung injury.

TNF-α in Cancer

TNF-α as a cancer inhibitor/promoter

The mechanisms by which TNF-α exerts antitumor effects have been studied extensively. TNF-α inhibits tumor-induced vascularization by damaging the tumor-associated vasculature. It blocks blood flow and causes ischaemia of the tumor cells (Watanabe et al., 1988a). TNF-α also induces antitumor inflammatory responses through activation of NK cells and CD8 T cells (Prevost-Blondel et al., 2000). Furthermore, TNF-α can have a direct effect on tumor cells by increasing lysozymal enzymes and hydroxyl radicals, and inducing cytochrome c release from the mitochondria and apoptosis (Watanabe et al., 1988b) (Figure 4a). Although high doses of TNF-α have antitumor activity, there is growing data to suggest that endogenous TNF-α acts as a tumor promoter.

The first published data that linked TNF-α to cancer reported that treatment with TNF-α induced production of more TNF-α in breast cancer cell lines (Spriggs et al., 1987). Following this discovery, multiple studies have demonstrated that TNF-α is not only produced by inflammatory cells within the tumor, but many human cancer cells constitutively produce small amounts of TNF-α as well, and that TNF-α has a significant pro-tumerigenic role (Beissert et al., 1989; Naylor et al., 1990; Naylor et al., 1993; Szlosarek et al., 2006). Both *in vivo* mouse model studies and data from cancer patients suggest a significant role for TNF-α in tumor promotion. In mouse models, deletion or inhibition of TNF-α reduces the incidence of cancer and even induces resistance to

chemically induced carcinogenesis of the skin (Arnott et al., 2004; Moore et al., 1999). Furthermore, in a mouse model of liver cancer induced by spontaneous cholangitis, inhibition of stromal cell TNF-α production decreased the incidence of liver tumors (Pikarsky et al., 2004).

Direct evidence for the involvement of TNF-α in cancer comes from observations that TNF-α knockout mice on 4 different genetic backgrounds were 10-fold more resistant to chemical carcinogenesis of the skin (Arnott et al., 2002; Arnott et al., 2004; Scott et al., 2003). Mice deficient in TNFR-1 and TNFR-2 also were resistance to skin cancers (Arnott et al., 2004) and TNFR-$1^{-/-}$ mice showed reduced liver tumorigenesis and liver metastasis (Kitakata et al., 2002). In addition, knockdown of TNF-α in ovarian cancer cell lines led to diminished growth and vascular density (Kulbe et al., 2007).

Tumor cells expressing low (picogram) quantities of sTNF-α induce tumor growth in mouse models by recruiting myeloid cells and educating them to demonstrate a tumor-promoting phenotype (Li et al., 2009; Pollard, 2004). Absence of TNF-α signaling in TNF-α receptor deficient mice (TNFR-KO) results in abolishment of TNF-α-induced tumor growth and overall reduction of tumor associated myeloid cells. Subsequent studies did not show a decrease in the rate of proliferation or apoptosis in these TNF-α-expressing tumor cells (Kulbe et al., 2007; Li et al., 2009). Endogenous TNF-α production by cancer cells positively correlates with increased expression of cytokines and chemokines such as CXCL12, CCL8, VEGF, matrix metalloproteinases (MMPs) as well as TNF-α itself, leading to neovascularisation, angiogenesis and metastasis (Balkwill,

2004b; Nabors et al., 2003). TNF-α production via the NF- κB pathway in tumor cells is also central to the induction of cell survival and proliferation of malignant cells (Pikarsky et al., 2004) (Figure 4b).

Once the protumorigenic properties of TNF-α were discovered, targeting TNF-α activity seemed to be a promising step toward tumor therapy. Several Phase I/II clinical trials have been undertaken with TNF-α antagonists in cancer patients (Brown et al., 2008; Harrison et al., 2007; Madhusudan et al., 2004). In these clinical trials, TNF-α antagonist treatment resulted in a period of disease stabilization in 20% of patients with advanced cancer. Based on a few other trials however, anti-TNF-α therapy has been found to potentially promote skin cancer (Mercer et al., 2012) and lymphoma (Keystone et al., 2004) and even increase thrombotic events (Lee et al., 2009). Collectively, these data demonstrate the complex contradictory role of TNF-α and its large spectrum of activities, including both antitumorigenic and protumorigenic functions.

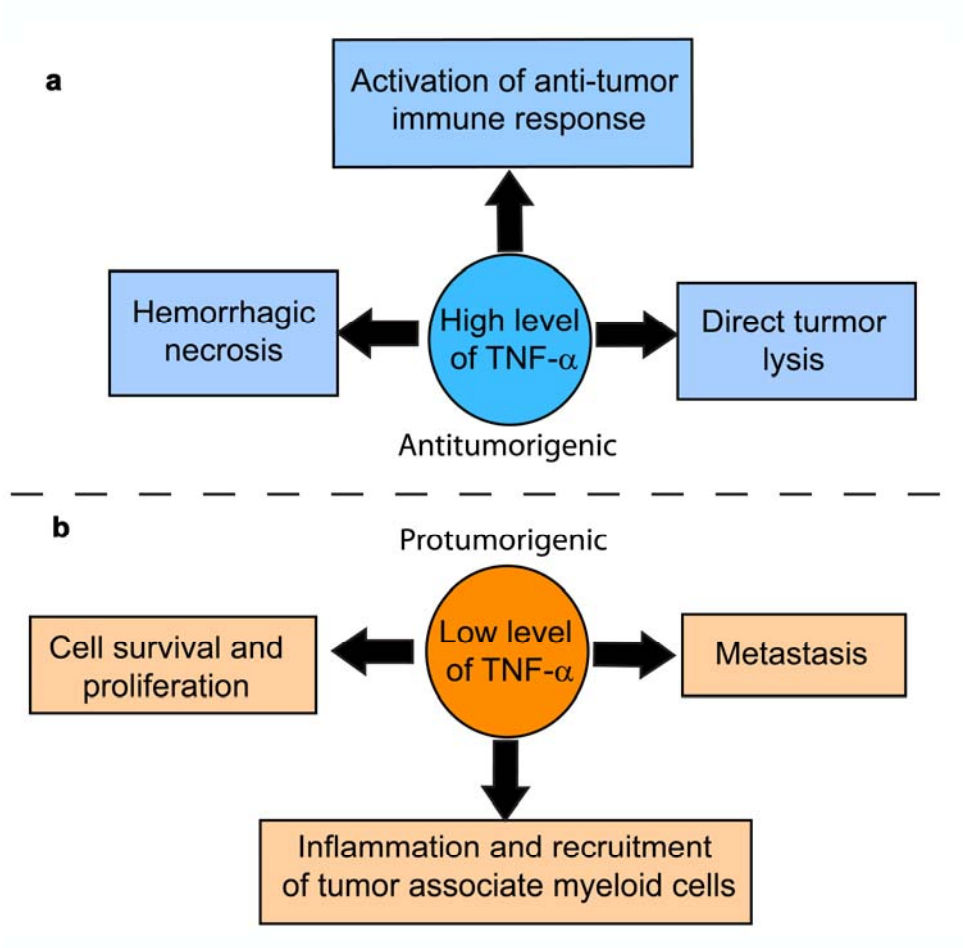

Figure 4. Schematic diagram showing the antitumorigenic/protumorigenic effects of TNF-α. (a) TNF-α causes hemorrhagic necrosis *in vivo* through the destruction of tumor vasculature. It also promotes tumor lysis by activating anti-tumor immune responses and can lead to direct tumour lysis via hydroxyl radicals and lysosomal enzymes. TNF-α can act synergistically with a variety of other cytokines to activate anti-tumor immune response by T-cells.
(b) TNF-α can induce cancer by inducing tumor proliferation and tumor metastasis through remodeling of the extracellular matrix (ECM) as well as driving tumor associated myeloid cells towards a protumorigenic phenotype (i.e. vascular leukocytes).

Summary, Statement of the Problem and Goal of the Study

Myeloid cells, a heterogeneous population of bone marrow-derived cells, play a critical role during growth and metastasis of malignant tumors. The tumor promoting property of myeloid cells is induced by the production of proangiogenic and immunosuppressive factors at the tumor site. It is widely believed that the generation of the pro-tumorigenic phenotype of myeloid cells results from cross-talk between myeloid cells and tumor cells. TNF-α has been identified as an important tumor-derived factor that educates tumor associated myeloid cells towards a protumorigenic subtype, and orchestrates the interplay between malignant cells and myeloid cells, which have been linked to tumor growth and metastasis.

TNF-α has been shown to promote both the death and survival of tumor cells under different circumstances. TNF-α has also been identified as a key regulator of the immune and inflammatory responses to cancer. Whether TNF-α primarily acts in a cell autonomous manner to promote cell survival and growth or through paracrine interactions with the tumor stroma is unclear.

The majority of studies that focus on the role of TNF-α in cancer biology have been mainly in the context of soluble TNF-α. As described in previous sections, TNF-α is initially expressed as a membrane bound protein with various biological functions. However, our knowledge of membrane TNF-α's role in modulating tumor inflammatory cells and its effect on tumor growth is limited.

There have been very few studies that suggest membrane TNF-α has a role in tumor biology. Monocytes primed with cytokines demonstrated increased

apoptosis of tumor cell lines as well as primary acute myeloid leukaemia blasts through a mechanism dependent on transmembrane TNF-α (Williams et al., 2000). Monocytes (effector cells) that express either sTNF-α or only the mTNF-α and incubated with TNF-α-sensitive tumor cells (target cells), resulted in target cell death, albeit through different mechanisms, as assessed by a target cell cytokine profile (Yang Lin, 2007). In a study by Perez et al., myeloid-derived mTNF-α was demonstrated to possess an ability to kill sTNF-α resistant liver cancer cells by inducing apoptosis (Perez et al., 1990).

These reports suggest that mTNF-α and sTNF-α have distinct effects on tumor biology which may explain the contradictory nature of current findings on the role of TNF-α in tumor growth and promotion. This underscores the importance of delineating the roles of TNF-α isoforms in tumor progression.

The goal of this study is to understand the cellular and molecular differences between mTNF-α- and sTNF-α-expressing tumor cells and their roles in modulating host derived myeloid cells and their functions. Our proposed project may aid in understanding the mechanisms of mTNF-α and sTNF-α regulation, which could give insights into the prognosis in cancer patients and provide a key therapeutic approach in cancer treatment. The next chapter will include an in-depth discussion of the effects of tumor derived membrane and soluble TNF-α on tumor growth and inflammation. In chapter III, the molecular mechanisms that distinguish membrane TNF-α from the soluble form will be evaluated.

CHAPTER II

MEMBRANE VERSUS SOLUBLE TNF-ALPHA ISOFORMS EXERT OPPOSING EFFECTS ON TUMOR GROWTH AND SURVIVAL OF TUMOR ASSOCIATED MYELOID CELLS

Introduction

TNF-α is a major inflammatory cytokine expressed within the tumor microenvironment. TNF-α is not normally detected in the serum of healthy individuals, but elevated levels have been detected in patients with prostate, pancreatic, renal cell, hematopoietic and metastatic breast cancers (Balkwill, 2006; Bozcuk et al., 2004; Ferrajoli et al., 2002; Michalaki et al., 2004; Pfitzenmaier et al., 2003; Yoshida et al., 2002). The role of TNF-α in cancer progression is conflicting. Multiple studies have demonstrated a pro-tumorigenic role of TNF-α *in vivo*, in part by inhibiting necrosis and by stimulating a proangiogenic myeloid phenotype (Balkwill et al., 2005; Li et al., 2009). Despite the growing body of evidence showing that TNF-α can function as a tumor promoter, there remain conflicting findings. Several case reports describe a temporal relationship between development of skin malignancies and lymphoma and the use of TNF-α inhibitors (Brown et al., 2002; Chakravarty et al., 2005). Moreover, the use of infliximab, which prevents binding of TNF-α to its receptors, does not improve clinical outcome in renal cell carcinoma (Larkin et al., 2010). Collectively, these data demonstrate the complexity of TNF-α in cancer pathogenesis.

The majority of studies to date focus on the 17-kDa soluble moiety of TNF-α, which is released after proteolytic cleavage of the 26-kDa type II transmembrane isoform by TNF-α-converting enzyme (TACE; ADAM-17) (Kriegler et al., 1988). The role of membrane form of TNF-α and its expression pattern in different tissue is poorly understood. Cardiac-restricted expression of membrane versus soluble TNF-α isoform has been shown to have adverse effect in cardiac remodeling (Dibbs et al., 2003; Diwan et al., 2004). Expression of sTNF-α in cardiomyocytes can cause dilation of left ventricle in mice whereas the mTNF-α results in a concentric hypertrophic cardiac phenotype. Increased mTNF-α expression on T-cells is shown to modulate monocytes IL10 production (Parry et al., 1997). In spite of these findings, the role of mTNF-α in tumor biology is unknown (Diwan et al., 2004). Thus far it is not known whether tumors can express both isoforms. In addition there is little understanding of the difference in the mechanism of action of sTNF-α versus mTNF-α in regulating tumor behavior or impact on the tumor inflammatory stroma.

The goal of this chapter is to assess if the conflicting data regarding the association of TNF-α with tumor progression is due to distinct effects of tumor expression of membrane vs. soluble isoforms. In this chapter, we will show that different TNF-α isoforms have distinct effect on tumor phenotype. Whereas sTNF-α expression promotes tumor growth, mTNF-α-expressing tumors exhibit reduced growth and are largely devoid of myeloid cells. Furthermore, human non-small cell lung cancer (NSCLC) tissues exhibit differential expression of membrane versus soluble TNF-α and patients with lung tumors predicted by the

molecular signature that have higher mTNF-α show a survival rate when compared to patients with tumors with higher soluble form of TNF-α expression (Ardestani et al., 2013).

Materials and Methods

Mice and cell lines. Wild-type C57Bl/6 (WT) mice were purchased from Jackson Laboratory. Homozygous mutants for TNFR-1 and R-2 knockout (TNFR-DKO) on a C57Bl/6 background were a generous gift from Dr. D. Polk. Lewis Lung Carcinoma (LLC), B16F10 melanoma, and RAW 264.7 cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM supplemented with glucose (4.5 g/l) along with penicillin (10 U/L), streptomycin (10 µg/ml), plasmocin (25 µg/ml), amphotericin-B (2.5 µg/ml). H520, HCC95, SW900, H157, HCC15, and A549 were provided by Dr. P. P. Massion and were maintained in RPMI 1640 medium (Gibco). A549 cells were maintained in Ham's F-12K medium (Gibco). All cell lines were supplied with 10% (v/v) fetal bovine serum and incubated at 37°C in 5% CO_2.

Constructs and retroviral transductions. Secretable TNF-α was generated by replacing amino acids -76 to -1 containing the cytoplasmic signal-anchor for type II membrane protein and a short extracellular region with a sequence coding for the IL-2 signal peptide (IL2sp, amino acids -20 to -1) that directs the transport of TNF-α to the outer cellular space and produces a solely secretable form of 17-kDa TNF-α. This was done by amplifying mouse wild-type TNF-α cDNA using forward primers flanked by BamH1-IL2sp. The amplified fragment was isolated

and purified by gel electrophoresis. The restriction sites at each end allowed ligation of the IL2sp-TNF-α fragment into the BamH1-EcoR1 site of LZRS-IRES-Neo retroviral vector, conferring neomycin resistance. The mTNFΔ1-9, K11E sequence encoding a mutant transmembrane TNF-α molecule with a deletion at the cleavage site between presequnce and mature membrane TNF-α (BCCM/LMBP plasmid collection, Ghent University) was also cloned into LZRS. This mutation prevents cleavage of the 26-kDa membrane TNF-α into secretory TNF-α isoform. An empty LZRS vector was used as a control vector.

Surface expression of TNF-α. Cells were detached from tissue culture plate and incubated with anti-TNF-α antibody (1 µl/2.5 x 10^4 cells, Southern Biotech) for 30 minutes on ice without permeabilization. PE conjugated secondary antibody (0.125 µg/10^6 cells/100 µl) was added for 30 min on ice. Surface expression of TNF-α was measured using flow cytometry. Data are presented as percent of viable cells.

***In Vivo* murine tumor model.** Control, IL2spTNF-α, and mTNF-α cell lines (10^6 cells in 100 µl of PBS) were implanted subcutaneously into WT, TNFR-DKO or TNFR-DKO-BMT mice. Mice were sacrificed 15 days post-implantation, tumors were excised and the volume was calculated by multiplying tumor length by width by height.

BrdU assay. The BrdU ELISA was performed according to the manufacturer's instructions. Briefly, 1000 cells/well were seeded in triplicate in a 96-well plate. Cells were allowed to attach for 8 hours. BrdU label was added to each well and incubated for an additional 24 hours. Absorbance was analyzed at 450-540 nm.

Cell viability assay. Cell viability was measured by seeding 5000 cells/well in a 96-well plate for 48 hours. Cells were labeled with 100 µl of PBS containing 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl] 2,5,-diphenyltetrazolium bromide (MTT) (Sigma). After 2 hours of incubation at 37°C, cells were lysed with 0.1 ml DMSO. Photometric measurement was carried out at 540 nm.

Leukocyte quantification in tumors. Tumor tissues were finely minced and incubated in 5 mL dissociation solution (RPMI medium supplemented with 5% FBS, and 1 mg/mL of Collagenase type IV (Worthington)) for 30 min at 37°C. To obtain a single-cell suspension, cells were passed through 70-µm nylon cell strainer (Becton Dickinson, NJ). Cells were washed with FACS buffer (PBS, 2 mM EDTA, 0.5% BSA) and incubated for 5 min in RBC lysis buffer solution (155 mM NH4Cl, 12 mM NaHCO3, 0.1 mM EDTA). Cells were washed twice in FACS buffer and incubated with anti-CD3 (Biolegend), -Ly6G (BD Pharminogen), -F480 (eBioscience) and -CD11b+ (BD Pharminogen). After two washes, labeled cells were resuspended in vital dye 7-AAD (BD Pharminogen) and subjected to flow cytometry on LSRFortessa flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ) and analyzed by using FlowJo software (TreeStar, Ashland, OR).

Myeloid cell-trafficking to tumor. Control vector or mTNF-α-expressing tumor cells were injected subcutaneously into wild-type mice. After 12 days, freshly isolated myeloid cells were labeled with 5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) fluorescent tracking dyes and injected into retro-orbital space (5×10^6 cells/animal). Eighteen hours later, tumors were

harvested and single-cell suspensions were made. CFSE labeled cells were detected using flow cytometry.

Caspase 3/7 activity. Freshly isolated CD11b+ (10^5 /96-well) were cocultured with fixed control, control+recTNF-α (100 U/mL), and mTNF-α B16F10 cells at CD11b+/tumor cells ratio of 1:10 for 5 hours. Apoptosis was quantified in the form of caspase-3/7 activation using the Apo-One fluorometric assay system from Promega Corporation (Madison, WI) according to the manufacturer's protocol.

Measurement of intracellular ROS. The oxidant-sensing probe CM-H2DCFDA (Invitrogen) was used to detect intracellular reactive oxygen species (ROS). Freshly isolated CD11b+ cells were loaded with 10 μM CM-H2DCFDA, and cocultured with fixed B16F10 control, control+100 U/ml of recombinant TNF-α, mTNF-α or mTNF-α+2 mM N-acetyl-cysteine (NAC) for 8 hours. Fluorescence was determined using a luminescence spectrophotometer (Spectra max, Molecular Devices) with an excitation wavelength of 429 nm and emission wavelength of 517 nm.

Migration assay. Three-μm-pore-size transwells (Costar Corp) were coated with fibronectin for 30 min and blocked with 2% (w/v) BSA for 1 hour at room temperature. CD11b+ cells isolated from the bone marrow of wild-type mice (EasySep, StemCell Technologies) were placed in the upper chambers (105 cells/well). Tumor-conditioned media (600 μl/well) from each tumor cell line (2x106 cells, 48 hours, 37°C, 5% CO2) were added to the lower chamber. After 7 hours of incubation, filters were fixed in 10% formalin and stained in 0.5%

crystal violet-0.2M Boric Acid for 30 minutes at room temperature. Migrated cell were counted under a high power (40x) lens.

ROS imaging using confocal microscopy. RAW 264.7 cells were plated overnight at a density of 106. Next day cells were incubated with fixed B16F10 control, control+100 U/ml of recombinant TNF-α, mTNF-α or mTNF-α+2 mM N-acetyl-cysteine (NAC). After 8 hours of incubation, cells were washed with PBS and loaded with 10 μM solution of CM-H2DCFDA for 20 minutes at 37°C. After PBS wash, the remaining attached RAW 264.7 cells were fixed in 1% paraformaldehyde and mounted. Cellular fluorescence was monitored at 480/30 nm (excitation) and 535/40 nm (emission).

TNF-α cytotoxicity assay. Overnight cultured CD11b+ cells or RAW 264.7 cells (2.5x104/100 μL/well, target) were cocultured with Paraformaldehyde-fixed (Zhang et al., 2008) control, control+100 U/ml of recombinant TNF-α, mTNF-α or mTNF-α+2 mM N-acetyl-cysteine (effector) at target:effector ratio of 1:10 and incubated for 48 hours. Cells were labeled with 100 μl of PBS containing 0.5 mg/mL of MTT for 2 hours at 37°C then lysed with 0.1 ml DMSO and photometric measurement was carried out at 540 nm.

P65/Caspase-3/Bax/Bcl-2 immunoblot analysis. RAW 264.7 cells were plated at a density of 106 cells overnight (37°C, 5% CO2). Fixed control, control + recTNF-α (100 U/mL), and mTNF-α B16F10 cells were co-cultured with RAW 264.7 at T/E ratio of 1:10. Cells were incubated at 37°C in 5% CO2 for 10 minutes (p65), 30 minutes (caspase-3), and 6/24 hours (Bax/Bcl-2). After each incubation period, RAW 264.7 cells were collected and the whole cell lysate was

evaluated for total phospho-NF-κB-p65, total NFκB-p65, caspase-3 (Cell Signaling Technology) and Bax/Bcl2 (eBioscience) by Western blot analysis.

TNF-α ELISA and immunoblotting. The expression of secretory levels of TNF-α in human lung caner cell lines was measured in cell cultured supernatant of 106 cells incubated for 24 hours, using ELISA kit (R&D Systems). Transmembrane TNF-α on cell surface of transduced LLC tumor cells was analyzed by isolating the membrane fraction protein and subjecting to western blot. Cells were lysed in 500 µl of membrane buffer [250 mM Sucrose, 20 mM HEPES (7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1mM DTT, PI Cocktail (III)]. Cells were scraped immediately and placed in 1.5 ml eppendorf tube. The lysate was passed through a 25 G needle 10 times and incubated on ice for 20 minutes. Lysate was spun at 10000 G for 10 min. The supernatant was spun in an ultracentrifuge at 100,000 G for 1 hour. The pellet was resuspended in 50 µl of RIPA buffer [3M NaCl, 1M Tris, 0.5M EDTA, 10% SDS, 1% NP40 substitute].

Immunohistochemistry/fluorescence and morphometry. Paraffin-embedded tumor tissues were used for determination of Ki67 (Novocastra), platelet endothelial cell adhesion molecule-1 (PECAM-1; clone 557355, Pharmingen), CD3 (Santa Cruz), B220b (Pharmingen), ER-HR3 (Santa Cruz), F4/80 (eBioscience). For immunofluorescence analysis, nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; Sigma). The images were visualized using a Ziess Axioplan 2 microscope (Carl Ziess MicroImaging). Images were photographed with a CoolSNAP Hq CCD camera (Photometrics). For confocal

analysis, the LSM510 (Zeiss) microscope was used to capture 1 μm optical slices (z stack); the images were analyzed with Metamorph v5.0 (Universal Imaging Corp.). Morphometry was performed by obtaining 5 digital images at defined magnification taken at random from each section. For morphometric evaluation care was taken to exclude regions of necrosis within each field.

Tissue microarray. The paraffin-embedded tissues were sampled from archived conventional tissue blocks. The tissue microarrays were constructed with a Beecher instruments tissue arrayer by sampling the three representative areas (0.6 mm) of tumor from the original blocks of tumor and transferring them into a new array block. TNF-α immunoreactivity was evaluated semiquantitatively based on the intensity of staining. It was scored as 1+ (low-moderate), and 2+ (intense). Samples with no staining or very weak staining were considered negative, and samples with moderate to intense staining were considered positive.

Survival analysis. A cohort published by Shedden et al. was analyzed for disease-free survival (Shedden et al., 2008). The data set included gene-expression profiles for 442 lung adenocarcinomas with high-quality gene-expression data, pathological data and clinical information. The association between gene expression and the survival was examined using the Cox proportional hazard model. Kaplan-Meier curves were generated to visualize the survival pattern by dichotomizing the gene-expression. The subgroup analysis of mTNF-α and TACE was done by dividing the cohort into 4 groups of high TNF-α with high/low TACE or low TNF-α with high/low TACE. Log-rank overall tests

were performed for the 4 groups. All statistical analyses were performed using R (www.r-project.org).

Statistical analysis. The statistical significance between experimental and control groups was determined by Student's *t-test* or ANOVA followed by Tukey's post-test using Prism software (Graphpad, San Diego, CA). A *P*-value of <0.05 was considered statistically significant.

Results

Membrane TNF-α isoform reduces tumor growth

The effects of different TNF-α isoforms on malignant tumor phenotypes were investigated using murine Lewis Lung Carcinoma (LLC) sublines expressing either sTNF-α (LZRS-IRES-IL2spTNF-α) or mTNF-α (LZRS-IRES-mTNFΔ1-9) by retroviral transduction (Figure 5A). Cells transduced with empty vector (LZRS-IRES-Neo) were used as control. Untransduced LLC cells exhibited undetectable levels of TNF-α expression as determined by ELISA. The relative expression of sTNF-α and cell surface expression of TNF-α by transduced cells were confirmed by ELISA and flow cytometry respectively. sTNF-α expression was detected in IL2spTNF-α-expressing cells at 5 ng/ml. Surface expression of TNF-α was not detected in control and IL2spTNF-α tumor cells (mean fluorescent intensity of 150 and 202 respectively). In contrast, mTNF-α-expressing LLC cells displayed 7.1- and 5.3-fold increase in surface TNF-α as compared with control and IL2spTNF-α, respectively (Figure 5B).

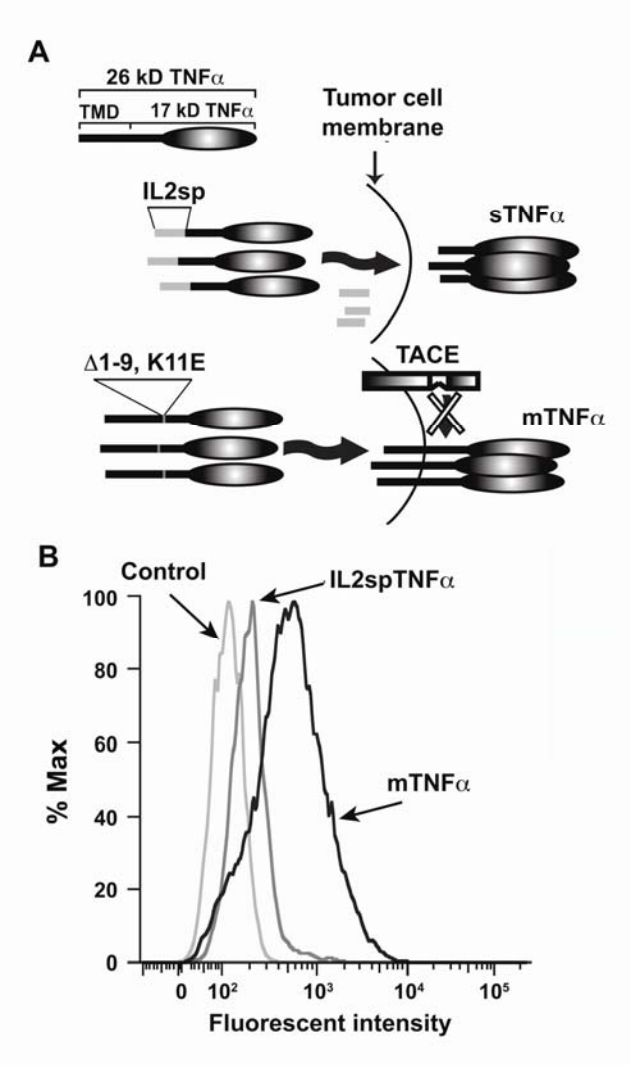

Figure 5. Generation of membrane and soluble TNF-α-expressing cells. **(A)** Schematic representation of TNF-α mutant which has the region coding for the TNF-α transmembrane domain (TMD) replaced with region coding for interleukin-2 (IL-2) signal peptide to generate soluble TNF-α (sTNF-α) and TNF-α lacking TACE cleavage site (Δ1-9, K11E) to generate membrane TNF-α (mTNF-α). **(B)** Expression of transmembrane TNF-α on the surface of LLC tumor cells transduced with empty vector (control), IL2spTNF-α or mTNF-α vectors was analyzed by flow cytometery.

To evaluate whether overexpressing various TNF-α isoforms affected growth or survival of tumor cells, *in vitro* proliferation and viability was assessed using the BrdU incorporation and MTT assay, respectively. Both IL2spTNF-α- and mTNF-α-expressing cells exhibited similar *in vitro* growth rates compared with control LLC lines (P>0.05; Figure 6A). IL2spTNF-α and mTNF-α LLC cell lines tested for viability also displayed similar levels of survival rate compared to control (P>0.05; Figure 6B).

To gain additional evidence that the membrane isoform did not reduce survival or viability and that these observations were not cell specific, B16F10-melanoma cell lines were transduced with retroviral constructs, containing mTNF-α or an empty construct as control cells. Similar to LLC lines, the proliferation rate (P>0.05) and viability (P>0.05) were not affected in mTNF-α-expressing B16F10 cells compared to control cells (Figure 7A and B). These findings are consistent with earlier studies in which it was shown that overexpressing the wild-type TNF-α in both LLC and B16F10 melanoma does not alter *in vitro* growth (Li et al., 2009).

LLC cell lines expressing soluble (IL2spTNF-α) or membrane (mTNF-α) isoforms were implanted subcutaneously into the flank of wild-type C57Bl/6 mice (WT). The same number of cells transduced with empty vector was implanted as a control. After 14 days, LLC tumors expressing IL2spTNF-α were ~7 fold (1214±122 mm^3) larger compared to control tumors (124.4±92 mm^3; n=5, P<0.0005; Figure 8A). By contrast, tumors expressing mTNF-α exhibited 65% reduction in tumor volume (105.8±29.3 mm^3) compared to control tumor

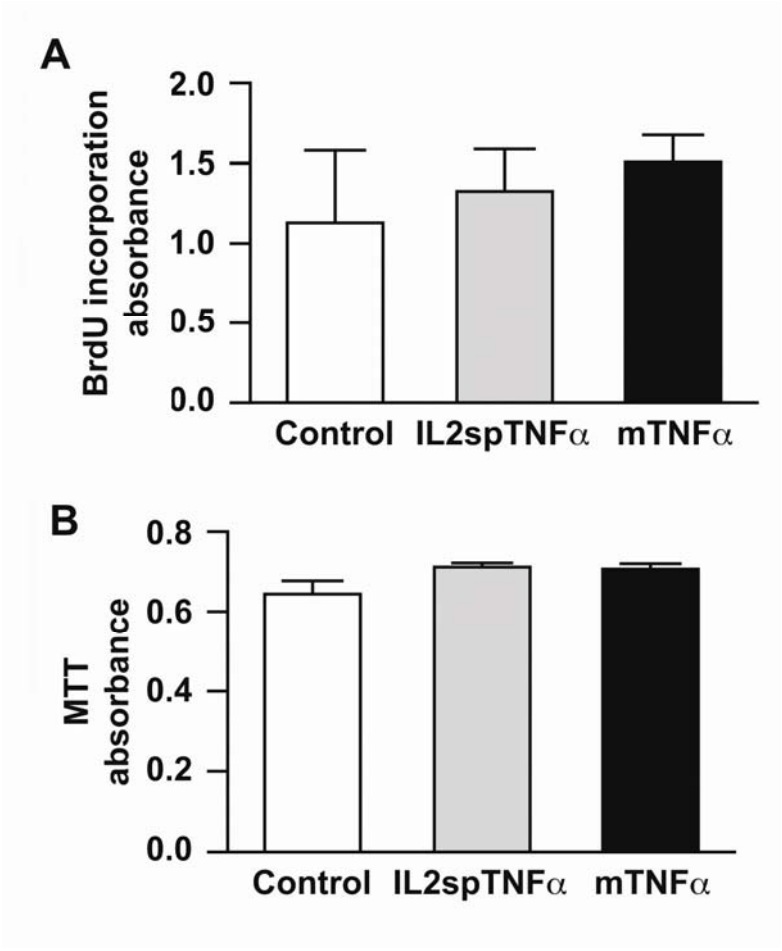

Figure 6. Expression of mTNF-α/sTNF-α does not affect proliferation and viability in LLC cells. (A and B) Proliferation rate and the viability of transduced tumor cells were determined by BrdU and MTT labeling assays respectively. All cell lines showed no significant difference in proliferation or viability. Data is representative of three independent experiments expressed as the mean±SEM.

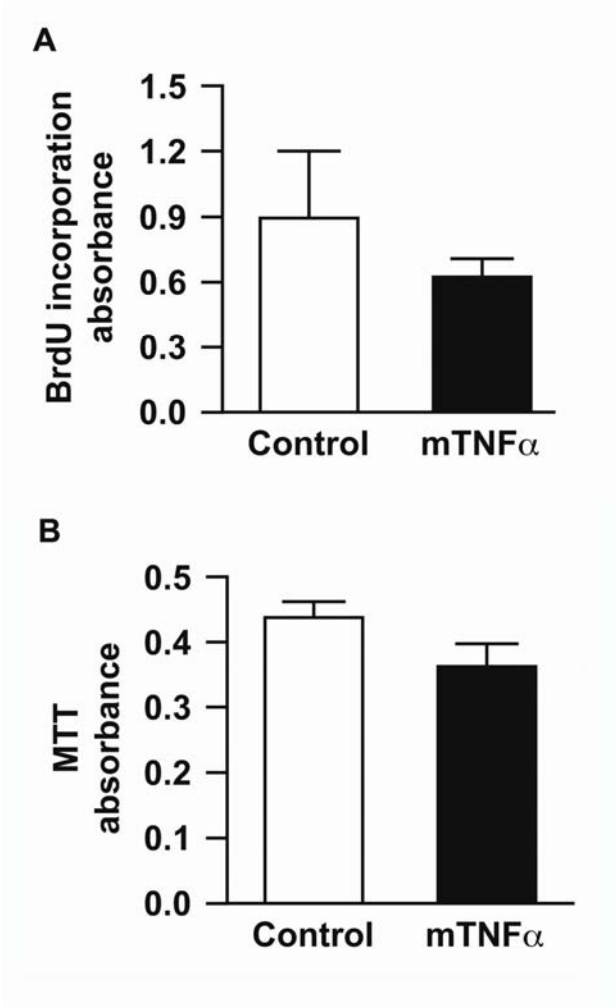

Figure 7. Expression of mTNF-α does not affect proliferation and viability in B16F10 melanoma cells. (A and B) *In vitro* proliferation **(A**) and the viability **(B)** of B16F10 cells transduced with control or mTNF-α vectors were determined by BrdU and MTT labeling assays, respectively. All cell lines showed no significant difference in proliferation (*P*=0.42) and viability (*P*=0.16). Data is representative of three independent experiments expressed as the mean±SEM.

(294.1±35.9 mm^3) (n=12, *P*<0.0005; Figure 8B). Similar growth reduction was observed with mTNF-α-expressing B16F10 tumors cell line when compared with matched controls (n=7, *P*<0.05; Figure 8C). In addition, tumor weight measurement followed similar pattern to tumor volume as presented in Figure 8D-F. These data suggested that different TNF-α isoforms have opposing effects on tumor size.

Expression of mTNF-α does not affect tumor proliferation or vascularity

To evaluated tumor vascular density and tumor cell proliferation rate, histological sections from control, IL2spTNF-α and mTNF-α LLC tumor were immunostained with platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody to evaluate microvessel density, and anti-Ki67 to assess tumor cell proliferation (Figure 9A). Histomorphometry of PECAM-1-positive areas showed no difference in vascular density amongst the tumors expressing different TNF-α isoforms versus control (*P*>0.05; Figure 9B). Furthermore, IL2spTNF-α- and mTNF-α-expressing LLC tumors showed no significant difference in immunoreactivity with the Ki-67 antibody compared to control tumors (*P*>0.05; Figure 9C).

Vascular density and cell proliferation analyses were also performed between B16F10 melanoma-derived tumors expressing mTNF-α versus control (Figure 10A). Similar to LLC tumors, no significant difference was observed in microvessel density and *in vitro* proliferation between the control and mTNF-α-expressing melanoma tumors (*P*>0.05; Figure 10B and C).

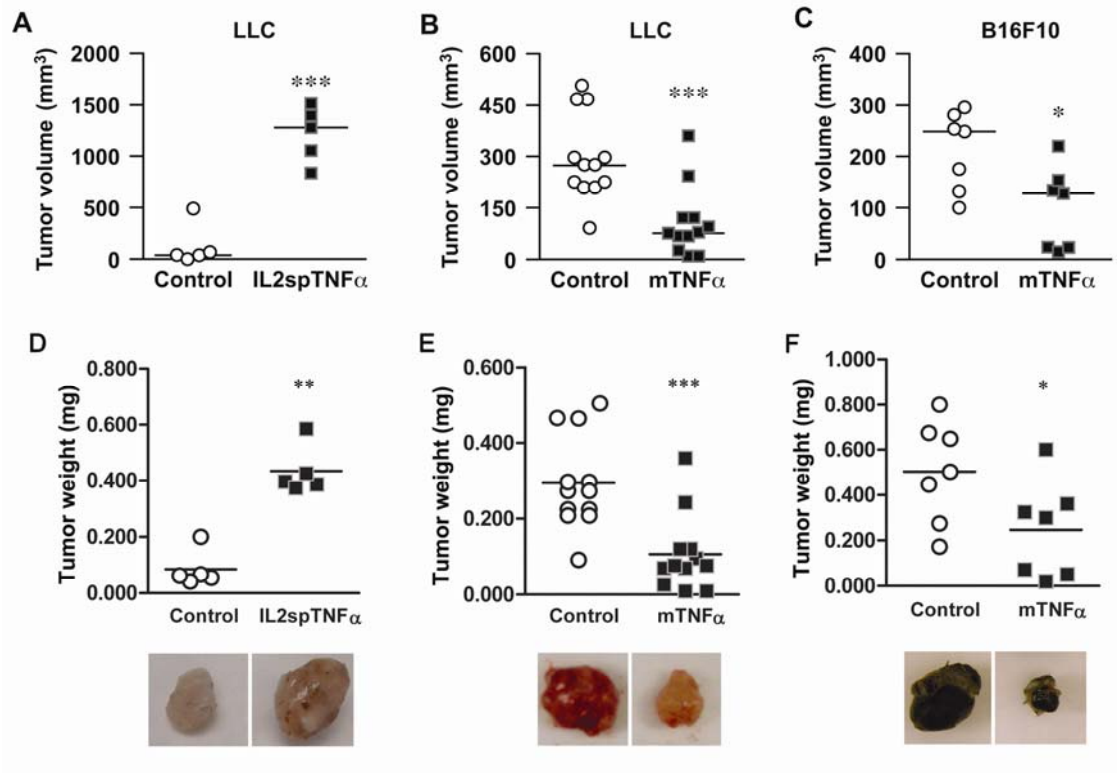

Figure 8. Membrane TNF-α-expressing tumor cells demonstrate delayed tumor growth. (A-C) *In vivo* growth of tumor cells transduced with control vector was compared to LLC lines expressing IL2spTNF-α **(A)**, or mTNF-α **(B)**, and B16F10 expressing mTNF-α **(C)**, by subcutaneous implantation in WT C57Bl/6 mice for 14 days. Tumor weight of **(D)** LLC control vector/IL2spTNF-α, **(E)** LLC control vector/mTNF-α and **(F)** B16F10 control/mTNF-α implanted in WT bl/6 mice. Photomicrograph of each tumor is shown with each experimental group. Each point represents an individual animal and the horizontal bar is the median. $*P<0.05$, $**P<0.005$,$***P<0.0005$, Student's *t*-test.

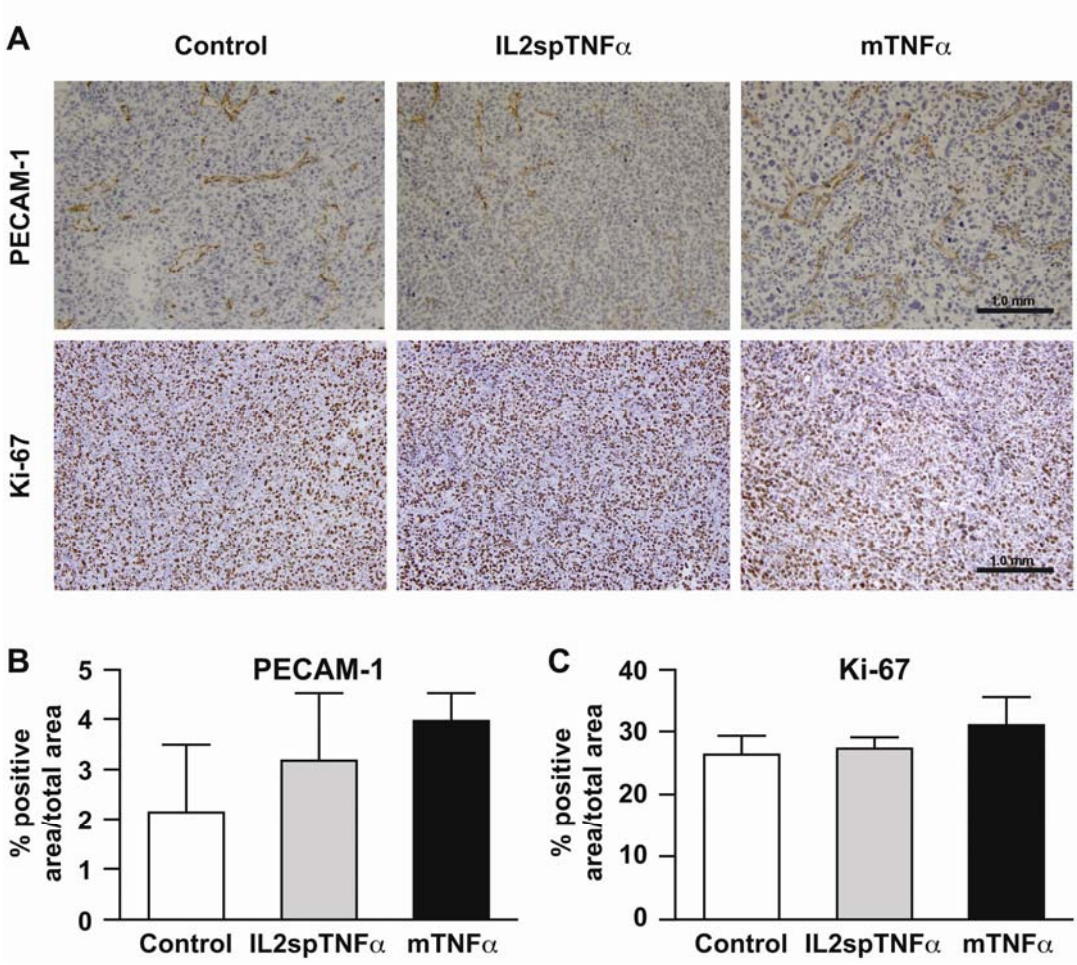

Figure 9. Expression of mTNF-α does not affect tumor proliferation or vascularity *in vivo*. (A) Representative sections of LLC tumors transduced with control, IL2spTNF-α and mTNF-α constructs were analyzed by immunohistochemistry for PECAM-1 or Ki-67 staining to define vascularity or proliferation, respectively. **(B and C)** Percentage of PECAM-1-positive **(B)** or Ki-67-positive area **(C)** in control, IL2spTNF-α and mTNF-α in LLC tumors was quantitated. There was no significant difference between the cohorts for either PECAM-1-positive or Ki-67-positive cells ($P>0.05$), 1-way ANOVA with Tukey's post-test.

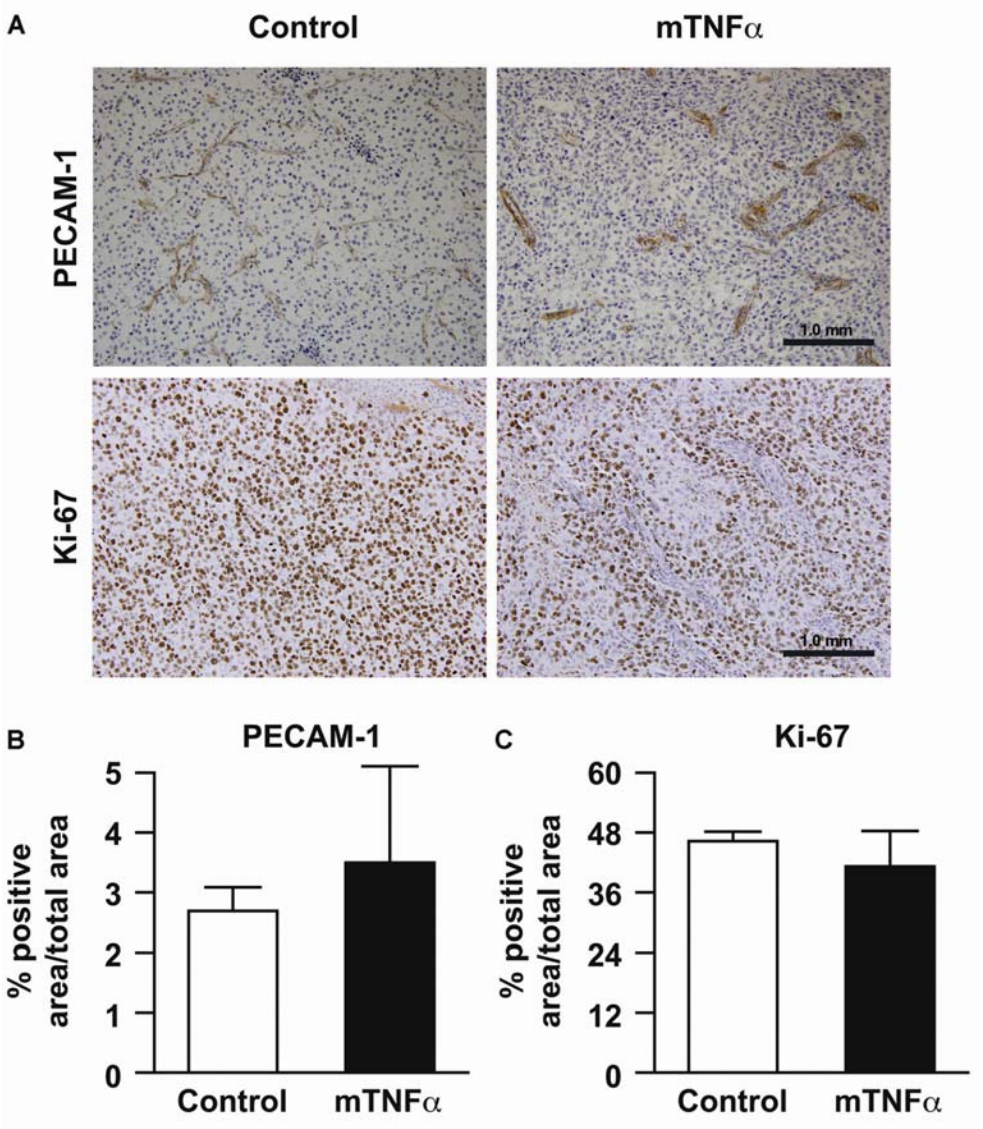

Figure 10. Expression of mTNF-α does not affect proliferation and vascularity in B16F10 melanoma tumors. (A) Representative sections of control, IL2spTNF-α and mTNF-α LLC tumors were analyzed by immunohistochemistry for PECAM-1 or Ki-67 staining to define vascularity or proliferation, respectively. **(B and C)** Number of PECAM-1-positive **(B)** or Ki-67-positive **(C)** cells in control, IL2spTNF-α and mTNF-α in LLC tumors were quantitated. There was no significant difference between the cohorts for either PECAM-1-positive (*P*=0.37) or Ki-67-positive (*P*=0.17) cells.

Membrane TNF-α-expressing tumors are devoid of tumor associated myeloid cells

It is often assumed that TNF-α-mediated tumor promotion is secondary to TNF-α-mediated inflammation. To determine whether mTNF-α-expressing tumors had altered composition of inflammatory cells, we quantified LLC tumor-associated T-cells (anti-CD3), B-cells (anti-B220b), neutrophils (anti-Ly6G) and myeloid-monocytic lineage (anti-ER-HR3, CD11b, F4/80) using immunohistochemistry staining and flow cytometric analysis of single-cell suspension of tumors. Both immunostaining and flow cytometric analysis showed no significant difference in T-cell content among control, IL2sp TNF-α and mTNF-α-expressing tumor cells (Figure 11A-C). Anti-B220b staining revealed only rare, infiltrating B-cells for all tumor groups (data not shown). Flow cytometric analysis of anti-Ly6G showed no significant difference in neutrophils population within control and mTNF-α-expressing tumors (P>0.05; Figure 12).

Interestingly when tumor-associated myeloid cell population was evaluated a significant difference was observed in mTNF-α-expressing tumors compared to control and IL2spTNF-α tumors. Histological staining of mTNF-α-expressing LLC tumors for ER-HR3, a myeloid marker reactive to ~70% of circulating monocytes and a subset of mature tissue macrophages (de Jong et al., 1994), had fewer number of infiltrated ERHR3+ cells (0.04±0.02%) versus control (6.4±0.37%) or IL2sp-TNF-α tumors (16±1.03%) (P>0.005; Figure 13A and B).

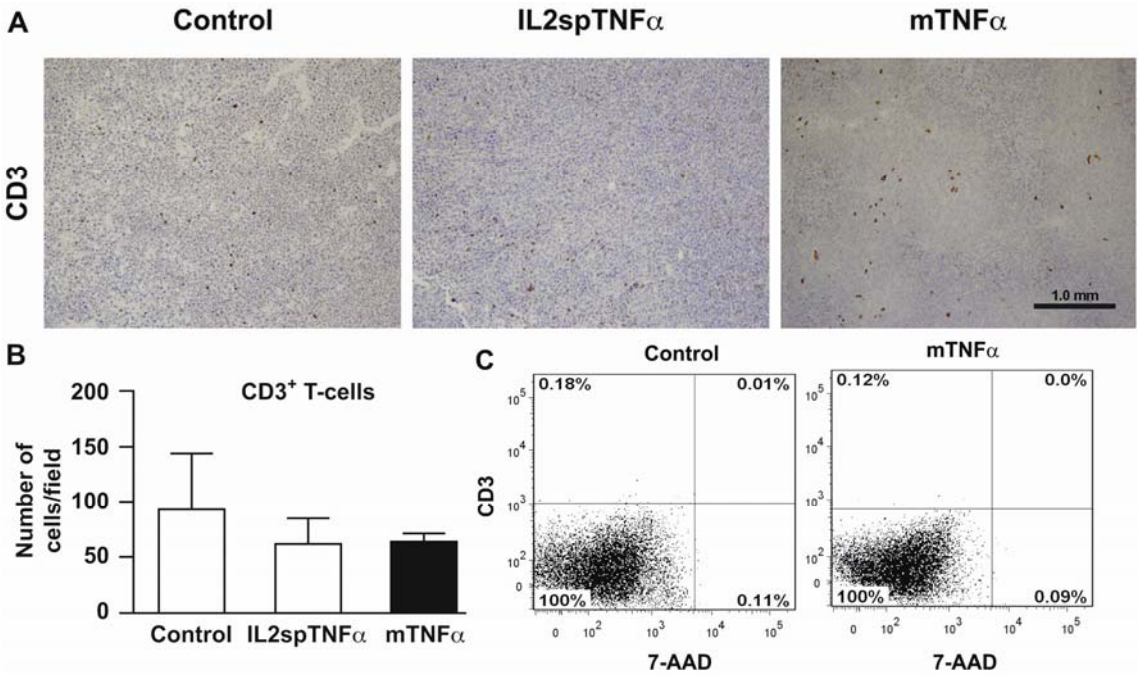

Figure 11. Tumor derived mTNF-α does not affect T-cell content in tumor. **(A)** Representative sections of control, IL2spTNF-α and mTNF-α-transfected LLC tumor cells were analyzed by immunohistochemistry for CD3-positive T-cell staining. **(B)** Number of CD3-positive cells in control, IL2spTNF-α and mTNF-α in LLC tumors was quantitated. There was no significant difference between the cohorts for CD3-positive cells. **(C)** Representative flow cytometric analysis of CD3-positive T-cells in LLC tumor cell suspension. Dot plots show CD3/7-AAD population in control (left) and mTNF-α (right) tumors.

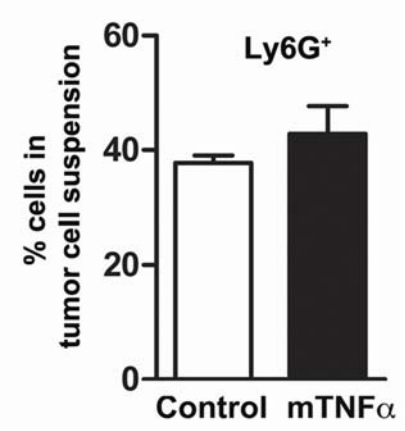

Figure 12. Tumor derived mTNF-α does not affect neutrophil content in tumor. Quantification of flow cytometeric analysis of Ly6G-positive neutrophils in LLC tumor cell suspension.

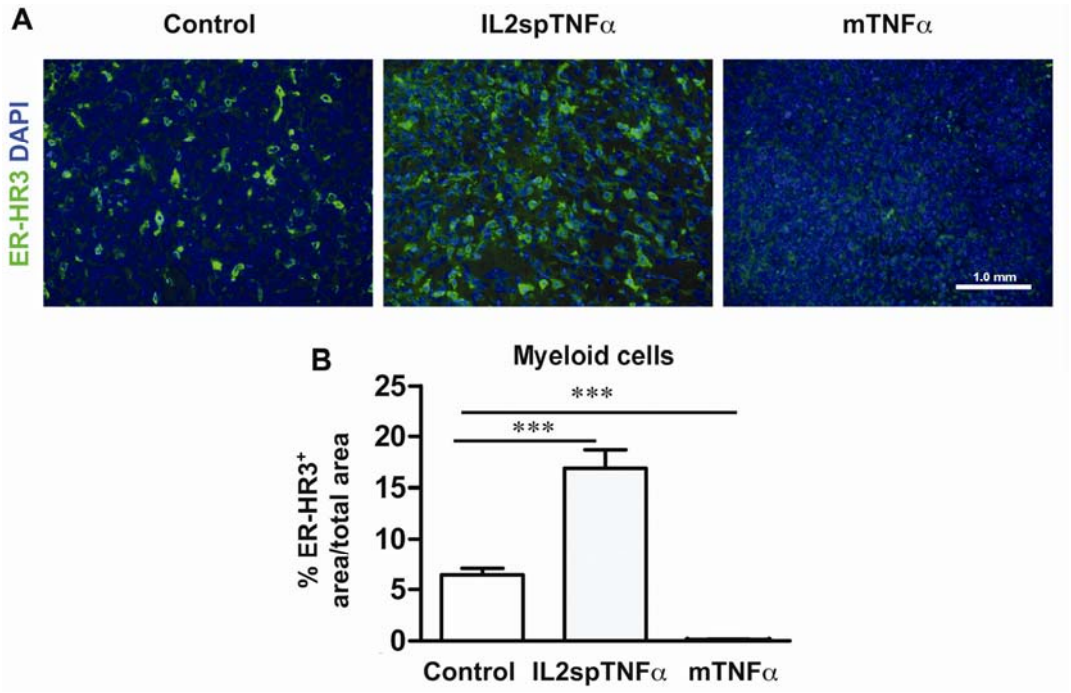

Figure 13. Membrane TNF-α-expressing tumors are devoid of ER-HR3-positive myeloid cells. (A) ER-HR3 staining of LLC tumor cells, expressing various TNF-α isoforms. Control (left), IL2spTNF-α (middle), and mTNF-α (right) LLC tumor sections from wild-type bl/6 were stained with ER-HR3 myeloid markers (green). **(B)** Percentage of ER-HR3-positive cells in LLC tumors transduced with control or different of TNF-α isoforms was quantitated. There was a significant decrease in the number of ER-HR3-positive cells in LLC tumors expressing mTNF-α isoform compared to control tumors.

Further evaluation of single-cell suspension of tumors showed significantly lower number of CD11b+ myeloid cells (52.17±6.1%; *P*>0.005) and F4/80 macrophages (3.75±2.4%; *P*>0.005) in mTNF-α-expressing tumors as compared to control (Figure 14A-D). Anti-F4/80 staining of tumor sections further confirmed the significant reduction of F4/80-positive macrophages in mTNF-α expressing tumors (Figure 14E and F).

To test whether the tumor inhibitory effects of mTNF-α required the presence of TNF-α receptors, tumor growth was assessed in TNF-α receptors deficient mice (TNFR-1 and TNFR-2 double knockout, TNFR-DKO). Mice were implanted with LLC cell line expressing various TNF-α isoforms. LLC lines expressing mTNF-α isoform did not generate significantly smaller tumors (110.5±17.6 mm^3) when compared with their paired control tumors (161.7±29.6 mm^3, n=5) (*P*>0.05; Figure 15A). In addition, implantation of mTNF-α-expressing LLC tumor cells in TNFR-DKO mice restored the ER-$HR3^+$ myeloid population in mTNF-α-expressing tumors (0.91±0.16% ER-HR positive area/total area in mTNF-α vs. 0.67%±0.16% in control; *P*>0.05; Figure 15B). Similar results were observed in B16F10 line (data not shown).

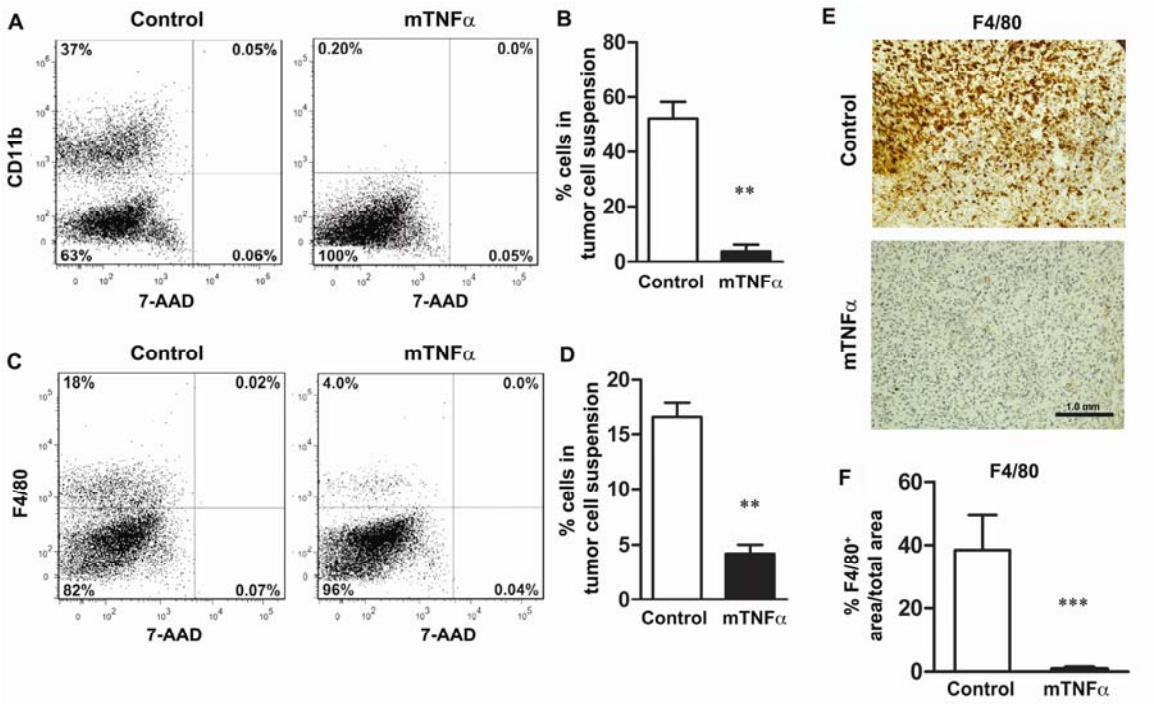

Figure 14. Membrane TNF-α-expressing tumors are devoid of CD11b- and F4/80-positive myeloid cells. (A and C) Representative flow cytometric analysis of CD11b- and F4/80-postitive population in LLC tumor cell suspension. Dot plots show CD11b/7-AAD **(A)** and F4/80/7-AAD **(C)** from one representative animal for each group. **(B and D)** Percentage of CD11b- and F4/80-positive cells were quantitated in control and mTNF-α tumor cell suspensions. **(E)** Representative sections of control and mTNF-α-transfected LLC tumors were analyzed by immunohistochemistry for F4/80+ macrophages. **(F)** Number of F4/80-postivie cells in control and mTNF-α in LLC tumors was quantitated. Data are presented as mean±SEM, ***P*<0.005, ****P*<0.0005; Student's *t*-test.

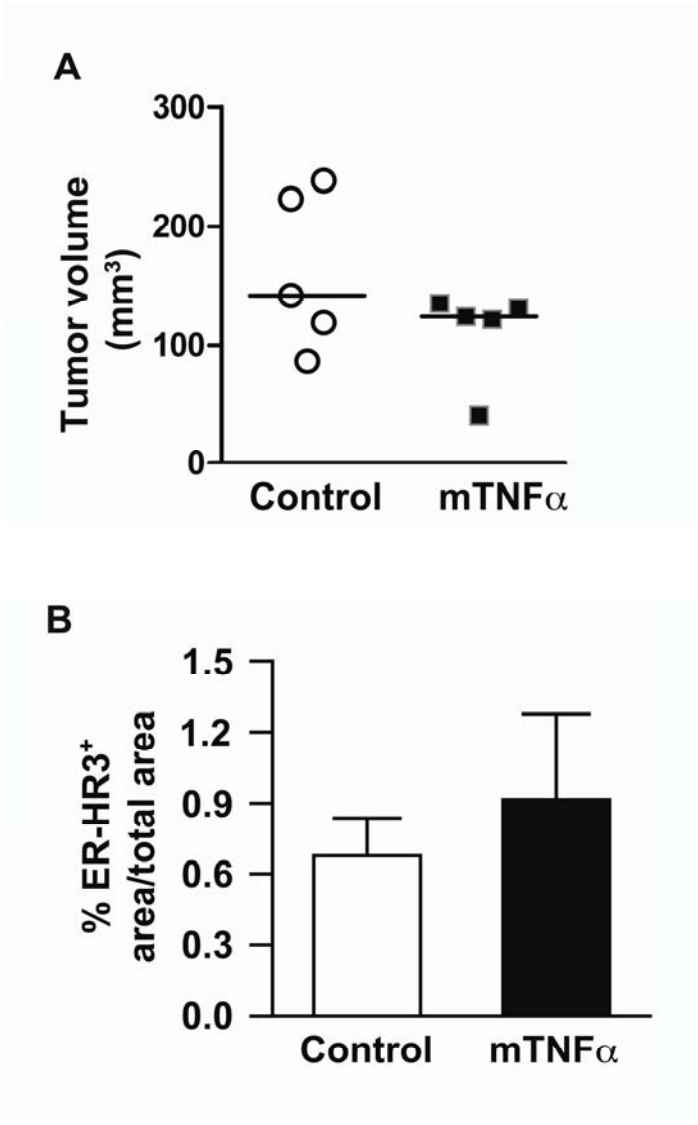

Figure 15. Tumor inhibitory effect of mTNF-α requires the presence of TNF-α receptors. (A) Control and mTNF-α-expressing LLC tumor cells were implanted subcutaneously in TNFR-DKO mice for 14 days. The average±SEM are shown for each group (n=5 animals). **(B)** Morphometric quantification of ER-HR3 staining of control or mTNF-α-expressing LLC tumor, from TNFR-DKO mice. Student's *t*-test.

Restoration of myeloid cell population in mTNF-α-expressing LLC tumors in TNFR-DKO host prompted us to further evaluate the requirement of TNFR signaling in inflammatory cells (i.e bone marrow-derived cells). Therefore, tumor growth was assessed in WT mice receiving bone marrow (BM) transplants from TNFR-DKO mice (referred to as BMT-TNFR-DKO mice). Similar to experiments in TNFR-DKO host, LLC line expressing mTNF-α isoform, implanted into BMT-TNFR-DKO, did not generate smaller tumors in mice engrafted with TNFR-deficient BM (497.2±137.6 mm^3) as compared with control tumors (387.9±95.94 mm^3) (*P*>0.05; Figure 16A). Furthermore, tumor associated myeloid cell populations were quantified in BMT-TNFR-DKO mice. The overall percentage of myeloid populations in mTNF-α-expressing LLC tumors (7.8±0.4%) was similar to control tumors (7.5±1.4%) (*P*>0.05; Figure 16B and C). These data suggested that tumor derived mTNF-α significantly reduced myeloid population within the tumor microenvironment. Since this effect was abrogated in WT mice transplanted with TNF-α receptor deficient BM, it could be concluded that intact TNF-α signaling through its receptor in bone marrow derived cells was required for this effect, and that this effect was not mediated by secondary factors from the tumor cells.

Membrane TNF-α-derived soluble factors do not affect CD11b+ myeloid cell migration/recruitment

One possible explanation for the reduction of myeloid cells observed in mTNF-α-expressing tumors was that such tumors exhibited reduced expression of necessary signals for myeloid recruitment. Using a modified Boyden chamber

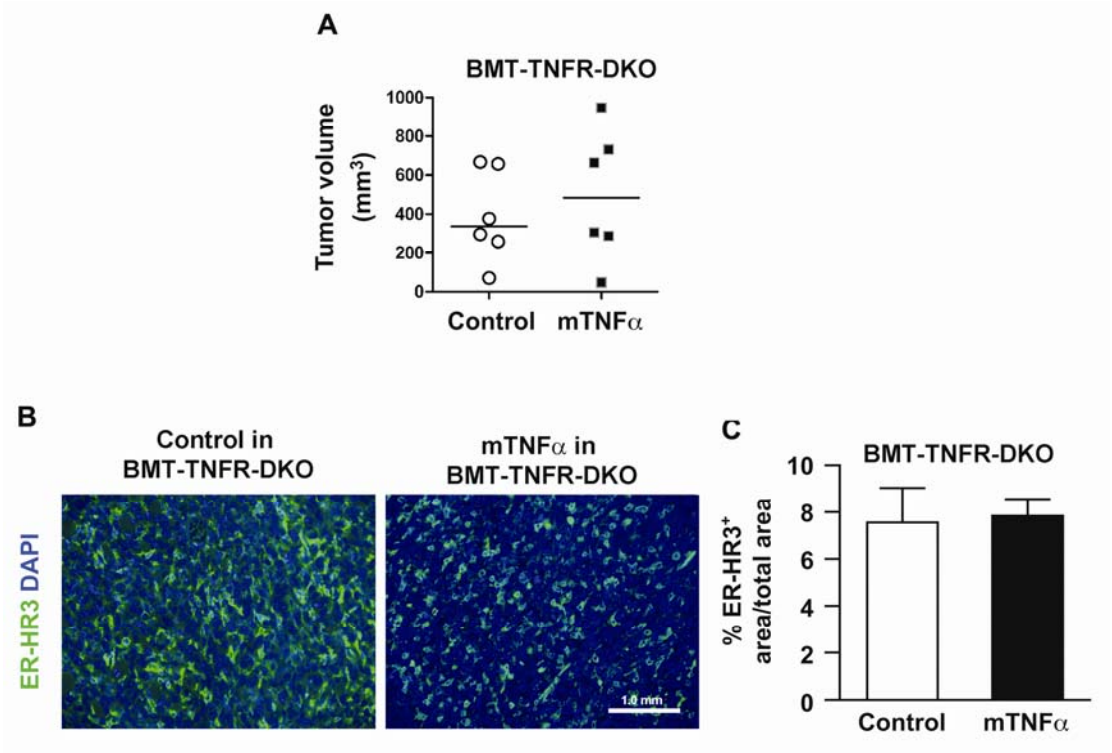

Figure 16. Tumor-inhibitory effects of mTNF-α requires signaling between TNF-α and its cognate receptors. (A) Control and mTNF-α-expressing LLC tumor cells were implanted subcutaneously in WT bl/6 mice received bone marrow (BM) transplant from TNF-α receptors1/2 knockout donor (BMT-TNFR-DKO mice) for 14 days. The mean is shown for each group (n=6 animals). **(B)** Representative ER-HR3 immunofluorescence staining from control and mTNF-α-transduced tumors. **(C)** Percentage of ER-HR3-positive cells in control and mTNF-α-expressing LLC tumors from WT mice with BMT from TNFR-DRKO donor was quantitated. There was no significant difference between the cohorts for ER-HR3-positive cells (n=3). Data are presented as mean±SEM, Student's *t*-test.

assay, the ability of conditioned media (CM) from LLC and B16F10 melanoma cell lines expressing various forms of TNF-α to promote migration (i.e. recruitment) of primary murine CD11b+ myeloid cells was evaluated. CM from mTNF-α did not inhibit migration of CD11b+ as compared to control-CM in both LLC and B16F10 melanoma line (Figure 17A and C). An increase in CD11b+ myeloid cells migration was observed in CM derived from both IL2spTNF-α-expressing LLC (~1.5-fold; Figure 17B) and B16F10 line (~4-fold; Figure 17D). This may be attributed to the presence of TNF-α itself, which is known to induce chemotactic response (de Jong et al., 1996; Torrente et al., 2003). These results suggested that the relative paucity of myeloid cells in mTNF-α-expressing tumors was likely not due to reduced expression of key cytokines, necessary for myeloid extravasation and migration into the tumor.

Next, the ability of control and mTNF-α-expressing LLC tumors to effectively recruit myeloid cell *in vivo* by adoptive transfer of CFSE labeled CD11b+ into tumor bearing mice was evaluated. After 18 hours post injection CFSE-positive myeloid cells were quantified in each tumor type by flow analysis of single-cell suspension of tumor digests. The overall number of CFSE-positive cells in mTNF-α-expressing LLC tumors (58±21) was similar to control tumors (68±12.57) ($P>0.05$; Figure 18A and B). These data demonstrate that reduced myeloid cells in mTNF-α-expressing tumors was not due to impaired recruitment of circulating cells.

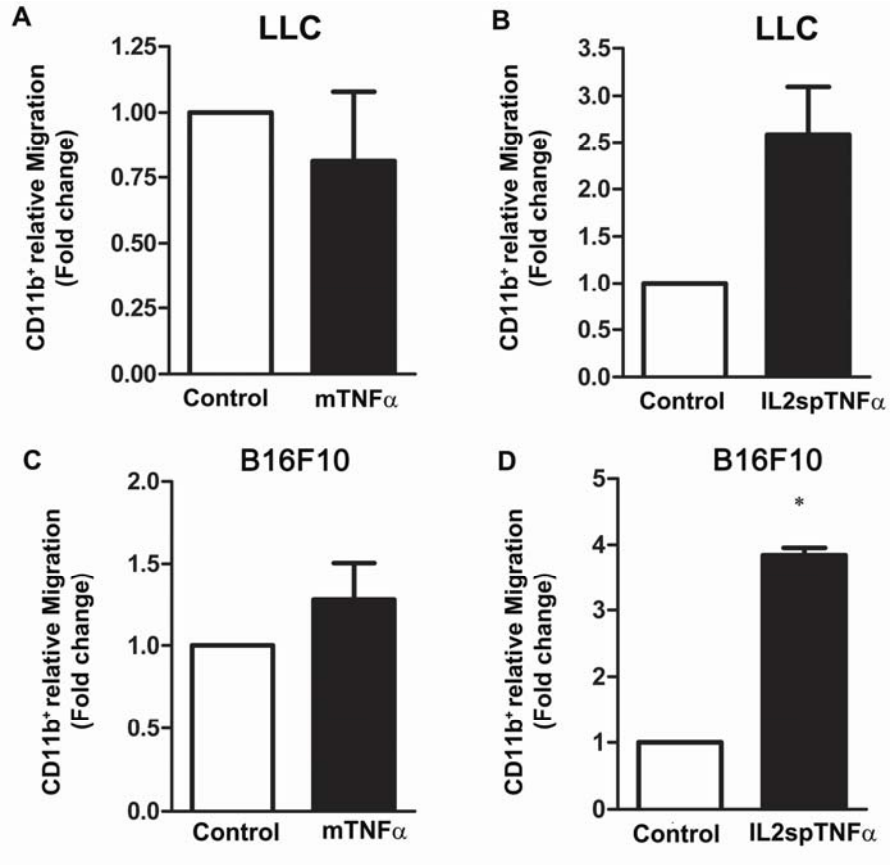

Figure 17. Soluble factors derived from mTNF-α do not affect the rate of CD11b+ myeloid cell migration compared to control. (A and B) Transwell migration assay of primary CD11b+ cells treated with conditioned media derived from LLC tumor cells transduced with control/mTNF-α **(A)** or cotnrol/IL2spTNF-α **(B)** constructs. **(C and D)** Transwell migration assay of primary CD11b+ cells treated with conditioned media derived from B16F10 tumor cells transduced with control/mTNF-α **(C)** or cotnrol/IL2spTNF-α **(D)** constructs. Data presents the mean±SEM.

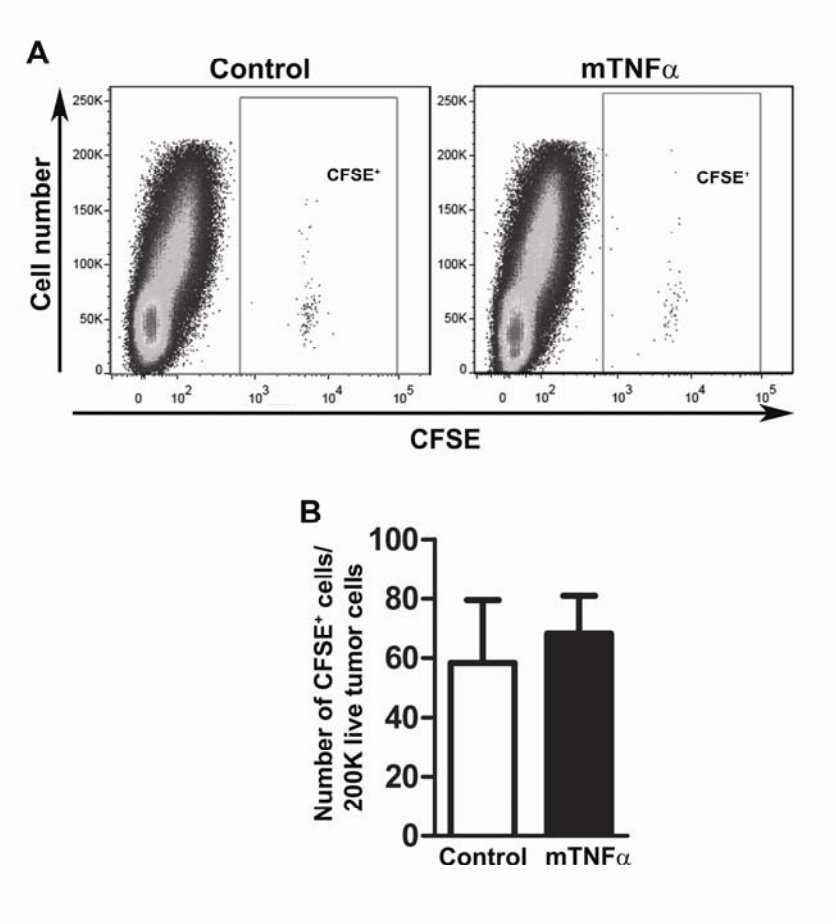

Figure 18. Reduced myeloid cells in mTNF-α-expressing tumors is not due to impaired recruitment of circulating cells. (A) Representative flow cytometric analysis of CFSE-positive cells presented in LLC tumor suspension expressing either control (left) or mTNF-α (right) isoform. **(B)** Quantification of CFSE-positive cells detected in a given number of tumor suspension (n=3 for each tumor type). Data are presented as mean±SEM, *P*>0.05.

Membrane TNF-α induces cell death through apoptosis-independent pathway

To investigated if the distinct TNF-α isoforms exerted cytotoxic effects on myeloid cells, freshly isolated CD11b+ cells (target) were mixed with 1% paraformaldehyde-fixed B16F10 melanoma cells (effector) expressing empty vector with or without 100 U/ml recombinant murine TNF-α, ($FxB16_{cont}$ or $FxB16_{cont}$+rTNF-α) or fixed mTNF-α-expressing B16F10 cells ($FxB16_{mTNF}$) at an effector:target ratio of 10:1. As measured by the MTT assay (Figure 19A), $FxB16_{mTNF}$ resulted in more than 60±29% cytotoxicity of CD11b+ myeloid cells after 48 hours of incubation, as compared to CD11b+ cells incubated with $FxB16_{cont}$ (P<0.005). CD11b+ in the presence of $FxB16_{cont}$+rTNF-α showed less than 1% cytotoxicity in compared to control. The activation of apoptotic pathway as the mechanism of mTNF-α-induced myeloid cell death by determining the caspase-3/7 enzymatic activity in CD11b+ cells was assessed. Compared to control, CD11b+ myeloid cells cocultured with $FxB16_{cont}$+rTNF-α or $FxB16_{mTNF}$ did not show any significant increase in the level of caspase 3/7 activity (P>0.05, Figure 19B).

To assess the activation of apoptotic pathway in RAW 264.7 cells, the level of Bax/Bcl-2of and cleavage/activation of caspase-3 proteins in RAW 264.7 was determined. The data suggested that the Bax/Bcl-2 ratio (Figure 20A) and active-caspase-3 (Figure 20B) proteins in RAW 264.7 cells treated with both soluble and membrane TNF-α had no significant changes when compared to control. Together these findings are indicative of another death pathway independent of apoptosis.

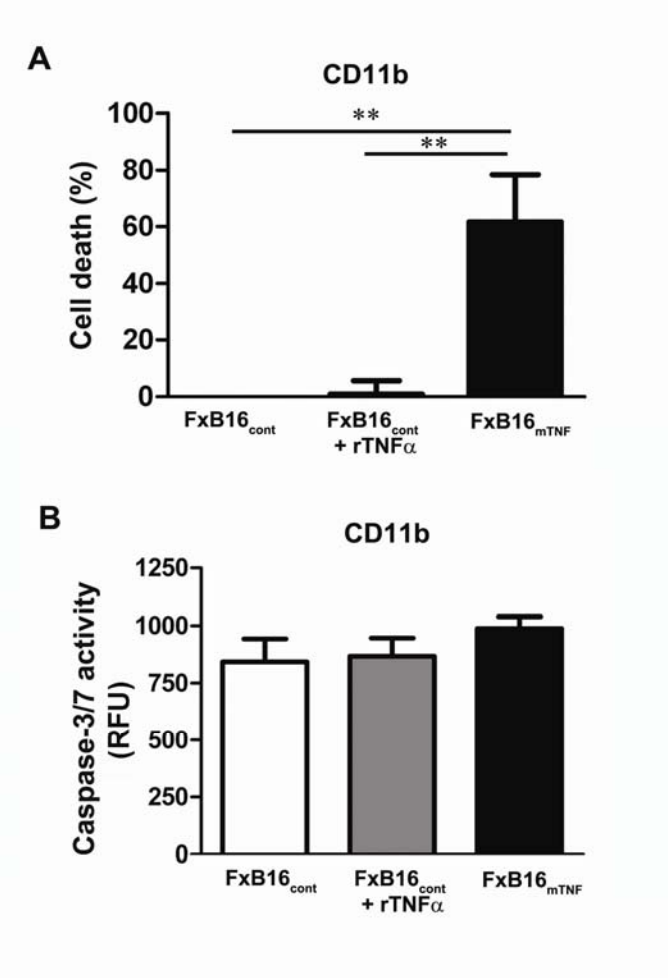

Figure 19. Membrane TNF-α induces cell death through apoptosis-independent pathway. (A) Cytotoxic effect of sTNF-α and mTNF-α on CD11b+ cells measured by MTT assay. **(B)** Caspase-3/7 activity (relative fluorescence unit [RFU]) in CD11b+ cocultured with Paraformaldehyde-fixed control (FxB16cont), control+rTNF-α (FxB16cont+TNF-α), or mTNF-α ($FxB16_{mTNF}$). Data is representative of three independent experiments expressed as the mean±SEM. $**P<0.05$, 1-way ANOVA with Tukey's post-test.

NF-κB is a critical factor in the determination of cell death versus survival and proliferation (Karin and Lin, 2002; Senftleben and Karin, 2002). In cases of failed NF-κB activation, TNF-α can induce either programmed cell death or necrosis through complex signal transduction cascades (Gupta, 2002). To evaluate whether soluble versus mTNF-α isoforms induced distinct cellular responses via regulation of NF-κB, we tested NF-κB activity in RAW 264.7 cells co-cultured with $FxB16_{cont}$, $FxB16_{cont}$+rTNF-α, or $FxB16_{mTNF}$. As shown in Figure 20C, the level of NF-κB p65 phosphorylation activity in RAW 264.7 cells stimulated with both $FxB16_{cont}$+rTNF-α and $FxB16_{mTNF}$ was similar to $FxB16_{cont}$, suggesting that mTNF-α isoform did not affect the activity of NF-κB p65 compared to sTNF-α.

Membrane TNF-α-induced cell death occurs through induction of ROS

TNF-α can induce cell death by induction of intracellular reactive oxygen spices (ROS)(Corda et al., 2001; Deshpande et al., 2000). To test the possibility that soluble versus membrane TNF-α isoforms induced distinct cellular responses via regulation of intracellular ROS, we evaluated ROS levels in CD11b+ myeloid cells incubated with different TNF-α isoform by measuring CM-H2DCFDA fluoresce. In CD11b+ cell incubated with $FxB16_{cont}$+rTNF-α, the CM-H2DCFDA fluorescence did not differ from control, whereas in cells with $FxB16_{mTNF}$ a 1.6-fold increase was observed after 8 hours of incubation ($P<0.05$; Figure 21A). Addition of N-acetyl-cysteine into cells cultured with $FxB16_{mTNF}$

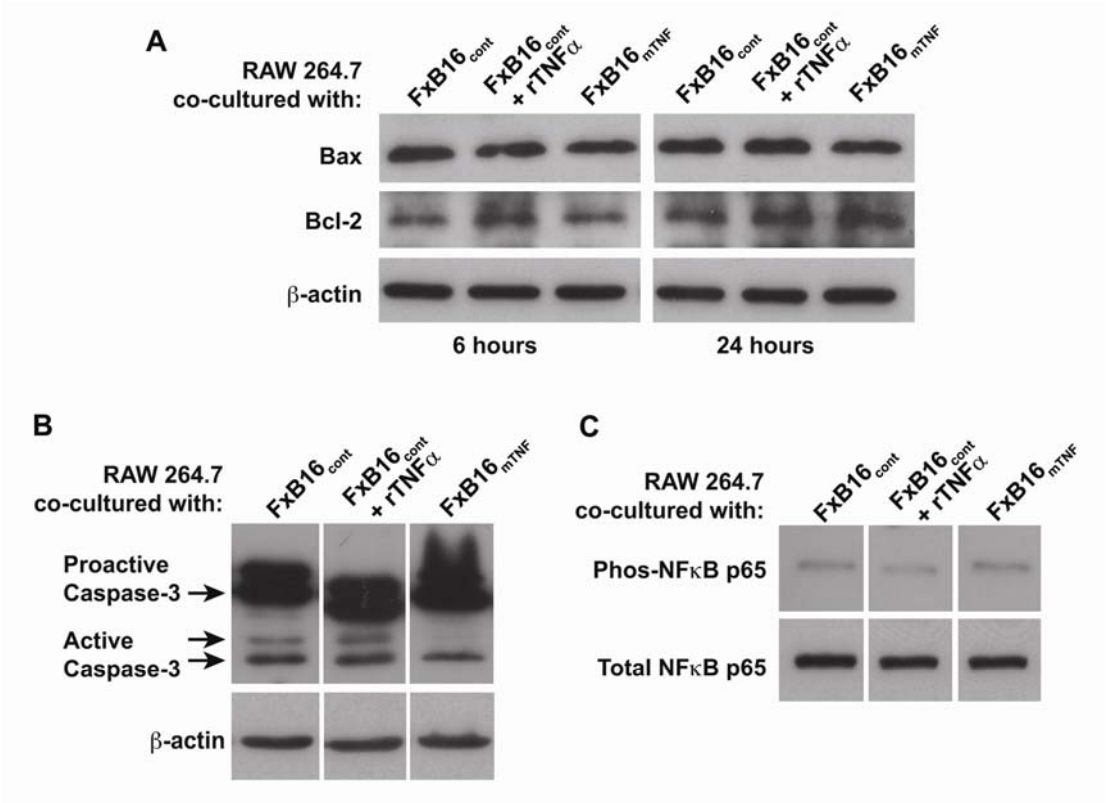

Figure 20. Kinetics of NFκB, caspase-3 and BAX/Bcl-2 activation in mTNF-α stimulated RAW 264.7. FxB16cont, FxB16cont+rTNF-α, or $FxB16_{mTNF}$ was added for indicated incubation period. RAW 264.7 cells were harvested and total cellular protein was analyzed for Bax/Bcl-2 ratio **(A)**, activated caspase-3 **(B)**, and total and phopho- NF-κB p65 **(C)**. Immunoblot analysis showed no differences in Bax/Bcl-2 ratio or caspase-3 and NF-κB pathway activation with different TNF-α isoforms compared to control.

significantly decreased the intensity of CM-H2DCFDA fluorescence indicating decrease in the level of ROS (P>0.05). NAC treatment of $FxB16_{mTNF}$ treated CD11b+ reduced mTNF-α induced cytotoxicity ($FxB16_{mTNF}$, 61.57±29.12% cytotoxicity; $FxB16_{mTNF}$+NAC, 10.64±29.17% cytotoxicity; P<0.05; Figure 21B).

In addition, ROS generation in individual RAW 264.7 by CM-H2DCFDA fluorescent staining assay was evaluated (Kim et al., 2005). The fluorescent intensity in RAW 264.7 co-cultured with $FxB16_{cont}$+rTNF-α was similar to the basal level (4.6±3%). However in the presence of $FxB16_{mTNF}$, we observed an induction of ROS intensity (82.5±15%) as detected by the presence of green fluorescence staining (Figure 22A and B). Treatment of RAW 264.7 cocultured with $FxB16_{mTNF}$ cells supplied with the ROS scavenger N-acetyl-cysteine ($FxB16_{mTNF}$+NAC) diminished mTNF-α-induced accumulation of intracellular ROS in RAW 264.7(0.5±0.4%; Figure 22A and B) and led to abolition of mTNF-α induced cytotoxicity ($FxB16_{mTNF}$, 34±9% cytotoxicity; $FxB16_{mTNF}$+NAC, 0.1±7% proliferation; P<0.05; Figure 22C).

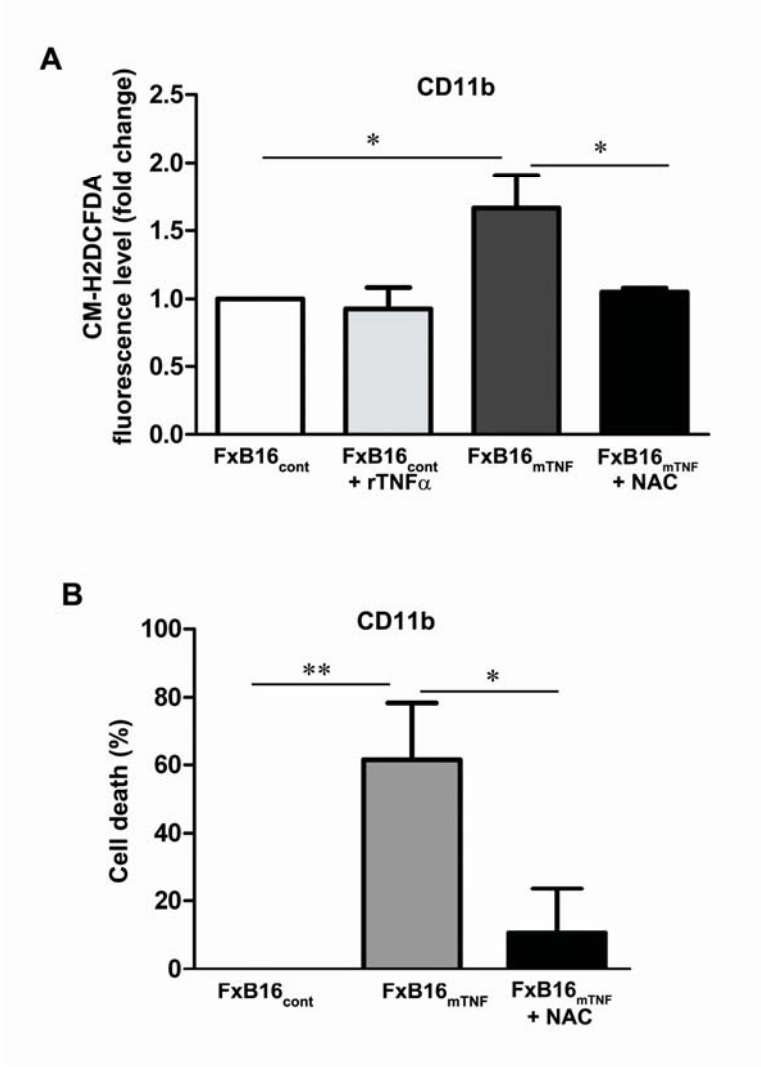

Figure 21. Membrane TNF-α-induced cell death occurs through induction of ROS. (A) Effects of various TNF-α isoforms on intracellular ROS generation in CD11b+ cells. Cells were labeled with ROS detection reagent, CM-H2DCFDA, and incubated with $FxB16_{cont}$, $FxB16_{cont}$+TNF-α, or $FxB16_{mTNF}$, FxB16mTNF+N-acetyl-cysteine (NAC, 2mM) and then ROS level was quantitatively analyzed. **(B)** Cytotoxic effect of mTNF-α on CD11b+ cells decreased in the presence of ROS scavenger NAC. Data is representative of three independent experiments expressed as the mean±SEM. $*P<0.05$ and $**P<0.005$, 1-way ANOVA with Tukey's post-test.

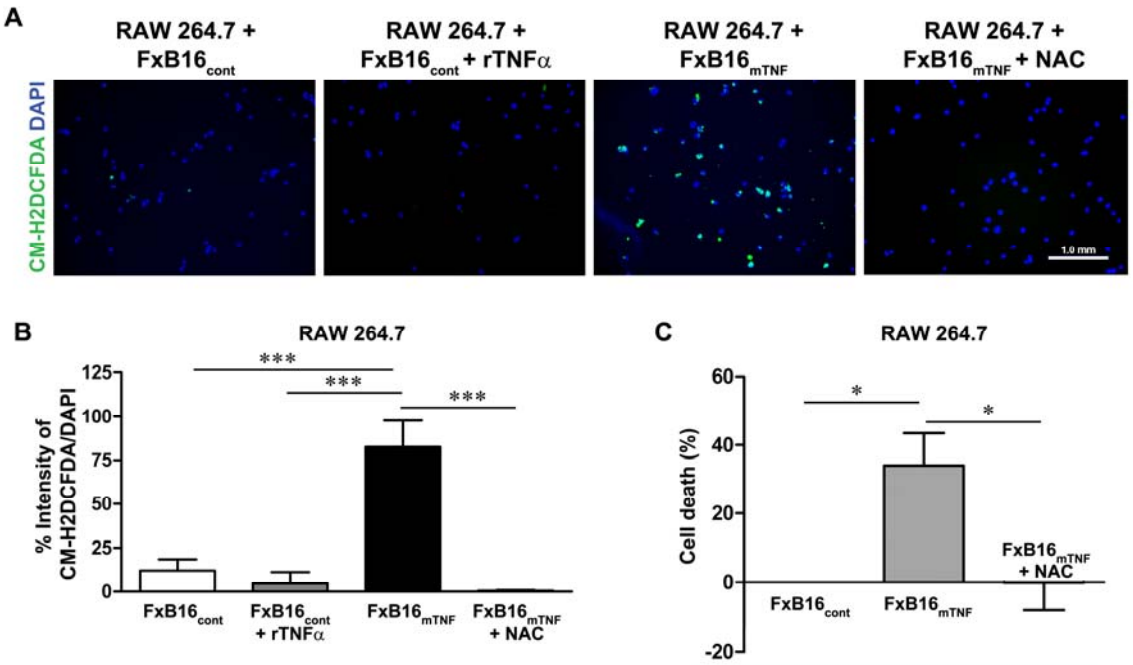

Figure 22. ROS level in mTNF-α treated RAW 264.7.
(A) Fluorescent micrographs of RAW 264.7 (blue: DAPI nuclear staining) incubated for 8 hours with FxB16cont, FxB16cont+rTNF-α, or $FxB16_{mTNF}$, $FxB16_{mTNF}$+NAC (2mM) and subsequently treated with ROS detection reagent, CM-H2DCFDA (green). **(B)** Intensity of CM-H2DCFDA was quantitated. Increase in number of ROS generating cell was detected in RAW 264.7 cocultured with mTNF-α-expressing B16F10 cells which was reversed by addition of NAC. **(C)** Cytotoxic effect of mTNF-α on RAW 264.7. $*P<0.05$, and $***P<0.0005$, 1-way ANOVA with Tukey's post-test.

Relative expression pattern of TNF-α/TACE correlates with survival probability in lung cancer patients

In most studies, the role of TNF-α in cancer has only been investigated in murine models or in modified cell lines and to our knowledge the expression level of mTNF-α has not been evaluated in human tumors. To extend the relevance of these findings from the murine model to human cancer, 62 tissue cores available in a single human non-small cell lung carcinoma (NSCLC) tissue array containing squamous cell carcinoma, large cell carcinoma, and adenocarcinoma were examined.

Using co-immunofluorescent staining analysis with anti-TNF-α and carcinoma-enriched membrane antigen (EMA) the distribution of both degree (high- or low-expressors) and localization (membrane, cytoplamic or both) of TNF-α staining were evaluated (Figure 23A). Forty of 62 individual tumors were positive for TNF-α expression. Among 40 tumors, expressing either high or low TNF-α, tumors presenting high levels of membrane-localized TNF-α, tumors presenting high cytoplasmic TNF-α (i.e. tumors with higher expression of sTNF-α) or tumors with cytoplasmic and membrane-localized TNF-α were detected. Eighteen of the 40 tumors (45%) were high expresser with 27.8% cytoplasmic localization, 16.7% localized on the membrane and 55.5% were positive for both membrane and cytoplasmic TNF-α. Twenty two (65%) showed low expression of TNF-α with 18.3% cytoplasmic, 31.8% membrane and 50% with both membrane and cytoplasmic (Figure 23B). These data are the first evidence for the existence of mTNF-α in human tumors and that its level varies significantly from patient to patient. Moreover, its level and localization varies significantly from

patient to patient, which is likely an important consideration in predicted therapeutic response to anti-TNF-α agents based on our pre-clinical observations.

To provide further evidence that human tumors exhibit varying expression of TNF-α isoforms, the expression of sTNF-α was determined by ELISA of CM and the expression of mTNF-α was evaluated by immunoblot analysis of cell membrane fraction. The analysis showed significant variation in the relative expression of membrane versus soluble TNF-α among different human lung cancer-derived cell lines (Figure 23C).

The possible association of sTNF-α versus mTNF-α ratio with patient outcome was investigated (Shedden et al., 2008). It has been shown that there is a positive correlation between TACE surface expression and TNF-α cleavage. Upregulation of TACE protein has also been shown to be associated with a decline in mTNF-α level and increased soluble level and vice versa (Armstrong et al., 2006). Using publicly accessible NSCLC microarray database (n=442 patients), the gene expression data were divided into four groups. The first two groups featured low TNF-α gene expression and low or high TACE. The third and fourth groups demonstrated high TNF-α with either low or high TACE. Over all higher TACE level was significantly correlated to lower survival probability. Expression of high TNF-α/low TACE — representing tumors with high mTNF-α:sTNF-α relative expression — was associated with longer survival than expression of high TNF-α/high TACE — representing tumors with low mTNF-α:sTNF-α relative expression (log rank P=0.035; Figure 24).

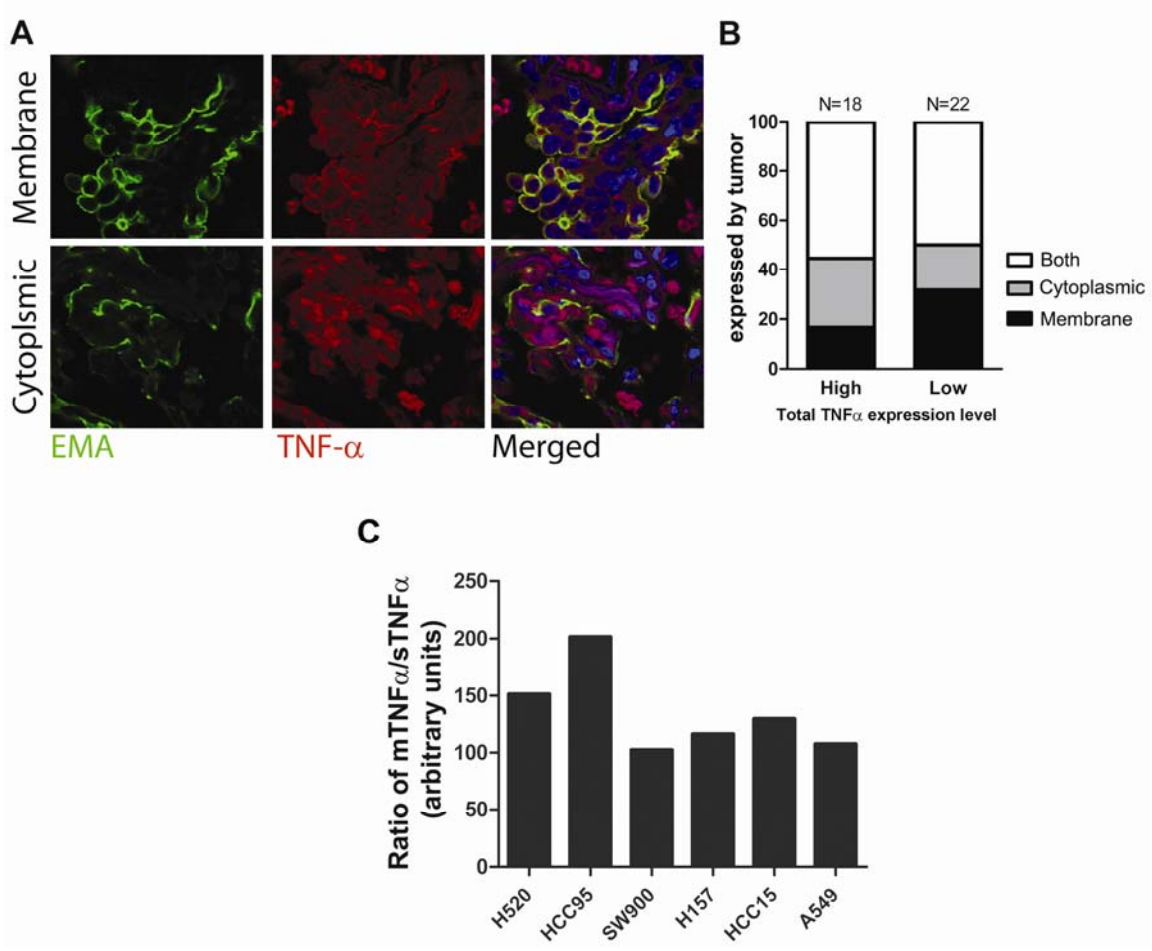

Figure 23. Human NSCLC express various levels of membrane and soluble TNF-α. Analysis of TNF-α expression from 40 human non-small cell lung carcinoma (NSCLC) tissue array. Using co-immunofluorescent staining analysis the distribution of both degree (high- or low-expressors) and localization (membrane, cytoplamic or both) of TNF-α staining were evaluated. **(A)** Selected example of a membrane TNF-α expression (top) and a cytoplasmic TNF-α expression (bottom) in tumors using co-immunofluorescent staining analysis with anti-TNF-α (red) and carcinoma-enriched membrane antigen (EMA-green). **(B)** Graph displays the distribution of both degree and localization of TNF-α staining 40 NSCLC patient samples. **(C)** Expression pattern of sTNF-α and mTNF-α in human NSCLC cell lines measured by ELISA and immunoblotting methods, respectively. Variation was observed in the ratio of mTNF-α to sTNF-α expressed by each cell line.

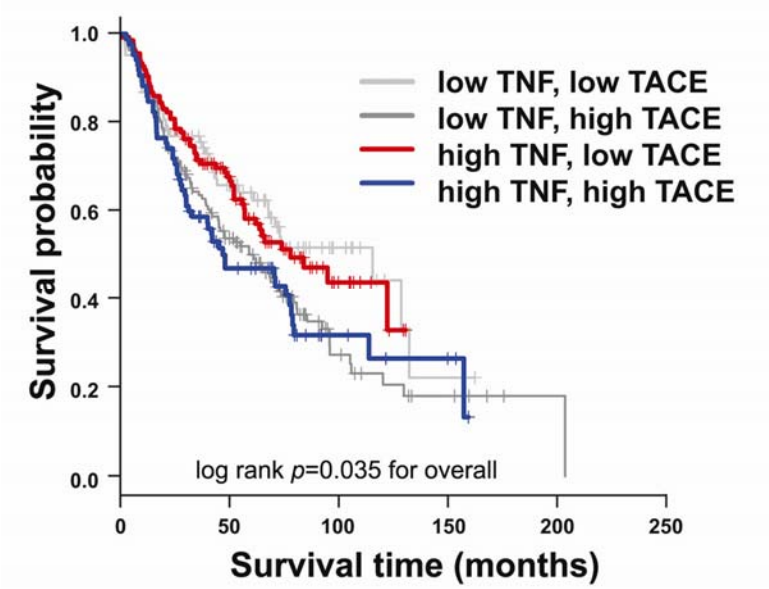

Figure 24. Relative expression pattern of TNF-α/TACE correlates with survival probability in lung cancer patients.
Association between TNF-α and TACE co-expression pattern and survival probability in patients with NSCLC. Analysis of the publicly available data from the Shedden cohort was used to correlate TNF-α/TACE expression pattern with survival probability (n=442, log rank *P*=0.035).

Discussion

Data from both experimental and human cancers have identified TNF-α as key cytokine modulating tumor progression, yet its effects are incompletely understood. In murine models, deletion or inhibition of TNF-α reduces the incidence of cancer formation and even increases resistance to chemically induced carcinogenesis of the skin (Arnott et al., 2004; Moore et al., 1999). Consistent with this, there was a positive correlation between level of TNF-α expression and tumor grade in ovarian tumors (Naylor et al., 1993). On the other hand, anti-tumorigenic properties of TNF-α are also well-documented. In a study by Boldrini et. al, assessment of TNF-α expression in 61 NSCLC samples demonstrated expression of TNF-α in 45.9% of cases and directly correlated with a better clinical outcome (Boldrini et al., 2000).

Many soluble proteins such as TNF-α are originally expressed as a membrane-bound form and then processed to a secretory form through proteolytic cleavage. Some of these proteins such as Fas ligand, a member of TNF-α super family, have been described to have distinct biological effects on disease process as a membrane isoform when compared to the soluble isoform (LA et al., 2009). In this chapter we sought to better understand the role of different TNF-α isoforms in the modulation of tumor progression and to determine if some of the reported opposing effects can be attributed to distinct effects of these isoforms.

Here, it was shown that subcutaneous implantation of mTNF-α expressing LLC and B16F10 cancer cell lines expressing uncleavable mTNF-α generates

significantly smaller tumors and this was not the result of impaired angiogenesis or reduced tumor cell proliferation but was driven by components of the host derived cells. This idea was further strengthened by significant reduction of tumor associated myeloid cell content in mTNF-α-expressing tumors which were restored in tumor cells transplanted in TNFR-DKO mice. Numerous studies have shown critical roles for tumor-associated stromal cells, specifically, bone marrow-derived myeloid cells, in tumor growth (De Palma et al., 2005; Shojaei et al., 2008). Upon activation by cancer cells, tumor-associated macrophages can release growth factors, cytokines and inflammatory mediators that may facilitate cancer cell invasion, migration, angiogenesis, tumor progression or metastasis (Condeelis and Pollard, 2006; Wang et al., 2005; White et al., 2001). Furthermore, systemic depletion (Zeisberger et al., 2006) or inhibition (Allavena et al., 2005) of tumor associated myeloid cells migration into the tumor has shown to significantly reduce tumor growth. In light of our findings, it would be of great interest to determine the precise role of myeloid cells in mTNF-α-mediated tumor growth.

The presented data in this chapter revealed that tumor cell expression of the membrane isoform of TNF-α resulted in tumor associated myeloid cell death through increased ROS production. It has been shown that TNF-α has the ability to induce necrotic cell death by utilizing death domain-containing adaptor proteins such as RIP1, TRADD and FADD upon TNFR activation. Once recruited to the TNFR death domain further downstream events lead to ROS generation and cell death (Festjens et al., 2006; Lin et al., 2004; Morgan et al., 2008).

Multiple pathways have been shown to lead to ROS generation upon TNFR activation (Corda et al., 2001; Vanden Berghe et al., 2007). Necrotic cell death induced by TNFR has been associated with generation of ROS derived from either mitochondrial or non-mitochondrial sources (Schwandner et al., 1998; Vanden Berghe et al., 2007). Mitochondrial complex I-mediated generation of ROS has been linked to direct activation by TNFR and ceramide mediated activation (Festjens et al., 2006; Morgan et al., 2008). In a study by Kim et al., TNFR was reported as an activator of Nox1 NADPH oxidase complex in a TRADD-and RIP1-dependent recruitment (Kim et al., 2009). The presented findings suggest that the membrane form of TNF-α is very efficient at stimulating ROS generation and initiating necrotic cell death. This could be due to the ability of mTNF-α to recruit death domain-containing adaptor proteins more efficiently or mTNF-α activates a pathway that is more efficient in ROS generation. The mechanistic pathway(s) which leads to mTNF-α induced ROS generation requires further investigation.

There has not been any study evaluating the relative expression of soluble and membrane TNF-α during tumor progression in human cancer, including analyses designed to determine if there is any correlation between the level of sTNF-α versus mTNF-α and the cancer outcome. Here, the *in vivo* tissue array staining and *in vitro* assessment of soluble and membrane TNF-α expression in human NSCLC cell lines showed that the ratio of soluble to membrane TNF-α varies among different tumor cell types. Furthermore, we verified this by immunofluorescence staining of tumor section for TNF-α. The fact that mTNF-α

is present in tumor and at different levels and subcellular localization may provides important clues to divergent outcomes seen in TNF-α positive tumor phenotype seen in different patients.

In order to generate sTNF-α, the membrane associated TNF-α is cleaved through proteolytic activity of TACE. Although it has been suggested that other proteinases are capable of TNF-α cleavage it has been shown that TACE has the highest affinity for TNF-α ectodomain shedding among the other known substrate (Armstrong et al., 2006). Level of TACE present on the surface of the membrane has been inversely correlated with the level of membrane associated TNF-α and inhibition of TACE by MMP inhibitors has demonstrated a transient increase in mTNF-α surface expression (Armstrong et al., 2006; Solomon et al., 1997). These studies suggest that the regulation of TACE activity and subsequent alteration of the sTNF-α to mTNF-α ratio could have a great impact on tumor growth. The association between higher TACE and higher TNF-α gene expression in NSCLC and decreased survival further confirms the importance of different TNF-α isoforms availability on tumor regulation.

As discussed in previous chapter antitumor therapy in clinical trial has been unsuccessful, suggesting that in order to take this forward, we need to identify those patients who are most likely to benefit from TNF-α antagonist treatment. Perhaps determining the predominant form of TNF-α expressed by tumor in these patients would be beneficial for a more effective treatment with TNF-α inhibitors which can block both soluble and membrane isoforms.

In summary, it was shown that TNF-α membrane versus soluble isoforms have opposing effects on cancer growth. Expression of both forms of TNF-α in NSCLCs indicates that this finding is relevant to human malignancies and that isoform analysis should be applied to identify candidates for which anti-TNF-α agents are likely to be beneficial versus detrimental.

CHAPTER III

MEMBRANE TNF-ALPHA-ACTIVATED PROGRAMMED NECROSIS IS MEDIATED BY CERAMIDE-INDUCED REACTIVE OXYGEN SPECIES

Introduction

Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine, that activates cell inflammation, proliferation, survival and cell death depending on autocrine/paracrine signals, and on the cellular context (Rangamani and Sirovich, 2007; Wallach et al., 1999). The soluble homotrimeric form of TNF-α (sTNF-α) that is released from the cell surface activates multiple signal transduction pathways including NF-κB survival pathway. In addition to activation of cell survival pathways, sTNF-α can induce cell death (Balkwill, 2009; Baud and Karin, 2001). Activation of caspases and initiation of apoptosis has been described as the classic form of TNF-mediated cell death. Recent evidence suggests that sTNF-α can also trigger an alternative form of cell death that is distinct from apoptosis. This form of cell death is referred to as “programmed necrosis” and is dependent on the generation of reactive oxygen species (Morgan et al., 2008; Wu et al., 2012).

So far, in the majority of studies TNF-α-mediated programmed necrosis have been attributed to the biological and mechanistic function of sTNF-α and its interaction with TNFR-1 in the presence of pharmacological or genetic inhibition of apoptosis (Arnott et al., 2004; Chan et al., 2003). TNF-α can also exist as a membrane-anchored protein. (Horiuchi et al., 2010; Perez et al., 1990). Like

sTNF-α, membrane TNF-α is biologically active and binds either of the two TNF-receptors. The study presented in the previous chapter indicated that human lung NSCLC express both soluble and membrane isoforms. Using a murine lung cancer model it was shown that unlike sTNF-α, mTNF-α exhibits inhibitory effects on tumor growth and myeloid content. We demonstrated that mTNF-α efficiently induced myeloid cell death through induction of ROS-mediated necrosis in the absence of any apoptosis inhibitors. Currently nothing has been reported on how mTNF-α mediates programmed necrosis.

Soluble TNF-α-induced programmed necrosis typically occurs where apoptosis is inhibited, and is mediated through a few defined pathways. In all cases, the serine/threonine kinase receptor interacting protein-1 (RIP-1) has been shown to play a central role in initiation of programmed necrosis (Li et al., 2012; Moquin and Chan, 2010), mainly through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) or mitochondria (Fan et al., 2002; Frey et al., 2002; Hordijk, 2006; Kim et al., 2007). Recent studies have also described a role for ceramide-mediated programmed necrosis. An increase in the level of intracellular ceramide has been linked to increased redox reaction within the cell (Garcia-Ruiz et al., 1997; Huwiler et al., 1999), suggesting the potential for crosstalk between ceramide, ROS, and TNF-α pathways in this process. In spite of these observations, the specific mechanism by which ceramide signaling leads to increased redox reactions is not fully understood.

In this chapter we will determine the molecular pathway involved in mTNF-α-mediated oxidative stress-induced cell death. Using inhibitors targeting

mitochondrial electron transport chain and NADPH oxidase we present evidence that mitochondrial-dependent oxidative stress is the major source of mTNF-induced intracellular ROS generation, regulated by ceramide-activated protein kinase (CAPK) activity (Ardestani, under review).

Materials and Methods

Mice, cell lines and materials. Wild-type C57Bl/6 (WT) mice were purchased from Jackson Laboratory. Homozygous mutants for TNFR-1, TNFR-2, and TNFR-1/2 double knockout (TNFR-DKO) on a C57Bl/6 background were a generous gift from Dr. D. Polk. B16F10 melanoma, RAW264.7 and L929 cells were purchased from American Type Culture Collection (ATCC) and were maintained in DMEM with 10% (v/v) fetal bovine serum, heat inactive fetal bovine serum and MEM with 10% (v/v) horse serum, respectively. N-Acetyl-L-cysteine (NAC), 2-Thenoyltrifluoroacetone (TTFA), myxothiazol and 4-(Dimethylamino)pyridine (DMAP) were purchased from Sigma-Aldrich and prepared fresh on the day of the experiment.

Constructs. Membrane TNF-α-expressing cells were generated by cloning the mTNFΔ1-9, K11E sequence encoding a mutant transmembrane TNF-α protein with a deletion at the cleavage site between pre-sequence and mature membrane TNF-α (BCCM/LMBP plasmid collection, Ghent University) into the BamH1-EcoR1 site of LZRS-IRES-Neo retroviral vector, conferring neomycin resistance. This mutation prevents cleavage of the 26-kDa membrane TNF-α into secretory TNF-α isoform. An empty LZRS vector was used as a control vector.

Surface expression of TNF-α. Trypsinized cells were incubated with anti-TNF-α antibody (1 µl/2.5 x 10^4 cells, Southern Biotech) for 30 minutes on ice. PE conjugated secondary antibody (0.125 µg/10^6 cells/100 µl) was added for 30 minutes on ice. Surface expression of TNF-α was measured using flow cytometry.

TNF-α cytotoxicity assay. Overnight cultured RAW 264.7, L929 or freshly isolated bone marrow CD11b cells (EasySep, StemCell Technologies) ($2.5x10^4$/100 µL/well, target) were cocultured with Paraformaldehyde-fixed (Zhang et al., 2008) control, control+100 U/ml of recombinant TNF-α or mTNF-α (effector) at target:effector ratio of 1:10 and incubated for 48 hours. Cells were labeled with 100 µl of PBS containing 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl] 2,5,-diphenyltetrazolium bromide (MTT) (Sigma) for 2 hours at 37°C then lysed with 0.1 ml DMSO. Photometric measurement was carried out at 540 nm. Percentage of cell death was calculated by using the following formula: Cell death (%) = $(1 - OD_{sample}/OD_{control}) \times 100$.

Lactate dehydrogenase (LDH) assay. Early cell damage was determined using the LDH cytotoxicity detection kit (Promega, Madison, WI), which quantifies the LDH release from the cells into the culture medium. Cells were seeded in a 96-well plates at a density of $2.5x10^4$ cells/well overnight to promote adherence. Cells were cocultured with fixed B16F10 control or mTNF-α in the absence or presence of the indicated treatments for 24 hours. Supernatants from the cultures were collected and used in the LDH assay as instructed by the manufacturer. LDH activity was detected separately in the supernatant and cell

lysate. The percentage of LDH leakage was calculated as 100 x ($LDH_{supernatant}$ / ($LDH_{supernatant + lysate}$)).

Measurement of intracellular ROS. The oxidant-sensing probe CM-H2DCFDA (Invitrogen) was used to detect intracellular reactive oxygen species (ROS). An overnight culture of cells ($2.5x10^4$) were loaded with 10 μM CM-H2DCFDA for 30 minutes and cocultured with $2.5x10^5$ fixed B16F10 control or mTNF-α-expressing cell in the absence or presence of the indicated treatments for 6 hours. Fluorescence was determined using a luminescence spectrophotometer (Spectra max, Molecular Devices) with an excitation wavelength of 495 nm and emission wavelength of 525 nm.

Immunoblot analysis. Target cells were plated at a density of 10^6 cells overnight (37°C, 5% CO_2). Cells then were treated with fixed control or mTNF-α-expressing B16F10 at target cell/fixed tumor cell ratio of 1:10. After 30 minutes incubation at 37°C, target cells were lysed with RIPA buffer [3 M NaCl, 1 M Tris, 0.5 M EDTA, 10% SDS, 1% NP40 substitute and 1× Complete Protease Inhibitor Cocktail (Roche)]. Whole cell lysate was evaluated for caspase-3 cleavage (Cell Signaling Technology) and RIP-1 (BD biosciences) by Western blot analysis.

Statistical analysis. The statistical significance between experimental and control groups was determined by Student's *t-test* or ANOVA followed by Tukey's post-test using Prism software (Graphpad, San Diego, CA). A *P*-value of <0.05 was considered statistically significant.

Results

Membrane TNF-α is an inducer of cell death

To investigate the ability of the membrane versus soluble TNF-α isoforms to induce cell death, RAW 264.7 cells, a line derived from murine leukemic monocytes/macrophage cells (target), were mixed with 1% paraformaldehyde-fixed B16F10 melanoma cells (effector) expressing empty vector, ±100 U/ml rTNF-α ($FxB16_{cont}$ or $FxB16_{cont}$+rTNF-α), or fixed mTNF-α-expressing B16F10 cells ($FxB16_{mTNF}$) at an target:effector ratio of 1:10. Paraformaldehyde-fixed tumor cells were used to eliminate the endogenous sTNF-α (Figure 25A). As measured by the MTT dye reduction assay, incubation with $FxB16_{mTNF}$ resulted in more than 70±12% cytotoxicity of RAW 264.7 myeloid cells after 48 hours of incubation, as compared to RAW 264.7 cells incubated with $FxB16_{cont}$ (Figure 25B, $P<0.05$). In contrast $FxB16_{cont}$+rTNF-α increased RAW 264.7 cell survival compared to control.

To determine the molecular pathway leading to mTNF-α-induced cell death, we utilized the highly TNF-α-sensitive L929 fibrosarcoma cell line. As shown in Figure 25C, mTNF-α isoform resulted in more than 50% cell death compared to L929 cocultured with $FXB16_{cont}$ as determined by MTT reduction assay. Cellular toxicity causes membrane damage and results in the release of lactate dehydrogenase (LDH) from the cytoplasm and thus LDH in the media can also be used to measure cell death. To confirm the results obtained with MTT assay, we measured LDH release in L929 cell in the presence of control or

mTNF-α-expressing tumor cells. The mTNF-α isoform increased the level of LDH leakage by 12% over control (Figure 25D).

Membrane TNF-α-induced cell death was mediated through either TNFR-1 or TNFR-2

Membrane TNF-α signal transduction has been linked to a cooperative signaling between TNFR-1 and TNFR-2 (Chan et al., 2003; Lazdins et al., 1997). Next we sought to determine whether mTNF-α-mediated cell death was dependent on a specific TNF receptor. Primary CD11b myeloid cells were isolated from wild-type (WT), TNFR-1 knockout (TNFR-1KO), TNFR-2KO or TNFR-1 and TNFR-2 double knockout (TNFR-DKO) and cocultured with fixed control tumor cells with or without rTNF-α or fixed mTNF-α-expressing tumor cells for 48 hours. Cell cytotoxicity was determined by MTT assay. As shown in Figure 26, presence of either TNFR-1 or TNFR-2 resulted in increased levels of mTNF-α-induced cytotoxicity similar to WT-CD11b (~17% cell death in WT-CD11b, ~20% in TNFR-1KO-CD11b and TNFR-2KO-CD11b). In contrast, control cells treated with rTNF-α improved cell survival in WT- and TNFR-1KO-CD11b and resulted only in ~4% cell death in TNFR-2KO-CD11b and ~5% cell death in TNFR-DKO-CD11b. Interestingly, mTNF-α-mediated cell cytotoxicity was reversed in TNFR-DKO-CD11b cells. These findings suggested that mTNF-α-induced cell death can be mediated through both TNFR-1 and TNFR-2.

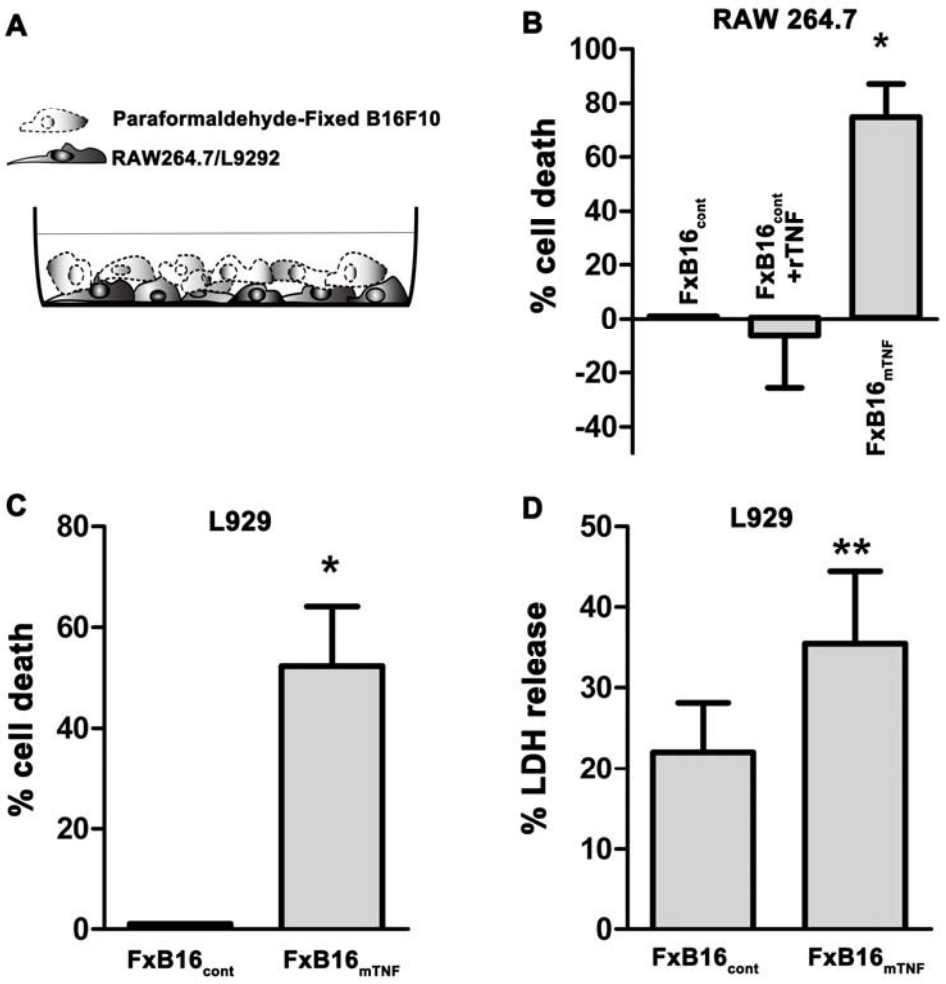

Figure 25. Membrane TNF-α isoform effectively induces cell death. (A) Schematic diagram of fixed B16F10 cells coculture with L929/RAW264.7. **(B)** RAW cells were cocultured with paraformaldehyde-fixed control ($FxB16_{cont}$), control+rTNF-α ($FxB16_{cont}$+TNF-α), or mTNFα ($FxB16_{mTNF}$) for 24 hours. Cell death rate was measured by MTT assay. **(C)** MTT assay showing the cytotoxic effects of mTNF-α isoform on L929. **(D)** LDH assay measuring L929 cells %LDH leakage in the presence of control or mTNF-α-expressing fixed B16F10 tumor cells. Data show the percentage of LDH leakage into media to total LDH (media + cells). Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. Data are expressed as average ± S.E. $*P<0.05$ and $**P<0.005$, 1-way ANOVA with Tukey's post-test **(B)**, students' *t-test* **(C and D)**.

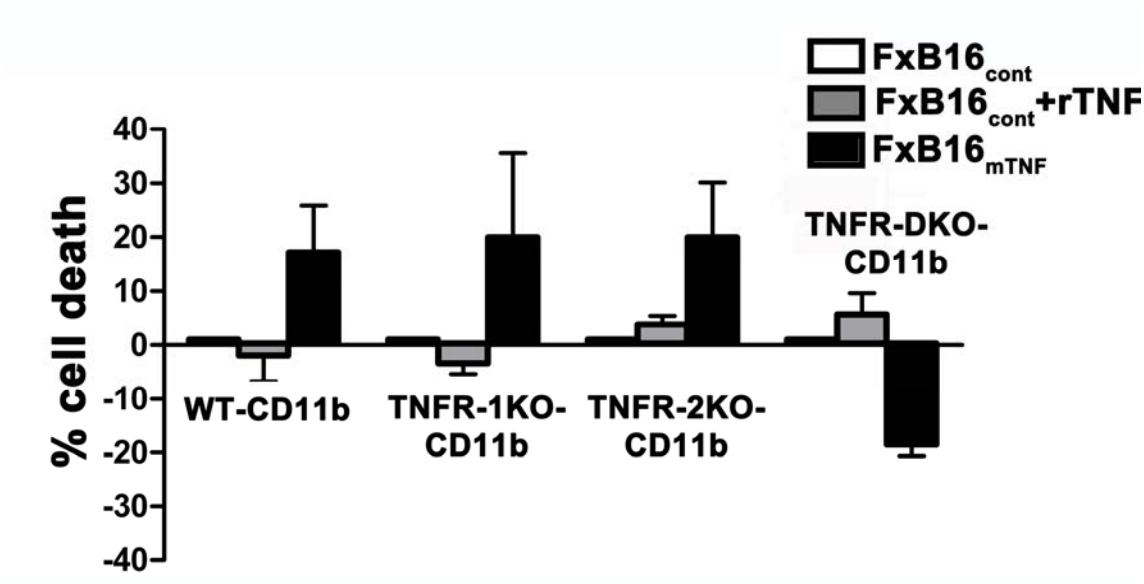

Figure 26. Membrane TNF-α-induced cell death can be mediated through both TNFR-1 or TNFR-2. Freshly isolated CD11b myeloid cells from wild type mice (WT-CD11b), mice deficient in TNF receptor 1 (TNFR-1KO-CD11b), TNFR-2KO-CD11b or both receptors (TNFR-DKO-CD11b). Cells were cocultured with paraformaldehyde-fixed control ($FxB16_{cont}$), control in the presence of 100 U/ml recombinant TNF-α ($FxB16_{cont}$+rTNF-α), or mTNF-α ($FxB16_{mTNF}$) for 24 hours. Percentage of cell death was measured by MTT assay. Data present mean percentage (bars, mean ± S.E.) of three replicates from 3 independent experiments.

Membrane TNF-α exerts cell cytotoxicity by increasing intracellular ROS production

Induction of cell death by sTNF-α occurs mainly through activation of caspases leading to apoptosis. To test whether the mTNF-α isoform exerts its cell toxicity in part by activating the caspase pathway, we determined the level of cleavage/activation of caspase-3 proteins in L929 cells treated with fixed control or mTNF-α-expressing tumor cells. As presented in Figure 27A, treatment of L929 cells with $FxB16_{mTNF}$ did not result in an increased level of active-caspase-3.

In the previous chapter we showed that mTNF-α-treated myeloid cells exhibit increased intracellular ROS and decreased cell survival. To demonstrate an association between intracellular ROS level and cell death in L929 fibrosarcomas, cells incubated with different TNF-α isoforms were measured using CM-H2DCFDA (which quantitatively reacts with oxygen species to produce a highly fluorescent dye). L929 cells incubated with $FxB16_{mTNF}$ resulted in a 60% increase in CM-H2DCFDA fluorescence, indicating an increase in the level of ROS (P>0.05; Figure 27B). Furthermore, incubation of L929 cells with ROS scavenger N-acetyl-cysteine (NAC) reduced mTNF-α-mediated ROS level (130% of control in $FxB16_{mTNF}$; 94% of control in $FxB16_{mTNF}$+NAC; Figure 27C, P<0.5). This was followed by 4-fold decrease in LDH release in mTNF-α-treated L929 cells supplied with NAC (P<0.05; Figure 27D).

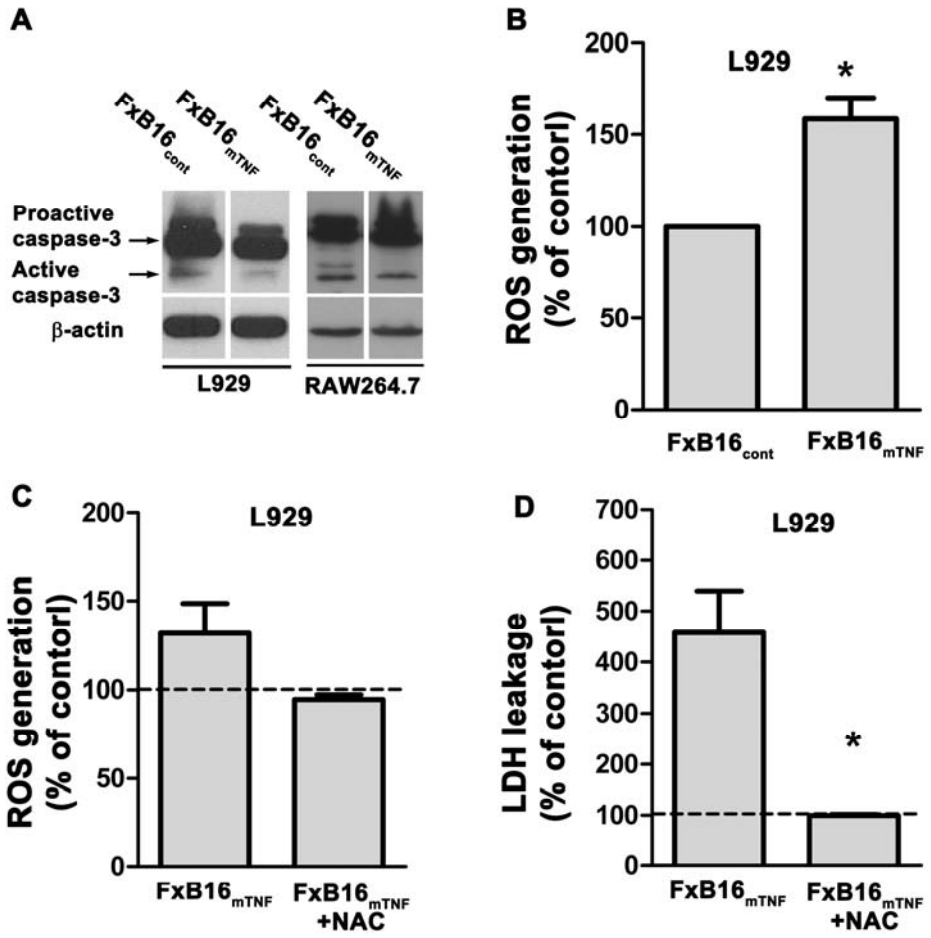

Figure 27. Membrane TNF-α exerts cell cytotoxicity by increasing intracellular ROS production. (A), caspase-3 activity in L929 and RAW 264.7 cells after incubation with paraformaldehyde-fixed control ($FxB16_{cont}$) or mTNF-α ($FxB16_{mTNF}$) for 30 minutes. L929 cells were harvested and total cellular protein was analyzed for active caspase-3. **(B)** ROS production measured by CM-H2CDFDA intensity in L929 cocultured with fixed control or mTNF-α-expressing B16F10. **(C and D***)* Addition of N-acetyl cysteine (2 mM) reduced ROS level **(C)** and decreased LDH leakage into the media **(D)** in L929 cells. Data are represented as percent of CM-H2DCFDA intensity **(C)** or LDH in media/total LDH **(D)** to L929 cells cocultured with $FxB16_{cont}$ cells with or without NAC. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. Data are expressed as average ± S.E. $*P<0.05$. Students's *t-test*.

Inhibition of mitochondrial respiratory chain decreased mTNF-α-mediated ROS generation

Next we sought to determine the source of ROS in response to mTNF-α in L929 cells. There is report of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1 (NOX1) and mitochondria as the two major source of TNF-α induced ROS production (Bedard and Krause, 2007; Liu et al., 2002). To do so, the NADPH-dependent oxidase and the mitochondrial respiratory chain complex II were blocked using DPI (2 μM) and TTFA (0.5 μM) respectively. The NADPH-dependent oxidase inhibitor DPI did not inhibit the ROS production induced by mTNF-α (156.7±8.3% of control in $FxB16_{mTNF}$ and 155.4±14.4% in $FxB16_{mTNF}$+DPI; $P<0.05$; Figure 28A) and further had no effect on the LDH leakage (270% of control in $FxB16_{mTNF}$ and 280% in $FxB16_{mT}$+DPI; $P<0.05$; Figure 28B). However, addition of TTFA into L929 cells cocultured with mTNF-α-expressing tumor cells, reduced CM-H2DCFDA oxidation (108% of control) and LDH release (10% of control, Figure 28B). These data suggested that mitochondria are the source of mTNF-induced ROS generation and cell death.

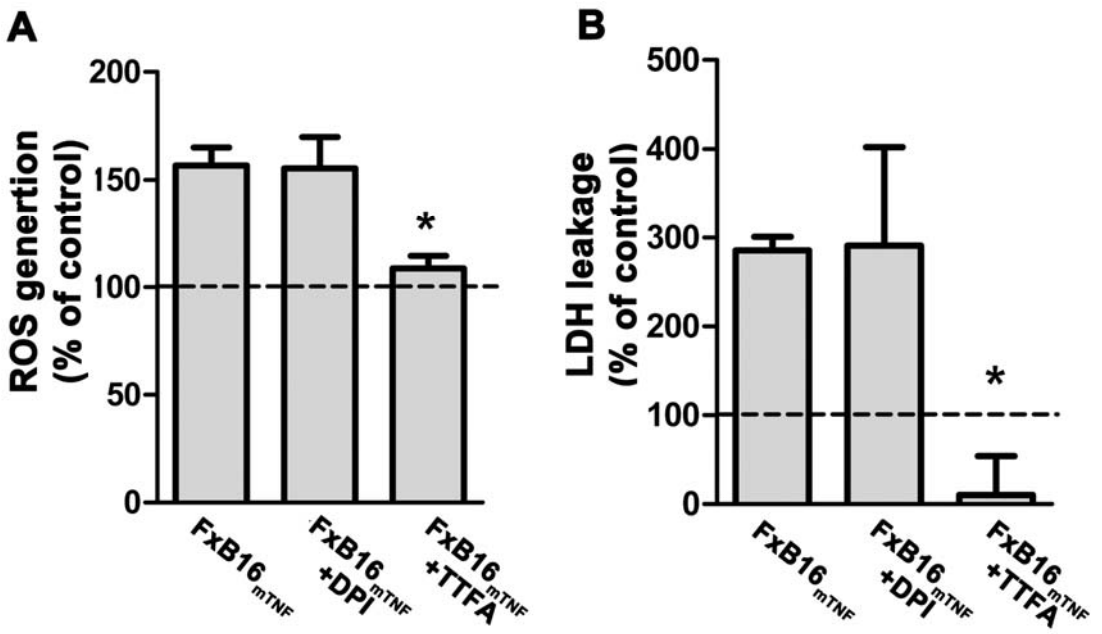

Figure 28. Inhibition of mitochondrial respiratory chain decreases mTNF-α-mediated ROS generation. (A and B) L929 cell cocultured with mTNF-α-expressing tumor cells in the absence or presence of NOX inhibitor-DPI (2 μM) and mitochondrial complex II inhibitor-TTFA (0.5 μM) for 24 hours. TTFA reduced ROS level, shown by reduction of CM-H2DCFDA intensity **(A)** and LDH leakage into media **(B).** Addition of NOX inhibitor-DPI had no effects on both ROS generation of LDH level. Data are represented as % of CM-H2DCFDA intensity **(A)** or LDH in media/total LDH **(B)** to L929 cells cocultured with control expressing tumor cells ($FxB16_{cont}$) with or without DPI or TTFA. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. Data are expressed as average ± S.E. $*P<0.05$, 1-way ANOVA with Tukey's post-test.

Membrane TNF-α-mediated ROS production involves ceramide pathway

TNFR-mediated mitochondrial ROS generation can be induced through RIP-1 kinase activity or through a ceramide-dependent signaling pathway initiated by sphingomyelinases (SMases) activity (Figure 29). To determine the specific pathway responsible for activation of mitochondrial ROS production we analyzed level of active RIP1 by evaluating its phosphorylation in L929 or RAW 264.7 in the presence of $FxB16_{cont}$ or $FxB16_{mTNF}$ by immunoblot analysis. As shown in Figure 30A, treatment of both cell lines with fixed membrane expressing tumor cells, $FxB16_{mTNF}$, did not increase the level of RIP-1 phosphorylation.

There is evidence to support the role of ceramide as a second messenger of TNF-α activated cells involved in activation of programmed necrosis (Corda et al., 2001). Next we evaluated the role of ceramide signaling in TNF-α-induced ROS production and survival. Addition of DMAP (1 mM), a CAPK inhibitor, reduced mTNF-α-induced ROS by 60% (138±15.6% of control in $FxB16_{mTNF}$; 80±2.9% in $FxB16_{mTNF}$+DMAP; Figure 30B). Percentage of LDH leakage was also reduced from 276% in mTNF-α-treated cells to 163% in mTNF-α-treated cells supplied with DMAP ($P<0.005$, Figure 30C). These findings suggested that mTNF-α-induced mitochondrial ROS generation requires protein kinase activity associated with ceramide. This was further confirmed in RAW 264.7 cell lines. Similar to L929 cells, inhibition of CAPK in mTNF-α treated Raw 264.7 cells reduced LDH leakage (Figure 30D).

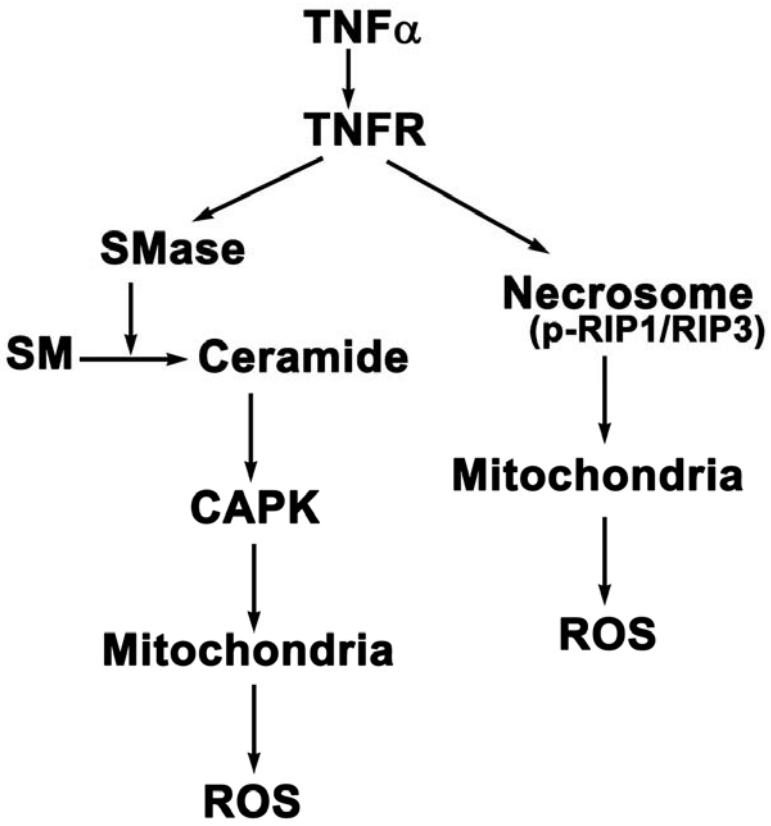

Figure 29. Schematic diagram of TNF-α-activated pathways leading to ROS generation. Binding of TNF-α to its receptor activates sphingomyelinases (SMase) which convert sphingomyelin (SM) to ceramide. Ceramide activates ceramide-activated protein kinase (CAPK) and induces mitochondrial reactive oxygen species (ROS) generation. Alternatively, TNFR activation leads to autophosphorylation between RIP-1 with RIP-3 result in formation of the necrosome. Necrosome induces ROS production via direct effects in the mitochondria.

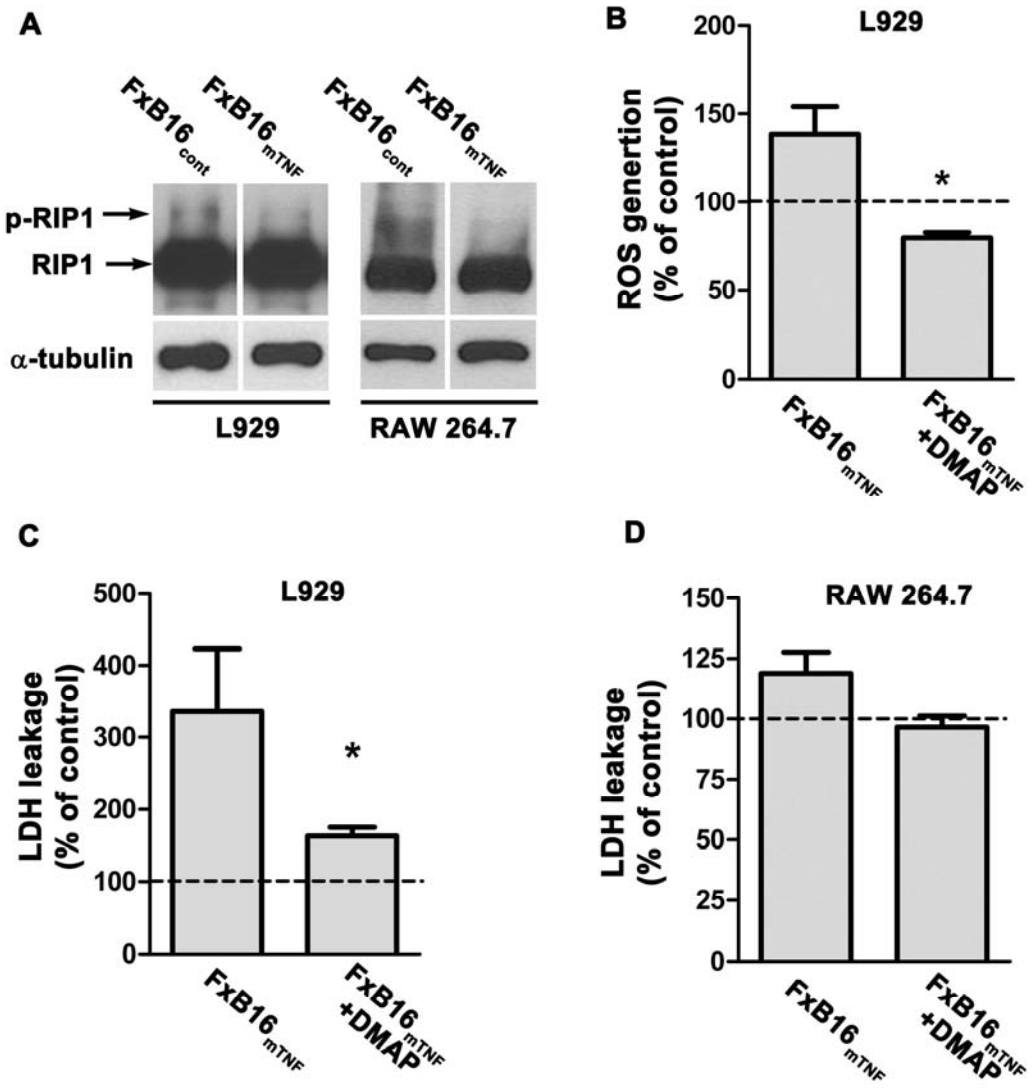

Figure 30. Membrane TNF-α-mediated ROS production involves ceramide pathway. (A) Level of phospho-RIP1 in L929 or RAW 264.7 cells treated with fixed B16F10 control cells ($FxB16_{cont}$) or B16F10 mTNF-α ($FxB16_{mTNF}$) cells. After 30 min incubation, L929 cells were harvested and total cellular protein was analyzed for RIP-1. Inhibition of CAPK reduced mTNF-α-mediated ROS generation **(B)** and LDH release in L929 **(C)** and LDH release in RAW26.7 **(D)**. Data are represented as % of CM-H2DCFDA intensity **(B)** or LDH in media/total LDH **(C and D)** to cells cocultured with control expressing tumor cells ($FxB16_{cont}$) with or without DMAP. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. Data are expressed as average ± S.E. Student t-test. $P<0.05$.

Discussion

Previously necrotic cell death has been defined as a sudden, unregulated form of cell death which leads to inflammation and tissue damage. However, in recent years accumulating evidence suggests that not all form of necrotic cell death is accidental but can instead be a programmed event (Morgan et al., 2008; Wu et al., 2012). There have been several reports of TNF-α-induced programmed necrosis, mainly in the context of the soluble form of TNF-α. Importantly, induction of programmed necrosis by sTNF-α typically requires the presence of inhibitors of caspases (Robaye et al., 1991; Zhang et al., 2009). Here, we present for the first time that the lesser-known membrane form of TNF-α has the ability to induce programmed necrosis through ROS generation, independent of caspase inhibitors. In this study we explored the mechanism of mTNF-α-mediated ROS generation and programmed necrosis.

In our study treatment of mTNF-α-induced L929 cells with mitochondrial inhibitor complex II increased ROS reduction and improved survival, suggesting a role for mitochondrial complex II in mTNF-α-mediated programmed necrosis. The plasma membrane-associated NADPH oxidases (NOX) have been proposed as an alternate source of ROS production (Fan et al., 2002; Hordijk, 2006; Quinlan et al., 2012). In contrast to what we observed with mitochondrial inhibitor, inhibition of NOX failed to inhibit ROS generation and to increase cell viability.

The involvement of the mitochondria in sTNF-α induced ROS production has been demonstrated in several studies (Meier et al., 1989; Shoji et al., 1995).

The mechanisms by which sTNF-α induces mitochondrial ROS are complex and not fully understood. Multiple pathways have been proposed in which activated TNFR increases the activity of metabolic enzymes such as glutamine synthase (GLUL), glutamate dehydrogenase 1 (GLUD1), and glycogen phosphorylase (PYGL) (Zhang et al., 2009). These metabolic enzymes eventually stimulate the TCA cycle and oxidative phosphorylation, which leads to enhanced mitochondrial ROS production (Mates et al., 2009). In several studies, inhibition of mitochondrial electron transport, using specific inhibitors of mitochondrial complex I, II, or III have shown to reverse oxidative stress (Corda et al., 2001; Quinlan et al., 2012) .

Although, RIP1/RIP3 kinases have been shown to orchestrate the programmed necrosis pathway activity of sTNF-α, new pathways, such as the ceramide pathway, has emerged as alternative mechanism for induction of programmed necrosis (Vandenabeele et al., 2010). An enhanced level of ceramide has been shown to contribute to depletion of ROS scavenger, glutathione (Mari et al., 2004) and increasing mitochondria susceptibility to GD3, a ceramide-derived ganglioside. GD3 traffics to the mitochondria and directly induces ROS production (Garcia-Ruiz et al., 2002; Garcia-Ruiz et al., 2000). In our study mTNF-α-induced ROS and cell death seems to be regulated through activity of ceramide since the inhibitor of CAPK, blocked mTNF-α-mediated ROS and cell death. Both membrane bound neutral sphingomyelinase (N-SMase) and the endosomal acid SMase (A-SMase) shown to be the target of TNFR signal transduction in ceramide formation pathway (Cutler and Mattson, 2001; Won and

Singh, 2006). TNF-α-induced activation of both A-SMase and N-SMase has been implicated in hepatocytes, endothelial (Corda et al., 2001), monocytes (Wiegmann et al., 1994) and pre-B cells (Adam et al., 1996). Whether, mTNF-α targets N-SMase or A-SMase remains to be determined.

Unlike soluble TNF-α which its signal transduction is mediated mainly through TNFR-1 (Morgan et al., 2008; Rodriguez-Berriguete et al., 2012), it is evident from our study that mTNF-α is efficient in activation of both TNFR-1 and TNFR-2. Members of the tumor necrosis factor receptor superfamily (TNFR-1, Fas,TRAIL) previously shown to be capable of inducing cell death (Shen and Pervaiz, 2006). These receptors, including TNFR-1 contain a conserved DD in the intracellular region that is required for activation of caspases (Gupta, 2002; Rangamani and Sirovich, 2007). However induction of cell death by DD-lacking TNF receptor superfamily has also been reported. For instance, TNFR-2 shown to trigger cell death in the rhabdomyosarcoma cell line KYM-1 (Grell et al., 1993) and the stimulation of CD30 induces cell death in T cell hybridomas (Lee et al., 1996). It is not yet clear how TNF receptor superfamily members lacking a death domain (i.e. TNFR-2) execute their death inducing capability. This effect could be FADD-dependent (Depuydt et al., 2005) or could be mediated through cooperative activity with other receptors such as Fas/FasL (Teh et al., 2000).

In conclusion we have demonstrated that mTNF-α can induce cell death independent of caspase inhibitors by increasing ROS. This occurs through RIP-1-independent, ceramide-dependent activation of mitochondrial ROS. Molecular

mechanism, leading to mTNF-α-induced ceramide formation and mitochondrial-ROS generation, remains to be investigated.

CHAPTER IV

DISSCUSSIONS AND FUTURE DIRECTIONS

Summary

In this body of work, we showed that membrane and soluble TNF-α isoforms have diametrically opposing effects on both tumor growth and myeloid content. Mouse lung and melanoma tumor lines expressing mTNF-α, generated smaller tumors devoid of monocytes versus respective control lines or lines expressing sTNF-α. The lack of myeloid cells was due to a direct effect of mTNF-α on myeloid survival via induction of cell necrosis by increasing reactive oxygen species (ROS) (Figure 31). Next, a novel mechanism by which mTNF-α induces programmed cell death was identified (Chapter III). Using a cultured RAW 264.7 monocytic cell line and L929 fibroblasts, we found that mTNF-α increased ROS-mediated cytotoxicity independent of RIP-1, a serine/threonine kinase that serves as a main adaptor protein of sTNF-α induced programmed necrosis. Instead, mTNF-α induced ROS generation and cell death through the activity of the ceramide pathway as determined by the use of ceramide-activated protein kinase (CAPK) inhibitor which prohibited both ROS level and cell death.

These findings demonstrate that there are significant differences in the role of various TNF-α isoforms in tumor progression and that the mTNF-α isoform is a more effective inducer of programmed necrosis. This is the first report identifying the mTNF-α isoform as a potent activator of programmed necrosis,

even in the absence of inhibitors of apoptosis, via ceramide-dependent mitochondrial ROS generation. The molecular mechanism by which the two different TNF-α isoforms exert distinct biological effects remains elusive. It is interesting that although sTNF-α and mTNF-α have similar structures and are able to interact with both TNF-α receptors, they exert opposing effects on tumor growth and cell survival. This raises questions on the plausible molecular mechanisms to account for these differences and how two different isoforms with similar structures can elicit such contrasting biological responses.

In the following sections we will use published literature to establish a link between the signaling events leading to programmed necrosis, starting with TNFR stimulation which marks the programmed necrosis initiation followed by activation of the secondary messenger ceramide and the execution of programmed necrosis by increasing mitochondrial ROS. We will discuss the mechanisms by which TNF receptor activation increases ceramide level and mitochondrial ROS generation and how different TNF-α isoforms may modulate this process differently. Finally, we will discuss whether activation of mitochondrial ROS through ceramide is sufficient to initiate cell death or perhaps there are other pathways contributing to these events (Figure 31).

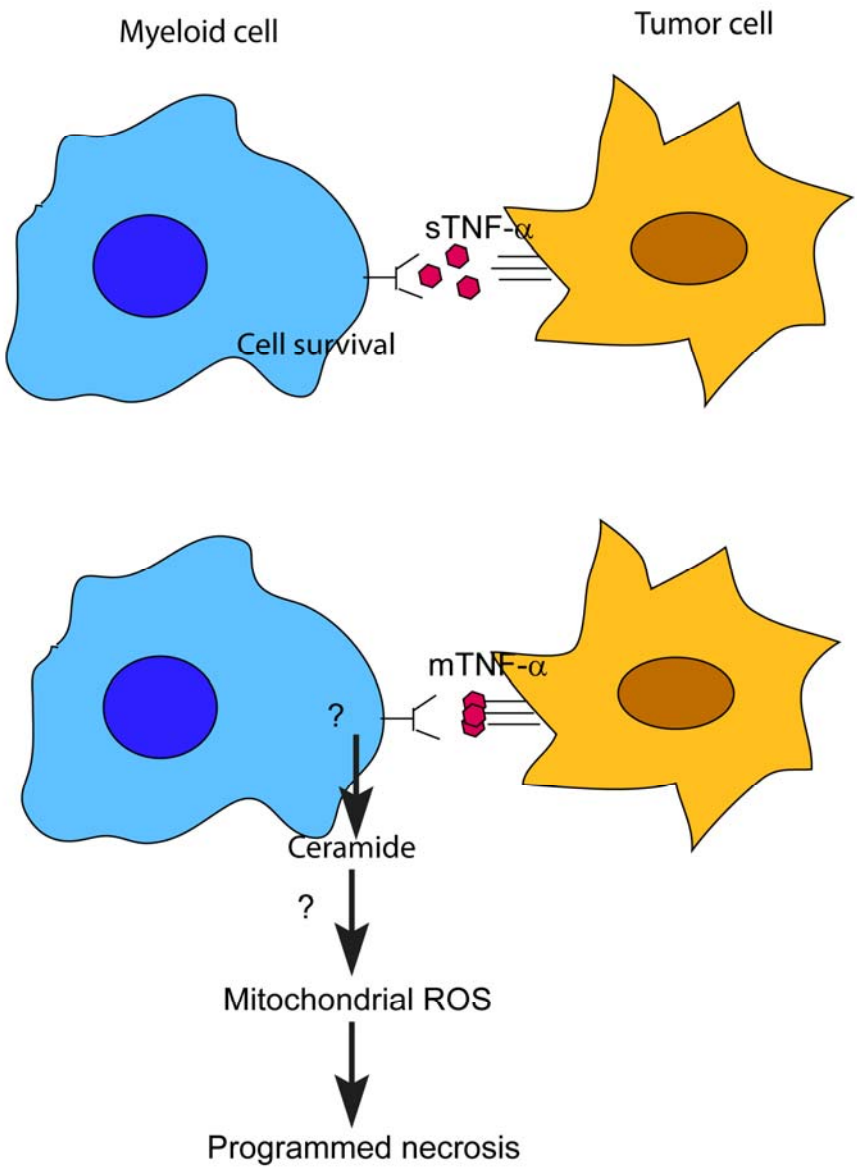

Figure 31. Schematic overview of tumor-derived soluble and membrane TNF-α effects on tumor associated myeloid cells. Soluble TNF-α expression by tumor cells results in survival of tumor associated myeloid cells. In contrast, membrane TNF-α increases programmed necrosis through increased ceramide-mediated mitochondrial ROS generation.

Role of Ceramide in Programmed Necrotic Cell Death

TNF receptor superfamily is well known for their ability to induce cell death. As discussed in chapter I, some of these receptors contain a cytosolic death domain which regulates cell death. Programmed necrosis stimulated by TFNR-1, Fas and TNF-related apoptosis-inducing ligand receptor-1 and -2 (TRAIL-R1/2), usually require presence of apoptosis inhibitors or the absence of the caspase-8-activating adaptor, FADD. Once programmed necrosis is initiated, multiple factors contribute to the final execution of cell death. One of the most studied regulators of necrosis is receptor interacting protein-1 (RIP-1). This pathway was discussed in chapter I. Another factor that induces TNF-α-mediated necrosis is ceramide, which in our study was identified as the mediator of mTNF-α-induced mitochondrial ROS and programmed necrosis. Although the mechanism by which ceramide induced mitochondrial ROS was not determined, some of the necessary molecular events in ceramide-induced mitochondrial ROS have been characterized. Interestingly, most of these studies have been done in relation to the soluble form of TNF-α and its activation of TNFR-1 and therefore, our understanding of mTNF-α mediated ceramide activation and TNFR-2 activity is limited. We hypothesize that the downstream events leading to ceramide-mediated mitochondrial ROS and cell death are shared by both TNF-α isoforms albeit with different initial activation mechanism. In the following section, we will discuss the current knowledge of mechanisms by which ceramide contributes to programmed cell necrosis and how its activity is linked to TNF-α-mediated ROS

production. These pathways could serve as a model for membrane TNF-α-activated programmed necrosis.

Ceramide as a signaling molecule

Signaling through the sphingomyelin pathway and the generation of the second messenger ceramide is ubiquitous and evolutionarily conserved. Most mammalian cells appear capable of signaling through the sphingomyelin pathway (Kolesnick and Kronke, 1998). The sphingomyelin pathway has been implicated as a major signaling mechanism that modulated the action of a number of receptors such as Fas, CD28, CD95, IL-1β and progesterone, causing the activation of sphingomyelinases (SMases) (Kolesnick and Kronke, 1998). Activated SMases hydrolyze the phosphodiester bond of sphingomyelin to yield the second messenger ceramide and phosphorylcholine (Won and Singh, 2006). For some of these receptors, ceramide signals immediately after cellular activation and hence appears to fulfill the role of a classic second messenger, whereas in other instances, ceramide generation is a later response downstream of a complex set of interacting signals (Kolesnick and Kronke, 1998).

Ceramide-mediated mitochondrial ROS and programmed necrosis

Studies have shown that naturally occurring C16 ceramide or addition of exogenouse C2-ceramide causes an increase in ROS generation through mitochondria (Di Paola et al., 2000; Garcia-Ruiz et al., 1997). Ceramide may generate ROS from mitochondria as a consequence of cytochrome c release, an

electron carrier of the respiration chain between complexes II and III in mitochondria (Cai and Jones, 1998). This was further demonstrated by Ghafourifar et al. who showed that C2- and C6-ceramide induce release of cytochrome c from isolated mitochondria (Ghafourifar et al., 1999). This led to a decrease in mitochondrial oxygen consumption, mitochondrial inner transmembrane potential, Ca_2^+ retention, and finally to mitochondrial dysfunction and ROS generation (Ghafourifar et al., 1999). Ceramide was also reported to disturb the respiratory chain through direct interaction (Gudz et al., 1997; Schulze-Osthoff et al., 1992) as C2- and C6-ceramide treatment induced large pores in phospholipid planar membranes (Siskind and Colombini, 2000). Dynamic changes in the ceramide content of mitochondrial membranes by vesicular transport or local production could possibly regulate mitochondrial integrity and ROS generation.

TNFR-1-mediated ceramide generation

TNFR-1-mediated ceramide signaling is regulated through independent activation of two distinct forms of sphingomyelinases (SMases), a membrane-associated neutral SMase (N-SMase) and an acid SMase (A-SMase), found in caveolae and in the endosomal-lysosomal compartment (Liu and Anderson, 1995; Wiegmann et al., 1994). N-SMase and A-SMase are activated independently by a distinct cytoplasmic domain of TNFR-1(Wiegmann et al., 1994). The domain of TNFR-1 activating the A-SMase pathway corresponds to the death domain (DD) (Tartaglia et al., 1993; Wiegmann et al., 1994). This

region binds the cytoplasmic protein TRADD which serves as an adapter in recruitment of other proteins to the cytoplasmic TNF receptor complex (Hsu et al., 1996a; Shu et al., 1996). In contrast, N-SMase activation is mediated by neutral sphingomyelinase domain (NSD), which is adjacent to the death domain of TNF-R1 (Adam-Klages et al., 1996). The NSD binds FAN (factor associated with N-SMase activation) that mediates activation of N-SMase. Ceramide generated by N-SMase at the plasma membrane directs the activation of ceramide-activated protein kinase (CAPK) (Mathias et al., 1991).

The described pathways would explain the ceramide-induced programmed necrosis in cells expressing DD-containing TNFR-1. However, in our study mTNF-α-mediated cell necrosis also occurred in TNFR-1 knockout cells, indicating the presence of another distinct pathway independent of the death domain region. In the following sections we will propose possible mechanisms by which DD-lacking TNFRs can induce cell death. Furthermore, we will discuss models by which different TNF-α isoforms can elicit different biological responses.

Proposed Models Describing Differential Activity Between Soluble and Membrane TNF-α

Receptor/ligand stability

The variation in biological response to the soluble versus the membrane form of cytokine receptors activation has been observed in many TNF superfamily. The membrane form of molecules such as Fas ligand have been

shown to have an opposing role in modulating cell death when compared to their soluble form (Date et al., 2003). In a study by Hohlbaum and colleagues it was shown that in glaucoma, full-length FasL (i.e. membrane form) accelerates retinal ganglion cell death. By contrast, FasL-deficiency or administration of soluble FasL has a protective effect (Hohlbaum et al., 2000). TRAIL, which has important functions in inducing apoptosis, has been shown to have differential activation capacity toward TRAIL-R1 and R2 in a soluble form versus the membrane form (Wajant et al., 2001).

The molecular mechanisms accounting for these differences, remains unknown. A model proposed by Grell links mTNF-α isoform differential signaling pattern to its ability to form a more stable interaction with TNFRs (Grell et al., 1995). This model suggests that mTNF-α signaling depends on cell to cell contact which creates a juxtaposition of mTNF-α/TNFR, allowing formation of ligand-receptor complexes of greater stability with a different quality and quantity of the induced cellular response compared to sTNF-α. It is possible that the variation in ligand/receptor complex stability, and their half-life, may contribute to the different biological responses observed between different isoforms. This models remains to be validated.

Differential receptor conformation changes induced by different isoforms

TNF-R1 and TNF-R2 each contain four cysteine-rich repeats in their extracellular domains and form elongated shapes which interact with the lateral grooves of the trimeric ligand formed between each two of its three protomers

(Banner et al., 1993; Naismith and Sprang, 1998). Ligand-dependent trimerization of the receptors has been known as the key event for signal initiation. Ligand binding to the preformed TNFR complex induces conformational change and activates the receptor and then acquires signal competence (Wajant et al., 2003). Given the diverse events modulated by changes in the spatial proximity of cell surface receptors and the trimeric nature of TNF-α, it is possible that anchored mTNF-α encourages further modification or spatial arrangement of receptors on adjacent cells which result in induction of different signaling pathway. This may lead to differential recruitment of TNFRs adaptor molecules.

Inhibition of TNFR/TNF-α Endocytosis

Receptor internalization is a widely used mechanism to modulate signaling. Binding of hormones and growth factors to their cognate receptors typically trigger internalization, leading to transient receptor clearance from the surface, followed by either recycling to the membrane or lasting clearance through degradation in lysosomes (Bonifacino and Traub, 2003). Endocytosis also is an important mechanism to regulate TNF-α signaling. TNFR/TNF-α internalization and lysosomal degradation prohibits TNF signaling pathway and subsequent biological responses. Engagement of membrane TNF-α and formation of a stable complex may prevent cells from internalizing the TNFRs and this may serve as a stress signal which could ultimately induce a necrotic cell death.

Clustering of TNFRs and formation of lipid rafts

The mechanism by which the membrane TNF-α isoform induces cell death through DD-lacking TNFR-2 remains unknown. As discussed earlier, the cell death inducing property of TNFR-2 has been related to its cooperative nature with TNFR-1, through common signal intermediates such as TRAF1 and TRAF2 (Hsu et al., 1996b; Rothe et al., 1994). Therefore mTNF-α induced cell death observed in TNFR-1 knockout cell (chapter III) is indicative of the existence of an alternate mechanism that allows DD-lacking receptors to initiate a cell death signal.

It is well known that most TNF receptor superfamily members are able to induce cell death; CD40 and CD30 are among these receptors. This is intriguing, given the fact that similar to TNFR-2, the cytoplasmic C terminus of CD30 and 40 lack a death domain homology with the cytotoxic members of the TNFR superfamily, such as Fas, TNFR1, and TNF-related apoptosis-inducing ligand (TRAIL) receptors (Eliopoulos et al., 2000; Gulbins and Grassme, 2002).

How these events regulate programmed cell necrosis through ceramide could be explained by the formation of lipid rafts. A variety of receptors including CD30, CD40, CD95 and TNFR from the TFNR superfamily, have been shown to cluster upon stimulation (Gulbins and Grassme, 2002). Often these clusters are formed in cholesterol- and sphingolipid-rich domains of the cell membrane referred to as rafts. Upon activation of these receptors, ASMase is recruited from intracellular compartments to the cell surface which results in the release of ceramide in rafts, the generation of signaling platforms and the clustering of

receptors. Furthermore, rafts often shown to consist of multiple receptor types. It is also possible that upon stimulation of TNF receptors by mTNF-α other members of TNFR superfamily is recruited into the lipid raft. This has been confirmed by showing the localization of TNFR-1 to the CD40 cluster and induction of cell death. This process is dependent on the activation of SMase and ceramide production (Grassme et al., 2002). Interestingly, disruption of TNFR1 recruitment to the raft prevented TNF-α-induced cell death in Jurkat cells (Ko et al., 1999).

The intermediates between receptor stimulations and recruitment of ASMase are unknown; involvement of factors such as G-protein Ras or Rac which have been shown to be activated by TNF receptor superfamily is suspected (Brenner et al., 1997). Collectively, these studies provide evidence that receptor clustering and rafts are the specific sites of ceramide generation, which appears essential in modulation of signaling for some TNF receptor superfamily members (Cremesti et al., 2002). It is possible that mTNF-α-mediated ceramide production is regulated through formation of a cluster of receptors in these lipid rich rafts, multiple TNF receptor cluster formation, or formation of a receptor cluster with other TNFR superfamily members.

Is mTNF-α-Activated Mitochondrial ROS Sufficient for Induction of Programmed Necrosis?

In addition to their role as an inducer of cell death, reactive oxygen species are also produced during normal physiological events, and are important

in carrying various biological processes (Droge, 2002; Morel and Barouki, 1999; Sauer et al., 2001). Therefore, the intracellular levels of ROS are kept tightly regulated by numerous ROS defense systems. This raises the question of whether mitochondrial ROS generated by mTNF-α- induced ceramide is sufficient to disrupt the balance in the redox state of the cell and to initiate programmed necrosis or whether there any other factor(s) contributing to the final execution of cell death.

Depletion of ROS scavengers

ROS generation alone is not the source of oxidative stress, but cellular stress is ultimately driven by an imbalance in ROS production and detoxification. One way to disrupt the ROS homeostasis of the cells is to allow the accumulation of ROS by eliminating the scavangers present in the cell. This causes the imbalance of free radicals and their scavengers, increasing the level of ROS and inducing DNA (Marnett, 2000), protein (Berlett and Stadtman, 1997) and lipid (Noguchi et al., 2002) damage. Interestingly, an enhanced level of ceramide has been shown to contribute to depletion of the ROS scavenger, glutathione (Mari et al., 2004) and increasing mitochondria susceptibility to GD3, a ceramide-derived ganglioside. GD3 trafficking to the mitochondria directly induces ROS production (Garcia-Ruiz et al., 2002; Garcia-Ruiz et al., 2000). It would be interesting to see whether the availability of antioxidant enzymes in mTNF-α-treated cells are affected. This could be evaluated by assessing the level of different scavengers in mTNF-α treated cells.

Sustained JNK activation

A significant increase in ROS level is necessary to disturb the intracellular ROS balance and initiate programmed necrosis. It is plausible that in addition to direct mTNF-α-activation of mitochondria, activation of other ROS inducing pathways also contribute to this event. It has been well established that ROS plays a critical role in TNF-α-mediated c-Jun NH2-terminal kinase (JNK, known as stress-activated protein kinases) activation and TNF-α induced necrotic cell death. Elevated levels of ROS immediate prolonged JNK activation and TNF-α-induced ROS, cause oxidation and inhibition of JNK-inactivating phosphatases by converting their catalytic cysteine to sulfenic acid. This results in sustained JNK activation; activated JNK further promotes ROS production from mitochondria, forming a positive feedback loop, enhancing necrosis (Shen and Pervaiz, 2006; Wu et al., 2012). It is possible that mTNF-α induced ROS and programmed cell necrosis is also mediated in part by further activation of JNK and related genes further increasing ROS levels (Figure 32).

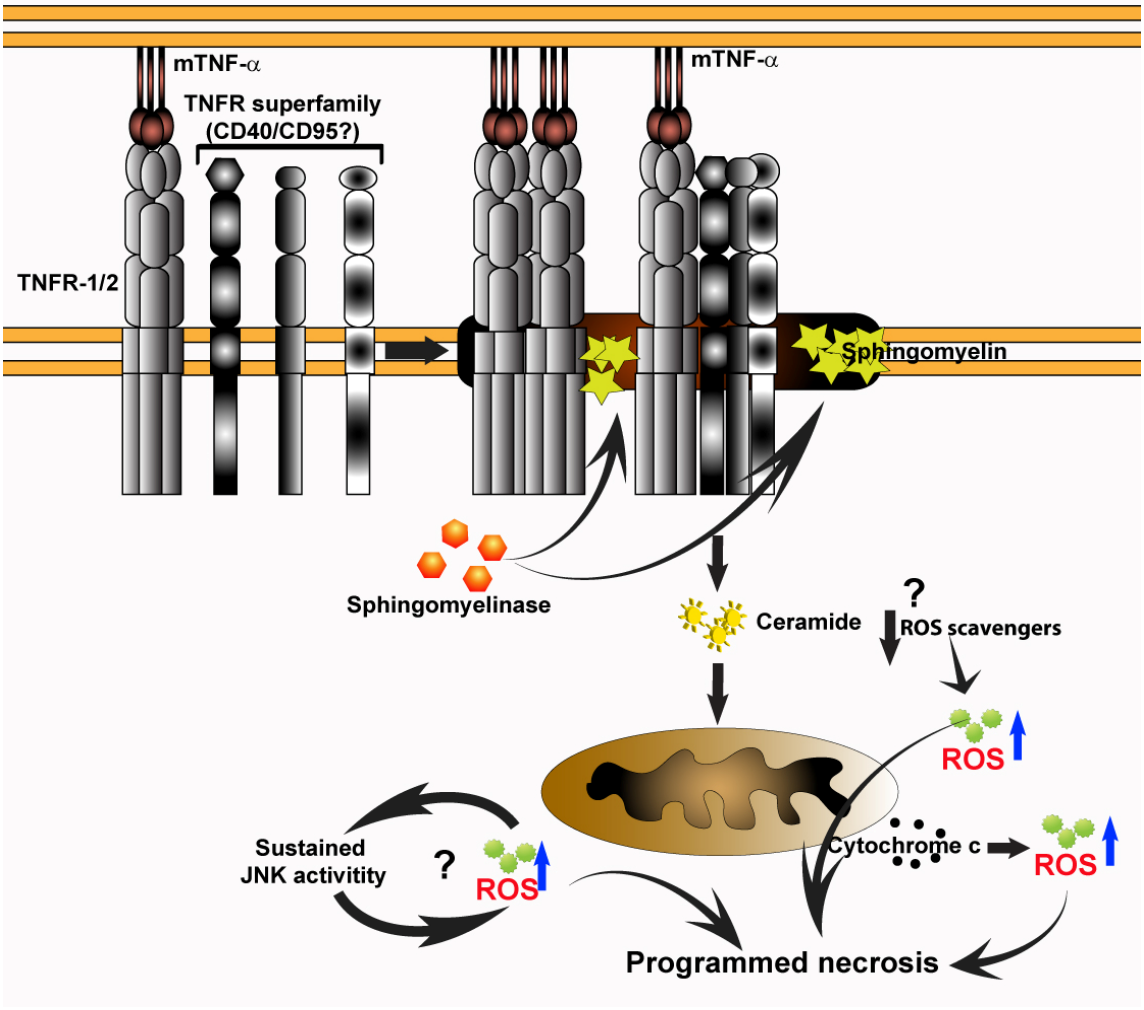

Figure 32. Proposed mechanism of mTNF-α activated ceramide pathway and mitochondrial ROS generation. Membrane anchored TNF-α creates a more stable contact with TNFR1/2 with a longer half-life. This allows the formation of higher-order receptor complexes (receptor clustering). These clusters of receptors are formed in lipid rafts abundant in sphingomyelin which are the precursor of ceramide. This activates ASM and triggers a translocation of the enzyme onto the sphinomyelin rich lipid rafts leading to an increase in intracellular ceramide level. Ceramide will decrease mitochondrial membrane integrity and reactive oxygen species (ROS) leakage which could increase DNA, protein, and organelle damage or further increase JNK activity and in return create a positive feedback and further increasing mitochondrial ROS.

Significance

For many years interactions between tumor-infiltrating myeloid and tumor cells have been of great interest since they both promote and inhibit tumor formation, growth and progression. This study further highlights the importance of tumor derived cytokines and their contribution to malignant growth by modulating inflammatory cells in cancer.

In chapter II it was shown that human NSCLC tumor cell express both soluble and membrane TNF-α at varying levels. Analysis of Human none-small-cells lung carcinomas (NSCLCs) microarray database showed that TNF-α and TACE expression patterns favoring mTNF-α were predictive of improved lung cancer survival. These findings suggest that the bioavailability of each isoform may distinctly regulate tumor progression and analysis of TNF-α gene expression and the level of soluble and membrane TNF-α could potentially be used as a prognostic/therapeutic tool. This study is the first to suggest that TNF-α isoform analysis should be applied to identify candidates for which anti-TNF-α agents are likely to be beneficial vs. detrimental. These finding further allows us to determine the utility of novel cancer treatments that inhibit TNF-α dependent tumor growth such as development of drugs that inhibit cleavage of membrane TNF-α as an alternate cancer treatment.

Despite the large body of published studies on the role of this important cytokine in cancer, the molecular and physiological context, necessary for optimal targeting of TNF in malignant disease, is still not clear. This has posed a challenge in using many of the FDA approved anti-TNF in effective cancer

therapy. Our work provides new insights on this paradox and on how we may rationally move TNF-α-targeted therapy forward. This information is of great interest as anti-TNF-α agents have recently been used in phase I/II clinical trials without significant benefit.

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