

# Reduced Tumor Necrosis Factor Receptor–Associated Death Domain Expression Is Associated with Prostate Cancer Progression

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## Abstract

**By using LNCaP and its derivative cell lines, we first observed an association between tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) resistance and hormone independence. Moreover, we found that the expression of tumor necrosis factor receptor–associated death domain (TRADD) was reduced in androgen deprivation–independent cells compared with that in androgen deprivation–dependent cells. TRADD is a crucial transducer for TNF- $\alpha$ –induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation. Knocking down TRADD expression in LNCaP cells impaired TNF- $\alpha$ –induced NF- $\kappa$ B activation and androgen receptor repression, whereas overexpression of TRADD in C4-2B cells restored their sensitivity to TNF- $\alpha$ . Finally, we found that androgen deprivation reduces TRADD expression *in vitro* and *in vivo*, suggesting that androgen deprivation therapy may promote the development of TNF- $\alpha$  resistance by reducing TRADD expression during prostate cancer progression. [Cancer Res 2009;69(24):9448–56]**

## Introduction

Prostate cancer is the most common malignancy and one of the leading causes of cancer death in North American men. Initially, most prostate cancers are responsive to androgen deprivation therapy, which is achieved by chemical or surgical castration. However, these cancers relapse eventually and ultimately progress to castration-recurrent disease that is resistant to not only hormone deprivation therapy but also other systemic chemotherapy (1, 2). Nonetheless, androgen receptor (AR) signaling remains critical for castration-recurrent prostate cancers (3, 4).

The tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) pathway is also aberrantly regulated during prostate cancer progression (5). TNF- $\alpha$  is a pleiotropic cytokine that exerts a variety of functions in inflammation, immunity, cell differentiation, cell proliferation, and, paradoxically, cell death (6). TNF- $\alpha$  is primarily produced by immune cells. However, it is also produced by numerous other cell types, including epithelial cells of human prostate cancer (7). TNF- $\alpha$  binds to the TNF- $\alpha$  receptor (TNFR1), leading to phosphorylation, ubiquitination, and proteasome-mediated degradation of the inhibitor of  $\kappa$ B (I $\kappa$ B), which binds to and inhibits nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation by forming a complex in the cytoplasm. Degradation of I $\kappa$ B results in the release of NF- $\kappa$ B, which then translocates to

the nucleus. In the nucleus, NF- $\kappa$ B regulates the transcription of target genes, which promote cell proliferation or inflammatory responses. In most cells, TNF- $\alpha$  activates caspase-8 and induces apoptosis only when NF- $\kappa$ B activation is hampered (8). However, in prostate cancer LNCaP cells, even if NF- $\kappa$ B signaling is activated, TNF- $\alpha$  can still induce cell death in a dose-dependent manner (9). This apparent discrepancy may be explained by the negative regulation of AR expression and activity by NF- $\kappa$ B (10, 11). Interestingly, androgen deprivation–independent (AI) prostate cancer cells are mostly resistant to TNF- $\alpha$ , whereas androgen deprivation–dependent (AD) cells are sensitive to it, suggesting an association between androgen deprivation and TNF- $\alpha$  susceptibility (12). However, it remains unclear how prostate cancer cells lose their sensitivity to TNF- $\alpha$  as they progress.

Tumor necrosis factor receptor–associated death domain (TRADD) was one of the first identified TNF-R1–associated proteins (13). TRADD contains a COOH-terminal death domain that is 23% identical to the death domain of TNFR1 and can directly interact with TNFR1 (13). The NH<sub>2</sub> terminus of TRADD interacts with the TNFR-associated factors TRAF1 (14), TRAF2 (15), and TRAF3 (16). Thus, TRADD mediates TNF-R1–induced apoptosis as well as NF- $\kappa$ B activation (13). Recent knockout studies also revealed TRADD-mediated functions in Toll-like receptor signaling and antiviral immune responses (17, 18).

In this article, we report an association between reduced TRADD expression and TNF- $\alpha$  resistance in AI prostate cancer cell lines. Our data show that TRADD is critical for TNF- $\alpha$ –induced NF- $\kappa$ B activation and AR suppression in prostate cancer. Furthermore, we show that androgen deprivation decreases TRADD expression *in vitro* and *in vivo*, suggesting that prostate cancer cells may lose their TNF- $\alpha$  sensitivity after androgen deprivation therapy.

## Materials and Methods

**Materials.** LNCaP were purchased from the American Type Tissue Collection. C4, C4-2, and C4-2B cells were purchased from ViroMed Laboratories.

pcDNA wild-type (wt)–TRADD and TRADD $\Delta$ 301 were gifts from Dr. T. Tsuruo (University of Tokyo, Tokyo, Japan). pcDNA I $\kappa$ B $\alpha$  SS/AA was a gift from Dr. D.D. Billadeau (Mayo Clinic, Rochester, MN). siRNA-A (no target siRNA), RIP siRNA (h2), and TRADD siRNA were purchased from Santa Cruz Biotechnology.

The following antibodies were used in this study: anti-AR (p21; Millipore); anti–prostate-specific antigen (PSA; c-19), anti-ERK2 and anti-receptor-interacting protein 1 (RIP1; k-20; Santa Cruz Biotechnology); anti-phospho-I $\kappa$ B $\alpha$  (Ser32/36; 5A5), anti-Lamin A/C, and anti- $\alpha$ -tubulin (Cell Signaling); anti-TRADD, anti-I $\kappa$ B $\alpha$ , and anti-NF- $\kappa$ B p65 (BD Transduction Laboratory); anti-FAS-associated death domain (FADD; BD Pharmingen); and anti-TNF $\alpha$  neutralizing and anti-TNF-R1 (R&D Systems).

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Recombinant human TNF- $\alpha$  was purchased from Chemicon. Dutasteride was obtained from GlaxoSmithKline. Methyltrienolone (R1881) was purchased from DuPont.

**RNA isolation, amplification, and quantitative reverse transcriptase-PCR.** Total RNA from cultured cells was isolated by Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 5  $\mu$ g total RNA using the SuperScript III first-strand synthesis system (Invitrogen) following the manufacturer's instructions.

Tissues obtained at prostatectomy were frozen in optimal cutting temperature (OCT; Tissue Tek OCT, Sakura Finetek). Frozen prostate samples embedded in OCT were used for separate laser capture microdissection of stroma, benign and malignant epithelium as indicated. Approximately 2,000 to 3,000 cells per sample were collected using the Arcturus Veritas LCM System and used for isolation of total RNA followed by two rounds of linear amplification as we have previously described (19, 20). Primers specific for genes of interest were designed using the National Center for Biotechnology Information Web-based primer design tool Primer-BLAST.<sup>5</sup> First-strand cDNA templates were generated from 1  $\mu$ g of amplified RNA using 1  $\mu$ g of random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen).

Quantitative reverse transcriptase-PCR (qRT-PCR) was performed with SYBR green PCR Master Mix (Applied Biosystems) on an Applied Biosystems System Sequence Detector 7700HT. All reactions were assessed for quality by examination of both amplification and dissociation curves. Samples were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or RPL13A.

Primer sequences used in this study are the following: AR Fwd, GGAA-CAGCAACCTTCACAGC; AR Rev, CTTTAAGGTCACGGAGCAG; TRADD Fwd, CGCATACCTGTTTGTGGAGTC; TRADD Rev, CGGTGGATCTTCAG-CAATCTG; RPL13A Fwd, CCTGGAGGAGAAGAGGAAGAGA; RPL13A Rev TTAGAGGACCTCTGTGTATTGTCAA. Human GAPDH primers were purchased from Applied Biosystems.

**Cell lysate preparation and Western blotting assays.** Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer with a complete protease inhibitor cocktail (Santa Cruz Biotechnology). Nuclear and cytoplasmic protein fractions were prepared with a Nuclear Extraction kit according to the manufacturer's instructions (Millipore). Equal amounts of protein (30–80  $\mu$ g) were loaded onto 10% NuPage Bis-Tris gels (Invitrogen), and electrophoresis was performed according to the manufacturer's instructions. Proteins were blotted onto nitrocellulose membranes. Blots were probed with antibodies to evaluate protein expression.

**Transfection and luciferase assay.** Transfections by electroporation were performed as described previously (21). For luciferase assays, cells were harvested and lysed 2 to 3 d after transfections. Firefly luciferase and Renilla luciferase activities in cell lysates were determined using a dual luciferase reporter assay (Promega). Relative luciferase units were calculated as indicators of transcriptional activity.

**Cell viability assay.** Cells were seeded in 96-well tissue culture plates at a density of 3,000 or 5,000 per well and treated as indicated. Cell viability was assessed with the Cell Titer 96 Aqueous One solution cell proliferation assay (Promega) according to the manufacturer's instructions.

**Clinical protocols.** All procedures involving human subjects were approved by institutional review boards of participating institutions; all subjects signed written informed consent. Prostate tissue samples were obtained from a clinical trial evaluating dutasteride (GlaxoSmithKline) before radical prostatectomy as previously described (22). Eighty-one men ages 45 to 80 y with clinically localized prostate cancer (T1c-T2b), Gleason scores of <7, and serum PSA of 2.5 to 10 ng/dL were randomized to immediate radical prostatectomy ( $n = 25$ ) or 4 mo of 0.5 mg ( $n = 26$ ) or 3.5 mg ( $n = 24$ ) of dutasteride orally daily preceding radical prostatectomy. Prostate tissue samples were also obtained from a recently completed clinical trial of neoadjuvant castration therapy before prostatectomy. Fifteen men ages 18 y or older with clinically localized prostate cancer (T1c-T2b), Gleason scores of <7, and serum PSA of <20 ng/dL were randomized to

3 wk of castration with either leuprolide and bicalutamide (7.5 mg injection and 50 mg orally daily, respectively;  $n = 7$ ) or castration with transdermal estradiol (E2; 0.6 mg every 3 d;  $n = 8$ ). Untreated prostate tissue samples were obtained from men undergoing standard radical prostatectomy as described previously (23).

**Prostate cancer xenograft tumors and immunohistochemistry staining.** AD and AI versions of LuCaP 23.1 and LuCaP 35 prostate cancer xenografts were generously provided by Dr. Robert L. Vessella (University of Washington, Seattle, WA). Tumors were propagated in severe combined immunodeficient mice as described (24). Formalin-fixed, paraffin-embedded tissues were cut into 5- $\mu$ m sections, deparaffinized, and rehydrated in a graded series of ethanol dilutions. Immunohistochemistry was performed with the IHC Select Immunoperoxidase Secondary Detection System (Millipore) according to the manufacturer's instructions.

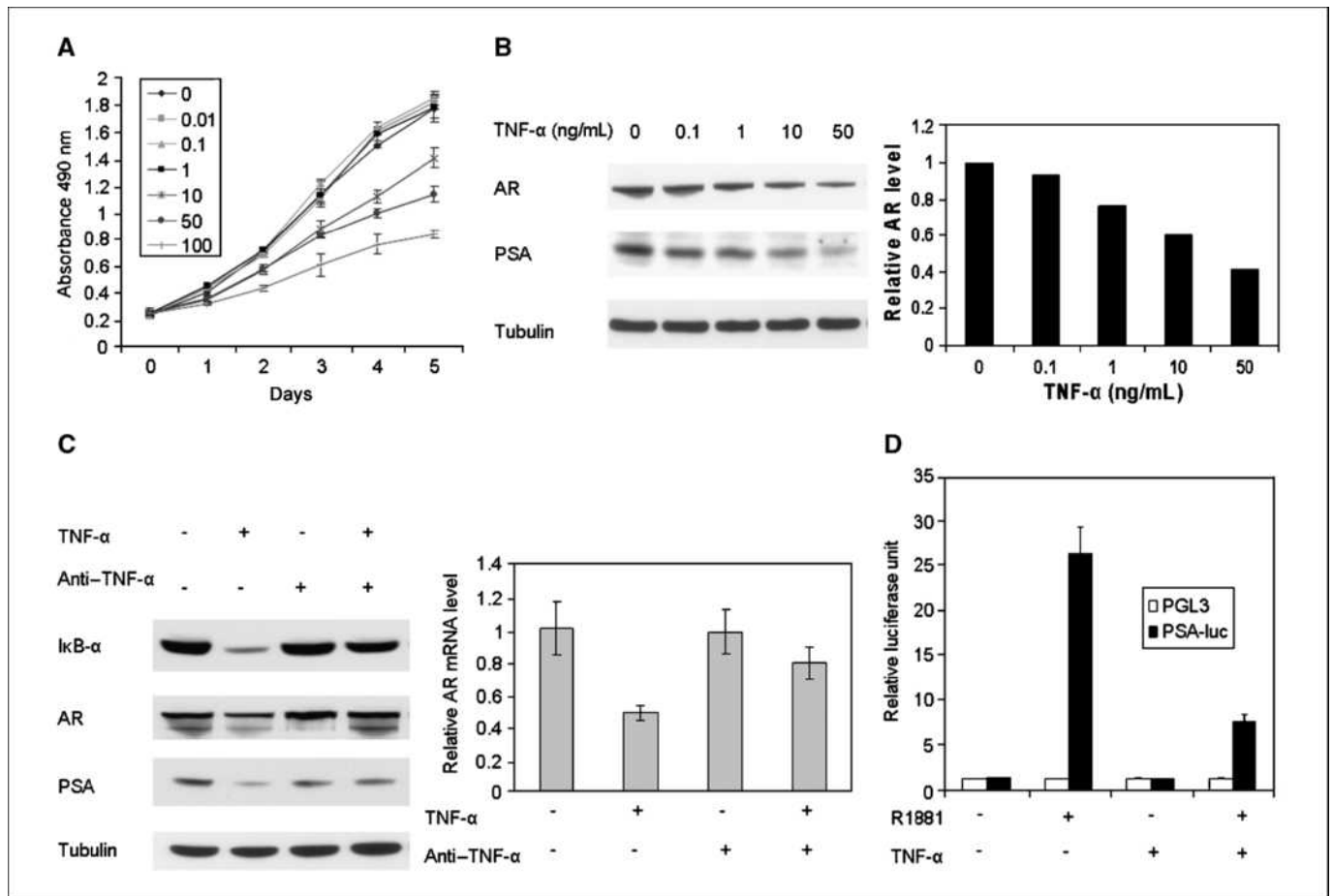
## Results

**TNF- $\alpha$  reduces prostate cancer cell viability through inhibition of AR expression and activity.** Although elevated TNF- $\alpha$  has been documented in prostate cancers (7), the effects of TNF- $\alpha$  on prostate cancer cells still mainly remain unclear. Thus, we investigated the effect of TNF- $\alpha$  on the viability of LNCaP cells. The results showed that TNF- $\alpha$  reduced LNCaP cell viability in a dose-dependent manner (Fig. 1A). Given the fact that TNF- $\alpha$  activates NF- $\kappa$ B in LNCaP cells, it is paradoxical that TNF- $\alpha$  reduces LNCaP cell viability. Mizokami and colleagues (7) first reported that TNF- $\alpha$  represses AR expression in LNCaP cells and suggested that this might be the underlying mechanism by which TNF- $\alpha$  inhibits prostate cancer cell growth and survival. To confirm the effect of TNF- $\alpha$  on AR expression, we examined AR expression by Western blotting after treating LNCaP cells with different doses of TNF- $\alpha$ . As expected, TNF- $\alpha$  reduced AR and PSA expression in a dose-dependent manner (Fig. 1B). To confirm that AR reduction was indeed caused by TNF- $\alpha$ , we used a TNF- $\alpha$ -specific antibody to neutralize TNF- $\alpha$ . TNF- $\alpha$ -neutralizing antibody totally abolished the TNF- $\alpha$ -induced reduction of I $\kappa$ B $\alpha$ , AR, and PSA expression (Fig. 1C, left). Real-time qRT-PCR also showed that the TNF- $\alpha$ -neutralizing antibody blocked the TNF- $\alpha$ -induced reduction of AR at mRNA level (Fig. 1C, right). To assess whether TNF- $\alpha$ -induced downregulation of AR leads to a suppression of AR transcriptional activity, we used a PSA promoter-driven luciferase reporter to monitor AR transcriptional activity. R1881, a synthetic androgen, greatly induced AR transcriptional activity but cotreatment with TNF- $\alpha$  significantly suppressed androgen-induced AR transactivation (Fig. 1D), indicating that TNF- $\alpha$  negatively regulates AR transcriptional activity.

**TNF- $\alpha$ -induced AR repression requires NF- $\kappa$ B activation.** Because NF- $\kappa$ B has been suggested to mediate TNF- $\alpha$ -induced AR repression (11), we blocked NF- $\kappa$ B activation with an I $\kappa$ B $\alpha$  super-suppressor (pcDNA-I $\kappa$ B $\alpha$ SS/AA-HA) to test whether TNF- $\alpha$ -induced AR suppression requires NF- $\kappa$ B activation (25). TNF- $\alpha$ -induced reduction of AR and PSA expression was totally blocked by an I $\kappa$ B $\alpha$  super-suppressor (Supplementary Fig. S1), suggesting that NF- $\kappa$ B is required for TNF- $\alpha$ -induced downregulation AR expression and activity.

**AD and AI prostate cancer cells differentially respond to TNF- $\alpha$ .** To determine whether TNF sensitivity is altered during prostate cancer progression, we used a prostate cancer cell line progression model. The model is composed of LNCaP, C4, C4-2, and C4-2B cell lines (26–28). LNCaP cells are sensitive to androgen deprivation and have limited metastatic ability. On the other hand, C4, C4-2, and C4-2B are resistant to androgen deprivation

<sup>5</sup> <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>



**Figure 1.** TNF- $\alpha$  reduces prostate cancer cell viability through AR suppression. *A*, LNCaP cell viability was measured by the MTS assay at the indicated time points after indicated amount (ng/mL) of TNF- $\alpha$  treatment. The results are representative of three independent experiments. *B*, LNCaP cells were treated with indicated doses of TNF- $\alpha$  for 24 h; AR and PSA expression was analyzed by Western blotting (*left*). AR band density was quantitated by Molecular Analyst Alias (Bio-Rad) and the relative level was illustrated (*right*). *C*, LNCaP cells were treated with 20 ng/mL TNF- $\alpha$  and/or 5  $\mu$ g neutralizing anti-TNF- $\alpha$  for 24 h. *Left*, Western blotting with indicated antibodies; *right*, real-time qRT-PCR for AR mRNA levels. The results are representative of three independent experiments. *D*, PSA-luc luciferase reporter assay. LNCaP cells transfected with PGL3-basic or PGL3-PSA-luc were cultured in CSS medium and treated with 20 ng/mL TNF- $\alpha$  and/or 1 nM R1881. Luciferase activity was measured after 24 h. The results are representative of three independent experiments. *Columns*, mean values; *bars*, SD.

and display more aggressive phenotypes. In many respects, the development of these AI cell lines recapitulates the progression of prostate cancer *in vivo*. Consistent with previous reports (9, 11), C4-2 and C4-2B are mostly resistant to TNF- $\alpha$  (Fig. 2A). In contrast to a nearly 50% reduction in LNCaP cells, TNF- $\alpha$  only reduced C4-2 and C4-2B cell viability by <20%, suggesting that prostate cancers may develop TNF- $\alpha$  resistance as they progress.

We then asked whether TNF- $\alpha$ -induced AR suppression is absent or limited in AI cell lines. We treated LNCaP and C4-2 cells with or without 20 ng/mL TNF- $\alpha$  and evaluated the expression of AR and PSA. In contrast to that in LNCaP cells, TNF- $\alpha$ -induced AR and PSA reduction was almost absent in C4-2 cells (Fig. 2B, *left*). A TNF- $\alpha$  dose-response assay indicated that even as high as 50 ng/mL, TNF- $\alpha$  still did not significantly reduce AR or PSA expression in C4-2B cells (Fig. 2B, *right*), confirming the resistance to TNF- $\alpha$  in AI cells. Given the critical role of NF- $\kappa$ B in TNF- $\alpha$ -induced AR repression, we speculated that NF- $\kappa$ B activation induced by TNF- $\alpha$  might be blocked or reduced in AI cells. Therefore, we compared the phosphorylation levels of I $\kappa$ B $\alpha$  in LNCaP and C4-2B cells after 1 hour of treatment with 20 ng/mL TNF- $\alpha$ . Consistent with the results of the NF- $\kappa$ B luciferase assay, TNF- $\alpha$ -induced

phosphorylation of I $\kappa$ B $\alpha$  at serine 32 and serine 36 was significantly decreased (59%) in C4-2B cells compared with that in LNCaP cells (Fig. 2C). Accordingly, the reduction of total I $\kappa$ B $\alpha$  in LNCaP cells (51%, *second lane* versus *first lane*) was higher than that in C4-2B cells (34%, *fourth lane* versus *third lane*). We then compared TNF- $\alpha$ -induced NF- $\kappa$ B activity between these cell lines by a reporter luciferase assay. Consistent with the results of I $\kappa$ B $\alpha$  phosphorylation, TNF- $\alpha$  strongly induces NF- $\kappa$ B activation in LNCaP cells. Although TNF- $\alpha$ -induced NF- $\kappa$ B activation was not totally blocked in C4-2 and C4-2B cells, the amplitude of NF- $\kappa$ B activation was much lower than that in LNCaP cells (Fig. 2D), suggesting that TNF- $\alpha$ /NF- $\kappa$ B signaling pathway is impaired in AI cells. Collectively, these studies indicate that the TNF- $\alpha$  resistance in AI cells might be due to the impairment of the TNF- $\alpha$ /NF- $\kappa$ B signaling pathway.

**TRADD expression level is correlated with TNF- $\alpha$  sensitivity in AD and AI prostate cancer cells.** To explore the underlying mechanism by which AI cells develop increased resistance to TNF- $\alpha$ , we next examined the expression of TNFR1 in AD and AI cells. Consistent with previous studies (11, 29), TNFR1 expression was comparable at the protein level in all these cell lines (Fig. 3A). We then examined the expression of other important

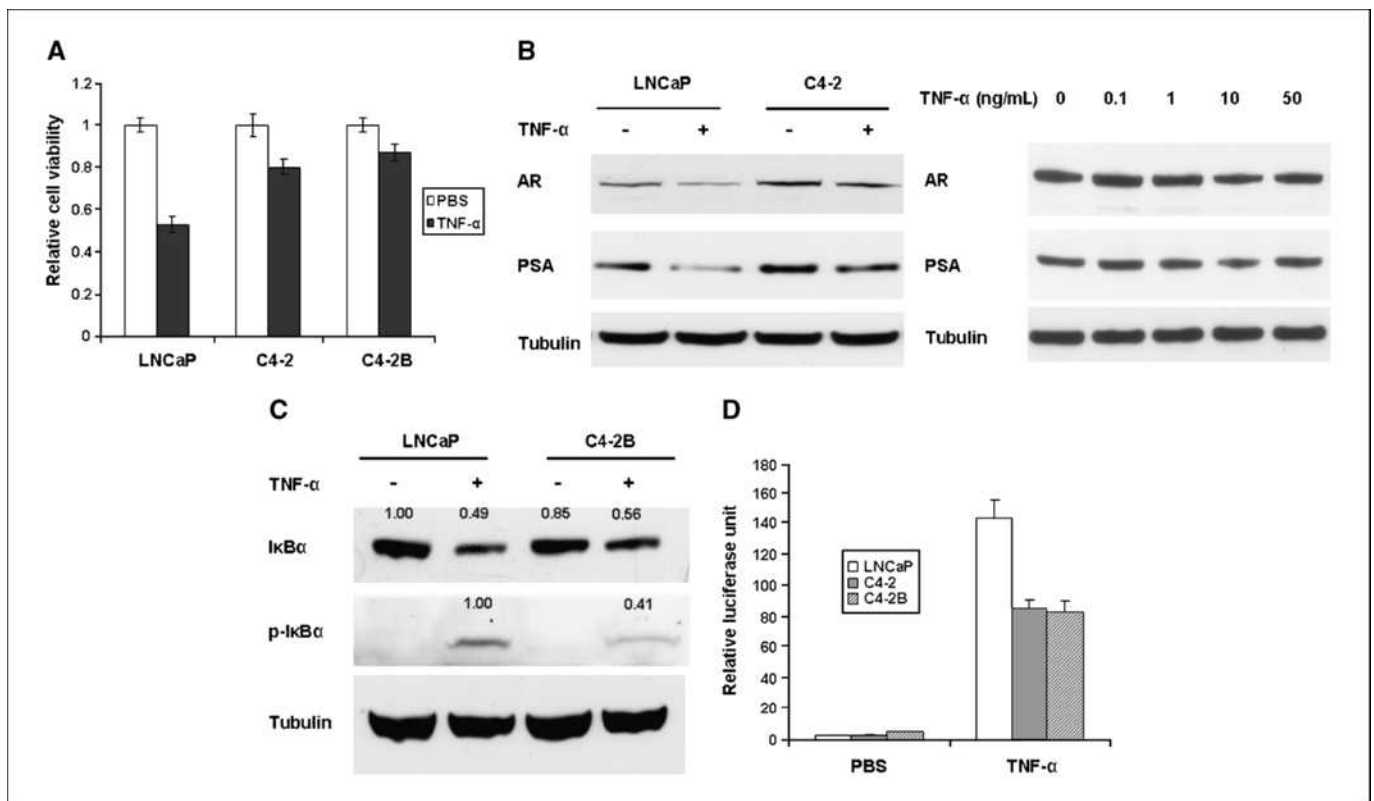
signal transducers in the TNF- $\alpha$  signaling pathway, including TRADD, FADD, and RIP1. Interestingly, TRADD expression was significantly decreased in C4-2 and C4-2B cells compared with LNCaP cells, whereas RIP1 and FAS-associated death domain levels were comparable (Fig. 3A). To evaluate the mRNA level of TRADD, we designed a pair of TRADD-specific primers for real-time qRT-PCR assays. As shown in Fig. 3B, overexpressing TRADD increased both protein and mRNA levels of TRADD, whereas knocking down TRADD reduced both, suggesting that these primers are specific for TRADD. Using these primers, we then compared TRADD mRNA levels among these cell lines. Consistent with the protein level, the mRNA level of TRADD declined from LNCaP to C4-2B (Fig. 3C), suggesting a transcriptional regulation on TRADD during prostate cancer progression.

**TRADD is critical for TNF- $\alpha$ -induced NF- $\kappa$ B activation and AR suppression.** To test whether TRADD plays a critical role in TNF- $\alpha$ -induced NF- $\kappa$ B activation and AR suppression, we knocked down TRADD expression by TRADD siRNA in LNCaP cells. In TRADD-competent LNCaP cells, TNF- $\alpha$  treatment resulted in a strong I $\kappa$ B $\alpha$  phosphorylation, which consequentially reduced its expression (Figs. 2C and 4A). However, in TRADD knocked down cells, the TNF-induced I $\kappa$ B $\alpha$  phosphorylation was inhibited significantly (56% reduction). Accordingly, the total I $\kappa$ B $\alpha$  reduction was not as significant as that in TRADD-competent cells (Fig. 4A), suggesting that NF- $\kappa$ B activation is impaired in TRADD-deficient cells. To further confirm the role of TRADD in TNF- $\alpha$ -induced NF- $\kappa$ B

activation, we evaluated NF- $\kappa$ B activity with a luciferase reporter assay. As shown in Fig. 4B, TNF- $\alpha$ -induced NF- $\kappa$ B activity was greatly inhibited in TRADD-deficient cells, demonstrating that TRADD is important for TNF- $\alpha$ -induced NF- $\kappa$ B activation.

However, knocking down TRADD did not completely block TNF- $\alpha$ -induced I $\kappa$ B $\alpha$  phosphorylation and NF- $\kappa$ B activation. It might be due to the limited efficiency of TRADD siRNA. More importantly, recent studies on TRADD knockout mice suggest that a strict dependence on TRADD for TNF- $\alpha$ -induced NF- $\kappa$ B activation is cell type specific (30). In cells with very low levels of RIP1, such as fibroblasts, TRADD deficiency almost completely abolishes TNFR1-mediated signaling. However, in cells with abundant RIP1 expression, such as macrophages, RIP1 is sufficient to mediate TNFR1 signaling in the absence of TRADD (17, 18). As shown in Fig. 3A, prostate cancer cells express abundant RIP1. Therefore, it is likely that RIP1 enables TNF- $\alpha$  to activate NF- $\kappa$ B at some level even in the absence of TRADD.

To determine whether TRADD is critical for the effects of TNF- $\alpha$  on AR suppression, we next evaluated the AR expression and activity after knocking down TRADD in LNCaP cells. As expected, knocking down TRADD almost abolished the AR reduction induced by TNF- $\alpha$  (Fig. 4C). Accordingly, PSA reduction was also abolished in TRADD-deficient cells. Furthermore, we evaluated the nuclear AR levels in TRADD-competent and TRADD-deficient cells. LNCaP were cultured in medium containing 9% charcoal-stripped serum (CSS) and treated with or without androgens and



**Figure 2.** AD and AI prostate cancer cells differentially respond to TNF- $\alpha$ . *A*, relative cell viability after 50 ng/mL TNF- $\alpha$  treatment for 96 h in LNCaP, C4-2, and C4-2B cells. *Columns*, mean values from three experiments; *bars*, SD. *B*, Western blotting assays. *Left*, LNCaP and C4-2 cells treated with or without 20 ng/mL TNF- $\alpha$  for 24 h; *right*, C4-2B cells treated with indicated doses of TNF- $\alpha$  for 24 h. *C*, Western blotting detecting total and phosphorylated I $\kappa$ B $\alpha$  after 1 h of 20 ng/mL TNF- $\alpha$  treatment. Relative band density is indicated above the bands. *D*, NF- $\kappa$ B-luciferase reporter assays after 24 h of 10 ng/mL TNF- $\alpha$  treatment. *Columns*, mean values from samples in triplicate; *bars*, SD.

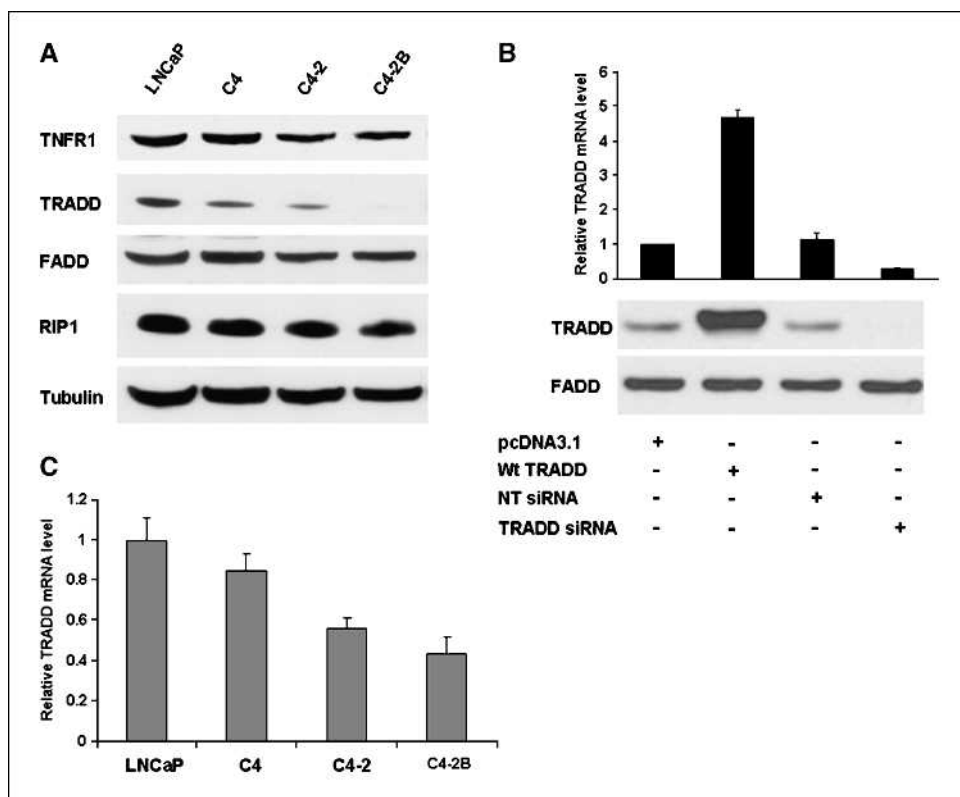
TNF- $\alpha$ . Nuclear AR levels were assessed as indicators for AR activity. As expected, androgens greatly increased nuclear AR levels in cells transfected with control siRNA, but cotreatment with TNF- $\alpha$  significantly reduced the nuclear AR accumulation induced by androgens (Fig. 4D). However, in cells transfected with TRADD siRNA, TNF- $\alpha$  did not significantly reduce nuclear AR levels. To further confirm that TRADD is important for TNF- $\alpha$ -induced AR suppression, we performed immunofluorescence staining with an AR-specific monoclonal antibody to visualize AR expression and localization. As shown in Supplementary Fig. S2, AR was localized primarily within the cytoplasm in the absence of androgens. Androgen treatment resulted in increased AR expression and also translocation of AR into the nucleus. Cotreatment with TNF- $\alpha$  significantly suppressed androgen-induced AR translocation in TRADD-competent cells but not in TRADD-deficient cells, which is consistent with what we observed by nuclear AR Western blotting.

Collectively, these data suggest that TRADD plays a critical role in TNF- $\alpha$ -induced NF- $\kappa$ B activation and AR suppression. Interestingly, the TNF- $\alpha$  response pattern in TRADD knockdown LNCaP cells was very similar to that in C42B cells, implying that TNF- $\alpha$  resistance in AI cells may be due to loss of TRADD expression.

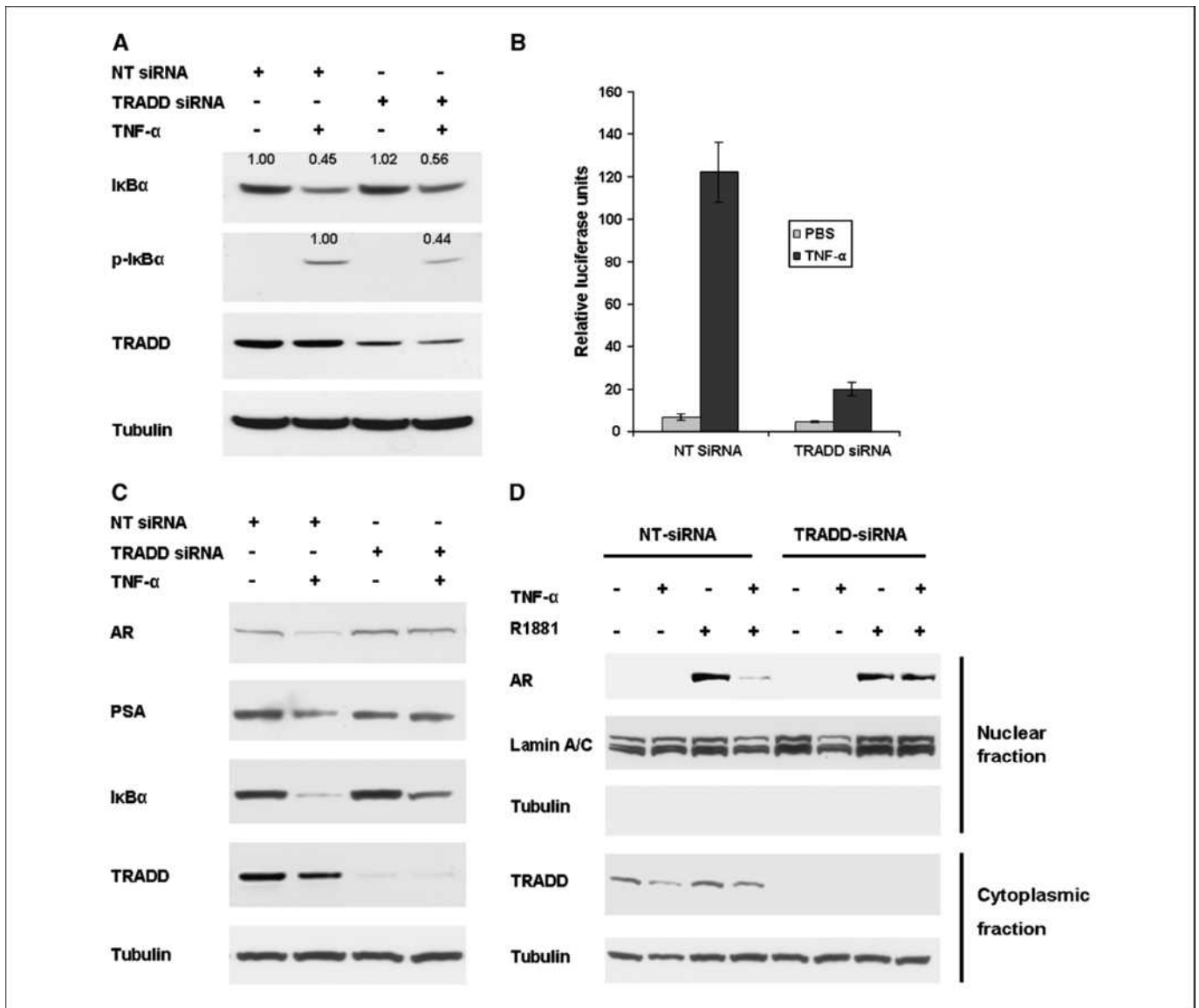
**Ectopic expression of TRADD restores the TNF- $\alpha$ -induced AR repression in AI cells.** To confirm that the reduced TRADD expression in AI cells contributes to the resistance to TNF- $\alpha$ -induced AR repression, we tested whether overexpression of TRADD in C4-2B cells could restore their TNF- $\alpha$  sensitivity. Because TRADD signals not only NF- $\kappa$ B activation but also cell apoptosis (13), we used two different TRADD constructs: wt-TRADD and TRADD $\Delta$ 301. In the latter construct, 12 amino acids

at the COOH-terminal end were deleted (31). Deletion of the COOH-terminal 12 amino acids abolishes its apoptosis-inducing ability but does not abolish the ability to activate NF- $\kappa$ B (13). As expected, overexpression of either wt-TRADD or TRADD $\Delta$ 301 in C4-2B cells potentiated TNF- $\alpha$  to activate NF- $\kappa$ B (Fig. 5A), confirming that TRADD-mediated activation is independent of apoptosis. Similarly, ectopic expression of either wt-TRADD or TRADD $\Delta$ 301 both enabled TNF- $\alpha$  to repress AR and PSA expression (Fig. 5B), suggesting that NF- $\kappa$ B activation, instead of apoptotic signals, mediates TNF- $\alpha$ -induced AR repression. Furthermore, ectopic expression of either wt-TRADD or TRADD $\Delta$ 301 both re-sensitized C4-2B cells to TNF- $\alpha$  treatment (Fig. 5C). Because TRADD $\Delta$ 301 lost its ability to directly induce apoptosis (31), the reduction of cell viability is most likely due to AR repression instead of TNF- $\alpha$ -induced apoptosis.

**Androgen deprivation suppresses TRADD expression *in vitro* and *in vivo*.** The next issue we addressed is to determine the mechanism underlying the reduced TRADD expression in AI cell lines. Because these cell lines were developed under selective pressure of androgen deprivation, we questioned whether androgen deprivation affects TRADD expression. We investigated TRADD expression in cells cultured in hormone-free medium and found that removal of hormone significantly reduced TRADD expression in LNCaP cells. Likewise, treatment with the antiandrogen bicalutamide or the SRD5A inhibitor dutasteride also significantly decreased TRADD expression (Fig. 6A, left), suggesting that androgens are required to maintain a high level of TRADD expression. Conversely, treatment with 1 nmol/L R1881 for 48 hours greatly increased TRADD expression, which could be blocked by bicalutamide (Fig. 6A, right).



**Figure 3.** TRADD expression level is correlated with TNF- $\alpha$  sensitivity. *A*, Western blotting assay with the corresponding antibodies. Tubulin serves as loading control. *B*, confirmation of specificity of TRADD primers. *Top*, real-time qRT-PCR results; *bottom*, Western blotting. *C*, TRADD mRNA levels assessed by real-time qRT-PCR. GAPDH was used as internal control. *Columns*, mean values from samples in triplicate; *bars*, SD.

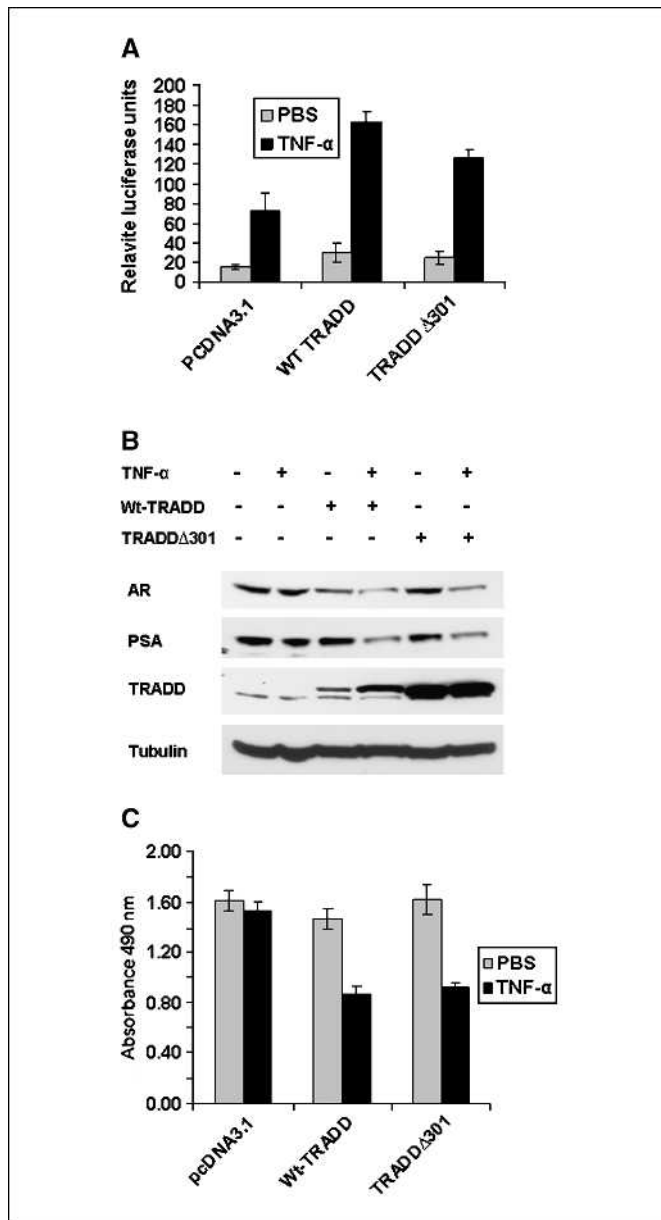


**Figure 4.** TRADD is critical for TNF- $\alpha$ -induced NF- $\kappa$ B activation and AR suppression. **A**, Western blotting evaluating total and phosphorylated IkB $\alpha$  levels in TRADD knockdown LNCaP cells in response to 20 ng/mL TNF- $\alpha$  treatment for 1 h. Relative band density was indicated above the bands. **B**, NF- $\kappa$ B-luc reporter assay on TRADD knockdown LNCaP cells in response to 10 ng/mL TNF- $\alpha$  treatment for 24 h. *Columns*, mean values from samples in triplicate; *bars*, SD. **C**, Western blotting evaluating indicated protein expression after knocking down TRADD by siRNA in LNCaP cells. **D**, Western blotting evaluating nuclear AR levels after the indicated treatments in LNCaP cells. Lamin A/C serves as a control for nuclear protein and tubulin serves as a control for cytoplasmic protein.

To determine whether TRADD expression is affected by androgen deprivation *in vivo*, we evaluated TRADD expression in prostate tissue samples from a neoadjuvant clinical trial of dutasteride. As expected, following 4 months of dutasteride treatment, tissue dihydrotestosterone levels were significantly lower than that found in untreated prostates, with concomitant increases in tissue testosterone. However, TRADD transcript levels were not significantly different between prostate epithelial cells acquired from untreated and dutasteride-treated prostate tissue samples (Supplementary Fig. S3). As documented previously, although dutasteride lowers dihydrotestosterone levels, there are concomitant higher levels of testosterone. Thus, the lack of a dutasteride effect on TRADD *in vivo* might be due to high levels of testosterone. We next measured TRADD expression in radical prostatectomy samples resected from men treated with standard

androgen deprivation therapy with the LHRH agonist luprolide and the antiandrogen bicalutamide, or with high doses of E2. Compared with transcript levels measured in benign untreated prostate epithelium, TRADD transcript levels in both benign untreated prostate epithelium, TRADD transcript levels in both benign untreated prostate epithelium ( $P < 0.001$ ) and neoplastic epithelium ( $P < 0.04$ ) treated with luprolide + bicalutamide or E2 were significantly lower than TRADD levels in untreated benign epithelium (Fig. 6B), confirming a critical role of androgen on TRADD expression. Interestingly, we also observed that in either the luprolide + bicalutamide or the E2 group, levels of TRADD transcripts in benign epithelium was lower than that in neoplastic epithelium (Fig. 6B), suggesting that TRADD is differentially expressed between normal and cancerous prostate tissues.

**Aberrant TRADD expression is associated with prostate cancer progression.** To further assess the expression of TRADD



**Figure 5.** Ectopic expression of TRADD restores the TNF- $\alpha$ -induced AR suppression in AI cells. *A*, C4-2B cells transfected with pcDNA3.1, pcDNA-wt-TRADD, or pcDNA-TRADD $\Delta$ 301 were subjected to NF- $\kappa$ B-luc luciferase reporter assay after 10 ng/mL TNF- $\alpha$  treatment for 24 h. Columns, mean values from samples in triplicate; bars, SD. *B*, Western blotting evaluating AR and PSA levels in C4-2B cells overexpressed wt-TRADD or TRADD $\Delta$ 301 after 10 ng/mL TNF- $\alpha$  treatment for 24 h. *C*, cell viability was evaluated by the MTS assay in C4-2B cells transfected with indicated constructs after 50 ng/mL TNF- $\alpha$  treatment for 96 h. Columns, mean values from samples in triplicate; bars, SD.

in human prostate cancers, we used laser capture microdissection to acquire benign and cancerous prostate epithelial cells from 14 individuals with localized prostate adenocarcinoma. We quantitated TRADD transcripts by qRT-PCR using the RPL13 mRNA as a reference control. Compared with benign epithelium, the expression of TRADD was significantly lower in cancerous epithelium ( $P = 0.0138$ ; Fig. 6C).

Data from LNCaP cell line model suggested that TRADD expression might be altered as prostate cancers progress. To test whether

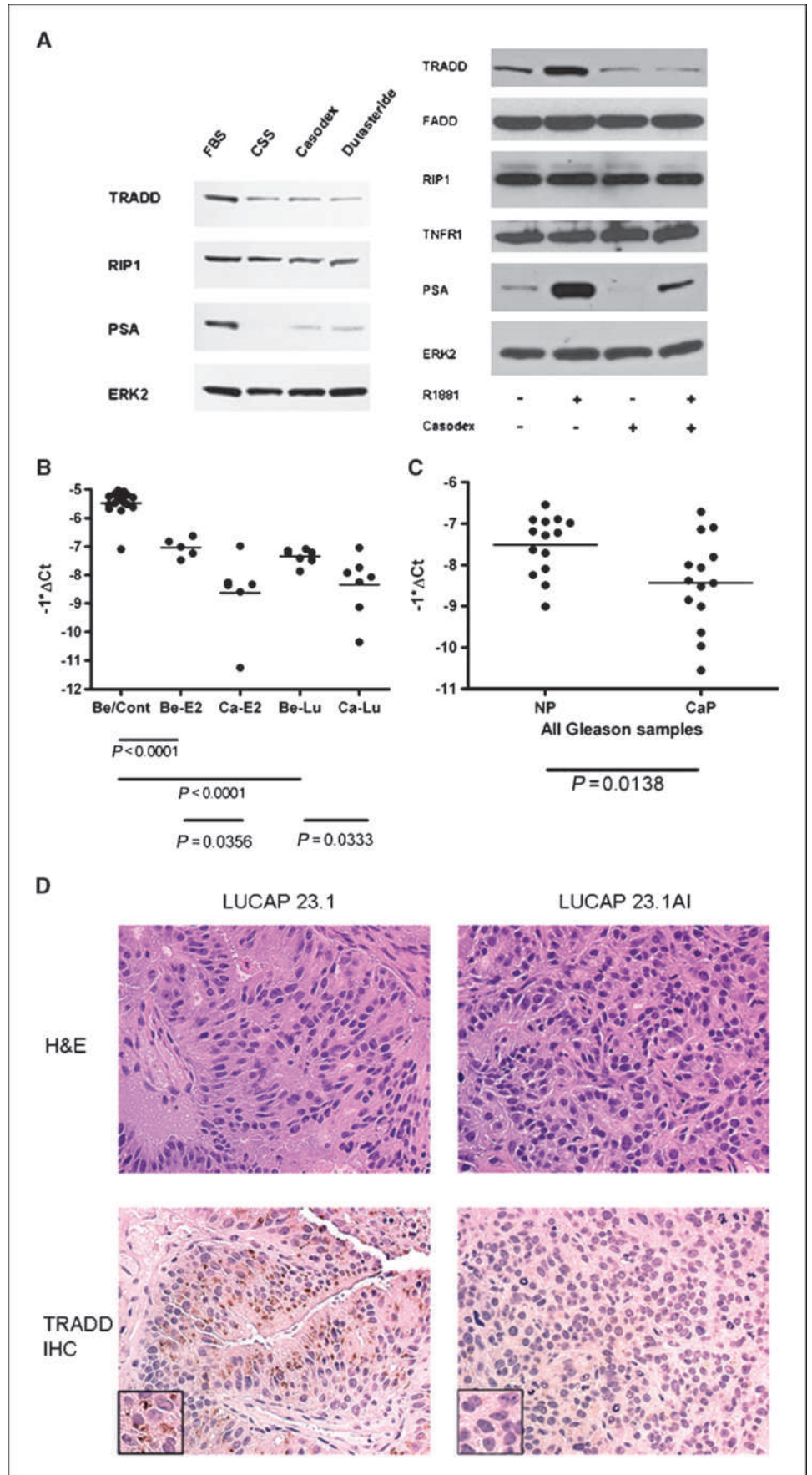
TRADD expression is decreased during prostate cancer progression *in vivo*, we used the LuCaP 23.1 and LuCaP 35 prostate cancer xenograft models. LuCaP 23.1 and LuCaP 35 tumors grow in intact immunocompromised mice and serve as a model for prostate cancer progression (32, 33). Both original LuCaP 23.1 and LuCaP 35 tumors regressed after castration but eventually recurred and became AI tumors. TRADD immunohistochemistry staining show that the original LuCaP 23.1 and LuCaP 35 tumors (AD tumors) express relatively high levels of TRADD, but castration-recurrent LuCaP 23.1 AI and LuCaP 35 AI tumors express very little TRADD (data not shown; Fig. 6D). Thus, both cell line and xenograft models strongly suggest that TRADD expression might be reduced as prostate cancer progresses.

## Discussion

It is well established that both androgen/AR and TNF- $\alpha$ /NF- $\kappa$ B signaling pathways are important for prostate cancer growth. The mutual inhibition between these two pathways has been documented (34–36). Consistent with these studies, we showed that TNF- $\alpha$  induces AR suppression in prostate cancer cells through NF- $\kappa$ B, which may mediate TNF- $\alpha$ -induced reduction in cell viability. Unlike in many other cell types, activation of NF- $\kappa$ B by TNF- $\alpha$  in prostate cells does not promote cell proliferation. Conversely, it has a proapoptotic effect (37). The mechanisms underlying this phenomenon are largely unknown. One potential mechanism is that NF- $\kappa$ B induces DR4 expression, which promotes TNF-related apoptosis inducing ligand (TRAIL)-induced cell death in prostate cancer cells (38). Our data suggest that TNF- $\alpha$ -induced AR suppression may be another important underlying mechanism.

In contrast to our finding, some other studies suggested that AR and NF- $\kappa$ B might enhance each other's activity (39, 40). In a recent report, Jin and colleagues (41) observed that constitutive activation of NF- $\kappa$ B in prostate cancer xenografts (in which I $\kappa$ B $\alpha$  was knocked down) is correlated with increased AR expression, and their data implied that NF- $\kappa$ B RelA increases AR expression and contributes to prostate cancer progression. Chen and Sawyers (42) identified four NF- $\kappa$ B binding sites in the PSA core enhancer and showed that these sites are directly bound by NF- $\kappa$ B and activate PSA expression in the absence of androgen, suggesting that constitutively active NF- $\kappa$ B might contribute to prostate cancer progression. However, Cinar and colleagues (43) soon reported a fifth NF- $\kappa$ B binding site in the core of the PSA promoter, which is suppressive to AR transcriptional activity. Binding of p65, but not p50, to this site represses, instead of activates, AR-mediated PSA transcription. Collectively, the precise effect of TNF- $\alpha$ /NF- $\kappa$ B on AR activity still remains unclear and it is likely dependent on the specific cellular milieu.

The correlation between androgen deprivation resistance and TNF- $\alpha$  resistance in prostate cells has been documented (11, 12). Ko and colleagues (11) reported that increased B-Myb (a NF- $\kappa$ B coregulator) expression in AI cells affects the inhibitory effect of NF- $\kappa$ B on the AR promoter, providing a potential mechanism for TNF- $\alpha$  resistance in AI prostate cancer cells. In our study, we found an association between reduced TRADD expression and TNF- $\alpha$  resistance in prostate cancer cell lines. We also showed that TRADD plays an important role in mediating TNF- $\alpha$ -inducing AR suppression and that loss of TRADD expression is associated with TNF- $\alpha$  resistance. Finally, we provided evidence that androgen deprivation leads to a reduction of TRADD both *in vivo* and *in vitro*. Considering the critical role of TRADD in



**Figure 6.** Androgen deprivation reduces TRADD expression *in vitro* and *in vivo*. **A**, Western blotting evaluating TRADD expression in LNCaP cells. *Left*, cells were cultured in CSS medium, treated with 10 μmol/L bicalutamide or 1 μmol/L dutasteride for 72 h as indicated; *right*, cells were grown in CSS medium and treated with or without R1881 and bicalutamide for 24 h. ERK2 serves as a loading control. **B**, qRT-PCR quantitates TRADD mRNA levels in benign (*Be*) and cancerous (*Ca*) epithelium from radical prostatectomy samples resected from men treated with leuprolide (*LU*) or E2. RPL13 mRNA was used as a reference control. **C**, TRADD mRNA levels in benign and cancerous prostate epithelial cells were evaluated by qRT-PCR. RPL13 mRNA was used as a reference control. **D**, immunohistochemistry (IHC) staining for TRADD (*bottom*) was performed in both LUCAP 23.1 AD (*left*) and LUCAP 23.1 AI (*right*) tumors along with H&E staining (*top*). Images were captured through a Zeiss Axioplan 2 light microscope (Carl Zeiss) under x40 objective magnification by using a Zeiss Axiocam. Close-up images are shown at the bottom-left corners.

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TNF- $\alpha$ -mediated signaling, we speculate that androgen deprivation therapy contributes to the development of TNF- $\alpha$  resistance during prostate cancer progression. Therefore, our findings provide a potential mechanism by which prostate cancer develops TNF- $\alpha$  resistance after androgen deprivation therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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