Functional Toll-like Receptor 4 Overexpression in Papillary Thyroid Cancer by MAPK/ERK–Induced ETS1 Transcriptional Activity

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Abstract

Emerging evidence suggests that unregulated Toll-like receptor (TLR) signaling promotes tumor survival signals, thus favoring tumor progression. Here, the mechanism underlying TLR4 overexpression in papillary thyroid carcinomas (PTC) mainly harboring the BRAFV600E mutation was studied. TLR4 was overexpressed in PTC compared with nonneoplastic thyroid tissue. Moreover, paired clinical specimens of primary PTC and its lymph node metastasis showed a significant upregulation of TLR4 levels in the metastatic tissues. In agreement, conditional BRAFV600E expression in normal rat thyroid cells and mouse thyroid tissue upregulated TLR4 expression levels. Furthermore, functional TLR4 expression was demonstrated in PTC cells by increased NF-κB transcriptional activity in response to the exogenous TLR4-agonist lipopolysaccharide. Of note, The Cancer Genome Atlas data analysis revealed that BRAFV600E-positive tumors with high TLR4 expression were associated with shorter disease-free survival. Transcriptomic data analysis indicated a positive correlation between TLR4 expression levels and MAPK/ERK signaling activation. Consistently, chemical blockade of MAPK/ERK signaling abrogated BRAFV600E-induced TLR4 expression. A detailed study of the TLR4 promoter revealed a critical MAPK/ERK–sensitive Ets-binding site involved in BRAFV600E responsiveness. Subsequent investigation revealed that the Ets-binding factor ETS1 is critical for BRAFV600E-induced MAPK/ERK signaling-dependent TLR4 gene expression. Together, these data indicate that functional TLR4 overexpression in PTCs is a consequence of thyroid tumor-oncogenic driver dysregulation of MAPK/ERK/ETS1 signaling.

Implications: Considering the participation of aberrant NF-κB signaling activation in the promotion of thyroid tumor growth and the association of high TLR4 expression with more aggressive tumors, this study suggests a prooncogenic potential of TLR4 downstream signaling in thyroid tumorigenesis.

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Introduction

The emerging role of Toll-like receptors (TLR) in the maintenance of tissue homeostasis as critical regulators of inflammatory processes and tissue regeneration response under physiologic conditions has led to the identification of aberrant functions of these receptors in different diseases, including inflammatory and infectious disorders, autoimmunity, and cancer (1). Particularly, unregulated TLR signaling has been described in several neoplastic processes associated with exacerbated production of proinflammatory cytokines involved in tumor progression (1, 2). Indeed, TLR signaling–deficient mice are resistant to tumor development in different experimental models (3, 4). Likewise, transgenic mice overexpressing constitutively active TLR4 under the intestine-specific villin promoter are highly susceptible to colitis-associated cancer (5). The close link between dysregulated TLR function and cancer progression has set the molecular basis for the development of potential therapeutics targeting TLRs or TLR-triggered intracellular signaling pathways (6).

Chronic activation of the immune system and tissue response might generate an inadequate negative regulation of TLR signaling pathways, leading to an excessive proinflammatory microenvironment that facilitates the promotion of neoplastic processes (7). Different TLR polymorphisms have been associated with increased susceptibility or severity of infections (8). Of note, the...
polymorphism rs4986790 in TLR4 gene was identified as a risk factor for the development of gastric cancer in Helicobacter pylori–infected patients (9).

Functional TLR expression in tumor cells has been linked to tumor survival and progression, evasion of immune system, and resistance to apoptosis. TLR4 engagement in lung cancer cells induces the expression of immunosuppressive and proangiogenic cytokines (10). Further studies in prostate cancer cells showed that TLR4 activation promotes the secretion of proangiogenic cytokines in response to NF-kB signaling (11). However, discrepancies regarding tumor cell survival in response to TLR4 activation in different cell models have been observed. Particularly, TLR4 signaling improves survival in ovarian cancer cells (12) and promotes the invasive ability of colon cancer cells (13). Conversely, studies in pituitary tumors showed that TLR4 activation inhibited tumor growth (14).

In the thyroid tissue, functional TLR3 expression was reported in normal thyrocytes. McCall and colleagues (15) demonstrated high constitutive TLR3 signaling in papillary thyroid carcinomas (PTC) compared with normal thyroid tissue and follicular thyroid carcinomas (FTC). Moreover, phenylmethimazole, a small molecule that decreases TLR3 expression and signaling, inhibited PTC cell growth and migration (15). In addition, we demonstrated functional TLR4 expression in normal murine thyrocytes (16). Using the exogenous TLR4-agonist lipopolysaccharide (LPS), we demonstrated that NF-kB signaling plays a central role in the regulation of TLR4-induced thyroid differentiation marker gene expression (17, 18). Interestingly, Hagström and colleagues (19) reported a significant correlation between high TLR4 protein levels and metastatic potential in FTCs. Moreover, Dang and colleagues (20) demonstrated that TLR4 is abundantly expressed in FTCs and stimulation of the PTC cell line W3 with the colistin did not show any significant changes in TLR4 expression (16).

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Here, we investigated the molecular events downstream thyroid cancer–driving oncogenes involved in the upregulation of TLR4 gene expression in differentiated thyroid carcinomas. As previously described, we show that TLR4 is abnormally overexpressed in PTCs and FTCs. Moreover, TLR4 expression is functional as its exogenous agonist LPS increased NF-kB transcriptional activity in PTC cell models. Consistent with transcriptomic data of PTC showing a significant correlation between TLR4 mRNA expression levels and ERK activation score, chemical blockage of MAPK/ERK signaling abrogated BRAFV600E-induced TLR4 expression. MAPK/ERK signaling–dependent BRAFV600E-induced TLR4 gene expression relies on a distal E26 transformation-specific (Ets)-binding site identified in the TLR4 promoter. Moreover, we show that the Ets-binding factor ETS1 is critical for BRAFV600E-induced MAPK/ERK signaling–mediated TLR4 gene expression in thyroid carcinomas. In summary, high TLR4 levels in PTC cells are the consequence of dysregulated MAPK/ERK signaling as a result of thyroid tumor drivers, such as BRAFV600E. Altogether, our evidence suggests a prooncogenic potential of TLR4 signaling in thyroid tumorigenesis, further considering the oncogenic potential of NF-kB signaling in the promotion of thyroid cancer (21).

Materials and Methods

IHC

TLR4 and ETS1 expression in clinical thyroid samples was studied using low- and high-density human thyroid tissue arrays (US Biomax). Endogenous peroxidase activity was blocked by treatment with H2O2 in methanol for 15 minutes. Thyroid sections were then incubated for 30 minutes in 5% normal rabbit serum to block nonspecific binding, followed by overnight incubation with 1 μg/mL goat polyclonal anti-TLR4 antibody (sc-16240, Santa Cruz Biotechnology), 5 μg/mL mouse monoclonal anti-TLR4 antibody (ab-22048, Abcam), or 3.3 μg/mL rabbit polyclonal anti-ETS1 antibody (sc-350, Santa Cruz Biotechnology) at 4°C in a humidified chamber. For negative controls, the primary antibody was replaced by the corresponding purified nonreactive IgG in a separate slide containing paraffin-embedded thyroid carcinoma tissue samples. Afterward, thyroid sections were incubated with biotinylated secondary antibody (Santa Cruz Biotechnology) and ABC complex (Vector Laboratories). 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) was used as the chromogenic substrate for 10 minutes at room temperature, and the sections were rinsed in running water and counterstained with Harris hematoxylin. Digital images were captured at ×400 magnification using a Nikon Eclipse TE2000-U light microscope (Nikon Instruments). Quantification of TLR4 and ETS-1 protein levels was performed by assessing DAB staining intensity relative to the number of cells in the histologic sections to correct differences in tissue architecture. DAB staining intensity and the number of nuclei seen in one randomly selected area of each tissue section were quantified using Imagej software (NIH, Bethesda, MD).

Plasmids

The expression vectors encoding human BRAFV600E and dominant-negative human A152–296 MyD88 were as previously described (22, 23). pCMV-mFlagEts1 (plasmid #86099) was from Addgene. The NF-kB reporter vector containing five kB consensus sites linked to the luciferase coding sequence (5x kB-Luc) and the expression vector encoding dominant-negative mouse A670-B35 TLR4 were from Clontech and InvivoGen, respectively. Mouse Th4 promoter constructs +52/+223, −104/+223, −336/+223, and −608/+223 were as previously reported (24). Disruption of the distal Ets-binding site in the mouse Th4 promoter was performed by PCR with the oligonucleotide 5′-TGGGTITTTATTCTCAGCTTAGAGAAATATGTAGTTCTAGTCTGAAAC-3′ carrying the desire mutation (underlined) using KOD Hot Start DNA polymerase (EMD Millipore) as described previously (25). The artificial Ets reporter 3x Ets2-Luc contains the luciferase gene under the control of a promoter containing three copies in tandem of the flanking region of the distal Ets-binding site (AAAATATGGTCCTCAGTCTGAAAC) of mouse Th4 promoter upstream the adenosine E1B core promoter. The normalization reporter pCMV-β-galactosidase was obtained from Promega. All constructs were sequenced to verify nucleotide sequence (Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina).

Cell culture

The Fischer rat thyroid cell line PCCl3 was kindly provided by Dr. Roberto Di Lauro (Università degli Studi di Napoli Federico II, Naples, Italy). PCCl3 thyroid cells engineered to obtain doxycycline-inducible expression of the oncogenes BRAFV600E (PC/ BRAFV600E), RET/PTC3 (PC/PTC3), and HRasG12V (PC/HRasG12V) were generously provided by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center).
Kettering Cancer Center, New York, NY). PCC13 and PCC13-derived cells were cultured in DMEM/Ham F-12 medium supplemented with 5% calf serum (Thermo Fisher Scientific), 1 mM/L bovine TSH (National Hormone and Peptide Program, Harbor-UCLA Medical Center), 10 µg/mL bovine insulin, and 5 µg/mL bovine transferrin (Sigma-Aldrich) and treated with 1 µg/mL of doxycycline (Sigma-Aldrich) to induce oncogene expression (26). Nontumoral human thyroid cell line Nthy-ori 3-1 was obtained from the European Collection of Authenticated Cell Cultures and human PTC-derived cell line BCPAP, harboring the BRAFV600E mutation, was provided by Dr. Massimo Santoro (Università degli Studi di Napoli Federico II, Naples, Italy). Human thyroid cells were cultured in DMEM medium supplemented with 10% FBS (PAA Laboratories). Human cell lines were authenticated using short tandem repeat genotyping using PowerPlex Fusion System (Promega) at Centro de Excelencia en Genes y Productos de Células (Seville, Spain), and human BRAFV600E-expressing human and human BRAFV600E-expressing transgenic mice were purchased from Sigma-Aldrich.

RNA isolation and real-time PCR
Total RNA purification, cDNA synthesis, and qPCR were performed as described previously (28). Gene-specific primer sets were as follows: mouse/rat TLR4 (180 bp) 5′-CACAAGACCTTGAGYCTGAGGC (forward) and 5′-TCTGGAAGGGTGGTTCCGTTC (reverse), human TLR4 (180 bp) 5′-CGAAGAACTCGGGAGCTTGAAGG (forward) and 5′-TCTGAGTGGGTTCCGTTCGTC (reverse), rat IL-6 (109 bp) 5′-GACAAAGCAGGATCTCACCAAGG (forward) and 5′-CAGATGTTAAGGTTGCTAG (reverse), rat inducible nitric oxide synthase (iNOS; 137 bp) 5′-CGAGTTTCTGGCAGCAG (forward) and 5′-CTTCCGA-GATGACGGCACCG (reverse), rat and mouse monoclonal anti-β-Tubulin (T6074, Sigma-Aldrich) antibodies. After washing, membranes were further incubated with IRDye 680 RD or IRDye 800 CW–linked secondary anti-rabbit and anti-mouse antibodies (LI-COR Biotechnology), protected from light. Membranes were visualized and quantified by Odyssey Infrared Imaging System (LI-COR Biotechnology). Equal loading was assessed by stripping and reprobing the same blot with 0.1 µg/mL rabbit polyclonal anti-GAPDH (sc-25778, Santa Cruz Biotechnology).

Immunoblotting
SDS-PAGE, electrophoresis to nitrocellulose membranes, and immunoblotting were as described previously (29). Membranes were incubated with 0.2 µg/mL mouse monoclonal anti-TLR4 (ab-22048, Abcam), 0.2 µg/mL rabbit polyclonal anti-BRAF (sc-166, Santa Cruz Biotechnology), 0.2 µg/mL mouse monoclonal anti-p-ERK (sc-7383, Santa Cruz Biotechnology), 0.2 µg/mL rabbit polyclonal anti-ERK (sc-94, Santa Cruz Biotechnology), 0.2 µg/mL rabbit polyclonal anti-ETS1 (sc-350, Santa Cruz Biotechnology), 0.2 µg/mL rabbit polyclonal anti-PARP-1 (sc-7150, Santa Cruz Biotechnology), and 0.4 µg/mL mouse monoclonal anti-α-Tubulin (T6074, Sigma-Aldrich) antibodies. After washing, membranes were further incubated with IRDye 680 RD or IRDye 800 CW–linked secondary anti-rabbit and anti-mouse antibodies (LI-COR Biotechnology), protected from light. Membranes were visualized and quantified by Odyssey Infrared Imaging System (LI-COR Biotechnology). Equal loading was assessed by stripping and reprobing the same blot with 0.1 µg/mL rabbit polyclonal anti-GAPDH (sc-25778, Santa Cruz Biotechnology).

Immunofluorescence
Cells seeded onto glass coverslips were fixed in 2% paraformaldehyde (30). Cells were immunostained with 10 µg/mL mouse monoclonal anti-TLR4 (ab-22048, Abcam) or 4 µg/mL rabbit polyclonal anti-ETS1 (sc-350, Santa Cruz Biotechnology) in PBS containing 0.2% BSA. For analysis under permeabilized conditions, an additional 0.1% Triton was added. Secondary staining proceeded with 50 nmol/mL anti-mouse Alexa-594 antibody (Thermo Fisher Scientific). Nuclear DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted with FluorSave Reagent (EMD Millipore), and images were acquired on a Leica DMi8 epifluorescence microscope (Leica Microsystems). The number of nuclei and the intensity of TLR4 or ETS1 immunostaining were analyzed using ImageJ software.

Transient transfection and reporter gene assay
Cells were transiently cotransfected with 0.64 µg/well of expression vectors and 0.16 µg/well of luciferase reporter vectors using Lipofectamine 2000 (Thermo Fisher Scientific; ref. 29). The luciferase activity was measured using a Luciferase Assay System (Promega) according to the manufacturer’s instructions and normalized relative to that of β-galactosidase.

siRNA
Negative control siRNA pool (D-001206-13) and rat siRNA specific for ETS1 (sc-1557602) were from Dharmacon and Santa Cruz Biotechnology, respectively. PC12 cells were seeded at a density of 5 × 10^4 cells per well onto 6-well plates and transfected with 10 nmol/L of each siRNA pool using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. ETS1 protein expression was monitored by immunoblotting 48 hours after transfection.

Chromatin immunoprecipitation
Cells were crosslinked in culture media containing 1% formaldehyde and nuclei were purified and lysed in 50 mmol/L Tris-
HCl (pH 8), 10 mmol/L EDTA, and 1% SDS (31). Genomic DNA was broken by sonication and 10-fold diluted in IP Dilution Buffer [30 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and 0.5% Nonidet P-40]. Immunoprecipitation was performed with 2 μg nonspecific mouse IgG or rabbit polyclonal anti-ETS1 antibody (sc-350, Santa Cruz Biotechnology; ref. 32). Immune complexes were purified with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) preblocked with sonicated salmon sperm DNA (Sigma-Aldrich). Immunoprecipitates were washed four times with IP Dilution Buffer containing 0.1% SDS, twice with High Salt IP Wash Buffer [30 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 5 mmol/L EDTA, 0.1% SDS, and 1% Triton X-100], and once with TE [10 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA]. DNA was purified using Chelex-100 (Bio-Rad). Immunoprecipitated DNA was quantified by qPCR using the primer set 5'–GAGAGAGGTCTATTGCCCCATG (forward) and 5'–AGCGTTTGCTGACCAGCTTC (reverse) as described. Relative fold of increase were calculated according to the equation: $2^{-[(C_t\text{input} - C_t\text{target}) – (C_t\text{input} - C_t\text{mock})]}$.

**Statistical analysis**

Results are reported as the mean ± SEM from at least three independent experiments. Multiple group analysis was conducted by one-way ANOVA with Newman–Keuls multiple comparisons posttest. Comparisons between two groups were performed using unpaired Student $t$ test or nonparametric Mann–Whitney test. IHC quantifications were analyzed by the nonparametric Kruskal–Wallis test with Dunn multiple comparison post hoc tests. Correlation and disease-free survival analysis using molecular data derived from The Cancer Genome Atlas (TCGA) were analyzed using Spearman correlation coefficient and Mantel–Cox test, respectively. Statistical analyses were performed using GraphPad Prism (GraphPad Software). Differences were considered statistically significant at $P < 0.05$.

**Results**

TLR4 is overexpressed in differentiated thyroid carcinomas

We studied TLR4 expression in normal ($n = 13$) and malignant thyroid sections, including PTC ($n = 50$) and FTC ($n = 32$)
by IHC. In agreement with previous observations (16, 19, 20), TLR4 expression was detected on normal thyrocytes (Fig. 1A). Interestingly, TLR4 protein levels were significantly higher in differentiated, follicular and papillary, thyroid carcinomas compared with normal thyroid tissue (Fig. 1A). Moreover, similar changes in TLR4 protein expression were evident in independent assays using a different anti-TLR4 antibody (Supplementary Fig. S1). Of note, comparison of TLR4 expression levels between matched samples of tumor-adjacent normal thyroid tissue and PTCs (n = 4) showed a significant upregulation of TLR4 levels in the neoplastic tissue (Fig. 1B). No significant correlation was evidenced between TLR4 protein immunostaining and tumor, node, and metastasis staging in PTCs or FTCs (Supplementary Fig. S2). Significantly, TLR4 protein expression was also detected in lymph node metastasis derived from PTCs (Fig. 1C). Comparison of TLR4 expression levels between matched samples of primary PTCs and its lymph node metastasis (n = 8) showed a significant upregulation of TLR4 levels in the metastatic tissue (Fig. 1C).

Thereafter, we used molecular data derived from The Cancer Genome Atlas (TCGA) study of PTC to perform combined analysis of TLR4 mRNA expression as a function of the most frequent driver oncogenes identified in PTCs, including point mutations and gene fusions (33). Although TLR4 mRNA levels were found unaltered among PTCs harboring different tumor-driving oncogenes, we noticed a highly heterogeneous TLR4 expression in PTCs harboring the same oncogene (Fig. 1D). However, a significant positive correlation was evidenced between TLR4 mRNA expression levels and ERK activation score (rs = 0.1280, P = 0.0189), an evaluation of the activation status of MAPK/ERK signaling (33), suggesting that oncogene-stimulated MAPK/ERK signaling is an important event driving TLR4 gene expression in PTCs (Fig. 1E). In agreement, the Gene Expression Omnibus (GEO) dataset GSE33630 analysis also

**Figure 2.** Conditional oncogene expression induced TLR4 expression in PTC models. **A**, RT/qPCR assessing TLR4 mRNA expression levels in response to doxycycline (Dox)-induced BRAFV600E, HRasG12V, or RET/PTC3 expression at 48 hours in PCC13 cells. **P** < 0.05 and **P** < 0.01 versus vehicle-treated cells (one-way ANOVA, Newman-Keuls test). **B** and **C**, Immunoblot assessing TLR4 protein expression levels in response to conditional BRAFV600E expression for 48 hours in PC/BRAF cells (B) or transient transfection of BRAFV600E into nontumoral Nthy-ori 3-1 thyroid cells (C). Fold change indicates relative TLR4 abundance. **P** < 0.05 versus vehicle-treated cells or empty vector–transfected cells (unpaired Student t test). **D**, Immunofluorescence under nonpermeabilized conditions analyzing TLR4 protein expression at the plasma membrane in response to BRAFV600E expression for 48 hours in PC/BRAF cells. Scale bar, 10 μm. **P** < 0.001 versus vehicle-treated cells (unpaired Student t test). **E** and **F**, Analysis of TLR4 mRNA and protein expression in the thyroid tissue of transgenic mice conditionally expressing BRAFV600E in the thyroid follicular cell and littermate control mice. Scale bar, 20 μm. Quantification of TLR4 protein expression was expressed relative to the number of cells in the tissue sections. **P** < 0.05 and **P** < 0.001 (Mann-Whitney t test).
showed a significant positive correlation between TLR4 mRNA expression levels and ERK activation score in PTC (Supplementary Fig. S3). Therefore, we focused our analysis on PTCs harboring the oncogene BRAFV600E as this mutation has been reported to occur in 30% to 80% of PTCs (34). We evidenced a significant positive correlation between TLR4 mRNA expression and the thyroid differentiation score \( r_s = 0.1436, P = 0.0302 \), an evaluation of the dedifferentiation status of the tumor (33), supporting that TLR4 gene expression is higher in more differentiated PTCs (Fig. 1F). Moreover, we observed a nonsignificant positive association between TLR4 mRNA expression and MACIS score \( r_s = 0.1039, P = 0.1548 \), an outcome predictor in patients with PTC (Fig. 1G). The modest association may be due in part to the characteristics of the patients, as most of them fall into the low-risk category (MACIS score < 6). Thereafter, patient samples harboring the oncogene BRAFV600E (wild-type TERT status) were divided into the high or low cohort based on the median expression of TLR4 mRNA levels within the group. Interestingly, patients whose tumors showed high TLR4 expression, based on TLR4 expression above the median, had a lower median disease-free survival than those who showed TLR4 expression below the median (TLR4\textsubscript{high} = 26.63 months vs. TLR4\textsubscript{low} = 31.24 months, \( P = 0.0071 \); Fig. 1H). Together, these data suggest that TLR4 mRNA expression constitutes a potential marker of PTC aggressiveness.

PTC-driving oncogenes induce TLR4 expression

To study oncogene-driving TLR4 gene expression, we analyzed TLR4 mRNA expression in PCCl3 thyroid cell conditionally expressing the oncogenes BRAFV600E, HRAS\textsubscript{G12V}, or RET/PTC3 in response to doxycycline. In PCCl3 cells, doxycycline treatment did not upregulate TLR4 mRNA expression. Conversely, in PC/BRAFV600E, PC/HRAS\textsubscript{G12V}, and PC/PTC3 cells, doxycycline-induced oncogene expression was associated with a significant increase of TLR4 mRNA expression (Fig. 2A). In addition, we assessed TLR4 protein expression in PC/BRAFV600E cells in response to doxycycline. BRAFV600E expression significantly increased TLR4 protein levels in PC/BRAFV600E cells (Fig. 2B). Furthermore, transient transfection of BRAFV600E into nontumoral Nthy-ori 3-1 thyroid cells significantly increased TLR4 protein expression levels compared with those of empty vector–transfected cells (Fig. 2C). Analysis of ERK phosphorylation was used to assess BRAFV600E oncogenic activity. Complementarily, immunofluorescence performed under nonpermeabilized conditions showed increased TLR4 protein levels at the plasma membrane in response to BRAFV600E expression (Fig. 2D). Similar observations were done in PCCl3 cells conditionally expressing the oncogenes HRAs\textsubscript{G12V} and RET/PTC3 (Supplementary Fig. S4).

To further confirm our findings, TLR4 mRNA expression was studied in 5-week-old transgenic mice conditionally expressing the oncogene BRAFV600E in the thyroid follicular cell (27). The
thoracic tumors of transgenic mice showed significantly higher TLR4 mRNA expression levels compared with that of nontransgenic littermates controls (Fig. 2E). In addition, the expression of TLR4 protein levels in paraffin-embedded normal and malignant mouse thyroid tissues was assessed by IHC. Accordingly, TLR4 immunostaining was significantly increased in the thyroid tumor tissue of BRAFV600E-expressing mice compared with littermate controls (Fig. 2F).

**Functional TLR4 protein expression in cell models of PTC**

All TLR signaling pathways culminate in the activation of the transcription factor NF-κB (35). Therefore, the functionality of TLR4 overexpression in thyroid carcinomas was assessed by analyzing NF-κB signaling activation in response to the TLR4 agonist LPS. Consistently with previous observations indicating the activation of NF-κB signaling in response to BRAFV600E expression (34), doxycycline-treated PC/BRaFV600E cells showed a significant induction of the NF-κB reporter 5x κB-Luc (Fig. 3A). Interestingly, LPS treatment induced a significant upregulation of NF-κB transcriptional activity in doxycycline-treated PC/BRaFV600E cells (Fig. 3A). The NF-κB inhibitor BAY 11-7082 was used to investigate the specificity of activation of NF-κB pathway (Fig. 3A). Moreover, consistently with the activation of NF-κB signaling in response to TLR4 activation, we observed a significant BAY 11-7082-sensitive increase in the mRNA expression of the well-known TLR4-induced NF-κB targets IL6 and iNOS in response to LPS stimulation in doxycycline-treated PC/BRaFV600E cells (Fig. 3B and C).

To reinforce our observations, we further studied the activation of NF-κB signaling in response to the TLR4-agonist LPS in BCPAP cells harboring the BRAFV600E mutation. LPS stimulation of NF-κB reporter transiently transfected BCPAP cells showed a significant induction of NF-κB transcriptional activity (Fig. 3D). As mentioned, assay specificity was verified by inhibiting the activation of NF-κB signaling using BAY 11-7082 (Fig. 3D).

In addition, we evidenced that BCPAP cells transiently expressing a nonsignaling TLR4 mutant missing the carboxy-terminus or a nonsignaling MyD88, intracellular TLR4 signaling adaptor, mutant missing the TLR4-interacting domain were not responsive to LPS stimulation (Fig. 3D). Consistently, LPS-induced TLR4-dependent signaling increased IL6 and iNOS mRNA expression in BCPAP cells (Fig. 3E and F). Together, this evidence suggests that TLR4/MyD88/NF-κB signaling is functionally conserved in the PTC cell line BCPAP.

**BRAFV600E-induced TLR4 gene expression involves MEK/ERK signaling pathway**

The oncogene BRAFV600E leads to constitutive activation of MAPK/ERK pathway by directly phosphorylating MEK, resulting in thyroid follicular cell transformation (23, 34). To uncover the signal pathway involved in the regulation of TLR4 gene expression in response to BRAFV600E activity, we explored the effect of the specific BRAFV600E inhibitor PLX4032 and the MEK1/2 inhibitor U0126 on TLR4 expression in PC/BRaFV600E cells under doxycycline treatment. As expected, BRAFV600E inhibition reduced the upregulated TLR4 expression in response to doxycycline treatment (Fig. 4A). In line with the observed positive correlation between TLR4 expression and MAPK/ERK activation score, chemical inhibition of MAPK/ERK signaling activation reduced TLR4 expression in response to BRAFV600E induction (Fig. 4A). We analyzed ERK phosphorylation status by immunoblot to assess the inhibition of BRAFV600E-induced MAPK/ERK signaling in response to PLX4032 or U0126 treatment (Fig. 4A). Complementarily, immunofluorescence performed under non-permeabilized conditions showed that chemical inhibition of MAPK/ERK signaling abrogated BRAFV600E-induced cell surface TLR4 protein expression levels (Supplementary Fig. S5). Moreover, chemical inhibition of MAPK/ERK signaling activation reduced TLR4 mRNA expression in response to BRAFV600E induction (Fig. 4B). To confirm this finding, we studied BRAFV600E-induced TLR4 gene expression in response to doxycycline treatment. As expected, BRAFV600E inhibition reduced the upregulated TLR4 expression in response to doxycycline treatment (Fig. 4A).
regulated TLR4 gene expression in BCPAP cells. Interestingly, BCPAP cells treated with the chemical inhibitors PLX4032 or U0126 showed a significant reduction of TLR4 protein and mRNA expression levels (Fig. 4C and D). Altogether, these observations reinforce the involvement of MAPK/ERK signaling in the BRAF\textsuperscript{V600E}-regulated TLR4 gene expression.

A distal Ets-binding site is crucial for TLR4 gene transcription in response to constitutive BRAF\textsuperscript{V600E} signaling

To uncover the molecular mechanism involved in the BRAF\textsuperscript{V600E}-induced TLR4 gene expression, we studied the transcriptional activity of sequential mouse Tlr4 promoter deletions linked to luciferase in doxycycline-treated PC/BRAG\textsuperscript{V600E} cells. The Tlr4 promoter constructs –336/+223 and –608/+223 showed a significant transcriptional response to the induction of BRAF\textsuperscript{V600E} expression (Fig. 5A). However, the promoter constructs +52/–223 and –104/+223 abrogated BRAF\textsuperscript{V600E}-induced TLR4 transcriptional activity (Fig. 5A). As reported, the sequence of the Tlr4 promoter comprising nucleotides –336 and –104 contains a distal Ets-binding site (Ets\textsuperscript{d}) critical for constitutive TLR4 expression in mouse macrophages (24). Thereafter, we performed site-directed mutagenesis of the Ets\textsuperscript{d} DNA-binding site within the –336/+223 promoter construct. Significantly, Ets\textsuperscript{d} mutagenesis reduced BRAF\textsuperscript{V600E}-induced Tlr4 promoter activity (Fig. 5B). Similar observations were done in PCCl3 cells conditionally expressing the oncogenes HRas\textsuperscript{NL6} and RET/PTC3 (Supplementary Fig. S6).

To further test our observations, we constructed an artificial reporter containing three copies in tandem of the flanking region of the Ets\textsuperscript{d}-binding site linked to luciferase (3x Ets\textsuperscript{d}-Luc). Significantly, BRAF\textsuperscript{V600E} expression increased the luciferase activity of PC/BRAG\textsuperscript{V600E} cells transiently transfected with the trimeric Ets\textsuperscript{d} reporter construct (Fig. 5C). Moreover, we observed that the transcriptional activity of the –336/+223 Tlr4 promoter construct missing the Ets\textsuperscript{d}-binding site (Ets\textsuperscript{d} mut –336/+223) was significantly lower than that of the wild-type promoter in BCPAP thyroid cancer cells (Fig. 5D). Together, these results support the involvement of the distal Ets-binding site in the transcriptional upregulation of TLR4 in response to BRAF\textsuperscript{V600E} expression.

The Ets-binding protein ETS1 mediates BRAF\textsuperscript{V600E}-induced TLR4 gene expression

Ets-binding proteins (ETSs) are a family of transcription factors that share a conserved approximately 85 amino acid sequence called Ets DNA-binding domain (36). ETS transcription factors are involved in a wide variety of functions, including differentiation, proliferation, migration, apoptosis, and angiogenesis. Significantly, RAS/RAF signaling increases the transcriptional activity of some, but not all, ETS transcription factors through direct phosphorylation of MAPK/ERK signaling (36).

To uncover the downstream BRAF\textsuperscript{V600E}-induced ETS factors required for TLR4 gene overexpression, we used transcriptomic data derived from TCGA of PTC (33) to correlate the mRNA expression of TLR4 and different members of the ETS family in the subset of PTCs harboring the oncogene BRAF\textsuperscript{V600E}. Interestingly, although a significant positive correlation with ELF1, ETV3, and GABPA, and negative correlation with ETV4 and ELK1 were evidenced (Supplementary Fig. S7A), ETS1 showed the highest positive association with TLR4 mRNA levels \((r = 0.5788, P < 0.0001;\) Fig. 6A), suggesting a potential role for ETS1 in the BRAF\textsuperscript{V600E}-induced TLR4 gene overexpression in PTCs. In agreement, GEO dataset GSE33630 analysis also showed that the ETS family member ETS1 has the highest positive correlation with TLR4 mRNA expression in PTCs (Supplementary Fig. S7B). Supporting these findings, ETS1 mRNA expression is upregulated in PTCs harboring the oncogene BRAF\textsuperscript{V600E} (37) and constitutes a critical genome-wide transcriptional effector of RAS/ERK signaling in tumor epithelial cells (32).

We further explored ETS1 protein expression in tissue microarrays containing unmatched normal thyroid tissue \((n = 9)\) and PTCs \((n = 24)\) by IHC. Although in the normal thyroid tissue, ETS1 protein expression was detected at low levels, its expression was significantly upregulated in PTC (Fig. 6B). In tumor tissues, assessment of high-magnification images suggested that ETS1 was localized in both the cytoplasm and nucleus. Moreover,
ETS1 mediates BRAFV600E-induced TLR4 gene expression. A, Correlation analysis of TLR4 mRNA expression as a function of ETS1 mRNA expression in the subset of PTC harboring the oncogene BRAFV600E (n = 234, Spearman correlation test). B, Representative unmatched sections of normal thyroid tissue and PTC showing ETS1 protein expression levels assessed by IHC using a rabbit polyclonal anti-ETS1 antibody (sc-350, Santa Cruz Biotechnology). Scale bar, 20 μm. Quantification of ETS1 protein levels was expressed relative to the number of cells in the tissue sections and plotted as median with interquartile range. ***, P < 0.001 versus normal tissue (Mann-Whitney test). C, Correlation analysis of TLR4 and ETS1 protein expression assessed by IHC in equivalent PTC tissues (n = 24, Spearman correlation test). D, Immunoblot analysis assessing nuclear ETS1 protein levels in response to BRAFV600E expression in PC/BRAFV600E cells. PARP-1 and α-tubulin were used as nuclear and cytoplasmic loading markers, respectively. Fold change indicates relative ETS1 abundance. **, P < 0.05 (unpaired Student t test). E and G, MAPK/ERK-dependent ETS1 protein expression in response to doxycycline (Dox) treatment in PC/BRAFV600E cells. Scale bar, 10 μm. *P < 0.05 (unpaired Student t test). F and H, MAPK/ERK-dependent ETS1 protein expression in response to BRAFV600E oncogenic activity in PC/BRAFV600E and BCPAP cells. Fold change indicates relative ETS1 abundance. **, P < 0.05 versus vehicle-treated cells (one-way ANOVA, Newman-Keuls test). I, Immunoblot analysis showing TLR4 protein expression in scramble (SCR) or ETS1 siRNA-transfected PC/BRAFV600E cells. Fold change indicates relative ETS1 abundance. **, P < 0.05 (unpaired Student t test). J, TLR4 mRNA expression in doxycycline (Dox)-treated scramble (SCR) or ETS1 siRNA-transfected PC/BRAFV600E cells. ***, P < 0.001 versus vehicle-treated cells; ###, P < 0.01 versus DOX-treated siSCR-transfected cells (one-way ANOVA, Newman-Keuls test). K, Transcriptional activity of the Tlr4 promoter construct −336/+223 in doxycycline (Dox)-treated scramble (SCR) or ETS1 siRNA-transfected PC/BRAFV600E cells. ***, P < 0.001 versus vehicle-treated cells; ###, P < 0.01 versus Dox-treated siSCR-transfected cells (one-way ANOVA, Newman-Keuls test). L, Transcriptional activity of the Tlr4 promoter construct −336/+223 in empty or ETS1 expressing vector-transfected normal PCC13 cells. Fold change indicates relative TLR4 abundance. ***, P < 0.001 (unpaired Student t test). M, ChIP analysis assessing ETS1 binding to Tlr4 promoter region −365 to −24 (relative to the transcription start site) in response to BRAFV600E induction in PC/BRAFV600E cells. ***, P < 0.01 versus vehicle-treated cells (unpaired Student t test).
correlation analysis demonstrated a significant positive correlation between TLR4 and ETS1 protein levels, reinforcing a potential involvement of ETS1 transcriptional activity in TLR4 expression levels (Fig. 6C).

To elucidate the involvement of ETS1 as downstream target of BRAF\(^{V600E}\)-oncogenic activity, we studied ETS1 protein expression in response to doxycycline treatment in PC/BRAF\(^{V600E}\) cells. Conditional BRAF\(^{V600E}\) expression promoted an increase of ETS1 protein expression and its nuclear accumulation (Fig. 6D and E). In addition, we evidenced the involvement of MAPK/ERK signaling in the BRAF\(^{V600E}\)-dependent ETS1 protein expression in PC/BRAF\(^{V600E}\) and BCPAP cells (Fig. 6F and G).

Moreover, to analyze the involvement of ETS1 in TLR4 gene expression, we studied BRAF\(^{V600E}\)-induced TLR4 mRNA expression in ETS1 knockdown PC/BRAF\(^{V600E}\) cells. Immunoblot analysis showed a successful knockdown of ETS1 protein levels in ETS1 siRNA-transfected cells compared with scrambled (SCR) siRNA-transfected cells (Fig. 6H). Interestingly, we evidenced a significant inhibition of BRAF\(^{V600E}\)-induced TLR4 mRNA expression levels in ETS1 knockdown cells compared with those of SCR siRNA-transfected cells (Fig. 6I). Consistently, analysis of Tlr4 promoter activity showed that ETS1 knockdown repressed the BRAF\(^{V600E}\)-increased TLR4 transcriptional activity (Fig. 6J). In agreement, TLR4 protein expression (Fig. 6K) and its nuclear accumulation (Fig. 6D and E) showed a significant upregulation in PCC3 normal thyroid cells transiently transfected to achieve ETS1 overexpression.

Therefore, we studied ETS1 binding to the Tlr4 promoter in response to BRAF\(^{V600E}\)-induced TLR4 expression in PC/BRAF\(^{V600E}\) cells using chromatin immunoprecipitation (ChIP) assays. In detail, one putative Ets-binding site located between –240 and –233 (relative to the transcription start site) in the rat Tlr4 promoter was evidenced when its sequence was analyzed for putative Ets-binding sites using MatInspector software (Genomatix AG). Interestingly, ChIP assay with an anti-ETS1 antibody of doxycycline-treated PC/BRAF\(^{V600E}\) cells revealed a significant enrichment in the Tlr4 promoter sequence flanking the Ets-binding site (Fig. 6M). These data indicated that BRAF\(^{V600E}\)-induced activation of MAPK/ERK signaling upregulated directly ETS1 expression and binding to the TLR4 promoter that, in turn, modulated TLR4 gene expression.

Discussion

Accumulating evidence indicates the association between overactivation of TLR signaling and cancer progression (1). Indeed, dysregulated TLR signaling has been reported in several human carcinomas and related to an exacerbated production of a proinflammatory tumor microenvironment that provides the necessary context for tumor progression, angiogenesis, invasion, metastasis, and evasion of immunosurveillance (2). The biological relevance of TLR expression in tumor cells seems complex, and the regulatory mechanisms leading to differential TLRs expression in cancer cells remains unclear.

In the thyroid tissue, we demonstrated functional TLR4 expression in normal murine thyocytes as TLR4 engagement increased the expression of differentiation markers involved in thyroid hormonogenesis (16–18). Here, in agreement with previous studies (19, 20), we evidenced the presence of TLR4 protein overexpression in well-differentiated thyroid carcinoma compared with that of normal thyroid tissue. Moreover, we observed a significant upregulation of TLR4 protein levels in lymph node metastasis derived from PTCs. Unfortunately, a limitation of our IHC study of thyroid tumor sections is the unavailability of information regarding the oncogenic driver promoting thyroid tumorigenesis. Therefore, we were unable to assess whether upregulated TLR4 protein expression is restricted to a particular oncogenic context. However, we did not evidence different TLR4 mRNA expression patterns among PTCs harboring different tumor driving oncogenes. In addition, integrative analysis of clinical and transcriptomic data derived from TCGA of PTC (33) demonstrated that patients whose BRAF\(^{V600E}\)-positive tumor showed high TLR4 expression, based on TLR4 expression above the median, had a lower median disease-free survival than those who showed TLR4 expression below the median. In close correlation, Dang and colleagues (20) demonstrated a higher incidence of lymph node and distant metastasis in patients with PTC showing high TLR4 expression levels. Mechanistically, these authors demonstrated that TLR4 engagement with low molecular weight hyaluronic acid induced a significant upregulation of CXCR7, the receptor of the chemokine CXCL12 involved in the promotion of tumor cell proliferation and migration (20). Although these findings support a potential prometastatic role for TLR4-mediated signaling in thyroid tumorigenesis, its participation in PTC aggressiveness and metastatic potential should be further evaluated.

The ETSs regulate a plethora of processes, including differentiation, proliferation, apoptosis, and angiogenesis downstream MAPK/ERK signaling activation (36). Significantly, aberrant ETS activation has been reported in several solid tumors (36). Particularly, the transcriptional activity of ETS1 and ETS2 is required for thyroid follicular cell transformation (38). Here, we provided novel evidence regarding the mechanisms that result in functional TLR4 overexpression in PTCs. Our data indicate that TLR4 gene overexpression in PTC is the consequence of dysregulated MAPK/ERK signaling due to thyroid cancer driver oncogenes, such as BRAF\(^{V600E}\). Roger and colleagues (24) carried out a detailed study of the mechanism controlling TLR4 gene expression regulation in mouse macrophages, evidencing a distal Ets-binding site indispensable for constitutive TLR4 gene transcription, which may be recognized by epithelium-specific ESE1 transcription factor. In this study, we demonstrated that the same Ets-binding site in the TLR4 promoter is critical for its transcriptional upregulation in response to BRAF\(^{V600E}\)-oncogenic activity. Moreover, conducting bioinformatics analysis and biochemical approaches, we revealed the direct role of ETS1 as a transcriptional regulator of TLR4 gene expression downstream to BRAF\(^{V600E}\)-induced MAPK/ERK signaling. In agreement, a comprehensive gene expression analysis of ETS1-regulated genes in PC3 prostate cancer cells demonstrated that ETS1 knockdown decreases TLR4 mRNA expression, suggesting that ETS1 constitutes a positive regulator of TLR4 gene expression in cancer cells (39).

Several studies indicate that SNPs in TLRs are associated with immune system dysregulated and cancer development (8). Polymorphisms in TLR genes may shift the balance between pro- and anti-inflammatory cytokines, modulating the risk of infection, chronic inflammation, and cancer (8). Although TLR4 has been described as a highly polymorphic gene, certain TLR4 polymorphisms have been associated with a significant risk to develop epithelial cell–derived neoplasia. Particularly, the polymorphisms rs10116253 and rs1927911 in TLR4 gene were associated with decreased risk to develop gastric cancer.
(40), whereas the polymorphisms rs4986790, rs4986791, and rs10759932 appear to be positively associated with the development of gastric cancer (41). Moreover, the polymorphism rs11536889 located in the 3'-untranslated region of the TLR4 was associated with increased prostate and colon cancer risk (42, 43). In thyroid carcinomas, however, the presence of TLR4 polymorphisms remains to be investigated. Significantly, Kim and colleagues (44) reported a case–control association study of the polymorphisms rs3804099 and rs8304100 in TLR2 gene in patients with PTC and control subjects, describing a significant association between polymorphisms in the TLR2 gene and bilateral thyroid tumors, a surgical criterion to perform total thyroidectomy. More recently, Kim and colleagues (45) reported a significant association of the missense polymorphism rs11466653 in TLR10 with a higher risk for the development of thyroid papillary microcarcinomas than in the control group. Together, these findings support that abnormal TLR signaling in PTC may be involved in the interplay between inflammation and tumorigenesis.

TLRs recognize molecules that are broadly shared by pathogens but, sometimes, indistinguishable from host proteins released by stressed cells undergoing necrosis or proteolytic products derived from structural components of the extracellular matrix, collectively referred to as damage-associated molecular patterns (DAMP). Here, we demonstrated that malignant thyroid follicular cells themselves are responsive to exogenous TLR4 agonists. However, the question regarding the endogenous molecules that engage TLR4 in the tumor environment to induce a signaling cascade leading to the NF-kB activation remains unanswered. We hypothesize that TLR4 signaling–activating DAMPs, such as high mobility group box-1 (HMGB1), which is usually observed at high levels in the tumor microenvironment or hyaluronan-derived proteolytic products considering that hyaluronan is highly expressed in the tumor stroma of differentiated thyroid carcinomas (46), are likely candidates. Moreover, regarding the importance of DAMPs in thyroid tumor progression, recent evidence suggested that the multiligand receptor for advanced glycation end products (RAGE) mediates the promigratory response of extracellular small calcium-binding protein (S100A4) in human thyroid cancer cells (47).

TLRs-triggered signal pathways culminate in the activation of the transcription factor NF-kB, which induces the expression of proinflammatory cytokines, antiapoptotic factors, and proangiogenic factors (35). Recent evidence indicates that NF-kB signaling, as well as the pathways that culminate in its activation, are crucial players in many steps of carcinogenesis and tumor development (48). Genetic alterations leading to constitutive MAPK/ERK signaling have a fundamental role in thyroid carcinogenesis (34). However, additional molecular derangements are associated with tumor progression and aggressiveness. Particularly, aberrant NF-kB signaling activation is triggered by different oncogenes involved in thyroid carcinogenesis associated with a papillary phenotype (49) and linked to anaplastic thyroid tumorigenesis (21). NF-kB signaling activation has been associated with tumor progression, resistance to chemotherapeutic agent–induced apoptosis, and invasion associated with metalloproteinases expression (21). In close relation, the polymorphism rs2233406 in NFKBIA gene, an NF-kB signaling inhibitor, was associated with a proinflammatory tumor microenvironment resulting from the exacerbated TLR4-dependent secretion of IL1β that could favor tumor progression (50).

Overall, our findings raise an intriguing question regarding the pronocogenic potential of TLR4 downstream signaling in thyroid tumorigenesis, further considering that dysregulated NF-kB signaling has been implicated in thyroid cancer process (21). The understanding of TLR4 function in differentiated thyroid tumor development and growth as well as the mechanisms involved in these processes could have great clinical relevance. Further uncovering the mechanisms leading to TLR4 engagement and signaling in thyroid tumors may provide novel alternatives for the establishment of future therapies for thyroid cancer, and the design of new diagnostic procedures to identify a subset of potentially aggressive PTCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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