REGULATION OF TISSUE RESPONSES: THE TWEAK/FN14 PATHWAY AND OTHER TNF/TNFR SUPERFAMILY MEMBERS THAT ACTIVATE NONCANONICAL NFκB SIGNALING

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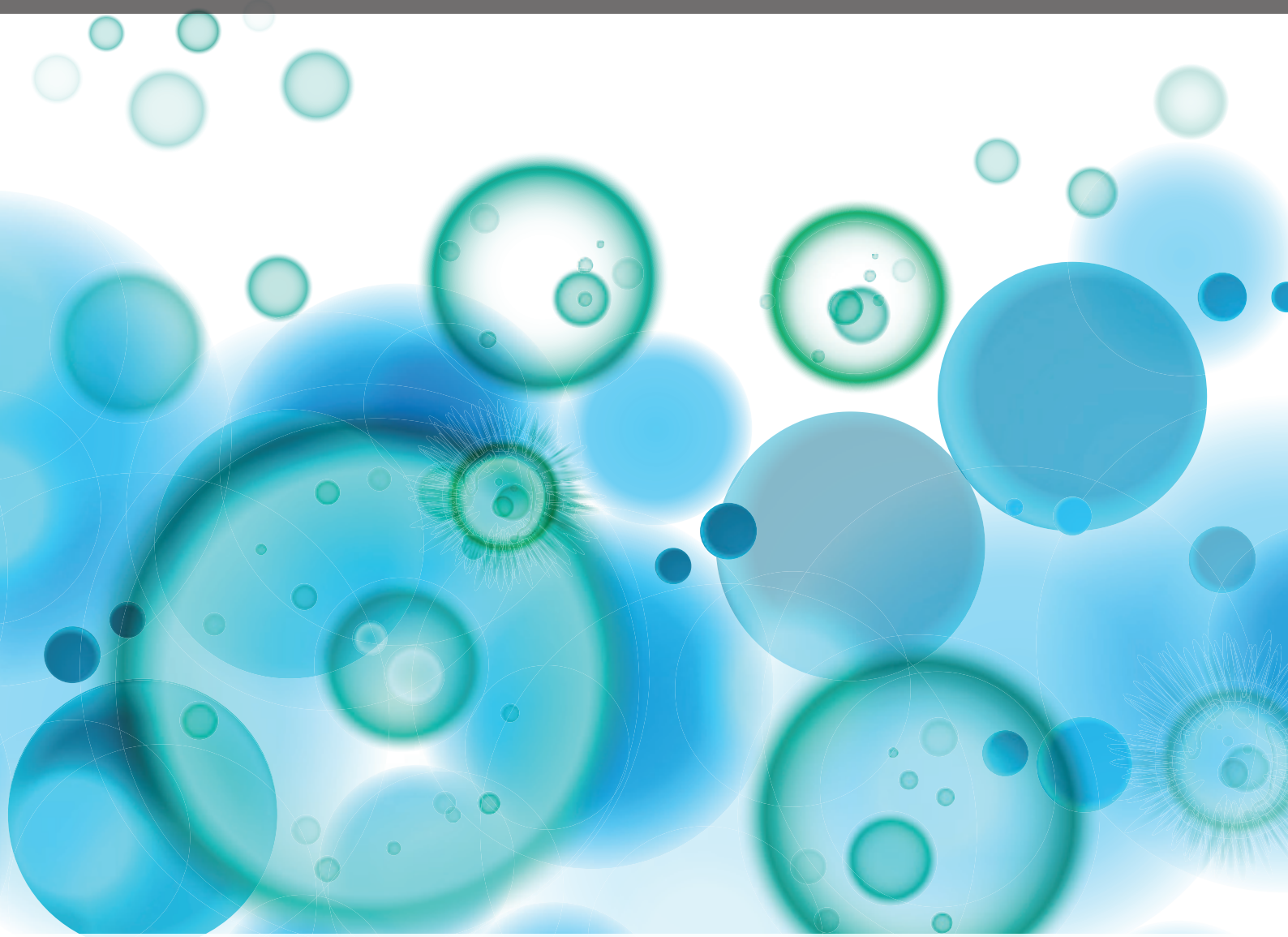

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REGULATION OF TISSUE RESPONSES: THE TWEAK/FN14 PATHWAY AND OTHER TNF/TNFR SUPERFAMILY MEMBERS THAT ACTIVATE NONCANONICAL NFκB SIGNALING

Topic Editors:
Linda C. Burkly, Biogen, Inc., USA
Timothy S. Zheng, Biogen, Inc., USA
John Silke, The Walter and Eliza Hall Institute, Australia

The immune system mediates tissue responses under both physiological and pathological conditions, impacting the inflammatory, fibrogenic and regenerative components. In addition to various leukocyte subsets, it is now recognized that epithelial, endothelial and other non-hematopoietic tissue cell types actively contribute to the interplay shaping tissue responses. Further understanding the molecular pathways and mechanisms mediating these tissue responses is of great interest.

In the past decade, TNF-like weak inducer of apoptosis (TWEAK) and its receptor, FGF-inducible molecule-14 (Fn14), members of the TNF/TNFR superfamily, have emerged as a prominent molecular axis regulating tissue responses. Generally leukocyte-derived, TWEAK signals through Fn14 which is highly induced in injured and diseased tissues on the surface of parenchymal, stromal and progenitor cells, thereby orchestrating a host of tissue-shaping responses, including inflammation, angiogenesis, cell proliferation or death, and the regulation of progenitor cells. Compelling preclinical results indicate that whereas transient TWEAK/Fn14 activation promotes productive tissue responses after acute injury, excessive or persistent TWEAK/Fn14 activation drives pathological tissue responses, leading to progressive damage and degeneration in target organs of injury, autoimmune and inflammatory diseases and cancer. Given that the highly inducible pattern of Fn14 expression is well conserved between mouse and man, the role of TWEAK/Fn14 in human disease is an area of intense investigation. Recent findings have also begun to shed light on how the TWEAK/Fn14 pathway fits into the immune network, interplaying with other well-established pathways, including TNFa, IL-17, IL-13 and TGFb, in regulating tissue responses.

The noncanonical nuclear factor kB (NFκB) pathway plays a role in immunity and disease pathologies and appears to be activated by only a subset of TNF/ TNFR superfamily members. Of the various signaling pathways downstream of TWEAK/Fn14, particular attention has been placed on the noncanonical NFκB pathway given that given that TWEAK induces acute activation of canonical NFκB but prolonged activation of noncanonical pathway. Thus dovetailing of the TWEAK/Fn14 axis with noncanonical NFκB pathway activation may be a key mechanism underlying tissue responses. Also of great interest is a deeper understanding of where, when and how tissue responses are regulated by other TNF/ TNFR superfamily members that can signal through noncanonical NFκB.

This Research Topic issue will cover:

1. TWEAK/Fn14 pathway biology, role in tissue responses, injury, and disease pathogenesis
2. Role of noncanonical NFκB signaling cascade in tissue responses
3. Translational studies of relevance of TWEAK/Fn14 and noncanonical NFκB in human disease
4. Other TNF superfamily members' signaling through noncanonical NFκB in the regulation of tissue responses
5. Reviews and Perspectives on the above

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Regulation of tissue responses: the TWEAK/Fn14 pathway and other TNF/TNFR superfamily members that activate non-canonical NFκB signaling

Linda C. Burkly*

Department of Immunology, Biogen Idec, Inc., Cambridge, MA, USA
**Correspondence: linda.burkly@biogenidec.com*

Edited and reviewed by:
Pietro Ghezzi, Brighton and Sussex Medical School, UK

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The immune system mediates tissue responses under both physiological and pathological conditions. In addition to leukocyte subsets, non-hematopoietic tissue cell types actively contribute to shaping tissue responses, including the inflammatory, fibrogenic, and regenerative components. TWEAK and its receptor Fn14, members of the TNF/TNFR superfamily, have emerged as a prominent molecular axis regulating tissue responses (1). Generally leukocyte-derived, TWEAK signals through Fn14, which is highly induced in injured and disease tissues on the surface of parenchymal, vascular, stromal, and progenitor cells, thereby orchestrating a host of tissue-shaping processes, including inflammation, angiogenesis, cell proliferation, and death, and regulation of progenitor cells. Of the downstream signaling pathways, particular attention has been given to the non-canonical NFκB pathway, given that TWEAK induces acute activation of canonical NFκB but prolonged non-canonical NFκB activation. Thus, non-canonical NFκB signaling may be a key mechanism underlying TWEAK/Fn14-induced tissue responses. The non-canonical NFκB pathway is known to play a role in immunity and disease pathologies and is typically activated by only a subset of TNFR superfamily members, including Fn14, TNFR2, BAFFR, CD40, LTβR, and RANK. Thus, there is also broad interest in the role of this subset of TNFR superfamily members and their downstream signaling potentials in the regulation of processes underlying tissue remodeling in health and disease. This Research Topic is addressed in a compilation of 19 expert reviews and 1 original research article.

The TWEAK/Fn14 pathway as an injury-inducible mediator of pleiotropic responses is introduced in a review of the work implicating sustained Fn14 signaling in disease pathogenesis and encompassing the current TWEAK/Fn14-targeting approaches for treatment of human disease (2). Broad relevance to neurological diseases is supported by a basic TWEAK/Fn14 role in regulating the structure and function of the neurovascular unit, thereby regulating blood–brain barrier (BBB) permeability (3). Furthermore, BBB damage appears to be an important component of neuropsychiatric systemic lupus erythematosus, and there is emerging evidence for a role for TWEAK/Fn14 in compromising the BBB in lupus (4). Also relevant to lupus is the pathogenic role of TWEAK/Fn14 in the renal manifestation of lupus nephritis. Indeed, evidence supporting TWEAK/Fn14-mediated pathological mechanisms in contexts of acute kidney injury and chronic kidney diseases is substantial and clinical targeting of TWEAK is ongoing in lupus nephritis (5). Also addressed in this Research Topic is the role of TWEAK/Fn14 in the pathological remodeling underlying other inflammatory diseases, namely cardiovascular diseases and obesity-associated Type-2 diabetes (6, 7), as well as in myocardial remodeling leading to heart failure (8), and a common theme also addressed in these articles is the potential use of soluble TWEAK as a biomarker for cardiovascular diseases. The expression of soluble TWEAK in biological fluids of patients with autoimmune/chronic inflammatory diseases and its potential as a biomarker of these diseases is also more broadly discussed (9). Given that TWEAK has emerged as a major cytokine regulating skeletal muscle biology, two articles are dedicated to its role in muscle wasting and mitochondrial dysfunction, and its complex regulation of myogenesis where distinct roles for canonical versus non-canonical NFκB signaling have been delineated (10, 11). Besides Fn14 upregulation in contexts of injury/disease, it is also highly expressed on tumor cells relative to normal tissue making it an attractive therapeutic target. Purcell et al. (12) review the growth inhibitory activity of an Fn14-specific antibody on an array of human tumor cells, differentially dependent on canonical and/or non-canonical NFκB, an approach that is currently being pursued as a novel cancer treatment.

In summary, there is substantial evidence implicating TWEAK/Fn14 in the regulation of physiological and pathophysiological tissue responses, though there is still an incomplete understanding of the role of TWEAK/Fn14-induced canonical versus non-canonical NFκB in these various contexts. Since soluble TWEAK and membrane TWEAK differ in their capacity to induce canonical NFκB, distinct biological responses may reflect spatial and temporal differences in sources of TWEAK (13). New studies continue to inform the understanding of TWEAK-induced signaling, including ubiquitination events that are key to orchestrating canonical and non-canonical NFκB activation (14).

The role of other TNF/TNFR superfamily members in shaping tissue responses in health and disease is also reviewed, concentrating on those that can activate non-canonical NFκB. In a review on TNFα signaling through TNFR2, Faustman and Davis (15) discuss the concept of leveraging TNFR2 agonism to reshape the T cell compartment in autoimmune disease, and to promote tissue regenerative processes. On the other hand, Gardam and Brink (16) review the importance of BAFF/BAFFR-mediated non-canonical

NFκB signaling in peripheral B cell survival and maturation. Likewise, CD40L–CD40-mediated activation of non-canonical NFκB appears to be critical for B cell survival and possibly contributes to development of B cell malignancies (17). Both BAFFR and CD40-mediated non-canonical NFκB activation in B cells is restrained by the adaptor protein TRAF3, and the relationship between TRAF3 degradation and non-canonical NF-kB2 activation is delineated in an original research article (18). Beyond T and B cell activation, positioning cues are critical for proper immune system development and function. In this regard, LTβR plays a critical role in lymph node development and remodeling through its delivery of differentiation signals for reticular networks and vasculature (19). Tissue remodeling in the context of chronic liver diseases also features a prominent role for both LTβ and TWEAK in the crosstalk between liver progenitor cells and hepatic stellate cells, which can evolve into pathological fibrosis and hepatocellular carcinoma (20). Finally, Walsh and Choi (21) review the RANKL–RANK–OPG system, a preeminent player in the bone homeostasis, pathologies including mammary gland tumorigenesis, and in the interplay between bone and the immune system. This collection of expert reviews provides a current perspective of the role of this particular subset of TNF/TNFR family members that can activate non-canonical NFκB signaling in shaping tissue responses in contexts of development, homeostasis, and remodeling.

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TWEAK/Fn14 axis-targeted therapeutics: moving basic science discoveries to the clinic

Emily Cheng†, Cheryl L. Armstrong†, Rebeca Galisteo and Jeffrey A. Winkles*

Department of Surgery, Center for Vascular and Inflammatory Diseases and Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA

Edited by:
Linda C. Burkly, Biogen Idec Inc., USA

Reviewed by:
Fulvio D'Acquisto, Queen Mary University of London, UK
Miriam Wittmann, University of Leeds, UK
Nicolas Wisniacki, Biogen Idec Ltd, UK

***Correspondence:**
Jeffrey A. Winkles, Department of Surgery, University of Maryland School of Medicine, UMB BioPark Building One, Room 320, 800 West Baltimore Street, Baltimore, MD 21201, USA
e-mail: jwinkles@som.umaryland.edu

† Emily Cheng and Cheryl L. Armstrong have contributed equally to this work.

The TNF superfamily member TWEAK (TNFSF12) is a multifunctional cytokine implicated in physiological tissue regeneration and wound repair. TWEAK is initially synthesized as a membrane-anchored protein, but furin cleavage within the stalk region can generate a secreted TWEAK isoform. Both TWEAK isoforms bind to a small cell surface receptor named Fn14 (TNFRSF12A) and this interaction stimulates various cellular responses, including proliferation and migration. Fn14, like other members of the TNF receptor superfamily, is not a ligand-activated protein kinase. Instead, TWEAK:Fn14 engagement promotes Fn14 association with members of the TNFR associated factor family of adapter proteins, which triggers activation of various signaling pathways, including the classical and alternative NF-κB pathways. Numerous studies have revealed that Fn14 gene expression is significantly elevated in injured tissues and in most solid tumor types. Also, sustained Fn14 signaling has been implicated in the pathogenesis of cerebral ischemia, chronic inflammatory diseases, and cancer. Accordingly, several groups are developing TWEAK- or Fn14-targeted agents for possible therapeutic use in patients. These agents include monoclonal antibodies, fusion proteins, and immunotoxins. In this article, we provide an overview of some of the TWEAK/Fn14 axis-targeted agents currently in pre-clinical animal studies or in human clinical trials and discuss two other potential approaches to target this intriguing signaling node.

Keywords: TWEAK, Fn14, inflammatory disease, cancer, clinical trial

INTRODUCTION

The TNF superfamily member TWEAK was first described in 1997 (1), and since that initial publication dozens of research groups throughout the world have studied the biological properties of this multifunctional cytokine. One of the major early milestones in the TWEAK research arena was the cloning of the human TWEAK receptor by Wiley et al. (2), which had 100% predicted sequence identity to a previously reported growth factor-inducible type I transmembrane protein named Fn14 (3, 4). TWEAK is the only TNF superfamily member that can bind Fn14 (5). The TWEAK and Fn14 genes are expressed at low levels in most normal tissues but upregulated in damaged tissue [reviewed in Ref. (6, 7)]. Moreover, the Fn14 gene is highly expressed in both primary tumors (8–14) and tumor metastases (11). TWEAK is synthesized as a type II transmembrane protein but it can be cleaved to generate a soluble cytokine (1, 15, 16). TWEAK:Fn14 engagement promotes TNFR associated factor (TRAF) binding to the Fn14 cytoplasmic tail (17), activation of downstream signaling pathways (e.g., NF-κB, MAPK, PI3K/Akt), and triggering of various cellular responses, including proliferation, survival, migration, differentiation, or death [reviewed in Ref. (6, 7)]. Interestingly, binding of the membrane-bound and soluble TWEAK isoforms to Fn14 can trigger differential downstream outputs; for example, membrane TWEAK is a more potent activator of the classical NF-κB signaling pathway (16).

One important aspect of Fn14 biology that may have clinical implications is the concept of TWEAK-independent Fn14 signaling. Experimental manipulation of Fn14 expression levels in cancer cells cultured *in vitro* can regulate signal transduction and cellular properties; for example, cell migration and invasion (8, 10, 18–20). These findings have led our group to propose that when Fn14 expression in cells reaches a certain threshold level it may signal on its own, even without ligand engagement (6). Recent studies in which we transiently expressed a mutant Fn14 protein that is unable to bind TWEAK support the notion that Fn14 can in fact signal in a ligand-independent manner (21). This signaling mechanism may be particularly important in injured tissues and cancers where Fn14 levels are high but TWEAK levels are low [e.g., in glioblastomas (22) and melanomas (unpublished data)]. We hypothesize that the most likely explanation for TWEAK-independent Fn14 activation is that when Fn14 is expressed at high levels in cells it spontaneously multimerizes, and this will trigger TRAF association, downstream signaling, and cellular responses.

A second critical milestone in the TWEAK-Fn14 research arena was the generation of TWEAK- or Fn14-deficient mice by groups at Genentech (23) and Biogen Idec (24, 25). Studies using these mice, in conjunction with studies testing the effects of TWEAK-neutralizing biologics in mouse models of human tissue injury and disease, have been instrumental in establishing the generally accepted view that TWEAK/Fn14 signaling is important for

effective wound repair following acute tissue injury and that chronic Fn14 signaling can promote pathological tissue responses [reviewed in Ref. (6, 7, 26, 27)].

Basic science studies using cells in culture, expression profiling studies using normal and diseased tissue specimens, and *in vivo* studies using wild-type (WT) or genetically engineered mice have all indicated that the TWEAK/Fn14 axis may play an important role in the pathophysiology of several different human diseases [reviewed in Ref. (6, 7, 26–28)]. In general, this axis seems to be primarily involved in disease progression and maintenance, not initiation. Numerous academic and industrial research laboratories have initiated programs to develop biologics or small molecule compounds that activate or inhibit this signaling axis, depending on the disease target [reviewed in Ref. (28)]. Remarkably, the first two TWEAK/Fn14 axis-targeted Phase I clinical trials began recruiting in 2008, only 7 years after the initial report demonstrating that TWEAK and Fn14 were a ligand-receptor pair (2). In this article, we provide an overview of some of the TWEAK- or Fn14-directed therapeutic agents that are presently in pre-clinical development or have entered clinical trials.

TWEAK/Fn14 AXIS-TARGETED THERAPEUTICS: INFLAMMATORY AND/OR NEURODEGENERATIVE DISEASES

Inflammation is a complex, dynamic process that occurs in tissues following traumatic, infectious, toxic, or autoimmune injury [reviewed in Ref. (29, 30)]. This physiologic response is critical for our ability to heal wounds and fight off pathogens. Inflammation is normally very tightly controlled but when this process is excessive or prolonged it contributes to the pathogenesis of numerous diseases, including atherosclerosis, ischemic stroke, rheumatoid arthritis (RA), and inflammatory bowel diseases [reviewed in Ref. (30–32)]. Persistent TWEAK/Fn14 signaling has been implicated in the pathogenesis of these and other related diseases [reviewed in Ref. (7, 27)] and in this section we summarize some of the TWEAK-targeted therapeutic agents under development for these conditions (**Table 1**).

Fn14-Fc FUSION PROTEIN

Ischemic stroke ranks third among the most common causes of mortality and is a leading cause of long-term disability in the world (33, 34). The onset of cerebral ischemia triggers a number of pathophysiological events, including oxidative stress, glucose deprivation, disruption of the architecture of the neurovascular unit [which results in an increase in the permeability of the blood brain barrier (BBB) and the development of cerebral edema], and brain inflammation [reviewed in Ref. (32, 34)]. Intravenous administration of the thrombolytic agent tissue plasminogen activator (tPA) within 3–4.5 h of stroke onset is the only FDA-approved treatment for stroke patients (34).

Numerous studies have indicated that TWEAK is a potential novel therapeutic target for treatment of acute cerebral ischemia. Both TWEAK and Fn14 expression have been detected in astrocytes, microglia, endothelial cells, and neurons *in vitro* and *in vivo*. TWEAK expression is more prominent in astrocytes, and Fn14 expression is found predominantly in neurons [reviewed in Ref. (35)]. Early studies using murine models of focal cerebral ischemia showed that both TWEAK and Fn14 mRNA expression in ischemic brain tissue increases within hours after an ischemic insult (36–38). Importantly, there is also an increased level of these mRNAs in human infarct tissue obtained from deceased stroke patients and TWEAK levels are elevated in the serum of stroke patients (39). Functional studies using Fn14-deficient mice have indicated that TWEAK/Fn14 signaling contributes to the cell death and edema that occurs following acute cerebral ischemia (38, 40). Accordingly, Yepes and colleagues tested whether administration of a soluble Fn14-Fc decoy protein shortly after ischemia onset could inhibit TWEAK activity in mouse brain tissue and thereby attenuate cell death and edema. In their initial studies they found that Fn14-Fc injection significantly reduced infarct volume, microglia activation, and apoptotic cell death in the ischemic penumbra (37). Subsequent research found that Fn14-Fc delivery could also improve animal motor activity and reduce the extent of cerebral ischemia-triggered BBB breakdown and edema (40, 41). Thus, TWEAK neutralization may be an effective therapeutic strategy for acute cerebral ischemia.

Fn14-TRAIL FUSION PROTEIN

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS) that causes progressive neurological deterioration [reviewed in Ref. (42, 43)]. MS is characterized by

Table 1 | Examples of TWEAK-targeted therapeutic agents for inflammatory and/or neurodegenerative diseases.

Agent	Developer	Type of agent	Status	Disease	Reference(s)
Fn14-Fc	EUSOM/UMSOM	Decoy receptor	Pre-clinical	Cerebral ischemia and edema (stroke)	(37, 40, 41)
Fn14-TRAIL (KAHR-101)	KAHR Medical	Signal converter protein	Pre-clinical	EAE (model of multiple sclerosis)	(52, 54)
BIIB023	Biogen Idec	Neutralizing mAb	Phase I trial Start date: October 2008 Completion date: April 2011	Rheumatoid arthritis	ClinicalTrials.gov NCT00771329 (68)
BIIB023	Biogen Idec	Neutralizing mAb	Phase II trial Start date: July 2012 (recruiting)	Lupus nephritis	ClinicalTrials.gov NCT01499355

EUSOM, Emory University School of Medicine; UMSOM, University of Maryland School of Medicine; EAE, experimental autoimmune encephalomyelitis; mAb, monoclonal antibody.

the presence of focal demyelinated lesions in the white and gray brain matter, the accumulation of inflammatory cells, and BBB disruption with edema. A number of effective anti-inflammatory and immunomodulatory treatments are now available that reduce disease activity for patients in the relapsing-remitting stage of the disease [reviewed in Ref. (43, 44)]. The primary first-line treatments for MS are interferon-β (IFN-β) glatiramer acetate (Copaxone), a synthetic protein whose mechanism of action is not fully understood. Over time, however, autoantibodies can develop in the IFN-β-treated patients, resulting in decreased efficacy. Skin conditions such as erythema were the most common side effects in Copaxone-treated patients [reviewed in Ref. (43)]. If disease stabilization fails to be achieved using these first-line therapies, a number of second-line treatments are available. These second-line treatments leave MS patients at risk for a number of side effects including CNS infection, skin cancer, and cardiac toxicity [reviewed in Ref. (43)]. Therefore, new targeted therapies are needed for MS patients and several studies have indicated that TWEAK deserves consideration as a potential molecular target [reviewed in Ref. (45)].

Experimental autoimmune encephalitis (EAE) is a Th1-mediated autoimmune disease of the CNS that is the most commonly used experimental model for human CNS demyelinating diseases, including MS [reviewed in Ref. (43, 46)]. EAE is induced by inoculating mice with myelin oligodendrocyte glycoprotein (MOG). This leads to a disease course characterized by an acute paralytic phase followed by a moderate remission and finally a chronic phase associated with demyelination. Two independent studies showed that TWEAK/Fn14 pathway inhibition decreased disease severity in the EAE model. In one study, Desplet-Jego et al. found that intraperitoneal administration of an anti-TWEAK-neutralizing monoclonal antibody (mAb) after the priming phase reduced leukocyte CNS infiltration and clinical score (47). In the second study, Mueller et al. showed that intraperitoneal injection of recombinant rat soluble TWEAK and Fn14 extracellular domain resulted in induction of neutralizing antibodies, causing a decrease in CNS inflammatory infiltration and EAE severity in both rat and mouse models (48). It has also been reported that soluble TWEAK overexpression, accomplished either by injection of a TWEAK expression plasmid (48) or the generation of TWEAK transgenic mice (49), increases EAE severity.

Another animal model relevant to MS is cuprizone-induced demyelination. It is an ideal system to study neuroinflammation, demyelination, and remyelination [reviewed in Ref. (46)]. Cuprizone is a copper chelating agent, which causes oligodendrocyte cell death and microglial infiltration into the damaged site in the murine CNS. Whereas in the EAE model demyelination is dependent upon $CD4^+$ T cells, demyelination in the cuprizone model is mediated by microglia with little contribution from peripheral macrophages. Iocca et al. showed that modest upregulation of Fn14 and TWEAK occurred during the demyelination phase of cuprizone treatment (50). Furthermore, TWEAK-deficient mice exhibited a delay in demyelination of the corpus callosum after 4 weeks of cuprizone treatment as compared to WT animals.

These studies, in combination with research demonstrating that TWEAK and Fn14 expression is upregulated in human brain specimens from MS patients compared to controls (51) support the contention that TWEAK-neutralizing agents could be effective therapeutics for this disease. In this regard, Razmara et al. reported that transposon-mediated expression of an Fn14-TRAIL fusion protein in mice reduced the incidence and severity of EAE (52). Fn14-TRAIL consists of the Fn14 extracellular domain fused to the soluble form of the TNF superfamily member TRAIL. This "signal converter protein" is designed to both inhibit TWEAK/Fn14 signaling and promote TRAIL/TRAILR signaling, with this second functional attribute included on the basis of earlier work showing that TRAIL attenuates MOG-induced EAE (53). A more recent study by Prinz-Hadad et al. found that purified Fn14-TRAIL protein limited T cell responses and alleviated EAE severity only when administered 10 days after EAE induction as compared to Fn14-TRAIL treatment starting on the day of MOG immunization (54). This suggests that Fn14-TRAIL has a more significant impact on the propagation phase of EAE rather than during the induction phase. Fn14-TRAIL (KAHR-101) is currently under further preclinical development by KAHR Medical Ltd., for MS and cancer (see below).

TWEAK mAb BIIB023

Rheumatoid arthritis is a progressive autoimmune disease characterized by joint inflammation and tissue destruction that leads to disability and a decrease in quality of life. While the cause of RA is unknown, many genetic determinants and environmental factors that contribute to disease susceptibility have been identified [reviewed in Ref. (55, 56)] and the pathogenesis of the disease has been well characterized [reviewed in Ref. (57)]. RA development involves a cellular and humoral immune response to auto-antigens, with patients developing a range of autoantibodies [reviewed in Ref. (58)]. The disease is usually concentrated in the joint regions of the hands, knees, or feet, where the inflamed synovial membrane, caused by activated leukocytes and angiogenesis, contains hyperplastic synoviocytes, pro-inflammatory cytokines, cartilage-degrading enzymes, and the osteoclast-containing pannus that breaks down bone. RA treatment options include corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate (MTX) and biologics including TNF-α or IL-6 inhibitors [reviewed in Ref. (57, 59)].

Numerous studies have implicated the TWEAK/Fn14 axis as a potential contributor to RA pathophysiology. When TWEAK and Fn14 expression were evaluated by immunohistochemistry of synovial tissue (ST) biopsies, TWEAK expression was higher in patients with RA compared to patients with psoriatic arthritis (PsA) (60) or osteoarthritis (OA) (61, 62). TWEAK levels are higher in serum obtained from RA patients versus control (63) and in both serum and synovial fluid of RA versus OA patients (62, 64). Furthermore, TWEAK expression was detected in newly diagnosed, DMARDs-naive patients as well as TNF-α mAb (Infliximab)-treated patients (60). While Fn14 expression was found elevated in RA patients compared to normal, healthy controls (62) there was no increase relative to PsA patients and no difference between active and inactive RA (60). Co-staining experiments revealed that TWEAK expression was found on fibroblast-like synoviocytes, $CD22^+$ B cells, macrophages, plasma cells, multi-nucleated cells, and ST blood vessels (60, 62). *In vitro*

studies have shown that TWEAK may contribute to RA pathogenesis through multiple mechanisms such as promoting bone resorption (61, 62) and joint tissue destruction (65), inhibiting bone and cartilage repair mechanisms (65), and promoting joint inflammation (66).

In vivo studies using mouse models of RA have shown that the TWEAK/Fn14 signaling pathway is a significant contributor to RA pathogenesis. An initial report showed that TWEAK levels were elevated in the sera of mice with collagen-induced arthritis (CIA) and that the disease severity, assessed by evaluating inflammation in the mouse paws, was decreased when the mice were treated with an anti-TWEAK-neutralizing mAb (65). The TWEAK antibody treatment also resulted in reduced inflammation and reduced cartilage and bone loss according to histological analysis. Furthermore, the TWEAK antibody-treated mice had reduced synovial angiogenesis and a reduction in the serum levels of inflammatory cytokines and chemokines. A second independent study also showed that an anti-TWEAK-neutralizing mAb attenuated the severity of CIA by reducing synovial angiogenesis and inflammatory chemokine levels in the serum and joints of CIA mice (67). Both studies showed that T- and B-cell responses to collagen II were maintained in the CIA experimental animals that were treated with TWEAK antibody, indicating that TWEAK/Fn14 signaling blockade does not have an effect on the adaptive immune response.

Recently, the results of a Phase I clinical trial (ClinicalTrials.gov Identifier NCT00771329) testing the TWEAK-blocking mAb BIIB023 in patients with RA were reported by Wisniacki et al. (68). BIIB023 was well tolerated in RA patients that received a single dose up to 20 mg/kg either alone or as an add-on to TNF-α inhibitor administration, with all patients taking background MTX. The single BIIB023 dose did not increase the incidence of adverse events when added to the TNF inhibitor treatment, and all adverse effects (AEs) were mild to moderate in severity. Additionally, there were no serious infections in this trial, consistent with the idea that blocking TWEAK/Fn14 signaling does not inhibit the adaptive immune response. BIIB023 showed low immunogenicity, favorable pharmacokinetics, and at high doses, was able to suppress serum soluble TWEAK levels for 28 days. BIIB023 is currently under investigation in a Phase II trial in patients with lupus nephritis (LN) (see below).

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with a relapsing-remitting course and a diverse array of clinical manifestations, including LN, cutaneous lupus, neuropsychiatric lupus, and atherosclerosis, that contribute to a decrease in quality of life and an increase in mortality [reviewed in Ref. (69)]. SLE genetic risk factors and disease pathogenesis have been well studied [reviewed in Ref. (70)]. SLE patients develop antibodies against an array of auto-antigens, including nuclear antigens, which can lead to inflammation and injury in many organs. SLE is primarily treated with glucocorticoids, cyclophosphamide, B cell-targeted therapy (e.g., anti-CD20 mAbs), and immunosuppressive drugs that are associated with a risk of infection, adverse events such as infusion reactions (characterized by fever, chills, rash, and swelling), and organ damage. These serious side effects have lead more recently to the development of targeted, biologic therapies [reviewed in Ref. (59)], but biologic and traditional therapies for SLE are not always effective and there is still a need for the development of novel, targeted therapies.

The TWEAK/Fn14 signaling pathway has been identified as a potential therapeutic target for LN because of its role in promoting inflammation, angiogenesis, cell proliferation, and fibrosis: all biological processes that contribute to LN pathology [reviewed in Ref. (71)]. TWEAK expression has been examined in clinical specimens and while there have been conflicting reports about TWEAK mRNA and protein levels in the peripheral blood mononuclear cells and serum of SLE patients (72–75), there have been consistent reports of elevated TWEAK levels in the kidney and urine of LN patients (73, 76–78). The urinary TWEAK levels in LN patients were found to be increased relative to healthy control donors, SLE patients without LN, RA patients, OA patients, and patients with kidney disease due to diabetes or hypertension. These data suggest that increased urinary TWEAK levels are due to the specific pathology of LN, and not due to a non-specific effect of systemic autoimmunity or decreased kidney function.

Animal studies also support the idea that TWEAK is involved in the local kidney pathology seen in LN. In a chronic graft-versus-host (cGVH) mouse model of SLE, splenocytes are transferred from a donor mouse strain with an MHC class II mutation into a coisogenic recipient mouse. This results in the activation of the alloreactive donor T cells, which in turn costimulate recipient B cells and promote the production of autoantibodies and subsequent kidney damage. When SLE was induced in Fn14-deficient mice, there was a reduction in renal disease as measured by glomerular IgG deposition and urinary protein levels (proteinuria) compared to SLE-induced WT mice (79). There was also a reduction in pro-inflammatory cytokines and chemokines, a decrease in kidney-infiltrating macrophages, and a decrease in renal cellular proliferation. There were no differences in serum IgG levels or serum autoantibody levels between the Fn14-deficient and WT mice, indicating that TWEAK/Fn14 signaling does not play a role in the induction of SLE. When a murine neutralizing anti-TWEAK antibody was used in the same SLE mouse model, a similar reduction in renal disease was observed (79).

In another study using bone marrow chimeric mice, the relative contributions of Fn14 expression in the resident kidney cells and bone marrow in the development of LN were evaluated using the cGVH mouse model of SLE (80). Mice with $Fn14^+$ non-bone marrow-derived cells, mice with the $Fn14^+$ bone marrow-derived cells, and control non-chimeric cGVH mice displayed the same elevation in serum antinuclear autoantibodies. In contrast, the mice with $Fn14^+$ non-bone marrow-derived cells showed a more rapid and stronger nephritis disease course than mice with $Fn14^+$ bone marrow-derived cells, as measured by urine albumin levels. These results indicate that Fn14 expression does not impact SLE induction, but that resident kidney cells, and not the bone marrow-derived cells, play a prominent role in LN pathology.

Another model of LN, nephrotoxic nephritis (NTN), was used to evaluate the role of TWEAK and Fn14 in LN pathology (81). NTN is induced by passive transfer of preformed rabbit anti-mouse glomerular antibodies into mice pre-immunized with rabbit IgG, which promotes the deposition of immune complexes and macrophage-mediated injury in the kidney. Fn14-deficient mice with NTN developed reduced renal disease compared to WT

mice. Similarly, when NTN was induced in WT mice treated with anti-TWEAK antibody, the mice displayed decreased proteinuria and improved kidney pathology, including a decrease in glomerular proliferation. Additionally, TWEAK antibody-treated mice had decreased mRNA levels of pro-inflammatory mediators, reduced levels of glomerular immune complex deposition, less fibrosis, and less macrophage infiltration of kidneys.

The TWEAK/Fn14 pathway was also shown to promote the neuropsychiatric symptoms of SLE in an MRL lymphoproliferative (lpr) mouse strain that contains a mutation in the Fas gene that results in the spontaneous development of SLE (82). TWEAK and Fn14 mRNA expression were upregulated in the brains of MRL/lpr mice compared to MRL mice and Fn14 protein expression was mostly detected in the endothelial cells. There were no differences found between Fn14-deficient and WT mice in the quantity of a range of autoantibodies. Fn14-deficient mice showed less depression-like behavior in a forced swim test and a saccharin-drink reward test and improved spatial memory in an object placement test, while there was no difference in motor ability detected by a balance beam coordination test. The mRNA expression of pro-inflammatory mediators and RANTES protein expression were decreased in Fn14-deficient brain compared to WT brain. Fn14-deficient mice had decreased CSF albumin relative to WT mice, a measure of BBB integrity. Additionally, anti-dsDNA antibodies, shown to be similar in serum, were increased in the WT brain compared to the Fn14-deficient brain. These data indicate that the TWEAK/Fn14 pathway may promote neuropsychiatric lupus by compromising the BBB.

The anti-TWEAK mAb BIIB023 is currently being evaluated in a Phase II clinical trial (ClinicalTrials.gov Identifier NCT01499355) to test its efficacy against LN, which as mentioned above, is a common manifestation of SLE that can lead to kidney failure. While the primary endpoint of the trial is to test the renal response of LN patients to BIIB023, secondary objectives include assessment of extrarenal SLE disease activity and manifestations [reviewed in Ref. (71)].

TWEAK/Fn14 AXIS-TARGETED THERAPEUTICS: CANCER

Cancer is the second leading cause of death in the USA, with ~580,000 deaths predicted to occur in 2013 (83). The vast majority of these deaths are due to metastasis, a complex multi-step process whereby primary tumor cells travel to distant organs and form secondary tumors that can impair critical organ functions [reviewed in Ref. (84, 85)]. Significant progress has recently been made in identifying the molecular genetic abnormalities that drive the growth and spread of some cancer types, and these findings have led to the development of new targeted therapeutics that dramatically improve the survival of those patients with tumors harboring these particular genetic alterations [reviewed in Ref. (86, 87)]. However, the main treatment options for most cancer patients with metastatic disease continue to be chemotherapy and/or radiation therapy. Thus, there is an urgent need to identify new molecular targets for anti-cancer drug development, and in this section we summarize some of the drug development efforts that target the TWEAK and Fn14 proteins (**Table 2**).

Table 2 | Examples of TWEAK- or Fn14-targeted therapeutic agents for cancer.

Agent	Developer	Type of agent	Target	Status	Comments	Reference
RG7212 (RO5458640)	Hoffmann–La Roche	Neutralizing mAb	TWEAK	Phase I trial Start date: 2011 July Completion date: 2013 March	Various cancer types; only patients with Fn14$^+$ tumors; no toxicities noted; disease stabilization in some patients	ClinicalTrials.gov NCT01383733 (88, 89)
Fn14-TRAIL (KAHR-101)	KAHR Medical	Signal converter protein	TWEAK	Pre-clinical	Inhibits tumor growth in xenograft model	(90)
BIIB036 (P4A8)	Biogen Idec	Agonistic mAb	Fn14	Pre-clinical	Inhibits tumor growth in xenograft models	(91, 92)
18D1	UHW/arGEN-X	Agonistic mAb	Fn14	Pre-clinical	Inhibits tumor formation in experimental metastasis assays	(93)
PDL192 (enavatuzumab)	Abbott	Agonistic mAb	Fn14	Phase I trial Start date: 2008 July Completion date: 2011 October	Various cancer types; liver and pancreatic enzyme toxicity	ClinicalTrials.gov NCT00738764 (9, 12, 94)
ITEM4-rGel	MD Anderson Cancer Center/UMSOM	Immunotoxin conjugate	Fn14	Pre-clinical	All three agents inhibit tumor growth in xenograft models	(11, 95, 96)
hSGZ		Immunotoxin fusion protein				(11, 96)
GrB-TWEAK		Ligand-apoptotic factor fusion protein				2013 AACR Meeting Abstract #2185

UHW, University Hospital of Würzburg; UMSOM, University of Maryland School of Medicine; rGel, recombinant gelonin; GrB, granzyme B; mAb, monoclonal antibody.

STRATEGY #1: INHIBITION OF TWEAK:Fn14 ENGAGEMENT

TWEAK mAb RG7212

Both TWEAK and Fn14 expression has been detected in tumors and TWEAK/Fn14 signaling may promote tumor growth *in vivo* through a variety of mechanisms [reviewed in Ref. (6, 97) and see Ref. (2, 8, 10, 20, 98–100)]; accordingly, agents that inhibit TWEAK binding to Fn14 may have potential therapeutic utility. In a recent report, Yin et al. described the development, functional characterization, and anti-cancer properties of a humanized anti-TWEAK-neutralizing mAb named RG7212 (RO5458640) (88). This antibody blocks TWEAK-stimulated proliferation, NF-κB activation, and cytokine secretion when added to cells cultured *in vitro*. Also, tumor growth inhibition was demonstrated in mouse models in which human cancer cell lines or patient-derived tumorgrafts were xenotransplanted into immunodeficient mice, or alternatively, when syngeneic mouse cancer cell lines were allotransplanted into immunocompetent mice. The greatest tumor growth inhibition was detected in xenograft models of renal cell carcinoma (ACHN, Caki-1), breast carcinoma (MDA-MB-231), and non-small cell lung cancer (Calu-3). TWEAK mAb-treated mice had decreased tumor mRNA levels of various TWEAK-inducible genes and decreased serum levels of mouse (m) MMP9, (m) EGF, (m) bFGF, human (h) CCL2, (h) IL-8, and (h) IL-6. Western blot and immunohistochemistry analyses of tumor tissue showed that TWEAK mAb-treated mice had decreased cell proliferation and increased apoptotic signaling. Depletion of specific immune cells in a syngeneic mouse model showed that $CD8^+$ T cells and NK cells, but not $CD4^+$ T cells, were required for RG7212-mediated tumor growth inhibition. Taken together, these data demonstrate that TWEAK/Fn14 signaling supports tumor growth by promoting cell proliferation and survival and also by shaping the tumor microenvironment by stimulating the expression of chemokines, cytokines, and inflammatory mediators.

In consideration of the RG7212 pre-clinical data summarized above, a Phase I trial was initiated in 2011 July (ClinicalTrials.gov Identifier NCT01383733). Thirty-eight patients with Fn14-positive tumors were treated with RG7212 (89). No dose-limiting toxicities were observed and most adverse events were considered low-grade. One patient with refractory melanoma showed tumor regression, while another eight patients, including patients with refractory non-small cell lung cancer, renal cell carcinoma, mesothelioma, and metastatic breast cancer, had stable disease for over 3 months. Thus, RG7212 demonstrated tolerability and some efficacy in patients with advanced solid tumors.

Fn14-TRAIL fusion protein

The Fn14-TRAIL protein described above in the context of MS has recently been shown to induce hepatocellular carcinoma (HCC) cell apoptosis *in vitro* in a dose-dependent manner (90). In contrast, the fusion protein did not have a significant impact on cell viability in non-malignant hepatocyte cell lines. Interestingly, HCC cells treated with Fn14-TRAIL underwent higher rates of apoptosis than when treated with soluble TRAIL or soluble Fn14 extracellular domain either alone or in combination. Furthermore, subcutaneous administration of Fn14-TRAIL significantly inhibited HCC xenograft growth *in vivo* (90). It is unclear at his time whether TWEAK neutralization is contributing to the anti-tumor effects noted with this agent.

Comments on strategy #1

The main concern with this strategy is that TWEAK is expressed at low levels in many Fn14-overexpressing tumors and in this situation it is likely that TWEAK-independent Fn14 signaling occurs, which will not be inhibited by these agents.

STRATEGY #2: ACTIVATION OF THE Fn14 SIGNALING PATHWAY

Fn14 mAbs BIIB036, 18D1, and PDL192

Fn14 gene expression is elevated in over a dozen different solid tumor types compared with matched adjacent normal tissue or normal tissues from non-diseased donors (8–14); consequently, Fn14 is also a potential target for cancer therapy. TWEAK is a pro-apoptotic factor for some human cancer cell lines [reviewed in Ref. (6, 101)] and several studies have revealed that TWEAK-stimulated cell death occurs via an indirect mechanism; in particular, via TWEAK-triggered upregulation of other cytokines [e.g., TNF-α (102–105) and IFN-β (106)]. The ability of TWEAK to promote tumor cell death has provided the rationale for several groups to explore the potential of agonistic Fn14 mAbs as anti-cancer therapeutics. Scientists at Biogen Idec have developed a humanized anti-Fn14 antibody named BIIB036 (P4A8) that binds with high affinity to Fn14 in BiaCore and FACS assays (91). BIIB036 treatment of $Fn14^+$ human cancer cell lines activated the NF-κB signaling pathway, stimulated IL-8 production, and in some cases induced cell death, indicating that this mAb exhibits agonistic activity. This mAb can also inhibit the binding of both membrane-bound and soluble TWEAK to Fn14 (91, 107). Intraperitoneal injection of BIIB036 inhibited WiDr colon carcinoma, NCI-N87 gastric carcinoma, MDA-MB-231 breast cancer, and patient-derived colorectal tumor cell growth in xenograft assays (91, 92). Subsequent studies indicated that the optimal dosing for BIIB036 in tumor-bearing mice was twice weekly and tumor growth inhibition persisted up to 50 days after the termination of dosing (92). Finally, BIIB036 exhibited antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* and maximal BIIB036 anti-tumor activity *in vivo* required full Fc effector function, indicating that ADCC was one of the mechanisms mediating the observed inhibitory effects (91). BIIB036, as well as the 18D1 and PDL192 mAbs described below, exhibits the strongest agonistic activity upon oligomerization with protein G or binding to Fcγ receptors (93, 107).

Researchers at the University Hospital of Würzburg and arGEN-X recently reported the generation and characterization of three llama-derived anti-Fn14 mAbs (93). Two of these mAbs, 18D1 and 4G5, blocked TWEAK:Fn14 binding and all exhibited agonistic activity (including cell death induction in the presence of cycloheximide). The 18D1 antibody inhibited metastatic colony formation in both RENCA cell and HCT116 cell experimental metastasis assays, and the anti-metastatic effect noted in the RENCA cell experiments was predominantly mediated via ADCC.

Facet Biotech, which was acquired by Abbott Laboratories in 2010, has also developed an agonistic Fn14 antibody for potential therapeutic use (9). The murine form of this antibody, 19.2.1, was shown to inhibit the growth of multiple cancer cell lines in a dose-dependent manner, including HSC3 oral squamous carcinoma

cells, A253 salivary gland cells, A375 melanoma cells, and Mia-PaCa2 pancreatic cells. Its humanized counterpart, PDL192 (Enavatuzumab), also exhibited growth inhibitory activity *in vitro* (9). Studies using $HER2^+$ SKBR3 breast cancer cells revealed that PDL192 had a synergistic effect on growth inhibition when combined with trastuzumab (Herceptin), the standard treatment for $HER2^+$ breast cancer patients (12). PDL192 also exhibited anti-tumor activity in multiple xenograft models of breast cancer, including the MCF-7, HCC70, and MDA-MB-231 models (12). However, the antibody had little to no effect on the *in vivo* growth of other $Fn14^+$ cell lines, including Calu6 lung cancer cells and HCT116 colorectal cancer cells (9). As found for BIIB036, the observed growth inhibitory effects of PDL192 on xenograft tumor growth in mice are mediated, at least in part, by ADCC (9). In contrast to BIIB036 and 18D1, PDL192 does not recognize murine Fn14 and does not interfere with TWEAK:Fn14 binding (93, 107). Abbott Laboratories initiated a Phase I clinical trial in 2008 July to test the effects of PDL192 administration (six dosage levels) in 30 patients with advanced solid tumors (ClinicalTrials.gov Identifier NCT00738764). The trial was completed in 2011 October and the results were disclosed in Abstract form at an international meeting (94). No patients showed partial or complete tumor regression but two patients had stable disease for 2–4 months duration. However, patients treated with PDL192 at doses demonstrating anti-tumor efficacy in mice had elevated liver and pancreatic enzyme levels, indicating significant drug toxicity in these organs.

Comments on strategy #2

Monoclonal antibodies that target specific cell surface receptors on tumor cells may attenuate tumor growth *in vivo* via multiple mechanisms, including receptor activation, blockade of ligand:receptor engagement, and stimulation of immune cell Fc receptor-mediated pathways such as ADCC. All three of the Fn14-specific mAbs described above have complex biological properties when characterized *in vitro* (93, 107) and thus the precise mechanisms responsible for their apparent anti-tumor growth (PDL192, BIIB036) and anti-metastatic (18D1) activity is not clear. The main concern with the use of Fn14 mAbs that may exhibit agonistic activity within the tumor milieu is that Fn14 activation in most "normal" and cancer cell lines does not cause cell death. Indeed, Yin et al. found that TWEAK treatment had no effect on the viability of 299 tumor cell lines as assessed by CellTiter-Glo or MTT assays (88). In general, most studies support the view that TWEAK/Fn14 signaling in the tumor microenvironment will most likely stimulate pro-tumorigenic/metastatic cellular responses, including tumor cell growth (88, 108), invasion (8, 10, 18–20), and resistance to chemotherapeutic agents (109). There is also evidence that TWEAK can act on $Fn14^+$ endothelial cells and promote angiogenesis (2, 100, 110); thus, if TWEAK is expressed by tumor cells or tumor-associated stromal/immune cells *in vivo* it could promote tumor vascularization. Agonistic Fn14 mAbs could have a similar biological effect.

STRATEGY #3: DIRECT KILLING OF Fn14-POSITIVE CANCER CELLS

Fn14 mAb-based immunotoxins

Targeted toxins are a class of cancer therapeutics that internalize into malignant cells to deliver a cytotoxic payload. These agents consist of a targeting polypeptide (e.g., an antibody, an antibody fragment, or a receptor ligand) that is either chemically conjugated or covalently linked to a toxin, which is usually of bacterial or plant origin [reviewed in Ref. (111, 112)]. Targeted toxins that utilize a mAb or the variable fragment (Fv) of an antibody as the targeting moiety are generally referred to as immunotoxins. Typically, targeted toxins bind specific cell surface receptors, become internalized via receptor-mediated endocytosis, and then the toxin moiety is eventually delivered to the cytosol, its site of action. Immunotoxins targeting a variety of cell surface proteins are currently being evaluated in human clinical trials [reviewed in Ref. (112)].

Two types of Fn14-targeted immunotoxins have been developed and tested in pre-clinical cancer studies by Rosenblum and colleagues (11, 95, 96). Both agents are based on the anti-Fn14 antibody ITEM4 and the plant toxin gelonin. ITEM4 was raised against the human Fn14 extracellular domain (113). It binds both human and murine Fn14 and has weak agonistic activity (11, 113, 114). Gelonin is a ~29-kDa *N*-glycosidase found in the seeds of *Gelonium multiflorum* plants that cleaves 28S rRNA and thereby inhibits protein synthesis (115). The first immunotoxin, ITEM4-rGel, was composed of the ITEM4 mAb chemically conjugated to a recombinant form of gelonin (rGel) purified from *E. coli* cells. This agent was highly cytotoxic when added to $Fn14^+$ cancer cell lines *in vitro* and inhibited human T-24, MDA-MB-435, and MDA-MB-231 cancer cell growth in xenograft assays after intravenous administration (11, 95, 96). The second immunotoxin was a humanized, bivalent single-chain protein named hSGZ. The N-terminal region of this protein consists of a single-chain Fv (scFv) fragment of ITEM4 in which the V_H and V_L sequences from the hypervariable region were linked by a flexible peptide. These two domains were "humanized" by site-specific mutagenesis to alter certain amino acid residues within the framework domain without impacting affinity or specificity. The C-terminal region consists of rGel followed by a short dimerization domain to produce a bivalent immunotoxin. Recombinant hSGZ protein purified from *E. coli* cells is also highly cytotoxic to $Fn14^+$ cells *in vitro* and therapeutic efficacy studies showed significant growth inhibition in both MDA-MB-435 melanoma and MDA-MB-231 breast cancer xenograft models (11, 96). No overt toxicity was noted in mice treated with either the ITEM4-rGel or hSGZ immunotoxins, although as mentioned above, ITEM4 can bind murine Fn14 (114). Thus, these two immunotoxins appear to be effective anti-cancer agents in pre-clinical studies.

Granzyme B-TWEAK fusion protein

A completely human fusion protein designed to kill $Fn14^+$ cancer cells was recently described by Zhou et al. (96) at the 2013 AACR Annual Meeting (Abstract #2185). This construct, named granzyme B (GrB)-TWEAK, uses the human TWEAK receptor-binding domain as the targeting moiety and GrB as the cell-killing agent. GrB is a serine protease stored in the secretory granules of cytotoxic T lymphocytes and NK cells [reviewed in Ref. (116, 117)]. It normally enters target cells (e.g., non-self or virally infected) via the granule exocytosis pathway and

promotes apoptotic cell death [reviewed in Ref. (116, 117)]. GrB-TWEAK is expressed in transfected HEK293 cells as a catalytically inactive protein, purified from conditioned media using nickel-NTA affinity chromatography, and then activated by *in vitro* incubation with enterokinase. Initial studies have revealed that GrB-TWEAK is rapidly internalized by $Fn14^+$ cancer cells and is highly cytotoxic in the low nanomolar range. Also, GrB-TWEAK administration (iv) inhibited HT-29 colon carcinoma cell growth in immunodeficient mice (Abstract #2185 Poster).

Comments on strategy #3

Importantly, the therapeutic efficacy of these agents does not depend on promoting or inhibiting intracellular signaling pathways or on immune cell function (i.e., ADCC). Also, since these agents directly kill cancer cells, vulnerable cells cannot activate compensatory signaling cascades that promote drug resistance. Nevertheless, there are a number of obstacles that limit the clinical application of this class of therapeutic agents; for example, bacterial or plant toxins can cause vascular leak syndrome and elicit immune responses in patients [reviewed in Ref. (112, 118)]. However, with respect to rGel immunogenicity, in a recent Phase I trial in which an anti-CD33 mAb-rGel immunoconjugate was administered to 28 patients with advanced myeloid malignancies, the immunogenicity of the rGel moiety appeared to be low, as only 2 patients developed detectable levels of anti-rGel antibodies during the course of treatment (119).

TWEAK/Fn14 AXIS-TARGETED THERAPEUTICS: ADDITIONAL APPROACHES

Protein-based therapeutics like those listed in **Tables 1** and **2** can only act on cell surface or secreted molecules, are costly to produce, and have other inherent disadvantages; for example, they generally have to be administered intravenously and they have poor tissue penetration [reviewed in Ref. (120)]. Here, we describe two additional classes of therapeutic agents that may be suitable for inhibition of chronic TWEAK/Fn14 signaling in diseased tissue.

RNAi-BASED THERAPEUTICS TO DOWN-REGULATE TWEAK OR Fn14 GENE EXPRESSION

RNA interference (RNAi) is a potent mRNA sequence-specific post-transcriptional gene silencing mechanism that is currently being evaluated for use as a therapeutic strategy for a wide range of human disorders [reviewed in Ref. (121, 122)]. The goal is to down-regulate the expression of cellular genes involved in disease by administration of either synthetic small interfering RNAs (siRNAs) or gene constructs encoding short hairpin RNAs (shRNAs). Numerous siRNA/shRNA therapeutics have already advanced into clinical trials [reviewed in Ref. (123)], but there are still significant challenges to overcome for safe and effective use of these agents. Some of these challenges include ineffective delivery into target cells, the potential for off-target effects, and immune system-mediated toxicities [reviewed in Ref. (121, 122, 124)]. Although there have been no reports to date describing TWEAK or Fn14 depletion *in vivo* after localized or systemic delivery of either siRNA duplexes or shRNA constructs, this is an approach that should be considered in the near future as progress continues to be made on the development of RNAi-based drugs for treatment of human diseases.

SMALL MOLECULE THERAPEUTICS TO INHIBIT TWEAK TRIMERIZATION, TWEAK:Fn14 BINDING, Fn14 MULTIMERIZATION, OR Fn14:TRAF INTERACTION

The classic model of TNF:TNFR superfamily signaling is that the binding of homotrimeric ligands to receptor monomers promotes receptor trimerization (and most likely multimerization), which in turn leads to the recruitment of intracellular adaptor proteins to the cytoplasmic domains of the receptors. Thus, effective signal transduction depends on a series of highly specific protein-protein interactions. Although targeted disruption of protein-protein interfaces by small organic molecules is generally regarded as a challenging goal [reviewed in Ref. (125)], there have been some successes with this approach in the TNF/TNFR superfamily field. For example, compounds have been identified that bind to trimeric TNF-α (126) or CD40L (127), thereby causing conformational perturbations that inhibit receptor binding and functional activity. Small molecule inhibitors of the TNF-α:TNF-R1 interaction have also been reported (128).

TWEAK/Fn14 pathway-specific small molecule inhibitors could potentially be identified using several experimental strategies, including: (i) the use of cell- or recombinant protein-based high throughput screening assays for testing of synthetic chemical or natural product libraries, and (ii) computational methods such as virtual screening using X-ray- or NMR-derived protein structures and chemical compound databases (*in silico* drug discovery). In regard to the first strategy, a high throughput screen measuring the effect of ~60,000 compounds on soluble TWEAK binding to the Fn14 extracellular domain was conducted by Benicchi and colleagues (129). Fifteen primary hit compounds were identified and subsequent counterassays revealed that two were potent but partial inhibitors and two were weak inhibitors (overall hit rate of 0.007%). However, the inhibitory activity of these molecules was not confirmed using other types of assays, and experiments were not conducted to determine the actual protein target (TWEAK or Fn14). In regard to the second strategy, structures of the human Fn14 extracellular domain (130, 131) and of the soluble TWEAK trimer (in complex with the Fab fragment of a neutralizing mAb) (132) have been determined via NMR and x-ray crystallography, respectively. This information, in conjunction with TWEAK (131, 133) and Fn14 (131, 134) mutagenesis data and the available structures of several closely related TNF:TNFR superfamily member complexes, has been used by three groups to derive molecular models of the TWEAK:Fn14 complex (131–133). Dhruv et al. (133) successfully utilized their validated model for the *in silico* identification of small molecule inhibitors of TWEAK:Fn14 binding. Briefly, 60 compounds scoring positive in the virtual screen were obtained and rescreened for inhibitory activity using an ELISA assay. Subsequent studies indicated that one of the compounds, L524-0366, was a potent inhibitor of TWEAK-stimulated NF-κB pathway activation and glioma cell migration *in vitro*. It is anticipated that more small molecule drugs disrupting either TWEAK:Fn14 binding or other critical protein-protein interactions associated with this signaling node will be discovered in the next few years.

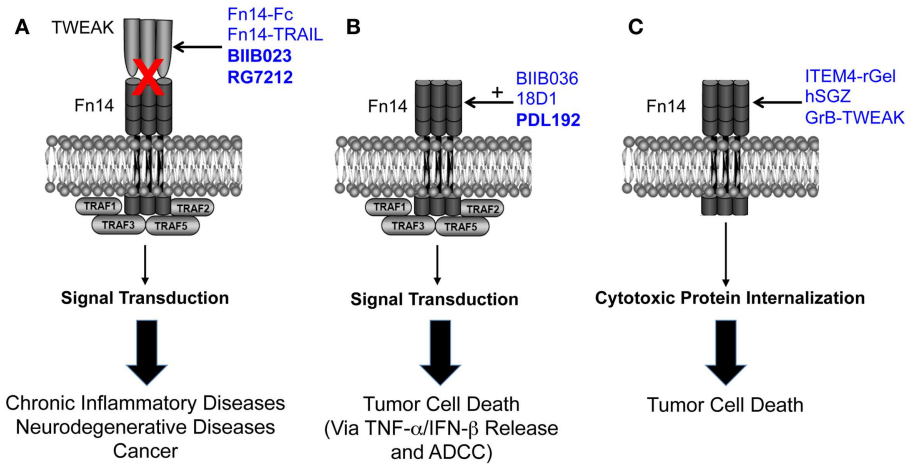

FIGURE 1 | Summary of the three TWEAK/Fn14 axis-targeted therapeutic strategies discussed in this review. **(A)** Four TWEAK-neutralizing agents have been developed to prevent TWEAK:Fn14 engagement and thereby inhibit disease progression. **(B)** Three Fn14-directed agonistic mAbs have been developed to activate Fn14 signaling in tumor cells and promote cell death. Studies have shown that TWEAK-induced cell death *in vitro* is an indirect effect mediated by other cytokines, so the anti-tumor efficacy of these agents may occur via this mechanism as well as by ADCC. The agonistic activity of these mAbs is potentiated by oligomerization with protein G or binding to Fcγ receptors. BIIB036 and 18D1, but not PDL192, can also inhibit TWEAK:Fn14 binding. **(C)** Three Fn14-directed agents have been developed that use Fn14 as a portal to deliver toxins or pro-apoptotic proteins into tumor cells. All of the agents shown in this figure have therapeutic efficacy in animal models, and the three agents in bold type have been or are currently under investigation in human clinical trials. Drawing adapted from Figure 3 in Ref. (135).

SUMMARY

The TWEAK/Fn14 axis was first described ~12 years ago, and since that time studies from around the globe have revealed that Fn14-triggered signal transduction and downstream cellular responses could potentially play a role in the pathophysiology of many major human diseases, including cardiovascular disease, stroke, and cancer. These studies have led to drug development efforts and the first TWEAK- or Fn14-targeted therapeutic agents (**Figure 1**). Three of these agents have already entered clinical trials, and this is remarkable progress. However, only the collaborative efforts of basic, translational and clinical researchers will continue to move the TWEAK/Fn14 axis field forward in the coming years, and it is anticipated that this research will ultimately lead to safe and effective therapeutics for clinical use.

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TWEAK and Fn14 in the neurovascular unit

Manuel Yepes[1,2]*

[1] Department of Neurology and Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA, USA
[2] Department of Neurology, Veterans Affairs Medical Center, Emory University School of Medicine, Atlanta, GA, USA

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Sophie Desplat-Jégo, Aix Marseille University, France
Chaim Putterman, Albert Einstein College of Medicine of Yeshiva University, USA

***Correspondence:**
Manuel Yepes, Department of Neurology and Center for Neurodegenerative Disease, Emory University, Whitehead Biomedical Research Building, 615 Michael Street, Suite 505J, Atlanta, GA 30322, USA
e-mail: myepes@emory.edu

The neurovascular unit (NVU) is a dynamic structure assembled by endothelial cells (EC), a basement membrane (BM), perivascular astrocytes (PA), pericytes, and surrounding neurons. The NVU regulates the passage of substances and cellular elements from the intravascular space into the brain parenchyma. This function, also known as blood-brain barrier (BBB), is regulated by the integrity of tight junctions proteins between EC, and the interaction between PA and the basal lamina. The cytokine tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (Fn14) are abundantly expressed in the NVU. Here we will review data indicating that the interaction between TWEAK and Fn14 in the endothelial cell-BM-astrocyte interface regulates the function of the BBB following an ischemic/hypoxic injury, and that pharmacological inhibition of TWEAK-Fn14 is a promising target for the treatment of patients with neurological diseases that have a direct impact on the structure and function of the NVU.

Keywords: cerebral ischemia, neurovascular unit, blood-brain barrier, middle cerebral artery occlusion, tumor necrosis factor-like weak inducer of apoptosis, fibroblast growth factor-inducible 14, cerebral edema, nuclear factor kappa-light-chain-enhancer of activated B cells

THE TWEAK/Fn14 PATHWAY

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumor necrosis factor (TNF) superfamily of cytokines that is synthesized as a type II transmembrane protein from which a soluble ∼17 kDa ligand factor with biological activity can be released [soluble TWEAK (sTWEAK)] (1). The membrane-bound form of TWEAK has an extracellular TNF homology domain (THD) that mediates the assembly of homotrimeric proteins, and a stalk region that contains the recognition site for proteolytic processing by serine proteases of the furin family. TWEAK mRNA is expressed in a variety of tissues and cells, including brain, heart, and lung (2), as well as human endothelial and smooth muscle cells. Soluble TWEAK induces various cellular responses *in vitro*, including cell proliferation (3–5), migration, differentiation (6), angiogenesis (3), and the expression of pro-inflammatory molecules such as IL-8, MCP-1, ICAM-1, and E-selectin in human umbilical endothelial cells (EC) (7), and IL-6, IL-8, and ICAM-1 in astrocytes (8).

Tumor necrosis factor-like weak inducer of apoptosis activity is mediated via binding to fibroblast growth factor-inducible 14 (Fn14), a 14-KDa member of the TNF receptor superfamily (9). Fn14 has a 28 amino acids residues-cytoplasmic tail without a death domain. Instead, Fn14 contains a single binding site for adapter proteins of the TNF receptor-associated factors (TRAF) family (9–11). Fn14 is expressed in a variety of cells and tissue types including tumor cell lines of non-lymphoid origin, fibroblasts, and endothelial and epithelial cells. Furthermore, Fn14 expression is up-regulated following growth factor stimulation of quiescent cell cultures, exposure to hypoxia (12, 13), oxidative stress, chemical and mechanical injuries, inflammation, and tumor growth (14). The TWEAK-Fn14 signal transduction pathway is not fully understood, but it has been shown that TWEAK binding to Fn14 activates the NF-κB (10), extracellular signal-regulated kinase (ERK) (15), and c-Jun NH_2-terminal kinase (JNK) (15) signal transduction pathways.

In this review we will use the conceptual model of the neurovascular unit (NVU) to discuss the role of TWEAK and Fn14 in the response of the brain to a hypoxic/ischemic injury, and will discuss TWEAK/Fn14 as a potential target for the development of therapeutic strategies to promote cell survival and improve the neurological outcome in patients suffering from acute ischemic stroke and other diseases of the central nervous system (CNS) that affect the NVU.

THE NEUROVASCULAR UNIT

The NVU is a dynamic structure assembled by EC ensheated by a basal lamina, and surrounded by astrocytic end-feet processes, pericytes, and neurons (**Figure 1**). One of the main functions of the NVU is to regulate the passage of plasma components and cellular elements from the intravascular space into the brain (16). This barrier function, also known as the blood-brain barrier (BBB), is determined not only by the integrity of the endothelium, but most significantly by a functional interplay between EC, the basal lamina, and perivascular astrocytes (PA).

The permeability of the endothelium is restricted by junctional complexes assembled by adherens and tight junctions proteins (AJ and TJ, respectively). AJ's are formed mostly by vascular endothelial (VE)-cadherin that mediates cell–cell adhesion via its interaction with the actin cytoskeleton. TJ's are located in the apical region of the intercellular cleft and function as a "zipper" between the apical and basolateral cell membranes. The transmembrane components of the TJ include junctional adhesion molecule (JAM)-1, occludin, and claudins. A set of accessory proteins, known as zonula occludens (ZO)-1 and ZO-2, link these proteins with

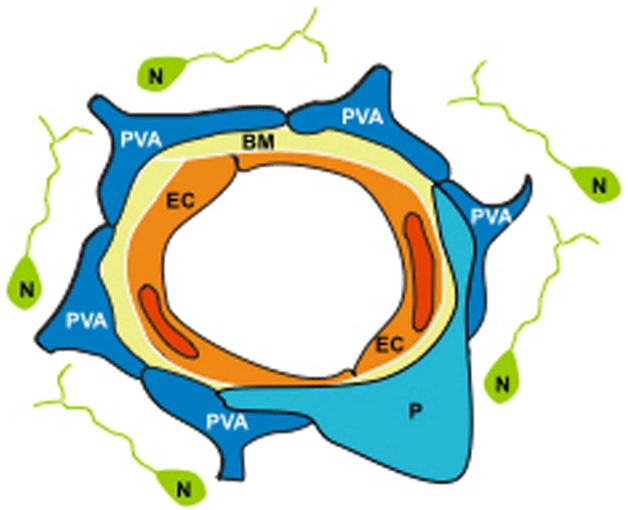

FIGURE 1 | The neurovascular unit (NVU) is assembled by endothelial cells (EC) ensheated by a basement membrane (BM), embraced by perivascular astrocytes (PVA) and pericytes (P), and surrounded by neurons (N).

the cytoskeleton. Importantly, although disruption of AJ's may increase the permeability of the NVU, it is primarily the TJ's that confer the low paracellular permeability and high electrical resistance that characterize the cerebrovascular endothelium.

The basement membrane (BM) forms an interface between EC and PA, pericytes and neurons. It is composed of extracellular matrix proteins such as laminin, collagen IV, fibronectin, and perlecan. About ∼95% of the basal lamina that encases the capillary network is embraced by astrocytic end-feet processes (17), and the opposite pole of each perivascular astrocyte contacts ∼30,000 synapses (18).

The adhesion of EC and astrocytes to the basal lamina requires the interaction of cell-adhesion receptors and their ligands in the matrix. These receptors, known as integrins, play a key role in the activation of cell signaling pathways in response to changes in the extracellular environment, and in the regulation of the interaction between EC and PA. In the NVU the integrin sub-unit β_1 is found in EC and astrocytes, whereas the integrin $\alpha_6\beta_4$ is found in astrocytic end-feet processes (19). These receptors interact with laminin-1 and laminin-5 that are found in the basal lamina.

As it will be discussed below, the binding of TWEAK to Fn14 has a direct effect on the composition of the basal lamina and on the interaction between astrocytic end-feet processes and the BM. The interplay between PA and the basal lamina is an important determinant of the permeability of the NVU (20). Indeed, astrocytic-derived factors have a direct effect on the attachment of PA to the BM and on the integrity and distribution of interendothelial TJs proteins, and detachment of astrocytic end-feet processes from the basal lamina result in decreased transendothelial electrical resistance (TEER) across the NVU with increased permeability of the BBB.

Here we will review experimental data indicating that the interaction between TWEAK and Fn14 regulate the interaction between astrocytic end-feet processes and the basal lamina, and that TWEAK/Fn14 play a central role in the regulation of the permeability of the BBB and the survival of neurons associated with each NVU. Furthermore, we will discuss the role of TWEAK and Fn14 in the induction of a pro-inflammatory response in EC and astrocytes, and the infiltration of inflammatory cells from the intravascular space into the brain parenchyma.

BIOLOGICAL EFFECTS OF TWEAK-Fn14 ON THE NEUROVASCULAR UNIT

TWEAK AND Fn14 EXPRESSION IN THE NEUROVASCULAR UNIT

Tumor necrosis factor-like weak inducer of apoptosis and Fn14 are abundantly expressed in the NVU. Indeed, TWEAK and Fn14 have been found in EC of the microcirculation (blood vessels of $<100\,\mu m$ of diameter), in PA, and in neurons of the cerebral cortex, caudate nucleus, putamen, substantia nigra, cerebellar Purkinje cells, and spinal cord (12, 13). Interestingly, *in vivo* studies suggest that the expression of TWEAK and Fn14 in the NVU is cell type-specific. Accordingly, TWEAK appears to be expressed primarily by EC while the highest level of Fn14 expression is detected in PA and neurons. Furthermore, TWEAK has also been found in pericytes (unpublished data), which not only have contractile properties but also secrete several factors, such as angiopoietin-1, that induce the expression of the TJ protein occludin in EC, maintaining the high TEER characteristic of the BBB (21).

THE INTERACTION BETWEEN TWEAK AND Fn14 ACTIVATES THE NF-κB PATHWAY IN THE NEUROVASCULAR UNIT

The NF-κB family includes homo- and heterodimers assembled by the five members of the Rel transcription factor family (22). NF-κB functional complexes are found in EC, PA, and neurons, where they can be activated via two different pathways. In the classic or canonic pathway, IκBα phosphorylation results in the release of NF-κB, leading to its nuclear translocation and DNA binding. In the alternative pathway, IKK1-mediated phosphorylation of the p100 precursor form of the p52 sub-unit leads to proteosomal processing of p100–p52, and nuclear translocation of p52-containing NF-κB dimers (22, 23). Although it has been proposed that TWEAK and Fn14 are able to activate both pathways, *in vitro* and *in vivo* studies suggest that in the brain TWEAK/Fn14-induces NF-κB pathway activation via the canonic pathway. Indeed, incubation of brain microvascular EC, neurons and astrocytes with TWEAK, or the intracerebral injection of TWEAK, leads to IκBα phosphorylation with nuclear translocation of p65 but not p100 phosphorylation (24). However, it has been reported that in other systems such as myoblasts and renal tubular cells, TWEAK may also signal via the non-canonical NF-κB pathway (25, 26). In the brain, treatment with TWEAK induces the expression of ICAM-1, IL-6, and IL-8 in astrocytes (5), and TNF-α in astrocytes and neurons (27). Importantly, the pro-inflammatory effect of TWEAK in the NVU is not limited to astrocytes. Indeed, recent evidence indicates that TWEAK induces an inflammatory response in human cerebral microvascular EC that is associated with increase in the permeability of the BBB (28). Together, these data indicate that the interaction of TWEAK with Fn14 has an NF-κB-mediated pro-inflammatory effect in the different components of the NVU and, as it will be analyzed below, this has a direct effect on the

permeability of the BBB, the development of cerebral edema, and neuronal survival.

NEURONAL DEATH

Tumor necrosis factor-like weak inducer of apoptosis can induce cell death in different tumor cell lines via TNF-α-dependent and -independent mechanisms (29). However, this effect is weak and in several experimental systems it requires co-incubation with gamma interferon (1). In contrast, incubation with TWEAK seems to have a stronger effect on neuronal death. Accordingly, treatment of cerebral cortical neurons with TWEAK induces apoptotic cell death (30) and the cleavage and accumulation of poly(ADP-ribose) polymers. This effect is not mediated by TNF-α and instead requires activation of the NF-κB pathway and a functional Fn14 receptor (31). These data suggest that, in contrast with tumor cell lines, the interaction between TWEAK and Fn14 has a direct effect on cell survival in cerebral cortical neurons.

TWEAK AND Fn14 IN THE ISCHEMIC BRAIN

ISCHEMIC STROKE

Ischemic stroke is the third cause of mortality a leading cause of disability in the US. About ∼795,000 people suffer a stroke every year, from which 600,000 are first attacks and 195,000 are recurrences (32). Worldwide, it is estimated that 15 million people suffer stroke each year. However, it is important to keep in mind that in many countries ischemic stroke is underreported, and thus this number may be significantly higher. A growing body of experimental evidence in cell cultures (30), rodents (12, 13), and humans (33) indicate that the interaction between TWEAK and Fn14 plays a pivotal role in the response of the brain to an ischemic injury. More importantly, the TWEAK/Fn14 pathway has emerged as a target for the development of pharmacological strategies for the treatment of acute ischemic stroke patients.

TWEAK AND Fn14 IN THE ISCHEMIC BRAIN

Studies with a murine model of cerebral ischemia indicate that the expression of Fn14 mRNA increases in the ischemic tissue as early as 30 min after the occlusion of the middle cerebral artery (hereinafter referred to as MCAO). This effect peaks at 6–24 h, and declines again 48–72 h later. In contrast, the effect on TWEAK mRNA is less well characterized, and while some studies have failed to detect a significant increase in TWEAK mRNA expression in response to the ischemic injury (13), later observations have shown a transient increase in TWEAK mRNA between 30 min and 3 h of the onset of the ischemic injury. Additionally, it has also been reported an effect of cerebral ischemia on TWEAK and Fn14 protein expression. Interestingly, these changes have been detected in the area surrounding the necrotic core, also known as ischemic penumbra, and in those zones of the brain with developing cerebral edema (12, 13). The translational importance of these reports is underscored by recent studies demonstrating that cerebral ischemia also induces the expression of TWEAK and Fn14 in the brain of ischemic stroke patients (33).

INHIBITION OF TWEAK – Fn14 INTERACTION HAS A PROTECTIVE EFFECT IN THE ISCHEMIC BRAIN

The effect of TWEAK/Fn14 inhibition on the outcome of the ischemic injury has been studied with three different strategies: the use of mice genetically deficient on either TWEAK ($^{-/-}$) or Fn14 (Fn14$^{-/-}$), or treatment with either a soluble Fn14-Fc fusion protein, or anti-TWEAK monoclonal antibodies. The first published studies reported that either the intraperitoneal administration of anti-TWEAK monoclonal antibodies 10 min before the induction of cerebral ischemia (30), or the intracerebroventricular injection of an Fn14-Fc decoy receptor immediately after (12, 13, 34) following the onset of ischemic stroke results in a ∼30 and 40% decrease in the volume of the ischemic lesion, respectively. These observations were supported by later studies indicating that when compared to their littermate controls, Fn14$^{-/-}$ mice exhibit ∼60% decrease in the volume of the ischemic lesion following MCAO (34, 35). Additionally, it has been reported that the neuroprotective effect of Fn14$^{-/-}$ deficiency in the ischemic brain is mediated by the induction of the granulocyte-colony stimulating factor (G-CSF) pathway (35). Interestingly, the effect of genetic deficiency of TWEAK on the volume of the ischemic lesion is less dramatic (∼20–40% decrease compared to littermate controls). Together, these data indicate that pharmacological inhibition of TWEAK/Fn14 with Fn14-Fc decoy or TWEAK monoclonal antibodies may be an effective strategy for the treatment of acute ischemic stroke. However, future studies should define if the intravenous administration of these inhibitors also has an effect on the volume of the ischemic lesion, and if so, how far after the onset of the ischemic insult they can be administered.

THE NEUROVASCULAR UNIT IN ISCHEMIC STROKE

Cerebral ischemia has a profound impact on the structure and function of the NVU. Indeed, shortly after the onset of the ischemic injury, those neurons located more distantly from a blood vessel exhibit signs of irreversible injury and death. This is followed by a rapid decrease in the expression of endothelial-β_1 and astrocytic-β_1 and -$\alpha_6\beta_4$ integrins, and degradation of laminin in the BM, redistribution of interendothelial TJs proteins, and increase in the permeability of the NVU, with the sub-sequent development of cerebral edema and hemorrhagic transformation (36, 37). This process is accompanied by the passage of inflammatory cells from the intravascular space into the ischemic tissue where they further increase the permeability of the BBB and have a deleterious effect on the survival of those neurons located more proximally to the blood vessel. As it will be discussed below, the interaction between TWEAK and Fn14 in the NVU plays a pivotal role in the development of cerebral edema, the infiltration of inflammatory cells into the ischemic tissue, and neuronal death. These data indicate that inhibition of TWEAK/Fn14 is an important target for the development of therapeutic strategies aimed at maintaining the structural and functional integrity of the NVU during ischemic stroke.

TWEAK AND Fn14 EXPRESSION IN THE NEUROVASCULAR UNIT UNDER ISCHEMIC CONDITIONS

Experimental data with an *in vitro* model of hypoxia show that deprivation of oxygen and glucose increases the expression of TWEAK and Fn14 in astrocytes, EC, and neurons. However, hypoxia has an effect primarily on the expression of TWEAK in EC and Fn14 in PA and neurons (24).

THE INTERACTION BETWEEN TWEAK AND Fn14-INDUCES THE PASSAGE OF INFLAMMATORY CELLS THROUGH THE ENDOTHELIAL CELL-BASEMENT MEMBRANE-ASTROCYTE INTERFACE

NF-κB pathway activation plays a pivotal role in the development of an inflammatory response in the ischemic brain. Earlier studies have demonstrated that TWEAK induces NF-κB activation in EC, astrocytes, and neurons, and that either treatment with an Fn14-Fc decoy, or genetic deficiency of Fn14, abrogates cerebral ischemia-induced Iκκβ and p65 phosphorylation in the ischemic brain (24). Two events of special pathophysiological significance mediated by the NF-κB pathway are the activation of matrix metalloproteinase-9 (MMP-9) in PA, and the passage of inflammatory cells into the ischemic brain parenchyma, both of which have direct effect on the composition of the basal lamina and the permeability of the BBB (see below).

MMP-9 is an NF-κB-regulated pro-inflammatory matrix metalloproteinase that has a direct effect on the composition of the basal lamina (38). MMP-9 expression in the ischemic tissue not only induces proteolytic degradation of the basal lamina but also degradation of TJ proteins, which leads to the development of cerebral edema, hemorrhagic transformation, and cell death. The intracerebral injection of TWEAK induces MMP-9 activation, and either genetic deficiency of Fn14, or treatment with Fn14-Fc decoy inhibits cerebral ischemia-induced MMP-9 activation. Importantly, the effect of Fn14-Fc decoy on MMP-9 is dose-dependent (24).

The monocyte chemoattractant protein-1 (MCP-1) is an NF-κB-regulated cytokine that has chemoattractant properties for monocytes, memory T-lymphocytes, and natural killer cells. Astrocytes are the main source of MCP-1 in the brain. The onset of cerebral ischemia induces a progressive increase in MCP-1 expression in the ischemia tissue, associated with the passage of leukocytes from the intravascular space into the ischemic brain (39). Studies with an *in vitro* model of the BBB and an *in vivo* model of cerebral ischemia indicate that the interaction between astrocytic-derived TWEAK and Fn14-induces the expression of MCP-1 in PA, leading to the recruitment of neutrophils from the intravascular space into the ischemic tissue. This effect, mediated by NF-κB pathway activation, is abrogated by treatment with Fn14-Fc decoy (40). Together, the available data suggest not only that TWEAK plays a central role regulating the process of ischemic inflammation in the brain, but also indicate that TWEAK inhibition with Fn14-Fc decoy may have a role for the treatment of acute ischemic stroke.

THE TWEAK-Fn14 PATHWAY MEDIATES THE DEVELOPMENT OF ISCHEMIC EDEMA

The structural integrity of the NVU is compromised when the cerebral blood flow falls below 10–15 ml/100 g/min, allowing the passage of fluid, and proteins from the intravascular space into the brain parenchyma (36, 37), with the development of cerebral edema, which is a leading cause of death in the early phases of ischemic stroke (41). Among the most important events underlying the development of ischemic cerebral edema are changes in the composition of the basal lamina with detachment of astrocytic end-feet processes and redistribution of interendothelial TJs proteins (42, 43). A role for TWEAK in the development of cerebral edema was suggested by the observation that the injection of TWEAK directly into the cerebral cortex is followed by detachment of PA from the BM, and the development of areas of focal cerebral edema (24, 34). Sub-sequent studies demonstrated that either genetic deficiency of Fn14 or treatment with Fn14-Fc decoy inhibit MCAO-induced degradation of laminin in the basal lamina (34). Importantly, the effect of TWEAK on the permeability of the NVU in the ischemic brain is mediated by MMP-9 activation (24).

TWEAK AND NEURONAL DEATH: A DOUBLE EDGE SWORD

TWEAK and Fn14 induce neuronal death in the ischemic brain

A growing body of experimental evidence indicates that TWEAK induces neuronal death. Early *in vitro* studies showed that 24 h of incubation with TWEAK induces apoptotic cell death in neurons and that this effect is mediated by NF-κB pathway activation (30). A role for TWEAK on neuronal death in the ischemic brain was later demonstrated by the observation that treatment with either anti-TWEAK neutralizing antibodies (30) or Fn14-Fc decoy (13), or genetic deficiency of Fn14 (34), reduces the volume of the ischemic lesion following MCAO. Sub-sequent studies reported that the interaction between TWEAK and Fn14-induces poly(ADP-ribose) polymerase-1 (PARP-1) activation and that this effect leads to caspase-3 cleavage and apoptotic neuronal death via a TNF-α-independent mechanism (31).

TWEAK induces tolerance in the ischemic brain

Ischemic tolerance is an endogenous neuroprotective mechanism whereby exposure to hypoxia/ischemia for a length of time or intensity that is not severe enough to cause cell death (preconditioning event) renders neurons resistant to a sub-sequent lethal hypoxic/ischemic injury (44). The preconditioning event can protect the brain either very soon after its application (early preconditioning) or after a delay of 24–72 h (delayed preconditioning). Studies with an *in vitro* model of hypoxia and an *in vivo* model of cerebral ischemia demonstrated that either treatment with sub-lethal doses of TWEAK, or induction of endogenous of TWEAK and Fn14 expression with sub-lethal hypoxia, renders neurons tolerant to a lethal hypoxic or ischemic injury applied 24 h later. This protective effect is mediated by TWEAK's ability to induce neuronal TNF-α expression and ERK 1/2 activation, and is mediated by phosphorylation of the B-cell lymphoma 2-associated death promoter protein (Bad) (27).

CONCLUSION AND FUTURE DIRECTIONS

In the brain TWEAK and Fn14 play a pivotal role in the response of the NVU an ischemic injury. Accordingly, the interaction between TWEAK and Fn14 in the endothelial cell-BM-astrocyte interface mediates the passage of inflammatory cells into the ischemic tissue. Likewise, *in vivo* and *in vitro* data indicate that TWEAK binding to Fn14 is followed by detachment of astrocytic end-feet processes from the BM, which leads to increase in the permeability of the BBB and cerebral edema. In neurons, TWEAK and Fn14 have a dual role. Hence, while the interaction between TWEAK and Fn14 during the acute stages of the ischemic injury induces neuronal death, exposure to TWEAK at doses that do not produce cell death, renders neurons resistant

against a sub-sequent hypoxic/ischemic injury (ischemic tolerance). Taken together, the data published to this data indicate that TWEAK/Fn14 inhibitors may play a role in the treatment of neurological conditions associated with increase in the permeability of the BBB and hypoxia/ischemia-induced neuronal death. The role of TWEAK as an inductor of ischemic tolerance is intriguing and future studies should define whether this cytokine has a role protecting the brain of patients at high risk of ischemic stroke.

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Neuropsychiatric lupus, the blood brain barrier, and the TWEAK/Fn14 pathway

Ariel D. Stock*[1], *Jing Wen*[1] *and Chaim Putterman*[1,2]

[1] *Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA*
[2] *Division of Rheumatology, Albert Einstein College of Medicine, Bronx, NY, USA*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Juan C. Chavez, Biogen Idec, Inc., USA
George Bertsias, University of Crete, Greece

****Correspondence:***
Chaim Putterman, Division of Rheumatology, Albert Einstein College of Medicine, F701N, 1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: chaim.putterman@einstein.yu.edu

Patients with systemic lupus erythematosus (SLE) can experience acute neurological events such as seizures, cerebrovascular accidents, and delirium, psychiatric conditions including depression, anxiety, and psychosis, as well as memory loss and general cognitive decline. Neuropsychiatric SLE (NPSLE) occurs in between 30 and 40% of SLE patients, can constitute the initial patient presentation, and may occur outside the greater context of an SLE flare. Current efforts to elucidate the mechanistic underpinnings of NPSLE are focused on several different and potentially complementary pathways, including thrombosis, brain autoreactive antibodies, and complement deposition. Furthermore, significant effort is dedicated to understanding the contribution of neuroinflammation induced by TNF, IL-1, IL-6, and IFN-γ. More recent studies have pointed to a possible role for the TNF family ligand TWEAK in the pathogenesis of neuropsychiatric disease in human lupus patients, and in a murine model of this disease. The blood brain barrier (BBB) consists of tight junctions between endothelial cells (ECs) and astrocytic projections which regulate paracellular and transcellular flow into the central nervous system (CNS), respectively. Given the privileged environment of the CNS, an important question is whether and how the integrity of the BBB is compromised in NPSLE, and its potential pathogenic role. Evidence of BBB violation in NPSLE includes changes in the albumin quotient (Q_{alb}) between plasma and cerebrospinal fluid, activation of brain ECs, and magnetic resonance imaging. This review summarizes the evidence implicating BBB damage as an important component in NPSLE development, occurring via damage to barrier integrity by environmental triggers such as infection and stress; cerebrovascular ischemia as result of a generally prothrombotic state; and immune mediated EC activation, mediated by antibodies and/or inflammatory cytokines. Additionally, new evidence supporting the role of TWEAK/Fn14 signaling in compromising the integrity of the BBB in lupus will be presented.

Keywords: TWEAK, Fn14, blood brain barrier, neuropsychiatric lupus, MRL/lpr

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by hyper-activation of B and T lymphocytes resulting in the overproduction of autoantibodies, tissue deposition of immune complexes, and high levels of inflammatory cytokines, cumulatively resulting in a systemic pro-inflammatory state (1). SLE patients may suffer from skin, joint, hematologic, and renal disease, the latter being a predominant contributor to morbidity and mortality. Treatments have traditionally consisted of corticosteroids and potent immunosuppressive agents such as cyclophosphamide, though biologic medications targeting particular cytokines may eventually prove to be promising alternatives. Additionally, the course of the disease is highly variable between patients, with certain manifestations more common than others, and the overall impact on quality of life dependent on the individual patient's circumstances and particular disease manifestations.

Central nervous system (CNS) presentations in SLE patients consist of a broad array of symptoms, which can be generally divided between focal neurological and diffuse psychiatric manifestations. Focal episodes may include seizures and cerebrovascular events, while psychiatric presentations often consist of anxiety and depression (2). A neuropsychiatric SLE (NPSLE) phenotype can be a presenting feature of lupus, and is eventually found in up to 40% of SLE patients (3). Research into the underlying mechanisms of NPSLE has taken several different and potentially complementary directions. Human, murine and *in vitro* systems have all been utilized to examine the effects of autoantibodies, cytokines, vascular disease, and cellular effectors in the development of NPSLE symptoms. TNF-like weak inducer of apoptosis, better known as TWEAK, is a TNF family member cytokine which together with its sole confirmed receptor Fn14 have recently shown to be instrumental in the pathogenesis of murine NPSLE. Furthermore, it is increasingly evident that blood brain barrier (BBB) disruption is an essential component of NPSLE pathogenesis (4), and that TWEAK may play an important role in this process (5).

NPSLE IN HUMAN LUPUS AND EXPERIMENTAL MODELS

Studying NPSLE in humans poses some obvious limitations, including the scarcity of CNS tissue samples and the heterogeneity

of NPSLE presentations. Most available data from NPSLE patients consist of blood and cerebrospinal fluid (CSF) analysis, and radiologic imaging, including magnetic resonance imaging (MRI). CSF is often remarkable for the presence of increased immunoglobulins, elevated concentrations of cytokines, and evidence of BBB disruption, as measured by increased albumin concentrations. MRI data has additionally proven useful in identifying the brain regions most frequently involved in NPSLE, as well as which CNS tissues are affected (6). Additionally, extensive work has gone into the correlation between certain systemic autoantibody titers and NPSLE phenotypes (7).

There are several spontaneous mouse models of SLE, including the NZB × NZW F1 (NZB/W F1), BXSB, and MRL/*Tnfrsf6*$^{lpr/lpr}$ (MRL/lpr) strains. These three models all develop some measure of neuropsychiatric disease. BXSB mice, for example, demonstrate problems with both spatial and non-spatial learning tasks (8). One issue with the BXSB model, however, is the sex bias toward males, which is inconsistent with the strong female predominance found in human SLE. Additionally, BXSB and NZB/W F1 mice may have congenital structural abnormalities of the brain (9), potentially confounding structure-function analyses. Both NZB/W F1 and MRL/lpr mice demonstrate neurological deficits (10, 11), though the MRL/lpr model has a greater incidence of neuropsychiatric disease (12). The MRL/lpr has the added benefit of a congenic control (MRL$^{+/+}$), which does not develop disease.

The MRL/lpr strain develops a disease phenotype consistent with the affective and behavioral pathologies seen in human lupus (13). Gao et al. found that depressive symptoms appear as early as 6 weeks of age in female MRL/lpr mice, preceding onset of renal pathology. Additionally, we found a correlation between depressive symptoms and several autoantibodies, including anti-NMDAR and anti-dsDNA (14). Similarly, depression and other neuropsychiatric symptoms can appear early in the disease course in human disease (15). MRL/lpr mice demonstrate increased immobility on the forced swim test, which is a widely accepted indicator of depression in rodents (if strength and locomotion are otherwise normal). Additionally, MRL/lpr mice display decreased preference for sweetened water (anhedonia), as well as an acquired anosmia, both manifestations of murine depressive-like behavior (16, 17). Finally, cognitive tests in MRL/lpr mice reveal clear deficits in the object placement task indicating deficits in spatial memory, relatable to the cognitive decline found in NPSLE patients.

Another experimental model of NPSLE is induced by treatment with anti-*N*-methyl-D-aspartate (anti-NMDA; also known as anti-NR2) receptor antibodies coupled with BBB disruption in BALB/c mice (18). Depending on the method of BBB disruption, resulting symptoms may include impaired performance on memory tasks or altered fear responses (19, 20). More recently, Kivity et al. showed that intracerebroventricular transfer of the 16/6 idotype (a human anti-ssDNA antibody) into C3H mice resulted in hippocampal inflammation and decreased performance in memory tasks (21).

NEUROPATHIC ANTIBODIES IN NPSLE

As previously mentioned, NPSLE deficits can be defined as either focal or diffuse in nature. Focal findings in NPSLE are most readily associated with the presence of antiphospholipid (aPL) antibodies, including anti-cardiolipin antibodies, anti-β2glycoprotein I antibodies, and lupus anticoagulant (22). These antibodies dramatically increase susceptibility to thrombosis, resulting in an increased rate of cerebrovascular accidents (CVA) and transient ischemic attacks (TIA). It is thought that aPL antibodies may act through increasing oxidative stress, as measured by increased level of oxidized low-density lipoprotein, which itself is associated with atherosclerosis and thrombosis (23). While these patients will present with typical focal findings, such as motor and cranial nerve deficits, seropositivity for aPL antibodies is not typically associated with diffuse psychiatric and cognitive presentations (7).

Other circulating and intrathecal antibodies are also associated with NPSLE manifestations. Anti-ribosomal-P (anti-P) antibodies have long been associated with NPSLE presentations (24–26), and more recently have been found to induce depression in mice when injected intraventricularly (16). Recent work by Matus et al. found that anti-P antibodies from human lupus serum induced calcium influx and subsequent apoptosis in p331 positive neurons in rats, which they characterized as a new P-antigen. Death of these neurons, found in the hippocampus, amygdala, and certain neocortical layers, account for a broad range of potential symptoms, including depression, memory deficits, and cognitive decline (27). Anti-NMDA receptor antibodies are also associated with psychiatric symptoms, such as depression and memory dysfunction in SLE patients, and altered fear responses when transferred to mice, due to excitotoxic glutamatergic effects on neurons (14, 19, 28–30). Anti-U1-RNP antibodies have been reported by Sato et al. as a more specific marker of NPSLE than anti-P or anti-NR2 antibodies (31), and may potently induce expression of interferons (32). Anti-ganglioside (anti-GM1) antibodies were once thought to be associated with NPSLE pathogenesis (4, 33) through disruption of voltage gated Na^+ channels found near nodes of Ranvier (34), though it has since been found that they are more likely associated with peripheral neuropathies than central NPSLE presentations (35). Other antibodies associated with NPSLE include anti-dsDNA, anti-Microtubule-associated Protein 2, anti-Triosephosphate isomerase, and brain reactive autoantibodies (BRAA) (36–38). The reader is referred to a number of excellent reviews for further detail regarding these and other neuropathic antibodies (39–42). Regardless of the antigenic specificity of these neuropathic autoantibodies, the question remains how they gain entry into the central nervous system (CNS) from the systemic circulation, implicating a failure in the BBB as a key component of NPSLE pathogenesis.

THE BLOOD BRAIN BARRIER

The CNS is maintained as a privileged environment due to the combination of tight junctions between endothelial cells (ECs) limiting paracellular transport, and astrocytic processes, which regulate transcellular transport from systemic circulation. The choroid plexus (CP) and arachnoid epithelia similarly maintain this barrier through tight junctions, as well as secrete and reabsorb CSF, respectively. Another element that seems essential to a competent blood brain barrier is the presence of resident microglia. Microglia populations of monocytic origin are found perivascularly within the CNS parenchyma, limit paracellular transport

across ECs, and may serve as a bridge between CNS and systemic immune activity (43).

The isolating nature of brain microvascular ECs is attributable to the tight junctions, including members of the zonula occludens (ZO-1) and claudin (claudin-5) families, resulting in impermeability to macromolecules (44). Additionally, these multilaminated junctional complexes provide very high electrical resistance, dependent on the basal parenchymal presence of astrocytes, yielding remarkably low permeability to smaller and ionic molecules (45, 46). Astrocytes are also required to provide the environmental cues ECs need to develop and maintain their unique CNS phenotype, and are involved in bidirectional cytokine signaling with ECs (47). Microglia are involved as well in modulating EC through secretion of TNF, which upregulates MHC-I presentation on ECs and promotes entry of T-cells into brain parenchyma during pathological states (48).

Beyond serving as a barrier, ECs are directly responsible for regulating the immune response in the brain. ECs help maintain the CNS in its basal state of suppressed immunity through secretion of TGFβ (49) and soluble cellular adhesion molecules (50). Brain ECs can, however, activate an immune response, such as under LPS stimulation, which induces production of IL-6 and GM-CSF. Interestingly, Verma et al. showed that LPS itself does not appear to induce disruption of EC junctional complexes; rather, further signaling by luminal and parenchymal effector cells likely potentiates BBB disruption following LPS stimulation (51).

The CP vasculature is unique within the CNS, as it consists of fenestrated ECs necessary for CSF volume maintenance. This vasculature is isolated from brain parenchyma by a blood-CSF barrier (BCSFB). The BCSFB consists of cuboidal CP epithelial cells, which are interconnected by tight junctions, effectively providing for a barrier similar to the BBB (52, 53). CSF not only provides physical support to CNS tissue by reducing its apparent weight, it is also able to rapidly transmit hormonal signals within the CNS (54) and provide for drainage, much as the lymphatics do systemically (55). Additionally, CSF is enriched with memory T-cells due to one of its primary roles, immune surveillance of the CNS. Normally, non-autoreactive T-cells are allowed to pass through the CP epithelial barrier unhindered (56). It is believed, however, that through increased expression of cellular adhesion molecules, CP ECs are involved in the pathogenesis of certain autoimmune conditions, such as multiple sclerosis (57).

EVIDENCE OF BBB DAMAGE IN SLE

There are several markers available to monitor the integrity of the BBB. Albumin is a large and extensively charged protein that is not synthesized intrathecally, and whose transport into the CNS is tightly regulated. As such, an elevated albumin concentration gradient (Q_{alb}) between CSF and plasma (normal $[Alb]_{CSF}/[Alb]_{Plasma} \leq 7.6 \times 10^{-3}$) serves as an indicator of BBB disruption, and has been found repeatedly in NPSLE patients (31, 58, 59) and MRL/lpr mice (60). While Q_{alb} provides a useful measurement of relatively large scale leakage across the BBB, it lacks the finer resolution needed to appreciate small and transient leakage (4). The IgG index $[CSF_{(IgG/Albumin)}]/[Serum_{(IgG/Albumin)}]$ is another useful measure of BBB permeability that can also identify the relative intrathecal vs. systemic origin of IgG within the CNS (61, 62), and is found elevated in both NPSLE patients and experimental models (31, 60). Sato et al. found both an elevated Q_{alb} and an elevated IgG index in 8 and 9 of 14 NPSLE patients, respectively (31). Similarly, Sidor et al. found elevated Q_{alb} and IgG index measurements in MRL/lpr mice when compared with $MRL^{+/+}$ mice, along with increased neurodegeneration in those mice with a disrupted BBB (60). Jacob et al. further demonstrated that IgG enters CNS parenchyma in MRL/lpr mice (63). Finally, Ma et al. have shown extensive penetration of $CD3^+$ cells into the CP and in brain parenchyma, as well as the presence of $CD19^+$-B-cells in MRL/lpr mice, providing further evidence of BBB failure in NPSLE (64).

The availability of new and improved modalities with finer resolution will likely continue to be an asset in measuring BBB function non-invasively in NPSLE. Since the calcium binding protein S100B is predominately found in astrocytes, its presence in serum serves as valuable indicator of BBB injury (65–67). Schenatto et al., examining a cohort of 89 SLE patients, identified elevated S100B levels in NPSLE vs. non-NPSLE, a finding which was even more prominent during acute episodes (68). Evolution of MRI is also proving to be useful in the clinical characterization of BBB breaches. The increasing availability of 3.0 T MRI magnets and newer gadolinium (Gd) containing contrast agents, which visualizes contrast flow into CNS parenchyma with T1-weighted imaging, are improving the ability to highlight areas of BBB insufficiency (69). Recently, Toledano et al. utilized multiple MRI modalities in imaging NPSLE patient brains, and found vascular damage in a third of patients with the vast majority consisting of small vessel damage (70). One could reasonably speculate that evidence of BBB disruption may be obscured due to transient changes in BBB integrity, or the reparative effects of treatment. Indeed, findings of BBB disruption by Gd-contrast enhancement are likely under-reported, since the frequent use of corticosteroids in SLE treatment likely results in stabilization of BBB damage (71).

MECHANISMS OF BRAIN EC ACTIVATION AND BBB DISRUPTION IN SLE

EC ACTIVATION IN SLE

As described above, brain ECs are not mere bystanders in the regulation of the CNS environment; they play an active role in concert with both CNS and luminal effector cells and molecules. Infection and systemic inflammation are potent activators of ECs, resulting in upregulated expression of cytokines and chemokines, thereby potentiating a local immune response (51, 72). IL-1, TNF, and LPS signaling each result in upregulation of E-selectin, ICAM-1, and VCAM-1 in microvascular ECs *in vitro* (73). MRL/lpr mice are found to have elevated expression of ICAM-1 and VCAM-1 in predominately CP associated ECs when compared to congenic controls (74). Sun et al. have recently shown that immune complexes in SLE induce production of inflammatory cytokines and cellular adhesion molecules in ECs via NF-κB signaling, due to HMGB1-RAGE axis activation (75).

TREX1 is a major endogenous 3′–5′ DNA exonuclease. Mutations in TREX1 are associated with chilblain lupus erythematosus, a rare form of cutaneous disease, as well as with sporadic SLE (76). In addition, TREX1 variants are found in two other diseases with neurological manifestations, autosomal dominant retinal

vasculopathy with cerebral leukodystrophy and Aicardi–Goutieres syndrome (77). TREX1 deficiency or dysfunction may lead to accumulation of cytosolic DNA and enhanced alpha-interferon signaling. Furthermore, TREX1 deficiency in lymphocytes modulates vascular EC angiogenesis (78), suggesting an interesting possible link between the genetic susceptibility for lupus associated with TREX1, ECs, and CNS disease (76). Irrespective of the cause, endothelial activation results in increased vascular permeability and diapedesis, and increased local aggregation of humoral and cellular effectors.

ENVIRONMENTALLY INDUCED BBB DISRUPTION

Diamond et al. have done extensive work on anti-NMDA receptor antibodies as effectors of NPSLE symptomatology. In their early studies, CNS disease was induced by direct injection of anti-NMDA receptor antibodies into the cerebral cortices of healthy mice, thereby bypassing the BBB (79). They then utilized several models of extraneous BBB disruption, including LPS as model of infection and epinephrine as a model of stress. When LPS was used to induce BBB disruption, mice showed poorer performance on the Morris water maze and T-maze tasks, indicative of learning and memory deficits, and consistent with hippocampal damage. Neuron loss in the hippocampus was found histologically and was evident as structural abnormalities on MRI (80). When using epinephrine, neuron loss occurred selectively in the amygdala, inducing alterations in conditioned fear responses in mice, while sparing hippocampal neurons (81). These findings provide a possible explanation for the phenotypic differences between NPSLE patients, and the role environmental mediators may play.

ANTI-ENDOTHELIAL CELL ANTIBODIES

Conti et al. found that 64.7% of NPSLE patients sera are positive for anti-endothelial cell antibodies (AECAs), as compared to only 29.4% of non-NPSLE patients (82). AECAs have been previously characterized as inducers of increased EC cellular adhesion molecules, including E-selectin, ICAM-1, and VCAM-1. Nara et al. stimulated HUVEC with monoclonal antibodies targeted toward thrombomodulin, a proposed antigenic substrate of some AECAs in SLE patients (83), and found increased endothelial production of IL-6 and IL-8 mediated through the NF-κB pathway (84). More recently, Yoshio et al. found that anti-NR2 antibodies recognize antigenic targets on HUVECs, and can induce IL-6 and IL-8 expression with IL-1β co-treatment (85). Collectively, AECAs may induce endothelial activation, which is pivotal in many inflammatory processes, but in the brain is an important component of BBB disruption as well (86).

COMPLEMENT

One of the hallmarks of increased disease activity in SLE patients is the depletion of complement components from serum, due to consumption by circulating immune complexes, deposition in target tissues and targeting by anti-C1q antibodies (87, 88). Low circulating levels of complement components C3 and C4 have also been suggested as potential biomarkers of human NPSLE activity (89). Alexander et al. first demonstrated that complement inhibition can attenuate NPSLE presentations in MRL/lpr mice (90). Jacob et al. then showed that C5aR activation induces EC cytoskeletal alteration *in vitro* and laminin disruption in MRL/lpr brain vasculature, both indicators of BBB damage. Additionally, C5aR activation results in increased CCL2 and CXCL2 production by mouse microvascular ECs, when pretreated with IL-6 (91). Finally, in further characterizing the BBB disruption that occurs with C5aR activation, Jacob et al. found increased expression of MAP-kinase, increased nuclear NF-κB translocation and decreased zona occludin (ZO) levels, indicative of EC junctional complex interruption, which would dramatically increase BBB permeability (92).

CYTOKINES AND CHEMOKINES

Stimulation of human brain microvessel ECs (HBMEC) with cytokines such as IL-1β, IL-8, TNF, and IFN-γ are known to induce increased permeability across monolayers (86, 93). CCL2 signaling has been shown to play a role in BBB disruption both *in vitro* and in $CCR2^{-/-}$ mice (94, 95). Zameer et al. found elevated expression levels of ICAM-1 and VCAM-1 in the CNS of MRL/lpr mice, providing further evidence of an inflammatory process involving the BBB (74, 96). Trysberg et al., analyzing CSF from lupus patients, found elevated levels of IL-6 and IL-8 which were correlated with elevated MMP-9 levels, the latter associated with degradation of BBB extracellular matrix (97).

In most, if not all of the above models of BBB disruption, it is clear that the cytokine and chemokine environment is critical. While complement and AECAs may indeed be effectors of BBB disruption, they cannot do so on their own; cytokines are needed to fully activate and disrupt the BBB. Amongst these cytokines, TWEAK has recently been demonstrated as a potent effector of multiple downstream pathways needed for BBB disruption, including activating intracellular signaling cascades and inducing production of additional cytokines, chemokines, and metalloproteinases.

The causes and consequences of EC activation are summarized in **Table 1**.

TWEAK, NPSLE, AND BBB DISRUPTION

TWEAK is a pro-inflammatory cytokine member of the TNF superfamily. Through activation of its sole receptor, Fn14, TWEAK variably induces cellular proliferation, angiogenesis, inflammation, and apoptosis (98). As previously mentioned, MRL/lpr mice develop a neuropsychiatric phenotype remarkably similar to human SLE. We recently found increased TWEAK and Fn14 expression in the cerebral cortices of MRL/lpr mice. Furthermore, in MRL/lpr Fn14-knockout (Fn14KO) mice we found significantly improved cognitive function, decreased depression, and less anhedonia, as demonstrated by object placement tasks, the forced swim test, and preference for sweetened water, respectively (17). The attenuated neuropsychiatric phenotype in Fn14 deficient lupus mice may be due to decreases in brain expression of CCL5 and C3, which have been found associated with depression and cognitive decline (99–101). Other mechanisms by which the Fn14KO is protective of NPSLE development in MRL/lpr mice are under active investigation.

Fn14 is expressed both in the human cerebral microvascular EC line (hCMEC/D3) and astrocytes, while TWEAK is secreted by only the latter (5, 102). As described above, we found decreased

Table 1 | Potential routes of endothelial cell activation in SLE.

Cause of endothelial activation	Model	Findings
Environmental mediators	BBB disruption by treatment with LPS or epinephrine, in models of infection and stress, respectively (19, 81)	Treatment with human lupus serum containing anti-NMDAR antibodies following BBB disruption results in IgG deposition, hippocampal neuron loss, and memory impairment (LPS), or amygdala neuron loss and altered fear responses (epinephrine)
AECAs	*In vitro* treatment of HUVEC with anti-thrombomodulin (83, 84) or anti-NR2 antibodies (85)	Increased IL-6 and IL-8 expression
Complement	Mouse brain endothelial cells and MRL/lpr mice (63, 92)	C5aR activation yields increased CCL2 and CXCL2, NFκB signaling, and decreased ZO expression
Cytokine and chemokines	MRL/lpr mice (74) and CSF from human lupus patients (97)	Elevated ICAM-1 and VCAM-1 in MRL/lpr CNS; increased IL-6, IL-8, and MMP-9 in lupus CSF
TWEAK	*In vitro* hCMEC/D3 cells (5)	Elevated ICAM-1, CCL2, IL-6, IL-8, and MMP-9, ZO-1 degradation and decreased occludin levels

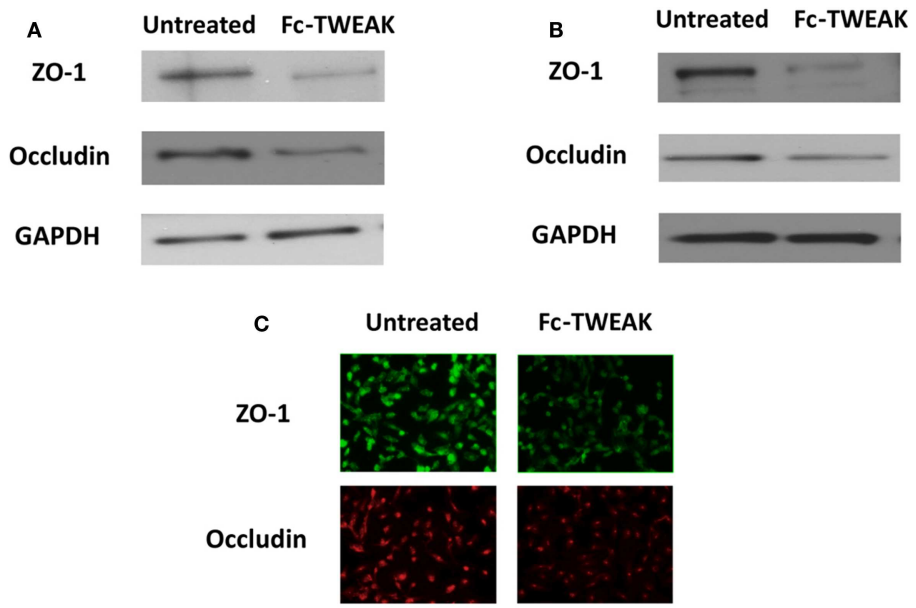

FIGURE 1 | ZO-1 and occludin expression after treatment with Fc-TWEAK. ZO-1 and occludin expression was measured by western blot. Treatment with Fc-TWEAK (100 ng/mL, 48 h) decreased ZO-1 and occludin expression in hCMEC/D3 cells **(A)**. ZO-1 and occludin expression was decreased in hBMEC cells after treatment with Fc-TWEAK (100 ng/mL, 24 h) as well **(B)**. Similarly, immunofluorescent detection of ZO-1 and occludin in hBMECs was reduced after treatment with Fc-TWEAK (100 ng/mL, 24 h) **(C)**.

Q_{alb} ratios in MRL/lpr Fn14KO mice as well as decreased CSF titers of anti-dsDNA antibodies (17). More recently, we characterized the effects of TWEAK on endothelial cytokine expression and barrier disruption using hCMEC/D3 brain ECs, and found TWEAK-induced increases in ICAM-1, CCL2, IL-6, and IL-8 (5). Furthermore, TWEAK-induced activation of the MAPK pathway yielded increased expression of MMP-9, which degraded ZO-1, decreased occludin expression (**Figure 1**), and increased permeability (5). A similar finding was seen by Polavarapu et al. in their investigation of cerebral ischemic injury, where intracerebral injection of TWEAK in wild type mice increased MMP-9 activity and BBB permeability (103). Furthermore, TWEAK induced expression of ICAM-1, IL-8, and IL-6 in cultured astrocytes, typical of reactive astrocytes, another effector of BBB disruption (104). Together, this data supports the conclusion that TWEAK is instrumental in the development of lupus associated neuropsychiatric disease, with BBB disruption as an important mechanistic contribution of this cytokine. The lack of effect of TWEAK on systemic autoantibodies (17) and our current understanding of the mechanism of action of the TWEAK/Fn14 signaling pathway also suggest that

attenuated neuropsychiatric disease in MRL/lpr Fn14 KO mice is not due to a reduced systemic autoantibody response, but rather local (i.e., brain) effects of blocking this pathway such as the effect on the BBB discussed here. Finally, it is important to note that not only is TWEAK/Fn14 signaling involved in NPSLE, but this cytokine/receptor pair has been implicated in the pathogenesis of injury in other major lupus target organs, including the kidney [for which there is evidence both in murine models (105, 106), and in human disease (107)], and in cutaneous lupus as well (108).

The success of mAb treatment targeting pathogenic cytokines such as TNF, IL-1, and BLyS in inflammatory rheumatic diseases, together with the data presented above, strongly suggest the need to examine anti-TWEAK antibody treatment as novel treatment approach in NPSLE. Antibodies given intravenously can have therapeutic effects on the brain, especially if the blood brain barrier is already breached (as in NPSLE) (109). Alternatively, it would be necessary to bypass the BBB, or develop a delivery system to deliver the antibody to the CNS despite the compartmentalization of the brain from the blood (e.g., bispecific antibodies using the transferrin receptor) (110).

CONCLUSION

Neuropsychiatric SLE is often associated with the presence of neuropathic antibodies within the CNS, making the question of how they gain entry into this anatomically privileged space increasingly important. Evidence points to entry of autoantibodies across the BBB, with entry into different brain regions and specific autoantibody subtypes potentially associated with the variable phenotypes found in both murine experimental models and NPSLE patients. There is strong support for the roles of AECAs, complement components, and environmental mediators in increasing permeability across the BBB, though in each of these cases, cytokines and chemokines have an essential role as well. TWEAK appears to be one such cytokine that is necessary for the development of NPSLE; one mechanism central to the contributions of the TWEAK/Fn14 axis appears to be its role in BBB disruption.

Interestingly, TWEAK/Fn14 signaling has been implicated in other neurologic diseases besides NPSLE, including hypoxic brain damage and autoimmune brain disease. Elsewhere in this special issue, Yepes describes how TWEAK/Fn14 signaling in middle cerebral artery occlusion (a model of ischemic stroke) induces inflammation and MMP-9 mediated basal lamina disruption (111). Desplat-Jego et al. demonstrated the involvement of TWEAK in the development of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, and that TWEAK over-expression in transgenic mice further exacerbates the EAE phenotype (102). In both these disease models, preventing TWEAK signaling via Fn14 deficiency, treatment with a Fn14-Fc decoy receptor, or treatment with anti-TWEAK monoclonal antibodies results in attenuated disease. Ameliorating the disruption of the BBB may be a valuable tool in the control of NPSLE as well other neurologic disorders, and targeting the TWEAK pathway may be one way to do so.

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TWEAK/Fn14 and non-canonical NF-kappaB signaling in kidney disease

Jonay Poveda[1†], Luis C. Tabara[1†], Beatriz Fernandez-Fernandez[1], Catalina Martin-Cleary[1], Ana B. Sanz[1], Rafael Selgas[2], Alberto Ortiz[1] and Maria D. Sanchez-Niño[2]*

[1] *Department of Nephrology, IIS-Fundacion Jimenez Diaz, Universidad Autonoma de Madrid and IRSIN, Madrid, Spain*
[2] *Department of Nephrology, IdiPAZ, Madrid, Spain*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Chaim Putterman, Albert Einstein College of Medicine of Yeshiva University, USA
Nicolas Wisniacki, Biogen Idec, Inc., UK
Joel M. Weinberg, University of Michigan Medical Center, USA

***Correspondence:**
Maria D. Sanchez-Niño, Department of Nephrology, IdiPAZ, Paseo de la Castellana 260, Madrid 28046, Spain
e-mail: dolores.sanchez@idipaz.es

[†] *Jonay Poveda and Luis C. Tabara have contributed equally to this work.*

The incidence of acute kidney injury (AKI) and chronic kidney disease (CKD) is increasing. However, there is no effective therapy for AKI and current approaches only slow down, but do not prevent progression of CKD. TWEAK is a TNF superfamily cytokine. A solid base of preclinical data suggests a role of therapies targeting the TWEAK or its receptor Fn14 in AKI and CKD. In particular TWEAK/Fn14 targeting may preserve renal function and decrease cell death, inflammation, proteinuria, and fibrosis in mouse animal models. Furthermore there is clinical evidence for a role of TWEAK in human kidney injury including increased tissue and/or urinary levels of TWEAK and parenchymal renal cell expression of the receptor Fn14. In this regard, clinical trials of TWEAK targeting are ongoing in lupus nephritis. Nuclear factor-kappa B (NF-κB) activation plays a key role in TWEAK-elicited inflammatory responses. Activation of the non-canonical NF-κB pathway is a critical difference between TWEAK and TNF. TWEAK activation of the non-canonical NF-κB pathways promotes inflammatory responses in tubular cells. However, there is an incomplete understanding of the role of non-canonical NF-κB activation in kidney disease and on its contribution to TWEAK actions *in vivo*.

Keywords: acute kidney injury, fibrosis, inflammation, kidney, lupus nephritis, podocyte, proteinuria

UNSOLVED ISSUES IN KIDNEY DISEASE

Acute kidney injury (AKI) and chronic kidney disease (CKD) are the most severe forms of kidney disease (1, 2). AKI is characterized by a sudden loss of renal function. AKI patients have increased short- and long-term mortality and risk of CKD progression. However, there is no therapy that accelerates recovery from AKI. CKD is a major healthcare problem, with more than 20 million aged 20 years or older affected in the United States. Diabetic kidney disease is the leading cause of end stage renal disease in the Western Countries. However, current treatments based on blockade of the renin-angiotensin system are not sufficient to prevent progression of diabetic kidney disease (3).

Recent evidence suggests a role for TNF superfamily member Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, or TNFSF12) in both AKI and CKD, where it has been shown to regulate cell death, inflammation, and fibrosis through activation of the TWEAK receptor Fn14 and a variety of intracellular signaling pathways, including the transcription factor nuclear factor-kappa B (NF-κB) (4, 5) (**Figure 1**). Clinical trials are testing anti-TWEAK neutralizing antibodies[1,2]. One key difference between TWEAK and the best characterized member of the family, TNF, is that TWEAK activates the non-canonical NF-κB pathway. We now review current information on TWEAK, non-canonical NF-κB activation, and kidney disease.

TWEAK

TWEAK may be membrane-bound or soluble, although most functional studies have been performed with soluble TWEAK. Soluble TWEAK is thought to be generated from full-length TWEAK by furin-mediated cleavage of the extracellular domain (6).

The TWEAK receptor, Fn14 (TNFRSF12a), is the smallest member of the TNF receptor superfamily. Fn14 is a type I transmembrane protein which has 102 aa in its mature isoform. The extracellular domain has 53 aa and harbors a cysteine rich domain required for TWEAK binding (7). Interestingly, the Fn14 intracellular domain (29 aa) lacks the characteristic death domain of TNFRSF receptors but contains TNFR-associated factor (TRAF) binding sites. Fn14 trimerization recruits TRAF2 and TRAF3 upon TWEAK binding (8).

TWEAK may regulate cell proliferation, cell death, cell differentiation, and inflammation (4, 6).

TWEAK may trigger cell death or proliferation processes, depending on cell type and microenvironment; TWEAK promotes proliferation of numerous cell types including quiescent renal tubular cells through activation of NF-κB, MAPK, and phosphatidyl-inositol 3-kinase (PI3K)/AKT pathways (9). In addition TWEAK was described as a weak inductor of apoptosis which required special microenvironment (such as the presence of interferon-γ – IFN-γ) to induce cell death (10–12). Under certain circumstances TWEAK can induce apoptosis without co-treatment with other cytokines. It has been proposed that levels of Fn14 expression may sensitize cells to TWEAK but it is also

[1] http://clinicaltrials.gov/ct2/show/NCT00771329
[2] http://clinicaltrials.gov/ct2/show/NCT01499355

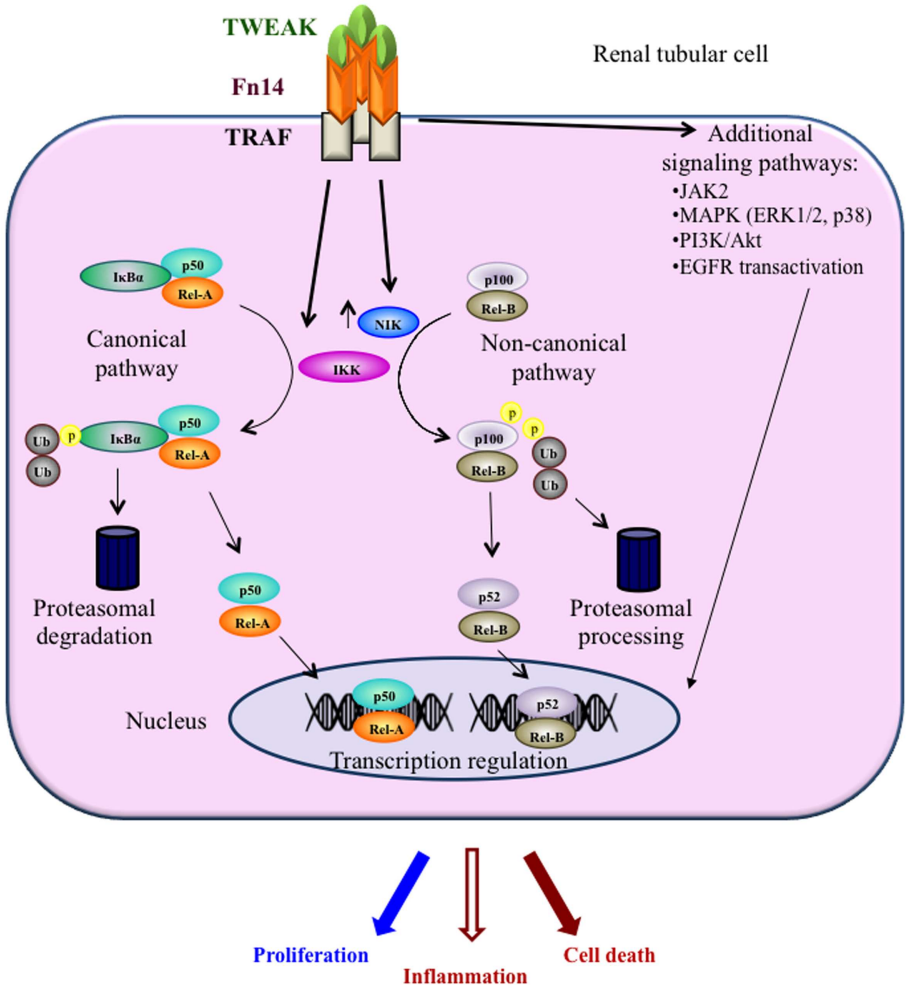

FIGURE 1 | Key intracellular pathways activated by TWEAK engagement of Fn14 in kidney tubular cells. TWEAK signaling in kidney cells has been characterized most in detail in tubular cells. TWEAK engages both the canonical and the non-canonical NFκB pathways and kinase signaling mechanisms.

clear that this cannot be the only mechanism (9). Indeed, the signaling cascade which triggers cell death following Fn14 activation remains poorly understood as Fn14 does not contain a death domain (7). Induction of TNF expression by TWEAK has been reported in certain cell types. In immortalized tumor cells, TWEAK activation of Fn14 recruits a TRAF2/cellular inhibitor of apoptosis 1 (cIAP1) complex that results in the lysosomal degradation of cIAP1-TRAF2 in a cIAP1-dependent manner (13). TWEAK depletion of cIAP1 and TRAF2 activates non-canonical NF-κB signaling. However the function of non-canonical NF-κB signaling was not explored.

TWEAK is expressed in many tissues. High levels are found in the pancreas, intestine, heart, brain, lung, ovary, vasculature, and skeletal muscle, and lower levels in the liver and kidney (4). Fn14 is expressed by many cell types, including epithelial, mesenchymal, and endothelial cells. In healthy tissues Fn14 expression is low. However, cellular Fn14 levels are increased in response to stress or injury.

TWEAK/FN14 IN KIDNEY DISEASE

Fn14 is expressed in kidney tubular cells, mesangial cells, and podocytes (10). Fn14 expression by kidney endothelium has not been well characterized. Renal infiltrating cells such as macrophages also express Fn14 (14). Fn14-expressing cells are potentially responsive to TWEAK. In addition TWEAK and Fn14 expression is increased in kidney injury and targeting of the system is beneficial in different models of kidney injury.

TWEAK/FN14 ACTIONS ON RENAL CELLS

Potential kidney sources of TWEAK include infiltrating monocytes and T lymphocytes and local cells such as mesangial and tubular cells (10, 15–17).

During glomerular injury both mesangial cells and podocytes may be targets of the inflammatory response. Mesangial cell injury is observed in proliferative glomerulonephritis, while podocyte injury is characteristic of proteinuric kidney diseases. TWEAK promotes the expression of chemokines, adhesion molecules,

and matrix metalloproteinases in human and murine mesangial cells (17, 18). TWEAK also increases mesangial cells proliferation, but TWEAK combined with IFN-γ promotes mesangial cells apoptosis (17, 18). In human and murine podocytes TWEAK induces the expression of proinflammatory mediators in an NF-κB-dependent manner (18, 19). TWEAK also promotes nephrin expression and human podocyte proliferation (18). Expression of nephrin and proliferation are not usually associated *in vivo*. In fact, podocytes are terminally differentiated cells that do not divide. Podocyte proliferation is only observed under very specific pathological circumstances and is usually associated with dedifferentiation and loss of podocyte markers including nephrin.

In murine and human renal tubular cells TWEAK also promotes the expression of cytokines and chemokines (20). TWEAK also increases tubular cell proliferation through recruitment of the mitogen-activated protein kinases ERK and p38, the PI3K/Akt pathway and the canonical NF-κB pathway (9). Similar to observations in mesangial cells, in a proinflammatory milieu TWEAK induces apoptosis of tubular cells (10). By contrast to mesangial cells, the lethal action of TWEAK in tubular cells requires the simultaneous presence of TNFα and INFγ. Surprisingly, caspase inhibition prevented the features of apoptosis induced by the cytokine cocktail but increased overall cell death through a reactive oxygen species-dependent necrotic pathway (10). More recently, TWEAK/TNFα/INFγ-induced cell death in tubular cells was shown to have features of necroptosis (21). Necroptosis is an active form of cell death that requires the kinase activity of receptor-interacting protein 1 (RIP1) and RIP3.

TWEAK also promotes murine renal fibroblasts proliferation through activation of the Ras/ERK pathway (22). The proliferative effect of TWEAK on fibroblasts overrides its negative effect on extracellular matrix production. Thus, the overall effect of TWEAK targeting in experimental renal fibrosis is decreased fibrosis (22). In addition, TWEAK also promotes the expression of inflammatory cytokines in renal fibroblasts (22).

So far, the proinflammatory effect of TWEAK on mesangial cells, podocytes, and fibroblasts have been shown to proceed through canonical NF-κB activation involving RelA migration to the nucleus and expression of canonical RelA targets such as MCP1, RANTES, and others (18, 19, 22). By contrast, both canonical and non-canonical NF-κB activation by TWEAK have been observed in tubular cells (20, 23). The known consequences of non-canonical NF-κB activation are discussed below.

TWEAK/FN14 EXPRESSION IN KIDNEY INJURY

TWEAK and Fn14 expression is increased in experimental animal models of AKI, lupus nephritis, albumin overdose-induced proteinuria, kidney fibrosis induced by unilateral ureteral obstruction and anti-GBM nephritis (10, 19, 20, 22, 24, 25). High levels of tubular Fn14 expression have been also observed in human ischemic AKI and in acute or chronic human tubulointerstitial inflammation (24, 26). In human lupus nephritis glomerular Fn14 mRNA expression was increased and was higher in proliferative than in membranous lupus nephropathy (27, 28). Urinary TWEAK has been proposed as a biomarker of lupus nephritis activity (29–32).

THERAPEUTIC MODULATION OF TWEAK OR FN14 IN EXPERIMENTAL KIDNEY INJURY

Therapeutic modulation of the TWEAK/Fn14 pathway has been successful in experimental models of AKI, kidney fibrosis, lipid-induced kidney injury, proteinuria-induced kidney injury, and immune-mediated glomerular injury, including lupus nephritis. The TWEAK/Fn14 pathway was modulated in mice either by gene targeting of TWEAK/Fn14, by neutralizing anti-TWEAK antibodies or by blocking anti-Fn14 antibodies.

Mice with experimental ischemic or folic acid-induced AKI displayed a variety of benefits from TWEAK targeting that included better histological parameters and renal function, and reduction of chemokine expression, tubular cell apoptosis, and renal fibrosis, while the anti-inflammatory and anti-aging hormone klotho was increased (4, 9, 20, 23, 24, 26, 33). TWEAK downregulates Klotho in normal kidneys (33).

Fn14-deficient mice show decreased kidney damage, inflammation, and fibrosis in models of lupus nephritis (5, 34). Anti-TWEAK neutralizing antibodies reduced inflammatory gene expression and renal damage in lupus nephritis (34). Reduced residual fibrosis was observed in mice which had been protected from the acute phase of ischemia reperfusion by anti-Fn14 blocking antibodies (24). Protection from fibrosis by interfering with TWEAK/Fn14 is not limited to residual fibrosis following amelioration of the initial injury. TWEAK knockout mice were protected from fibrosis in the unilateral ureteral obstruction of model of persistent kidney insult while overexpression of TWEAK causes renal fibrosis in normal previously normal kidneys (22).

Fn14-deficient mice were protected from anti-GBM induced glomerulonephritis (25). In addition, neutralizing anti-TWEAK antibodies improved nephritis in wild type mice without altering the adaptive immune response, indicating that TWEAK/Fn14 directly regulates the inflammatory response (25). In this regard, anti-TWEAK antibodies decreased hyperlipidemia-induced kidney inflammation and injury (35).

Experimental kidney diseases in which TWEAK/Fn14 targeting has been successful share the presence of diverse degrees of local inflammation. Thus, the kidney milieu to some extent reproduces the cell culture conditions under which TWEAK promotes kidney cell death. However, the environment also influences TWEAK actions in the kidney *in vivo*. The TWEAK/Fn14 pathway may contribute to tissue regeneration (9, 36, 37). In experimental, inflammation-free unilateral nephrectomy TWEAK promotes remnant kidney growth and tubular cell proliferation (9). However, TWEAK knockout mice have decreased remnant kidney size and tubular cell proliferation (9). This information may be useful in the context of regenerative medicine. However, the regenerative potential of TWEAK was not apparent in animal models of inflammatory kidney injury, where the injurious effect was observed in all models studied so far.

NON-CANONICAL NF-κB SIGNALING

The NF-κB transcription factor binds to the κB enhancer in DNA to control transcription of over 400 genes. NF-κB controls immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. Dysregulation of NF-κB has been linked to cancer, inflammatory, and autoimmune diseases (9, 23, 38).

The mammalian NF-κB family has five members, RelA/p65, RelB, c-Rel, NF-κB1 p50, and NF-κB2 p52 (39, 40). All share a highly conserved DNA-binding/dimerization domain called the Rel homology domain (RHD), through which they form homo or heterodimers. RelA, c-Rel, and RelB contain a C-terminal transactivation domain (TAD) with multiple ankyrin repeats. In order to activate transcription, they form dimers with either p50 or p52.

Nuclear factor-kappa B activation does not require the novo synthesis of NF-κB proteins. In most cells, NF-κB proteins are present as an inactive complex in the cytoplasm. The activity of NF-κB is regulated by its interaction with inhibitory IκB proteins. The IκB proteins include p105, p100, IkBα, IκBβ, IκBγ, IκBε, IκBz, and Bcl-3 (41–43), NFκB1 and NFκB2 are synthesized as precursors, p105 and p100, respectively. These precursors contain an IκB-like C-terminal portion and function as NF-κB inhibitors. Ubiquitin/proteasome processing results in selective degradation of the C-terminal ankyrin repeats, disrupts the IκB-like function and generates the active NF-κB subunits p50 and p52 (44, 45).

Nuclear factor-kappa B activation in response to extracellular signals can proceed through classical/canonical, alternative/non-canonical, or hybrid pathways (4, 38, 46–49). Classical NF-κB activation is a rapid and transient response to a wide range of stimuli, while the alternative pathway involves slow activation of the p100/RelB heterodimer leading to the generation of p52/RelB and prolonged activation of NF-κB target genes in response to a more limited set of stimuli (45, 50). There is interplay between both pathways. Thus, classical NF-κB activation-induced transcription of NF-κB2 and RelB favors activation of the non-canonical pathway. Both pathways converge on the activation of a complex that contains a serine-specific IκB kinase (IKK). IKK contains at least, three distinct subunits: the catalytic kinase subunits IKKα (IKK1) and IKKβ (IKK2) and the regulatory subunit, IKKγ (NEMO).

Nuclear factor-kappa B inducing kinase (NIK, MAP3K14) is the apical kinase triggering non-canonical NF-κB activation. NIK belongs to the family of MAP3Ks that are known to be activated through T-loop phosphorylation. Upon activation, NIK activates IKKα and serves as a docking molecule that recruits IKKα to p100, facilitating ubiquitination by the β-TrCP ubiquitin ligase and subsequent proteasomal processing into the mature p52 subunit in a manner dependent on IKKα-dependent p100 phosphorylation (50–52). This allows the RelB/p52 heterodimer to translocate to the nucleus and to activate transcription of target genes (53). p100 processing is regulated by a short list of activators known to signal through NIK (53–57). This list includes TWEAK (58).

A variety of functions have been described for NIK including generation and/or maintenance of memory T cells (59), the formation of Th17 cells (60), promotion of glucagon responses (61), and the pathogenesis of chronic inflammation and insulin resistance in type 2 diabetes (62). Some of these functions may be independent from activation of IKKα and the non-canonical NF-κB pathway (63) and for others the relationship to non-canonical NF-κB was not explored. Thus, NIK modulates melanoma survival and growth through a β-catenin-mediated transcription way (64), is recruited to the promoters of pro-inflammatory genes to induce H3K9 histone acetylation in response to TNFα (65) and may favor or repress Smac mimetic induced death depending on the cell context. NIK upregulation in response to Smac mimetics/TNF repressed apoptosis induced by this combination, likely by maintaining FLICE inhibitory protein (c-FLIP) levels to suppress caspase-8 activation. Thus, resistant cells were sensitized to cell death by NIK depletion. NIK was required for activation of both canonical and non-canonical NF-κB pathways but their relative contribution to the protective effect was not explored (66).

NON-CANONICAL NF-κB ACTIVATION AND KIDNEY DISEASE

There is little information on the occurrence and role of non-canonical NF-κB activation in kidney disease. Few studies have addressed the overall regulation of the pathway. However, a few reports have explored individual molecules participating in non-canonical NF-κB activation, frequently without exploring function.

In diabetic mice kidney cortex NIK and RelB are upregulated several fold and phosphorylation of IKK alpha was increased (67). Non-canonical NF-κB components were predominantly located in tubular epithelial cells (67). NIK overexpression in cultured human proximal tubular cells increased RelB/p52 nuclear levels and DNA-binding activity and expression of inflammatory cytokines such as IL-6, IL-8, and MCP1 (68). TRAF3 silencing also increased nuclear RelB/p52 and transcription of proinflammatory cytokines. AGEs increased NIK and nuclear RelB/p52 in cultured proximal tubular cells (68).

In human kidney graft biopsies with delayed graft function NIK was increased in proximal tubular, interstitial, and mesangial cells and was observed in nuclei. In pig ischemia-reperfusion tubular and glomerular NIK phosphorylation was increased as observed by immunohistochemistry. In cultured proximal tubular cells thrombin induced NIK phosphorylation (69). However, no functional study addressed the consequences of NIK phosphorylation.

RelB targeting by siRNA may protect mice against lethal kidney ischemia (70). Mice injected with RelB siRNA had lower serum creatinine, histological tissue injury, and TNF expression as compared to controls. Furthermore, RelB targeting increased survival (70).

In cultured proximal tubular cells, lentiviral small hairpin RNA (shRNA)-mediated knockdown of RelB, abrogated the excess apoptosis induced by TNF in combination with cisplatin. Thus, cells with targeted RelB exposed to TNF/cisplatin have the same apoptosis rate as cells treated only with cisplatin. RelB targeting protection from apoptosis was associated with phenotypic markers of epithelial-to-mesenchymal transition. A transcriptomics analysis disclosed that knockdown of RelB was associated with upregulation of Snai2 and Rho GTPases. Targeting Rho kinase prevented the protective action of RelB knockdown (71).

The uremic toxins *p*-cresylsulfate and indoxylsulfate increased NF-κB2 expression by 50–80% in cultured proximal tubular cells (72). However, whether this was associated with increased protein levels or the functional consequences of this observation for the tubular cell cytotoxicity or inflammatory response elicited by these toxins (73) were not explored.

TWEAK AND NON-CANONICAL NF-κB ACTIVATION IN KIDNEY DISEASE

A sustained NF-κB activation, persistent for up to 24 h, was observed in tubular cells exposed to TWEAK, consistent with activation of the non-canonical pathway in addition to the already

Table 1 | TWEAK actions on kidney cells involving NF-κB activation and evidence for the role of canonical or non-canonical pathways.

Cell type	Effect	Functional modulation	NF-κB pathway involved	Reference
Mesangial cells	Inflammation	BAY11-7082	Canonical	Gao et al. (18)
Podocytes	Inflammation	Parthenolide	Canonical	Sanchez-Nino et al. (19)
Tubular cells	Inflammation	Parthenolide	Canonical	Sanz et al. (20)
	Proliferation	Parthenolide		Sanz et al. (9)
	Inflammation: CCL21, CCL19	NIK siRNA, RelB siRNA	Non-canonical	Sanz et al. (23)
Renal fibroblasts	Inflammation	Parthenolide	Canonical	Ucero et al. (22)

characterized canonical NF-κB activation (20) (**Table 1**). In this regard, in cultured renal tubular cells TWEAK increases nuclear RelB/p52 accumulation, RelB and p52 DNA-binding activity, and NIK- and RelB-dependent CCL21 and CCL19 expression (23). Nuclear RelB/p52 migration and CCL21/CCL19 expression peaked at 24 h and, thus, were delayed as compared to RelA nuclear migration and expression of canonical RelA-dependent genes such as MCP1 and RANTES that peak at 3 and 6 h, respectively. By contrast, parthenolide, which inhibits the degradation of IκBα and RelA nuclear translocation, did not prevent CCL21 upregulation (20, 23, 74). Furthermore, TWEAK administration *in vivo* to healthy mice resulted in nuclear translocation of RelB and p52 in tubular cells and in increased renal CCL21 expression. Conversely, neutralizing anti-TWEAK antibodies prevented both RelB/p52 accumulation and increased expression of CCL21 in mice with folic acid-induced AKI (20). CCL21 expression had been previously shown to be dependent on non-canonical NF-κB activation in non-renal cells (53). CCL21 is T-cell and fibrocyte chemotactic factor that plays a role in renal tubulointerstitial fibrosis (75, 76).

In summary, TWEAK is the only cytokine known to activate the non-canonical NF-κB pathway in tubular cells, both in cell culture and *in vivo*. Activation of the non-canonical NF-κB pathway is a key difference with TNF. However, whether TWEAK activates the non-canonical NF-κB pathway in mesangial cells, podocytes, or kidney fibroblasts and the functional in these cells remains unexplored.

CONCLUSION

Accumulating evidence suggests a role for TWEAK in the pathogenesis of diverse forms of kidney injury, thus making TWEAK an attractive therapeutic target. Indeed, ongoing clinical trials are targeting TWEAK in kidney disease. Recently, a phase I clinical trial of anti-TWEAK neutralizing antibodies in rheumatoid arthritis was completed.[1] Intravenous administration of anti-TWEAK resulted in undetectable serum-TWEAK for a month and in decreased levels of several inflammatory biomarkers. An ongoing phase II trial in lupus nephritis patients is testing the nephroprotective effect of BIIB023 anti-TWEAK antibody.[2] TWEAK is one of a handful of cytokines that activate the non-canonical NF-κB pathway and the only one to have been explored with respect to non-canonical NF-κB pathway activation in kidney cells. Functional studies suggest that non-canonical NF-κB activation is a relevant action for TWEAK-induced kidney inflammation. Potential therapeutic approaches include both the simultaneous inhibition of both NF-κB pathways when targeting TWEAK as well as the eventual independent regulation of canonical and non-canonical NF-κB responses by designing differential inhibitors. While these non-canonical NF-κB inhibitors are not yet ready for human use, progress is being made on the design of NIK inhibitors (77). However, there is little functional information on the overall role of NIK and non-canonical NF-κB activation in kidney disease and on the consequences of differential therapeutically manipulation of canonical and non-canonical NF-κB responses. Clearly, more research is needed in this area.

AUTHOR CONTRIBUTIONS

Maria D. Sanchez-Niño and Alberto Ortiz devised the structure and overviewed and directed the effort. Luis C. Tabara, Jonay Poveda, Beatriz Fernandez-Fernandez, and Catalina Martin-Cleary reviewed the TWEAK and the non-canonical NF-κB literature, respectively. Ana B. Sanz and Rafael Selgas contributed to the final form.

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TWEAK/Fn14 axis: a promising target for the treatment of cardiovascular diseases

Luis M. Blanco-Colio*

Vascular Research Laboratory, IIS-Fundación Jiménez Díaz, Madrid, Spain

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Maureen McMahon, University of California Los Angeles, USA
Ronglih Liao, Brigham and Women's Hospital and Harvard Medical School, USA

****Correspondence:***
Luis M. Blanco-Colio, Vascular Reseach Laboratory, IIS-Fundación Jiménez Díaz, Avenue Reyes Católicos 2, Madrid 28040, Spain
e-mail: lblanco@fjd.es

Cardiovascular diseases (CVD) are the first cause of mortality in Western countries. CVD include several pathologies such as coronary heart disease, stroke or cerebrovascular accident, congestive heart failure, peripheral arterial disease, and aortic aneurysm, among others. Interaction between members of the tumor necrosis factor (TNF) superfamily and their receptors elicits several biological actions that could participate in CVD. TNF-like weak inducer of apoptosis (TWEAK) and its functional receptor and fibroblast growth factor-inducible molecule 14 (Fn14) are two proteins belonging to the TNF superfamily that activate NF-κB by both canonical and non-canonical pathways and regulate several cell functions such as proliferation, migration, differentiation, cell death, inflammation, and angiogenesis. TWEAK/Fn14 axis plays a beneficial role in tissue repair after acute injury. However, persistent TWEAK/Fn14 activation mediated by blocking experiments or overexpression experiments in animal models has shown an important role of this axis in the pathological remodeling underlying CVD. In this review, we summarize the role of TWEAK/Fn14 pathway in the development of CVD, focusing on atherosclerosis and stroke and the molecular mechanisms by which TWEAK/Fn14 interaction participates in these pathologies. We also review the role of the soluble form of TWEAK as a biomarker for the diagnosis and prognosis of CVD. Finally, we highlight the results obtained with other members of the TNF superfamily that also activate canonical and non-canonical NF-κB pathway.

Keywords: atherosclerosis, stroke, biomarker, TWEAK, TNF superfamily

INTRODUCTION

Cardiovascular diseases (CVD) claim more lives worldwide than any other, causing an estimated 17 million deaths worldwide each year due to heart attacks and strokes. CVD included several pathologies such as coronary heart disease, stroke or cerebrovascular accident, congestive heart failure, peripheral arterial disease, and aortic aneurysm, among others.

Cumulative evidence supports the important role of the tumor necrosis factor (TNF) superfamily of proteins in the development of CVD. The majority of ligands included in this family are synthesized as type II transmembrane proteins with a common structural motif (THD) that mediates self-trimerization and receptor binding (1). The extracellular domain of ligands can be cleaved to generate soluble cytokines. Receptors are usually type I transmembrane glycoproteins characterized by the presence of extracellular cysteine-rich domains (1). As their ligands, functional receptors are also usually trimerics. Many TNF members, including TNF-like weak inducer of apoptosis (TWEAK), activate the nuclear factor kappaB (NF-κB) family of transcription factors (2). NF-κB DNA-binding complex are homo- or heterodimers of five Rel proteins: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. NF-κB dimers translocate to the nucleus and bind to DNA by two different pathways, canonical or non-canonical NF-κB activation. Binding of NF-κB to DNA activates the transcription of several target genes that are implicated in the inflammatory response as well as in cell proliferation, migration, and differentiation. All these processes are closely related to pathological vascular remodeling.

TWEAK AND Fn14: STRUCTURE, EXPRESSION, AND FUNCTION

The human TWEAK gene is located at chromosomal position 17p13.1 and encodes a 249-amino acid (aa) type II transmembrane protein (3). TWEAK is expressed as a full-length, membrane-bound protein (mTWEAK) and then is proteolytically processed by furin, leading to the release of a 156-aa, 18 kDa soluble form (sTWEAK) (3). The extracellular domain contains the receptor-binding site and the intracellular domain contains a putative serine phosphorylation site. In 2001, Fn14 was identified as the functional TWEAK receptor using a cDNA expression library screening approach (4). The human Fn14 gene is located at the chromosomal position 16p13.3 (5), and encodes a 129-aa type I transmembrane protein of 14 kDa that is processed into a mature form of 102-aa (4). The extracellular domain contains the ligand-binding site and the intracellular domain contains a TNFR-associated factor (TRAF)-binding site (6) implicated in signal transduction induced by TWEAK (7). A second receptor for TWEAK, CD163, has recently been identified (8, 9). CD163 is a hemoglobin scavenger receptor that is exclusively expressed by monocytes/macrophages (10). It has been proposed that CD163 acts as a scavenger receptor for TWEAK, thus preventing TWEAK from exerting its biological actions by sequestering it from the environment. However, it has been reported that recombinant CD163 failed to decrease cell death induced by TWEAK in macrophages (11). The relevance of TWEAK/CD163 interaction needs to be confirmed and more studies are needed in order to determine whether this interaction

takes place either *in vitro* or *in vivo*. In addition, the existence of a third alternative receptor for TWEAK has been proposed, since murine RAW264.7 cells differentiation induced by TWEAK occurs in an Fn14-independent manner (12). However, no reports describing this alternative receptor have been published and we have observed that this monocytic/macrophage cell line expresses functional Fn14 (Blanco-Colio, unpublished observation).

TNF-like weak inducer of apoptosis is expressed in several cell types and tissues including the intestine, pancreas, lung, brain, ovary, skeletal muscle, and vasculature, and to a lesser degree in kidney and liver (3). Although TWEAK can be upregulated after injury (13), changes in TWEAK gene expression are usually moderated. By contrast, Fn14 expression in healthy tissues, including the vasculature and heart, is usually low or undetectable, although it is rapidly and highly upregulated under pathological conditions as demonstrated in experimental models of chronic liver injury (14), myocardial infarction (15), colitis (16), denervation-induced skeletal muscle atrophy (17), restenosis after balloon injury (4), atherosclerosis (18), autoimmune encephalomyelitis (19), acute kidney injury (20), and cardiac dysfunction (21). Once Fn14 is upregulated, TWEAK binds and causes Fn14 trimerization and signal transduction (7). Although soluble TWEAK is responsible for the responses associated with Fn14, it has been recently reported that full-length, membrane-anchored TWEAK can, in a juxtacrine manner, bind to Fn14 on neighboring cells and activate the NF-κB signaling pathway, thus initiating the cellular response (22).

Fn14 is upregulated by several growth factors, cytokines, and interleukins in cells present in the injured vascular wall such as endothelial cells, vascular smooth muscle cells (SMCs), and monocyte/macrophages, but not in T and B lymphocytes (4, 18, 23, 24). However, little is known of the regulatory mechanisms of Fn14 expression, and only the RhoA/ROCK pathway has been related to Fn14 upregulation in cardiomyocytes (25). TWEAK protein can be upregulated by PMA and IFN-γ in cultured peripheral mononuclear cells and natural killer cells (24, 26). Fn14 trimerization induces the recruitment of TRAF2 and TRAF5 through its TRAF-binding motif (PIEET). This motif is responsible for activating different signaling pathways such as NF-κB and mitogen-activated protein kinases (MAPK) (**Figure 1**) (7, 27). Activation of NF-κB by TWEAK participates in the upregulation of several cytokines implicated in the recruitment of inflammatory cells within the injured vessel wall. Thus, TWEAK increases MCP-1 and RANTES in SMCs (28). TWEAK also activates NF-κB in cultured Thp-1 monocytic cell line (29). In addition, TWEAK induces the expression of CCL19 and CCL21 in murine tubular cells (30), and both cytokines are also expressed in atherosclerotic plaques of ApoE-deficient mice, a model of hyperlipidemic-induced atherosclerosis (31). TWEAK also activates MAPK, although activation of ERK, c-Jun N-terminal kinase (JNK), or p38 pathways is context-dependent. MAPK activation has been reported in several cell lines, including Thp-1 monocytic cell line, endothelial cells, cardiomyocytes, fibroblast, and others (23, 29, 32–34). There are also different reports indicating that TWEAK activates PI3K/AKT

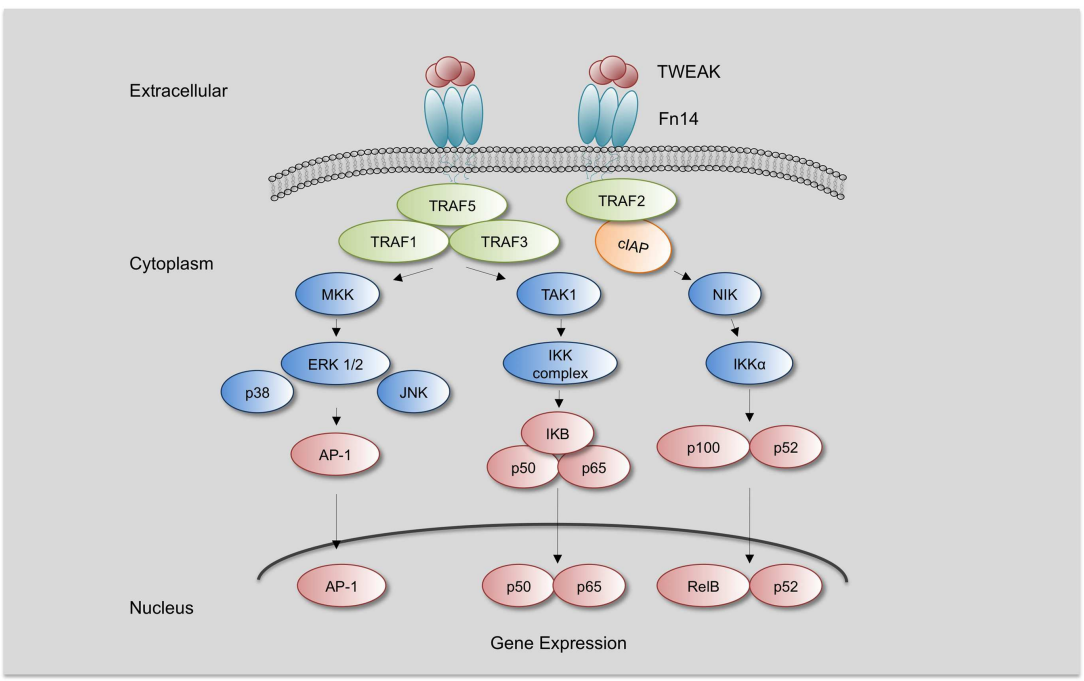

FIGURE 1 | TWEAK/Fn14 Signaling. TWEAK/Fn14 binding induces the recruitment of cIAP and TRAF-1, 2, 3, and 5 and leads to activation of different kinases such as mitogen-activated protein kinase kinases (MKK), transforming growth factor β-activated kinase 1 (TAK1), and NF-κB inducing kinase (NIK). TAK1 activates IKKβ and NIK activates IKKα, leading to the activation of canonical or non-canonical NF-κB pathway, respectively. MMK are responsible for the activation of c-Jun N-terminal kinase 1 (JNK1) and p38 MAPK, which activate transcription factor activator protein-1 (AP-1). Increased activation of NF-κB and AP-1 leads to the expression of specific target genes responsible for TWEAK-mediated responses.

in different cell types. Thus, TWEAK increases HMGB1 secretion by cultured monocytes through PI3K activation (29). In addition, TWEAK also activates transforming growth factor-β activated kinase 1 (TAK1), implicated in NF-κB activation (35, 36) and JNK, related with AP-1 activation (37). Overall, TWEAK activates several signaling pathways that participate in the inflammatory response of the injured tissues.

Several functions with potential pathological significance have been related to TWEAK/Fn14 interaction and are dependent on the cell type, microenvironment, and cell activation. However, the basis for these differential responses is poorly understood. TWEAK can regulate cell proliferation, migration, differentiation, and death as well as tissue inflammation, angiogenesis, and regeneration (**Figure 2**) (3, 5, 38–40). The precise role of TWEAK in different pathological situations needs to be characterized, since TWEAK has beneficial or deleterious effects depending on the stage of the disease (13, 41).

TWEAK AND ATHEROSCLEROSIS

Atherosclerosis is a multifactorial disease characterized by chronic inflammation and excessive cell proliferation (**Figure 3**). Vascular lesions begin as a fatty streak in the subendothelial space of large arteries. Endothelial cells acquire an activated phenotype and express adhesion molecules such as intercellular adhesion molecules (ICAMs), selectins, and vascular adhesion molecules (VCAMs) that act as receptors for proteins expressed by leukocytes (monocytes and lymphocytes and neutrophils). Recruitment of monocytes to the subendothelial space causes their differentiation to macrophages that uptake oxidized low density lipoproteins (ox-LDL). In addition, chemokines and cytokines are secreted by inflammatory cells and induce proliferation and migration of SMCs from the media forming the neointima (42). The transition of relatively early lesions to more advanced lesions is characterized by the proliferation of SMCs and continuous uptake of ox-LDL by macrophages, forming foam cells. In addition, SMCs synthesize extracellular matrix proteins that lead to the development of the fibrous cap. This cap confers resistance to rupture by the accumulation of collagen synthesized by SMCs. The continuous ingestion of ox-LDL by foam cells induces death of these cells, releasing of insoluble lipids and contributing to the formation of the necrotic core characteristic of advanced lesions. Expression of different proteases by macrophages and SMCs leads to degradation of the fibrous cap, promoting plaque instability and subsequent plaque rupture. Rupture of an atherosclerotic plaque may result in the occlusion of an artery by the formation of a thrombus over an atherosclerotic lesion, causing myocardial infarction, stroke, or peripheral vascular disease (42).

The TWEAK/Fn14 axis plays an important role in several steps of atherosclerotic plaque development including initiation, progression, destabilization/rupture, and subsequent thrombosis. As commented, TWEAK is expressed in both the normal and pathological arterial wall (18), but Fn14 is almost absent in healthy arteries and its expression is highly upregulated in the carotid artery (18), femoral atherosclerotic plaques (43), and in abdominal aortic aneurysms (44). Different stimuli induce Fn14 expression in resident and inflammatory cells present in the vascular wall. Thus, pro-inflammatory cytokines (IL-1β and INF-γ), growth factors (PDGF-BB, EGF, FGF-2), Angiotensin II, or α-thrombin increase Fn14 expression in human and rat aortic SMCs (4, 18). In addition, VEGF-A and FGF-2 increase Fn14 expression in human umbilical

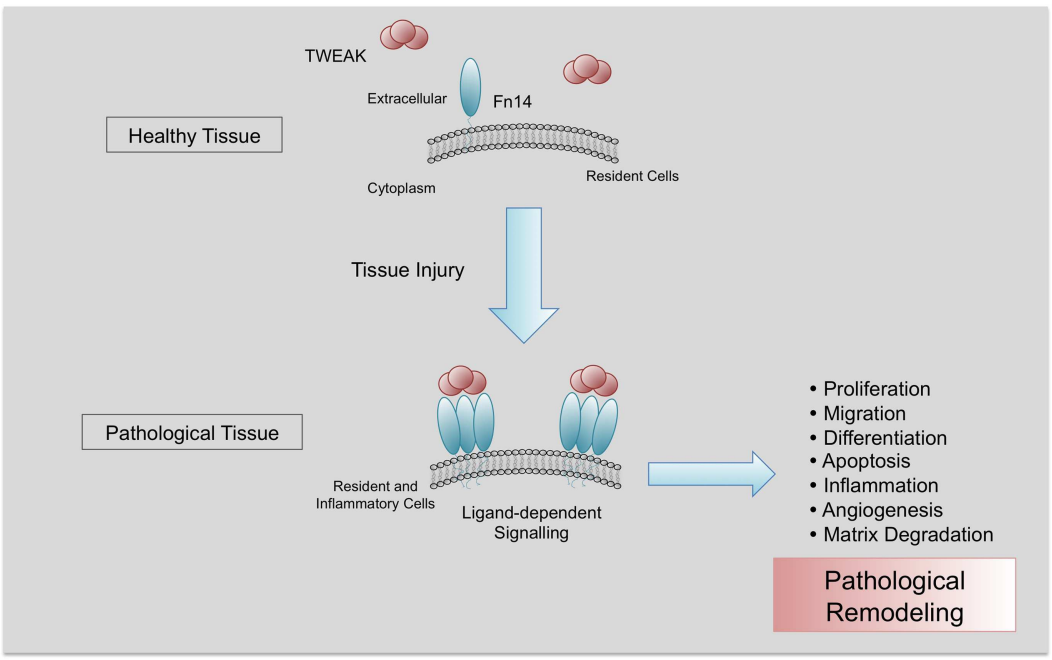

FIGURE 2 | Pathological actions of TWEAK/Fn14 interaction. Fn14 is almost absent in healthy tissues. After injury, Fn14 is upregulated, facilitating the interaction with its ligand TWEAK and its trimerization. In a context of chronic injury, TWEAK/Fn14 interaction participates in pathological tissue remodeling, promoting proliferation, migration, differentiation, apoptosis, inflammation, angiogenesis, and matrix degradation.

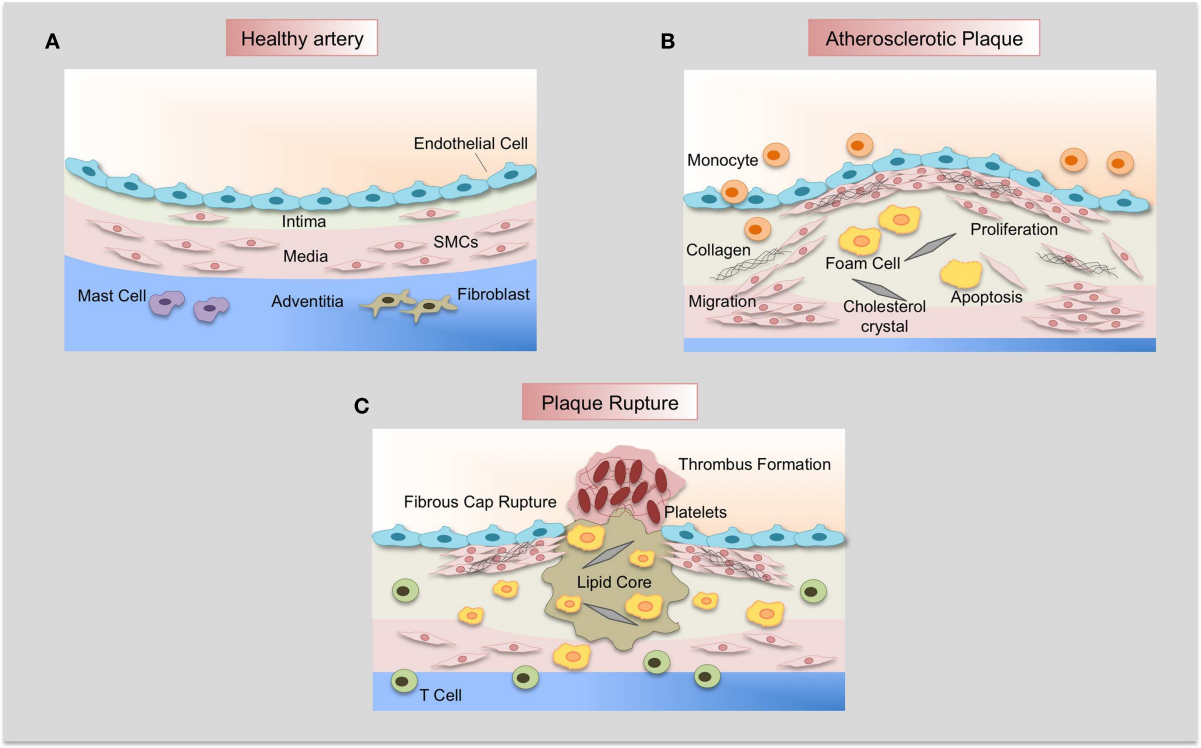

FIGURE 3 | Atherosclerotic plaque development. **(A)** Normal artery is formed by a monolayer of endothelial cells, the tunica media of resident smooth muscle cells embedded in an extracellular matrix, and the adventitia that contains mast cells, fibroblasts, and microvessels. **(B)** Adhesion of leukocytes to dysfunctional endothelium leads to their migration into the vascular wall, forming the neointima. Monocytes differentiate to macrophages and uptake lipids yielding foam cells. SMCs migrate from the media, proliferate, and synthesize extracellular matrix proteins such as collagen and elastin. SMCs and macrophages can die and lipid derived from dead cells accumulates in the central region of the plaque, the necrotic core that is covered by a fibrous cap. **(C)** Finally, when the fibrous cap is broken, blood coagulant components trigger thrombus formation, which occludes the lumen and interrupts the blood flow.

endothelial cells (23) and human $CD14^+$ monocytes express Fn14 in response to INF-γ or PMA stimulation (24).

In the first steps of atherosclerosis development, adhesion molecules and chemokines are responsible for the recruitment of the inflammatory cells to the injured tissue. *In vitro* studies have demonstrated that TWEAK/Fn14 interaction induces expression of adhesion molecules such as ICAM-1 and E-selectin in human umbilical endothelial cells (45). Furthermore, TWEAK increases interleukin-8 and MCP-1 secretion by endothelial cells (45). Infiltrating cells secrete many cytokines that contribute to SMCs migration and proliferation, favoring plaque progression. In this sense, TWEAK/Fn14 interaction directly induces proliferation and migration of human and rat aortic SMCs (39, 45, 46) and human endothelial cells (4, 23, 39, 45). These suggest that the TWEAK/Fn14 axis could participate in neointimal thickening of the pathological arterial wall. In fact, an *in vivo* study has reported that Fn14 is upregulated in SMCs after balloon injury in mice (4).

The presence of a chronic inflammatory response is an important phenomenon implicated in the development and progression of atherosclerotic plaque. A key transcription factor implicated in vascular inflammation is NF-κB. Activation of signal transduction mediated by NF-κB has been demonstrated at different stages of atherosclerotic lesion development, from plaque formation to plaque rupture (47). NF-κB is activated in SMCs, macrophages, and endothelial cells in human atherosclerotic plaques (48–50). Several molecules can activate this transcription factor in the context of atherogenesis. Inflammatory stimuli such as members of the TNF-α superfamily, IL-1, and ox-LDL induce NF-κB activation, and in consequence amplifying and maintaining a vascular inflammatory response that facilitates atherosclerosis progression. Activation of this transcription factor in endothelial cells enhances the expression of adhesion molecules, chemokines, and metalloproteinases (MMP). These molecules coordinate the invasion of inflammatory cells into the vascular wall, and enhance migration and proliferation of SMCs as well as the remodeling of the extracellular matrix. Inflammatory cells and SMCs also increase cytokine and MMP expression through NF-κB activation, perpetuating the inflammatory response. In particular, TWEAK activates NF-κB in several cell types and increases the expression of pro-inflammatory proteins such as IL-6, IL-8, MCP-1, and RANTES (7, 18, 27, 28, 46, 51, 52); these pro-inflammatory proteins are implicated in atherogenesis. In addition, recombinant TWEAK injection increases atherosclerotic lesion size and inflammatory cell content as well as NF-κB activation in the aortic root of hyperlipidemic ApoE-knockout mice (28). Moreover, anti-TWEAK monoclonal antibody (mAb) therapy diminishes NF-κB activation as

well as inflammatory response in ApoE-null mice, indicating that endogenous TWEAK participates in atherogenesis (28). In addition, genetic deletion of TWEAK or treatment with anti-TWEAK mAb diminished NF-κB activation, chemokines secretion and inflammatory response in ApoE-deficient mice (53). The activation of NF-κB by TWEAK observed in this experimental model was related to the canonical pathway, since p50/p65 dimers were detected in the nuclei of cells within atherosclerotic plaques. Until now, non-canonical NF-κB activation induced by TWEAK has not been reported in atherosclerotic plaques.

TWEAK also increases the secretion of HMGB1 through NF-κB activation in human M1 macrophages (29). HMGB1 is a DNA-binding cytokine that activates endothelial cells and monocytes/macrophages to express pro-inflammatory cytokines, chemokines, and adhesion molecules functioning as a critical mediator of inflammation (54). HMGB1 colocalizes with Fn14 in the shoulder region of human atherosclerotic plaques, a macrophage-rich area (29). In addition, systemic injection of recombinant TWEAK augmented HMGB1 expression in atherosclerotic plaques of hyperlipidemic ApoE-null mice (29). The importance of the finding that NF-κB can regulate HMGB1 release induced by TWEAK is because secreted HMGB1 may in turn induce NF-κB activation, forming a loop between NF-κB and HMGB1 that perpetuates vascular pro-inflammatory effects related to TWEAK. These data support the notion that TWEAK/Fn14 interaction has deleterious consequences in the injured vascular wall.

Interestingly, it has been reported that TWEAK can modulate macrophage size within atherosclerotic plaques (55). This finding is related to the capacity of TWEAK to modulate lipid uptake by macrophages. In fact, ApoE-deficient mice treated with Fn14–Fc protein present smaller macrophages in their atherosclerotic plaques, and treatment with anti-Fn14 or anti-TWEAK antibodies or Fn14–Fc protein diminished macrophage uptake of modified lipids *in vitro* (55).

The stability of the advanced atherosclerotic plaque depends on the integrity of the fibrous cap that encloses its lipid core. Established atherosclerotic lesions usually have a dense fibrous cap. However, areas with sustained inflammation, macrophage accumulation, and apoptosis are prone to rupture due to a weakening of the fibrous cap. Deterioration of the fibrous cup is dependent on the activity of MMP, which are collagen-degrading endopeptidases that are secreted by SMCs and macrophages (56). As commented, TWEAK and Fn14 are expressed in macrophages/foam cells rich regions of atheroma plaques and colocalized with MMP (57). Moreover, an activating anti-Fn14 antibody increases the expression of MMP-9 and MMP-1/13 in cultured monocytes (57). In addition, anti-TWEAK mAb treatment diminishes MMP activity in atherosclerotic plaques present in the aortic root of ApoE-deficient mice (53). In addition, features of greater plaque stability included augmented collagen/lipid ratio, reduced macrophages content, and less presence of lateral xanthomas, buried caps, medial erosion, intraplaque hemorrhage, and calcium content have been observed in TWEAK/ApoE-double knockout mice or in anti-TWEAK mAb treated ApoE-deficient mice (53). These data indicate a potential role of TWEAK in extracellular matrix degradation, which favors plaque instability.

Plaque rupture or erosion, and subsequent thrombosis, represent the main complications of atherosclerosis and could lead to an acute cardiovascular event. Different molecules, such as plasminogen activator inhibitor 1 (PAI-1) and tissue factor (TF), are responsible for hemostasis and thrombosis (58). TF is the principal initiator of the clotting cascade, while PAI-1 plays a critical role in inhibiting fibrinolysis, and thereby the activity of both molecules promotes thrombotic states and plays a crucial role in vascular diseases (59). Fn14 colocalizes with PAI-1 and TF in human carotid atherosclerotic plaques (60). In addition, TWEAK increases TF and PAI-1 mRNA and protein expression as well as activity in cultured human aortic SMCs (60). Furthermore, systemic injection of recombinant TWEAK augmented TF and PAI-1 expression in atherosclerotic plaques of ApoE-deficient mice and, conversely, anti-TWEAK treatment diminished the expression of both prothrombotic proteins (60). These data indicate that TWEAK favors thrombus formation after plaque rupture.

Overall, data obtained from *in vitro* and *in vivo* studies indicate that TWEAK participates in different stages of atherosclerotic plaque development from early stages to progression and subsequent plaque rupture that lead to an acute cardiovascular event, such as myocardial infarction or stroke. Anti-TWEAK treatment has the capacity to diminish pro-inflammatory response associated with atherosclerotic plaque progression and to alter plaque morphology toward a stable phenotype.

TWEAK AND STROKE OUTCOME

Stroke is the third most common cause of death in the world (61). A stroke, or cerebrovascular accident, causes rapid loss of brain function due to a lack of oxygen; sudden death of brain cells also takes place. The two main types of stroke include ischemic (when a blood clot or thrombus forms) and hemorrhagic stroke. The outcome after a stroke depends on where it occurs and how much of the brain is affected. Smaller strokes may result in minor problems, such as weakness in an arm or leg. Larger strokes may lead to paralysis or death. Ischemic stroke triggers a cascade of pathophysiological events such as energy depletion, excitotoxicity, peri-infarct depolarization, inflammation, and apoptotic cell death (62). The onset of the ischemic insult is followed by an increase in the expression of pro-inflammatory molecules in the ischemic tissue, which has been associated with neuronal death and poor outcome.

Recent reports have shown the role of TWEAK/Fn14 axis after an ischemic stroke. In fact, it has been reported that ischemic stroke in humans (63) and experimental middle cerebral artery occlusion (MCAO) in mice (64, 65) increase the expression of both TWEAK and Fn14 in the ischemic tissue. In the central nervous system (CNS), TWEAK and Fn14 are expressed mainly in endothelial cells, perivascular astrocytes, microglia, and neurons. There are two principal mechanisms by which TWEAK participates in stroke pathogenesis: neuronal apoptosis and breakdown of the blood–brain barrier (BBB) (64, 66). It has been demonstrated that in response to hypoxia/ischemia, TWEAK induces cell death in neurons via NF-κB activation and PARP-1 and caspase-3 cleavage (64, 67). However, oxygen–glucose deprivation conditions did not affect cell survival in neurons from Fn14- or TWEAK-deficient

mice, indicating that cell death is mediated by the TWEAK/Fn14 interaction (67).

On the other hand, during cerebral ischemia, disruption of the architecture of the neurovascular unit (NVU) results in an increase in the permeability of the BBB with the development of cerebral edema, which is a major cause of mortality among patients with acute stroke. NVU is a dynamic structure consisting of endothelial cells, the basal lamina, astrocytic end-feet processes, pericytes, and neurons (68). The permeability of BBB is increased by pro-inflammatory cytokines that act on the NVU under ischemic conditions (69). It has been demonstrated that TWEAK has a detrimental effect on the structure of the NVU and the permeability of the BBB in the early stages of cerebral ischemia. Recombinant TWEAK injection directly into the brain induces activation of NF-κB and MMP-9 expression, resulting in the disruption of the structure of NVU and an increase in the permeability of BBB (70). Furthermore, inhibition of TWEAK actions by Fn14–Fc decoy receptor or Fn14 deficiency diminished cerebral ischemia-induced increase in the permeability of the NVU. This protection was associated with a faster recovery of locomotor activity (66).

Finally, intraperitoneal administration of anti-TWEAK monoclonal antibodies (64) or intracerebroventricular administration of Fn14–Fc decoy receptor (65) diminished the infarct size by around 30–40% after 48 h of MCAO. In addition, Fn14-deficient mice exhibited a 60% reduction in the volume of the ischemic lesion following MCAO compared to wild-type animals (66). Overall, TWEAK may play an important role during ischemia-induced brain injury and its inhibition in the brain could be a novel neuroprotective strategy for the treatment of ischemic stroke.

DIAGNOSTIC AND PROGNOSTIC VALUE OF SOLUBLE TWEAK FOR CARDIOVASCULAR DISEASES

As commented above, TWEAK is expressed as a full-length, membrane-bound protein and then is proteolytically processed by furin, leading to the release of a 156-amino acid, 18 kDa soluble form (sTWEAK) (3). Among the potential biomarkers that could be differentially secreted by the pathological arterial wall, sTWEAK was identified as a protein that is highly released by normal arteries in comparison with carotid atherosclerotic plaques (71). Plasma levels of sTWEAK were also found to be diminished in patients with atherosclerosis compared to control subjects. The association of sTWEAK with the presence of CVD or CVD-related diseases has been extensively validated in other cohorts of individuals (**Figure 4**). Thus, sTWEAK concentrations were significantly reduced in patients with chronic kidney disease (CKD) and/or type II diabetes (72). In addition, sTWEAK plasma concentrations were diminished in patients undergoing hemodialysis compared to healthy subjects (73). In addition, a gradual decrease in sTWEAK along with a reduction in estimated glomerular filtration rate in CKD patients has been observed (74–76). Reduced levels of sTWEAK have also been associated with the presence of coronary artery disease (CAD) (25, 77), systolic heart failure (78), peripheral artery disease (PAD) (43), and aortic abdominal aneurysm (AAA) (44). Finally, elevated circulating sTWEAK levels have been described in patients after myocardial infarction (25) and stroke (63). However, although the study of circulating proteins in subjects suffering an acute event could unveil novel proteins implicated in atherothrombosis, some of these proteins could be

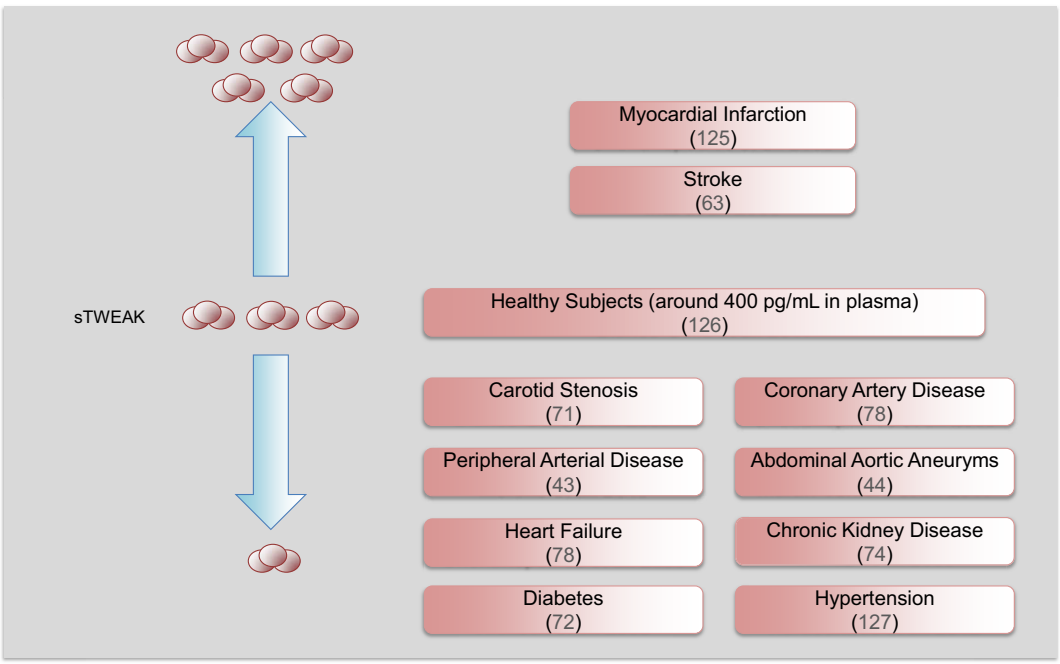

FIGURE 4 | Soluble TWEAK as a biomarker of cardiovascular diseases. Circulating sTWEAK concentrations are diminished in subjects with chronic vascular damage including carotid stenosis, coronary artery disease, peripheral artery disease, abdominal aortic aneurysm, heart failure, and chronic kidney disease. Soluble TWEAK levels are also diminished in subjects with cardiovascular risk factors such as diabetes or hypertension. However, circulating sTWEAK levels are increased in patients suffering an acute cardiovascular event (myocardial infarction or stroke).

released by the tissue necrosis that takes place during an acute event.

Two different surrogate markers of atherosclerosis such as flow-mediated dilation (FMD) and intima/media thickness (IMT) have been negatively associated with sTWEAK concentrations (71, 74, 75). As mentioned previously, endothelial dysfunction participates in the formation of vascular lesion (79). FMD described the vasodilation of a conduit artery following an augmentation in shear stress induced by an ischemia period. FMD provides us information about the integrity of the endothelium (80). It has been reported that sTWEAK plasma concentrations are negatively associated with FMD in patients with CKD (74), persisting after adjustment for factors related to FMD in CKD subjects such as blood pressure, C-reactive protein, and estimated glomerular filtration rate. This result was later confirmed in hypertensive CKD patients (75). In addition, IMT can be measured non-invasively by means of B-mode ultrasound, and increases in IMT have been associated with an augmentation for future cardiovascular outcomes (81). Thus, IMT has been negatively associated with sTWEAK concentrations in asymptomatic subjects (71) and in patients with CKD (76, 82, 83), even after adjustment for traditional risk factors and inflammatory biomarkers. Moreover, sTWEAK is associated with atherosclerotic burden in CKD patients (83). However, IMT was positively correlated with sTWEAK in renal transplant patients (84, 85). Overall, the association of sTWEAK levels with different surrogate markers of atherosclerosis indicates that this protein could be a novel and independent biomarker of CVD.

Finally, different reports have indicated the potential use of sTWEAK as a prognostic biomarker of CVD or CVD-related diseases as well as its impact on survival. Thus, individuals in the upper two tertiles of sTWEAK concentrations presented a lower incidence of PAD (43). In addition, a decreased sTWEAK concentration was significantly and independently associated with long-term cardiovascular mortality in patients with lower-extremity PAD (86). sTWEAK levels were also negatively related with AAA size and AAA expansion rate after a 5-year follow-up, and sTWEAK concentrations were predictive for subjects expanding more than 2 mm/year in AAA size (44). As for CKD, decreasing sTWEAK concentration was associated with increased risk of cardiovascular events independently of basic confounders (age, gender, estimated glomerular filtration rate, C-reactive protein, diabetes, and cardiovascular comorbidity) (76). However, high levels of sTWEAK were associated with atherosclerosis in patients with systemic lupus erythematosus (SLE), but not in control subjects (87). In addition, although sTWEAK plasma levels were diminished in HD patients compared with controls, subjects belonging to the upper tertile of sTWEAK presented a higher risk of all-cause and cardiovascular mortality (73). This discrepancy could be due to the existence of the known reverse epidemiology observed in HD patients. Finally, sTWEAK also provides prognostic information on subjects with heart failure. Subjects with chronic stable heart failure with reduced sTWEAK plasma concentrations (78) present a higher mortality rate than those with elevated sTWEAK levels. In addition, the increase of sTWEAK concentrations diminishes the risk of mortality in subjects with non-ischemic heart failure (88).

The mechanism/s by which sTWEAK is diminished in subjects with vascular damage should be related to the expression of their receptors. As commented, Fn14 expression is undetectable in the vasculature in normal conditions (18). However, under pathological conditions including systemic inflammatory states, Fn14 is highly upregulated in the vasculature, favoring sTWEAK binding and retention in the pathological tissues (18, 57). In addition, the expression of CD163 by M2 macrophages in pathological tissues could be responsible for the decrease in sTWEAK, since CD163 can bind and internalize sTWEAK *in vitro* (8). On the basis of this preceding literature, we speculate that the reduction in sTWEAK concentrations in cardiovascular-related diseases could potentially reflect either Fn14 binding or CD163 degradation (**Figure 5**). However, this hypothesis needs to be tested in future studies. Overall, all these data reveal that sTWEAK could be a novel biomarker of CVD. More large-scale studies to consolidate its usefulness are required.

OTHER TNF SUPERFAMILY MEMBERS IMPLICATED IN ATHEROTHROMBOSIS THAT ACTIVATE BOTH CANONICAL AND NON-CANONICAL NF-κB PATHWAY

In recent years, the number of TNF receptors that are known to potentially activate the non-canonical NF-κB pathway has increased. These include, in addition to Fn14, CD40, B-cell activating factor receptor (BAFFR), lymphotoxin β receptor (LTβR), receptor activator of NF-κB (RANK), and CD27 (89–93). Some of these receptors have been implicated in different functions related to the pathogenesis of atherosclerosis (**Table 1**).

The role of *CD40* and its ligand CD40L in atherosclerosis has been extensively studied. Both proteins are expressed in various cell types implicated in atherogenesis such as platelets, endothelial cells, monocytes/macrophages, and SMCs (110). CD40L induces a broad inflammatory response in these cell types, including increased expression of adhesion molecules, pro-inflammatory cytokines, matrix degrading enzymes, and pro-coagulants (111, 112). Different groups have analyzed the role of CD40 and CD40L in athero-prone mice by using diverse strategies such as gene modification, blocking antibody treatment, or bone-marrow transplantation (BMT). Thus, CD40L and ApoE double deficient mice develop markedly reduced atherosclerosis (96, 98, 100). In addition, treatment with neutralizing anti-CD40L antibodies diminished atherosclerotic lesion size (98, 99) in LDLR-deficient mice but failed to modify plaque size in ApoE-null mice (97). The decrease of atherosclerotic plaques was associated with features of higher plaque stability such as reduced macrophage and lipid content as well as increased collagen deposition. The effect of CD40L on atherosclerotic plaque progression seems to be related with resident cells (endothelial and SMCs), since BMT failed to modify atherosclerosis plaque size in $LDLR^{-/-}$ mice (100, 101). However, a recent study describes that transfer of $CD40L^{-/-}$ platelets into ApoE-null mice diminished atherosclerotic burden, an effect that results from the capacity of platelets to synthesize higher amounts of CD40L (102). The role of CD40 in atherosclerosis remains controversial. It has been demonstrated that CD40 and ApoE double deficient mice develop reduced levels of atherosclerosis when given a normal chow diet compared with control animals (94). However, a similar atherosclerotic burden was observed in $CD40^{-/-}LDLR^{-/-}$ mice on a high-cholesterol diet (95). These data could suggest

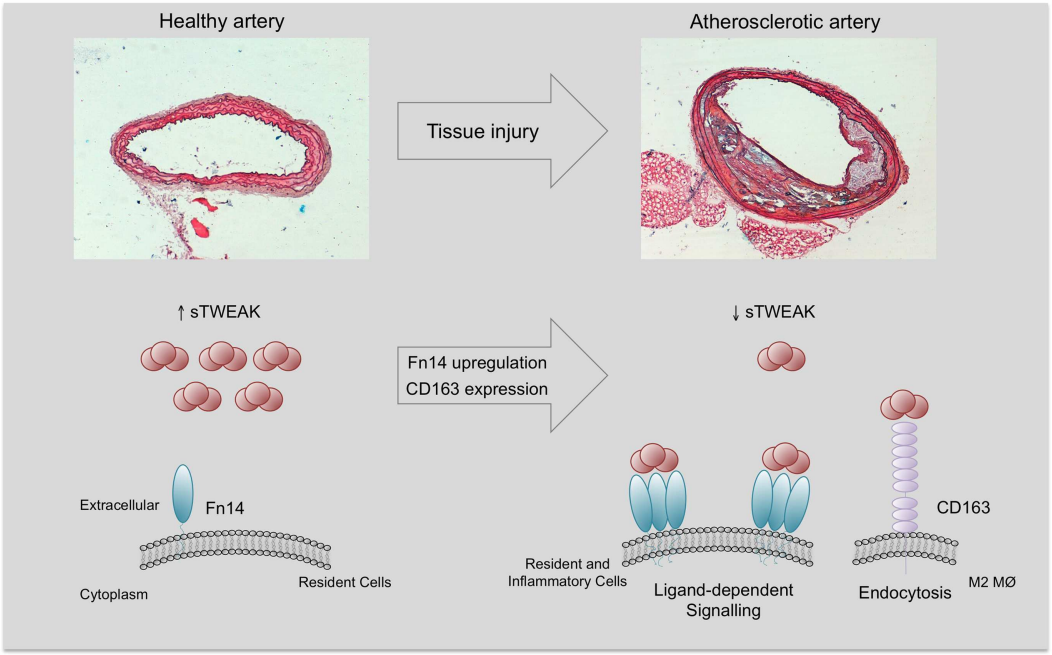

FIGURE 5 | Soluble TWEAK in health and disease. Fn14 expression is almost absent and minimal Fn14 activation is expected in the healthy arterial wall. However, under pathological conditions, Fn14 expression is highly upregulated in resident (smooth muscle cells) and inflammatory cells (e.g., M1 macrophages). This increase facilitates the interaction with sTWEAK and would trigger sTWEAK-dependent Fn14 signaling. On the other hand, pathological tissues are infiltrated by anti-inflammatory macrophages (M2) that express CD163, a scavenger receptor of sTWEAK. The tissue consumption of sTWEAK by Fn14 interaction and CD163 degradation could be responsible for the decrease in circulating sTWEAK levels observed in subjects with atherosclerostic complications.

Table 1 | Effect of TNF superfamily members that activate non-canonical NF-κB in experimental models of murine atherosclerosis.

TNF member	Experimental approach/animal model	Plaque size	Reference
TWEAK	Systemic rTWEAK injection in ApoE $^{-/-}$ mice	Increased	Muñoz-García et al. (28)
	Blocking TWEAK in ApoE$^{-/-}$ mice	Reduced	Muñoz-García et al. (28)
Fn14	Blocking Fn14-Fc in ApoE $^{-/-}$ mice	Reduced	Schapira et al. (55)
CD40	CD40$^{-/-}$/ApoE$^{-/-}$ double deficient mice	Reduced	Lutgens et al. (94)
	CD40$^{-/-}$/LDLR$^{-/-}$ double deficient mice	No change	Zirlik et al. (95)
	CD40-TRAF6 blocking interaction in ApoE $^{-/-}$ mice	Reduced	Lutgens et al. (94)
CD40L	CD40L$^{-/-}$/ApoE$^{-/-}$ double deficient mice	Reduced	Lutgens et al. (96)
	Blocking CD40L in ApoE$^{-/-}$ mice	No effect	Lutgens et al. (97)
	Blocking CD40L in LDLR$^{-/-}$ mice	Reduced	Mach et al. (98), Schonbeck et al. (99)
	BMT: CD40L$^{-/-}$ to LDLR$^{-/-}$ mice	No change	Bavendiek et al. (100), Smook et al. (101)
	Transfer CD40L$^{-/-}$ platelets into ApoE$^{-/-}$ mice	Reduced	Lievens et al. (102)
BAFFR	BAFFR$^{-/-}$/ApoE$^{-/-}$ double deficient mice	Reduced	Kyaw et al. (103)
	BMT: BAFFR$^{-/-}$ to LDLR$^{-/-}$ mice	Reduced	Sage et al. (104)
	Blocking BAFFR antibody in ApoE$^{-/-}$ mice	Reduced	Kyaw et al. (105)
LTβR	Blocking LTβR in ApoE$^{-/-}$ mice	No change	Gräbner et al. (106)
RANK	OPG$^{-/-}$/ApoE$^{-/-}$ double deficient mice	Increased	Bennett et al. (107)
	Blocking Fc-OPG in ApoE$^{-/-}$ mice	No change	Morony et al. (108)
CD70	CD70 tg/ApoE*3 Leiden mice	Reduced	van Olffen et al. (109)

BMT, bone-marrow transplantation; OPG, osteoprotegerin.

that CD40L mediates atherosclerosis development independently of CD40 (95). However, specific interruption of CD40/TRAF6 interaction in ApoE-deficient mice diminished atherosclerotic plaque size, indicating that CD40L/CD40 interaction participates in atherosclerotic plaque development (94).

It has been demonstrated that the depletion of B cells diminished atherosclerosis in mice (113). The proatherogenic effect of B cells is mainly driven by the B2 subset, which responds to T-cell-dependent antigens and is part of the adaptive immune response (114), while the atheroprotective effect is attributed to the B1 subset, which responds to T-cell-independent antigens (115). The survival and maturation of B2 lymphocytes depends on the interaction of BAFF with its receptor, *BAFFR* (116). Genetic disruption of BAFFR induces a significant reduction in mature B2 cells without affecting B1a cells (117) and BAFFR/ApoE double deficient mice present a reduced atherosclerotic plaque size and macrophage content in their aortic root; this effect is also related to a decrease in the number of B2 cells (103). In addition, BMT of BAFFR deficient cells to LDLR$^{-/-}$ mice also leads to a reduction in plaque size and inflammation (104). These data could suggest that BAFFR is an interesting therapeutic target to limit the development of atherosclerosis. Indeed, atherosclerosis development is diminished in ApoE$^{-/-}$ treated with a BAFFR blocking mAB (105).

Until now, the role of *LTβR* in atherosclerotic plaque development is unclear. As commented above, cells present in atherosclerotic lesions elicit persistent inflammation and trigger immune adaptive response toward arterial wall-derived autoantigens, such as ox-LDL or heat shock proteins. Coronary patients with atherosclerosis present infiltrates of leukocytes in the adventitia, and the presence of adventitial B-cell follicle-like aggregates in human aorta has been demonstrated (118). Moreover, adventitia of ApoE-deficient mice also contains T- and B-cell aggregates. These aggregates are the precursors of fully structured aorta tertiary lymphoid organs (ATLOs) that contain a high number of germinal centers, endothelial venules, regulatory T cells, and LN-like conduits that connect ATLOs to medial SMCs (106). The recruitment of T- and B-cell aggregates is dependent on CXCL13 and CCL21 secretion by SMCs through an LTβR-dependent signaling pathway. The formation of ATLOs in ApoE-deficient mice is restricted to abdominal aorta, and ATLOs are communicated with medial SMC by conducts that serve as channels for both transport of molecules (e.g., cytokines and chemokines) and soluble antigens (119). Although it is conceivable that ATLOs play a role in atherosclerotic plaque progression, ApoE-null mice treated with anti-LTβR have not modified their atherosclerotic plaques. Future gene deletion studies would help to understand the role of LTβR and ATLOs in atherogenesis.

RANKL, which is expressed in human atherosclerotic plaques (120), is capable of modulating different cell-type activities through its receptor, RANK. RANK/RANKL interaction activates several intracellular signal transduction pathways such as MAPKs and NF-κB (121). Several proatherogenic actions of RANKL have been described. For example, RANKL induces MCP-1 expression and secretion and matrix MMP activity in SMCs (122). In addition, RANKL induces TF expression in macrophages mainly through the cooperative action of NF-κB, AP-1, and Egr-1, supporting a role of RANKL in the thrombogenicity of atherosclerotic plaques (123). Furthermore, RANK, RANKL, and the decoy receptor for RANKL osteoprotegerin (OPG) have been related with vascular calcification, a crucial step for plaque destabilization and rupture. In the absence of OPG, mice display vascular calcification and present increased atherosclerotic plaque size (107). However, treatment of LDLR-deficient mice with Fc-OPG diminished vascular calcification but failed to reduce atherosclerotic plaque size (108).

Finally, *CD27* is mainly found in T cells, and its ligand CD70 is expressed in B cells, activated T cells, and dendritic cells. Interaction of both proteins is necessary for the generation and long-term maintenance of T-cell immune responses (124). Overexpression of CD70 in B cells leads to an expansion of Th1 T cells in mice. This indicates that these mice should have a proatherogenic phenotype. However, mice overexpressing CD70 are protective against atherosclerosis development, possibly due to a reduced viability of circulating monocytes (109). After these results, further research is clearly needed to clarify the relevance of CD27 in atherosclerosis.

CONCLUDING REMARKS

The evidence gathered to date supports a role of the TWEAK/Fn14 axis in the development and outcome of atherosclerosis and ischemic stroke. Data from experimental models make TWEAK and its functional receptor Fn14 a promising target for the treatment of patients with different CVD. Treatment with the TWEAK neutralizing antibody or Fn14–Fc decoy protein has demonstrated a beneficial effect on the development and progression of atherosclerotic plaques in mice. Furthermore, Fn14 deletion or anti-TWEAK administration diminished the volume of the ischemic lesion after stroke, a related complication of atherosclerotic plaque rupture. Although the use of monoclonal antibodies offers – unlike small-molecule drugs – high target specificity and allows less frequent, albeit parenteral, administration, there is a need for further drug development in this area, including Fn14-specific antagonists.

The role of the TWEAK/Fn14 axis on pathological vascular remodeling is not completely understood, and many questions need to be answered. Could TWEAK induce vascular remodeling in contexts other than atherosclerosis? As in stroke, could TWEAK participate in the outcome of myocardial infarction? Could anti-TWEAK therapy prevent atherosclerotic plaque development and progression in the presence of different cardiovascular risk factors such as diabetes or hypertension? Since TWEAK is also able to interact with CD163 (8), could overexpression of CD163 diminish the proatherogenic effect of TWEAK? Statins can diminish Fn14 expression in cultured SMCs (18), so could statins inhibit atherosclerotic plaque progression induced by TWEAK? In the near future, these and other potential questions could help us to understand the role of this axis in different cardiovascular pathologies.

Finally, the evidence accumulated indicates that sTWEAK could be a biomarker for the diagnosis and prognosis of CVD. The reduction of sTWEAK plasma levels has been demonstrated in subjects with different vascular affectations and its association with total and cardiovascular morbidity and mortality has been reported in different cohorts. However, after an acute cardiovascular event,

sTWEAK concentrations are increased. Current and future studies in large-scale populations will help us to determine the relevance of sTWEAK as a CVD biomarker and its potential implementation in clinical practice.

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TWEAK: a new player in obesity and diabetes

Joan Vendrell and Matilde R. Chacón*

Research Unit, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Hospital Universitari de Tarragona Joan XXIII, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Tarragona, Spain

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Martin S. Obin, Tufts University, USA
Andreas Tsapis, U976 Institut national de la santé et de la recherche médicale, France

***Correspondence:**
Matilde R. Chacón, Research Unit, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas CIBERDEM, Hospital Universitari de Tarragona Joan XXIII, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, C/Dr. Mallafré Guasch 4, Tarragona 43007, Spain
e-mail: mrodriguez.hj23.ics@gencat.cat

Obesity and type 2 diabetes (T2D) are associated with chronic low-grade inflammation. Mounting evidence suggests the involvement of an inflammatory switch in adipose tissue, both in mature adipocytes and immune-competent cells from the stromal vascular compartment, in the progression of obesity and insulin resistance. Several inflammatory cytokines secreted by obese adipose tissue, including TNFα and IL-6 have been described as hallmark molecules involved in this process, impairing insulin signaling in insulin-responsive organs. An increasing number of new molecules affecting the local and systemic inflammatory imbalance in obesity and T2D have been identified. In this complex condition, some molecules may exhibit opposing actions, depending on the cell type and on systemic or local influences. Tumor necrosis factor weak inducer of apoptosis (TWEAK), a cytokine of the tumor necrosis (TNF) superfamily, is gaining attention as an important player in chronic inflammatory diseases. TWEAK can exist as a full-length membrane-associated (mTWEAK) form and as a soluble (sTWEAK) form and, by acting through its cognate receptor Fn14, can control many cellular activities including proliferation, migration, differentiation, apoptosis, angiogenesis, and inflammation. Notably, sTWEAK has been proposed as a biomarker of cardiovascular diseases. Here, we will review the recent findings relating to TWEAK and its receptor within the context of obesity and the associated disorder T2D.

Keywords: TWEAK, obesity, type 2 diabetes, adipose tissue, TNFα, insulin resistance, inflammation

INTRODUCTION

A characteristic feature of obesity is a low-grade level of inflammation, likely originating in the expanding adipose tissue, which is illustrated by infiltration of immune cells, including macrophages, lymphocytes, and leukocytes (1). Pro-inflammatory cytokines released by activated immune cells and adipocytes can impair insulin signaling in insulin-responsive organs, promoting systemic insulin resistance, which increases the risk of developing type 2 diabetes (T2D) (2).

Tumor necrosis factor alpha (TNFα) was the first described cytokine to have a relevant role in obesity and an associated insulin-resistant state. Studies first conducted by Hotamisligil et al. (3), demonstrated an upregulation of TNFα in adipose tissue from obese patients. Since then, many members of the TNF superfamily have been shown to participate in obesity related diseases, including TNF-related apoptosis inducing ligand (TRAIL) (4), B cell activating factor (BAFF) (5), Lymphotoxin-α (LTα) (6, 7), Lymphotoxin β receptor (LTβR) (8), and Tumor necrosis factor ligand superfamily member 14 (TNFSF14) (9) among others. Recently, Tumor Necrosis Factor Weak Inducer of Apoptosis (TWEAK) has gained attention as a potentially important regulator of the inflammatory/anti-inflammatory equilibrium which takes place in the insulin-resistant milieu. TWEAK is a cytokine belonging to the TNF superfamily and triggers multiple, and often seemingly conflicting, cellular activities in a wide variety of cells, ranging from proliferation to cell death (10, 11). Like most TNF members, TWEAK protein exists as a membrane-bound (mTWEAK) form, and also as a soluble variant (sTWEAK), formed after proteolytic cleavage by a furin endoproteinase. Both forms are biologically active and can bind to Fibroblast growth factor-inducible 14 (Fn14), its only *bona fide* signal transducing receptor (12). Studies *in vitro* suggests that mTWEAK, can function as a juxtacrine signaling molecule and sTWEAK can elicit qualitatively different states of activity through Fn14 (12–14). Moreover, some authors have proposed sTWEAK as a potential biomarker in human cardiovascular diseases (CVDs) (15).

The cytoplasmic domain of Fn14 contains a TNF Receptor-Associated Factor (TRAF) binding site allowing recruitment of TRAF adapter proteins. This interaction is shared by most members of the TNF receptor family including TNFα, and plays a pivotal role in activating the nuclear factor κB (NF-κB) or mitogen-activated protein kinase (MAPK) pathway, which can be also activated by TWEAK (16). In particular, TRAF2 is implicated in the activation of TWEAK signaling in several human cell lines (17).

Interestingly, new data from human studies points to the TWEAK/Fn14 axis as a component of the network that contributes to the inflammatory imbalance occurring in insulin

Abbreviations: BMI, body mass index; CD163, cluster of differentiation 163; CVDs, cardiovascular disease; ERK, extracellular signal-regulated kinases; Fn14, fibroblast growth factor-inducible 14; HOMA-IR, homeostasis model assessment of insulin resistance; IL-6, interleukin 6; MAPKs, mitogen-activated protein kinases; mTWEAK, full-length TWEAK isoform; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38, mitogen-activated protein kinase; PP2A, protein phosphatase 2A; sTWEAK, soluble TWEAK isoform; SVF, stromal vascular fraction; T2D, type 2 diabetes; TNFα, tumor necrosis factor alpha; TRAF2, TNF receptor-associated factor 2; TWEAK, tumor necrosis factor weak inducer of apoptosis.

resistance-associated diseases (18–22). Here we review the role of TWEAK in adipocyte biology, and the prognostic and diagnostic value of its soluble form sTWEAK, within the context of insulin-resistant associated diseases, obesity, and diabetes.

TWEAK AND ADIPOCYTE BIOLOGY

Tumor necrosis factor weak inducer of apoptosis mRNA expression was initially described in multiple human tissues (23). However, the first data concerning the expression of this cytokine in adipose tissue revealed mRNA gene expression of both *TWEAK* and its receptor *Fn14* in human fat depots from subcutaneous and visceral origin (18, 19, 24). Furthermore, altered patterns of TWEAK and Fn14 protein expression were observed in subcutaneous adipose samples from severely obese subjects when compared to healthy subjects (21).

In addition to adipocytes, adipose tissue contains a heterogeneous population of cells including preadipocytes, mesenchymal stem cells (MSC), endothelial cells, and macrophages among others cell types (25). Together, this collection of cells is termed the stromal vascular fraction (SVF). From this compartment, TWEAK expression has been detected mainly on the surface of macrophages and lymphoid cells (21, 26), whereas Fn14 expression has been detected in mature adipocytes (18, 21, 27, 28), preadipocytes (28, 29), MSC (29), and endothelial cells (30).

It is well recognized that Fn14 is a highly inducible receptor (11). Inflammation can regulate the expression of Fn14 in adipocytes and TWEAK in macrophages (19). In agreement with this data, isolated adipocytes from severely obese subjects exposed to a striking inflammatory environment, displayed increased levels of Fn14 (18, 21). Independently of inflammation, hypoxic stress is suggested to be a contributing factor in the adipocyte metabolism in the setting of obesity (31). Insufficient oxygen supply can lead to endoplasmic reticulum (ER) stress and mitochondrial dysfunction, and hypoxia alters the balance between pro- and anti-inflammatory activities in adipose tissue (32, 33). Although an up-regulation of the TWEAK/Fn14 axis, in parallel with hypoxia and ER-specific genes has been observed in the adipose tissue of severely obese subjects, studies emulating hypoxia and ER stress *in vitro* do not report any change in *TWEAK/Fn14* gene expression in isolated adipocytes or macrophages (19).

The pro-inflammatory milieu facilitated by hypoxia in adipose tissue may represent an important stimulus for macrophage attraction (34, 35). These cells may have a different polarized state (termed M1 for the pro-inflammatory and M2 for the anti-inflammatory subtypes) (35). Although there is a controversy regarding the balance between M1 and M2 macrophages in human obesity, and in obese mice models (36), many authors agree that the M2 type constitute the most abundant infiltrating macrophages found in human obesity (37, 38). In SVF from obese subjects mTWEAK protein has been found over-expressed. Since macrophages are an important component of the SVF cells, this finding points to macrophages as one of the cells that expresses this cytokine in the adipose tissue of obese subjects. In this regard, *in vitro* studies describe a higher level of mTWEAK expression in M2 human monocyte derived-macrophages when compared to M1 cells (21).

Tumor necrosis factor weak inducer of apoptosis stimulus may induce a pro-inflammatory activity in several human cell types including endothelial, kidney, synoviocytes, and muscle cells, among others (11). In human adipocytes, TWEAK stimulus alone resulted in a modest pro-inflammatory state, with up-regulation of the cytokines IL-6 and MCP-1, whereas leptin and adiponectin expression were unaltered (19, 28). This inflammatory effect seemed to be mediated through both canonical and non-canonical NF-κB pathway activation. Whereas the canonical pathway was activated in subcutaneous adipocytes, the non-canonical pathway appeared activated only in visceral adipocytes (21, 22). Furthermore, a moderate induction of the MAPKs ERK1/2 and p38 after TWEAK stimulus, has also been observed in both subcutaneous and visceral adipocytes (19, 22).

It is known that TWEAK can interfere with the differentiation ability of several cell types, including myogenic, osteoblast, chondrocyte, and erythroblast lineages (11). In addition, TWEAK can also inhibit adipocyte differentiation at an early stage, as indicated by a rapid reduction of the key adipogenic transcription factors Peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/Enhancer Binding Protein α (C/EBPα) (28, 39).

In contrast to its effects on the differentiation capacity of adipocytes, TWEAK does not influence the metabolic function of these cells, such as glucose uptake and lipolysis. Furthermore, distinct from its clear apoptotic effect in neurons, monocytes, and tumor cell lines (40), a TWEAK stimulus does not induce apoptosis in adipocytes (22, 28).

Overall, the main impact of TWEAK on adipocytes appears to be an inhibition of adipocyte differentiation and the induction of a moderate inflammatory response.

CIRCULATING LEVELS OF sTWEAK IN OBESITY AND DIABETES

Diabesity is a new term coined to describe the common clinical associations between obesity and T2D, and highlights the close relationship between both states and their shared pathophysiological mechanisms. Chronic and subtle inflammation is usually documented in both states and can markedly influence cardiovascular (CV) outcomes (41).

Since the observation of lower levels of sTWEAK in patients with atherosclerosis and a negatively correlation between circulating sTWEAK levels and the intima/media thickness in asymptomatic subjects (15), decreased sTWEAK levels have been confirmed in many other CVD indications (40, 42–51). Consistent with these findings, obese individuals also present a similar trend of decreased sTWEAK in peripheral blood (21). Reinforcing the hypothesis that low sTWEAK levels associate with a poor CV profile, circulating levels of sTWEAK have also been found negatively associated with levels of glucose, glycated hemoglobin (HbA1), and also insulin resistance index (HOAM-IR) and central obesity; all of which are well known CV risk factors (20, 21, 46, 52). In contrast, the atherogenic lipid profile does not show a clear inverse association with circulating sTWEAK, and opposing data are reported in the literature. Whereas some studies describe a negative relationship with total cholesterol and triglycerides in severe obesity (21), other authors describe either a positive relationship with triglycerides (53) or no association at all (45). In

severe obesity, changes in the levels of Free Fatty Acids were found to negatively influence circulating sTWEAK, indicating that lipotoxicity could be a modulator of sTWEAK levels. The observation of a lower rate of release of sTWEAK in carotid atheroma plaques, compared to normal arteries, supports a link between the lipotoxic effects of abnormal lipid accumulation and TWEAK synthesis (15).

Some of the evidence mentioned may lead to speculation about the potential anti-inflammatory behavior of sTWEAK, at least in high-risk atherogenic conditions. Indeed, the inverse relationship with inflammatory markers or surrogate inflammatory scores lends support to this hypothesis (20, 53). The rise of circulating sTWEAK levels after massive weight loss in severely obese subjects reinforces the parallelism between expression of this cytokine and other well recognized anti-inflammatory molecules such as adiponectin (21). Along similar lines, a trend toward positive correlation between levels of sTWEAK and adiponectin in patients on chronic hemodialysis has been reported (52).

Recently, a new study highlighted the relevance of decreased serum sTWEAK as a predictive marker of T2D. Interestingly this study was conducted in a high CV risk population, in which the incidence of T2D was assessed during a follow up (54). In this large prospective nested case-control study lower sTWEAK serum levels were found in incident cases compared to matched controls. Indeed, previous cross-sectional studies have also proposed a link between sTWEAK concentration and T2D (52).

The rationale that low levels of sTWEAK, in contrast to other cytokines, appears as protective in conditions with high CV risk diseases associated with an increased of chronic inflammatory activity, is incompletely understood. Several conceivable explanations have been proposed. A reduction of sTWEAK in serum, due to uptake by the Fn14 receptor has been postulated. Endothelial dysfunction is the initial pathophysiological step in the progression of vascular damage that precedes and leads to clinically visible CVD (55). Under these conditions, Fn14 expression is increased in the endothelium. Recently, we reported increased Fn14 expression in human adipocytes from severely obese subjects. These cells also showed an increase in Fn14 expression after inflammatory stimulation, thus increasing the availability for sTWEAK ligand, which could lead to a peripheral reduction of serum sTWEAK (19, 21).

An alternative hypothesis proposes the involvement of CD163, a monocyte-macrophage surface receptor which has been suggested to act as a scavenger receptor for sTWEAK (56). Soluble CD163 (sCD163) is a macrophage-specific serum marker which is elevated in inflammatory conditions (57). Circulating levels of sCD163 and sTWEAK are expressed in an opposite trend in human carotid atherosclerotic plaques (58). Moreover, CD163-expressing macrophages can bind and internalize sTWEAK *in vitro* (58). Thus, the reduction of sTWEAK could be related to the presence of sCD163, which is up-regulated both in patients with chronic kidney disease (CKD), and in obese subjects (51, 59–61). This incremental increase could enable sTWEAK degradation by inflammatory macrophages, leading to decreased sTWEAK levels, represented by the reduction in the sTWEAK/sCD163 ratio observed in some diseases such CKD (51). Thus, low sTWEAK levels may be related to the degree of macrophage activation. However, these observations are not fully coincident in TD2 patients. High serum levels of sCD163 have been reported as a useful predictive biomarker of T2D (62), but a more recent study reveals no association between circulating sCD163 and the incidence of T2D (54).

In contrast to the hypothesis of the potential anti-inflammatory behavior of sTWEAK, animal studies with different approaches to investigate the role of TWEAK/Fn14 axis in the development and progression of atherosclerosis, gain of function, or loss of function, have showed that TWEAK participates in the atherogenic process (63, 64) indicating that the "net" effect of the pathway is damaging rather than protective in this condition.

sTWEAK MODULATES TNFα ACTIVITY

To date, TNFα has focused the attention as a preponderant inflammatory cytokine with important implications both at local and systemic levels in obesity and related diseases. The action of TNFα on adipose tissue can alter the production of many adipokines, and this is relevant for the systemic effects of TNFα on insulin sensitivity and whole body energy homeostasis (65). sTWEAK and TNFα co-exist within the obese adipose tissue milieu, and both cytokines have a pro-inflammatory potential, although at the same concentrations TNFα is a much more potent and rapid inflammatory mediator than TWEAK (66).

Examination of the biological mechanisms through which sTWEAK improves insulin sensitivity has demonstrated that, in visceral adipocytes, treatment with sTWEAK ameliorates TNFα-induced insulin resistance on glucose uptake. This occurs by abolishing the stimulatory effect of TNFα on JNK1/2 kinase, which is directly involved in the development of insulin resistance (67). This effect is produced, at least in part, through a reduction in the cellular concentration of TRAF2, leading to a curbing of TNFα intracellular signaling events. Furthermore, this modulation of TNFα-induced changes in insulin sensitivity was found to be associated with an increase in the activity of PP2A, a Ser/Thr protein phosphatase known to negatively regulate cytokine signaling (22). Additionally, in human subcutaneous adipocytes, sTWEAK exerts a modulator effect over TNFα-induced cytokine production by inhibiting the MAPK and NF-κB signaling cascades commonly used by TNFα (21).

This protective/modulatory effect of sTWEAK on TNFα activity has been observed in different cell types such cultured fibroblast like synoviocytes obtained from synovial tissues of rheumatoid arthritis patients (68), in mouse cerebral cortical neurons (69), and also in several tumor human epithelial cell lines (70), suggesting a broader and general competitive behavior between sTWEAK and TNFα.

CONCLUDING REMARKS

The duality between inflammatory and anti-inflammatory activity seems to be one of the major elements in the evolution of high CV risk diseases, such as obesity and T2D. In this scenario, some molecules may display contradictory actions, depending on the cell type and the location, and on the systemic, or local influence.

Here we have summarized emerging data on the role of TWEAK within the context of metabolic inflammation. Despite

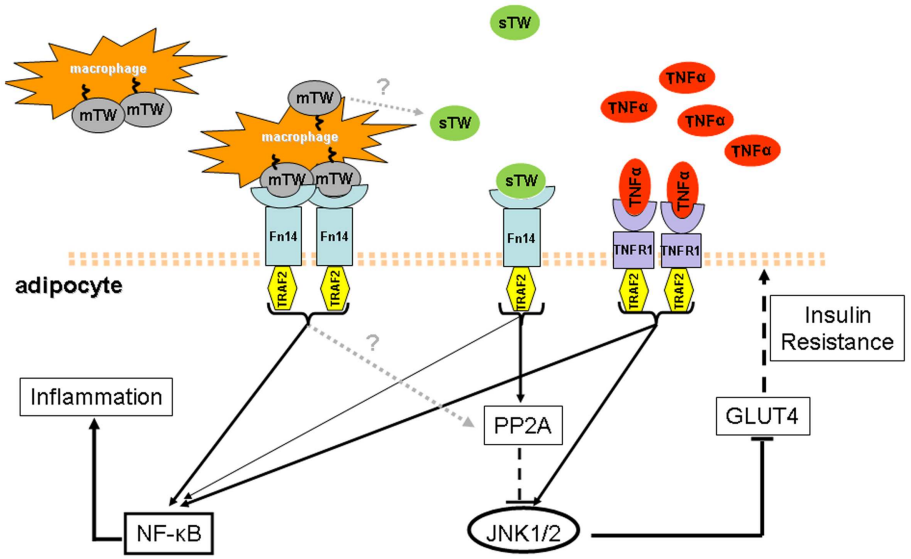

FIGURE 1 | Model of the state of the art of TWEAK and TNFα in obesity and T2D conditions. In obesity and T2D states, higher circulating levels of TNFα and lower levels of sTWEAK have been observed. Additionally, obese adipose tissue is highly infiltrated with macrophages that could be a source of sTWEAK production and also express mTWEAK on the surface. The latter may establish a strong pro-inflammatory response in adipocytes where the Fn14 receptor is over-expressed and activates the NF-κB pathway. In contrast, sTWEAK has been identified as a negative regulator of TNFα signaling since both signal via similar TNFR-associated factors, including TRAF2. Amelioration of TNFα-induced insulin resistance by sTWEAK in obesity and T2D could be a consequence, at least in part, of its direct regulation of JNK1/2 phosphorylation, controlled by PP2A phosphatase which is known to negatively regulate cytokine signaling. PPA2 activation could thereby be linked to the protective role of sTWEAK during the development of insulin resistance. Thus it is tempting to speculate that in obesity and T2D, the increase of mTWEAK and the decrease of sTWEAK may help to maintain the pro-inflammatory effect of TNFα-driven response.

the moderate inflammatory activity of the sTWEAK cytokine in adipocytes, a competitive interference ability of sTWEAK on TNFα signaling in the adipocyte has been revealed. Contrary to that observed with TNFα in obese and T2D patients, circulating sTWEAK appears as a protective element under these conditions. Interestingly, mTWEAK and sTWEAK have been shown to have different effects on signal transduction pathways. Since mTWEAK appears to be mainly expressed in macrophages, the metabolic effects of TWEAK may therefore differ in cells having contact with macrophages (e.g., adipocytes in an obesity context) and in more distant cells living in a macrophage-free environment. Thus, it is tempting to speculate that the decrease in sTWEAK levels, together with an increase of mTWEAK, may help to maintain the local pro-inflammatory effect of the TNF-α-driven response in obese and T2D conditions (**Figure 1**). Hence, the use of recombinant sTWEAK or Fn14 agonists to manipulate the TWEAK/Fn14 axis offers an exciting perspective for the treatment of insulin resistance and should be explored further.

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TWEAK-Fn14 cytokine-receptor axis: a new player of myocardial remodeling and cardiac failure

Tatyana Novoyatleva[1], Amna Sajjad[1,2] and Felix B. Engel[3]*

[1] *Department of Cardiac Development and Remodelling, Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany*
[2] *Government College University Faisalabad, Faisalabad, Pakistan*
[3] *Department of Nephropathology, Experimental Renal and Cardiovascular Research, Institute of Pathology, University of Erlangen-Nürnberg, Erlangen, Germany*

Edited by:
Timothy S. Zheng, Biogen Idec, Inc., USA

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Hartmut Kleinert, Johannes Gutenberg University Mainz, Germany
Kottarappat N. Dileepan, University of Kansas Medical Center, USA

***Correspondence:**
Felix B. Engel, Department of Nephropathology, Institute of Pathology, Friedrich-Alexander-University Erlangen-Nürnberg, Universitätsstraße 22, 91054 Erlangen, Germany
e-mail: felix.engel@uk-erlangen.de

Tumor necrosis factor (TNF) has been firmly established as a pathogenic factor in heart failure, a significant socio-economic burden. In this review, we will explore the role of other members of the TNF/TNF receptor superfamily (TNFSF/TNFRSF) in cardiovascular diseases (CVDs) focusing on TWEAK and its receptor Fn14, new players in myocardial remodeling and heart failure. The TWEAK/Fn14 pathway controls a variety of cellular activities such as proliferation, differentiation, and apoptosis and has diverse biological functions in pathological mechanisms like inflammation and fibrosis that are associated with CVDs. Furthermore, it has recently been shown that the TWEAK/Fn14 axis is a positive regulator of cardiac hypertrophy and that deletion of Fn14 receptor protects from right heart fibrosis and dysfunction. We discuss the potential use of the TWEAK/Fn14 axis as biomarker for CVDs as well as therapeutic target for future treatment of human heart failure based on supporting data from animal models and *in vitro* studies. Collectively, existing data strongly suggest the TWEAK/Fn14 axis as a potential new therapeutic target for achieving cardiac protection in patients with CVDs.

Keywords: cardiovascular disease, fibrosis, proliferation, hypertrophy, extracellular matrix, Toll-like receptors

INTRODUCTION

Most cardiovascular diseases (CVDs) result in heart failure due to the death of heart muscle cells, the cardiomyocytes. This leads to pathological remodeling, which triggers additional cardiomyocyte loss resulting finally in a diminished quality of life and inevitable in heart failure (1, 2). Thus, the most efficient way to prevent most CVDs appears to be preventing cardiomyocyte loss. One aim in cardiovascular medicine is therefore monitoring and targeting risk factors. These efforts resulted, for example, in the addition of antiplatelet therapy (3–6), introduction of reperfusion therapy with thrombolysis (7, 8), and acute percutaneous coronary intervention (9). The current optimal treatment regimen after the discovery that neurohormones contribute to the progression of heart failure is the use of angiotensin-converting-enzyme-inhibitors or angiotensin receptor blockers, beta-blockers, aldosterone antagonists as well as implantable automatic cardiac defibrillators. However, despite these advances, the prevalence of heart failure has increased in the last decades, remaining one of the leading causes of death worldwide (10, 11). This suggests that this treatment regimen does not target all pathological mechanisms in heart failure.

Already two decades ago, the observation of increased tumor necrosis factor (TNF) levels in patients with heart failure linked inflammation to CVDs (12). Meanwhile, a large number of reports have established the essential role of inflammatory cytokines in the progression of heart failure contributing to the processes of cardiac hypertrophy, fibrosis, and apoptosis (13–15). Recently, other TNFSF/TNFRSF members than TNF have been implicated in the pathophysiology of heart failure. Here, we review the role of the members of the TNFSF/TNFRSF in heart failure focusing on TWEAK and its receptor. In addition, we will explore their potential as biomarker for CVDs as well as therapeutic target for the future treatment of human heart failure.

TWEAK AND ITS COGNATE RECEPTOR Fn14

The member TWEAK of the TNFSF was discovered in 1997 (16). Like the other ligands of the TNFSF, TWEAK is primarily synthesized as a type II transmembrane receptor and then further processed by a furin endoprotease into the soluble cytokine sTWEAK (16–18). Cells can co-express both plasma membrane-anchored and soluble TWEAK (19, 20). However, membrane-anchored TWEAK is due to its efficient cleavage, which is rarely detectable (e.g., in monocytes and macrophages) (21). TWEAK expression was reported in a wide variety of different tissues and cells, including tumor cell lines and specimens (16, 19, 22–30).

TWEAK is known to be the sole TNFSF that signals through the cell surface receptor Fn14, an unusual small TNFRSF member (31). Fn14 was discovered in 1999 as Fibroblast Growth Factor 1 (FGF1)-inducible, immediate-early response gene in murine NIH3T3 fibroblasts (32) and is induced by a large variety of other growth factors including FGF2, Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF) and Vascular Endothelial Growth Factor (VEGF) as well as cytokines such as tumor necrosis factor alpha (TNFα), Interleukin-1beta (IL-1β), Interferon gamma (IFNγ), and transforming growth factor-beta (TGF-β) (32–35). Fn14 is a type I transmembrane protein expressed on a broad variety of different cell types (18, 32, 33). It contains a single cysteine-rich domain (CRD) in its ectodomain while most other TNF receptors have two to six copies of this

characteristic motif (36). The cytoplasmic domain of Fn14 contains only 28 amino acid residues lacking a death domain. Like other TNFRSF members lacking a death domain, Fn14 trimerizes upon ligand binding recruiting subsequently E3 ligase/adapter proteins of the tumor necrosis factor receptor-associated factor (TRAF) family to its cytoplasmic domain (21, 36). Several members of the TRAF family (TRAF1, TRAF2, TRAF3, and TRAF5) have been shown to be able to bind to Fn14 (37, 38).

TWEAK/Fn14 AXIS IN CARDIOMYOCYTE PROLIFERATION

The heart grows during embryonic development mainly due to cardiomyocyte proliferation. Shortly after birth, however, cardiomyocytes stop to proliferate and the heart continues to grow through the increase in cardiomyocyte cell size (i.e., hypertrophy) (39). Consistent with its role in proliferation in a number of cell types, such as smooth muscle cells (40), myoblasts (28, 41), astrocytes (42), liver progenitor cells (29), epithelial (43), and tubular cells (44), Fn14 expression correlates with the rate of cardiomyocyte proliferation during heart development (45). However, neither Fn14 nor TWEAK knockout mice exhibit a heart phenotype suggesting that the TWEAK/Fn14 axis is not essential for cardiomyocyte proliferation or heart development (28–30). Yet, TWEAK stimulation of neonatal rat cardiomyocytes, expressing Fn14 endogenously, induced cardiomyocyte proliferation (45). TWEAK activated extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) but not p38 mitogen-activated kinase (p38) signaling. In addition, TWEAK inhibited glycogen synthase kinase-3beta (GSK-3beta) (**Figure 1**) (45). The effect of TWEAK on several pathways has been described also for other cell types. In tubular cells, TWEAK activated, for example, ERK, p38, PI3K, and NF-κB signaling (44). TWEAK-induced proliferation in tubular cells and cardiomyocytes was prevented by inhibitors of ERK and PI3K (44, 45). In contrast, inhibition of p38 blocked only tubular cell proliferation. A general role of TWEAK-induced NF-κB signaling in cell proliferation remains unclear as it has not yet been determined in cardiomyocytes. In tubular cells, inhibition of NF-κB signaling blocked proliferation.

In contrast to neonatal rat cardiomyocytes, TWEAK had a negligible effect on adult cardiomyocyte proliferation, possibly due to the developmental downregulation of Fn14. However, ectopic expression of Fn14 enabled TWEAK-induced DNA synthesis in adult cardiomyocytes. To date, activation of TWEAK/Fn14 signaling is by far the most potent inducer of adult cardiomyocyte cell cycle re-entry but fails to promote progression into mitosis (45). This is important, as induction of cardiomyocyte proliferation is considered to be a potential future therapy to CVDs. Adult zebrafish and newt as well as newborn mice can all regenerate their heart through cardiomyocyte proliferation (46–48). Moreover, several studies have demonstrated that adult mammalian cardiomyocyte cell division can be induced, even though induction efficiency is relatively low (49, 50). Finally, recent reports utilizing carbon-14 isotope labeling due to atomic bomb tests in the 60s suggest that also human adult mammalian cardiomyocytes, at least a sub-set, might maintain the competence to proliferate (51). Thus, in the future it will be important to elucidate the TWEAK-mediated signaling that induces rat neonatal cardiomyocyte proliferation and to determine if reinstatement of these signaling modalities allows also adult mammalian cardiomyocyte proliferation.

THE TWEAK/Fn14 SIGNALING PROMOTES CARDIAC HYPERTROPHY AND HEART FAILURE

Pathological cardiac hypertrophy is a key risk factor for heart failure. Cardiac hypertrophy describes the enlargement of the heart due to the increase in cell size of cardiomyocytes. For example, physical exercise and pregnancy can lead to cardiac hypertrophy (52). This form of hypertrophy is considered physiological cardiac hypertrophy as heart function is not affected or improved. In contrast, hypertrophy induced by chronic pressure or volume overload results under certain disease conditions such as hypertension, valvular heart disease, and coronary artery disease, in cardiac dysfunction or heart failure (52). Thus, it is called pathological cardiac hypertrophy.

Tumor necrosis factor alpha was the first member of the TNFSF shown to induce cardiomyocyte hypertrophy (53). Cardiomyocyte-specific overexpression as well as infusion of TNFα causes dilated cardiomyopathy (DCM) suggesting that both circulating and locally produced TNFα induces myocardial dysfunction (54, 55). In recent years, animal experiments have suggested that also other TNFSF ligands can mediate cardiac hypertrophy and heart failure. For example, transgenic overexpression of FasL (TNFSF6) resulted in cardiac hypertrophy with pro-inflammatory consequences (56). That also the TWEAK/Fn14 axis is involved in cardiac hypertrophy was supported by the discovery that transgenic overexpression of full length-TWEAK (fl-TWEAK) in mice resulted in DCM with markedly increased heart to body weight ratio and severe cardiac dysfunction. Moreover, cardiomyocytes from fl-TWEAK-overexpressing mice displayed cellular hypertrophy characterized by pronounced cellular elongation (57). It has also been demonstrated that endogenous Fn14 is required for cardiac hypertrophy. Fn14 deletion attenuated right ventricular (RV) hypertrophy caused by pulmonary artery banding (PAB), a mouse model of pressure-overload-induced RV hypertrophy while TWEAK/Fn14 signaling promoted cardiomyocyte hypertrophy *in vitro* (58). However, the upstream and downstream signaling pathways regulating TWEAK/Fn14-mediated hypertrophy *in vivo* remain unclear. It has been shown that hypertrophic agonists including Angiotensin II (Ang II), Phenylephrine (PE), and Endothelin-1 (ET-1) induce Fn14 expression (59). Furthermore, TRAF2 and TRAF5, possible downstream targets of TWEAK/Fn14 signaling, have been implicated in cardiac hypertrophy (**Figure 1**). Cardiomyocyte-specific TRAF2 transgenic mice developed a time-dependent increase in cardiac hypertrophy, left ventricular (LV) dilation, and adverse LV remodeling, and a significant decrease in heart function (60). Moreover, deficiency of TRAF5 substantially aggravated cardiac hypertrophy and cardiac dysfunction in response to pressure overload after transverse aortic constriction (TAC) (61).

TWEAK IS AN EXTRACELLULAR MATRIX MODULATING FACTOR IN THE HEART

Heart failure describes a condition when the heart fails to pump sufficient blood to meet the metabolic demand of the body. It is caused by the loss of cardiomyocytes through necrosis, apoptosis,

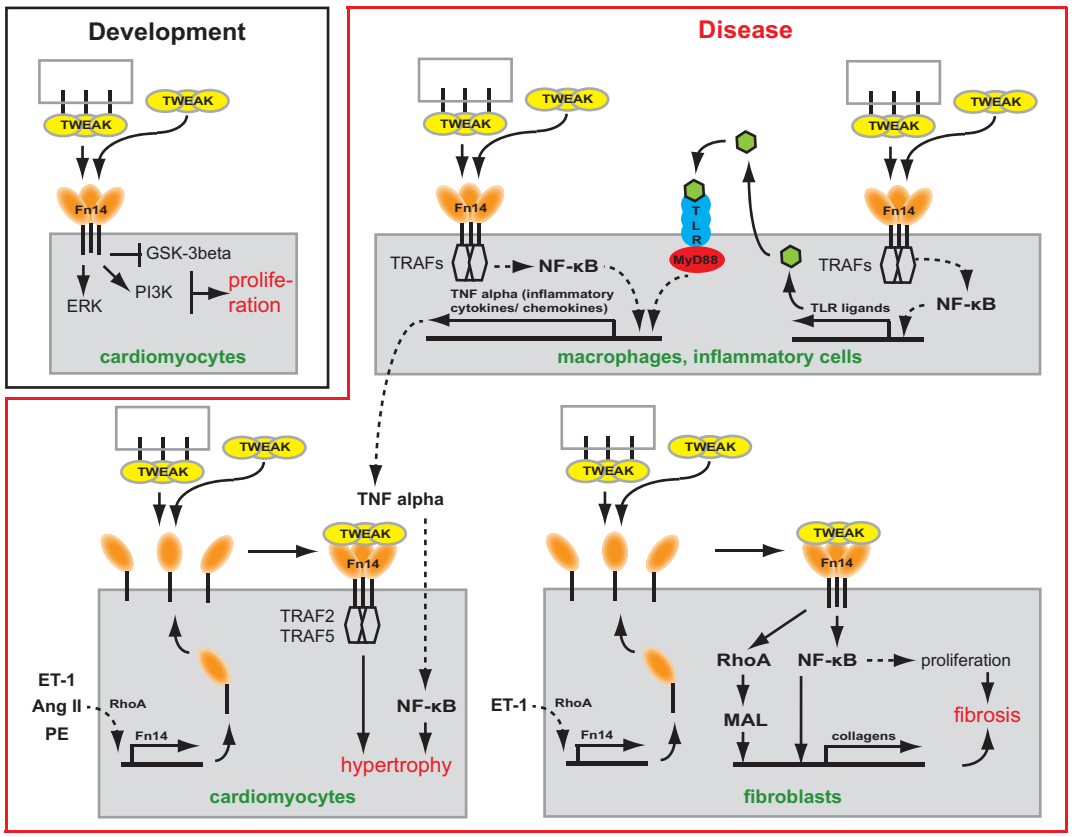

FIGURE 1 | Scheme depicting the potential role of TWEAK/Fn14 signaling in cardiac development and myocardial remodeling and cardiac failure. TWEAK might be presented to Fn14 as membrane-bound or secreted form. TWEAK stimulation induces *in vitro* proliferation of neonatal cardiomyocytes. Thus, TWEAK/Fn14 signaling might contribute to developmental heart growth. In CVDs, it has been shown that TWEAK has the potential to affect inflammatory cells, cardiomyocytes as well as fibroblasts. In inflammatory cells, TWEAK can enhance secretion of inflammatory cytokines/chemokines by enhancing their expression directly or by increasing the expression of TLR ligands. In cardiomyocytes, TWEAK induces via TRAF hypertrophy. In fibroblast, TWEAK induces the expression of collagens via RhoA and NF-κB and stimulates via NF-κB proliferation leading to cardiac fibrosis.

and autophagy (62, 63), which results in hypertrophy, myocardial fibrosis (fibrillar collagen deposition), and maladaptive extracellular matrix (ECM) remodeling, all characteristics of end-stage heart failure (64). ECM remodeling leads to cardiomyocyte slippage, ventricular dilatation, increased ventricular stiffness as well as impaired diastolic and systolic function (65).

As described above, TNFSF members are involved in the early stages of CVDs such as increased inflammation and hypertrophy (56, 66). However, there is clear evidence that TNFSF members such as TNFα or FasL are also directly involved in myocardial fibrosis (56, 66). First evidence that the TWEAK/Fn14 axis may play a role in ECM remodeling came from the analysis of fl-TWEAK overexpressing mice. These mice develop DCM exhibiting progressive myocardial and perivascular fibrosis (57). Recently, it has been shown that PAB-induced fibrosis was significantly reduced in Fn14 global knockout mice (30). Cell culture experiments demonstrated that TWEAK/Fn14 signaling promotes cardiac mouse fibroblast proliferation and collagen synthesis (30), major sources of fibrillar collagen in the heart under pathophysiological conditions (67, 68). Collagen expression induced by TWEAK/Fn14 signaling was mediated via RhoA-dependent nuclear translocation of the myocardin-related transcription factor-A (MRTF-A)/MAL. Interestingly, upregulation of Fn14 expression in cardiomyocytes due to stretch or stimulation with Ang II or norepinephrine was mediated by RhoA/ROCK signaling, too (69). Furthermore, Chen and colleagues independently demonstrated that TWEAK induces proliferation and collagen synthesis of rat cardiac fibroblasts (70). However, they showed that proliferation and enhancement of collagen synthesis was mediated by the activation of NF-κB signaling (**Figure 1**). Collectively, these data demonstrate that the TWEAK/Fn14 axis is involved in cardiac ECM remodeling. Importantly, Fn14 knockout mice were protected from PAB-induced RV dysfunction (30, 58) as well as TWEAK-induced cardiac dysfunction and dilation (57).

An essential prerequisite for the formation of fibrotic scar tissue and ECM remodeling is, besides the elevated production of ECM proteins, the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (71–73). Previously, it has been demonstrated that TNFα-induced cardiac remodeling and dysfunction depends on MMP activation (74). In addition, it has been shown that also FasL and other TNFSF members such as LIGHT, RANKL, and CD40L can potentially regulate

MMP activity (75–77). Finally, both TNFα and FasL overexpression is associated with increased levels of TGF-β1, an important inducer of myocardial fibrosis (56, 66). Taken together, these data suggest that besides TNFα several other members of the TNFSF might play important roles in ECM remodeling in cardiac disease.

TWEAK AND OTHER TNFSF SIGNAL VIA NF-κB

NF-κB has been shown to be cardio-protective (78). However, prolonged activation of NF-κB appears to promote heart failure. For example, cardiomyocyte-specific IκB kinase (IKK)/NF-κB activation induces reversible inflammatory cardiomyopathy and heart failure (79). In addition, it has been demonstrated in animal models, such as TAC-induced pressure-overload (80, 81) and monocrotaline (MCT)-induced RV hypertrophy (82), that inhibition of NF-kB signaling prevents heart failure (cardiac hypertrophy and/or cardiac remodeling).

NF-κB transcription factors are activated through cytokines, pathogens, injuries, and other stressful conditions. Mammalian cells express five NF-κB family members (RelA, RelB, c-Rel, NF-κB2/p100/p52, and NF-κB1/p105/p50) (83–85), that regulate the expression of a large variety of genes which are involved in a number of processes like inflammatory and immune responses of the cell, cell growth, and development. In unstimulated cells, NF-κB is bound to an inhibitory protein, IκB. Binding to IκB masks the nuclear localization signal of NF-κB, sequesters the NF-κB/IκB complex in the cytoplasm, and prevents NF-κB from binding to DNA. Canonical NF-κB signaling culminates in the activation of IKK, which phosphorylates the inhibitory IκB subunit of the NF-κB/IκB complex in the cytoplasm resulting in the proteasomal degradation of IκB. This releases NF-κB resulting in the translocation of NF-κB into the nucleus. In contrast, the non-canonical NF-κB signaling pathway mediates activation of the p52/RelB NF-κB complex. This NF-κB pathway relies on the inducible processing of NF-κB2 precursor protein, as opposed to the degradation of IκBα. A central signaling component of the non-canonical NF-κB pathway is NF-κB-inducing kinase (NIK), which functions together with the inhibitor of NF-κB kinase α (IKKα), to induce phosphorylation-dependent ubiquitination and processing of NF-κB2. Under normal conditions, NIK is continuously degraded. In response to signals mediated by a sub-set of TNFSF members such as Lymphotoxin-β (LT-β), B-cell activating factor (BAFF), and CD40 ligand (CD40L) (86–89), NIK becomes stabilized leading to the activation of non-canonical NF-κB (90, 91).

TWEAK/Fn14 axis has been shown to activate several different signaling cascades, though activation of NF-κB signaling appears to be the major and predominant cellular response through which TWEAK/Fn14 signals. TWEAK/Fn14 has been demonstrated to activate canonical NF-κB signaling in a large variety of cell types (38, 41, 70, 92–96). Interestingly, TWEAK can also signal via Fn14 through the non-canonical NF-κB pathway, which is dependent on the TRAF-binding site of Fn14 as well as TRAF2 and TRAF5 (97). Membrane-bound and oligomerized sTWEAK are superior to soluble TWEAK trimers in regard to the activation of the classical NF-κB pathway. In contrast, both TWEAK variants are equally potent inducers of the non-canonical NF-κB pathway (98). That TWEAK/Fn14 mediates its detrimental effect on heart function at least in part through NF-κB signaling has been supported by several studies. TWEAK-induced proliferation and collagen synthesis of rat cardiac fibroblasts *in vitro* was mediated by the activation of NF-κB signaling (70). Moreover, DCM induced through elevated circulating TWEAK levels occurred via an FN14-TRAF2-NF-κB-dependent signaling pathway (99). In addition, cardiomyocyte-specific TRAF2 overexpressing mice provoked adverse cardiac remodeling associated with elevated NF-κB signaling (60). These data suggest that the members of the TNFSF mediate their detrimental effects in the heart through TNFRSF members via TRAF2, which is associated directly or indirectly with the majority of TNFSFR members expressed in the heart (TNFR1, TNFR2, RANK, and Fn14), through non-canonical NF-κB signaling (77).

POTENTIAL INTERACTIONS OF TOLL-LIKE RECEPTORS AND TWEAK/Fn14 SIGNALING IN CVD

Toll-like receptors (TLRs) are a family of single, membrane-spanning, non-catalytic receptors, which are expressed on various immune cells, such as macrophages, dendritic cells, and neutrophils, as well as on non-immune cells, such as fibroblast cells and epithelial cells. Most commonly, they are known as key activators of the innate immune system as they are responsible for the synthesis and secretion of various inflammatory cytokines by the cells of this system (100). Upon detection of distinct pathogen-associated molecular patterns (PAMPs) of protozoa, virus, and bacteria origin, different members of the TLR family activate signaling pathways that result in the activation of NF-κB-dependent and interferon regulatory factor (IRF)-dependent molecular mechanisms. In addition, TLRs may also be activated by endogenous ligands named damage-associated molecular patterns (DAMPs), which allow the immune system to sense tissue injury in the absence of an infection.

TLR2 and TLR4 activation resulting in NF-κB-dependent release of inflammatory cytokines plays an important role in CVD (101–103). For example, it has been demonstrated that TLR2-deficient mice exhibit higher fractional shortening and survival after myocardial infarction in comparison to wild-type animals (104). In addition, both knockout of TLR2 and inhibition of TLR2 by neutralizing antibodies significantly reduced Ang II-induced cardiac fibrosis, which was associated with a reduction in the infiltration of macrophages, the production of inflammatory cytokines and chemokines, and the activation of NF-κB (103). However, TLR2 deletion in a hypertrophy model (TAC) revealed that TLR2 is required for adaptive cardiac hypertrophy through IL-1β upregulation via NF-κB activation (102). Similar to TLR2, TLR4-deficient mice show after myocardial infarction enhanced LV function and improved remodeling leading to significantly increased survival of TLR4-deficient mice (105, 106). Finally, adenoviral overexpression of dominant-negative MyD88, a common adaptor of TLR2 and TLR4, significantly reduced cardiac hypertrophy and cardiac fibrosis in an aortic constriction model improving cardiac function (107). Taken together there is accumulating evidence for detrimental effects of TLR signaling on cardiac remodeling, cardiac function, and fibrosis upon injury (**Figure 1**) (108).

Interestingly, it has recently been indicated that TWEAK has the ability to potentiate the pro-inflammatory effects of TLR ligands. For instance, TWEAK has been shown to cooperate with

the TLR2 ligand Pam_3CysSK_4 on the stimulation of IL-8 synthesis by epithelial cells (109). Furthermore, TWEAK is able to stimulate the secretion of HMGB1 (110, 111), another postulated TLR ligand, that contributes to the inflammation in various injury models via signaling through TLR2, TLR4, and RAGE in inflammatory cells (112, 113). Collectively, these data suggest that the TWEAK/Fn14 signaling pathway may also interact with TLR signaling in promoting acute inflammation in CVD.

PROGNOSTIC VALUE OF TWEAK/Fn14 EXPRESSION FOR HEART FAILURE

During recent years, evidence has accumulated that other members of the TNFRSF/TNFSF than TNFα/TNFR might play important roles in the development and progression of heart failure as they are regulated in both experimental and clinical heart failure. Expression analyses of TNFSF ligands and co-stimulatory molecules have revealed that cardiomyocytes of patients with acute myocarditis and DCM express high levels of CD27L, CD30L, and 4-1BBL and exhibit weak to moderate expression of OX40L (114). In heart failure post-myocardial infarction, RANKL was upregulated in both fibroblasts and cardiomyocytes (77). In addition to cardiac cells, elevated expression of TNFSF members were also observed in T lymphocytes in DCM (CD40L) (115) and peripheral blood mononuclear cells in chronic heart failure (4-1BBL, APRIL, CD27L, CD40L, FasL, LIGHT, and TRAIL-receptor 4) (116). Notably, receptors for several of these ligands (e.g., FasL, LIGHT, TNFα, RANKL, and TRAIL) have been reported to be expressed in the heart and enhanced levels of some of these TNF-related molecules also have been found within the failing myocardium (e.g., RANKL, OPG) (77, 117–124). That the members of the TNFSF, can be utilized as prognostic markers, is exemplified by OPG (125), whose plasma level correlated in apparently healthy patients with greater LV mass and lower LV ejection fraction (126). OPG also has been shown to be a reliable predictor of long-term mortality and heart failure development in patients with acute coronary syndrome (127), all-cause mortality in patients with symptomatic severe aortic stenosis (128) or even mixed etiology (129), and with hospitalization of patients with ischemic heart failure due to worsening of heart failure. Another example is BAFF, which is elevated in patients with acute myocardial infarction predicting increased risk of death or recurrent myocardial infarction (130).

In contrast to the poor prognosis found in relation to elevated TNFα levels in heart failure, increased levels of sTWEAK appear to be a good predictor of an adverse short-term outcome after severe type of myocardial infarction (ST-elevation myocardial infarction, STEMI) correlating with hospital duration time of the patients (131). In contrast, TWEAK protein levels were lowered in patients with chronic stable heart failure (132) or advanced non-ischemic heart failure (133). sTWEAK levels were inversely correlated with the severity of the disease and allowed prediction of patient's mortality, respectively. Importantly, the predictive value was also verified after adjustment for clinical and biochemical variables including the state of the art biomarker, NT-proBNP.

However, sTWEAK alone appears not to be an optimal predictor of heart disease in general as Fn14 gene expression, in contrast to other members of the TNFRSF, is highly regulated *in vivo*. Under physiological conditions, Fn14 is expressed at relatively low levels but its expression is elevated in several experimental models of injury and inflammation (18, 28, 29, 94, 134, 135). The predictive value of sTWEAK levels is also complicated by the fact that TWEAK and Fn14 can be expressed by a wide variety of cell types. Both are expressed in cardiomyocytes (30, 45, 57, 131) and cardiac fibroblasts (30, 70). In addition, their expression was observed also for macrophages and smooth muscle cells of carotid atherosclerotic plaques (134). Finally, TWEAK is also expressed in endothelial cells of coronary arteries (59) and Fn14 expression was upregulated in proliferating endothelial and smooth muscle cells of injured rat arteries (18). Thus, changed levels of plasma sTWEAK might be difficult to interpret. For example, plasma sTWEAK levels are decreased in patients with pulmonary arterial hypertension (PAH), which results in RV failure (136). This might suggest that sTWEAK has positive adaptive functions. However, animal experiments have demonstrated that Fn14 expression in the heart is highly upregulated after PAB- or MCT-induced PAH (30). In these animal models, TWEAK blood plasma levels were unchanged (PAB) or significantly reduced (MCT) while TWEAK mRNA expression in RVs was elevated. Thus, reduced TWEAK blood levels might be due sequestration of circulating TWEAK by the upregulated Fn14 receptor or might be a compensatory mechanism to protect from the consequences of Fn14 activation. Collectively, sTWEAK appears to be a promising biomarker if combined with clinical parameters.

BLOCKING OF Fn14 SIGNALING AS POTENTIAL THERAPEUTIC APPROACH

In addition to novel candidates for new biomarkers, several TNF-related molecules also could be attractive targets for cardiac therapy. Cell culture as well as *in vivo* experiments have indicated that TWEAK/Fn14 signaling is involved in cardiac hypertrophy, cardiac remodeling, and heart failure, identifying TWEAK and Fn14 as promising targets to treat CVDs (30, 45, 58, 59, 69, 70, 99, 137). Targeting TWEAK and Fn14 has been also considered in various other pathophysiological conditions. Blocking of TWEAK or Fn14 has successfully been demonstrated to be beneficial in preclinical models of Collagen-Induced Arthritis (138, 139), Experimental Autoimmune Encephalitis (140), Middle Cerebral Artery Occlusion (94, 135, 141), Ischemia Reperfusion Injury (142), and atherosclerosis (143, 144). Furthermore, therapeutical efficacies of TWEAK and Fn14 blocking antibodies were determined in tumor growth inhibition assays, utilizing TWEAK and Fn14-expressing human esophageal and pancreatic cell lines, as well as in a murine gastrointestestinal cancer model (145). Anti-TWEAK and anti-Fn14-specific antibodies are at the moment under clinical investigations in phase I studies in patients suffering on Rheumatoid Arthritis, lupus or solid tumors (http://clinicaltrials.gov/; NCT00771329, NCT001499355 and NCT00738764). Additionally to the usage of antibodies, the employment of the fusion proteins Fn14-Fc and Fc-TWEAK as well as soluble TWEAK provide alternative approaches (137). Taken together, therapies targeting TWEAK and/or Fn14 appear to be a realistic approach and thus warrant future preclinical studies.

CONCLUDING REMARKS

The members of the TNFSF and TNFRSF have been shown to be involved in the progression of CVDs to heart failure and thus they appear to be promising prognostic and therapeutic targets. However, the past has shown that correlations of cytokine blood levels to heart disease can be misleading (146). For example, TNFα mediates both adaptive and maladaptive effects on the myocardium. On the one hand it activates via NF-κB expression of anti-apoptotic and cytoprotective genes, but on the other hand it induces also inflammation (147). This explains why clinical trials with anti-TNF therapies were disappointing although overexpression of TNF, which is positively correlated with heart failure in patients, leads to experimental heart failure (146, 148). Thus, it will be important to consider this issue when designing new treatment strategies in heart failure that target members of the TNFSF or TNFRSF.

Based on the disappointing results from anti-TNF trials, the TWEAK/Fn14 axis may represent new targets for heart failure therapies. Fn14 appears to be an excellent therapeutic target as Fn14 knockout mice are viable and show no obvious phenotype under physiological conditions. In addition, Fn14 is upregulated in the myocardium of diseased hearts (30). However, at present the precise role of the TWEAK/Fn14 axis is still poorly understood, and it is unclear whether it has a positive, adaptive role in cardiac disease. One important issue is that the TWEAK/Fn14 axis regulates the behavior of several different cell types. Yet, genetic models inhibiting the TWEAK/Fn14 axis were beneficial in experimental models of heart disease (30, 58). Thus, it is important to test next treatment strategies such as anti-TWEAK antibodies in experimental heart failure models.

Cardiovascular diseases resulting in heart failure are highly complex diseases. Thus, in an ideal case a biomarker should be involved in several pathways of these multiple-pathway diseases reflecting several important pathophysiologies such as hypertrophy, fibrosis, remodeling, and inflammation. As the members of the TNFSF are involved in several of these processes, they appear to be promising biomarkers. However, it might be impudent to assume that a single member of the TNFSF is sufficient. For example, although NT-proBNP is a strong biomarker in heart failure it has recently been shown in patients with symptomatic aortic stenosis that the combination of high levels of both OPG and NT-proBNP was strongly associated with all-cause mortality, thus providing more information together than when either of these markers was used alone (128). Yet, a combination of TNFSF members that provides a "signature of disease" appears likely to be a suitable tool for risk prediction.

AUTHOR CONTRIBUTIONS

Tatyana Novoyatleva and Felix B. Engel wrote the manuscript. Felix B. Engel generated **Figure 1**. Amna Sajjad contributed to the literature search. All authors proof read the manuscript.

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Is TWEAK a biomarker for autoimmune/chronic inflammatory diseases?

Daniel Bertin[1,2], Delphine Stephan[1], Michel Khrestchatisky[1] and Sophie Desplat-Jégo[1,2]*

[1] *Aix-Marseille Université, NICN, CNRS, UMR7259, Marseille, France*
[2] *Service d'Immunologie, Pôle de Biologie, Hôpital de la Conception, Assistance Publique – Hôpitaux de Marseille, Marseille, France*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

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Massimo Gadina, National Institutes of Health, USA
Meena Subramanyam, Biogen Idec, Inc., USA

***Correspondence:**
Sophie Desplat-Jégo, Service d'Immunologie, Pôle de Biologie, Hôpital de la Conception, Assistance Publique – Hôpitaux de Marseille, 147 Bd Baille, 13005 Marseille, France
e-mail: sophie.jego-desplat@ap-hm.fr

The TWEAK/Fn14 pathway is now well-known for its involvement in the modulation of inflammation in various human autoimmune/chronic inflammatory diseases (AICID) including lupus, rheumatoid arthritis, and multiple sclerosis. A panel of data is now available concerning TWEAK expression in tissues or biological fluids of patients suffering from AICID, suggesting that it could be a promising biological marker in these diseases. Evidences from several teams support the hypothesis that blocking TWEAK/Fn14 pathway is an attractive new therapeutic lead in such diseases and clinical trials with anti-TWEAK-blocking antibodies are in progress. In this mini-review we discuss the potential use of TWEAK quantification in AICD management in routine practice and highlight the challenge of standardizing data collection to better estimate the clinical utility of such a biological parameter.

Keywords: TWEAK, biomarker, auto-immunity, disease monitoring, serum levels, urinary levels

INTRODUCTION

Autoimmune and chronic inflammatory diseases (AICID) including rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), or systemic sclerosis (SSc) constitute an important medical, social, and economic problem. The prevalence of AICID is estimated to be more than 3% in the adult population (1) and despite scientific progress in the past decade, identification of new reliable markers for diagnosis, prognosis, and prevention of hospitalization is still necessary.

TWEAK is a pleiotropic and multifunctional cytokine that regulates inflammatory pathways by inducing multiple cellular responses depending on the cell type and its micro-environment. During tissue repair and remodeling, the biological activity of TWEAK is complex and even dual: after acute injury, TWEAK promotes tissue regeneration especially by stimulating progenitor cells, but in chronic diseases where TWEAK is persistently activated, it alters tissue repair in part by inhibiting differentiation of the same progenitor cells (2). A growing body of data points to the involvement of the TWEAK/Fn14 pathway in inflammation in various human AICID including SLE, RA, and MS. It is now admitted that TWEAK plays a role in the physiopathology of such diseases and the first clinical trials are in progress, based on anti-TWEAK-blocking therapies during RA or SLE (3, 4). Nevertheless it remains to be proven that the assessment of TWEAK levels in tissues or biological fluids is of interest for the management of patients suffering from AICID. In this article we propose to review the available data on TWEAK quantification in human AICID and to discuss the potential place and the modalities of TWEAK evaluation in the diagnosis and/or the follow-up of AICID.

TWEAK EVALUATION IN AICID: WHY, WHEN, AND WHAT FOR?

Biomarkers in medicine have gained immense scientific and clinical interest in recent years. Biomarkers are potentially useful in the context of primary, secondary, and tertiary prevention. An ideal biomarker should be safe to assess, easy to measure and associated with acceptable costs. Additionally, the biomarker should have "good performance characteristics" (i.e., sensitivity, specificity, positive- and negative-predictive values) and there should be scientific evidence to suggest that biomarker modification influences disease outcome.

In AICID, various serum circulating auto-antibodies are widely used as reliable markers for diagnostic or sometimes prognostic evaluation. Nevertheless the identification of new markers to evaluate inflammatory activity of the disease, to predict disease flare or to monitor the clinical response to biotherapy remains necessary.

The idea that TWEAK could be such a biomarker in AICID is suggested by its role in the modulation of inflammation in AICID both in animal models and in human pathologies. Moreover, in the past decade, various research groups have shown modulation of TWEAK expression in tissue and biological fluids of patients suffering from AICID.

FOR ESTABLISHING A DIAGNOSIS OF AICID

Very few data are available concerning the diagnostic potential of soluble TWEAK in AICID. Among them, urinary TWEAK (uTWEAK) has been proposed by Schwartz and colleagues as a biomarker for lupus nephritis (LN) as they showed that it is elevated in subjects with LN at diagnosis compared with those with SLE but no renal disease (5). Moreover uTWEAK levels were better at distinguishing LN and non LN-SLE than anti-DNA antibodies and complement levels. Schwartz et al. also reported that serum TWEAK (sTWEAK) levels and the urine/sTWEAK ratio were not better markers for LN than the less invasive uTWEAK. It is intriguing to note in this work that sTWEAK levels were significantly lower in SLE patients than in healthy individuals. Later, El-Shehaby et al. have also shown that uTWEAK levels positively correlate with

renal involvement during SLE with a positive predictive value of the marker of 93% for kidney inflammation (6).

FOR FOLLOWING-UP DISEASE ACTIVITY

Autoimmune and chronic inflammatory diseases are chronic diseases that usually fluctuate between periods of remission (little/no symptoms) and unpredictable flare-ups (worsening symptoms). In this context, the ability to predict the severity of disease and the outcome of flares should allow tertiary prevention of disease complications. In LN, renal biopsy remains the gold standard for assessment of disease activity but because of its invasive modalities, this approach cannot be used repeatedly in clinical practice to follow-up disease activity. In this context, uTWEAK has been shown to correlate with the degree of clinical disease activity during LN (5) and fluctuations of uTWEAK levels were found to reflect LN flares. uTWEAK could thus represent an interesting biological tool if the diagnosis of a flare is in doubt.

In RA, auto-immunity and chronic inflammation leads to the destruction of cartilage and bone in the joints. The relationship between TWEAK and arthritis has been supported by anti-inflammatory and anti-angiogenic effects of TWEAK inhibition in a mouse model of collagen-induced arthritis (7, 8). Park et al. showed for the first time that sTWEAK levels were higher in RA patients than in healthy controls (9). Moreover, sTWEAK levels in RA patients positively correlated with the DAS 28 disease activity scores (9).

In 2009, Yanaba et al. described for the first time elevated sTWEAK levels in patients suffering from another AICID, SSc, a generalized connective tissue disorder characterized by sclerotic changes in the skin and internal organs. The longitudinal study of sTWEAK levels performed in this study showed that these high soluble TWEAK levels were associated with a lower prevalence of pulmonary fibrosis and better pulmonary function in SSc patients. This perturbed expression of TWEAK suggested some involvement of the TWEAK pathway in the appearance of pulmonary fibrosis, a well-known complication of the disease. Nevertheless the mechanisms and the meaning of the elevation of sTWEAK levels in this context remain to be determined (10).

FOR MONITORING THERAPIES TARGETING TNF SUPERFAMILY MEMBERS

Currently, few autoimmune diseases can be cured with treatment. In the last decade, TNF blocking agents have been developed for the treatment of human diseases and have been very successful in ameliorating disease signs and symptoms especially in patients suffering from AICID like RA.

According to the work of Park et al. high sTWEAK levels have been shown to correlate with TNF alpha levels in RA patients and to reflect short-term response to etanercept, a biotherapy targeting TNF alpha (9). In fact, responders to etanercept showed a significant decrease in sTWEAK levels at the 12th week of treatment, whereas TWEAK levels in non-responders were not different from their baseline levels. TWEAK is a member of TNF family. Like TNF inhibition, TWEAK inhibition is an attractive therapeutic option in AICID. The results of the first study exploring the safety and tolerability (phase I) of a TWEAK-blocking therapy using the monoclonal antibody BIIB023 in patients with RA have been recently published (3). They demonstrated that a single-dose administration of BIIB023 was well tolerated in patients with RA and was associated with a suppression of sTWEAK levels for up to 28 days in the case of BIIB023 high dose. The efficacy of BIIB023 (phase II) is now explored in the ongoing clinical trial ATLAS (Anti-TWEAK in Lupus Nephritis patients) for patients with lupus nephritis, a pathology that requires a more innovative treatment than RA. In this context, quantification of soluble TWEAK in anti-TWEAK treated patients is an interesting way for managing BIIB023 administration.

TWEAK EVALUATION IN AICID: HOW TO PROCEED?

WHICH FORM OF TWEAK TO QUANTIFY: MEMBRANE AND/OR SOLUBLE FORM?

TWEAK expression has been studied in various AICID animal models and in humans (11–14). The main cellular sources of TWEAK are cells from the monocyte/macrophage family including immune infiltrating cells in tissues during inflammation. TWEAK can expressed as a type II transmembrane protein or efficiently cleaved to generate sTWEAK. Elevated levels of sTWEAK can be found locally at the site of diseased tissue during AICID. The presence of membrane-bound TWEAK has also been described in human cells (15–17) but not in other studies of freshly isolated peripheral blood mononuclear cells (PBMCs) from normal subjects or patients with RA or SLE (Linda Burkly, personal communication). Nevertheless the relevance of one form versus the other is not yet elucidated. Modulation of the soluble form levels rather than the membrane form is more frequently studied and published.

WHICH SAMPLE TO COLLECT AND TO USE FOR TWEAK QUANTIFICATION?

Soluble TWEAK is currently quantified in patient serum due to the facility of routine blood collection by venous puncture. But depending on the target tissue involved in AICID soluble TWEAK could be quantified in various other body fluids such as synovial fluid (18), cerebrospinal fluid (17), or urine (19) and may better reflect inflammatory activity in respectively the joint, the brain, and the kidney. TWEAK expression during AICID has also been studied in PBMCs by western blot and PCR. In SLE, elevation of TWEAK in PBMC is correlated with disease activity and lupus nephritis (20). In SSc, the production of soluble TWEAK by cultured PBMC is significantly diminished in patients with more severe microvascular damage as indicated by capillaroscopy (21). In the context of neuroinflammation, Serafini et al. reported strong TWEAK expression on post-mortem MS brain tissue while TWEAK was generally undetectable by immunohistochemistry in the normal human brain white matter or cerebral cortex samples (11). Even if these results are very interesting for further evaluation of the role of TWEAK in MS physiopathology, these approaches are not appropriate for routine diagnosis of MS. Desplat-Jégo et al. have shown by using PBMC analysis after culture that TWEAK is expressed at the cell surface of circulating monocytes during MS and not during non-MS neurological inflammatory diseases (17). This study also showed that TWEAK was detectable in the serum and CSF of MS patients but found no significant difference between the MS group and other clinical groups.

Table 1 | "Normal" mean values and ranges for serum soluble TWEAK in humans determined by ELISA.

Publication reference	ELISA kit manufacturer	Number of control healthy subjects tested	Serum soluble TWEAK levels (pg/ml) Mean ± SD (ranges)
Park et al. (9)	Bender Medsystems	40	42.7 ± 14.5
Yanaba et al. (10)	Bender Medsystems	31	41.5 ± 33.5
Yilmaz et al. (26)	Bender Medsystems	55	445 (326–634)
Chorianopoulos et al. (27)	Bender Medsystems	30	377 (310–432)
Xia et al. (28)	Bender Medsystems	45	34.8 (15–54)
Kowal-Bielecka et al. (25)	Bender Medsystems	48	294 ± 147 (132–1015)
Turkmen et al. (29)	Bender Medsystems	25	457 (320–538)
Desplat-Jégo, personal results	Bender Medsystems	58	467 (200–866)
Schwartz et al. (5)	Home-made ELISA	19	23.5 (17.3–27.1)
Llaurado et al. (30)	R&D Systems	68	1636 (1146–3754) for men and 1401 (788–2422) for women

SD, standard deviation.

In RA, data on TWEAK/Fn14 expression in the synovium of patients are available (12, 18) and support a high level of TWEAK in RA synovial tissue concordant with the numerous activated macrophages found in synovium during the disease and with the beneficial effect of TWEAK blockade in a mouse model of RA.

TO QUANTIFY TWEAK ALONE OR ASSOCIATED WITH OTHER(S) BIOLOGICAL PARAMETER(S)?

Panel-based approaches measuring a combination of biological parameters are more and more used for routine biological diagnosis of human diseases. Very few studies have addressed the diagnostic performance of associating TWEAK with one or several other markers.

Moreno et al. have reported that soluble CD163/soluble TWEAK ratio in peripheral blood is a more sensitive biomarker of subclinical atherosclerosis than soluble CD163 or soluble TWEAK alone (22). CD163 is a member of the scavenger receptor cysteine-rich family now recognized as an immunomodulator of the atherosclerotic plaque. It has been previously proposed that TWEAK could specifically bind and neutralize TWEAK and that CD163-expressing monocytes/macrophages were able to bind and internalize exogenous TWEAK (23). However this interaction has recently failed to be confirmed (24). Nonetheless, Kowal-Bielecka et al. have recently shown that a high serum soluble CD163/soluble TWEAK ratio is associated with lower risk of digital ulcers but with a more severe skin disease in patients with SSc (25).

In the case of spot urine exploration of soluble TWEAK, it is recommended to normalize TWEAK levels to urinary creatinine measured in the same spot urine since creatinine excretion rate into the urine is relatively constant and allows to compensate for the variability in the volume of urine and the concentrations of soluble TWEAK from void to void (5).

TWEAK EVALUATION IN AICID: HOW TO DEFINE PATHOLOGICAL VALUES?

In the first place, normal value ranges have to be determined prior to the definition of pathological values for sTWEAK levels in humans. A threshold for pathological TWEAK levels has to be established. It is not an easy task. Different ranges of normal values issued from various laboratory teams are available (**Table 1**) and there is obviously a lack of homogeneity. The differences observed in soluble TWEAK levels in healthy control subjects depend presumably on various parameters: age, sex, ethnic origin of the recruited control group, pre-analytical conditions especially sample storage conditions before analysis, the technical characteristics of the ELISA kits, nature of TWEAK epitopes recognized by anti-TWEAK antibodies, capacity of soluble TWEAK to trimerize etc. **Table 1** summarizes the data obtained by 10 teams and shows the difficulty in obtaining to date consensual normal values for sTWEAK levels.

In urine, LN patients have been compared with control subjects but data obtained in this latter group was detailed by only one team in 23 healthy volunteers with a reference value for uTWEAK of 5.67 pg/mg of creatinine (interquartile range: 3.10) (5, 6, 19).

In the case of other biological fluids (CSF, synovial fluid) data are lacking to establish normal ranges especially because collections of such fluids are invasive and not allowed in control healthy subjects by ethic committees.

IN CONCLUSION

On the one hand, quantification of uTWEAK in AICID seems to be an interesting tool in the follow-up of disease activity especially in lupus nephritis. On the other hand it is premature to propose sTWEAK as a diagnostic tool of AICID due to the lack of specificity for one disease in particular. Moreover studies analyzing diagnostic performance of serum sTWEAK are still needed. sTWEAK assay is nevertheless useful in the follow-up of anti-TWEAK biotherapies and perhaps could be useful in the follow-up of anti-TNFα therapies but this latter point needs to further be evaluated. At this point, the most important criticism concerning TWEAK evaluation in AICID is the difficulty in obtaining consensual ranges for normal values of sTWEAK. Reagents for the assessment of TWEAK levels are now commercially available for research purposes only. However, standardization of the proposed kits is absolutely required all the more because free soluble TWEAK is a "sticky" protein difficult to manipulate based on experience from our team

and others (31). Moreover comparison between studies using different reagents must be conducted carefully. It is our opinion that the standardization of data collection is critical to further evaluate the clinical utility of TWEAK as a promising biomarker.

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TWEAK/Fn14 signaling axis mediates skeletal muscle atrophy and metabolic dysfunction

Shuichi Sato, Yuji Ogura and Ashok Kumar*

Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, KY, USA

Edited by:
Timothy S. Zheng, Biogen Idec Inc., USA

Reviewed by:
Fulvio D'Acquisto, Queen Mary University of London, UK
Takayuki Yoshimoto, Tokyo Medical University, Japan

Correspondence:
Ashok Kumar, Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, 500 South Preston Street, Louisville, KY 40202, USA
e-mail: ashok.kumar@louisville.edu

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) through binding to its receptor fibroblast growth factor inducible 14 (Fn14) has been shown to regulate many cellular responses including proliferation, differentiation, apoptosis, inflammation, and fibrosis, under both physiological and pathological conditions. Emerging evidence suggests that TWEAK is also a major muscle wasting cytokine. TWEAK activates nuclear factor-κB signaling and proteolytic pathways such as ubiquitin–proteasome system, autophagy, and caspases to induce muscle proteolysis in cultured myotubes. Fn14 is dormant or expressed in minimal amounts in normal healthy muscle. However, specific atrophic conditions, such as denervation, immobilization, and starvation stimulate the expression of Fn14 leading to activation of TWEAK/Fn14 signaling and eventually skeletal muscle atrophy. TWEAK also causes slow- to fast-type fiber transition in skeletal muscle. Furthermore, recent studies suggest that TWEAK diminishes mitochondrial content and represses skeletal muscle oxidative phosphorylation capacity. TWEAK mediates these effects through affecting the expression of a number of genes and microRNAs. In this review article, we have discussed the recent advancements toward understanding the role and mechanisms of action of TWEAK/Fn14 signaling in skeletal muscle with particular reference to different models of atrophy and oxidative metabolism.

Keywords: TWEAK, Fn14, skeletal muscle, atrophy, oxidative phosphorylation

INTRODUCTION

Skeletal muscle is the largest tissue of the human body, which provides posture, ensures basic functions such as locomotion and respiration, and plays a vital role in whole body metabolism. Skeletal muscle is also one of the most highly plastic tissues in the body. A number of extracellular and intracellular signals have now been identified, which cause physiological adaptations including enhanced substrate metabolism, mitochondrial biogenesis, angiogenesis, muscle growth, and regeneration (1, 2). For example, exercise training causes improvement in muscle mass and contractility, and metabolic function resulting in enhanced force generation capacity and resistance to fatigability. Conversely, inactivity and many chronic disease states result in loss of skeletal muscle mass and metabolic dysfunction (3, 4). Maintenance of skeletal muscle mass and function are prerequisites for whole body health throughout life.

Skeletal muscle mass is maintained through a delicate balance between protein synthesis and degradation. Resistance exercise, hormones, and nutritional uptake increase rate of protein synthesis resulting in increased muscle mass (5). Insulin-like growth factor-1 (IGF-1)/phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR is one of the most important signaling pathways, which increases protein synthesis leading to skeletal muscle hypertrophy (6, 7). This pathway also inhibits muscle protein degradation through distinct mechanisms (8, 9). By contrast, many catabolic stimuli increase the activity of various signaling intermediates, such as extracellular-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), and TNF receptor-associated factors (TRAFs) and transcription factors, such as nuclear factor-κB (NF-κB), activator protein 1 (AP1), p53, FoxO1, and FoxO3a resulting in the activation of ubiquitin–proteasome system (UPS) and autophagy–lysosomal system (ALS), the two major proteolytic mechanisms in skeletal muscle (1, 10, 11). Recent studies also suggest that activating transcription factor 4 (ATF4), growth arrest and DNA damage-inducible 45α (GADD45α), histone deacetylase 4 (HDAC4), and myogenin mediate skeletal muscle atrophy under specific conditions (12–14). Furthermore, changes in the mitochondrial content, integrity, and function play a critical role in regulation of skeletal muscle mass and function (15, 16). The biogenesis of new mitochondria and clearance of defunct mitochondria are essential to meet cellular energy demand especially during endurance exercise and to protect from many chronic diseases such as diabetes, heart failure, obesity, and cancer (17, 18). Impairment in mitochondrial function is also an important facet of aged skeletal muscle. However, it is not yet clear whether dysfunctional mitochondria are a cause or consequence of aged skeletal muscle (19).

Classical proinflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6, and interferon-γ have been suggested as important mediators of catabolic response (protein loss and insulin resistance), contractile dysfunction, and disruption of muscle regenerative ability in many chronic disease states (20–24). Although, neutralization of some of these

cytokines attenuates muscle wasting, blocking their activity failed to preserve skeletal muscle mass in some conditions where even their abundance was significantly elevated suggesting that there are additional mediators of muscle wasting (24). TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily, has recently been identified as an important mediator of skeletal muscle wasting and metabolic dysfunction. While the levels of several classical muscle wasting cytokines are increased in various chronic disease states, there is no evidence about their potential role in the loss of muscle mass in disuse conditions (e.g., denervation, immobilization, and unloading). Our studies have shown that TWEAK/Fn14 system also mediates skeletal muscle wasting in disuse conditions and even in response to starvation. Moreover, increased levels of TWEAK inhibit skeletal muscle regenerative capacity through affecting self-renewal of satellite cells and proliferation, fusion, and differentiation of myoblasts into multinucleated myotubes both *in vitro* and *in vivo* (25–29). The role of TWEAK/Fn14 signaling in regulation of muscle progenitor cell biology has been discussed in another article in this issue (30). We have focused our review article to discuss the recent advancement toward understanding the role and mechanisms of action of TWEAK in adult skeletal muscle.

BRIEF OVERVIEW TWEAK/Fn14 DYAD

TWEAK is a member of TNF super family (TNFSF) cytokines, which is initially synthesized as type II transmembrane protein (249 amino acids) similar to TNF-α. However, membrane-bound TWEAK is cleaved to its soluble form (156 amino acids) by furin, a calcium-dependent serine endoprotease (31–33). Fn14, a type I transmembrane protein, was first recognized by differential display analysis and later identified as the unique TWEAK receptor (34–36). Fn14 is the smallest member (102 amino acids) of TNF receptor super family (TNFRSF), which is expressed at relatively low levels in normal healthy tissues except in progenitor cells. However, the expression of Fn14 is drastically increased in response to tissue injury and various pathological conditions (31, 33). Similar to other members of TNFRSF, the cytoplasmic domain of Fn14 contains a TRAF binding site, which allows downstream signaling upon stimulation by TWEAK (37). By contrast, Fn14 lacks a death domain and does not show any association with death-inducing signaling complex (33). Furthermore, some studies suggest that Fn14 can also function by clustering themselves on the cell membrane under low TWEAK condition. TWEAK/Fn14 system plays an important role in tissue homeostasis through regulating cell survival, proliferation, migration, and angiogenesis (31, 33). Conversely, aberrant regulation of TWEAK/Fn14 signaling causes many pathological consequences including cancer, rheumatoid arthritis, systemic lupus erythematous, multiple sclerosis, skeletal muscle wasting, and cardiac dysfunction and failure (31).

Like other members of TNFSF, TWEAK mediates unique and context-dependent pleiotropic effects. In contrast to TNF-α, TWEAK attenuates the transition from innate to adaptive immunity by suppressing the production of interferon-γ and IL-12 cytokines (38). Moreover, TWEAK activates multiple intracellular pathways such as MAPK, PI3K/Akt, and canonical and non-canonical NF-κB signaling in various cell types (39–42). Binding of TWEAK leads to the formation of TRAF2/cIAP1 (cellular inhibitor of apoptosis protein 1) complex at Fn14 cytoplasmic domain that results in the activation of various signaling proteins including TRAF6, transforming growth factor-β activated kinase1 (TAK1), I kappa β kinase (IKK), NF-κB-inducing kinase (NIK), and MAPKs, which regulate expression of several molecules involved in various cellular responses (**Figure 1**) (33, 39, 43–45). Moreover, the activation of TWEAK/Fn14 is coupled with TNFα–TNFR1 signaling. For example, the activation of TWEAK/Fn14 signaling sensitizes TNF-α signaling by exhausting cytosolic TRAF2/cIAP1 complex (44).

TWEAK CAUSES ATROPHY IN CULTURED MYOTUBES

C2C12 as well as mouse primary myoblasts differentiate into myotubes upon incubation in low serum conditions. These cultured myotubes serve as an excellent model to study atrophy in response to exogenously added molecules. We have previously reported that treatment of cultured myotubes with even low concentrations of TWEAK protein causes severe atrophy (46, 47). Multiple studies have shown that skeletal muscle atrophy is associated with rapid degradation of selective muscle proteins including myosin heavy chain (MyHC) (48–50). Consistent with effects on myotube size, addition of TWEAK led to significant reduction in overall protein content and levels of MyHC in cultured myotubes (46, 47). Moreover, at equimolar concentration, TWEAK was found to be more potent in inducing MyHC degradation compared with its structural homolog TNF-α suggesting that TWEAK is a potent muscle wasting cytokine (47).

The UPS is one of the most important proteolytic systems that cause selective degradation of muscle structural proteins (1, 11, 51). Two muscle-specific E3 ubiquitin ligases, muscle RING-finger 1 (*MuRF1*) and muscle atrophy F-box (*MAFBx*; also known as Atrogin-1) are the key enzymes of the UPS involved in degradation of muscle proteins. Levels of both MuRF1 and MAFBx are drastically increased in atrophying skeletal muscle, where disruption of these genes in mice preserves skeletal muscle mass in many catabolic conditions, such as hind limb unloading, cast immobilization, and denervation (1, 11, 52). ALS is another proteolytic mechanism that contributes to degradation of muscle proteins and cellular organelles in skeletal muscle in response to various atrophic stimuli. In autophagy, cytosolic proteins and organelles are sequestered by an isolation-membrane structure to form autophagosome. The autophagosome then fuses with lysosome, where the autophagosome is degraded to amino acid or peptide (53). Autophagy constitutively takes place at lower levels in most cells under normal condition and is an essential component of homeostasis machinery in different tissues including skeletal muscle (53, 54). However, excessive activation of autophagy leads to muscle wasting in many conditions such as starvation and denervation (8, 55).

Our studies have shown that TWEAK increases the gene expression of MuRF1 and MAFBx and stimulates the conjugation of ubiquitin with MyHC in cultured myotubes (46, 47). Furthermore, TWEAK also induces the expression of several components of ALS and activates caspases in cultured myotubes (46). Indeed, inhibition of the activity of either UPS, autophagy, or caspases

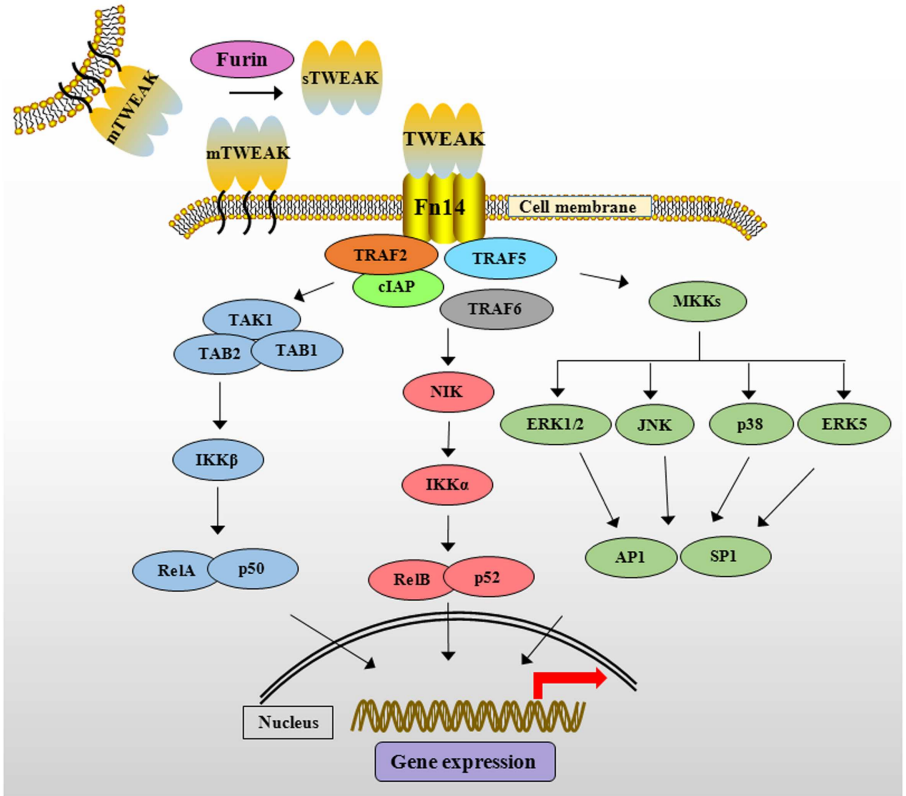

FIGURE 1 | The TWEAK/Fn14 signaling cascade. TWEAK cytokine is initially synthesized as membrane-anchored protein (mTWEAK). mTWEAK is then cleaved by furin into soluble form of TWEAK (sTWEAK). Binding of trimeric mTWEAK or sTWEAK to Fn14 receptor leads to the recruitment of cIAPs and various TRAFs resulting in the activation of multiple downstream signaling cascades. TWEAK stimulates canonical NF-κB pathway through activation of TAK1 and IKKβ and phosphorylation and degradation of IκB protein. TWEAK activates non-canonical NF-κB pathway through activation of NIK and IKKα leading to proteolytic processing of p100 protein into p52. TWEAK-mediated signaling also causes the activation of various MKKs, which phosphorylate ERK1/2, ERK5, JNK, and p38MAPK resulting in the activation of downstream transcription factors such as AP1 and SP1. TWEAK, TNF-like weak inducer of apoptosis; Fn14, fibroblast growth factor inducible 14; cIAP, cellular inhibitor of apoptosis; TRAF, TNF receptor-associated factor; TAK1, transforming growth factor β-activated kinase 1, TAB, TAK1 binding protein; IKK, IκB kinase β; IκB, inhibitor of nuclear factor of kappa B; NIK, NF-κB-inducing kinase; MAPK, mitogen-activated protein kinase; MKKs, mitogen-activated protein kinase kinases; ERK, extracellular signal-regulating kinase; JNK, c-Jun N-terminal kinase; AP1, activator protein 1; SP1, specificity protein 1.

significantly reduced TWEAK-induced degradation of MyHC and myotube atrophy suggesting that TWEAK induces muscle proteolysis through coordinated activation of multiple proteolytic systems (46).

NF-κB is a major proinflammatory transcription factor, which is strongly linked to skeletal muscle wasting not only in chronic diseases but also in disuse conditions (10). Inhibition of NF-κB has been found to rescue skeletal muscle atrophy in response to cytokines, tumor growth, denervation, and unloading (10, 56). By contrast, forced activation of NF-κB is sufficient to cause severe muscle wasting in mice (56). NF-κB induces muscle atrophy through augmenting the expression of MuRF1 and several other components of UPS (10). It has been recently reported that MuRF1 causes breakdown of MyHC and other components of the thick filament of the sarcomere during atrophy (57, 58). Our studies have shown that TWEAK causes sustained activation of NF-κB. Furthermore, inhibition of NF-κB through pharmacological or molecular approaches prevents the degradation of MyHC and atrophy in cultured myotubes (46, 47, 59, 60). Although, TWEAK did not inhibit protein synthesis, it inhibited the activation of PI3K/Akt/mTOR signaling pathway in cultured myotubes (47). Similarly, TWEAK has also been found to inhibit the insulin-induced Akt phosphorylation in hepatocytes (61). Since Akt signaling inhibits the activity of FoxO family of transcription factors, which are required for the expression of MuRF1 and MAFBx (1), it is likely that the inhibition of Akt signaling is another mechanism by which TWEAK augments the gene expression of MuRF1 and MAFBx in cultured myotubes. Collectively, these observations suggest that TWEAK causes atrophy in cultured myotubes through the activation of NF-κB and various proteolytic systems such as UPS, ALS, and caspases (**Figure 2**).

The effect of TWEAK on global gene expression and microRNAs (miRs) in cultured myotubes has also been investigated. Pathway analysis of microarray gene expression data suggested that TWEAK affects the activation of several toxic pathways including those involved in initiation and manifestation of fibrosis, oxidative stress, and mitochondrial dysfunction in skeletal muscle (62). For example, TWEAK reduces the expression of structural molecules

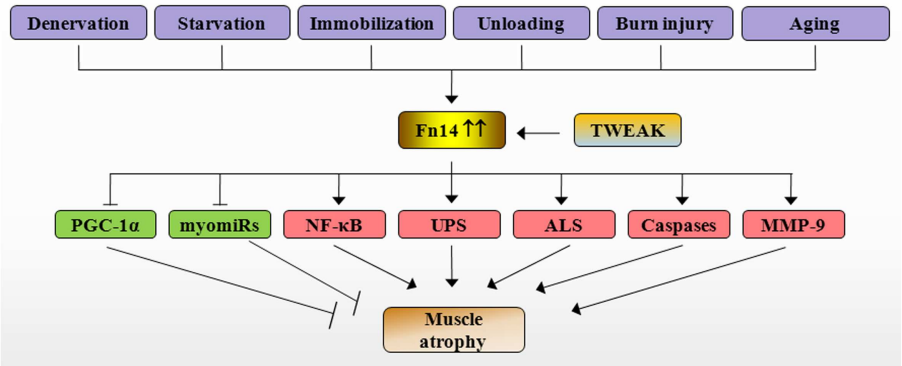

FIGURE 2 | Mechanisms of action of TWEAK/Fn14 system in skeletal muscle atrophy. Specific catabolic conditions such as denervation, starvation, immobilization, unloading, burn injury, and aging augments the expression of Fn14 resulting in stimulation of TWEAK/Fn14 signaling in skeletal muscle. TWEAK-Fn14 signaling represses the levels of PGC-1α and myomiRs and increases the activation of NF-κB, UPS, ALS, caspases, and MMP-9, which eventually cause loss of skeletal muscle mass. Fn14, fibroblast growth factor inducible 14; TWEAK, TNF-like weak inducer of apoptosis; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-kappa B; UPS, ubiquitin–proteasome system; ALS, autophagy–lysosome system; myomiRs, muscle microRNAs.

(e.g. MyHC, TCap, and ankyrin repeat domain 2), metabolic enzymes (e.g., phosphoglycerate mutase 2), and signaling proteins (e.g., Notch1 and TRAF6) in C2C12 myotubes. By contrast, TWEAK induces the expression of multiple components of NF-κB signaling pathways and expression of matrix metalloproteinase-9 (MMP-9) in myotubes (62). Recently, a few muscle-specific miRs such as miR-1, miR-133a, miR-133b, and miR-206 (also called myomiRs) have been identified, which are essential for muscle progenitor cell proliferation, differentiation, and maintenance. Expression of miR-1 and miR-133a in embryonic stem cells and other non-muscle cell types showed that they promote the differentiation into the skeletal muscle lineage. Unlike other myomiRs, which are also expressed in cardiac tissues, miR-133b and miR-206 are predominantly expressed in skeletal muscle (63). Interestingly, microarray analysis of miRs has shown that TWEAK reduces the expression levels of miR-1, miR-133a, miR-133b, and miR-206 in cultured myotubes (62). Recent studies have demonstrated that myogenic transcription factors, such as myocyte enhancer factor 2c (MEF2c) and MyoD control the expression of myomiRs in skeletal and cardiac muscles (63). Interestingly, TWEAK diminishes the levels of both MEF2c and MyoD (25, 62) suggesting that reduced expression of myomiRs could be a result of diminished abundance of MEF2c and MyoD in TWEAK-treated myotubes. TWEAK also regulates the expression of a number of other miRs, which are involved in distinct biological responses. Using differentially regulated miR data set, we generated a network of pathways from differentially regulated genes in cDNA microarray data set. This analysis showed that miRs network overlaps with mRNA networks suggesting that miRs may play important roles in the regulation of gene expression and skeletal muscle structure and function in response to TWEAK (62).

TWEAK CAUSES SKELETAL MUSCLE ATROPHY *IN VIVO*

In vivo effects of TWEAK on skeletal muscle were first investigated by systemic and chronic administration of soluble TWEAK proteins in wild-type mice. Chronic administration of TWEAK in mice led to a significant reduction in whole body weight, individual muscle mass, and fiber-cross-sectional area (47). Furthermore, transgenic (Tg) overexpression of full-length TWEAK using muscle creatine kinase (MCK) promoter resulted in smaller muscle size and neonatal lethality in mice (47). TWEAK-Tg mice, which survived due to relatively low expression of TWEAK (four to sixfolds higher than littermate controls) showed muscle atrophy and interstitial fibrosis at around 4–6 months of age (43). Consistent with *in vitro* studies, transcript levels of MuRF1 and ubiquitination of MyHC were significantly elevated in skeletal muscle of TWEAK-Tg mice compared with their littermate controls. By contrast, there was no significant difference in mRNA levels of MAFBx in skeletal muscle of wild-type and TWEAK-Tg mice indicating that TWEAK causes muscle atrophy *in vivo* through augmenting the expression of MuRF1 (43, 62). Furthermore, the gene expression of several components of NF-κB signaling pathway and DNA-binding activity of NF-κB were significantly increased in skeletal muscle of TWEAK-Tg mice (43). These findings suggest that the activation of NF-κB pathway is one of the important mechanisms for TWEAK-induced muscle proteolysis (**Figure 2**).

Accumulating evidence suggests that peroxisome proliferator-activated receptor γ (PPAR-γ) coactivator 1α (PGC-1α) plays a key role in preserving skeletal muscle mass and mitochondrial content in atrophic conditions (64). Levels of PGC-1α are repressed in multiple atrophy conditions whereas muscle-specific overexpression of PGC-1α rescues the loss of skeletal muscle mass in catabolic states (65, 66). Our recent studies demonstrate that TWEAK represses the expression of PGC-1α in cultured myotubes (67). Overexpression of PGC-1α inhibits TWEAK-induced atrophy, NF-κB activation, and expression of MAFBx and MuRF1 in cultured myotubes. Moreover, progressive muscle atrophy observed in TWEAK-Tg mice is significantly attenuated in TWEAK-PGC-1α double Tg mice suggesting that PGC-1α plays an important role in TWEAK-induced muscle atrophy (67).

Another potential mechanism by which TWEAK causes muscle atrophy is through deregulation of matrix metalloproteinases

(MMPs). Protein levels as well as enzymatic activity of MMP-9 are significantly elevated in skeletal muscle of TWEAK-Tg mice (59, 62). Since, muscle atrophy in response to chronic administration of TWEAK is rescued to some extent in *Mmp9*-knockout (KO) mice, it is likely that MMP-9 is involved in TWEAK-induced skeletal muscle wasting *in vivo*. Elevated levels of MMP-9 can potentially induce muscle atrophy through breakdown of extracellular matrix and proteolytic activation of other catabolic cytokines (59). In agreement with microarray data in TWEAK-treated myotubes, we have found that the levels of many structural molecules such as MyHC, TCap, and ankyrin repeat domain 2 and myomiRs were reduced in skeletal muscle of TWEAK-Tg mice suggesting that TWEAK also causes atrophy through repression of specific muscle proteins (62).

TWEAK/Fn14 SYSTEM MEDIATES MUSCLE ATROPHY IN CATABOLIC CONDITIONS

Muscle atrophy can be observed in various physiological and pathophysiological conditions such as bed-rest, space flight, aging, cancer, severe burn injury, starvation, high dose glucocorticoid therapy, and neurotmesis (2). Our group and others have recently investigated whether the gene expression of TWEAK or Fn14 is affected in skeletal muscle in different atrophy conditions. Interestingly, the gene expression of Fn14 is dramatically induced in skeletal muscle in disuse conditions such as denervation and immobilization (43) and in response to starvation (48). Wu et al. studied the global gene expression in skeletal muscle of mice in response to hind limb suspension, a model to elicit unloading-induced skeletal muscle atrophy. Microarray and quantitative real-time PCR assays showed that the gene expression of Fn14 is significantly increased in skeletal muscle of mice (68). Moreover, a recent study has also demonstrated that the levels of TWEAK and Fn14 are increased in human skeletal muscle in response to severe burn injury (69). By contrast, mRNA levels of TWEAK and Fn14 remained unaffected in skeletal muscle of mice in response to high doses of glucocorticoids (43) and in skeletal muscle of patients with chronic obstructive pulmonary disease (70) indicating that TWEAK/Fn14 system may not be the mediator of muscle atrophy in all conditions.

The physiological role of TWEAK/Fn14 system in muscle atrophy has been investigated through a series of experiments using denervated hind limb muscle as a model of neurotmesis (43). Levels of Fn14 protein are dramatically increased upon denervation regardless of muscle fiber type (43). Denervation-induced loss of skeletal muscle mass and fiber cross-sectional-area were significantly exaggerated in TWEAK-Tg mice but ameliorated in TWEAK-KO mice compared with wild-type mice. Denervated soleus muscle of TWEAK-KO mice showed improved absolute force compared with corresponding wild-type mice. Skeletal muscle of TWEAK-Tg mice also showed increased gene expression of collagen I and collagen III and interstitial fibrosis upon denervation indicating that TWEAK can directly induce fibrosis in skeletal muscle tissues. Similar to cell culture studies, TWEAK stimulated the DNA-binding activity of NF-κB and increased the gene expression of MuRF1 in denervated skeletal muscle (43). Furthermore, starvation-induced loss of skeletal muscle mass was reduced in TWEAK-KO mice compared with wild-type mice (43). Collectively, these studies provide strong evidence that TWEAK/Fn14 system mediates muscle atrophy in catabolic conditions (**Figure 2**). It is noteworthy that denervation and starvation did not affect the expression TWEAK in skeletal muscle of mice. Similar to studies in rodents, a recent study reported that chronic hemiplegia-induced muscle atrophy was not accompanied with changes in expression of TWEAK in humans (71).

While TWEAK affects the activation of a number of signaling pathways and proteolytic systems in cultured myotubes, only a few of them appear to be regulated by TWEAK in skeletal muscle of mice in atrophic conditions. For example, TWEAK inhibits the activity of PI3K/Akt pathway in cultured myotubes; however, there was no difference in level of phosphorylation of Akt in denervated muscle of wild-type, TWEAK-Tg and TWEAK-KO mice. Furthermore, while denervation increased expression of several autophagy-related genes (such as LC3B, Beclin1, Atg5, Atg12, and Gabarapl1), their expression levels were comparable in denervated skeletal muscle of wild-type, TWEAK-Tg and TWEAK-KO mice (43). These results suggest that denervated skeletal muscle is enriched with certain growth factors, which neutralize some of the actions of TWEAK. However, we cannot rule out that TWEAK/Fn14 signaling also affects the activation of PI3K/Akt and ALS in other conditions of muscle atrophy.

REGULATION OF Fn14 EXPRESSION IN SKELETAL MUSCLE

While, it is increasingly clear that the expression of Fn14 is a rate-limiting step, molecular mechanisms leading to its increased expression in skeletal muscle in atrophic conditions remain poorly understood. Both human and mouse Fn14 promoters lack a typical TATA box, which is generally required for the expression of mammalian genes. However, the promoter region of Fn14 contains consensus sequence for several transcription factors (72). Recent studies in our laboratory have shown that mouse and human Fn14 promoters contain a cytosine–guanine dinucleotide (CpG) island close to transcription start site. Fn14 promoter also contains consensus DNA sequence for AP1 and specificity protein 1 (SP1) transcription factors. Our studies suggest that denervation causes hypomethylation at specific CpG sites in mouse Fn14 promoter in skeletal muscle. Interestingly, while all three DNA methyltransferases (Dnmts) interact with Fn14 promoter in naïve muscle, the levels of DNA methyltransferase 3a (Dnmt3a) are repressed in denervated skeletal muscle of mice. The role of Dnmt3a in Fn14 expression has been supported by our findings that overexpression of Dnmt3a inhibits the expression of Fn14 and attenuates denervation-induced muscle atrophy. We have also found that denervation increases the activation of MAPK, AP1, and SP1 and they are involved in the expression of Fn14 in denervated skeletal muscle.

TWEAK PROMOTES SLOW-TO-FAST FIBER TYPE TRANSITION IN SKELETAL MUSCLE

Rodent skeletal muscle contains four fiber types based on the primary expression of MyHC: I, IIA, IIX, and IIB (73, 74). Type I fibers are slow to twitch by stimuli, display a two to three-fold higher mitochondrial content, and rely largely on oxidative metabolism to produce ATP. In contrast, type II fibers are fast

in responding to stimuli and rely on glycolytic metabolism as a major energy substrate (75). Type IIB fibers have relatively few mitochondria and store large amounts of glycogen; therefore they are glycolytic in metabolism. Types IIA and IIX fibers have physiologically intermediate properties of slow and fast twitch fibers, which are also rich in mitochondria and possess a relatively high capacity to generate ATP by oxidative metabolism (76, 77). Moreover, it is now increasingly evident that different fiber types display different sensitivity to atrophy stimuli. Oxidative fibers are somewhat resilient to atrophy upon denervation (78) whereas glycolytic fibers undergo more rapid atrophy in response to starvation or sepsis (79, 80).

TWEAK/Fn14 axis appears to be an important regulator of fiber type composition in skeletal muscle. Tg overexpression of TWEAK in mice causes a significant reduction in proportion of type I fibers with a concomitant increase in type II fibers in both soleus and extensor digitorum longus muscle (43). Moreover, hind limb muscle of the founder TWEAK-Tg mice, which could not survive beyond neonatal age, appeared pale compared with wild-type littermates indicating lower myoglobin abundance and fast muscle phenotype in TWEAK overexpressing muscle (47). By contrast, proportion of types I and IIA muscle fibers and skeletal muscle mitochondrial content was found to be increased in skeletal muscle of TWEAK-KO mice (43, 81). These results suggest that TWEAK favors fast-type fiber phenotype even under normal conditions.

Recent studies have shown that PGC-1α plays an important role in regulating skeletal muscle fiber composition, mitochondrial content, and oxidative metabolism in both physiological and pathophysiological conditions (64, 82, 83). PGC-1α is expressed preferentially in skeletal muscle enriched in type I fibers. Positive role of PGC-1α in inducing type I fibers is supported by the findings that Tg overexpression of PGC-1α in skeletal muscle increases the proportion of types I and IIA fibers (84). Conversely, muscle-specific PGC-1α KO mice exhibited a shift from oxidative types I and IIA toward types IIX and IIB muscle fibers (85) while global PGC-1α KO mice did not show a marked skeletal muscle phenotype (85, 86). PGC-1α drives the transcription of type I muscle fibers through co-activation of myocyte enhancer factor 2 (MEF2) family members such as MEF2c and MEF2d (84). MEF2 and PGC-1α also regulate the expression of PGC-1α in an auto-regulatory loop (87). Interestingly, our recent studies have shown that TWEAK suppresses the expression of MEF2c in C2C12 myotubes (62). Therefore, it is likely that TWEAK increases the number of type II fibers through reducing MEF2/PGC-1α signaling (**Figure 3**). Supporting this notion, our recent study has demonstrated that transcript levels of PGC-1α and several molecules involved in mitochondrial oxidative metabolism are significantly increased in skeletal muscle of 5-month-old TWEAK-KO mice. Furthermore, treatment of cultured primary myotubes with TWEAK drastically reduced levels of PGC-1α and other mitochondria-related genes (81). Likewise, an inverse relationship between TWEAK and PGC-1α has been observed in denervated skeletal muscle of mice (67). Since type II muscle fibers are more susceptible to atrophy compared to type I muscle fibers in many chronic diseases, it will be interesting to investigate whether slow- to fast-type fiber transition is essential for TWEAK-induced muscle atrophy.

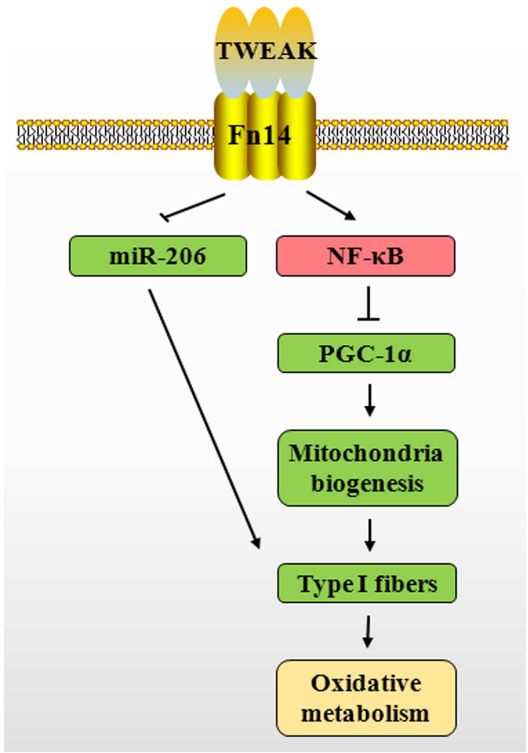

FIGURE 3 | Putative mechanisms by which TWEAK–Fn14 system inhibits skeletal muscle oxidative phosphorylation capacity. TWEAK/Fn14 system activates canonical NF-κB signaling pathway, which represses PGC-1α levels in skeletal muscle. This leads to reduced expression of several mitochondrial genes and proportion of type I fibers. TWEAK also decreases the expression of miR-206, a positive regulator of type I fibers. TWEAK, TNF-like weak inducer of apoptosis; Fn14, fibroblast growth factor inducible 14; NF-κB, nuclear factor-kappa B; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; miR-206, microRNA-206.

TWEAK INHIBITS SKELETAL MUSCLE OXIDATIVE METABOLISM

Skeletal muscle, due to its large mass, is the principal organ for glucose disposal in the body and therefore even a small reduction in skeletal muscle glucose uptake capacity or its ability to metabolize glucose is sufficient to cause metabolic abnormalities and obesity. The decline in skeletal muscle oxidative phosphorylation capacity during aging has been recognized as a major cause for increased fatigability, lowered quality of life, and morbidity (19). The loss of oxidative capacity with elevated levels of proinflammatory cytokines is also implicated in development of metabolic syndrome in many chronic disease states, while the maintenance with exercise has been found to be beneficial to some extent (88, 89).

We have recently studied the role of TWEAK in exercise tolerance and skeletal muscle oxidative phosphorylation capacity. TWEAK-KO mice run longer and with higher speed in a treadmill exercise tolerance test (81). Furthermore, TWEAK-KO mice show augmented levels of subsarcolemmal and intermyofibrillar mitochondria, increased succinate dehydrogenase (SDH)-positive

myofibers, and elevated gene expression of metabolic proteins such as PGC-1α, PPARδ, and mCPT-1. Moreover, oxidative phosphorylation is also increased in exercised TWEAK-KO mice compared with wild-type mice. Consistent with studies in TWEAK-KO mice, treatment of cultured myotubes with TWEAK decreased mitochondrial biogenetic capacity and maximal respiratory activity. TWEAK also reduced the expression of PGC-1α and several mitochondrial genes in cultured myotubes (81). Recent studies also suggest that TWEAK represses PGC-1α in cultured cardiomyocytes and myotubes through the activation of canonical NF-κB signaling pathway (67, 90). Collectively, these studies suggest that the repression of PGC-1α is an important mechanism by which TWEAK reduces mitochondrial content and oxidative phosphorylation capacity in skeletal muscle and in other cell types.

There is also a possibility that TWEAK inhibits skeletal muscle oxidative phosphorylation capacity through affecting levels of various miRs. The expression of miRs is sensitive to cytokine levels and alternation of miRs in response to inappropriate cytokine simulation may result in disrupting metabolic homeostasis (91). A subset of muscle-specific miRs has been shown to play an important role in skeletal muscle development and metabolic adaptation (92). Abundance of miR-206 is significantly higher in slow-type skeletal muscle compared with fast-type muscle (93). Levels of miR-206 are diminished during slow-to-fast fiber type transition in response to unloading (94). Furthermore, levels of miR-206 in vastus lateralis have been found to be significantly decreased in patients with type II diabetes compared with healthy individuals (95). Our low-density miRNA array analysis has shown that TWEAK inhibits the expression of miR-206 in C2C12 myotubes (62). These findings suggest that TWEAK may also inhibit skeletal muscle oxidative metabolism through down-regulation of miR-206 levels (**Figure 3**).

CONCLUDING REMARKS

The studies summarized above indicate that TWEAK/Fn14 system plays an essential role in skeletal muscle remodeling and metabolism. Most of these observations suggest that TWEAK/Fn14 signaling causes loss of skeletal muscle mass and decreases skeletal muscle oxidative metabolism implying that the inhibition of this cytokine-receptor axis can be used as a therapy to maintain skeletal muscle mass and metabolic function. We believe that TWEAK/Fn14 system is among the most attractive drug targets to combat muscle wasting. TWEAK being an extracellular protein *per se*, TWEAK-dependent signaling can be blocked using a TWEAK neutralizing antibody or soluble Fn14-Fc decoy protein, which prevents TWEAK binding to Fn14 cell surface receptors. Indeed, these two reagents have been found to be effective in improving pathologic condition in mouse models of some other diseases where TWEAK/Fn14 signaling is elevated. Alternatively, small-molecule antagonists that prevent Fn14 trimerization or interaction of TWEAK with Fn14 can also be used to block catabolic actions of TWEAK in skeletal muscle.

Whereas the role of TWEAK/Fn14 signaling in skeletal muscle has become increasingly clear, there are still some outstanding questions that need to be addressed. For example, it is important to identify other conditions where TWEAK/Fn14 axis is a mediator of muscle wasting. Furthermore, it is important to investigate the mechanism by which TWEAK causes metabolic abnormalities and whether the inhibition of TWEAK/Fn14 signaling can prevent type II diabetes and obesity in response to high fat diet. It will also be interesting to examine whether TWEAK affects mitochondrial content through regulating mitochondria biogenesis, fusion, fission, or mitophagy. The role of various miRs in regulation of TWEAK-induced muscle atrophy and metabolic dysfunction also needs more investigation. Nevertheless, recent studies have provided strong evidence that TWEAK/Fn14 system is a major regulator of skeletal muscle mass and function.

AUTHOR CONTRIBUTIONS

Ashok Kumar conceptualized the study. Shuichi Sato, Yuji Ogura, and Ashok Kumar wrote the manuscript.

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Role of the TWEAK-Fn14-cIAP1-NF-κB signaling axis in the regulation of myogenesis and muscle homeostasis

Emeka K. Enwere[1], Eric C. LaCasse[2], Nadine J. Adam[2,3] and Robert G. Korneluk[2,3]*

[1] *Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada*
[2] *Solange Gauthier Karsh Molecular Genetics Laboratory, Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada*
[3] *Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada*

Edited by:
Timothy S. Zheng, Biogen Idec Inc., USA

Reviewed by:
Daisuke Kamimura, Osaka University, Japan
Hideki Ogura, Osaka University, Japan

***Correspondence:**
Robert G. Korneluk, Solange Gauthier Karsh Molecular Genetics Laboratory, Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute, 401 Smyth Road, Ottawa, ON K1H 8L1, Canada
e-mail: bob@arc.cheo.ca

Mammalian skeletal muscle maintains a robust regenerative capacity throughout life, largely due to the presence of a stem cell population known as "satellite cells" in the muscle milieu. In normal conditions, these cells remain quiescent; they are activated upon injury to become myoblasts, which proliferate extensively and eventually differentiate and fuse to form new multinucleated muscle fibers. Recent findings have identified some of the factors, including the cytokine TNFα-like weak inducer of apoptosis (TWEAK), which govern these cells' decisions to proliferate, differentiate, or fuse. In this review, we will address the functions of TWEAK, its receptor Fn14, and the associated signal transduction molecule, the cellular inhibitor of apoptosis 1 (cIAP1), in the regulation of myogenesis. TWEAK signaling can activate the canonical NF-κB signaling pathway, which promotes myoblast proliferation and inhibits myogenesis. In addition, TWEAK activates the non-canonical NF-κB pathway, which, in contrast, promotes myogenesis by increasing myoblast fusion. Both pathways are regulated by cIAP1, which is an essential component of downstream signaling mediated by TWEAK and similar cytokines. This review will focus on the seemingly contradictory roles played by TWEAK during muscle regeneration, by highlighting the interplay between the two NF-κB pathways under physiological and pathological conditions. We will also discuss how myogenesis is negatively affected by chronic conditions, which affect homeostasis of the skeletal muscle environment.

Keywords: TWEAK, Fn14, cIAP1, NF-κB signaling, myogenesis, myoblast fusion, muscle regeneration

INTRODUCTION

Skeletal muscle is comprised of multinucleated fibers that result from the fusion of hundreds or thousands of individual mononucleated progenitor cells. In addition to their highly specialized roles in the generation of force, individual muscle fibers are capable of extensive metabolic and functional plasticity. Skeletal muscle also exhibits robust regenerative capacity, as a means to recover from injury as well as to adapt to changing physical demands (1). A population of muscle-resident stem cells, known as satellite cells, resides within the laminin sheath encasing each muscle fiber, and is responsible for regeneration of muscle in the adult. These normally quiescent cells enter the cell cycle upon muscle injury, producing a transient and rapidly expanding population of committed progenitors or myoblasts. After several rounds of proliferation, the myoblasts enter a highly orchestrated differentiation program, wherein most exit the cell cycle, adopt biochemical and physiological characteristics of mature muscle, and fuse with each other to replace or repair the damaged tissue (**Figure 1**). The multiple steps in the process of muscle regeneration, beginning with satellite cell activation and ending with myoblast fusion, are all subject to separate levels of regulation, and are affected by a variety of muscle disorders and myopathies.

Investigation of the intracellular signaling pathways involved in muscle repair has been traditionally hampered by difficulties in accurately modeling the regenerative context *in vitro*. Recent developments in genetic and imaging techniques, however, have allowed new and detailed insights into many aspects of the repair process. These insights can be summarized under three major themes. First, myogenesis is comprised of several processes with distinct, and not necessarily complementary, regulatory, and signaling requirements. For instance, a pathway that promotes myoblast proliferation may have different and even inhibitory effects on the subsequent steps of myoblast differentiation, fusion, and muscle growth. Second, several cell populations co-exist with satellite cells in skeletal muscle; these other cells are either myogenic precursors [as in the case of pericytes surrounding blood vessels (2)] or non-myogenic contributors to the regenerative process, such as macrophages. Third, skeletal muscle engages in active signaling interplay with other tissue systems to maintain physiological homeostasis. Evidence in recent years has implicated the TWEAK-NF-κB signaling axis in several important functions associated with muscle damage and repair. This short review highlights both known and putative roles of TWEAK signaling in muscle biology. We emphasize recent discoveries that reflect the diverse and highly context-dependent effects of TWEAK on muscle regeneration and homeostasis. A companion article in this research topic by Sato et al. further discusses the critical importance of TWEAK signaling in skeletal muscle atrophy (3).

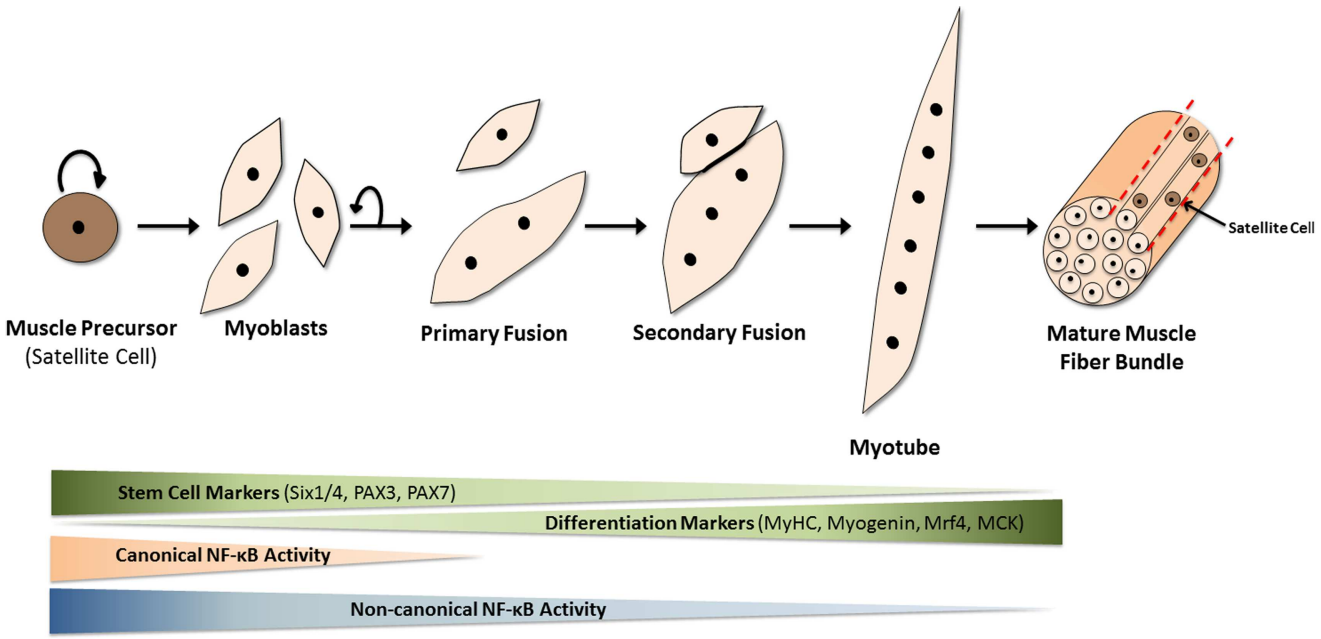

FIGURE 1 | Mammalian skeletal myogenesis. Upon muscle injury, a resident population of quiescent myogenic precursor cells, known as satellite cells (because they encircle muscle fibers), start to proliferate and differentiate into myoblasts. These mononuclear cells proliferate and fuse together (primary fusion step) to create multinucleated myocytes or myotubes over the course of several days. Additional myoblasts fuse to the existing myotubes in the secondary fusion step to produce even larger myotubes which eventually align to form muscle fibers. This differentiation process is regulated by many internal and external factors. Over the course of myogenesis, expression of stem cell markers such as Pax7 is gradually lost, while the appearance of differentiation markers, such as myosin heavy chain (MyHC), muscle-regulatory factor 4 (Mrf4), and muscle creatine kinase (MCK) gradually increases. The NF-κB pathways are now known to play significant roles in this differentiation process. The canonical (or classical) NF-κB pathway is needed for myogenic cell proliferation. However, upon the loss of canonical NF-κB signaling and the activation of non-canonical (or alternative) NF-κB signaling, myoblasts stop dividing and start fusing to form multinucleated myotubes, a key event in myogenesis.

REGULATION OF NF-κB SIGNALING BY TWEAK

Fibroblast growth factor-inducible protein 14 (Fn14/TNFRSF12A) is classified as a member of the tumor necrosis factor receptor (TNFR) superfamily based on its ability to bind TWEAK (TNFSF12), although it bears minimal sequence homology to other TNFR superfamily members (4, 5). Fn14 is also the smallest member of the TNFR superfamily; the proteolytically processed form that is present as a transmembrane receptor has only 102 amino acids (4, 6). Furthermore, the cytoplasmic tail contains a single TNF receptor-associated factor (TRAF)-binding domain but lacks a death domain motif normally found in several other TNFR superfamily members. The adaptor proteins TRAF-1, -2, -3, and -5 are able to bind to this site, and are essential for downstream pathway activation (5, 7, 8). Given the lack of other functional domains it is likely that all TWEAK–Fn14 signal transduction is due to interaction of Fn14 with one or more of these TRAF adaptors (9). While a comprehensive screen of TNF superfamily cytokines identified TWEAK as the only ligand able to interact with Fn14 (10), a number of reports suggests that the TWEAK–Fn14 pairing is not exclusive. For example, the scavenger receptor CD163 can bind and internalize TWEAK, though there is no evidence of signal transduction activity resulting from this interaction (11). A 2003 study (12) demonstrated the ability of TWEAK to induce robust differentiation of RAW264.7 murine macrophages, which do not express Fn14 (12, 13). Other studies have demonstrated the ability of Fn14 to activate canonical NF-κB signaling in the absence of TWEAK (7, 14). Furthermore, three separate studies reported that down-regulation of Fn14 severely attenuates myoblast fusion, even in the absence of TWEAK (15–17). Nevertheless, the interaction of TWEAK with Fn14 is sufficient to activate canonical and non-canonical NF-κB pathways (9, 18, 19), so we will focus hereafter on signaling mediated by the binding of TWEAK to Fn14.

TWEAK, THE cIAP PROTEINS, AND CANONICAL NF-κB SIGNALING

The NF-κB family consists of five transcription factor subunits, as well as a plethora of inhibitors, activators, and signal transduction molecules that function as both pathway regulators and mediators of inter-pathway cross-talk. The NF-κB subunits are RelA/p65, RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2) (20). All subunits contain a Rel-homology domain near their N-termini, which confers protein dimerization and DNA-binding capabilities; however, only RelA, RelB, and c-Rel contain C-terminal transactivating domains. An NF-κB complex consists of a homodimer or heterodimer of any pair of subunits. These dimers are normally retained in the cytoplasm in an inactive state by an array of inhibitor of κB (IκB) repressor proteins. One of the better-studied NF-κB signaling axes, known as the classical or canonical pathway, principally involves signal transduction through the p50:RelA heterodimer. Upon pathway stimulation by ligands such as TNFα

(**Figure 2**), a signaling complex forms, which leads to the activation of IκB kinase α, β, and NEMO complex (IKK). IKK catalyzes the phosphorylation and subsequent degradation of IκBα, thus allowing nuclear translocation of p50:RelA and transcriptional activation of NF-κB target genes.

Several studies over the past two decades have identified the cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) proteins as regulators of multiple signal transduction pathways, including NF-κB, that are activated by TNF superfamily cytokines (19, 21–29). The inhibitors of apoptosis (IAP) were identified based on their homology to viral IAPs (30–32), and contain one or more N-terminal baculoviral IAP repeat (BIR) homology domains. In addition, cIAP1/2 were independently identified based on their association with the TNF receptor 2 (TNF-R2) and the adaptors TRAF1 and TRAF2 (21). These two IAPs are recruited to the various TNF superfamily receptors through direct interaction with TRAF1, TRAF2, or TRAF6 (19, 33–36). The cIAP1/2 proteins contain single C-terminal RING E3 ubiquitin ligase moieties (37), as well as ubiquitin-binding UBA domains (38), whose functions have been the focus of considerable research interest. In most cells, cIAP1 triggers the constitutive lysine-48 (K48)-ubiquitination and degradation of cIAP2 (39); thus, cIAP2 expression is low in most non-lymphoid tissues unless cIAP1 is absent, or unless cIAP2 expression is induced (40–42). Nevertheless, in many scenarios cIAP1 and cIAP2 function redundantly (28). In the context of NF-κB signaling induced by a prototypical ligand such as TNFα, the TNF receptor 1 (TNF-R1) recruits a signal transduction complex consisting of TNF-R1-associated DEATH domain (TRADD), the kinase/scaffold protein receptor-interacting protein 1 (RIP1), and TRAF2. TRAF2 recruits cIAP1/2, which in turn catalyze the polyubiquitination of RIP1 by K63- and K11-mediated linkages (43). The K63- and K11-linked chains on RIP1 serve as scaffolds for the assembly of an IKK complex consisting of TAB1, TAK1, NEMO, and IKKα,β. This complex phosphorylates IκBα, thus completing the signal transduction process upstream of the NF-κB transcription factors (**Figure 2**). When cIAP1/2 are depleted, either by genetic or pharmacological means (such as through the use of IAP antagonists known as SMAC mimetic compounds or SMCs), RIP1 instead activates a pro-apoptotic complex, referred to as the ripoptosome. This death complex consists of de-ubiquitinated RIP1, the DEATH domain-containing adaptor protein FADD, and caspase-8 (44, 45) (**Figure 2**).

The receptor Fn14 is an effector of signaling through its ability to recruit TRAF-1, -2, -3, and -5. At sufficiently high concentrations, soluble TWEAK triggers IκB phosphorylation and degradation, as well as p65 phosphorylation and nuclear translocation, events that are typical of the canonical NF-κB pathway activation (7). As Fn14 lacks a death domain, it is unable to directly recruit a death-signaling complex. Instead, apoptosis results from the NF-κB-stimulated release of TNFα, which induces apoptosis in a manner requiring RIP1 and FADD (46). Cancer cells that have been "primed" with TWEAK are sensitized to TNFα-induced cell death owing to the depletion of cIAP1 and TRAF2 proteins (18). Evidence is accumulating that canonical NF-κB activation and cell death are consequences of pathological, rather than physiological, levels of TWEAK or Fn14. At low (physiological) concentrations, TWEAK is unable to activate canonical NF-κB, but still produces robust activation of the non-canonical pathway (17, 47), as described in the next section. Remarkably however, membrane-bound TWEAK is highly capable of activating canonical NF-κB signaling (47), suggesting that juxtacrine signaling may produce considerably different effects on target cells. Furthermore, Fc-TWEAK or Fc-Fn14 fusion constructs, which have a high propensity to multimerize, are able to activate Fn14 and cause significant canonical NF-κB activation with pathological outcomes (47–49). These negative consequences of TWEAK signaling are also seen upon Fn14 upregulation due to stress or injury, even when TWEAK levels remain unchanged. This is due to the greater chance for receptor oligomerization and clustering to occur, which is needed to induce downstream signaling events (50).

During myogenesis, canonical NF-κB activity promotes myoblast proliferation and inhibits differentiation [reviewed elsewhere (51–53), and see **Figures 1** and **4** for illustration]. These effects are important during the early phases of muscle regeneration, where efficient repair necessitates rapid expansion of the myoblast population. Notably, following muscle damage, the first wave of inflammatory cells release a plethora of inflammatory cytokines, such as TNFα, IL-6, and TWEAK, which are potent activators of NF-κB signaling (54, 55). During chronic regenerative cycles observed in certain muscle disorders, such as Duchenne muscular dystrophy, the continued presence of such inflammatory cytokines both impairs muscle repair and aggravates the resulting pathology (56). This differential effect of transient and chronic cytokine signaling will be discussed later in this review, in the context of non-myogenic contributors to myogenesis.

TWEAK AND NON-CANONICAL NF-κB SIGNALING

Transcriptional activity in the non-canonical NF-κB pathway is mediated by the p52:RelB heterodimer. This signaling axis is tightly regulated by the controlled processing of p100 into p52. Under basal conditions, a ubiquitin ligase complex consisting of TRAF2, TRAF3, and cIAP1/2 catalyzes the constitutive K48-ubiquitination and consequent degradation of the NF-κB inducing kinase (NIK), which is essential for activation of the non-canonical pathway (33, 57). Upon non-canonical NF-κB stimulation by a variety of ligands [including TWEAK, BAFF, CD40 ligand (CD40L), and RANKL], TRAF2, TRAF3, and cIAP1/2 are sequestered to the corresponding membrane-bound TNF superfamily receptor. Here, K48-ubiquitination of TRAF3 by cIAP1/2 leads to autoinactivation of the complex and stabilization of cytosolic NIK (58). NIK in turn phosphorylates IKKα, which activates p100 and leads to its partial proteasomal processing to p52. The p52:RelB dimer is then released for nuclear translocation and gene transactivation (**Figure 3**). TWEAK signaling subsequently triggers the degradation of TRAF2, TRAF3, and cIAP1/2 through both proteasomal and lysosomal pathways (17, 18, 59, 60). The lysosomal degradation mechanism may represent a separate mode of NF-κB activation unique to TWEAK, since inhibiting lysosomal protein degradation is sufficient to completely prevent p100 processing (18). Unlike the stimulation of the canonical pathway, which is quite rapid, the non-canonical pathway is gradually activated over several hours, possibly due to the requirement for *de novo* NIK translation and accumulation. The lysosomal degradation of cIAP1 and TRAF2 by TWEAK impairs NF-κB activation

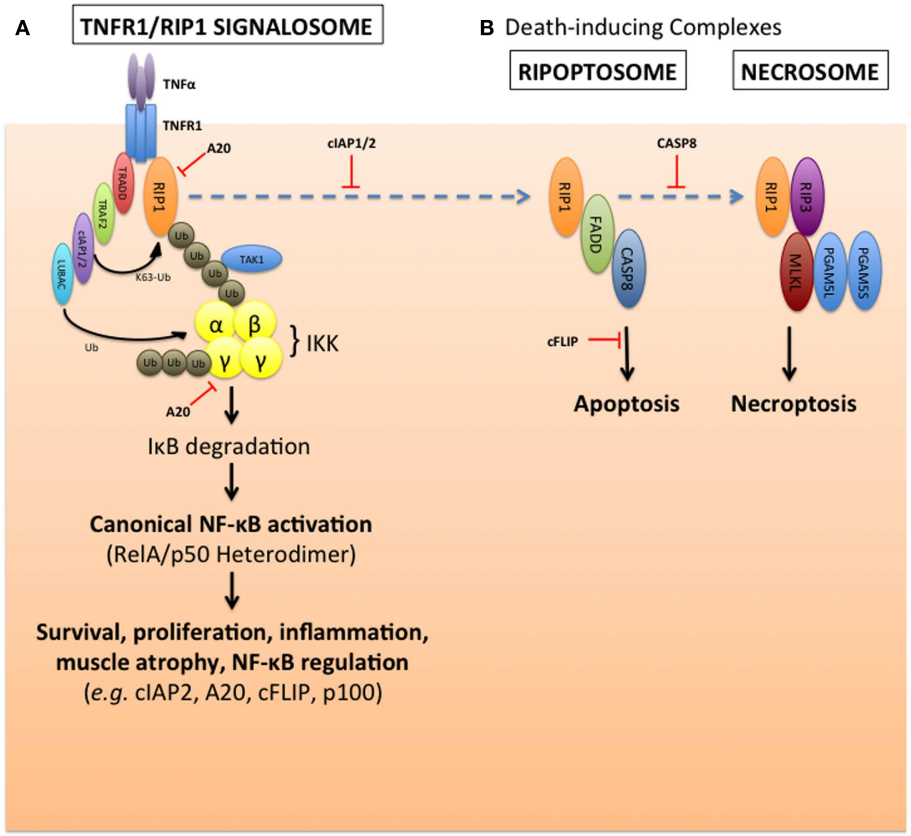

FIGURE 2 | cIAP1/2 regulation of TNFα-induced canonical NF-κB pathway activation and suppression of default death pathways. **(A)** The E3 ubiquitin ligases cIAP1 and cIAP2 are required for TNFα activation of canonical NF-κB signaling, and to suppress TNFα-induced caspase-8 death pathway. These two cIAPs are recruited to the TNFα receptor, TNF-R1, via their association with the adaptor protein TRAF2. Upon ligand stimulation, the cIAPs promote K63-linked polyubiquitination of RIP1, which creates a signaling scaffold (or signalosome complex) to recruit the TAK1 kinase, LUBAC, otherwise known as the linear ubiquitin chain assembly complex (consisting of Sharpin, HOIL-1L, and HOIP), and the trimeric IKK kinase complex (consisting of IKKα, IKKβ, and IKKγ/NEMO). This signalosome formation results in the activation of the IKK complex, and subsequent phosphorylation and degradation of the NF-κB inhibitor, IκB, which occurs within minutes. This allows canonical NF-κB heterodimers to form and translocate into the nucleus to alter gene expression profiles over several hours, which affects many pathways such as survival, proliferation, inflammation, muscle atrophy, and NF-κB signaling itself. This pathway is subject to negative regulation by the deubiquitinase, A20, for example. **(B)** In the absence of the cIAPs, the unmodified RIP1 can form a death complex with FADD and caspase-8 known as the ripoptosome. This apoptotic death pathway can also default to a necrotic-like pathway in the absence of caspase-8, through another complex called the necrosome which involves the kinases RIP1 and RIP3, mixed lineage kinase domain-like (MLKL) and the short and long forms of the phosphatase PGAM5.

by other cytokines that require these adaptors; thus, TWEAK sensitizes cancer cells to TNFα-induced apoptosis through activation of caspase-8 (8, 18, 61). The cIAPs are thus considered to be negative regulators of the non-canonical NF-κB pathway, through their constitutive effects on NIK degradation. The binding of TWEAK to Fn14 then relieves this cIAP1/2 suppression by recruiting the TRAFs and cIAPs to the receptor, away from NIK. This membrane receptor sequestration of the cIAPs and TRAFs may be sufficient for NIK stabilization, or may require further degradation and loss of those factors to fully activate NIK as illustrated in **Figure 3**.

TWEAK AND cIAP1 AS REGULATORS OF MYOBLAST FUSION

While the functions of canonical NF-κB signaling in muscle regeneration and atrophy have been investigated extensively over the years (52, 62, 63), very few studies have examined the role of non-canonical NF-κB in skeletal muscle. In 2001, a paper (64) suggested that NIK and IKKα promote differentiation of the rat L6E9 myoblast cell line. More recently, the non-canonical NF-κB signaling was implicated in muscle resistance to metabolic stress (65), and as a factor specifying the oxidative mode of glucose metabolism in muscle fibers (66). We had observed that primary myoblasts from cIAP1$^{-/-}$ mice [note that skeletal muscle does not express cIAP2 (28)] exhibit constitutively elevated canonical and non-canonical NF-κB activity. We reasoned that upon differentiation of cIAP1$^{-/-}$ myoblasts into myotubes, which are the *in vitro* analogs of muscle fibers, both canonical and non-canonical NF-κB pathways should produce separate respective phenotypes.

The initial outcome of our experiments was unexpected. While there was a clear delay in cell cycle exit and differentiation of cIAP1$^{-/-}$ myoblasts, the resulting myotubes were characterized by significant hypernucleation and increased myotube size, indicative of a robust fusion response. Subsequent experiments showed

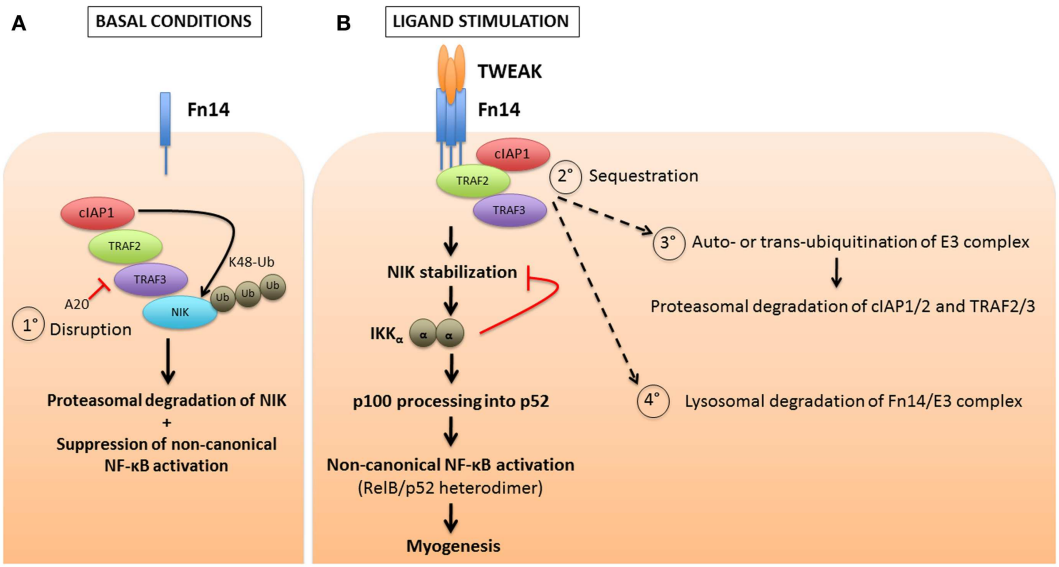

FIGURE 3 | cIAP1/2 regulation of TWEAK-induced non-canonical NF-κB pathway activation. **(A)** Contrary to the canonical NF-κB pathway for which cIAP1/2 are positive regulators, these two E3 ubiquitin ligases act, via the bridging molecules TRAF2 and TRAF3, as negative regulators of the non-canonical NF-κB by continuously degrading the NF-κB-inducing kinase, NIK. This occurs through the attachment of K48-linked polyubiquitin chains and the targeting of NIK to the proteasome, under basal or non-stimulated conditions. One mechanism (process 1°) to reverse this inhibitory effect is through A20 mediated disruption of the cIAP-TRAF complex, which would presumably lead to ligand-independent activation of the non-canonical NF-κB pathway. **(B)** In most instances, upon stimulation of a TNF receptor superfamily member by its ligand, the cIAPs and TRAFs are recruited away from the cytosolic reactions and sequestered at the plasma membrane (process 2°). This allows for the stabilization of NIK, the formation of IKKα homodimers, and ultimately the partial processing of p100 into p52. RelB and p52 then dimerize to form an active, functional NF-κB transcription factor complex. Several models of receptor-mediated non-canonical NF-κB activation have been proposed, which include the cIAPs inducing K48-linked ubiquitination of themselves and the TRAFs, resulting in their proteasomal degradation (process 3°). Alternatively, the receptor-mediated endocytosis of the TWEAK-Fn14 complex results in lysosomal degradation of the cIAPs and TRAFs (process 4°). This loss of cIAP and TRAF adaptors may impact other pathways, such as CD40L signaling through CD40, that also require these adaptors.

that the elevated non-canonical NF-κB activity, resulting from the loss of cIAP1, was responsible for this effect (17). This observation highlighted a disparity between immortalized myoblast cell lines (C2C12) and primary myoblasts. In C2C12 cells, p100 processing to p52 increases over the time course of differentiation (65). In contrast, primary cells exhibit the greatest p100 processing at the myoblast stage; processing diminishes markedly as muscle fibers form (17). The increased myoblast fusion observed in cIAP1$^{-/-}$ cells could be recapitulated using low doses of TWEAK. At low concentrations, exogenous TWEAK led to robust activation of the non-canonical NF-κB pathway. At higher concentrations, TWEAK activated both canonical and non-canonical pathways. The requirement for high TWEAK concentrations to activate canonical NF-κB suggests that this pathway represents a secondary mode of signaling for TWEAK. In order to further investigate the physiological consequences or TWEAK activity *in vivo*, we employed the snake venom cardiotoxin (CTX) model of muscle injury, which involves the direct injection of CTX into muscle. This treatment causes rapid development of focal necrotic lesions, but also initiates a robust regenerative response. Following CTX injection, the cIAP1$^{-/-}$ muscle exhibits a slight increase in average muscle fiber size as compared to wildtype controls, but not to the same robust extent observed *in vitro* (17). This is likely an outcome of the interplay and functional antagonism between both NF-κB pathways. TWEAK administered by micro-osmotic pump produced greater increases in fiber size than did the loss of cIAP1, further indicating the preference of TWEAK for non-canonical NF-κB signaling. In regenerating muscle, the window of regenerative opportunity is very narrow; the majority of myogenesis occurs within 4 days of the injury. A delay in the development of fusion competence – as can be caused by elevated canonical NF-κB activity – may be sufficient to reduce the muscle's regenerative potential. Collectively, the data indicate that enhanced myogenesis is best achieved by attenuating the canonical NF-κB pathway, and promoting fusion through the non-canonical corollary [(17); see also **Figure 4**].

The evidence to date shows that both canonical and non-canonical NF-κB pathways are concurrently active in proliferating and differentiating mouse myoblasts. During the process of muscle differentiation, both pathways are similarly inactivated. However, these NF-κB pathways clearly have complementary but opposing functions in muscle regeneration (**Figures 1** and **4**). While the canonical pathway is important during myoblast proliferation (67), the reported effects of non-canonical NF-κB signaling on stress resistance (65), metabolism (66), and fusion (17) are all features specific to developing muscle fibers, rather than to myoblasts. Therefore, a model can be proposed in which canonical NF-κB activity is switched off to suppress myoblast proliferation thereby allowing for their differentiation. At this point, non-canonical NF-κB predominates, likely driven by TWEAK stimulation, to promote

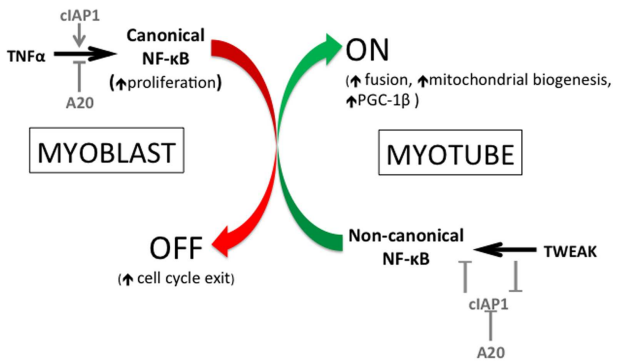

FIGURE 4 | Roles of TNFα, TWEAK, cIAP1/2, and A20, in myogenesis in the regulation of switching from canonical to non-canonical NF-κB signaling. The transition from myoblast to myotube has been known for several years to involve a switch from canonical to non-canonical NF-κB signaling. More recently, several of the key players and the mechanisms of action for this switch have been identified. The promotion of TNFα-mediated canonical NF-κB signaling involves the positive regulation by the E3 ubiquitin ligase cIAP1/2 and the negative regulation by the deubiquitinase A20. The non-canonical pathway is oppositely controlled: it is negatively regulated by cIAP1/2 and positively regulated by A20. Interestingly, the cytokine TWEAK (a pathological factor in muscle atrophy) has been recently shown to stimulate non-canonical NF-κB signaling by removing the negative regulation of cIAP1/2 on that NF-κB pathway. Physiological levels of TWEAK, in fact, promote myogenesis through the enhancement of myoblast fusion amongst other things. The non-canonical NF-κB pathway has been shown previously to regulate mitochondrial biogenesis and to promote myotube maintenance, and recently shown to do so through the NF-κB-mediated induction of the mitochondrial regulator PPAR-γ co-activator 1β (PGC-1β). Figure adapted from Ref. (65).

the formation of myotubes, while also aiding in their maintenance. The canonical NF-κB pathway may in fact prime the non-canonical NF-κB pathway by inducing the expression of p100, the precursor to p52. It is thus possible that p52:RelB (non-canonical NF-κB) activity then serves to further push myoblast fusion, by inducing expression of transcriptional targets whose functions are only observed in later stages of the differentiation process. With the exception of PGC-1β (66), the relevant transcriptional targets of non-canonical NF-κB in this context, and the effectors of TWEAK-driven myoblast fusion, are unknown. Another possibility is that, though p100/52 expression is high in myoblasts, activity of this pathway may be inhibited in myoblasts, and subsequently de-repressed during differentiation. A potential mechanism for the switch between canonical and non-canonical pathways involves the deubiquitinase A20. The A20 protein inhibits canonical NF-κB signaling by removing the K63-ubiquitin chains on RIP1 that are essential for its function as an adaptor (68). Conversely, A20 disrupts the cIAP1/2–TRAF2/3 ubiquitin ligase complex, thus preventing NIK degradation and promoting non-canonical NF-κB signaling (69). Since A20 has been shown to be upregulated during muscle cell differentiation (70), it is possible that A20 is essential for normal regeneration.

Knockout studies have revealed disparities between the muscle-intrinsic effects of TWEAK and Fn14 on regeneration. Upon muscle injury, TWEAK$^{-/-}$ mice exhibit more rapid muscle regeneration than wildtype controls; in contrast, mice over-expressing TWEAK under a muscle-specific promoter exhibit slower regeneration (71). However, TWEAK is not normally expressed in myoblasts either *in vivo* or *in vitro* (15, 17), suggesting that the phenotype observed in the knockout mice may result from decreased paracrine signaling from other TWEAK-producing cells such as macrophages. Fn14 expression in muscle is clearly induced during regeneration and atrophy (16, 50), and mice lacking Fn14 exhibit considerably impaired regeneration. This effect can be recapitulated *in vitro* in the absence of TWEAK (16, 17), highlighting the possibility that Fn14 may play essential roles independent of TWEAK.

The application of TWEAK/Fn14 therapeutics in the context of muscle regeneration may at first appear somewhat counterintuitive, until the specific mechanistic intent is examined closely, and hypotheses are tested. Most of the published reports on TWEAK highlight its aggravating role in muscle (72, 73), liver (74), kidney (75) and neurological (76) regeneration or repair. A consistent feature of these studies however, is the pathologically elevated levels of TWEAK signaling, due either to the experimental intervention (transgenic over-expression of TWEAK) or a chronic, localized over-production of cytokine (77). However, we propose that transient TWEAK/Fn14 activation at physiological levels may prove beneficial (**Figure 5**). Consistent with this idea, a number of studies have shown that TWEAK can act as a mitogen to stimulate proliferation of progenitor cells (15, 78–81). In particular, soluble TWEAK, particularly at low concentrations, preferentially activates the non-canonical NF-κB pathway, whereas high concentrations are sufficient to mobilize both canonical and non-canonical pathways (17, 48). Therapeutic activation of the non-canonical pathway has been suggested in a number of separate contexts. For example, TWEAK promotes lymphocyte and T cell recruitment to the kidney following renal injury by inducing expression of the chemokine CCL21 in a non-canonical NF-κB-specific manner (82, 83). TWEAK is also important to prevent certain lymphoproliferative disorders that lead to impaired antibody responses (84, 85). A better understanding of the mechanisms of TWEAK signaling should permit an informed tailoring of its uses, such as with agonistic and antagonistic antibodies (48), for particular therapeutic applications.

NF-κB, NFAT SIGNALING, AND THE PROMOTION OF MYOBLAST FUSION

Extracellular calcium is one of the earliest known regulators of myoblast fusion. In 1969, Shainberg and colleagues (86) demonstrated that fusion of chick myoblasts could be reversibly blocked by removing Ca^{2+} ions from the growth medium. These results were confirmed in multiple species (87–89). Importantly, it was shown that extracellular calcium is not required for myoblast cell cycle exit or for muscle-specific gene expression (90–93). Calcium was subsequently identified as a potent activator of the nuclear factor of activated T cells (NFAT) transcription factor pathway. Out of five proteins in this family, three – NFAT-c1, -c2, and -c3 – are regulated by calcium and expressed in skeletal

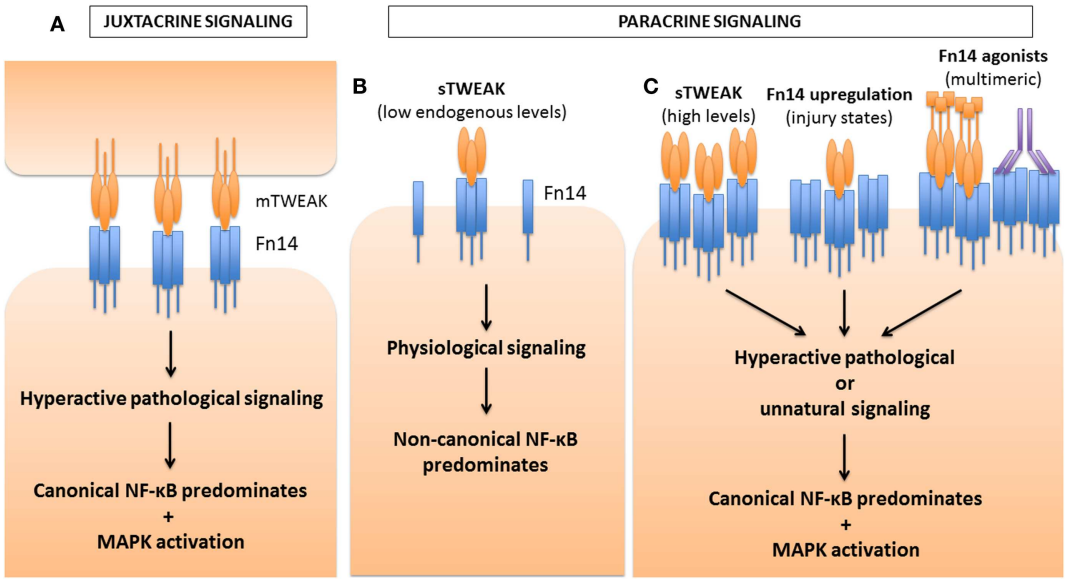

FIGURE 5 | Differential activation of NF-κB pathways by TWEAK based on strength of Fn14 signaling. TWEAK can signal either in a juxtacrine manner, as a membrane-bound ligand, mTWEAK **(A)**, or in a paracrine manner as a soluble ligand, sTWEAK **(B,C)**. Depending on the concentration of ligand or receptor, or the propensity of the Fn14 ligand to oligomerize the receptor and form signaling clusters, differential activation of the NF-κB pathways occurs. **(A)** During juxtacrine signaling, mTWEAK favors the clustering of Fn14 on the opposite cell, leading to hyperactive signaling for which canonical NF-κB signaling predominates over non-canonical signaling. MAPK activation also occurs. **(B)** Under physiological conditions, low endogenous levels of sTWEAK signal in a paracrine manner, leading predominantly to non-canonical NF-κB activation. **(C)** However, under certain pathological or experimental conditions, sTWEAK can also lead to hyperactive signaling leading to canonical NF-κB and MAPK signaling. For example, high concentrations of TWEAK, applied to myoblasts or myotubes *ex vivo*, hinder differentiation and cause atrophy, respectively. Transgenic over-expression of TWEAK in mice also leads to pathological consequences. In a variety of injury states (such as denervation-induced muscle atrophy), the receptor Fn14 is induced, which then is followed by and pathological responses to endogenous levels of TWEAK. Furthermore, recombinant TWEAK fusion proteins with the ability to multimerize, or Fn14 agonistic antibodies, can both promote receptor clustering and the activation of the canonical NF-κB pathway. The pathways leading to pathological activation of the canonical NF-κB pathway are still poorly defined, but are thought to arise either from pathway cross-talk, such as that seen with NIK or IKK activation of the canonical mediators, or through the adaptor and E3 ubiquitin ligase TRAF6. In addition, activation of MAPK pathways may also contribute to the pathology observed.

muscle (93). Ca^{2+}-activation of the phosphatase calcineurin leads to the dephosphorylation of NFAT proteins. These then translocate to the nucleus where they activate the transcription of a range of NFAT target genes (94). Multiple stimuli that potentiate the Ca^{2+}-calcineurin-NFAT signaling axis, such as calcium ionophores (95), increase myoblast fusion; conversely, substances that deplete intracellular or extracellular calcium stores, such as EDTA and thapsigargin, impair myoblast fusion (96, 97). A notable aspect of NFAT transcription factors is that they mediate different aspects of myoblast fusion. Whereas NFATc3 is calcium-responsive in myoblasts, NFATc1 and NFATc2 are active in nascent myotubes (93). This suggests that they differentially regulate primary fusion (myoblast–myoblast) and secondary (myoblast–myotube) fusion events (**Figure 1**). Consistent with this hypothesis, the protein four-and-a-half LIM 1 (FHL1) is a co-factor of NFATc1; over-expression of FHL1 increases myoblast fusion *in vitro* and *in vivo* (98). Muscle fiber formation is also impaired in *NFATc2*$^{-/-}$ mice (99). Collectively the data indicate a hierarchical process of fusion, whereby primary myotubes, formed under control of NFATc3, recruit further myoblasts in an NFATc1- or NFATc3-specific manner for formation of secondary myotubes and continued myogenesis.

Evidence from some published reports suggest that the TWEAK/Fn14/cIAP signaling axis may act through one or more NFAT pathways to regulate myoblast fusion. Upon inactivation of the non-canonical NF-κB pathway in wildtype myoblasts, myotubes still form, but are small and have reduced numbers of nuclei (17). This phenotype is similar to that observed with *NFATc2*$^{-/-}$ cells (99), where myogenesis stalls at the primary myotube phase. The existence of cross-talk between the structurally similar NFAT and NF-κB transcription factors is well-established (100–102). A recent study indicated that RANKL, which like TWEAK activates both canonical and non-canonical NF-κB pathways in osteoclasts, induces expression of NFATc1 in a manner that requires NIK (103), suggesting that NFATc1 may be a target of the non-canonical pathway. Furthermore, cIAP1/2 was shown to suppress NFATc1 expression in response to RANKL; conversely, loss of cIAP1/2 led to upregulation of NFATc1in osteoclasts (104).

Our understanding of molecular triggers and signaling pathways that are critical to myoblast fusion is still very limited. While the list of known effectors of fusion is extensive [as has been categorized in other recent reviews (105, 106)], a coherent picture of timing, mechanism, and relative importance has yet to emerge. It

seems likely that the TWEAK-NF-κB signaling axis converges with the transcriptional upregulation of one or more muscle-derived cytokines or chemokines (referred to as "myokines"). A search for known targets of the non-canonical NF-κB pathway, including such molecules as CCL19 and CCL21 (83), should provide further insight into the placement of NF-κB within the stepwise processes of fusion.

INNATE IMMUNITY AND MUSCLE REGENERATION MEDIATED BY THE CYTOKINES IL-4, IL-10, AND IL-13

In the context of regenerating muscle, a number of recent papers have examined the interplay between muscle cells and multiple non-muscle lineages that participate in the regenerative process. Following muscle injury, an inflammatory response emerges, which involves the infiltration of myeloid cell types such as eosinophils, basophils, mast cells, macrophages, and leukocytes (107, 108). These cells release a medley of cytokines and chemokines; the leukocytes in particular are robust sources of TWEAK (109, 110). Broadly speaking, the damage-associated innate response is structured such that the early infiltrating cells, dominated by CD68-expressing "M1" macrophages, produce pro-inflammatory cytokines such as IL-6, TNFα, and IL-1β (54). The timing of M1 influx correlates with the activation of satellite cells and proliferation of myoblasts, a process that is enhanced by canonical NF-κB activation. The M1 macrophages are subsequently replaced with CD163-expressing "M2" macrophages, which promote muscle growth through the secretion of IL-4, IL-10, and IL-13. The M2 response occurs during the phase of muscle regeneration predominated by myoblast fusion, which may be enhanced by M2-derived cytokines such as IL-4 and IL-13.

The various contributions of inflammatory and lymphoid cells to the course of muscle regeneration have been assessed in injury models. A commonly used animal model of muscle injury is the *mdx* mouse, which exhibits many of the hallmark symptoms and pathology of the human disorder known as Duchenne muscular dystrophy. In mice and humans, the disease results from loss of the structural protein dystrophin, which leads to increased muscle fragility and continued cycles of injury and regeneration (111). This creates a chronic inflammatory milieu in muscle, which both aggravates and perpetuates the pathology. In *mdx* mice depleted of macrophages during the early stages of the disease, muscle injury is significantly reduced (112). Similar outcomes are observed in *mdx* mice depleted of CD8-positive cytotoxic T cells alone (113), or of both CD4-positive helper and CD8-positive T cell populations (114). An important point of note, however, is that these studies describe the outcome of short-term depletion of these cell populations on the dystrophic phenotype. In an analysis of *mdx* mice crossed with *scid* mice (lacking both mature T and B cells), no differences in muscle fiber size, percentage of regenerated fibers, or muscle force were observed as compared to immunocompetent *mdx* mice (115). In contrast, mice depleted of monocytes and macrophages, using a targeted cytotoxic diphtheria toxin approach, exhibit severely impaired muscle regeneration (116). These observations are consistent with the biphasic and important roles of M1 and M2 macrophages in the regenerative process.

Also relevant to this stage of regeneration is a recently identified population of fibro/adipogenic progenitors (FAPs), which are essential contributors to normal muscle regeneration following acute trauma (117–122). These FAPs, like satellite cells, are activated following muscle injury, and proliferate in response to IL-4 and IL-13 secreted by eosinophils (123). IL-4 also specifies the fate of FAPs as phagocytes rather than fat-generating adipocytes. In the absence of IL-4/IL-13-secreting eosinophils or in an IL-4 receptor alpha-knockout ($IL\text{-}4R\alpha^{-/-}$) background, muscle regeneration is severely impaired, at least in part due to excessive deposition of FAP-generated brown fat. There are currently no studies explicitly examining the relationship between TWEAK and FAPs during myogenesis; nevertheless corollary evidence from other tissue systems suggests that TWEAK may promote muscle regeneration through regulation of FAP differentiation. TWEAK and Fn14 are expressed in adipocytes (124, 125), and TWEAK inhibits adipocyte differentiation (126). This occurs at least in part through the blunting of pro-inflammatory and pro-adipogenic signaling induced through the canonical NF-κB pathway by TNFα (125–127). Still further, TWEAK synergizes with IL-13 as a fibroblast mitogen (127, 128). Given the overlap between the influx of TWEAK-expressing M2 macrophages and FAP activation during regeneration, it seems likely that the pro-regenerative context established by both cell types may involve low levels of secreted TWEAK as a paracrine regulator of muscle regeneration.

Given the upregulation of Fn14 following muscle injury, and the influx of TWEAK-expressing myeloid cells, it is likely that the non-canonical NF-κB signaling has a direct influence on the course of muscle repair. The highly orchestrated nature of the innate immune response in damaged muscle, which occurs in synchrony with the course of muscle differentiation and fusion, is critical to normal muscle regeneration. This timing is controlled by IL-10 (129), which deactivates M1 macrophages; and by AMPKα1, which is required for macrophage acquisition of an M2 phenotype (130). Given that such timing is disrupted in chronic degenerative muscle diseases and myopathies, factors that skew the population distribution in favor of an M2 phenotype may improve muscle regeneration in disease conditions. This hypothesis is supported by certain acute experiments in which administration of exogenous IL-4 (123) or IL-10 (131) was found to promote necrotic cell clearance and muscle regrowth.

Recently we evaluated the effect of cIAP1 loss on muscle function in the *mdx* mouse model of Duchenne muscular dystrophy (132). In $cIAP1^{-/-}$ *mdx* double-mutant mice, muscle degeneration was attenuated in some muscle groups, particularly the soleus and diaphragm. The outcome was that double-mutant mice exhibited improved muscle resiliency and exercise endurance as compared to the *mdx* controls. These results were accompanied by a reduction in pro-inflammatory M1 macrophages, and an increase in pro-regenerative M2 macrophages in muscle tissue. These results suggest that non-canonical NF-κB activation, through loss of cIAP1 can mediate diverse effects that converge to improve muscle regeneration and function. It remains to be seen if low doses of TWEAK, which would more specifically target the non-canonical pathway, can recapitulate these positive effects on muscle regeneration.

TWEAK SIGNALING IN DIABETES AND MUSCLE REGENERATION

Skeletal muscle is responsible for the uptake of 80% of blood glucose (133–135); consequently, the outcome of prolonged insulin resistance in muscle is primarily type 2 diabetes (136). The presence of excessive fat deposits is associated with both onset and progression of type 2 diabetes (137). It was recently discovered that high-glucose diets trigger the differentiation of multipotent myoblasts into adipocytes (138). While the resulting fat deposits can accelerate insulin resistance through autocrine release of TNFα, TWEAK can inhibit this process by blocking TNF-mediated activation of JNK (139). TWEAK is constitutively expressed in adipose tissue (124, 125), suggesting that it actively antagonizes the process of insulin resistance. Evidence for this is shown by a recent study demonstrating that reduced levels of TWEAK correlate with increased risk of diabetes (140), at least in part by reducing autocrine release of TNFα from adipocytes (141). Overall, these findings suggest that TWEAK can operate in a feed-forward mechanism to both promote muscle regeneration and attenuate the pathogenesis of diabetes.

While TWEAK-expressing adipocytes may be beneficial for the purposes of insulin tolerance, it is naturally preferable to reduce fat deposition in muscle altogether. This is consistent with a beneficial role for FAPs in muscle regeneration when the differentiation choice toward adipocytes is blocked (123, 142, 143). Remarkably, TWEAK is a potent inhibitor of adipocyte differentiation and functions, unlike TNFα, without affecting glucose uptake or cytokine release (126). Collectively, the data show that TWEAK signaling can positively regulate homeostasis by improving glucose tolerance in muscle, reducing fat deposition, and reducing adipocyte differentiation of FAP cells during muscle regeneration.

CONCLUSION

The array of recent discoveries on the functions and mechanisms of action of TWEAK offer several intriguing possibilities into both the frontiers of new biology and the potential for therapeutic interventions. The ability of TWEAK/Fn14 to preferentially activate the non-canonical over the canonical NF-κB pathway (17) places TWEAK in a category of TNF superfamily members along with BAFF, CD40L, RANKL, and lymphotoxin β (144). This implicates TWEAK in immunological functions that have, to date, been explored only briefly. The preferential activation of the canonical pathway by membrane-bound TWEAK and the upregulation of Fn14 upon injury are likely the causes of most TWEAK-associated pathology (47, 50). Thus, the paradox of TWEAK as a beneficial and deleterious cytokine becomes a matter of degree: whereas low concentrations of soluble TWEAK can be beneficial for immunological and regenerative purposes, high levels of TWEAK or Fn14 may have pathological consequences, and require intervention using neutralizing antibodies or TWEAK inhibitors.

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Nuclear Factor κB is required for tumor growth inhibition mediated by enavatuzumab (PDL192), a humanized monoclonal antibody to TweakR

James W. Purcell[1]*†, Han K. Kim[1]†, Sonia G. Tanlimco[1], Minhtam Doan[1], Melvin Fox[2], Peter Lambert[1], Debra T. Chao[1], Mien Sho[1], Keith E. Wilson[1], Gary C. Starling[2] and Patricia A. Culp[2]

[1] *Department of Biologics Technologies, AbbVie Biotherapeutics, Redwood City, CA, USA*
[2] *Department of Oncology Biologics, AbbVie Biotherapeutics, Redwood City, CA, USA*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Nhan Le Tran, Translational Genomics Research Institute, USA
Harald Wajant, University Hospital Würzburg, Germany

****Correspondence:***
James W. Purcell, Department of Biologics Technologies, AbbVie Biotherapeutics Inc., 1500 Seaport Blvd., Redwood City, CA 94063, USA
e-mail: james.purcell@abbvie.com

† James W. Purcell and Han K. Kim have contributed equally to this work.

TweakR is a TNF receptor family member, whose natural ligand is the multifunctional cytokine TWEAK. The growth inhibitory activity observed following TweakR stimulation in certain cancer cell lines and the overexpression of TweakR in many solid tumor types led to the development of enavatuzumab (PDL192), a humanized IgG1 monoclonal antibody to TweakR. The purpose of this study was to determine the mechanism of action of enavatuzumab's tumor growth inhibition and to provide insight into the biology behind TweakR as a cancer therapeutic target. A panel of 105 cancer lines was treated with enavatuzumab *in vitro*; and 29 cell lines of varying solid tumor backgrounds had >25% growth inhibition in response to the antibody. Treatment of sensitive cell lines with enavatuzumab resulted in the *in vitro* and *in vivo* (xenograft) activation of both classical (p50, p65) and non-classical (p52, RelB) NFκB pathways. Using NFκB DNA binding functional ELISAs and microarray analysis, we observed increased activation of NFκB subunits and NFκB-regulated genes in sensitive cells over that observed in resistant cell lines. Inhibiting NFκB subunits (p50, p65, RelB, p52) and upstream kinases (IKK1, IKK2) with siRNA and chemical inhibitors consistently blocked enavatuzumab's activity. Furthermore, enavatuzumab treatment resulted in NFκB-dependent reduction in cell division as seen by the activation of the cell cycle inhibitor p21 both *in vitro* and *in vivo*. The finding that NFκB drives the growth inhibitory activity of enavatuzumab suggests that targeting TweakR with enavatuzumab may represent a novel cancer treatment strategy.

Keywords: enavatuzumab, monoclonal antibody, TweakR, Fn14, NFκB, p21

INTRODUCTION

TweakR (Fn14, TNFRSF12A) is a member of the TNF receptor superfamily which is activated by its ligand, the cytokine TWEAK (TNFSF12). TweakR is the smallest member of the TNFR superfamily (1). It lacks the death domain associated with other TNFR members such as TNFR1, Fas, and TRAIL-R1, but it does contain a cytoplasmic TNFR-associated factor (TRAF) binding site allowing recruitment of TRAF adapter proteins which are vital for many of the intracellular signaling events that occur downstream of the TNFR family (2, 3).

TweakR was initially described as an inducer of apoptosis in certain cancer cell lines upon stimulation with its ligand TWEAK (4). TweakR also has a role in diverse biological processes such as inflammation, tissue repair, angiogenesis, and cell migration (5–8). The signaling pathways downstream of TweakR have been elucidated for some of these biological functions. The ERK, JNK, and NFκB pathways have been shown to be upregulated by TWEAK in endothelial cells, while the NFκB pathway appears to be involved in TWEAK-stimulated inflammation and cell survival (9–11). TWEAK and agonist TweakR antibodies have also been shown to induce cell death in certain tumor cell lines through multiple mechanisms, including caspase-dependent and -independent apoptosis and necrosis (3, 12–14). However, in other tumor cell lines, TweakR stimulation leads to a slowed growth effect, not cell death (15). The signaling pathways mediating that phenotype have not been determined.

The ability of TweakR stimulation to inhibit the growth of certain cancer cell lines, as well as the observation that TweakR is over-expressed in many cancers (3, 16) suggested that an antibody targeting TweakR could be a potential therapeutic agent. Enavatuzumab (formerly PDL192), a humanized monoclonal IgG1, which binds to and activates TweakR, has been shown to have growth inhibitory activity in multiple solid cancer models both *in vitro* and *in vivo* and has been evaluated in a Phase 1 study (15, 17). In preclinical studies, the *in vivo* activity of enavatuzumab was attributed to both direct stimulation of TweakR and Fc-mediated antigen dependent cellular cytotoxicity (ADCC). The mechanism of how enavatuzumab directly inhibited the growth of tumor cells, and the cell signaling events occurring downstream of enavatuzumab binding to TweakR were undefined, and therefore became the primary focus of this study.

In this report we show that enavatuzumab activates the NFκB pathway, and that its growth inhibitory activity is dependent on NFκB. The finding that NFκB activation induced by the TweakR

pathway drives the growth inhibitory activity of enavatuzumab provides an interesting function for the NFκB family which is more frequently associated with growth and survival of cancer cells than their inhibition (18, 19).

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Enavatuzumab (PDL192), 19.2.1, and the human IgG1 control used in this study have been described previously (17). The enavatuzumab-Fc mutant contains the L234A, L235A mutations that reduce FcγR binding and ADCC. PDL400 (human IgG1) is a humanized version of the previously described ITEM-4 (13). Mouse anti-TweakR antibodies 136.1 (IgG1) and 18.3.3 (IgG2a) were generated using the same strategy as that described for 19.2.1 (17). Antibodies were used at 10 μg/mL for *in vitro* studies, unless otherwise stated, and crosslinked with $F(ab')_2$ goat anti-human IgG (Fc_γ specific) from Jackson ImmunoResearch at 3.5 μg/mL. Recombinant human TWEAK was purchased from R&D Systems.

siRNAs were purchased from Thermo Scientific Dharmacon and included the following: non-targeting control siRNA pool (D-001810-10-05), p65 (RelA) siRNA pool (L-003533-00-0002) and set of four individual siRNAs (LQ-003533-00-0002), p52/p100 (NFκB2) siRNA pool (L-003918-00-0002) and set of four individual siRNAs (LQ-003918-00-0002), p50/p105 (NFκB1) siRNA pool (L-003520-00-0002) and set of four individual siRNAs (LQ-003520-00-0002), RelB siRNA pool (L-004767-00-0002) and set of four individual siRNAs (LQ-004767-00-0002), IKKα (CHUK) siRNA pool (L-003473-00-0002) and set of four individual siRNAs (LQ-003473-00-0002), IKKβ siRNA pool (L-003503-00-0002) and set of four individual siRNAs (LQ-003503-00-0002), p21 (CDKN1A) set of four individual siRNAs (LQ-003471-00-0002).

CELL LINES

All cell lines were obtained from the American Tissue Culture Collection (ATCC) or National Cancer Institute (NCI), except HSC-3 which was purchased from the Japan Health Science Foundation and the MB231 variant cell line, which was derived from the MDA-MB-231 cell line for its increased metastatic potential *in vivo*.

CELL VIABILITY ASSAY

The 105 cell line panel was cultured at 500 cells per well in triplicate with enavatuzumab or IgG1 control in the presence of $F(ab')_2$ goat anti-human IgG (Fc_γ specific) for 5 days in 96 well plates. Relative cell viability was determined using CellTiter-Blue™(Promega). Fluorescence emitted at 590 nm was used to calculate the growth effect relative to the IgG1 control antibody treatment. Each cell line was tested twice with the average growth inhibition reported.

LUCIFERASE TRANSCRIPTIONAL REPORTER ASSAY

The Cancer 10-Pathway Reporter Luciferase Kit was purchased from SA Biosciences [CCA-101L (Plate Format)] and reverse transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were treated with the indicated antibodies for 24 h following transfection. Cells were harvested an additional 24 h later and reporter activity was measured using the Dual-Luciferase Reporter Assay (Invitrogen).

WESTERN BLOT ANALYSES

Whole cell protein lysates were generated using Cell Signaling protein lysis buffer containing protease inhibitors (Roche) and phosphatase inhibitor cocktail (Sigma), quantified using BCA reagent (Pierce), and protein expression was detected using ECL Plus Chemiluminescence kit (GE-Amersham). P-IκBα (Ser32/36) 5A5, p21 WAF1/CIP1 (DCS60), p65, p100/p52, p105/p50, and RelB antibodies were purchased from Cell Signaling. GAPDH antibodies were from Santa Cruz. Mouse and rabbit secondary antibodies were from GE-Amersham.

NFκB TRANSCRIPTION FACTOR ELISA

TransAM™ NFκB family ELISA kit (Active Motif) was used to determine NFκB subunit DNA binding and functional activity in response to enavatuzumab treatment. Nuclear protein extracts were generated and the subunit ELISAs (p65, p50, RelB, p52) performed according to the manufacturer's guidelines.

GENE CHIP ANALYSIS

RNA was isolated and purified with RNeasy Micro kits (Qiagen) and analyzed using an Agilent 2100 Bioanalyzer RNA 6000. cDNA was prepared from the RNA template, and used for *in vitro* transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated RNA targets were fragmented, and hybridized to a customized Affymetrix genechip platform (Eos Hu03plus) using standard Affymetrix protocols. Genechips were performed in duplicate and stained with streptavidin phycoerythrin (SAPE) and scanned on an Affymetrix GeneChip Scanner 3000. Raw data files were obtained after analysis of scanned images with GCOS (GeneChip Operating Software, Affymetrix). Gene chip expression data was generated according to previously described methods (20). The microarray data contained within this manuscript is registered in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and can be accessed using the GEO submission GSE51934.

siRNA TRANSFECTIONS

OnTarget Plus pooled siRNA (15 nM) (Thermo Scientific) against the target gene of interest or non-targeting pool control siRNA were reverse transfected with Lipofectamine RNAiMax (Invitrogen) according to manufacturer's protocol. A positive control siRNA for successful transfection was also included (Kinesin Spindle Protein, KSP). Target knockdown was maximally achieved 2 days post transfection. Cells were then treated with enavatuzumab or IgG1 control + crosslinking antibody for the time indicated. The relative viability of the cells was determined, and cells were also harvested for Western blot analyses.

IKK16 NFκB INHIBITION

The IκB kinase (IKK) selective inhibitor IKK16 (Tocris Bioscience) was used at 160 nM (21). The IKK16 was added to cells either alone or 1 h prior to addition of enavatuzumab (10 μg/mL) + crosslinking antibody (3.5 μg/mL). Cell viability was determined 5 days post treatment.

CELL DIVISION ANALYSES

HT3 cells (30,000 cells per six-well) were siRNA transfected as described above. Two days post transfection, CellTrace™ violet

(Invitrogen) was added to the cells at 5 μM for 30 min, cells were washed according to the manufacturer's instructions, and treated with enavatuzumab or IgG1 control in the presence of crosslinking antibody for 5 days. Cells were trypsinized and then fixed in 2% paraformaldehyde. The amount of CellTrace™ dye within the cells was analyzed by flow cytometry using a violet laser (CyAn, Dako).

XENOGRAFT GENERATION

ICR SCID mice (Taconic) were injected subcutaneously with 10^7 cells in RPMI media. Mice were randomized into groups when the average tumor volume reached ~100 mm^3. The animals were injected with 10 mg/kg enavatuzumab or IgG1 control three times per week (i.p.). Where p21 expression was to be analyzed by IHC, mice received only a single dose of antibody. Animals were sacrificed at the various times indicated post-dose. Tumors were harvested and flash frozen for protein or fixed in buffered formalin and paraffin embedded. All animal protocols and procedures were approved by the vivarium's Institutional Animal Care and Use Committee consistent with The U.S. Public Health Service *Policy on Humane Care and Use of Laboratory Animals* (Office of Laboratory Animal Welfare, National Institutes of Health).

IMMUNOHISTOCHEMISTRY

Xenograft samples were formalin fixed and paraffin embedded. Tissue sections (5 μm) were cut, mounted on slides, deparaffinized and ethanol rehydrated. Antigen retrieval was performed using BORG Decloaker RTU (Biocare Medical). Primary antibodies were p21 mIgG1 (Dako M7202) and cytokeratin18 rIgG1 (Abcam). Secondary antibodies were AlexaFluor488 goat anti-rabbit and AlexaFluor594 goat anti-mouse (Invitrogen). Slides were mounted in Vector Lab DAPI mounting medium and imaged on a Zeiss Axioskop two fluorescent microscope. The number of p21-positive cells in response to enavatuzumab or IgG1 control was determined by counting three 40× magnification fields for each xenograft tested.

RESULTS

ENAVATUZUMAB DISPLAYS BROAD GROWTH INHIBITORY ACTIVITY *IN VITRO*

A panel of 105 cancer cell lines representing the majority of solid tumor types was tested with enavatuzumab for *in vitro* growth inhibitory activity in a 5-day proliferation assay, in the presence of an anti-human $F(ab')_2$ to provide crosslinking of enavatuzumab (**Figure 1**). Cell surface TweakR expression was confirmed in 103 out of 105 cell lines by flow cytometry (data not shown). Of the 105 cell lines tested, enavatuzumab displayed ≥20% growth inhibitory activity in 38 of the cell lines (37%). Of these cell lines, 29 (28%) had ≥25% growth inhibition compared to cells treated with an isotype control antibody. Sixty-five TweakR-expressing cell lines were not sensitive to enavatuzumab, as indicated in **Table 1**. Enavatuzumab displayed activity in a broad range of tumor types with no single disease indication having notably superior sensitivity. However, two of the small cell lung cancer lines tested, NCI-H69 and NCI-H146 did not express TweakR and were not sensitive to enavatuzumab, demonstrating target specific activity.

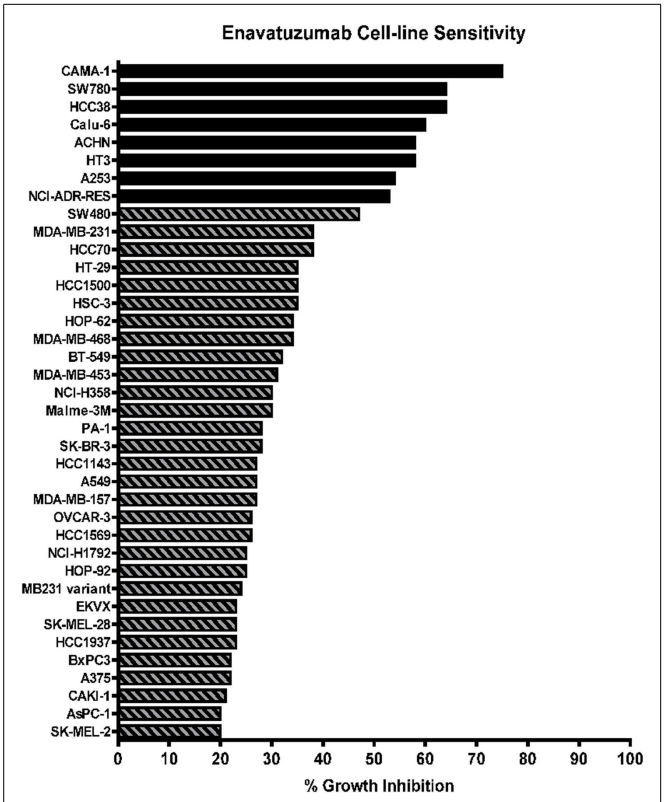

FIGURE 1 | Enavatuzumab has growth inhibitory activity in a broad range of cancer cell lines *in vitro*. One hundred and five cell lines representing multiple solid tumor types were tested for sensitivity to enavatuzumab in the presence of anti-human crosslinking antibody. The 38 cell lines that had ≥20% growth inhibition after 5-day treatment with enavatuzumab are shown.

NFκB PATHWAY IS ACTIVATED IN RESPONSE TO ENAVATUZUMAB TREATMENT

Multiple signaling pathways have been shown to be activated downstream of TweakR, including NFκB, ERK, and JNK. NFκB, in particular, has been suggested to play a pivotal role in the context of tumor cell growth, as TWEAK upregulates the NFκB signaling pathway in glioma cells where TWEAK also mediates resistance to cytotoxic agents (22). To determine if NFκB signaling might also be upregulated by TweakR agonists that inhibit tumor growth, we transfected enavatuzumab sensitive cells with a NFκB luciferase transcriptional reporter construct. Upon enavatuzumab treatment, we observed a marked increase in NFκB-driven luciferase expression in several cell lines, BT549 (**Figure 2A**), HCC38, A375, H358, and HT3 (data not shown). To investigate further the activation of the NFκB pathway by enavatuzumab, we next assessed the levels of phosphorylation of IκBα, the cytoplasmic inhibitor of classical NFκB subunits. Phosphorylation of IκBα on Serine 32/36 has been shown to reflect NFκB pathway activation, as it allows the cytoplasmic release and migration of NFκB dimers into the nucleus to initiate transcription (18, 23, 24). In enavatuzumab sensitive cell lines, we consistently observed phosphorylation of IκBα following enavatuzumab treatment (**Figure 2B**). Degradation of IκBα was observed 30 min

Table 1 | Enavatuzumab sensitivity did not correlate with TweakR expression.

Sensitive lines		Resistant lines			
Cell line	**Expression**	**Cell line**	**Expression**	**Cell line**	**Expression**
CAMA-1	5.8	786-0	7.5	NCI-H187	3.9
SW780	16.1	A2058	3.8	NCI-H209	2.4
HCC38	10.7	A498	9.4	NCI-H226	4.8
Calu-6	14.2	BT20	5.3	NCI-H23	8.4
ACHN	9.8	BT-474	4.7	NCI-H292	12.0
HT3	15.2	BT-483	2.6	NCI-H322M	1.4
A253	11.6	C32	17.1	NCI-H345	2.4
NCI-ADR-RES	72.2	Calu-3	4.8	NCI-H460	3.7
SW480	55.0	COLO-205	1.9	NCI-H520	1.9
MDA-MB-231	4.8	DMS-79	2.1	NCI-H522	3.6
HCC70	2.1	DU-4475	3.9	NCI-H596	2.9
HT-29	18.5	HCC-1428	4.0	*NCI-H69*	1.2
HCC1500	5.2	HCC2998	5.4	NCI-H82	2.0
HSC-3	8.3	HCT-116	26.3	OVCAR4	15.9
HOP-62	10.3	HCT-15	16.0	OVCAR-5	1.4
MDA-MB-468	2.6	Hs 578T	5.1	OVCAR-8	55.3
BT549	5.6	HT1376	21.6	RXF393	6.2
MDA-MB-453	1.8	HT-144	5.0	SK-MEL-5	3.6
NCI-H358	11.6	IGROV-1	8.5	SKOV3	19.4
Malme-3M	11.4	KM12	2.2	SN12C	8.2
PA-1	17.4	LoVo	3.7	SR	1.7
SK-BR-3	4.2	LOXIMVI	11.3	SW48	8.9
HCC1143	10.2	M14	10.1	SW620	4.5
A549	16.4	MCF-7	11.3	SW626	9.2
NCI-H157	4.9	MDA-MB-157	6.3	SW948	12.8
OVCAR-3	9.2	MDA-MB-175-VII	2.3	T47D	4.7
HCC1569	3.9	MDA-MB-361	6.2	TK10	18.4
NCI-H1792	1.9	MDA-MB-435	4.7	U031	15.4
HOP-92	19.7	MDA-MB-435S	28.6	UACC-257	2.8
MB231 variant	19.8	MIA-PaCa-2	6.9	UACC62	11.0
EKVX	26.1	MX-1	11.9	WM-115	7.9
SK-MEL-28	14.2	NCI-H1155	2.0	ZR-75-1	3.1
HCC1937	5.3	*NCI-H146*	1.2	ZR-75-30	4.2
BxPC3	21.4	NCI-H1838	4.6		
A375	8.9				
CAKI-1	9.0				
AsPC-1	3.6				
SK-MEL-2	6.2				

*Sensitive lines (n = 38) were defined as exhibiting at least 20% growth inhibition, and are arranged in order of sensitivity to enavatuzumab (see **Figure 1**). Resistant lines showed <20% growth inhibition and included 65 TweakR-expressing lines and 2 non-expressing lines, italicized (NCI-H146 and NCI-H69). TweakR expression was measured by enavatuzumab binding by flow cytometry and is reported as the fold increase in mean fluorescence intensity versus control antibody stained cells.*

post enavatuzumab treatment, but this degradation was transient, with IκBα expression being quickly re-established (**Figure 2C**). The phosphorylation of IκBα was observed at later time points 4 and 24 h, indicating sustained activation of the NFκB pathway, but this phosphorylation was not maintained at 48 and 72 h timepoints (**Figure 2D**). This phosphorylation event was seen in all sensitive cell lines tested, with no marked change in total IκBα expression at these time points (data not shown). In HT3 cells, cleavage of p100 to the activated subunit p52 was observed at 4 h and this activation was prolonged out to 72 h (**Figures 2C,D**).

ENAVATUZUMAB INDUCES ELEVATED NFκB SUBUNIT ACTIVATION AND INCREASED DOWNSTREAM TRANSCRIPTIONAL CHANGES IN SENSITIVE COMPARED TO RESISTANT CELL LINES

Having established that NFκB was activated in response to enavatuzumab treatment, we next performed NFκB functional ELISAs across multiple cell lines to determine which specific NFκB

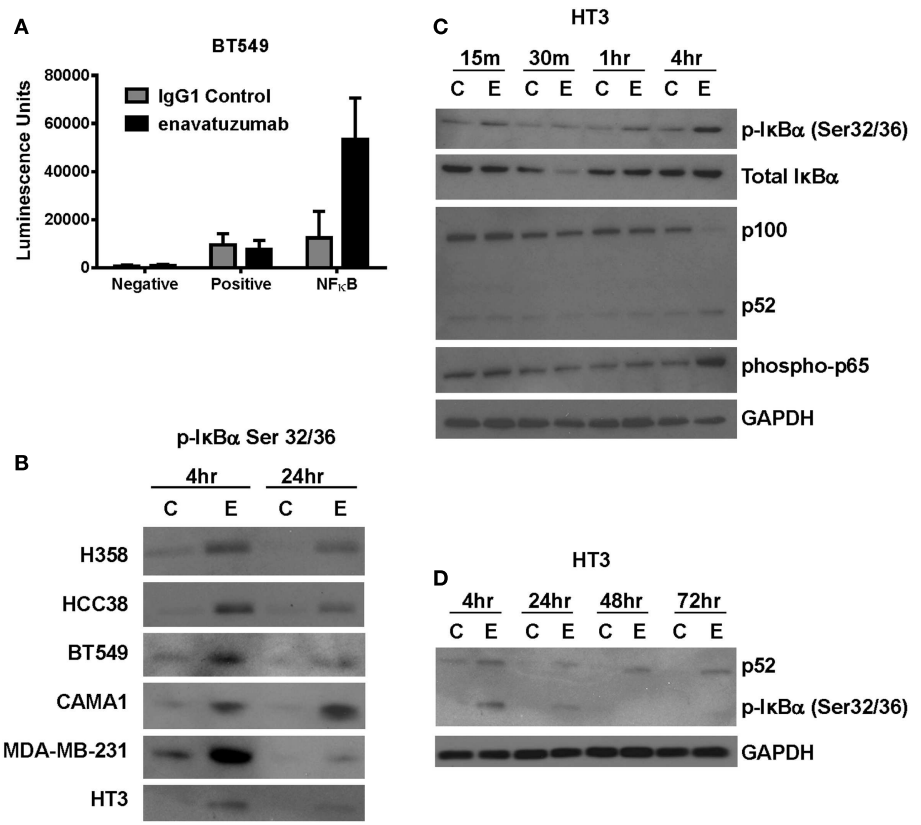

FIGURE 2 | The NFκB pathway is activated in response to enavatuzumab treatment *in vitro*. (A) A NFκB luciferase reporter construct was transfected into BT549 cells, then treated with antibody/crosslinker. Luciferase expression was measured 24 h post antibody treatment. **(B)** NFκB activation was examined by Western blotting for p-IκBα expression. Cell lysates were prepared at 4 and 24 h post antibody treatment. C, samples treated with control antibody/crosslinker; E, enavatuzumab/crosslinker-treated samples. **(C,D)** Enavatuzumab-induced the NFκB pathway in HT3 cells. HT3 cells were treated with enavatuzumab (E) or IgG1 control antibody (C) in the presence of crosslinking antibody for the times indicated. Cell lysates were prepared and activation of NFκB pathway members was analyzed by Western blot.

subunits were activated in response to enavatuzumab treatment. The NFκB DNA binding ability of six sensitive cell lines (A375, HCC38, H358, BT549, MDA-MB-468, and HT3) and three resistant cell lines (UACC62, T47D, BT20) were examined by ELISA post treatment (**Figure 3A**). Induction of all NFκB subunits (p50, p65, p52, RelB, and c-Rel) was observed in sensitive cell lines following treatment. This indicates that both classical (p50, p65) as well as non-classical (p52, RelB) NFκB pathways could be activated by enavatuzumab treatment. Interestingly, resistant cell lines showed significantly less induction of all NFκB subunits, with little or no subunit induction occurring in UACC62, T47D, and BT20.

The differential pattern of NFκB activation between enavatuzumab sensitive and resistant lines was further confirmed by gene chip microarray analysis. The number of genes up- or down-regulated >2-fold was greater in sensitive lines (BT549, MDA-MB-468) compared to those in resistant lines (T47D, BT20) (**Table 2**). For example, 408 genes were up- or down-regulated in sensitive BT549 cells after 24 h of treatment, while the expression of only 9 genes changed in the resistant T47D cells at this timepoint. Many of the transcriptional changes were for genes known to be regulated by NFκB (**Figure 3B**). Known NFκB pathway members and NFκB-regulated genes, as defined by GSES, are induced by enavatuzumab in BT549 cells. In contrast, few NFκB-regulated genes were induced in the T47D resistant cell line. Upregulation of NFκB-regulated genes was seen across all the time points in the BT549 gene chip, while for T47D, activation of the few NFκB-regulated genes appeared to be more delayed, and were often different genes than those in the sensitive lines.

NFκB ACTIVATION IS SEEN *IN VIVO* FOLLOWING ENAVATUZUMAB TREATMENT

To determine whether NFκB was activated *in vivo* in response to enavatuzumab treatment, we selected the enavatuzumab sensitive H358 xenograft model, as H358 cells displayed strong NFκB activation and growth inhibition by enavatuzumab *in vitro*. In addition, enavatuzumab exhibited 70% tumor growth inhibition (TGI) of H358 tumors *in vivo*. In this model, response to enavatuzumab was dependent on signaling through TweakR, as a version of enavatuzumab containing a mutation in the Fc region that prevents antibody-dependent cellular cytotoxicity, exhibited equivalent activity as wild-type enavatuzumab (**Figure 4A**). Other sensitive lines, including HT3 and BT549, did not form xenografts in

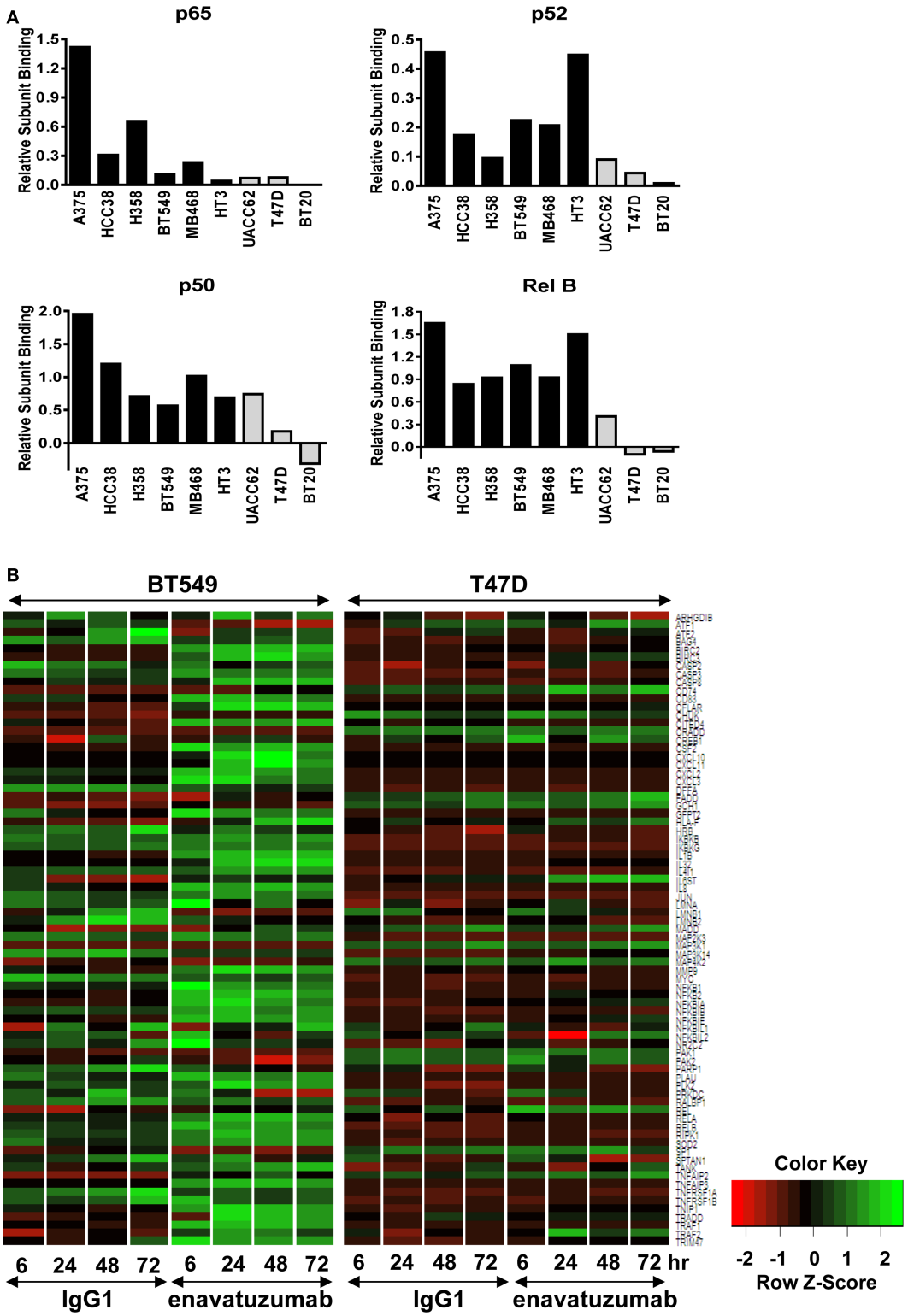

FIGURE 3 | Differential NFκB pathway activation in sensitive compared to resistant cell lines. **(A)** NFκB functional DNA binding ELISAs were performed on sensitive (black bars) and resistant (gray bars) cell lines. The relative level of subunit binding (*y*-axis) was calculated by subtracting the OD_{450} of the control samples from that of the enavatuzumab-treated samples. **(B)** Enavatuzumab-induced changes in gene expression were measured 6–72 h after antibody treatment by gene expression profiling. The average of duplicate samples is shown for each time point treated with enavatuzumab or IgG1 control in the presence of a crosslinking antibody. Changes in NFκB responsive genes are shown for a sensitive (BT549) and resistant (T47D) cell line.

Table 2 | Enavatuzumab treatment induced more gene expression changes in sensitive cell lines than in resistant lines.

	Number of genes up or down >2-fold			
	Sensitive lines		Resistant lines	
	MDA-MB-468	BT549	T47D	BT20
6 h	51	126	1	nt
24 h	50	408	9	nt
48 h	329	648	15	3
72 h	445	766	13	nt

The total number of genes whose expression is altered more than twofold up or down following enavatuzumab treatment at each time point is displayed.
nt, not tested. Analysis of BT20 cells was performed only at 48 h.

our hands. In H358 tumors harvested from enavatuzumab-treated mice, an increase in the levels of NFκB subunits RelB, p105/p50, and p52 was observed (**Figure 4B**), indicating activation of the NFκB pathway. No change in p65 expression was observed following enavatuzumab treatment in this model.

INHIBITION OF NFκB PATHWAY MEMBERS REDUCES ENAVATUZUMAB-DRIVEN CANCER GROWTH INHIBITION

Having shown that enavatuzumab treatment upregulates NFκB pathway activation, we next assessed whether NFκB signaling was important for enavatuzumab's growth inhibitory activity. siRNA experiments were performed to knock down components of the NFκB pathway, after which cells were assessed for sensitivity to enavatuzumab. siRNAs to multiple NFκB pathway members, including the upstream kinases IKKα (CHUK) or IKKβ significantly reduced the sensitivity of cell lines to enavatuzumab (**Figure 5A**). To confirm the role of the NFκB pathway in growth inhibition by enavatuzumab, a small molecule inhibitor (IKK16) of the NFκB upstream kinases IKKα/β was also found to reduce growth inhibition by enavatuzumab in multiple cell lines (**Figure 5B**). Assessing the impact of siRNA knockdown of individual NFκB subunits (p65, p52, RelB, and p50) showed a differential reduction in growth inhibition by enavatuzumab. siRNA inhibition of p52 expression reduced enavatuzumab growth inhibition in MDA-MB-468 cells, while BT549 cells primarily showed a dependency on p50 and p65 for enavatuzumab activity (**Figure 5C**). Knockdown of RelB was found to reduce enavatuzumab growth inhibition in other cell lines, including HT3 (data not shown). The importance of each particular subunit for the observed growth inhibition varied between cell lines and did not necessarily correlate with induction of subunit expression or activation by enavatuzumab (**Figures 3A,B**).

We next investigated the mechanism by which enavatuzumab inhibits tumor cell growth inhibition through NFκB. The rate of cell proliferation in enavatuzumab-treated cells was assessed using CellTrace™ dye. The dye contained within the labeled cells is diluted when passed to daughter cells during cell division, resulting in reduced fluorescence in the progeny. HT3 cells treated with enavatuzumab exhibited higher fluorescence than control-treated cells, suggesting that they divided less frequently (**Figure 5D**). Inhibition of cell division by enavatuzumab was shown to be NFκB-dependent by using siRNAs against individual NFκB subunits (p50, p65, RelB, p52). Knockdown of each of these NFκB subunits allowed HT3 cells to divide more frequently, thereby overcoming inhibition of cell division by enavatuzumab.

ENAVATUZUMAB CAUSES AN NFκB-DEPENDENT UPREGULATION OF THE CELL CYCLE INHIBITOR p21

To characterize further the mechanism of cell growth inhibition by enavatuzumab, we next assessed whether inhibition of cell division might be achieved by altering cell cycle regulators. One well characterized NFκB-regulated cell cycle inhibitor is p21 (WAF1/CIP1) (25, 26). The expression of p21 was found to be upregulated in multiple sensitive cell lines after enavatuzumab treatment (**Figure 6A**). To confirm that p21 upregulation by enavatuzumab was mediated by NFκB, siRNAs against p65 and p52 were transfected into HT3 cells, after which they were treated with enavatuzumab. While siRNAs to both p65 and p52 downmodulated expression of their cognate proteins, they had little/no effect on the expression of the other subunit (**Figure 6B**). However, p21 upregulation by enavatuzumab was blocked by siRNAs to both p65 and p52, suggesting that p21 induction was NFκB-dependent. The ability of other TweakR agonists to effect p21 upregulation was also evaluated. Multiple TweakR agonists, including the ligand, TWEAK, and TweakR targeting antibodies with differing signaling potentials, including weak (PDL400, 18.3.3), moderate (19.2.1 and enavatuzumab), and strong (136.1) agonists, were all able to upregulate p21 in HT3 cells (**Figure 6C**), suggesting that TweakR stimulation by a variety of agonists activates common downstream signaling pathways.

To determine whether p21 upregulation was important for growth inhibition mediated by enavatuzumab, BT549 cells transfected with p21 siRNAs were treated with enavatuzumab. Knockdown of p21 expression reduced the ability of enavatuzumab to inhibit the growth of BT549 cells by ~20% (**Figure 6D**). Taken together, these results suggest that growth inhibition by TweakR agonists is mediated by p21 upregulation, which is, in turn, driven by NFκB pathway activation.

Having shown that enavatuzumab-mediated growth inhibition by upregulating p21 *in vitro*, enavatuzumab was next evaluated for its ability to induce p21 *in vivo*. H358 xenografts were treated with a single dose of enavatuzumab, after which tumors were harvested and stained for p21 by immunohistochemistry (**Figure 7A**). Enavatuzumab treatment resulted in an increase in p21-positive cells that was observed 3 days after dosing, and was maintained for at least 1 week after a single i.p. dose of enavatuzumab (**Figure 7B**).

DISCUSSION

In this study we have demonstrated that enavatuzumab caused a growth inhibitory effect on many cancer cell lines, across a range of tumor types. This agrees with the broad *in vivo* activity seen in xenograft models (17) and supports the investigation of enavatuzumab as a therapeutic modality in solid tumors. Growth inhibition and signaling was enhanced through the crosslinking of enavatuzumab. This implies that TweakR oligomerization is important for enavatuzumab-mediated growth inhibition and for the signaling that occurs downstream of the receptor. This agrees

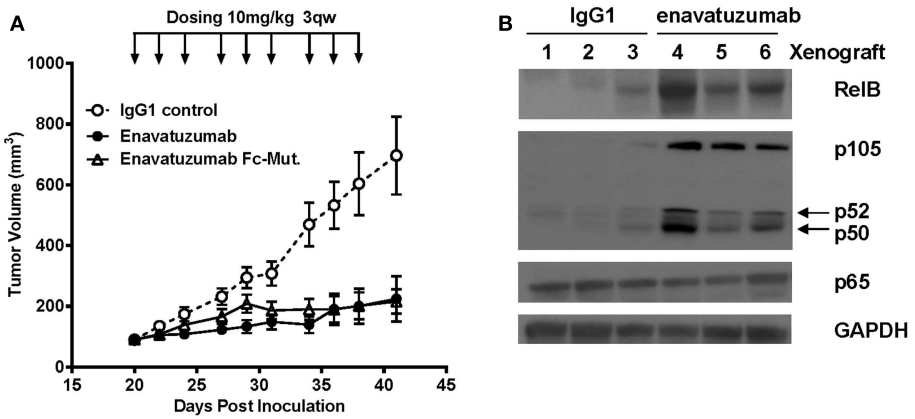

FIGURE 4 | ***In vivo*** **efficacy and NFκB activation in H358 xenografts in response to enavatuzumab**. **(A)** H358 xenograft tumors at ~100 mm³ in ICR SCID mice were treated i.p. with enavatuzumab, enavatuzumab-Fc mutant, or IgG1 control at 10 mg/kg q3w as indicated. **(B)** H358 xenografts were treated on days 0 and 3 with enavatuzumab or control antibody. Tumors from three mice from each treatment group were excised on day 4, after which cell lysates were prepared and analyzed by Western blot. Activation of NFκB was examined by measuring RelB, p105/p50, p52, and p65 levels in the tumors.

with previous reports assessing TweakR activation with agonist antibodies (15, 27).

It should be noted that the extent of cell surface expression of TweakR did not correlate with *in vitro* activity, as discussed previously (15, 17). Therefore the highest TweakR-expressing cell lines were not necessarily the most enavatuzumab sensitive. This is likely due to the pleiotropic functions of TweakR and reveals a degree of complexity regarding the relationship between receptor expression and function that requires further investigation.

The induction of NFκB in response to enavatuzumab stimulation of TweakR agrees with findings in the literature for the receptor's natural ligand TWEAK (2, 9, 28). Like TWEAK, enavatuzumab activated both classical and non-classical NFκB pathways. Previous reports have demonstrated transient activation of p50/p65 NFκB subunits and a more sustained activation of the non-classical p52/RelB subunits in response to TWEAK (29, 30). Interestingly we observed phosphorylation of the NFκB inhibitor IκBα up to 24 h post enavatuzumab treatment. This indicates prolonged activation and continual re-expression of IκBα despite NFκB activation. This contrasts with what is observed with TNFα, where IκBα is rapidly phosphorylated and degraded leading to transient activation of NFκB (31). We also saw activation of p52 up to 72 h post addition of enavatuzumab. The finding that NFκB pathway induction was observed several days post enavatuzumab treatment by Western blot and microarray analyses indicates some possible differential activity between that of TweakR's ligand TWEAK and enavatuzumab.

Transient versus persistent NFκB activation can result in different gene induction profiles in response to TNFα and LPS, resulting in contrasting protein expression and varying growth and survival phenotypes (32, 33). Therefore continual signaling through TweakR by enavatuzumab may induce different NFκB-regulated genes than when TWEAK binds TweakR. Also, it has been previously reported that TWEAK can be internalized (10, 34), and like other TNF family members, the cell surface expression of the receptor TweakR is likely to be tightly regulated/internalized upon ligand stimulation (35, 36). In contrast, antibody binding to TweakR maintains cell surface expression of TweakR (data not shown), which may lead to the observed prolonged receptor activation and sustained stimulation of downstream signaling pathways, such as NFκB. Alternatively, the crosslinking of enavatuzumab may aid in prolonging the signaling downstream of TweakR which could explain differences in persistence of signaling seen with TWEAK ligand versus enavatuzumab. Such a relationship between antibody crosslinking and downstream signaling has been described previously (37).

Cell lines sensitive to enavatuzumab displayed more NFκB activation, as seen by subunit functional ELISA, compared to resistant cell lines. Resistant cell lines also showed a delayed and reduced induction of NFκB-regulated genes. Gene expression analysis revealed strong induction of many NFκB-regulated genes across all time points tested (6–72 h), while T47D cells had little induction of the same NFκB-regulated genes, especially at the earlier time points. Sensitive cell lines showed 400–700 genes up or down-regulated >2-fold following enavatuzumab treatment, while resistant cells exhibited changes in expression in fewer than 20 genes, indicating that significant transcriptional activity occurred in sensitive cell lines, which was not observable in the resistant setting. A small number of NFκB-regulated genes were induced in the T47D cell line, which were not induced in BT549 cells, which may suggest that some of these genes may play a role in the resistant phenotype, but their role, if any, is unclear. In general, it appears that sensitive cell lines exhibit increased overall activation of NFκB signaling by enavatuzumab, and these changes could potentially be useful when selecting patients and measuring their response. Therefore, understanding the molecular changes downstream of TweakR signaling in the sensitive/resistant settings is of great interest.

We demonstrated that the NFκB activation observed in response to enavatuzumab was essential for its growth inhibitory activity. siRNA targeting of specific NFκB subunits (p50, p65, p52, RelB) abrogated the growth inhibition, however different cell lines

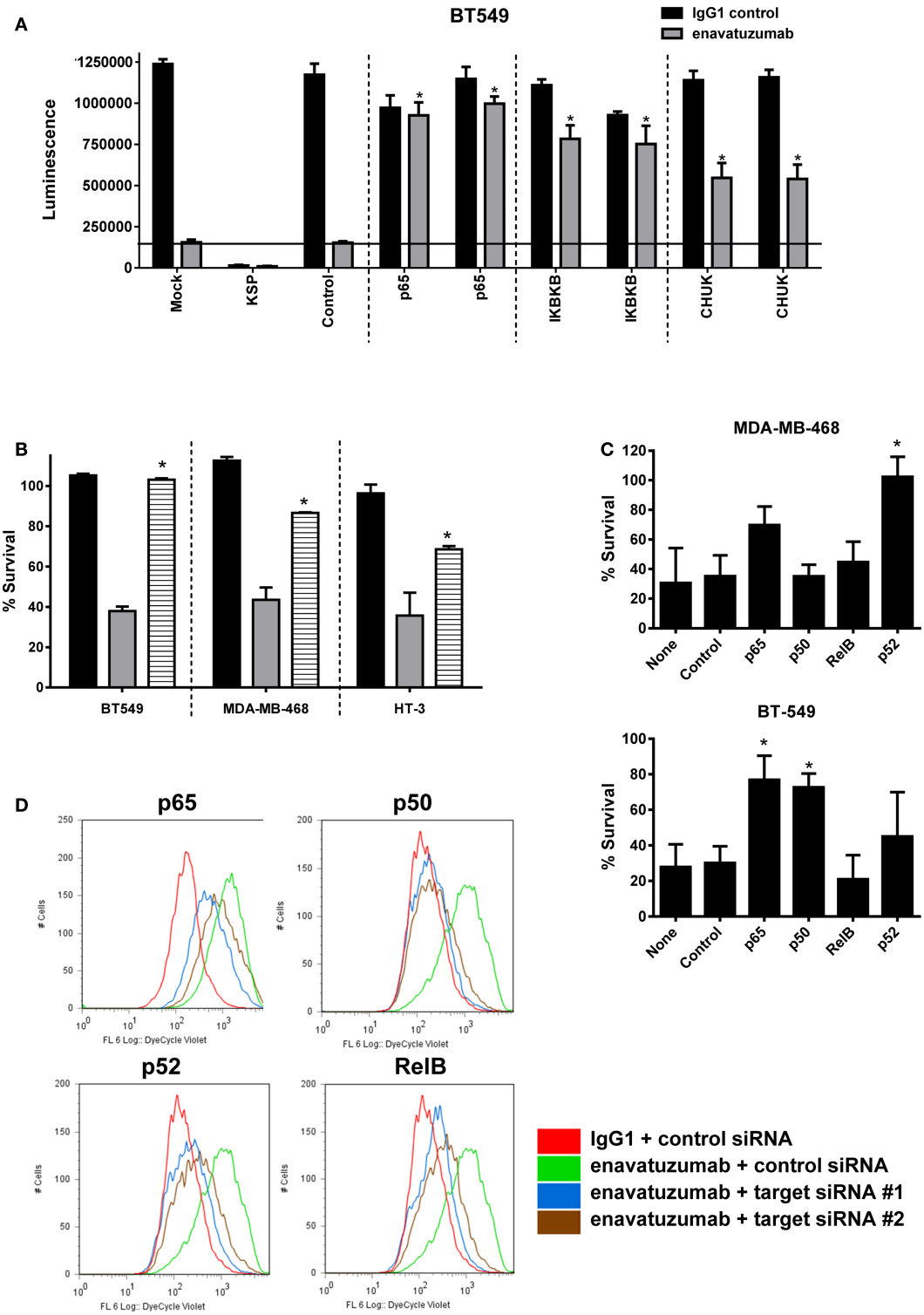

FIGURE 5 | Inhibition of NFκB activation prevents the growth inhibitory activity of enavatuzumab. **(A)** BT549 cells were transfected with two different siRNAs against p65 or upstream kinases IKK1 (IκBKβ), IKK2 (CHUK). Transfection controls were non-targeting control (negative) or KSP (positive control for transfection efficiency). After 48 h, cells were treated with enavatuzumab or IgG1 control at 10 μg/mL for an additional 5 days in the presence of anti-human crosslinking antibody (3.5 μg/mL), and the cell viability was determined. siRNA significantly reduced growth inhibition by enavatuzumab, compared to mock or control siRNA transfected cells (horizontal line) (*p-value < 0.05). **(B)** Enavatuzumab sensitive lines were treated with IKK inhibitor IKK16 at 160 nM (black bars), enavatuzumab/crosslinker (gray bars), or IKK16 plus enavatuzumab/crosslinker (striped bars) and % survival was measured after 5 days. IKK16 significantly blocked growth inhibition by enavatuzumab compared to enavatuzumab alone (*p-value < 0.05).

(Continued)

FIGURE 5 | Continued
(C) Targeting individual NFκB subunits (p50, p65, RelB, p52) by siRNA reduced enavatuzumab activity in sensitive cell lines (MDA-MB-468 and BT549). Cells were transfected with pooled targeting siRNA or control siRNA for 48 h, prior to treatment with enavatuzumab or IgG1 control for 5 days in the presence of a crosslinking antibody. Percent survival was calculated from the relative viability of cells treated with enavatuzumab versus control-treated cells (*p-value < 0.05). **(D)** The effect of NFκB induced by enavatuzumab on cell division was investigated by pre-labeling HT3 cells with the Cell Trace™ reagent. Cells treated with enavatuzumab/crosslinker (green line) are compared to the IgG1 control-treated cells (red line). Cells transfected with two different siRNAs to p65, p50, p52, or RelB and treated with enavatuzumab/crosslinker (blue and brown lines) are also displayed.

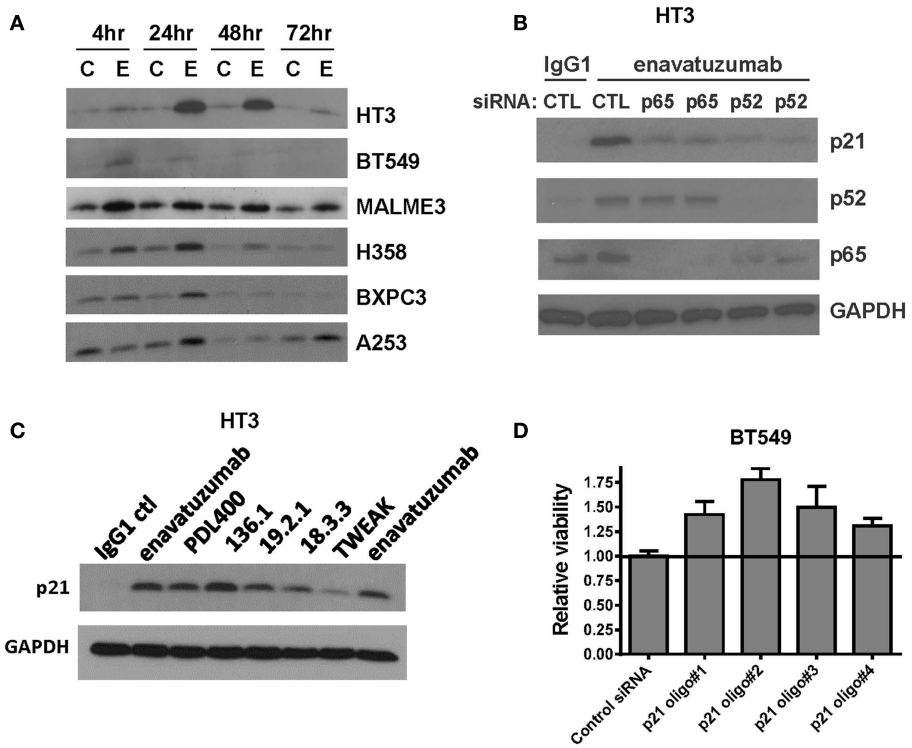

FIGURE 6 | Enavatuzumab inhibits cell division and upregulates p21 *in vitro* and *in vivo* in an NFκB-dependent manner. **(A)** Sensitive cell lines HT3, BT549, MALME-3M, H358, BXPC3, and A253 were treated with IgG1 control antibody (C) or enavatuzumab (E) for 4–72 h in the presence of crosslinking antibody, after which p21 expression was assessed by Western blot. **(B)** The role of NFκB in enavatuzumab-induced p21 expression was examined by knocking down the expression of p65 or p52 with two different siRNAs to each. Samples were transfected with siRNA for 24 h, then treated with IgG1 control or enavatuzumab for an additional 24 h in the presence of crosslinking antibody. Cell lysates were analyzed by Western blot for p21, p52, and p65 expression. **(C)** p21 is activated by multiple antibodies targeting TweakR targeting and TWEAK ligand. HT3 cells were treated (10 μg/mL) with antibodies that bind and activate TweakR including enavatuzumab, PDL400, 136.1, 19.2.1, 18.3.3, or TWEAK ligand (300 ng/mL) for 24 h in the presence of crosslinking antibody. p21 expression was then assessed by Western blot. **(D)** siRNA inhibition of p21 reduces the relative growth inhibition caused by enavatuzumab treatment. BT549 cells were transfected with four different siRNA oligos targeting p21. Two days post transfection, cells were treated with enavatuzumab for 5 days in the presence of crosslinking antibody and cell viability was determined. Relative viability of 1.0 represents the viability of cells transfected with the non-targeting control siRNA and treated with enavatuzumab. An increase above 1.0 in cells transfected with p21 siRNAs indicates an increase in cell viability relative to the control siRNA transfected cells ($p < 0.05$). Control transfected cells treated with the IgG1 control antibody exhibited a relative viability of 3.75.

were more sensitive to inhibition of certain subunits than others. For example, HT3 cells were dependent on non-classical (p52/Rel B) NFκB activation, while BT549 cells relied more on the classical NFκB (p50/p65) pathway for enavatuzumab growth inhibition. All sensitive cell lines showed a marked increase in the expression of NFκB subunits by enavatuzumab, but there was no clear pattern of subunit induction in response to enavatuzumab treatment, and the induction did not always correlate with siRNA subunit sensitivity. It should be noted however, that we were able to reduce enavatuzumab activity in every sensitive cell line by inhibiting at least one or more NFκB subunits. Therefore, there does not appear to be a single reliance on either the classical or non-classical NFκB pathways for enavatuzumab activity, but is likely to comprise a complex combination of cellular events involving cross-talk and interdependency between both pathways that requires further examination.

Enavatuzumab-induced growth inhibition of many cancer cell lines, but the characteristics of this growth inhibition had not

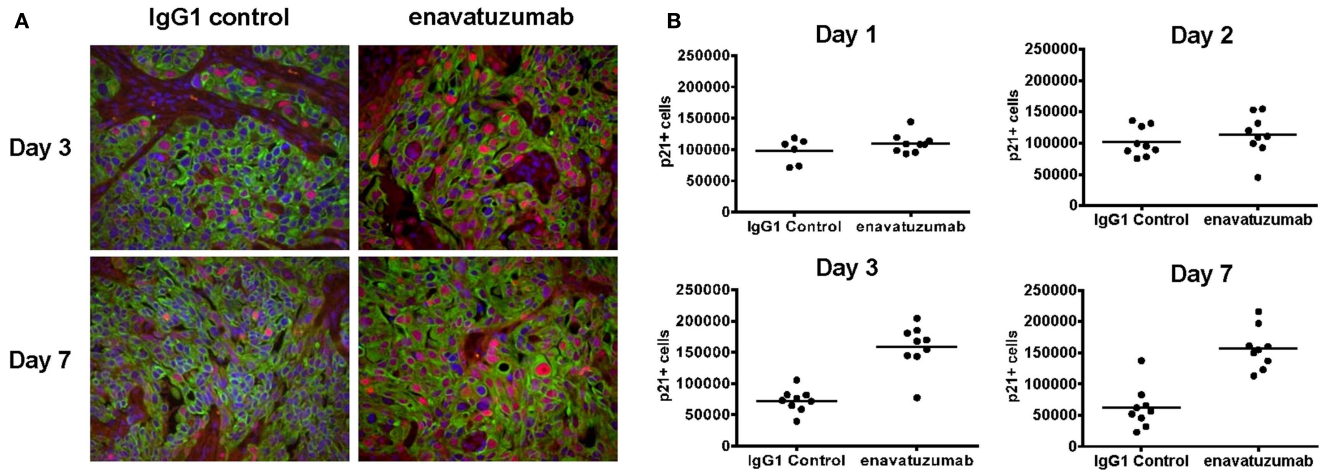

FIGURE 7 | Enavatuzumab treatment of H358 xenograft tumors results in p21 upregulation *in vivo*. (A) H358 xenografts were grown in ICR SCID mice to ~100 mm^3 and mice were then treated with a single i.p. injection of IgG1 control or enavatuzumab at 10 mg/kg. Tumors were excised and the levels of p21 were determined by multi-color IHC. A representative image with p21 expression in red, cytokeratin in green, and nuclei in blue is shown (40× magnification). **(B)** Quantification of p21 expression from H358 xenografts excised on days 1, 2, 3, and 7 post treatment. Data points represent a single 40× magnification field with three fields for three individual xenografts analyzed per time point for each treatment ($p < 0.001$ IgG1 control versus enavatuzumab days 3 and 7). The *y*-axis represents quantification of p21+ cells in arbitrary units.

previously been described. We found that enavatuzumab treatment resulted in a marked reduction in cell division. In HT3 cells, siRNA inhibition of p50, p52, and RelB in the context of enavatuzumab treatment helped restore the number of cell divisions to that seen with IgG1 control. This further confirmed NFκB's critical role in enavatuzumab's growth inhibitory activity.

When we evaluated the possible causes of the cell division block caused by enavatuzumab, we found that the key cell cycle regulator p21 was induced following treatment in many responder cell lines both *in vitro* and in H358 xenografts *in vivo*. The prolonged *in vivo* upregulation of the cell cycle inhibitor p21, up to 7 days after a single antibody dose, is a compelling growth inhibitory mechanism of enavatuzumab and is a novel activity for a TweakR-targeted therapeutic. p21 was found to be upregulated by multiple TweakR-targeted molecules that have different levels of agonism, suggesting a common mechanism of growth inhibition by diverse agents that activate the TweakR signaling pathway. We were able to block enavatuzumab-driven p21 upregulation by inhibiting specific NFκB subunits. Inhibition of p52 in particular, resulted in complete loss of p21 induction in HT3 cells, further supporting the importance of the non-classical NFκB pathway for enavatuzumab's activity in this cell line.

It has been reported that TweakR activation can result in growth inhibition by NFκB-mediated upregulation of TNF and subsequent apoptosis via TNFR1 in some cell lines (e.g., Kym-1, OVCAR4, SKOV3) (38, 39). Interestingly, two of these cell lines, OVCAR4 and SKOV3 were included in our screen and were found not to be sensitive to enavatuzumab. However, because not all enavatuzumab sensitive cell lines were assessed for p21 upregulation, it is possible that additional mechanisms, including TNF-mediated apoptosis, may be involved in the growth inhibition by enavatuzumab. This question warrants further evaluation.

The NFκB-dependent growth inhibitory activity of enavatuzumab is an interesting finding for a family of transcription factors frequently associated with growth and survival in cancer (40–42). However, the diversity and complexity of NFκB signaling suggests that NFκB can inhibit the growth of tumor cells under certain circumstances (43).

Therefore, our finding that the growth inhibitory activity of enavatuzumab is driven in large part by NFκB suggests that the targeted activation of the NFκB pathway may be a novel therapeutic approach for treating cancer. In summary we have outlined how NFκB activation and induction of downstream signaling events, including p21, are essential for the TGI mediated by enavatuzumab.

AUTHOR CONTRIBUTIONS

All authors are employes or former employes of AbbVie Biotherapeutics Inc., or Abbott Biotherapeutics. The design, study conduct, and financial support for this research was provided by AbbVie. James W. Purcell and Han K. Kim participated in the design and execution of experiments; interpretation of results; and drafted, revised, and approved the final manuscript. Sonia G. Tanlimco and Minhtam Doan participated in the design and execution of experiments, interpretation of results; and revised and approved the final manuscript. Melvin Fox participated in the execution of experiments, interpretation of results; and revised and approved the final manuscript. Peter Lambert participated in the interpretation of results; and revised and approved the final manuscript. Debra T. Chao participated in the design and execution of experiments, interpretation of results; and revised and approved the final manuscript. Mien Sho participated in the design and execution of experiments; and revised and approved the final manuscript. Keith E. Wilson participated in the design of experiments; interpretation

of results; and revised and approved the final manuscript. Gary C. Starling participated in the design of experiments; interpretation of results; and revised and approved the final manuscript. Patricia A. Culp participated in the design of experiments; interpretation of results; and revised and approved the final manuscript. AbbVie participated in the review and approval of the manuscript.

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TNF receptor-associated factor 1 is a major target of soluble TWEAK

José Antonio Carmona Arana[1], Axel Seher[2], Manfred Neumann[1], Isabell Lang[1], Daniela Siegmund[1] and Harald Wajant[1]*

[1] *Division of Molecular Internal Medicine, Department of Internal Medicine II, University Hospital Würzburg, Würzburg, Germany*
[2] *Department of Oral and Maxillofacial Plastic Surgery, University Hospital Würzburg, Würzburg, Germany*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Gail Abendroth Bishop, The University of Iowa, USA
Domagoj Vucic, Genentech, Inc., USA
John Silke, Walter and Eliza Hall Institute, Australia

****Correspondence:***
Harald Wajant, Department of Internal Medicine II, Division of Molecular Internal Medicine, University Hospital Würzburg, Röntgenring 11, 97070 Wüerzburg, Germany
e-mail: harald.wajant@mail.uni-wuerzburg.de

Soluble tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), in contrast to membrane TWEAK and TNF, is only a weak activator of the classical NFκB pathway. We observed that soluble TWEAK was regularly more potent than TNF with respect to the induction of TNF receptor-associated factor 1 (TRAF1), a NFκB-controlled signaling protein involved in the regulation of inflammatory signaling pathways. TNF-induced TRAF1 expression was efficiently blocked by inhibition of the classical NFκB pathway using the IKK2 inhibitor, TPCA1. In contrast, in some cell lines, TWEAK-induced TRAF1 production was only partly inhibited by TPCA1. The NEDD8-activating enzyme inhibitor MLN4924, however, which inhibits classical and alternative NFκB signaling, blocked TNF- and TWEAK-induced TRAF1 expression. This suggests that TRAF1 induction by soluble TWEAK is based on the cooperative activity of the two NFκB signaling pathways. We have previously shown that oligomerization of soluble TWEAK results in ligand complexes with membrane TWEAK-like activity. Oligomerization of soluble TWEAK showed no effect on the dose response of TRAF1 induction, but potentiated the ability of soluble TWEAK to trigger production of the classical NFκB-regulated cytokine IL8. Transfectants expressing soluble TWEAK and membrane TWEAK showed similar induction of TRAF1 while only the membrane TWEAK expressing cells robustly stimulated IL8 production. These data indicate that soluble TWEAK may efficiently induce a distinct subset of the membrane TWEAK-targeted genes and argue again for a crucial role of classical NFκB pathway-independent signaling in TWEAK-induced TRAF1 expression. Other TWEAK targets, which can be equally well induced by soluble and membrane TWEAK, remain to be identified and the relevance of the ability of soluble TWEAK to induce such a distinct subset of membrane TWEAK-targeted genes for TWEAK biology will have to be clarified in future studies.

Keywords: CD40, NFκB, TNF, TRAF1, TWEAK

INTRODUCTION

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor (FGF)-inducible molecule-14 (Fn14), alternatively termed TweakR or TNFRSF12, form a phylogenetically well conserved ligand–receptor pair of the TNF family. Fn14 is highly expressed during development and in cancer but is also strongly induced in a variety of cell types of the adult organism upon tissue damage (1, 2). Accordingly, Fn14 has been implicated in tissue repair-associated processes, such as control of proliferation and differentiation of mesenchymal progenitor cells, orchestration of immune reactions, cell migration, and angiogenesis. However, if not held in check, Fn14 activity can also have detrimental effects contributing to autoimmune-related pathologies but also to muscle atrophy, ischemic tissue damage, and fibrosis (1, 2). Typically, stimulation of Fn14 results in the activation of various proinflammatory signaling pathways such as the classical and alternative NFκB pathway and the various MAPK pathways (3, 4). Like most other members of the TNF receptor family, Fn14 activates proinflammatory signaling pathways with the help of adapter proteins of the TNF receptor-associated factor (TRAF) family, especially TRAF2 (5–7). Indeed, TRAF2 and the TRAF2-associated E3 ligases cellular inhibitor of apoptosis-1 (cIAP1) and cIAP2 are not only required by Fn14 for the activation of the classical NFκB pathway but are also crucially involved in TWEAK-induced stimulation of MAP kinases (4, 8). There is furthermore evidence that TRAF2 is also required for TWEAK/Fn14-mediated activation of Rho-GTPases by recruiting the Src homology 3 domain-containing guanine nucleotide exchange factor [SGEF; (9)]. Noteworthy, TWEAK-induced activation of Rho-GTPases appears particularly relevant for cell migration of glioblastoma cells (9–11). In general, however, the question how the various Fn14-associated signaling pathways contribute, in which cell type, to the aforementioned biological effects of TWEAK and Fn14 is poorly understood. Indeed, the complexity of Fn14 signaling is particularly surprising against the background of the simple structure of the molecule. Two major mechanisms that contribute to the plasticity and variability of TWEAK/Fn14-mediated effects are based on the capability of Fn14 to adopt different states of activity and to modulate signaling by other members of the TNF receptor family.

Like other members of the TNF ligand family, TWEAK acts as a membrane-bound trimeric protein but also in a soluble form that is released from the membrane-bound molecule by proteolytically processing (12–15). It is noteworthy that the two forms of TWEAK trigger partly different patterns of intracellular signaling events. So far investigated, membrane TWEAK or oligomerized soluble TWEAK, which mimics the activity of membrane TWEAK, efficiently activate all known Fn14-associated signaling pathways including the classical NFκB pathway and proteolytic degradation of TRAF2 (15–17). Soluble TWEAK, however, fails to trigger these responses or does it only very moderately (15). Membrane and soluble TWEAK, however, are both comparably effective with respect to activation of the alternative NFκB pathway and enhancement of TNFR1-mediated cell death (15, 17). The different activity of soluble and membrane TWEAK correlates with a different effect on complexes of TRAF2 with cIAP1 or cIAP2. Both forms of TWEAK induce efficient interaction of Fn14 with TRAF2–cIAP1/2 complexes. However, in addition to this, Fn14 also triggers transactivation of the TRAF2-associated cIAPs and thus activation of the classical NFκB pathway, TRAF2 degradation, and cIAP autodegradation when stimulated by membrane TWEAK or oligomerized soluble TWEAK. The sole recruitment of TRAF2–cIAP1/2 complexes to Fn14 is typically sufficient to reduce the cytosolic pool of TRAF2–cIAP1/2 complexes (16, 17). The cytosolic pool of TRAF2–cIAP1/2 complexes, however, is crucially involved in the constitutive inhibition of the alternative NFκB pathway and also antagonizes caspase-8 activation upon TNFR1 stimulation (2). Thus, TWEAK-induced Fn14-mediated depletion of cytosolic TRAF2–cIAP1/2 complexes, irrespective of subsequent cIAP1/2 activation, can adequately explain the similar activity of soluble and membrane TWEAK regarding activation of the alternative NFκB pathway and sensitization for TNF-induced cell death. As TRAF2–cIAP1/2 complexes are also of relevance for classical NFκB signaling and MAPK activation in response to stimulation of various members of the TNF receptor family, TRAF2–cIAP1/2 depletion is presumably also responsible for the attenuated proinflammatory activities of TNFR1, TNFR2, and CD40 that has been observed in TWEAK-primed cells (17, 18–20).

TNF receptor-associated factor 1 forms heterocomplexes with TRAF2 at the expense of the formation of TRAF2 homotrimers (21). As TRAF1 and TRAF2 can differ in their affinity for the TRAF2 binding site of a particular TRAF2-interacting TNF receptor, TRAF1–TRAF2 heteromer formation can modify TRAF2-mediated TNF receptor signaling by changing the efficacy/strength of receptor–TRAF2 interaction. Indeed, the TNF receptor family member CD40 recruits TRAF2–TRAF1 heteromers less efficient than TRAF2 homotrimers and this correlates with reduced activation of the classical NFκB pathway (22). TRAF1 is furthermore a well-established target of classical NFκB-stimulating cytokines, such as TNF and IL1. In light of a possible role of TRAF1 in the crosstalk of Fn14 with other TRAF2-interacting receptors and against the background of the different effects of soluble and membrane TWEAK on the alternative and classical NFκB pathway, we evaluated here the ability of different forms of TWEAK to induce TRAF1 expression. Unexpectedly, we found that soluble TWEAK, which poorly stimulates the classical NFκB pathway, induces strong TRAF1 expression in transformed and non-transformed cells. We furthermore obtained initial evidence that soluble TWEAK-induced TRAF1 expression contributes to the ability of the TWEAK/Fn14 system to modulate the activity of CD40 signaling.

RESULTS

SOLUBLE TWEAK IS SUPERIOR TO TNF IN TRAF1 INDUCTION

The capabilities of soluble TNF and soluble TWEAK to activate the classical NFκB pathway were analyzed in various cell lines. As expected, TNF efficiently triggered phosphorylation and degradation of IκBα, two hallmarks of the classical NFκB pathway, in all cell lines investigated within 5–15 min including transformed cell lines of different origin (**Figure 1A**) and primary cells (Figure S1 in Supplementary Material). In contrast, in the 2-h time window investigated, soluble TWEAK showed only delayed barely detectable IκBα phosphorylation and IκBα degradation was practically not evident (**Figure 1A**). This furthermore correlated with the fact that production of IL8, a cytokine that is dominantly regulated by the classical NFκB pathway, was much stronger induced by TNF than by soluble TWEAK (**Figure 1B**; Figure S1 in Supplementary Material).

To rule out that the observed limited effects of soluble TWEAK were related to a general poor TWEAK responsiveness of the cell lines investigated or low specific activity of the soluble TWEAK batch used for stimulation, we also assayed activation of the alternative NFκB pathway. Indeed, already at low concentrations soluble TWEAK triggered accumulation of the NFκB inducing kinase (NIK) and processing of p100, two events indicative for stimulation of the alternative NFκB pathway (23), while TNF had no effect in this regard (**Figures 2A,B**). We also analyzed induction of TRAF1, a well-recognized NFκB-regulated target of TNF (24–26). According to the literature, there was only weak expression of TRAF1 in unstimulated cells, but TRAF1 expression was readily induced by treatment with TNF. Surprisingly, however, soluble TWEAK was as efficient as, or even superior to TNF in TRAF1 induction (**Figures 2A,B**; Figure S1 in Supplementary Material). Although, soluble TWEAK-induced TRAF1 expression with somewhat slower kinetics than TNF, the maximal TRAF1 levels reached were regularly significantly higher (**Figures 2A,B**). Noteworthy, soluble TWEAK-induced TRAF1 production occurred with some delay with respect to NIK accumulation and p100 processing. (**Figures 2A,B**). NIK and IKK1 can also crosstalk into the classical NFκB pathway, e.g., at the level of IκBα phosphorylation (27). We thus re-analyzed phosphorylation of IκBα in the samples of, in comparison to **Figure 1A**, the extended time course used for evaluation of TRAF1 induction and activation of the alternative NFκB pathway. Indeed, there was now significant phosphorylation of IκBα in soluble TWEAK-treated cells (**Figure 2B**). However, analysis of total IκBα levels showed no major changes in the amount of this molecule and there was only a minor fraction of the slower migrating phosphorylated IκBα species. As the gene encoding IκBα itself is a bona fide target of the classical NFκB pathway, this argues for weak but persistent stimulation of the downstream steps of the classical NFκB pathway resulting in a balance between degradation and resynthesis of IκBα. However, as soluble TWEAK, in contrast to TNF, failed to trigger robust

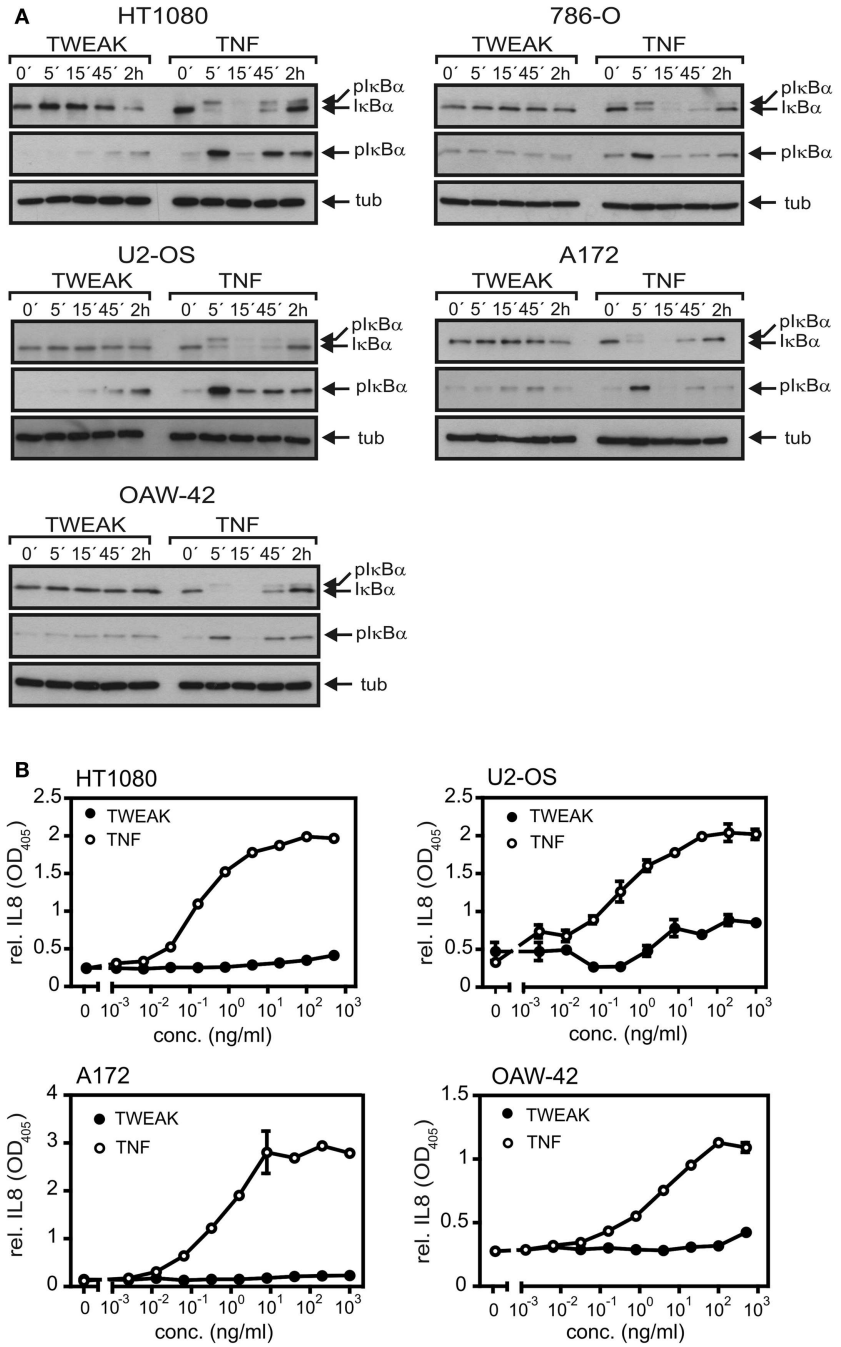

FIGURE 1 | TNF is superior to soluble TWEAK in activation of the classical NFκB pathway. **(A)** The various cell lines were stimulated for the indicated times with Flag-TNF (100 ng/ml) and Flag-TWEAK (200 ng/ml) and total cell lysates were then analyzed for the presence of phospho-IκBα and total IκBα. **(B)**. Cells were challenged overnight in triplicates with the indicated concentrations of Flag-TNF and Flag-TWEAK and supernatants were assayed for IL8 by ELISA. 786-O cells display strong constitutive expression of IL8 and were thus not assayed for IL8 production.

production of IL8 (**Figure 1B**), this hardly explains the superior induction of TRAF1 by soluble TWEAK.

One possible explanation for the observation that the capabilities of TNF and soluble TWEAK to trigger the classical NFκB pathway does not correlate with the TRAF1 expression levels induced by these ligands is that in the case of TNF, the accumulation of TRAF1 is limited by post-translational mechanisms. Indeed, it has been reported that TRAF1 can be processed at D163 by caspase-3 and caspase-8 in death receptor-stimulated cells resulting in a NFκB inhibitory fragment (28–30). Thus, this mechanism could possibly not only reduce the amount of TRAF1 full-length molecules but could also terminate sustained TRAF1 induction via

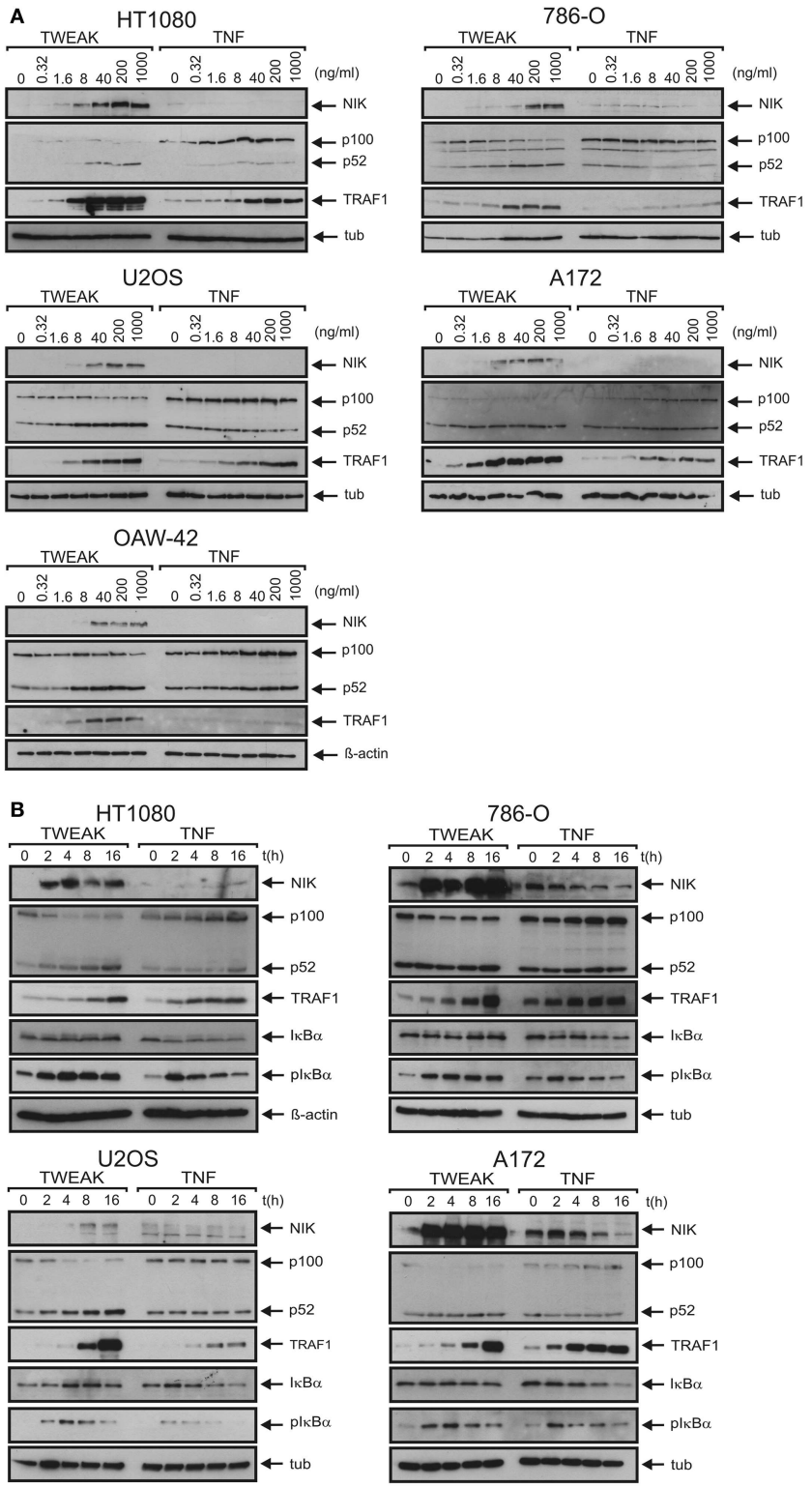

FIGURE 2 | Soluble TWEAK is superior to TNF in TRAF1 induction. **(A,B)** Cells were either stimulated overnight with the indicated concentrations of Flag-TWEAK and Flag-TNF **(A)** or were incubated with a constant amount of these cytokines (100 ng/ml Flag-TNF, 200 ng/ml Flag-TWEAK) for varying times **(B)**. Total cell lysates were finally analyzed by western blotting with respect to the indicated proteins.

the classical NFκB pathway. There is furthermore evidence that cIAP2-mediated ubiquitination of TRAF1 results in proteasome-dependent degradation (31). Thus, to evaluate the possibility that differences in the post-translational regulation of TRAF1 expression between TNF- and TWEAK-treated cells are responsible for the seemingly superior TRAF1 induction by TWEAK, we analyzed TRAF1 stability in TNF- and TWEAK-stimulated cells. For this purpose, cells were treated upon TRAF1 induction with the proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide (CHX) (**Figure 3A**). As TNF has a weak apoptosis-inducing effect on HT1080 cells, we investigated in this cell line also the effect of the pan-caspase inhibitor z-VAD-fmk on TRAF1 induction (**Figure 3B**). However, we found no evidence in these experiments that the aforementioned post-transcriptional mechanisms differentially limit TNF- and TWEAK-induced TRAF1 expression. Furthermore, more efficient TRAF1 induction by soluble TWEAK was also evident from RT-PCR analysis of TNF- and TWEAK-treated cells arguing again against a major role of post-transcriptional mechanisms in TRAF1 production by TNF and soluble TWEAK (**Figures 3C,D**).

SOLUBLE TWEAK INDUCES TRAF1 BY CLASSICAL NFκB PATHWAY-DEPENDENT AND -INDEPENDENT MECHANISMS

The superior ability of soluble TWEAK compared to TNF to induce TRAF1 as well as the kinetics of TWEAK-induced TRAF1 expression suggest that classical NFκB pathway-independent mechanisms play here a crucial role. Indeed, oligomerization of soluble TWEAK, a way to enhance the ability of soluble TWEAK to stimulate the classical NFκB pathway, which, however, has practically no effect on the stimulation of the alternative NFκB pathway (15), showed no major enhancing effect on TRAF1 induction (**Figure 4A**). The ability of soluble TWEAK to induce the classical NFκB target IL8, however, was strongly enhanced by oligomerization (**Figure 4B**). As, on the one hand, oligomerization enhances the ability of soluble TWEAK to trigger the classical NFκB pathway, and as, on the other hand, oligomerization has no effect on the dose response of TWEAK-induced TRAF1 induction, the latter seems to be controlled to a significant extent by mechanisms independent from classical NFκB signaling. In line with our previous finding that oligomerized soluble TWEAK mimics the activity of membrane TWEAK (15, 32), we furthermore observed that cells expressing a non-cleavable mutant of membrane TWEAK efficiently trigger IL8 and TRAF1 production, while soluble TWEAK producing cells showed strong TRAF1 induction but only very moderate IL8 induction (**Figures 4C,D**).

We have recently shown that TNF-induced IKK2-mediated activation of the classical NFκB pathway is strongly inhibited without a significant effect on TWEAK-induced IKK1-mediated activation of the alternative NFκB pathway in cells treated with the IKK2-specific inhibitor TPCA1 (33). Under such conditions, TNF-induced TRAF1 production was blocked in all cell lines investigated (**Figure 5A**). In some cell lines, the minor levels of basal TRAF1 expression were also reduced by treatment with TPCA1. In these cases, TPCA1 treatment reduced TNF-induced TRAF1

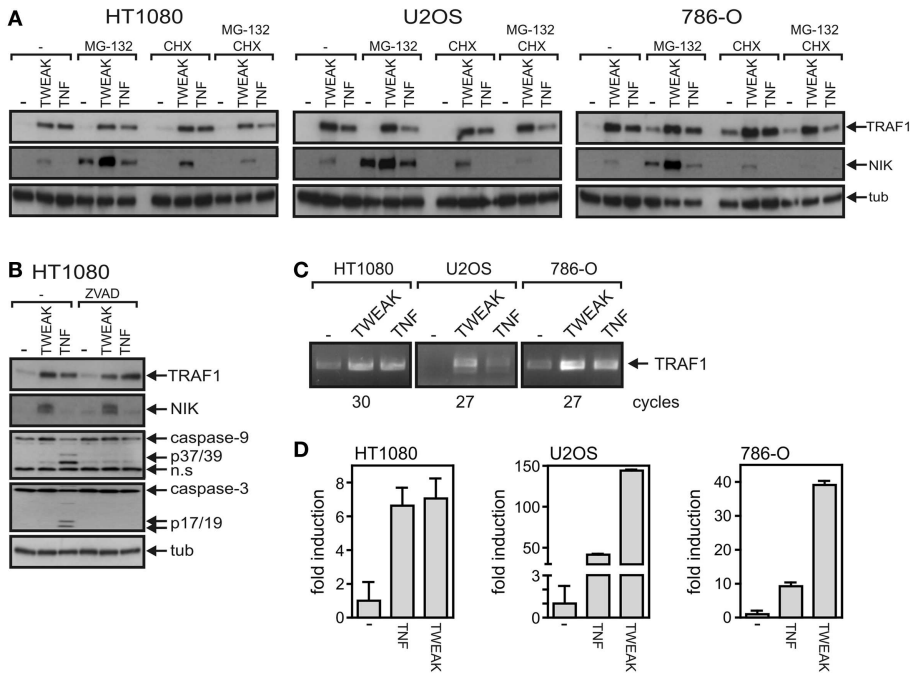

FIGURE 3 | Soluble TWEAK is superior to TNF in *TRAF1* mRNA induction. **(A,B)** TRAF1 expression was induced in the indicated cell lines by overnight stimulation with Flag-TWEAK (200 ng/ml) or Flag-TNF (100 ng/ml). Cells were then treated for additional 4 h with the proteasome inhibitor MG132 (20 μM) and/or the protein synthesis inhibitor cycloheximide (25 μg/ml). **(B)** HT1080 cells were stimulated overnight with TNF and TWEAK in the presence and absence of z-VAD-fmk (20 μM) and total cell lysates were analyzed by western blotting for TRAF1 induction. **(C,D)** Total RNA was isolated from cells stimulated with Flag-TNF (100 ng/ml) or Flag-TWEAK (200 ng/ml) overnight and subjected to analysis by reverse transcriptase PCR **(C)** and quantitative real time PCR. The average fold induction calculated from normalized data derived of three fully independent experiments is shown **(D)**.

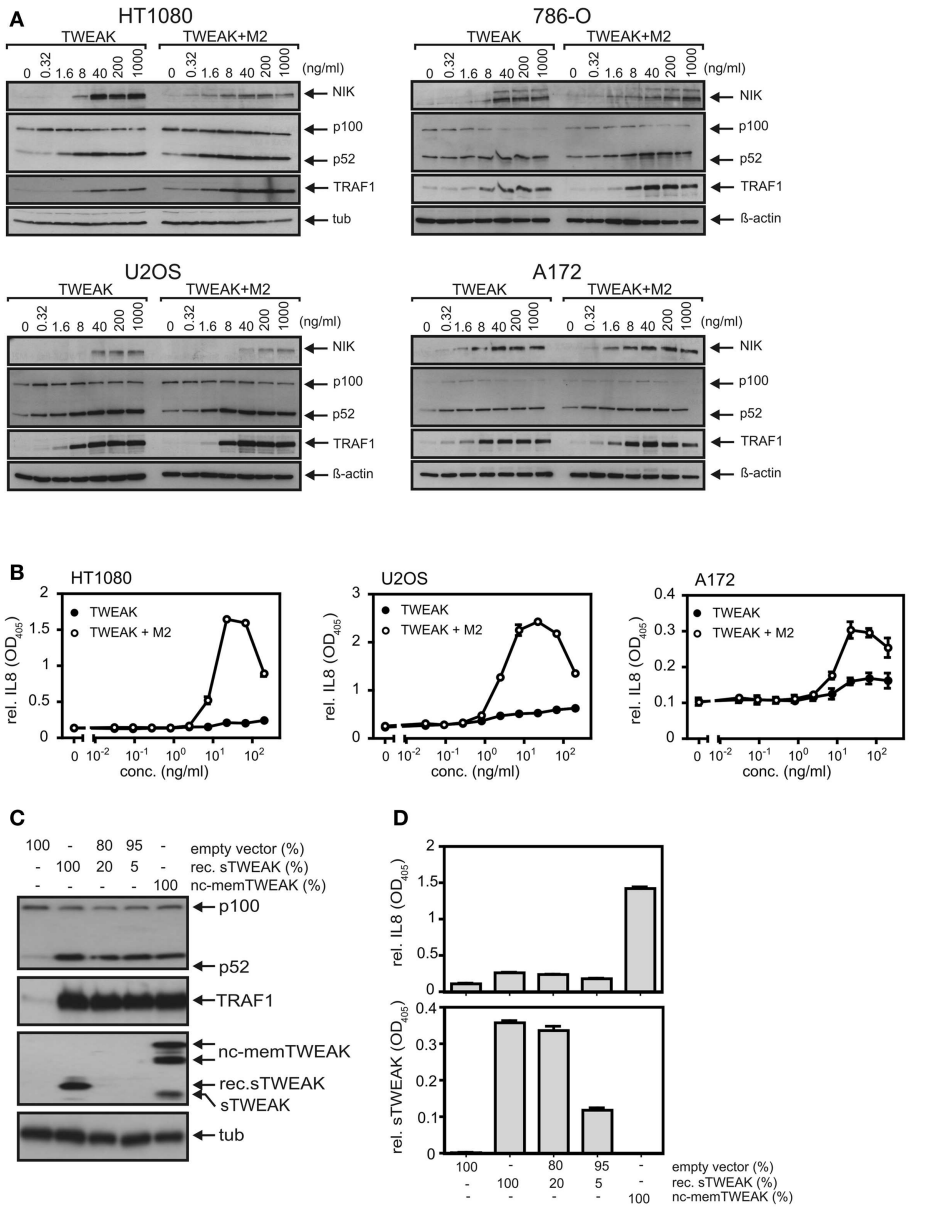

FIGURE 4 | Oligomerization of soluble TWEAK results in enhanced induction of IL8 but has no major effect on TRAF1 induction and alternative NFκB signaling. **(A)** Cells were stimulated overnight as indicated with Flag-TWEAK and 1 μg/ml of the Flag-specific mAb M2 and TRAF1 expression in total cell lysates were analyzed by western blotting. **(B)** Cells were stimulated in triplicates with increasing concentrations of Flag-TWEAK in the presence and absence of M2 (1 μg/ml). The next day, supernatants were assayed for their IL8-content by ELISA. Prior stimulation cell culture medium was replaced to reduce the background caused by constitutive IL8 production. Please note, the decline in IL8 production observed at high concentrations of anti-Flag oligomerized Flag-TWEAK presumably reflects suboptimal complex formation by M2 and Flag-TWEAK. **(C,D)** HT1080 cells were transiently transfected with the indicated mixtures of expression plasmids encoding soluble Flag-TWEAK (rec. sTWEAK) or a non-cleavable mutant of membrane TWEAK (nc-memTWEAK) and empty vector. Next day, cells were harvested and seeded in 6-well and 96-well plates. After an additional day, total lysates derived of transfected cells (6-well) were analyzed by western blotting for expression of the indicated proteins **(C)** and supernatants (triplicates) were analyzed with respect to their IL8 and TWEAK content by ELISA **(D)**.

expression to the level or even below the level of basal expression of the untreated cells (**Figure 5A**). This emphasizes the efficacy of the inhibitory effect of TPCA1 and indicates that basal TRAF1 expression is at least partly maintained in some cell lines by weak constitutive classical NFκB signaling. The effect of TPCA1 on TWEAK-induced TRAF1 production, however, varied dependent on the cell line considered. TWEAK-induced TRAF1 expression was fully inhibited in TPCA1-treated A172 cells and likewise in IκBα-SR expressing A172 transfectants (**Figures 5A,B**). However, in the other cell lines investigated there was only partial inhibition

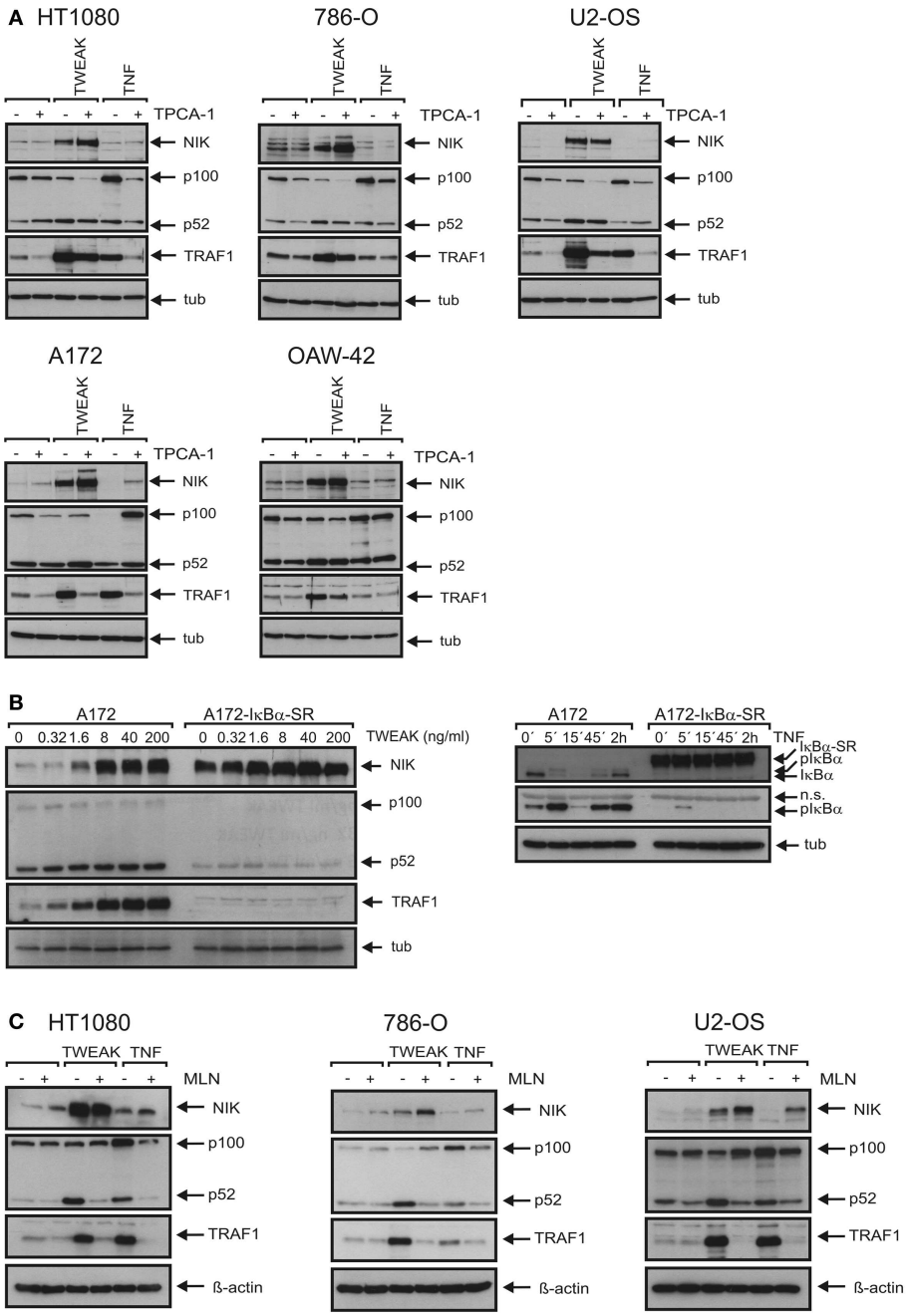

FIGURE 5 | The two NFκB signaling pathways are of varying cell type-specific relevance for TWEAK-induced TRAF1 expression. **(A)** Cells were stimulated overnight with Flag-TWEAK (200 ng/ml) and Flag-TNF (100 ng/ml) and total cell lysates were analyzed by western blotting for NIK accumulation, p100 processing and expression of TRAF1, where indicated cells were pretreated for 30 min with 10 μM TPCA1. **(B)** Control vector and IκBα-SR transduced A172 cells were stimulated overnight with increasing concentrations of Flag-TWEAK and were assayed by western blotting of total cell extracts for TRAF1 production and activation of the alternative NFκB pathway (left panel). Efficacy of IκBα-SR-mediated repression of the classical NFκB pathway was proved by analyzing phosphorylation and degradation of IκBα in cells stimulated with TNF (100 ng/ml) (right panel). **(C)** The indicated cell lines were pretreated or not with 10 μM MLN4924 for 30 min and then stimulated with Flag-TWEAK (200 ng/ml) and Flag-TNF (100 ng/ml). The next day, total cell lysates were prepared and analyzed for the presence of the indicated proteins by western blotting.

of TWEAK-induced TRAF1 expression by TPCA1 (**Figure 5A**). This also argues for a capability of TWEAK to induce TRAF1 by classical NFκB pathway-independent mechanisms. Moreover, in all cell lines analyzed, including those where soluble TWEAK induces significant TRAF1 expression in the presence of TPCA1, the NEDD8-activating enzyme (NAE)-inhibitor MLN4924 (34),

which interferes with IκBα degradation and p100 processing (33), inhibited upregulation of TRAF1 (**Figure 5C**). Although, MLN4924 also targets other signaling pathways and the cell cycle, this points to an important role of the alternative NFκB pathway in soluble TWEAK-induced TRAF1 expression.

TWEAK PRIMING AND ECTOPIC TRAF1 EXPRESSION INTERFERE WITH CD40 SIGNALING

We observed recently that priming of cells with soluble TWEAK for a few hours strongly inhibits CD40 signaling and traced this back to impaired formation of TRAF2–cIAP1/2 containing CD40 signaling complexes (20). Noteworthy, TRAF1 forms with high efficiency heterotrimers with TRAF2 and this heteromeres interact much stronger with cIAP2 than homotrimeric TRAF2 (21). On the other side, however, there is evidence that TRAF1-TRAF2 heteromers bind weaker to CD40 than TRAF2 homotrimers (22). To evaluate a possible role of TRAF1 induction in the crosstalk of TWEAK and CD40, we took advantage of 786-O and U2-OS cells that we have stably transfected for another project with a caspase cleavage-resistant TRAF1 variant with unchanged protein–protein interaction properties. In accordance with our previous findings (20), priming with soluble TWEAK diminished CD40L-induced upregulation of the classical NFκB-controlled cytokine IL8 in U2-OS cells as well as induction of the likewise classical NFκB-regulated cytokine IL6 in 786-O cells (**Figure 6A**).

More important, analysis of the TRAF1 transfectants also revealed reduced CD40L-stimulated cytokine induction (**Figure 6A**). A similar inhibitory effect of TWEAK priming and ectopic TRAF1 expression was also evident from western blot analysis of CD40L-induced phosphorylation and degradation of IκBα. In accordance with the delayed weak activation of the classical NFκB pathway already observed in **Figure 2B**, 786-O and U2-OS cells that were primed overnight with soluble TWEAK showed increased levels of phosphorylated IκBα compared to non-primed cells (**Figure 6B**). More important, however, while rapid (5–15 min) CD40L-induced phosphorylation and degradation of IκBα were detectable in non-primed 786-O and U2-OS cells, CD40 stimulation failed to trigger a significant reduction in the IκBα levels in TWEAK-primed cells as well as in TRAF1 transfectants. Moreover, there was no further CD40L-induced increase in "basal" IκBα phosphorylation in the TWEAK-primed cells and no or only a minor effect on IκBα phosphorylation in the TRAF1 transfectants (**Figure 6B**). As already observed earlier (20), priming with soluble TWEAK showed neither a major effect on CD40 cell surface expression nor on the affinity of CD40L for CD40 (**Figures 6C,D**). Similarly, TRAF1 transfectants showed no disturbance of cell surface expression of CD40 and CD40L–CD40 interaction (**Figures 6C,D**). Thus, changes in CD40 expression cannot explain the inhibitory effect of TWEAK priming and ectopic TRAF1 expression on CD40 signaling.

DISCUSSION

The proteins of the NFκB family (RelA/p65, RelB, cRel, p50, and p52) form homo- and heterodimeric transcription factors whose nuclear translocation and activity are controlled by two prototypic signaling pathways: the classical (canonical) and the alternative (non-canonical) NFκB pathway (23, 35). In non-stimulated cells NFκB dimers are retained in the cytoplasm either by interaction with proteins of the IκB family or due to the activity of an inhibitory domain, which is part of the p50 and p52 precursor proteins p100 and p105. The classical NFκB pathway can be activated by various stimuli, including proinflammatory cytokines. The latter initially trigger, with the help of E3 ligases TRAF2, cIAP1, cIAP2, and TRAF6, activation of a subset of MAP3Ks, e.g., MEKK3 and TAK1. These MAP3Ks stimulate then the IκB kinase (IKK) complex, which is composed of the regulatory scaffold protein IKKγ/NEMO and the kinases IKK1 and IKK2. IKK complex-catalyzed phosphorylation of IκBs leads next to the ubiquitination and proteasomal degradation of this inhibitory proteins and thus to release and nuclear translocation of IκB-bound NFκB dimers (35). The signaling events of the classical NFκB pathway are fast and typically result in IκB degradation and NFκB nuclear translocation in <1 h. Activation of the alternative NFκB pathway is primarily stimulated by some TNF receptors and crucially bases on the accumulation of the constitutively active MAP3K NIK. In non-stimulated cells, there is ongoing proteasomal degradation of NIK which is triggered by the concerted action of the aforementioned E3 ligases cIAP1, cIAP2, and TRAF2 and the TRAF2-related E3 ligase TRAF3. Stimulation of TNF receptors that activate the alternative NFκB pathway results in depletion and/or degradation of the NIK inhibitory E3 ligases and thus in accumulation of NIK. The latter now activates IKK1 and IKK1 in turn triggers processing of p100 to p52 and nuclear translocation of the remaining p52 containing NFκB dimers (23).

NFκB-mediated induction of TRAF1 is well established for a variety of inducers of the classical NFκB pathway, including TNF, IL1, T-cell and B-cell receptor-stimulating antibodies, LPS, and phorbol-12-myristate-13-acetate (PMA) (24–26, 36). Soluble TWEAK furthermore has been recognized as poor inducer of genes regulated by the classical NFκB pathway particular in comparison with TNF [(12, 15, 32); see also **Figure 1**]. Against this background, it was surprising to note in this study that soluble TWEAK is superior to TNF in TRAF1 induction, although it barely induced other classical NFκB-regulated genes, such as the gene encoding IL8 (**Figures 1** and **2**). This suggests that soluble TWEAK utilizes classical NFκB-independent mechanisms for TRAF1 induction. So far the only Fn14-associated signaling events for which robust triggering by soluble TWEAK had been demonstrated are depletion of TRAF2–cIAP complexes along with stimulation of the alternative NFκB pathway and enhancement of TNFR1-induced apoptosis (15, 17). It is therefore tempting to speculate that the alternative NFκB pathway plays an important role in TWEAK-induced TRAF1 expression. This idea is supported by several lines of evidence. First, the kinetic of TWEAK-induced TRAF1 expression is delayed in comparison to the kinetic of TRAF1 induction by TNF and follows the activity of the alternative NFκB pathway (**Figure 2**). Second, oligomerization of soluble TWEAK, which converts soluble TWEAK trimers into a molecule species with membrane TWEAK-like activity and thus a strongly enhanced capacity to trigger the classical NFκB pathway, has no major effect on TRAF1 induction although it leads to potentiation of induction of IL8 (**Figure 4**). Third, p80HT, a lymphoma-associated truncated mutant of p100 lacking parts of the inhibitory domain of the molecule, interacts with the TRAF1 promoter and induces TRAF1

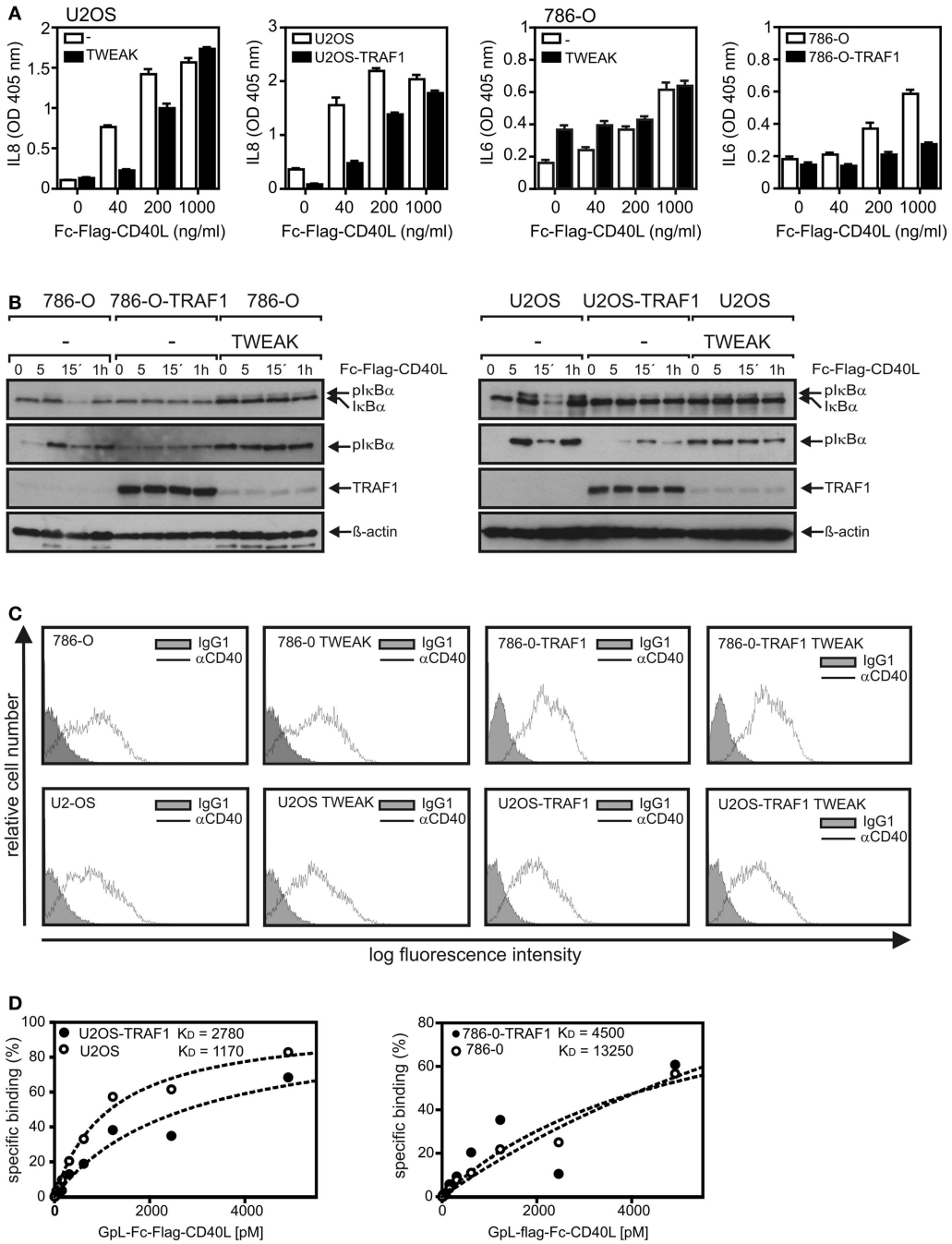

FIGURE 6 | TRAF1 expression interferes with CD40-induced signaling. **(A)** U2OS and 786-O cells with and without TWEAK priming (200 ng/ml, 6 h) and U2OS-TRAF1 and 786-O-TRAF1 transfectants were stimulated in 96-well plates in triplicates with the indicated concentrations of Fc–Flag-CD40L. Next day, supernatants were assayed for production of IL8 or IL6 by ELISA. Prior stimulation cell culture medium was replaced to reduce the background caused by constitutive cytokine production. **(B)** U2-OS and 786-O cells with and without TWEAK priming and TRAF1 expressing U2-OS and 786-O transfectants were stimulated with Fc–Flag-CD40L for 5 and 15 min and were finally analyzed by western blotting to detect the indicated molecules. Please note, in the case of the TRAF1 western blots a relatively short exposure time is shown to ensure reasonable visibility of overexpressed and TWEAK-induced TRAF1. **(C)** 786-O and U2-OS cells and their corresponding TRAF1 transfectants were primed overnight with soluble TWEAK or remained untreated and were then analyzed by FACS for CD40 cell surface expression. **(D)** Cells (2×10^5 cells/well) were seeded in 24-well plates. Next day, half of the samples of each cell type were pretreated for 1 h at 37°C with 2 μg/ml of Fc–Flag-CD40L. Next, untreated and Fc–Flag-CD40L pretreated cells were incubated pairwise with increasing concentrations of GpL–Fc–Flag-CD40L (1 h, 37°C), a fusion protein of Fc–Flag-CD40L with the luciferase from *Gaussia princeps*. After removal of unbound CD40L molecules, cells were scratched in 50 μl medium to measure cell-associated luciferase activity. Specific binding values were obtained by subtraction of the non-specific binding values derived of Fc–Flag-CD40L pretreated cells from the corresponding total binding values. Specific binding values were fitted by non-linear regression using the GraphPad Prism5 software. Specific binding values were normalized according to the maximum binding value obtained from the linear regression.

expression (37). Fourth, the IKK2-selective inhibitor TPCA1, which only blocked activation of the classical NFκB pathway, inhibited TNF-induced TRAF1 production completely but showed in most cell lines, investigated here only a partial inhibitory effect on TRAF1 expression (**Figure 5A**). In contrast, the NAE inhibitor MLN4924, which inhibits signaling by both NFκB pathways, completely blocked TWEAK-induced TRAF1 expression (**Figure 5C**). The results of the inhibitor studies indeed argue for a role of the alternative NFκB pathway in TWEAK-induced TRAF1 expression but also point to a contribution of the classical NFκB pathway. This becomes particularly evident in the A172 cell line where TWEAK-induced TRAF1 production was completely abrogated by TPCA1 treatment (**Figure 5A**) or IκBα-SR expression (**Figure 5B**) despite poor activation of the classical NFκB pathway (**Figure 1**). In sum, our data suggest that both the classical and the alternative NFκB pathway act together to realize the strong induction of TRAF1 by TWEAK. In this context, it is worth mentioning that various cross-talk mechanisms have been identified in the recent years that link the two NFκB signaling pathways. These mechanisms reach from NIK- and IKK1-mediated phosphorylation of IκBα (27) over phosphorylation and transactivation of c-Rel by NIK (38) to IKK1-mediated chromatin remodeling (39, 40). Moreover, the NIK/IKK1-regulated p52 precursor protein p100 can inhibit NFκB dimers intermolecularly with the result that NFκB dimers, which are actually regulated by the classical pathway, come under the control of the alternative pathway (41).

We and others have observed that priming with soluble TWEAK for a few hours can induce a state of reduced responsiveness for proinflammatory signaling by TNFR1, TNFR2, and CD40 in a variety of cell lines and cell types including adipocytes and fibroblast-like synoviocytes of patients suffering on rheumatoid arthritis [(17, 18–20); see also **Figure 6**]. Although, the molecular basis of the desensitizing effect of soluble TWEAK has not been intensively studied so far, there is good evidence that TWEAK-induced Fn14-mediated depletion of cytosolic complexes of TRAF2 and cIAP1/2 plays a major role by limiting the availability of these proteins for the aforementioned receptors. For example, TRAF2 and cIAPs are recruited to the TNFR1 signaling complex and are required there for ubiquitination of RIP and subsequent recruitment of the classical NFκB-stimulating IKK complex [for review, see (42)]. However, recruitment of TRAF2 and especially of the TRAF2-interacting cIAPs and the IKK complex to the TNFR1 signaling complex as well as RIP ubiquitination are severely reduced in TWEAK-primed cells (17). Reduced recruitment of cIAP1 and cIAP2 and the IKK complex along with reduced classical NFκB signaling in TWEAK-primed cells have also been observed for TNFR2 (20). Depletion of cytosolic TRAF2–cIAP1/2 complexes straightforwardly explains the desensitizing effect of soluble TWEAK on activation of the classical NFκB pathway by TNFR1, TNFR2, and CD40 because all these receptors exploit TRAF2 and the cIAPs for activation of this pathway (4, 42). In the case of CD40, however, there is evidence for an additional mechanism. Immunoprecipitation of ligand-associated receptor signaling complexes in TWEAK-primed cells resulted for TNFR1 and TNFR2 in practically unchanged amounts of co-precipitated receptor and only showed a reduction in the amount of co-precipitated TRAF2, cIAPs, and IKK molecules (17, 20). In contrast, CD40L immunoprecipitates of TWEAK-primed cells contained only minor amounts of co-immunoprecipitated CD40 despite unchanged CD40 expression and normal CD40L–CD40 interaction on intact cells (20). It is thus tempting to speculate that TRAF2 trimers "crosslink" neighboring CD40L–CD40 complexes and that this result in supramolecular CD40–CD40L clusters that counteract the dissociation of CD40–CD40L complexes in the disintegrated cell during immunoprecipitation. Indeed, previous findings showed that CD40 poorly interacts with TRAF2–TRAF1 heteromers and preferentially binds TRAF2 homotrimers whereas TNFR1 and TNFR2 recruit TRAF2–TRAF1 heteromers with high efficacy. Against the background that we observed strong induction of TRAF1 already by soluble TWEAK (**Figures 1–3**), it appears possible that soluble TWEAK-induced TRAF1 expression affects recruitment of TRAF2 to CD40 and the formation/stability of supramolecular CD40L–CD40 clusters. Indeed, it has been found that TRAF1 and TRAF2 preferentially form $TRAF1_1$–$TRAF2_2$ heterotrimers and that these $TRAF1_1$–$TRAF2_2$ heterotrimers have a significantly higher affinity for cIAP2 than TRAF2 homotrimers (21). The favored formation of $TRAF1_1$–$TRAF2_2$–cIAP complexes along with their weak affinity for CD40 might result in reduced recruitment of cIAP-containing TRAF complexes to CD40 and could further enhance the effect of Fn14-mediated depletion of TRAF2-containing complexes. As TRAF2–TRAF1 heteromers strongly interact with TNFR2 and TNFR1-associated TRADD [e.g., Ref. (43)] this mechanism would not be of relevance for the crosstalk of TWEAK with TNFR1 and TNFR2. In line with the set fourth hypothesis, we observed in this study that ectopic expression of TRAF1 has principally a similar effect on CD40 signaling as priming with soluble TWEAK. Both TWEAK priming and ectopic TRAF1 expression inhibited CD40L-induced activation of the classical NFκB pathway (**Figures 6A,B**) without disturbing CD40 expression (**Figure 6C**) or CD40L–CD40 interaction [Ref. (20) and **Figure 6D**].

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The human renal cell carcinoma cell line 786-O, the human osteosarcoma cell line U2-OS, the human fibrosarcoma cell line HT1080 were from the American Tissue Culture Collection. The human ovarian cancer cell line OAW-42 were from the European Typical Culture Collection and vector and IκBα-SR transduced A172 cells were a kind gift of Prof. Simone Fulda (University of Frankfurt) that has been characterized in detail elsewhere (44). With exception of 786-O cells, all cells were cultivated in RPMI1640 medium (Sigma) supplemented with 10% fetal calf serum (Invitrogen). 786-O cells were maintained in 10% FCS supplemented DMEM (Sigma). The characterization of HT1080-CD40 transfectants as well as cloning, production, and purification of Flag-TWEAK, Flag-TNF, and Fc–Flag-CD40L have been described elsewhere (15, 45). MG132 and cycloheximide were purchased from Sigma and z-VAD-fmk from Bachem. Human skeletal muscle progenitor cells including growth medium and supplements were from PeloBiotech GmbH and hMSCs were a kind gift from Dr. Joachim Nickel (University Hospital of Würzburg).

TRANSFECTION OF HT1080 CELLS

HT1080 cells were transfected with expression plasmids encoding Flag-tagged soluble TWEAK (Flag-sTWEAK) or non-cleavable full-length TWEAK (nc-memTWEAK) harboring mutations (R80A–R93A–R97A) that strongly reduce processing by furin proteases using Lipofectamine according to the suppliers protocol (Invitrogen). Next day, cells were harvested and seeded in 6-well and 96-well plates. After an additional day, cells from 6-wells were used for western blot analysis of total cell lysates and cell culture supernatants of cells cultivated in 96-well plates were subjected to ELISA to determine the content of IL8 (see below) and soluble TWEAK (ELISA Duo Set; R&D Systems).

EQUILIBRIUM BINDING STUDIES WITH GpL–Flag-CD40L

The interaction of CD40L with cell surface expressed CD40 was investigated by equilibrium binding studies at 37°C with a *Gaussia princeps* luciferase (GpL) fusion protein of Fc–Flag-CD40L as recently described elsewhere (20).

WESTERN BLOTTING

For analysis of NIK accumulation, p100 to p52 processing, expression of TRAF1 and TWEAK, and phosphorylation and degradation of IκBα cells were washed once with PBS, harvested using a rubber policeman, centrifuged, and then directly lysed in 4× Laemmli sample buffer (approximately 1×10^6 cells per 100 µl buffer; 5 min, 95°C) supplemented with complete protease inhibitor from Roche Applied Science and phosphatase inhibitor mixtures I and II from Sigma. Lysates were sonicated for 15 s with maximum amplitude (UP100H Ultrasonic Processor, Hielscher, Germany), heated for 5 min at 95°C and centrifuged for 3 min (Eppifuge, full speed) to remove residual insoluble debris. Lysates were further processed using standard protocols for SDS-PAGE and immunoblotting using horse radish peroxidase-conjugated secondary antibodies (Dako) and the ECL Western blotting detection reagents and analysis system (Amersham). Primary antibodies used were anti-IκBα (clone C35A5, Cell Signaling), anti-pIκBα (clone 14D4, Cell Signaling), anti-NIK (#4994, Cell Signaling), anti-p100/p52 (#05-361, Upstate), anti-TRAF1 (H-132, Santa Cruz), anti-TWEAK (#AF1090, R&D Systems,) and anti-tubulin (clone DM1A, Neomarker).

RT-PCR AND qPCR

Total RNA was purified using the RNeasy Mini kit from Qiagen according to the protocol of the supplier. For reverse transcription PCR, 1 µg of total RNA were used to synthesize cDNA with the QuantiTect Reverse Transcription kit (Qiagen) which were then subjected to conventional PCR using the TRAF1-specific primers 5′-GCCCCTGATGAGAATGAGTT-3′ (forward) and 5′-CCTGGTGACATTGGTGATCTT-3′ (rewind) and the following program: I. 95°C, 2 min; II. 20 s at 95°C followed by 60 s at 63°C and 45 s at 70°C; III. 5 min, 72°C. For quantitative real time PCR 2% of the reverse transcription PCR reaction obtained with 1 µg total RNA were subjected to real time PCR using the BioRad Cycler CT1000 with the CFX96 Real Time System and the QuantiTect SYBR Green PCR Kit (Qiagen). Following program was used: I. 95°C, hot start; II. 40 Cycles of 15 s at 95°C followed by 30 s at 52°C and 30 s at 72°C and a sample without cDNA served as a negative control. TRAF1 primers used for amplification were 5′-CATGAGAGGGGAGTATGATG-3′ (forward) and 5′-GAAGAAGAGTGGGCATCCAC-3′ (rewind). PCR Amplification of ß-actin using commercially available primers (Qiagen) was used for normalization. Fold-induction values for TRAF1 were calculated as follows: the CT-values of the β-actin PCR reactions of the TNF and TWEAK-treated samples were set to the CT-value of the β-actin PCR reaction of the cDNA derived of untreated cells. The differences between the latter and the CT-values of the β-actin PCR reactions of the TNF and TWEAK-treated samples were furthermore used to correct the corresponding CT-values of the TRAF1 PCR reactions. The differences between the corrected CT-values of the untreated and TNF- or TWEAK-treated samples (ΔCT) were finally transformed into fold induction (fold induction $= 2^{\Delta CT}$).

ANALYSIS OF IL8/IL6 PRODUCTION

Cells were seeded with a density of 2×10^4 cells/well in 96-well tissue cultures plates and cultivated for a day. To reduce the background of basal IL8 production, medium was replaced by fresh medium prior overnight stimulation with Flag-TNF or Flag-TWEAK. Supernatants were cleared by centrifugation and assayed for IL8/IL6-production by help of the BD OptEIA Human IL8 ELISA Kit or the BD OptEIA Human IL6 ELISA Kit according to the protocol of the manufacturer (BD Biosciences).

STATISTICAL ANALYSES

Statistical analyses were performed with the help of the program GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Error bars shown in ELISA and RT-PCR experiments represent the standard error of mean (SEM) of triplicates. ELISA data shown are representative for two or more independent experiments. The comprehensive side by side analyses of the dose response and kinetics of TNF- and TWEAK-induced TRAF1 expression were performed only once for most cell lines whereby the basic findings (no enhancing effect of oligomerization of soluble TWEAK, comparable or stronger TRAF1-inducing activity of soluble TWEAK compared to TNF) were previously observed in two or more experiments with slightly varying conditions (stimulation time, concentrations used for stimulation, and the like).

AUTHOR CONTRIBUTIONS

José Antonio Carmona Arana and Manfred Neumann performed the western blot analysis of TNF- and TWEAK-stimulated TRAF1 induction and the ELISA experiments. Axel Seher did the qPCR analysis, Isabell Lang produced the recombinant proteins, Daniela Siegmund analyzed the stability of TRAF1, and Harald Wajant wrote the paper and supervised the project.

ACKNOWLEDGMENTS

The IκBα-SR expressing A172 cells and the corresponding control cells were a kind gift of Prof. Dr. Simone Fulda (University of Frankfurt). This work was supported by Deutsche Forschungsgemeinschaft (grants Wa 1025/19-2 and Wa 1025/21-1) and Deutsche Krebshilfe (grant 109922). We further thank Mrs. Daniela Weisenberger (Division of Molecular Internal Medicine, Department of Internal Medicine II) for preparation of total RNA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00063/abstract

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The role of ubiquitination in TWEAK-stimulated signaling

Domagoj Vucic*

Department of Early Discovery Biochemistry, Genentech, Inc., South San Francisco, CA, USA

Edited by:
John Silke, Walter and Eliza Hall Institute, Australia

Reviewed by:
John Silke, Walter and Eliza Hall Institute, Australia
Harald Wajant, University Hospital of Würzburg, Germany

****Correspondence:***
Domagoj Vucic, Department of Early Discovery Biochemistry, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA
e-mail: domagoj@gene.com

Tumor necrosis factor superfamily ligands and receptors are responsible for development, immunity, and homeostasis of metazoan organisms. Thus, it is not surprising that signals emanating from these receptors are tightly regulated. Binding of TNF-related weak inducer of apoptosis (TWEAK) to its cognate receptor, FN14, triggers the assembly of receptor-associated signaling complex, which allows the activation of canonical and non-canonical nuclear factor kappa B (NF-κB) as well as mitogen-activated protein kinase signaling pathways. Ubiquitin ligases cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and 2) and adaptor proteins TNFR-associated factors 2 and 3 (TRAF2 and TRAF3) are crucial for the regulation of TWEAK signaling as they facilitate the recruitment of distal signaling components including IKK and linear ubiquitin chain assembly complex complexes. At the same time c-IAP1/2, together with TRAF2 and TRAF3, promote constitutive ubiquitination and proteasomal degradation of NF-κB inducing kinase (NIK) – a kinase with critical role in the activation of non-canonical NF-κB signaling. While c-IAP1/2 mediated ubiquitination allows the activation of TWEAK-stimulated canonical NF-κB signaling, these E3 ligases are negative regulators of non-canonical signaling. TWEAK stimulation prompts the recruitment of c-IAP1/2 as well as TRAF2 and TRAF3 to the FN14 signaling complex leading to c-IAP1/2 autoubiquitination and degradation, which stabilizes NIK and allows subsequent phosphorylation of IKKα and partial proteasomal processing of p100 to activate gene expression. Recent studies have revealed that the spatio-temporal pattern of TWEAK-stimulated ubiquitination is a carefully orchestrated process involving several substrates that are modified by different ubiquitin linkages. Understanding the significance of ubiquitination for TWEAK signaling is important for the overall understanding of TWEAK biology and for the design of therapeutics that can be used in the treatment of human pathologies that are driven by TWEAK/FN14 expression and activity.

Keywords: TWEAK, ubiquitin, NF-κB, IAP, c-IAP1, TRAF2, TRAF3, NIK

UBIQUITINATION SYSTEM

The regulated posttranslational modification and degradation of cellular proteins by the ubiquitin-proteasome system impacts a wide range of crucial processes in normal and diseased cells (1). Tumor necrosis factor (TNF) superfamily ligands, including TNF-related weak inducer of apoptosis (TWEAK), rely extensively on ubiquitination to promote activation of non-canonical and canonical nuclear factor kappa B (NF-κB) signaling as well as mitogen-activated protein kinase (MAPK) pathways. Ubiquitination requires the activity of ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s) (2). Coordinated activity of these components results in the covalent ligation of ubiquitin to the acceptor lysine, or less frequently amino-terminal, residues of the substrate protein (**Figure 1**). Covalent attachment of a single ubiquitin molecule to the substrate is referred to as monoubiquitination (3). However, the presence of seven lysines and available amino-terminus within a ubiquitin molecule enables the formation of a variety of ubiquitin–ubiquitin linkages and polyubiquitin chains (3). The varied topologies of different polyubiquitin chains provide means for communicating complex biological information that is vital for many cellular functions (4). For example, K63-linked chains, amino-terminally linked chains, and in some cases K11-linked chains, provide a platform for the assembly of signaling complexes (5–7). On the other hand, K48-linked chains mostly target substrate proteins for proteasomal degradation (1).

Hundreds of E3 ligases that ensure substrate specificity and tens of E2 enzymes that dictate the type of the ubiquitin chain assembly present the ubiquitination processes with a remarkable combinatorial potential (8). Numerous ubiquitin-binding domains that recognize different ubiquitin chains and transmit encoded biological information decipher the information embedded in diverse ubiquitin modifications (9). Just like many complex biological systems, ubiquitination is a reversible process. A separate class of enzymes, called deubiquitinases (DUBs), carries out the removal and depolymerization of ubiquitin chains (10). All together, E1–3 enzymes that promote ubiquitination, ubiquitin-binding domains that recognize different ubiquitin moieties, and DUBs that eliminate ubiquitin modifications afford a powerful molecular set of tools for fine-tuning intricate signaling messages.

SIGNALING PATHWAYS INDUCED BY TWEAK

Tumor necrosis factor superfamily ligands are homotrimeric type 2 transmembrane proteins that are either membrane-embedded or

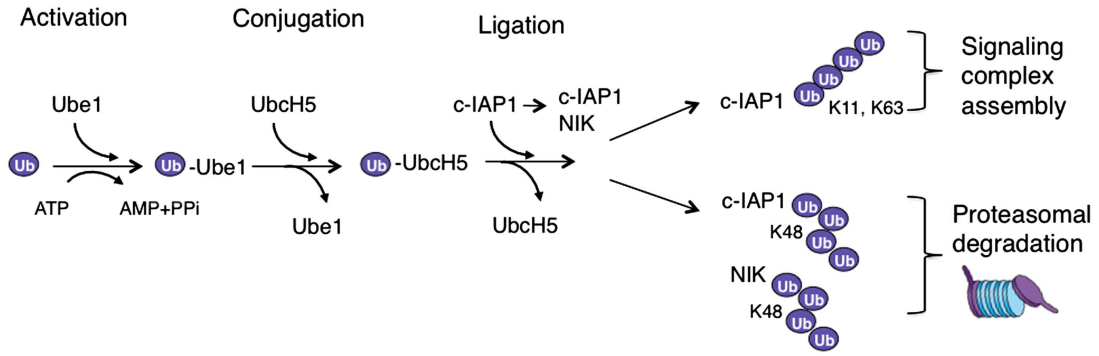

FIGURE 1 | Ubiquitination cascade in TWEAK signaling. In the activation reaction ubiquitin is transferred to an E1 enzyme (Ube1) in an ATP-dependent fashion, which leads to the transfer of activated ubiquitin to an E2 enzyme (UbcH5) in the conjugation reaction. The E2 with ubiquitin binds E3 ubiquitin ligase (c-IAP1), which can also bind a substrate – often through a different protein interaction domain – and thus allows the ubiquitin ligation to occur. When polyubiquitin chains are assembled this process is repeated with a lysine (K) residue of the ubiquitin molecule itself serving as a substrate. The assembly of K63- or K11-link polyubiquitin chains on c-IAP1 promotes the formation of signaling complexes, while K48-linked ubiquitination of NIK or c-IAP1 targets them for proteasomal degradation.

cleaved to generate soluble proteins (11). These ligands bind one or more members of TNF receptor (TNFR) superfamily, type 1 transmembrane proteins, by interacting with the cysteine-rich domain in the extracellular region of TNFRs (11). Ligands and receptors of TNF/TNFR superfamily are vital for the proper functioning and organization of the immune system (12). Thus, it is no surprise that these proteins are implicated in a variety of genetic or acquired human diseases (12). Binding of TNF ligands to their cognate receptors triggers the assembly of receptor-associated signaling complexes and activates multiple signaling pathways, including the NF-κB, MAPKs c-Jun N-terminal kinase (JNK) and p38, and in some instances cell death (13–15). NF-κB transcription factor family members (NF-κB1 or p105/p50, NF-κB2 or p100/p52, RelA or p65, RelB, and cRel) operate as homodimers or heterodimers and can be activated through the canonical (classical) or non-canonical (alternative) pathways (16). In unstimulated cells inhibitor of canonical NF-κB signaling (IκB) keeps p50/Rela dimer in cytoplasm until IκB kinase β (IKKβ) phosphorylates it marking it for ubiquitination by SCF-βTrCP and subsequent proteasomal degradation (17). Without IκB around NF-κB dimers translocate to the nucleus and stimulate transcription of a series of proinflammatory and anti-apoptotic proteins (17). In the non-canonical pathway NF-κB inducing kinase (NIK) is the primary kinase that phosphorylates IKKα leading to the phosphorylation of the C-terminal domain of NF-κB precursor protein p100 (18). This triggers SCF-βTrCP dependent ubiquitination and partial proteasomal degradation of p100 to yield the mature p52 protein, which together with RelB moves to the nucleus and stimulates the gene expression with largely overlapping pattern with canonical pathway (18).

TNF-related weak inducer of apoptosis (also known as TNFSF12) is a TNF family cytokine that binds FN14 receptor (also known as TNFRSF12A) to promote proliferation but also apoptosis in a wide variety of epithelial and endothelial cells (19, 20). Association of TWEAK with FN14 leads to FN14 oligomerization and the recruitment of signaling adaptor proteins TNFR-associated factors 2 and 3 (TRAF2 and TRAF3) (19, 21–23). The presence of TRAF2 in FN14 complex is instrumental for the recruitment of ubiquitin ligases cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and 2), and for the activation of canonical NF-κB and MAPK signaling (24–26) (**Figure 2**). Adaptor protein TRAF2 exists in cells as a trimer that binds a single molecule of monomeric c-IAP1 or c-IAP2 (27, 28). Engagement of TRAF2 to TWEAK-stimulated signaling complex promotes receptor-mediated TRAF2 aggregation resulting in dimerization/oligomerization of c-IAP proteins thereby upregulating their E3 ligase activity (28, 29). Consequently, c-IAPs ubiquitinate themselves and TRAF2 to enable the recruitment of the IKK complex as well as the linear ubiquitin chain assembly complex (LUBAC) to FN14 (29, 30). The assembly of the TWEAK-FN14-associated receptor complex leads to the rapid activation of canonical NF-κB and MAPKs JNK and p38 signaling pathways within minutes of TWEAK treatment (26, 29) (**Figure 2**).

Following this first wave of signaling, non-canonical NF-κB signaling gets activated few hours after the formation of TWEAK-FN14 complex (21). The reason for the extended length of time needed for the activation of this pathway lies within the mechanism of activation (18). In the canonical NF-κB all signaling components are poised for action once inhibitory factor IκB has been phosphorylated and degraded. However, in the non-canonical NF-κB pathway, central regulator, kinase NIK, needs to be newly synthesized for the signaling to occur. NIK is kept at extremely low levels in cells by continuous ubiquitination and consequent proteasomal degradation. The protein complex responsible for keeping NIK suppressed consists of adaptor proteins TRAF2 and TRAF3 and E3 ligases c-IAP1 and 2 (31–35). Within this complex TRAF3 at the same time binds NIK and TRAF2 with its constitutive partners c-IAP proteins. This way, TRAF2 and TRAF3 juxtapose E3 ligases c-IAP1/2 and their substrate NIK to enable efficient NIK ubiquitination and suppression of signaling (31) (**Figure 2**). TWEAK binding to FN14 recruits TRAF2, TRAF3, and with them c-IAP1/2 from the cytoplasm to the membrane-associated receptor complex thereby liberating NIK from degradative control (25, 29, 31). Within hours NIK

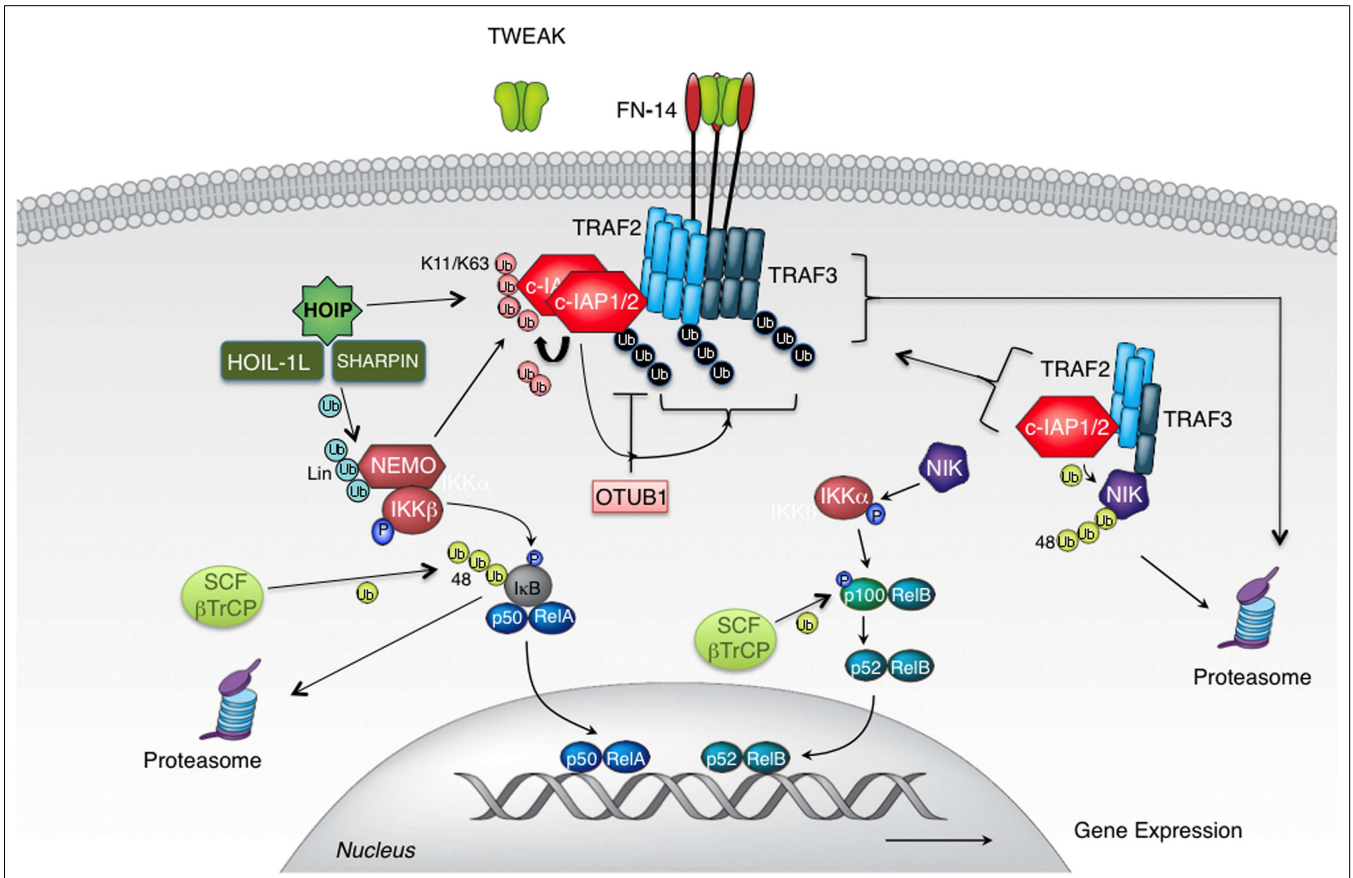

FIGURE 2 | TWEAK-stimulated activation of canonical and non-canonical NF-κB signaling pathways. Cellular IAP proteins are positive regulators of canonical and negative regulators of non-canonical NF-κB signaling pathways. c-IAPs, cellular IAPs; FN14, fibroblast growth factor-inducible 14; HOIL-1, heme-oxidized IRP2 ubiquitin ligase-1; HOIP, HOIL-1L-interacting protein/RNF31; Sharpin, SHANK-associated RH domain-interacting protein; IKK, IκB kinase; NEMO, NF-κB essential modulator; NIK, NF-κB inducing kinase; SCF-bTrCP, Skp1/Cul1/F-box β transducin repeat-containing protein; TRAF, tumor necrosis factor (TNF) receptor-associated factor; TWEAK, TNF-related weak inducer of apoptosis; Ub, ubiquitin.

accumulates and triggers phosphorylation of IKKα, p100, and signaling ensues. However, transient recruitment of TRAF2, TRAF3, and c-IAP proteins at the receptor complex would not permit efficient induction of non-canonical NF-κB signaling. To prevent them from re-entering the cytoplasmic complex with NIK, TRAF2, TRAF3, and c-IAPs need to be sequestered and/or eliminated. For this reason, following receptor engagement these adaptors and E3 ligases relocalize from the cytoplasm into membrane-associated cellular fractions where they undergo ubiquitination in c-IAP E3 ligase dependent manner (25, 29, 36). Consequently, autoubiquitinated c-IAP proteins, as well as ubiquitinated TRAF2 and TRAF3 undergo proteasomal, and in some cases also lysosomal degradation (25, 29). This relocation-ubiquitination-degradation process ensures their efficacious depletion from the cytoplasmic cellular compartment and allows the activation of non-canonical NF-κB signaling.

Similar mechanism for the activation of non-canonical NF-κB signaling is employed by vast majority of TNF family ligands and receptors (for example LT-βR, CD40, CD30) (29). The only exception is B cell-activating factor receptor 3 (BR3 or BAFFR), a receptor for BAFF that exclusively mediates activation of the non-canonical NF-κB pathway (37, 38). Just like the other TNFR family members that activate this signaling pathway, BR3 binds TRAF3 (39, 40). However, BR3 does not bind any other TRAF molecules as other TNFRs do (39, 40). As a result, BR3 does not recruit E3 ligases c-IAP1 and 2 or TRAF6. Being devoid of ubiquitin ligases in its signaling complex, BR3 signaling does not utilize ubiquitination but rather, it relies on the translocation of TRAF3 to the insoluble membrane compartment (29). This sequestration of TRAF3 from the soluble cytoplasmic compartment eliminates the physical link between c-IAPs and NIK and enables activation of the non-canonical NF-κB signaling.

Thus, ubiquitination is instrumental for the proper regulation of the non-canonical NF-κB pathway and TWEAK/FN14 and most of the related TRAF3-binding TNFR family members depend on ubiquitination for effective activation of signaling (29).

E3 LIGASES AND UBIQUITIN LINKAGES IN TWEAK SIGNALING

Several ubiquitin ligases have been implicated in TWEAK signaling but probably the most important ones are c-IAP1 and 2 as the elimination or reduction of c-IAP levels severely diminishes canonical NF-κB and MAPK signaling (29). Cellular IAP proteins regulate the activation of canonical NF-κB and MAP kinases following

initiation of TWEAK signaling by aggregation and dimerization and induced autoubiquitination within FN14-associated complex (29). Autoubiquitination of c-IAP proteins, and potentially also ubiquitination of adaptor protein TRAF2, provides a platform for the assembly of distal signaling complex that includes NEMO, IKKβ, and HOIL-1L-interacting protein (HOIP) (29). Cellular IAPs promote the assembly of a variety of polyubiquitin linkages on themselves with Lys11-, Lys63-, and Lys48-linked chains being the best studied (41) (**Figures 1** and **2**). Other polyubiquitin chain linkages as well as branched polyubiquitin chains (involving a mixture of several chain types) possibly also play important function in TWEAK signaling but their role(s) have not been well established yet. Binding of NEMO to these polyubiquitin chains allows the recruitment of IKK complex where activated IKKβ phosphorylates IκBα to stimulate IκBα proteasomal degradation (5, 29). At the same time, association of HOIP with autoubiquitinated c-IAP proteins leads to the engagement of LUBAC complex (29, 42) (**Figure 2**).

Linear ubiquitin chain assembly complex is an E3 ligase complex that consists of HOIP/RNF31, Heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1L), and/or SHANK-associated RH domain-interacting protein (Sharpin) (6, 30). It is unique among E3 ligases as it promotes the assembly of linear or Met-linked ubiquitination on NEMO, RIP1, itself, and several other signaling molecules (6). Given that RIP1 does not participate in TWEAK signaling, the activity of LUBAC is likely restricted to NEMO and itself although additional substrates potentially await discovery. Linear ubiquitination stabilizes signaling complexes, and decrease in LUBAC levels, and consequent absence of linear ubiquitination, negatively impacts TWEAK-stimulated canonical NF-κB and MAPK activation (29). Another ubiquitin ligase that has been associated with TWEAK signaling is TRAF6, although not through direct participation in TWEAK-stimulated pathways (43). Starvation of skeletal muscles induces TRAF6 dependent expression of FN14, an event that is critical for the regulation of skeletal muscle atrophy (43).

Non-canonical NF-κB signaling is also critically regulated by ubiquitination and proteasomal degradation. Cytoplasmic complexes consisting of ubiquitin ligases c-IAP1 and 2 and adaptor proteins TRAF2 and TRAF3 promote constitutive ubiquitination and proteasomal degradation of kinase NIK (31–33). Before the enzymatic role of cellular IAP proteins has been demonstrated, it was believed that TRAF2 and TRAF3 possess E3 ligase activity and that TRAF3 is a ubiquitin ligase for NIK (44, 45). However, recent cellular and biochemical studies have indicated that TRAF2 and TRAF3 are not functional E3 ligases and that their RING domains cannot interact with ubiquitin conjugating enzymes or promote ubiquitination (46–48). On the other hand, discoveries that multiple myeloma patients with inactivating mutations in c-IAP1 and 2, as well as knockouts and knockdowns of c-IAP1/2, have constitutive activation of non-canonical NF-κB pathway definitively demonstrated the seminal role of c-IAP E3 ligase activity in the suppression of NIK and non-canonical NF-κB signaling (49–52). In addition, discovery of SMAC-mimicking IAP antagonist compounds that target c-IAP protein for proteasomal degradation greatly aided the affirmation of c-IAPs as E3 ligases for NIK (31, 32). IAP antagonist treatment triggers rapid conformational change in c-IAP proteins that elevates their ubiquitin ligase activity leading to proteasomal degradation (53, 54). Some cells treated with these agents secrete a variety of NF-κB regulated inflammatory cytokines over a prolonged period even in the absence of cell death (55, 56). This cytokine production coincides with processing of p100 to p52 and NIK stabilization thus linking IAP antagonist treatment with the activation of non-canonical NF-κB signaling (55, 56). Presently, several IAP antagonists are undergoing clinical evaluations for anti-cancer treatments and all of them were found to stimulate c-IAP degradation and associated activation of non-canonical NF-κB signaling (57). The exact nature of ubiquitin chains assembled on endogenous NIK has never been determined, probably because of the extremely low levels of ubiquitinated NIK in unstimulated cells. Nevertheless, given that c-IAP1 and 2 can efficiently promote Lys48-linked polyubiquitination that is closely associated with proteasomal degradation, NIK is most likely modified with this chain linkage.

Interestingly, the activation of the non-canonical NF-κB also relies on the c-IAP ubiquitin ligase activity. Stimulation of cells with TWEAK, as well as with the related TNF family ligands that trigger recruitment of TRAF2 and TRAF3 – LIGHT, CD30L, or CD40L leads to the aggregation of c-IAP proteins within the receptor-associated membrane fraction (29). There, c-IAP proteins mediate autoubiquitination of themselves as well as ubiquitination of TRAF2 and TRAF3 causing their proteasomal and lysosomal degradation (25, 29) (**Figure 2**). TWEAK treatment promotes Lys11-, Lys48-, and Lys63-linked polyubiquitin chain assembly on c-IAP1, and TRAF2 and TRAF3 are likely modified in a similar fashion (41). Sequestration and degradation of c-IAP1/2, TRAF2, and TRAF3 ensures that NIK can accumulate to trigger phosphorylation-mediated activation of signaling. Simultaneous elimination of all NIK regulating components is probably excessive since depletion of any one of those components can break this degradation-promoting circle as seen in multiple myeloma patients that harbor mutations in individual components, IAP antagonists that specifically target c-IAP1/2 proteins or BR3 signaling that selectively recruits TRAF3 (29, 31, 32, 38, 49, 50). Nevertheless, concomitant removal of c-IAP1/2 and TRAF2/3 likely provides added guarantees that ensure the liberation of NIK and activation of signaling. An additional consequence of TWEAK mediated elimination of TRAF2 and c-IAP proteins is the diminished activation of TNF or CD40L stimulated canonical NF-κB and MAPK signaling (36, 58). Since TNFR1 and CD40 rely on c-IAPs and TRAF2 for the assembly of signaling complex and the activation of the canonical NF-κB and MAPK pathways, TWEAK can negatively impact the signaling downstream of TNFR1 and CD40 by depleting critical E3 ligases and adaptors (25, 36, 58).

Processing of p100 to p52 is one of the final steps in the activation of non-canonical NF-κB signaling, and it is also regulated by ubiquitination. IKKα mediated phosphorylation of p100 recruits SCF-βTrCP E3 ligase complex, which promotes p100 ubiquitination and partial proteasomal degradation to yield a p52 form (59–61). In addition, Fbw7, another substrate-binding component of SCF ubiquitin ligase complex, can also regulate proteasomal processing of p100. However, in this case the kinase that provides phosphorylation trigger for ubiquitination is not IKKα but GSK3 (62–65). Thus, multiple kinases and ubiquitin ligases control p100

processing to ensure proper control of the non-canonical NF-κB pathway activation.

DEUBIQUITINASES IN TWEAK SIGNALING

Given the importance of ubiquitination for TWEAK mediated signaling it is no surprise that deubiquitination also plays a functional role for TWEAK biology. DUBs are enzymes that remove ubiquitin moieties from substrate proteins and allow reversal or inhibition, but in some cases also activation of signaling that is regulated by ubiquitination (7, 10). Recently, the Lys48-specific DUB OTUB1 has been identified as c-IAP1 interacting DUB that can regulate c-IAP1 protein stability following TWEAK stimulation (41, 66). TWEAK stimulates Lys48-linked polyubiquitination of c-IAP1 that ultimately leads to c-IAP1 degradation and the activation of non-canonical NF-κB signaling (31, 41). However, elimination of c-IAP1 also diminishes TWEAK-stimulated activation of canonical NF-κB and MAPK signaling (41). OTUB1 is recruited to TWEAK induced FN14-associated signaling complex where it regulates c-IAP1 Lys48-linked polyubiquitination (41) (**Figure 2**). In the absence of OTUB1, treatment with TWEAK promotes enhanced c-IAP1 degradation resulting in reduced activation of canonical NF-κB and MAPK pathways (41). However, OTUB1 does not seem to have significant effect on the non-canonical NF-κB pathway, most likely because even in the presence of OTUB1 TWEAK induces c-IAP1 degradation ultimately leading to NIK de-suppression and activation of NF-κB signaling. Another DUB from the ovarian tumor (OTU) domain family of DUBs, A20, potentially regulates non-canonical NF-κB signaling in non-enzymatic fashion by disrupting interaction between c-IAP1 and TRAF2/TRAF3, thereby breaking the link between E3 ligase c-IAP1 and its substrate NIK (67). In the absence of A20, TWEAK-stimulated NIK accumulation and p100 processing were diminished suggesting that A20 is a positive regulator of non-canonical NF-κB signaling (67).

An additional DUB candidate for the regulation of TWEAK-stimulated non-canonical NF-κB signaling is OTUD7B or Cezanne (68). OTUD7B regulates TRAF3 ubiquitination and in particular Lys48-linked polyubiquitination of TRAF3 following stimulation with LT-β or CD40L (69). Through TRAF3 interaction OTUD7B is recruited to CD40 and LT-βR where it regulates TRAF3 ubiquitination and stability. In the absence of OTUD7B TRAF3 is more heavily ubiquitinated with Lys48 linkages leading to its faster degradation (69). As TRAF3 is dispensable for the activation of canonical NF-κB and MAPK signaling OTUD7B does not affect these pathways. However, expedited removal of TRAF3 in the OTUD7B knockouts allows faster activation of non-canonical NF-κB and results in B cell hyper-responsiveness to antigens (69). Although the role of OTUD7B in TWEAK signaling has not been examined yet, striking similarities in the activation of non-canonical NF-κB pathway by TWEAK, LIGHT, and CD40L suggest the OTUD7B might influence TWEAK-stimulated non-canonical NF-κB signaling as well.

CONCLUSION

The controlled posttranslational modification of signaling adaptors and effectors has a great potential to regulate signaling outcomes (6, 7, 9, 30). Ubiquitination is one such modification that impacts diverse aspects of TWEAK signaling with direct consequences for the production of inflammatory cytokines and cellular survival and proliferation. TWEAK and FN14 employ signaling principles that significantly rely on ubiquitination for the regulation of signaling complexes and investigation of ubiquitination processes has greatly aided our understanding of the fascinating biology of TWEAK signaling (19). In recent years several agents have been developed that specifically target therapeutically attractive proteins, such as IAP antagonists for E3 ligases c-IAP1/2 and kinase inhibitors for their substrate NIK, in a number of cellular pathways (7, 70). An improved understanding of ubiquitin networks and molecular and physiological mechanisms that control them should reveal novel modalities for targeting TWEAK and FN14 regulated pathways and pathologies.

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TNF receptor 2 and disease: autoimmunity and regenerative medicine

***Denise L. Faustman*[1]* and *Miriam Davis*[2]**

[1] *Immunobiology Laboratory, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA*
[2] *Immunobiology Laboratory, Massachusetts General Hospital, Boston, MA, USA*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Andrey Kruglov, German Rheumatism Research Center, Germany
Eleni Douni, Agricultural University of Athens, Greece

***Correspondence:**
Denise L. Faustman, Immunobiology Laboratory, Massachusetts General Hospital and Harvard Medical School, Building 149, 13th Street, Room 3601, Boston, MA 02129, USA
e-mail: faustman@helix.mgh.harvard.edu

The regulatory cytokine tumor necrosis factor (TNF) exerts its effects through two receptors: TNFR1 and TNFR2. Defects in TNFR2 signaling are evident in a variety of autoimmune diseases. One new treatment strategy for autoimmune disease is selective destruction of autoreactive T cells by administration of TNF, TNF inducers, or TNFR2 agonism. A related strategy is to rely on TNFR2 agonism to induce T-regulatory cells (T_{regs}) that suppress cytotoxic T cells. Targeting TNFR2 as a treatment strategy is likely superior to TNFR1 because of its more limited cellular distribution on T cells, subsets of neurons, and a few other cell types, whereas TNFR1 is expressed throughout the body. This review focuses on TNFR2 expression, structure, and signaling; TNFR2 signaling in autoimmune disease; treatment strategies targeting TNFR2 in autoimmunity; and the potential for TNFR2 to facilitate end organ regeneration.

Keywords: TNF, TNF receptor 2, autoimmune disease, type 1 diabetes, regeneration

INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic cytokine involved in regulating diverse bodily functions including cell growth modulation, inflammation, tumorigenesis, viral replication, septic shock, and autoimmunity (1). These functions hinge upon TNF's binding to two distinct membrane receptors on target cells: TNFR1 (also known as p55 and TNFRSF1A) and TNFR2 (also known as p75 and TNFRSF1B). TNFR1 is ubiquitously expressed on the lymphoid system and nearly all cells of the body, which likely accounts for TNF's wide-ranging functions. TNFR2 is expressed in a more limited manner on certain populations of lymphocytes, including T-regulatory cells (T_{regs}) (2, 3), endothelial cells, microglia, neuron subtypes (4, 5), oligodendrocytes (6, 7), cardiac myocytes (8), thymocytes (9, 10), islets of Langerhans (personal communication, Faustman Lab), and human mesenchymal stem cells (11). Its more restricted cellular expression makes TNFR2 more attractive than TNFR1 as a molecular target for drug development. Activation of TNFR1 alone by exogenous TNF is systemically toxic (12, 13).

As a general rule, TNF depends on TNFR1 for apoptosis and TNFR2 for any function related to cell survival, although there is some degree of overlapping function depending upon the activation state of the cell and a variety of other factors (14). Likewise, TNFR1 and TNFR2 have distinct intracellular signaling pathways, although there is some overlap and crosstalk (15). TNF binding to TNFR1 triggers apoptosis through two pathways, by activation of the adaptor proteins TNFR1-associated death domain (TRADD) and Fas-associated death domain (FADD). In contrast, TNFR2 signaling relies on TRAF2 and activation and nuclear entry of the pro-survival transcription factor nuclear factor-kB (NFkB) (16–18). TNFR2 expression on T_{regs} is induced upon T-cell receptor activation (19).

While the etiologies of autoimmune disorders vary, there is some degree of overlap in their genetic, post-translational, and environmental origins. One overlapping feature is that various defects in TNF signaling pathways, acting through the TNF receptors and NFkB in autoreactive T cells, occur in both human and mouse models of various autoimmune disorders, including Crohn's disease, Sjogren's syndrome, multiple sclerosis, ankylosing spondylitis, and type I diabetes (20–39). The defects range from defects in the proteasome in both the non-obese diabetic (NOD) mouse model and humans with Sjogren's syndrome, to specific polymorphisms in the TNFR1 or TNFR2 receptors themselves, to punitive interruptions in genes that control the ubiquitination of the NFkB pathway.

TNFR EXPRESSION, STRUCTURE, AND SIGNALING

As noted above, TNFR1 and TNFR2 possess different patterns of expression. TNFR1 is found on nearly all bodily cells, whereas TNFR2 is largely found on certain immune cells (CD4+ and CD9+ lymphocytes), certain CNS cells, and endothelial cells, among others. Neither receptor is located on erythrocytes. Typically, cells that express TNFR2 also express TNFR1, with the ratio of expression varying according to cell type and functional role. Because TNFR1 typically signals cell death, while TNFR2 typically signals cell survival, the ratio of their co-expression will shift the balance between cellular survival and apoptosis.

TNFR1 and TNFR2 have extracellular, transmembrane, and cytoplasmic components. The extracellular component of both receptors is rich in cysteine, which is characteristic of the TNF superfamily. TNFR1 contains 434 amino acids. Its intracellular region of 221 amino acids contains a death domain that binds TRADD or FADD. In T cells, activation of TRADD or FADD activates the caspases, resulting in apoptosis (**Figure 1**).

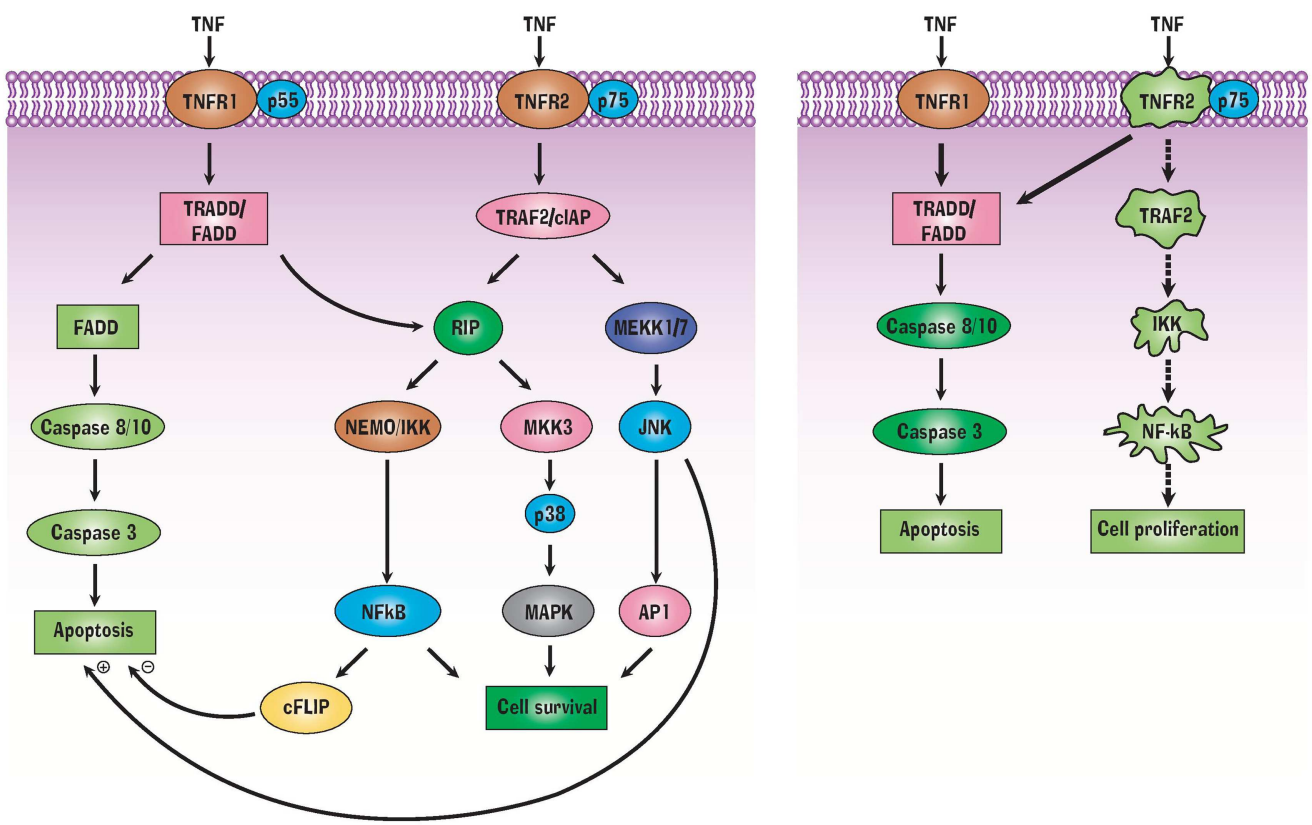

FIGURE 1 | TNF Signals through TNFR1 and TNFR2 receptors (A) but abnormalities in this signaling pathway in autoimmunity (B) can favor a pathway of selective apoptosis due to a variety of protein signaling defects.

A second apoptotic pathway relies on TRADD's activation of RIP (receptor interacting protein) (**Figure 1A**). In contrast to TNFR1, TNFR2 does not have a cytoplasmic death domain. The receptor consists of 439 amino acids. Its extracellular domain is formed by the first 235 amino acids, its transmembrane domain is formed by 30 amino acids, while its cytoplasmic domain is formed by 174 amino acids. TNFR2's cytoplasmic domain has a TRAF2 binding site. TRAF2, in turn, binds TRAF1, TRAF3, cIAP1, and cIAP2 (17, 18). These signaling proteins activate several other signaling proteins, yielding cell survival (**Figure 1A**). Cell survival is ensured when the transcription factor NFkB is liberated from its inhibitor protein IkBα in the cytoplasm and translocates to the nucleus where it activates pro-survival target genes (40). Both TNFR1 and TNFR2 can bind monomeric TNF or trimeric soluble TNF although soluble TNF induces no or weak signaling for TNFR2. This may be related to altered association or dissociation kinetics or more optimal kinetics with pre-formed transmembrane TNF (41). TNFR2 also preferentially binds transmembrane TNF (42). Transmembrane TNF is a trimer on the cell surface and transmits signals to the cell where it is contained, i.e., reverse signaling. It is thought that TNFR2 preferentially binds transmembrane bound TNF (43). Solution of the crystal structure of the TNF-TNFR2 complexes demonstrated that these interactions also result in the formation of aggregates on the cell surface and this likely promotes signaling (44).

Transgenic mice have been produced to try to understand better the function of TNFR2 (45). TNFR2$^{-/-}$ mice homozygous for TNFR2$^{-/-}$ are viable and fertile. They also show normal T-cell development and activity and are resistant to TNF-induced death. The T-cell proliferation responses are diminished and they also show abnormal central nervous system regeneration (JAX Mice Database – 00260).

TNF IN DEVELOPMENT AND AUTOIMMUNITY

Tumor necrosis factor, and its signaling through the two receptors, plays several crucial roles during normal development. It shapes the efficacy of the immune system and protects against infectious disease, cancer, and autoimmune disease (46). Upon release, TNF proceeds throughout the lifecycle to exert regulatory roles over immune cells by triggering transcription of genes responsible for inflammation, proliferation, differentiation, and apoptosis. To counter a pathological infection, TNF facilitates proliferation of immune cell clones. To continue to fight against the infection, TNF stimulates differentiation and recruitment of naïve immune cells. Subsequently, TNF orchestrates destruction of superfluous

immune cell clones to reduce inflammation and tissue damage once the infection is resolved.

In the process of developing autoimmunity, abnormal progenitors to T cells and other immune cell types proliferate and begin to mature in the thymus. T-cell education occurs through two parallel pathways for CD4 and CD8 T cells through either HLA class II or HLA class I cell surface structures. For almost all autoimmune disease there is strong genetic linkage to the HLA class II region. This genetic region is rich in immune response genes and contains not only the class II genes themselves but also the HLA class I assembly genes such as the tap transporters (Tap1/Tap2) and proteasome genes (that control self peptide presentation) such as LMP2 (PSMB9), LMP7 (PSMB8), and LMP10 (PSMB10) (47). During T-cell education, the vast majority of immature immune cells die by apoptosis, which serves to remove defective progenitors. The process is not foolproof, however. Failures in T-cell education in humans perhaps driven by defective antigen presentation allow autoreactive but still immature T cells defined as CD45RA+ (2H4) and lesser numbers of CD45RO+ (4B4) to enter the circulation (36, 48, 49). In humans and autoimmune animal models diverse mutations and polymorphisms drive altered proteasome function with varying phenotypes of autoimmunity (50–55). Once in the circulation, the cells differentiate into mature autoreactive T cells when they encounter specific self-antigens (56). The failure of T-cell education of autoreactive CD8 T cells, due to HLA class I interruption, yields self-reactive T cells directed at specific self-antigens. This failure underlies various immune diseases, including type I diabetes, Crohn's disease, multiple sclerosis, and Sjogren's syndrome (50).

TNFR2 SIGNALING AND BENEFITS TO HEALTH

TNFR2 signaling pathways appear to offer protective roles in several disorders, including autoimmune disease, heart disease, demyelinating and neurodegenerative disorders, and infectious disease. According to *in vitro* and *in vivo* studies, TNF or TNFR2 agonism is associated with pancreatic regeneration (57–59), cardioprotection (60, 61), remyelination (5, 6), survival of some neuron subtypes (5, 62, 63), and stem cell proliferation (11, 64–66).

Knockout of the *tnfr2* gene in a mouse model produces a higher rate of heart failure and reduced survival after myocardial infarction (60). TNFR1 signaling is deleterious and TNFR2 signaling is protective in regeneration and repair processes following infarcted myocardium in female mice (61).

An agonist for TNFR2 selectively destroys autoreactive T cells but not healthy T cells in blood samples from type I diabetes patients, as well as multiple sclerosis, Graves, Sjogren's autoreactive T cells (57). Animal models of type I diabetes exhibit massive regeneration of the pancreas after elimination of autoreactive T cells with low-dose TNF (58, 59). TNFR2 is crucial for TNF-induced regeneration of oligodendrocyte precursors that make up myelin (6), a finding that may be important in the treatment of multiple sclerosis and other demyelinating disorders, regardless of whether they have an autoimmune etiology. In viral encephalitis-infected knockout mice, the TNFR2 pathway is relied upon to repair the brain's hippocampus, and TNFR1 is relied upon to repair the brain's striatum (63). Oligodendrocyte regeneration appears to occur as a result of TNFR2 activation on astrocytes, which promotes oligodendrocyte proliferation through the induction of chemokine CXCL12 in an animal model of demyelination (67). Lastly, TNFR1 promotes neurodegeneration while TNFR2 promotes neuroprotection in an animal model of retinal ischemia in knockout mice (68).

TNF RECEPTOR AND AUTOIMMUNE DISEASE

A variety of defects in TNFR2 and downstream NFKB signaling are found in various autoimmune diseases. The defects include polymorphisms in the TNFR2 gene, upregulated expression of TNFR2, and TNFR2 receptor shedding. A recently published study implicates a new decoy splice variant of the TNFR1 receptor in multiple sclerosis. This causes a relative deficiency in TNF with inadequate TNFR2 signaling for autoreactive T-cell selection and induction of beneficial T_{regs} (39). Polymorphisms in TNFR2 have been identified in some patients with familial rheumatoid arthritis (69–71), Crohn's disease (72), ankylosing spondylitis (38), ulcerative colitis (73), and immune-related conditions such as graft versus host disease associated with scleroderma risk (74). Common to several autoimmune diseases, with the notable exception of type I diabetes, is a polymorphism in which the amino acid methionine is substituted for arginine at position 196 in exon 6 of chromosome 1p36 (16). This polymorphism may alter the binding kinetics between TNF and TNFR2, the result of which may reduce signaling through NFkB.

Upregulated expression of TNFR2 is also found in several immune diseases (16, 75). Higher systemic levels of soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2) are produced by administration of TNF to patients, likely by shedding of receptors into the extracellular space (76, 77). The greater the TNF stimulation, the greater is the increase in sTNFR1 and sTNFR2. Higher levels of sTNFR2 but not sTNFR1 are found in serum and bodily fluids of patients with familial rheumatoid arthritis (78) and systemic lupus erythematosus, both of which are marked by polymorphisms in TNFR2. TNFR2, but not TNFR1, is upregulated in the lamina propria of mice with Crohn's disease, and it causes *in vivo* experimental colitis (79). Decreasing the concentration of TNFR2, via receptor shedding or other means, is a possible compensatory mechanism to lower inflammation. The extracellular component of TNFR2 is proteolytically cleaved to produce sTNFR2. This component binds to TNF in the extracellular space, yielding lower concentrations of TNF available for binding to functional T cells (80, 81). The development of the first anti-TNF medications, including soluble TNFR2 fusion proteins like Enbrel, were therapeutic for some patients with rheumatoid arthritis but consistently worsened or induced new autoimmune diseases like type 1 diabetes, lupus, or multiple sclerosis. The human data are consistent with past mouse data where overexpression of TNFR2 triggered multi-organ inflammation especially in the presence of TNF.

To achieve cell survival, the final steps in the TNFR2 pathway rely on NFkB mobilization and translocation to the nucleus. This can only occur with an intact proteasome, which is responsible for cleaving the bond between NFkB and its inhibitor protein IKBA. A defect that inhibits proteasomal-driven cleavage of NFkB is seen in the type I diabetes-prone and Sjogren's syndrome-prone NOD mouse (33). A protein subunit of the proteasome, LMP2, is lowered

in all patients with Sjogren's syndrome (36, 52, 82). The LMP2 subunit of the proteasome is necessary for intracellular activation of NFkB in highly activated T cells (33).

TNF AS TREATMENT FOR AUTOIMMUNE DISEASE

Given the commonality of TNFR signaling abnormalities in autoimmune diseases, the administration of TNF has emerged as a common treatment strategy. Low-dose TNF exposure, acting through its receptors, selectively destroys autoreactive, but not healthy, CD8+ T cells in blood samples from patients with type I diabetes (57). Low-dose TNF also kills autoreactive T cells in an animal model of Sjogren's syndrome (83). A similar result with TNF exposure is achieved in blood samples from patients with scleroderma (84). A sustained effect need not require continuous dosing, unlike treatment with anti-cytokines or immunosuppressive drugs: TNF can be effective when administered intermittently (33). However, the administration of TNF is not feasible in humans because it is systemically toxic when given to cancer patients who already have high TNF levels due to an intrinsic defense system (12, 13, 85). The toxicity of TNF likely stems from the ubiquitous cellular expression of TNFR1. Because TNFR2 is more restricted in its cellular expression, TNFR2 agonism may offer a safer therapeutic approach than administration of TNF. The possibility of intermittent exposure would also enhance the safety profile. As noted earlier, upregulated expression of TNFR2 in the target tissue is observed in several autoimmune disorders on the target; this target tissue expression may be responsible for the growth-promoting and regenerative properties of TNF agonism. In a baboon study, TNFR2 agonism was generally safe but exhibited adverse effects in the form of thymocyte proliferation, a febrile reaction, and a small, transient inflammation caused by mononuclear cell infiltration (86). Not all TNFR2 antibodies are the same, however, as some can bind to the receptor without eliciting an immune response. It may well be the case that tissue-specific or cell-specific therapies afford a better safety profile. Many factors have profound effects on the nature of TNFR signaling with antibody agonists. Their safety and efficacy are affected by changes in the ligand, receptor, adapter proteins, or other members of the signaling pathway. Findings may also vary depending on culture conditions, origin of cells, and activation state.

The rationale for TNFR2 agonism as therapy for autoimmune disease was first shown in type I diabetes. TNFR2 agonism or induction of TNF is an effective means of selectively killing autoreactive CD8+ T cells in animal models, in human cells *in vitro* (33, 58, 83, 87, 88) and in blood samples taken from patients with type I diabetes (57). In the latter study, there was a dose-response relationship between TNFR2 agonism and CD8+ T-cell toxicity. The CD8+ T cells were autoreactive to insulin, a major autoantigen in type I diabetes.

How is TNF effective at killing autoreactive T cells? A variety of TNFR2 signaling defects prevent liberation of NFkB from IkB, precluding transcription of pro-survival genes. This in turn biases autoreactive T cells to shift to the TRADD/FADD cell death signaling pathway which leads to apoptosis (**Figure 1B**). In other words, NFkB dysregulation makes autoreactive T cells selectively vulnerable to TNF-induced apoptosis (20). T cells, unlike B cells and other immune cells, do not constitutively express the active form of NFkB. Only this active form can translocate to the nucleus in order to transcribe pro-survival genes.

THERAPEUTIC STRATEGIES FOR AUTOIMMUNE DISEASE

SMALL-MOLECULE AGONISTS

Medicinal chemists have found it challenging to create receptor-specific agonists for the TNF superfamily. Developing an antagonist is generally accomplished more readily than developing an agonist. That said, peptides, antibodies, and small molecules have been developed as TNFR2 agonists (89, 90). Of these types, antibody agonists have been more effective at engaging a specific signaling pathway (57). In a labor-intensive process, TNFR2 agonists have been developed by point mutations in the TNF protein by site-directed mutagenesis (90). Our laboratory has recently generated a TNFR2 agonist that activates TNF signaling pathways and suppresses CD8 T cells (91). The advantage of this agonist is that it also induced proliferation of T_{reg} cells that exert an immunosuppressive function. TNFR2 agonists, while less toxic than TNFR1 agonists, still may have toxicities, especially to cells within the CNS (16). For that reason it may be desirable to develop agonists that do not succeed at crossing the blood-brain barrier.

TNF INDUCERS

The foremost inducer of TNF is the mycobacterium bovis bacillus Calmette–Guerin (BCG), which has been on the market for decades as a vaccine for tuberculosis and as a treatment for bladder cancer. Its chemical equivalent that does not meet FDA's standards for purity is complete Freund's adjuvant (CFA). In an early double blinded placebo-controlled Phase I clinical trial, BCG administration produced a transient increase in TNF in the circulation (92). BCG or CFA have been successfully used in animal models of type I diabetes to either prevent onset of diabetes or kill autoreactive T cells, leading to the restoration of pancreatic islet cell function and normoglycemia (58, 59, 93–95). Furthermore, in a proof-of-concept randomized, controlled clinical trial, BCG killed the insulin-autoreactive T cells in the circulation of patients with type I diabetes (92). With the removal of insulin-autoreactive T cells, pancreatic islets managed to regenerate to the extent that there was a transient rise in C-peptide, a marker for insulin production. The transient rise in C-peptide was striking, considering that patients in the trial averaged 15 years of disease. This clinical trial data repudiated the presumption that loss of pancreatic function is irreversible. Although BCG and CFA release TNF and therefore are not specific for TNFR2, they have low toxicity and thereby may be safe for treating autoimmune disease by virtue of inducing low levels of TNF.

NFKB PATHWAY MODULATION

Nuclear factor-kB is thwarted from entering the nucleus to transcribe pro-survival genes in autoimmune diseases featuring defects in TNF signaling (33, 34). Instead of being cleaved, NFkB remains bound in the cytoplasm to its inhibitory chaperone protein IkBa. A genetic defect in type I diabetes-prone and Sjogren's syndrome-prone NOD mouse blocks the proteasome from cleaving NFKB from IkBa (34). Patients with Sjogren's syndrome also exhibit this defect (52). Consequently, inhibiting NFkB's translocation to the nucleus offers another therapeutic approach to autoimmune disease if it could be done in the select cells that are disease causing.

TNFR1 ANTAGONISM

Tumor necrosis factor binds to TNFR1 and TNR2. Another way to make TNF selective for TNFR2 signaling, an effect that could promote tissue regeneration and remove autoimmunity, is to create a TNFR1 antagonist. This strategy would bias TNF to act solely through the TNFR2 receptor. This strategy also appears promising for hepatitis or autoimmunity in murine models (96). A humanized TNFR1-specific antagonistic antibody for selective inhibition of TNF action has been tested with promising results (96–98).

EXPANSION OF T-REGULATORY CELLS VIA TNFR2

T-regulatory cells are a type of immunosuppressive cell that displays diverse clinical applications in transplantation, allergy, infectious disease, GVHD, autoimmunity, and cancer (99). T_{regs} co-express CD4+ and the interleukin-2 receptor alpha chain CD25 hi and feature inducible levels of intracellular transcription factor forkhead box P3 (FOXP3). Naturally occurring T_{regs} appear to express TNFR2 at a higher density than TNFR1 (3, 100, 101). There is evidence from animal models that TNF signaling through TNFR2 promotes T_{reg} activity: TNFR2 activates and induces proliferation of T_{regs} (100) and TNFR2 expression indicates maximally suppressive T_{regs} (102).

T-regulatory cells have been proposed to prevent or treat autoimmune disease, but the rate-limiting problem has been obtaining sufficient quantities, whether by generating them *ex vivo* or stimulating their production *in vivo*. *In vivo* stimulation turns out to be too toxic with standard expansion agents IL-2, anti-CD3, and anti-CD28. These expansion agents can be used to generate large quantities of T_{regs} *ex vivo*, but the problem is that they produce heterogeneous progeny consisting of mixed CD4+ populations. Heterogeneous progeny carry risk: they are capable of releasing pro-inflammatory cytokines and consist of cell populations with antagonistic properties. Some new approaches are being attempted, including expansion of T_{regs} *in vivo* with TL1A-Ig, a naturally occurring TNF receptor superfamily agonist (103). Additionally, our laboratory has developed a method of *ex vivo* expansion using a newly synthesized TNFR2 monoclonal antibody agonist that produces homogeneous progeny expressing a uniform phenotype of 14 cell surface markers (91). The TNFR2-agonist expanded T_{regs} are capable of suppressing CD8+ T cells. In healthy humans, the TNF inducer BCG causes transient expansion of T_{regs} (91). In a clinical trial, BCG triggers T_{reg} production in patients with type I diabetes (92), which appears to contribute to the suppression of disease and temporary restoration of islet cell function.

USE OF TNFR2 FOR TISSUE REGENERATION

When type 1 diabetes was first reversed in end-stage diabetic mice with boosting of TNF, the research showed an unexpected outcome (59). The pancreas of the treated diabetic mice had regenerated their islets and the original islet transplants that were performed to restore blood sugars were not needed (59). The histologic shape of the reappearing insulin secreting islets was also remarkable. The newly regenerated islets were larger in size than unaffected, untreated NOD mouse cohorts, and contrasted greatly from islets of NOD mice that had received immunosuppressive drug strategies, such as anti-lymphocyte serum or anti-CD4 or anti-CD3 antibodies, to avert diabetes (104, 105). Past autoimmune treatments of diabetic NOD mice worked almost only in pre-diabetic mice or early new-onset diabetic mice (106). Also the rescued islets of NOD mice, commonly treated with anti-CD3 immunosuppressive antibodies, were small in size, and demonstrated no or limited regeneration. The immunosuppressive drug was best administered to pre-diabetic mice or to mice with recent onset hyperglycemia. In total, this data strongly suggested that administration of TNF directly or boosting TNF indirectly with BCG or the heat-killed equivalent, CFA, had a dual mechanism of action – a direct killing of the autoreactive T cells and also a TNF effect directly on the target organ to promote healing and regeneration. Also the TNF effect on the target tissue indicated that even late stage diabetes could be reversed in large part due to the regenerative effect in contrast to a pure rescue effect, survival of existing islets without expansion, of standard immunosuppressive strategies.

The effect of TNF on the pancreas was not the only tissue showing possible regeneration with TNF stimulation. In the field of neuroregeneration, the Ting laboratory showed TNF similarly promoted proliferation of oligodendrocytes progenitors and remyelination (6). Gradually the broader literature reported the regenerative effect of TNF and TNFR2 agonism on heart regeneration, bone marrow stem cells, and even neuron regeneration in the setting of Parkinson's disease model in mice (11, 60, 66, 107).

CONCLUSION

An overlapping feature across autoimmune disorders is various defects in TNF signaling through its two receptors. TNFR2 is a more attractive molecular target than TNFR1 because of its limited cellular expression. A variety of strategies utilizing TNFR2 agonism can be pursued for treatment of autoimmune disease and also used for regenerative medicine therapies. TNFR2 agonism has been associated with selective death of autoreactive T cells in type 1 diabetes and with induction of T_{regs}. It holds promise for treating other autoimmune disorders featuring dysregulation of NFkB, which is a key component of the TNFR2 signaling pathway.

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Non-canonical NF-κB signaling initiated by BAFF influences B cell biology at multiple junctures

Sandra Gardam [1] **** and Robert Brink*** [1,2]

[1] *Immunology Division, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia*
[2] *St. Vincent's Clinical School, University of New South Wales, Darlinghurst, NSW, Australia*

Edited by:
John Silke, Walter and Eliza Hall Institute, Australia

Reviewed by:
Hideki Ogura, Osaka University, Japan
Kai Fang, University of California Los Angeles, USA

****Correspondence:***
Sandra Gardam, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia
e-mail: s.gardam@garvan.org.au

It has been more than a decade since it was recognized that the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) transcription factor family was activated by two distinct pathways: the canonical pathway involving NF-κB1 and the non-canonical pathway involving NF-κB2. During this time a great deal of evidence has been amassed on the ligands and receptors that activate these pathways, the cytoplasmic adapter molecules involved in transducing the signals from receptors to nucleus, and the resulting physiological outcomes within body tissues. In contrast to NF-κB1 signaling, which can be activated by a wide variety of receptors, the NF-κB2 pathway is typically only activated by a subset of receptor and ligand pairs belonging to the tumor necrosis factor (TNF) family. Amongst these is B cell activating factor of the TNF family (BAFF) and its receptor BAFFR. Whilst BAFF is produced by many cell types throughout the body, BAFFR expression appears to be restricted to the hematopoietic lineage and B cells in particular. For this reason, the main physiological outcomes of BAFF mediated NF-κB2 activation are confined to B cells. Indeed BAFF mediated NF-κB2 signaling contributes to peripheral B cell survival and maturation as well as playing a role in antibody responses and long term maintenance plasma cells. Thus the importance BAFF and NF-κB2 permeates the entire B cell lifespan and impacts on this important component of the immune system in a variety of ways.

Keywords: BAFF, BAFFR, NF-κB2, B lymphocyte, signaling

INTRODUCTION

In 2001 it was recognized that nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) signaling actually consisted of two distinct pathways which have become known as the canonical (classical/NF-κB1) and non-canonical (alternative/NF-κB2) pathways (1). The canonical NF-κB pathway involves the constitutive processing of full length NF-κB1 (p105) to its active form p50, which readily forms dimers with RelA (also known as p65) or c-Rel. These dimers are retained in the cytoplasm by the actions of the inhibitors of NF-κB (Iκ-B proteins), which mask their nuclear translocation signals. Signal-induced activation of this pathway leads to phosphorylation by the Iκ-B kinase (IKK) complex (consisting of IKKα, IKKβ, and IKKγ) of the Iκ-B proteins, subsequently leading to their degradation and allowing nuclear translocation of p50 containing dimers [reviewed in Ref. (2)]. In the non-canonical NF-κB pathway, NF-κB2 (p100) acts as the Iκ-B by retaining itself and RelB in the cytoplasm. Processing of NF-κB2 to its active form, p52 only occurs upon signal-induced activation of the pathway (3) and it is the loss of the carboxy-terminus of p100, which facilitates translocation of active p52/RelB dimers to the nucleus. In both pathways the presence of NF-κB dimers in the nucleus initiates specific transcription programs via the binding of dimers to κB sequences in the promoters of various genes. In this way the NF-κB signaling pathways are able to exert a variety of effects on tissues throughout the body. Interestingly, the non-canonical NF-κB pathway appears to activate a relatively small number of downstream genes compared to the canonical pathway (4), with the specific genes activated potentially varying depending on the cell type involved.

The major class of ligand/receptors pairs responsible for activating NF-κB2 signaling are the receptors of the tumor necrosis factor (TNF) family. Whilst the members of the TNFR superfamily that carry cytoplasmic death domains do not typically trigger non-canonical NF-κB signaling (e.g., TNFR1, Fas), those that lack death domains are invariably found to stimulate this pathway to some degree (5). Non-death domain members of the TNFR superfamily for which strong NF-κB2 activation has been demonstrated include CD40 (also known as TNFRSF5), lymphotoxin beta receptor (LT-βR also known as TNFRSF3), receptor activator of NF-κB (RANK also known as TNFRSF11a), and B cell activating factor of the TNF family receptor (BAFFR also known as TNFRSF13c). This review focuses on NF-κB2 activation by BAFFR and its ligand BAFF and the specific outcomes for tissues which express this receptor. The BAFF and BAFFR families of molecules will be described as well as the proximal signaling events which have been linked to this ligand/receptor pair. Finally, given the almost complete confinement of BAFFR expression to B cells, the effects of BAFF/BAFFR induced NF-κB2 on B cell survival, maturation, and responses will be described.

INTRODUCING THE MAIN PLAYERS

THE LIGANDS: BAFF AND APRIL

B cell activating factor of the TNF family (BAFF, also known as TNFSF13B) was identified simultaneously by several groups in

1999, who variously named it BAFF, Blys, TALL-1, THANK, and zTNF4 (6–10). BAFF was soon recognized to be a factor essential to the survival of mature, conventional B lymphocytes. Like other members of the TNF ligand family, BAFF is type II transmembrane protein which forms a constitutive trimer. However, it can be readily cleaved by furin to release as a soluble factor. In soluble form BAFF can persist as a trimer or assemble into a 60mer, consisting of 20 trimers, which maintains receptor binding capabilities and may indeed bind to more than one receptor at a time (11, 12). Despite the crystallographic evidence for this multimeric form, little is understood about its functional significance. A splice variant of BAFF has also been identified, ΔBAFF, which opposes the actions of BAFF by sequestering full length BAFF in heteromultimers. Unlike full length BAFF, ΔBAFF is confined to the membrane (13, 14).

A proliferation inducing ligand (APRIL, also known as TNFSF13) is closely related to BAFF and they share some receptor specificity. Cleavage of APRIL by furin convertase occurs at the Golgi apparatus (15) and soluble trimeric APRIL is subsequently secreted from the cell. As such, membrane bound forms of APRIL are not observed at the cell surface. However a fusion protein formed from trans-splicing of TNF-related weak inducer of apoptosis (TWEAK, also known as TNFSF12) and APRIL, known as TWE-PRIL is membrane bound and displays the APRIL receptor binding domain at the cell surface (16). TWE-PRIL is biologically active, however its physiological role is yet to be identified. Soluble APRIL trimers have been shown to interact via non-receptor interacting sites with cell surface heparin sulfate proteoglycans (HSPG), which is thought to create multimeric forms that are more biologically active than cell-free APRIL trimers (17–19). Heterotrimers of BAFF and APRIL have also been identified (20) and have been shown to be present in the sera of patients with various autoimmune diseases, though their contribution to disease or any physiological function has not yet been elucidated.

Cells of the innate immune system, including neutrophils, macrophages, monocytes, and dendritic cells, are the main producers of BAFF and APRIL. More recently a number of non-hematopoietic cells have been identified which also produce BAFF and/or APRIL, including osteoclasts, some epithelial cells, and astrocytes to name a few. Many of these have been identified at sites of disease and as such may be responsible for maintaining a local B cell population in response to disease [reviewed in Ref. (21)].

THE RECEPTORS: TACI, BCMA, AND BAFFR

BAFF and APRIL share binding to two TNFR family members: transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI, also known as TNFRSF13B) and B cell maturation antigen (BCMA, also known as TNFRSF17). Additionally BAFF, but not APRIL, is also able to interact with a third receptor, BAFFR (TNFRSF13C). However a splice variant of APRIL has been detected in mice which shows some affinity for BAFFR (22). All three receptors display the trimeric structure common to TNFR members and contain TNF receptor associated factor (TRAF) binding sites in their cytoplasmic domains but lack death domains. Amino acid residues in BAFF that are involved in TACI binding have been identified (23), although the extent to which these are also required for binding to BAFFR and BCMA is yet to be determined. The expression of all three receptors is restricted mainly to B lymphocyte lineage cells. Both BAFFR and TACI are widely expressed on all B cells, with BAFFR levels increasing as the B cells mature. TACI is particularly high on marginal zone (MZ) and B1 B cells in the mouse and $CD27^+$ memory B cells in humans. BCMA expression is restricted to plasma cells (PCs) in the mouse, though in humans it is also expressed on some germinal center (GC) and memory B cells (24, 25). On non-B cells, BAFFR is expressed on activated T cells and T regulatory cells (26), whilst TACI is expressed on dendritic cells and monocytes (27, 28). However in contrast to their roles in B cell biology, little is known about their roles in these other cells types.

TACI is able to recruit TRAFs 2, 5, and 6 to its cytoplasmic domain (29) and has been shown to activate NF-κB1, AP-1, and NFAT signaling pathways (30). BCMA has binding sites for TRAFs 1, 2, and 3 in its cytoplasmic tail and is capable of activating NF-κB1, Elk-1, p38 MAPK, and JNK signaling pathways (31). BAFFR contains only a single TRAF binding site, specific for TRAF3 and efficiently activates the NF-κB2 signaling pathway (32). Given these characteristics of the receptors, the majority of this review will focus on BAFFR and it role in activating non-canonical NF-κB signaling.

BRIDGING THE GAP FROM RECEPTOR TO TRANSCRIPTION FACTOR

An outline of NF-κB2 signaling was given in the introduction, however a more in depth description of the proximal signaling events which lead to the activation of NF-κB2 transcription programs in response to BAFF/BAFFR ligation is given below (**Figure 1**). These events have been recently elucidated using mainly *in vitro* systems employing on both CD40 and BAFFR as the activating receptors. A more complete understanding of the molecular events facilitating NF-κB2 activation in response to BAFFR ligation will aid in understanding how the molecules involved have been manipulated *in vivo* in order to reveal the tissue specific outcomes of BAFF/BAFFR-mediated NF-κB2, which will be discussed in Section "Tissue Responses and Effector Functions: The Outcomes of NF-κB2 Signaling in Response to BAFF."

THE ABSENCE OF BAFFR LIGATION: KEEPING NF-κB2 SWITCHED OFF

In contrast to many other signaling pathways, the initiation of NF-κB2 signaling by BAFFR actually results from the de-repression of the pathway, rather than its activation. The key kinase in the pathway, NF-κB inducing kinase (NIK) is constitutively degraded by the proteasome in the absence on BAFFR ligation (33). A complex consisting of TRAF2, TRAF3 and the cellular inducer of apoptosis proteins 1 or 2 (cIAP1/2) is responsible for this degradation. While all three components of the complex have ubiquitin ligase capability, only the cIAPs have been shown to mediate the attachment of K48 ubiquitin linkages, which direct proteins to the proteasome for degradation (34, 35). Both TRAF2 and TRAF3 harbor RING domains in their N termini, however their ubiquitin ligase activity is thought to be restricted to K63 ubiquitin linkages which are involved in signaling interactions rather than degradation of proteins (36, 37). Thus the role of TRAF2 and TRAF3 is thought to be acting as a molecular bridge. TRAF3 is able to directly interact

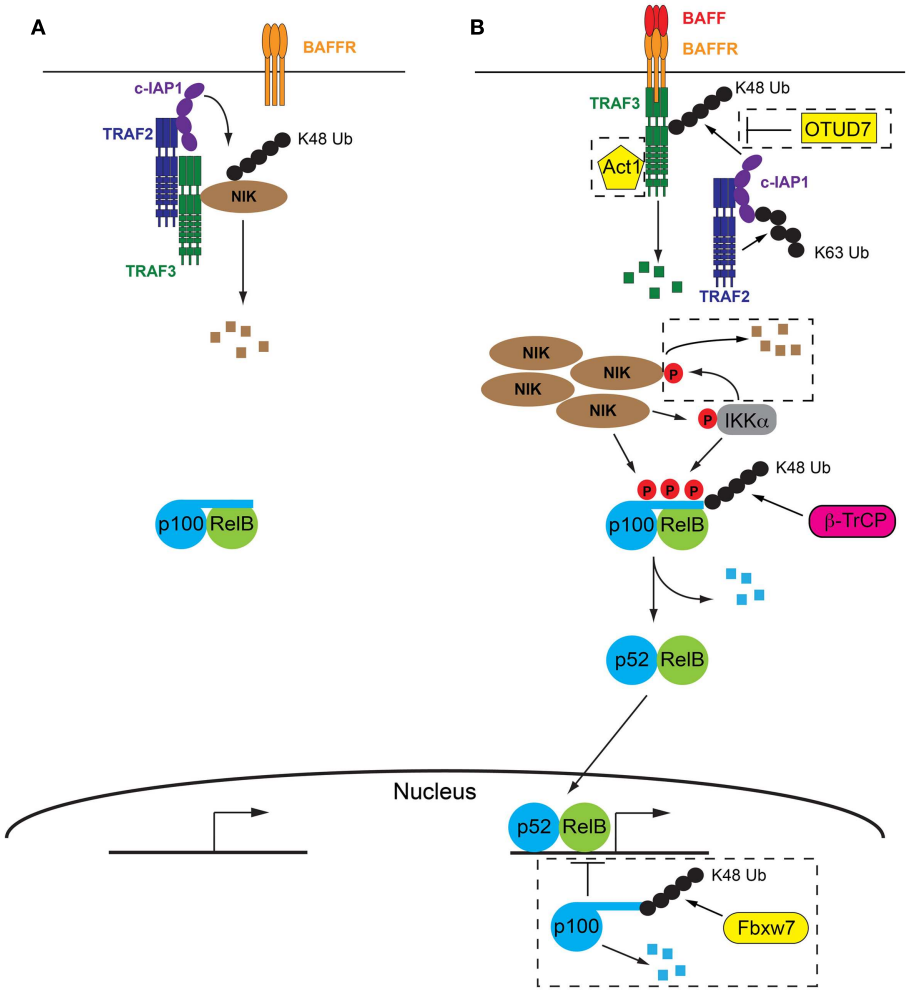


FIGURE 1 | The molecular details of BAFF/BAFFR-mediated activation of NF-κB2 signaling pathway. **(A)** In the absence of BAFF a complex consisting of TRAF2, TRAF3, and cIAP1/2 facilitate the degradation of NIK, the key kinase involved in activation of NF-κB2 signaling. p100 inhibits NF-κB2 activation by sequestering RelB in the cytoplasm. **(B)** Following BAFF ligation of BAFFR, TRAF3 is recruited to the receptor and subsequently degraded by the combined actions of TRAF2 and cIAP1/2. Lack of TRAF3 deactivates the TRAF/cIAP complex, releasing NIK from degradation and allowing it to accumulate in the cell. NIK then facilitates degradation of p100 via direct phosphorylation and phosphorylation of IKKα. p100 is subsequently partially degraded and active p52/RelB dimers are able to migrate to the nucleus and initiate NF-κB2 specific gene transcription programs. Refer to Sections "The Absence of BAFFR Ligation: Keeping NF-κB2 Switched Off" and "Turning NF-κB2 on in Response to BAFFR Ligation" of text for further details. Negative control mechanisms which impact on NF-κB2 activation are indicated within dashed boxed, including OTUD7, Act1, IKKα, and nuclear p100, refer to Section "Negative Control Mechanisms Limiting BAFFR Induced NF-κB2" of the text for further details. Small black circles represent ubiquitin, small red circles with P are phosphorylations.

with NIK and it has long been recognized that this interaction is followed by the ubiquitylation and subsequent degradation of NIK (33). The interaction between TRAF2 and cIAP1/2 was more recently demonstrated to be essential for K48 ubiquitylation of NIK and the cIAP proteins were identified as the ubiquitin ligases responsible (38, 39). Interaction between TRAF2 and TRAF3 is the final step that brings the ubiquitin ligase, cIAP1/2 into close proximity with its target, NIK (40, 41). Indeed a fusion protein consisting of the RING and zinc finger domains of TRAF2 and the TRAF domain of TRAF3 was able to compensate for both TRAF2 and TRAF3 in the ubiquitin ligase complex and, along with cIAP1/2, facilitate the degradation of NIK (41).

TURNING NF-κB2 ON IN RESPONSE TO BAFFR LIGATION

The extracellular interaction between BAFF and BAFFR facilitates the recruitment of TRAF3 to the cytoplasmic domain of BAFFR, via a PVPAT binding site (32) which is unable to recruit other TRAF family members (42). Following recruitment to BAFFR, TRAF3 undergoes proteasomal degradation (33), a process which requires TRAF2 and cIAP1/2. Indeed cell line studies using CD40 engagement as a stimulus indicated that the K63 ubiquitylation of cIAP1/2 by TRAF2 contributed to enhanced activity of the cIAP's own ubiquitylation action. The target of cIAP1/2's K48 ubiquitylation action was TRAF3, resulting in its degradation by the proteasome (40). No direct interaction between cIAP1/2

and TRAF3 has ever been shown, which suggests that TRAF2 plays a dual role in this process: it both recruits the ubiquitin ligase, cIAP1/2 to its target, TRAF3 via direct interaction with both proteins, as well as activating cIAP1/2, by K63 ubiquitylation, which optimizes the subsequent K48 ubiquitylation and degradation of TRAF3. It is likely a similar process occurs at the BAFFR and this is supported by *in vivo* evidence showing that TRAF3 is not efficiently degraded in TRAF2-deficient B cells, which express BAFFR and are subject to continual BAFFR ligation (43). It is not currently understood how the K63 polyubiquitination of cIAP1/2 promotes its own K48 ubiquitylation activity. It is tempting to speculate that this molecular mark on cIAP1/2 may contribute to directing its activity away from NIK and toward TRAF3. However it is equally possible that this change in specificity is due to conformational changes in TRAF3 caused by binding to BAFFR (42) or even the subcellular location of the event, that is proximal to BAFFR and thus the cell membrane. It is equally possible that K63 ubiquitination of cIAP1/2 results in the subsequent recruitment of another protein that acts to modulate the specificity of cIAP1/2, however as yet no such protein has been identified.

The recruitment to BAFFR and subsequent degradation of TRAF3 disrupts the cytoplasmic complex of TRAF2/TRAF3/cIAP1/2, not only by the removal of TRAF3, but also by the recruitment of TRAF2 and cIAP1/2 to the vicinity of the BAFFR in order to mediate the degradation of TRAF3. These events mean that NIK is no longer targeted for constitutive degradation and subsequently accumulates within the cell. The requirement for NIK accumulation explains the slower kinetics of the non-canonical NF-κB pathway compared to canonical NF-κB signaling (44) and is thought to result in the phosphorylation of NIK, possibly via autophosphorylation (45). Recent evidence suggests that this phosphorylation of NIK is not necessary for its activity as the kinase domain is in the active conformation even in the absence of phosphorylation (46). Thus it appears that it is the rescue of NIK from degradation and its subsequent accumulation in the cell that is the critical step in activating NF-κB2 signaling. NIK is capable of both phosphorylating p100 directly, at serines 866 and 870 (3), as well as phosphorylating another p100 kinase, IKKα (47). IKKα phosphorylates p100 at serine 822 (1) and it is thought that the combination of all three p100 phosphorylations is required to initiate the processing of p100 to p52 (48, 49). This final processing step is mediated by beta-transducin repeat containing protein (β-TrCP), a component of the SCF (Skp1-Cullin-1/Cdc53-F box protein) ubiquitin ligase complex (50). Active p52/RelB dimers are then free to migrate to the nucleus to initiate gene transcription programs (**Figure 1**).

A number of other positive mediators of NF-κB2 signaling have recently been identified whose contribution is not well understood. While the BAFFR cytoplasmic domain appears to contain only one TRAF binding site, specific for TRAF3, recent *in vitro* evidence suggests that it may also interact with TRAF1 (51). The presence of TRAF1 was demonstrated to decrease TRAF3, stabilize NIK, and increase p100 processing, though these functions were not a result of competing with TRAF3 for receptor binding. More recently TRAF1 has been shown to form heterotrimers with TRAF2, which display enhanced interaction with cIAP2 over the TRAF2 homotrimers (52). Thus TRAF1 may contribute to NF-κB2 activation by helping TRAF2 to enhance cIAP-mediated TRAF3 degradation. A further alternative is that TRAF1 may directly interact with NIK, stabilizing it and interfering with its TRAF2/TRAF3/cIAP1/2-mediated degradation, though the study suggesting this mechanism used TNFα as a stimulus, presumably acting through its non-death domain receptor TNFR2 (53).

It has been suggested that Mucosal associated lymphoid tissue lymphoma translocation gene 1 (MALT1) is required for p100 phosphorylation, optimal p100 processing and p52/RelB nuclear translocation (54). MALT1 was shown to interact with TRAF3 and was therefore proposed to act as a scaffold for the TRAF/cIAP complex. However, molecular evidence for this is currently lacking. Interestingly the MALT1 binding partner B cell CLL/lymphoma 10 (Bcl10) has also been implicated both directly (55) (though through LPS stimulation, rather than BAFF stimulation) and indirectly (56, 57) to contribute to NF-κB2 signaling. Whether these contributions also require MALT1 is largely unknown and requires further investigation.

NEGATIVE CONTROL MECHANISMS LIMITING BAFFR INDUCED NF-κB2

A number of mechanisms have been identified which target various components of this pathway as a way of limiting ongoing NF-κB2 signaling (**Figure 1**).

A further layer of control exists with respect to NF-κB2 or p100 itself: the presence of nuclear p100, which inhibits RelB binding to DNA. The ubiquitylation and subsequent degradation of nuclear p100 has recently been shown to be mediated by a different subunit of the SCF ubiquitin ligase complex, F box/WDF-repeat containing protein 7 (Fbxw7) and is thought to be constitutive (58, 59). Thus mechanisms affecting the function of Fbxw7 may impact on the efficiency of NF-κB2 activation.

Recently the deubiquitinating enzyme (DUB) ovarian tumor domain containing 7B (OTUD7B, also known as Cezanne) has been identified as the DUB responsible for removing degradative K48 ubiquitin chains from TRAF3 (60). OTUD7B was shown to be indirectly recruited to the receptor along with the TRAF3, TRAF2, and cIAP proteins in response to receptor ligation. Thus it is proposed to provide a negative feedback loop to oppose signal-induced activation of NF-κB2.

TRAF3 interacting protein 2 (TRAF3IP2, also known as Act1) is recruited to BAFFR, via its interaction with TRAF3. Mice lacking TRAF3IP2 exhibit B cell hyperplasia (61), suggesting that TRAF3IP2 is a negative regulator of B cell survival possibly via modulation of NF-κB2 signaling (see next section for a description of the contribution of NF-κB2 to B cell survival). However its mechanism of action is currently not understood and whether it exhibits its function independently of BAFFR ligation or in response to it, is unknown. The B cell hyperplasia phenotype is partially B cell extrinsic as B cell specific deletion of TRAF3IP2 produces a milder phenotype (61).

NF-κB inducing kinase is also subject to negative feedback control in response to receptor ligation, which was independent of the TRAF/cIAP complex. NIK can be phosphorylated by IKKα which results in its destabilization and subsequent degradation by the proteasome. Whether this involved ubiquitylation and which ligase was involved has not be determined (62).

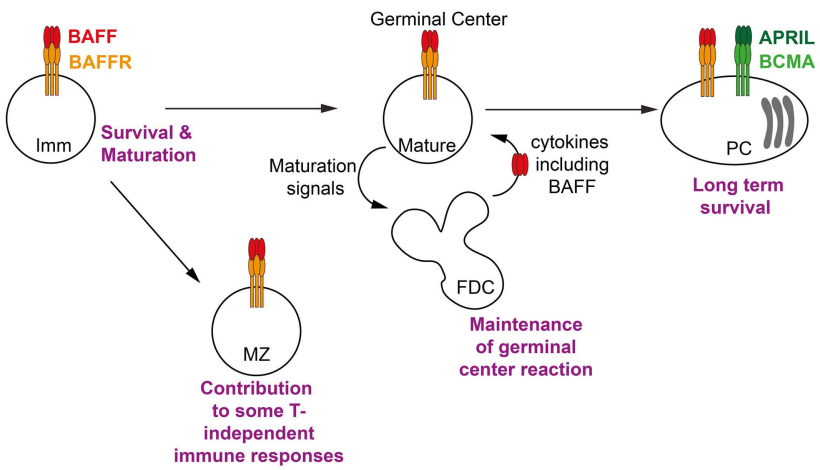
TISSUE RESPONSES AND EFFECTOR FUNCTIONS: THE OUTCOMES OF NF-κB2 SIGNALING IN RESPONSE TO BAFF

Many of the studies investigating the role of TRAFs and cIAPs in NF-κB2 activation were performed *in vitro* using cell lines, MEFs and in some cases utilized synthetic antagonists. These studies in many cases and did not examine BAFFR-mediated NF-κB2 activation, but rather utilized a variety of other TNFRs including CD40 and LT-βR in order to study NF-κB2 activation. Thus while these studies have allowed us to delineate the cytoplasmic events which contribute to NF-κB2 activation, they have not contributed greatly to our knowledge of the physiological outcomes of NF-κB2 activation, especially downstream of BAFF/BAFFR ligation in primary B cells. Importantly *in vivo* work has provided much greater insight in this respect, in addition to supporting much of the mechanistic data obtained *in vitro*.

Although the non-canonical NF-κB pathway has been clearly linked to a number of physiological responses, in some cases the activating receptor/ligand pair has not been clearly identified. Given the restriction of the expression of BAFF receptors to the lymphoid compartment, it is unsurprising that most identified roles for BAFF are in lymphocytes. Indeed the largest body of evidence for the role of BAFF mediated NF-κB2 signaling is in relation to B cell biology, including peripheral B cell survival and maturation, the generation of antibody response and the maintenance of PCs, all of which are further discussed below (**Figure 2**). Interestingly, NF-κB2 signaling is also essential for the development and organization of secondary lymphoid organs such as spleen, lymph node and Peyer's patches [reviewed in Ref. (63)]. However these functions of NF-κB2 appear to be mediated entirely by LT-βR signaling, and do not involve BAFF or its receptors and thus will not be further discussed here.

PERIPHERAL B CELL SURVIVAL AND MATURATION

B cells, like all cells of the hematopoietic lineage develop from hematopoietic stem cells in the bone marrow. The defining feature of B cell development is the expression of a B cell receptor (BCR), composed of immunoglobulin heavy and light chains derived from V(D)J recombination, which combine to define the unique specificity of each BCR and also of the antibody that the B cell will secrete should it eventually differentiate into a plasma cell. Pre-B cells in the bone marrow express only a recombined immunoglobulin heavy chain along with a pseudo-light chain. Final rearrangement of the light chain immunoglobulin genes and expression of a fully functional BCR is the defining feature of an immature B cell in the bone marrow which is then ready to egress into the periphery. In the periphery immature B cells have been further categorized into transitional subsets T1, T2, and T3. There is much controversy of the number of these subsets (some researchers defining two T2 subsets, a T2 follicular and T2 MZ), their position in the schema of development and even their functionality (it is possible that the T3 population represent not a transitional subset of immature B cells, but rather an anergic population). So while the exact developmental pathways of immature B cells in the periphery are not well defined, their outcomes are. Two subsets of mature B cells can be found in the periphery: follicular B cells, found in the spleen, lymph nodes and circulating in the



Cell Type:	Immature	Marginal Zone	GC B cell	Plasma Cell
Outcome of BAFF mediated NF-κB2:	survival maturation	contributes to T independent immune response	maintenance of GC reactions	long term survival

FIGURE 2 | Phenotypic outcomes of B cells in response to BAFF/BAFFR-mediated NF-κB2 activation. Activation of non-canonical NF-κB signaling in response to BAFF contributes to key events throughout the lifespan of a B cell. These include facilitating the survival of immature (Imm) B cells in the periphery and activating transcriptional programs which allow them to mature into follicular and marginal zone (MZ) B cells; contributing to some T-independent immune response; extending the duration of germinal center (GC) reactions; and maintaining long lived plasma cells (PCs) in the bone marrow. Refer to Section "Tissue Responses and Effector Functions: The Outcomes of NF-κB2 Signaling in Response to BAFF" of text for further details.

blood, and MZ B cells, a sessile population found only in the spleen. The various genetically modified mice lines discussed below have demonstrated the importance of BAFF/BAFFR-mediated NF-κB2 signaling to the survival of mature peripheral B cell subsets and their maturation (**Figure 2**). It should be noted that a further population of B cells exists, known as B1 B cells. These arise in the fetal liver and mainly populate the peritoneal cavity, though some are also found in the spleen. Their survival appears to be independent of BAFF and BAFFR signaling and thus they will not be further discussed here.

BAFF-deficient (64, 65) and BAFFR-deficient (66, 67) mice exhibit very similar phenotypes, with normal early B cell development in the bone marrow, but a paucity of peripheral B cell populations after transitional stage 1, suggesting that BAFF/BAFFR signaling is essential for B cell maturation and survival in the periphery. In addition a naturally occurring mutant strain A/WySnJ, which lacks peripheral B cells, was identified to have a mutation in the Tnfrsf13c gene that encodes BAFFR (68, 69) further validating the role of BAFF and BAFFR in supporting peripheral B cell survival. In contrast, BAFF transgenic mice display expanded mature B cells in the periphery, demonstrating that the constitutive production of BAFF is limiting and restricts total B cell numbers in wild-type mice (70, 71). It was of course possible that signaling through the other BAFF receptors may contribute to B cell survival. However, the phenotype of BCMA- and TACI-deficient mice, demonstrate that BAFF/BAFFR signaling is the main contributor to this phenotype. BCMA-deficient mice do not display any B cell specific phenotype (65, 72) and TACI-deficient mice actually exhibit a slight hyperplasia of B cells, suggesting negative regulatory role for this receptor in maintaining B cell populations (73, 74).

The accumulation of B cells in BAFF transgenic mice is associated with autoantibody production and autoimmune manifestations (70, 71) suggesting that induced or increased production of BAFF may rescue some self-reactive B cells from elimination from the repertoire. This was verified to be the case using transgenic mouse models of B cell self-reactivity (75, 76) and may contribute to the onset of disease in a number of human autoimmune diseases associated with increased levels of circulating BAFF (21).

As described above, BAFF/BAFFR signaling efficiently activates the NF-κB2 pathway and genetic evidence also demonstrates that it is this pathway that primarily supports peripheral B cell survival. Thus NF-κB2-deficient mice also display a deficit in peripheral B cells (77, 78) and more importantly the survival *ex vivo* of those that do exist cannot be rescued by addition of BAFF, emphasizing that this pathway is required to facilitate BAFF's survival effect (79). The survival of some periphery B cells to maturity in NF-κB2-deficient mice suggests that other signaling pathways also contribute to B cell survival and maturation. Indeed NF-κB1/NF-κB2 double-deficient mice show a more severe loss of peripheral B cells (80) and certainly NF-κB1 signaling activated by the BCR contributes to B cell survival. At least part of the canonical pathway's contribution is indirect, as p100 and RelB are NF-κB1 regulated genes (81–83). Thus canonical activation may be required to furnish the cell with sufficient components of the non-canonical pathway to allow it to promote B cell survival (84). Over activation of the canonical pathway can substitute for loss of BAFFR (85). However, it is likely that under physiological conditions both pathways contribute to B cell survival and that the BCR is the primary activator of canonical NF-κB signaling, whilst BAFFR is primarily responsible for activating NF-κB2 signaling.

NF-κB inducing kinase is the central kinase controlling NF-κB2 activity. Deficiency of NIK (86) or mutation in its kinase domain (alymphoplasia – aly mice) (87) results in severe lymphoid abnormalities including loss of lymph nodes and Peyer's patches as well as B cell lymphopenia and splenic disorganization. As mentioned above control of lymphoid organ formation and organization has been attributed to LT-βR mediated NF-κB2 signaling and indeed these mice phenocopy LT-βR-deficient mice with respect to these phenotypes (88). These abnormalities are also more similar to NF-κB1/NF-κB2 double-deficient mice than either of the single deficient mice alone. Indeed it has been suggested that, in addition to its role in NF-κB2 signaling, NIK may also contribute to NF-κB1 signaling. Despite the many additional phenotypes present in the aly mice, they retain the B cell lymphopenia common to mice models with defects in BAFF/BAFFR signaling and NF-κB2 signaling. Thus the phenotype of NIK-deficient mice also reflects the role of BAFFR-mediated NF-κB2 activation in B cell survival and maturation.

In contrast to deletion of other components of the NF-κB2 pathway, deletion of IKKα results in perinatal lethality (89) suggesting that this kinase has additional roles outside NF-κB signaling. Bone marrow chimeras for IKKα-deficient fetal liver cells or mice expressing a kinase-inactive version of IKKα, do display B cell abnormalities similar to other mice models with defective NF-κB2, including a deficit in peripheral B cell numbers and impaired GC formation (90, 91). However, given that non-NF-κB2 related roles for IKKα exist and may confound NF-κB2 related phenotypes, further detailed description of these mice will not be undertaken here.

One outcome of NF-κB2 signaling which is thought to promote B cell survival is the upregulation of anti-apoptotic molecules such as B cell CLL/lymphoma 2 (Bcl-2) (92, 93). Indeed transgenic overexpression of Bcl-2 in mice models lacking functional BAFFR signaling was sufficient to restore mature B cell populations (66, 94, 95). However while follicular B cells were observed in these models, MZ B cells were not restored. This demonstrated that in addition to providing survival signals to peripheral B cells, BAFF/BAFFR signaling is also essential for the complete maturation of B cells into MZ phenotype.

In line with this finding, mouse models with hyperactive NF-κB2 signaling display an expansion of the MZ B cell population, confirming that NF-κB2 signaling promotes this phenotypic outcome. These models include BAFF transgenic mice (71), as well as mice lacking components of the ubiquitin ligase complex which facilitates NIK degradation, namely TRAF2, TRAF3, or the cIAPs (43, 96–98). TRAF2 and TRAF3 play co-operative, but distinct roles in facilitating both the constitutive suppression of NF-κB2 signaling and the BAFFR-mediated activation of this pathway, the molecular details of which are described above. Mice completely lacking either TRAF are perinatally lethal (99, 100). Thus the use of a B cell specific deletion system to investigate their role in B cells has been important in furthering our understanding of these molecules. Lack of either TRAF2 or TRAF3 specifically in B cells

led to increased NF-κB2 signaling and an enlarged mature B cell population, with the largest expansion being in the MZ compartment of the spleen (43). While loss of TRAF2 or TRAF3 from B cells produced a similar phenotype, the implication from *in vitro* work that the two molecules are not performing the same function in regulating BAFFR signaling is supported by evidence from *in vivo* work. Firstly, the fact that loss of either TRAF resulted in the same phenotype, that is one was not able to completely compensate for the loss of the other. Secondly, mice lacking both TRAF2 and TRAF3 from their B cells did not display a more extreme phenotype in terms of levels of NF-κB2 activation or expansion of mature B cell populations, as would be expected if TRAF2 and TRAF3 were able to partially compensate for each other (43).

In contrast to the TRAF proteins, cIAP1 and cIAP2 are able to compensate for each other in their roles in BAFFR signaling. Thus mice completely deficient in cIAP2 or mice lacking cIAP1 in B cells, both displayed normal NF-κB2 activation in their B cells and consequently normal B cell survival and maturation. However mice lacking both cIAP1 and cIAP2 from their B cells had hyperactive NF-κB2 and expanded mature B cell populations (98). cIAP1/2-deficient B cells also contained high levels of TRAF3 (98). This demonstrated definitively that *in vivo* cIAP1 and cIAP2 are able to compensate for each other in facilitating both the degradation of NIK to suppress NF-κB2 activation and the degradation of TRAF3 in order to activate it. Indeed the ubiquitin ligase activity of cIAPs was shown to be essential *in vivo* to mediate these processes by the development of mice in which cIAP2 contained mutations which inactivated its E3 ligase activity (101). These mice displayed increase peripheral B cells and expanded MZs in the spleen. The authors proposed that mutant cIAP2 also had the affect of inhibiting the association of cIAP1 with TRAF2 and thus with the ubiquitin ligase complex which regulates NIK, thus explaining why in this case cIAP1 was not able to compensate for cIAP2 as it is able to in the complete absence of cIAP2.

The loss of either BAFF (43), or BAFFR (98) can be completely compensated for in terms of B cell survival and maturation by disruption of the TRAF/cIAP ubiquitin ligase complex and thus constitutive hyperactivation of NF-κB2. Whilst other evidence presented above shows that BAFF, BAFFR and NF-κB2 signaling are all able to individually contribute to B cell survival and maturation, it is these experiments which definitively demonstrate that activation of NF-κB2 sufficiently compensates for loss of BAFF or BAFFR. That is the primary, perhaps even the exclusive purpose of BAFF/BAFFR signaling in B cells is the activation of NF-κB2 signaling and it is this pathway which facilitates the transcriptional effects required in order for B cells to survive and mature in the periphery. However, because of the tight link between BAFF signaling and B cell survival, the specific genes that are upregulated by BAFF in B cells and their roles in B cell physiology have been difficult to verify and are not well understood.

B CELL ANTIBODY RESPONSES

The main function of B cells is to protect the body from foreign invasion by the production of antibodies. A B cell encountering a foreign antigen that matches the specificity of its BCR can ultimately differentiate into a plasma cell, a specialized antibody-producing factory capable of making large amounts of secreted antibodies. Most foreign antigens illicit a T cell dependent antibody response, in which activated B cells form GCs where they are able interact with cognate T follicular helper cells as well as receive survival signals from support cells such as follicular dendritic cells (FDC) in order to select cells with high affinity for the foreign antigen that have been generated by somatic hypermutation (SHM) of the immunoglobulin genes [reviewed in Ref. (102)]. Alternatively some antigens are able to illicit maximal responses from B cells even in the absence of T cells and germinal center formation – that is they illicit T cell independent antibody responses. These antigens tend to be generic antigens, for example the repeating carbohydrate units which make up bacterial cell walls. BAFF/BAFFR signaling has been implicated in both these processes (**Figure 2**).

The germinal center reaction

Despite the severe restriction on the survival of peripheral B cell in mice deficient for either BAFF or BAFFR, those remaining are able to form of relatively normal GCs in which SHM can occur (67, 103, 104). Thus BAFF/BAFFR signaling activating NF-κB2 is not required for these processes. However it appears they do play a role in the maintenance of GCs as GCs in mice lacking BAFF/BAFFR signaling dissipate a few days after they form. Interestingly the reasons for the instability differed between the two models. A lack of BAFF meant the FDC reticulum, important for trapping and presenting immune complexes failed to mature, probably leading to a lack of stimulation being received by the B cells present in the GC and subsequent breakdown of the GC (103, 104). However the FDC reticulum was normal in A/WySnJ (BAFFR mutant) mice, and B cells instead exhibited a proliferation defect after initial germinal center formation (103). The most obvious explanation for this discrepancy is that BAFF acts directly on FDCs to promote their maturity and does so through a receptor other than BAFFR. In addition BAFF acting through the BAFFR on B cells is also required for germinal center maintenance. However this explanation is unlikely as both TACI and BCMA-deficient mice (72, 73) display normal GC formation, and therefore were unlikely to be the receptors responsible for the failure of FDC maturation in BAFF-deficient mice. Whatever the precise roles of BAFF are in supporting GC persistence, the fact that activated FDCs are a rich source of BAFF (105) makes them the most likely source of the BAFF required to support the GC.

A second explanation is that the maturity of the B cells entering the GC may impact on FDC maturation as this process is known to require interaction with GC B cells and those lacking signals from BAFF may be too immature to provide these signals to the FDCs. Indeed some B cells in A/WySnJ are able to mature past the transitional stages in contrast to BAFF- or BAFFR-deficient mice (106), thus the greater maturity of the these cells may explain their ability to support maturation of the FDC reticulum, where the B cells from BAFF-deficient mice cannot. The increased maturity of B cells in A/WySnJ mice may be due to the ability of the mutant BAFFR to retain some signaling, as only the last 8 amino acids of this mutant receptor have been replaced with an unrelated transposon sequence (69). An examination of GC formation in BAFFR-deficient mice would help to answer this question and indeed like BAFF-deficient and A/WySnJ mice GCs in BAFFR-deficient mice form, but fail to be maintained (67), However,

whether this is due to lack of FDC maturation or a B cell proliferation defect has unfortunately not been investigated. The authors did suggest that the defect they observed in BAFFR-deficient mice was less severe than that observed in BAFF-deficient mice. If this is the case it does suggest that BCMA or TACI, while not playing any essential role in GC maintenance as indicated by the phenotypes of their respective knockout mice, may be able to compensate for some of the functions of BAFFR in its absence.

It is highly likely given the involvement of BAFFR and its strong activation of the NF-κB2 pathway, that NF-κB2 signaling is involved in GC maintenance. However this has been difficult to confirm as NF-κB2-deficient mice completely fail to form GCs and FDC networks suggesting the NF-κB2 is a key signaling pathway involved in the initiation of GCs as well as their maintenance (77, 78). Neither BAFF nor BAFFR are involved in initial GC formation, suggesting that in this case the NF-κB2 pathway is being activated through a different ligand, possibly lymphotoxin αβ. Of course without formation of GC it is impossible to assess their maintenance. Adoptive transfer experiments in RelB-deficient mice showed that in order to restore GC formation, RelB was required in radioresistant stromal cells rather than hematopoietic cells (107). However, as GCs were only examined on day 10 post-immunization, it is impossible to say whether GC maintenance was also defective when RelB was absence from hematopoietic cells. In alymphoplasia (*aly* – NIK mutant) mice, similar adoptive transfer experiments revealed that functional NIK was indeed required in the stromal cells for FDC formation, but was also required in hematopoietic cells for GC formation (108). These apparently conflicting results as to the requirement for NF-κB2 signaling in hematopoietic cells may be explained by functional redundancy of RelB with another member of the NF-κB family, whereas NIK, the central kinase of the NF-κB2 pathway is absolutely required. While these results attempt to identify the cell types in which NF-κB2 signaling is required, it remains difficult to link its usage with the activating receptor, as indeed it is likely that more than one receptor contributes at various stages in GC formation and maintenance. Thus the questions surrounding GC maintenance remain.

Whilst normal NF-κB2 appears necessary for GC formation, even hyperactive NF-κB2 cannot rescue GC formation in the absence of other signals required, for example T cell help in the form of CD40-CD40L interactions. The adapter molecules TRAF2, TRAF3, and cIAP1/2 are all involved in CD40 signaling in addition to their regulation of BAFFR signaling. While indeed they maintain their role as gatekeepers of NF-κB2 signaling, almost certainly through analogous activation methods as BAFFR, they are also variously involved in the JNK, MAPK, and NF-κB1 pathways. Generally TRAF2 and cIAP1/2 are positive mediators of these pathways, whereas TRAF3 is a negative regulator (40, 98, 109). Thus in mice lacking these molecules in their B cells, TRAF3-deficient B cells are able to form and maintain GCs as they retain competent CD40 signaling, whereas GCs are stunted or almost completely absent in the TRAF2 and cIAP1/cIAP2-deficient mice, due to a lack of CD40 signaling, despite high NF-κB2 signaling (98). Likewise over expression of BAFF and thus NF-κB2 in BAFF transgenic mice is unable to rescue GC formation in CD40-deficient mice (110). These results emphasize the complex nature of the signaling required in order to establish and maintain GC reactions. BAFF/BAFFR signaling contributes to these processes, but it is not a master regulator of them, as it is with B cell survival and maturation.

T-independent antibody responses

The involvement of both TACI and BAFF in T-independent antibody responses seems to be quite clear [reviewed in Ref. (111)]. It is possible that the multimeric BAFF 60mer is the ligand responsible for these functions (112). However, as TACI is not a strong inducer of NF-κB2 either via its interaction with BAFF or APRIL (84), it is unlikely that NF-κB2 signaling plays a role in these processes. For some T-independent antigens the BAFFR, and thus possibly NF-κB2 signaling, do appear to be involved. Antibody titers were lower in BAFFR-deficient mice compared to wild-type mice in response to NP-Ficoll or TNF-Ficoll but similar in response to Pneumovax vaccine (66, 67), suggesting that certain specific responses may require BAFFR signaling. In addition, some human patients with BAFFR deficiency also showed defects in mounting T-independent responses (113). However it remains unclear if BAFF/BAFFR signaling is directly required during a T-independent antibody response. It is possible that the defects observed in the absence of BAFFR, may be ascribed at least in part to a dramatic decrease in MZ B cells as this subset is recognized to be the origin of the early responders to T-independent antigens (114).

PLASMA CELL MAINTENANCE

Plasma cells are highly differentiated B cells capable of secreting large amounts of antibody and, along with the production of memory B cells, are the main B cell outcome of an immune response. Whilst large numbers of antigen specific PCs exist during and immediately following an immune response, over time these numbers greatly reduce. However, it is thought that a small number of the PCs resulting from a particular immune response survive long term and are the source of "basal" immunoglobulin found circulating within the body. The bone marrow has been identified as a survival niche for these long lived PCs and a variety of cells including stromal cells, myeloid cells and granulocytes all contribute to the production of factors which attract and promote the survival of PCs in these sites [reviewed in Ref. (115)]. Among these survival factors are APRIL and BAFF. Neutralization of both ligands is required in order to ablate plasma cell survival in the bone marrow (19, 116), however it is likely that under physiological conditions APRIL plays a greater role than BAFF. This is supported by the findings that APRIL is better than BAFF at supporting plasma cell survival *in vitro* (117) and *in vivo* plasma cell survival in APRIL-deficient mice was greatly diminished, whereas it was normal in BAFF-deficient mice (117, 118). BCMA is the receptor which is most thought to contribute to plasma cell survival, with BCMA-deficient mice failing to sustain long lived PCs in the bone marrow (119). It is possible, however, that TACI and/or BAFFR may contribute to these responses.

Although BCMA is not thought to be a strong activator of NF-κB2 signaling, there is evidence that NF-κB2 signaling can contribute to plasma cell survival, at least in the disease state. Multiple myeloma is late stage B cell malignancy that arises from PCs in the bone marrow. Whilst the primary genetic lesions are immunoglobulin gene translocations and hyperdiploidy, a large number of

secondary genetic mutations also characterize the progression of the disease and it is amongst these that the NF-κB pathways have been strongly implicated (120–122). Mutations that activate NF-κB2 signaling including inactivating mutation in *TRAF2*, *TRAF3*, *cIAP1*, and *cIAP2*, as well as activating mutations or duplications of *NIK* and *NF-κB2* have been identified in patients with multiple myeloma. These mutations are thought to contribute to the ability of the tumor to become independent in terms of its survival from the bone marrow microenvironment, that is no longer requiring BAFF and APRIL and other survival factors produced in the bone marrow. This in turn implies that NF-κB2 signaling is either normally involved in these processes for non-malignant PCs, or at least it can compensate for the signaling pathways involved under non-cancerous conditions. Thus it seems that BAFF induced NF-κB2 signaling plays a role at almost every stage of a B cell's life (**Figure 2**).

CONCLUSION

In addition to multiple myeloma, BAFF signaling as been implicated in a variety of autoimmune disorders, B cell malignancies and immunodeficiency disorders. It is also emerging that BAFF plays a role in regulating immune responses to infections [reviewed in Ref. (123)]. While in many of these cases there is quite strong evidence that BAFF contributes to disease, further molecular details, such as whether BAFFR is the responsible receptor and whether activation of NF-κB2 also contributes to disease, require additional investigation. Given the central role of BAFF mediated NF-κB2 activation in the life span of B cells and the importance of B cells in attempting to control (in the case of infection) or potentially contributing to (in the case of autoimmunity) disease, it is likely that this signaling pathway indeed impacts greatly on our state of health and disease.

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CD40-mediated activation of the NF-κB2 pathway

Bruce S. Hostager [1,2] **** and Gail A. Bishop*** [2,3,4,5] ******

[1] *Department of Pediatrics, The University of Iowa, Iowa City, IA, USA*
[2] *The Graduate Program in Immunology, The University of Iowa, Iowa City, IA, USA*
[3] *Department of Microbiology, The University of Iowa, Iowa City, IA, USA*
[4] *Department of Internal Medicine, The University of Iowa, Iowa City, IA, USA*
[5] *Iowa City VA Medical Center, Iowa City, IA, USA*

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Fulvio D'Acquisto, Queen Mary University of London, UK
Ellen D. Cahir-McFarland, Biogen Idec, Inc., USA

****Correspondence:***
Bruce S. Hostager, Department of Pediatrics, The University of Iowa, Iowa City, IA 52242, USA
e-mail: bruce-hostager@uiowa.edu;
Gail A. Bishop, Department of Microbiology, The University of Iowa, Iowa City, IA 52242, USA
e-mail: gail-bishop@uiowa.edu

CD40 is a critical stimulatory receptor on antigen-presenting cells of the immune system. CD40-mediated activation of B cells is particularly important for normal humoral immune function. Engagement of CD40 by its ligand, CD154, on the surface of activated T cells initiates a variety of signals in B cells including the activation of MAP kinases and NF-κB. The transcriptional regulator NF-κB is in reality a family of factors that can promote B cell activation, differentiation, and proliferation. Complex – and only partially understood – biochemical mechanisms allow CD40 to trigger two distinct NF-κB activation pathways resulting in the activation of canonical (NF-κB1) and non-canonical (NF-κB2) NF-κB. This brief review provides a summary of mechanisms responsible for activation of the latter, which appears to be particularly important for enhancing the viability of B cells at various stages in their life cycle and may also contribute to the development of B cell malignancies. CD40 is also expressed by various cell types in addition to B cells, including T cells, macrophages, dendritic cells, as well as certain non-hematopoietic cells. Here too, while perhaps less extensively studied than in B cells, the CD40-mediated activation of NF-κB2 also appears to have important roles in cellular physiology.

Keywords: CD40, NF-κB, signal transduction

The transcriptional regulator NF-κB participates in many important activation events in B cells, including B cell proliferation and differentiation in response to signaling by tumor necrosis factor receptor (TNFR) family members or toll-like receptor (TLR) proteins (1). NF-κB is not a single transcription factor, but rather a family of factors composed of homo- and hetero-dimers of p50, p52, c-Rel, RelA (p65), and RelB. Activation of these dimer pairs occurs via two general mechanisms, sometimes referred to as the canonical and non-canonical pathways. Canonical (NF-κB1) activation is typically rapid as it does not usually require new protein synthesis. Activation of this pathway is mediated largely by degradation of inhibitors present in the cytoplasm, which allows transit of NF-κB dimers (typically p50/RelA or p50/c-Rel heterodimers) into the nucleus. In contrast, activation of non-canonical NF-κB (NF-κB2) often occurs after activation of the canonical pathway, may require new protein synthesis, and ultimately results in the nuclear localization of predominantly p52/RelB heterodimers, although p52/p65 and p52/c-Rel hetero-dimers have also been described (2). Excellent recent reviews discuss current general models of NF-κB2 activation (2–5). Briefly, the activation of the NF-κB2 pathway is largely regulated by the production and posttranslational processing of its precursor, p100. Its proteolytic processing is regulated by IκB kinase α (IKKα), which is in turn regulated by NF-κB-inducing kinase (NIK).

CD40-MEDIATED NF-κB2 ACTIVATION IN B CELLS

CD40 signaling in B cells can modulate the activation of NF-κB2 at several points. First, CD40 signaling strongly activates NF-κB1 in B cells. This activation leads to the enhanced production of p100 (4). In addition, CD40 can regulate the posttranslational processing of p100 by regulating the activity of NIK (6). In resting cells, NIK activity is inhibited by a protein complex that includes TNFR-associated factor (TRAF)2, TRAF3, and cellular inhibitors of apoptosis (cIAP)1/2 (5). The major function of this complex appears to be in mediating the ubiquitination and degradation of NIK in resting B cells. Within the complex, TRAF3 appears to interact with NIK, while TRAF2 mediates interactions between TRAF3 and the cIAP molecules. Engagement of CD40 by its ligand leads to recruitment of TRAF proteins, including TRAFs 2 and 3, to the cytoplasmic domain of CD40. This event disrupts the NIK regulatory function of the TRAF2/TRAF3/cIAP complex, perhaps by promoting the ubiquitination, and degradation of TRAF3. With disruption of the NIK regulatory complex, NIK begins to accumulate (via new protein synthesis) in the cytoplasm to levels where it promotes the phosphorylation and activation of IKKα. In turn, IKKα activity mediates the phosphorylation of p100, which targets the protein for processing by the proteasome, resulting in p52 production (3–5).

A number of genetically modified mouse strains and cell lines have contributed to our understanding of NF-κB2 regulation by CD40 in B cells (7–11). TRAF2, TRAF3, and TRAF6 all significantly contribute to this CD40 signal transduction. Mouse B cell lines deficient in TRAF2 or TRAF6 exhibit little or no defect in CD40-mediated NF-κB2 activation (10). However, B cell lines doubly deficient in TRAF2 and TRAF6 appear defective in the activation of NF-κB2, suggesting that the two molecules have

overlapping functions in the activation of this pathway. Potentially, these results are explained by the ability of CD40, through TRAF2 and TRAF6, to activate the NF-κB1 pathway, which in turn activates p100 transcription. Primary B cells from TRAF2-deficient mice exhibit elevated basal NF-κB2 activation (not observed in the cell line studies), which is only weakly augmented by CD40 signaling (9), consistent with a role for TRAF2 in the NIK regulatory complex. In the cell line studies, TRAF2 deficiency did not appear to augment the basal level of p52 production (10), although this may have been due to limited production of p100 in non-activated cells. B cells from mice conditionally deficient in TRAF3 specifically in B cells also exhibit strong constitutive activation of the NF-κB2 pathway (7, 11), which is again consistent with the model in which TRAF3 is a major component of the NIK regulatory complex. While the phenotypes of TRAF2-, cIAP-, and TRAF3-deficient primary B cells are somewhat similar with respect to the constitutive activation of NF-κB2, there are instructive differences between the strains [reviewed in (5)]. CD40-mediated differentiation of B cells into germinal center B cells is augmented in TRAF3-deficient mice (7, 11), but TRAF2- or cIAP-deficient mice have a significant defect in germinal center B cell development (8). This likely reflects the role of TRAF2/cIAP in activation of the NF-κB1 pathway, which TRAF3 does not share.

Interestingly, in B cell-specific TRAF3-deficient mice, the elevated constitutive activation of NF-κB2 can be enhanced somewhat by CD40 stimulation (11), indicating the existence of TRAF3-independent mechanisms of NIK/NF-κB2 regulation. This possibility is further supported by the observation that CD40 signaling can activate NF-κB2 in TRAF3-deficient B cell lines reconstituted with a mutant TRAF3 molecule that binds NIK robustly, but is not degraded following CD40 stimulation (Lin et al., this issue). These observations demonstrate that the regulation of NF-κB2 by CD40 in B cells is only partially understood. Recently, the protein kinase TANK-binding kinase 1 (TBK1) was shown to negatively regulate NF-κB2 activation and IgA Ig isotype switching in primary B cells, potentially through phosphorylation/degradation of NIK (12). The de-ubiquitinating enzyme OTUD7B was also shown recently to potentially negatively regulate NF-κB2 (and primary B cell activation) by inhibiting activation-induced degradation of TRAF3 (13). Additionally, zinc finger protein 91 has recently been shown to promote NIK stability in a CD40-stimulated epithelial tumor cell line, perhaps through K63-linked polyubiquitination of NIK (14). Whether this mechanism is relevant to the CD40-mediated activation of B cells remains to be demonstrated. It is likely that additional regulatory mechanisms will come to light in the next few years, and will contribute to our understanding of normal B cell biology as well as the physiology of B cell malignancies.

CD40-MEDIATED NF-κB2 ACTIVATION IN NON-B CELLS

Compared to the extensive research performed in B cells, a cell type in which NF-κB activation is a major regulatory pathway, relatively little investigation has been performed on CD40-mediated NF-κB2 signaling in other cell types. Most studies of CD40 functions in non-B cells do not examine NF-kB activation. In those that do, canonical NF-κB1 activation is typically the focus, and/or methods used (reporter gene activation or electrophoretic mobility shift assay, EMSA) do not allow results to distinguish between NF-κB1 and NF-κB2 induction. We summarize below the information that is currently available on CD40-mediated induction of the NF-κB2 pathway in non-B cells.

OTHER HEMATOPOIETIC CELLS

T lymphocytes

The physiologic importance of direct signaling to T cells by CD40 has at times been a controversial topic (15). However, there is no doubt that normal activated $CD4^+$ and $CD8^+$ T cells can express CD40, and it appears to play significant biological roles in mouse models of T cell-dependent immune responses (16, 17), including autoimmune responses [reviewed in Ref. (15)]. However, the role of CD40-mediated NF-κB activation, and in particular NF-κB2 activation in these roles is unclear, and may be context-dependent. $CD3^+CD8^+CD40^+$ T cells in Balb/c mice exhibit cytotoxic activity toward $CD4^+CD25^+$ T regulatory cells during *Leishmania* infection – this activity is CD40-dependent, but unaffected by inhibitors of NF-κB (18). A $CD4^+$ mouse T cell line stably expressing transfected CD40 utilizes CD40 as an effective co-stimulatory signal with T cell receptor signals to activate T cell functions. In this model, both NF-κB1 and NF-κB2 pathways in T cells are activated by CD40 (19). The ultimate biological importance of NF-κB2 activation in T cell CD40 signaling remains to be explored.

Myeloid cells

Monocytes and macrophages, like B cells, constitutively express CD40, which activates mitogen-activated protein (MAP) kinases and NF-κB in these cells [reviewed in Ref. (20)]. However, very little is known about the potential involvement of the NF-κB2 pathway in macrophage CD40 signaling. Revy et al. performed a direct comparison between human monocytes and B cells activated through CD40. EMSA demonstrated that nuclear translocation of NF-κB binding activity is induced by CD40 in both cell types, and the p50 subunit involved in NF-κB1 activation is part of these nuclear complexes. Interestingly, the p65 subunit was only present in complexes from B cells (21). However, as the CD40 stimulation was only of short duration in these experiments (30 min); the activation of NF-κB2 was not assessed at later times.

CD40 also delivers potent and important signals to dendritic cells (DC) [reviewed in Ref. (22)]. Following signals from CD40 that stimulate the ability of DC to cross-present antigen to T cells, the nuclear translocation of p52 characteristic of NF-κB2 signaling is seen. Mice bearing a mutant NIK that disrupts NF-κB2 activation by various signals, the $NIK^{aly/aly}$ mouse, show DC functional defects (23). CD40 fails to induce cross presentation of antigens to CD8 T cells in the $NIK^{aly/aly}$ mouse, implicating NF-kB2 in this particular DC function. However, the evidence is indirect, as the NIK mutation is expressed in all cells of the mouse, and will impact multiple pathways of cell development and function. In another experimental model, human monocyte-derived DC treated with siRNA for NIK or IKKα are unable to produce the cytokines IDO and IL-6 following *in vitro* stimulation with the CD40 ligand, CD154. This also renders the treated DC unable to promote the

induction of T regulatory cells (24). Thus, NF-κB2 signaling plays important roles in CD40 functions in DC.

NON-HEMATOPOIETIC CELLS

Epithelial cells

CD40 was first described as an antigen expressed on the surface of bladder carcinoma cells (25), and can be expressed on a variety of types of epithelial cells under particular circumstances [reviewed in (26, Lin et al., this issue)]. Unfortunately, by far the most commonly used cells of epithelial origin used to study CD40 signaling are the transformed cell lines HEK 293 and HeLa. In the majority of studies using these cell lines, CD40 and/or the signaling proteins studied are exogenously expressed at highly non-physiologic levels. Thus, because such models can provide misleading results [recently reviewed in (27)], we will not discuss this body of work in this article. Very little is known about the usage of the NF-κB2 pathway by endogenous CD40 expressed by normal epithelial cells. An interesting report using airway epithelial cells, which produce the cytokine RANTES upon CD40 stimulation, examined NF-κB activation by EMSA. Nuclear binding complexes for probes with sites characteristic of both NF-κB1 and NF-κB2 were identified, with the former peaking at 30 min of stimulation, and the latter at 2 h. The p65 subunit was present in both types of complexes (28). The functional importance of this NF-κB2 activation remains to be defined.

Fibroblasts

Although CD40 can be expressed by fibroblasts, no information is currently available on the extent or importance of NF-κB2 activation by CD40 in this cell type. A recent report made the interesting observation that TWEAK, a molecule implicated in various fibrotic processes [reviewed in (29)] inhibits CD40 signaling in the HeLa cell line (30). However, neither fibroblasts nor NF-κB2 pathways were examined in this study.

Vascular endothelium

It is well-known that CD40 can be expressed on activated endothelium, and its interaction with CD154 has been implicated in the pathogenesis of atherosclerosis [reviewed in Ref. (31)]. A specific role for NF-κB2 activation has yet to be explored in this CD40 function. In human vascular endothelial cells, stimulation of CD40 with soluble CD154 stimulates the phosphorylation and nuclear translocation of the p65 subunit of NF-κB at 6 h (32). However, it is unclear whether this represents activation of the NF-κB2 pathway.

While it is clear that the mechanisms of CD40-mediated NF-κB activation are complicated (and still incompletely characterized), the consequences of NF-κB activation in B cells and other cells are orders of magnitude more complex. Many of the genes regulated by NF-κB have been identified by experiment and by computer analysis, but the work of understanding the subsequent roles of these genes and their interactions in cellular physiology is only in its infancy. Nevertheless, continued characterization of NF-κB activation mechanisms and consequences of NF-κB-regulated transcription will lead to a more complete understanding of basic immune system biology as well as the identification of molecular events and interactions that can be exploited in the treatment of immune disorders and cancer.

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A complex relationship between TRAF3 and non-canonical NF-κB2 activation in B lymphocytes

Wai W. Lin[1], Joanne M. Hildebrand[2†] and Gail A. Bishop[1,2,3,4]*

[1] *Graduate Program in Immunology, University of Iowa, Iowa City, IA, USA*
[2] *Department of Microbiology, University of Iowa, Iowa City, IA, USA*
[3] *Department of Internal Medicine, University of Iowa, Iowa City, IA, USA*
[4] *VA Medical Center, Iowa City, IA, USA*

Edited by:
John Silke, The Walter and Eliza Hall Institute, Australia

Reviewed by:
Christine Loh, Biogen Idec Inc., USA
John Silke, The Walter and Eliza Hall Institute, Australia

****Correspondence:***
Gail A. Bishop, Department of Microbiology, University of Iowa, 375 Newton Road, 2193 MERF, Iowa City, IA 52242, USA
e-mail: gail-bishop@uiowa.edu

*†**Present address:***
Joanne M. Hildebrand, The Walter and Eliza Hall Institute for Medical Research, Parkville, Australia

The adaptor protein TRAF3 restrains B cell activating factor receptor (BAFFR) and CD40-mediated activation of the NF-κB2 pathway in B cells. Mice lacking TRAF3 specifically in B cells revealed the critical role of TRAF3 in restraining homeostatic B cell survival. Furthermore, loss-of-function mutations of the *traf3* gene have been associated with human B cell malignancies, especially multiple myeloma (MM). It has been proposed that receptor-induced TRAF3 degradation leads to stabilization of the NF-κB inducing kinase (NIK), and subsequent NF-κB2 activation. However, it is unclear how receptor-mediated TRAF3 degradation or loss-of-function contributes to B cell-specific NF-κB2 activation. In the current study, we employed two complementary models to address this question. One utilized a mutant *traf3* gene found in a human MM-derived cell line called LP1. The LP1 mutant TRAF3 protein lacks the TRAF-N and TRAF-C domains. Consistent with the paradigm described, expression of LP1 TRAF3 in B cells promoted higher basal levels of NF-κB2 activation compared to Wt TRAF3. However, LP1 did not associate with TRAF2, CD40, or BAFFR, and no LP1 degradation was observed following receptor engagement. Interestingly, LP1 showed enhanced NIK association. Thus, TRAF3 degradation becomes dispensable to activate NF-κB2 when it is unable to associate with TRAF2. In a second model, we examined several mutant forms of BAFFR that are unable to induce NF-κB2 activation in B cells. Signaling to B cells by each of these BAFFR mutants, however, induced levels of TRAF3 degradation similar to those induced by Wt BAFFR. Thus, in B cells, receptor-mediated TRAF3 degradation is not sufficient to promote NF-κB2 activation. We thus conclude that there is not a simple linear relationship in B lymphocytes between relative levels of cellular TRAF3, induced TRAF3 degradation, NIK activation, and NF-κB2 activation.

Keywords: TRAF3, NF-κB, B cell, CD40, BAFF

INTRODUCTION

Tumor necrosis factor receptor (TNFR) associated factor 3 (TRAF3) is an important adaptor molecule shown to regulate NF-κB2 activation induced by the TNFR superfamily molecules CD40 and B cell activating factor receptor (BAFFR), as well as other immune receptors (1–3). In B lymphocytes, CD40 and BAFFR-mediated signaling is important for regulation of maturation, survival, Ig class switch recombination, and antibody production (4, 5). Engagement of BAFFR with BAFF or interaction between CD40 and its ligand CD154 triggers the non-canonical NF-κB or NF-κB2 pathway [reviewed in Ref. (6)]. TRAF3 negatively regulates NF-κB2 activation (7, 8), and mouse B cells deficient in TRAF3 have substantially enhanced survival, which correlates with constitutive activation of the NF-κB2 pathway (9, 10). Understanding how TRAF3 regulates both NF-κB2 activation and homeostatic B cell survival is thus of great importance.

The NF-κB2 pathway is tightly regulated at steady state, and dysregulation of the pathway is frequently observed in hematological malignancies (11, 12). Gain or loss-of-function mutations of genes encoding key signaling molecules implicated in the NF-κB2 pathway have been observed in such tumors. In particular, mutations in NF-κB inducing kinase (NIK), TRAF3, TRAF2, and cellular inhibitors of apoptosis 1 and 2 (cIAP1, cIAP2) are highly associated with human multiple myeloma (MM) and MM-derived cell lines. Loss-of-function mutations of the *traf3* gene have been identified in 9–17% of MM patient cohorts (13, 14). Such mutations have also been identified in different subtypes of B cell lymphoma and Waldenström's macroglobulinemia (15–17). Consistent with this, a portion of older mice deficient in TRAF3 specifically in B cells develop B cell lymphomas (18). Taken together, these studies provide strong evidence that loss or reduced expression of TRAF3 contributes to B cell malignancies.

A widely held paradigm suggests that the physical association between TRAF3, TRAF2, and NIK allows the TRAF2-cIAP E3 ubiquitin ligase complex to polyubiquitinate NIK (19, 20). This promotes proteasome-mediated degradation of NIK, and by so doing, restrains NF-κB2 activation (21). Engagement of CD40 or BAFFR on B cells is known to recruit TRAFs 2 and 3 to the plasma membrane lipid-raft compartment (22, 23). This recruitment initiates TRAF2-mediated polyubiquitination of both

TRAFs 2 and 3, and their subsequent proteasome-mediated degradation (22, 24, 25). The paradigm mentioned above posits that this receptor-mediated TRAF3 degradation allows NIK stabilization, and thus NF-κB2 activation (26, 27). However, it remains unclear whether TRAF3 degradation is necessary and sufficient for NF-κB2 activation, in response to CD40 and BAFFR signaling, in B lymphocytes. The present study was designed to address this important mechanistic question. Utilizing two complementary approaches, we found that association between TRAFs 2 and 3 is important for CD40 and BAFFR-mediated TRAF3 degradation. However, degradation of endogenous TRAF3 was neither sufficient nor always required to induce CD40 or BAFFR-mediated activation of NF-κB2 in B cells.

MATERIALS AND METHODS

MICE

A/J, BAFFR$^{-/-}$, and Bcl2 transgenic (tg) mice with A/J congenic backgrounds were kindly provided by Dr. Colleen Hayes (University of Wisconsin, Madison, WI, USA). A/WySnJ mice, also on the A/J genetic background, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). A/J, A/WySnJ, and BAFFR$^{-/-}$ mice were bred with Bcl2 tg mice to circumvent mature B cell developmental defects in A/WySnJ and BAFFR$^{-/-}$ mice (28, 29). All mice had one allele of the *Bcl2* transgene, and were used at 10–12 weeks of age as a source of B cells for experiments. Mice were maintained under pathogen-free conditions at the University of Iowa. Use of mice in this study was according to a protocol approved by The University of Iowa Animal Care and Use Committee.

CELL LINES

The mouse B cell lines M12.4.1 (30), CH12.LX, and its TRAF3-deficient subclones have been previously described (31, 32). B cell lines stably transfected with hybrid human CD40-mouse BAFFR constructs described below were maintained in B cell medium containing RPMI 1640 (Life Technology, Grand Island, NY, USA) with 10 μM 2-βmercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), 10% heat-inactivated FCS (Atlanta Biologicals, Atlanta, GA, USA), 2 mM L-Glutamine (Life Technologies), 100 U/ml of Penicillin Streptomycin antibiotics (Life Technologies) (BCM10). Medium additionally contained 400 μg/ml of G418 disulfate (Research Products International, Mount Prospect, IL, USA) for subclones expressing transfected hCD40-BAFFR constructs, and both G418 and 200 μg/ml of hygromycin (Life Technologies) for subclones expressing FLAG-tagged TRAF3 or the LP1 mutant TRAF3.

DNA CONSTRUCTS AND TRANSFECTIONS

Plasmids encoding a mutant BAFFR from the A/WySnJ mouse and a mouse BAFFR lacking the C-terminal eight amino acids (ΔC) were kindly provided by Drs. Colleen Hayes and Christopher Mayne. These plasmids were used as a source of BAFFR to produce cDNA encoding a chimeric molecule consisting of the extracellular portion of human CD40 and the transmembrane and intracellular portions of mouse Wt BAFFR, the BAFFR mutant of the A/WySnJ mouse, or mouse BAFFR lacking the C-terminal 8 amino acids (ΔC). These chimeric constructs were subcloned into the mammalian expression vector pRSV5.neo for stable expression in CH12.LX or M12.4.1 cells (33). cDNAs encoding FLAG-tagged human Wt TRAF3, or the truncated TRAF3 mutant identified in the human MM cell line LP1 (14) were subcloned into a variant of the pRSV5.neo plasmid with a sequence containing the binding element of the repressor of the bacterial Lac operon in the promoter, as previously described (33). This allows Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible, stable TRAF3 expression in a CH12.TRAF3$^{-/-}$ cell line that stably expresses the Lac repressor (32, 33). There is thus no endogenous TRAF3 in cells expressing Wt or LP1 TRAF3 in these studies.

ANTIBODIES AND REAGENTS

Isopropyl β-D-1-thiogalactopyranoside was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-phospho-jun kinase (JNK) Ab, rabbit anti-phospho-p38 Ab, rabbit anti-pIκBα Ab, rabbit anti-total IκBα Ab, rabbit anti-NIK Ab, and rabbit anti-p100/p52 Ab were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-TRAF2 Ab was purchased from Medical and Biological Laboratories (Woburn, MA, USA). Anti-FLAG M2 Ab for immunoprecipitation, anti-FLAG M2-HRP Ab for immunoblotting, and mouse anti β-actin Ab were purchased from Sigma. Rabbit anti-JNK Ab, mouse anti-YY1 (H-10) Ab, and rabbit anti-mouse CD40 (M-20) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Production of Hi Five (HiV) strain insect cells infected with Wt baculovirus or baculovirus encoding human CD154 or mouse CD154 is described in (34). Rat anti-mouse CD40 mAbs (1C10 and 4F11) were produced in our laboratory from their respective hybridomas as described (32), kindly provided by Dr. Frances Lund (University of Alabama, Birmingham, AL, USA). The isotype control rat total IgG Ab was purchased from Southern Biotech (Birmingham, AL, USA) and mouse IgG1 control Ab from eBioscience (San Diego, CA, USA). Goat anti-BAFFR Ab for immunoprecipitation was purchased from R&D Systems (Minneapolis, MN, USA), and rabbit anti-BAFFR Ab for immunoblotting was purchased from Abcam (Cambridge, MA, USA). HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG secondary Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

CELL ISOLATION AND ASSAYS TO MEASURE RECEPTOR SIGNALING AND TRAF3 DEGRADATION

Resting splenic B cells were obtained as previously described (9). Briefly, high density splenic B cells were isolated by centrifugation through a Percoll density gradient (GE Life Sciences, Pittsburg, PA, USA) followed by anti-CD43 Ab-mediated negative selection, using a magnetic bead kit (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's protocol. 5×10^6 splenic B cells or 2×10^6 CH12.LX or M12.4.1 cells were stimulated as indicated in the Figure legends. After stimulation, cells were lysed and cytosolic and nuclear extracts were prepared as described in Xie et al. (35) to detect NF-κB2 activation via nuclear translocation of p52. For proximal signaling assays and TRAF3 degradation assays, 1–2 $\times 10^6$ cells were stimulated for indicated times at 37°C. After stimulation, cells were lysed in 100 μl 2× SDS-PAGE loading buffer (1% SDS, 2% β-mercaptoethanol, 62.5 mM Tris, pH 6.8). Lysates were sonicated using a Branson Sonifier 250 (VWR International, Radnor, PA, USA) with 10 pulses at 90% duty cycle, output 1.5. Samples were boiled for 10 min at 95°C prior to gel loading.

IMMUNOPRECIPITATION

CH12.TRAF3$^{-/-}$ cells expressing LacR and stably transfected with inducible FLAG-tagged Wt or LP1 mutant TRAF3 were cultured with BCM10 containing 100 μM IPTG overnight to induce TRAF3 expression. 2×10^7 cells were resuspended in 1 ml BCM10 and stimulated with 10 μg/ml of hamster anti-mouse CD40 mAb (HM40.3, eBioscience) or 500 ng/ml of recombinant BAFF (Peprotech, Rocky Hill, NJ, USA) for the indicated times. Cells were pelleted and lysed in lysis buffer (0.5% Triton X, 40 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing complete protease inhibitor cocktail (Roche, Germany), 0.05 mg of DNAse I (Roche), and 2 mM Na_3VO_4. Whole cell lyses were incubated with mouse anti-FLAG Ab (Sigma) or control IgG1 Ab overnight at 4°C with constant agitation. The immune complex was precipitated with Dyna Protein G beads (Life Technologies), washed and resuspended in SDS-PAGE loading buffer, and heated to 95°C for 10 min.

IMMUNOPRECIPITATION FROM DETERGENT-INSOLUBLE PLASMA MEMBRANE FRACTIONS

3×10^7 CH12.LX cells were stimulated at 37°C with 500 ng/ml recombinant BAFF (Peprotech) or 3×10^6 HiV insect cells infected with Wt baculovirus, or baculovirus expressing mouse CD154 (34). These insect cells, which grow at 25°C, die and serve as a source of CD154-expressing membranes at 37°C. Cells were lysed using buffer containing 1% Brij58 (Thermo Scientific, Rockford, IL, USA), 150 mM NaCl, 20 mM Tris, 50 mM β-glycerophosphate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, protease inhibitor cocktail, and 2 mM Na_3VO_4. The Brij58-insoluble cell fractions were separated by centrifugation at $14{,}000 \times g$ for 30 min at 4°C and solubilized using 1% Triton X-100 and 0.1% SDS-containing buffer. This solubilized cholesterol-rich membrane fraction (22) was subjected to immunoprecipitation using rat anti-mouse CD40 mAbs (an equal mixture of the clones 1C10 and 4F11) (36), goat polyclonal anti-BAFFR Ab, or appropriate isotype control antibodies. To immunoprecipitate the receptor signaling complex, Protein G Dyna beads, pre-incubated with goat anti-rat Ab (Jackson) for 30 min at room temperature, or uncoupled beads were incubated with solubilized membrane fractions for 2 h at 4°C with constant agitation. After washing, the immunoprecipitate was deglycosylated using PNGaseF (NEB) and boiled in SDS-PAGE loading buffer.

IMMUNOPRECIPITATION OF HCD40-BAFFR CHIMERIC MOLECULES

3×10^7 cells CH12.LX cells that stably express hCD40-BAFFR (Wt), hCD40-A/WySnJ (A/WySnJ), hCD40-ΔC, or hCD40-BAFFR chimeric molecules with a mutated TRAF3 binding site (AVAAA) were stimulated with Protein G Dyna Beads (Life Technologies) conjugated with agonistic mouse anti-hCD40 mAb or isotype control Ab for the indicated time points at 37°C. Abs were crosslinked to the beads using DSS crosslinker (Thermo Scientific) according to the manufacturer's recommended protocol. Immunoprecipitation of hCD40-BAFFR chimeric molecules was performed as previously described (37).

WESTERN BLOTS

Proteins were resolved on SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk in TBST (120 mM NaCl, 0.08% Tween 20, and 40 mM Tris) for 1 h at room temperature, and incubated overnight at 4°C in primary Ab. Blots were washed in TBST, incubated with secondary Abs for 2 h at room temperature and developed using Supersignal West Pico (Thermo Scientific). Western blot chemiluminescence was read with an LAS-4000 low-light camera and analyzed with Multi Gage software (Fujifilm Life Science, Edison, NJ, USA).

STATISTICAL ANALYSIS

p-Values were generated by Student's *t*-test (unpaired, two-tailed, at 95% confidence interval).

RESULTS

RECEPTOR-INDUCED DEGRADATION OF Wt VS. LP1 TRAF3

We and others previously reported that B cell-specific loss of the *traf3* gene enhances the survival of B cells independent of cell proliferation (9, 10). We also demonstrated that this enhanced B cell-specific survival renders B cells independent of the soluble factor BAFF, and results in constitutive activation of the non-canonical NF-κB2 pathway (9). At the same time, several groups reported that loss-of-function mutations in the *traf3* gene are associated with human MM and enhanced NF-κB2 activity (13, 14). These studies identified various forms of TRAF3 mutations, from point mutations leading to single amino acid substitutions to large truncations. These mutations of *traf3* are highly correlated with frequent chromosomal translocations of chromosome 14, commonly seen in MM (13, 14). Among these is a TRAF3 mutation identified in the human MM LP1 cell line. The LP1 TRAF3 missense mutation results in production of a truncated TRAF3 lacking the TRAF-N and TRAF-C domains (**Figure 1A**), which we predict would fail to associate with TRAF2 (19) and thus not undergo receptor-mediated degradation.

To test our prediction that the LP1 TRAF3 mutant fails to undergo receptor-mediated degradation, we established CH12.TRAF3$^{-/-}$ subclones with stable, IPTG-inducible expression of FLAG-tagged Wt or LP1 TRAF3. Cells were stimulated via CD40 or BAFFR, known strong activators of TRAF2-dependent TRAF3 degradation (22, 24, 25). Based on the results shown in **Figures 1B,C**, LP1 was only minimally degraded after stimulation with CD40 or BAFFR, compared to Wt TRAF3.

RECEPTOR-INDUCED NF-κB2 AND JNK ACTIVATION IN LP1-EXPRESSING CELLS

A widely held paradigm is that TRAF3 degradation induced by CD40 or BAFFR ligation allows NIK stabilization, which then activates the NF-κB2 pathway (20, 26, 27). This paradigm predicts that TRAF3$^{-/-}$ cells expressing LP1 would fail to undergo receptor-induced NF-κB2 activation, as LP1 is not able to undergo receptor-induced degradation. To investigate whether LP1 is inducing NF-κB2 activation independent of receptor-induced degradation, we provided CD40 or BAFF stimuli to CH12.TRAF3$^{-/-}$ B cells induced to express either Wt or LP1 TRAF3. Consistent with TRAF3's proposed role in NF-κB2 activation, induction of Wt TRAF3 expression reduced nuclear p52 from its elevated levels in TRAF3$^{-/-}$ B cells. Because the LP1 TRAF3 mutant is a loss-of-function mutation, we predicted that LP1 TRAF3-expressing

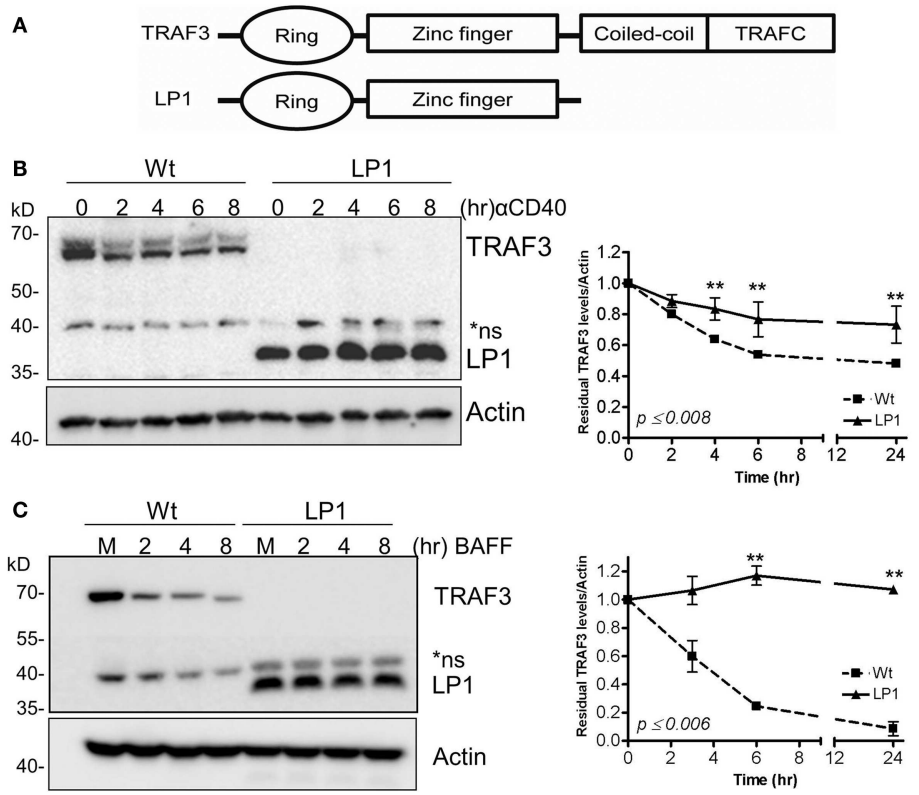

FIGURE 1 | Dissociation between CD40 and BAFF-mediated NF-κB2 activation and TRAF3 degradation. **(A)** Schematic presentation of Wt and LP1 mutant TRAF3. **(B,C)** CH12.TRAF3$^{-/-}$ cells stably transfected for inducible expression of FLAG-tagged Wt or LP1 mutant TRAF3 were treated overnight with IPTG to induce TRAF3 expression, as described in Section "Materials and Methods." Induced cells were either untreated (M) or treated with isotype control Ab (C) or stimulated with 5 μg/ml of anti-CD40 mAb **(B)** or 200 ng/ml of recombinant BAFF **(C)** for the indicated times at 37°C. Whole cell lysates were resolved by SDS-PAGE and Western blots of gel probed with anti-FLAG Ab to assess relative cellular levels of Wt and LP1 TRAF3. Graphs depict mean values ± SEM of TRAF3 band intensities, normalized to actin, from immunoblots of three experiments. **Statistically significant differences between Wt and LP1 TRAF3, analyzed as in Section "Materials and Methods." Numbers on the graph indicate the highest *p* values for individual time points.

B cells will have constitutive nuclear p52 and there will be no further activation of the NF-κB2 pathway upon stimulation. Interestingly, however, induction of LP1 expression in TRAF3$^{-/-}$ B cells also reduced nuclear p52 levels (**Figures 2A,B**). Induction of LP1 expression reduced basal nuclear p52 levels in TRAF3$^{-/-}$ B cells. However, p52 levels remained ~twofold higher in LP1 compared to Wt TRAF3-expressing cells (**Figures 2A,B**), which is consistent with previous observations in myeloma cells and fibroblasts that the presence of LP1 TRAF3 mutant led to higher p52 levels (8, 14). Surprisingly, unlike TRAF3-deficient B cells, which further activate NF-κB2 in response to CD40 but not BAFFR stimulation (9), LP1-expressing B cells responded to both CD40 and BAFF stimulation with further nuclear p52 increases (**Figures 2A,B**). These data suggested that the TRAF3 mutant LP1 partially restrains NF-κB2 activation, and upon stimulation activates the NF-κB2 pathway independent of receptor-mediated LP1 degradation. Collectively, published studies and results presented here lead us to propose that induction of NF-κB2 activation in B cells does not solely depend on the absence of TRAF3, whether TRAF3 reduction is caused by receptor-induced degradation, mutation, or gene-targeted deletion.

LP1 expression also resulted in enhanced c-JNK activation following BAFF stimulation (**Figure 2D**), and sustained and significantly enhanced JNK activation in response to CD40 signals (**Figure 2C**). In contrast, neither phosphorylation of the mitogen-activated kinase (MAPK) p38 nor activation of classical NF-κB1 was impacted by the LP1 mutation compared to Wt TRAF3 (**Figure 3**). These data indicate that the C-terminal domains of TRAF3 are important for restraining activation of the JNK pathway. Consistent with our prior report that complete loss of TRAF3 does not impact CD40-mediated NF-κB1 activation (9), LP1 expression did not result in enhanced NF-κB1 or p38 activation in response to CD40 or BAFFR signals. Canonical NF-κB1 activation was unaffected by LP1 TRAF3 expression, indicating that increased nuclear p52 levels in LP1-expressing cells were not due to NF-κB1-induced enhanced p100 expression.

ASSOCIATION OF TRAF3 WITH KEY SIGNALING COMPONENTS IN NF-κB2 ACTIVATION

TRAF3 is typically recruited to the cytoplasmic domains of CD40 and BAFFR, together with TRAFs, to mediate downstream signaling and biological functions (22, 23, 38). Recruitment to receptor

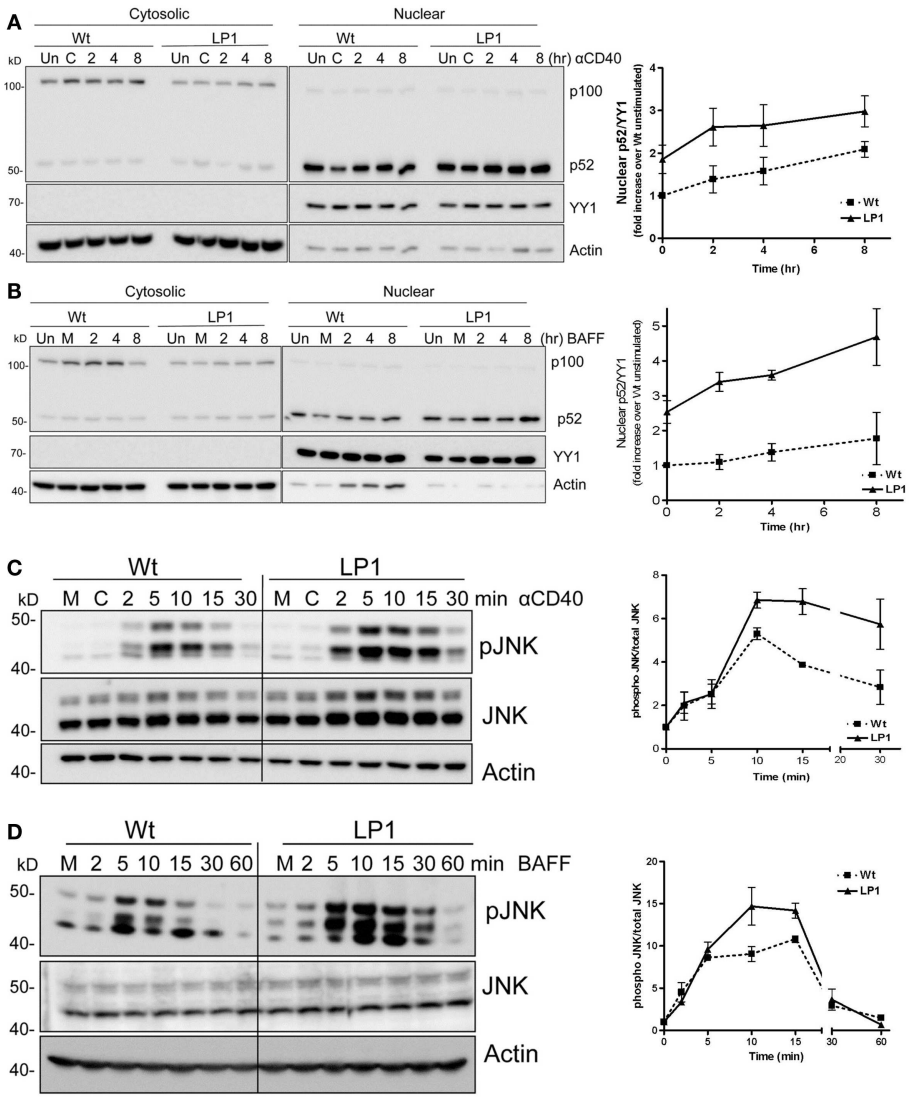

FIGURE 2 | Impact of TRAF3 on CD40 and BAFFR-mediated NF-κB2 and JNK activation. CH12.TRAF3$^{-/-}$ cells were induced to express Wt or mutant LP1 TRAF3 as in **Figure 1**. **(A,B)** Cells were untreated (M) or treated with isotype control Ab (C) or stimulated with 5 μg/ml of anti-mouse CD40 mAb (HM40.3) **(A)** or 200 ng/ml of recombinant BAFF **(B)** for the indicated times at 37°C. Uninduced cells were also included as control (Un). Cytosolic and nuclear extracts of cell lysates were prepared and immunoblotted for the NF-κB2 precursor protein p100 and its cleavage product p52, with probes for actin and YY1 serving as loading controls for cytoplasmic and nuclear protein levels respectively. YY1 levels were used to normalize nuclear p52 values shown quantitatively in graphs. Representative blots from cells stimulated via CD40 **(A)** or BAFFR **(B)** are shown. Graphs in **(A,B)** show mean values ± SEM of fold increases in normalized band intensities of nuclear p52 from five (CD40) or four (BAFF) independent experiments. Values from LP1-expressing cells were divided by values from Wt TRAF3-expressing unstimulated cells. **(C,D)** 1×10^6 cells were stimulated with 10 μg/ml of anti-mouse CD40 mAb or 500 ng/ml of BAFF for the indicated times. Whole cell lysates were prepared, and Western blots probed for phosphorylated JNK (pJNK), total JNK (JNK), and actin. Representative blots are presented. The corresponding graphs represent mean values for band intensities of pJNK divided by total JNK ± SEM from two (BAFF) or three (CD40) independent experiments.

cytoplasmic domains and association with TRAF2 and/or cIAP1/2 are thought to be required for K48-mediated ubiquitination, followed by proteasomal degradation of TRAF3 after receptor engagement (24, 25, 39, 40). To investigate the reason for the lack of receptor-mediated LP1 degradation, we assessed whether LP1 can associate with TRAF2. CH12.TRAF3$^{-/-}$ B cells induced to express Wt or LP1 TRAF3 were stimulated through CD40, and Wt or LP1 TRAF3 was immunoprecipitated from cell lysates. Consistent with a previous report (19), the C-terminal TRAF-N and TRAF-C domains of TRAF3 were required for TRAF3 to associate with TRAF2, with or without CD40 stimulation (**Figure 4A**). Similar results were obtained with BAFF stimulation (data not shown).

To assess whether LP1 associated with either CD40 or BAFFR after stimulation, we isolated the lipid-raft enriched detergent-insoluble plasma membrane fractions of cells as described in Section "Materials and Methods," and immunoprecipitated the

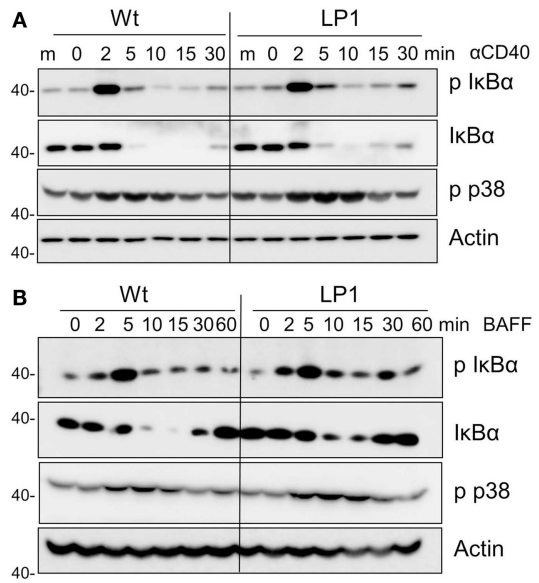

FIGURE 3 | Impact of TRAF3 on CD40 and BAFFR-mediated NF-κB1 and p38 MAP kinase activation. CH12.TRAF3$^{-/-}$ cells were induced to express Wt or mutant LP1 TRAF3 as in **Figure 1**. Cells were stimulated as described in **Figures 2C,D** via CD40 **(A)** or BAFFR **(B)**, and immunoblots of whole cell lysate samples were probed for phosphorylated IκBα (p IκBα), total IκBα, phospho p38 (p-p38), and actin (used as a control for p-p38 because antibodies to total p38 also recognize the phosphorylated form). Representative blots from three independent experiments are shown.

receptor signaling complex. **Figures 4B,C** reveal that LP1 was not part of the CD40 or BAFFR signaling complexes, but Wt TRAF3 associated with the receptors upon stimulation. These data suggest that the observed lack of receptor-induced LP1 degradation reflects a requirement for recruitment of TRAF3 to the receptor cytoplasmic domains and association with TRAF2, to initiate the degradation process.

Strikingly, despite lack of receptor-mediated degradation of LP1 TRAF3, LP1-expressing B cells were capable of inducing NF-κB2 activation above the already high basal levels following receptor stimulation. To address this discrepancy with the prevailing paradigm, we investigated whether LP1 associates with NIK, a MAP kinase implicated as key to NF-κB2 activation (21). A previous report showed that TRAF3 physically interacts with NIK when both molecules are overexpressed in transformed epithelial cells, and this interaction requires the C-terminal residues from 424 to 543 of TRAF3 (20). The interaction between TRAF3 and NIK is proposed to allow the TRAF2 E3 ubiquitin ligase activity to constantly ubiquitinate NIK for proteosomal degradation (20, 26, 27). Hence, although LP1 was not degraded after receptor stimulation (**Figure 1**), if NIK fails to associate with LP1, this could stabilize NIK in LP1-expressing cells, ultimately leading to enhanced NF-κB2 activation. However, immunoprecipitated LP1 TRAF3 from unstimulated or BAFF-activated B cells displayed 10–16 fold *increased* association with NIK, compared to Wt TRAF3 (**Figure 5**). Notably, there was no drastic difference in total NIK accumulation in LP1 vs. Wt TRAF3-expressing B cells. It is important to note that the function of TRAF3 is highly context-dependent; TRAF3 plays very different roles with regards to distinct receptors and cell types [reviewed in Ref. (2)]. Thus, the discrepancy between the present findings and previous conclusions based upon experiments performed in epithelial cell lines (20) are likely due to these contextual differences. Collectively, the present results suggest that when tumor-derived mutant TRAF3 becomes resistant to receptor-mediated degradation, stabilization of cellular NIK levels is controlled by TRAF3 degradation-independent mechanisms to regulate NF-κB2 activation in B cells.

DEFECTIVE NF-κB2 ACTIVATION VIA THE MUTANT BAFFR OF A/WySnJ B CELLS

B cell activating factor receptor provides an important B cell survival signal, thought to require activation of the non-canonical NF-κB2 pathway (5). As a complementary experimental model to further examine the importance of receptor-mediated TRAF3 degradation for NF-κB2 activation in B cells, we isolated B cells from the A/WySnJ mouse. This mouse contains a spontaneous mutation in the *Baffr* gene called *Bcmd1* (B cell maturation defect-1) that leads to defects in survival and maturation of peripheral B cells (41). *Bcmd1* encodes a protein that shares ≥95% sequence homology with Wt BAFFR. Notably, the TRAF3 binding motif PVPAT remains intact in this mutant BAFFR (**Figure 6A**) (42). However, the last eight amino acids of Wt BAFFR are replaced by a 22-amino acid peptide sequence resulting from a proviral Intracisternal A-particle (IAP) retro-transposon insertion event (41). Although A/WySnJ mice have considerably reduced peripheral B cell numbers due to reduced B cell survival, the expression of tg Bcl2 restores the peripheral B cell compartment to near-normal levels (29). We thus utilized A/WySnJ-Bcl2 Tg mice for these experiments (designated A/WySnJ in Figures for simplicity). When we stimulated their splenic B cells with BAFF, the NF-κB2 pathway showed markedly defective activation compared to control mice of the parent strain (A/J) expressing tg Bcl2 (**Figure 6B**). Consistent with the concept that BAFF binding to BAFFR preferentially activates the NF-κB2 pathway (43), BAFFR$^{-/-}$ B cells were not able to active NF-κB2 in response to BAFF stimulation (**Figure 6B**). In **Figure 6B**, we also noted that A/WySnJ BAFFR signaling induced TRAF3 degradation to a similar extent as did Wt BAFFR. We also examined the endogenous NIK protein levels in B cells of Wt and BAFFR mutant mice, and found that there were no differences in cellular NIK levels in A/WySnJ B cells as compared to Wt B cells (**Figure 6C**). These data suggest that TRAF3 degradation, leading to NIK stabilization, is not sufficient in itself to activate the non-canonical NF-κB2 pathway.

Because BAFF binds to three receptors expressed on B cells, BAFFR (44), BCMA (B cell maturation antigen) (45), and TACI (transmembrane activator and calcium modulator and cyclophilin ligand) (46), it is important to verify that the defective BAFF response of splenic B cells from the A/WysnJ mouse is due to its mutant BAFFR. We thus generated CH12.LX and M12.4.1 mouse B cell lines stably expressing chimeric receptors, containing the human (h)CD40 extracellular and transmembrane domains fused to the cytoplasmic domain of Wt BAFFR (Wt), the A/WySnJ mutant BAFFR, or BAFFR that lacks the last eight

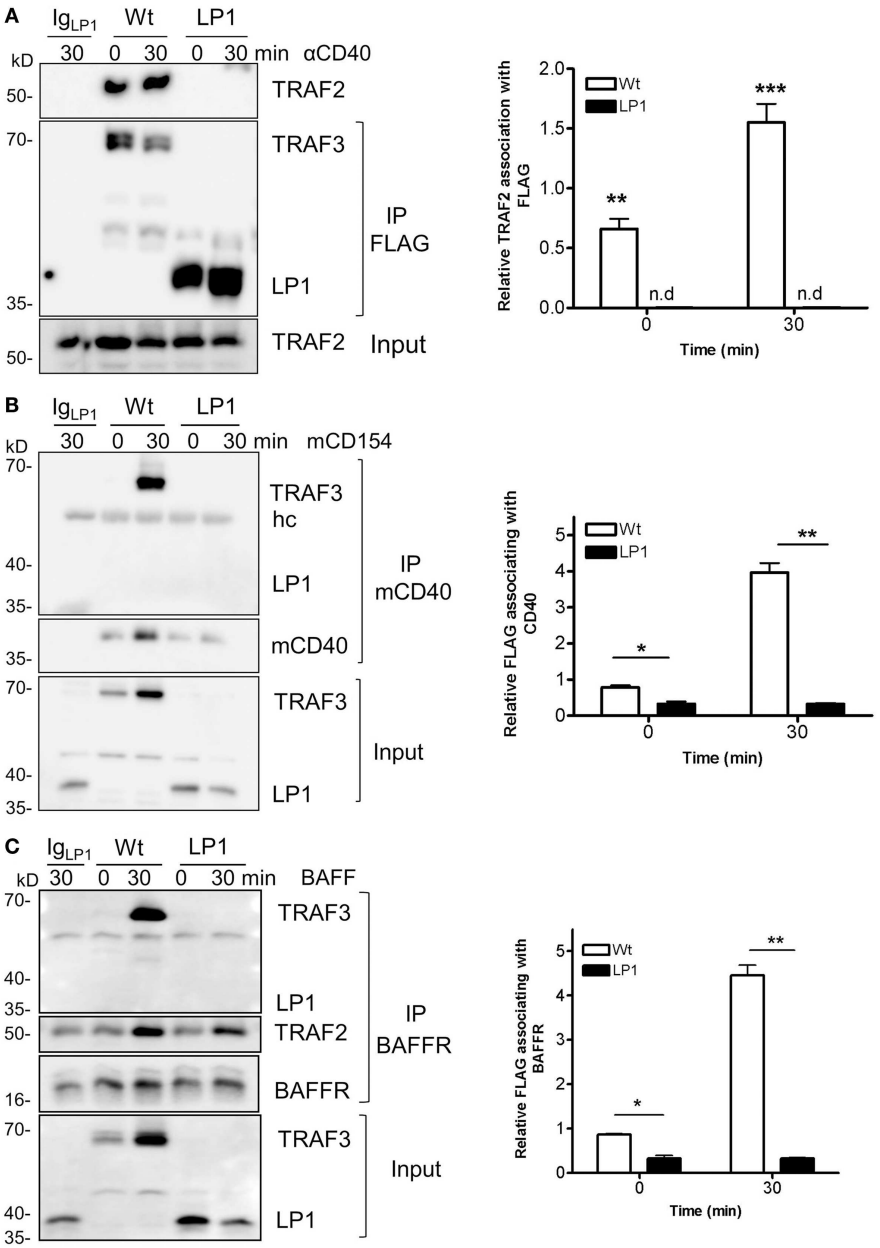

FIGURE 4 | Impaired association of LP1 TRAF3 with TRAF2, CD40, and BAFFR. **(A)** CH12.TRAF3$^{-/-}$ cells induced to express FLAG-tagged Wt or LP1 as in previous Figures were untreated or stimulated with 10 μg/ml of anti-CD40 mAb. Whole cell lysates were prepared as described in Section "Materials and Methods" and immunoprecipitated with anti-FLAG Ab and Western blots were probed for TRAF2 and FLAG. The bar graph presents quantification of relative amounts of TRAF3-associated TRAF2 from three independent experiments. $^{**}p = 0.001$; $^{***}p = 0.0005$; n.d. = none detected. **(B)** Cells were stimulated with HiV insect cells infected with Wt baculovirus, or HiV cells expressing baculovirus-encoded mouse CD154. Detergent-insoluble plasma membrane fractions were prepared from cell lysates and subjected to immunoprecipitation with anti-mCD40 Ab as in Section "Materials and Methods." Western blots were probed for FLAG and CD40. Graph represents quantification of mean values of relative amounts of TRAF3 associating with membrane CD40 from two different experiments. $^{*}p = 0.03$; $^{**}p = 0.005$. **(C)** Cells were untreated or cultured with 500 ng/ml of BAFF as indicated. Detergent-insoluble membrane fractions were isolated as above and anti-BAFFR Ab were used to immunoprecipitate the receptor signaling complex. Western blots of immunoprecipitates were probed for FLAG and BAFFR. The bar graph shows quantification of mean relative amounts of TRAF3 associated with BAFFR, from two different experiments. $^{*}p = 0.03$; $^{**}p = 0.003$.

amino acids of the cytoplasmic domain (ΔC) (**Figure 6D**). The chimeric receptors allowed us to be certain that we were examining consequences of BAFFR signaling in these B cells. B cells were stimulated with HiV insect cells expressing hCD154, and NF-κB2 activation was analyzed. Stimulation with anti-mouse (m)CD40 agonistic Ab served as an internal control, showing that there was

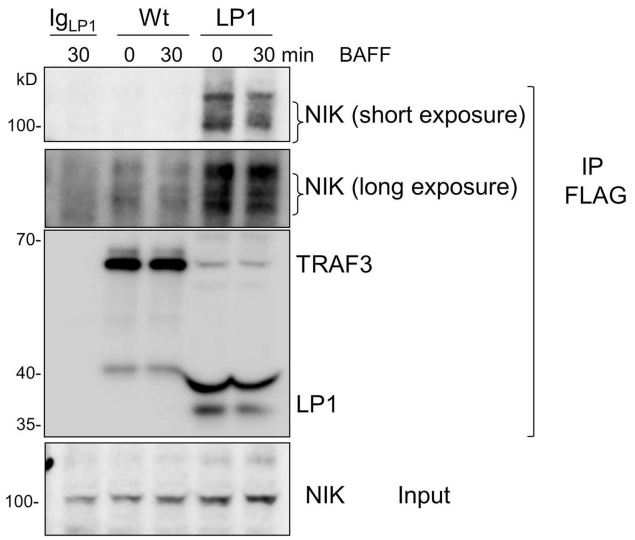

FIGURE 5 | Enhanced association of LP1 TRAF3 with NIK. CH12.TRAF3$^{-/-}$ cells expressing FLAG-tagged Wt or LP1 TRAF3 were untreated or stimulated with 500 ng/ml of BAFF. Whole cell lysates were prepared and immunoprecipitated with anti-FLAG Ab. Western blots of immunoprecipitates were probed for NIK and FLAG. Both short and long exposures of the blot are shown due to differences in NIK association between Wt and LP1 TRAF3. Data shown are representative of two similar experiments.

no global defect in CD40 signaling of cells expressing various chimeric molecules. Consistent with results from primary splenic B cells from A/WySnJ or control mice, B cells expressing hCD40-A/WySnJ BAFFR displayed defective NF-κB2 activation compared to hCD40-Wt BAFFR (**Figure 6E**). This result indicates that the signaling defect observed in splenic B cells is due to the dysfunctional BAFFR. Impaired activation of NF-κB2 was also observed with hCD40-ΔC BAFFR, indicating that the last eight amino acids of BAFFR are necessary for NF-κB2 activation, although they have been shown to be dispensable for B cell survival (29). This reveals the interesting information that pathways alternative to NF-κB2 can mediate BAFFR-induced B cell survival.

TRAF3 DEGRADATION MEDIATED BY MUTANT BAFFRS

To explore whether TRAF3 degradation status correlates with the impaired activation of NF-κB2 in cells expressing mutants of BAFFR, we stimulated B cell transfectants shown in **Figure 6C** through hCD40-BAFFR to induce BAFFR-mediated TRAF3 degradation (**Figure 7A**). TRAF3 was degraded similarly, regardless of which BAFFR cytoplasmic domain was expressed. This result reveals that TRAF3 degradation is not sufficient to induce BAFFR-mediated NF-κB2 activation. Results presented in **Figures 6B** and **7A** led us to predict that TRAF recruitment to the A/WySnJ BAFFR cytoplasmic tail would be comparable to TRAF recruitment to Wt BAFFR. Indeed, cells expressing hCD40-A/WySnJ chimeric molecules not only were able to recruit both TRAF2 and 3 to the BAFFR, this mutant BAFFR showed enhanced TRAF recruitment compared to Wt BAFFR (**Figure 7B**). Consistent with previous studies, when the TRAF3 binding site (PVPAT) on BAFFR was disrupted (AVAAA), there was no recruitment of TRAFs to the receptor's cytoplasmic domain (**Figure 7B**). These results show that the defective BAFF-stimulated NF-κB2 activation in A/WySnJ B cells is not due to a failure to induce TRAF3 recruitment or degradation.

Taken together, results from experiments presented in this study indicate that receptor-mediated TRAF3 degradation is not sufficient to induce NF-κB2 activation in B cells and TRAF3 degradation-independent mechanisms can also regulate the NF-κB2 pathway.

DISCUSSION

Functions of TRAF molecules vary both between cell types, and between individual types of receptors even within the same cell. This is particularly true for TRAF3 (2). There are many disadvantages of using the experimental model of transient overexpression in transformed cell lines, usually epithelial or fibroblast, to understand how endogenous signaling pathways work in specific hematopoietic cell types [reviewed in Ref. (47)]. Unfortunately, a great deal of the experimental investigation of TRAF3 functions to date has relied upon overexpression models. Largely based upon such models, it was concluded that receptor-induced TRAF3 degradation leading to NIK stabilization is a crucial requirement for downstream activation of the non-canonical NF-κB2 pathway (20, 26, 27).

We wished to further explore this model in B lymphocytes, as our previous findings, and those of others, indicate that TRAF3 plays uniquely important roles in regulation of this cell type. Our results presented here using complementary experimental systems of primary B cells and B cell lines, demonstrate that TRAF3 degradation is not sufficient for downstream NF-κB2 activation. The results from our study further emphasize the importance of studying TRAF functions in a cell type and receptor-specific manner. In this study, we also demonstrated TRAF-N and TRAF-C domains are required for recruitment of TRAF3 to CD40 and BAFFR, and that association with the TRAF2 complex is necessary for receptor-mediated TRAF3 degradation (**Figures 1** and **4**). However, the lack of degradation of the MM-derived TRAF3 mutant LP1 did not prevent activation of downstream signaling pathways; in fact, LP1-expressing B cells showed similar ability to undergo receptor-induced NF-κB2 activation, and enhanced JNK activation. In contrast to a previous study performed in non-hematopoietic cells (20), we found that TRAF3 association with the NF-κB2-activating kinase NIK in B cells does not require the TRAF-N and TRAF-C domains. Although lacking these domains, the LP1 mutant TRAF3 molecule actually bound substantially more NIK than Wt TRAF3. While the explanation for this enhanced binding is currently unknown, it is possible that one or more additional protein–protein interactions are involved. A slight increase in total NIK protein in LP1-expressing cells also suggested that NIK stabilization can be controlled by additional mechanisms. IKKα phosphorylation of NIK also destabilizes NIK (48). Additionally, TBK1 stabilizes NIK protein levels independent of TRAF3 degradation, while in contrast the protein OTUD7B regulates NIK protein levels through TRAF3 degradation in B cells (49, 50). Unlike TRAF3-deficient B cells, LP1 TRAF3-expressing

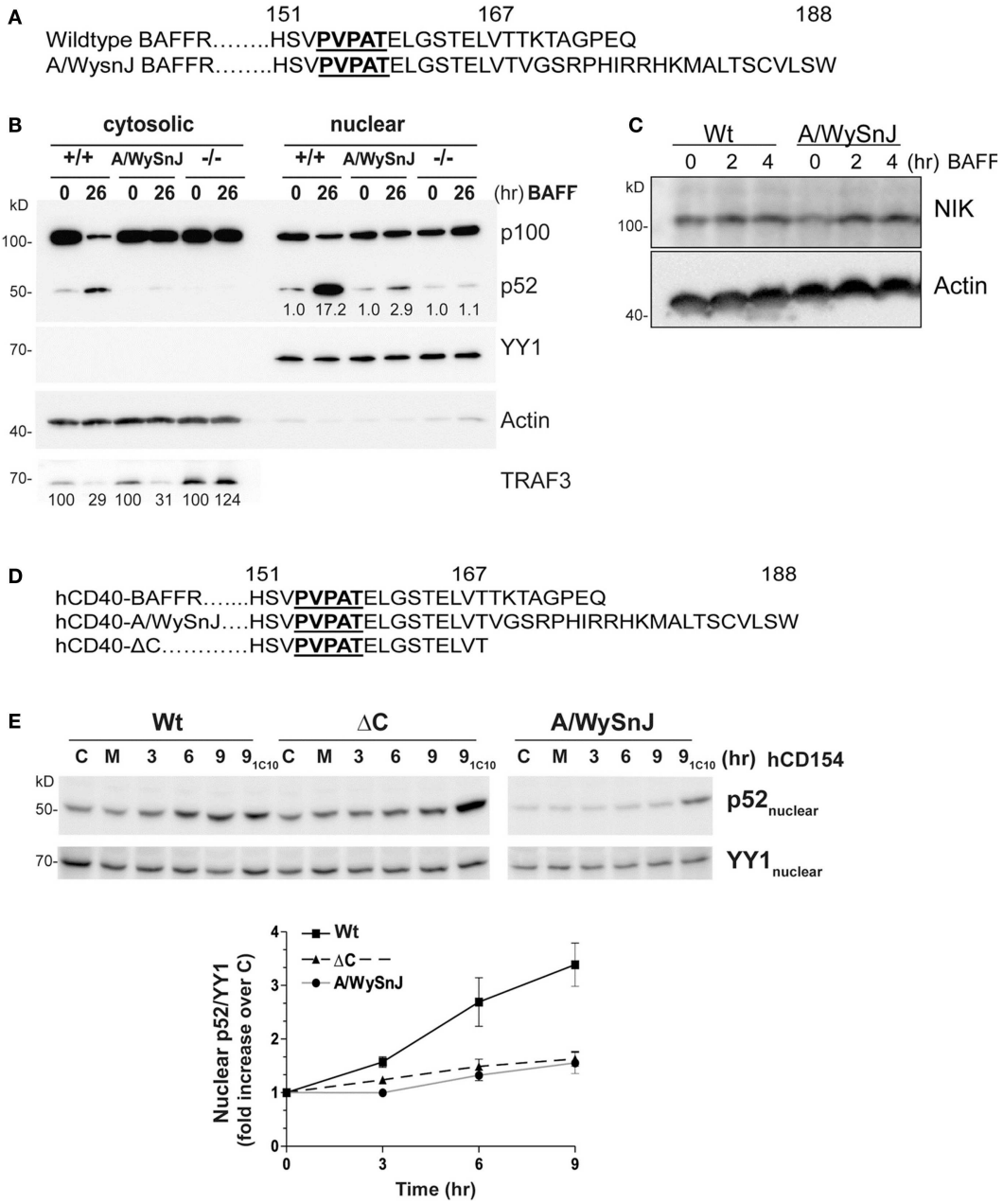

FIGURE 6 | Impaired NF-κB2 activation induced by mutant BAFFRs. **(A)** Comparison of the C-terminal signaling domain amino acid sequences of Wt and A/WySnJ BAFFRs. Bold and underlined sequences indicate the TRAF3 binding motif, numbers above the sequences indicate the position of the amino acids. **(B)** Equal numbers of resting splenic B cells isolated from Wt (+/+), A/WySnJ, and BAFFR$^{-/-}$ (−/−) mice were stimulated with 100 ng/ml of BAFF for 26 h. Cytosolic and nuclear fractions were prepared and subjected to SDS-PAGE as in Section "Materials and Methods." Western blots of samples were probed for p100/p52, TRAF3, actin, and YY1, as in **Figure 1**. Numbers on the TRAF3 blot indicate mean values of the proportion of remaining TRAF3 relative to unstimulated controls of each genotype, from two independent experiments. Numbers on the blot of nuclear extracts represent averages of the fold increase over unstimulated control within each strain of mouse, from two independent experiments. **(C)** Equal numbers of splenic B cells isolated from Wt or A/WySnJ mice were stimulated with 200 ng/ml of BAFF for the indicated times. Whole cell lysates were immunoblotted for NIK and actin. Representative blots from two independent experiments are shown. **(D)** C-terminal amino acid sequences encoded by chimeric constructs of hCD40-BAFFR, used to stably transfect CH12.LX cells as described in Section "Materials and Methods." **(E)** CH12.LX B cell subclones stably expressing various hCD40-BAFFR chimeras were treated with medium only (M), control HiV insect cells (C), or HiV insect cells expressing hCD154 for the indicated times. Anti-mCD40 agonistic mAb (1C10) treatment for 9 h served as an internal control for each subclone (9_{1C10}). Nuclear extracts were prepared and Western blots probed for p100/p52, and YY1. Representative blots of three similar experiments are shown. The corresponding graph represents mean band intensity values of treated/control samples ± SEM of three independent experiments.

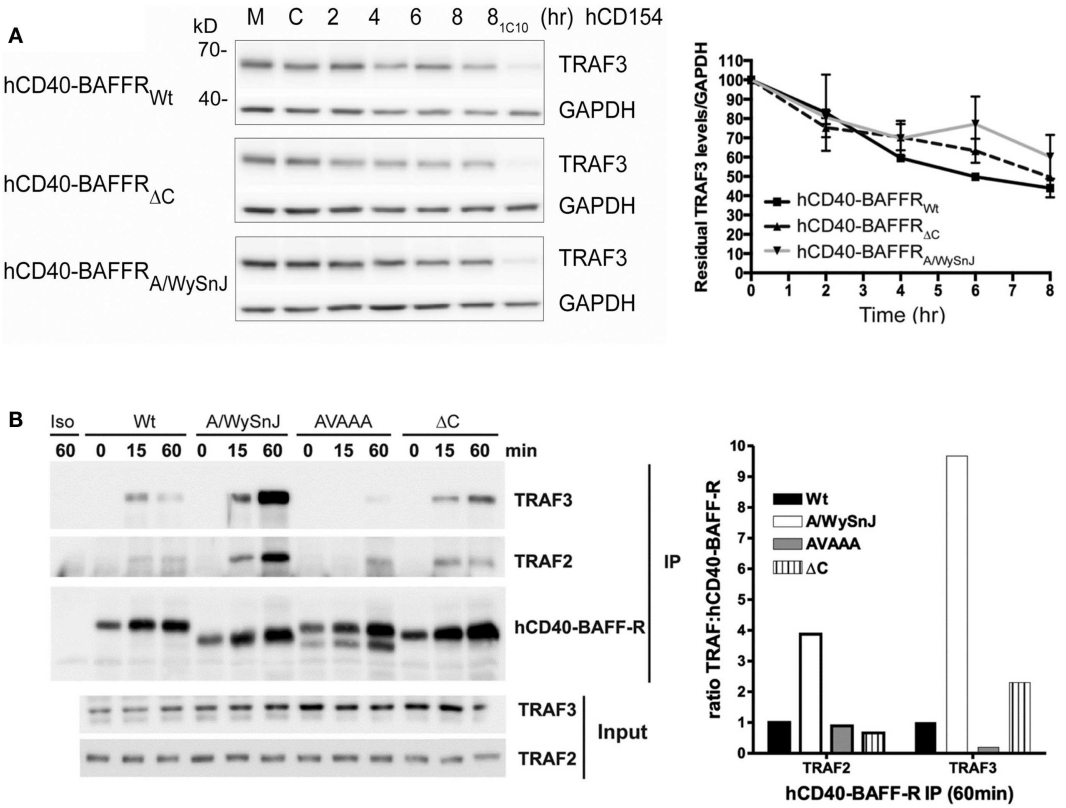

FIGURE 7 | Dissociation between relative TRAF3 degradation and NF-κB2 activation mediated by Wt vs. mutant BAFFRs. (A) Subclones of the M12.4.1 B cell line stably transfected with various forms of hCD40-BAFFR chimeras shown in **Figure 6C** were untreated (M), or treated with HiV insect cells (C), or HiV insect cells expressing hCD154 for the indicated times, or anti-mCD40 agonistic Ab 1C10 for 8 h (8_{1C10}). Whole cell lysates were prepared and subjected to SDS-PAGE as in Section "Materials and Methods." Representative immunoblots of samples, probed for TRAF3 and GAPDH are presented. The graph depicts relative amounts of TRAF3, normalized to GAPDH. Values presented are mean ± SEM of data combined from three independent experiments. **(B)** CH12.LX B cells subclones as in **Figure 6D** and CH12.LX B cells that expressed hCD40-BAFFR with disrupted TRAF3 binding sites (AVAAA) were stimulated with anti-hCD40 mAb or isotype control (Iso) coated beads and immunoprecipitated as mentioned in Section "Materials and Methods." The bead bound protein complexes were separated on SDS-PAGE and immunoblotted for TRAF2 and TRAF3. Representative blots from three independent experiments were shown. Bar graph represents relative levels of TRAF2 and TRAF3 normalized to hCD40-BAFFR chimera molecules.

B cells responded to both BAFF and CD40 stimulation with further increases in nuclear p52 levels. The current proposed model is that TRAF3 association with the TRAF2-cIAP complex prevents activation of the NF-κB2 pathway. Results presented here indicate that the mechanism by which TRAF3 regulates NF-κB2 activation is more complex than this. The degradation-resistant LP1 mutant TRAF3, without complexing with TRAF2-cIAP, can still regulate NF-κB2 activation. Our results also showed that the amino terminal RING and Zinc finger domains of TRAF3 are also quite important for TRAF3 to restrain NF-κB2 activation, in accordance with a published study (8). Taken together, the published and present data demonstrate that there are multiple mechanisms by which TRAF3 regulates the NF-κB2 pathway in B cells, both dependent upon, and independent of receptor-mediated TRAF3 degradation.

B cell activating factor receptor-mediated activation of signaling pathways is important for B cell homeostasis and survival, as exemplified by the deficiencies in mature B cell numbers and function observed in BAFF$^{-/-}$ and BAFFR$^{-/-}$ mice, as well as the spontaneous BAFFR mutant-expressing mouse, A/WySnJ (5). The NF-κB2 pathway is robustly activated by BAFFR, and is thought to play a major role in BAFFR-mediated B cell survival signals (21). Thus, the previously presented paradigm of NF-κB2 activation predicts that loss-of-function mutants of BAFFR would show reduced ability to induce TRAF3 degradation in B cells. Consistent with previous reports (28, 51), we observed impaired BAFF-mediated NF-κB2 activation in B cells isolated from A/WySnJ and BAFFR$^{-/-}$ mice, as well as with B cell lines expressing mutant hCD40-BAFFR chimeric receptors. We further demonstrated that the last eight amino acids of the BAFFR cytoplasmic domain are required for NF-κB2 activation in B cells. This is particularly interesting, because Mayne and Hayes reported that these residues are NOT required for BAFFR-mediated B cell survival (29). Together with the present results, this indicates that NF-κB2 activation is not the only important pro-survival pathway induced by BAFFR signaling.

Several studies have concluded that receptor-induced TRAF3 degradation is sufficient to activate the NF-κB2 pathway (20, 26, 27). Because the A/WySnJ BAFFR retains the TRAF3 binding motif PVPAT, we compared BAFFR-inducedTRAF3 degradation using B cells expressing different forms of BAFFR. Again in contrast to the current paradigm, we found that signaling through the A/WySnJ or ΔC BAFFRs induced TRAF3 degradation to levels similar to Wt BAFFR, leading to the conclusion that receptor-induced TRAF3 degradation is not sufficient to drive NF-κB2 activation. Additionally, the A/WySnJ BAFFR recruits more TRAF2 and 3 to its cytoplasmic tail, so it is possible that the mutant BAFFR is recruiting important additional, unidentified signaling molecules that regulate NF-κB2 activation. We also found that the last eight amino acids of the C terminus of BAFFR were not required for either receptor-mediated TRAF3 degradation or TRAF recruitment to the receptor, but the impact of this region upon recruitment of other unidentified factors are not yet known.

In summary, our data demonstrate that the relationship between TRAF3 degradation and activation of NF-κB2 in B cells is neither simple nor linear. These findings support the need for further investigation into both how NF-κB2 activation is regulated in B cells, as well as how TRAF3 may regulate B cell survival via mechanisms additional to NF-κB2 activation. The identification of loss-of-function TRAF3 mutations in various types of human B cell malignancies underlines the importance of reaching a clearer and more detailed understanding of these relationships.

AUTHOR CONTRIBUTIONS

Wai W. Lin, Joanne M. Hildebrand, and Gail A. Bishop designed research; Wai W. Lin and Joanne M. Hildebrand performed experiments. Wai W. Lin, Joanne M. Hildebrand, and Gail A. Bishop prepared the manuscript.

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Role of the lymphotoxin/LIGHT system in the development and maintenance of reticular networks and vasculature in lymphoid tissues

Theresa T. Lu*[1,2]* and *Jeffrey L. Browning*[3]

[1] *Autoimmunity and Inflammation Program and Pediatric Rheumatology, Hospital for Special Surgery, New York, NY, USA*
[2] *Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY, USA*
[3] *Department of Microbiology and Section of Rheumatology, Boston University School of Medicine, Boston, MA, USA*

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Linda C. Burkly, Biogen Idec, Inc., USA

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Sanjiv A. Luther, University of Lausanne, Switzerland
Jorge Caamano, University of Birmingham, UK

***Correspondence:**
Theresa T. Lu, Autoimmunity and Inflammation Program and Pediatric Rheumatology, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021, USA
e-mail: lut@hss.edu;
Jeffrey L. Browning, Department of Microbiology and Section of Rheumatology, Boston University School of Medicine, 72 East Concord Street, E-5, Boston, MA 02118, USA
e-mail: browninj@bu.edu

Lymphoid organs are meeting zones where lymphocytes come together and encounter antigens present in the blood and lymph or as delivered by cells migrating from the draining tissue bed. The exquisite efficiency of this process relies heavily on highly specialized anatomy to direct and position the various players. Gated entry and exit control access to these theaters and reticular networks and associated chemokines guide cells into the proper sections. Lymphoid tissues are remarkably plastic, being able to expand dramatically and then involute upon resolution of the danger. All of the reticular scaffolds and vascular and lymphatic components adapt accordingly. As such, the lymph node (LN) is a wonderful example of a physiologic remodeling process and is potentially a guide to study such elements in pathological settings such as fibrosis, chronic infection, and tumor metastasis. The lymphotoxin/LIGHT axis delivers critical differentiation signals that direct and hone differentiation of both reticular networks and the vasculature. Considerable progress has been made recently in understanding the mesenchymal differentiation pathways leading to these specialized networks and in the remodeling that occurs in reactive LNs. In this article, we will review some new advances in the area in terms of developmental, differentiation, and maintenance events mediated by this axis.

Keywords: lymphotoxin-beta receptor, reticular stroma, high endothelial venules, reactive lymph node, follicular dendritic cell, fibroblastic reticular cells

INTRODUCTION

The functional specialization of the reticular scaffolds and the vascular and lymphatic vessels in lymphoid organs is essential for the exquisite sensitivity and efficiency of the immune system. These various stromal elements have been increasingly well studied over the last decade with major advances occurring in the description of their developmental lineages and differentiation as well as their activation and adaptation in reactive lymphoid organs. This field has been extensively reviewed (1–12). In general, the stromal cells in the secondary lymphoid organs (SLO) include reticular cells of which there are at least two different kinds, the follicular dendritic cell (FDC) and the fibroblastic reticular cell (FRC). Additional fibroblastoid cells are present in splenic red pulp and in the medullary sinuses of the lymph nodes (LN) (2). Cells of endothelial origin include blood endothelial cells of which there are both conventional "flat" forms as well as high endothelial venules (HEV) and the lymphatic endothelium that lines the afferent and efferent lymphatics, the subcapsular sinus, and the various lymphatic sinuses within the cortex, paracortex, and medulla (13). These structures are relatively stable in the resting state but undergo dynamic remodeling in the case of reactive LN. Reactive LN normally involute and presumably restore homeostasis once the activating trigger is resolved.

The lymphotoxin (LT) system, a member of the TNF family, is a key regulator of lymphoid architecture. For almost two decades now, the roles played by this pivotal system have been studied in the context of the development and maintenance of lymphoid reticular networks, HEVs, neo-angiogenesis, and lymphangiogenesis as well as their impact on immunological function. This pathway has been reviewed in detail (14–20). With a focus on recent advances, we highlight here the role of the LT system in the control of the reticular and endothelial elements within organized lymphoid structures.

THE LYMPHOTOXIN SYSTEM

The original LT protein, called LTα, is a member of the TNF family of ligands and is secreted as a homotrimeric molecule. Like TNF, LTα binds to the TNF receptors. An additional membrane bound form of LTα is composed of a heterotrimeric complex with a second protein called LTβ in a LTα1/β2 stoichiometry (LTα/β). LTα/β binds uniquely to the lymphotoxin-beta receptor (LTβR) (**Figure 1**). A second ligand, LIGHT (TNFSF14), binds to three different receptors; LTβR (TNFRSF3) with high affinity, another TNF family receptor called HVEM (TNFRSF14) and a soluble receptor named DcR3 (TNFRSF6B). LIGHT is found in both membrane tethered and secreted forms. Dimerization of LTβR by either LIGHT or LTα/β is sufficient to induce signal transduction (21, 22); however, it is likely that higher order oligomerization states of the receptor lead to either more intense or qualitatively different signaling (23). HVEM also interacts with several non-TNF family

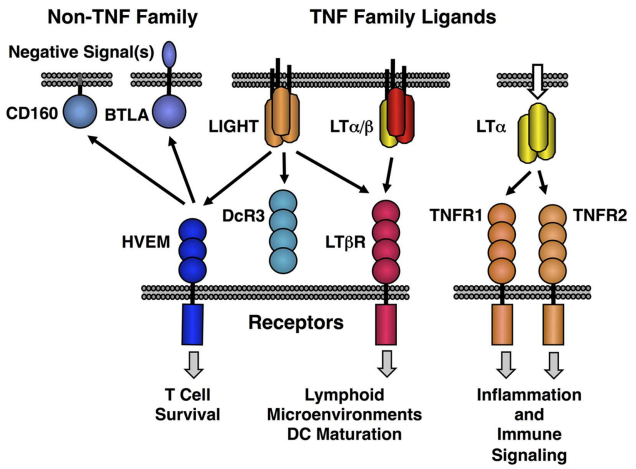

FIGURE 1 | Map of the interactions between LTβR, HVEM, and TNF receptors with the LT/LIGHT family and the Ig-super family members CD160 and BTLA. LIGHT is shown in its membrane bound form, but it is also readily secreted like homotrimeric LTα.

members, i.e., BTLA and CD160 and these proteins have context-specific regulatory effects on T and NK cell function (20, 24, 25). Human but not murine LTα can bind to HVEM and LTα binds to Troy (TNFRSF19) (26). However, these LTα interactions are low affinity and their physiological significance is questionable (27).

The soluble human LTα trimer appears to have specific functions. Most notably, LTα and LTβ knockout animals differ in that $LT\beta^{-/-}$ mice retain the mesenteric lymph node (mLN) while $LT\alpha^{-/-}$ mice lack all nodes (19). Likewise, the two LT knockout animals differ in their ability to survive cerebral malaria (28). The consequences of ectopic LTα gain-of-function in transgenic mice differ depending on whether or not LTβ is expressed with peripheral lymph node addressin (PNAd) display requiring LTβ (29). More recently, a specific role for LTα in the induction of gut IgA responses was defined (30). Indeed, differences between LTα and LTβ deficient mice have been attributed to additional and unique roles for the LTα homotrimer. Whether such LTα-specific functions are physiological can be questioned as the $LT\beta^{-/-}$ mouse represents potentially an LTα gain-of-function mouse. In a study using transfected cells, LTβ expression dramatically rerouted LTα expression into the membrane form to the detriment of secreted LTα (31). Thus, the loss of LTβ could initiate an LTα-driven inflammatory response especially in mucosal environments when TNF expression is minimal. In general, it is believed that the LTα/β heteromeric ligand represents a direct cell-to-cell communication system, whereas LTα3 is only secreted. In early studies, efficient secretion or shedding of the LTα/β complex was not observed (31–33); however, recently small amounts of shed LTα/β were detected in the blood and synovial fluid from rheumatoid arthritis patients using a highly sensitive assay (34).

The LTα/β-LTβR system has been implicated in many immunological events including maintenance of FDC networks, HEV, specialized macrophages capable of antigen capture in the splenic marginal zone and subcapsular sinus of the LN as well as dendritic cell (DC) homeostasis and differentiation (19, 35). This system also plays major roles in mucosal microenvironments with multiple consequences for epithelial cell biology and host defense/repair programs (36). LTβR signaling affects the state of specialized epithelial cells within the medulla of the thymus (37). The role of LT in epithelial cell interactions is beyond the scope of this review. Both LTβR and TNF-receptors activate canonical NFκB signaling, yet LTβR is distinguished by the efficient activation of the alternative NFκB pathway (19). Importantly, receptor internalization is required to shift NFκB activation from the classical to the alternative pathway and the nature of the ligand, e.g., soluble LIGHT vs. membrane bound forms of LIGHT or LTα/β may affect the ability to drive receptor internalization (38). The alternative pathway is typically linked to developmental and cell differentiation programs in contrast to the predominantly inflammatory role of the canonical pathway (39). The centerpiece of the alternate NFκB pathway is NIK kinase activation and NIK is required for many if not all of the developmental and differentiation events associated with LTβR (19).

Lymphotoxin-beta receptor is readily observed on most non-hematopoietic cells and on monocytic and DC lineages. Historically expression of the LT ligand was considered to be unique to hematopoietic lineages, especially B cells, activated T cells including Th1 and Th17 subsets, NK cells and innate lymphoid cells (40). This picture has become fuzzier of late, since LTβ RNA is readily observed in several other cell types such as in DC, hepatocytes, and hepatic oval cells (13, 41, 42). As LTβ RNA appears more abundant, LTα expression has been viewed as the limiting element in controlling membrane LTα/β display. It is less clear how often LTβ RNA reflects actual functional membrane LT, which requires expression of both LT proteins. Bona fide surface LTβ protein was observed on DC by histology and FACS; however, surface heteromeric LT α/β protein capable of binding LTβR actually has not been described outside of the hematopoietic system (43). As non-hematopoietic lineage cells and myeloid lineages usually express LTβR, it is theoretically possible that functional ligand produced within such a cell rapidly complexes with receptor, initiates signal transduction and the complex is processed. In such a case, cell autonomous LTβR signaling could be possible without actual transit to the surface and, moreover, surface ligand detection would be difficult. Furthermore, in cells with a high density of LTβR, there is evidence for autonomous signaling in the absence of any ligand (44).

The LT genes lie within a cluster with TNF in the class III region of the MHC and epigenetic control and chromatin organization are key elements of the regulation of cytokine expression within the cluster (45, 46). In general, understanding of the physiological transcriptional control of the LT and LIGHT genes remains incomplete. The LTβ promoter uses Ets1, Sp1, NFκB, and Egr-1/Sp1 transcription factors to drive RNA expression and expression was induced by TNF in a hepatocyte cell line (47). Furthermore, a regulatory element in exon 4 of the LTβ gene appears to interact with multiple sites within the TNF gene cluster (48). In general, LTα is expressed by activated lymphocytes and NFAT, NFκB, Sp1, and STAT elements are found within the promoter region. LTα expression can be induced by interleukin-12 p40 homodimer in myeloid lineages (49). Non-hematopoietic cells can also make LTα, for example, vascular smooth muscle cells secrete LTα in response

to extracellular nucleotide activation of the $P2Y_2$ receptor (50). Adding to this complexity, LTα RNA can be found in several variants. Interestingly, an alternate core promoter residing within the intron between exons 1 and 2 was predominantly utilized in T cells stimulated with TGFβ1 or FGF-7 (51). Clearly, a better understanding of the physiological regulation of the LT locus is needed.

LYMPHOTOXIN AND THE DIFFERENTIATION STATES OF RETICULAR FIBROBLASTS IN RESTING LYMPHOID TISSUES

The cells forming the lymphoid reticular scaffolds are of mesenchymal lineage and generally are grouped into two broad categories based on their locations within organized lymphoid organs (**Figure 2**). FDC scaffold the B cell follicles and perform a multitude of functions related to antigen-presentation, cell survival, and handling of apoptotic cells (9–12). They possess a unique recycling mechanism to protect captured antigen from degradation and retain it for long-term presentation (52). FDC express the chemokine CXCL13 required for follicular compartmentalization of B cells (10). The second major category of reticular networks encompasses the T cell region of both LN and the spleen and is formed by FRC (2, 5, 8). FRC have multiple functions including the attraction of CCR7-expressing T cells and DC by expression of CCL19 and CCL21, T cell survival signals, and formation of collagen fibers. As such, they are fundamentally different from the FDC networks. A third category called a marginal reticular cell (MRC) has received recent attention. This cell sits under the subcapsular sinus close to B cell follicles, combines elements of both FDC and FRC and has been proposed to be a progenitor cell to the two major lineages (5, 53). Within the FRCs, there is heterogeneity, for example, VEGF-expressing FRC are enriched in vessel-rich areas (54). Also, using a lineage-tracing system for past expression of CD21, a population of mesenchymal cells in the T zone was identified that are similar to FRCs, yet express higher levels of TGFβ and lower amounts of CCL19 and CCL21 relative to FRCs (55). How these cells are related in lineage to the classical FRCs remains to be determined.

To understand reticular cell differentiation pathways, it is important to appreciate the embryological underpinnings of LN development (16). Early LN anlagen are formed from buds of endothelial and mesenchymal tissue. The mesenchymal cells progress through an ICAM1/VCAM1+ intermediate phenotype and at this stage, upon LTβR activation by hematopoietic lymphoid tissue inducer (LTi) cells, they acquire an ICAM1/VCAM1/MAdCAM1+ stromal organizer phenotype (56). This cell is often referred to as a lymphoid tissue organizer (LTo). The LTo cells now express CCL19, CCL21, CXCL13, IL-7, and RelB, and these markers will typify future specialized stromal elements in the mature organ. The reliance on LTβR signaling at the stromal organizer switching point is believed to account for the impaired development of LN in mice deficient in components of the LT pathway. Importantly, ontogeny has provided an important guide in understanding lymphoid tissue changes as embryological paradigms are largely recapitulated during inflammation (57, 58). Studies on the role of the LT system have always been plagued by the complexity of the direct knockout animals, i.e., the lack of LN and the issues of dissociating embryological programs including postnatal events from physiological maintenance/homeostasis. Historically, pharmacological inhibition of LTβR signaling has been the most direct method to achieve dissection, however, cell-specific or inducible genetic manipulations now provide powerful tools.

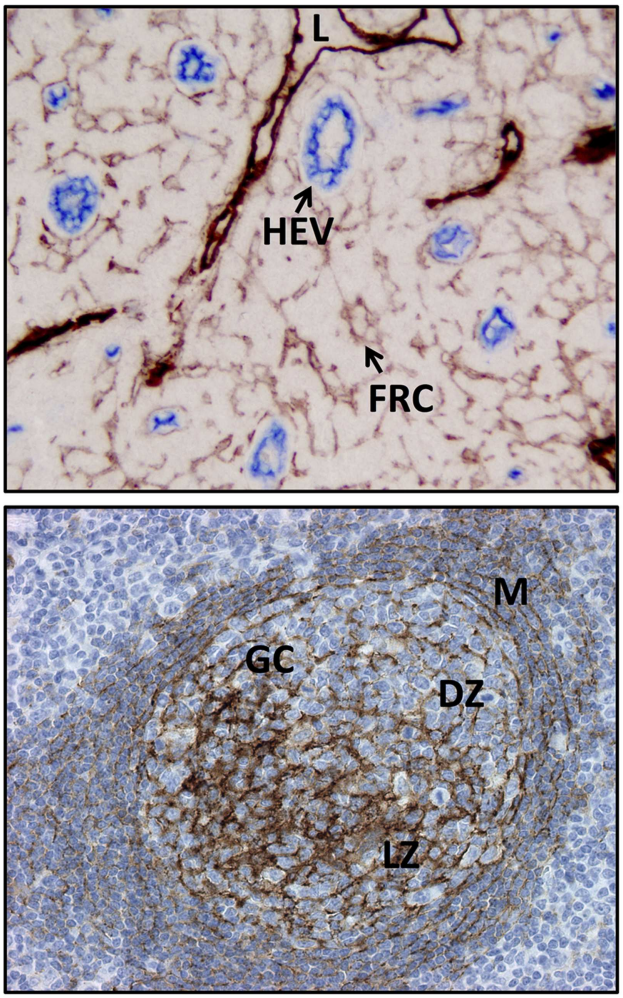

FIGURE 2 | Examples of FDC and FRC networks in the human tonsil. Top panel shows the podoplanin positive FRC network and lymphatic vessels (both brown) and the surrounding HEV and non-HEV vessels (CD34, blue). Bottom panel shows the FDC network (CD35, brown) in a secondary follicle with a GC. Note light and dark (CD35 positive) regions are readily discerned along with the residual primary follicle or mantle zone surrounding the GC. L, lymphatic vessel; FRC, fibroblastic reticular cell, HEV, high endothelial venule; M, mantle zone or primary follicle; GC, germinal center or secondary follicle, DZ, dark zone; LZ, light zone.

RETICULAR NETWORK PROGENITORS

The current paradigms for reticular cell specialization in the SLO invoke an early progenitor cell that is somewhat similar to a mesenchymal stem cell (MSC) (**Figure 3**) (6). Signals early in development provided by retinoic acid or RET ligand depending on the context lead to an immature organizer cell (16). In most contexts, LTβR signaling is required for progression to the

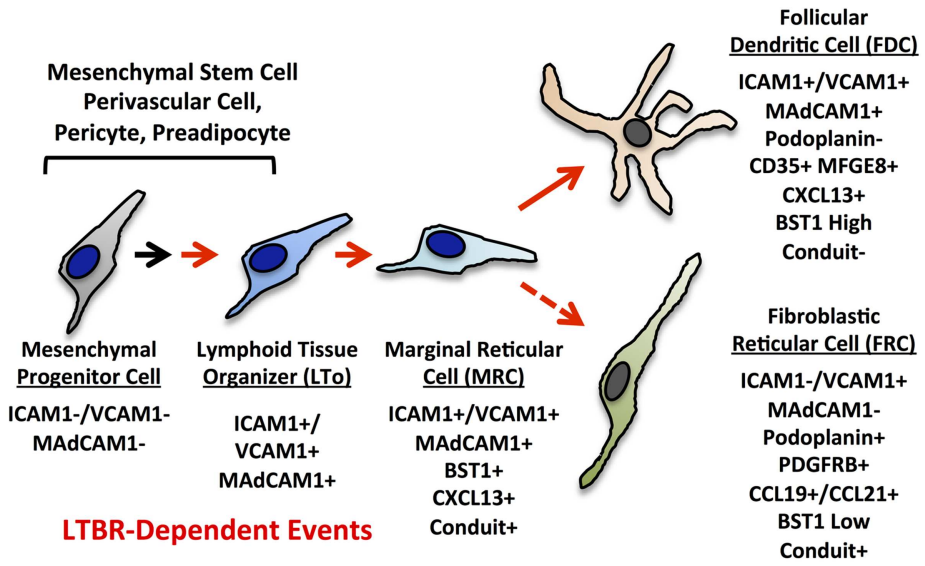

FIGURE 3 | Putative differentiation pathways for the FDC and FRC networks showing major phenotypic changes. The MRC is speculated to be a progenitor for FRC and FDC lineages based on phenotype, but this relationship has not been formally proven.

mature organizer or LTo that releases chemokines and displays surface adhesion molecules to nucleate the emerging LN anlage. Brendolan and colleagues recently were able to use lineage-tracing methods to map this pathway using the transcription factors Nkx2–5 and Islet1 (59). The Nkx2–5/Islet1 cells differentiated into all three major reticular lineages, MRC, FDC, and FRC as well as into classical mural cells or pericytes, but not the endothelial lineages.

FDC NETWORKS

Lymphotoxin-beta receptor signaling is essential for the development, differentiation, and maintenance of FDC networks in the SLO, in mucosal compartments and in chronically inflamed sites, i.e., so-called tertiary lymphoid tissues/organs (TLTs/TLOs) (9–12). TNF signaling is also critical for the differentiation of the FDC phenotype, but not FDC maintenance at least in a secondary follicle. Surface LT on B cells is crucial for the development and maintenance of the FDC networks in the spleen as shown by the use of classical bone marrow chimeras and more specifically with a conditional B cell selective knockout of LTβ (60–63).

Historically, the term "dendritic cell" in this setting was an unfortunate misnomer and contributed to the confusion surrounding their true origin. Obscuring their origin even further, FDC "precursors" could be transferred from bone marrow; however, this observation preceded the discovery of MSCs in the bone marrow. In a breakthrough, the precursor cells to FDCs were recently identified as perivascular cells resembling pericytes (64). Pericytes are members of a subset of fibroblastoid cell types including classical pericytes also called mural cells, vascular smooth muscle cells, adventitial fibroblastoid cells, and stellate cells (hepatic, pancreatic, pituitary, astrocytes, etc.). MSCs are progenitors of both this immediate "pericyte" family as well as a much wider group of cell types including adipocytes, chondrocytes, osteoblasts, fibroblasts, myofibroblasts, synoviocytes, and podocytes. The exact differences between MSC and perivascular cells or pericyte progenitors remain murky (65, 66). There is some semantic confusion as a pure classical definition of pericytes would include direct contact with an endothelial cell without interruption by a basement membrane. In practice, lacking unique cellular markers, there appears to be a spectrum of related perivascular cells.

Aguzzi and colleagues used multiple approaches to understand the origin of FDCs. They were able to lineage map PDGFRβ+ precursors into FDC and ablation of these same cells led to the loss of FDC. Additionally, transfer of adipose-derived perivascular cells into the renal capsule resulted in the formation of a local FDC network (64). B cells were the sources of LT and TNF for FDC development, yet in the absence of B cells, LTi cells were implicated as a key source of LT. It should be noted that these approaches were not completely definitive, e.g., the markers PDGFRB and MFGE8 are not specific for pericytes and FDC, respectively. This limitation in the study highlights how the pericyte field suffers from the lack of absolutely unique markers (67). Nonetheless, PDGFRβ positive cells in adipose tissue are generally accepted to be a pericyte/MSC and these cells were able to give rise to FDCs. Therefore, the differentiation of mesenchymal progenitor cells into a reticular cell that is highly honed to retain antigen within the follicular microenvironment, requires either LT or TNF signaling provided by local lymphoid cells. This system provides an excellent example of the ability of the lymphoid cells to locally shape mesenchymal lineages.

Established FDC networks exist in both a primary form, i.e., in a follicle lacking a germinal center (GC) reaction or in GC containing secondary follicles wherein the network is further differentiated (10, 68). Maintenance of established networks absolutely requires LTβR signaling as shown by pharmacological inhibition of the pathway (69–73). Mice genetically deficient in elements of the

LT system have been instrumental in defining the roles of splenic FDC. For example, spleens from LT-deficient mice displayed vastly reduced CXCL13 expression and pharmacological inhibition gave roughly similar results (74). Using various LT pathway deficient mice or bone marrow chimeric mice, lack of FDC resulted in profound impairment in both class switch recombination and affinity maturation. These results were probably complicated by additional defects in the splenic marginal zone, trafficking of cells to the correct compartments, and DC biology, etc. (14, 75). For example, the impressive loss of immune complex trapping on splenic FDCs in monkeys following LTβR inhibition was potentially a combination of both degradation of antigen trapping machinery in the primate marginal zone as well as FDC collapse (71).

The impact of FDC disruption has been studied by many approaches, but a selective depletion system described recently by Cyster and colleagues provided a relatively clean analysis (76). Loss of FDC networks collapsed follicular organization, reduced CXCL13 chemokine production and led to abortive GC reactions probably due to lack of long-term antigen retention (76). In monkeys, splenic FDC networks were substantially collapsed after only 1 month of pharmacological inhibition of LTβR signaling (71). Curiously, GC reactions in the mLN may be less dependent on proper FDC networks than in the spleen or peripheral LN (pLN) (76). mLN are clearly different from pLN in terms of their developmental requirements (76–79). In general, the role of LTβR signaling in FDC maintenance during a GC reaction remains poorly described.

FRC NETWORKS

Fibroblastic reticular cell are reticular cells that scaffold the T cell rich regions of organized lymphoid structures (1, 2, 4, 7, 8). The chemokines CCL19 and CCL21 are produced by FRC and orchestrate the movement and attachment of lymphocytes along this backbone with DCs being more tightly attached and T cells loosely marching along the network to spatially facilitate T-DC encounters. Clean experiments to dissect immune function in the presence and absence of FRC are lacking and indeed some have questioned how much the FRC actually facilitates encounters (80). FRC also provide the IL-7 for T cell survival and the control of homeostatic compartment size in a manner reminiscent of BAFF and B cell survival (81). FRC have the capacity to directly present antigen to promote peripheral tolerance and they can respond with nitric oxide production during acute inflammation thereby dampening T cell responses (82–84). The scaffold itself is composed of collagen fibers that are made and wrapped by the FRC, forming a functional unit called a "conduit" (85). These conduits are found in all lymphoid tissues, e.g., spleen, thymus, LN, and TLT and appear intrinsic to an organized microenvironment. The conduits allow for very fast transport of low molecular weight proteins, i.e., up to roughly 70 kDa from the lymph or blood to the LN parenchyma. DC residing on the FRC can sample the conduit contents, raising the potential for direct detection of small antigens. Additionally, it is reasonable to propose that conduits allow the LN to sense chemokine/cytokine signals emanating from an inflamed tissue bed.

Recently, three studies have contributed greatly to understanding FRC differentiation. First, the Turley group in conjunction with the Immunological Genome project purified and analyzed the transcriptomes of FRC, blood endothelial, and lymphatic endothelial cells along with a fourth so-called double-negative cell (DNC) population that was negative for the endothelial marker CD31 and the FRC marker podoplanin (86). The DNC population was pericyte-like and most closely resembled FRC. This work provided a unique molecular fingerprint of these stromal populations and suggested a lineage relationship between pericytes and FRC. Caamano and colleagues showed that embryonic progenitor cells resembling preadipocytes can differentiate into lymphoid stromal cells (87). LTβR signaling, via the alternate NFκB pathway, was required to stop a progenitor cell from embarking on an adipocyte program and to direct a shift toward a lymphoid stromal cell differentiation pathway. This capacity was demonstrated using both embryonic and adult progenitors derived from adipose tissue. Moreover, they hypothesized that adipose tissue is a source of lymphoid stromal cells. Consistent with these observations, LIGHT-LTβR interactions can inhibit the program of adipocyte differentiation (88, 89). Third, Ludewig and coworkers exploited a unique CCL19-Cre mouse with expression limited to the FRC (90). Using this tool, LTβR could be selectively deleted from FRC-like cells. Surprisingly, these mice developed LN and FRC/conduits normally; however, the LTβR-deficient FRC displayed reduced CCL19, CCL21 chemokine, and podoplanin expression. It is likely that insufficient CCL19 driven Cre is expressed at the early progenitor phase to remove LTβR prior to LTo generation and hence LN development proceeds normally. The mice bearing LTβR-deficient FRC poorly managed a murine herpes virus infection suggesting defective FRC diminished the immune response. These studies indicate that some aspects of FRC are under LT control, both developmentally and upon ectopic relocation, i.e., a setting similar to the induction of TLT.

Therefore, it appears that, like FDC, *de novo* development of FRC requires LTβR signaling. This result was best illustrated in the earlier observations on the formation of FRC networks in the spleen and TLT (74, 91). Despite these elegant studies, the basic question is less clear as to whether established FRC networks in either resting or reactive LN are under continuous LTβR control as observed with FDC. In prior analyses by the Cyster group, there was a clear developmental dependence on LT positive B cells for the splenic T zone FRC and their expression of CCL21 (74, 92). The development of splenic FRC, like FDC, continues from birth out for several weeks and indeed neonatal inhibition of LTβR signaling reduced CCL21 levels (a surrogate for FRC maturation) (92, 93). However, neither pharmacological inhibition of LTβR in adult mice nor transfer of LT-deficient lymphocytes appreciably reduced CCL21 levels in either spleen or mLN (74, 92). As in the spleen, FRC in LN continue to develop for 2–6 weeks post gestation based on CCL19 expression (90). The loss of LTβR on FRC using the CCL19-Cre system caused major disruptions in the nature of the FRC network (90). Likewise, combined TNF and LTβR signaling was synergistic leading to generation of collagen containing conduit-like fibrils in cultures of LN-derived stromal cells (94). One interpretation of this result is that canonical NFκB signaling by TNF is required to resupply the components to drive long-term alternate NFκB signaling by LTβR. In the light of these various observations, it is perhaps perplexing that pharmacological

LTβR inhibition in adult mice did not affect CCL21 levels. However, these observations are consistent with a developmental role for LTβR signaling in FRC development, but not in the maintenance of established networks. Further histological analysis of non-matrix markers such as podoplanin, PDGFRβ, etc., expression in FRC networks following LTβR-Ig treatment need more careful examination. Therefore, in contrast to FRC development and FDC networks, the existing data indicate that the maintenance of established FRC networks is not under LT control.

THE ROLE OF THE LYMPHOTOXIN SYSTEM IN REGULATION OF THE RESTING LYMPHOID ENDOTHELIUM

HIGH ENDOTHELIAL VENULES

A prominent feature of LN is the presence of specialized postcapillary venules called HEVs that allow for the transit of lymphocytes from the blood into the LN parenchyma (13). The selective display of various adhesion molecules, coupled with specific chemokine triggers for integrin activation differentially gate access to the unique microenvironments of the skin, mucosa, and various LN. The robust transit of lymphocytes through the endothelium presses the endothelial cells into plump shapes giving the venule its characteristic "high" status (95). Development and maintenance of the HEV specifically requires LTβR signaling via the alternative NFκB pathway with a dialog occurring between LT positive DC and LTβR positive endothelial cells to promote the "HEV program" (96–98). The role of LTβR in HEV maintenance was conclusively demonstrated by pharmacological intervention of LTβR signaling in adult mice (99, 100). Developmentally, the requirement for LTβR signaling was less obvious since LN development itself is arrested in knockout mice, although addressin expression was reduced both in the mLN in LTβ deficient mice and in the occasional LN that develop following gestational inhibition of LTβR signaling (101, 102). Recently, deletion of LTβR expression selectively in endothelial lineages curtailed development of a normal repertoire of LN's, yet HEV development was blocked in remaining rudimentary LN (103). This study proved that the HEV program was directly under LTβR control and not a consequence of more indirect events related to a disrupted lymphoid architecture. Although some HEV are also associated with the pericyte-like DNCs (86), FRC appear to extend directly to the HEV, and wrap the abluminal face (104). It is conceivable that the FRC/HEV acts as a unit comparable to the astrocyte/pericyte/endothelial cell neurovascular unit or the relationship between stellate cells, pericytes. and endothelial cells in the liver sinusoids.

The PNAd molecule on HEV is a complex sulfated glycan assembled on specific scaffold proteins in both N- and O-linked forms. PNAd binds to L-selectin on the lymphocyte triggering the initial rolling. The basic LTβR-controlled "HEV program" encompasses the direct induction of scaffold gene expression in LN such as MAdCAM1, GLyCAM1, and CD34, as well as the biosynthetic machinery needed to assemble on the scaffold proteins the unique sulfated glycans that bind to L-selectin (13). MAdCAM1 is an addressin for the integrin α4β7, as well as itself being a scaffold for PNAd attachment. Vascular MAdCAM1 expression in the mLN is LTβR-dependent, although inflammatory signals such as TNF or secreted LTα can also induce MAdCAM1 expression (13). MAdCAM1 expression on the sinus floor of the splenic marginal zone and on FDCs is also LTβR-dependent as is mostly likely the expression on MRCs and the LN subcapsular sinus (105). Vascular MAdCAM1 is elevated in colitis in an LTβR-dependent manner; however, it is unresolved whether this component is indirect and secondary to effects on the disease processes (14).

HEV can readily accumulate radiolabeled sulfate and contain elevated levels of the machinery involved in sulfate uptake and transport (106, 107). In addition to specific sulfated glycan that comprise PNAd, sulfation is also involved in chemokine retention. Heparin sulfate is crucial for endothelial chemokine binding and lymphocyte trafficking as well as DC recruitment to the lymphatics (108). Whether there is any dependence on LTβR signaling for endothelial sulfate capture or sulfated matrix components has not been explored.

While considerable progress has been made uncovering the role of the LT pathway in lymphoid vascular biology, there remain murky aspects of HEV development and specialization within unique environments, e.g., mucosal, peripheral, TLT, tumor, etc. For example, the homeodomain transcription factor Nkx2.3 controls vascular compartmentalization in the spleen yet its deletion led to the formation of splenic HEV-like vasculature complete with expression of PNAd and CCL21 (109). HEV formation remained LTβR-dependent in line with the appearance of splenic PNAd positive structures in a mouse with a constitutive gain in alternative NFκB signaling (110). Nkx2.3 deficiency also led to altered splenic FRC structures and the presence of LYVE1 positive lymphatic-like structures thus pivoting the spleen toward an "LN architecture" (111).

LYMPHATICS

The afferent lymphatics transport cells and soluble substances from the draining tissue to the subcapsular sinus. From the subcapsular sinus, DC find their way into the T zone parenchyma while T cells either flush through or they can enter the LN parenchyma via peripheral medullary sinuses (112). Recirculating lymphocytes leave the LN parenchyma by entering cortical sinuses that feed into medullary sinuses and efferent lymphatics (113). The lymphatic endothelial cells are an important source of sphingosine-1-phosphate that elicits lymphocytes to leave the parenchyma and enter the sinuses (114). Whether LTβR signaling is crucial for lymphatic function is less clear, although defects in lymphatic function were observed in LT-deficient mice (115).

LYMPHOTOXIN PATHWAY AND ADAPTATION IN THE REACTIVE STATE

During immune responses, the reticular networks and the endothelium undergo growth and remodeling with an overall increase in LN cellularity. Such enlarged LN are termed "reactive" and normally the LN involute and return to the resting state once the triggering stimulus is resolved. Experimentally, reactive LN are induced by immunization with an adjuvant such as alum, complete Freund's adjuvant (CFA) or Montanide, or by infection with a pathogen. Total LN cellularity in both the resting and hypertrophic reactive state is LTβR-dependent (99, 116, 117). Whether the stroma or vasculature bears an imprint of prior reactivity is unknown, although gross lymphangiogenesis following inflammation is certainly reversible (118).

RETICULAR NETWORK

Within the T zone, the reticular network expands with the enlarging T zone and the stromal cells undergoing proliferative expansion (119–121). The concentration of reticular fibrils under the follicles where T cells frequently interact with DC is known as the cortical ridge and this ridge becomes more prominent after immunization with ovalbumin (OVA) in alum (119). Following immunization with OVA in CFA, the stromal cells undergo an initial proliferative burst between days 0 and 2 that is dependent on CD11c+ DC cells and independent of lymphocytes. This burst is followed by continued proliferation and expansion in cell numbers that is detectable by day 5. The expansion was abrogated in the absence of lymphocytes and B cells contributed to the full expansion, while T cells contributed to the continued high proliferation rate (120). Recently, Luther and colleagues carefully analyzed changes in the FRC network following immunization with OVA in the adjuvant Montanide in mice containing transferred OVA specific T cells (121). Similar to changes induced with OVA/CFA, OVA/Montanide-induced a rapid CD11c+ cell-dependent stromal proliferation, and modest stromal expansion could be detected within 40 h. Stromal cells continued to proliferate and expand, and the expansion by day 5.5–6 was abrogated in RAG2$^{-/-}$ mice. Significantly, this study revealed that LTβR-Ig treatment did not affect the modest expansion at day 3, but did reduce FRC expansion at day 5.5–6 suggesting a role for LTα/β expressed by lymphocytes. A number of genes were previously shown to be altered in activated FRC and in this recent study, these cells displayed elevated levels of podoplanin and smooth muscle actin (86, 121). While maintenance of the resting FRC is not obviously under LTβR control, it is clear that events occurring in reactive FRC are LT/LIGHT-dependent and this dependence may underlie partially the profound effects of LTβR-Ig treatment on reactive LN. Again, the reticular changes occurring during inflammation appear to recapitulate programs that are utilized during development.

As hinted above, reticular networks may differ in their response to various immunological challenges including acute vs. chronic settings. For example, in contrast to Alum, CFA as an adjuvant leads to disruption of normal ER-TR7 patterning (ER-TR7 is a marker of collagen fibrils that is usually a surrogate for FRC presence), with loss of distinct B and T zones (119). A more detailed analysis of the time course is needed to determine whether the distinctions between Alum and CFA may in part reflect differences in kinetics. During a viral response, such as with LCMV infection, CCL21 and CXCL13 chemokine expression is downregulated during the first 8 days in the spleen and this response was partly dependent on interferon-γ but was minimally impacted by LTβR-Ig (119, 122, 123). The same process occurs in draining LN (123). Although, the LCMV clone-13 strain can infect the FRCs, this phenomenon of chemokine downregulation also occurred with OVA/LPS suggesting that this is a general phenomenon in inflamed lymphoid tissues (123). The loss of chemokine expression with viral infection may be in part attributable to loss of stromal cells (122). After this initial viral-induced stromal disruption, stromal cells recover and lymphoid organization is gradually restored. It is this process of recovery that is accelerated by LTi cells and is at least partially dependent on LTβR (122).

During LN hypertrophy, some of the most notable changes occur in the medulla (121, 124). This compartment normally appears condensed at homeostasis, but upon immune stimulation, the region swells and fills with lymphocytes. By day 6 after NP-OVA/CFA immunization, the parenchyma is enlarged relative to the area covered by the lymphatic marker LYVE1, and areas of collagen IV reticulum-rich and reticulum-poor areas can be identified. B cells are preferentially localized to the reticulum-poor area and newly generated plasma cells localize to the reticulum-rich areas. The significance of this compartmentalization remains to be elucidated. Medullary remodeling was reduced by LTβR-Ig treatment in a manner that appeared independent of its effects on FDC and HEV (124).

VASCULATURE

Following immunization, the vasculature also undergoes proliferative expansion that is accompanied by major phenotypic changes in the HEV. HEV, non-HEV blood endothelial cells, and lymphatic endothelial cells all proliferate and expand coordinately with remodeling of the feeding arteriole to deliver more blood flow (100, 120, 125–128). Similar to stromal cell growth, endothelial cells in LNs stimulated with OVA/CFA undergo initial CD11c+ cell-dependent proliferation followed by lymphocyte-dependent expansion (120, 125). HEVs can be seen to grow in length, width, and branching as imaged using optical projection tomography (116, 129). The HEV expansion triggered by LCMV infection is sensitive to LTβR-Ig and partially dependent upon B cell-derived LT. As B cells are not required for HEV maintenance at homeostasis (99, 100), this role of B cell-derived LTβ appears to be specific to the inflamed LN. With inflammation, there is the phenomenon of venularization whereby adjacent endothelium adopts a post-capillary venule phenotype (130). In inflamed LN, this change seems to occur as HEV (i.e., PNAd+) endothelial cells expand to a greater degree than PNAd-blood endothelial cells (120). One unresolved question is whether the reduction of HEV expansion in the absence of LTβR signaling reflects inhibition of HEV differentiation or inhibition of HEV proliferation? Anderson and colleagues observed that endothelial cell proliferation occurred frequently at transitions between high and flat endothelium, consistent with simultaneous proliferation and differentiation (131). Another area that is still poorly understood is whether the LTβR-dependent HEV expansion reflects LTβR function in reticular cells, DC, or the endothelial cells.

In the reactive LN, numbers of both blood vessels and lymphatic sinuses increase and this expansion is accompanied by lymphatic endothelial cell proliferation (100, 120, 126). The increase in the number of lymphatic structures was partly sensitive to LTβR-Ig (100), but it is unknown whether LTβR-Ig blocked proliferation or impacted other facets of lymphatic growth. Somewhat surprisingly, LTβ$^{-/-}$ mice showed greater lymphangiogenesis and angiogenesis in inflamed lung and skin and hence, the general role of LTβR in LN vascular growth remains ill-defined (115). In contrast, LTα$^{-/-}$ mice showed reduced lymphatic function and reduced lymphangiogenesis with skin inflammation, and transgenic LTα expression drove lymphangiogenesis, suggesting a pro-lymphangiogenic role for LTα3 as well as a role for LTαβ in "sequestering" LTα subunits. In agreement with a role for LTα3,

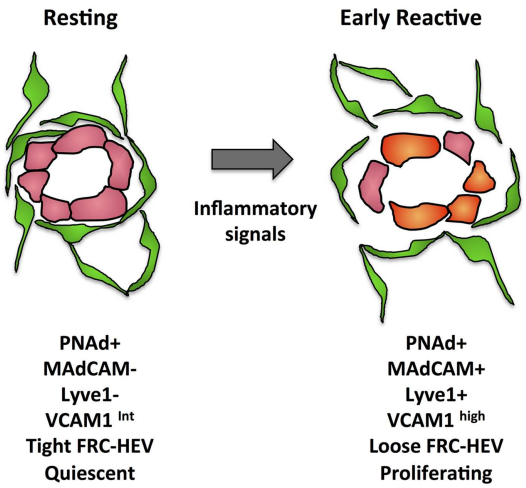

FIGURE 4 | Illustration of some of the changes that occur in the FRC-vascular units during the resting to reactive transition in a lymph node.

TNF inhibition or TNFR1 deficiency were shown to partially reduce lymphangiogenesis in lung (132).

In addition to proliferative expansion, the HEVs also undergo phenotypic alterations. The phenotypic changes seen in reactive HEV are illustrated in **Figure 4**. At day 1 after injection of bone marrow derived DCs or OVA/CFA immunization, VCAM1 is upregulated and there is a greater trafficking of lymphocytes inwards via HEVs (104). This early change corresponds to disruption of the tight sheath of FRCs around vessels and the HEV activation is similar to the early ICAM1 upregulation seen after fever-range thermal stress (104, 133). By day 4 after oxazalone stimulation, HEV endothelial cells show reduced expression of proteins associated with the mature HEV program, i.e., LTβR, the scaffold protein GlyCAM1, and the sulfo-transferase HEC-GlcNAc6ST (100, 134). The downregulation of these HEV program proteins was accompanied by upregulation of MADCAM1 perhaps reflecting an immature HEV, since MADCAM1 is normally only expressed in peripheral nodes during development and in mucosal lymphoid tissues (100, 135). In the same time frame, LTβR expression decreases and PNAd and LYVE1 were colocalized despite LYVE1 being a typical lymphatic marker (100). It was unclear from this study whether this colocalization resulted from lymphatics that expressed PNAd or HEVs that expressed LYVE1. However, vessels with the morphology of HEVs in non-Hodgkins lymphoma patients expressed LYVE1 and blood vessels during development can express LYVE1, suggesting that the colocalization of PNAd with LYVE1 represents HEVs that have upregulated LYVE1 (136, 137). This PNAd/LYVE1 colocalization at day 4 was blocked by LTβR-Ig treatment at day 0 (100). By day 7, the mature HEV markers began to rebound while MADCAM1 and LYVE1 regressed, and this rebound phase was dependent on B cells and also sensitive to LTβR-Ig.

The reprograming of the HEV differentiation state as a LN enters the reactive state was noted to be similar to changes occurring after surgical interruption of afferent lymphatic flow (99, 100, 138). Consistent with this notion, Ruddle and colleagues showed a transient reduction in LN accumulation of subcutaneously injected Evans blue and skin-derived DC in the first 4 days after the onset of inflammation, which was followed by increased lymphatic flow (100). In contrast, Randolph and colleagues showed increased LN accumulation of skin-derived DC in the same time frame (126). These studies both applied FITC epicutaneously in pre-immunized mice and then tracked FITC+ DC, and it remains unresolved whether the differing results reflected different preimmunization strategies (oxazalone application vs. KLH/CFA injection) or different kinetics of these strategies. In human subjects, Braathen and colleagues measured lymphatic flow via cannulation of a dermal lymphatic, and found that application of a skin irritant resulted in a transient reduction in dermal lymphatic drainage before the drainage increased to above baseline (139). Whether the transient decrease was related to the trauma of skin cannulation 2 days prior is a caveat in interpreting the findings, although the trauma itself could be taken as a form of immune stimulation. However, with regard to the idea of HEV reprograming, the basic biological event that underlies this phenomenon remains a question, i.e., is it the disrupted flow, the inflammation or a dilution of LTβR signaling due to loss of ligand and/or receptor? Do the HEV in the inflamed LN need transport of soluble factors or cells via the lymphatics to prevent the reprograming?

PUTTING IT TOGETHER IN THE REACTIVE NODE: COORDINATE REGULATION OF VASCULATURE AND STROMAL ALTERATIONS AND THE ROLE FOR LTβR SIGNALING

Figure 5 presents a generalized view of some of the changes occurring in the LN as it cycles between resting and reactivity. In the first 2 days following immunization with OVA/CFA or bone marrow derived DCs, there is a lymphocyte-independent initiation phase marked by endothelial and reticular cell proliferation with parallel VCAM1 upregulation on HEV. From days 3–5, there is an expansion phase whereby endothelial cells and stromal cells continue to show high levels of proliferation and now noticeably expand in number. This expansion in most cases is at least partly dependent upon B cells and T cells contribute partially to the continued high level of endothelial and stromal cell proliferation during this phase (100, 120, 121, 124–126). The expansion phase correlates well with the morphologic HEV expansion that is dependent on B cell-derived LTβ and the LTβR-Ig sensitive OVA/Montanide-induced FRC expansion (100, 116, 121). In this context, the upregulation of MADCAM1 and downmodulation of GlyCAM1 and HEV-GlcNA6ST observed by day 4 and could potentially represent a reinstatement of the HEV programing that typically occurs during early LN anlage development either in early activated HEV cells or in newly expanded cells (100). Assuming that the colocalization of LYVE1 with PNAd by day 4 represents upregulation on HEV cells, consideration of the potential significance of LYVE1 alterations may provide some clues. LYVE1 on developing skin collecting lymphatic vessels is downregulated with the recruitment of smooth muscle cells (140), correlating vascular destabilization/immaturity with LYVE1 expression. During the initiation phase, the tight, presumably stabilizing sheath

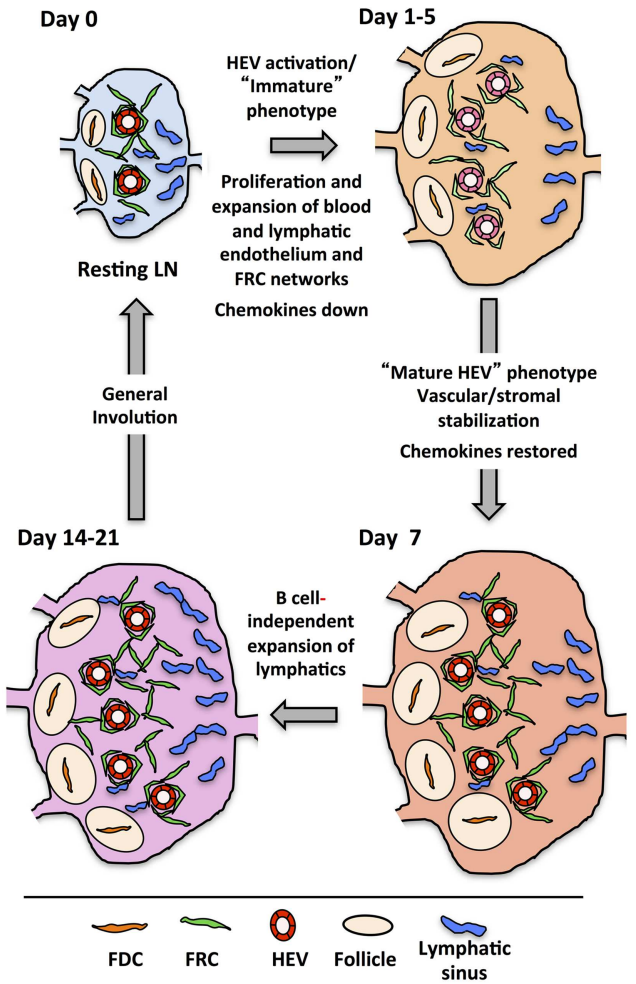

FIGURE 5 | Drawing shows the cycle that lymph nodes transit from resting to reactivity with some of the changes occurring at each stage. The actual timing of each stage will depend on the nature of the reactive trigger, e.g., immune complex, innate stimuli, virus, etc., and its subsequent clearance.

of FRCs around the vessels is disrupted and vascular permeability is increased (104, 131). It is conceivable that upregulation of LYVE1 on HEV cells, like VCAM1 upregulation is an early activation marker that reflects a state of HEV destabilization. Taking the LYVE1 upregulation as a marker of early HEV activation, then, both early HEV activation as well as subsequent expansion appears to be dependent on LTβR, and we would predict that the MADCAM1, GlyCAM1, and HEC-GlcNA6ST alterations are also LTβR-dependent.

The disrupted organization of FRCs around vessels during the initiation phase probably reflects a more general reticular network disorganization that was seen with OVA/CFA or, with a mild delay, with viral infection. The viral-induced stromal dysfunction was minimally sensitive to LTβR-Ig (123) suggesting that the reticular network disruption may be relatively LTβR-independent. Thus, early vascular and stromal changes in the reactive node may be differentially regulated by LTβR signals. This is in contrast to the later, LTβR-dependent reticular remodeling.

After the expansion phase, the vasculature undergoes a phase wherein quiescence and stabilization are re-established. By day 7, mature HEV markers rebound, robust endothelial cell proliferation slows and FRCs re-organize more tightly around the vessels (100, 104). Thus, restoration of mature HEV markers parallels the re-establishment of vascular quiescence/stabilization and stromal integrity. The LTβR-dependent stromal recovery after viral infection that was studied in the spleen may correspond to this phase in the LN, and, if so, would further support the idea of a global vascular-stromal re-stabilization. Whether, LTβR signals are needed for all the vascular changes and how the medullary remodeling is connected is unknown.

Following the vascular quiescence and stabilization phase, there is additional growth, at least in the lymphatic vasculature as well as considerable medullary remodeling (100, 124, 141). Lymphatic expansion is B cell dependent up to at least day 7, but by day 14, lymphangiogenesis is occurring in the absence of B cells (100). This late lymphangiogenesis coincides with expansion of cortical and medullary lymphatic sinus relative to that of subcapsular sinuses, which expanded at an earlier time point (141). This observation suggests that some cortical and medullary expansion is B cell-independent. LTβR signaling appears to be important for medullary remodeling, but the actual specifics of which events are being regulated needs further investigation. Any role for the LT pathway in the restoration of homeostasis or the "involution phase" is also unexplored.

In conclusion, LTβR signals in the reactive LN are needed for multiple phases of vascular and stromal alterations, yet major elements of this picture remain out-of-focus. How exactly does LTβR control the initial growth and phenotypic alterations? Do these requirements reflect homeostatic functions of LTβR or are these new functions in the context of an inflamed LN? Fu and coworkers recently showed that LIGHT-LTβR signaling was important for the growth of reactive nodes but not for homeostatic LN cellularity suggesting that additional LTβR ligands and functions are engaged in the reactive setting (117). Lastly, does the rebound of mature HEV markers and the stromal re-organization indicate an increased level of LTβR signaling during the phase of re-established quiescence and stabilization?

LTβR AND TISSUE REMODELING IN DISEASE

CHRONIC REACTIVITY IN LYMPH NODES

Chronic LN reactivity can change lymphoid architecture and this phenomenon has been well described especially in the context of viral infection (2, 3, 142). In primates, both HIV and SIV infection led to altered FRC function and a lack of T cell survival support. Interestingly, the chronic reactivity in HIV infected LN culminates in fibrosis perhaps mimicking aspects of other fibrotic diseases (143). This observation is consistent with increased smooth muscle actin expression in the FRC in reactive murine LN (121). Reactive LNs, hyperplasia, lymphadenopathy, and probably imbalanced reticular networks often characterize autoimmune disease (144). For example, abnormal FDC networks were noted in some SLE patients, albeit there is considerable heterogeneity in this population (145, 146). Additionally in rodent models of lupus, FDC

networks are altered in the MRL.lpr mouse (147). Aging is also associated with some degradation of lymphoid architecture (148). In most of these cases, the relationship to potentially impoverished LTβR signaling is unknown. Given the need for organized lymphoid architecture in a well-functioning immune system, this remains an important area for further investigation.

TERTIARY LYMPHOID TISSUES

Chronic inflammation drives the formation of semi-organized lymphoid structures in basically all organ settings including lungs, heart, stomach, intestine, kidney, CNS, glands, skin, joints, and vasculature (149–153). These TLT are also present physiologically in the gut where they develop upon colonization with the microbiome (154). A wide spectrum of structures can be observed ranging from relatively poorly organized perivascular aggregates to more complete LN-like structures that display HEV development, T/B cell segregation, GC formation, and the presence of specialized FDC and FRC reticular networks (152, 153). For example mature TLT are found in salivary glands in female NOD mice, yet a B cell dominated structure lacking FDC networks is observed in the young male NOD lacrimal glands (155, 156). TLT can accompany Th1 and Th17 driven responses and even assemble next to classical *Mycobacterium*-driven granulomas (157–159). Notably, TLT are induced by ectopic expression of LTα or combined LTα and LTβ in multiple organ systems and these studies have provided considerable insight into the biology of these structures (42, 149, 160). There appear to be multiple routes to TLT formation including those induced by RORγt positive cells such as type III innate lymphoid cells (ILC-3) in the gut as well as RORγt independent events (161). Simple ectopic overexpression of several chemokines is sufficient to culminate in TLT formation (152). In general, regardless of the inducing trigger, signaling by LTβR is essential for the formation of mature TLT in most settings with the exceptions being relatively small, more poorly organized structures (14, 91, 152, 162). FDC and especially HEV formation in TLT is clearly reduced following pharmacological inhibition of the LT pathway (155, 156, 162). In a mouse pancreas model, large TLT were dissociated by LT pathway inhibition, yet in the small remaining residual T cell rich zones, the FRC networks appeared fully developed with conduits (91). This result is consistent with the viewpoint that FRC maintenance is LT pathway independent.

In a major knowledge gap, the contribution of TLT to pathology is defined predominately by correlation and association. The presence of TLT in man is associated with more severe disease, e.g., in juvenile dermatomyositis, Sjogren's syndrome, and multiple sclerosis (163–165). In rodents, TLT can enhance viral defense in lung infections, naïve T cell recruitment, and epitope spreading in diabetes and exacerbate heart allograft rejection (152, 166–168). Furthermore, HEV can be found in the absence of mature TLT and, indeed, simply the emergence of cardiac HEV is a strongly prognostic for pending heart graft rejection (169).

TUMORS

The role of the LT pathway in tumor biology has become an active area. LTβR signaling can promote tumor metastasis by maintaining a level of pro-inflammatory signaling (170–173). In preexisting tumors, the formation of HEV within tumors appears to be beneficial presumably by allowing for entry of a wider range of lymphocyte subsets and enhanced tumor immunity (174). HEV are found in melanoma tumors often in close proximity to LTβ-expressing DC suggesting that processes are in play analogous to those maintaining HEV in LN (43).

FUTURE DIRECTIONS

A clear picture of the integration of the vascular and reticular networks in the primary, secondary, and tertiary immune organs in resting, reactive, and pathological states is essential to an understanding of how the immune system maintains optimal sensitivity and selectivity. It is also reasonable to assume that many pathological events revolve not only around altered adaptive and innate immunology, but also on altered stromal elements, e.g., HIV infection. The LT system is clearly interwoven in some of these processes, yet many of the studies have blurred the distinction between developmental and maintenance controls. More work is required especially at the clinical level to understand the contributions of the LT system in human disease and it is fortuitous that various clinical interventions are targeting LT (anti-LTα antibody), LIGHT (anti-LIGHT antibody), and both LT and LIGHT (LTβR-Ig).

Certainly studies of stromal cell states in lymphoid tissues serve to expand the basic knowledge of these differentiation programs and the reactive state is a model of controlled inflammation in a lymphocyte-rich environment. Importantly, the LN can return to the resting state – a perfect example of physiological tissue remodeling. Studying the lymphoid setting may help one comprehend the interplay between chronic inflammation, vascular damage, and stromal cell activation present in many diseases. Given the success of recent immunomodulatory strategies in oncology, manipulation of the HEV entry portals for lymphocytes as well as modification of lymphocyte–tumor stroma interactions could be very productive approaches (174, 175). There is considerable interest in the control of the differentiation pathways leading from mesenchymal lineages into the myofibroblastoid cells driving pathological tissue remodeling and fibrosis (176). For example, vascular disruption and dysfunctional pericyte-endothelial cell interaction appears to be occurring in many pathological conditions including scleroderma, interstitial lung disease, lupus, multiple sclerosis, some neurodegenerative diseases and diabetic retinopathy, to name a few (67). It is possible that important lessons can be gleaned from the analysis of these cells in lymphoid microenvironments.

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TWEAK and LTβ signaling during chronic liver disease

Benjamin J. Dwyer[1], John K. Olynyk[1,2,3,4], Grant A. Ramm[5,6] and Janina E. E. Tirnitz-Parker[1,2]*

[1] School of Biomedical Sciences, CHIRI Biosciences Research Precinct, Curtin University, Bentley, WA, Australia
[2] School of Medicine and Pharmacology, University of Western Australia, Fremantle, WA, Australia
[3] Department of Gastroenterology, Fremantle Hospital, Fremantle, WA, Australia
[4] Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, WA, Australia
[5] Faculty of Medicine and Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia
[6] QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia

Edited by:
Linda C. Burkly, Biogen Idec, Inc., USA

Reviewed by:
Urs Christen, Klinikum der Goethe Universität Frankfurt, Germany
Takayuki Yoshimoto, Tokyo Medical University, Japan
Yury Popov, Beth Israel Deaconess Medical Center, USA

***Correspondence:**
Janina E. E. Tirnitz-Parker, Liver Disease and Regeneration Laboratory, School of Biomedical Sciences, CHIRI Biosciences Research Precinct, Curtin University, Kent Street, Bentley, WA 6102, Australia
e-mail: n.tirnitz-parker@curtin.edu.au

Chronic liver diseases (CLD) such as hepatitis B and C virus infection, alcoholic liver disease, and non-alcoholic steatohepatitis are associated with hepatocellular necrosis, continual inflammation, and hepatic fibrosis. The induced microenvironment triggers the activation of liver-resident progenitor cells (LPCs) while hepatocyte replication is inhibited. In the early injury stages, LPCs regenerate the liver by proliferation, migration to sites of injury, and differentiation into functional biliary epithelial cells or hepatocytes. However, when this process becomes dysregulated, wound healing can progress to pathological fibrosis, cirrhosis, and eventually hepatocellular carcinoma. The other key mediators in the pathogenesis of progressive CLD are fibrosis-driving, activated hepatic stellate cells (HSCs) that usually proliferate in very close spatial association with LPCs. Recent studies from our group and others have suggested the potential for cytokine and chemokine cross-talk between LPCs and HSCs, which is mainly driven by the tumor necrosis factor (TNF) family members, TNF-like weak inducer of apoptosis (TWEAK) and lymphotoxin-β, potentially dictating the pathological outcomes of chronic liver injury.

Keywords: liver progenitor cells, hepatic stellate cells, regeneration, fibrosis, cancer, TWEAK, LTβ, NFκB

INTRODUCTION

The liver possesses an extraordinary ability to orchestrate hepatocyte-mediated regeneration from acute injuries such as tissue resection or hepatic necrosis. However, hepatocyte proliferation is impaired or ablated by severe and chronic injury, dictating the need for an alternative liver regeneration pathway. Through a complex network of chemical and cellular mediators, the liver regenerates via the activation of a progenitor cell compartment, which retains proliferative and restorative capacity under severe injury conditions. Early chronic liver disease is typified by hepatocellular necrosis, hepatic inflammation, and release of immunomodulatory molecules by resident and recruited inflammatory cells, and dying hepatocytes, which activate the regenerative and fibrogenic wound healing responses.

The regenerative response consists of the "ductular reaction" (DR), in which bile ductules and liver progenitor cells (LPCs) proliferate from the Canals of Hering, the interface between the hepatocyte canaliculi and the biliary tree (1), resulting in biliary hyperplasia and the appearance of intermediate hepatocytes (2). Recent lineage-labeling studies have demonstrated that LPCs arise from a population of Sox9-expressing ductal cells that are activated to proliferate and differentiate into hepatocytes under certain chronic liver injury conditions (3, 4). This expansion is initiated and maintained by inflammatory cell-derived stimuli such as tumor necrosis factor (TNF) (5, 6) and interferon (IFN)-γ (7). Importantly, IFNγ has been shown to promote LPC expansion and inhibit proliferation of hepatocytes in concert with TNF stimulation (7). Additionally, transforming growth factor (TGF)-β suppresses liver epithelial cell proliferation. However, LPCs are significantly less sensitive to TGFβ-mediated growth inhibition during chronic liver injury and *in vitro* (8). In addition to stimuli from inflammatory cells, dying hepatocytes release hedgehog ligand (9), which has recently been shown to signal via the canonical Smoothened-dependent signaling cascade in primary cilium-positive LPCs, promoting their proliferation (10). Thus, the consequence of chronic hepatocyte injury and the subsequent inflammatory response is a liver microenvironment, which supports LPC expansion, while disabling hepatocyte-mediated regeneration.

LPC expansion occurs almost synchronously with fibrogenic wound healing, which is primarily driven by the action of hepatic stellate cells (HSCs). During chronic liver disease, quiescent HSCs are "activated" by inflammatory cytokines and begin to express α-smooth muscle actin (αSMA), signifying their transition to a myofibroblastic phenotype (11). Following this transition, activated HSCs drive fibrosis by depositing extracellular matrix (ECM) proteins, which assists to control LPC proliferation and differentiation. Accumulation of ECM is supported by the expression of tissue inhibitor of metalloproteinase (TIMP) proteins that inhibit matrix metalloproteases (MMPs), which function to degrade ECM proteins (12). Activated HSCs further reinforce regenerative and repair responses by expressing chemotactic factors such as intercellular adhesion molecule 1 (ICAM-1) and regulated upon activation, normal T-cell expressed, and secreted (RANTES), which attract additional inflammatory and progenitor cells to the site of injury (13). If the hepatic insult is resolved, LPCs mature to replace the lost epithelial cell types, hepatocytes, and/or cholangiocytes, depending on the underlying pathology. At the same time fibrosis recedes restoring structural and functional

integrity of the liver. However, if injury persists, the regenerative and wound healing processes spiral out of control and become pathological. Chronic stimulation of HSCs results in fibrosis from excessive matrix deposition. With further impaired regeneration, this may progress to cirrhosis and liver failure. Furthermore, the prolonged stimulation of LPCs by pro-proliferative/survival cytokines from inflammatory cells generates a niche favoring the accumulation of genetic and epigenetic alterations, which can lead to the malignant transformation of LPCs and ultimately the formation of hepatocellular carcinoma (HCC).

A combination of association studies in patients and pathway manipulation experiments in rodents implicate LPCs as key regulatory cells in progressive chronic liver injury. LPCs are observed in many diseases with a predisposition to HCC, including chronic hepatitis B (HBV) and C virus (HCV) infection (14, 15), non-alcoholic fatty liver disease (16), alcoholic liver disease, and genetic hemochromatosis (14). Importantly, the numbers of LPCs increase as liver fibrosis progresses to cirrhosis, regardless of the underlying liver pathology (14, 17). Since LPCs proliferate at various stages of human liver tumorigenesis, ranging from preneoplastic lesions (18) to well-developed HCCs (19–21), they have been suggested as causative players during tumor development and maintenance. Supporting evidence comes from murine intervention studies where selective inhibition of c-kit$^+$ LPCs by imatinib mesylate resulted in reduced tumor formation (6). These studies suggest that LPCs either represent direct cellular precursors of HCC or they crucially influence disease development by regulating other contributing cells such as fibrosis-driving HSCs. Either way they represent ideal chemotherapeutic targets for HCC prevention strategies in chronic liver injury.

The processes of inflammation, fibrosis, and LPC induction are tightly regulated and occur in close spatial association (**Figure 1**), suggesting the potential for cellular communication (**Figure 2**). Cross-talk between the hepatic wound healing and regenerative responses occurs via several factors including ECM proteins, growth factors, and cytokines, particularly of the TNF superfamily. In this review, we will discuss the role of two TNF superfamily members, lymphotoxin-β (LTβ), and TNF-like weak inducer of apoptosis (TWEAK), in regulating liver regeneration and wound healing.

TWEAK/FN14 SIGNALING

TWEAK/FN14 SIGNALING INDUCES LPC PROLIFERATION VIA NFκB ACTIVATION

TWEAK ligand was first identified as a novel cell-associated and secreted factor with TNF family homology, which induced cytotoxicity in the human adenocarcinoma cell line HT29 in combination with IFNϒ treatment (28). TWEAK interacts with target cells via its receptor, fibroblast growth factor-inducible 14 (Fn14) (29), which is highly homologous in mouse and human tissues, and is upregulated in HCC lines and tissues (30). Biologically, TWEAK has been shown to regulate numerous cellular processes including proliferation, differentiation, migration, and cell survival and has also been described as a pro-angiogenic and

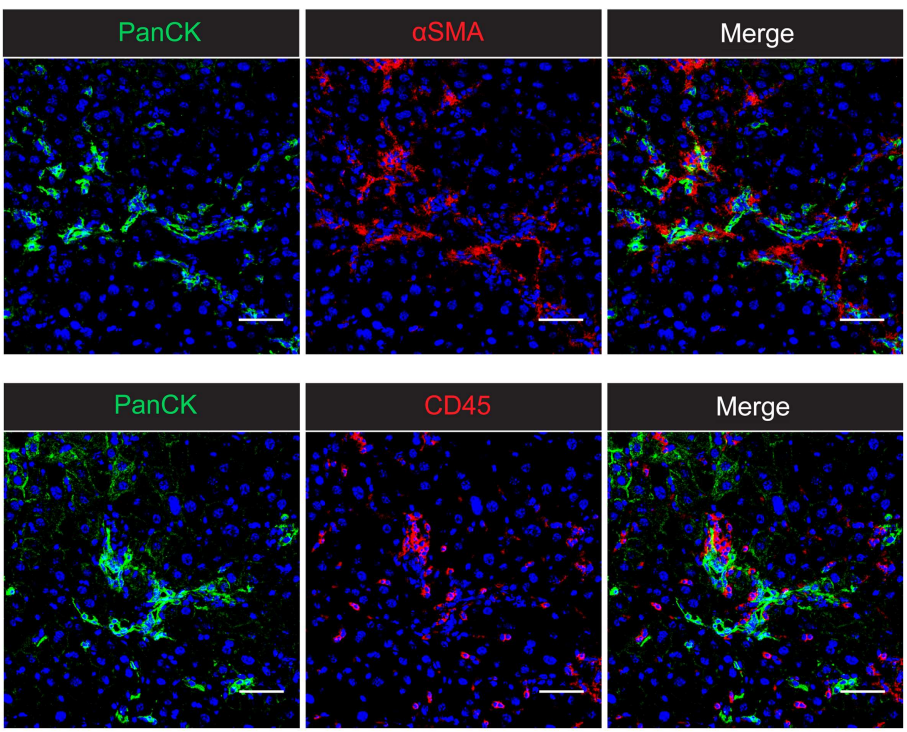

FIGURE 1 | The intrahepatic wound healing and regenerative niche in CDE-induced chronic liver injury in mice. Confocal microscopy of 2-week CDE-injured mouse liver shows the close association of PanCK$^+$ liver progenitor cells with both αSMA$^+$ activated hepatic stellate cells and CD45$^+$ inflammatory cells. The spatial proximity of all three cell types suggests the potential for cellular cross-talk and co-regulation of the inflammatory, fibrogenic, and progenitor cell responses. Nuclei are stained with DAPI (blue). Scale bars represent 50 μm.

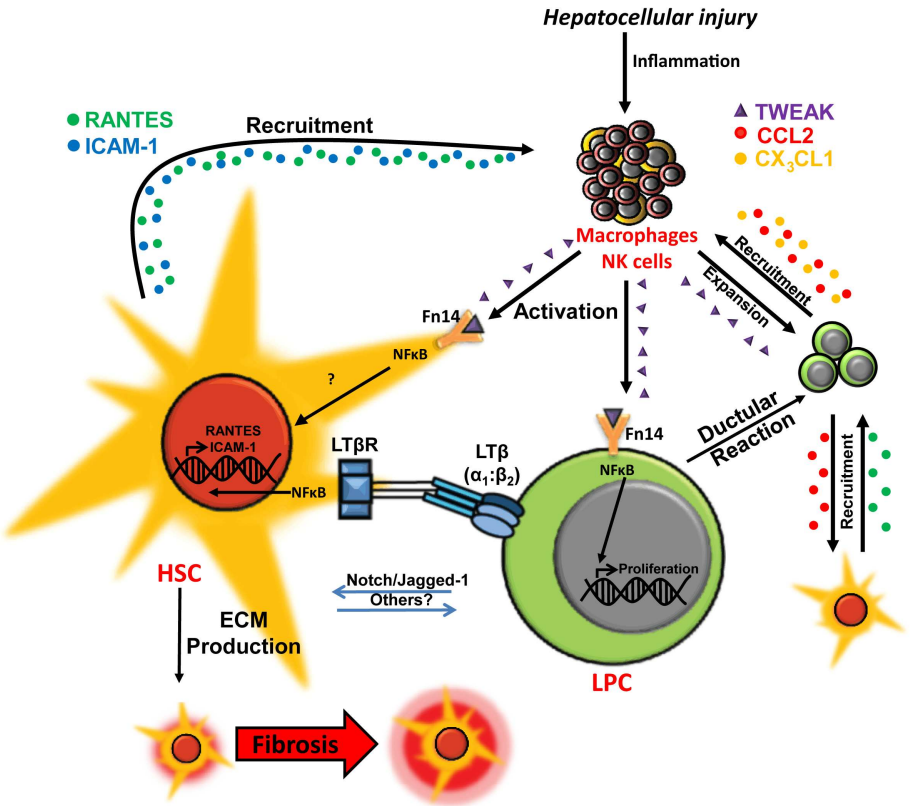

FIGURE 2 | Co-regulation of hepatic wound healing and regenerative responses via TWEAK- and LTβ-mediated cellular cross-talk. In chronic liver injury liver progenitor cell (LPC) proliferation is induced via macrophage/natural killer (NK) cell-produced TWEAK activation of the NFκB pathway. Interaction between LPCs and activated hepatic stellate cells (HSCs)/myofibroblasts via Notch pathway and other potential mediators may drive LPC differentiation to cholangiocytes. LPCs and ductular reaction cells produce chemokines, such as CCL2 (red) and CX_3CL1 (orange), which promote chemotaxis of inflammatory/myofibroblastic cells. Interactions between lymphotoxin-β (LTβ) expressed on LPCs and the LTβ receptor (LTβR) on activated HSCs triggers NFκB-driven expression of chemotaxis-associated factors ICAM-1 (blue) and RANTES (green) by HSCs, which may play a role in mediating recruitment of LPCs, HSCs, and leukocytes for wound healing, fibrogenesis and regeneration during chronic liver injury (13, 22–27).

pro-inflammatory factor (31). In chronic liver injury and repair, the principal function of TWEAK appears to initiate ductal proliferation and LPC expansion via activation of NFκB signaling. Ductal hyperplasia is observed in livers of mice overexpressing TWEAK under the control of the liver-specific α1-antitrypsin promoter, demonstrating the ability of TWEAK signaling, in isolation, to initiate ductal expansion (32). Conversely, ductal cell expansion is mitigated after pharmacological blocking of TWEAK signaling as well as in Fn14-knockout mice subjected to either 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or choline-deficient, ethionine-supplemented (CDE)-induced chronic liver injury (22, 32). Furthermore, macrophages and natural killer cells have been shown to be the primary producers of TWEAK ligand in chronic liver injury, providing an important link between the inflammatory response and TWEAK-mediated ductal expansion (33). In the CDE model, TWEAK-producing macrophages have been observed in close association with expanding ductal cells (33), suggesting a mechanism whereby focal points of inflammatory cells signal via the TWEAK/Fn14 pathway to promote LPC expansion. Accordingly, transplantation of bone marrow-derived macrophages into normal liver stimulates a TWEAK-dependent response in LPCs and biliary cells, demonstrating a primary role of macrophage-generated TWEAK in initiating the DR (23). Previous studies indicate that this effect is mediated via TWEAK-induced activation of NFκB signaling (22).

The NFκB family comprises five monomers, which interact via N-terminal Rel homology domain (RHD) polypeptides. These monomers, RelA, RelB, cRel, p50, and p52, interact to form an active NFκB homo- or heterodimer and translocate to the nucleus to activate transcription of genes involved in numerous biological processes including, but not limited to, inflammation, proliferation, and cell survival (34). Regulation of nuclear localization occurs by sequestering of NFκB dimers through binding with inhibitory proteins (IκBs). Inhibition is released through phosphorylation and subsequent proteolytic degradation of IκB. Phosphorylation of IκB is initiated by binding of extracellular mediators to their receptors, which lead to activation of IκB-kinase (IKK) via NFκB essential modulator (NEMO)-dependent (canonical) or independent (non-canonical) mechanisms (35). NFκB plays a vital role in all aspects of chronic liver diseases (CLD), acting as

a transducer of cytokine-mediated signals promoting inflammation and fibrosis (36), particularly in the survival and activation of HSCs (37, 38). NFκB signaling is activated upon TNF treatment of LPCs, promoting its mitogenic effects (39), and also regulates IL-6/TNF-mediated upregulation of LTβ in LPCs (40, 41).

TWEAK has been shown to activate NFκB via canonical (42) and non-canonical mechanisms (43). Accordingly, strong cytoplasmic and nuclear NFκB is observed in proliferating pan cytokeratin-expressing cholangiocytes and LPCs following recombinant TWEAK administration in CDE-injured mice (22). *In vitro* studies in the LPC lines BMOL and BMOL-TAT demonstrated dose-dependent proliferation following TWEAK stimulation. Mitogenesis was inhibited by transfection with siRNA targeting the NFκB p50 subunit, confirming the pro-proliferative effect of TWEAK in LPCs is NFκB-dependent (22). The ability of TWEAK to promote proliferation and self-renewal of LPCs appears to be vital in allowing LPCs to survive and thrive in the chronic liver injury setting. Interestingly, this modulation also affects the dynamics of hepatic fibrosis and inflammation.

TWEAK AS A REGULATOR OF FIBROGENESIS

Assessing the cellular players involved in TWEAK signaling provides unique insights into its versatile role in establishing a niche conducive to wound healing, regeneration as well as carcinogenesis. In addition to stimulating ductal/LPC expansion, one of the therapeutically interesting consequences of altering TWEAK signaling in chronic liver injury is the co-modulation of liver fibrosis. Experiments utilizing TWEAK pathway knockout mice and exogenous TWEAK stimulation/inhibition have shown a positive correlation between LPC proliferation and the fibrogenic response to chronic liver injury. Collagen deposition and the expression of TIMPs are reduced in Fn14-knockout mice on the CDE diet (22). Similarly, a recent study of TWEAK modulation in CCl_4-stimulated fibrosis with partial hepatectomy (PHx)-induced hepatocyte deficit has shown that pharmacological TWEAK inhibition decreases collagen deposition during LPC expansion following PHx (44).

Given that the main function of TWEAK signaling appears to be in activating LPC proliferation, how might the TWEAK pathway regulate the fibrotic response of the liver? One possibility that is yet to be investigated is that TWEAK could act directly on fibrosis-driving HSCs. Supportive evidence for this possible scenario is provided by results showing that at least a subset of freshly isolated, activated HSCs express the receptor Fn14 and are therefore potentially TWEAK-responsive in CDE-induced liver injury (22). Another intriguing possibility is that modulating TWEAK/Fn14 signaling could influence the potency of the fibrogenic and inflammatory responses by modulating ductal/LPC cross-talk with leukocytes and/or HSCs. In the next section of this review, we will explore the mechanisms by which TWEAK signaling might regulate the inflammatory and fibrogenic responses through its effects on the LPC compartment.

TWEAK REGULATION OF HEPATIC INFLAMMATION

Through its effects on LPC numbers, TWEAK/Fn14 signaling could affect the dynamics of inflammation in chronic liver injury via LPC-mediated recruitment of inflammatory cells. Fn14-deficient mice fed a CDE diet display a delayed response of $CD45^+$ (general inflammatory) and $F4/80^+$ (macrophage) leukocytes. Conversely, stimulation of LPC proliferation and the DR with recombinant TWEAK injections results in an increase in $CD45^+$ cells (22), suggesting that the amplitude of the DR might have an impact on the level of inflammatory cell recruitment. Given that LPCs express chemokine (C–C motif) ligand 2 (CCL2), also referred to as monocyte chemotactic protein-1 (MCP-1), and chemokine (C-X3-C motif) ligand 1 (CX_3CL1), and have a demonstrated ability to attract $CD11b^+$ macrophages isolated from normal and CDE-injured livers *in vitro* (33), the dynamics of the DR may play a role in regulating the inflammatory response. Since inflammatory cells, particularly the macrophage compartment, support the establishment of fibrosis in chronic liver injury by affecting HSC activation (45), influences on inflammatory cell recruitment may also affect the dynamics of liver fibrosis initiation and injury progression. Consequently, targeting the TWEAK/Fn14 pathway may be an effective way to alter the wound healing response by influencing the dynamics of LPC/inflammatory cell/HSC cross-talk.

LPC/HSC CROSS-TALK VIA LTβ SIGNALING

In an effort to understand the opposing roles of LPCs and HSCs in controlling regeneration and wound healing versus fibrogenesis progression and carcinogenesis, pathways involved in LPC/HSC cross-talk are currently under investigation. As we have discussed, modulating the LPC response via TWEAK signaling affects the dynamics of liver fibrosis mediated by HSCs. Additionally, Boulter *et al.* demonstrated regulation of LPC differentiation by activated HSCs through expression of the Notch ligand Jagged 1 and Notch-dependent biliary specification in adjacent LPCs (24). Conversely, expression of MCP-1 by cholangiocytes within hyperplastic or mature bile ducts was shown to drive HSC/myofibroblast chemotaxis in chronic cholestatic liver disease (25). These studies demonstrate the clear interactions of LPCs and HSCs during progressive chronic liver injury. Thus, interventions targeting pathways modulating LPC/HSC interactions might be of therapeutic benefit in patients with chronic liver disease. One such novel target is the TNF family member LTβ, which was recently discovered as a key regulator of LPC/HSC cross-talk, facilitated by NFκB-dependent downstream signaling (13).

LTβ is a type II transmembrane protein that signals as a cell surface-anchored heterotrimer with LTα (i.e., LTα2:β1 or predominantly LTα1:β2) (46). LTβ levels are increased in various animal chronic liver injury models including bile duct ligation (47) and CDE-induced injury (48, 49) and its expression correlates with the severity of fibrosis in chronic HCV infection in humans (50). It has been demonstrated on the surface of cells of the lymphocytic lineage, including activated B and T cells as well as natural killer cells (51) but interestingly also on small portal hepatocytes and proliferating LPCs during chronic liver injury (50). In our CDE injury model, these $LT\beta^+$ LPCs are observed in close proximity to activated HSCs, which express the LTβ receptor (LTβR). *In vitro* studies revealed that upon receptor binding, LTβ initiates a NFκB-dependent signaling cascade in HSCs that

results in expression of chemotaxis-associated mediators ICAM-1 and RANTES, which in turn recruit RANTES receptor (C–C chemokine receptor type 5, CCR5)-positive LPCs (13). Hence this paracrine cytokine/chemokine cross-talk has the capacity to facilitate LPC and HSC migration through the liver parenchyma to sites of injury in addition to promoting wound healing by recruitment of new leukocytes, LPCs and HSCs. The LTβ signaling pathway also plays a role in fibrogenesis, since chronically injured LTβR knockout mice show reduced numbers of αSMA^+ HSCs and decreased collagen deposition as evidenced by reduced Sirius Red staining (13). Concurrently, numbers of $A6^+$ and muscle pyruvate kinase 2 $(M_2PK)^+$ LPCs are reduced (48), which once again suggests the co-regulation of the fibrogenic and the progenitor cell response in chronic liver injury. The significance of the LTβ pathway in liver disease and hepatocarcinogenesis was highlighted by Haybaeck et al. who analyzed tg1223 mice, which overexpress lymphotoxin in the liver, and showed that sustained expression leads to chronic hepatitis and eventually to HCC. To confirm results, LTβ overexpression was neutralized by pharmacological blocking of LTβR, which drastically reduced liver injury and prevented HCC formation (19). Hence, suppression of this pathway might be beneficial in liver diseases with a chronic overexpression of cytokines that signal through LTβR, including LTα and LTβ or LTβ-related inducible ligand competing for glycoprotein D binding to herpes virus entry mediator on T cells (LIGHT), such as seen in chronic HBV or HCV infection.

THERAPEUTIC POTENTIAL AND FUTURE DIRECTIONS

In the Western world, chronic liver injury is ever increasing in prevalence. A variety of etiologies including chronic HBV/HCV infection, and non-alcoholic fatty liver disease can cause fibrosis and, subsequently, cirrhosis and HCC. The World Health Organization ranks CLD as the ninth commonest global cause of death with end organ failure as a result of cirrhosis and HCC, accounting for half the mortality each (52). Most HCC cases arise in the setting of established cirrhosis, with a median survival of 6–16 months, if untreated (53). At present, a range of primary treatment options including antiviral therapy and weight reduction strategies are variably effective in these conditions. Unfortunately, a significant number of patients still progress to end-stage liver disease and many require orthotopic liver transplantation. However, limited availability of donor organs and religious and/or economic reasons may restrict access to transplantation surgery. Prolonged waiting times for donor organs often result in disease progression and death of patients with initially treatable disease. Thus, the development of new therapeutic strategies for the prevention or treatment of hepatic fibrosis and its sequelae of cirrhosis and HCC are urgently required.

The carcinogenic and fibrogenic processes are amenable to manipulation by agents, which interfere with these processes, however to date approaches have been limited, and new targeted therapies such as tyrosine kinase inhibitors have not significantly improved survival (54, 55). Identification of new cellular targets for preventative therapies that minimize fibrosis or carcinogenesis represent the future for advancement of therapy, and knowledge of cross-talk and signaling pathways such as those defined here are critical for such advancement. The key will be balancing beneficial effects on reduced fibrosis and carcinogenesis against detrimental effects consequential to impaired replacement of healthy hepatocytes or cholangiocytes, as can be seen following IFN-based therapies for chronic viral hepatitis (56).

SUMMARY

The liver responds to chronic injury by initiating an inflammatory response, which enables the dual action of resolving injury, through the activation of fibrosis, and regeneration of injured tissue comprising the activation and differentiation of LPCs. These processes are inextricably linked and act in concert to reinforce and progress the chronic injury response until such a time that injury abates. Since these processes are linked, affecting the biology of one necessarily affects the dynamics of the whole system. As we have discussed in this review, TWEAK/Fn14 signaling plays a primary role in regulating LPC expansion and in doing so, affects both the inflammatory and wound healing responses of the liver through cellular cross-talk. Through interactions between LPCs and the immune system, TWEAK modulation of LPC numbers may act to enhance the inflammatory response at the site of regeneration, reinforcing LPC expansion. Likewise, LPC interactions with HSCs via LTβ signaling may also promote recruitment of leukocytes to the site of injury, amplifying the fibrogenic response of the liver. Accordingly, TWEAK signaling may be an important "valve" with which we might modulate the landscape of chronic liver injury. Therefore, we propose that therapies targeting TWEAK signaling in chronic liver injury may be useful for reducing the severity of fibrosis through its dual action on the inflammatory and HSC compartments via LPCs. In doing so, it may be possible to diminish progression to cirrhosis and liver failure, as well as limiting the pro-tumorigenic environment existing in the regenerative niche supporting LPCs.

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Biology of the RANKL–RANK–OPG system in immunity, bone, and beyond

Matthew C. Walsh and Yongwon Choi*

Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

Edited by:
Linda C. Burkly, Biogen Idec Inc., USA

Reviewed by:
Hiroki Yoshida, Saga University, Japan
Jaewoo Hong, National Institutes of Health, USA
Steven L. Teitelbaum, Washington University, USA

***Correspondence:**
Yongwon Choi, Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, 421 Curie Blvd, BRBII/III RM 308, Philadelphia, PA 19104, USA
e-mail: ychoi3@mail.med.upenn.edu

Discovery and characterization of the cytokine receptor-cytokine-decoy receptor triad formed by receptor activator of nuclear factor kappa-B ligand (RANKL)–receptor activator of NF-κB (RANK)–osteoprotegerin (OPG) have led not only to immense advances in understanding the biology of bone homeostasis, but have also crystalized appreciation of the critical regulatory relationship that exists between bone and immunity, resulting in the emergence of the burgeoning field of osteoimmunology. RANKL–RANK–OPG are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies, and share signaling characteristics common to many members of each. Developmentally regulated and cell-type specific expression patterns of each of these factors have revealed key regulatory functions for RANKL–RANK–OPG in bone homeostasis, organogenesis, immune tolerance, and cancer. Successful efforts at designing and developing therapeutic agents targeting RANKL–RANK–OPG have been undertaken for osteoporosis, and additional efforts are underway for other conditions. In this review, we will summarize the basic biology of the RANKL–RANK–OPG system, relate its cell-type specific functions to system-wide mechanisms of development and homeostasis, and highlight emerging areas of interest for this cytokine group.

Keywords: osteoimmunology, TRAF6, TRANCE, RANKL, TNFSF11, TNFRSF11, mTECs, rheumatoid arthritis

INTRODUCTION

Functional diversity typifies the cytokines and receptors of the tumor necrosis factor (TNF) and tumor necrosis factor receptor (TNFR) superfamilies. TNF superfamily (TNFSF) members are broadly expressed in a variety of tissues and organ systems, and are commonly associated with expression on cells of the immune system. In recent years, studies of TNF/TNFR superfamily members have been responsible for elucidating previously unrecognized linkages between the immune system and other biological systems, as well as previously unrealized networks controlling various disease conditions (1, 2). An important example is the system consisting of the cytokine receptor activator of nuclear factor kappa-B ligand [RANKL; additionally identified as TNF-related activation-induced cytokine (TRANCE), osteoclast differentiation factor (ODF), and TNFSF11], its signaling receptor receptor activator of NF-κB (RANK), and the soluble decoy receptor osteoprotegerin (OPG). The RANKL–RANK–OPG system was originally discovered through parallel efforts in the late 1990s that identified it as important to immunity, primarily via actions on dendritic cells (DCs) (3, 4), and as important to bone homeostasis through regulation of osteoclasts (OCs) (5, 6). Work employing various genetically deficient mouse models has shown the critical role of the RANKL–RANK–OPG system plays in bone and immunity – significantly contributing to the emergence of the field of osteoimmunology – as well as organogenesis, and disease conditions including cancer and rheumatoid arthritis (RA). In this review, we will provide a summary of current understanding of the biological functions of the RANKL–RANK–OPG system in development, homeostasis, immunity, and disease, as well as ongoing efforts to target RANKL–RANK–OPG to prevent or fight diseases like osteoporosis and cancer.

RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND

Receptor activator of nuclear factor kappa-B ligand (3), which was independently discovered by four groups, is alternatively named TRANCE (4), ODF (6), osteoprotegerin ligand (OPGL) (5), and designated TNFSF11. The gene encoding the RANKL protein, *Tnfsf11*, is located on human chromosome 13q14, and a conserved syntenic region on mouse chromosome 14 (4). RANKL protein is a type II membrane protein bearing close homology to TNFSF members TRAIL, FasL, and TNF-a (4). Full-length murine RANKL is 316 amino acids, shares 83% sequence homology with human RANKL (4), and consists of a C-terminal extra-cellular receptor-interacting domain and a transmembrane domain, but is found in both membrane-bound and soluble forms (7). Cleavage of soluble RANKL from the membrane-embedded portion is mediated by the metalloprotease–disintegrin TNF-a convertase (TACE) (8, 9). Further, three distinct isoforms of RANKL message have been identified, the shortest of which – lacking the intracellular and transmembrane domains – may have inhibitory function (10). These data comport with observations that soluble RANKL is less efficient at mediating osteoclastogenesis (11). RANKL expression has been detected in various tissues including T lymphocytes (4), osteoblasts (OBs), osteocytes and bone stroma, and lung (5, 6, 12, 13). Throughout development RANKL mRNA can be detected in the brain, heart, kidneys, skeletal muscle, and skin of mouse embryos, and has been specifically identified in E15 chondrocytes

(5, 12). Expression of RANKL is highly inducible, and is regulated by various osteoactive factors including glucocorticoids (14), Vitamin D3 [1,25(OH)2D3] (6, 15), IL-1 (16), TNF-a (16), TGF-b (17), Wnt ligands (18), and LPS (19). Binding studies show that RANKL can bind to both the functional receptor RANK and the decoy receptor OPG (3, 5).

RECEPTOR ACTIVATOR OF NF-κB

Receptor activator of NF-κB [(3); alternatively identified as TNF-related activation-induced cytokine receptor (TRANCE-R) (20) or osteoclast differentiation and activation receptor (ODAR) (21)] is the signaling receptor for RANKL. RANK has been designated TNFRSF11A, and is a type I 616 amino acid homo-trimerizing transmembrane protein containing four extra-cellular cysteine-rich pseudorepeats. Trimerization is promoted by interaction with RANKL (22). The human gene that encodes RANK, *Tnfrsf11a*, is located on chromosome 18q22.1 (3), and RANK message is detected in thymus, liver, colon, mammary glands, prostate, pancreas, bone marrow, heart, lung, brain, skeletal muscle, kidney, liver, and skin (3, 7, 23). RANK is strongly induced, especially on OC precursor cells, by M-CSF (24). Typical of TNFRSF members, RANK lacks intrinsic kinase activity and must rely on recruiting factors capable of activating downstream signaling pathways (summarized in **Figure 1**). As such, RANK intracellular signal transduction is mediated first through direct interaction with tumor necrosis factor receptor-associated factors (TRAFs), which are recruited upon receptor activation (25, 26). RANK interacts with TRAFs 1, 2, 3, 5 in a membrane-distal region of the 383 amino acid cytoplasmic tail, and with TRAF6 at a distinct membrane-proximal Pro-X-Glu-X-X-(aromatic/acid residue) binding motif (25–27). TRAF6 is critical activating mitogen-activated protein kinases (MAPKs) p38 and JNK, as well as the canonical NF-κB pathway in response to RANK signaling (8, 28, 29). TRAF6 utilizes the adapter TAB2 to interact with the MAPK kinase TAK1 to mediate RANK signaling (30). While RANK binds similar TRAFs as the related receptor CD40, it has been shown that stronger activation of TRAF6 may account for unique RANK function (31). Other interacting factors that may modulate RANK signaling include Grb2-associated binding protein 2 (Gab2) (32), epidermal growth factor receptor (EGFR) (33), four-and-a-half LIM domain 2 (FHL2) (34), Lyn (35), CYLD de-ubiquitinase (36), and TRAF family member-associated NF-κB activator (TANK) (37). It has been shown that RANK signaling can regulate calcium oscillation through downstream activation of regulator of G-protein signaling 10 (RGS10) (38), and that RANK-mediated calcium flux is itself regulated by transmembrane protein 64 (TMEM64) interaction with sarcoplasmic endoplasmic reticulum Ca(2+) ATPase 2 (SERCA2) (39). RANK also activates Src family kinase signaling in a manner that leads to Akt/PKB activation through interactions between TRAF6 and Cbl scaffolding proteins (40, 41). TRAF6-dependent RANK signaling has been shown to be negatively regulated via cross-talk with the IFN-g signaling pathway, employing a mechanism leading to TRAF6 ubiquitination and degradation (42). More recently, it has been shown that TRAF3 plays a key role in negatively regulating RANK-mediated activation of the non-canonical NF-κB pathway (43). One of the signaling properties of RANK that may distinguish it functionally from some other TNFR superfamily members is its capacity to activate both the canonical and non-canonical NF-κB pathways. At the level of gene regulation, RANK signaling is crucial for induction of the transcription factors c-fos and NFATc1/NFAT2 (44–46). The functional effects of RANK signal transduction are discussed further below.

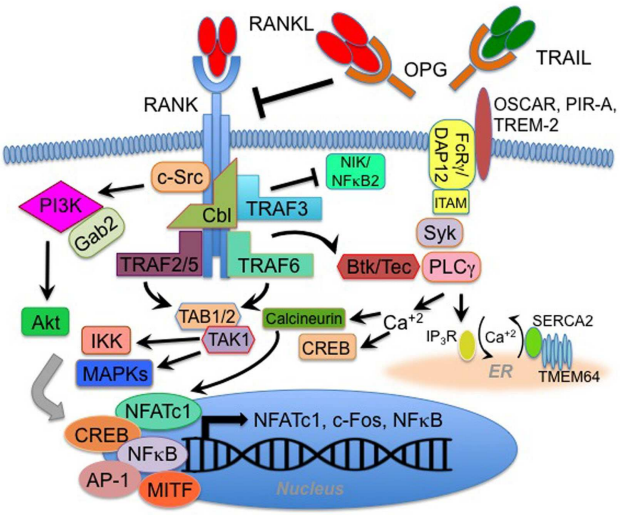

FIGURE 1 | RANK signaling pathways. The RANK receptor lacks intrinsic enzymatic activity and therefore utilizes interaction with adaptor and docking proteins, including TRAFs 2, 3, 5, and 6, Gab2, and Cbl to activate downstream signaling. Gab2 and Cbl are associated with RANK-mediated activation of c-Src, PI3 kinase (PI3K), and Akt, while TRAFs 2 and 6 can activate the TAB1/TAB2/TAK1 complex, which (along with other upstream kinases) leads to activation of IKKβ and MAP kinases (MAPK). Activation of these pathways promotes translocation and activation of transcription factors including NFATc1, CREB, NFκB, AP-1, and MITF. Specific RANK-activated gene transcription varies depending on cell-type, but often involves feed forward expression of NFATc1, c-fos, and NFκB-related genes. RANK-associated TRAF3 has been implicated in negative regulation of the non-canonical NFκB2 pathway through regulation of the upstream kinase NIK. Inhibition of NIK is mediated by the TRAF3 RING finger domain, and is overcome when RANK activation by RANKL triggers autophagic/lysosomal degradation of TRAF3. While many of these mechanisms may be generalizable to various RANK-expressing cell-types, some mechanisms appear thus far to be osteoclast (OC) lineage-specific. The best characterized of these OC-associated mechanisms involves synergistic signaling between RANK and ITAM motif-containing proteins DAP12 and FcRγ (which associate with cell surface receptors OSCAR, PIR-A, or TREM-2) to activate the Syk-PLCγ pathway and flux calcium. This activity enhances NFATc1 and CREB activities. Synergy with RANK occurs via coordinate activation of Btk/Tec. RANK further regulates calcium flux in OC lineage cells by a mechanism involving transmembrane protein 64 (TMEM64) interaction with the sarcoplasmic endoplasmic reticulum Ca(2+) ATPase 2 (SERCA2). This mechanism further promotes CREB and NFATc1 activity.

OSTEOPROTEGERIN

Osteoprotegerin, now designated as TNFRSF11B, was first identified through a discovery effort targeting TNFRSF homologs in rat, and human and mouse OPG homologs subsequently identified exhibited >85% homology (47). Independently discovered and alternatively named osteoclastogenesis inhibitory factor (OCIF) (48), TR1 (49), and follicular DC-derived receptor-1 (FDCR-1)

(50) were found to be identical to OPG. OPG mRNA is detected in the calavaria, skin, liver, lung, and heart of the adult mouse, and peaks at days 7 and 15 in fetal tissue (47). OPG is expressed primarily by bone marrow stromal cells, but can be induced in B lymphocytes, DCs, and follicular DCs (50). OPG expression regulated both positively (e.g., TGF-b, IL-1, TNF, estrogen, and Wnt ligands) and negatively (e.g., prostaglandin E2 (PGE2) and glucocorticoids) by a wide array of factors, most of which are associated with bone homeostasis (51). Full-length OPG protein is 401 amino acids long, which is signal peptidase-cleaved to a 380 amino acid form containing four cysteine-rich N-terminal domains (domains 1–4), two death domain homologous regions (domains 5 and 6), and a C-terminal heparin-binding domain (domain 7) (52, 53), and then N-linked glycosylated, and secreted as a disulfide-linked homodimer (47–49). As such, OPG is believed to function primarily as a decoy receptor, modulating interactions between ligands and signaling receptors. The high-affinity binding partner for OPG appears to be RANKL, but it has also been shown to bind with low affinity (3.0 nM at 4°C and 400 nM at 37°C) to the TNFSF member and pro-apoptotic factor, TRAIL (54). *In vitro* and pre-clinical studies suggest that OPG–TRAIL interactions may be relevant to apoptosis of tumor cells (52). Additional potentially physiologically relevant ligands of OPG include syndecan-1, glycosaminoglycans (GAGs), von Willebrand Factor, and Factor VIII von Willebrand Factor complex (52).

ORGANOGENESIS

Receptor activator of nuclear factor kappa-B ligand–receptor activator of NF-κB has emerged as a critical signaling pathway for the cellular differentiation and development of epithelial tissues in various organs. These RANK-dependent processes have important implications not only for development, but also for regulating immunity and cancer.

LYMPH NODES

Characterization of RANKL- and RANK-deficient mice revealed failed or abnormal development of secondary lymphoid tissues, including lymph nodes, Peyer's patches, cryptopatches, and spleen (55–58). During embryogenesis, secondary lymphoid tissue development occurs as RANKL signals through RANK on lymphoid tissue inducer (LTi) cells recruited to a rudimentary anlage composed of lymphoid tissue organizer (LTo) progenitor cells. LTi cells then transmit lymphotoxin a/b (LTa/b) to LTo progenitors that express LTbR, which drives development of mature LTo cells. This event triggers a feedback loop of RANKL and RANK expression by mature LTo cells, which amplifies LTi growth and tissue organization (59). The relevant RANK signaling in this context appears to be mediated by the transcriptional regulator Id2 (60).

MAMMARY GLANDS

Receptor activator of nuclear factor kappa-B ligand- and RANK-deficient mice also exhibit defective mammary gland development (61, 62), which requires RANK-triggered activation of the IKK-alpha kinase domain in order to activate the non-canonical NF-κB pathway (63). RANK signaling in the context of mammary gland development is also associated with expression of cyclin D1, Id2, and Id4 (59). It has recently been shown that RANK signaling is required for mammary epithelial stem cell activation (64, 65). Further, RANK signaling has been shown to be required for progesterone-driven proliferation of normal breast tissue (66).

medullary thymic epithelial cells

A critical role for RANKL–RANK signaling has been established in thymic organ development, and specifically for the epithelial lineage cells required for negative selection of developing T cells (67, 68). The thymus educates self-tolerant T cells by eliminating those expressing potentially self-reactive TCRs and by generating the immunosuppressive T cells that are essential for preventing autoimmune disease. Epithelial cells localized in the thymic medulla called medullary thymic epithelial cells (mTECs) are non-hematopoietic in origin and are essential for negative selection (68). Recent studies have revealed that mTECs also contribute to the selection and survival of immunosuppressive Foxp3-positive regulatory T cells (Tregs) (68, 69). The significant decrease in mTECs in the absence of RANKL provision by thymocytes suggests that mTEC differentiation is actually driven by the cells that mature mTECs subject to negative selection (68). In fact, this process appears to be initiated in the neo-natal period by innate RANKL-expressing gamma-delta lineage cells before the appearance of alpha-beta cells in the thymus (70). mTECs require signaling through each of the TNFRSF members LTbR, CD40, and RANK in order to induce sufficient expression of the critical factors autoimmune regulator (Aire) and tissue-specific antigens (TSAs), but it had been unclear whether downstream activation by those receptors was qualitatively or quantitatively distinct. Cell-specific deletion of the non-canonical NF-κB inhibitor TRAF3 suggests that LTbR and CD40 are similar in their provision of non-canonical NF-κB activation, but that RANK apparently provides additional requisite signals during mTEC development (71). From genetic experiments, however, it is known that the relevant RANK signals are transduced by TRAF6 (67). Together, these data show that RANK-mediated signaling is required for developmental processes that critically affect immune regulation and homeostasis.

M CELLS

Microfold cells are specialized intestinal epithelial-derived cells that make up roughly 10% of the follicle-associated epithelial (FAE) cells covering the gut-associated lymphoid tissues (GALT). Microfold (M) cells exhibit some morphological distinctions compared to neighboring enterocytes, but are most important for their enhanced capacity for phagocytosis and transcytosis of macromolecules, particulate antigens, and micro-organisms residing in the gut lumen (9). M cells are essential for transfer of antigens from orally acquired pathogens, such as Salmonella Typhimurium, to antigen-presenting cells, and subsequently, for optimal immune responses (9). Because of their importance to mucosal immunity, various efforts have aimed at determining the mechanisms of differentiation of M cells. Within the villous crypts of the FAE reside leucine-rich repeat-containing G-protein-coupled receptor- (Lgr5) expressing epithelial stem cells that are capable of differentiating into the various sub-lineage found in the gut, including enterocytes, goblet cells, enteroendocrine cells, tuft cells, Paneth cells, and M cells (9). Signals received depending on micro-anatomic location and neighboring cells can determine lineage specificity.

It has been shown that stromal cells in the subepithelial dome of the FAE provide RANKL to RANK-expressing stem cells to induce M cell differentiation, and that in RANKL-deficient mice, M cells fail to develop unless sufficient exogenous RANKL is provided (57, 72). This data demonstrated an absolute requirement for RANK signaling during M cell development. This work further suggested that RANK signaling on M cell precursors is triggered by membrane-bound RANKL on stromal cells, such that cell–cell contact and FAE micro-architecture may be critical to M cell differentiation (57). RANKL has been shown to specifically induce the markers annexin A5 and myristoylated alanine-rich C-kinase substrate)-like protein 1 (MARCKS) on M cell precursor stem cells, but their significance in this context remains unclear (9, 73, 74). RANK signaling apparently acts in a critical manner to upregulate the ETS (E26 transformation-specific) transcription factor Spi-B, which was shown to be required for M cell maturation, and is responsible for cell-intrinsic expression of genes including glycoprotein 2 (Gp2), TNF alpha-induced protein 2 (Tnfaip2), and C–C motif chemokine ligand 9 (Ccl9) (73, 74). Finally, it remains to be determined why only a small proportion of Lgr5+ stem cells in the subepithelial dome of the FAE develop into stem cells despite ample exposure to stromal RANKL (9, 57). A possibility recently put forward might involve cross-talk between the RANK and Notch signaling pathways during M cell development, such that availability of given Notch ligands to M cell precursors determines whether RANK signaling is sufficient for M cell differentiation to proceed (9, 75).

BONE HOMEOSTASIS

Bone is essential for providing skeletal strength, vital organ protection, a mineral reservoir for calcium, and a site for immune cell development. Bone cell homeostasis is maintained by the balanced functions of primarily two cell-types: OBs, which build bone, and OCs, which resorb bone (76, 77). In a continuous cycle, OCs remove bone by sealing off sections and secreting digestive acid into the lacunae, followed by OBs filling the cavity with new bone (76, 77). This constant remodeling of the bone matrix is necessary to maintain both skeletal strength and a reservoir for hematopoiesis. There is intense interest in understanding the mechanisms governing OC development and function. While OBs are mesenchymal lineage cells, OCs arise from myeloid hematopoietic precursor cells (76, 77). Interestingly, new OCs are generated largely through cell contact-dependent interactions between OC precursors and OBs in the bone stroma (76, 77). RANKL–RANK signaling was shown early on to be a potent driver of OC differentiation even under minimal *in vitro* conditions (5). OC precursors require the growth factor M-CSF for OC precursor growth and RANK induction (78, 79), and then the RANK-induced transcription factors c-fos (80), NFATc1/NFAT2 (44, 46), and NF-κB members p50 and p52 (81, 82) [which also activated c-fos and NFATc1/NFAT2 in pre-OCs (83)] for OC development. Mice genetically deficient for any of these factors exhibit osteopetrosis, a thickening of the bones, due to reduced OC numbers and/or activity. RANKL–RANK-mediated activation of these pathways and induction of c-fos, NFATc1/NFAT2, and canonical and non-canonical NF-κB pathways during OC development is necessary for activation of OC-critical genes, including tartrate-resistant acid (TRAP), cathepsin-K, calcitonin receptor, as well as c-myc, to promote OC proliferation (76). RANKL–RANK signaling also plays a crucial role in the bone-resorbing function of mature OCs, which after αVβ3-mediated attachment of OCs to bone surfaces, leads to activation of Src-dependent pathways, including Syk, through complex formation involving RANK, Slp-76, Vav3, and Rac (76). Activation of these pathways and cytoskeletal rearrangements promote ruffled border formation via fusions between lysosomal secretory vesicles and the cytoplasmic membrane (76, 84). RANKL-deficient mice are severely osteopetrotic due to a cell non-autonomous defect in OC development, and additionally exhibit failed tooth eruption (common in developmental osteopetrosis), and diversion of hematopoiesis to the spleen and liver due to failed bone marrow cavity formation (58). Therefore, new bone formation occurs in the absence of RANK signaling, including intramembranous and endochondral ossification processes during fetal development, but bone remodeling is severely diminished. Further, RANK- and RANKL-deficient mice are exact phenocopies with respect to OC development, suggesting an exclusive relationship between the RANK–RANKL receptor–ligand pair (55). The critical source(s) of RANKL during normal bone remodeling is still debated, but evidence suggests that the source changes over development, such that hypertrophic chondrocytes provide RANKL for removal of trabecule during endochondral ossification, and trabecular osteocytes provide most RANKL for both mature bone remodeling and in response to mechanical stress (85, 86). Additionally, many well known osteotropic factors, including IL-1, IL-6, and IL-11 are believed to exert osteoclastogenic activity simply by inducing RANKL expression on OBs (78). The cytokine TNF-a promotes osteoclastogenesis, not only via direct stimulation of OC precursors (87), but also by inducing RANKL expression on stromal cells and RANK expression on OC precursor cells (88). TRAF6-deficient mice also exhibit severe osteopetrosis, confirming the critical relationship between this signaling adapter and RANK signaling (28, 29). Another study examining TRAF binding site in the RANK receptor suggests that TRAF6 might be most important for OC function and normal F-actin ring formation, and implying that signals through other TRAFs may also make important contributions to RANK-mediated osteoclastogenesis (89). However, a study using TRAF2-deficient OC precursors showed that while TRAF2 made a minor contribution to RANK signaling, it is primarily required for TNF-a-dependent osteoclastogenesis (90). Downstream of TRAF6, RANK signaling in OCs has been shown to activate JNK1 (91), Akt/PKB (41), p44/42 ERK (41), p38 MAPK (92), and the canonical NF-κB pathway (26). RANK-mediated activation of the c-src pathway links it to a critical requirement c-src for normal OC development (40, 41, 93, 94). Studies of OC differentiation have also identified a critical costimulatory pathway for RANK signaling involving immunoreceptor tyrosine based activation motif (ITAM)-containing receptors DNAX activation protein of 12 kDa (DAP12) and Fc-receptor g subunit (FcRg) (95). This costimulatory pathway is required for RANK-mediated osteoclastogenesis, and signals downstream through the protein kinase Syk (96, 97), which activates phospholipase Cg (PLCg) and the BTK and Tec kinases (98), eventually leading to calcium-mediated activation of NFATc1/NFAT2 (95). OPG functions as a soluble decoy-like factor for RANKL, and thus as a

negative regulator of RANK signaling, and is capable of inhibiting osteoclastogenesis *in vitro*, and of inducing osteoporosis when transgenically overexpressed in mice (47). Furthermore, OPG-deficient mice are described as osteoporotic, with excessive numbers of OCs (99). OPG has further been shown to inhibit mature OC function *in vitro* (100). In addition to OPG, other negative regulatory mechanisms of RANK signaling have been described that inhibit osteoclastogenesis. For instance, though T cells can express RANKL, there is a negative correlation between T lymphocyte activation and signaling through RANK on OC precursors, as T cell-derived IFN-g drives proteasomal degradation of TRAF6 (42). In this way, a productive immune response is prevented from having an overlapping, deleterious effect on bone in the surrounding environment. TRAF6 activates downstream signaling via non-degradative ubiquitination (101). It has been demonstrated that TRAF6-mediated RANK signaling in pre-OCs is negatively regulated de-ubiquitinase CYLD, and that CYLD-deficient mice exhibit osteoporosis due to increased OC activity (36). Another regulatory mechanism involves negative feedback via by RANK-mediated upregulation of IFN-b, which mediates a feedback mechanism that blocks further c-fos-dependent activity (102). In support, it is reported that c-fos-deficient OC precursors exhibit deficient RANKL-mediated IFN-b production, and that mice deficient for the IFNa/b receptor (IFNAR1) exhibit osteoporosis characterized by an increase in OCs (102). A more recently characterized negative regulatory mechanism of RANK signaling in OC precursor cells involves the formation of a TRAF3-containing complex on the RANK intracellular domain that inhibits both canonical and non-canonical NF-κB pathways (43, 103). The physiologic relevance of this complex is demonstrated by TRAF3 conditionally deficient mice, which exhibit mild osteoporosis (43). Finally, an example of negative regulation of RANK-mediated ITAM activation has recently been described, which involves semaphorin 3A (Sema3A) interaction with neuropilin-1, and is supported by the finding that Sema3a-deficient mice are osteopenic (104). These data highlight the multiple levels of control that bone requires for proper homeostatic function, and suggest the potential that RANKL signaling has as a therapeutic target in treating bone-related ailments. In fact, denosumab, an anti-RANKL antibody is now in clinics for use in treating osteoporosis and shows promise for treating additional OC-related conditions (105).

INHERITED BONE DISEASES

Though typically rare, various heritable mutations linked to the bone pathologies have been identified in the genes encoding the RANKL–RANK–OPG system. Familial expansile osteolysis (FEO) and Paget's disease are rare autosomal dominant conditions characterized by enhanced bone remodeling and osteolytic lesions present in the long bones. Short in-frame duplications in exon 1 of the gene encoding RANK have been linked to FEO and Paget's disease of the bone (PDB) (106). It has been shown that these mutations disrupt function of the RANK signal peptide and result in constitutive RANK activity (106). Expansile skeletal hyperphosphatasia (ESH) is a genetic disorder characterized by early onset deafness, premature loss of teeth, progressive hyperostotic widening of long bones causing painful phalanges in the hands, accelerated bone remodeling, and episodic hypercalcemia. While ESH is distinguished phenotypically from FEO by the presence of hypercalcemia and the absence of large osteolytic lesions with cortical thinning in major long bones, it appears also to occur from an activating mutation – in this case a 15-bp tandem repeat – in the region encoding the RANK signal peptide (107). Multiple additional mutations have been characterized in the gene encoding RANK that result in varied forms of osteopetrosis, but despite early onset, it has been shown in some cases that disease can be cured by hematopoietic stem cell transplant even when carried out in late infancy (108). Juvenile Paget's disease is a rare autosomal recessive bone disease in which children are normal at birth, but then experience rapidly remodeling woven bone, osteopenia, fractures, and progressive skeletal deformity. Genetic analysis determined that this disorder is the result of an inactivating mutation in the gene encoding OPG, and that serum levels of OPG are undetectable in affected individuals (109). Cherubism is a rare autosomal dominant disease of the lower jaw characterized by excessive OC-mediated bone resorption and associated with mutations in the gene Sh3bp2 (110). While the mechanism(s) disease onset was not initially understood, more recent work has demonstrated that the mutations appear to impact regions of scaffolding protein encoded by Sh3bp2 that coordinate signals converging from RANK and M-CSFR to activate Syk, PLCg2, and Vav (111). These alterations in signaling complexes lead to increased TNF-a expression and augmented OC activity (111). Together these genetic cases confirm the critical role of the RANKL–RANK–OPG system in human bone development and function.

ACQUIRED BONE PATHOLOGIES

Much more common than bone pathologies caused directly by genetic lesions to the RANKL–RANK–OPG systems are bone-related pathologies that arise later due to environmental factors, homeostatic dysregulation, hormonal changes, or other disease sequelae. The most common of these is post-menopausal osteoporosis, a skeletal disorder characterized by weakening of the bones and predisposition to fracture due to bone loss caused by an imbalance in OC activity versus new bone formation (112, 113). Osteoporosis is associated with hormonal changes, such as decreased estrogen levels in post-menopausal women, and has been linked to increased RANKL levels on bone marrow cells of women exhibiting osteoporosis (113, 114). Similarly, patients receiving hormone ablation therapy for breast cancer (estrogen suppression) or prostate cancer (chemical or surgical castration for testosterone suppression) may also suffer osteoporotic bone loss due to increased RANKL expression (112, 113). These findings correlate with the osteoporotic phenotype observed in the OPG-deficient mouse model, in which RANKL–RANK interactions are enhanced due to the absence of the OPG-mediated inhibition (99). Cancer is another area where the RANKL–RANK–OPG system may affect bone health. In patients with bone metastases, skeletal complications caused by increased OC activity may result in pathological fractures, spinal cord compression, and the need for radiotherapy to the bone or orthopedic surgery [collectively known as skeletal-related events (SREs)] (115). Increased bone turnover may even enhance tumor growth in bone by facilitating the early establishment, as well as later progression, of bone metastases (116). Cancer metastases to bone result from

engagement by tumor cells with non-malignant resident cells of the bone microenvironment, including OCs, stromal cells, and vascular cells, through cell–cell, paracrine, and/or endocrine interactions. The role of RANKL–RANK–OPG in metastasis can be divided into its contribution to enhanced osteolysis and to its effects on promoting metastasis. In the former case, some examples and mechanisms have been described. For instance, bone pain and excessive OC activity are the primary complication for multiple myeloma patients, with increased levels of RANKL often found in bone stromal cells (117). With respect to mechanisms of tumor-driven increases in RANK activity, one study showed tumor cell expression of metalloproteases ADAMTS1 and MMP1, factors associated with increased risk of metastasis in breast cancer, alter secretion of epidermal growth-like factors in a manner that suppresses OPG expression by resident OBs (118). In another study, it was shown that prostate cancer cells expressing a soluble form of RANKL could directly induce osteoclastogenesis from precursor cells in the absence of stromal accessory cells (119). With respect to promoting metastasis, interest in a potential role for RANKL–RANK was triggered by observations relating to its role in epithelial organogenesis, specifically mammary stem cell development, which could be envisioned as contributing to carcinogenic events (65, 115, 120). During tumor formation, RANKL is found to increase proliferation and survival of both normal and pre-neoplastic breast in addition to expansion of mammary stem/progenitor cells (65, 120). Progesterone and prolactin, which have been implicated in mammary tumorigenesis, both trigger RANKL expression in the mammary gland (121). In one study using a hormone-triggered mammary tumor model in mice, specific deletion of RANK in mammary epithelial cells significantly delays tumor onset (65, 121). Further, RANKL treatment is shown to protect mammary epithelial cells from γ-irradiation-induced cell death, one indicator of malignancy (65, 121). In a complementary study, transgenic mammary gland overexpression of RANK was shown to exacerbate medroxyprogesterone acetate-induced mammary tumor formation, and that systemic RANKL blockade resulted in a 90% reduction in hormone-induced mammary tumor onset (120, 121). With respect to showing the contribution of local differentiation factors, in addition to chemotactic factors, to metastasis, it was shown that RANKL stimulation directly triggered metastasis of melanoma cell lines and breast cancer in patients in a manner that is independent of pro-osteoclastic activity (122). A study investigating sources of RANKL outside of bone that may trigger metastasis showed that pulmonary metastasis of breast cancer may be driven by RANKL expressed on infiltrating Tregs, implicating the role of inflammatory factors in RANKL-driven metastasis (123). Finally, important work to determine the key molecular pathways downstream of RANK signaling in metastatic tumor cells showed a correlation between metastatic potential and RANK-induced IKK-a activation (124).

IMMUNITY AND OSTEOIMMUNOLOGY

The RANKL–RANK–OPG system was initially discovered through multiple independent efforts, some interested in discovering new genes relevant to bone biology, and others initially focused on the immune system. As such, while initial efforts to characterize the role of RANKL–RANK–OPG in controlling osteoclastogenesis were ongoing, parallel efforts were underway showing that RANKL provided by T cells can significantly enhance immunity by promoting the survival and function of DCs, the most potent professional antigen-presenting cells, in the context of an immune response (4, 125, 126). Emerging understanding that key cellular regulators of the immune and bone systems were responsive to the same cytokine systems and derived from common progenitors (127) was one of the key impetuses in developing a new field of study, osteoimmunology, which seeks to examine the interactions between the bone and immune systems. Studies of RANK intracellular signaling pathways and regulatory mechanisms have further demonstrated the extent to which bone and immune cells overlap in these areas. Osteoimmunologic mechanisms are relevant to diseases including RA, periodontal disease, osteoporosis, osteoarthritis, multiple myeloma, and metastatic bone tumors, all of which are associated with bone breakdown (128). The most prominently studied example of the pathologic relationship between bone and immune cells is RA, but many RANKL–RANK–OPG-driven mechanisms of pathologic bone–immune cell interaction are common between different diseases (129) (depicted in **Figure 2**). RA is an autoimmune disease that is characterized by inflammation of the synovial joints, leading to severe structural damage including bone destruction. RANKL is highly expressed in the synovium of RA patients and is largely responsible for RA-related bone destruction (129). The source of pathogenic RANKL in RA synovium is still debated, as T cells may express high levels, but the osteoclastogenic action of T cells can be counteracted by IFN-g production (42). Instead, it appears that synovial fibroblasts are the primary RANKL source in RA (42). It has further been determined that Th17 helper T cells are responsible for inducing RANKL expression on synovial fibroblasts via expression of IL-17 as well as IL-1, TNF-a, and IL-6 (129). At the same time, a recent fate mapping study showed that more a potently osteoclastogenic version of pathogenic Th17 cells are those that were previously Foxp3-expressing Tregs, but that converted phenotypes, and gained RANKL expression in response to synovial fibroblast-derived IL-6 (130). Another study suggests that OC differentiation activity may not be the only function of RANKL in bone–immune cell interactions. Multi-photon microscopy was employed to perform intravital imaging of bone tissue in the context of RANKL-mediated OC activation, and it was showed that RANKL-expressing Th17 cells were able to stimulate mature but non-resorptive OCs to begin resorbing bone, suggesting that Th17-mediated bone pathology may not necessarily generate need OCs, but simply increase activity of mature resident OCs (131). In another model system, a recent study showed that RANKL expression by B cells drive OC formation in an ovariectomy (ovx) model of osteoporosis, suggesting that B cells should be examined more closely in bone–immune cell interactions (132). For normal bone remodeling, OB or bone stromal cells have long been considered the major sources of RANKL, but recent work employing cell-specific RANKL deletion suggests that osteocytes are in fact the critical providers of RANKL to OC precursors (86, 133). It is therefore clear that the cellular source of RANKL is critical to the context in which it is acting, and whether it primarily affects bone or immune cells. In addition to activating the immune system through RANK signaling on DCs, RANKL is conversely important

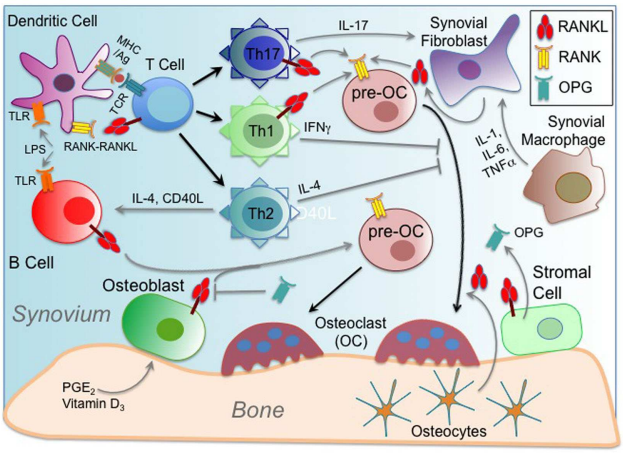

FIGURE 2 | Osteoimmunology and RANKL–RANK–OPG. Osteoimmunology involves cross-regulation between cells of the bone and immune systems, and in some cases in the source of pathogenic conditions like rheumatoid arthritis (RA). The interface between the synovium and bone joints is where RA occurs, and where many cellular interactions typical of osteoimmunity have been characterized. The unifying characteristic of many of these cellular interactions is often the interplay between sources of RANKL and RANK-expressing cells. Secondarily, there are factors secreted or provided through cell contact that promote RANKL and/or RANK expression. The net effect of osteoimmune interactions is largely tallied according to increased (or regulation of) bone loss due to enhanced RANKL-mediated osteoclast (OC) differentiation from pre-OCs. In addition to the usual sources of RANKL available to pre-OCs from bone-associated cells including bone stromal cells, osteoblasts (OBs), and osteocytes, an inflammatory environment provides other sources. B cells activated by TLR ligands, such as LPS, and expanded by T cell help induce RANKL expression. T cells, which are activated by dendritic cells (DCs) through MHC/Antigen (Ag)–TCR interactions, can also express RANKL, which can both act on pre-OCs, but can also act on DCs to promote their survival and to prolong T–DC interactions. DC interactions with helper T cells influence their differentiation into subsets such as Th1, Th2, and Th17. Th1 and Th2 cell elaboration of IFNγ and IL-4, respectively, exhibit modulating effects on RANK-mediated osteoclastogenesis. However, IL-17 produced by Th17 cells can act to induce RANKL, especially by synovial fibroblasts under inflammatory conditions. Synovial macrophages may also enhance fibroblast expression of RANKL through secretion of inflammatory cytokines like IL-1, IL-6, and TNF-α. At the same time, mitigation of potentially deleterious effects of osteoimmune interactions may be provided by secretion of OPG, which attenuates the potency of available RANKL.

for inducing immune tolerance by promoting Treg differentiation in certain autoimmune contexts. RANKL is required for Tregs that prevent cytotoxic destruction of pancreatic beta islet cells in a mouse type-1 diabetes model (134), as well as for Treg-mediated control of a colitis model (135). RANKL may promote peripheral immune tolerance. For instance, it has been reported that RANKL-expressing keratinocytes in inflamed skin trigger epidermal DCs to induce a Treg phenotype in infiltrating T cells (136). At the same time, another autoimmune disorder has recently been revealed, at a clinical level, to harbor a deleterious role for RANKL. High serum levels of soluble RANKL apparently correlate with risk for development of type-2 diabetes mellitus (T2DM). A recent study has identified a mechanism underlying this risk factor as hepatic insulin resistance induced by the RANK–NF-κB signaling pathway (137). Blockade of hepatic RANKL was able to ameliorate disease and lower plasma glucose levels, highlighting a potential strategy for treating T2DM (137). Finally, an example of modulation of RANKL function at the level of central immune tolerance may have important clinical implications. It was recently shown that mTEC inhibition via blockade of RANKL may represent a viable approach to boosting anti-tumor T cell responses by temporarily disrupting thymic negative selection to TSAs expressed by tumors (138). Together these examples show that RANKL–RANK either activating or suppressive to an immune response depending on the context, and that while important roles for other cytokines have been identified [e.g., IL-1, IL-6, IL-17, IL-23, TNF-a, and TGF-b (129)], RANKL remains the most critical means of communication between cells of the osteoimmunologic network.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

It is difficult to overstate the importance of the discovery of the RANKL–RANK–OPG system with respect to understanding how bone homeostasis is controlled. The hundreds of studies it spurred have uncovered a much more vast biological network of regulation involving RANKL–RANK in and across other organ systems, and have as an additional benefit, demonstrated previously unknown ways in which organ systems interact and cross-regulate at a molecular level. Now that RANKL–RANK has been successfully harnessed for purposes of therapeutic treatments of osteoporosis, bone loss, and bone metastasis, it will be important to answer additional questions – specifically with respect to how RANK signaling is modulated, and how and on what cells RANKL is physiologically expressed under normal versus disease conditions – if therapeutic RANKL–RANK targeting is to be refined and potentially applied to additional disease conditions.

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EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org