Lentiviral interferon: A novel method for gene therapy in bladder cancer

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Interferon alpha (IFNα) gene therapy is emerging as a new treatment option for patients with non-muscle invasive bladder cancer (NMIBC). Adenoviral vectors expressing IFNα have shown clinical efficacy treating bacillus Calmette-Guerin (BCG)-unresponsive bladder cancer (BLCA). However, transient transgene expression and adenoviral immunogenicity may limit therapeutic activity. Lentiviral vectors can achieve stable transgene expression and are less immunogenic. In this study, we evaluated lentiviral vectors expressing murine IFNα (LV-IFNα) and demonstrate IFNα expression by transduced murine BLCA cell lines, bladder urothelium, and within the urine following intravesical instillation. Murine BLCA cell lines (MB49 and UPPL1541) were sensitive to IFNα-mediated cell death after LV-IFNα, whereas BBN975 was inherently resistant. Upregulation of interleukin-6 (IL-6) predicted sensitivity to IFNα-mediated cell death mediated by caspase signaling, which when inhibited abrogated IFNα-mediated cell killing. Intravesical therapy with LV-IFNα/Syn3 in a syngeneic BLCA model significantly improved survival, and molecular analysis of treated tumors revealed upregulation of apoptotic and immune-cell-mediated death pathways. In particular, biomarker discovery analysis identified three clinically actionable targets, PD-L1, epidermal growth factor receptor (EGFR), and ALDH1A1, in murine tumors treated with LV-IFNα/Syn3. Our findings warrant the comparison of adenoviral and LV-IFNα and the study of novel combination strategies with IFNα gene therapy for the BLCA treatment.

INTRODUCTION

Approximately 70% of newly diagnosed bladder cancers (BLCAs) are non-muscle invasive (NMIBC). Conventional treatment of these tumors includes transurethral resection followed by intravesical therapy. Bacillus Calmette-Guerin (BCG) has been the mainstay of intravesical therapy for patients with high-risk disease for decades.1 Although this treatment achieves initial clinical responses in the majority of patients, disease recurrence and progression to a BCG-unresponsive state are common.2 Currently, the optimal management of BCG-unresponsive disease is radical cystectomy, an invasive procedure associated with significant morbidity and potential mortality.2

Several agents have and continue to be tested in the BCG-unresponsive setting.1 To date, few have provided durable disease responses. Immunostimulatory gene therapy is a novel therapeutic strategy with promise to improve the durability of disease responses after intravesical therapy through immune-mediated mechanisms of tumor killing. Since the first successful therapeutic gene transfer over 30 years ago, gene therapy has evolved into an asset in our fight against cancer.1,4,5

Adenoviral interferon-alpha 2b (Ad-IFNα2b) combined with a novel excipient (Syn-3), an excipient that facilitates gene transfer across the urothelium, has shown promising clinical activity in phase 1, 2, and 3 trials.7,8 In these studies, Ad-IFNα2b/Syn3 appeared to be well tolerated, provided sustained urinary IFNα levels, and demonstrated a 35% 1-year recurrence-free survival (RFS) in patients enrolled in a phase 2 trial. Accordingly, the recently concluded phase 3 trial showed a 60% response rate at 3 months with maintenance of complete response in 51% of patients at 12 months.9 These findings represent favorable improvement in the management of BCG-unresponsive disease compared with currently approved agents.4,10

The anti-tumor efficacy of Ad-IFNα is the result of IFNα’s pleiotropic anti-tumor effects. Preclinical studies demonstrated that IFNα directly induced apoptosis in some human BLCA cells by inducing
autocrine tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) production. Furthermore, Ad-IFNz treatment inhibited angiogenesis in human xenografts, and studies conducted in immune-competent syngeneic murine models demonstrated that the immune response to IFN was mediated by activation of both innate and adaptive immunity. Also, type I IFN signaling is important for initiating anti-tumor responses in dendritic cells.

While Ad-IFNz gene therapy is clinically efficacious, IFN transgene expression from this DNA viral vector is transient. In contrast, lentiviral vectors can integrate their genomes into the DNA of host cells and achieve stable immunostimulatory transgene expression from both dividing and non-dividing tumor cells. In addition, whereas adenoviral vectors are highly immunogenic, lentiviral vectors are less immunostimulatory and hosts carry lower levels of pre-existing humoral immunity. Lentiviruses have been used as vectors for transgene delivery in several clinical trials. The studies presented here are the first to evaluate lentiviral IFNz gene therapy (LV-IFNz) for BLCA.

RESULTS

Expression of IFNz protein and its target genes in syngeneic mouse BLCA cell lines after LV-IFNz transduction

The anti-tumor efficacy of LV-IFNz was tested in the murine BLCA cell lines BBN975 and UPPL1541, which recapitulate NMIBC, along with the carcinogen-induced cell line MB49 that is frequently used in preclinical studies. GFP expression confirmed complete transduction of all cells by 48–72 h after treatment using increasing multiplicities of infection (MOIs) (2, 6, and 8; Figure S1). Cell lines were then treated with lentiviral control vector (LV-Ctrl) or LV-IFNz at a MOI of 2. IFNz-specific ELISA performed on cell-free supernatants collected 24 and 72 h after transduction with LV-IFNz showed robust and significantly higher IFNz protein expression in all three cell lines compared with LV-Ctrl (Figures 1A and 1B). Quantitative real-time PCR using RNA from transduced cells showed upregulation of the IFNz target genes IRF7, PD-L1, and TRAIL (Figure 1C). IRF7 expression was significantly induced in all the three cell lines but was highest in MB49 (1,564-fold), followed by BBN975 (138-fold) and UPPL1541 cells (134-fold). PD-L1 was comparably upregulated in all the three cell lines (9.165-fold in BBN975, 11.7-fold in MB49, and 16.35-fold in UPPL1541). TRAIL expression was highest in MB49 (3,677-fold), followed by UPPL1541 (282.5-fold) and BBN975 (228.3-fold).

Cytotoxic effects of IFNz protein on BLCA cell lines after transduction with LV-IFNz

To determine whether IFNz gene therapy induced direct cytotoxic effects, we assessed cell viability of BLCA cell lines by Trypan blue dye exclusion after LV-IFNz transduction. In two of the three cell lines (MB49 and UPPL1541), viable cell counts decreased after LV-IFNz and 100 U of recombinant IFNz (rIFNz) treatment in a time-dependent manner (Figures 2A and 2B). This result was confirmed by MTT assay at 72 h after treatment, which demonstrated that MB49 cell proliferation was significantly lower after LV-IFNz treatment compared with LV-Ctrl (Figure S2A). BBN975 cells showed an initial response to LV-IFNz and 100 U rIFNz, but by 72 h, the viable cell count was comparable to controls (saline or LV-Ctrl; Figure 2C). Recombinant IFNz dose-response curves illustrated that BBN975 cells were resistant to rIFNz (up to 600 U/mL conc.; Figure S2B), whereas MB49 and UPPL1541 were sensitive at all dose levels tested (Figures S2C and S2D). The cytotoxicity of IFNz was mediated by cell-death pathways, as demonstrated by increased annexin V staining in MB49 and UPPL1541 cells following treatment with LV-IFNz and 100 U of rIFNz compared with controls (Figure 2D). Following the observation that IFNz gene therapy promoted PD-L1 expression through IFNAR1/STAT1 signaling, we performed western blot analysis on lysates from our three cell lines after treatment with LV-IFNz, rIFNz, LV-Ctrl, or vehicle, confirming that PD-L1, STAT1, and p-STAT1 were all upregulated following LV-IFNz of MB49, UPPL1541, and BBN975 (Figure 2E).

Cellular pathway discovery

To better understand how LV-IFNz affects global gene expression, we performed RNA sequencing (RNA-seq) on cell lines treated with rIFNz, LV-Ctrl, or LV-IFNz and compared them with untreated control cells (Ctrl). Hierarchical clustering and principal-component analyses showed distinct gene expression patterns for the four groups (Figures S3A and S3B). To identify candidate genes contributing to IFNz-mediated effects on cell viability, we compared differentially expressed genes between the rIFNz/Ctrl and LV-IFNz/LV-Ctrl pairs and generated heatmaps using a false discovery rate (FDR) cutoff of 0.05 and fold change of 2 (Figures 3A–3F). The gene lists are provided in Table S1.

We then used a more inclusive fold change cutoff of 1.5 to identify genes that are commonly altered among the three cell lines tested. We generated Venn diagrams that identified 506, 603, and 869 differentially expressed genes between LV-IFNz and LV-Ctrl treatment of BBN975, MB49, and UPPL1541, respectively, with 90 genes common to all cell lines (Figure 3G). Similarly, 455, 730, and 88 genes were differentially expressed across the respective cell lines between 100 U rIFNz-treated and untreated controls (Ctrl), with 70 genes common to all cell lines (Figure 3H). The complete gene list for the three cell lines is included in Table S2.

To identify major pathways activated in these cells, we used the sequencing data to perform gene set enrichment analysis (GSEA). Top GSEA hallmark pathways are shown in Table S3. As expected,
Figure 2. Cytotoxic effect of LV-IFNα on BLCA cell lines

(A–C) Murine cell lines were exposed to murine recombinant IFNα (rIFNα) or transduced with LV-Ctrl or LV-IFNα and cell counts were measured using Trypan blue dye exclusion method. At 72 h, MB49 (A) and UPPL1541 (B) showed significant reduction in cell counts, while BBN975 (C) showed no change in cell numbers when compared with the

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GSEA showed enrichment of the IFNz response pathway in both LV-IFNz- and rIFNz-treated MB49 cells when compared with respective controls (Figures 3I and 3J). GSEA of top pathways for BBN975 and UPPL1541 is shown in Figure S3C. We also performed ingenuity pathway analysis (IPA) on differentially expressed genes between LV-IFNz- and LV-Ctrl-treated cells in all three cell lines. IFN signaling and activation of IRF by cytosolic pattern recognition receptors were commonly enriched in all three cell lines with a positive Z score whereas retinoic-acid-mediated apoptosis signaling pathway and death receptor signaling were enriched only in the IFNz-sensitive MB49 and UPPL1541 (Figures 4A–4C) lines. PARP proteins and TRAIL (Tnfsf10) were commonly enriched among the two apoptotic pathways, whereas DAXX was specific to the death receptor signaling pathway and was upregulated >2-fold in both MB49 and UPPL1541 cells. The complete gene list for the apoptotic pathways is provided in Table S4.

**Mechanism underlying IFNz-mediated cell death**

Given that our sequencing studies confirmed increased expression of TRAIL in all three cell lines (Figure 1C), we next evaluated caspase 8 expression, which has been shown to mediate TRAIL-dependent cell death in human BLCA cell lines.12 Caspase 8 inhibition in MB49 (Figures 5A and 5B) and UPPL1541 (Figures 5C and 5D) partially rescued TRAIL-mediated cell death. In contrast, TRAIL-mediated cell death was not observed in BBN975, which is resistant to IFNz treatment (Figures S4A and S4B). Cell-surface expression of TRAIL was also increased in MB49 following treatment with LV-IFNz, which was rescued following caspase 8 inhibition (Figure S4C). In addition to caspase 8, in MB49 cells, we detected caspase 4 and 12 overexpression (2.0- and 2.2-fold, respectively), genes involved in endoplasmic reticulum (ER) stress response and whose expression has been linked to cell death (Table S1). Treatment of MB49 with tunicamycin to induce ER stress also resulted in cell death in a dose-dependent manner (Figure 5E), suggesting that MB49 cells are sensitive to ER stress induction. Treatment with IFNz together with caspase 12 inhibition partially rescued cell death in MB49 cells (Figure 5F). Interestingly, caspase 12 expression was downregulated in UPPL1541 (2.2-fold), suggesting that this caspase may not be involved in death of these cells (Table S1).

We recently reported that interleukin-6 (IL-6) was important for IFNz-mediated death in MB49 cells and that upregulation of IL-6 in clinical samples collected within the phase 2 trial correlated with response to IFNz gene therapy.13 We therefore evaluated the relative expression of IL-6 in all cell lines following treatment with LV-IFNz by ELISA. IL-6 protein expression was detected at 72 h after LV-IFNz treatment in MB49 and UPPL1541 (Figures 5G and 5H) cells, whereas its expression was undetectable in BBN975 (data not shown). This underscores the link between the induction of IL-6 and response to IFNz.

**Transduction of the bladder urothelium with LV-IFNz**

We next tested the transduction efficiency of LV-IFNz after murine intravesical instillation. Following pre-administration of the excipient Syn3,24 LV-IFNz transduction of murine urothelium was confirmed by electron microscopy (EM), with viral particles detectable inside vacuoles as early as 4 h post-instillation and persisting for up to 96 h post-instillation (Figures 6A–6D). Sequential instillations resulted in deeper vector penetration of the urothelium (Figures 6E and 6H) and apoptotic urothelial cells with shrunken cytoplasm and viral particles detected in the nuclei (Figures 6F and 6G). In addition to EM, we verified urothelial transduction using a lentivirus-beta-galactosidase (LV-βgal) vector (Figure 6I). Histological sections showed multifocal regions of βgal staining throughout the urothelium (Figures 6J and 6K). Finally, expression of IFNz in urine from treated mice measured by ELISA confirmed LV-IFNz transduction of and IFNz transgene expression from murine urothelium first detectable at 48 h and maintained for up to 96 h post-treatment, with protein expression of IFNz in bladder lysates peaking at 72 h post-transduction (Figures 6L and 6M).

**Efficacy of LV-IFNz in murine model for BLCA**

The anti-tumor efficacy of LV-IFNz was tested in the syngeneic MB49 murine BLCA model after intravesical administration. LV-IFNz treatment resulted in a significant improvement in median survival compared with LV-Ctrl (26 and 19 days, respectively; p < 0.001; Figure 7A). Histological examination of murine bladders treated with LV-IFNz revealed reduced tumor burden and decreased tumor proliferation (Figure 7B). To investigate intratumoral immune responses induced by LV-IFNz, we performed immunohistochemistry (IHC) on treated tumors and confirmed decreased numbers of tumor-infiltrating CD4 cells following LV-IFNz treatment compared with LV-Ctrl treatment. CD8 cells were not significantly altered (Figures 7C and 7D).

**In vivo gene expression profiles induced by LV-IFNz treatment**

Finally, we sequenced murine MB49 bladder tumors treated with LV-IFNz and compared their expression profile with those from LV-Ctrl and vehicle-treated bladder tumors. Hierarchical clustering analysis was performed using log-transformed normalized count data. The clustered dendrogram is presented with a heatmap of variable expressed genes (top 1,500) in Figure S5. Using an FDR cutoff of 0.05 and log2 fold change of 1, this analysis identified 190 genes differentially expressed between LV-IFNz and LV-Ctrl treatment groups (Figure 7E), with the complete gene list provided in Table S4. Specifically, we found upregulation of several granzyme genes that are positive regulators of apoptosis in LV-IFNz-treated tumors.25 Consistent with our in vitro results (Table S4), we also identified upregulation of caspase 12 in LV-IFNz-treated tumors. GSEA identified greater expression of apoptotic pathways in LV-IFNz-treated tumors when compared with LV-Ctrl tumors.
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Immune-related pathways, such as natural-killer-cell-mediated cytotoxicity and Fc-gamma-receptor-mediated phagocytosis pathways, were also enriched in LV-IFNα-treated tumors compared with LV-Ctrl.

In order to identify biomarkers associated with IFNα sensitivity, we used IPA with sequencing results from LV-IFNα-treated tumors using an FDR cutoff of 0.05 and fold change of 2. We identified 11 genes associated with cancer, inflammatory disease, and renal and urological disease (Table 1). The biomarker application for each of these genes, fold change (expr log ratio), and p value is listed (Table 1). We also evaluated expression of genes involved in cytotoxicity and immune cell recruitment. Directionality of expression of most genes involved in cytotoxicity and immune cell recruitment was consistent with their previously described functions (Table 2).

We also evaluated the differential expression of candidate genes associated with immune cell subtypes following LV-IFNα versus LV-Ctrl or vehicle treatment (Figures 8A and 8B; Table S5). The most differentially expressed genes included SPIB (B cell marker), granzyme A and B (cytotoxic cell marker), and CD163 and MS4A4A (macrophage markers), suggesting involvement of these immune cell subtypes in IFNα-mediated cytotoxicity.

DISCUSSION

Adenoviral-based IFNα2b gene therapy has shown promising efficacy in clinical trials. In addition to being well tolerated with a convenient dosing schedule, it achieved an RFS rate of 30.5% at 12 months. Although this represents a significant improvement over the agents currently approved for BCG-unresponsive disease, there remains a need to understand and improve upon intravesical IFNα gene therapy in clinical non-responders. This can be accomplished by identifying biomarkers predictive of sensitivity or resistance, by better understanding its mechanisms of action, and through the development of more efficient gene therapy vectors. We report here the initial study evaluating LV-IFNα therapy against BLCA, identifying mechanisms driving IFNα’s...
direct anti-tumor activity and its role(s) in immune modulation of the tumor microenvironment. Given the established clinical efficacy of IFN\(\alpha\) gene therapy, improved intensity and duration of IFN\(\alpha\) transgene expression following lentiviral transduction has the potential to improve the rate and durability of clinical responses.\(^{17}\)

Unlike adenoviruses, lentiviruses can efficiently transduce both dividing and non-dividing cells and achieve stable transgene expression, as shown in several clinical trials.\(^{20-22}\) Additional advantages to lentiviral vectors include reduced baseline seropositivity and decreased immunogenicity following sequential administrations.\(^{19,26,27}\) One significant practical limitation to the development of lentiviral vectors for clinical use was the challenge for large-scale manufacturing and purification. This limitation has been overcome using bioreactors for clinical vector production.\(^{28}\) The lentiviral vector appears to be more efficient than adenoviral vectors, as higher IFN levels were generated at a much lower MOI than required with the adenovirus (data not shown). We observed little to no toxicity in normal cells infected with the adenoviral-IFN\(\alpha\) and similarly have not observed any obvious evidence of toxicity in animals treated with lentiv-IFN\(\alpha\). However, if lenti-IFN\(\alpha\) is developed for clinical use, more formal toxicity studies will be performed. One concern with lentiviral gene therapy is the potential risk for integration into the host’s genome and the theoretical risk for insertional mutagenesis and development of a secondary malignancy. However, the benefits of long-term IFN\(\alpha\) production may outweigh this theoretical risk. Using the lentiviral vector, we observed robust expression of the IFN transgene in multiple cell lines in vitro, from murine urothelium, and from syngeneic murine bladder tumors after intravesical therapy. LV-IFN\(\alpha\) treatment resulted in significantly improved survival in our BLCA murine models, which also correlated with upregulation of IFN-target genes, including PD-L1 and TRAIL, and multiple cellular pathways involved in cytotoxicity and immune cell recruitment.

Interestingly, despite comparable expression levels of IFN\(\alpha\) following LV-IFN\(\alpha\) transduction in BBN975 cells, only MB49 and
Figure 6. Electron microscopy and transduction of bladder urothelium treated with LV vectors
(A) Normal mouse urothelium at 50,000×.
(B) Mouse urothelium 4 h post-LV-IFNγ instillation demonstrating one vacuole containing a virus particle inside the cytoplasm (red circle) at 100,000× is shown.
(C) Twenty-four hours post-instillation of LV-IFNγ demonstrates four vacuoles with LV vectors within them at 50,000×.
(D) Ninety-six hours post-LV-IFNγ instillation, four vacuoles containing LV vectors adjacent to the nucleus at 75,000× are shown.
(E) After five weekly instillations, at 10,000×, there are numerous vector-filled vacuoles outside the nucleus in a second cell layer (* denotes vacuoles and arrow denotes cell layer).
(F) Mouse urothelium after 3 months of BBN followed by
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UPPL1541 cells lines were sensitive to IFNz-mediated cell death. We found that this sensitivity correlated with TRAIL expression and activation of death receptor signaling and retinoic-acid-mediated apoptotic signaling pathways. Our group has previously reported TRAIL-mediated cell death in human BLCA cells mediated by caspase 8.12 Consistent with this mechanism, we observed that caspase 8 inhibition rescued TRAIL-mediated cell death in MB49 and UPPL1541 cells after exposure to rIFNz, whereas in the resistant BBN975 cells, recombinant TRAIL failed to induce cell death. We acknowledge that this is indirect evidence, and more detailed studies are being carried out to molecularly tease out the pathways contributing to cell death.

Molecular analyses of IFNz-treated cells clarified some of the mechanisms driving the therapeutic efficacy of and cellular resistance to LV-IFNz. Ninety IFNz response genes were upregulated in all three cell lines and included CD274 (PD-L1); chemokines, such as CXCL10 and CXCL5; GBP family proteins; IRF7; USP18; and the SLFN family known to be activated by IFN treatment.15 When restricting the comparison to the cell lines sensitive to LV-IFNz (MB49 and UPPL1541), TLR3, IL-15, CXCL11, TNFSF10 (TRAIL), and caspase 12 were differentially expressed. Of these, only caspase 12, which is involved in ER stress response,11,32 was differentially upregulated in MB49 and downregulated in UPPL1541. Caspase 12 inhibition rescued cell death in MB49, suggesting that at least two caspases, caspase 8 and caspase 12, are involved in cell death pathways in at least a subset of BLCAs. Notably, MB49 cells were also sensitive to cell death by tunicamycin, an ER-stress-inducing compound, suggesting ER-mediated stress may be a novel mechanism of direct toxicity associated with IFNz gene therapy. It remains to be determined whether ER-mediated cellular stress responses are specific to IFNz gene therapy or mediated by lentiviral transduction.

In earlier work, we identified IL-6 as an important mediator of anti-tumor effects of IFNz in our murine MB49 subcutaneous model.11 Here, we found that IL-6 was detectable at the protein level upon treatment with LV-IFNz in only the two sensitive cell lines MB49 and UPPL1541 and not in the resistant BBN975 line. This further supports the observation that IL-6 induction predicts response to IFNz.17 This result was confirmed by MTT assay at 72 h after treatment, which demonstrated that MB49 cell proliferation was significantly lower in LV-IFNz-treated cells compared with LV-Ctrl (Figure S2A). This is supported by other studies showing STAT1-mediated upregulation of IL-6 results in apoptosis in different cell types.33,34 STAT1 was upregulated and phosphorylated in all three cell lines; however, IL-6 was detectable only in the IFNz-sensitive cell lines. Taken together, IFNz-mediated cell death in culture may therefore be occurring by at least three mechanisms: (1) TRAIL-mediated cell death through caspase 8 in MB49 and UPPL1541, (2) ER-stress-induced cell death mediated by caspase 4 and 12 in MB49, and (3) IL-6 induction that induces cell death via STAT1 activation in MB49 and UPPL1541 cells.

GSEA of mouse tumors confirmed that in vivo efficacy resulted from activation of apoptotic pathways, natural-killer-cell-mediated cytotoxicity, and Fc-gamma-receptor-mediated phagocytosis in LV-IFNz-treated tumors. Accordingly, IPA showed increased expression of cytotoxicity genes, such as KlrA3, Gzma, Kitl, and Lamp1.35–38 In addition, when immune cell populations were analyzed by whole-tumor RNA-seq, we identified enrichment of genes identifying cytotoxic immune cell populations, suggesting multiple distinct mechanisms of tumor killing (apoptosis, ER stress, and cytotoxic immune cell recruitment) may be driving the observed in vivo efficacy of LV-IFNz. Future studies utilizing single-cell sequencing techniques and fluorescence-activated cell sorting (FACS) analysis will be required to conclusively identify the tumor-associated immune cell populations necessary for IFNz efficacy.

Finally, we identified three biomarkers that are part of clinically targetable molecular pathways using the biomarker filter of IPA. Aldh1a, an enzyme that has been associated with stem cell properties in cancer and resistance to drugs, is upregulated and can be targeted by disulfiram and chlorpropanamide.39,40 Epidermal growth factor receptor (EGFR) has been targeted in BLCA, with both cetuximab and erlotinib demonstrating effective anti-tumor activity in preclinical models.41 Finally, PD-L1 targeting has revolutionized cancer treatment in multiple disease settings, including BLCA, and there is great clinical potential for synergistic gene therapy and checkpoint inhibitor regimens currently being evaluated in clinical trials. Biomarker-driven combination strategies may provide therapeutic benefit for patients who would otherwise fail to respond or develop resistance to IFNz monotherapy.

One concern with LV-IFNz treatment includes the potential for local toxicity. We did not identify morphological changes to the urothelium or clinical symptoms in mice treated with LV-IFNz, suggesting good local tolerance. Additional areas for future investigation include identifying optimal dosing regimens, the long-term safety and durability of lentiviral-mediated IFNz expression by transduced urothelium, and the systemic immune responses elicited against both the lentiviral vector and tumor neoantigens following intravesical LV-IFNz therapy.

In summary, our investigation demonstrates the feasibility of using lentiviral vectors for the treatment of BLCA. We also improved our mechanistic understanding of LV-IFNz-mediated cytotoxicity within BLCA cell lines and murine preclinical disease models. Future studies...
will directly compare the efficacy of adeno- and lentiviral-based IFNα gene therapy and the therapeutic potential of combination therapy regimens.

MATERIALS AND METHODS

Cell lines

Mouse urothelial cell line MB49/GFP-luciferase was a generous gift from Dr. Robert Svatek (The University of Texas Health, San Antonio), and UPPL1541 (derived from UPII PTEN/p53 null) and BBN975 (p53−/− mice treated with BBN) lines were a generous gift from Dr. William Kim (University of North Carolina). Cells were grown in minimum essential medium (MB49) or Dulbecco’s modified Eagle medium (BBN975 and UPPL1541) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin). For viral transduction, cells were seeded in 6-well culture dishes at varying densities (MB49 50,000; UPPL1541 75,000; and BBN975 75,000). After overnight attachment, media were replaced with polybrene-containing media (4 μg/mL) and viral particles were added at desired MOI. Recombinant proteins murine IFNα (100 U/mL), TRAIL (100 ng/mL), caspase 8 inhibitor (50 mM), and pancaspase inhibitor (50 mM) were also added to seeded cells. Trypan blue dye exclusion method (ViCell, Beckman Coulter Genomics) was used to determine cytotoxicity. Annexin V allophycocyanin and propidium iodide staining (Thermo Fisher Scientific, 88-8102-72) were used to determine percentage of apoptotic cells.

Table 1. Biomarkers identified by IPA in mouse tumors

| Symbol   | Entrez gene name | Family      | Drug(s)                                          | Expr log ratio | Expr p value | Biomarker application(s) | Diseases (relevant)                                      |
|----------|------------------|-------------|-------------------------------------------------|----------------|--------------|--------------------------|----------------------------------------------------------|
| ALDH1A1  | aldehyde dehydrogenase | enzyme | disulfiram, chlorpropamide                      | 3.437          | 0.041        | diagnosis and disease progression | cancer, immunological disease, inflammatory disease, inflammatory response, and renal and urological disease |
| ALDH3A1  | aldehyde dehydrogenase | enzyme |                                                  | 2.577          | 0.012        | diagnosis                | cancer, immunological disease, and renal and urological disease |
| CARD9    | caspase recruitment domain | other |                                                  | 3.979          | 0.000        | diagnosis                | cancer, inflammatory disease, inflammatory response       |
| CAV1     | caveolin 1        | receptor   | hemay020, cetuximab and erlotinib, CK-101, etc.  | 1.839          | 0.047        | diagnosis                | cancer                                                  |
| EGFR     | epidermal growth factor | kinase |                                                  | 2.162          | 0.045        | diagnosis, disease progression, efficacy, prognosis, response to therapy, safety | cancer, immunological disease, inflammatory disease, inflammatory response, and renal and urological disease |
| ERCC5    | ERCC excision repair 5, endonuclease | enzyme |                                                  | 2.105          | 0.016        | diagnosis and response to therapy | cancer, immunological disease, inflammatory disease, inflammatory response, and renal and urological disease |
| HP       | haptoglobin       | peptidase  |                                                  | 4.224          | 0.004        | diagnosis and efficacy   | cancer, immunological disease, inflammatory response, and renal and urological disease |
| IL-23A   | interleukin-23 subunit alpha | cytokine | LY307/4828, tildrakizumab, and guselkumab        | −3.196         | 0.016        | diagnosis                | cancer                                                  |
| LGALS7/ LGALS7B | galectin 7 | other |                                                  | −5.229         | 0.005        | disease progression       | cancer and renal and urological disease                  |
| PPL      | periplakin        | other      |                                                  | 2.072          | 0.049        | diagnosis                | cancer and renal and urological disease                  |
| VEGFD    | vascular endothelial growth factor D | growth factor |                                                  | 2.867          | 0.014        | diagnosis, disease progression, efficacy, and prognosis | cancer, hypersensitivity response, immunological disease, inflammatory disease, and inflammatory response |

Figure 7. Efficacy of LV-IFNα in murine MB49 intravesical model

(A) Kaplan-Meier plot showing percent survival in C57Bl/6, MB49 intravesical disease model treated with vehicle (Syn3), LV-Ctrl, and LV-IFNα (p = 0.0004). (B) H&E staining of whole-mount bladder tissues with corresponding Ki67 staining in MB49 is shown. (C) IHC for CD4 and CD8 T cells on MB49 intravesical tumors treated with Syn3 (vehicle), LV-Ctrl, and LV-IFNα vectors is shown. (D) Quantification of CD4 and CD8 cells in the MB49 intravesical model is shown. (E) Heatmap of 190 significantly altered genes at an FDR cutoff 0.05 and log2 fold change of one between LV-IFNα and LV-Ctrl groups. (F) GSEA analysis shows enrichment of apoptosis, natural-killer-cell-mediated cytotoxicity, and Fc-gamma-receptor-mediated cytotoxicity (top panel) and EGFR tyrosine kinase inhibitor resistance, platinum drug resistance in LV-IFNα-treated tumors, and enrichment of bladder cancer gene set in LV-Ctrl group.
was used to determine relative gene expression. StepOnePlus Real-Time PCR instrument. Comparative Ct method TRAIL (Mm01283606_m1), and GAPDH (Mm99999915_g1) on probes to detect IRF7 (Mm00516788_m1), PD-L1 (Mm00452054), qPCR analysis (LV-GFP, LV-Ctrl, and LV-IFN). FKD Therapies, University of Finland, provided lentiviral vectors. Viral vectors subcloned into pSWOP shuttle vector to generate lentiviral murine IFN11 vector. Viral particles with the vector were used to transduce HeLa cells to check efficiency of IFN11 production. After confirmation of protein expression, the LV-IFNz was used to transduce BLCA cell lines.

**Viral vectors**
FKD Therapies, University of Finland, provided lentiviral vectors (LV-GFP, LV-Ctrl, and LV-IFN). Briefly, Invitrogen’s Gateway cloning technology was used for cloning murine IFNz11 gene into pDONR211 vector to obtain pENTRY mIFNz11 and then subcloned into pSWOP shuttle vector to generate lentiviral murine IFNz11 vector. Viral particles with the vector were used to transduce HeLa cells to check efficiency of IFNz11 production. After confirmation of protein expression, the LV-IFNz was used to transduce BLCA cell lines.

**qPCR analysis**
RNA was extracted using Ambion miRVANA kit (AM1561) and quantified using NanoDrop ND-1000 spectrophotometer. Twenty nanograms of RNA was used along with AgPAth-ID One-Step RT-PCR kit (Thermo Fisher Scientific, 4387391) with Taqman probes to detect IRF7 (Mm00516788_m1), PD-L1 (Mm00452054), TRAIL (Mm01283606_m1), and GAPDH (Mm99999915_g1) on a StepOnePlus Real-Time PCR instrument. Comparative Ct method was used to determine relative gene expression.

**ELISA for murine IFNz**
Cell-free supernatants from cells treated with the lentiviral vectors were collected, and levels of murine IFN were measured by ELISA (PBL, 42115–1). Urine IFNz levels were measured by Cloud Clone Corp murine IFNz11 kit (SE090Mu).

**Gene expression profiling**
Whole transcriptome RNA-seq was performed on Ion Gene Studio S5 (Thermo Fisher Scientific). RNA was isolated from cell lines using miRVANA miRNA isolation kit (Thermo Fisher Scientific) using manufacturer’s instructions. Quality of RNA was assessed using Nanodrop ND-1000 spectrophotometer and by 4200 Tapestation (Agilent Technologies). Twenty nanograms of RNA was transcribed into cDNA using Ion AmpliSeq transcriptome mouse gene expression chef-ready kit (A36412). cDNA was amplified and subsequently ligated with adapters and barcode. Purified libraries were quantified using Ion library quantitation kit (Thermo Fisher Scientific) and pooled in a set of eight followed by enrichment on IonChef (Thermo Fisher Scientific). Enriched samples were then loaded onto Ion 540 chips and run on the Ion Gene Studio S5. Primary analysis of RNA-seq data was performed using AmpliseqRNA analysis plugin in the Torrent Suite software. Raw reads were aligned with mouse reference genome Ampliseq_Mouse_Transcriptome_V1_ Reference. Raw counts and normalized reads per gene per million mapped reads (rpm) were generated, which was used for subsequent analysis. Unsupervised analysis was performed to identify outliers and assess overall similarities and differences among the samples. Differential expression analysis was performed using t tests, and FDR was estimated using the beta-uniform mixture method.

**Electron microscopy**
Samples were fixed with a solution containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.3 and then washed in 0.1 M sodium cacodylate buffer and treated with 0.1% Millipore-filtered cacodylate buffered tannic acid, post-fixed with 1% buffered osmium, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 60°C oven for approximately 3 days. Ultrathin sections were made using a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques, Danvers, MA).

**Western blotting**
Cells were grown in monolayer and treated with lentiviral vectors. After 48 h, cells were washed once with phosphate-buffered saline (PBS) and scraped off the plates into whole-cell lysis buffer (50 mM Tris-HCl [pH 7.4]; 150 mM NaCl; 5 mM EDTA; 25 mM NaF; 1% Triton X-100; 1% NP-40; 0.1 M Na3VO4; 12.5 mM β-glycerophosphate; 1 mM PMSP) containing protease and phosphatase inhibitors. Cell lysates were prepared by incubation on ice for 30–40 min with

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**Table 2. IPA of gene sets involved in cytotoxicity and recruitment of neutrophils and granulocytes**

| Gene ID | Prediction (based on measurement direction) | Log2Fold change | Findings from other studies |
|--------|---------------------------------------------|-----------------|----------------------------|
| Klr3   | increased                                    | 5.743           | increased                  |
| Gzma   | increased                                    | 4.611           | increased                  |
| Sgk1   | decreased                                   | 3.628           | decreased                  |
| Krl    | increased                                   | 2.536           | increased                  |
| Lamp1  | increased                                   | 2.027           | increased                  |
| Il23a  | decreased                                   | -3.196          | increased                  |
| Recruitment of neutrophils |
| Il21r  | affected                                    | -4.028          | affected                   |
| Lcn2   | increased                                   | 3.693           | increased                  |
| Adrb2  | increased                                   | 3.284           | increased                  |
| Cxcl1  | increased                                   | 3.165           | increased                  |
| Xdh    | increased                                   | 2.688           | increased                  |
| P2rx1  | decreased                                   | -3.127          | increased                  |
| Recruitment of granulocytes |
| Agt    | increased                                   | 7.097           | increased                  |
| Card9  | decreased                                   | 3.979           | decreased                  |
| Lcn2   | increased                                   | 3.693           | increased                  |
| Adrb2  | increased                                   | 3.284           | increased                  |
| Cxcl1  | increased                                   | 3.165           | increased                  |
| Xdh    | increased                                   | 2.688           | increased                  |
| Krl    | increased                                   | 2.536           | increased                  |
| P2rx1  | decreased                                   | -3.127          | increased                  |
| Il21r  | decreased                                   | -4.028          | increased                  |
Figure 8. Expression of cell-type-specific immune cell markers in MB49 tumors
Heatmap of expression comparing LV-IFNα versus LV-Ctrl (A) and LV-IFNα versus vehicle (B) in MB49 tumors. Asterisk indicates significantly altered genes.
intermittent vortexing every 10 min. Protein concentration was measured (Micro BCA protein assay kit, Thermo Fisher Scientific), and 30 μg of protein was resolved on 4%–15% gradient gel (Bio-Rad) and transferred to a nitrocellulose membrane. The blots were blocked in 10% mild powder in PBS and probed with antibodies against PD-L1 (AF1019, Bio-Techne), STAT1 (33–1400, Invitrogen), pSTAT1 (Cell Signaling Technology, no. 9171), and β-actin (AC-74). Species-specific secondary antibodies were used to detect proteins bands using an enhanced chemiluminescence detection kit (GE Healthcare).

In vivo experiments
All animal experiments were conducted in compliance with the Institutional Animal Care and Use Committee guidelines at MD Anderson Cancer Center (Houston, TX). For checking gene transfer efficiency in murine bladder, 6- to 8-week-old female C57Bl/6 mice were anesthetized using isoflurane and the urethra was catheterized using a 20G angiocatheter. Bladders were flushed with PBS and instilled with lentiviral vectors/Syn3 (1 mg/mL) for 40 min. Mice were allowed to recover and returned to their cages. Urine was collected after instillations, and mouse bladders were collected for βgal staining or IFN ELISA.

Superficial bladder tumors were established using previously published protocols. Briefly, after anesthetizing mice with isoflurane, mouse urethra was catheterized and instilled with 100 μL of poly(L-lysine) (PLL) (0.01 μg/mL) for 15 min. After emptying the bladder, 25,000 MB49 cells in 100 μL of HBSS were instilled into mouse bladders for 30 min. Mice were allowed to recover, and tumor burden was assessed by IVIS Spectrum Imaging System and Living Image Software (PerkinElmer). Mice were treated intravesically with either Syn3 (vehicle), LV-Ctrl (3 × 107 virus particles), or LV-IFN (3 × 107 virus particles, treatment), and tumor growth was monitored. Mice with significant reduction in body weight, lethargy, and hematuria were deemed as moribund and were humanely euthanized. All experiments were repeated thrice.

Histology and immunostaining
Mouse tissues were fixed in buffered formalin, embedded in paraffin, and sectioned at the Research Histology core laboratory at MD Anderson Cancer Center. Immunostaining was performed with the specified antibodies, and species-specific horseradish-peroxidase-conjugated secondary antibodies were used to detect proteins using the 3,3-diaminobenzidine substrate kit (Vector Laboratories). Sections were counterstained with hematoxylin and mounted in Permount. Images were captured using Nanozoomer image scanner (Hamamatsu). For βgal staining, mouse bladders were fixed and stained as described earlier.

Statistical methods
Statistical analysis was performed using GraphPad Prism 7 software. ANOVA was used to make multiple group comparisons with Tukey’s test, and results were considered significant when p < 0.05. Log rank test was used to perform the survival analysis.

Data availability statement
The RNA-seq data are available through NCBI Gene Expression Omnibus (GEO): GSE205493 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205493).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.06.005.

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AUTHOR CONTRIBUTIONS
C.P.D., D.J.M., N.R.P., S.Y.-H., and S.M. conceived the project and wrote the manuscript. S.M., V.M.N., A.H.L., J.J.D., A.K., T.S.M., A.P.M., D.P., S.S.A., M.J.M., K.D., and T.N. performed experiments, developed reagents, and analyzed data. B.J. and K.S.S. helped in data interpretation.

DECLARATION OF INTERESTS
C.P.D. received personal compensation from FKD Therapies, Oy for consulting and advisory services, including serving as the Independent Chairperson of the steering committee for the phase 3 nadofaragene firadenovec (rAd-IFNα2b/Syn3) trial.

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