TARGETING TNF-RECEPTOR ASSOCIATED FACTORS (TRAFs) FOR THERAPEUTIC INTERVENTION.

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Abstract

TNF-Receptors Associated Factors (TRAFs) are the molecules that upon engagement of the TNF-receptor (TNFR) by a TNF-family ligand come first in contact with the activated TNFR, initially acting as docking molecules for kinases and other effector proteins that are recruited to the activated receptor. TRAFs later regulate the subcellular relocalization of the receptor-ligand complex and finally they modulate the extent of the response by controlling the degradation of key proteins in the pathway.

In this chapter, we review the involvement of different TRAF family members in the etiology of a variety of pathologies and address the question of whether the use of TNFR-mimic-peptides or small molecule modulators targeting TRAFs might be suitable for therapeutic intervention, discussing the advantages and disadvantages of this strategy.

TNF-Receptor Associated Factors (TRAFs)

A total of seven TRAF-family members participate in the regulation of as many as 20 TNFRs. TRAF3 and TRAF6 are also involved in the regulation of different members of the Toll-like Receptor (TLR) and interleukin-1 receptor (IL-1R) family. Furthermore, TNFR-family members generally utilize more than one TRAF family member for signaling, often activating similar pathways and even the same downstream effectors. Therefore, the levels of expression of the different TRAF-family members and downstream effectors will likely play an important role in the outcome of the response.

The consensus amino-acid motif supporting binding of TRAF1, TRAF2, TRAF3 and TRAF5 to TNFR-family proteins is (P/S/A/T)x(Q/E)E [1, 2], implying that TRAF1, 2, 3 and 5 potentially interact with the same TNFR family members and that they might compete among themselves for the binding. In contrast, the consensus sequence for TRAF6 is PxExx(Ar/Ac) (where the last amino-acid residue is aromatic or acidic) [3]. The binding motif for TRAF4 is yet to be identified. TRAF7 lacks a TRAF domain and does not directly interact with TNFRs [4].

The crystal structures of TRAFs bound to different TNFR family members have confirmed that the peptide core motif provides the specificity of the binding. However, the actual composition of the core motif as well as other amino-acids adjacent to this core can affect the interaction, by establishing molecular interactions with residues in the

TRAF-domain, by decreasing the binding affinity by steric impediments or electrostatic repulsions, or by intramolecular interactions that affect the conformation of the TRAF-binding peptidyl motifs. These results provide a molecular explanation for the differences in binding specificity and affinity of the members of the TRAF family for the different TNFR family members [3, 5-9]. These results also imply that it would be conceivable to design peptides that could act as agonist or antagonist of the function of different TNFR family members, either by modulating the binding of particular TRAF proteins to those receptors; or by activating TRAF-signaling pathways independently of the activation of the TNFR. In this regard, it has been shown that 11-residue linear peptides bearing the intracellular CD40/TRAF binding motif were sufficient to induce NF-κB activation in WEHI-231 lymphoma cells [10], thus indicating that small peptides can mimic TNFR signaling. Also, Ye *et al.* [3] using RANK peptides mimicking its TRAF6 docking site could block osteoclast differentiation *in vitro* in both primary cells and cell lines, without affecting cell viability. These results support the suitability of using peptides mimicking TRAF-binding motifs to modulate TRAF-family signaling and associated biological functions.

No non-peptidyl small molecules that bind TRAFs have been described to date, though it conceivably should be possible to generate such molecules. Non-peptidyl molecules could afford the advantage of superior cell permeability compared to peptides, and also probably better pharmacological properties in terms of half-life, bioavailability and biodistribution. Structural studies however reveal that the pocket on the surface of TRAFs responsible for binding peptidyl motifs found in the cytosolic tails of TNF-family receptors is somewhat shallows[3, 5-9], which may hinder the ability to generate high affinity antagonists. In this regard, peptides representing core motifs of the TRAF-binding sites of TNFRs typically bind to TRAFs with low affinity. For instance, the interaction between TRAF2 and monomeric receptors is relatively weak (K_d = 0.04–1.5 mM) which ensures that TRAFs do not interact with nonactivated receptors and implyes that multivalency of TRAFs (note that TRAFs and TNFRs are functional as trimeric molecules) may play a large role in generating sufficient free energy to account for binding *in vivo* [1].

TRAFs and disease

TRAFs are emerging as essential components of the TNFR-family signaling, acting as coordinators of the downstream signaling pathways and consequently having a key role in the outcome of the response. Not surprisingly, growing evidence is pointing out a direct involvement of TRAFs in different pathologies. An overview of some of the pathologies where manipulation of TRAF activities might have therapeutic interest is discussed below.

TRAF2 and Chronic Lymphocytic Leukemia

Recent results from our laboratory have revealed a tumor suppressor role for TRAF2 in B lymphocytes. Transgenic mice with B cells lacking functional TRAF2 and over-expressing Bcl-2 developed Small B cell lymphoma/Chronic Lymphocytic leukemia (SBL/CLL) with high incidence [11]. The mechanism underlying the tumor suppressor function of TRAF2 might involve its role in the control of apoptosis in B cells. In this regard, we and others have shown that TRAF2-deficient B cells are more resistant to various apoptotic stimuli [11, 12] and accordingly, the absence of functional TRAF2 increases B cells numbers *in vivo* [13]. These results support an important role for TRAF2 in B cell homeostasis. In our transgenic mouse model of SBL/CLL, deregulation of TRAF2 might increase the resistance of subsets of B cells to apoptosis induced by TNF-family members, while over-expression of Bcl-2 increases the resistance of these cells to stimuli involving the mitochondrial pathway of apoptosis, ultimately resulting in the development of malignancies.

Interestingly, TRAF2 is over-expressed in Reed-Sternberg cells from Hodgkin lymphoma patients [14, 15] where it is located in cytosolic aggregates [16]. However, TNF failed to induce both TRAF2 translocation to the insoluble fraction and JNK activation in Hodgkin Reed-Sternberg L-428 cells [17], strongly suggesting that TRAF2 is not fully functional in these cells. In contrast, Reed-Sternberg cells have aberrant constitutive activation of both the canonical and non-canonical NFκB pathways [17-19], which is also similar to what has been observed in TRAF2-deficient B cells [12].

Altogether, these results suggest a role for TRAF2 in controlling B cell homeostasis and indicate that inhibition of TRAF2 increases development of B cell malignancies. Consequently, devising strategies aimed to restore TRAF2 expression or function might prove useful for the treatment of certain types of cancer.

TRAF1 in B cell leukemia and lymphoma

Among the members of the TRAF family, TRAF1 shows the most striking deregulation of its expression in B cell malignancies. In normal physiological conditions, expression of TRAF1 has a very restricted pattern. It is only found in some epithelia, dendritic cells and activated lymphocytes [20, 21]. In contrast, TRAF1 expression is upregulated in a variety of hematopoietic malignancies, such as chronic lymphocytic leukemias (CLL) [20], non-Hodgkin lymphomas (NHL) [22], Reed-Sternberg cells of Hodgkin disease [14, 15], LMP-1 positive post-transplant lymphoproliferative disease and HIV-associated lymphoma [23], strongly suggesting a possible role for TRAF1 in the etiology of these B cell malignancies.

In this regard, the known functions of TRAF1 are consistent with a role in tumorigenesis. First, TRAF1 protects against apoptosis. TRAF1's anti-apoptotic role might be mediated by its interaction with various anti-apoptotic proteins that it helps to recruit to the activated TNFRs, including the NF-κB inhibitory protein A20, the inhibitor of apoptosis proteins (cIAP-) 1 and 2, and FADD-like interleukin-1β converting enzyme (FLICE)-like inhibitory protein (FLIP) [24]. Indeed, TRAF1 recruitment of cIAP1 and cIAP2 to TNFR1 seems to inhibit receptor-mediated caspase-8 activation [25]. Consistent with the anti-apoptotic function of TRAF1, epithelial cells lacking TRAF1 were more sensitive to apoptosis induced by TNF [26], and TRAF1-deficient dendritic cells displayed severely impaired survival in response to TNF and CD40L [27]. Furthermore, enforced expression of TRAF1 in T cells blocks apoptosis of reactive T cells thus preventing antigen-induced tolerance [28]. TRAF1 over-expression was also able to partially protect TRAF2 -/- MEF cells from TNF-mediated apoptosis [29].

Second, considerable evidence supports a role for TRAF1 in the regulation of TRAF2 activities, with TRAF1 primarily operating as an antagonist of TRAF2. In this regard, TNF was able to induce NF-κB and JNK activation more efficiently in *TRAF1*-

deficient T cells than in normal T cells, an effect that was dependent on TNFR2 [26] and would likely involve a more efficient TRAF2 recruitment to the activated receptor in the absence of TRAF1. Furthermore, an excess of TRAF1 abrogated the interaction of TRAF2 and CD40, with the consequent inhibition of CD40-dependent NF-κB activation [30]. Conversely, down-regulation of TRAF1 with small interfering RNAs enhanced CD40/CD40L-induced NF-κB activation. Interestingly, TRAF1 expression disrupted the subcellular relocalization of TRAF2 and its association to cytoskeleton in CD40-activated cells [27].

In summary, TRAF1's upregulation in leukemia and lymphoma, its anti-apoptotic functions, and its role as a TRAF2 inhibitor make it a likely candidate to be implicated in the etiology of B cell malignancies. Therefore, development of peptiomimectics or small molecule inhibitors that interfere with TRAF1 functions might be useful for treating those leukemias where upregulation of TRAF1 is a hallmark, although additional research is needed to elucidate the actual role of TRAF1 in the etiology of these diseases.

Caveats of targeting TRAF1 and TRAF2

As indicated above, interfering with TRAF1 function in B cell malignancies could hypothetically improve the outcome of the disease by, for instance, sensitizing these malignancies to apoptosis-inducing cytokines and possibly other types of apoptosis inducers. However, mice deficient in TRAF1 are hyper-responsive to TNF and, as a result, they display hyper-proliferation of T cells and suffer from skin epithelium apoptosis [26], as well as developing TNF-mediated acute liver injury [31]. Interfering with TRAF1 function might consequently enhance TNFR1 and TNFR2 responses and thus predispose to autoimmunity and chronic inflammation. In this regard, increased TNF produced by reactive leukocytes is a common feature of several autoimmune diseases, including rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis and other chronic inflammatory diseases. For example, excessive production of TNF can drive synovial inflammation and degradation of articular cartilage and bone, which are common features of RA (reviewed in [32]). In Crohn's disease, high levels of TNF cause inflammation of the digestive track [33]. Thus, even if TNF levels remain normal, targeting TRAF1 might increase the responsiveness of T lymphocytes (and maybe other cell types) to this

lymphokine, causing autoimmunity. These potential side-effects of TRAF1 antagonists might be counteracted by treating patients with commercial biological anti-TNF agents, such as etanercept, infliximab and adalimumab, but are nevertheless worrisome.

TRAF2-deficient and TRAF2-dominant-negative (DN) mice have severe defects in T cell function, and fail to mount a cytotoxic response in mixed lymphocyte reaction assays [34-36], thus highlighting an important role for TRAF2 in the control of cytotoxic T responses. Therefore, it is conceivable that blocking TRAF2 function might have positive implications for transplantation, ameliorating host versus graft disease [36]. However, TRAF2-deficient macrophages produce increased amounts of nitric oxide and TNF in response to TNF stimulation [35] and mice lacking TRAF2 also develop cachexia as a result of the increased levels of TNF [34, 35]. Thus, enhancing the pro-inflammatory effects of macrophages by targeting TRAF2 would not be an acceptable outcome.

In addition, it is important to mention that the mechanism by which TRAF2 operates as a tumor suppressor in B cells is unknown, but could be related to its role as a regulator of TNFR-mediated apoptosis [2, 37]. However, the role of TRAF2 controlling apoptosis might be cell dependent and/or TNFR dependent. In this regard, there is evidence supporting an anti-apoptotic function for TRAF2 in thymocytes [34], muscle [38] and fibroblasts [39, 40], further cautioning about the use of TRAF2 modulators in therapy.

TRAF3 and EBV-mediated diseases

Epstein Barr virus (EBV) is a member of the herpes virus family that infects over 90% of the world adult population. It persistently infects B lymphocytes, although rarely causing disease. However, immunosuppressed carriers infected with EBV might be prone to develop different pathologies of lymphoid origin, such as infectious mononucleosis, X-linked lymphoproliferative disease, B lymphoproliferative disease, Burkitt's lymphoma Hodgkin's disease and nasopharyngeal carcinoma, among others [41]. Different proteins encoded by the EBV genome are involved in the control of proliferation and survival of the infected cell, and therefore are essential for the persistence of the infection and eventually for the development of the overt pathology. However, latent membrane protein (LMP)-1 is the only EBV-encoded protein that seems to be sufficient to induce oncogenic transformation of mammalian cells [42, 43] and to sustain the development of

lymphoma in at least one transgenic mouse model [44, 45]. Furthermore, ample evidence exists supporting a key role for LMP-1 in the etiology of EBV-associated lymphoproliferative disease and lymphomas [41, 43, 46, 47].

Several reports demonstrate a role for TRAF-family members in LMP-1 signaling. TRAFs associate with LMP-1 through its C-terminal activating region (CTAR)-1, encompassing amino-acids 194 to 232 [48-50]. It has been suggested that LMP-1 signaling mimics CD40 and utilizes similar signal transduction pathways (reviewed in Refs. [47, 51, 52]). However, LMP signals in a seemingly deregulated manner, leading to amplified and sustained B cell activation [53, 54]. Both CD40 and LMP-1 recruit TRAFs to lipid rafts, a class of nonionic detergent-insoluble, sphingolipid-enriched membrane microdomains [54-57]. However, recent investigations have highlighted significant differences in the usage of TRAFs by CD40 and LMP-1. In this regard, TRAF3 is more efficiently recruited to LMP-1 than to CD40, while TRAF2 seems the opposite [49, 57]. Furthermore, the crystal structure of the LMP-1 peptide ^{204}PQQATDD^{210} encompassing the CTAR-1 bound to TRAF3 [58] shows that it binds the same TRAF3 crevice as CD40 [7]. However, CTAR-1 also forms additional hydrogen bonds that stabilize its interaction with TRAF3. Thus, LMP-1 has a higher affinity for TRAF3 than CD40. These observations suggest that LMP-1 mimicking peptides might be more potent as competitive antagonists of TRAF3, compared to peptidyl inhibitors based on the sequence of various TNF-family receptors.

TRAF1 [59] and TRAF6 [60-62] have been also implicated in LMP-1 signaling, but additional *in vivo* data are necessary to determine the actual roles of these two TRAFs in LMP-1 signaling under physiological conditions. Overall, the available data are consistent with the critical role played by TRAF3 in LMP-1 signaling, as illustrated by the abrogation of LMP-1 signaling in TRAF3 deficient cells [63-65]. If the essential role of TRAF3 in LMP-1 signaling is confirmed, targeting TRAF3 binding to LMP-1 would be a reasonable strategy for treating EBV-related diseases.

TRAF3 and Mantle Cell Lymphoma

It has been recently reported that TRAF3 and TRAF5 are upregulated in splenic marginal zone lymphoma (MZL) [66]. TRAF3 has been shown to be an inhibitor of TNFR-

family mediated NF-κB activation [67]. However, TRAF3 can form heterotrimers with TRAF5 [68], and TRAF5 is able to induce NF-κB activation [69, 70]. Therefore, since both TRAF3 and TRAF5 are upregulated in MZL, the formation of these heterotrimers might be favored and support the induction of NF-κB activity. Also, it is important to note that TRAF3 seems to work as an inhibitor of various TRAF2-mediated functions [71] and in some context, it might have functions similar to TRAF1.

TRAF3 and autoimmunity

Immune tolerance ensures an inability of the cellular components of the immune system to react to self-antigens while preserving defenses against pathogens. Several safeguard mechanisms are in place to protect the organism from autoreactive lymphocytes and autoantibodies, and their failure results in autoimmune diseases. One of these control mechanisms is the elimination of autoreactive B and T cells by apoptosis. Blockage of cell death pathways in the immune cells can therefore result in autoimmunity and/or cancer.

The autoimmune pathologies caused by BAFF deregulation deserve special mention. BAFF (TNFSF13B) is a TNF-family member required for survival of transitional and mature B cells [72, 73] and which is essential for later stages of B cell maturation and for Mantle Zone (MZ) B cell differentiation (reviewed in [74]). BAFF expression is deregulated in several autoimmune diseases and other pathologies. For instance, BAFF levels are elevated in sera from patients with severe B cell autoimmune disorders, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome [74]. Higher levels of BAFF are also found in the sera of human immunodeficiency virus (HIV) patients, which are prone to develop SLE [75]. Furthermore, BAFF and BAFF-R (TNFRSF13C) over-expression has been also described in several B cell malignancies, such as multiple myeloma [76, 77], non-Hodgkin's lymphoma [78, 79] and B-cell chronic lymphocytic leukemia (B-CLL). Indeed, most B-CLL cells express BAFF-R mRNA and a subset display BAFF on the surface, suggesting that BAFF might operate as an autocrine survival factor for B-CLL [80-82], in addition to promoting autoimmune manifestations observed in B-CLL patients (review in [83]). Furthermore, chronic infection may also lead to the sustained release of BAFF and thus the emergence of autoimmunity.

Consistent with these results, BAFF-transgenic mice developed immunoglobulin-based autoimmune disorders similar to systemic lupus erythematosus (SLE) and Sjögren's syndrome [84-87], thus proving the direct involvement of BAFF in the development of autoimmunity.

BAFF-mediated autoimmunity seems to be result of the preservation of maturing autoreactive T2 B cells which colonize forbidden follicular and marginal zone microenvironments [88, 89]. Survival of these cells causes a dramatic alteration of peripheral tolerance and the development of autoimmunity. Several lines of evidence indicate that among the different TNF-family receptors that can interact with BAFF, the BAFF-R (TNFRSF13C) protein is the one primarily responsible for increasing B cell survival (reviewed in [74]).

Little is known about the signal transduction pathways utilized by BAFF-R. TRAF3 might be the only member of the TRAF family that interacts with BAFF-R [90, 91]. The specificity of this interaction seems to be mediated by the sequence motif ^{162}PVPAT^{166}, which is different from the canonical TRAF1/2/3/5-binding motif. Furthermore, other amino-acids in the cytosolic tail of BAFF-R participate in the stabilization of the complex [9, 90, 91]. It is well established that BAFF-R signaling induces the activation of the non-canonical NF-κB pathway [92, 93]. However, the role of TRAF3 in this process is conflicting. Experiments involving TRAF3 over-expression indicate that it inhibits BAFF-R-mediated NF-κB activation and IL-10 production, thus supporting a role for TRAF3 as a negative regulator of at least some of the signaling events mediated by BAFF-R [90]. Conversely, mutations in the ^{162}PVPAT^{166} motif that abolished TRAF3 interaction with BAFF-R abrogated BAFF-R ability to activate the non-canonical NF-κB pathway [91]. These seemingly opposite results could be explained if the activation of the non-canonical NF-κB pathway by BAFF-R requires receptor-mediated degradation of TRAF3 [94].

It is worth noting that Hauer and coworkers [67] have recently shown that TRAF3 is a general inhibitor of TNFR-mediated non-canonical NF-κB activation, which may preclude its use as a drug target. However, if TRAF3 is indeed the only member of the TRAF family that regulates BAFF-R signaling, then TRAF3 would be a worthy target for

therapeutic intervention against SLE and Sjögren's syndrome. Resolution of the question of whether TRAF3 is the only TRAF-family member capable of binding BAFF-R thus is required to direct future possible therapeutic strategies.

Caveats of targeting TRAF3

Mice lacking TRAF3 have hypoglycemia and high glucocorticoid levels in serum, which results in depletion of peripheral white cells. These mice also develop cachexia and die by day 10 after birth. TRAF3 is prominently expressed in adrenocorticotropin hormone (ACTH)-secreting cells in the hypophysis [95]. Altogether, these results strongly support a role for TRAF3 in the regulation of ACTH production. Consequently, targeting TRAF3 might result in severe alterations in the metabolism of glucocorticoids.

TRAF3 and TRAF6 in infections and septic shock

Toll Receptors (TLR) are key players in the regulation of innate immune responses [96-98]. Ten TLR family members have been identified in humans. These receptors recognize pathogen-associated molecular patters (PAMPs), triggering host defense responses as part of innate immunity. Different TLRs recognize distinct PAMPs. Thus, bacterial lipoproteins are recognized by TLR2, double stranded DNA by TLR3, bacteria lipopolysaccharide by TLR4, flagellin by TLR5, single-stranded viral RNA by TLR7, and unmethylated CpG DNA of bacteria and viruses by TLR9 (reviewed in [97]). Important for the host responses against pathogens are also the members of the IL-1R family, which regulate inflammation responses [99, 100].

Alterations in TLR structure, expression, and function have been implicated in several diseases. In this regard, polymorphisms of proteins in the TLR pathways are related to anomalous responses against pathogens, and have been correlated with immunoinsufficiency (i.e., chronic infection), atherosclerosis, cancer, and asthma [101].

TRAF6 is a common and critical mediator of signal transduction by the TLR/IL-1R family [96, 102]. This is well illustrated in *traf6* deficient mice, which have severely impaired TLR-mediated responses to various PAMPs [103, 104] and fail to properly respond to IL-1 stimulation [103, 105].

TRAF6 does not directly interact with either TLRs or IL-Rs. Instead, MyD88, Tollip and IRAK-family proteins mediate its recruitment to the receptors. Then, IRAKs and TRAF6 dissociate from the complex, allowing TRAF6 to interact with ubiquitin conjugating enzymes Ubc13 and Uev1A. These enzymes covalently attach non-canonical poly-ubiquitin chains to TRAF6, in which the isopeptide bond occurs at the lysine 63 residue in ubiquitin, instead of lysine 48. This form of polyubiquitin is not a substrate for the proteasome, and does not target TRAF6 for degradation, but rather induces TRAF6 to associate with a complex composed by TAB1, TAB2 and transforming growth factor β activating kinase (TAK)-1, resulting in TAK1 phosphorylation and activation. Activated TAK-1 then activates the IκB kinase kinase (IKK) complex and also activates MAP kinase kinase (MKK)-6, resulting in NF-κB and c-JUN (AP-1) activation, and the induction of expression of multiple proinflammatory genes [106, 107].

Because TRAF6 is an essential common mediator of TLR/IL1R family signaling, it stands as an attractive drug target for possible use in treatment of a wide variety of acute and chronic inflammatory conditions. Septic shock provides a good example. Studies in traf6-/- mice have shown profound impairment of TLR-mediated responses to different PAMPs [103, 104] supporting the notion that TRAF6 might be a suitable target in severe cases of infection. In this regard, the lethal consequence of systemic bacterial invasion have been linked to over-stimulation of the TLR pathways, resulting in massive production of pro-inflammatory cytokines, causing severe systemic inflammation that may progress to multiple organ failure and death even after the bacterial infection has been clinically controlled [108-111]. Septic shock is associated with a 30-50% death rate in severe cases [109, 110], accounting for over 100,000 deaths annually in the United States alone [101].

Interestingly, recently it was reported that TRAF3 deficient cells fail to induce type I interferons and anti-inflammatory cytokines in response to TLR activation, which has led to the identification of a new TRAF3 dependent pathway involved in the control of innate immunity [112, 113]. Similar to TRAF6, the TRAF3 protein is also recruited to the TLRs through MyD88 and IRAK1 and 4, but rather than activating MAP3K and IKK,

which induce pro-inflammatory cytokines, TRAF3 engages TRIF-dependent signaling pathways leading to activation of TBK-1 and IKK-ε, inducing the expression of type I interferons and the anti-inflammatory IL-10 [112, 113]. Thus, TRAF3 may play important roles both in interferon-dependent responses to viral pathogens, as well as in down-regulating innate immune responses via its effects on IL-10 production. Therefore, by pharmacologically modulating the recruitment of either TRAF3 or TRAF6 to the activated TLR, or by interfering with their downstream functions, it may be possible to manipulate the type of response emanating from TLRs, depending on the pathogen, stage of infection, or other scenarios.

TRAF4 might also function as a silencer of TLR-signal transduction through its association to TRAF6 and TRIF [114], but additional *in vivo* data using TRAF4 deficient cells or TRAF4 knock-out mice would be required to ascertain the role of TRAF4 in innate immunity.

TRAF6 and other diseases

The analysis of the phenotypes developed by TRAF6-deficient mice has highlighted a seminal role of TRAF6 in the regulation of signaling by various TNFR-family members. These results suggest additional avenues for the usage of TRAF6 agonists and/or antagonists as therapeutics. For instance, TRAF6 is a critical regulator of RANK. This TNFR family member is essential for the differentiation and activation of osteoclasts, the cells responsible for bone resorption [115, 116]. This is demonstrated by the phenotype developed by mice deficient in RANK or its ligand (RANKL), which are osteopetrotic as the result of lack of bone resorption and remodeling caused by functionally deficient osteoclasts [116]. TRAF6 is essential for RANK signaling and consequently it is required for osteoclast cytoskeletal organization and resorptive function [117]. Accordingly, TRAF6 deficient mice lack functional osteoclasts and develop severe osteopetrosis [103, 105].

X-linked hypohidrotic ectodermal dysplasia is a genetic disorder characterized by lack or anomalous formation of hair follicles, teeth and sweat and sebaceous glands. Affected children have a reduced ability to sweat, which can result in life-threatening high fever [118, 119]. This disease is caused by mutations of the ectodysplasin A gene (Eda)

encoding the TNF family ligands EDA-1 and EDA-2, which interact with the TNFR-family members EDAR and XEDAR, respectively [120, 121, 122]. Besides EDAR and XEDAR, the TNFR family member TROY might also regulate the development of these epidermal appendages [123]. TRAF6 deficient mice also develop a phenotype similar to hypohidrotic ectodermal dysplasia [124]. In this regard, TRAF6 interaction and regulation of XEDAR and TROY has been reported [123, 125]. However, given the total absence of sweat glands in *traf6* -/- mice, it is suspected that TRAF6 might also participate in the control of EDAR activities [124].

Caveats of targeting TRAF6

In summary, the key role of TRAF6 in innate immune responses, as well as in bone formation and resorption, and hair follicle formation opens the possibility of using TRAF6 modulators for treating diseases such as septic shock, osteoporosis, arthritis, periodontal disease, cancer-induced bone lesions and even alopecia [126]. However, blocking TRAF6-mediated signaling would increase the risk of opportunistic bacterial infections, which might preclude the use of drugs targeting TRAF6 for chronic diseases and immunosuppressed patients. On the other hand, as a short-term treatment, it might prove helpful for reducing the mortality associated with septic shock by shutting down TLR-mediated induction of pro-inflammatory cytokines.

Perspectives

The phenotypes of the TRAF-specific knock-out and TRAF-transgenic mice have brought to light the pleiotropic roles of TRAFs in cell physiology and have warned of the adverse effects of dysregulating their expression and function. Studies of genetically engineered mice, however, have also uncovered the participation of TRAFs in processes relevant to several human diseases for which new therapeutic approaches are desperately needed (Figure 1).

Despite the difficulty in identifying small molecule modulators that can either disrupt or enhance specific protein-protein interactions, the development of new screening and structure-based drug design technologies raises optimism. Thus, the application of high throughput screening technologies to test large synthetic and natural

chemical compound libraries, as well as structured-based drug design will likely identify compounds capable of interfering with the functions of specific members of the TRAF family or other proteins in the pathways that are dependent on TRAFs. In this regard, recent articles have shown the potential of these technologies for modulating the activity of TNF-family proteins. Thus, Takasaki and coworkers [127] have identified exocyclic small peptidomimetics corresponding to critical binding sites in the TNFR1 that prevent TNF-mediated apoptosis. He and coworkers [128] have identified a small-molecule inhibitor of TNF that binds trimeric TNF and promotes subunit disassembly and its functional inhibition. Also, Fournel and coworkers [129] have reported the structure-based design of small molecules with C3 symmetry that mimic CD40L and act as agonist of CD40 functions. Altogether, these results provide proof of concept that similar approaches could result in the identification of compounds that modulate TRAF-trimerization or their association with TNFRs and other proteins in the pathway.

Development of TNFR-mimic peptides that target the function of specific members of the TRAF-family is a complementary approach that might yield significant success. Indeed, the suitability of TNFR-mimic peptides to interfere with TRAF activities has been already shown in cell cultures [3, 10]. The crystal structures of different TRAF-family members bound to TRAF-binding peptides from several members of the TNFR family support the notion that development of peptidomimetics that preferentially interact with and modulate the function of particular members of the TRAF family is feasible and worth exploring for therapeutic purposes. Recent advances in cell permeable peptide technology, improving cellular penetration and stability [130-132] also raises optimism that peptidomimetics could be eventually translated to the clinic.

Alternatively, enzymes that associate with TRAFs may be attractive and more pharmaceutically tractable targets for drug discovery. For instance, inhibitors of Ubc13, the unique E2 that associates with the RING domains of TRAF, would be predicted to short-circuit signal transduction mediated by many of these adapter proteins. Similarly, the protein kinases recruited to TRAFs could also be targeted. The relative advantages and disadvantages of these various targets from the perspective of efficacy and toxicity, however, are beyond the scope of this review.

While the pleiotropic effects of TRAF-family proteins and the partner proteins with which they associate caution against the use of pharmacological TRAF modulators, at least for chronic diseases, rapidly evolving new drug delivery systems and nanodevices that restrict drugs to sites of disease forecast emerging opportunities to consider therapeutic approaches for either enhancing or inhibiting the activities of TRAFs for future drug development.

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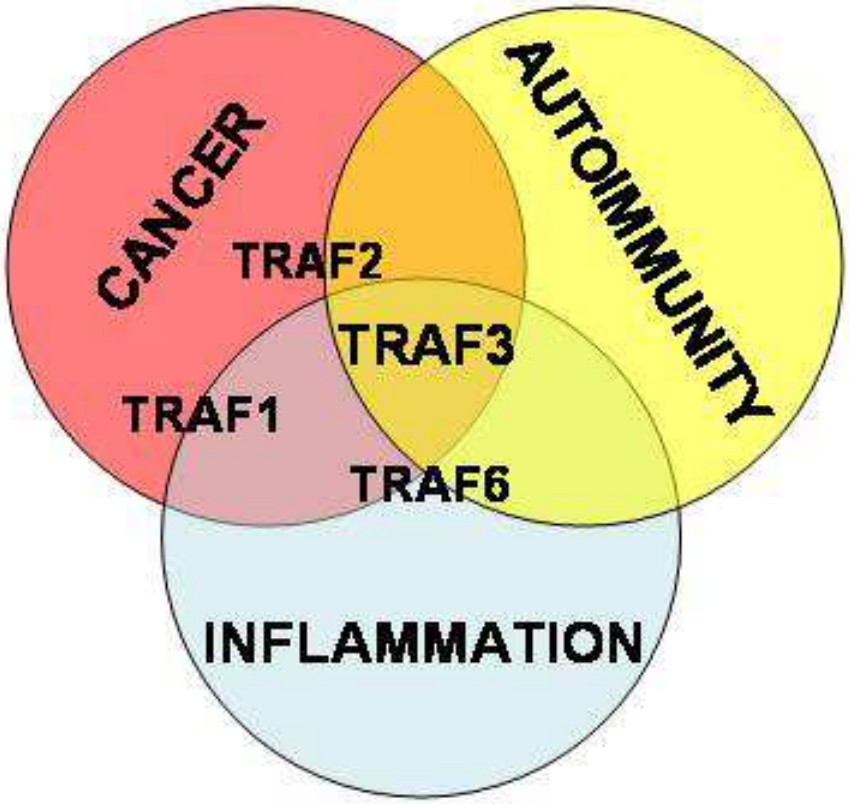
Figure 1.

TRAFs regulate both the acquired and innate immune systems, as well as certain additional physiological processes. Deregulation of these immune pathways is causative of cancer, autoimmunity and inflammation. Targeting the function of specific TRAF family members could provide novel approaches to restoring normal immune system function, but caution must be taken to avoid unwanted side-effects.



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