Identification of Functionally Distinct TRAF Proinflammatory and Phosphatidylinositol 3-Kinase/Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase (PI3K/MEK) Transforming Activities Emanating from RET/PTC Fusion Oncoprotein*

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Background: Follicular cell-derived thyroid carcinomas harboring RET/PTC oncoproteins are immunostimulatory and highly curable, despite activating RAS/BRAF/MEK/ERK and PI3K/AKT.

Results: RET/PTC oncoproteins associate with TRAFs to mediate cytokine production but not transformation.

Conclusion: The RET/PTC-TRAF pathway is functionally separable from the RET/PTC-induced MEK/ERK and PI3K/AKT pathways.

Significance: Understanding RET/PTC-mediated immunostimulation could provide new strategies for treating more aggressive forms of thyroid carcinoma.

Thyroid carcinomas that harbor RET/PTC oncogenes are well differentiated, relatively benign neoplasms compared with those expressing oncogenic RAS or BRAF mutations despite signaling through shared transforming pathways. A distinction, however, is that RET/PTCs induce immunostimulatory programs, suggesting that, in the case of this tumor type, the additional pro-inflammatory pathway reduces aggressiveness. Here, we demonstrate that pro-inflammatory programs are selectively activated by TRAF2 and TRAF6 association with RET/PTC oncoproteins. Eliminating this mechanism reduces pro-inflammatory cytokine production without decreasing transformation efficiency. Conversely, ablating MEK/ERK or PI3K/AKT signaling eliminates transformation but not pro-inflammatory cytokine secretion. Functional uncoupling of the two pathways demonstrates that intrinsic pro-inflammatory pathways are not required for cellular transformation and suggests a need for further investigation into the role inflammation plays in thyroid tumor progression.

Inflammation can contribute to late phases of cancer progression by enhancing viability, angiogenesis, and metastasis (1, 2). The notion that inflammation can also be an important early event during neotransformation is based upon the positive correlation observed between pre-existing chronic inflammatory conditions and cancer incidence (1–3) and the observed effects of anti-inflammatory compounds modulating cancer development (4). Thus, it has been suggested that activation of the NF-κB family of transcription factors promotes mitogenicity by triggering cytokine secretion from neoplasia and inflammatory infiltrates while supporting tumor viability through inhibition of apoptotic pathways (5). Additionally, inflammatory infiltrates have the potential to induce reactive oxygen species, and such an oxidative environment could promote oncogenic mutations (6). Yet NF-κB itself is not an oncogene, and tissue-specific autoimmunity in the absence of cancer often exists in patients for extensive periods. Therefore, the requirement for inflammation during initiation and early stages of tumorigenesis remains unclear (1).

Papillary thyroid carcinoma (PTC), the most prevalent endocrine cancer worldwide (7), is particularly attractive for exploration of this issue. PTCs that progress to become more aggressive follicular cell-derived thyroid carcinomas (FDTCs), such as follicular thyroid carcinoma and anaplastic thyroid carcinoma (ATC), are generally associated with point mutations in RAS small GTPases or BRAF serine/threonine kinases, respectively (2, 8). The more indolent PTCs are associated with radiation-induced RET/PTC oncogenes that encode fusions of the RET receptor kinase domain with one of several different dimerizing proteins, resulting in a constitutively active kinase (9). The most prevalent RET/PTC isoforms are RET/PTC1 (RP1) and RET/PTC3 (RP3) consisting of either H4/CCDC6 or ARA70/ELE1 as the respective N-terminal dimerizing partner (10–12). RET/PTC oncogenes activate both RAS/BRAF/MEK/
Diverse RET/PTC Proinflammatory and Transforming Pathways

ERK and PI3K/AKT pathways that are crucial for thyrocyte transformation (13–15) yet are associated with a high cure rate and low tumor recurrence. In contrast, the more aggressive FDTCs usually harbor oncogenic RAS or BRAF point mutations and are associated with a poorer prognosis and higher recurrence rate (2, 8).

Notably, FDTCs harboring RET/PTC oncogenes display an immunostimulatory profile (14, 15) and are associated with the development of autoimmune thyroiditis (16–21). Conversely, the more aggressive and poorly differentiated FDTCs, expressing oncogenic RAS or BRAF point mutations, are characterized by tumor-promoting immune responses such as the infiltration of immunosuppressive macrophages (22). Although the mechanistic basis for RET/PTC-induced immunostimulation is currently unclear, it is thought to involve members of both the classical and alternative pathways of NF-κB through the stabilization of NIK kinase (23–25).

Because RET/PTC oncoproteins activate RAS/BRAF/MEK/ERK, PI3K/AKT, and NF-κB signal transduction pathways, this tumor type might be expected to be highly proliferative and readily progress to a less differentiated cancer such as anaplastic carcinoma. However, RET/PTC-expressing PTCs tend to be rather indolent, and expression in poorly differentiated and anaplastic thyroid carcinomas is rare (26). Thus, in the case of PTC, one interpretation of this process is that the additional immunostimulatory program is detrimental to the progressing tumor. To resolve this conundrum, our primary goal was to determine whether proinflammatory cytokine release and cellular transformation proceed along the same signaling pathways or whether the two processes are functionally distinct and separable. The latter would permit investigation of whether the RET/PTC-induced proinflammatory program is necessary for transforming events as well as exploration of the mechanistic link between RET/PTC expression and NF-κB activation. The results of our investigations provide insight into the early stages of thyroid oncogenesis that could influence future approaches to the treatment of all types of FDTC.

EXPERIMENTAL PROCEDURES

Chemical Reagents

All cell culture and chemical reagents were purchased from Sigma unless stated otherwise.

Cloning

mRP3.51 (RP3) was previously constructed and cloned into a bacterial expression vector (27). To perform the following studies, RP3 was excised from the pET29a vector and cloned into the mammalian expression vectors Rc/CMV and MSCV.IRES.GFP. A Kozak consensus and a TAA stop sequence were placed to the treatment of all types of FDTC.

Cell Culture

TPC-1 and PCCL3 cells were kindly provided previously by Dr. Massimo Santoro. NIH-3T3 (kindly provided by Dr. Tschiklis, Tufts University), 293T (ATCC), and TPC-1 cell lines were maintained in DMEM with 10% FBS (D10). The rat PCCl3 thyroid cell line was maintained in F-12 media containing 5 × 10^{-3} IU/ml bovine TSH, 5 μg/ml bovine insulin, 10 ng/ml Gly-His-Lys, 10 ng/ml somatostatin, 5 μg/ml apotransferrin, 10 nm hydrocortisone, and 10% FBS (F-12+). PCCl3 cells require TSH for cell growth under basal conditions and exhibit TSH-independent growth upon expression of RET/PTC. However, because removing TSH can alter the ability of thyrocytes to produce inflammatory cytokines (28–30), all PCCl3 treatments were maintained in the same culture conditions. For all experiments, PCCl3 cells were treated in a 1:1 ratio of F-12 media and D10. All cells were maintained in a 37 °C incubator at 9% CO₂.

Generation of Stable Lines—Retrovirus was prepared by co-transfecting 293T cells with pCL-Eco and indicated MSCV.IRES.GFP or MSCV.IRES.mRFP constructs with FuGENE 6 (Roche Applied Science). At 24 h post-transfection, fresh medium was replaced, and viral supernatant was collected at 24, 48, and 72 h. For viral transduction, cells were plated at 5 × 10⁴ cells/well in 6-well plates and treated with pre-filtered viral supernatant diluted 1:2 in DMEM containing 10% FBS and 8 μg/ml Polybrene. At 24 h post-transduction, viral supernatant was replaced with fresh 10% media and sorted for GFP or monomeric RFP expression on day 3.

Maintenance of Cell Lines—NIH-3T3 transductants were maintained in D10, and all PCCl3 transductants were maintained in 1:5 ratio of F12+/D10. TSH, insulin, Gly-His-Lys, somatostatin, apotransferrin, and hydrocortisone were all from Sigma. U0126 (Cell Signaling Technology) was chosen based on its high selectivity to both MEK1 and MEK2 (versus PD098059 which inhibits MEK1) and used at 10 μM at all indicated times based on previously published results (31). LY294002 (Cell signaling Technology) was chosen based on its high selectivity to PI3K and used at 10 μM to demonstrate PI3K/AKT inhibition in the absence of apoptosis/toxicity (32, 33).

Transient Transfection and siRNA Knockdown

For cytokine analysis of PCCl3 cells, 10⁵ cells/well were plated in 6-well tissue culture plates. The next day cells were transfected with Genejuice (EMD Chemicals) at a 3:3 ratio of reagent (μl)/DNA (μg). After 48 h, total RNA (Macherey & Nagel) or cytosolic protein was harvested for RT-PCR or Western analysis. For TRAF knockdown of TPC cells, TRAF2 siRNA and TRAF6 siRNA (Santa Cruz Biotechnology) were transfected at 25 nM with siRNAmax (Invitrogen) and on day 2 harvested for ELISA, RT-PCR, or Western analysis or subsequently treated and harvested on day 4 as indicated.

RT-PCR

Total RNA was extracted using the Nucleospin RNA II kit (Macherey & Nagel). Reverse transcriptase-PCR was performed on 0.5–2 μg of total RNA using Superscript II (Invitrogen) according to the manufacturer’s instructions. PCR was performed for 30 cycles at 60 °C.
**Western Blot Analysis and Immunoprecipitations**

All cells were lysed with 150 mM NaCl, 20 mM Tris-Cl, 1% Triton X-100, pH 7.2, containing 1× HALT protease inhibitors with EDTA (Pierce) and 1× HALT phosphatase inhibitors (Pierce). Lysates were quantified using BCA kit (Pierce).

**Immunoprecipitation**—Pre-cleared 500 µg of total protein was incubated overnight with anti-RET antibody and protein G-Sepharose beads (Pierce). Subsequently, beads were washed with cell lysis buffer three times and eluted with 100 mM glycine, pH 2.7. Eluted material was neutralized with 1 M Tris, pH 8.8, combined with 6× loading buffer, and run on 12% SDS-polyacrylamide gel.

**Western Analysis**—10 µg of total protein was loaded onto a 12% SDS-polyacrylamide gel. Gels were blotted for 1 h at 100 V onto nitrocellulose membranes (GE Healthcare) and blocked for 30 min with 5% milk/TBS + 0.1% Tween. All blots were incubated overnight with the indicated antibody, washed, and stained with anti-rabbit HRP (Cell Signaling), donkey anti-mouse HRP, or donkey anti-goat HRP (Jackson Immunoresearch). All indicated antibodies were from Cell Signaling Technology, except for anti-phospho-RET (1062), anti-RET (C-20), and anti-TRAF6 (H274) that were from Santa Cruz Biotechnology.

**Macrophage Chemotactic Protein MCP-1 ELISA**

A 96-well Maxisorp plate (Millipore) was coated at 2 µg/ml with anti-CCL2 antibody (eBioscience) overnight 4°C. The plate was blocked with 1% BSA/PBS, and cell culture supernatants were incubated in duplicate wells overnight at 4°C. The next day, plates were washed with PBS, 0.05% Tween 20 and incubated with biotinylated anti-CCL2 antibody (eBioscience) for 2 h at 27°C and washed, stained with 1:1000 streptavidin-HRP (BD Bioscience) for 1 h at 27°C, developed with 2 mg/ml 3-phenylenediamine dihydrochloride (Sigma) according to manufacturer’s instructions, and visualized at 405 nm in a spectrophotometer (VICTOR2 by Wallac 1420 multilabel counter).

**Cell Proliferation**

PCCL3 and TPC-1 transductants were cultured in triplicate at 1000 cells per well in 96-well flat bottom plates in D10 media in the absence of hormones. Viable cells were counted daily by trypan blue exclusion.

**Soft Agar Assay**

5 × 10³ stable GFP-expressing NIH-3T3 lines were suspended in 0.25% agarose in 1× MEM + 10% FBS with 1× penicillin/streptomycin (Invitrogen) and 1× gentamicin (Invitrogen) and plated in triplicate over a layer of pre-set 0.5% agarose in 1× MEM + 10% FBS with 1× penicillin/streptomycin and 1× gentamicin in 6-well plates. 20 days after plating, the cells were photographed and analyzed for colony number and size or stained for 1 h at 27°C with crystal violet in PBS and photographed over a light box. For quantification of colony size, five photographs were taken per well using a Nikon Eclipse TE 2000-U camera attached to a confocal microscope. One photograph/1-cm² block was taken using a grid that was partitioned in 1-cm² sections. The longest length of the colonies was measured in micrometers using the NIS Elements Version 2.3 program. The sum of the number and length of the colonies was calculated per well, and the mean ± S.D. of triplicate wells was calculated per condition (total of 15 photographs taken per condition).

**RESULTS**

**Chemical Inhibition of P38K and MEK1/2 Does Not Decrease RET/PTC-mediated Cytokine Induction**—Both RP1 and RP3 activate similar oncogenic and inflammatory programs (14, 15, 25, 34), and this trait is attributed to the constitutively active C-terminal RET kinase domain that is shared by all RET/PTC variants (35, 36). Therefore, we utilized an immortalized rat thyroid cell line, PCCL3, transiently transfected with RP3 to examine early activation of proinflammatory cytokine transcription. Early phase induction occurs within the first 48 h of expression and drives ectopic production of MCP1 in abundant quantities and IL6 to a lesser extent (34). To determine whether this early cytokine induction is dependent upon P38K/AKT or MEK/ERK signaling, PCCL3 cells were transfected with the highly related mouse homologue of RP3 (RP3WT) and treated with selective pathway inhibitors. As demonstrated in Fig. 1A, the P38K inhibitor LY294002 (LY) or the MEK1/2 inhibitor U0126 (U0) did not disrupt early transcriptional induction of MCP1 or IL6 by RP3. As a control for protein expression, RP3K284M, which is devoid of all kinase activity (37, 38), was examined and demonstrated no increase in AKT, ERK, or pro-inflammatory cytokine activity. Furthermore, RP3-transfected cells treated with LY or U0 and cells transfected with RP3K284M exhibited less cell scattering, indicative of increased cell mobilization and less transformation when phenotypically compared with cells transfected with RP3WT (supplemental Fig. S1A). The inhibition of DUSP6 induction by U0 and activation of the sodium iodide symporter expression by U0 and LY, demonstrate that the inhibitors were effective at disrupting their respective pathways (Fig. 1A). Furthermore, Western blot analysis revealed that treatments with LY and U0 led to a dramatic decrease in phosphorylation of AKT and ERK, respectively (Fig. 1B).

Similar experiments were then performed to determine the influence of the AKT and ERK pathways on late phase cytokine expression. After long term expression in thyocytes, RET/PTC induces a milieu of proinflammatory cytokines that include MCP1, IL6, IL8, TNFα, IL1α, IL1β, CXCL10, M-CSF, and GM-CSF (14). The TPC-1 cell line is derived from human papillary thyroid carcinoma, constitutively expresses RP1, and produces an altered phenotype, and decreased cell proliferation (supplemental Fig. S1, B and D). We did observe decreased MCP-1
secretion from LY-treated cells (supplemental Fig. S1C); however, this decrease was attributable to a reduction in total cell number (supplemental Fig. S1D). Together, these results support the idea that ectopic cytokine secretion is a common feature of RET/PTC variants (14, 34) and support the notion that this genetic program is not dependent upon PI3K/AKT and MEK/ERK.

**Proinflammatory and Mitogenic Signaling Pathways Originate from Distinct Regions of the RP3 Oncoprotein**—The observation that RP3-mediated early and late inflammatory programs are not dependent upon AKT and ERK signaling led us to investigate whether the inflammatory and mitogenic pathways could be uncoupled through genetic approaches. Mass spectrometric analysis had previously determined that at least 8 of 16 key tyrosine residues are autophosphorylated during RET activation (39), and RP3 contains all 8 proposed signaling tyrosines. Therefore, we generated a panel of Tyr face receptors such as NGF receptor and TGFβ promote proinflammatory cytokine production (47).

The GIPPERLF site is situated within a surface-soluble disordered loop (45) suggesting that this region is accessible to cytosolic binding partners, and the SEEE motif also appears to be located within disordered regions according to the ELM site (http:// Elm.u-strasbg.fr). Consequently, the sequence 480–487 (GIPPERL), adjacent to Tyr-478, was identified as a potential binding site for TRAF, and subsequently harvested for total RNA followed by reverse transcription and PCR for human-specific gene products. D, TPC-1 cells were treated as in C and harvested at 48 h for total protein and Western blot analysis. A–D, data representative of two independent experiments. RevT, reverse transcriptase; LY, LY294002; U0, U0126.

**FIGURE 1. Inhibitors of PI3K or MEK do not inhibit RP3-mediated proinflammatory cytokine transcription.** A, PCCL3 cells were transiently transfected with RP3WT or RP3K284M in the presence of 0.1% DMSO alone, 10 μM LY294002, or 10 μM U0126 for 48 h, and cells were lysed for total RNA followed by reverse transcription and PCR for rat-specific gene products. B, PCCL3 cells were treated as in A and harvested at 48 h for total protein and Western blot analysis. C, TPC-1 cells were treated for 48 h with 0.1% DMSO alone, 10 μM LY294002, or 10 μM U0126 and subsequently harvested for total RNA followed by reverse transcription and PCR for human-specific gene products. D, TPC-1 cells were treated as in C and harvested at 48 h for total protein and Western blot analysis. A–D, data representative of two independent experiments. RevT, reverse transcriptase; LY, LY294002; U0, U0126.
sell/Linding set parameters (48). To determine whether TRAF proteins associate with RET/PTC, 293T cells were transiently transfected with RP3, and cell lysates were subjected to immunoprecipitation with an anti-RET antibody specific for the last 51 amino acids of the C terminus. As shown in Fig. 3B, TRAF2 and TRAF6 co-precipitate with RP3WT without additional cross-linking indicating a specific association. Additionally, TRAF2 and TRAF6 binding to RP3Y588F is equivalent to that of RP3WT, although RP3Y478F exhibits decreased association to TRAF6 (Fig. 3, C and D). Of note, TRAF2 and TRAF6 bind equally well to the kinase-dead mutant, RP3K284M, indicating that binding is independent of kinase activity (Fig. 3, B–D). To determine the requirements for TRAF interaction, we created truncated mutants of RP3 (Fig. 3A). RP3/H9004N lacks the autodimerizing N-terminal segment (residues 1–238), and RP3/H9004TK2 lacks the second tyrosine kinase domain of RET (residues 338–589), in which the TRAF-binding motifs are predicted to reside. No association was detected with RP3/H9004TK2, although association of TRAF2 and TRAF6 was detectable with RP3/H9004N, only after prolonged exposure of the blot but beyond the linear range of detection compared with the RP3WT signal (Fig. 3B). These findings suggest that, although TRAF binding is independent of kinase function, TRAF association is still dependent upon RP3 dimerization (model in Fig. 7). Furthermore, the absence of TRAF association with RP3/H9004TK2 suggests that TRAF or TRAF-binding adaptor proteins associate within residues 338–589. We confirmed the specificity of the association by examining the endogenous interaction of TRAF2 and TRAF6 to RP1 in the TPC-1 cell line. Fig. 3G demonstrates that immunoprecipitation of RP1 can pull down both TRAF2 and TRAF6, suggesting that TRAF association is most likely occurring in the C terminus derived from RET.

To dissect the specificity of TRAF2 and TRAF6 binding, the putative sites were altered from SEEE to SEAA (RP3T2mut) and GIPPERLF to GIQPARLA (RP3T6mut). Examination of RP3T2mut showed decreased binding to TRAF2 compared with RP3WT with no effect on TRAF6 association (Fig. 3D), although RP3T6mut demonstrated decreased binding to both TRAF2 and TRAF6 (Fig. 3E). In contrast to the/H9004N and/H9004TK2 truncations, none of the tested mutations completely ablated TRAF association, suggesting that there are additional points of contact within the binding sites or that RP3 contains additional binding sites for TRAFs and/or TRAF-associated adaptors. Overall, these findings further support the idea that proinflammatory signaling is initiated at TRAF-binding sites through a mechanism that is distinct from Tyr-588-mediated pathways.

RP3-induced TRAF-mediated Production of Proinflammatory Cytokines Is Independent of the RAS/BRAF/MEK/ERK and PI3K/AKT Pathways—Because TRAF oligomerization is known to activate NF-κB family members and subsequent cytokine induction, we investigated whether TRAF signaling was responsible for production of cytokines from RET/PTC-trans-
formed cells. As shown in Fig. 4, A–C, siRNA knockdown of either TRAF2 or TRAF6 substantially decreased MCP1 production from TPC-1 cells. Induction of other cytokines was also reduced, GMCSF > IL8 > IL6 > TNFα. This change in inflammatory profile was regulated mainly, if not entirely, at the transcriptional level as demonstrated by RT-PCR (Fig. 4, A and B).

To confirm the reduction of cytokine secretion, supernatants were harvested from TPC-1 cells on day 2 and 4 after siRNA treatment and tested in a specific MCP1 ELISA. As shown in Fig. 4C, MCP1 protein secretion by TRAF siRNA-treated cells was greatly reduced in a time-dependent manner compared with siRNA control.

Additionally, we examined the ability of TRAF-inhibiting peptide sequences to knock down cytokine secretion over a longer period of time. The TRAF2 peptide sequence designed from CD40, AYPIQETA (T2pep), and the TRAF6 sequence designed from RANK, APTEDEYA (T6pep), as well as their controls (AYAIAATA and AATADAYA) were overexpressed using MSCV.IRES.mRFP viral vectors, and stable transductants were selected by cell sorting for RFP. Fig. 4D demonstrates that inhibition of TRAF2 oligomerization via overexpression of the TRAF2-binding motif greatly reduce cytokine expression in TPC-1 cells. Overexpression of T6pep did not reduce MCP1 induction to the same extent as T2pep and may reflect a decreased affinity of this peptide for its target.

We further examined the cytokine profile of PCCL3 cells stably expressing the RP3 mutants. As observed in Fig. 4E, it appears that ablation of MCP1 production was not maintained with sustained expression of RP3Y478F or RP3T2mut; however, this is in contrast to the RP3T6mut, in which the long term production of MCP1 was greatly reduced. This result may reflect the fact that the RP3Y478F or RP3T2mut mutations do not completely ablate TRAF binding, although the association is reduced.

We next determined whether TRAF2 or TRAF6 contributes to either AKT or ERK signaling. As predicted by our original findings, TPC-1 cells treated with siRNA specific for TRAF2 or TRAF6 showed no decrease in the phosphorylation levels of...
Indeed, it appears that TRAF2 signaling negatively regulates PI3K/AKT and RAS/RAF/MEK/ERK pathways because there is increased phosphorylation of AKT and ERK in the absence of TRAF2 (Fig. 5A). In addition, long term expression of TRAF2 and TRAF6 blocking peptides does not affect AKT and ERK activity or cellular proliferation relative to the peptide controls (Fig. 5, B and C). Furthermore, it appears that TRAF2 knockdown (Fig. 5A) or T2pep expression (Fig. 5C) leads to decreased NIK kinase stability. This finding is consistent with recent studies showing that RP3 promotes the stabilization of NIK to activate the canonical pathway of NF-κB (23). Together these findings suggest that RET/PTCs induce a TRAF-dependent proinflammatory program in thyroid cells, and this pathway is independent of PI3K/AKT and RAS/BRAF/MEK/ERK activity.

**TRAF-deficient RP3 Mutants Maintain Transforming Properties**—Our data indicate that the TRAF-mediated pathway is spatially and functionally distinct from the PI3K/AKT or RAS/BRAF/MEK/ERK pathways. Previous results have reported that point mutations within RAS or BRAF are sufficient to drive transformation of thyroid follicular cells (8). Based on our results above, we hypothesized that disrupting the TRAF pathway should have little to no impact on transformation. To test this prediction, we examined whether the TRAF-deficient mutants were capable of maintaining hormone-independent growth. In accordance with our observation that
TRAF-deficient mutants maintained ERK and AKT activity, PCCL3 cells stably transduced with RP3Y478F, RP3T6mut, and RP3T2mut demonstrated hormone-independent growth comparable with RP3-transduced cells (Fig. 5D). We further examined the ability of the TRAF-deficient mutants to promote anchorage-independent growth. NIH-3T3 cells were transduced with GFP-tagged retroviral constructs encoding wild type or mutated RP3, sorted for GFP expression, and examined for colony growth in soft agar. After 20 days of growth, the colonies were stained with crystal violet (supplemental Fig. S2) or photographed for measurement of colony size (Fig. 6, A and B). Most notably, the TRAF-deficient mutants, RP3T6mut and RP3T2mut, induced colonies in size and number comparable with wild type RP3 (Fig. 6, A and B). In contrast, RP3K284M, RP3Y431F, and RP3Y588F produced little to no colonies. We confirmed that the lack of colony formation was not due to loss of gene expression (supplemental Fig. S3). Interestingly, the Tyr-478 mutation resulted in transductants with compromised transformation properties compared with RP3WT (Fig. 6, A and B).

The lack of inflammatory cytokine production by RP3T6mut and RP3T2mut transfectants may limit their capacity to form tumors in vivo because of the lack of induced stromal support (49). To test this notion, immunodeficient RAG1/H11002/H11002 mice were subcutaneously injected with the NIH-3T3 transfectants described above and monitored for tumor growth. The supplemental Fig. S6 shows that mice injected with RP3WT, RP3T6mut, or RP3T2mut transfectedants rapidly developed palpable tumors after 13 days, whereas mice injected with NIH-3T3 expressing GFP alone showed no tumor growth. Mice that received RP3K284M or RP3Y588F cells did not grow significantly measurable tumors. Interestingly, it appears that the RP3T6mut transductants produced smaller tumors that may reflect the smaller colony size in Fig. 6B, a decreased influx of innate cells, or decreased stromal support and angiogenesis. Further investigation that includes immunocompetent mice and increased animal numbers may allow for more detailed insight into the role that an intrinsic TRAF pathway plays on in vivo tumor progression. In summary, the results indicate that RET/PTC-induced TRAF-mediated proinflammatory cytokine production is not required for cellular transformation driven by the PI3K/AKT and RAS/BRAF/MEK/ERK pathways.

**DISCUSSION**

The unusual pleiotropic signaling properties of the RET/PTC oncogenes (14, 24, 34) provide an attractive model for studying the mechanistic relationships between oncogene-induced cellular transformation and proinflammatory pathways. RET/PTC translocation is thought to be one of the earliest events in the initiation of papillary thyroid cancer (9, 50), and ectopic pro-inflammatory mediators have been presumed to contribute to the cellular transformation process (2, 3), but this has not been formally tested for this cancer type. We report here that TRAF-mediated cytokine induction and cellular transformation are functionally distinct in neoplastic thyrocytes, a finding that will enable a finer resolution of the components necessary to drive cancer progression. These data also point to one explanation for why oncogenes such as mutant

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**FIGURE 5. Disruption of TRAF2 and -6 pathways does not inhibit AKT and ERK pathways.**

A, TPC-1 cells were transfected with scrambled control, TRAF2, or TRAF6 siRNA and re-treated with siRNA and on day 2 and 4 were harvested for Western blot analysis. B, TPC-1 cells were transduced with MSCV.IRES.RFP constructs expressing T2- and T6-binding motifs and their peptide controls. Following selection for RFP, cells were harvested for Western blot analysis. C, cell proliferation assay of transduced TPC-1 cells from B that were plated at 1000 cells/well in 96-well culture dishes and counted daily. Data are representative of three independent experiments. Cntl, nontargeting siRNA control; T2, TRAF2; T6, TRAF6.
RAS or BRAF do not exhibit the same degree of immunostimulatory phenotype and progress along different pathologies despite utilizing the same transforming pathways as RET/PTC-based tumors. Although stimulatory cross-talk between the RAS/AKT and NF-κB pathways has been suggested for lung epithelial cell tumorigenesis (51), we did not observe positive feedback mechanisms in our experimental systems. If anything, TRAF2-mediated signaling appears to suppress the AKT and ERK signaling pathways (Fig. 5A). This is in accordance with the observation that TRAF6 negatively regulates PI3K activity during T cell activation (52). Additionally, the signaling dichotomy we demonstrate here has recently been shown for MIG-6 tumor suppressor expression in PTC. These studies demonstrate that MIG-6 overexpression in PTC tumor samples or the TPC-1 cell line activates NF-κB and inhibits ERK phosphorylation. Conversely, knockdown of MIG-6 decreased NF-κB nuclear localization and enhanced ERK activity (53).

PTC rarely harbors coexisting RET/PTC, RAS, and/or BRAF mutations suggesting that there is limited functional complementation between these oncogenes with respect to thyroid tumor initiation/progression (54–56). The experiments reported here demonstrate that RET/PTC-induced TRAF-mediated inflammation is not required for transformation. Evidently, only the PI3K/AKT and RAS/BRAF/MEK/ERK pathways are required to establish and maintain follicular cell transformation (57), although the inflammatory pathway that is unique to RET/PTC expression may serve to modulate the tumor environment. This conclusion is supported by recent evidence demonstrating that the MIG-6 tumor suppressor expression correlates directly with increased NF-κB activity and inversely with tumor recurrence, survival, and oncogenic BRAF mutations (53, 58).

Other studies have demonstrated functional separation in other cell types such as fibroblasts. For instance, the NF-κB members, p65 (RelA) and c-REL, are not required for RAS-mediated transformation in fibroblasts, although p65 and c-REL signaling can potentiate anchorage-independent growth (59). Furthermore, our studies showed that blockade of MEK/ERK and PI3K/AKT actually enhances inflammatory cytokine production from thyroid papillary carcinoma, compatible with the published finding that oncogenic RAS blocks TNF-induced NF-κB signaling (60).

Our investigation has revealed that TRAFs associate with RP3 and activate an inflammatory program that is independent from the PI3K/AKT and RAS/BRAF/MEK/ERK pathways. TRAF2 and TRAF6 appear to associate independently of kinase action (as observed with RP3K284M), whereas pro-inflammatory TRAF complexes are formed only upon a catalytically active conformational change of the kinase domain (as observed with RP3WT). This type of interaction has been described for TGFβ receptor, in which wild type, kinase-dead, and constitutively active TGFβ receptor all associate with TRAF6 (61). Because RET/PTC is constitutively expressed in transformed cells, it is possible that RET-TRAF complexes will be present at all times leading to aberrant regulation of NF-κB. This is distinct from the conditional activation observed with RET and its ligand GDNF under physiological conditions. A general model of RET/PTC signaling, based on our results, is depicted in Fig. 7. A key feature is the association of TRAF2 and TRAF6 only after dimerization of RP3. This is similar to TNF receptor family members BAFFR and CD40, which exhibit TRAF association only after ligand binding (62). In the case of RP3, conversion to the active dimeric conformation (Fig. 7) may result in increased surface exposure of the TRAF-binding segments. The observation that mutation of the putative TRAF6-binding site (RP3T6mut) resulted in reduced association with both TRAF2 and TRAF6 (Fig. 3, D and F) was also of interest. TRAF2 often forms heterocomplexes with other TRAF family members (63).
Therefore, it is possible that TRAF2 interacts directly with TRAF6 at this site or that this location contains binding motifs for both TRAF2 and TRAF6. This type of TRAF2/TRAF6 interaction has also been described for the proinflammatory receptor CD40 (64).

We observed that both TRAF2 and TRAF6 associate with RP3 independent of the Tyr-588 site. Previous reports suggest that proinflammatory cytokine production initiates via a Tyr-588-dependent signaling cascade (14, 25). It is interesting that in the absence of TRAF-mediated pathways, we observed the greatest difference in MCP1 production compared with other cytokines, whereas MCP1 was the least affected using Tyr-588 mutants (25). Further investigation is required to assess the relative involvement of a TRAF-, AKT-, and ERK-independent/Tyr-588-dependent pathway in cytokine induction. The RAS/BRAF pathway has also been implicated in secretion of IL-24, IL-8, and other chemokines (65, 66), and it is possible that the quality of the proinflammatory cytokine induction from a RAS-mediated pathway may lead to a more immune-suppressive/tumor-promoting environment. This could occur by several potentially cooperative mechanisms as follows. (a) Autocrine stimulation by cytokines may retard tumor growth. RET/PTC-expressing human thyroid cell lines and thyroids from transgenic animals show substantial up-regulation of many inflammatory mediators, including IL1α, IL1β, IL6, TNFα, IL8, GMCSF, and MCP1 (14, 24, 34), and it has been reported that many of these cytokines can inhibit thyroid tumor cell growth (72). (b) The composition of the infiltrate itself may lack the factors required for progression. More aggressive carcinomas that harbor RAS or BRAF mutations display a heavy influx of inflammatory cells, but these tend to be immunosuppressive in nature (22). Indeed, tumor-associated macrophages (TAMs) generally exhibit alternate (“M2”) activation and are mitogenic, angiogenic, and pro-metastatic through mechanisms that include IL10, TGFβ, VEGF, and matrix metalloprotease secretion, respectively (73). In contrast, the lymphocytic infiltration associated with indolent PTC is usually characterized by a Th1 phenotype (74, 75) that is not conducive to the production of immunosuppressive TAMs (73), and infiltrating TAMs are rarely found in less aggressive well differentiated thyroid carcinoma (22). (c) The proinflammatory environment may cause a break in peripheral tolerance and aid the adaptive arm in controlling/eliminating the tumor. For example, CXCR4, STAT1, CIITA, and MHC class II are also up-regulated by RET/PTC expression in human

![Model of RET/PTC-mediated TRAF activation](image-url)

**Figure 7.** Model of RET/PTC-mediated TRAF activation. RET/PTC dimers exhibit a greater affinity for TRAF2 and TRAF6 than RET/PTC monomers. The TRAF interaction with RET/PTC does not require an active conformation of the kinase domain. However, TRAF activation does require an active kinase domain conformation, which most likely induces TRAF oligomerization to promote NIK and NF-κB-mediated proinflammatory signaling. The RET/PTC-TRAF-NIK-NF-κB pathway is functionally distinct from Tyr-588-mediated RAS and PI3K signaling cascades that promote transformation.
thymus cells in vivo (34, 76, 77). Together, these molecules provide signals for leukocyte migration, dendritic cell differentiation, and antigen presentation. Consistent with this, tumors derived from RP3 transgenic animals are capable of rapid growth in SCID mice but not immunocompetent hosts (78).

Clinically, PTCs harboring active RET translocations are found coincident with autoimmune Hashimoto thyroiditis (HT) (16–21). This longstanding observation has led to the hypothesis that HT is a risk factor for PTC and furthermore that HT is a precancerous condition (2, 3). For the various reasons discussed, we suggest the converse, the autoimmune component is the result rather than the cause of RET/PTC transformation. Activation of innate and adaptive immunity via the TRAF signaling component of RET/PTC would explain the intense and persistent peritumoral lymphocytic infiltration and indolent growth that is often associated with PTC. The TRAF-mediated proinflammatory signaling linked to RET/PTC may also help explain the concurrent expression of RET/PTC (21) and the better overall prognosis of thyroid cancer in HT patients (79).

Although data indicate that TRAF-mediated pathways are not required for early events in transformation, the question remains whether these same TRAF-mediated pathways are required for later stages of tumor progression. Indeed the RP3\textsuperscript{Temut} or RP3\textsuperscript{T2mut} tumors were smaller than the comparable RP3\textsuperscript{WT} tumors suggesting that inflammatory mediators may contribute to, but are not necessary for, tumor growth. Accordingly, the lack of these cytokines may limit the recruitment of stromal elements and suppress the likelihood of growth variants (80). Consistent with this, TRAF-mediated NF-κB activation is known to control cell viability and angiogenesis in many tumor types (1).

Elucidating the influence of RET/PTC-induced inflammation on thyroid tumorigenesis may lead to more effective therapeutic strategies for PTC. For example, following surgery, any residual RET/PTC harboring tumors may be best approached by targeting only the PI3K/AKT- and RAS-mediated pathways, while preserving the inflammatory component. Conversely, induction of a “RET/PTC-like” TRAF signaling pathway within the more aggressive PTCs that feature only oncogenic RAS and BRAF mutations may convert such tumors to a more benign form. Alternatively, if TRAF-mediated NF-κB pathways are found to play a greater role in angiogenesis and escape during progression, then the therapeutic strategy should be to knock down both proinflammatory and oncogenic arms of RET/PTC signaling. Experiments are currently underway to test these possibilities.

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