Interleukin-17 Receptor-Like Gene Is a Novel Antiapoptotic Gene Highly Expressed in Androgen-Independent Prostate Cancer

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Abstract
We have recently identified a new gene, interleukin-17 receptor-like (IL-17RL), which is expressed in normal prostate and prostate cancer. This investigation is focused on the role of IL-17RL in prostate cancer. We found that IL-17RL was expressed at significantly higher levels in several androgen-independent prostate cancer cell lines (PC3, DU145, cdc1, cdc2, and cdc3) and tumors compared with the androgen-dependent cell lines (LNCaP and MLC-SV40) and tumors. In an in vivo model of human prostate tumor growth in nude mice (CWR22 xenograft model), IL-17RL expression in tumors was induced by androgen deprivation. The relapsed androgen-independent tumors expressed higher levels of IL-17RL compared with the androgen-dependent tumors. Overexpression of IL-17RL in tumor necrosis factor α (TNFα)–sensitive LNCaP cells inhibited TNFα-induced apoptosis by blocking activation of caspase-3 downstream to caspase-2 and caspase-8. Reciprocally, knocking down IL-17RL expression by small interfering RNA induced apoptosis in all the prostate cancer cell lines studied. Taken together, these results show that IL-17RL is a novel antiapoptotic gene, which may confer partially the property of androgen-independent growth of prostate cancer by promoting cell survival. Thus, IL-17RL is a potential therapeutic target in the treatment of prostate cancer. (Cancer Res 2006; 66(1): 175-83)

Introduction
Prostate cancers are initially androgen dependent but progressively become androgen independent, and the patients finally succumb to widespread metastases especially to bone (1, 2). The molecular mechanisms underlying the progression from the androgen-dependent (hormone sensitive) to the androgen-independent (hormone refractory) status of prostate cancer are increasingly being defined. The potential mechanisms involve mutations/amplifications of androgen receptor (AR) or its signaling pathways, neuroendocrine differentiation, and alterations of apoptosis-related genes (3–5). Up-regulation of antiapoptotic genes [e.g., Bcl-2, clusterin, and phosphatidylinositol 3-kinase (PI3K)/Akt] and inactivation of proapoptotic genes (e.g., p53) have been found to play an important role in the androgen-independent growth of prostate cancer (6–10). The results of this investigation show yet another novel mechanism of prostate cancer progression [i.e., inhibition of apoptosis by the gene encoding interleukin-17 receptor-like (IL-17RL) protein].

The IL-17 cytokine family has six members (i.e., IL-17A or IL-17, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F; refs. 11, 12). IL-17R is the receptor for IL-17A. Together with the other four homologous receptor-like molecules [i.e., IL-17Rh1 or IL-17BR, IL-17RL or IL-17RC, IL-17RD or similar expression to fng genes (Sef), and IL-17RE], IL-17R has formed a new receptor family that differs from the other cytokine receptor families. IL-17Rh1 binds to IL-17B but binds to IL-17E with higher affinity (13, 14). IL-17RD (or Sef) binds to fibroblast growth factor (FGF) receptors and inhibits the FGF receptor–mediated extracellular signal-regulated kinase (ERK) pathway (15, 16). The receptors for IL-17C, IL-17D, and IL-17F cytokines have not been identified nor the ligands for the receptor-like molecules IL-17RL, IL-17RD, and IL-17RE. In general, the IL-17 family members are involved in proinflammatory functions (17). However, depending on the tumor models used, IL-17A has been found to either promote (18, 19) or inhibit (20, 21) tumor growth. IL-17A expression is increased in 79% of benign prostate hyperplasia and 58% of prostate cancer specimens (22). Recently, IL-17Rh1 (or IL-17BR) has been found to be overexpressed in tamoxifen-treated nonrecurrent breast cancer cases, which may be used as a biomarker together with the HOXB13 gene (23).

The IL-17RL protein has 22% identity to IL-17R and is expressed in prostate cancer (24). The full-length IL-17RL protein is predicted as a type I transmembrane protein, although some of the 11 RNA splice variants may be secreted proteins due to the lack of transmembrane and intracellular domains. IL-17RL mRNAs have also been detected in kidney, cartilage, liver, heart, and skeletal muscle. During a systematic study to determine the function of IL-17RL, we unexpectedly found that overexpression of IL-17RL in 293 (human embryonic kidney) cells inhibited tumor necrosis factor α (TNFα)–induced apoptosis. This unexpected discovery led to a systematic investigation of the antiapoptotic function of IL-17RL and its role in the androgen-independent growth of prostate cancer.

Materials and Methods
Antibodies and reagents. Rabbit anti-IL-17RL cytoplasmic domain antibodies were from our lab (24). Anti-V5 antibodies were from Invitrogen (Carlsbad, CA). Anti-GAPDH antibodies were from Chemicon (Temecula, CA). Antibodies to Myc, TRAF2, caspase-2, and matrix-precoated six-well plates were from BD Biosciences (San Diego, CA). Antibodies to c-IAP1/2, pERK (pERK1/2), caspase-10, Bcl-2, and Bcl-XS/L were from Santa Cruz (Carlsbad, CA). Anti-GAPDH antibodies were from Chemicon (Temecula, CA). Antibodies to c-IAP1/2, pERK (pERK1/2), caspase-10, Bcl-2, and Bcl-XS/L were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to caspase-3, caspase-8, and Fas were from Upstate (Lake Placid, NY). TNFα, IL-17A, IL-17B-Fc, and TNFα–induced apoptosis

Note: The authors have no conflict of interest.

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

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antibodies to Bid were from R&D Systems, Inc. (Minneapolis, MN). IL-17A and IL-17F were from BioLegend (San Diego, CA). The rest of antibodies were purchased from Cell Signaling Technology (Beverly, MA). The Fc-tagged human recombinant IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IgG were from R&D Systems, Inc. (Minneapolis, CA). IL-17F was from Leinco Technologies (St. Louis, MO). The rest of chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell culture.** LNCaP, PC3, DU145, and 293 cells were from the American Type Culture Collection (Manassas, VA). LNCaP cells and the derivative LNCaP-C and LNCaP-BL cells were maintained in T-medium (custom formula 02-0056) with 5% fetal bovine serum (FBS). The androgen-independent LNCaP sublines cd1, cd2, and cd3 (25) were maintained in phenol red-free RPMI 1640 with 5% charcoal/dextran-treated FBS (HyClone, Logan, UT). PC3 cells were maintained in Ham's F12K medium with 10% FBS. DU145 cells were maintained in Earle's MEM with 10% FBS. 293 cells were maintained in DMEM with 10% FBS. MLC-SV40 (an immortalized human prostatic epithelial cell line, androgen dependent) cells (26) were maintained in PrEGM medium (Cambrex, Walkersville, MD). Medium and supplements were from Invitrogen, unless noted otherwise. The cells were cultured in a 37°C, 5% CO2 humidified incubator.

**Generation of plasmid constructs.** Full-length human IL-17R and IL-17Rh1 cDNAs were subcloned into pcDNA3/V5-His vector (Invitrogen) by PCR. The V5-His-tagged cDNAs were subcloned into pLNCX2 retrovirus vector (Clontech, Palo Alto, CA) by PCR and generated pLNCX2-IL-17R/V5-His and pLNCX2-IL-17Rh1/V5-His constructs. The insert sequences of all the constructs were confirmed by DNA sequencing (Davis Sequencing, Inc., Davis, CA). Detailed maps and sequences are available upon request.

**Establishment of stable cell lines.** LNCaP or 293 cells were transfected with pLNCX2-IL-17R-V5-His, pLNCX2-IL-17Rh1-V5-His, or pLNCX2 vectors by LipofectAMINE (Invitrogen)–mediated transfection. For transient expression, the cells were harvested 48 hours after transfection. For establishing stable cell lines, the transfected cells were selected with 0.6 mg/mL neomycin for 1 week. The resistant clones were pooled and confirmed by Western blot.

**Coimmunoprecipitation, pull-down assay, Western blot, quantitative reverse transcription-PCR, and immunohistochemistry.** Cell lysates from 293 cells overexpressing IL-17R or IL-17Rh1 were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer. Coimmunoprecipitation of IL-17R with IL-17F was done essentially as described (27), except that anti-IL-17F antibodies were used for immunoprecipitation and anti-V5 antibodies were used for Western blot. Pull-down assay was done by incubating the Fc-tagged IL-17 cytokines with the cell lysates and protein G-Sepharose beads. The rest of the Western blots were done as described (28). RNA isolation and quantitative reverse transcription-PCR (RT-PCR) were done as described (27). Human IL-17R primer sequences were 5'-CAGTGTCGCCACCGCTTT (forward) and 5'-GCTTTCAGCAATGG-GAAGTC (reverse). Mouse IL-17R primers and both human and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were from Applied Biosystems (Foster City, CA). CΔCt (cycle threshold) = C0, of IL-17R − C, of GAPDH. ΔΔCt = ΔCt of study group − ΔCt of control group. IL-17R mRNA fold change was calculated as 2ΔΔCt. The collection of the human bone metastatic prostate tumor tissues was approved by University of California Davis Institutional Review Board (29). Immunohistochemical staining of IL-17R was done on paraffin-embedded human prostate tumor sections as described (27), using rabbit anti-IL-17R antibodies.

**IL-17R oligomerization assay.** Full-length IL-17R with V5-His tag (IL-17R-V5) or with Myc tag (Myc-IL-17R) was transfected alone or cotransfected into 293 cells by LipofectAMINE-mediated transfection. Whole cell lysate was prepared in RIPA lysis buffer. Equal amounts of protein were precipitated with 1 μg of either anti-V5 or anti-Myc antibodies, together with Protein G-agarose beads for immunoprecipitation. Western blots were done using anti-Myc or anti-V5 antibodies, respectively. Whole-cell lysate was probed for the expression of individually tagged IL-17R.

**Cell viability, DNA fragmentation, and cell growth curve.** Cells were treated with 20 μM of TNFα unless noted otherwise for the indicated time. In some experiments, TNFα was added 30 minutes after addition of 20 μM/L. IL-294002, or 16 hours after transfection with small interfering RNA (siRNA) or C-siRNA. Digital images were captured by light microscopy at ×100 magnification. Cell viability was determined by the trypan blue exclusion assay, in which cell survival was calculated as (the living cell number of treated group ÷ the living cell number of untreated control group) × 100. The DNA fragmentation assay was done as described (28).

**Cell attachment assay.** As modified from (30), 2.5 × 104 per well of LNCaP-C or LNCaP-BL cells in 2-mL serum-free T-medium were plated in six-well plates in triplicate. The regular Costar plate was used as control, collagen type I–, collagen type IV–, fibronectin–, and laminin-precoated plates were used to test the cell attachment to these extracellular matrices. After 90-minute incubation at 37°C, the cells not attached were washed off with PBS. The attached cells were fixed with 5% glutaraldehyde for 30 minutes at room temperature. After three washes with PBS, the attached cells were stained with 0.1% crystal violet dye for 30 minutes. After five washes with water, the stain was dissolved in 10% acetic acid and read at 570 nm on a microplate reader. The crystal violet dye stains the cell protein that is proportional to the number of cells. The number of cells attached to the matrices was calculated as percentage of cells attached to the Costar plate.

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**Tumor xenografts.** The CWR22 model of human prostate tumors and relapsed tumors were generated in nude mice as described (31, 32). For the time course, tumors were harvested from intact mice (day 0) and from mice 3, 7, and 14 days after surgical castration. The animal experiment was approved by the University of California Davis Institutional Animal Care and Use Committee, according to NIH guidelines.

**Synthesis of siRNA and transfection.** Both IL-17R–specific siRNA and scrambled control C-siRNA were designed by Invitrogen's Block-iT RNAi Designer and synthesized with Invitrogen's proprietary Stealth technology. Their sequences were siRNA 5'-UCGGCCUUUGAGAUCUUA-CUACAA (sense) and C-siRNA 5'-UCGGGUUAGAGUCAACAUUCCCA (sense). Fifty percent confluent cells were transfected with the mixes of siRNA/LipofectAMINE 2000 (Invitrogen), C-siRNA/LipofectAMINE 2000, or LipofectAMINE 2000 only as control, according to the manufacturer's protocol. The final concentrations used were 100 nmol/L siRNA or C-siRNA and 5 μL/mL LipofectAMINE 2000.

**Statistical analysis.** Kruskal-Wallis midrank statistical test was used to compare the IL-17RL staining on human prostate tumors. Student's t test was used to analyze the remaining data.

**Results.** IL-17RL is a prosurvival molecule in 293 cells. Our initial approaches to identify a ligand for IL-17RL were not successful (Supplementary Fig. IA and B). We next overexpressed IL-17RL in 293 cells, expecting that overexpression of a “receptor” might cause autoactivation and initiate similar signaling pathways as those activated by IL-17/IL-17R (11, 12). We established a 293 cell line stably overexpressing IL-17RL (named 293RL) and a control cell line transfected with empty vector only (named 293C). Overexpression of IL-17RL in 293 cells did not activate nuclear factor-κB (NF-κB), ERK, c-Jun NH2-terminal kinase, p38, signal transducer and activator of transcription, protein kinase C (PKC), Src, or glycogen synthase kinase 3 β pathways, as shown by the absence of increase in the phosphorylated forms of the key proteins (Supplementary Fig. LB and C).

As TNFα is a strong activator of NF-κB pathway, it was used as a positive control for activation of NF-κB. We were surprised to find that 293RL cells were more resistant to the cell death induced by TNFα (Fig. 1A). TNFα killed 293C cells in a dose- and time-dependent manner, whereas overexpression of IL-17RL inhibited...
14% bone metastatic prostate tumor samples were collected.

Figure 1. Overexpression of IL-17RL in 293 cells inhibited TNFα-induced apoptosis. A, 293RL cells were 293 cells stably overexpressing IL-17RL, whereas 293C cells were control cells transfected with empty vector only. Cells were treated with or without 100 ng/mL TNFα for 3 days. The dead cells were counted by trypan blue exclusion assay. Cell survival was calculated as (the living cell number of untreated control group × 100). Column, mean of triplicate (same for all); bars, SD. P < 0.05 between the comparison groups. All in vitro experiments were repeated at least three times. Reproducible representative results. C, time course experiments in stable cell lines treated with 100 ng/mL TNFα. P < 0.05 between the comparison groups. D, 293 cells transiently overexpressing IL-17RL (IL-17RL group) or control 293 cells (control group) were treated with or without 100 ng/mL of TNFα for 2 days. P < 0.05 between the comparison groups.

IL-17RL is expressed at higher levels in androgen-independent prostate cancer cell lines. The LNCaP cell line is androgen dependent and TNFα sensitive. On the other hand, the PC3 cell line is androgen independent and TNFα resistant (33). Three androgen independent sublines of LNCaP (i.e., cdis1, cdis2, and cdis3) were generated recently (25). These three sublines of LNCaP were also resistant to TNFα, similar to PC3 (Fig. 2A). Because we previously showed that IL-17RL was expressed in prostate cancer and IL-17RL is a prosurvival molecule in 293 cells, we hypothesized that the increased expression of IL-17RL might be responsible for the survival advantage of the androgen-independent cell lines. As expected, we found that IL-17RL was expressed at significantly higher levels in androgen-independent prostate cancer cell lines than in androgen-dependent MLC-SV40 (26) and LNCaP cell lines at the mRNA level (Fig. 2B and C). As detected by quantitative real-time RT-PCR, the androgen-independent PC3, DU145, cdis1, cdis2, and cdis3 cells expressed 48-, 12-, 10-, 5-, and 2 fold, respectively, more IL-17RL mRNA than LNCaP cells. Consistently, IL-17RL protein expression was higher in the androgen-independent cells than in the androgen-dependent cells, as shown by Western blot (Fig. 2D).

IL-17RL is expressed at higher levels in androgen-independent prostate tumors. We next examined if IL-17RL expression is increased in androgen-independent human prostate tumors. Fourteen bone metastatic prostate tumor samples were collected either before androgen ablation (samples considered androgen dependent, n = 6) or at the time of relapse after androgen ablation (samples considered androgen independent, n = 8), as described previously (29). By immunohistochemical staining with rabbit anti-IL-17RL antibodies, we found that all six androgen-dependent samples were stained negatively or weakly. In contrast, two out of eight androgen-independent samples were stained moderately or strongly, whereas the remaining six samples were stained weakly (Fig. 3A). However, due to the small sample size, there was no statistical significance by Kruskal-Wallis midrank statistical test (P = 0.0519). As a complementary experimental approach, we used the well-established CWR22 androgen-dependent human prostate cancer xenograft mouse model (31, 32, 34, 35). We found that androgen-independent CWR22 tumors (relapsed tumors after castration) expressed higher levels of IL-17RL mRNA (ΔCt range, −7.9 to −4.3; average, −6.4; n = 4) compared with androgen-dependent CWR22 tumors from the intact mice (ΔCt range, −9.0 to −8.2; average, −8.6; n = 3; Fig. 3B). This indicated that the androgen-independent tumors on the average expressed 4.6-fold more IL-17RL than the androgen-dependent tumors, which is statistically significant (Student’s t test, P < 0.05). In addition, IL-17RL expression was induced in the CWR22 tumors upon castration, as 3, 7, and 14 days after castration, IL-17RL mRNA increased 3.3-, 4.7-, and 10.2 fold, respectively, compared with the tumors collected from the intact mice (Fig. 3C). As an internal control, the mouse IL-17RL expression in the stromal cells of mouse origin that infiltrated into the s.c. xenografted human tumors did not change significantly (Fig. 3C).

IL-17RL partially confers TNFα resistance in prostate cancer cells. To test if the increased IL-17RL in the androgen-independent cells confers TNFα resistance in prostate cancer cell lines, we established three independently pooled clones of LNCaP cells stably expressing IL-17RL (named LNCaP-RL) and control cells transfected with empty vector only (named LNCaP-C; Fig. 4A). Overexpression of IL-17RL in LNCaP cells inhibited about 20% of cell death induced by TNFα (Fig. 4B and C). Similar inhibition of cell death was observed in 293 cells transiently expressing IL-17RL (Fig. 1D), which ruled out the possibility that the survival advantage was due to clonal selection during the establishment of the stable cell lines. Taken together, these data implied that IL-17RL is a prosurvival molecule in 293 cells.
alone inhibited 50% of the cell death induced by 20 ng/mL of TNFα (Fig. 4C). Of note, in the absence of LY294002, the LNCaP cells were killed by TNFα at a slow rate. Significant cell death was observed 3 days after the treatment (Fig. 4B). In contrast, dramatic cell death was induced 24 hours after TNFα treatment in the presence of LY294002 (Fig. 4C). However, cell death was inhibited by over-expression of IL-17RL. These results were consistent among all three sets of independently pooled LNCaP-C and LNCaP-RL clones, which indicated that clonal selection of survival advantage was unlikely.

To address the potential bias that might arise from the overexpression model, we used siRNA (37) to knock down the endogenous and exogenous expression of IL-17RL in the cell lines. As a control, the same nucleotides were scrambled in sequence (C-siRNA). Both siRNA and C-siRNA were designed by Invitrogen’s Block-iT RNAi Designer and synthesized with Invitrogen’s proprietary Stealth technology to maximally avoid off-target and nonspecific IFN response. IL-17RL–specific siRNA specifically knocked down the expression of exogenous and endogenous IL-17RL in 293C, 293RL, LNCaP-C, LNCaP-RL, cds1, cds2, cds3, PC3, and DU145 cells (Fig. 5B; data not shown). Diminution of IL-17RL expression led to apoptosis of cells, as shown morphologically by microscopy (Fig. 5A) and biochemically by cleavage of poly(ADP-ribose) polymerase (PARP; Fig. 5B). Twenty-four hours after transfection, IL-17RL–specific siRNA killed about 30% of both the LNCaP-C and LNCaP-RL cells, whereas the scrambled control siRNA killed <5% of the cells possibly due to nonspecific stress response (Fig. 4D). Furthermore, addition of 20 ng/mL TNFα to the IL-17RL–specific siRNA-transfected cells killed 66% of LNCaP-C cells and 58% of LNCaP-RL cells, whereas TNFα alone killed only 15% of LNCaP-C cells and 3% of LNCaP-RL cells within 24 hours (Fig. 4D). The synergistic cell killing effects of siRNA and TNFα indicated that both the endogenous and overexpressed IL-17RL have antiapoptotic functions. Similar results were obtained in 293C and 293RL cells, as the combination of siRNA and TNFα induced dramatically more DNA fragmentation (shown as DNA ladder) compared with either treatment alone (Fig. 5C; similar results were obtained in LNCaP-C and LNCaP-RL cells). Taken together, these data suggested that IL-17RL is a bona fide antiapoptotic gene that provides a cell survival signal to 293 cells and prostate cancer cells.

IL-17RL inhibits apoptosis by blocking activation of caspase-3 downstream to caspase-2 and caspase-8. To examine further the mechanism of how IL-17RL inhibits TNFα-induced apoptosis, we treated LNCaP-C and LNCaP-RL cells with 20 ng/mL TNFα, with or without 20 μmol/L LY294002, for up to 48 hours. We found that two initiator caspases (caspase-2 and caspase-8) were activated similarly in LNCaP-C and LNCaP-RL cells, as shown by the cleavage of their proenzymes upon TNFα treatment (Fig. 6A). Addition of LY294002 increased the cleavage of procaspase-2 and procaspase-8, particularly at 24 to 48 hours after treatment. The third initiator caspase examined (caspase-10) was not activated in both cells under either treatment (Fig. 6A).

Figure 2. IL-17RL expression was increased in the androgen-independent and TNFα-resistant prostate cancer cell lines. LNCaP (a human prostate cancer cell line) and MLC-SV40 (an immortalized human prostatic epithelial cell line) cells are androgen dependent. Cds1, cds2, and cds3 are three androgen-independent cell lines derived from LNCaP cells. PC3 and DU145 cell lines are androgen-independent human prostate cancer cell lines. A, androgen-independent prostate cancer cells were resistant to TNFα-induced apoptosis. Cells were treated with 20 ng/mL TNFα for 3 days. Bar, 60 μm. B and C, IL-17RL mRNA levels detected by quantitative RT-PCR in the cells under normal culture conditions. IL-17RL mRNA fold change was calculated as $2^\Delta\Delta C_t$, where $\Delta C_t$ (cycle threshold) = $C_t$ of IL-17RL – $C_t$ of GAPDH. $\Delta\Delta C_t$ = $C_t$ of cancer cell line – $C_t$ of the MLC-SV40 cell line. D, IL-17RL protein levels detected by Western blot in the cells under normal culture conditions.
Caspase-3 is the executioner caspase downstream to caspase-2 and caspase-8 (38). In LNCaP-C cells, procaspase-3 was cleaved (thereby activated) by TNFα with or without LY294002 (Fig. 6A). This consequently led to apoptosis, which was shown by cleavage of PARP, with increased PARP cleavage upon addition of LY294002 (Fig. 6A). However, in LNCaP-RL cells, the cleavage of procaspase-3 and PARP was blocked when treated with TNFα alone or dramatically diminished with addition of LY294002 (Fig. 6A). The other executioner caspasas, such as caspase-7 and caspase-6, were not activated under current treatments (Fig. 6A; data not shown).

Bid has been reported to be activated upon caspase-2 or caspase-8 activation, which subsequently activates caspase-9 via inducing cytochrome c release from mitochondria (so-called intrinsic cell death pathway; refs. 39, 40). In both LNCaP-C and LNCaP-RL cells, Bid and caspase-9 were not activated under current treatments (Fig. 6A; data not shown), which implied that only the extrinsic cell death pathway was involved in our cell models. Of note, the cytomegalovirus promoter activity of the pLNCX2 vector carrying IL-17RL was inducible by TNFα and other stress conditions (data not shown), which led to the increase of IL-17RL expression upon TNFα treatment (Fig. 6A). In addition, we found that overexpression of IL-17RL did not change the expression of several proapoptotic or antiapoptotic genes, such as Fas, c-IAP1/2, TRAF2, Bcl-2, and Bcl-XL, either with or without dihydrotestosterone-one treatment (data not shown). These genes regulate apoptosis either above caspase-2/caspase-8, at mitochondria, or caspase-3 levels (39, 41). Furthermore, we found that overexpression of IL-17RL did not stimulate the cellular proliferation of either 293 cells or LNCaP cells (data not shown), which ruled out the possibility that the observed survival advantage of the cells overexpressing IL-17RL was due to enhanced cellular proliferation. Taken together, our data indicated that IL-17RL inhibits apoptosis by blocking activation of caspase-3 downstream to caspase-2 and caspase-8.

Overexpressed IL-17RL oligomerizes to induce gene expression that enhances cell attachment to the extracellular matrix. To further delineate the mechanism of how IL-17RL blocks caspase-3 activation, we tested if IL-17RL physically binds
to caspase-3. By coimmunoprecipitation assay, we found that IL-17RL did not bind to caspase-3 (data not shown). This indicates that IL-17RL might activate certain intracellular signaling pathways that are not the common pathways as we already tested (Supplementary Fig. 1C). By cotransfection of V5-His-tagged and Myc-tagged IL-17RL in 293 cells, we found that IL-17RL protein bound to its self as detected by coimmunoprecipitation assay (Fig. 6B). When the overexpressed IL-17RL was run on a nonreducing SDS-PAGE gel, a 203-kDa complex was detected by Western blot, which was not seen under reducing conditions (either with 25 mmol/L DTT or 7.5% β-mercaptoethanol; Supplementary Fig. 1D). These data indicate that IL-17RL may form a homotrimer that is bound together by disulfide bonds among the monomers. To identify the genes that are regulated by IL-17RL overexpression and mediate IL-17RL’s antiapoptotic function in LNCaP cells, we did gene microarray analysis on LNCaP-C and LNCaP-RL cells. We found that 74 genes were up-regulated, whereas 38 genes were down-regulated (Supplementary Table 1). Of particular interest was that integrin β5 and z-parvin genes were both up-regulated. As these two genes are involved in cell attachment to the extracellular matrix, we tested if IL-17RL overexpression could enhance cell attachment to the extracellular matrix. We found that there were 15%, 24%, and 26% more cells attached to the collagen type I, collagen type IV, and fibronectin matrices, respectively, in LNCaP-RL cells than in LNCaP-C cells (Fig. 6C). Meanwhile, there was no difference between the attachment of LNCaP-C and LNCaP-RL cells to laminin, to which few of both cells attached (Fig. 6C).

**Discussion**

The identification of IL-17RL as a novel antiapoptotic gene is an unexpected finding. First, the androgen-independent prostate cancer cell lines expressing high levels of IL-17RL were more resistant to TNFα-induced apoptosis than the prostate cancer cell line expressing low levels of IL-17RL. Second, overexpression of IL-17RL inhibited TNFα-induced apoptosis in both 293 cells and TNFα-sensitive LNCaP cells. Third, knocking down the expression of IL-17RL by IL-17RL–specific siRNA induced apoptosis in 293 cells and seven prostate cancer cell lines studied. Finally, TNFα killed more cells when IL-17RL expression was diminished by siRNA. We must point out that the antiapoptotic IL-17RL may not be the only cell survival signal in our models, as other antiapoptotic signals are also present, such as Bcl-2 and PI3K/Akt (6, 36, 42). The presence of these additional antiapoptotic signals may mask the effects of one particular antiapoptotic signal. This explains why overexpression of IL-17RL protected only 20% of cell death in the presence of other antiapoptotic signals, whereas it protected 50% of cell death in the absence of PI3K/Akt signal. These findings also highlight the necessity to block several or all survival signals in the design of new strategies to treat prostate cancer.

The TNFα-induced apoptosis signaling pathway has been well defined and is one of the paradigms widely used to identify novel antiapoptotic genes or mechanisms (38–40, 43, 44). To determine the mechanism underlying the inhibition of apoptosis by IL-17RL, we have systematically examined the apoptosis signaling pathways. We identified that IL-17RL did not inhibit the activation of caspase-2 overexpression of IL-17RL protected only 20% of cell death in the presence of other antiapoptotic signals, whereas it protected 50% of cell death in the absence of PI3K/Akt signal. These findings also highlight the necessity to block several or all survival signals in the design of new strategies to treat prostate cancer.

The TNFα-induced apoptosis signaling pathway has been well defined and is one of the paradigms widely used to identify novel antiapoptotic genes or mechanisms (38–40, 43, 44). To determine the mechanism underlying the inhibition of apoptosis by IL-17RL, we have systematically examined the apoptosis signaling pathways. We identified that IL-17RL did not inhibit the activation of caspase-2 but dramatically blocked the activation of caspase-3. By coimmunoprecipitation assay, we found that IL-17RL did not bind to caspase-3 (data not shown). This indicates that IL-17RL might activate certain intracellular signaling pathways that are not the common pathways as we already tested (Supplementary Fig. 1C). By cotransfection of V5-His-tagged and Myc-tagged IL-17RL in 293 cells, we found that IL-17RL protein bound to its self as detected by coimmunoprecipitation assay (Fig. 6B). When the overexpressed IL-17RL was run on a nonreducing SDS-PAGE gel, a 203-kDa complex was detected by Western blot, which was not seen under reducing conditions (either with 25 mmol/L DTT or 7.5% β-mercaptoethanol; Supplementary Fig. 1D). These data indicate that IL-17RL may form a homotrimer that is bound together by disulfide bonds among the monomers. To identify the genes that are regulated by IL-17RL overexpression and mediate IL-17RL’s antiapoptotic function in LNCaP cells, we did gene microarray analysis on LNCaP-C and LNCaP-RL cells. We found that 74 genes were up-regulated, whereas 38 genes were down-regulated (Supplementary Table 1). Of particular interest was that integrin β5 and z-parvin genes were both up-regulated. As these two genes are involved in cell attachment to the extracellular matrix, we tested if IL-17RL overexpression could enhance cell attachment to the extracellular matrix. We found that there were 15%, 24%, and 26% more cells attached to the collagen type I, collagen type IV, and fibronectin matrices, respectively, in LNCaP-RL cells than in LNCaP-C cells (Fig. 6C). Meanwhile, there was no difference between the attachment of LNCaP-C and LNCaP-RL cells to laminin, to which few of both cells attached (Fig. 6C).

**Figure 4.** IL-17RL conferred TNFα resistance in LNCaP cells. A, stable cell lines were established by selection with neomycin after transfection with pLNCX2–IL-17RL–V5-His (LNCaP-RL) or pLNCX2 (LNCaP-C). B and C, overexpression of IL-17RL in LNCaP cells inhibited TNFα-induced apoptosis. Cells were treated with 20 ng/mL TNFα alone for 3 days (B) or plus 20 μmol/L LY294002 for 24 hours (C). Cell survival was calculated as (the living cell number of treated group × the living cell number of untreated control group) / C4 / C2 × 100. P < 0.05 between the comparison groups. D, apoptosis was induced by transfection of the cells with 100 nmol/L siRNA for 24 hours, which was synergistically enhanced by adding 20 ng/mL of TNFα. Cell survival was calculated as (the living cell number of treated group / the living cell number of untreated control group) × 100. P < 0.05 between the treatment and control groups.
downstream to caspase-2/caspase8 but at or above the level of caspase-3. Currently, we do not know the precise action of IL-17RL in blocking the activation of caspase-3 despite considerable efforts. We have found that IL-17RL does not physically bind to caspase-3; therefore, IL-17RL must act through certain intracellular signaling pathways. We have ruled out the possibility that IL-17RL may act indirectly by modulating the expression of several proapoptotic and antiapoptotic genes, such as Fas, c-IAP1/2, TRAF2, Bcl-2, and Bcl-XL, or by activating the common signaling pathways as tested (Supplementary Fig. 1C).

However, we propose that the overexpressed IL-17RL forms a homotrimer complex via intermolecular disulfide bonds. This is based on our data that IL-17RL binds to its self, and the nonreduced complex’s molecular weight is about thrice of that of the reduced monomer (230 versus 85 kDa), although we can not rule out the possibility that the complex is formed by an IL-17RL homodimer and an unknown partner. Nevertheless, oligomerization may activate IL-17RL by autoactivation or by binding to an unknown ligand, which may activate the downstream signaling pathways that lead to regulation of gene expression. Indeed, we found a total of 112 genes with their expression being up-regulated or down-regulated by IL-17RL overexpression in LNCaP cells. Among them, integrin β3 and α-parvin are of particular interest, as both genes are involved in cell attachment and apoptosis (45, 46). These molecular changes are consistent with the biological response that IL-17RL overexpression enhances cell attachment to the extracellular matrixes. Cell attachment to the extracellular matrix is critical for cell survival (47–50). The enhanced cell attachment in LNCaP-RL cells may be partially responsible for the resistance to TNFα-induced apoptosis. In addition, α-parvin (also named as calponin homology domain-containing integrin-linked kinase–binding protein or actopaxin) has been found as an important component of the prosurvival signaling pathway functioning primarily by activation of PKB/Akt (46). α-Parvin acts in the cell survival pathway upstream of caspase-3, as depletion of α-parvin markedly activates caspase-3 and induces apoptosis (46). Therefore, we speculate that the inhibition of caspase-3 activation by IL-17RL is in part due to the induction of α-parvin expression.

The androgen-independent prostate cancer cell lines and tumors express higher levels of IL-17RL compared with their androgen-dependent counterparts, hinting at the possible role of IL-17RL in providing a cellular survival signal. The cancer cells need an additional survival signals to compensate for the deprivation of androgen. On the other hand, once the cancer cells establish this additional survival advantage, they are no longer dependent on androgen. Due to difficulties in obtaining large

Figure 5. IL-17RL–specific siRNA knocked down IL-17RL expression and induced apoptosis. A, apoptosis was induced by transfection of the cells with 100 nmol/L IL-17RL–specific siRNA for 48 hours but not by the control C-siRNA. Bar, 120 μm. B, IL-17RL–specific siRNA knocked down IL-17RL expression and induced cleavage of PARP in the cell lines in 48 hours as detected by Western blot (cell names of each panel matched vertically to A). C, DNA fragmentation was induced by 100 nmol/L IL-17RL–specific siRNA transfection in 293 cells in 24 hours, which was synergistically enhanced by adding 20 ng/mL TNFα.
numbers of androgen-independent human prostate tumors, the sample size was not large enough to attain statistical significance. Our in vivo xenograft study complemented this limitation by showing that IL-17RL expression was significantly increased in androgen-independent human prostate CWR22 tumors. Furthermore, we showed that IL-17RL expression was induced by androgen deprivation in a time-dependent manner.

The present study provides evidence that IL-17RL is a novel antiapoptotic gene. Because IL-17RL expression is induced by androgen deprivation and it is highly expressed in androgen-independent prostate cancer cell lines and tumors, it is reasonable to speculate that IL-17RL is potentially associated with androgen-independent growth of prostate cancer. It is likely that IL-17RL might be a potential therapeutic target in the treatment of androgen-independent prostate cancer.

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Figure 6. IL-17RL inhibited TNFα-induced apoptosis by blocking caspase-3 activation. A, cells were treated with 20 ng/mL TNFα, with or without 20 μM LY294002 for up to 48 hours. Western blots were done using the indicated antibodies. Of note, the decrease or disappearance of procaspases indicated their cleavage and activation. B, IL-17RL protein binds to its self. Full-length IL-17RL with V5-His tag (IL-17RL-V5) or with Myc tag (Myc-IL-17RL) was transfected alone or cotransfected into 293 cells by LipofectAMINE-mediated transfection. Equal amounts of cell lysate were precipitated with 1 μg of either anti-V5 or anti-Myc antibodies, together with Protein G-agarose beads for immunoprecipitation (IP). Western blots (WB) were done using anti-Myc or anti-V5 antibodies, respectively. Whole-cells lysates were probed for the expression of individually tagged IL-17RL. C, IL-17RL enhances cell attachment. Cells (2.5 × 10^5 per well) of LNCaP-C or LNCaP-RL cells in 2-mL serum-free T-medium were plated in triplicate in six-well Costar plate, or collagen type I (Col I), collagen type IV (Col IV), fibronectin, and laminin precoated plates. After a 90-minute incubation at 37°C, the attached cells were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet dye. The stain was dissolved in 10% acetic acid and read at 570 nm on a microplate reader. The crystal violet dye stains the cell protein that is proportional to the number of cells. The number of cells attached to the matrixes was calculated as percentage of cells attached to the Costar plate. Columns, mean; bars, SD. LNCaP-RL versus LNCaP-C: *, P < 0.05; **, P < 0.01.

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