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Inflammation and Pyroptosis Mediate Muscle Expansion in an Interleukin-1β (IL-1β)-dependent Manner*[S]

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Background: Hyperplasia is a common phenomenon in inflamed muscle.
Results: IL-1β secreted associated with pyroptotic cell death mediate IGF1-dependent detrusor expansion.
Conclusion: Bladder muscle hyperplasia resulting from NLRP3 inflammatory cascade can be attenuated by neutralization of IL-1β and IGF1.
Significance: Antagonizing IL-1β can have a therapeutic benefit for subjects with muscle hyperplasia resulting from chronic inflammatory diseases.

Muscle inflammation is often associated with its expansion. Bladder smooth muscle inflammation-induced cell death is accompanied by hyperplasia and hypertrophy as the primary cause for poor bladder function. In mice, DNA damage initiated by chemotherapeutic drug cyclophosphamide activated caspase 1 through the formation of the NLRP3 complex resulting in detrusor hyperplasia. A cyclophosphamide metabolite, acrolein, caused global DNA methylation and accumulation of DNA damage in a mouse model of bladder inflammation and in cultured bladder muscle cells. In correlation, global DNA methylation and NLRP3 expression was up-regulated in human chronic bladder inflammatory tissues. The epigenetic silencing of DNA damage repair gene, Ogg1, could be reversed by the use of demethylating agents. In mice, demethylating agents reversed cyclophosphamide-induced bladder inflammation and detrusor expansion. The transgenic knock-out of Ogg1 in as few as 10% of the detrusor cells tripled the proliferation of the remaining wild type counterparts in an *in vitro* co-culture titration experiment. Antagonizing IL-1β with Anakinra, a rheumatoid arthritis therapeutic, prevented detrusor proliferation in conditioned media experiments as well as in a mouse model of bladder inflammation. Radiation treatment validated the role of DNA damage in the NLRP3-associated caspase 1-mediated IL-1β secretory phenotype. A protein array analysis identified IGF1 to be downstream of IL-1β signaling. IL-1β-induced detrusor proliferation and hypertrophy could be reversed with the use of Anakinra as well as an IGF1 neutralizing antibody. IL-1β antagonists in current clinical practice can exploit the revealed mechanism for DNA damage-mediated muscular expansion.

Muscle inflammation and associated expansion is well recognized in multiple tissues with significant morbidity and mortality. Bladder inflammation is a model muscular tissue subjected to inflammation-associated expansion. Fortunately bladder cystitis is normally self limiting; however, it can endure in some patients (1). Conditions such as trauma, microbial, and non-microbial activators can contribute to inflammation. In the United States alone up to 7.9 million women have inflammation-associated bladder function impairment impacting the quality of life. Urothelial changes are striking in bladder inflammation, associated with denudation and ulceration (2). However, the lamina propria and detrusor muscle respond to inflammatory signals in controlling bladder compliance (3). The multifactorial physiological response to inflammation involves epigenetic mechanisms associated DNA and histone methylation, associated with the development of acute as well as chronic responses (4, 5). Pathways evolved to respond to infection are also used to respond to nonmicrobial triggers. Sterile triggers of bladder inflammation are more difficult to address clinically. Regardless of the trigger, an overriding observation in bladder inflammation and that of other muscular tissues include cell death accompanied by hyperplasia. The goal of this project was to better understand the mechanism of this apparent paradox.

The manifestation of bladder hyperplasia associated with inflammation can impact bladder function by reduced bladder compliance. Interestingly, acute inflammatory insults have the ability to impact its primary functions of storing large volumes of urine at low pressures, in protecting the kidneys from pressure-related damage, and efficiently emptying to completion under appropriate social conditions (6). Cyclophosphamide (CPX)[2]-induced urinary bladder inflammation is an established experimental model for bladder pain syndrome (2, 7–9). CPX is a chemotherapeutic agent that metabolizes to acrolein and accumulates in the bladder of patients (10–14). Mesna is administered to patients given CPX in order to sequester the acrolein to limit inflammatory bladder complications (15).

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[S] This article contains supplemental Table 1 and Figs. 1–5.
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[2] The abbreviations used are: CPX, cyclophosphamide; ROS, reactive oxygen species; 5meC, 5-methyl cytosine; 5azadC, 5-azadeoxycytidine; ANOVA, analysis of variance; 8-oxo-dG, 8-oxo-2′-deoxyguanosine.

Acrolein is a naturally occurring mutagenic breakdown product of both carbohydrates and lipids (associated with high temperature cooking); however, acrolein exposure through cigarette smoking is the largest proportion of human exposure (16). There are multiple reports of CPX-induced urinary bladder cystitis associated with an increase in voiding frequency and a decrease in urine volume per void (11, 17, 18). Long term urological complications from radiation have similarities to that of CPX treatment in detrusor DNA damage and symptomatic manifestations. In our study we find that DNA damage in the muscle is sufficient to cause pyroptotic cell death.

We hypothesized that death of a population of muscle cells potentiated the ultimate expansion of the muscle. The NLRP3 cascade product, IL-1β secretion, and its downstream mediator of muscle hyperplasia, IGF-1, was elevated by CPX and radiation. We revealed that DNA damage directly by radiation- or inflammation-induced epigenetic silencing of DNA damage repair enzymes could initiate IL-1β secretion and ensuing bladder expansion. However, another result of the NLRP3 cascade is caspase 1-dependent pyroptotic cell death. Thus, pyroptosis of muscle cell can be concomitant to the expansion of neighboring muscle cells in uncovering a new mechanism for a physiologic manifestation with broad implications. Together, we found that a common rheumatoid arthritis drug antagonizing IL-1 receptor activity could provide therapeutic benefit for bladder inflammation.

MATERIALS AND METHODS

Animal Experiments and Cultured Cells—Female C57BL/6 mice aged 7–10 weeks were housed in a pathogen-free environment at the Cedars-Sinai Medical Center Animal Facility under the approval of the Institutional Animal Care and Use Committee. Mouse bladder inflammation was developed by bilateral ovariectomy (day 0) followed by intraperitoneal injections of CPX (150 mg/kg) treatment on days 23 and 25. Nicotinamide (30 mg/kg, Thermo Fisher Scientific Inc., Waltham, MA) or Mesna (sodium 2-sulfanylethanesulfonate; 150 mg/kg, Sigma) or Anakinra (20 mg/kg, Amgen, Thousand Oaks, CA) treatment were started 3 days before CPX treatment and given up to day 26. All mouse bladders were harvested on day 27.

Primary mouse bladder detrusor cells culture was generated from wild type female C57BL/6 mice, aged 7–10 weeks. The cells were cultured in DMEMF-12 medium (Hyclone, Logan, UT) supplemented with 5% fetal bovine serum (Hyclone), 5% NuSerum (Hyclone), 5 μg/ml insulin (Gibco), and gentamycin (Gibco). The muscle cells were grown in the presence of nicotinamide 200 μM for 48 h before acrolein treatments. Cells were incubated with acrolein (10–50 μM) for 0–6 h.

Immunodetection—Paraffin-embedded tissue sections (5 μm) were deparaffinized and hydrated through xylene and graded alcohols using a standard protocol. Immunohistochemical localization was performed with antibodies against 5-methyl cytosine (5meC; EMD Chemicals, Billerica, MA), F4/80 (AbD Serotec), phosphorylated-p65 (Cell Signaling, Danvers, MA), NLRP3 (LSBio, Seattle, WA), caspase 1 (Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 and 8-oxo-2′-deoxyguanosine (8-oxo-dG; Abcam, Cambridge, MA), and TUNEL (Thermo Fisher Scientific Inc.) as described before (19, 20). All the slides were scanned using Leica SCN400 (Leica Micro System, Buffalo Grove, IL) and analyzed by Tissue IA Optimizer (Leica). The values of positively stained cells were measured in an unbiased manner. IL-1β concentration of cultured medium was assayed by sandwich ELISA using mouse IL-1β ELISA MAX Deluxe Absorbance kit (Biolegend) according to the manufacturer's instructions.

Mouse cytokine antibody arrays C4 (Ray Biotech Inc., Norcross, GA) were used according to the standard protocol (21). Cytokine array membranes were incubated in equal quantities of bladder tissue lysates from mice treated with mock (PBS), cyclophosphamide, and cyclophosphamide plus Anakinra.

Western blots separated by 10, 12, or 15% SDS-polyacrylamide gels were incubated with primary antibodies for γ-H2AX (Millipore, Billerica, MA), Mcl1 (Abcam), IL-1β (R&D Systems, Minneapolis, MN), p53 (Novus Biologicals, Littleton, CO), NLRP3, and caspase 1 (Genetex). The remaining antibodies were from Cell Signaling Inc. (phosphorylated-AKT, LC3, Beclin-1, Survivin) and Santa Cruz (Sirt1, and Bcl2, p16, p21, β-actin). Western blots were visualized using alkaline phosphatase-conjugated secondary antibodies (Sigma).

Measurement of Cell Death and Proliferation by Flow Cytometric Assay—Cell death and proliferation was quantified by flow cytometry (BD Biosciences). 7-Aminoactinomycin D (BD Biosciences) FACS staining was performed in wild type muscle after 6 h of acrolein treatment to examine cell death. Wild type bladder muscle cells were co-cultured with vital dye 5-chloromethylfluorescein diacetate-labeled (CMFDA; Life Technologies) *Ogg1*$^{-/-}$ counterparts in the ratios 1:0, 9:1, 4:1, and 1:1 in 2% serum-containing medium. Anakinra 500 ng/ml was applied every day for 72 h in co-cultured medium containing WT:*Ogg1*$^{-/-}$ muscle (10:1). WT muscle cells were sorted before Ki-67 (Abcam) staining for proliferation by flow cytometry. IGF1 neutralization antibody (R&D Systems) 400 ng/ml was added to conditioned media incubated with wild type bladder muscle cells for flow cytometric analysis for Ki-67 expression. FlowJo software (Tree Star Inc. Ashland, OR) was used for FACS analysis.

5-Azadeoxycytidine (5azadC) Treatment, RNA Isolation, and Real Time PCR—Wild type mouse bladder muscle cells were treated with 5 μM 5azadC (Sigma) for 5 days before acrolein treatment. RNA extraction and cDNA preparation were done by standard methods. Relative quantitation of mRNA expression was performed by $\Delta\Delta$Ct method after quantitative real-time PCR, where β-actin was use as an endogenous control (22–24). The list of primers used is found in the supplemental Table 1.

ChIP Analysis—Chromatin was immunoprecipitated either by normal rabbit IgG antibodies as a negative control or anti-NF-κB antibody similar to that previously reported (25). Non-immunoprecipitated chromatin was used as total input control. DNAs from either the input chromatin or immunoprecipitated chromatin were subjected to PCR analysis using the NF-κB transcription factor binding site (TFBS) targeting primers: 5′-CCCCTAAGAATTCCCATCAAGC-3′ and 5′-GAGCTGTGAAATTTTCCCTTGG-3′.

Statistical Analysis—Experiments were conducted a minimum of three times. Results are expressed as the mean ± S.D.

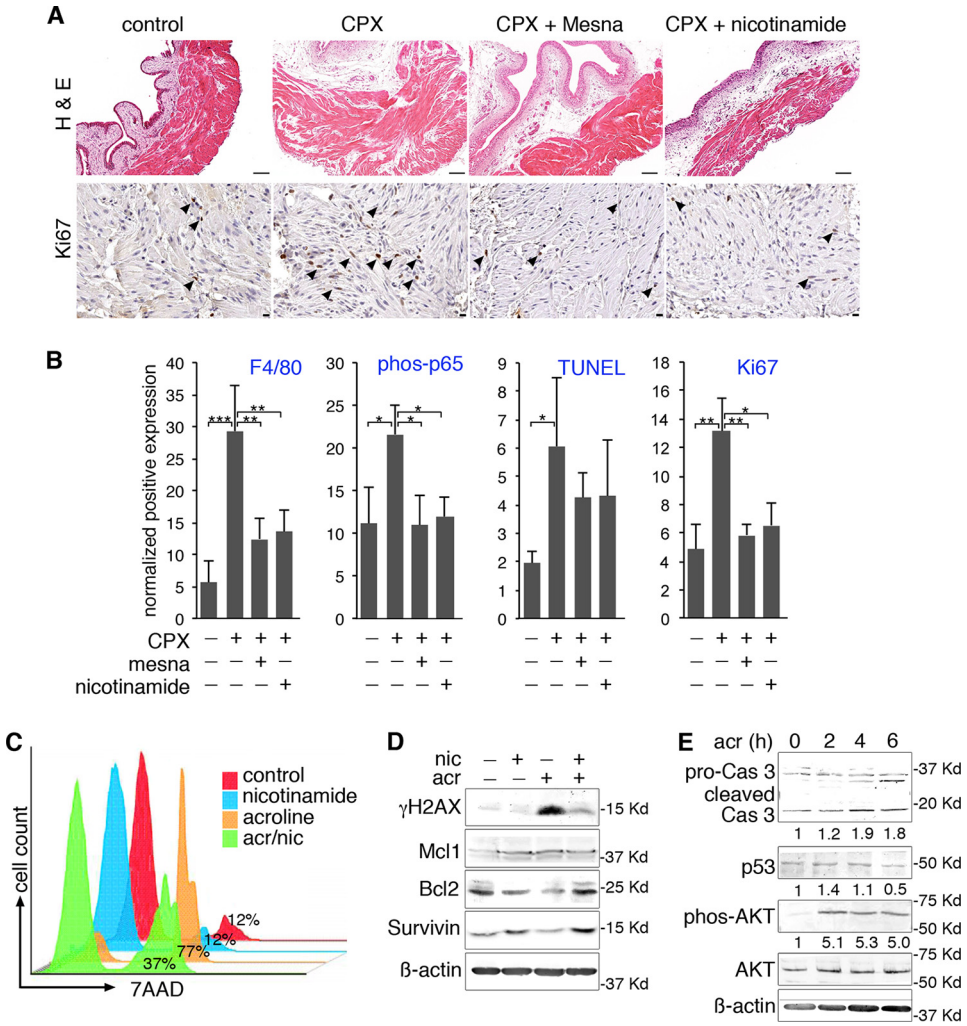

FIGURE 1. **Histological assessment of inflamed mouse bladder tissues and analysis of cell death signaling.** Mice were treated with CPX to induce bladder inflammation. Mesna or nicotinamide treatment was started 72 h before CPX administration. *A*, bladder detrusor expansion was determined by hematoxylin and eosin (H&E) staining (the *scale bar* represents 100 μm) and supported by immunohistochemical detection of Ki-67 expression (*arrowheads*) in the presence or absence of cyclophosphamide and Mesna or nicotinamide (the *scale bar* represents 10 μm). *B*, immunohistochemical localization of F4/80, phosphoryated-p65, TUNEL, and Ki-67 expression in the detrusor muscle was quantitated as a percentage of total cells per field. Data represent the mean ± S.D. ($n = 3$). *, p value < 0.05; **, p value < 0.01; ***, p value < 0.001, between groups by one-way ANOVA. *C*, FACS quantitation of 7-aminoactinomycin D (*7AAD*) staining of dead bladder detrusor cells was performed in control and in the context of 6 h of acrolein treatment in the presence or absence of nicotinamide (48 h). *D*, the presence or absence of nicotinamide (*nic*) affected acrolein (*acr*)-induced double-stranded DNA breaks as evidence by γ-H2AX expression. The expression of apoptotic effectors; Mcl1, Bcl2, and survivin were also subject to nicotinamide treatment. *E*, immunoblot for the expression of caspase 3 cleavage, p53, and phosphorylated-AKT expression was determined by the time-course of acrolein treatment. Densitometry of the bands were normalized to β-actin and mean -fold change over control ($n \geq 3$).

One-way ANOVA was used for comparisons between two groups. Statistical significance was defined as $p < 0.05$. Both heatmaps were generated with gene signatures under different experimental conditions. Clustergram function in bioinformatics toolbox of MATLAB (Natick, MA) was used for heatmap creation and gene-wise clustering. Genes with similar patterns were clustered together. The colors on the heatmap indicate genes expressed at different levels.

RESULTS

Bladder Inflammation Causes Detrusor Cell Death and Hyperplasia—Bladder inflammation is associated with cell death and muscular expansion. To determine the role of bladder inflammation on detrusor muscular expansion, we used the established cyclophosphamide model to induce inflammation in ovariectomized C57BL/6 female mice (19, 20). As expected, CPX induced chronic bladder inflammation as evidenced by urothelial ulceration, expansion of the lamina propria, edema, and detrusor muscle hyperplasia as compared with control mice administered saline or clinical standard of care, Mesna (Fig. 1*A*). We found that the pretreatment with the niacin (vitamin B3) metabolite, nicotinamide, markedly reduced bladder ulcerations, lamina propria expansion, and muscular hyperplasia induced by CPX. Immunohistochemical localization of macrophage by F4/80 staining revealed CPX treatment caused inflammatory infiltrate in the lamina propria and detrusor. Both Mesna and nicotinamide significantly limited macrophage recruitment to the bladder in CPX-treated mice (Fig. 1*B* and supplemental Fig. 1). The activation of NF-κB, localized by phosphorylated-p65 staining, paralleled the treatment conditions associated with macrophage recruitment. CPX-induced inflammation promoted detrusor cell death, as localized by TUNEL staining. Interestingly, the proliferation

marker, Ki-67, indicated a pattern of CPX-induced muscular hyperplasia that was limited by Mesna and nicotinamide. To better isolate the cause of cell death, cultured detrusor cells were treated with the CPX metabolite, acrolein, in the presence of nicotinamide. FACS quantitation of 7-aminoactinomycin D (*7AAD*) staining of dead cells paralleled that observed in mice where treatment with nicotinamide limited acrolein-induced cell death 4.5-fold (Fig. 1*C*). Measuring γ-H2AX-associated DNA double stranded breaks through Western blotting paralleled the 7-aminoactinomycin D FACS results (Fig. 1*D*). The expression of both Survivin and Bcl2, negative regulators of apoptosis, were down regulated by acrolein exposure but restored by nicotinamide. Closer examination of the role of acrolein on bladder fibroblasts revealed a time-dependent up-regulation of cleaved caspase 3 and phosphorylated AKT with a down-regulation of p53 (Fig. 1*E*). These initial observations, suggested detrusor expansion, and cell death could not be explained by previously reported apoptotic programmed cell death.

Pyroptotic Cascade Initiated in the Detrusor Muscle Can Be a Result of DNA Imprinting—In light of significant cell death of bladder detrusor resulting from inflammation, mechanisms of cell death were closely examined. CPX is a known reactive oxygen species (ROS)-producing agent and mediator of DNA damage. The markers of prototypical markers of senescence and autophagy were measured. Acrolein rapidly induced the up-regulation of p16 and p21 to support senescence induction. In parallel, autophagy was induced with the appreciable up-regulation of Beclin1 and LC3 II by acrolein (Fig. 2*A*). Senescence-associated secretome expression may explain some of the observed muscular expansion (26). However, detrusor cell death could also be explained by the induction of a pyroptotic cascade often associated with inflammasome multiprotein oligomer of caspase 1 and NLRP proteins in myeloid cells that result in the secretion of IL-1β (27). In testing the potential of pyroptotic death in our bladder muscle model system, we found the activation of signal I mediator NF-κB (phosphorylated-p65) to be up-regulated within 6 h of acrolein treatment of bladder muscle cells (Fig. 2*B*). However, in checking the expression of conventional upstream mediators, TLR2, TLR4, and TLR9, no statistically different changes were measured (supplemental Fig. 2 *A*). The signal II mediator, NLRP3, was up-regulated within 2 h of acrolein treatment as was the downstream cleavage of pro-IL-1β by active caspase 1. Mature IL-1β expression by wild type detrusor cells was proportional to the length of acrolein exposure time (Fig. 2*B*). Nicotinamide down-regulated acrolein-induced NLRP3, cleaved caspase 1, and activated IL-1β expression (Fig. 2*C*). The acrolein-induced secretion of IL-1β, measured by ELISA, was significantly down-regulated by nicotinamide (Fig. 2*D*). Further examination of the bladder tissue of mice treated with CPX had similar signal I and signal II activation with the up-regulation of phosphorylated-p65, NLRP3, cleaved-caspase 1, and active IL-1β as in cultured bladder muscle cells (supplemental Fig. 2*B*). ChIP analysis revealed the NF-κB loading on the IL-1β promoter upon acrolein treatment in cultured bladder muscle cells (Fig. 2*E*). Both Mesna and nicotinamide treatment limited CPX action reducing phosphorylated-p65 and NLRP3. However, Mesna treatment had little effect on preventing the CPX-mediated downstream activation of caspase 1 and IL-1β in mice. Much like the reported inflammasome response in immune cells, the bladder smooth muscle responded to inflammatory initiation by a 7-fold acrolein induction of IL-6 in culture and a 2-fold CPX-induction of IL-6 in mice (supplemental Fig. 2*C*). Parallel studies showed that nicotinamide limited acrolein-induced IL-6.

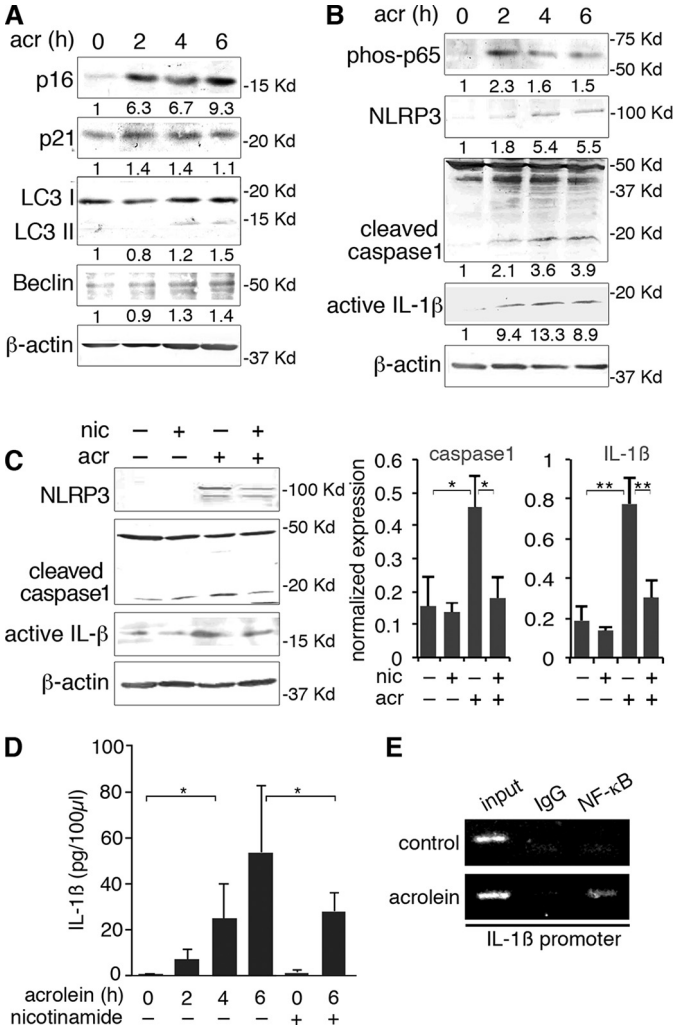

FIGURE 2. **Inflammation-mediated detrusor pyroptotic cell death.** Immunoblots quantitate the response to acrolein and CPX in cultured or *in vivo* mouse bladder detrusor, respectively. *A*, acrolein (*acr*)-induced senescence as determined by p16 and p21 expression as well as autophagy by LC3 I/LC3II and beclin-1 expression in a time-dependent manner. *nic*, nicotinamide. *B*, pyroptosis signaling molecules NF-κB-phosphorylated-p65, NLRP3, cleaved-caspase 1, and active IL-1β expression was measured in cultured bladder muscle after exposure to acrolein. *C*, nicotinamide antagonized acrolein-associated expression of NLRP3, active caspase 1, and IL-1β as quantitated in the *bar graph*. *D*, acrolein-induced detrusor secretion of IL-1β in a time-dependent manner was limited by nicotinamide as determined by ELISA. *E*, ChIP analysis of NF-κB binding to mouse IL-1β gene promoter; PCR-based analysis of DNA immunoprecipitated by ChIP targeting NF-κB transcription factor binding site (TFBS) within the *IL-1β* promoter region. PCR of input DNA shows equivalent starting material for the assay. Data represent the mean ± S.D. ($n \geq 3$). *, p value < 0.05; **, p value < 0.01 between groups by one-way ANOVA.

NLRP3 activation can be a result of DNA damage (28). CPX produced reactive oxygen-associated DNA damage as measured by the 20-fold induction of 8-oxo-dG, compared with saline-treated control mice (Fig. 3*A*). The pretreatment with either nicotinamide or Mesna significantly reduced 8-oxo-dG

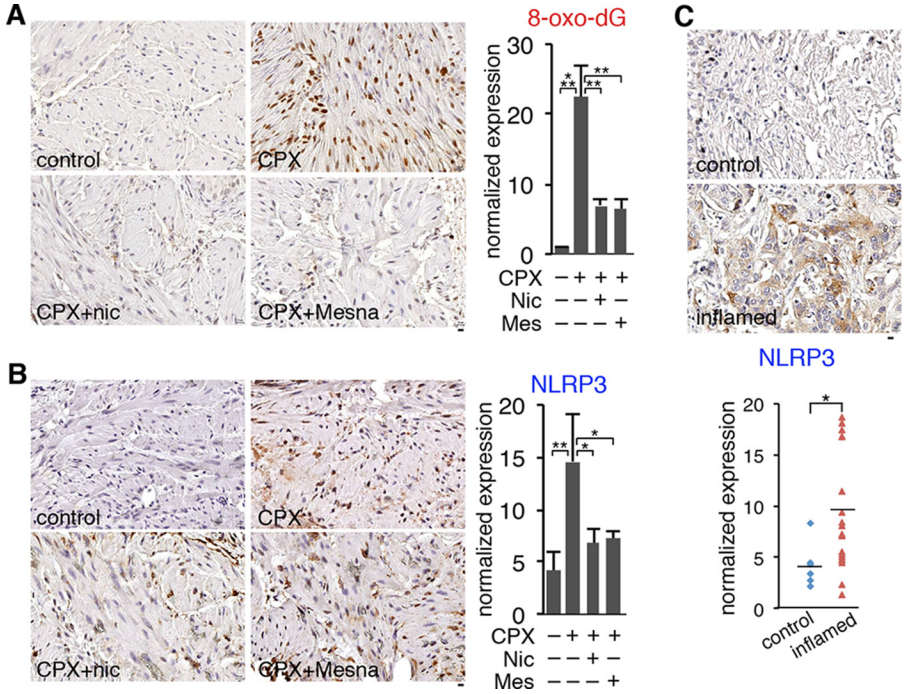

FIGURE 3. **Inflammation associated with DNA damage and NLRP3 activation.** 8-Oxo-dG (*A*) and NLRP3 (*B*) expression in mouse detrusor muscle. The corresponding *bar graphs* illustrate the mean expression of the respective staining ($n \geq 3$). *C*, detrusor NLRP3 expression by immunohistochemistry in control patients and those diagnosed with bladder inflammation was quantitated in the corresponding scatter plot (with the mean indicated by *a horizontal line*). The etiology of the inflammation was not known. The *scale bar* represents 10 μm. Data represent the mean ± S.D. ($n = 26$). *, p value < 0.05; **, p value < 0.01 between groups by one-way ANOVA. *nic*, nicotinamide; *Mes*, Mesna.

formation in CPX-exposed mice. The 8-oxo-dG binding partner, NLRP3, was similarly elevated in the detrusor of CPX treated mice (Fig. 3*B*). Both Mesna and nicotinamide significantly inhibited CPX-induced NLRP3 detrusor expression. The immunohistochemical down-regulation of CPX-induced total caspase 1 expression by Mesna and nicotinamide was observed but did not approach statistical significance (data not shown). NLRP3 expression was localized in human-inflamed and non-inflamed bladder tissues ($n = 26$). Quantitation of detrusor NLRP3 expression revealed NLRP3 induction in clinical bladder inflammation, with a >2.5-fold mean difference to non-inflamed bladder tissues (p value < 0.05, Fig. 3*C*). Together with the sterile-inflammation models studied, these novel findings suggest an intrinsic chronic nature to bladder inflammation and a possible means of resolution through nicotinamide administration.

Nicotinamide can down-regulate DNA methylation and reduce the chronic inflammatory phenotype through the inhibition of methionine metabolism (29, 30). In mice we found CPX-induced bladder inflammation promoted a doubling of global DNA methylation as measured by 5meC detrusor localization (Fig. 4*A*). Here we found the supplementation of nicotinamide to CPX treatment restored 5meC expression to that of control. The sequestration of acrolein by Mesna demonstrated similar efficacy as nicotinamide in reducing CPX-induced detrusor 5meC localization. After acute inflammatory initiation, a means of chronic disease progression could involve a deleterious imprinting mechanism. As evidence, we found that the inflamed human bladder tissue samples had significantly elevated global methylation in the detrusor compared with non-inflamed bladder (p value < 0.05; Fig. 4*B*). In light of observed DNA damage that persisted 72 h after inflammatory induction *in vivo* (data not shown), we tested the role of bladder muscle inflammation on DNA repair gene epigenetic imprinting. Fig. 4*C* illustrates *Ogg1*, *Neil1*, *Neil2*, *Brca1*, *Parp1*, and *Rad50* to be significantly down-regulated after a 6-h exposure of acrolein (p value < 0.05, supplemental Fig. 3). The pretreatment with demethylating agent 5azadC before acrolein treatment restored the expression of all six DNA repair genes to near control expression levels. Nicotinamide reactivated only three base excision repair genes, *Neil1* (p value < 0.01), *Ogg1* (p value < 0.05), and *Parp1* (p value < 0.01), as well as a homologous recombination repair gene, *Rad50* (p value < 0.001). The epigenetic silencing of *Ogg1*, responsible for mitochondrial DNA damage repair, could support the accumulation of 8-oxo-dG for associated NLRP3-mediated caspase 1 activation (28, 31, 32).

After CPX-induced inflammation-associated pyroptotic cell death, the manifestation of bladder detrusor hyperplasia contributes to poor bladder compliance and bladder over activity. To mimic the *Ogg1* epigenetic silencing, we expanded the bladder smooth muscle cells from *Ogg1*$^{-/-}$ mice in culture. *Ogg1* knock-out T cells are reported to undergo pyroptosis at an accelerated rate (33). We found that *Ogg1*$^{-/-}$ detrusor cells secreted 3-fold more IL-1β over that from wild type cells (Fig. 4*D*). To better understand the dynamic between pyroptotic bladder smooth muscle cells and non-effected neighboring cells, we co-cultured vital dye 5-chloromethylfluorescein diacetate (CMFDA)-labeled *Ogg1*$^{-/-}$ bladder cells with its wild type counterpart at varying ratios (Fig. 4*E*). Subsequently, proliferation of the wild type muscle cells was quantitated by Ki-67 expression through FACS analysis in co-culture percentages

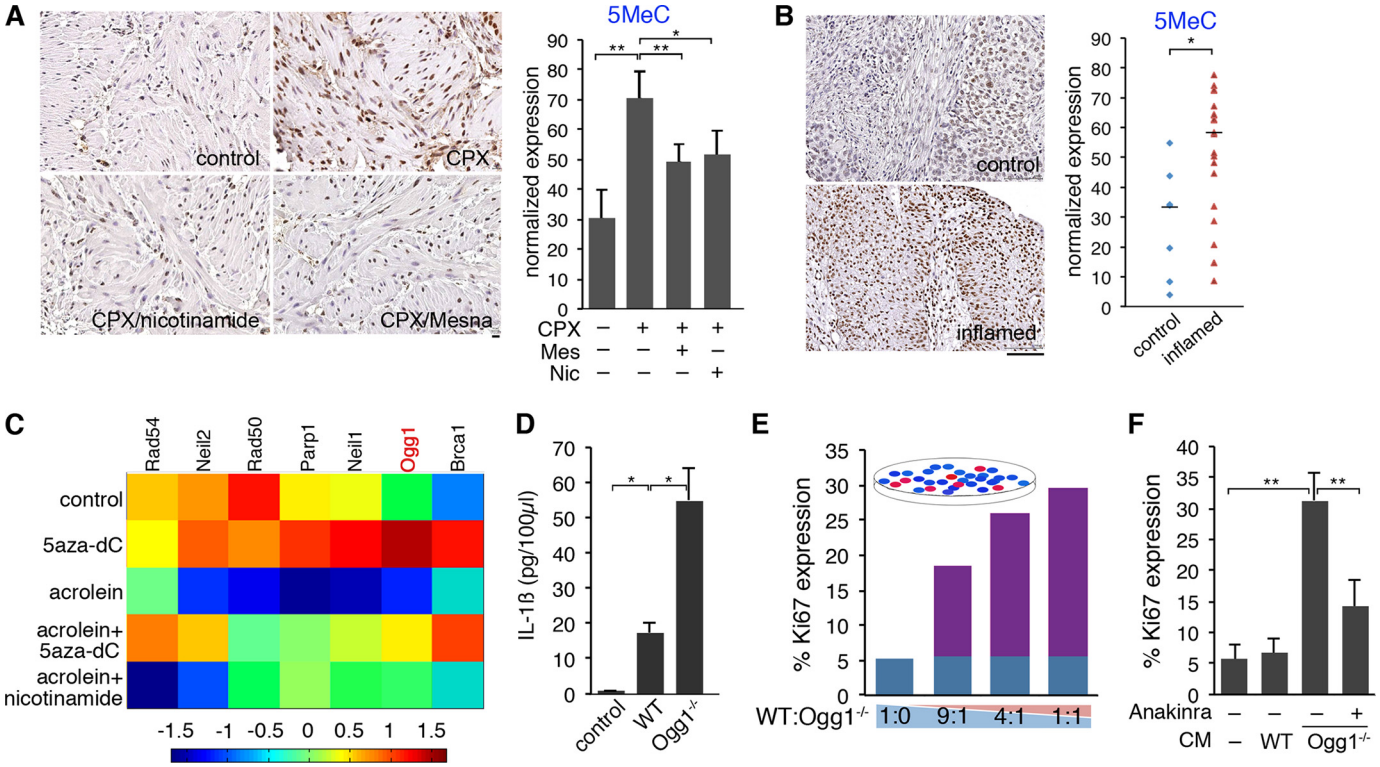

FIGURE 4. **Inflammation associated with bladder muscle imprinting and detrusor hyperplasia.** *A*, bladders of control and CPX-treated mice in the presence or absence of Mesna (*Mes*) or nicotinamide (*nic*) were evaluated for DNA methylation by immune-localization of 5meC expression. The *scale bar* represents 10 μm. *B*, Detrusor 5meC expression by immunohistochemistry in control patients and those diagnosed with bladder inflammation were quantitated in the corresponding scatter plot (with the mean indicated by a *horizontal line*). The etiology of the inflammation was not known. The *scale bar* represents 100 μm. *C*, mRNA expression profiles of the DNA damage repair enzymes in control mouse bladder detrusor cells or those exposed to acrolein for 6 h were presented in the heat-map ($n = 3$). 5azadC and nicotinamide treatment supported the role of promoter methylation in gene silencing associated with acrolein treatment. *D*, secreted IL-1β was measured in conditioned media from detrusor cells of WT and *Ogg1*$^{-/-}$ mice by ELISA. *E*, Ki-67-positive, proliferative WT (*blue*) bladder detrusor cells were measured by FACS after 72 h co-culture with *Ogg1*$^{-/-}$ cells (*red*) in the indicated ratios. Only WT cell proliferation was measured as a percent of total. *F*, conditioned media (*CM*) from WT and *Ogg1*$^{-/-}$ detrusor cells (72 h) promoted WT cell Ki-67 expression in the presence and absence of Anakinra. Data represent the mean ± S.D. ($n = 3$). *, p value < 0.05; **, p value < 0.01 between groups by one-way ANOVA.

comprising 0–50% *Ogg1*$^{-/-}$ cells. There was a striking wild type muscle proliferation induced by *Ogg1*$^{-/-}$ cells in a dose-dependent manner. Only 10% *Ogg1*$^{-/-}$ cells was required to more than triple the number of Ki-67 positive wild type cells compared with when the wild type cells were cultured alone. In a 1:1 ratio of *Ogg1*$^{-/-}$:wild type bladder muscle cell culture, the wild type cell proliferation expanded ~6-fold over when they were grown alone. To test if muscle proliferation was IL-1β-dependent, 72-h conditioned media from *Ogg1*$^{-/-}$ muscle were incubated with wild type cells in the presence and absence of the IL-1β antagonist, Anakinra (Kinret, Amgen Inc.). Bladder muscle-conditioned media of *Ogg1*$^{-/-}$ mice similarly induced wild type cell proliferation, significantly lessened by Anakinra incubation (p value < 0.01; Fig. 4*F*). Together, the data supporting inflammation-associated DNA methylation can promote IL-1β-dependent bladder muscle proliferation.

Bladder Detrusor Expansion Is IL-1β-dependent—Understanding the role of IL-1β in CPX-induced bladder muscle expansion could support therapeutic intervention for those with associated complications of bladder non-compliance. To this end mice were treated with CPX in the presence and absence of Anakinra. Bladder tissue lysates from these mice were subjected to a protein array to determine the IL-1β downstream secreted factors contributing to the detrusor phenotype. IGF1, Flt3 ligand, and osteoprotegerin were up-regulated >2-fold with CPX treatment and restored to near control levels with the addition of Anakinra (Fig. 5*A*, supplemental Fig. 4). Basic FGF (FGF2) emerged as a prominent secreted protein to be up-regulated by CPX. However, because Anakinra did not significantly alter its expression, FGF2 was not regarded as a critical mediator of the inflammatory bladder phenotype. IGF1 expression, however, was the only factor in the array that was significantly elevated with CPX treatment and restored to control levels by Anakinra. Not surprisingly, morphometric analysis indicated detrusor hypertrophy was induced by CPX (Fig. 5*A*). Antagonizing IL-1β restored CPX-induced bladder hypertrophy in mice to near control levels (Fig. 5*B*). In validating the small number of candidate IL-1β inducers of hypertrophy, we identified mRNA expression of *Igf-1* to follow the hypertrophic pattern resulting from CPX and down-regulation by Anakinra. The two components were independently measured and plotted together. Interestingly, 72-h-conditioned media from cultured *Ogg1*$^{-/-}$ bladder muscle significantly induced *Igf1* mRNA and protein in wild type bladder muscle cells, down-regulated by Anakinra (supplemental Fig. 5). Although by starkly different mechanisms, the treatment with Anakinra had comparable effects on bladder muscle hyperplasia and hypertrophy as treatments with Mesna or nicotinamide. Immunohistochemical localization of Ki-67 and TUNEL on control bladders and those exposed to CPX and Anakinra were evaluated.

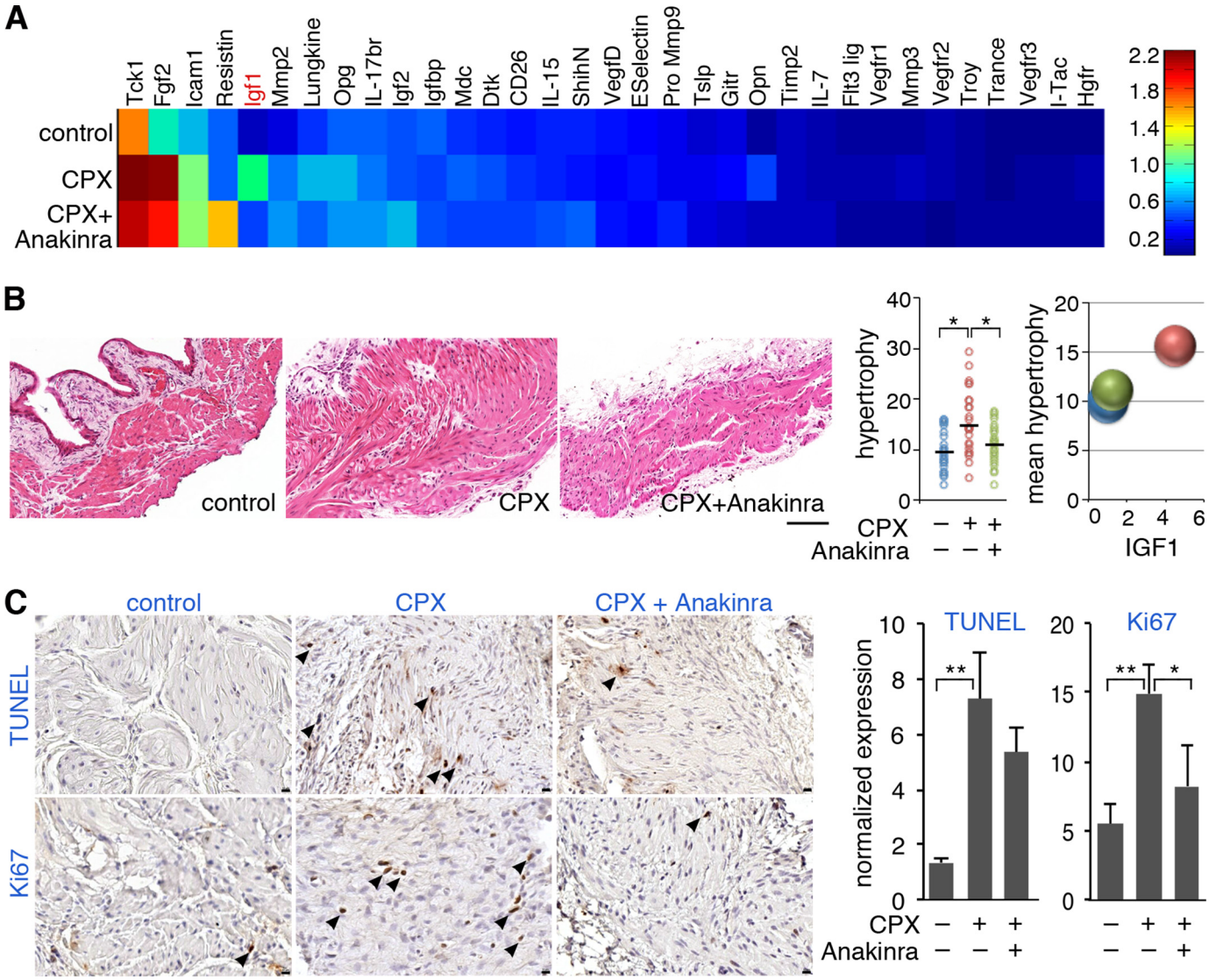

FIGURE 5. **IGF-1 mediates inflammation-associated detrusor expansion.** Mice were treated with CPX in the presence or absence of Anakinra to determine IL-1β-dependent growth factor induction by protein array analysis of bladder muscle tissue. The heatmap representation indicates IGF-1 (in *red*) to be the only factor significantly up-regulated by CPX and restored to control levels by Anakinra. *B*, bladder detrusor expansion was determined by H&E staining (the *scale bar* represents 100 μm). Muscle hypertrophy was quantitated by morphometric analysis and recorded in the scatter plot with mean values indicated by a *horizontal line*. Tissue mRNA expression of IGF-1 was quantitated by real time-PCR and plotted as a factor of muscle hypertrophy in the bubble plot. The colors associated with the treatment conditions in the scatter plot correspond to that of the bubble plot. *C*, immunohistochemical localization of TUNEL and Ki-67 (*arrowheads*) in detrusor tissues, quantitated in the *bar graph to the right* (the *scale bar* represents 10 μm). Data represent the mean ± S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$ between groups by one-way ANOVA.

Anakinra significantly down-regulated proliferative, Ki-67-positive bladder detrusor cells but did not significantly down-regulate the cell death induced by CPX (Fig. 5*C*). Together the data supported IL-1β as an important mediator of bladder muscle hyperplasia and hypertrophy, with IGF1 as a candidate downstream growth factor.

To determine if IGF1 could cause bladder muscle expansion, we chose another model to test NLRP3-dependent IL-1β activation. Because acrolein is toxic, we were not able to extend the *in vitro* experiments past 6 h. To better mimic the *in vivo* observation in the bladder, we chose to validate the critical role DNA damage has on the pyroptotic cascade in supporting the proliferation of the undamaged muscle. Irradiation was used as an independent means of causing DNA damage. Western blotting provided evidence of DNA double-stranded breaks was caused by 8 gray radiation of cultured bladder muscle cells through up-regulation of γ-H2AX and p53-binding protein (Fig. 6*A*). As another example of sterile inflammation, we found the increasing expression of NF-κB and NLRP3 within 6 h of irradiation. Cleaved caspase-1 was also elevated by irradiation. DNA damage and associated ROS induction, by either acrolein or -radiation, could initiate pyroptosis in bladder muscle.

Next, we designed a controlled experiment where media of 8 gray-irradiated wild type bladder muscle cells were transferred to a plate of similar cells never subjected to radiation. DNA double-stranded breaks were observed in the irradiated muscle by 6 h, as determined by γ-H2AX expression (Fig. 6*B*). The resulting expression of IL-1β and IGF1 was verified by Western blotting of the cell lysates of irradiated cells in a time course of 6–48 h. IL-1β was further found secreted in the media robustly

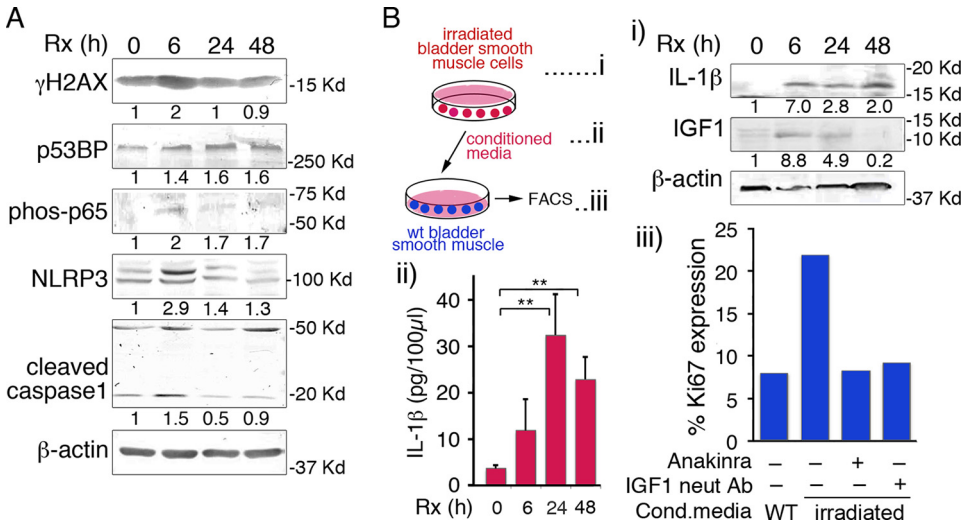

FIGURE 6. **Radiation-induced DNA damage can induce IGF-1-dependent detrusor proliferation.** *A*, protein analysis of mouse bladder detrusor cells after 8-gray radiation indicated changes in DNA damage markers (γ-H2AX and p53 binding protein (p53BP)), senescence (p16), and pyroptosis markers (NF-κB phosphorylated-p65, NLRP3, caspase-1, and active IL-1β). The densitometry of the blots indicate relative expression, normalized to β-actin and mean -fold change over control ($n = 3$). Bladders of control and CPX-treated mice in the presence or absence of Mesna or nicotinamide were evaluated for mitochondrial and nuclear DNA damage, and inflammasome activation by immune-localization of antagonizing IGF-1 by neutralizing antibody inhibits IL-1β-mediated proliferation (*B*). (i) The expression of γ-H2AX, IGF1, and IL-1β expression was immunoblotted 0–48 h after 8-gray irradiation of wild type bladder muscle cells. Densitometry of the bands was normalized to β-actin, and mean -fold change over control irradiation induced bladder detrusor secretion of IL-1β in a time-dependent manner as determined by ELISA (ii). Data represent the mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ between groups by one-way ANOVA test. (iii) Conditioned media from irradiated detrusor cells promoted non-irradiated wild type detrusor cell Ki-67 expression in the presence or absence of Anakinra and IGF-1-neutralizing antibody (*Ab*). *Rx,* radiation treatment.

in the same time course after irradiation, as was IGF1 to a lesser extent. FACS analysis of the target cells given 48 h of conditioned media for an additional 48 h demonstrated 3-fold greater Ki-67-positive proliferative cells over control. Both Anakinra- and IGF-1-neutralizing antibody independently added to the irradiated cell conditioned media restored proliferation to near control levels. These studies support the role of DNA damage on muscle expansion. DNA damage, both directly or through epigenetic silencing of DNA damage repair genes, was sufficient for pyroptosis and cause the adjacent cells not undergoing pyroptosis to proliferate.

DISCUSSION

Pyroptotic programmed cell death of the detrusor muscle cell has deleterious effects on bladder physiology. CPX-induced damage to the urothelium contributes to apoptosis and acute hemorrhagic cystitis (34, 35). However, CPX-induced cell death results in bladder detrusor expansion and manifestation of poor compliance. The urotoxic stress induced by ROS is implicated as a key component of acrolein accumulation in the bladders of CPX-treated patients (36, 37). However, necrosis, apoptosis, and autophagy, attributed to acrolein-induced urothelial death, is not replicated in the detrusor muscle (38). We demonstrated that despite the up-regulation of cleaved caspase 3 did not support of apoptosis-mediated death in the muscle in the context of the activation of AKT and down-regulation of p53 (Fig. 1). CPX-induced DNA damage induced the detrusor to express senescence (p16) and autophagy (Beclin and LC3II) markers as reported for radiation exposure of prostatic smooth muscle (Fig. 2) (26). However, the human manifestation of bladder inflammation, like that induced by CPX in mice, additionally demonstrate the activation of the NLRP3 cascade (Fig. 3). Macrophage and dendritic cells were originally reported to undergo pyroptosis in the potentiation of the inflammasome complex for the differentiation of T and B cells to facilitate an appropriate immune response (39, 40). Processes of sterile inflammation in diseases like gout, type 2 diabetes mellitus, atherosclerosis, and even myocardial infarction has emerged to involve pyroptotic cell death with pathologic recruitment of macrophage and neutrophil (41, 42).

Radiation and acrolein exposure to the bladder are examples of sterile inflammation causing muscular expansion. DNA damage was sufficient to initiate the NLRP3 activation and even potentiate NF-κB induction of IL-1β. Silencing of DNA damage repair genes can extend the efficacy of DNA-damaging mediators with differential implications in muscle cells compared with the urothelia. Although the phenotypic resolution of bladder inflammation, hyperplasia, and cell death by nicotinamide was similar to that with Mesna, the mechanism was quite different. In examining inflammation-induced epigenetic gene regulation, we focused on the expression of DNA damage repair genes (Fig. 4). We found that epigenetic silencing of a critical DNA (mtDNA) damage repair enzyme, *Ogg1*, was particularly silenced by acrolein and re-expressed by nicotinamide. The resulting mitochondrial damage would cause ROS accumulation and the observed DNA oxidation (8-oxodG) without a means of repair (Fig. 3), enabling the activation of NLRP3 in signal II (43, 44). Acrolein-mediated transcription silencing by promoter methylation of OGG1 resulted in reduced capacity to repair 8-oxo-dG (28, 31, 32). Activated NLRP3 can stimulate the complex with apoptosis-associated speck-like protein containing CARD (ASC) to cause the proteolytic maturation of caspase-1 (45). Activated caspase-1 cleaves pro- IL-1β to generate mature IL-1β (46, 47). For the initial expression of pro-IL-1β through signal I, CPX induction of NF-κB in the detrusor

could be due to uric acid crystal-mediated activation *in vivo* (48). Toll-like receptors are commonly attributed to the initiation of the pyroptotic pathway through the activation of NF-κB (49). However, both acrolein and radiation could stimulate ROS as a viable NF-κB activator candidate in cultured detrusor cells (50). Thus, a mechanism for the initiation of the pathophysiology of bladder inflammation may bypass the Toll-like receptor initiation reported for myeloid cells (supplemental Fig. 1) (51). However, the perpetuation of IL-1β secretion *in vivo* could be a result of inflammation-associated epigenetic imprinting of DNA damage repair genes (Fig. 4). The observed up-regulation of IL-6 and coincident recruitment of macrophage *in vivo* support a similar scenario bladder injury from CPX, where chronic maintenance of muscle pyroptosis may require traditional immune induction (Fig. 1*B*, supplemental Figs. 1 and 2*C*).

We found that the paradox behind bladder detrusor cell death and coordinated expansion associated with chemotherapeutic CPX treatment was the recognition of heterotypic interaction among detrusor muscle cells. The correlation of NLRP3 expression and human bladder inflammation support the model systems examined here. It is apparent that epigenetic abnormalities, in particular altered DNA methylation, play a crucial role in the development of acute to chronic inflammatory diseases (4, 52). Nicotinamide, able to cause global DNA hypomethylation, mirrored the effects of 5azadC in the re-expression of the DNA damage repair genes (Fig. 4) (53, 54). In parallel, global DNA methylation was significantly associated with patients with bladder inflammation. The antagonism of IL-1β reversed the proliferative effect of $Ogg1^{-/-}$-conditioned media. As we attempted to mimic through an add-mixing of labeled $Ogg1^{-/-}$ and wild type detrusor cells, inflammation-induced epigenetic imprinting could maintain mitochondrial damage and enable pyroptotic death of only a fraction of detrusor cells and enable IL-1β-dependent proliferation of neighboring muscle cells.

Based on these results, we proposed Anakinra could limit inflammation-associated detrusor expansion. Because IL-1β does not seem to have a direct role in muscle proliferation or hypertrophy, we looked to other inducible means of these phenotypic changes found to be mitigated by Anakinra. Protein array analysis supported IGF-1 as a viable IL-1β-dependent mediator of the phenotypic muscular changes associated bladder inflammation (Fig. 5). Anakinra only affected the proliferative and trophic factors, with little effect on cell death. We found the expression of the candidate downstream factor, IGF-1, to be correlated to bladder hypertrophy. Fig. 6 validated that an independent DNA damage mechanism, by radiation, could potentiate muscle hyperplasia in an IL-1β- and IGF-1-dependent manner, similar to that by acrolein. The three antagonists used in this study, Mesna, nicotinamide, and Anakinra, can impact the sequential steps of acrolein damage, DNA methylation, and the result of pyroptosis, respectively. The results have wider implications for the use of Anakinra in the inflammation cascade of muscular expansion.

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