

Article

Senescence-Driven Inflammation and Immune Dynamics in the Progression of Radiation Cystitis

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Abstract

Pelvic radiation therapy is an essential treatment for several pelvic malignancies, but it can lead to radiation cystitis (RC), a severe progressive inflammatory bladder disorder lacking effective diagnosis and therapeutic options. RC evolves through acute, latent, and chronic phases, ultimately resulting in bladder fibrosis, vascular damage, and hematuria. Here, we characterize the molecular and immunological features associated with RC progression using a preclinical mouse model. Building on a prior analysis of the acute and chronic phases, we examined the previously unanalyzed latent phase and integrated transcriptomics, immune cell profiling, inflammatory protein measurements, and bladder function assessments across all stages. Acute radiation injury was marked by the strong activation of apoptotic pathways, whereas latent and chronic phases were dominated by inflammatory signaling with distinct cytokine and chemokine signatures. The persistent upregulation of *Cdkn1a* (P21) was consistent with sustained senescence-associated signaling, while reductions in IL-27 and shifts in the granulocyte–lymphocyte-enriched immune population during the latent phase were consistent with altered immune regulatory states. At chronic stages, increased SASP-associated proteins and matrix remodeling mediators coincided with bladder functional decline. Together, these findings support a model in which radiation-induced senescence, coupled with immune dysregulation during the latent phase, are coordinated features accompanying inflammation, tissue remodeling, and bladder dysfunction in RC.

Keywords: radiation cystitis; cellular senescence; preclinical model; SASP



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1. Introduction

Aging is a significant risk factor for urological diseases, including underactive bladder and impaired bladder compliance, both of which are irreversible conditions associated with the development of tissue fibrosis [1–3]. Similarly, chronic radiation cystitis (RC)—a long-term bladder complication of pelvic radiation therapy (PRT)—shares similar pathological features. As RC advances, patients may develop fibrosis, vascular fragility, and severe hemorrhagic events [4–6]. Radiation exposure is known to accelerate aging phenotypes, particularly through the induction of cellular senescence, and promote fibrosis and tissue dysfunction across various tissues, including the bladder [7–11]. These parallels suggest that aging biology may play an underrecognized role in RC pathogenesis.

Cellular senescence is a fundamental hallmark of aging. It refers to a state of cell-cycle arrest. Previous studies [12–14] showed that this was triggered by diverse stressors, including DNA damage, mitochondrial dysfunction, and oxidative stress [15–17]. Although senescence initially serves protective functions, preventing the proliferation of damaged cells, suppressing tumors, aiding development, promoting wound healing, supporting tissue remodeling, and developing vasculature, its persistence becomes maladaptive. Accumulated senescent cells disrupt tissue homeostasis, promote chronic inflammation, and contribute to age-related diseases [18–21]. These dual roles underscore the importance of understanding how senescence is regulated in pathological contexts such as RC.

Senescent cells can acquire a senescence-associated secretory phenotype (SASP) characterized by the secretion of a complex mixture of inflammatory proteins, cytokines, chemokines, growth factors and proteases. These factors can interfere with the surrounding tissue by fostering inflammation, recruiting immune cells, and prompting tissue remodeling. Although SASP initially prevents the proliferation of damaged cells and facilitates short-term tissue repair, its chronic expression promotes sustained inflammation, aberrant immune cell recruitment, extracellular matrix degradation, and tissue dysfunction. Persistent SASP signaling is increasingly recognized as a driver of fibrosis and chronic inflammatory disease, positioning it as a potential contributor to RC progression [18,19,22,23]. RC presents as a spectrum of symptoms, ranging from mild dysuria and increased urinary frequency to severe hematuria and bladder dysfunction. Its pathophysiology involves complex inflammatory responses, vascular damage, and fibrosis [4,5,24]. Despite technological advances in radiation delivery and supportive care, RC remains a significant clinical challenge, with no reliable diagnostic, preventive, or therapeutic options, thereby profoundly impacting patients' quality of life [24–27]. A deeper mechanistic understanding is therefore essential to inform new therapeutic strategies. RC develops in patients receiving PRT for malignancies such as prostate, cervical or colorectal cancer. Clinically, RC is characterized by three phases: an acute phase lasting only several weeks after treatment completion, a symptom-free latent phase, and a chronic phase that can start months to years after PRT. The reported incidence of RC varies significantly due to differences in study design, patient populations, and the definitions of cystitis [28–30]. Acute RC is relatively common, with incidences as high as 50% during or shortly after treatment. Chronic RC, though less frequent, affects up to 15% of patients treated with PRT and may manifest months to years after treatment completion. Risk is in part influenced by radiation doses, treatment duration, and patient age, suggesting that both therapy-related and host-related factors shape disease susceptibility.

Using a CT-guided, multi-beam preclinical model of bladder-targeted irradiation, we previously demonstrated that mice develop progressive bladder fibrosis accompanied by functional impairments consistent with human RC [31,32]. Transcriptomic profiling revealed the marked activation of apoptotic pathways during the acute phase followed by increased activity in inflammatory and tissue remodeling pathways at chronic stages [33]. However, the latent phase remained largely unexplored. In this study, we integrate RNA sequencing to assess immune cell profiling, inflammatory protein measurements and bladder functional analysis to define the molecular and immunological mechanisms driving RC progression across all three phases.

We hypothesized that radiation induces persistent bladder senescence and that impaired immune surveillance during the latent phase permits senescent cell accumulation, thereby promoting SASP-driven inflammation, extracellular matrix remodeling, and eventual bladder dysfunction. By characterizing these mechanisms across acute, latent, and chronic phases, we aim to establish a mechanistic framework for RC progression and identify potential targets for therapeutic intervention.

2. Materials and Methods

2.1. Radiation Cystitis Preclinical Model

All in vivo experiments were performed under protocols approved by the Institutional Animal Care and Use Committee (AL-2020-04; AL-23-02) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed, treated, and cared for in an AAALAC-accredited facility under standard conditions. Female C57Bl/6 mice, 8 weeks old, were purchased from The Jackson Laboratory (Harbor, ME, USA) and assigned at random to irradiated or sham-treated cohorts. (Group sizes consisted of five animals per condition and time point, unless stated otherwise.) Female animals were selected to maintain consistency with previously established acute and chronic radiation cystitis datasets. Age-matched controls were used at each time point.

Bladder irradiation was performed using the Small Animal Radiation Research Platform (SARRP) with a two-beam approach, as previously described [31]. Briefly, mice were anesthetized with inhaled 2.5–3% isoflurane and maintained at 1.5–2% during the 30 min procedure. Mice were positioned for the CT-based anatomical localization of the bladder. A two-field irradiation configuration was implemented to confine radiation exposure to the bladder while minimizing dose delivery to adjacent organs. Critical structures, including the spinal cord, colon, and long bones, were excluded from beam paths. A total dose of 40 Gy was administered using a 5 × 5 mm collimator, divided evenly between the two beams.

Following irradiation, mice were monitored in a warmed recovery environment and returned to standard housing upon regaining mobility. Nutritional support was provided for seven days post-treatment using mash and hydrogel. Untreated control mice underwent anesthesia and handling identical to irradiated mice without radiation exposure. Bladder tissues were collected at 1 week, 3 months, and 6 months after irradiation to capture the acute, latent and chronic phases of RC respectively (Figure 1).

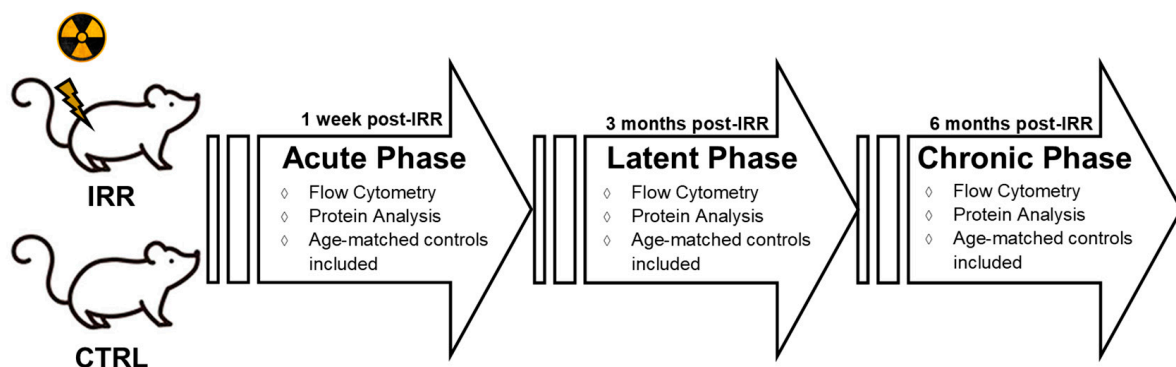


Figure 1. The experimental design for studying RC disease progression. Female mice received a single dose of irradiation (IRR). At indicated time points, bladders were harvested for protein or flow cytometer analysis. Age-matched controls were included at each time point.

2.2. RNA Sequencing

Transcriptomic analysis focused on bladder tissues collected during the latent phase of radiation cystitis and integrated these data with previously reported acute and chronic phase datasets [33]. Latent-phase samples had been sequenced previously but were not analyzed until the present study. As previously described, RNA isolation, library preparation, and sequencing were conducted at the University of Michigan Advanced Genomics Core [33]. Sequencing libraries were generated and run on an Illumina NovaSeq-6000 platform (Illumina, San Diego, CA, USA) with 151 paired-end or single-end cycles. Adapter

sequences were removed with Cutadapt (v2.3), and quality control was performed using FastQC (v0.11.8). Reads were aligned to the GRCh38 reference genome (ENSEMBL) using STAR (v2.6.1b), and gene counts were assigned with RSEM (v1.3.1). Alignment parameters followed ENCODE quality control standards to ensure data reliability prior to downstream analysis.

2.3. Differential Expression Analysis

Genes with expression fewer than 10 counts were excluded before statistical analysis to reduce background noise. Differential gene expression was assessed using DESeq2 (v1.50.2), a generalized linear modeling approach optimized for count-based RNA-seq data. Thresholds were set at a linear fold change >1.5 or <-1.5 , with a Benjamini–Hochberg FDR-adjusted p -value < 0.05 . Data visualization utilized DESeq2 plotting functions and additional R packages (v3.3.3). Genes were annotated with NCBI Entrez GeneIDs and associated text descriptions.

Functional analysis, including pathway activation/inhibition, p -values, and GO term enrichment, was performed using the iPathwayGuide scoring system (ADVITA Bioinformatics, Ann Arbor, MI, USA). Two pruning methods were applied to reduce redundancy in GO terms: high-specificity pruning, which identifies the most specific terms, and smallest common denominator pruning, which selects terms best summarizing the dataset.

2.4. Immune Cell Profiling

Bladders were collected and maintained in MACS Tissue Storage Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) for <12 h at 4°C until analysis. Tissue samples were processed to single cells using a GentleMACS dissociator (Miltenyi Biotec) according to the manufacturer's protocol for the dissociation of mouse kidney using the Multi Tissue Dissociation Kit 2 (Miltenyi Biotec).

Following dissociation, cells were washed, resuspended in BD Stain Buffer, counted, and assessed for viability. Cells were stained for 30 min at 4°C with antibodies against CD3e (BD, San Jose, CA, USA, Cat# 558214), CD19 (BD, Cat# 565473), CD31 (BD, Cat# 553372), CD44 (BD, Cat# 560568), CD45 (BD, Cat# 557235), CD54 (BD, Cat# 753779), CD117 (BD, Cat# 567818), CD127 (BD, Cat# 565490), CD206 (BD, Cat# 566813), CD324 (BD, Cat# 752470), CD1016 (BD, Cat# 740471) and amphiregulin (AREG; LS Bio, Seattle, WA, USA, Cat# LS-C696945-100). Cell viability was assessed using 7-aminoactinomycin D (7-AAD).

Viability was assessed via 7AAD staining. Samples were analyzed on a ZE5 flow cytometer (Bio-Rad, Hercules, CA, USA), and all analysis was performed via FlowJo (V10.8.1). All presented plots and data were gated on viable single cells. Endothelial cells were gated on CD31, urothelial cells were gated on CD324, fibroblasts were gated on CD44, and CD45 was used as a marker of immune cells, and the reported proportions are those of total viable single cells. CD45+ cells were further gated based on FSC and SSC to delineate crude surrogates for granulocytes (FSC high, SSC high), monocytes (FSC mid, SSC mid), and lymphocytes (FSC low, SSC low) with data presented as the relative proportion of CD45+ cells, as shown in representative plots.

2.5. Inflammatory Protein Profiling

Inflammatory mediator profiling was guided by transcriptomic signatures associated with senescence and immune modulation. A 19-multiplex R&D Systems magnetic bead-based immunoassay was used to quantify a panel of cytokines, chemokines, and matrix-associated proteins in bladder tissue lysates [34]. IL-27 was included based on its immunoregulatory role [35,36], while amphiregulin was measured separately using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA #DAR00).

Irradiated and age-matched control bladders (N = 4/group) were harvested at 1 week, 3 months, and 6 months post-treatment and snap-frozen in liquid nitrogen. Tissue lysates were prepared using a commercial Immunoprecipitation Kit (Abcam, Cambridge, UK #ab206996). Briefly, frozen tissues were pulverized with a stainless-steel tissue grinder and resuspended in a non-denaturing cell lysis buffer supplemented with protease inhibitors (50 mg tissue powder per 1 mL buffer). Samples were incubated at room temperature for 1 h and centrifuged ($10,000\times g$, 5 min, 4 °C) to remove debris, and the supernatant was transferred to fresh tubes. Samples were diluted 1:2 in assay buffer and analyzed in duplicate.

The proteins analyzed included MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), MCP-3 (CCL7), MCP-2 (CCL8), Eotaxin (CCL11), MCP-5 (CCL12), MDC (CCL22), GRO α (CXCL1), IP-10 (CXCL10), IFN- γ , TNF- α , ICAM-1 (CD54), IL-10, IL-13, IL-16, IL-27 and MMP-3. Data acquisition employed a Bio-Plex[®] Luminex[®] 200 IS System, with fluorescence intensity measured and converted to protein concentrations using 5-parameter logistic regression. Each plate was run with a standard curve to qualify assay performance.

2.6. Statistical Analysis

Immune cell populations and inflammatory protein levels were assessed via a two-way ANOVA with Sidak's multiple comparison test (GraphPad Prism, v9.5.1), with significance set at $p < 0.05$. Principal Component Analysis (PCA) was performed on detected inflammatory proteins from bladder tissue lysates to assess clustering and separation between irradiated and control samples across disease phases. Normality assumptions were assessed visually using Q-Q plots and frequency distributions.

3. Results

3.1. RC Latent-Phase RNA Sequencing

The RNA sequencing results for the acute and chronic phases were previously reported [33]. The transcriptional evaluation of latent-phase bladder samples demonstrated high sequencing quality with >30 million uniquely mapped reads per sample. Prior to multiple-testing correction, 68 pathways, 2261 Gene Ontology (GO) terms, two miRNAs, 119 gene upstream regulators, 270 chemical upstream regulators, and 85 diseases were significantly enriched, highlighting substantial transcriptomic remodeling during the latent phase.

Significant differentially expressed (SDE) genes were identified using a statistical threshold of $p < 0.05$ and a log fold change (logFC) with an absolute value of at least 0.585. A total of 457 SDE genes were identified (Table S1), comparing latent-phase to age-matched control samples. Pathway p -values were calculated using the iPathwayGuide scoring system, which employs the Impact Analysis method. The predominant impacted pathways are related to immune signaling, i.e., chemokine signaling, antigen processing and presentation, and cytokine–cytokine receptor interaction pathways (Table S2), and the immune response was the primary biological process altered in response to irradiation (Table S3).

RNA sequencing identified the upregulation of apoptotic pathway activity during the acute phase (Figure 2A,B). During the latent (Figure 2C,D) and chronic (Figure 2E,F) phases, activity in inflammatory pathways and processes was significantly increased. However, the immune response profile changed over time. Interestingly, a significant increase in *cyclin-dependent kinase inhibitor 1* (Cdkn1 α), which encodes for the P21 protein, persisted across the acute, latent, and chronic phases (Figure 2) with logFC values for acute, latent and chronic phases of 2.04, 1.41 and 1.38, respectively ($p \leq 0.000001$ at all time points).

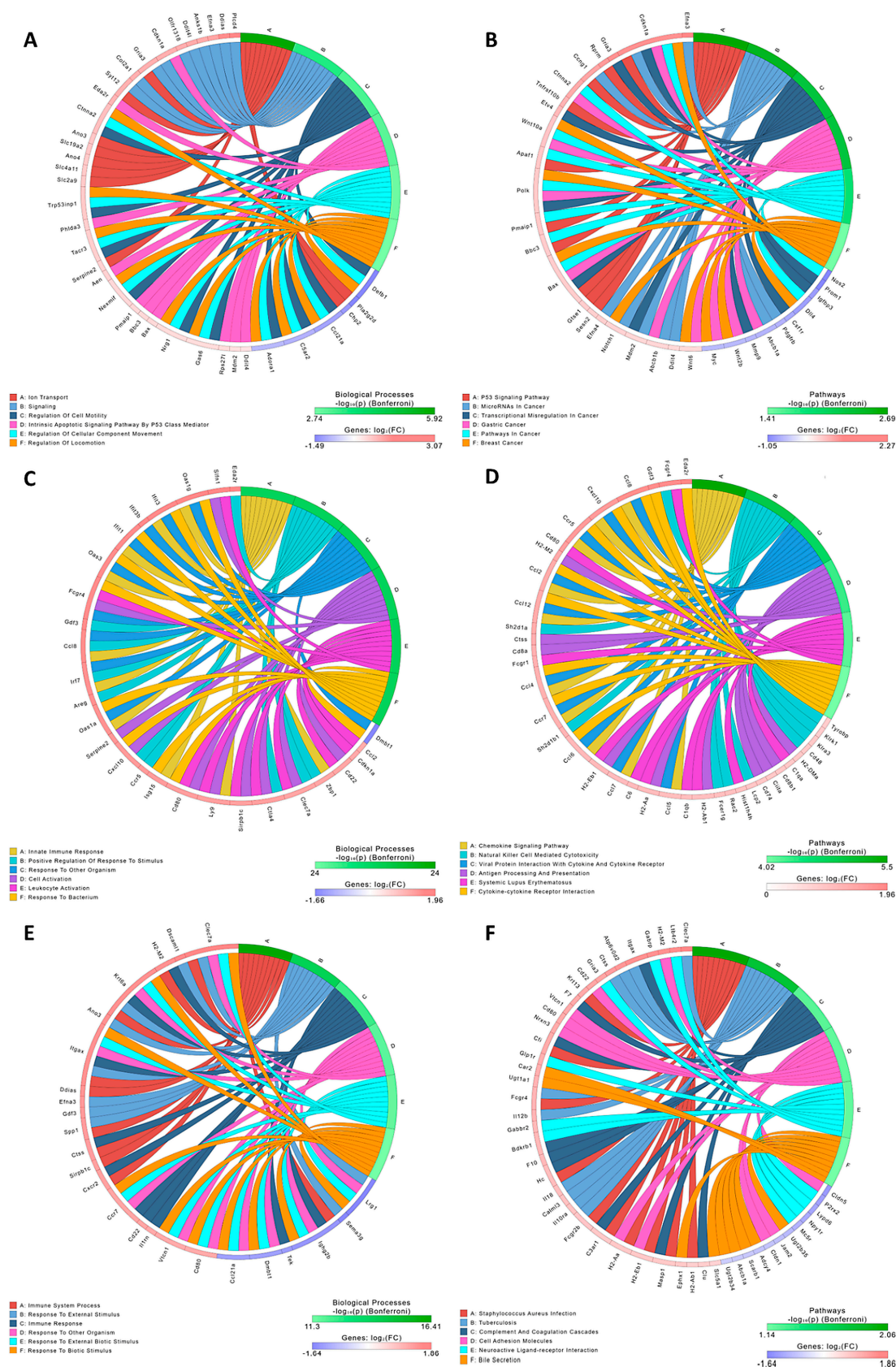


Figure 2. Chord diagram illustrating top genes associated with hallmarks and biological processes in response to irradiation. Here, 1-week (A,B), 3 months (C,D), and 6 months (E,F) post-IRR represent acute, latent and chronic phases of RC disease progression respectively. Acute and chronic phases previously published [33]. Threshold $p < 0.05$ and $\log FC \geq |0.585|$. Higher-magnification images can be found in Supplementary Figure S1.

3.2. Cell Profiling in RC Disease Progression

To characterize immune dynamics during RC progression, we performed flow cytometric profiling of dissociated bladder tissue across acute, latent, and chronic time points. Significant interactions between phase and treatment were detected for granulocyte-enriched and lymphocyte-enriched populations, mast cells and ILC2-associated cell populations (p -values 0.0205, 0.0283, 0.0037, and 0.0493, respectively) (Table S4). A comparison of CD45+ immune cells between control and irradiated bladders at the acute, latent, and chronic phases (representative plots, Figure S2) indicated temporal shifts in immune composition following irradiation. In particular, the relative proportion of granulocyte-enriched populations was inversely correlated to the proportion of lymphocyte-enriched populations after irradiation: during the acute and latent phases, a higher proportion of lymphocyte-enriched populations was observed, while during the chronic phase, granulocyte-enriched populations were more abundant than lymphocyte-enriched populations. No significant phase- or treatment-dependent differences were observed in endothelial, fibroblast, urothelial, or CD45+ cell abundance.

Consistent with these findings, each phase demonstrated unique shifts in immune composition over time (Figure 3). Bladder-resident cell phenotypes (Figure 3A,B) highlight the dynamic remodeling of the immune microenvironment following irradiation, supporting the notion that distinct immune states characterize each phase of RC. Distinct shifts in bladder-resident cell populations occurred, with significant phase-by-treatment interactions observed for granulocyte- and lymphocyte-enriched populations, mast cells, and ILC2-associated cells (Table S4). Comparisons of CD45+ subsets demonstrated that lymphocyte-enriched populations predominated earlier in disease progression, whereas granulocyte-enriched populations became more abundant during the chronic phase (Figure 3C).

3.3. Inflammatory Protein Profiling in RC Disease Progression

Gene expression analysis demonstrated phase-dependent changes in the RNA expression of inflammatory genes (Figure 4A). A total of 11 protein analytes were detected in bladder tissue lysate samples within assay limits (Figure 4B). These included MCP-3, Eotaxin, IL-27, TNF- α , MIP-1 α , MCP-2, MCP-5, GRO α , IL-16, MMP-3, and IL-13. A two-way ANOVA revealed a significant phase-by-treatment interaction for MCP-3, MCP-2, IL-27, and MMP-3 (p -value 0.0142, 0.0364, 0.0391 and 0.0462 respectively, Figure 4B, Table S1). Notably, IL-27 levels were significantly higher in control bladders compared with irradiated bladders at the latent (3-month) phase, consistent with impaired immune surveillance following irradiation. The chronic phase exhibited marked increases in the MCP-3, MCP-2, and MMP-3 mediators associated with leukocyte recruitment and extracellular matrix remodeling, indicating a transition toward a pro-inflammatory, fibrotic microenvironment.

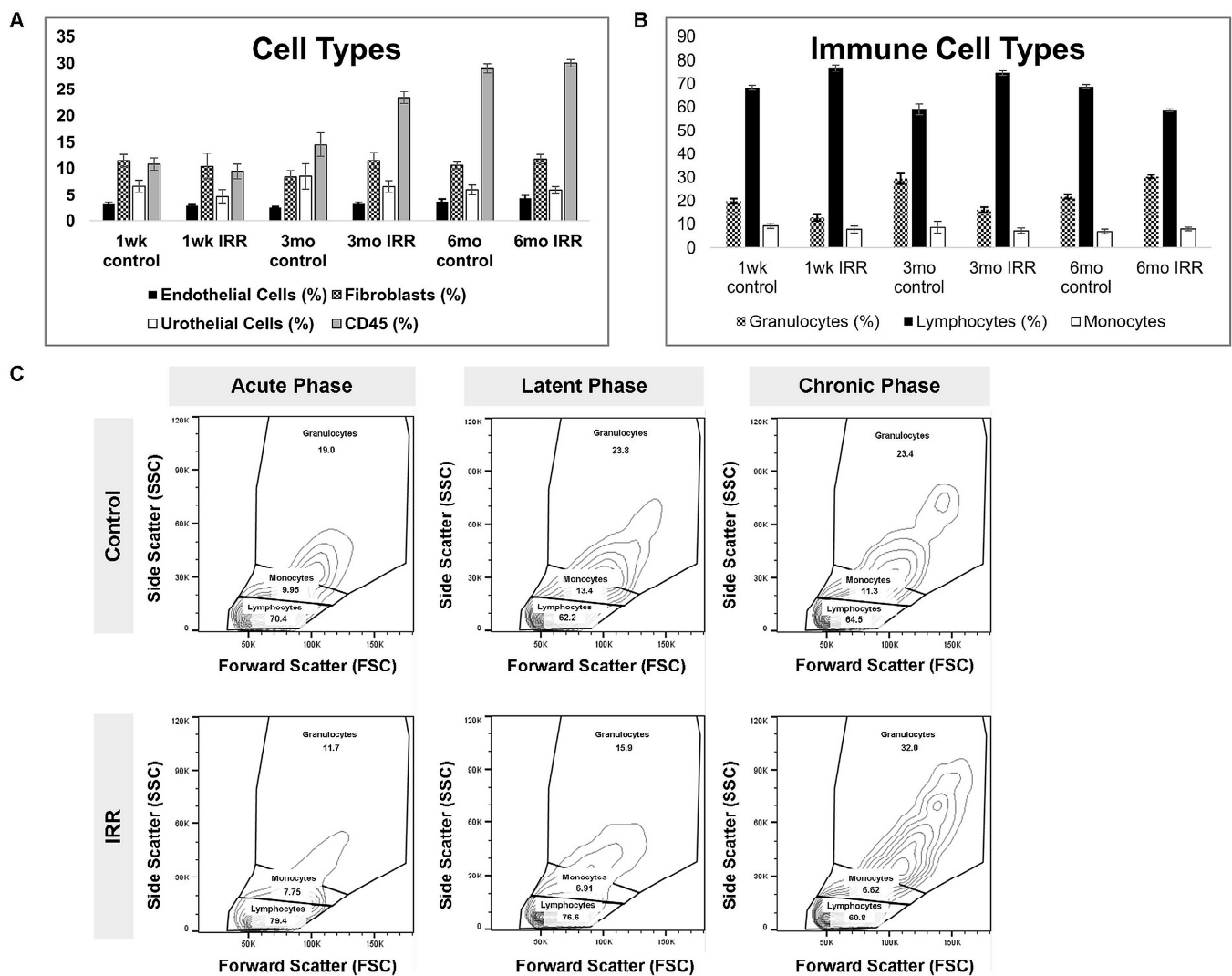


Figure 3. Immune cell profiling at 1 week, 3 months, and 6 months post-IRR to represent acute, latent and chronic phases of RC disease progression. (A) Breakdown of cell phenotypes, (B) immune phenotypes and (C) representative contour flow plots showing forward and side scatter plotting of CD45+ immune cells. Significant phase-by-treatment interactions were observed for granulocytes and lymphocytes.

A PCA of the 11 inflammatory proteins detected in bladder tissue samples (Figure 4C) revealed clear phase-dependent clustering between irradiated and control samples with higher precision in the chronic phase. At the latent phase, PC1 and PC2 accounted for 62.36% of the variability in the data, with MMP-3, Eotaxin, IL-16, and IL-27 exerting the strongest influence on sample separation. Discriminatory power increased in the chronic phase, with PC1 and PC2 explaining 73.26% of variance. Chronic-phase clustering was driven primarily by the elevated levels of the MMP-3, MIP-1 α , and GRO- α markers associated with chronic inflammation, extracellular matrix remodeling, and SASP activity.

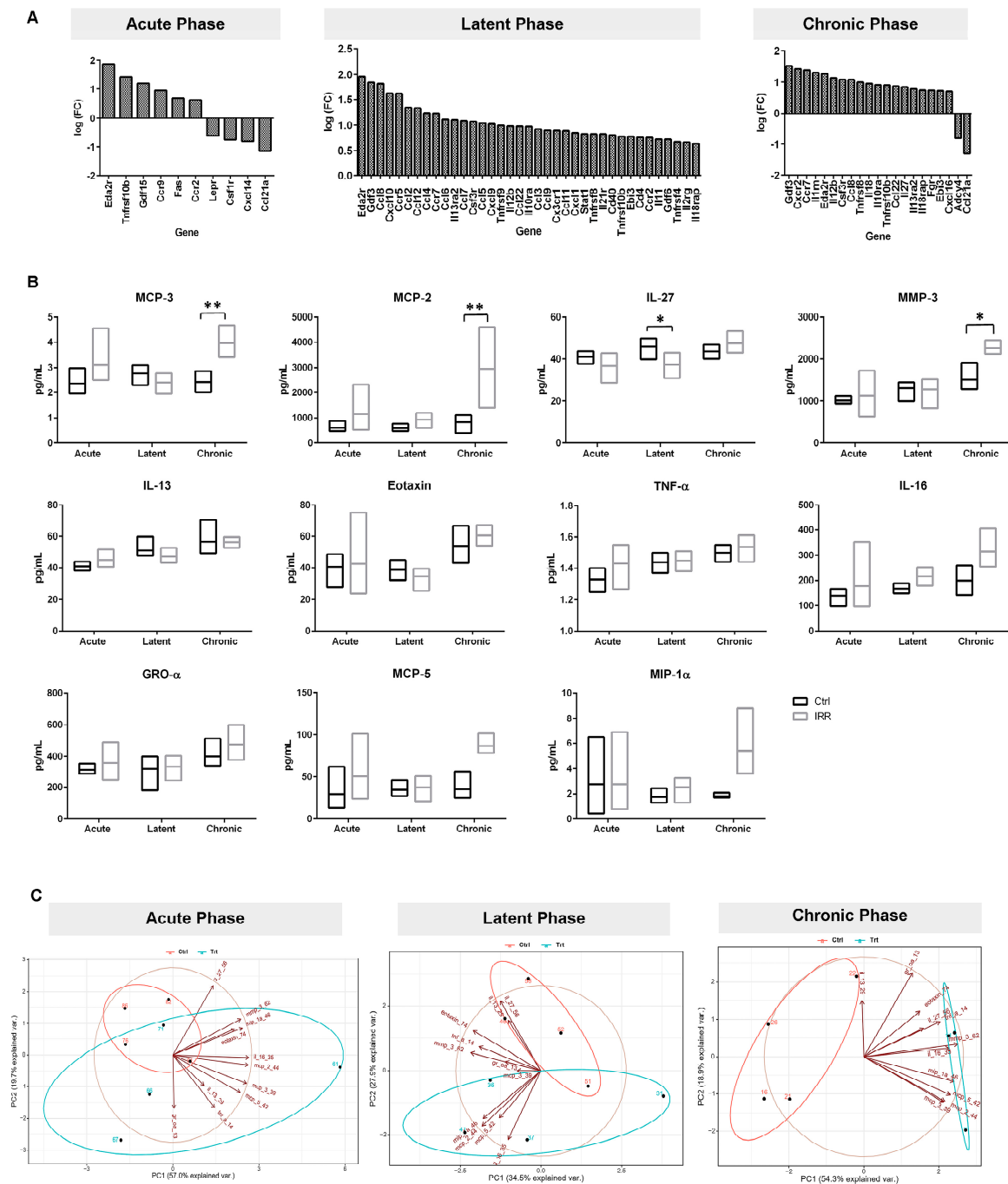


Figure 4. Inflammatory protein profiling at 1 week, 3 months, and 6 months post-IRR to represent acute, latent and chronic phases of RC disease progression. **(A)** RC gene expression, **(B)** representative protein profiling graphs and **(C)** PCA. SDE genes selected from RNA sequencing study previously published at acute, latent (data not previously published) and chronic phases [33]. Genes identified using iPathwayGuide scores. * and ** Significant differentially expressed genes if $p < 0.05$ and $p < 0.01$ respectively. Ctr = control; Trt = irradiation treatment.

4. Discussion

Radiation cystitis is a complex condition marked by acute and chronic inflammation, fibrosis, and vascular damage, progressing through three distinct phases. The initial phase involves bladder symptoms a few weeks after pelvic radiation therapy. This is followed by a symptom-free latent phase, which can last months to years, during which molecular changes may continue despite the absence of clinical signs. Ultimately, the final chronic phase is irreversible, marked by a spectrum of clinical symptoms, from mild dysuria to

impaired bladder compliance and hemorrhaging, for which no standard effective treatment exists. Understanding the biological mechanisms governing the transition between these phases is essential for developing strategies to prevent or mitigate chronic RC.

An established preclinical model of RC was employed to investigate RC disease progression [24,32]. This model resembles the clinical manifestation of RC, with 1 week, 3 months and 6 months post-IRR representing the acute, latent and chronic phases. As previously published, irradiated mice are asymptomatic in the latent phase and develop a significant increase in extracellular matrix stiffness and compromised bladder function 6 months post-IRR treatment [32]. This model therefore provides a controlled framework for dissecting phase-specific molecular and immunological events that precede overt functional decline.

RC RNA sequencing data for the acute and chronic phases were previously published [33], and here we report RNA sequencing data for the latent phase [33]. Transcriptomic analysis across disease stages revealed a dynamic shift in biological pathways in apoptotic and inflammatory pathways as the disease progresses with acute injury dominated by apoptosis-related gene expression, reflecting direct cellular damage immediately following irradiation. By contrast, both the latent and chronic phases showed a strong enrichment in inflammatory pathways, with each phase displaying distinct cytokine and chemokine profiles. Extracellular matrix remodeling genes were highly expressed in the chronic phase, consistent with fibrosis development, indicating an attempt to repair the damage induced by irradiation. Notably, the level of *Cdkn1a* (encoding P21), a key senescence mediator, was persistently elevated across all phases. Sustained P21 expression suggests prolonged senescence induction beginning early after irradiation and continuing into advanced disease stages.

Radiation is known to induce senescence through P21 overexpression in a dose-dependent manner [16,37–39]. Radiation triggers DNA damage response (DDR) pathways, activating key sensor proteins such as ATM and ATR, which in turn phosphorylate downstream effectors, including tumor suppressor p53. Activated p53 induces the transcription of *Cdkn1a* (P21), enforcing cell-cycle arrest and stabilizing the senescent phenotype. This mechanism has been reported across multiple cell types, including smooth muscle, fibroblasts, osteocytes, myeloid cells, B cells, and T cells, showing increased p21 expression after radiation exposure [40–43]. The sustained elevation of P21 observed over 6 months beyond tissue injury is therefore consistent with ongoing DDR activation and suggests a persistent senescence phenotype within the irradiated bladder.

In this study, we did not observe an increase in P16, which is another commonly assessed senescence marker. However, P16 and P21 have distinct mechanisms through which they induce cellular senescence, and high expression levels of either senescent marker are observed in distinct cell populations [44]. In addition, increased P16 and P21 result in different secretory profiles. P21's unique secretory profile plays a role in inducing the immune surveillance response for stressed cells and helps recruit macrophages through the release of chemokines [44].

Accumulating evidence indicates that radiation-induced tissue damage progresses through a self-amplifying feedback loop involving DNA damage, mitochondrial dysfunction, reactive oxygen species (ROS), and SASP proteins [43,45,46]. Under healthy conditions, senescent cells are efficiently eliminated by immune surveillance mechanisms. However, in disease states, senescent cells accumulate in the tissue due to inadequate immune surveillance, enhanced anti-apoptotic mechanisms, increased rates of senescence, or a combination of these factors [46–48]. Their SASP output can chronically inflame and remodel the tissue microenvironment, promoting fibrosis and functional deterioration. Our findings suggest that such mechanisms may underlie RC progression.

SASP proteins contribute to inflammation, regulate immune responses, alter the microenvironment, impair wound healing, and promote fibrosis [47,49]. Many cytokines and chemokines upregulated in our transcriptomic data are established SASP components [23]. By integrating cell and inflammatory protein profiling with bladder function and RNA sequencing, senescence-associated immune dysregulation contributions to RC progression were determined. As RC progresses, we observed a dynamic change in immune cell populations and inflammatory cytokines. Granulocyte-enriched populations; lymphocyte-enriched populations; CD117 and CD127 cells; and MCP-3, MCP-2, IL-27, and MMP-3 demonstrated significant differences between phases. A progressively inflamed and remodeling-prone tissue environment was highlighted by MCP-3 and MCP-2, indicating the enhanced inflammatory chemotaxis of cells such as monocytes, lymphocytes and granulocytes. In addition, increases in MMP-3 indicate that these alterations in the immune microenvironment are commensurate with alterations in extracellular matrix (ECM) remodeling. Transcriptomic and protein profiling revealed the increased expression level of chemokines involved in myeloid cell recruitment, including MCP-2 (CCL8), MCP-3 (CCL7), and MCP-5 (CCL12), across the latent and chronic phases. These changes were accompanied by a proportional expansion of granulocytes and a relative reduction in lymphocytes measured by flow cytometry, consistent with the chemokine-driven skewing of the bladder immune microenvironment. In parallel, the reduction in IL-27 expression during the latent phase, an immunoregulatory cytokine that supports cytotoxic lymphocyte activity and restrains neutrophil-dominated inflammation, aligned with the observed shift toward a granulocyte-dominant immune profile.

These findings indicate that SASP-associated chemokines identified by RNA sequencing and protein profiling provide a mechanistic basis for the immune cell redistribution detected by flow cytometry, linking molecular inflammatory signaling to tissue-level immune remodeling during RC progression.

A particularly notable finding was the significant reduction in IL-27 in irradiated bladders during the latent phase, due to its role in response to damaged cells by antigen-presenting cells which coordinate both innate and adaptive immunity by activating NK and cytotoxic T cells while limiting excessive granulocyte activity [35,50]. IL-27 acts as a negative regulator of granulocyte activity, interfering with both cytokine and ROS production [51]. These immune subsets play central roles in the clearance of senescent cells. Thus, reduced IL-27 at the latent phase is consistent with altered immune regulation that may allow senescence-associated inflammatory processes to persist. Consistent with this interpretation, immunophenotyping revealed a shift from lymphocyte predominance in earlier phases toward granulocyte enrichment in the chronic phase, a pattern compatible with diminished immunoregulatory signaling and progressive inflammatory dysregulation. These findings highlight the latent phase as a critical window during which immune surveillance failure may contribute to long-term RC pathology.

As the disease progresses to the chronic phase, IL-27 levels rebound, surpassing those in control samples, likely reflecting a compensatory or dysregulated immune response in the context of persistent inflammation. Chronic-phase clustering was driven by significant increased MMP-3, MCP-3 and MCP-2, with an increasing trend in MIP-1 α , MCP-5 (CCL12), and IL-16 mediators, which are associated with extracellular matrix turnover and chronic inflammatory signaling. These biochemical changes coincided with an increased infiltration of granulocyte-enriched and lymphocyte-enriched immune populations. Over time, MMP-3 levels were markedly elevated, which may reflect heightened tissue remodeling activity. Together, these data support a model in which early senescence induction, followed by latent-phase immune dysregulation, is associated with SASP amplification and tissue remodeling.

Understanding the mechanisms underlying radiation-induced bladder senescence and tissue fibrosis is crucial for developing preventive and targeted therapies for radiation cystitis. Potential options include the use of senotherapies, a group of chemical compounds that target senescent cells in aging and disease, or immunomodulatory therapies [52–54]. These therapies may offer a means to mitigate radiation-induced bladder fibrosis and dysfunction [9,10,22,55,56]. Collaboration among radiation oncologists, biologists, and clinicians is essential for translating these findings into clinical applications that can optimize treatment outcomes and enhance the quality of life for patients undergoing pelvic radiation. Further research employing single-cell resolution and longitudinal profiling will be essential for defining senescent cell subtypes, immune surveillance dynamics, and the temporal windows in which intervention is the most effective. Translating these insights into clinical strategies will require close collaboration among radiation oncologists, urologists, biologists, and immunologists to improve treatment outcomes and survivorship for patients receiving pelvic radiation therapy.

This study has several limitations that should be acknowledged. First, immune profiling was based on relative cell proportions rather than absolute cell numbers, which may underestimate changes in total immune cell burden within the irradiated bladder. Second, although immune composition and inflammatory signaling were comprehensively characterized, direct functional assays of immune activity, such as cytotoxic or phagocytic capacity, were not performed, and immune surveillance was therefore inferred rather than directly measured. Third, immune cell populations were analyzed at a broad compositional level, and the definitive identification of granulocyte and lymphocyte subsets (e.g., Ly6G/Ly6C or CD4⁺/CD8⁺ T cells) was not performed; thus, immune populations are interpreted as enriched fractions rather than discrete immune lineages. Fourth, the analyses were conducted on bulk tissue, and spatial relationships between senescence-associated signals, immune infiltrates, and tissue remodeling could not be resolved. Finally, only female mice were used in this study to maintain consistency with our previously published acute and chronic phase analyses; this limits the ability to assess sex-specific differences in immune responses and fibrosis. Future studies incorporating male mice and spatially resolved, functionally informative approaches will be essential to extend these findings.

5. Conclusions

Our findings demonstrate that radiation induces a bladder environment resembling accelerated aging, characterized by persistent senescence, altered immune regulation, and progressive tissue remodeling. Central to this process is the latent phase, during which reduced IL-27 and shifts in immune composition are consistent with altered immune regulation. Failure to eliminate senescent cells likely permits their accumulation, amplifying SASP-mediated inflammation and promoting extracellular matrix remodeling. By the chronic phase, this inflammatory–fibrotic loop manifests as structural and functional bladder decline. These results establish a framework in which senescence, immune dysregulation, and SASP activity are coordinated features that together contribute to radiation cystitis progression.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cells15040337/s1>, Figure S1: Cell profiling at 1 week, 3 months, and 6 months post-IRR to represent acute, latent and chronic phases of RC disease progression; Figure S2: Flow cytometric analysis of bladder cell and immune cell populations across acute, latent, and chronic phases after irradiation; Table S1: Significant differentially expressed genes at 3 months post-radiation treatment compared to age-matched controls; Table S2: Pathways and their calculated *p*-values at 3 months post-radiation treatment compared to age-matched control samples; Table S3: Biological processes and their calculated *p*-values at 3 months post-radiation treatment compared to

age-matched control samples; Table S4: Two-way ANOVA for bladder cells in irradiated samples versus time-matched controls harvested at 1 week, 3 months and 6 months post-IRR treatment.

Author Contributions: Conceptualization: S.M., M.B.C. and B.M.M.Z.; Data curation: E.P.W.; Formal analysis: S.M., A.G., E.P.W. and Y.-C.L.; Investigation: S.M., R.M., A.G., E.P.W., S.N.B. and A.M.; Methodology: S.M., B.M.M.Z. and M.B.C.; Project administration: S.M. and B.M.M.Z.; Supervision: B.M.M.Z.; Visualization: S.M. and A.G.; Writing—original manuscript: S.M.; Writing—reviewing and editing: R.M., A.G. and B.M.M.Z. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Corewell Health Research Institute (AL-2020-04, approved on 19 January 2021; AL-23-02, approved on 28 December 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

IRR	Irradiation
PRT	Pelvic Radiation Therapy
RC	Radiation Cystitis
RSEM	RNA-seq by Expectation–Maximization
SARRP	Small Animal Radiation Research Platform
SASP	Senescence-Associated Secretory Phenotype

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