Type III interferon inhibits bladder cancer progression by reprogramming macrophage-mediated phagocytosis and orchestrating effective immune responses

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Abstract

Background

Interferons (IFNs) are essential for activating an effective immune response and play a central role in immunotherapy-mediated immune cell reactivation for tumor regression. Type III IFN (λ), related to type I IFN (α), plays a crucial role in infections, autoimmunity, and cancer. However, the direct effects of IFN-λ on the tumor immune microenvironment have not been thoroughly investigated.

Methods

We used mouse MB49 bladder tumor models, constructed a retroviral vector expressing mouse IFN-λ3, and transduced tumor cells to evaluate the antitumor action of IFN-λ3 in immune-proficient tumors and T cell-deficient tumors. Furthermore, human bladder cancer samples (Cohort 1, n = 15) were used for IHC and mIF analysis to assess the expression pattern of IFN-λ3 in human bladder cancer and correlate it with immune cells’ infiltration. IHC analysis was performed in neoadjuvant immunotherapy cohort (Cohort 2, n = 20) to assess the correlation between IFN-λ3 expression and the pathologic complete response rate.

Results

In immune-proficient tumors, ectopic Ifnl3 expression in tumor cells significantly increased the infiltration of cytotoxic CD8+ T cells, Th1 cells, natural killer cells, M1-like macrophages, and dendritic cells. Transcriptomic analyses revealed significant upregulation of many genes associated with effective immune response, including lymphocyte recruitment, activation, and phagocytosis, consistent with increased antitumor immune infiltrates and tumor inhibition. Furthermore, IFN-λ3 activity sensitized immune-proficient tumors to anti-PD-1/PD-L1 blockade. In T cell-deficient tumors, increased Ly6G−Ly6C+I-A/I-E+ phagocytic macrophages still enhanced tumor cell phagocytosis in Ifnl3 over-expressing tumors. IFN-λ3 is expressed by tumor and stromal cells in human bladder cancer, and high IFN-λ3 expression was positively associated with effector immune infiltrates and the efficacy of immune checkpoint blockade therapy.

Conclusions

Our study indicated that IFN-λ3 enables macrophage-mediated phagocytosis and antitumor immune responses and suggests a rationale for using Type III IFN as a predictive biomarker and potential immunotherapeutic candidate for bladder cancer.

Background
Urothelial cell carcinoma of the bladder (UCB) is one of a few solid malignancies routinely treated with immunotherapy\textsuperscript{1,2}. The most exciting approach has been the immune checkpoint inhibitors, particularly programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) inhibitors, which represent a major treatment breakthrough for some patients with locally advanced or metastatic UCB\textsuperscript{3}. However, many patients still experience unsatisfactory clinical outcomes\textsuperscript{4}. A comprehensive understanding of the fundamental cellular and molecular mechanisms responsible for antitumor immunity could lead to optimized treatment strategies.

IFNs are required to activate effective immune responses during tumor progression\textsuperscript{5}. They play a central role in reactivating immune cells for immunotherapy-mediated tumor regression\textsuperscript{6−8}. Currently, IFNs are subdivided into three major classes: type I (α\textsubscript{1−13}, β, ε, κ, ω), type II (γ), and the recently described type III (λ)\textsuperscript{7,9}. Pre-existing IFN signatures, particularly type I IFNs, are highly associated with immune checkpoint inhibitor treatment efficacy in various human cancer types, including UCB\textsuperscript{10−13}. However, the limited efficacy and constitutional symptom-related adverse events associated with IFN-α treatment have restricted enthusiasm for its clinical application\textsuperscript{14}.

Type III IFN, which is related to IFN-α, consists of four IFNL genes (IFNL1, IFNL2, IFNL3, and IFLN4) in humans and only two functional Ifnl genes (Ifnl2 and Ifnl3) in mice\textsuperscript{7,9,15}. IFN-λ plays a crucial role in infections, autoimmunity, and cancer\textsuperscript{15−17}. The antitumor activity of IFN-λ has been reported in several mouse models, including melanoma, fibrosarcoma, and lung carcinoma\textsuperscript{18−20}. In humans, IFN-λ has been demonstrated to directly induce apoptosis in various tumor cells or stimulate monocyte-derived macrophage (Mφ) cytotoxicity, phagocytosis, and proinflammatory cytokine secretion to mediate immune response \textit{in vitro}\textsuperscript{21}. Although IFN-λ induces a weaker STAT1 activation than IFN-α, it can still trigger an effective immune response in a cell type-dependent manner, including in epithelial and myeloid cells\textsuperscript{21,22}. Therefore, IFN-λ-based therapies, alone or with other immunotherapies, could potentially overcome the adverse effects associated with IFN-α-based treatments.

Here, we aimed to elucidate IFN-λ function and mechanisms in bladder cancer progression and determine its clinical significance in human UCB tissues. We developed a retroviral vector encoding mouse IFN-λ3 and transduced tumor cells to evaluate the antitumor effect of IFN-λ3. IFN-λ3 secreted by transduced tumor cells markedly inhibited \textit{in vivo} subcutaneous tumor growth in the MB49 mouse bladder tumor model. We further confirmed that the effect of IFN-λ3 on tumor progression depended on repolarizing tumor-associated Mφs toward antitumoral phenotypes and functions, including tumor cell phagocytosis and improved adaptive immune responses. Furthermore, we found that PD-L1 blockade was more effective in leading to tumor regression in IFN-λ3\textsuperscript{high} than IFN-λ3\textsuperscript{low} tumors. Finally, we demonstrated that IFN-λ3 is expressed by human UCB tissues and stromal cells and that high IFN-λ3 expression was positively associated with high densities of infiltrating effector T, natural killer (NK), and antigen-presenting cells, and the efficacy of immune checkpoint blockade therapy. Thus, our findings indicate that IFN-λ uniquely activated innate and adaptive immune responses against tumor progression.
Methods

Mice

Female C57BL/6 and BALB/c nude mice aged 6–8 weeks were purchased from a certified vendor (SJA Laboratory Animal Co., Ltd, China). The mice were housed under specific pathogen-free conditions with a 12-h light-dark cycle in the South China University of Technology animal facility.

Cell line

The mouse bladder cancer MB49 cell line was used to investigate the *in vitro* and *in vivo* functions of IFN-λ3. Cells were tested yearly for mycoplasma contamination using a mycoplasma detection kit (Yeasen, China). The mouse *Ifnl3* plasmid was purchased from (IGEbio, China). For stable transfection, HEK-293T-derived lentivirus was used in conjunction with polybrene (Beyotime, China). Specifically, HEK-293T packaging cells were seeded at a density of $1 \times 10^6$ cells per medium-sized dish. The HEK-293T cells were transfected with 3 µg pCDH-IFN-λ3 vector, 2.25 µg psPAX2, and 0.75 µg pMD2G using 400 µl Opti-MEM and 9 µl x-tremeGENE (Roche, Switzerland). After collecting the supernatant with the viral particles over 48 hours, it was filtered through a 0.45-µm syringe filter and concentrated using 4 M NaCl and PEG 8000. The viral particles were resuspended in PBS and stored at − 80°C following centrifugation at 3200 $g$ for 20 min. The cells were cultured at 37°C under 5% CO$_2$ in DMEM (Gibco, USA) supplemented with 10% FBS (ExCell Bio, China), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco, USA).

Subcutaneous bladder cancer studies

Tumors were established by subcutaneous injection of $3 \times 10^5$ MB49-EV or transfected MB49-*Ifnl3*OE cells into the flank of C57BL/6 and $1 \times 10^5$ MB49-EV or transfected MB49-*Ifnl3*OE cells into the flank of BALB/c nude mice, respectively. Subcutaneous MB49-EV and MB49-*Ifnl3*OE bladder cancer models were established in 6–8-week-old C57BL/6 mice to evaluate the effect of anti-PD-L1 treatment. From the fifth day after implantation, the mice were administered four intraperitoneally injections, once every three days, of 50 µg anti-mouse PD-L1 antibody (BioXcell, USA) or Rat isotype IgG (BioXcell, USA) as control. The tumors were measured, and the volumes were calculated in mm$^3$ using the following formula:

\[
\text{Volume} = \frac{\text{[Length (mm) } \times \text{ Width (mm)}^2]}{2}.
\]

Human UCB samples

We enrolled 15 patients who underwent transurethral bladder tumor resection or radical cystectomy during 2020–2021 (Cohort 1) and 20 who underwent radical cystectomy for resectable UCB during 2021–2022 (Cohort 2) at the Sun Yat-sen Memorial Hospital. Cohort 1 UCB tissue samples were used for IHC and mIF analysis to assess the expression pattern of IFN-λ3 in UCB tissues and correlate it with
immune cells’ infiltration. Cohort 2 received at least three neoadjuvant therapy cycles (anti-PD-1, gemcitabine, and cisplatin) before cystectomy. Pathologic complete response was defined as pT0N0. IHC analysis was performed in Cohort 2 to assess the correlation between IFN-λ3 expression and the pathologic complete response rate. Tumors were graded according to the World Health Organization 2016 classification and staged using the TNM classification (8th edition, 2017). Follow-up was performed every three months in the first year, every six months in the second year, and annually thereafter. The patients’ clinicopathological characteristics are described in Table S1.

**Histology, IHC, and mIF**

Tumor samples fixed in formalin and embedded in paraffin were cut into 5-µm sections and stained by H&E, IHC, and mIF as previously described. H&E-stained MB49-EV and MB49-Ifnl3OE tumor sections were analyzed for immune cell infiltration using the Nuclear Phenotype feature on the HALO Image Analysis Platform (Indica Labs, USA).

For IHC, antigen retrieval was done with Tris-EDTA (pH = 9.0) in a high-pressure cooker for 15 min. Unspecifc protein binding sites were blocked with 5% BSA (Thermo Fisher Scientifc, USA) for one hour at room temperature. Slides were incubated with primary antibodies (listed in Table S2) at 4°C overnight. The sections were developed with peroxidase-conjugated secondary antibodies and stained with peroxidase and 3,3′-diaminobenzidine tetrahydrochloride in an Envision System (Agilent Dako, USA). Hematoxylin was used for counterstaining. After dehydration and mounting, animal slides were captured at 200× magnifed high-power elds by an ECLIPSE Ni-E/Ni-U microscope (Nikon Corporation, Japan) and analyzed by ImageJ software (http://imagej.nih.gov/ij/). Human slides were scanned by Vectra Polaris Automated Quantitative Pathology Imaging System (PerkinElmer, USA). Subsequently, InForm 2.5.0 software (PerkinElmer, USA) randomly deined and quantied 16 zones within the bladder cancer tissues, each measuring 0.64 mm².

For mIF, sections were incubated with anti-mouse or anti-human primary antibodies (Table S2), followed by incubation with appropriate peroxidase-conjugated secondary antibodies (Agilent Dako, USA). Detection was performed using a PANO 5-plex IHC kit (PANOVUE, China), which includes PPD520-, PPD570-, and PPD690-labeled Tyramide. The slides were counterstained with DAPI. Stained slides were scanned into images. Image analysis was performed using InForm 2.5.0 software (PerkinElmer, USA). The analysis procedures included tissue and cell segmentation and scoring. Tumor and blank areas were defied in tissue segmentation to accurately calculate the tumor areas. For cell segmentation, the nucleus was deined by a relative DAPI intensity of 0.1, a splitting sensitivity of 0.4, a minimum size of 20nm, and a cytoplasm thickness of 1.0nm. Positivity scoring thresholds for the various markers were determined by automatic calculations and manual adjustments.

**RNA and protein isolation**

The tumor RNA and proteins were extracted, purifed, and quantifed following previously established methods. For RNA extraction, bladder tumor samples were homogenized in RNAiso Plus (Takara,
Japan) using a Tissuelyser (Shanghai Jingxin, China) at 60 Hz, work 30 s, rest 30 s, five cycles for five minutes. After centrifugation, the supernatant was stored at −80°C until use. RNA was isolated using the RNA Quick Purification kit (ESscience, China), quantified using Nanodrop One (Thermo Fisher Scientific, USA), and stored at −80°C until use. For protein extraction, bladder tumor samples were homogenized in PBS containing 0.1% Tween 20 (Servicebio, China) and 4% protease inhibitor (CWBio, China) using a Tissuelyser at 60 Hz, work 30 s, rest 30 s, five cycles for five minutes. The homogenate was transferred to a new tube and snap-frozen in liquid nitrogen for one minute, followed by thawing in a 37°C water bath for three minutes. Subsequently, samples were sonicated for one minute and centrifuged at 13,000 g for ten minutes at 4°C. The supernatant was transferred to a new tube and stored at −80°C until use. Protein concentration was determined by a bicinchoninic acid (BCA) assay kit (CWBio, China). Specific protein levels in the TME were quantified as the rate of their concentration to the total protein concentration (ng/mg).

**Reverse transcription quantitative real-time PCR (RT-qPCR)**

Total RNA was extracted from culture cells or bladder tumor tissues using the RNA Quick Purification kit (ESscience, China). One microgram of total RNA was reverse transcribed to cDNA templates using a cDNA synthesis kit (Yeasen, China) and amplified following the manufacturer’s instructions. RT-qPCR was performed in triplicate using SYBR Green (Accurate Biology, China) and LightCycler 480 II (Roche, Switzerland) or ABI QuantStudio Dx (Thermo Fisher Scientific, USA) in a reaction volume of 10 µl. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The target genes’ expression was normalized to GAPDH and displayed as fold change relative to the MB49-EV control group for cells and tumor tissues. Primers were purchased from Tianyi Huiyuan Gene Technology (China) (Table S3).

**RNA sequencing**

Mouse bladder tumor total RNA samples were sent to HaploX Genomics Center (China) for RNA sequencing. Each group comprised three samples. The RNA samples were qualified and quantified using Agilent 4200 TapeStation, Qubit 3.0, and Nanodrop. Transcriptional libraries were prepared and sequenced using Illumina PE150 (USA). RNA sequencing data were aligned to the mouse reference genome (mm10). Raw sequencing data were demultiplexed and examined for quality. Differentially expressed genes (DEGs) were screened using the R package limma (https://bioconductor.org/) with a threshold of log2 fold change $\geq 1$ and adjusted $P<0.05$, and further analyzed by R packages clusterProfiler, DOSE, G0.db, and topGO (https://bioconductor.org/).

**Data retrieval and preprocessing**

RNA-sequencing expression data from human bladder cancer and normal samples were obtained from TCGA public database (https://tcga-data.nci.nih.gov/tcga/). Based on the Creative Commons 3.0 License, the complete expression data and detailed clinical information of the UCB immunotherapy-related IMvigor210 cohort were obtained from http://research-pub.gene.com/IMvigor210CoreBiologies/packageVersions/. 
Wilcoxon tests assessed whether the \textit{IFNL1}, \textit{IFNL2}, and \textit{IFNL3} genes were differentially expressed between tumor and adjacent normal samples in TCGA dataset. The same method was applied to assess differential expression in the response and non-response groups in the IMvigor210 immunotherapy dataset.

The MCP-counter method was used to estimate the relative abundance of several immune cell populations\textsuperscript{28}. Considering the multiple isoforms of the human \textit{IFNG} gene, we ran the MCP-counter method using an \textit{IFNL} gene set that included \textit{IFNL1}, \textit{IFNL2}, and \textit{IFNL3}. We also identified the effector genes of TIICs using previous studies (Table S4)\textsuperscript{29}. High (top 50%) and low (bottom 50%) \textit{IFNL} signature scores defined by the MCP-counter method were used for gene set enrichment analysis and correlation analysis with TIICs in human bladder cancers from TCGA dataset.

**Flow cytometry**

Fresh tumor tissues, lymph nodes, and spleens were harvested from tumor-bearing mice and used for flow cytometric analysis\textsuperscript{30}. Bladder tumors and tumor-draining lymph nodes were incubated with collagenase type II (Worthington, USA) and filtered through a 70-µm cell strainer to obtain single-cell suspensions. Spleen suspensions were also filtered through a 70-µm cell strainer and treated with an RBC lysis buffer (CWBio, China) to remove the red blood cells. The lymph node, spleen, and tumor cells were stained with live/dead fixable viability dye and subsequently with the following monoclonal surface antibodies: anti-CD45.2, anti-CD3ε, anti-NK1.1, anti-CD4, and anti-CD8α (Table S5). For intracellular and nuclear staining, cells were fixed, permeabilized with the Foxp3 Transcription Factor Staining Buffer Set (eBiosciences, USA), and stained with antibodies against T-bet, Foxp3, Ki-67, and Granzyme B (Table S5). Stained cells were assayed using a CytoFLEX flow cytometer (Beckman Coulter, USA). The single-cell populations were gated on the forward/side scatter plots for analysis. The live cell population was gated for the fixable viability dye-negative population. Gates for intracellular markers were determined using IgG isotypes. Data were analyzed using FlowJo software (TreeStar, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

The cytokines and chemokines were quantified by ELISA following the manufacturer's instructions (R&D Systems, USA). The Mouse IFN-λ2/3, CXCL9, and CXCL10 DuoSet ELISA Kits (R&D Systems, USA) were used to measure the expression of IFN-λ3, CXCL9, and CXCL10 in mouse bladder tumor tissues or cell line supernatants (Table S6). In the case of human samples, the expression of CXCL9 and CXCL10 in Mφs, supernatants stimulated with human IFN-λ1 (10 ng/ml) or IFN-α (10 ng/ml) were quantified using the human CXCL9 and CXCL10 DuoSet ELISA Kits (R&D Systems, USA; Table S6).

**In vitro proliferation assays**

CCK-8 and colony formation assays were conducted to compare the proliferation capacity of MB49-\textit{Ifnl3OE} and MB49-EV\textsuperscript{31}. In the CCK-8 assay, the transfected tumor cells were seeded in 96-well plates at a density of 1,000 cells per well for four days. Viable cells were incubated with 10% CCK-8 reagent (ApeXbio, USA) for two hours, followed by measurement of absorbance at 450 nm using a microplate
reader (TECAN Spark, Switzerland). In the colony formation assay, the transfected tumor cells were seeded in 6-well plates at a density of 1,000 cells per well and maintained in DMEM complete medium containing 10% FBS for 5–7 days. The cells were fixed with methanol, stained with 0.01% crystal violet for 15 mins, and imaged and analyzed using vSpot Spectrum (AID, Germany) to quantify colony formation.

**Generation of human polarized Mφs**

Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy donors using a Ficoll density gradient as previously described\(^{32,33}\). Monocytes were separated from the peripheral blood mononuclear cells using anti-CD14 magnetic beads (Miltenyi Biotechnology, UK) following the manufacturer’s instructions. Resting Mφs were obtained by culturing \(10^6/\text{ml}\) CD14\(^+\) monocytes for six days in RPMI 1640 containing 10% FBS with 25 ng/ml recombinant human M-CSF (PeproTech, USA). Mφs were polarized by stimulating them with 10 ng/ml recombinant human IFN-\(\alpha\) (rh IFN-\(\alpha\), R&D Systems, USA) or 100 ng/ml recombinant human IFN-\(\lambda\) 1 (rh IFN-\(\lambda\) 1, R&D Systems, USA; Table S6) for either 6 or 12 h.

**Generations of bone marrow-derived Mφs**

To generate BMDMs, bone marrow cells were collected from femurs and tibias of 6–8-week-old C57BL/6J mice\(^{34}\). After lysing the erythrocytes with RBC Lysis Buffer (CWBio, China), \(1 \times 10^6\) cells/ml were seeded in 6-well plates. BMDM culture medium comprised DMEM with 10% FBS, 1% penicillin/streptomycin (Gibco, USA), and 20 ng/ml murine M-CSF (PeproTech, USA; Table S6). The culture medium was replaced every three days. On days 6–7, the BMDMs were confirmed to be > 80% F4/80\(^+\) by flow cytometry, performed as described above, and used for *in vitro* phagocytosis assays. Cells were dissociated using a cell scraper and vigorous pipetting.

**Ex vivo phagocytosis assay**

BMDMs were cultured with live MB49 bladder tumor cells as a phagocytosis assay\(^{21,34}\). Phagocytosis of live tumor cells was measured by flow cytometry. Specifically, BMDMs were cultured with GFP-expressing MB49 cells at a 1:2 ratio for 18 h. Subsequently, the cells were washed and labeled with PE/Cyanine7 anti-mouse CD11b antibody (Biolegend, USA) or BV510 rat anti-mouse F4/80 antibody (Biolegend, USA) as outlined in Table S5. The resulting samples were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, USA).

**Statistical analysis**

Graphs were generated, and statistical analysis was performed using Prism 8.0 (GraphPad Software, USA). Two independent groups were compared using the Student’s *t*-test when the *F* test outcome was insignificant; otherwise, the Mann-Whitney *U* test was used. Means of three or more groups were compared by one-way ANOVA with Tukey’s multiple comparison test. Repeated measures two-way ANOVA compared the tumor growth and CCK-8 curves. Spearman’s rank correlation assessed protein
correlations in the TME. The individual data points shown in the graphs represent independent biological replicates. RT-qPCR results were normalized to the zero time point, set to a relative quantity of 1.0. IFN-λ3-positivity in human bladder cancer tissues was defined as $\geq 1\%$ IFN-λ3$^+$ cells. Two-tailed $P$-values $< 0.05$ were considered statistically significant. It should be noted that the TCGA and IMvigor210 datasets were analyzed separately from the other experiments described above.

**Results**

**Ectopic Ifnl3 expression significantly inhibits MB49 tumor progression**

We used the subcutaneous MB49 bladder cancer mouse model to evaluate the *in vivo* antitumor effect of IFN-λ3. Given the low endogenous expression of IFN-λ3 in the MB49 cell line, we ectopically overexpressed *Ifnl3* in MB49 cells (MB49-*Ifnl3*OE) and confirmed the altered expression by RT-qPCR and ELISA (Fig. 1a-b). We also confirmed high IFN-λ3 expression in MB49-*Ifnl3*OE tumor tissues by ELISA assay and immunohistochemistry (IHC) staining (Figure S1a-b). Although MB49-*Ifnl3*OE cells did not change tumor cell proliferation when compared to MB49 cells transfected with empty vector (EV), as indicated by Cell counting kit-8 (CCK-8) and colony-formation *in vitro* assays (Fig. 1c and Figure S1c-d), ectopic *Ifnl3* expression significantly reduced MB49 tumor growth and weight in immune-competent C57BL/6 mice (Fig. 1d–f). H&E staining of sections, analyzed by Nuclear Phenotype on the HALO software platform (Indica Labs, USA), revealed increased immune cell infiltration in MB49-*Ifnl3*OE tumors (Fig. 1g). These results demonstrated that IFN-λ3 induced a potent antitumor response against bladder cancer.

**Ectopic Ifnl3 expression promotes immune activation in MB49-*Ifnl3* OE tumors**

We compared RNA-Seq transcriptome analysis of MB49-*Ifnl3*OE and MB49-EV tumors 23 days after subcutaneous tumor cells injection to investigate the potential mechanisms underlying the *in vivo* antitumor effect of IFN-λ3. As expected, most upregulated genes were associated with Types I and III interferon responses, JAK-STAT signaling and lymphocyte activation in MB49-*Ifnl3*OE tumors (Fig. 2b-d and Figure S2c). Further analysis revealed significant upregulation of many genes associated with cytokine-cytokine receptor interactions, chemokine signaling pathways, and phagocytosis in MB49-*Ifnl3*OE tumors (Figure S2a-b and S2d-e). Lymphocyte exhaustion genes such as *Cd274* (PD-L1) and *Pdcd1* (PD-1) were also significantly upregulated at the detection time (Fig. 2a). Moreover, gene expressions of selected markers were confirmed by RT-qPCR in a larger group of specimens, including those used for RNA-Seq (Figure S3a). The expressions of T-cell maturation and activation markers, including *Ifng*, *Il15*, and *Il18*, were increased in MB49-*Ifnl3*OE tumors. Furthermore, the expressions of antitumoral Mφ markers (*Cd86*, *Tnfsf10*, and *Nos2*) and T cell-associated chemokines, such as *Cxcl9* and *Cxcl10*, were also increased in MB49-*Ifnl3*OE tumors. However, the pro-tumoral Mφ marker *Arg1* expression was comparable in MB49-*Ifnl3*OE and MB49-EV tumors. These data indicated that the effector T cell and antitumoral Mφ phenotypes were positively correlated with antitumor effects of IFN-λ3.
Ectopic Ifnl3 expression led to tumor cytotoxic T and myeloid cell infiltration

We used two complementary approaches, multicolor flow cytometry of cells harvested from the tumors, draining lymph nodes, and spleens, and IHC and multiplex immunofluorescence (mIF) of cells within the tumor, to assess the tumor-infiltrating cell phenotypes in the tumor microenvironment (TME). The flow cytometry gating strategy is illustrated in Figure S4. Compared with the MB49-EV group, ectopic Ifnl3 expression induced a significant increase in the proportion of tumor-infiltrating CD45+ leukocytes and NK1.1+ CD3− NK, CD3+ NK1.1− T, total CD4+ T, CD4+ T-bet+ Th1, CD4+ Foxp3+ regulatory T, and CD8+ effector T (Teff) cells. However, the Teff to regulatory T cell ratio and cytotoxic or proliferative NK cells were comparable in MB49-Ifnl3OE and MB49-EV tumors (Fig. 3a, Figure S5c-d). Among T cells, proliferation (Ki-67+) and activation (GZMB+) markers were significantly enhanced in CD4+ and CD8+ T cells in MB49-Ifnl3OE tumors (Fig. 3a). Detailed analysis of the immune cells in draining lymph nodes on the tumor side and the spleen showed markedly higher CD3+ T cells, total CD4+ T cells, CD4+ T-bet+ Th1 cells, and CD8+ Teffs in mice with MB49-Ifnl3OE tumors than in the MB49-EV mice (Figure S5a-b). Intriguingly, only CD8+ T cells in the draining lymph node, but not in the spleen, of mice with MB49-Ifnl3OE tumors showed increased Ki-67 expression and Teff to regulatory T cell ratio (Figure S5b and S5d), indicating that IFN-λ3 induced a systemic antitumor immune response.

We further assessed various immune cell markers expressed in the tumors using IHC, mIF, or the ELISA assay. We observed a three to fivefold increase in NCR1+ NK, CD4+ T, and CD8+ T cells in MB49-Ifnl3OE tumors compared to the MB49-EV group (Fig. 3b-c). Moreover, in myeloid cells, ectopic Ifnl3 expression resulted in a pronounced increase in F4/80+ Mφs infiltrate, and a smaller increase in CD11c+ F4/80− dendritic cell (DC) infiltrate. We questioned whether a Mφ phenotype transformation occurred in MB49-Ifnl3OE tumors. To address this, we first assessed the expression of known pro- and antitumoral Mφ genes in tumor tissues from both groups (Figure S3a-b). Our data revealed increased expression of antitumoral Mφ genes, including Nos2, Tnfsf10, Tnfa, Cd80, Cd86, Cxcl9, and Cxcl10, and some parallel expression of pro-tumoral Mφ genes (Arg1) in MB49-Ifnl3OE tumors. Additionally, we found that the number of iNOS+F4/80+ Mφs increased while that of Arg1+F4/80+ Mφs decreased (Fig. 3d-e), following the increase in CXCL9+ and CXCL10+ cells in the tumor region (Fig. 3f-g), indicating an effective reprogramming of Mφs toward antitumor functions. Moreover, protein levels of IFN-λ3, CXCL9, and CXCL10 were associated with each other in tumor tissues (Figure S6), suggesting that ectopic Ifnl3 expression increased CXCL9 and CXCL10 in the TME, contributing to increased tumor infiltration by T and NK cells. Furthermore, CD14+ monocytes isolated from human peripheral blood mononuclear cells were differentiated into resting Mφs in vitro (Figure S7a). We confirmed that IFN-λ1 could stimulate these cells to acquire antitumoral phenotypes, including CXCL9, CXCL10, CD80, and CD169 expression (Figure S7b-c) and CXCL10 secretion into the supernatant (Figure S7d). Taken together, these results indicated that ectopic Ifnl3 expression in tumors promoted T-cell infiltration and activation, an influx of myeloid cells, and antitumoral Mφ polarization.
The antitumor effect of IFN-λ3 in MB49 bladder tumors relies on T cells and Mφs

As CD8+ T cells within the TME are strongly associated with the T cell-based antitumor immune response following immunotherapy35, we hypothesized that tumor-infiltrating CD8+ T cells play a crucial role in mediating Ifnl3-driven antitumor immunity. To test this hypothesis, we compared MB49-Ifnl3OE and MB49-EV tumor inhibition rates in immune-deficient nude mice (lacking T and B cells) and immune-competent C57BL/6 mice. Although MB49-Ifnl3OE tumors in C57BL/6 mice exhibited greater suppression than in nude mice (Fig. 4a), a significant delay in tumor growth was observed in MB49-Ifnl3OE compared to MB49-EV tumors in nude mice (Fig. 4b-c), indicating a lymphocyte-independent antitumor component and function in MB49-Ifnl3OE tumors.

Consistent with immune-competent mice, we observed an increase in total F4/80+ and iNOS+F4/80+ Mφs and a decrease in Arg1+F4/80+ Mφs in MB49-Ifnl3OE tumors (Fig. 4d-e). Notably, a phagocytic Mφ subset, identified by the co-expression of the surface markers Ly6C and the MHC class I molecule IA/IE34, was highly enriched in MB49-Ifnl3OE tumors (Fig. 4f-j). However, we observed relatively balanced infiltrate distributions of NCR1+ NK and CD11c+ DC cells in MB49-Ifnl3OE and MB49-EV tumors in nude mice, while Ly6G+ neutrophils were significantly reduced at the detecting point in MB49-Ifnl3OE tumors (Figure S8a-f). These data indicated that Mφs play a role in the antitumor effect of IFN-λ3 in an immune-deficient MB49 bladder tumor model.

Mφs display enhanced phagocytosis of tumor cells with ectopic Ifnl3 expression

As Mφs can act as phagocytes and directly kill tumor cells, and RNA-Seq data indicated phagocytosis pathway augmentation in MB49-Ifnl3OE tumors (Figure S2e), we tested their phagocytic capacity in in vivo and in vitro experiments. We implanted nude mice with MB49-Ifnl3OE or MB49-EV tumors expressing green fluorescence protein (GFP) to measure in vivo phagocytosis. After the tumors were harvested, infiltrating Mφs were analyzed by flow cytometry (Figure S9a). The results showed increased phagocytosis in Ly6C+IA/IE+ phagocytic Mφs rather than CD11b+ monocyte and Mφ populations in MB49-Ifnl3OE tumors compared to MB49-EV tumors (Fig. 5a-d). Phagocytosis was directly visualized in excised MB49-Ifnl3OE tumor tissue in situ by mIF staining using antibodies to F4/80 and GFP, which the MB49 tumor cells expressed (Fig. 5e-f). The fraction of tumor cell-containing Mφs was significantly higher in MB49-Ifnl3OE than in MB49-EV tumors. To better model the effects of ectopic Ifnl3 expression in MB49 tumor cells on Mφ phagocytosis, a coculture of bone marrow-derived Mφs (BMDMs) and viable tumor cells was developed to conduct the in vitro phagocytosis assay. Flow cytometry showed that more MB49-Ifnl3OE than MB49-EV cells were phagocytized by the BMDMs (Fig. 5g-h). These findings demonstrated that ectopic Ifnl3 expression in MB49 tumors enhanced tumor cell uptake by mononuclear phagocytes.
PD-1/PD-L1 axis blockade leads to better antitumor effects in MB49-Ifnl3\textsuperscript{OE} tumors

As IFNs are known to contribute to the quality of antitumor immunity and response to immunotherapy\textsuperscript{7}, we evaluated the relationship between ectopic Ifnl3 expression and the PD-1/PD-L1 axis in a mouse model. Using IHC staining, we observed that the MB49-Ifnl3\textsuperscript{OE} tumors had a higher concentration of infiltrating PD-1\textsuperscript{+} cells than MB49-EV tumors (Figure S10a-b). We next investigated whether blockade of the PD-1/PD-L1 axis could improve IFN-\textlambda\ antitumor efficacy in MB49 tumors. As shown in Fig. 6a, MB49-Ifnl3\textsuperscript{OE} and MB49-EV tumors were generated in C57BL/6 mice. PD-L1-blocking and IgG control antibodies were injected intraperitoneally four times, once every three days, at a dose of 0.05 mg per mouse. As expected, we detected a significant delay in tumor progression in mice with MB49-Ifnl3\textsuperscript{OE} tumors. While no benefit was detected in MB49-EV tumors treated with anti-PD-L1 antibodies, a further reduction in tumor growth was observed in MB49-Ifnl3\textsuperscript{OE} tumors treated with anti-PD-L1 antibodies (Fig. 6b-c). These findings indicated that blocking the PD-1/PD-L1 axis led to a higher Ifnl3 antitumor efficacy in MB49 tumors.

IFN-\textlambda\ is associated with innate and adaptive immune responses in bladder cancer

The antitumor effect of ectopic Ifnl3 expression in MB49 tumors, which depends on T and myeloid cells, led us to investigate the relationship between IFN-\textlambda\ and the innate and adaptive immune response in human bladder cancer. Using data from The Cancer Genome Atlas Urothelial Bladder Carcinoma (TCGA-BLCA), we demonstrated that expression of the IFN-\textlambda\ genes (IFNL1, IFNL2, and IFNL3) was upregulated in tumor tissues compared to adjacent normal tissues (Fig. 7a). Using the microenvironment cell populations (MCP)-counter algorithm, we defined a human IFN-\textlambda\ signature that comprised the three IFN-\textlambda\ isoforms (IFN-\textlambda\1, IFN-\textlambda\2, and IFN-\textlambda\3). Based on the median level of the IFN-\textlambda\ signature, we divided the 412 patients into high and low IFN-\textlambda\ signature groups. We identified the tumor infiltrating immune cells (TIICs) from previous studies and calculated their infiltration level using the MCP-counter algorithm in the TCGA-BLCA dataset\textsuperscript{36,37}. Further analysis revealed that the IFN-\textlambda\ signature was positively associated with the infiltration levels of CD8\textsuperscript{+} T cells, Th1 cells, NK cells, M\textphi\s, and DCs (Fig. 7b). Gene set enrichment analysis showed that positive regulation of leukocyte activation and phagocytosis pathways was enriched in the high-IFN-\textlambda\ signature group (Fig. 7c). These results were validated by IHC and mIF staining in a human bladder cancer cohort ($n=15$). IHC staining showed that IFN-\textlambda\3 is expressed on tumor and stromal cells (Fig. 7d). mIF staining of serial sections was used to evaluate TIIC infiltration, including CD8\textsuperscript{+} T, CD4\textsuperscript{+} T, NCR1\textsuperscript{+} NK, and HLA-DR\textsuperscript{+} antigen-presenting cells. Consistently, tumors in which IFN-\textlambda\3 was detected appeared to have higher TIIC infiltration rates than tumors in which IFN-\textlambda\3 was not detected (Fig. 7e-f). These results were confirmed in a cohort of patients with bladder cancer receiving anti-PD-1 neoadjuvant immunotherapy ($n=20$). IHC staining data showed that IFN-\textlambda\3 detection was higher in responders than in non-responders in this cohort ($P<0.001$; Fig. 7g). Furthermore, we evaluated the IFN-\textlambda\ signature and anti-PD-L1 immunotherapy efficacy in the IMvigor210 cohort\textsuperscript{38}. The IFN-\textlambda\ signature was
positively associated with the immunotherapy efficacy ($P = 0.0342$; Figure S11). Taken together, these results indicated that IFN-λ is associated with innate and adaptive immune responses and the efficacy of immunotherapy in human bladder cancer.

**Discussion**

Our study highlighted the potential of type III interferon to induce Mφ-mediated immune responses against MB49 bladder tumor progression. In immune-proficient tumors, ectopic *Ifnl3* expression in tumor cells significantly increased the infiltration of cytotoxic CD8$^+$ T cells, Th1 cells, NK cells, Mφs, and DCs. Transcriptome analysis revealed significant upregulation of many genes associated with an effective immune response, including lymphocyte recruitment, activation, and phagocytosis. These findings were consistent with the increased antitumor immune infiltrates and tumor inhibition. Increased total Mφs and phagocytic Mφ subsets in T cell-deficient MB49-*Ifnl3*OE tumors enhanced tumor cell phagocytosis. As immunosuppressive regulatory T cells and immune checkpoint molecules also increase in the TME, PD-L1 blockade therapy proved more efficient in MB49-*Ifnl3*OE than in MB49-EV tumors. Furthermore, IFN-λ expression was positively associated with effector immune infiltrates and the efficacy of immune checkpoint blockade (ICB) therapy in patients with UCB. These results indicated that IFN-λ enables T cell- and non-T cell-mediated antitumor immunity and could serve as a candidate target for bladder cancer immunotherapy.

Type III interferon (IFN-λ) can inhibit virus dissemination by regulating adaptive immunity but plays dual roles in tumor progression.$^{16,22}$ IFN-λ showed significant benefits in a preclinical study for melanoma in which the antitumor effect was associated with cell cycle arrest and apoptosis$^{18}$. In contrast, IFN-λ induced matrix metalloproteinase 9 expression and promoted tumor migration and invasiveness in a bladder cancer model$^{39}$. In the current study, ectopic *Ifnl3* expression had no effect on tumor cell proliferation *in vitro*, but MB49-*Ifnl3*OE tumors showed heavy immune cell infiltration and grew significantly slower than MB49-EV tumors. Further investigation indicated that both innate and adaptive immune responses were activated by IFN-λ upregulation in MB49-*Ifnl3*OE tumors. Numasaki et al. confirmed the antitumor role of IFN-λ in a fibrosarcoma cell tumor model and showed that NK cells, T cells, and neutrophils were involved in the process.$^{19}$ Depletion of both NK cells and CD8 T cells at once almost completely abrogated the antitumor action of IFN-λ on fibrosarcoma progression.$^{19}$ In contrast, our study found that while neutrophils decreased, Mφs highly infiltrated the MB49-*Ifnl3*OE tumors and repolarized to antitumor phenotypes. This discrepancy is probably due to differences between tumor cell lines. In our study, the Mφs expressed high levels of T cell-associated chemokine and activating molecules *in vivo* and *in vitro*. Notably, Mφs enhanced phagocytosis of tumor cells and inhibited tumor growth, even in T cell-deficient nude mice with MB49-*Ifnl3*OE tumors. Overall, our study suggests that IFN-λ upregulation elicits Mφ-mediated phagocytosis and Teff and NK cell responses, resulting in MB49 tumor cell death.
Mφs are multi-functional innate immune cells comprising a continuum of phenotypes in TME, spanning from antitumor “defenders” to pro-tumor “remodelers”\textsuperscript{40,41}. We discovered that IFN-λ could redirect Mφ “programming” to the antitumor state in the MB49 model. Consistent with our findings, Scott et al. have shown that IFN-λ stimulates cytotoxicity and phagocytosis in human-derived Mφs and the secretion of pro-inflammatory cytokines \textit{in vitro}\textsuperscript{21}. Specifically, we found that phagocytic Mφs co-expressing MHC class II and Ly6C significantly increase and augment phagocytosis of tumor cells in MB49-\textit{Ifnl3}\textsuperscript{OE} tumors. Previous studies have shown that antagonism between Toll-like receptor 9 agonist and cellular inhibitor of apoptosis protein can enhance phagocytosis by reprogramming Ly6C\textsuperscript{+} Mφs towards a tumor-destructive phenotype\textsuperscript{34,42}. Moreover, our transcriptome analyses revealed that phagocytosis-associated molecules such as SLAMF7 and complement 3 were significantly upregulated in MB49-\textit{Ifnl3}\textsuperscript{OE} tumors. SLAMF7 associates with macrophage-1 antigen, composed of integrins CD11b and CD18, and forms a protein complex on the Mφ cell surface. This complex interacts with two immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors on the Mφ cell surface, FcRγ and DAP12, eliciting signaling via Src kinase, spleen tyrosine kinase, and Btk kinase to activate the phagocytic machinery\textsuperscript{43,44}. Additionally, macrophage-1 antigen is essential in inducing phagocytosis of complement fragment C3bi-opsonized pathogens and apoptotic cells\textsuperscript{44,45}. However, the mechanisms underlying IFN-λ-induced phagocytic repolarization of Mφs require further investigation.

Antitumor immune responses are frequently accompanied by immune suppressive mechanisms, which can be exploited by tumors cells to evade immune surveillance\textsuperscript{46,47}. We observed that the PD-1/PD-L1 axis was highly activated in MB49-\textit{Ifnl3}\textsuperscript{OE} tumors and that suboptimal PD-L1 blockade had a therapeutic effect on MB49-\textit{Ifnl3}\textsuperscript{OE} but not MB49-EV tumors. This finding suggests that IFN-λ upregulation can increase sensitivity to ICB therapy. Previous understanding of the IFN-λ system mostly came from mice and human cell line studies\textsuperscript{16,21}. In human breast cancer, IFN-λ plays a key role in inducing IL-12p70, IFN-γ, CXCR3 ligands, CX3CL1, cytokines, and chemokines involved in NK and Teff cell recruitment and activation\textsuperscript{37}. It was revealed that \textit{IFNL1} or \textit{IFNLR1} gene expression was associated with favorable patient outcomes\textsuperscript{37}. Our study of patients with UCB found that IFN-λ is present in tumor tissues, positively associated with tumor-infiltrating immune cells, and can predict ICB treatment efficacy. Similar results were confirmed in the TCGA-BLCA and IMvigor210 datasets. As only approximately 20% of patients with UCB show an effective response to anti-PD-1 or PD-L1 monotherapy, a combination of anti-PD-1 or PD-L1 therapy and other drugs is the key approach to overcoming ICB resistance\textsuperscript{48,49}. Unfortunately, most combination therapy clinical trials failed for the lack of rational mechanisms\textsuperscript{50}. The present study provides a rationale for combining IFN-λ with anti-PD-L1 drugs for UCB treatment. IFN-λ medication has been used in a clinical trial to treat patients with hepatitis C virus\textsuperscript{51}. TLR agonist can also stimulate the expression of IFN-λ\textsuperscript{52,53}. The rapid development and clinical deployment of COVID-19 vaccines worldwide has highlighted the potential of mRNA-based technologies as useful tools for cancer treatment\textsuperscript{54,55}. These potential methodologies to induce IFN-λ production could help develop new therapeutic strategies.

Conclusions
To our knowledge, this was the first study to demonstrate the expression of IFN-λ in UCB tissues and evaluate the relationship between its expression and tumor-infiltrating immune cells and immunotherapeutic efficacy. Furthermore, our study indicated that IFN-λ upregulation in MB49 tumors reprograms Mφs to an antitumor state, enhances phagocytosis and inflammatory cytokines secretion, and activates adaptive immunity to inhibit tumor progression. Since IFN-λ can promote phagocyte reprogramming in T-cell-deficient mice, we propose that IFN-λ-mediated antitumor immunity is a potential mechanism for controlling ICB refractory tumors.

**Declarations**

**Author Contributions**

B.W., B.Z., and J.C. designed and performed the experiments, analyzed the data, and wrote the manuscript; X.S., W.Y., T.Y., H.Y., P.C., K.C., X.H., X.F., and W.H. contributed to the experimental design and data analysis; J.H. and T.L. supervised the research and provided critical feedback on the experimental design, data analysis, and manuscript writing. All authors have read and approved the manuscript and agree with their inclusion as a co-author.

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**Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All human samples were anonymously coded following the local ethical guidelines as stipulated by the Declaration of Helsinki. Written informed consent was obtained from all patients, and the protocol was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital. All animal experiments were approved by the Institutional Animal Care and Use Committee of the South China University of Technology.
Consent for publication

Not applicable.

Conflict of Interest

The authors declare no potential conflicts of interest.

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**Figures**

**Figure 1**

[Images of figures a to g are not transcribed.]
Ectopic *Ifnl3* expression significantly inhibits MB49 tumor progression

IFN-λ3 is highly overexpressed at the mRNA (a) and protein (b) levels in MB49-*Ifnl3*OE compared to MB49-EV cells. (c) The CCK-8 assay indicates that MB49-*Ifnl3*OE and MB49-EV cells show similar proliferation ability *in vitro* (two-way ANOVA). Representative images (d; scale bar: 1 cm), growth kinetics (e; two-way ANOVA), and weight at harvest (f) of MB49-*Ifnl3*OE and MB49-EV tumors 20 days after implantation (*n = 8* each). (g) H&E staining for calculating the immune cell population in MB49-*Ifnl3*OE and MB49-EV tumors by the Nuclear Phenotype on the HALO software platform. The bar graph presents means ± SEM; *P* values were determined by Student’s *t*-test when the *F* test outcome was insignificant; otherwise, the Mann-Whitney *U* test was used.
Transcriptome analysis of MB49-Ifnl3OE and MB49-EV tumors

Subcutaneous MB49-Ifnl3OE and MB49-EV tumors were established in C57BL/6 mice. The tumors were harvested on day 23, and total RNA was extracted for RNA-Seq. (a) Volcano plot of differentially expressed genes (DEGs; threshold: log2 fold change $\geq 1$, adjusted $P < 0.05$) between MB49-Ifnl3OE and MB49-EV.
MB49-EV tumors. (b) Gene set enrichment analysis (GSEA) showed the enriched pathways in MB49-
Ifnl3OE tumors. Heatmaps of DEGs involved in types I and III Interferon response (c) and lymphocyte
activation (d) genes in MB49-Ifnl3OE and MB49-EV tumors. Data were generated from one experiment.

Figure 3

FC, IHC and mIF analysis of MB49-Ifnl3OE and MB49-EV tumors
Subcutaneous MB49-\textit{Ifnl3}\textsuperscript{OE} and MB49-EV tumors were established in C57BL/6 mice. The tumors were harvested on day 23, and multicolor FC, IHC, and mIF were performed. (a) Percentages of live CD45\textsuperscript{+}, NK1.1\textsuperscript{+}CD3\textsuperscript{−} natural killer, CD3\textsuperscript{+}NK1.1\textsuperscript{−} T, CD4\textsuperscript{+} T, CD8\textsuperscript{+} T, CD4\textsuperscript{+}T-bet\textsuperscript{+} Th1, CD4\textsuperscript{+}GZMB\textsuperscript{+} T, CD4\textsuperscript{+}Ki-67\textsuperscript{+} T, CD8\textsuperscript{+}GZMB\textsuperscript{+} T, and CD8\textsuperscript{+}Ki-67\textsuperscript{+} T cells were analyzed by multicolor FC in MB49-\textit{Ifnl3}\textsuperscript{OE} (n = 8) and MB49-EV (n = 7) tumors. (b, c) Infiltration of T cell subsets, NK cells, F4/80\textsuperscript{+} Mφs, and CD11c\textsuperscript{+}F4/80\textsuperscript{−} DCs in MB49-\textit{Ifnl3}\textsuperscript{OE} and MB49-EV tumor tissue sections (n = 8 each). Representative images with the positive cells stained green (CD4\textsuperscript{+}, NCR1\textsuperscript{+}), red (CD8\textsuperscript{+}, F4/80\textsuperscript{+}), and blue (Foxp3\textsuperscript{+}, CD11C\textsuperscript{+}), and their colocalization (examples indicated with arrows) are presented (b) and quantified (c). (d, e) Phenotype transformation detected by mIF. Pro-inflammatory (iNOS\textsuperscript{+}) and anti-inflammatory (Arg1\textsuperscript{+}) tumor-infiltrating F4/80\textsuperscript{+} Mφs evaluated in MB49-\textit{Ifnl3}\textsuperscript{OE} and MB49-EV tumors (n = 6 each). Representative images with the positive cells stained green (F4/80\textsuperscript{+}), red (Arg1\textsuperscript{+}), and blue (iNOS\textsuperscript{+}) and their colocalization (examples indicated with arrows) (d) and percentages of iNOS\textsuperscript{+} and Arg1\textsuperscript{+} in total F4/80\textsuperscript{+} Mφs (e) in MB49-\textit{Ifnl3}\textsuperscript{OE} and MB49-EV tumors are presented. Representative images (f) and bar graphs (g) of CXCL9\textsuperscript{+} and CXCL10\textsuperscript{+} cell densities in MB49-\textit{Ifnl3}\textsuperscript{OE} and MB49-EV tumors (n = 8 each) stained with IHC and assessed in 200× high power fields. Scale bar, 100 μm. Bar graphs show means ± SEMs; \( P \) values were determined by Student’s \( t \)-test when the \( F \) test outcome was insignificant; otherwise, the Mann-Whitney \( U \) test was used; FC, flow cytometry; IHC, immunohistochemistry; mIF, multiplex immunofluorescence.
Figure 4

The antitumor effect of IFN-λ3 in MB49 bladder tumors relies on T cells and Mφs

Tumors were established by subcutaneous injection of MB49-EV or transfected MB49-Ifnl3OE cells into the flanks of C57BL6 or BALB/c nude mice, respectively. (a) Inhibition rates of MB49-Ifnl3OE and MB49-EV tumors were analyzed in three independent experiments. Inhibition rates were calculated as follows:
inhibition rate (%) = (average MB49-EV tumor volume – average MB49-Ifnl3<sup>OE</sup> tumor volume) / average MB49-EV tumor volume × 100. The rates were used to compare the inhibition efficacy of ectopic Ifnl3 expression in the C57BL/6 and BALB/c nude mice tumor models. (b) Growth kinetics of MB49-Ifnl3<sup>OE</sup> and MB49-EV tumors (n = 6 each) in BALB/c nude mice (two-way ANOVA). (c) MB49-Ifnl3<sup>OE</sup> and MB49-EV tumor weights per BALB/c nude mouse at the endpoint. (d, e) Densities of antitumoral and protumoral Mφs in MB49-Ifnl3<sup>OE</sup> and MB49-EV tumor sections (n = 6 each) by multiplex immunofluorescence. Representative images with positive cells stained green (F4/80<sup>+</sup>), red (Arg1<sup>+</sup>), and blue (iNOS<sup>+</sup>) and their colocalization (examples indicated with arrows) are presented (d; scale bar: 100 μm). Arg1<sup>+</sup>F4/80<sup>+</sup> and iNOS<sup>+</sup>F4/80<sup>+</sup> Mφs in MB49-Ifnl3<sup>OE</sup> and MB49-EV tumors were quantified and compared (e). (f - j) Four Mφ subpopulations were evaluated by flow cytometry in MB49-Ifnl3<sup>OE</sup> and MB49-EV tumors (n = 5 each). Representative images of the four Mφ subpopulations (f) and their percentages in CD11b<sup>+</sup>Ly6G<sup>−</sup> monocyte and Mφ populations (g-j). Bar graphs show means ± SEMs; P values were determined by Student’s t-test when the F test outcome was insignificant; otherwise, the Mann-Whitney U test was used.
Figure 5

Mφs display enhanced phagocytosis of tumor cells with ectopic IfnI3 expression

(a, b) Green fluorescence protein (GFP)-positive MB49-EV or transfected MB49-IfnI3OE cells were injected subcutaneously into the flanks of BALB/c nude mice. Mφ phagocytosis of tumor cells was evaluated by flow cytometry on day 23. Representative plots showing tumor+ CD11b+Ly6G− monocyte and Mφ
populations (a) and their quantification in MB49-Ifnl3OE and MB49-EV tumors (b; n = 5 each). (c, d) Phagocytosis by phagocytic Ly6C⁺IA/IE⁺ Mφs. Representative plots showing tumor⁺ Ly6C⁺IA/IE⁺ Mφs (c) and their quantification in MB49-Ifnl3OE and MB49-EV tumors (d; n = 5 each). (e, f) Phagocytosis assessment by multiplex immunofluorescence in MB49-Ifnl3OE and MB49-EV tumors (n = 5 each). Representative images of GFP⁺ F4/80⁺ Mφs (e) and their quantification (f). (g, h) In vitro phagocytosis assays were assessed by flow cytometry after bone marrow-derived Mφs (BMDMs) were co-cultured with live MB49-Ifnl3OE and MB49-EV tumors (n = 5 each). Representative images of GFP⁺F4/80⁺ Mφs (g) and a bar graph (h) are presented. Each dot represents a biological replicate. Bar graphs show means ± SEMs; P values were determined by Student’s t-test when the F test outcome was insignificant; otherwise, the Mann-Whitney U test was used.

Figure 6
The antitumor effects in MB49-Ifn/Ifnl3OE tumors are better than in MB49-EV tumors after PD-1/PD-L1 axis blockade

Subcutaneous MB49-Ifn/Ifnl3OE and MB49-EV tumors were established in C57BL/6 mice. For the PD-1/PD-L1 pathway blockade experiment, the mice were injected intraperitoneally with 50 µg/dose of anti-PD-L1 antibodies or IgG on days 5, 8, 11, and 14 after tumor cell injection. The tumors were harvested on day 24, $n = 6$ per group. (a) Schematic diagram of the PD-L1 blockade experiment. Tumor growth kinetics (b; one-way ANOVA with Tukey's multiple comparisons test) and weight per mouse at harvest (c; one-way ANOVA with Tukey's multiple comparison test). Bar graphs show means ± SEMs; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1.
Figure 7

IFN-λ is associated with innate and adaptive immune responses in bladder cancer

(a) Box plots of the various IFN-λ isoforms (IFNL1, IFNL2, and IFNL3) expressed in bladder tumor tissues and adjacent normal tissues in The Cancer Genome Atlas (TCGA) datasets (Wilcoxon tests). (b) Heatmap of effector gene sets of the various immune cells determined by the microenvironment cell populations.
(MCP)-counter method in TCGA dataset. (c) Gene set enrichment analysis (GSEA) analysis of positive regulation of leukocyte activation (top) and phagocytosis (down) in a bladder cancer cohort from TCGA.
(d-f) Clinical bladder cancer samples were divided into IFN-λ3\textsuperscript{high} (\(n = 7\)) and IFN-λ3\textsuperscript{low} (\(n = 8\)) groups. IFN-λ3\textsuperscript{high} was defined as ≥ 5% IFN-λ3\textsuperscript{+} cells in human bladder cancer tissues using IHC staining. Representative images of IFN-λ3\textsuperscript{high} and IFN-λ3\textsuperscript{low} cells with IHC staining (d). Positive cells stained cyan (NCR1\textsuperscript{+}), green (CD4\textsuperscript{+}), red (CD8\textsuperscript{+}), and blue (HLA-DR\textsuperscript{+}) by multiplex immunofluorescence are presented (e) and quantified (f). IFN-λ3 expression and anti-PD-1 immunotherapy efficacy in the neoadjuvant immunochemotherapy cohort (g; IFN-λ3\textsuperscript{high} (\(n = 10\)) and IFN-λ3\textsuperscript{low} (\(n = 10\))). Bar graphs show means ± SEMs; \(P\) values were determined by Student’s \(t\) test when the \(F\) test outcome was insignificant; otherwise, the Mann-Whitney \(U\) test was used; PD-1, programmed cell death protein 1; IHC, immunohistochemistry.

**Supplementary Files**

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- Supplementaltables.docx