Published in final edited form as:

Sci Signal. ; 2(86): ra49. doi:10.1126/scisignal.2000338.

IL-1 induces IGF-dependent epithelial proliferation in organ development and reactive hyperplasia

Travis J. Jerde¹ and Wade Bushman¹

¹ University of Wisconsin School of Medicine and Public Health, Department of Urology, Madison, WI 53792

Abstract

Chronic inflammation and reactivation of developmental signaling pathways are both hallmarks of adenocarcinomas. However, developmental biology and inflammation are generally thought of as distinct and are believed to represent separate paths to carcinogenesis. Here, we show that the inflammatory cytokine IL-1 α plays a critical role in prostate development by activating insulin-like growth factor signaling; this process is reiterated during inflammatory reactive hyperplasia to elicit epithelial proliferation. The appearance of developmental signals during hyperplasia supports the hypothesis that reactivation of developmental signaling plays a role in the hyperplastic reaction to inflammation and suggests that there may be a conserved link between inflammatory signaling and canonical developmental pathways.

Introduction

Some of the cardinal features of embryonic development, including proliferation of epithelial cells and progenitor cells, and stromal remodeling,¹ are also characteristic of wound healing and cancer.² The morphological similarities among these three conditions have been echoed by molecular studies showing re-activation of various developmental signaling pathways in the response to epithelial injury and in cancer.^{3,4} This suggests a mechanistic link between the programmed growth of the embryo, the reactive growth response to injury or inflammation, and the dysregulated growth found in cancer. The proliferative response of a tissue to injury and inflammation is necessary to the survival of the organism;⁵ however, the association between chronic inflammation and cancer suggests that the proliferative response to injury may drive epithelial growth in neoplasia.⁶⁻⁹ Malignant transformation has been postulated to result from sustained epithelial proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents.⁶ However, inflammatory mediators are generally conceived as having a primary role in the inflammatory cascade and, although they may contribute to tumor development and progression, their effect on epithelial cell proliferation is considered a non-specific collateral effect of their action rather than the primary effect.⁷

Recognizing the parallels between development and regenerative repair, the association of chronic inflammation and cancer, and the trophic effects inflammatory mediators have on tumor cell proliferation, we postulated that inflammatory signaling mechanisms might play a fundamental role in organ development—and that this same pathway is reactivated in the adult in response to injury and inflammation. To investigate this possibility, we examined interleukin signaling in normal development and in the reactive hyperplasia associated with inflammation.

Although we used the prostate as a model for this analysis because of the known reactivation of established developmental cascades in prostate cancer¹⁰ and the high prevalence of inflammation juxtaposed to regions of prostate cancer and reactive hyperplasia,⁹ these findings are likely to have relevance to other organ systems, particularly those in which inflammation leads to reactive epithelial hyperplasia and the promotion of carcinoma.

Results

Developmental signaling is interrelated to inflammatory signaling

We performed a cytokine gene array (SuperArray Biosciences, Fredrick, MD) to screen for expression of cytokines associated with inflammation in the developing, adult, and inflamed mouse prostate. There were 128 genes on the array, 24 of which showed increased expression during inflammation. 20 of 24 genes that showed significantly increased expression during inflammation also showed increased expression in development compared to the normal adult. These included the genes encoding the interleukins IL-1 α , IL-1 β , IL-1F8, IL-6, IL-8, IL-12, IL-13 and IL-17 [Table S1]. Tumor necrosis factor (TNF) α , interferon γ , interleukin 2, and TNFSF5 [CD-40 ligand] showed increased expression during inflammation but not during development. The overall picture was consistent with increased expression of inflammatory cytokines during development and re-expression of many of these same factors during inflammation.

We focused on the interleukin-1 (IL-1) family of cytokines for detailed study because this family is generally regarded as being critical to inflammation and repair, and is abundant at the mRNA and protein level in chronic inflammation and numerous carcinomas.¹¹ Moreover, IL-1 signaling has been implicated in testicular development and in bone remodeling.¹²⁻¹⁴ Reverse transcription polymerase chain reaction (RT-PCR) confirmed the array results, indicating that expression of these inflammatory mediators was significantly increased during development [Fig. 1 A, B]. Peptide synthesis and release (as determined by ELISA) indicated that the pattern of IL-1 α peptide synthesis and release parallels expression of IL-1 α mRNA. [Fig. 1C] IL-1 β protein content and release remained relatively constant throughout development despite changes in mRNA expression; even so, total peptide was significantly higher at postnatal day 1 (P1) than in the adult [Fig. 1D]. Both IL-1 α and IL-1 β peptide content and release were markedly increased three days after bacterial infection. In contrast to the general pattern of greater IL-1-family expression during development and substantial induction during inflammation, one family member, IL-18, showed significantly lower expression during development but was highly inducible during inflammation [fig. S1].

Hematoxylin and eosin stained prostate sections showed no evidence of an inflammatory cell infiltrate, and immunostaining for CD-3, CD20, and F4-80 revealed no evidence of T cells, B cells, or macrophages during prostate development [fig. S2]. This eliminated inflammatory cells as the source of IL-1 ligand during development. Localization of *IL-1* mRNA by sectional in situ hybridization revealed that *IL-1 α* is expressed in the developing prostatic ducts during development [fig. S3A]. During inflammation, *IL-1 α* is expressed in both the adult ductal epithelium [fig. S3C] and in the inflammatory cell infiltrate. *IL-1 β* mRNA is present in the developing urethral epithelium [fig. S3B], and expression is limited to areas of inflammatory infiltrate [fig. S3D] during inflammation in the adult. These data demonstrate that, during development, the epithelium is the source of IL-1 α and IL-1 β .

Multiple developmental mediators are induced at the mRNA level by inflammation in tissues exhibiting reactive hyperplasia. [Fig. 1E] These include insulin-like growth factor-1 (*IGF-1*), platelet-derived growth factor (*PDGF*), sonic hedgehog (*Shh*) and its effector glioma-associated protein 1 (*Gli1*), and transforming growth factor- β (TGF β) 1 and 3. All of these factors contribute to the organogenesis of various structures, including the prostate.¹⁰

Furthermore, IL-1 stimulates the expression of *TGFβs* and *IGFs* in cultured stromal cells and organ cultures in a concentration-dependent fashion [Fig. 1 F, G, H]. These data suggest that there is a reactivation of developmental signaling pathways during inflammatory reactive hyperplasia and indicate that inflammatory mediators can directly activate these pathways. These data also support the hypothesis that the hyperplastic response to inflammatory damage is a repair mechanism that resembles developmental processes, and may represent a reactivation of developmental signaling.

IL-1 induces epithelial and stromal expansion and organogenesis

Development in the prostate is representative of development in many organs systems with branched tubular architecture, including airways, kidneys, mammary glands, and salivary glands. In the mouse prostate, development is first observed by the outgrowth of epithelial buds (embryonic day 17.5) from the endodermal urogenital sinus (UGS) into the supporting mesenchyme. These buds undergo branching morphogenesis to generate the secretory ductal network of the adult prostate.¹⁰ To examine the effect of IL-1 on organogenesis, we removed UGS from wild-type mice at E16 and cultured them in serum free media for seven days in the presence of dihydrotestosterone (DHT), IL-1s, or vehicle (control).^{15,16} Addition of DHT resulted in substantial epithelial outgrowth. [Fig. 2 A, B] IL-1 induced epithelial and stromal growth in the absence of androgen [Fig. 2 C, D]. Growth induction was concentration-dependent (1, 10, 100 ng/ml) and robust effects were seen at concentrations generally considered physiologic (1-10 ng/ml). IL-1-treated tissues showed growth responses earlier than did tissues treated with DHT but the response tapered off earlier and was less overall [Fig. S4]. IL-1-induced growth did not depend on androgen signaling; UGS tissues treated with the selective androgen receptor antagonist bicalutamide responded similarly to IL-1 stimulation as vehicle-treated (0.1% ethanol) tissues. [Fig. S5] In contrast to the well-formed ductal buds induced by the DHT, which showed a distinctive basal layer of P63⁺ cells (indicating basal epithelial prostatic cells), IL-1β-treated tissues showed non-cannulated, bulbous outgrowths consisting predominantly of P63⁺ cells [Fig. S6]. When the UGS was cultured in the presence of both DHT and IL-1, the epithelial outgrowths showed a mixed phenotype, with normal cannulated ducts, bulbous outgrowths, and ducts that contain bulbous expansions of P63⁺ cells. The selective expansion of P63⁺ cells by IL-1, even in the presence of DHT, suggests that IL-1 has a selective proliferative effect on this progenitor-containing cell population.

There are two receptors that bind IL-1α, IL-1β, and IL-1F10: IL-1R1 and IL-1R2.¹⁷ IL-1R1 is the active receptor for these ligands, whereas IL-1R2 lacks an intracellular signaling domain and is believed to be a decoy receptor.¹⁷ IL-1R1^(-/-) mice¹⁸ that lack IL-1R1, showed significantly decreased size and branching of the ventral and dorsolateral prostate lobes [Fig. 2 E-H]. There was no difference in size or branching of the coagulating gland (anterior prostate) but there was a seminal vesicle phenotype characterized by decreased curvature and a reduced number of folds. Despite these morphologic differences, histological examination revealed normal epithelial differentiation of the prostate and seminal vesicles. Sparing of coagulating gland development argues against hypogonadism as a cause for the observed abnormalities of prostate and seminal vesicle development; however we further confirmed the integrity of the androgen axis in IL1-R1 mice in several ways. First, there was no difference in testosterone production from the testes of IL-1R1^(-/-) and wild-type mice at various time-points during development [fig. S5]; the concentration of testosterone in adult IL1-R1 testes was equivalent to previously reported normal concentrations.¹⁹ Secondly, testicular histology, spermatogenesis and fertility in IL1-R1 null males was normal. Additionally, the selective androgen receptor antagonist bicalutamide (10 nM) did not inhibit IL-1α-dependent prostate growth [fig. S5].

IL-1 signaling is critical to inflammatory reactive hyperplasia

A mouse model of bacterially-induced prostatic inflammation shows substantial reactive hyperplasia.²⁰ To determine whether IL-1 signaling was necessary for the reactive hyperplasia associated with infection, we compared the response to bacterial infection in IL-1R1 knockout mice and wild type controls. Infection was verified three days after inoculation by standard plate colony counts on minced tissue from control and infected prostates harvested from wild-type and knockout animals grown on Levine eosin methylene blue (EMB) agar. Non-infected animals had no cultureable bacteria and there was no significant difference in the number of cultureable *E. coli* harvested from infected wild-type and knockout animals. Three days after inoculation, wild-type animals showed widespread inflammatory infiltrate and multi-layering of the epithelium [Fig. 3]. A significant hyperplastic response to acute inflammation was confirmed by BrdU labeling. IL-1R1 KO animals showed an equally severe immediate (acute) inflammatory response as characterized by neutrophilic infiltrate, edema and epithelial slough. However, the reactive hyperplastic response in these animals was reduced. The epithelium retained its psuedostratified histomorphology and the infection-associated increase of in BrdU labeling was significantly diminished [Fig. 3].

IL-1-mediated epithelial growth is mediated by STAT-dependent stromal IGF-1 induction

The comparable growth-related effects of IL-1 signaling suggest a conserved mechanism of action for IL-1 in both development and reactive hyperplasia. We pursued a two-pronged strategy to identify possible mechanisms of IL-1-induced growth and proliferation. As shown earlier, we found that IL-1 stimulated the canonical developmental IGF-1, IGF-2 and TGF β 1, and 3 signaling pathways [Fig. 1 F, G, and H]. We then examined the role of inflammation-related signal transduction pathways in IL-1 signaling. The primary receptor for IL-1 ligands, IL-1R1, was activated only in the stroma of the developing prostate as shown by staining for activated (tyrosine-phosphorylated) IL-1R1 in the stroma of E16 UGS and P5 prostate. [Fig. S7] Several pathways are known to be activated by IL-1.^{17, 21} These include: the Janus-activated kinase-signal transducer and activator of transcription (Jak-STAT) pathway—often stimulated in resident epithelial, stromal, or leukocytic cells; the phosphoinositide-3-kinase-Akt (PI3K-Akt) pathway—often stimulated in reactive epithelial cells; and the nuclear factor κ -B (NF κ B) signaling pathway—most known for its activation in leukocytes.¹⁷ We therefore investigated these pathways in cultured prostate stromal and epithelial cells treated with IL-1 α . IL-1 α treatment activated Jak-STAT signaling as indicated by phosphorylated STAT-3 in stromal cells isolated from both the ventral and dorsal-lateral prostate [Fig. 4]. No activation of Jak-STAT signaling was apparent in epithelial cells. The PI3K-Akt pathway was active in epithelial cells in culture as indicated by phosphorylated Akt; however IL-1 treatment did not increase Akt phosphorylation further [Fig. 4]. We did not observe activation of NF κ B signaling in either stromal or epithelial cells, as measured by phosphorylated I κ B or nuclear p65 localization. These data suggested that there is a direct, stromal-specific activation of Jak-STAT signaling as a primary response to IL-1. Activation of PI3 kinase signaling (Akt phosphorylation) is apparent in epithelial cells, but does not appear to be stimulated by IL-1 α in monolayer epithelial culture.

Jak-STAT signaling was apparent in the stroma of wild-type 5-day-old mice, but was decreased in IL-1R1 ($^{-/-}$) animals [Fig. 5 A, B]. Stromal activation of Jak-STAT signaling during inflammation was attenuated in IL-1R1 ($^{-/-}$) mouse prostates [Fig. 5 C-E], and IL-1 α treatment of the cultured UGS activated Jak-STAT signaling specifically in the mesenchyme [Fig. 5F]. In contrast to the cell culture studies described above, IL-1 treatment of the UGS also resulted in epithelial PI3K-Akt pathway activation, suggesting a paracrine mechanism of activation that depends on Jak-STAT signaling. This was confirmed by treating organ cultures with IL-1 α in

with or without inhibition of Jak or PI3K [Fig. 5 H-J]. Inhibition of PI3K blocked epithelial expansion whereas Jak inhibition decreased both epithelial budding and stromal expansion. The selective activation of IL-1R1 in the prostatic stroma, the cell and organ culture studies suggesting direct activation of stromal Jak-STAT and indirect activation of epithelial PI3K-Akt pathway signaling by IL-1, and the dichotomous effect of the Jak and PI3K inhibitors are most easily explained by a paracrine signaling loop in which IL-1 acts upon the stroma in a Jak-dependent fashion to stimulate epithelial PI3K-Akt signaling.

IL-1 treatment of UGS in organ cultures activated the IGF1R in the UGS epithelium as determined by tyrosine phosphorylation of the IGF1R [Fig. 6A]. This suggests that IL-1 stimulates paracrine IGF signaling and, consistent with this interpretation, concurrent treatment with Jak inhibitors abrogated epithelial IGF1R activation [Fig. 6B]. Developing prostates (P5) in vivo expressed epithelial-specific IGF1R activation, which was attenuated in the epithelium of IL-1R1 knockout mice [Fig. 6 C, D]. In cultured E16 UGS tissues treated with IL-1, antagonists to the primary IGF-1 receptor (IGF1R) attenuated IL-1-induced epithelial growth [Figure 6 E, F; fig. S8]. This is consistent with the observation that IGF-1 induces prostate epithelial growth in organ culture [Fig. 6G]. Using pathway inhibitors, we found that IGF-1-induced epithelial growth depends on PI3K but not on Jak-STAT signaling, echoing the selective effect of PI3K inhibition on IL-1 induced epithelial growth [Fig. 6 I, J; fig. S8].

In summary, the data presented in this section indicate that IL-1 ligands are produced by the epithelium and act on the stroma, leading to stromal production of IGF through the Jak-STAT pathway. Stromally-produced IGF acts directly on epithelial cells to stimulate their proliferation through the PI₃K pathway (Fig. 7).

Discussion

Various cytokines generally considered to be inflammatory mediators are present during organogenesis. They are found in the absence of inflammatory cells; rather, they are produced by the developing tissues themselves. The functional importance of interleukin expression in organ development is shown by the growth-inducing effects of IL-1-family cytokines in culture and the hypomorphic phenotype of the IL-1R KO mouse prostate. Thus, cytokines considered to have a primary role in inflammation may play a role as growth factors during development. In previous work that explored a distinct but potentially related observation, mice with the immune and inflammatory mediator colony stimulating factor (CSF) loss of function and associated macrophage deficiency showed decreased breast and brain development.²² CSF receptors are found in the developing prostate and in prostate cancer cells,²³ and our array showed significant expression of CSF-2 and 3 ligands during development.

IL-1 α , IL-1 β , and IL-18 mRNA expression, peptide content, and peptide release were markedly increased three days after bacterial infection. The inducibility of IL-1 β and IL-18 during inflammation, combined with their minimal expression during development relative to that of IL-1 α , suggests differential roles for these IL-1 peptides. This was supported by in situ hybridization studies showing differing expression patterns for the two peptides during prostate inflammation. We postulate that IL-1 α may be a primary growth mediator in the prostate whereas the role of IL-1 β and IL-18 may be limited to immune function and the inflammatory response. Future studies should elucidate the role of specific inflammatory mediators in development versus their role in the immune response.

Our data demonstrate that the mechanism by which IL-1 induces epithelial growth during development involves at least in part induction of pathways known to be involved in development. In particular, IGF stimulates epithelial growth, and IGF-1 null mice show multiple developmental phenotypes, including attenuated prostatic development, similar to the

phenotypic effects of IL-1R1 loss of function demonstrated here.^{24,25} Our data further indicate that the IL-1 to IGF signaling loop involves epithelial to stromal signaling interactions, in which IL-1 signals from epithelium to the stroma to produce IGF, which signals back to the epithelium to stimulate epithelial expansion [Fig. 7]. Epithelial-stromal signaling loops are common in developmental biology, and have also been implicated in cancer growth. Here, we add the premise of epithelial-leukocytic-stromal interactions during inflammation, resulting in reactive hyperplasia.

Castration and testosterone-implanted regrowth provides an interesting model to assess prostate growth. It would be intriguing to know how mice lacking the IL-1 receptor respond to castration, and to testosterone-mediated regrowth. However, this is actually rather complicated. Castration of wild-type mice elicits an inflammatory response in the mouse prostate²⁶, which may be due to loss of androgen-dependent tight-junction proteins.²⁷ IL-1 abundance is likely increased in this context; thus if IL-1R1 (^{-/-}) mice show decreased regrowth, it may reflect their inability to respond to inflammation, rather than an effect on growth.

Re-activation of developmental signaling pathways such as those mediated by Hedgehog and Wnt signaling occurs in the regenerative response to injury and plays an important role in the proliferation of putative progenitor cells.²⁸ Putative progenitor cells exist in the basal layer of the prostate epithelium, and their precisely regulated proliferation is responsible for epithelial expansion during development and for tissue regeneration in response to injury.²⁹ It is also these cells that have been proposed to retain potentially tumorigenic mutations due to their lack of differentiation.²⁹ Similarly, our studies suggest that IL-1-family cytokines, which mediate growth in normal development, exercise a similar activity in the inflammatory response to injury either when produced by the tissue itself (IL-1 α) or from infiltrating inflammatory cells such as macrophages, neutrophils, or lymphocytes. The ability of cytokines to directly stimulate P63⁺ prostate epithelial cell proliferation not only explains the progressive hyperplasia we observe in inflammation but also suggests a mechanism by which inflammation may expand the pool of progenitor cells. These findings, in the context of the link between inflammatory signaling and tumor growth,⁹ suggest that the hyperplasia associated with chronic inflammation, and the trophic effect of inflammatory mediators on tumor growth, may reflect a fundamental symmetry in the biological processes of development, inflammatory reactive hyperplasia, and cancer, and may depend on a conserved role of inflammatory mediators in promoting epithelial proliferation in all three.

Materials and Methods

Gene Array for analysis of inflammatory mediator mRNA expression

Prostate and urogenital sinus tissues were collected from CD-1 male mice at ages of embryonic day E16 (8 pooled per data point), postnatal day P1 (5 pooled per data point), P5 (3 pooled per data point), P21, and the dorsolateral lobe of 8 week old adults. Additional tissues were collected from 8 week-old adult mice infected with *E. coli* for 3 days as a positive control for interleukin expression. Total RNA was isolated from all tissues with Qiagen RNeasyTM kits according to the manufacturer (Qiagen, Valencia, CA), and cDNA was made using reverse transcription as previously described.¹⁶ We used the SuperArrayTM Inflammatory Cytokines and Receptors hybridization gene array for mouse (SuperArray Biosciences, Fredrick, MD); the assay was conducted according to manufacturer's directions, and development was performed using chemiluminescence and X-ray film as directed by the manufacturer. Quantification of spots was performed with GEarray expression analysis suite software (SuperArray). Densitometry readings are expressed as ratio of gene to ribosomal S27. Comparisons between developmental time points to 8 week-old adult prostates were made with analysis of variance (ANOVA), with $p < 0.05$ indicative of significant difference.

mRNA expression of IL-1 family members by RT-PCR

Prostate and UGS tissues were collected from CD-1 male mice at ages of E16 (8 pooled per data point), P1 (5 pooled per data point), P5 (3 pooled per data point), P21, and 8 week old naïve and infected adults. Total RNA was isolated from all tissues with Qiagen RNeasy™ kits according to the manufacturer (Qiagen), and cDNA was made and RT-PCR performed as previously described.¹⁶ Cycle to threshold was calculated as previously described, and expression of all interleukins of interest was calculated as a ratio to ribosomal S27 expression. Comparisons between developmental time-points to 8 week-old adult prostates were made with analysis of variance (ANOVA), with $p < 0.05$ indicative of significant difference.

Protein content and release of IL-1 family members by ELISA

Prostate and UGS tissues were collected from CD-1 male mice at ages of E16 (10 pooled per data point), P1 (6 pooled per data point), P5 (4 pooled per data point), P10 (3 pooled per data point), P15 (2 pooled per data point), P21, and 8 week-old naïve and infected adults. Mediator release was analyzed as previously described:³² tissues were equilibrated for 1 hour in aerated Krebs physiological salt solution, with buffer changes every 15 minutes. At the end of the equilibration period, tissues were incubated in fresh aerated Krebs for 30 minutes. Following the experiment, Krebs was collected and frozen as the released fraction. The tissue was then homogenized in fresh buffer; the resulting slurry was incubated with TritonX-100 at a final concentration of 0.1% and incubated on ice for 30 minutes. The homogenate was centrifuged at 16,000xG for 30 minutes and supernatant was collected as total tissue content. All collections were analyzed by enzyme-linked immunosorbent assay (ELISA) for IL-1 α , IL-1 β , and IL-18 concentrations as recommended by the manufacturer (Biosource; Camarillo, CA). Absorbance readings for each concentration were analyzed relative to a standard curve of known IL-1 concentrations. Comparisons between developmental time points to 8 week-old adult prostates were made with analysis of variance (ANOVA), with $p < 0.05$ indicative of significant difference.

mRNA localization by in situ hybridization

Urogenital sinuses or prostate lobes were fixed for 24 hours in 4% paraformaldehyde, stored in 100% methanol at -80°C, hydrated through methanol gradient to PBS, embedded in 4% agarose, and cut into 80 micron sections using a Vibratome™. Sectional ISH was performed as previously described.³³ Mouse-specific digoxigenin-incorporated probes were constructed using PCR of cDNA synthesized from each IL-1 mRNA and the Roche digoxigenin incorporation system (Mannheim, Germany). Following ISH, digoxigenin was visualized using immunohistochemistry with the Roche digoxigenin detection system (Mannheim, Germany). Stained sections were mounted on glass slides and visualized by light microscopy for cellular localization of IL-1 mRNA.

Protein localization by immunohistochemistry

Urogenital sinuses or prostate lobes were fixed in phosphate-buffered formalin overnight, processed and embedded in paraffin, and 5 micron sections were made with a microtome. Tissues were mounted and heat fixed to glass slides, deparaffinized in xylenes and methanol gradient, and subjected to heat-induced antigen retrieval in citrate buffer for 15 minutes. Sections were blocked with BSA/serum mixture for 3 hours and incubated with primary antibody overnight at 4°C. Antibodies and dilutions used for this study were: rabbit α CD-3 (1:200-Dako Pathology, Dako Denmark), rabbit α CD-20 (1:50, BD Pharmingen, San Jose, CA), rabbit α P63 (1:100, Santa Cruz, CA), α P-T705-STAT3 (Cell Signaling Technologies, Danvers, MA), P-T308-Akt (1:100, Cell Signaling), rabbit α P-I κ B (Imgenex, San Diego, CA), mouse α -p65 (1:100, Abcam, Cambridge, MA), rabbit α -P-Y495-IL-1R (1:50, Abcam), rabbit α -P-Y1161-IGFR1 (1:200, Abcam). Sections were washed with TBS-Tween and incubated

with Alexa-488-conjugated anti-rabbit secondary antibody for 1 hour at 20–25°C. Sections were washed and incubated with 4 mg/ml Hoechst counterstain for 10 minutes. Tissues were washed and covered with aqueous medium and glass coverslips. Tissue sections were analyzed by immunofluorescence and positive and negative cells were determined.

Protein quantification by immunoblotting

Whole prostate tissue or isolated prostate cells were homogenized in lysis buffer containing protease inhibitor (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM benzenesulfonyl fluoride, and 10 µg/ml each of aprotinin, bestatin, L-leucine, and pepstatin A). Triton X-100 was added to a concentration of 1%, and the homogenate was incubated on ice for 60 minutes, followed by centrifugation for 20 min at 14,100xG at 4°C. The supernatant was collected and total protein concentration was determined by BCA assay (Pierce, Rockford, IL). Proteins (20 µg/well) were resolved by electrophoresis in 4–20% gradient SDS-polyacrylamide electrophoresis gels. Proteins were transferred to PVDF membranes, blocked overnight [10 g/L nonfat dry milk, 10 g/L bovine serum albumin, and 0.5 g/L NaN₃ in 1× phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 136 mM NaCl, 8 mM Na₂HPO₄) + 0.05% (v/v) Tween 20] and incubated for 16 hours with one of the following primary antibodies: αP-T705-STAT3 (Cell Signaling Technologies, Danvers, MA), P-T308-Akt (1:100, Cell Signaling), rabbit αP-IκB (Imgenex, San Diego, CA), mouse α-p65 (1:100, Abcam, Cambridge, MA), rabbit α-P-Y495-IL-1R (1:50, Abcam), rabbit α-P-Y1161-IGFR1 (1:200, Abcam). After washing six times in PBS + 0.05% Tween 20, blots were incubated with goat anti-rabbit IgGs conjugated to horseradish peroxidase for one hour (1:200,000 dilution, Pierce, Rockford, IL) in 2.5 g/L nonfat dry milk, PBS, and 0.05% Tween 20. Peroxidase activity was detected via West Femto[®] chemiluminescence reagent as directed by the manufacturer (Pierce). Photo images were analyzed by densitometry and ratios of protein of interest to GAPDH were determined and compared between treatments.

Ex vivo analysis of IL-1-induced prostatic growth in organ culture

Urogenital sinus tissues were harvested from mouse embryos at embryonic day 16 and grown in organ culture conditions as previously described.¹⁵ Tissues were grown with serum free medium (DMEM/F12 media ± 10 µM cyclopamine supplemented with 2% insulin–transferrin–selenium (ITS), 25 µg/ml gentamycin, 0.25 µg/ml amphotericin B) for 1 to 7 days in the presence or absence of dihydrotestosterone (Sigma-Aldrich, St. Louis, MO) or interleukin-1 family members (1, 10, 100 ng/ml-Cell Signaling). Experiments were conducted with or without antagonism or pathway inhibition, in which case the antagonist was added 1 hour prior to addition of growth-inducing ILs. Growth rate was determined by inverted light microscopy and 2-dimensional growth was quantified by pixel counts. Following the experiments, tissues were either paraffin-embedded for histological evaluation or snap frozen for molecular evaluation. Total growth for each IL-1 and DHT was compared and statistical analysis by analysis of variance was performed.

Analysis of developmental prostate phenotype in IL-1R1 (–/–) mice

We dissected adult male C57BK or IL-1R1 (–/–) mice (The Jackson Laboratory, Bar Harbor, ME), at 21 days, 8 weeks, and 12 weeks of age. Each lobe of the prostate was removed and fixed in 70% ethanol overnight at 4°C. Each lobe was analyzed for wet weight and overall size. Lobes were incubated on ice in PBS + collagenase for 30 minutes and microdissected as previously described, and total number of duct tips and branch points were counted. Numbers for each lobe, WT versus IL-1R1 (–/–) were compared by unpaired student's T-test.

In vivo induction of inflammation and assessment of reactive hyperplasia

We instilled *E. coli* strain 1677 (2×10^6 /ml-100 μ l per mouse) through catheters into the urinary tract of C57BK WT and IL-1R1 ($^{-/-}$) mice at 8 weeks of age. Mice were inoculated with 100 mg of BrdU (Roche) 1 hour prior to sacrifice at 1, 2, 3, 5, 7, or 14 days post-infection and their prostates and urinary bladders were removed. Saline instilled animals were used as controls. Tissues were analyzed for validation of infection by colony counting as previously described. Additional tissues were paraffin-embedded for histological and immunohistochemical analysis, snap frozen for molecular analysis, or incubated in Krebs buffer for release experiments as described above.

Testicular testosterone production

The testicles of mice at embryonic day 17, post-natal day 1, 10, and adult were harvested, homogenized, and analyzed for total testosterone content by ELISA in conditions recommended by the manufacturer (Assay Designs, Ann Arbor, MI). Wild-type and IL-1R1 ($^{-/-}$) mice were compared at all time points of development by student's T-test.

Cell lines and cell culture experiments

E6 and **E7** prostate epithelial cells were kind gifts from Dr. David Jarrard, Department of Urology, University of Wisconsin Madison, as previously published.³⁰ Briefly, prostate tissue was minced with a scalpel, digested in a 500 units/ml collagenase solution, and plated on collagen-coated plates in Ham's F-12 medium (Invitrogen) supplemented with free insulin, 0.25 units/ml; human transferrin, 5 μ g/ml; dextrose, 2.7 mg/ml; nonessential amino acids, 0.1 mM; penicillin, 100 units/ml; streptomycin, 100 μ g/ml; L-glutamine, 2 mM; cholera toxin, 10 ng/ml; bovine pituitary extract, 25 μ g/ml; and 1% fetal bovine serum. These low serum conditions, and the presence of cholera toxin, selected against the culturing of stromal cells, as confirmed by RT-PCR for epithelial and stromal markers.³⁰ Infections and characterization of HPEC cell lines were carried out as described using retrovirus constructs carrying either the HPV16 E6 or E7 genes. Immortalized cell lines were screened for HPV16 E6 or E7 protein expression or both and for loss of p53 and pRB by Western blot analysis. All immortalized lines were cultured for over 20 passages to confirm their immortality. Stromal cells were generated from adult mouse prostate lobes using a previously published protocol.³¹ Briefly, immortalized stromal cells were derived from the ventral or dorsal-lateral lobes of INK4a $^{-/-}$ β -actin-tva transgenic mice, a kind donation from Bart Williams (Van Andel Research Institute, Grand Rapids, MI).³¹ UGS epithelium was separated from stroma following trypsin digestion and dissociated into single cells by digestion in 0.5% collagenase and dissociated stromal cells were grown in DMEM + 15% FBS + 1% pen/strep overnight to attachment. Prostatic epithelial cells failed to attach during this time frame, selecting for stromal cells. Stromal cells were grown to confluence in a 6-well plate. The stromal nature of the cells was confirmed by RT-PCR for smooth muscle actin, vimentin, and the complete lack of expression of probasin, cytokeratin 14, and cytokeratin 8, all epithelial markers. Thereafter cells were grown in DMEM/F12 + 10% FBS + 1% Pen/Strep + 1% ITS + 10 nM DHT (INK4 culture medium).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Donjacour AA, Cunha GR. Stromal regulation of epithelial function. *Cancer Treat Res* 1991;53:335. [PubMed: 1672086]
2. Krtolica A, Campisi J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *Int J Biochem Cell Biol* 2002;34:1401. [PubMed: 12200035]

3. Katoh M. WNT signaling in stem cell biology and regenerative medicine. *Curr Drug Targets* 2008;9:565. [PubMed: 18673242]
4. Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. *Stem Cell Rev* 2007;3:30. [PubMed: 17873379]
5. Puchelle E, Zahm JM, Tournier JM, Coraux C. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006;3:726. [PubMed: 17065381]
6. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357
7. Allavena P, Garlanda C, Borrello MG, Sica A, Mantovani A. Pathways connecting inflammation and cancer. *Curr Opin Genet Dev* 2008;18:3. [PubMed: 18325755]
8. Hold GL, El-Omar ME. Genetic aspects of inflammation and cancer. *Biochem J* 2008;410:225. [PubMed: 18254728]
9. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. *Nat Rev Cancer* 2007;7:256. [PubMed: 17384581]
10. Sciavolino PJ, Abate-Shen C. Molecular biology of prostate development and prostate cancer. *Ann Med* 1998;30:357. [PubMed: 9783834]
11. Ricote M, García-Tuñón I, Bethencourt FR, Fraile B, Paniagua R, Royuela M. Interleukin-1 (IL-1 α and IL-1 β) and its receptors (IL-1RI, IL-1RII, and IL-1Ra) in prostate carcinoma. *Cancer* 2004;100:1388. [PubMed: 15042672]
12. Sims NA, Jenkins BJ, Nakamura A, Quinn JM, Li R, Gillespie MT, Ernst M, Robb L, Martin TJ. Interleukin-1 receptor signaling is required for normal bone remodeling. *J Bone Miner Res* 2005;20:1093. [PubMed: 15940362]
13. Ishikawa T, Hwang K, Lazzarino D, Morris PL. Sertoli cell expression of steroidogenic acute regulatory protein-related lipid transfer 1 and 5 domain-containing proteins and sterol regulatory element binding protein-1 are interleukin-1 β regulated by activation of c-Jun N-terminal kinase and cyclooxygenase-2 and cytokine induction. *Endocrinol* 2005;146:5100.
14. Petersen C, Svechnikov K, Fröysa B, Söder O. The p38 MAPK pathway mediates interleukin-1-induced Sertoli cell proliferation. *Cytokine* 2005;32:51. [PubMed: 16181786]
15. Cook C, Vezina CM, Allgeier SH, Shaw A, Yu M, Peterson RE, Bushman W. Noggin is required for normal lobe patterning and ductal budding in the mouse prostate. *Dev Biol* 2007;312:217. [PubMed: 18028901]
16. Lamm ML, Podlasek CA, Barnett DH, Lee J, Clemens JQ, Hebner CM, Bushman W. Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate. *Dev Biol* 2001;232:301. [PubMed: 11401393]
17. Subramaniam S, Stansberg C, Cunningham C. The interleukin 1 receptor family. *Dev Comp Immunol* 2004;28:415. [PubMed: 15062641]
18. Abcouwer SF, Norman J, Fink G, Carter G, Lustig RJ, Souba WW. Tissue-specific regulation of glutamine synthetase gene expression in acute pancreatitis is confirmed by using interleukin-1 receptor knockout mice. *Surgery* 1996;120:255. [PubMed: 8751591]
19. Vezina CM, Allgeier SH, Moore RW, Lin TM, Bemis JC, Hardin HA, Gasiewicz TA, Peterson RE. Dioxin causes ventral prostate agenesis by disrupting dorsoventral patterning in developing mouse prostate. *Toxicol Sci* 2008;106(2):488–96. [PubMed: 18779384]
20. Elkahwaji JE, Zhong W, Hopkins WJ, Bushman W. Chronic bacterial infection and inflammation incite reactive hyperplasia in a mouse model of chronic prostatitis. *Prostate* 2007;67:14. [PubMed: 17075821]
21. Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochim et Biophys Acta* 2002;1592:265.
22. Pollard JW. Role of colony-stimulating factor-1 in reproduction and development. *Mol Reprod Dev* 1997;46:54–60. [PubMed: 8981364]
23. Ide H, Seligson DB, Memarzadeh S, Xin L, Horvath S, Dubey P, Flick MB, Kacinski BM, Palotie A, Witte ON. Expression of colony-stimulating factor 1 receptor during prostate development and prostate cancer progression. *Proc Natl Acad Sci U S A* 2002;99:14404. [PubMed: 12381783]

24. Ruan W, Powell-Braxton L, Kopchick JJ, Kleinberg DL. Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development. *Endocrinology* 1999;140:1984. [PubMed: 10218945]
25. Kleinberg DL, Ruan W, Yee D, Kovacs KT, Vidal S. Insulin-like growth factor (IGF)-I controls prostate fibromuscular development: IGF-I inhibition prevents both fibromuscular and glandular development in eugonadal mice. *Endocrinology* 2007;148(3):1080. [PubMed: 17138659]
26. Palapattu GS, Meeker A, Harris T, Collector MI, Sharkis SJ, DeMarzo AM, Warlick C, Drake CG, Nelson WG. Epithelial architectural destruction is necessary for bone marrow derived cell contribution to regenerating prostate epithelium. *J Urol* 2006;176:813. [PubMed: 16813953]
27. Meng J, Mostaghel EA, Marks LS, Nelson PS. The impact of androgen deficiency on tight junction architecture and prostate inflammation. *J Urol* 2009;181(4 supplement):A1414.
28. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. *Nature* 2004;432:324–331. [PubMed: 15549094]
29. Shen MM, Wang X, Economides KD, Walker D, Abate-Shen C. Progenitor cells for the prostate epithelium: roles in development, regeneration, and cancer. *Cold Spring Harb Symp Quant Biol* 2008;73:529–38. [PubMed: 19150960]
30. Schwarze SR, DePrimo SE, Grabert LM, Fu VX, Brooks JD, Jarrard DF. Novel pathways associated with bypassing cellular senescence in human prostate epithelial cells. *J Biol Chem* 2002;277(17):14877–14883. [PubMed: 11836256]
31. Shaw A, Papadopoulos J, Johnson C, Bushman W. Isolation and characterization of an immortalized mouse urogenital sinus mesenchyme cell line. *Prostate* 2006;66(13):1347–1358. [PubMed: 16752376]
32. Jerde TJ, Calamon-Dixon JL, Bjorling DE, Nakada SY. Celecoxib inhibits ureteral contractility and prostanoid release. *Urology* 2005;65(1):185. [PubMed: 15667901]
33. Vezina CM, Allgeier SH, Moore RW, Lin TM, Bemis JC, Hardin HA, Gasiewicz TA, Peterson RE. Dioxin causes ventral prostate agenesis by disrupting dorsoventral patterning in developing mouse prostate. *Toxicol Sci* 2008;106(2):488–96. [PubMed: 18779384]
34. The authors gratefully acknowledge Megan M. Landry and Eliza K. McFarland for conducting RT-PCR experiments, Drs. Chad M. Vezina, and Robert W. Moore for technical expertise and design of in situ hybridization experiments and probes, Crist H. Cook for expertise in conducting prostate organ culture experiments, Jee Hang Tse for optimizing immunohistochemical antibodies and procedures, Johnny Elkahwaji for demonstrations on prostatic instillations, Jonathan A. Ewald and David F. Jarrard for generation of immortalized prostate epithelial cells, Diana M. Pitterle and Aubie K. Shaw for development of immortalized prostate stromal cells, and Xu Dong Shi, Jerry J. Gipp, Robert Lipinski, Min Yu, Dale E. Bjorling, Richard E. Peterson, Norman Drinkwater, and John Fallon for discussions of the data and suggestions on experimental design. This work is funded by the Prostate Cancer Foundation, the Department of Defense, and National Institutes of Health-NIDDK. T.J.J. was also funded by the Department of Molecular and Environmental Toxicology through training grant from the National Institute of Environmental Health Sciences (NIEHS). T.J.J. and W.B. jointly conceived the hypothesis of this work. T.J.J. designed the experiments, analyzed the data, and wrote the manuscript with input and suggestions from W.B.

Fig. 1. Interrelationship between inflammatory and developmental signaling

mRNA encoding the inflammatory mediators IL-1 α [A] and IL-1 β [B] is more highly expressed during development (E16, P1, P5, P10) than in adulthood. Data are mean $\pm$ s.e.m. * p <0.05 versus 8 week adult; $\blacklozenge$ p <0.05 versus P10; comparisons using analysis of variance (ANOVA), n =6. [C] IL-1 α peptide synthesis and release are increased during development and inflammation. Total bar height reflects total amount of IL-1 peptide in tissue plus that released into the medium; the white portion reflects concentration in the tissue; the dark portion reflects concentration released. [D] IL-1 β synthesis is increased during inflammation. Released data are pg/g tissue protein per 30 minutes of release time; total tissue concentrations are pg/g tissue

protein. * $p < 0.05$ versus 8 week adult; $\blacklozenge p < 0.05$ inflamed versus normal adult (ANOVA), $n=4$, all data points. [E] *IGF-1*, *PDGF*, *Shh*, *Gli1*, and *TGF β 1* and 3 mRNA expression is reactivated during inflammatory reactive hyperplasia. * $p < 0.05$ inflamed versus normal adult (ANOVA), $n=4$, all data points. [F, G, H] IL-1 increases expression of developmental mediators TGF β s and IGFs in isolated prostate stromal cells [F,G] and in cultured whole tissue [H]. * $p < 0.05$ inflamed versus control adult; (ANOVA), $n=4$, all data points.

Fig. 2. Interleukin-1 promotes epithelial and stromal growth in cultured tissues and organ development in vivo

[A-D] Ex vivo organ culture of E16 urogenital sinus cultured for 7 days in serum free media supplemented with vehicle [A], 10 nM dihydrotestosterone [B], or 100 ng/ml IL-1 [C]. Dashed line in B and C shows boundary between epithelium and stroma. IL-1- induced growth was blocked by simultaneous addition of the IL-1R1 antagonist IL-1RA [D]. [E, F] Decreased growth and branching in ventral prostates from IL-1R1^{-/-} mice [F] relative to wild-type controls [E]. [G] Average lobe weights for all lobes. [H] Quantitative analysis of decreased ductal branching in the ventral prostate and dorsolateral prostate of IL-1R1^{-/-} mice determined by micro-dissection. Data presented are mean $\pm$ s.e.m. * $p < 0.05$ wild-type (WT) versus IL-1R1^{-/-}, ANOVA, $n = 6$, all groups.

Fig. 3. IL-1R1^{-/-} mice show a reduced hyperplastic proliferative response to inflammation
[A, D] Normal histologic architecture and low proliferation index was observed in the normal mouse prostate, determined by routine H&E staining and immunohistochemical staining for BrdU (green) and Pan-cytokeratin (red). Brackets indicate the range of epithelial thickness, demonstrating the thickness of the hyperplastic epithelium. Note also the cellular atypia of cells in inflamed prostatic ducts (arrows). **[B, E]** Wild-type mice show epithelial proliferation and hyperplasia 3 days after inoculation of *E. coli*. **[C, F]** Attenuated epithelial proliferation and hyperplasia was observed in IL-1R1^{-/-} mice 3 days after bacterial inoculation. Quantified proliferation rates are shown in panel **[G]** as the number of BrdU⁺ cells per duct in each lobe, and compared across inflamed and normal wild-type (WT) and IL-1R1^{-/-} mice. Data presented are mean ± s.e.m. *p < 0.05 inflamed WT versus control WT; ◆p < 0.05 inflamed IL-1R1^{-/-} versus inflamed WT, comparisons via ANOVA. Quantification of BrdU⁺ cells was determined by analyzing a minimum of 8 ducts per lobe and 6 lobes per data point (n=6).

Fig. 4. IL-1 α treatment activates Jak-STAT signaling in cultured stromal cells

Two prostate stromal cell lines (isolated from ventral [v] and dorsal-lateral [d] lobes) and two prostate epithelial cell lines (E6 and E7) were treated with 10 ng/ml IL-1 α or vehicle (Veh, 0.2% BSA), as indicated, for 1 hour. Optimal concentrations and time points were determined in preliminary experiments. Whole protein extracts were analyzed for Jak-STAT and Akt activation (phosphorylation) by phospho-specific antibodies (P-705-STAT-3 or P-S473-Akt). GAPDH used as a loading control.

Fig. 5. IL-1-induced growth involves Jak-STAT signaling in the mesenchyme and stroma, and PI3K signaling in the epithelium

Activation of STAT-3 as detected by phosphorylation of STAT-3 [green] is evident in the mesenchyme of developing (P5) wild-type mouse prostates [A]. Arrows indicate cells exhibiting positive staining. Dashed lines outline the epithelial ducts. P5 IL-1R1 ($^{-/-}$) mouse prostates show reduced STAT-3 activation [B]. Normal adult prostate [C] shows little STAT-3 phosphorylation, but activation is visible in inflamed adult mouse prostates [D]. STAT-3 phosphorylation is reduced in inflamed IL-1R1 ($^{-/-}$) mice [E]. Jak-STAT signaling is apparent in mesenchymal cells (Mes) of IL-1-treated organ cultures [F]. IL-1 treatment induced PI3K signaling in the epithelium of organ cultures as (Epi) measured by phosphorylated Akt [G, red cytoplasm]. Inhibition of PI3K results in a loss of IL-1-induced epithelial (Epi) expansion [I] relative to IL-1 alone [H], inhibition of Jak attenuates both stromal (Str) and epithelial (Epi) expansion [J]. Dashed lines outline the epithelial ducts.

Fig. 6. IL-1-driven epithelial expansion involves stromal IGF-1 induction and a signaling loop to the epithelium

Exogenous IL-1 activates IGF-1R (IGF-1R phosphorylation) specifically in the epithelium (Epi), as measured by phosphotyrosine 1161-specific antibodies to IGF-1R [A, green membranes]. Co-incubation with a Jak inhibitor attenuates IL-1-induced IGF-1R. Dashed lines outline the epithelial ducts [B]. Activation of IGF-1R is apparent in P5 developing prostate [C] but reduced in IL-1R1^{-/-} mice [D]. Epithelial (Epi) and stromal (Str) growth of UGS organ cultures following 7 days of incubation with 10 ng/ml IL-1α [E]. Inhibition of IGF-1 receptor signaling (picropodophyllin, 100 ng/ml) attenuates epithelial expansion but not stromal growth induced by IL-1 [F]. IGF-1 induces epithelial but not stromal expansion in

organ culture [G], and is antagonized by IGF-1R antagonism [H]. IGF-1 induced epithelial growth is attenuated by PI3K inhibition [I], but not by Jak-STAT inhibition [J].

Fig. 7.

Proposed model of IL-1 signaling in prostate. IL-1 family members expressed in the epithelium during prostate development or either epithelium or leukocytes during inflammation signal to the stroma where IL-1R1 is present. Stimulation of IL-1R1 activates Jak-STAT signaling, leading to the expression of genes, including (but not limited to) IGF. IGF signals to the epithelium in both developing and hyperplastic prostates, resulting in PI3K-Akt pathway activation, epithelial proliferation, and tissue expansion.