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Roles for TNF-Receptor Associated Factor 3 (TRAF3) in Lymphocyte Functions

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Abstract

TRAF3 is an adapter protein that serves and regulates the functions of several types of receptors, located both inside the cell and at the plasma membrane. These include members of the TNF receptor superfamily (TNFR-SF), toll-like receptors (TLR), and cytokine receptors. It has become increasingly evident that the roles and functions of TRAF3 are highly context-dependent. TRAF3 can serve distinct roles for different receptors in the same cell, and also has highly cell-type-dependent functions. This review focuses upon the current state of knowledge regarding how TRAF3 regulates the biology and effector functions of B and T lymphocytes, two major cell types of the adaptive immune response in which TRAF3 has markedly distinct roles.

Keywords

TRAF; B lymphocyte; T lymphocyte; signal transduction; lymphocyte activation

TRAF3 plays multiple roles in a large number and variety of signaling pathways in virtually all types of mammalian cells. In recent years, published reports have revealed that TRAF3 function can differ considerably, depending upon both the specific receptor and signaling pathway involved, as well as the specific cell type in which TRAF3 is expressed. The purpose of this focused review is to summarize the specific roles played by TRAF3 in both B and T lymphocytes. Consideration of these roles highlights both the distinct and varied functions of TRAF3.

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1. Roles of TRAF3 in B lymphocytes

1.1 TRAF3 in B cell biology

1.1.1 B cell homeostasis, development and survival—TRAF3 clearly plays critical roles in cellular physiology, because the original TRAF3-null mouse resulted in early lethality (1). Additionally, TRAF3 plays both positive and negative roles for various immune receptors expressed on B cells, and many of these receptors activate very similar signaling pathways, as discussed in the following section. For these reasons, it was very difficult to study the specific functions of TRAF3 in B cells, until mouse models of conditional deletion of the *traf3* gene became available. Two independent mouse strains with conditional deletion of *traf3* specifically in B cells reveal that TRAF3 is particularly important for restraint of B-cell homeostasis and survival, and in development of the marginal zone (MZ) B cell subset (2, 3). Unlike the TRAF3 null mouse, which has reduced spleen size and cellularity (1), B-cell specific TRAF3-deficient mice (B-TRAF3^{-/-}) display enlarged spleens and lymph nodes, with increased cellularity and expansion of MZ and follicular B cells (2, 3). Expansion of these B cell subsets accounts for the splenomegaly and lymphadenopathy, because there is no significant change in other immune cell types. B cells isolated from B-TRAF3^{-/-} mice display constitutive nuclear p52, discussed further below, which is seen in all TRAF3-deficient cell types to date (4). *Ex vivo* cultures of B cells isolated from B-TRAF3^{-/-} mice show enhanced factor-independent cell survival compared to littermate controls (LMC). While BAFF treatment markedly improves LMC B cell survival, it does not impact TRAF3^{-/-} B cell survival (2).

Xie et. al. also showed that without TRAF3, B cells no longer depend upon BAFF signaling for maturation into MZ B cells (2). The development of mature B cells requires BAFF-R induced activation of the non-canonical NF- κ B2 pathway (5). p52^{-/-}/p100^{-/-} mice show defects in splenic MZ architecture and a markedly decreased MZ B cell population (6, 7). This further supports the hypothesis that TRAF3-mediated regulation of the NF- κ B2 pathway is important for MZ B cell development. However, contrary to effects on MZ B cell development, hyper-survival of B cells due to lack of TRAF3 does not entirely rely on constitutive activation of NF- κ B2 (2, 3). T cells or dendritic cell-specific TRAF3 deletion leads to enhanced NF- κ B2 activation, but these other immune cell types do not survive longer than cells from their LMC (3, 8); J. Poovassery & GAB, data not shown). These data suggest that, in addition to NF- κ B2 activation, TRAF3 restrains additional pro-survival pathways, and/or promotes pro-apoptotic pathways in a B cell-specific manner.

1.1.2 B cell malignancies—Consistent with its demonstrated key role in restraint of normal B cell survival, loss-of-function mutations of the *traf3* gene are prevalent in the human B cell malignancies multiple myeloma (MM), Waldenström's macroglobulinemia, and different subtypes of B cell lymphoma. Dysregulation of NF- κ B pathways has been observed in many hematological cancers. Two reports showed that constitutive NF- κ B activation is very common among MM tumors from patients and in MM-derived cell lines (9, 10), and TRAF3 is identified as one of the common deleted genes. Keats et. al showed that ~19% of MM patients and 17% of MM cell lines have TRAF3 mutations. When Wt TRAF3 is reintroduced into MM cell lines with inactivated *traf3*, growth of 80% of the cell

lines tested is inhibited, with reduced levels of p52. Annunziata et. al further demonstrated that reintroduction of TRAF3 in MM cells decreases NF- κ B target gene expression. Monoallelic deletion of *traf3* is present in 15% of patients diagnosed with classical Hodgkin lymphoma (11) and biallelic deletion of *traf3* is also observed in different subtypes of B cell lymphoma (12). Taken together, these results indicate that TRAF3 plays an important tumor suppressor role in B lymphocytes. In support of this concept, ~ 30% of B- TRAF3^{-/-} mice spontaneously develop various B cell malignancies between 9-18 months of age (13). The frequent loss or truncation of the *traf3* gene observed in B-cell malignancies may occur in part because the *traf3* locus is near the *Igh* locus on chromosome 14 in human and chromosome 12 in mouse, a genetic region subject to frequent translocation.

It is thus evident that TRAF3 plays a major and unique role specifically in B lymphocytes, that of negative regulation of homeostatic survival, and this role also makes TRAF3 an important suppressor of B cell malignancies. It will thus be important to identify the specific pro-survival pathways regulated by TRAF3, in order to more effectively target and prevent recurrence of B cell cancers with inactivating *traf3* mutations.

1.2. Roles of TRAF3 in receptor-mediated signaling to B cells

In addition to its major role in restraining homeostatic survival of B cells, TRAF3 serves specific roles in regulating signaling and effector functions downstream of a number of distinct receptors of B cells. The following section will summarize the known roles and mechanisms of TRAF3 for each of these receptors.

1.2.1 CD40 and Latent Membrane Protein 1 (LMP1)—CD40 is the TRAF3-binding receptor that has been most frequently studied. This is not surprising when considering that TRAF3 was the first signaling protein found to bind the CD40 cytoplasmic domain, and TRAF3 was initially called ‘CD40 binding protein’ (14, 15). Here, we will focus upon the known functions of TRAF3 in interacting with, and mediating signals via B cell CD40. Induced overexpression of either full-length TRAF3 or a mutant lacking the N-terminal RING domain (DNTRAF3) in B cells inhibits CD40-induced antibody production, the first indication that TRAF3 serves as a negative regulator of CD40 biological functions in B cells (16). The similar inhibitory activities of Wt and DNTRAF3 suggested that this negative regulation was mediated at least in part by potential competition with a positive regulatory factor. Inasmuch as TRAFs 1, 2, 3, and 5 share a partially overlapping CD40 binding site (17), competition among them is plausible. This overlapping TRAF binding site also complicates interpretation of studies in which the site is mutated or in which one or more TRAFs is overexpressed relative to the others.

Mice totally deficient in TRAF3 succumb to early lethality (1), so cannot serve as an experimental model to study the functions of TRAF3 in mature B cells. To circumvent this obstacle prior to the availability of conditional knockout mice, Hostager et al. adapted the technique of gene targeting by homologous recombination to somatic cells, allowing for the first time production of viable B cells specifically and completely lacking distinct individual TRAFs (18). Studies with these cell lines demonstrated that TRAF3 competes with TRAF2 for CD40 binding, and thereby inhibits the TRAF2-dependent synergy between CD40 and B

cell antigen receptor signals (19). TRAF3^{-/-} B cell lines display enhanced CD40-mediated activation of the MAP kinase JNK and increased antibody production (20), consistent with the negative impact of overexpressed TRAF3 (16), but CD40-mediated activation of ERK, Akt or p38 kinases, and canonical NF- κ B1 are not significantly altered in the absence of TRAF3 (20). Similarly, CD40-mediated costimulatory molecule upregulation and production of IL-6 and TNF α in B cells appears to be TRAF3-independent (20). Subsequently, B cell-conditional TRAF3-deficient mice allowed re-assessment of the role of TRAF3 in CD40-mediated signaling to B cells. Although, as discussed above, the phenotype of these mice revealed a critical role for TRAF3 in restraining homeostatic B cell survival, their B cells do not show enhanced CD40-mediated NF- κ B1 activation (2), confirming results in TRAF3^{-/-} B cell lines, and leading to the conclusion that TRAF3 regulates a distinct subset of CD40 function in B cells (Fig. 1).

The Epstein-Barr virus (EBV)-encoded transforming protein LMP1 is a powerful mimic of CD40-induced effector functions and upstream signals in B cells, but results in amplified and sustained B cell activation compared to CD40 effects (reviewed in (21, 22). Although the C-terminal cytoplasmic signaling domain of LMP1 has very little sequence homology with that of CD40, it does contain a modified TRAF-binding site (TBS), and like CD40, B cell-expressed LMP1 associates with TRAFs 1, 2, 3, 5 and 6 (reviewed in (23, 24). It was thus reasonable to initially assume that both CD40 and its mimic LMP1 use each of the TRAFs similarly. Surprisingly, it has been learned over the past decade that CD40 and LMP1 both associate with and utilize individual types of TRAFs in unique and distinct ways in B cells (22). This is particularly striking in the case of TRAF3. B cell lines lacking only TRAF3 (described above) revealed that while TRAF3 deficiency has the expected positive impact on CD40 signals and function, the same signals and functions mediated by the LMP1 signaling domain are markedly compromised in the absence of TRAF3 (20). Also in contrast to CD40, LMP1 requires its association with TRAF3 to enable recruitment of TRAF5 (25), a TRAF that plays a much more important role in LMP1 biological effects in B cells than effects mediated by CD40 (26). The contrasting roles played by TRAF3 in CD40 vs. LMP1 functions in B cells correlate with substantially more avid binding of TRAF3 to LMP1 (20), which is in turn associated with additional binding contacts in the TRAF3-LMP1 association, compared to the association of TRAF3 with CD40 (27).

Another important way in which CD40 and LMP1 interact differently with TRAF3 in B cells involves TRAF3 regulation. While both receptors rapidly recruit TRAF3 to cholesterol-rich membrane lipid domains (28-30), CD40 signaling induces polyubiquitination and proteasome-mediated TRAF3 degradation, while LMP1 induces no degradation of TRAF3 (31). This may be because CD40-mediated TRAF3 degradation requires close association with TRAF2 and the E3 ubiquitin ligase activity of the TRAF2 RING domain (32), and LMP1 exhibits much weaker TRAF2 binding than CD40 in B cells (20). However, the regulation of TRAF3 function by receptor binding is complex. The multiple-TRAF associating TBS regions of CD40 vs. LMP1 contain unique as well as shared sequences (33). When B cells are transfected with CD40 or LMP1 molecules in which their respective TBS sequences are interchanged, the binding strength of TRAF2, but not of TRAF3, tracks with the TBS sequence and this is also true of receptor-mediated TRAF2 and 3 degradation (34). Similarly, a human polymorphism in the cytoplasmic

domain of CD40 that confers a gain of function phenotype in CD40 signaling and B cell function (35) results in enhanced TRAF3 binding; the increased signaling is abrogated in TRAF3^{-/-} B cells (36). However, CD40-mediated TRAF3 degradation is intact, and the single amino acid polymorphism occurs well outside of the TBS, and does not impact TRAF2 or TRAF6 binding (35, 36). The nature of TRAF3 association with distinct receptors thus clearly impacts its role in receptor function, but multiple mechanisms likely contribute to this, including specific associations of both TRAF3 and the receptor in question with other TRAFs.

1.2.2 BAFF-R, TACI, and BCMA—Three closely-related members of the TNFR superfamily (TNFR-SF) – BAFFR/BLyS/TALL-1, TACI and APRIL – play critical roles in B cell development, homeostasis, and function (reviewed in (37-39)). Initial binding studies of these receptors exogenously overexpressed in transfected cells indicated that all three receptors bind TRAF3, but - unusual among TNFR-SF members - BAFFR appeared to bind only TRAF3 (40, 41), while TACI and BCMA bind both TRAF3 and additional TRAFs (42-44). Subsequent studies of endogenous BAFFR in B cells revealed that this receptor in fact also binds to TRAFs 2 and 6; the latter is important in BAFFR-mediated B cell activation signals (45);(4). In contrast, TRAF3 emerged as a negative regulator of BAFFR-mediated NF- κ B activation in B cells (40). Like CD40, BAFFR can induce TRAF3 degradation in B cell lines (40, 46), although this is difficult to detect in primary B cells (2). The requirement of TRAF3 association with the TBS to observe BAFFR-mediated TRAF3 degradation in the B cells line M12.4.1 (40) suggests that, similar to CD40-mediated TRAF3 degradation, there is a requirement to associate with TRAF2 and its E3 ubiquitin ligase activity. Further evidence of this is provided by the lack of BAFFR-mediated degradation of a human TRAF3 mutant isolated from a multiple myeloma; this mutant TRAF3 fails to associate with TRAF2 following BAFFR signaling (47).

While competition with TRAF2 is an important mechanism by which TRAF3 negatively regulates CD40 signaling in B cells, it is not yet clear how TRAF3 inhibits BAFFR-mediated signals apart from those involved in B cell homeostasis, discussed above. TRAF3 coimmunoprecipitates with the NF- κ B pathway-associated protein Act1, in human B cells stimulated *in vitro* with BAFF (48). Like mice lacking TRAF3 in B cells (2), Act1-deficient mice have enhanced B cell survival, enlarged secondary lymphoid organs, and evidence of autoimmunity. However, TRAF3^{-/-} B cells, unlike Act1^{-/-} B cells, do not have enhanced responses to BAFFR or CD40, and do not have upregulation of Bcl-xL (2, 48). A further complication is that another strain of Act1^{-/-} mice does not display any abnormalities in B cell survival or BAFFR functions (49), although both strains share a hyper-responsiveness to IL-17 (50). This is consistent with a proposed role for both Act1 and TRAF3 in IL-17R signaling, as shown in epithelial cell lines and a mouse autoimmunity model (51, 52). This role for TRAF3 has not been investigated in B cells. The precise relationship between Act1, BAFFR, and TRAF3 *in vivo* thus remains incompletely resolved.

TRAF3 binds *in vitro* to TACI, as demonstrated in mouse and human B cell lines and primary mouse B cells stimulated with BAFF or APRIL (40, 45). However, little is currently known about the specific impact of TRAF3 association on TACI signaling, as opposed to the general ability of cytoplasmic TRAF3 to inhibit NF- κ B2 activation by various TNFR-SF

members, discussed above, including TACI (53). The TNF family ligand APRIL binds both TACI and BCMA, while BAFF binds both BAFFR and TACI, thus complicating interpretation of many experiments in which multiple receptors can be engaged by the same ligand. Similarly, little is known about the role(s) played by TRAF3 in BCMA signaling. In this case, TRAF3-BCMA binding remains to be confirmed in B cells, as to date this binding has only been demonstrated by exogenous overexpression in epithelial or fibroblast cell lines (42, 43). Understanding how TRAF3 regulates TACI and BCMA signals and functions in B cells is a knowledge gap that needs to be filled.

1.2.3 TNFR2/CD120b—As indicated by their name, the first two TRAFs (TNF-receptor associated factors 1 and 2) were initially identified by their association with CD120b (54). The majority of studies of CD120b signaling have not been performed with B cells, so it was not known for many years that CD120b in B cells also binds to TRAF3 (55). Interestingly, B cells express little if any CD120a/TNFR1, so CD120b mediates activating effects of TNF on B cells (56). The cytoplasmic domain of CD120b mediates NF- κ B2 activation in a TRAF2-dependent manner in B cells, and like CD40 and BAFFR, induces degradation of both TRAFs 2 and 3 (55). It is thus likely that TRAF3 negatively regulates CD120b-mediated activation signals to B cells, both by competing for TRAF2 binding to the receptor, and by inhibiting NF- κ B2 activation in the cytoplasm. However, these mechanisms remain to be tested.

1.2.4 Toll-like receptors (TLRs)—It has become increasingly evident over the past several decades that specific pattern-recognition receptors play critical roles in various aspects of many immune responses. These receptors have been most often studied on various types of myeloid cells, as well as non-immune cell types. However, the first family of innate immune receptors to be identified, the TLRs, are also expressed by B cells, which respond robustly to activation signals from these receptors (reviewed in (57-59). For some time, it was thought that TRAF6 was the only TRAF molecule participating in TLR signals (reviewed in (60). However, TRAF3 was subsequently identified as an important positive mediator of a subset of antiviral TLR signals in myeloid cells (61-63). Thus, when a rare human patient with a defective immune response to a Herpesvirus infection was found to possess a heterozygous germline TRAF3 mutation, reduced responses of the patient's myeloid and fibroblast cells to antiviral TLR ligands was the focus of investigation (64). The important role for TRAF3 in T cell function, discussed below, now indicates that this TRAF3 role may also have been involved in the patient's abnormal immune response, although the true function of this mutant TRAF3 molecule, and its interaction with Wt TRAF3 in the same cells, is likely complex. The overall theme of this review, that TRAF3 has very context-specific functions, prompted an investigation of the role played by TRAF3 in B cell responses to TLRs. In contrast to the positive role of TRAF3 in myeloid cell responses to TLRs, TRAF3 functions as a negative regulator of B cell TLR responses, including TLRs 3, 4, 7, and 9 (65). This role is independent of impact on B cell survival or proliferation, and is associated with enhanced TLR-mediated NF- κ B1 activation. In contrast, TLR-mediated MAPK activation is not affected by B cell TRAF3 (65). A recent study suggests a possible complementary role for TRAFs 3 and 5 in regulating TLR signals to B cells. TRAF5 was identified as another negative regulator of TLR-mediated B cell

activation, but in contrast to TRAF3, TRAF5 inhibits the MAPK pathway downstream of TLRs, but has no impact on the NF- κ B1 pathway (66). As TRAF3 and TRAF5 can associate in B cells (25), this raises the intriguing possibility that these two TRAFs use complementary mechanisms to restrain TLR signals in a B cell-specific manner.

2. Roles of TRAF3 in T lymphocytes

It is clear that TRAF3 plays critical roles in B cells, dendritic cells (DC) and macrophages. However, the roles of TRAF3 in T cells have only recently begun to be closely examined. Recent work has validated earlier suggestions that TRAF3 is a critical regulatory factor in T-cell function, and revealed that TRAF3 plays important roles in both TCR signaling and development of particular T cell subsets. Additionally, as in other cell types, TRAF3 plays receptor-specific roles in T cell biology.

2.1 TRAF3 in T cell biology and TCR signaling

2.1.1 T cell function—Approaches to study the role of TRAF3 in T cell biology *in vivo* were initially limited by the early lethality of mice lacking TRAF3 in all cells and tissues (1). To circumvent this major limitation, early studies used TRAF3-deficient liver stem cells to reconstitute the immune systems of irradiated Wt mice. It was found that T-cell-dependent (TD) IgG production is impaired in TRAF3-deficient chimeric mice, but T-cell-independent (TI) IgG production is unaffected. Interestingly, although TRAF3-deficient T cells are defective in antigen-specific recall responses, they proliferate normally upon anti-CD3 Ab stimulation in this study. It was also shown that TRAF3-deficient B cells proliferate normally in response to anti-IgM Ab and CD154 stimulation (1). These early results indicated that the function of TRAF3 deficient T cells is impaired. However, it is unclear how TRAF3 deficiency in all other immune cells contributed to the defective TD immune responses in these chimeric mice, as it is now evident that the function of B cells, DC, macrophages and possibly other immune cells are markedly altered by the absence of TRAF3 (4). In addition, T cell development in this special environment could also be altered. It is thus impossible to determine which features of the observed phenotype are due to T cell intrinsic vs. extrinsic effects. However, it could be concluded that TRAF3 plays important roles in T cell function. Specific mechanisms for these roles were not explored, however, for > 10 years.

To more clearly address the role of TRAF3 in T cell biology, CD4^{Cre} mice were bred with TRAF3^{flox/flox} mice (8). Studies with this new mouse model extend previous findings that TRAF3 is required for TD antibody responses, by showing that TRAF3 in T cells is particularly critical for the production of IgG1 (8). Further, antigen-specific CD8⁺ and CD4⁺ T-cell responses to infection with the intracellular pathogen *Listeria monocytogenes* are strikingly impaired (8), demonstrating that TRAF3 is required for optimal T-cell function *in vivo*. Interestingly, Foxp3⁺ regulatory T cells (Treg) in CD4^{Cre}TRAF3^{flox/flox} (T-TRAF3^{-/-}) mice are doubled in number. This finding, together with the well-established role of TRAF3 signaling by TNFR-SF members such as CD40 and BAFFR, led us to initially hypothesize that this impairment is attributable to the combined effects of increased Treg and the impaired activation by TNFR-SF TCR costimulatory that bind TRAF3 (e.g. OX40, 4-1BB, CD30, CD27). However, *in vitro* stimulation of purified Treg cell-depleted TRAF3^{-/-} T

cells with anti-CD3 and anti-CD28 Abs resulted in markedly reduced cytokine production and significantly less proliferation but more apoptosis, compared to LMC T cells (8). These results indicated that TRAF3 is intrinsically involved in TCR+CD28 mediated signaling, and this is an important and unanticipated mechanism by which TRAF3 deficiency impacts T cell functions.

2.1.2 TCR signaling—TRAF6-mediated IKK γ ubiquitination and IKK activation requires oligomerization of the Bcl-2 family proteins BCL10 and MALT1 (67). More recently, it was reported that TRAF6 can modulate ubiquitination of the adapter protein Linker of Activated T cells (LAT) in TCR signaling (68), indicating that TRAF6 plays roles at multiple stages of TCR signaling. There is thus precedent for the concept of TRAF involvement in TCR functions. To explore how TRAF3 affects TCR signaling, we examined different TCR signaling pathways in TRAF3-deficient Treg-depleted T cells. TCR/CD28-mediated early signaling events, including phosphorylation of the early TCR signaling molecules ZAP70, LAT, PLC- γ , and ERK, are impaired (8), and TCR-induced Ca⁺ influx is also defective in the absence of TRAF3 (ZY and GAB, data not shown). However, we observed no changes in TCR induced canonical NF- κ B1 activation (8), indicating that TRAF3 might differentially regulate different TCR signaling pathways. To explore the molecular mechanisms of how TRAF3 exerts its effect, the TCR complex was immuno-precipitated after stimulation. In Wt T cells, TRAF3 is recruited to the TCR complex in a CD28-dependent manner, as anti-CD3 or anti-CD28 Ab alone cannot induce the recruitment. Recent experiments reveal that TRAF3 modulated the activation status of Lck and CSK, as well as decreasing membrane CSK (ZY and GAB, data not shown). Future work will focus on how TRAF3 associates with the TCR complex, identification of its direct targets and how TRAF3 promotes TCR-mediated signaling.

2.1.3 Thymocyte development—In studies of CD4^{Cre}TRAF3^{flox/flox} mice, deficiency of TRAF3 in T cells does not affect the proportion of B cells or CD4⁺ and CD8⁺ conventional T cells. However, these mice display a 2-3-fold increase in Foxp3⁺ Treg (8) and a profound decrease in invariant Nature Killer T (iNKT) cells (69). The cellularity of CD4⁺ T cells, CD8⁺ T cells and B cells is normal or slightly increased in thymus, spleen and lymph nodes. Because TRAF3 deficiency impairs TCR signaling in mature T cells, we explored whether thymic selection is also affected. Our preliminary data show that neither positive nor negative selection in the thymus is altered in the absence of TRAF3 (ZY and GAB, data not shown). In contrast to findings in CD4^{Cre}TRAF3^{flox/flox} mice, Gardam et al. (3) reported that Lck^{Cre}TRAF3^{flox/flox} mice exhibit reduced numbers of peripheral CD4⁺ and CD8⁺ T cells. To explore this difference, we crossed Lck^{Cre} with TRAF3^{flox/flox} mice and found 2-3-fold fewer T cells in thymus and peripheral lymphoid tissues in Lck^{Cre}TRAF3^{flox/flox} mice than LMC mice or CD4^{Cre}TRAF3^{flox/flox} mice (ZY and GAB, data not shown). Lck^{Cre}-mediated *traf3* gene depletion occurs significantly earlier in T cell development than does CD4^{Cre}-mediated removal of *traf3* (70, 71), as TRAF3 protein is completely depleted at the double-negative (DN)3 stage in Lck^{Cre}TRAF3^{flox/flox} mice and at the double-positive (DP) stage of T cell development in CD4^{Cre}TRAF3^{flox/flox} mice (8, 69). These results suggest that TRAF3 plays critical roles in early stages of T cell development. TRAF3 may thus be required for pre-TCR signaling and early stage expansion of

thymocytes, but not after T cells attain the DP stage. Therefore, TRAF3 is differentially required at distinct T cell developmental stages.

2.1.4 iNKT cell development and function—Development of iNKT cells requires TCR signaling at early stages (stages 0 and 1) and IL-15 signaling at later stages 2 and 3 (72). We found that there are ~ 10-fold fewer iNKT cells in the spleen, liver and thymus of T-TRAF3^{-/-} mice than LMC. Interestingly, only later stages of development of iNKT cells are impaired, but not stages 0 and 1 (72), indicating that TCR signaling required for iNKT cell selection at early stages is unaltered. Those TRAF3-deficient iNKT cells that do develop fail to up-regulate T-bet and CD122, two critical molecules for IL-15 signaling following TCR engagement, during the transition from stage 1 to stage 2. This results in defective expansion at later stages. Therefore, TRAF3 plays a critical role in the transition between these two distinct signaling pathways and developmental stages. In addition, TRAF3 is also required for mature iNKT cell function, as defective TCR signaling and cytokine production is observed in the absence of TRAF3 in iNKT cells (69).

Upregulation of CD122 and T-bet requires TCR signaling (73-76). Results discussed above suggest that TCR signaling in stage 1 of iNKT cell development is impaired in the absence of TRAF3. The question then arises as to why TRAF3 affects TCR signaling in stage 1 and fully mature iNKT cells, but not earlier stages. One possibility is that this is due to the distinct maturation status of different developmental stages of iNKT cells. During iNKT cell development, CD24 is downregulated after stage 0, indicating the maturity of iNKT cells, which is equivalent to mature conventional T cells. Together with the impaired TCR signaling in mature conventional T cells and the unaltered thymic selection (discussed above), we propose that TRAF3 plays more important roles in mature T cells (Fig. 2). Taken together, these results further support our opinion that TRAF3 plays distinct roles in different T cell developmental stages.

2.1.5 Treg development—Treg are an essential component for preventing harmful activation of the immune system in both humans and mice (77-81). The size of the Treg population must be tightly controlled to keep self-reactive immune cells in check but avoid impairing normal immune responses. The increasing interest in clinical manipulation of Treg underlines the necessity of fully understanding the mechanisms of Treg development and function. A particularly interesting phenotypic feature of the T-cell-specific TRAF3-deficient mouse is that the number and percentage of Foxp3⁺ Treg cells are increased 2-3-fold compared to those of LMC mice (8). The similar percentage of Ki-67 and BrDU-positive Treg in T-TRAF3^{-/-} compared with LMC mice indicates that homeostatic proliferation of Treg is unaffected by the absence of TRAF3 (ZY and GAB, data not shown). Unaltered thymic selection in T-TRAF3^{-/-} mice indicates that alternative mechanisms are responsible for the impact of TRAF3 upon Treg development or maintenance. We are currently investigating how TRAF3 affects Treg cell development and whether TRAF3 is involved in Treg functions.

2.2 TRAF3 in signaling by additional receptors of T lymphocytes

2.2.1 TNFR-superfamily members—TNFR-superfamily members expressed on activated T cells include CD27, CD30, CD137 (4-1BB), GITR, OX40, and HVEM, all of which can bind TRAFs (reviewed in (82)). A subset of T cells important in autoimmunity expresses CD40 (reviewed in (83)). In a seminal report, Hauer et al (53), using an epithelial cell transfection system and in the absence of TNFR stimulation, showed that all members of the T cell-associated TNFR-superfamily can interact with TRAF3. CD30 and HVEM showed highest TRAF3 binding in this artificial system, and 4-1BB and OX40 bound the least TRAF3. However, this could differ in actual T cells. In this report, TRAF3 was implicated in the negative control of NF- κ B2 activation; in every case the absence of overexpressed TRAF3 led to increased levels of nuclear p52 (53), as discussed above. Subsequent reports of TNFR signaling in T cells have seldom considered TRAF3 effects on signaling, instead focusing mainly on TRAFs 1,2 and 5. These TRAFs in general promote positive signals through the TNFR-SF receptors, driving activation of the classical and alternative NF- κ B and MAPK pathways (reviewed in (82)). In mice with T cells deficient in TRAF3 (8), the T cell activation defect makes it difficult to obtain cells expressing the inducible forms of the TNFR -SF members in sufficient numbers for biochemical studies (YZ and GAB, data not shown), so there is a knowledge gap in the understanding of the impact of TRAF3 deficiency on TNFR signaling in T cells. Here we will consider TNFR-SF members expressed in T cells. We will focus upon contexts where there is some evidence of TRAF3 involvement in signaling, or where TRAF2 is implicated and thus the shared binding site suggests that TRAF3 might also be involved.

2.2.1.1 OX40: The inducible costimulatory TNFR-SF member OX-40 is a potent inducer of NF- κ B in T cells (82, 84). OX40, expressed 24-48 hours after TCR stimulation, interacts with TRAFs 1,2, and 3 (85, 86). TRAF3 inhibits OX40 mediated NF- κ B activation in epithelial cells (87), but this effect of TRAF3 has not been studied in T cells. Recent work from the Croft lab shows that upon OX40 signaling, OX40 moves to the detergent insoluble plasma membrane fraction, where it organizes a signaling complex that includes TRAF2, PKC θ , IKKs and CARMA1 (84). In addition to NF- κ B, this complex activates PI-3 kinase, Akt and downstream survival factors (88, 89). So and Croft (90) speculate that TRAF adaptors may be involved in Akt signaling downstream of TNFR-SF members.

2.2.1.2 4-1BB: 4-1BB/CD137 is a costimulatory molecule induced on T cells by activation through the TCR (reviewed in (82, 91)). This expression appears to depend upon signaling through another TNFR-SF protein, GITR (92). Like OX40, 4-1BB is implicated in the survival of activated T cells (93, 94). 4-1BB has TRAF binding sites in its cytoplasmic domain that bind TRAFs 1,2 and 3 in human cells (85, 95, 96). Stimulation of 4-1BB in mouse CD8⁺ T cells induces the degradation of TRAF3 in a cIAP-dependent manner over a course of hours, though TRAF2 is not degraded by 4-1BB signaling (97). CD3 and 4-1BB costimulation leads to NF- κ B1 and 2 activation in mouse CD8⁺ T cells. TRAF1 is also implicated in the promotion of Erk signaling and Bim downmodulation (98). PI3K and Akt are activated by 4-1BB (99),(93), and Lee et al recently showed that 4-1BB is unique among the TNFR-superfamily members in activating TCF1 and β -catenin signaling in CD8⁺ T cells

(100). Effects of TRAF3 on Erk signaling, and the TCF1/ β -catenin pathway have not yet been reported.

2.2.1.3 HVEM: The herpesvirus entry mediator (HVEM) is expressed on most T cells (101). It binds TRAFs 1,2,3 and 5 in an epithelial cell overexpression system (102). HVEM interacts with several ligands, including LIGHT, Lymphotoxin receptor α/β and BTLA. CD4⁺ T cells in mice lacking the LIGHT-HVEM interaction show impaired T cell survival upon reactivation, and Akt activation downstream of HVEM appears to be critical (103). TRAF molecule contributions to HVEM signaling in T cells remain understudied.

2.2.1.4 GITR: Glucocorticoid induced TNFR family related gene (GITR) is constitutively expressed on T cells and can be upregulated upon TCR stimulation (104, 105). GITR is implicated in CD8⁺ T cell expansion and survival in viral infections (82, 106). It is also constitutively expressed on Tregs at a relatively high level, and blockade of GITR interaction with its ligand can prolong allograft survival(107). TRAF3 inhibits GITR-driven NF- κ B activation in a reporter assay in transfected epithelial cells (105), but TRAF3 has not been studied in GITR signaling in T cells in any detail.

2.2.1.5 CD30: TRAFs 1, 2, 3 and 5 interact with the cytoplasmic domain of CD30 in epithelial cell overexpression systems (108, 109). Like several of the other TNFR-SF molecules described above, CD30 promotes T cell survival, and is implicated in the maintenance of long-lived CD8⁺ memory (110) and Th17 generation (111).

2.2.2 TLRs—mRNAs encoding TLRs are expressed in T cells from mouse and human, but T cell TLR function has not been studied extensively. The TLRs known to utilize TRAF3 for signaling include TLR3, TLR4, TLR7 and TLR9 (62), all of which are expressed in T cells(reviewed in (112)). Activation of T cells can lead to upregulation of TLRs (113), and studies are supportive of the function of TLRs as costimulators for T cell proliferation, survival and cytokine production (114),(115). A recent review has covered the current understanding of the role of TLRs in T cell biology (112). Specific roles for TRAF3 in T cell TLR signaling have not been examined.

2.2.3 RIG-I-like Receptors—Retinoic acid inducible gene (RIG-I) and related receptors Melanoma differentiation associated protein 5 (MDA5) and LGP2 are RNA helicases that detect pathogen associated patterns in viral RNA (reviewed in (116)). The CARD containing RLRs, RIG-I and MDA5, signal through a pathway involving the mitochondrial anti-viral signaling protein (MAVS) to drive type I interferon production. Upon binding viral RNA, RIG-I assumes an altered conformation allowing the CARD domain to interact with MAVS, a CARD-domain containing adaptor molecule that localizes to the outer mitochondrial membrane, where it triggers activation of NF- κ B and IRF3. TRAF3 interacts with MAVS and positively affects type I IFN production in myeloid cells (117, 118). Triad3A negatively regulates RIG-I or MDA5 signaling by catalyzing the ubiquitination and degradation of TRAF3 (119). In West Nile virus infection, TCR or type I interferon stimulation leads to a T cell-intrinsic increase in the CARD-less RLR LGP2, and promotes CD8⁺ T cell survival by modulating response to death associated receptors (120). While MAVS can interact with LGP2, it is not required for this response. Other studies of RLR pathways have not focused

on T cell intrinsic effects, so the contribution of TRAF3 to RLR signaling in T cells remains unexplored.

2.2.4 Viral proteins—Several T cell-specific oncoproteins encoded by old world monkey viruses *Herpesvirus saimiri* and *Herpesvirus ateles* utilize TRAF molecules, including *H. saimiri*-encoded StpA, StpB, StpC and *H. ateles*-encoded Tio. All these proteins drive NF- κ B activation in a reporter assay conducted in transiently transfected Jurkat T cells (121, 122). P100 processing was also identified in lysates from these transfectants. In addition to NF- κ B activation, Tio expression also induces STAT1,3 and 5 phosphorylation and MAPK activation (122-124). deJong et al showed that Tio-induced NF- κ B activation is dependent on TRAF3 binding to a non-conventional TRAF binding site (125). TRAF3 is sequestered by Tio into membrane- and cytoskeleton-associated insoluble fractions. Interestingly, TRAF3 sequestration is not diminished by proteasome inhibition or SMAC mimetics, nor by inhibition of lysosomal enzymes. Tio did not enhance but in fact decreased the basal state of ubiquitination of TRAF3, and no ubiquitin activity co-localized with Tio and TRAF3 (125). Though Tio promotes T cell transformation, it has been demonstrated that Src kinase pathways are essential to this process (126). Thus, sequestration of cytoplasmic TRAF3 by this viral protein, though sufficient to induce activation of NF- κ B2, is not sufficient to induce T cell transformation.

The HIV encoded protein Nef is implicated in the disruption of TCR signaling by interfering with the interaction between LAT, Zap70 and SLP76 (127). Interestingly, Nef interacts with TRAF2 via a AQEEEE site (128), and TRAF3 could potentially interact with the same site. The TRAF2 interaction site was necessary for Nef induced NF- κ B and STAT activation in macrophages (128).

In microglial cells, exposure to HIV-TatC protein, a cell penetrating peptide, induces expression of host derived miR-32 (129). TRAF3 is a direct target of this microRNA, and TRAF3 mRNA and protein is decreased by miR-32, with resulting alterations in expression of IRF3 and IRF7 (129). miR-32 effects on TRAF3 in T cells have not been studied. Thus it seems plausible that a Tat-mediated effect on TRAF3 expression in T cells could contribute to T cell specific immune response defects caused by this virus.

2.2.5 T cell NF- κ B2 signaling—Activation of the NF- κ B2 pathway is important for lymphoid organogenesis and B cell survival, as discussed above. It has been suggested that the NF- κ B2 pathway plays important roles in autoimmune diseases in a T-cell intrinsic manner (130, 131). However, a recent report showed that in a mouse deficient in NIK, it is the thymic accessory cells, in particular DCs, rather than T cells that accounts for defective development of Treg and other CD4⁺ T cell lineages (132). Consistent with these findings, NIK and NF- κ B2 expression by stromal cells are required for normal development of thymic epithelial cells (133, 134). It was further demonstrated that NF- κ B2 signaling pathway in thymic epithelial cells, but not in T cells, is required for iNKT cell development (135, 136). In addition, T cell expression of NIK is not required for normal thymic Treg development (137).

TRAF3 deficiency results in enhanced basal activation of the NF- κ B2 pathway by blocking the constitutive degradation of NIK (138, 139), and this enhanced NF- κ B2 activation is seen in both TRAF3-deficient B and T cells (3, 8). Activation of NF- κ B2 is associated with increased survival of B cells, but not of T cells (2, 3, 8), indicating that in different cell types, TRAF3 and NIK play different roles. It is unclear whether constitutive activation of NF- κ B2 contributes to reduced iNKT cells, increased Treg or impaired T cell function in T-TRAF3^{-/-} mice. However, activation of the NF- κ B2 pathway occurs later than the early TCR signals impacted by TRAF3, so cannot account for this role. Nevertheless, it will be important to investigate the role of constitutively activated NF- κ B2, as well as various pathways downstream of T cell TNFR-SF members that bind TRAF3, to obtain a comprehensive picture of the roles of TRAF3 in T cell biology.

TRAF3 has been demonstrated to interact not only with the TCR, but with a host of costimulatory TNFRs, TLR pathway kinases, and RLRs. TRAF3 is thought to negatively regulate NF- κ B2 p100 processing, but its effects on other signal transduction pathways are either positive or not yet completely understood.

3. Conclusions

Although B and T lymphocytes are just two of the many cell types expressing TRAF3, the discussion of TRAF3 functions in these cells presented above demonstrates the tremendous variety of ways that this highly versatile signaling protein can impact cellular development, homeostasis, and activation. Although the role of TRAF3 in restraining NF- κ B2 activation is shared between T and B cells, the downstream impact of even this role differs between the two types of lymphocyte, and many additional functions are highly cell type and receptor-specific. Many receptor-specific functions of TRAF3 in B and T cells remain to be investigated, and should provide additional valuable information about the large repertoire of regulatory mechanisms possessed by TRAF3.

Biography

Gail Bishop received a Ph.D. in Cellular and Molecular Biology from University of Michigan in 1983, followed by postdoctoral work at University of North Carolina. She became Assistant Professor of Microbiology at University of Iowa in 1989, Associate Professor in 1994, and Professor in 1998. She is currently College of Medicine Distinguished Professor of Microbiology and Holden Chair of Cancer Biology. She also serves as Associate Director for Basic Science Research of the Holden Comprehensive Cancer Center. Dr. Bishop was as Associate and Section Editor of Journal of Immunology,

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Laura L. Stunz graduated with a B.S. in Biology from the University of Oregon in 1978. She received her Ph.D. from the University of Iowa Department of Microbiology in 1986. Her thesis advisor was Thomas Feldbush, and her thesis research dealt with polyclonal B cell activation induced by proteins isolated from gram negative bacteria. Dr. Stunz was a postdoctoral fellow in the laboratory of Richard A. Anderson in the Department of Internal Medicine at the University of Iowa and the VA Medical Center, working on MHC class II gene regulation. Following her postdoc, she joined the laboratory of Dr. Robert Ashman in the Division of Rheumatology as a Research Scientist, and also continued her work on MHC class II gene regulation in a VA funded project. While in Dr. Ashman's lab, she studied regulation of B cell apoptosis by cytokines, FcR signaling and TLR9 stimulation, helping to characterize inhibitory CpG oligonucleotides. Dr. Stunz joined Dr. Gail Bishop's laboratory in the Dept. of Microbiology at the University of Iowa in 2001, and since then has been involved in the characterization of LMP1 transgenic mice, and B cell and T cell specific TRAF3 deficient mice. She has served as an associate editor of the Journal of Immunology, and on the VA IACUC, including two years as chair. She has also served on the Autumn Immunology Council and is an associate member of the Holden Cancer Center.

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Fig. 1. Role of TRAF3 in early CD40 signaling to B cells

Engagement of CD40 induces association with TRAFs 1, 2, 3, 5 and 6. TRAF3 competes directly with TRAF2 for CD40 binding, as shown. TRAF3 inhibits TRAF2-mediated activation of MAPK and JNK signals and downstream antibody production. Association with TRAF2 and cIAP2 induces the ubiquitination and degradation of TRAF3, which restrains the degradation of the kinase NIK, and results in activation of the non-canonical NF-κB2 signaling pathway.

Fig. 2. Requirement for TRAF3 in iNKT development

During iNKT development, upregulation of T-bet and CD122 occurs at the transition from developmental stages 1 to 2, a transition required for the IL-15-mediated proliferation of iNKT cells. TRAF3 is essential for this upregulation, and hence the developmental transition. TRAF3 is also important for TCR signals and effector functions of mature iNKT cells.