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B cell–derived lymphotoxin promotes castration-resistant prostate cancer

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Abstract

Prostate cancer (CaP) progresses from prostatic intraepithelial neoplasia through locally invasive adenocarcinoma to castration resistant (CR) metastatic carcinoma[1]. Although radical prostatectomy, radiation and androgen ablation are effective therapies for androgen-dependent (AD) CaP, metastatic CR-CaP is a major complication with high mortality[2]. Androgens stimulate growth and survival of prostate epithelium and early CaP. Although most patients initially respond to androgen ablation, many develop CR-CaP within 12-18 months[2]. Despite extensive studies, the mechanisms underlying CR-CaP emergence remain poorly understood and their elucidation is critical for development of improved therapies. Curiously, CR-CaP remains androgen receptor (AR) dependent and potent AR antagonists induce tumor regression in castrated mice[3]. The role of inflammation in CR-CaP has not been addressed, although it was reported that intrinsic NF-κB activation supports its growth[4]. Inflammation is a localized protective reaction to injury or infection, but it also has a pathogenic role in many diseases, including cancer[5]. Whereas acute inflammation is critical for host defense, chronic inflammation contributes to tumorigenesis and metastatic progression. The inflammation-responsive IκB kinase (IKK) β and its target NF-κB have important tumor promoting functions within malignant cells and inflammatory cells[6]. The latter, including macrophages and lymphocytes, are important elements of the tumor microenvironment[7-9], but the mechanisms underlying their recruitment remain obscure, although thought to depend on chemokine and cytokine production[10]. We found that CaP progression is associated with inflammatory infiltration and activation of IKKα, which stimulates metastasis by an NF-κB-independent, cell autonomous, mechanism[11]. We now show that androgen ablation causes infiltration of regressing AD tumors with leukocytes, including B cells, in which IKKβ activation results in production of cytokines that activate IKKα and STAT3 in CaP cells to enhance hormone-free survival.

To determine whether IKKβ-driven NF-κB participates in development of CR-CaP, we conditionally deleted the *Ikkβ* gene in prostate epithelial cells of *TRAMP* mice, in which CaP is induced by prostate specific expression of SV40 T antigen[12]. Counter to previous expectations[4], IKKβ ablation in prostate epithelial cells had no effect on genesis and progression of AD-CaP (Fig. S1) or development of CR-CaP after castration (Fig. S2). To facilitate mechanistic analysis of CR-CaP development we used subcutaneous (SC) allografts of the mouse AD-CaP cell line myc-CaP, which is derived from the *FVB* genetic

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background[13]. Silencing of IKKβ in myc-CaP cells did not affect primary tumor growth or CR-CaP re-growth in castrated *FVB* mice (Fig. S3).

By contrast, deletion of IKKβ in interferon-responsive cells upon induction of *Mx1-Cre* expression prevented castration-induced metastatic spread in *TRAMP/Ikkβ*$^{F/F}$*/Mx1-Cre* mice (Fig. S4A). To narrow down the role of IKKβ to bone marrow (BM)-derived cells (BMDCs), we reconstituted irradiated *FVB* mice with BM from *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta/\Delta}$ mice [*Ikkβ*$^{F/F}$*/Mx1-Cre* mice injected with poly(IC) to induce *Mx1-Cre*] and inoculated the resulting chimeras (Fig. S4B) with myc-CaP cells. Absence of IKKβ in BMDC had no impact on primary tumor growth, but delayed CR-CaP emergence after castration (Fig. S4C). A similar delay in CR-CaP growth was seen in castrated tumor-bearing mice treated with specific IKKβ inhibitors[14, 15] (Fig. S4D and data not shown).

Dependence of CR-CaP emergence on IKKβ in BMDC, suggested that androgen deprivation elicits a tumor-associated inflammatory response. Castration of mice bearing myc-CaP tumors resulted in CaP cell death, peaking within one week (Fig. 1A). Concurrently, the regressing tumors were infiltrated with T and B lymphocytes, NK cells and myeloid cell types (Fig. 1B; Fig. S5). Infiltration was transient, declining by 2 weeks after castration. B and T lymphocyte infiltration was also detected in 100% of human CaP samples (untreated patients with Gleason scores of 6-8), but B cells were undetectable in normal prostate or benign prostatic hyperplasia (Fig. 1C). The mRNAs for many inflammatory chemokines were also upregulated in the myc-CaP allografts, but no changes in AR mRNA expression were found (Fig. S6A). These chemokines may recruit lymphoid and myeloid cells into the regressing tumor. Indeed, antibody mediated inhibition of CXCL13, a B cell chemoattractant[16], prevented castration-induced B cell recruitment (Fig. S6B,C). Inflammatory cytokine mRNAs, including IL-6, IL-12, TNF-α and lymphotoxin (LT), were also upregulated in the regressing myc-CaP allograft, but only LT expression was reduced upon CXCL13 inhibition (Fig. S7). Castration resulted in nuclear export of AR, but after 3 weeks AR was nuclear again (Fig. S8), suggesting it is activated at late phases of CR-CaP growth despite androgen depletion.

STAT3 was proposed to promote activation of unliganded AR[17]. Indeed, STAT3 was activated during CR-CaP emergence, faster than AR was (Fig. S9). *Mx1-Cre*-mediated IKKβ deletion, which was nearly complete in mature B and T lymphocytes (Fig. S10A), prevented STAT3 activation in regressing tumors, but did not affect ERK and AKT activation (Fig. S10B). Immunohistochemical analysis confirmed STAT3 activation in CaP cells, inhibitable by A490 (Fig. S10C), an inhibitor of STAT3 phosphorylation[18] that delayed appearance of CR-CaP (Fig. S10D) but did not inhibit IKK activation (Fig. S11A). Conversely, ML120B did not inhibit STAT3 activation (Fig. S11B).

Ablation of BMDC IKKβ did not prevent leukocyte recruitment into regressing tumors (Fig. S12) but did inhibit cytokine induction (Fig. S13). To further investigate the role of lymphocytes, we used chimeric mice generated by transplantation of BM from lymphocyte-deficient *Rag1*$^{-/-}$ mice. Although primary tumor growth was identical in mice receiving WT or *Rag1*$^{-/-}$ BM (Fig. S14A), CR-CaP growth was significantly delayed in mice receiving *Rag1*$^{-/-}$ BM (Fig. 2A), but not in mice reconstituted with BM from *Tcrβ*$^{-/-}$*/δ*$^{-/-}$ mice (Fig. S14B), which lack only mature T lymphocytes. CR-CaP growth was delayed in mice reconstituted with BM from $J_H^{-/-}$ mice (Fig. S14C), which lack mature B cells, or upon B cell depletion with CD20 antibody[19] (Fig. S14D). Reconstitution of *Rag1*$^{-/-}$ *FVB* mice with splenic B cells, but not T cells of *FVB* mice, restored rapid CR-CaP re-growth (Fig. 2A). Primary tumors, isolated from *Rag1*$^{-/-}$ chimeric mice one week after castration, did not show STAT3 activation (Fig. 2B), but reconstitution with B cells, rather than T cells, restored castration-induced STAT3 phosphorylation (Fig. 2C).

CaP allografts from castrated, but not sham-operated, mice exhibited IKKα nuclear translocation (Fig. 3A,B). Silencing of IKKα in myc-CaP cells using siRNA (Fig. S15A) had little effect on primary tumor growth, but delayed CR-CaP emergence (Fig. 3C). Nuclear translocation of IKKα was dependent on IKKβ in BMDC and on B cells, but not on T cells (Fig. 3D). IKKα nuclear translocation parallels progression of human and murine CaP and coincides with primary tumor infiltration with cells expressing IKKα-activating cytokines, RANK ligand (RANKL) and LTα[11]. Castration induced LTα and LTβ in regressing myc-CaP allografts, but did not alter RANKL expression (Fig. S7). LT expression in regressing tumors was absent in *Rag1*$^{-/-}$ mice (Fig. S16A) and flow cytometry localized it to tumor infiltrating B cells (TIBC; Fig. S16B). We characterized TIBC by 7-color flow cytometry with several markers and a LTβR-Ig fusion protein to detect LT. The typical TIBC was a conventional, mature B2 cell that expressed LT on its surface and was negative for B1 markers (Fig. S17). IKKβ deletion abolished LT expression by B cells (Fig. 4A), supporting the previously suggested[20] role of NF-κB in LTα/β induction. To examine whether LT production by tumor-infiltrating lymphocytes stimulates CR-CaP growth, we transplanted BM from B-*Ltβ*$^{-/-}$ or T-*Ltβ*$^{-/-}$ mice, which lack LTβ in either B or T cells[21], into lethally irradiated mice. LTβ ablation in B cells, but not in T cells, delayed growth of CR-CaP (Fig. 4B) and abolished LTβ expression within tumors but did not prevent B cell or macrophage infiltration (Fig. S18). Treatment of mice with the LTβR-Ig decoy[22] was as effective as B cell-specific LTβ ablation in delaying CR-CaP growth (Fig. 4C) and prevented IKKα and STAT3 activation (Fig. S19). Silencing of LTβR in Myc-CaP cells (Fig. S15B) also delayed CR-CaP growth (Fig. 4D). Exogenous LT maintained myc-CaP growth in the presence of flutamide, a clinically used AR antagonist[3], in a manner dependent on IKKα (Fig. 4E), whose nuclear translocation was LT inducible (Fig. 4F).

CR-CaP is a major complication that limits the success of androgen ablation therapy and is responsible for most prostate cancer mortality[2]. CR-CaP was studied mainly at the level of AR function, the central player in this process[4]. Our results suggest that an inflammatory response triggered by death of androgen-deprived primary cancer is another important contributor to emergence of CR-CaP. In addition to dying CaP cells, critical participants in this response are tumor infiltrating B cells, which produce LTα:β heterotrimers that stimulate LTβR on CaP cells to induce IKKα nuclear translocation and STAT3 activation, thereby enhancing androgen-independent growth (Fig. S20). Interference with any component of this response results in a significant and reproducible 3-4 week delay in appearance of CR-CaP. Although these inhibitory effects are not absolute, extrapolation from “mouse time” to “human time” suggests that interventions that prevent LT production or signaling may delay appearance of CR-CaP in patients undergoing androgen ablation therapy by 2.3 to 3.1 years. Importantly, our results suggest that, at least for CaP, the inflammatory response elicited by the dying primary tumor, contributes to the failure rather than the previously proposed success of anti-cancer therapy[23]. Although we have not determined how death of androgen-deprived CaP triggers the inflammatory response described above, necrotic cell death releases mediators, such as HMGB1[24] and IL-1α[25], that activate IKKβ and NF-κB and stimulate production of chemokines, one of which, CXCL13, recruits B cells into the regressing tumor. Notably, TIBC were detected not only in androgen-deprived mouse CaP, but also in human CaP. Although B cells were reported to promote progression of skin carcinomas[9] and exert immunosuppressive effects through activation of inhibitory Fc receptors on myeloid cells[26], the critical tumor promoting B cell function in our experimental model is production of LT, an IKKα-activating cytokine[27], which promotes survival of androgen-deprived CaP. Another important function of TIBC is activation of STAT3, an anti-apoptotic and pro-tumorigenic transcription factor[28]. Although the critical STAT3-activating cytokine in this system remains to be identified, castration induces expression of STAT3-activating IL-6 and IL-12 family members. Furthermore, CaP cells use autocrine IL-6 to stimulate their progression[29] and activated STAT3 promotes ligand-independent AR activation[29]. LT is also involved in the etiology of human

CaP. An epidemiological study revealed that reduced CaP risk due to consumption of non-steroidal anti-inflammatory drugs, such as aspirin, is limited to men who express a common polymorphic *LTα* allele that specifies high LT production[30]. Further work should examine the effect of *LTα* polymorphism on the response to androgen ablation. Our results predict that individuals who are high LT producers are more likely to develop CR-CaP and should therefore be the main beneficiaries of anti-LT therapy.

Methods Summary

A detailed Methods section is available in Supplementary Information. Mice were handled according to institutional and NIH guidelines. Tumors were grown in *FVB* mice. Where indicated, lethally irradiated *FVB* mice were reconstituted with BM from different strains that were backcrossed into the *FVB* background for at least two generations. *Ltβ* knockout strains were, however, in the *BL6* background which does not elicit a graft vs. host response in *FVB* mice. Conditions for antibody used were posted to http://biorating.com. Human material was obtained from the Cooperative Human Tissue Network (CHTN) along with pathology reports. Histology, gene expression and cell signaling were analyzed as described[11, 25].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

METHODS

Mice and cell culture

Ikkβ$^{F/F}$ (*BL6*) mice were crossed to *TRAMP* (*BL6*x*129*) mice[31] and *PB-Cre4* (*BL6*)[32] or *Mx1-Cre* (*BL6*) mice[33] to generate *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*PB-Cre4*$^{+/-}$ and *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*PB-Cre4*$^{+/+}$ or *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*Mx1-Cre*$^{+/-}$ and *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*Mx1-Cre*$^{+/+}$ progeny that were intercrossed with *TRAMP* mice for six generations. After that, *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*PB-Cre4*$^{+/-}$ and *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*PB-Cre4*$^{+/+}$ or *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*Mx1-Cre*$^{+/-}$ and *TRAMP*$^{+/-}$/*Ikkβ*$^{+/F}$/*Mx1-Cre*$^{+/+}$ mice were intercrossed to generate *TRAMP*$^{+/-}$/*Ikkβ*$^{F/F}$/*PB-Cre4*$^{+/-}$, *TRAMP*$^{+/-}$/*Ikkβ*$^{F/F}$/*PB-Cre4*$^{+/+}$, *TRAMP*$^{+/-}$/*Ikkβ*$^{F/F}$/*Mx1-Cre*$^{+/-}$ and *TRAMP*$^{+/-}$/*Ikkβ*$^{F/F}$/*Mx1Cre*$^{+/+}$ mice. Only male littermates were used. *FVB*, *Tcrβ*$^{-/-}$*δ*$^{-/-}$ *(BL6)*, *Mx1-Cre* and *Rag1*$^{-/-}$ (*BL6*x*129*) mice were from the Jackson Laboratory. $J_H^{-/-}$ mice *(FVB)* were kindly provided by L. Coussens (Cancer Research Institute and Anatomic Pathology, UCSF, San Francisco, CA). Bone marrow from B-*Ltβ*$^{-/-}$ or T-*Ltβ*$^{-/-}$ mice[21] was kindly provided by C.F. Ware (La Jolla Institute for Allergy and Immunology, La Jolla, CA). *PB-Cre4* and *TRAMP* mice were from MMHCC (Mouse Models of Human Cancer Consortium). Mice were maintained under specific pathogen-free conditions, and experimental protocols were approved by the UCSD Animal Care Program, following NIH guidelines. Radiation chimeras were generated as described[34]. In general, irradiated *FVB* mice were reconstituted with bone marrow from different strains that have been backcrossed to the *FVB* background for at least 2 generations. However, in the case of B-*Ltβ*$^{-/-}$ and T-*Ltβ*$^{-/-}$ mice, bone marrow donors were

of the *BL6* background, whose bone marrow did not lead to a graft vs. host response in irradiated *FVB* mice. Myc-CaP cells derived from the *FVB* background were provided by C. Sawyers (UCLA and Memorial Sloan Kettering Cancer Center)[35] and were cultured under standard conditions and confirmed to be mycoplasma free. Myc-CaP cells were injected subcutaneously into the flank of male *FVB* mice as described[35]. Tumor growth was measured with a caliper. Surgical procedures were as described[35].

Human specimens

Anonymous human prostate, benign prostatic hyperplasia and prostate cancer frozen sections were provided by the Cooperative Human Tissue Network (CHTN). Pathology reports were provided by CHTN for each sample.

CXCL13 and B cell depletion and LT inhibition

CXCL13 neutralizing antibody was purchased from R&D and administered i.p. at 200 mg/mouse as described[36]. Anti-CD20 was kindly provided by Genentech (Oceanside, CA) and was administered i.p. at 250 μg/mouse. LTβR-Ig fusion protein was a kind gift from Yang-Xin Fu (University of Chicago, Chicago, IL) and was administered as described[22]. hIgG and mouse IgG2a were purchased from Sigma-Aldrich and were used as controls.

IKKβ inhibitors

ML120 was kindly provided by Millenium Inc. and administered orally as described[37]. IKKβ Inhibitor IV was purchased from Calbiochem and was tail vein injected as described[14].

Histological procedures

Mouse prostate and CaP tissues and dissected metastatic tumors were immersed in 10% neutral buffered formalin before sectioning and paraffin embedding. Sections were stained and processed as described[11], using H&E stain, TUNEL assay kit or antibodies for IKKα (Imgenex), phospho-STAT3 (Cell Signaling) and CD19 (eBioscience) as described[38]. Frozen sections of human and mouse origins were fixed in acetone and processed as described [11], using antibodies for AR (Santa Cruz), B220 (BD),CD20 (BD), CD4 (BD) and CD8 (BD).

Analysis of RNA and protein expression

Total tissue RNA was prepared using RNAeasy (Qiagen). Quantitative PCR was performed as described[38]. Cells and tumors were lysed and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting[38] with antibodies to histone H3, α-tubulin, STAT3 (Santa Cruz Biotechnology), ERK, phospho-ERK, AKT, phospho-AKT, phospho-STAT3 (Cell Signaling). Nuclear extracts were prepared and analyzed for NF-κB DNA-binding as described[39].

Lentiviral and retroviral transduction

siRNAs to mouse IKKα, IKKβand LTβR mRNAs were cloned into pLSLPw, provided by I. Verma (The Salk Institute), and lentivirus stocks were prepared as described[11]. Virus-containing supernatants were added to myc-CaP cells for 2 days with polybrene, and transduced cells were selected in 5 μg ml^{-1} puromycin (Invitrogen).

Leukocytes purification and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a double-layered Histopaque-Ficoll (GE Lifescience) gradient. Splenic B and T lymphocytes were isolated by magnetic cell sorting (MACS) with CD4, CD8 or CD19 antibodies conjugated to

magnetic beads. Tumor infiltrating leukocytes were stained with CD45, B220, LTβR-Ig, TCRβ, Gr1, CD4 and CD8 fluorescent antibodies, as well as Aqua LIVE/DEAD dye (Molecular Probes) and analyzed on a flow cytometer (Accuri C6 or Becton Dickinson LSR II).

Statistical analyses

Results are expressed as means ± s.e.m. or s.d. Data were analyzed by Student's t-test and Kaplan-Meier survival analysis using GraphPad Prism statistical program. Error bars depict s.e.m. or s.d. P values >0.05 were considered insignificant (ns), 0.01 to 0.05 were considered significant (*), 0.001 to 0.01 were considered very significant (**) and < 0.001 were considered as highly significant (***).

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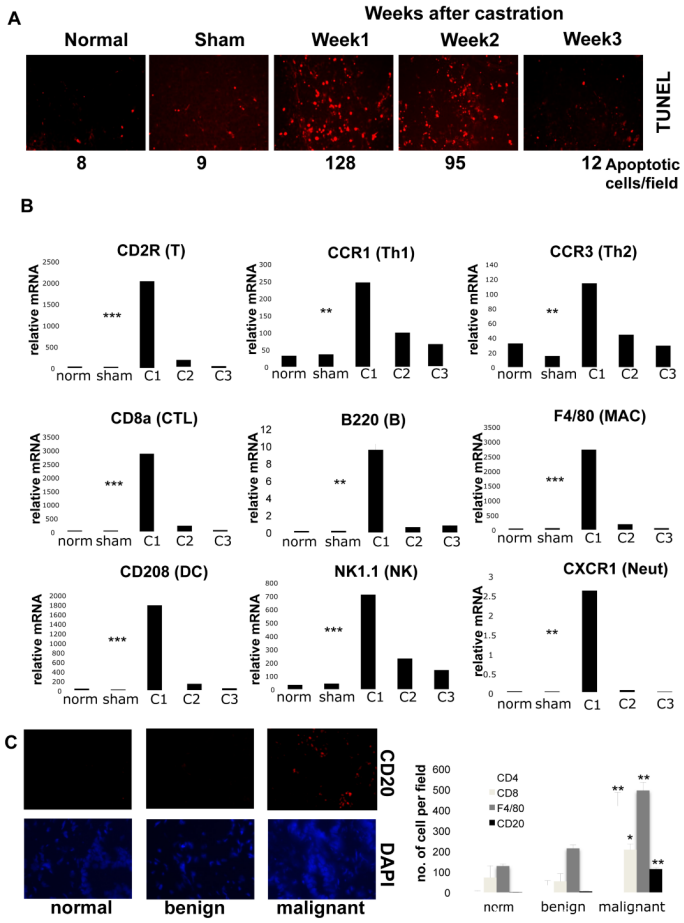

Figure 1. Androgen ablation induces tumor inflammatory infiltration

Six week old *FVB* males (n=10) were inoculated with myc-CaP cells. When tumors reached 1000 mm^3, mice were left untreated, castrated or sham operated. Tumors were collected when indicated for analysis. **A.** Paraffin-embedded tumor sections were TUNEL stained to determine apoptotic cell frequency (results are averages, n=3). **B.** Total RNA was isolated from tumor samples and expression of indicated cell marker mRNAs was quantitated and normalized to that of cyclophilin A (norm= normal, C1,2,3= mice analyzed 1,2,3 weeks after castration, sham= sham-operated). Results are averages ± s.d. (n=10). **C.** Frozen human prostate sections (normal tissue [n=3], prostatic hyperplasia [n=3], and malignant CaP with Gleason scores 6-8 [n=10]) were stained with CD4, CD8 and CD20 antibodies and DAPI and analyzed by immunofluorescent microscopy. The histogram denotes average frequencies of indicated cell types (n=3 per sample). P values were determined and are depicted as insignificant (ns), significant (*), very significant (**) or highly significant (***).

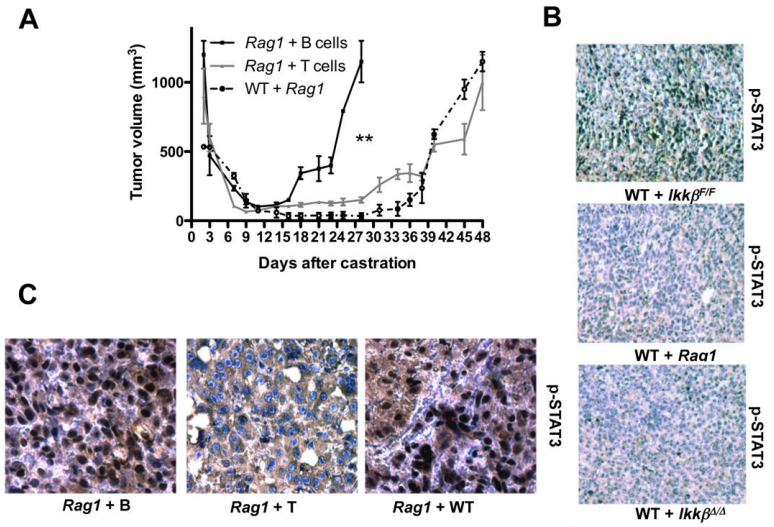

Figure 2. Role of B cells and IKKβin STAT3 activation and CR-CaP emergence

A. Myc-CaP tumors were established in WT mice reconstituted with BM from *Rag1*$^{-/-}$ males (n=10) or in *Rag1*$^{-/-}$ males. When tumors reached 1000 mm^3, mice were castrated. Three days before castration, *Rag1*$^{-/-}$ mice (n=10 per group) received via the tail vein purified splenic B or T cells. Tumor volume was measured. Results are averages ± s.e.m.. P values were determined and are indicated as above. **B.** Tumors were removed from radiation chimeras reconstituted with *Ikkβ*$^{F/F}$, *Ikkβ*$^{Δ/Δ}$, or *Rag1*$^{-/-}$ BM, one week after castration. STAT3 phosphorylation was analyzed by immunohistochemistry. **C.** Tumor-bearing *Rag1*$^{-/-}$ males were injected with WT splenocytes (*Rag1* + WT), or purified splenic B (*Rag1* + B) or T (*Rag1* + T) lymphocytes. One day later, mice were castrated and after one week, tumors were removed and analyzed for STAT3 phosphorylation.

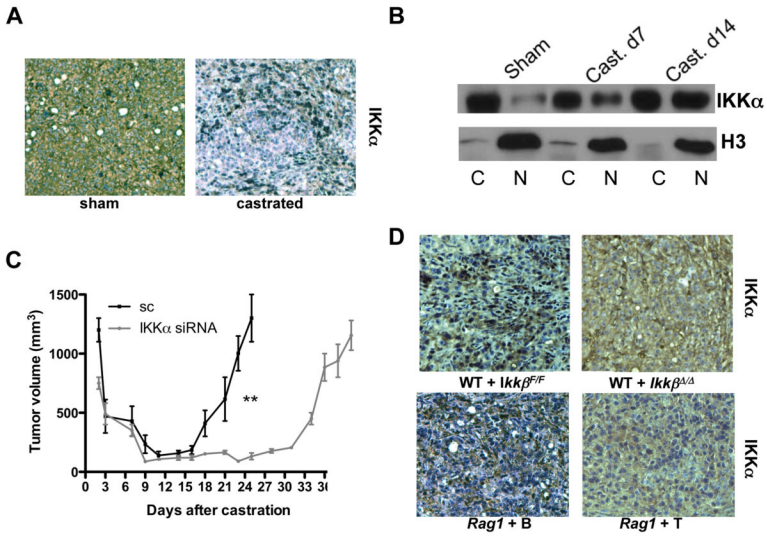

Figure 3. Role of IKKα in emergence of CR-CaP

Tumor-bearing mice were castrated or sham operated as above. **A.** Tumors were analyzed one week later for nuclear IKKα by immunohistochemistry. **B.** Tumors removed at indicated times were divided into cytosolic (C) and nuclear (N) fractions and IKKα and histone H3 distribution was determined. **C.** Tumors were established using myc-CaP cells transduced with lentiviruses expressing scrambled siRNA (sc) or IKKα-specific siRNA. Mice were castrated as above and tumor volume was measured. Results are averages ± s.e.m. (n=10). P values were determined and are indicated as above. **D.** Tumors were established in lethally irradiated *FVB* males reconstituted with *Ikkβ*$^{F/F}$ or *Ikkβ*$^{Δ/Δ}$ BM or in *Rag1*$^{-/-}$ males reconstituted with either B or T cells. *Ikkβ*$^{F/F}$ and *Ikkβ*$^{Δ/Δ}$ chimeras were i.p. injected three times with poly(IC) (250 μg) prior to castration to delete IKKβ. One week after castration, tumor samples were analyzed for IKKα distribution by immunohistochemistry. Nuclear IKKα results in punctuate staining, while cytoplasmic IKKα results in diffuse staining.

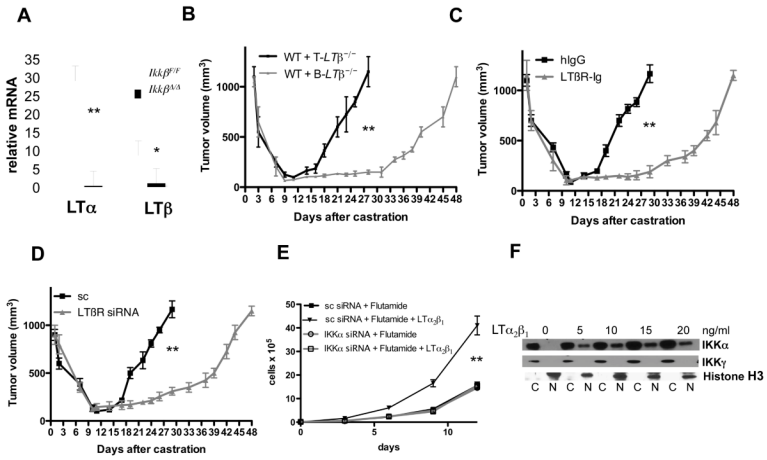

Figure 4. IKKβ-dependent lymphotoxin production by tumor-infiltrating B cells stimulates IKKα-dependent androgen-free survival

A. RNA from splenic B cells of *Ikkβ*$^{F/F}$ and *Ikkβ*$^{\Delta/\Delta}$ mice was analyzed for LTα and LTβ expression as above. Results are averages ± s.d. (n=3). **B.** Lethally irradiated *FVB* males were reconstituted with BM from B-*Ltβ*$^{-/-}$ or T-*Ltβ*$^{-/-}$ mice (n=6 per group). After 8 weeks, myc-CaP tumors were established, mice were castrated and tumor volume was measured as above. Results are averages ± s.e.m.. **C.** *FVB* mice (n=6 each group) bearing myc-CaP tumors were castrated and given hIgG or LTβR-Ig (100 μg) every 5 days, starting 4 days before castration. Tumor volume was measured as above. Results are averages ± s.e.m.. **D.** Tumors were established using myc-CaP cells transduced with lentiviruses expressing scrambled (sc) siRNA or LTβ-specific siRNA. Mice were castrated and tumor volume was measured. Results are averages ± s.e.m. (n=10). **E.** Myc-CaP cells (previously infected with lentiviruses expressing scrambled or IKKα siRNAs) were plated at 40% confluency. After 6 hrs, the cells were cultured with or without flutamide (10 μM) in the absence or presence of $LT\alpha_2\beta_1$, and cell number was determined. **F.** Myc-CaP cells were plated at 60% confluency. After 12 hrs, cells were stimulated for 1 hr with $LT\alpha_2\beta_1$, collected, divided into cytosolic (C) and nuclear (N) fractions and IKKα and histone H3 distribution was determined. In **A**, **B**, **C**, **D** and **E**, P values were determined and are indicated as above.