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Original article

Modulation of Cytokine Profiles in Macrophages and Exosomes by Ionizing Radiation

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ABSTRACT Low-dose radiation therapy (LDRT) represents a promising therapeutic approach for managing inflammatory diseases, effectively alleviating both inflammation and pain in patients. Although LDRT has been used clinically for a long time, the precise mechanisms by which LDRT exerts its anti-inflammatory effects remain largely unexplored. This study aims to elucidate the underlying mechanisms by which gamma irradiation, a key component of LDRT, alleviates inflammation. Cytokine analyses demonstrate a significant decrease in tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) levels following gamma irradiation. This observed decrease implies that the interferon-gamma (IFN-γ) signaling pathway may be important in mediating immune responses. Moreover, these findings suggest that these alterations in cytokine expression not only reduce inflammatory response but may also enhance the therapeutic potential of exosomes derived from macrophages, which are known to possess anti-inflammatory properties. These macrophage-derived exosomes, influenced by the cytokine modulation induced by gamma irradiation, could further contribute to the overall anti-inflammatory effect observed in this study. Overall, this research provides novel insights into the immunomodulatory capabilities of gamma irradiation, highlighting the critical role of IFN-γ regulation within the broader context of the anti-inflammatory response. This knowledge has the potential to enhance our understanding of how LDRT can be effectively utilized in clinical practice, thereby improving therapeutic strategies for patients with chronic inflammatory diseases.

Key words: Low-dose radiation therapy, Cytokine modulation, IFN-γ signaling, Exosomes, Anti-inflammatory response

1. Introduction

Very early after the discovery of ionizing radiation, medical applications of ionizing radiation were performed against cancer therapy [1]. However, despite its effectiveness, the risk of radiation-induced cancers and long-term adverse effects limited the applications of radiation therapy to other diseases [2,3]. However, several recent clinical trials have reported that low doses of ionizing radiation can effectively reduce the inflammation and pain of diseases [4]. For instance, Mansoor et al. reported that low-dose radiation therapy (LDRT) has potential efficacy in improving clinical outcomes for patients with severe COVID-19 pneumonia [5]. Yang et al. investigated the effects of LDRT on neuroinflammation in Alzheimer's disease. The LDRT-treated Alzheimer mouse showed reduced neuroinflammation and amyloid-beta accumulation, leading to improved cognitive function [6]. The findings suggest that LDRT could be a potential therapeutic strategy for modulating neuroinflammation and slowing disease progression in Alzheimer's patients [7].

In this context, abundant studies were conducted on the effectiveness of LDRT. However, the therapeutic mechanism of LDRT remains unclear. Therefore, a comprehensive analysis and preclinical studies of the immunological alterations induced by LDRT are required [8]. Cytokines are small proteins that mediate and control the immune and inflammatory responses [9]. Cytokine dysregulation is closely linked to numerous diseases, including autoimmune disorders, infectious diseases, and cancer [10, 11]. In disease states, overproduction of pro-inflammatory cytokines (e.g., TNF-α, IL-6) can lead to chronic inflammation and tissue damage [12,13]. Therefore, targeting cytokine expression can be a promising therapeutic approach [14]. Understanding the complex interaction between cytokine expression

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and LDRT could provide essential insights for developing therapeutic strategies.

In this study, we explored the effects of LDRT on the regulation of cytokine expression in macrophages. Additionally, we further examined the anti-inflammatory efficacy of exosomes derived from gamma-irradiated macrophages. Exosomes are cell-derived small vesicles, contain various types of nucleic acids, lipids, and proteins from the parent cells and are known to mediate intracellular communication [15]. Our previous research has shown that exosomes derived from gamma-irradiated macrophages can effectively promote various biological activities similar to those of their parent cells, suggesting their potential application in therapeutic settings [16]. The ultimate goal of this study was to elucidate the mechanism underlying LDRT-mediated cytokine modulation and investigate the anti-inflammatory properties of gamma-irradiated macrophage-derived exosomes.

2. Materials and Methods

2.1. Preparation and characterization of exosomes

Exosomes were isolated from the conditioned media of macrophage cells (RAW264.7 or polarized M1/IR-M1) using the Total Exosome Isolation Kit (Invitrogen, CA, USA) following the manufacturer's protocol. Cells were cultured in serum-free media for 12 hours, and supernatants were collected. Initially, cells and debris were removed by centrifugation at 2,000 × g for 30 minutes. The supernatant was then mixed with 0.5 volumes of exosome isolation reagent and incubated overnight at 4°C. Exosomes were pelleted by centrifugation at 10,000 × g for 1 hour at 4°C. The morphology of exosomes was analyzed using electron microscopy (EM) after negative staining with uranium acetate. The particle size distribution was measured using dynamic light scattering (DLS). Additionally, the expression of M1, M2 macrophage markers (iNOS and Arg-1) was verified by Western blotting.

2.2. Analysis of cell viability and NO production

Cell viability was assessed using the MTT assay. RAW264.7 macrophages were seeded at a density of 1×10^5 cells/well in a 96-well plate and incubated for 24 hours. Cells were treated with various compounds for 24 hours, after which MTT solution (10 μL of 0.5 mg/mL) was added to each well and incubated for an additional 3 hours. Formazan crystals were dissolved in 100 μL of DMSO, and absorbance was measured at 570 nm. The cell viability was calculated by comparing the absorbance of treated cells to that of the control. For nitrite (NO) production, RAW264.7 and DH82 cells were stimulated with 0.1 μg/mL LPS in the presence of compounds for 24 hours. The supernatant was mixed with an equal volume of Griess reagent, and absorbance was measured at 570 nm to quantify nitrite concentrations.

2.3. Measurement of PGE_2 and cytokine expression

Levels of PGE_2 and cytokines in the cell culture supernatant were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences) according to the manufacturer's instructions. RAW264.7 cells were treated with LPS (0.1 μg/mL) and the isolated compounds for 24 hours. Supernatants were collected and analyzed for PGE_2 and cytokine levels to evaluate the inflammatory response. Cytokine levels were quantified using standard curves generated from known concentrations.

2.4. Cytokine bead array

Cytokine profiling was performed using a cytokine bead array (CBA) kit (BD Biosciences). Supernatants from treated macrophages were collected, and the levels of specific cytokines were measured following the manufacturer's instructions. Data were analyzed using the CBA software, which provided quantification based on standard curves derived from known cytokine concentrations.

2.5. Western blot

Total proteins from RAW264.7 and exosomes were extracted using RIPA lysis buffer. Protein concentrations were quantified using a BCA protein assay kit (Thermo Fisher Scientific). Samples (10 μg of protein) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST for 1 hour at room temperature, then probed overnight at 4°C with primary antibodies against iNOS, COX-2, NF-κB, and GAPDH (Cell Signaling Technology). After washing, membranes were incubated with HRP-conjugated secondary antibodies for 2 hours at room temperature. Protein bands were visualized using an ECL detection system.

2.6. RNA preparation, RNA-seq, and q-PCR validation

Total RNA was isolated from polarized and irradiated macrophages using the WelPrep™ Total RNA Isolation Reagent (Welgene). RNA quality was assessed using a NanoDrop spectrophotometer. High-quality RNA samples were pooled

and sequenced using the HT-seq platform, with EdgeR used for differential gene expression analysis. Gene ontology and pathway analyses were conducted using DAVID. For quantitative real-time PCR (qRT-PCR), cDNA was synthesized from 2 μg of RNA using an M-MLV Reverse Transcriptase kit (Invitrogen). SYBR Green-based qPCR was performed with specific primers for target genes, and results were normalized to the expression of Gapdh using the ΔΔCt method.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences between two groups were assessed for significance using Student's t-test, while differences among three or more groups were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc tests as appropriate. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, CA, USA).

3. Results

3.1. Preparation and characterization of gamma-irradiated macrophage-derived exosomes

Exosomes were isolated from gamma-irradiated and non-irradiated macrophages using the ExoCAS-2 kit. NTA analysis revealed that the average particle size of exosomes was approximately ~150 nm. Interestingly, gamma irradiation can increase the size and concentration of exosomes. This result suggested a potential effect of gamma irradiation on exosome release. As shown in Fig, 1a, EM analysis showed that the exosomes derived from irradiated and non-irradiated macrophages have typical round or cup-shaped morphology. DLS analysis revealed that exosomes isolated from non-irradiated M0, M1, and M2 macrophages exhibited a hydrodynamic diameter ranging from 100-120 nm. In contrast, exosomes isolated from gamma-irradiated macrophages showed a slightly larger size, with a hydrodynamic diameter of 130-150 nm, indicating that gamma irradia-

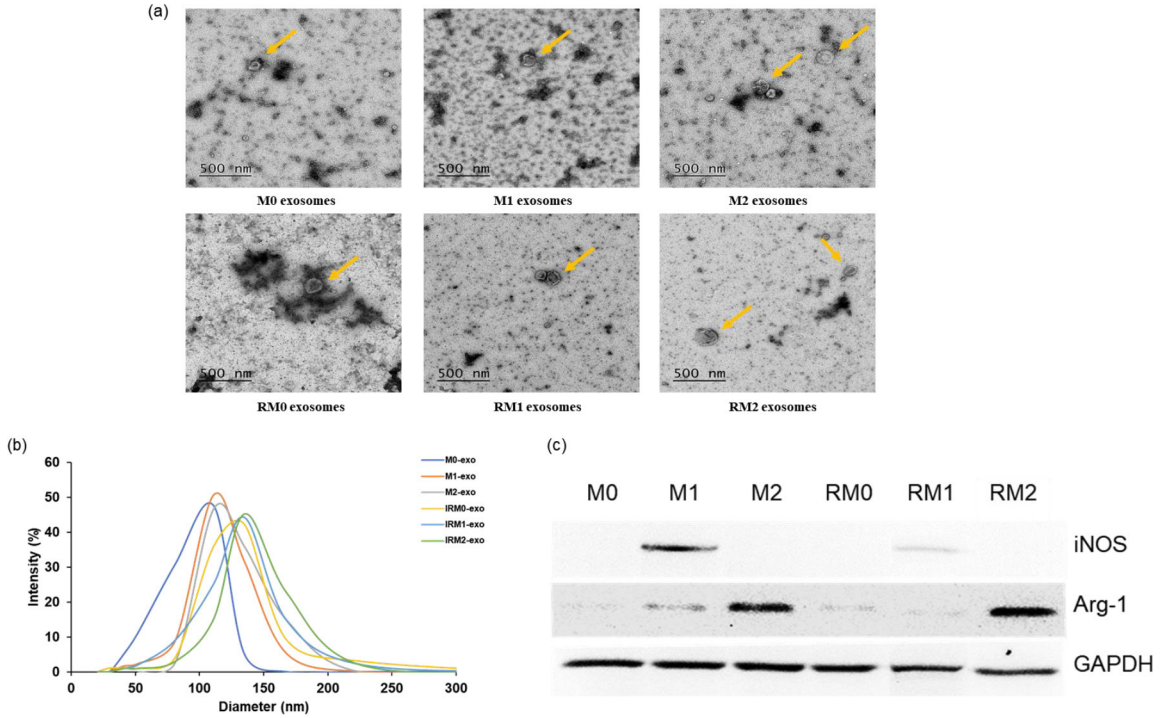

Fig. 1. Characterization of macrophages and macrophage-derived exosomes. (a) Representative electron micrographs showing the morphology of exosomes isolated from non-irradiated macrophages (M0, M1, M2) and gamma-irradiated macrophages (RM0, RM1, RM2). Yellow arrows indicate representative exosomes to aid visualization amidst background cellular debris (scale bar: 500 nm). (b) Hydrodynamic diameter of exosomes isolated from non-irradiated (M0, M1, M2) and gamma-irradiated (RM0, RM1, RM2) macrophages, analyzed by Dynamic Light Scattering. The gamma irradiation leads to a slight increase in exosome size compared to their non-irradiated groups. (c) Western blot analysis detecting the expression of M1 macrophage marker iNOS and M2 macrophage marker Arg-1 in corresponding macrophage lysates. GAPDH was used as a loading control.

tion leads to a slight increase in exosome size (Fig. 1b). Western blot analysis further demonstrated that upon gamma irradiation of M1 macrophages, the expression of the M1 marker iNOS was notably reduced. On the other hand, gamma irradiation of M2 macrophages increased the expression of the M2 marker Arg-1 (Fig. 1c). These findings suggest that gamma irradiation not only influences the size of exosomes but also modulates macrophage polarization markers, highlighting its potential role in immune modulation. These results suggest that gamma-irradiation not only affects the size and concentration of exosomes, but also modulates macrophage markers, highlighting a potential role for gamma-irradiation in immunomodulation. In particular, the decrease in iNOS in irradiated M1 macrophages and the increase in Arg-1 in irradiated M2 macrophages suggest a shift toward an anti-inflammatory or resolving phenotype in response to gamma-irradiation, which is consistent with the immunomodulatory effects of LDRT on macrophage plasticity.

3.2. In vitro immunomodulatory activity of macrophage-derived exosomes from irradiated and non-irradiated cells

To explore the biocompatibility of macrophage-derived exosomes, MTT assay was conducted. As shown in Fig. 2a, all exosome samples exhibited negligible cytotoxicity against macrophages, indicating their excellent biocompatibility. NO, and PGE_2 play a pivotal role in inflammation and immune responses [17]. Griess-reagent and ELISA analysis were conducted to examine the anti-inflammatory activity of gamma-irradiated cell-derived exosomes. Compared with the non-irradiated group, exosomes derived from gamma-irradiated M0 (referred to as IR-M0), M1 (IR-M1), and M2 (IR-M2) macrophages showed significantly reduced production of NO and PGE_2 (Fig. 2b-c). These results indicate that gamma irradiation modulates the immunomodulatory properties of macrophage-derived exosomes, particularly in downregulating inflammatory mediators. Exosomes derived from irradiated macrophages significantly

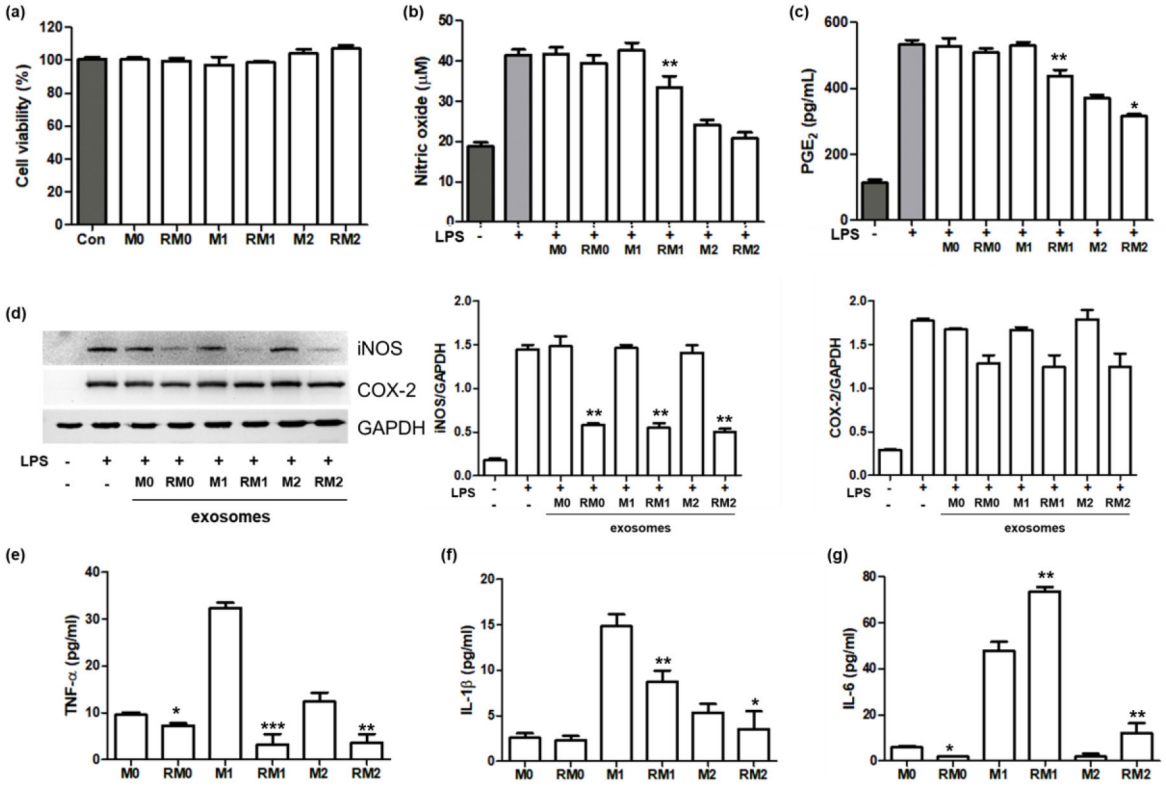

Fig. 2. Anti-inflammatory efficacy of irradiated macrophage-derived exosomes. (a) Cell viability of RAW264.7 macrophages treated with engineered exosomes was assessed using the MTT assay. (b) Nitric oxide (NO) production and (c) PGE_2 levels in LPS-stimulated RAW264.7 cells were measured following treatment with engineered exosomes. (d) Western blot analysis showing protein expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells with or without exosome treatment. Exosomal effects on inflammatory cytokine expression were evaluated by measuring (e) TNF-α, (f) IL-1β, and (g) IL-6 levels using ELISA.

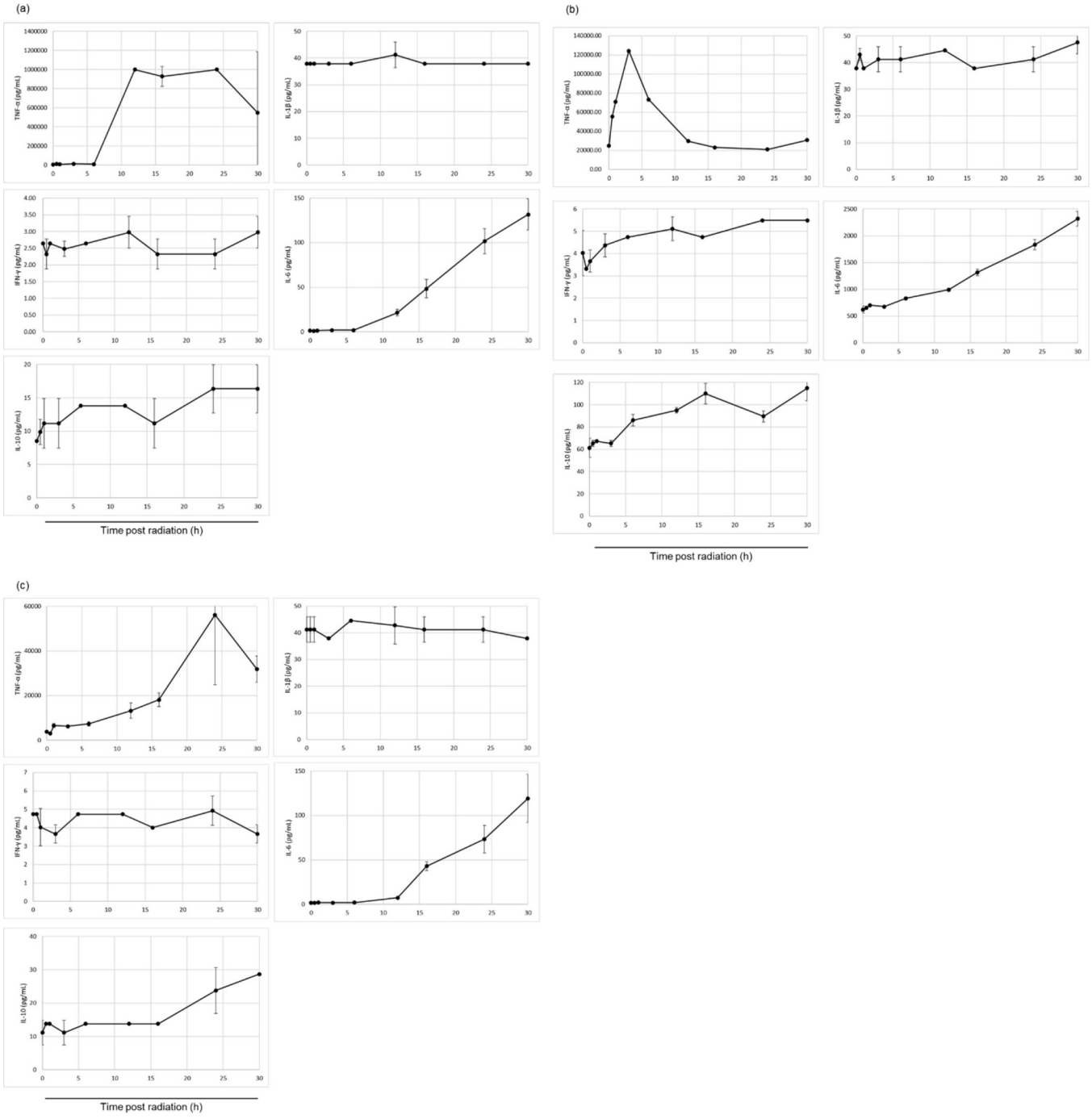

Fig. 3. Time-dependent modulation of inflammatory cytokine production in M0, M1, and M2 macrophages following gamma radiation. (a) Cytokine production profiles in M0 macrophages. Pro-inflammatory cytokines such as TNF-α and IL-1β show an initial increase, peaking around 6-12 hours post-irradiation, followed by a gradual decrease. In contrast, IL-6 exhibits a sustained increase over the 30-hour observation period, suggesting a prolonged inflammatory response or a role in resolution. (b) Cytokine production profiles in M1 macrophages. Similar to M0, TNF-α and IL-1β demonstrate an initial surge and subsequent decline. IL-6 also shows a continuous upward trend. (c) Cytokine production profiles in M2 macrophages. While the overall levels might differ, the general pattern of initial increase and later decrease for TNF-α and IL-1β is observed, aligning with the time-dependent regulation seen in other macrophage types.

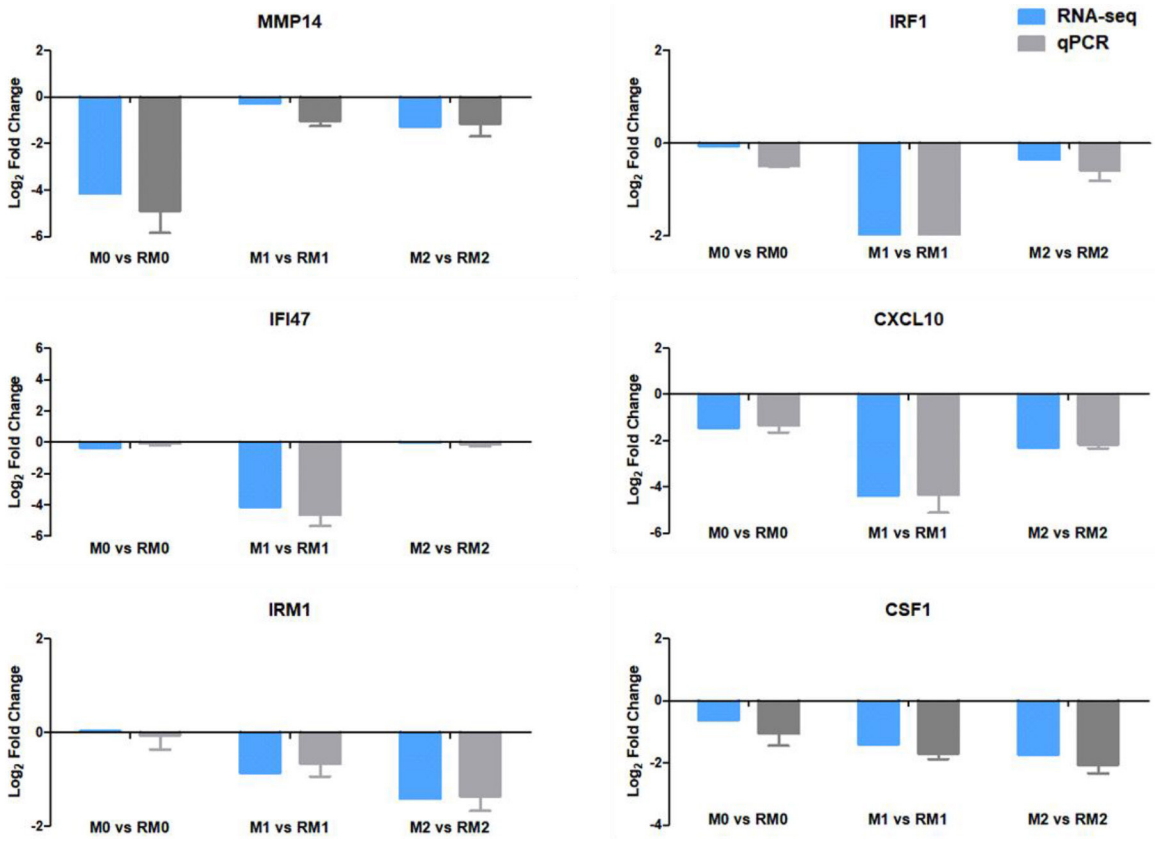

Fig. 4. Validation of RNA-seq data using qPCR for genes related to the IFN-γ pathway. The expression levels of selected genes associated with the IFN-γ pathway were measured by RNA-seq (blue columns) and validated using qPCR (gray columns).

reduced the protein expression of iNOS and COX-2 (Fig. 2d). Moreover, an in-depth analysis of the cytokine profiles demonstrated that gamma-irradiated exosomes are capable of decreasing pro-inflammatory cytokine levels, suggesting their potential application in therapies for treating inflammatory diseases (Fig. 2e-g).

3.3. Gamma irradiation can regulate cytokine expression in M0, M1, and M2 macrophages

Cytokine analysis was performed to assess the effect of gamma irradiation on cytokine release. As shown in Fig. 3, the expression levels of pro-inflammatory cytokines, including TNF-α and IL-1β, were observed to increase up to 12 hours post-irradiation. However, after this peak, a gradual decline in cytokine levels was detected, suggesting a time-dependent modulation of the inflammatory response following gamma irradiation. The initial spike suggests a rapid inflammatory response induced by gamma irradiation. After this peak, cytokine levels gradually decreased, indicating a time-dependent regulation of the inflammatory response. This pattern implies that gamma irradiation initially triggers an acute phase inflammatory response, which then transitions into a resolution phase characterized by decreasing pro-inflammatory cytokine levels. While TNF-α and IL-1β exhibited this transient increase followed by a decrease, it was observed that IL-6 displayed a sustained increase over the monitored period. This differential response suggests a complex and finely tuned immunomodulatory effect of gamma irradiation, where certain inflammatory mediators are acutely regulated while others may be involved in more prolonged signaling pathways.

3.4. RNA Profiling and qPCR Validation

RNA sequencing and subsequent qPCR validation were conducted to further explore the regulatory mechanisms affected by gamma irradiation. Analysis of cytokine-related pathways, specifically focusing on TNF-α and IL-1β, revealed that several genes associated with these pathways were downregulated. Among these, genes linked to the IFN-γ signaling pathway

exhibited significant reductions in expression (Fig. 4). This suggests that key genes involved in IFN-γ signaling and downstream inflammatory responses, IRF1, CXCL10, CSF1, IFI47, IRM1, and MMP14, were significantly downregulated. Considering that IFN-γ signaling is an important pathway for the induction and expression of various inflammatory cytokines and immune responses, the downregulation of these genes suggests that gamma irradiation may specifically attenuate IFN-γ-related inflammatory responses through gene regulation. This reduces the downstream inflammatory cascade. The observed transcriptional changes provide the molecular basis for the anti-inflammatory effects observed at the protein level, suggesting that gamma irradiation directly affects gene expression within key cytokine pathways to modulate the inflammatory response.

4. Discussion

This study aimed to investigate the impact of gamma radiation on macrophage-derived exosomes and their underlying mechanisms of cytokine profile modulation. LDRT is well known for its therapeutic potential in various inflammatory conditions, including arthritis, Alzheimer's disease, and pneumonia. However, its effect on cytokine modulation remains underexplored. By analyzing the differences in cytokine profiles post-irradiation, we investigate the biological effects of gamma irradiation at the cellular level.

After irradiation, the cytokine levels initially increased but gradually decreased over time. This result supports the potential of gamma irradiation as a promising immunomodulatory tool. The downregulation of IFN-γ-associated cytokines further suggests a targeted mechanism through which gamma irradiation influences immune pathways, offering insights into the therapeutic potential of gamma-irradiated macrophage-derived exosomes in treating inflammatory diseases. Furthermore, our results demonstrate that gamma irradiation significantly enhances the anti-inflammatory activity of isolated exosomes. It is notable that low dose gamma irradiation can modulate the cytokine profile of macrophages, conferring anti-inflammatory properties that can downregulate inflammatory mediators.

Regarding the observed differences in LDRT's effect on macrophage morphology and polarization states (M0, M1, M2), our findings suggest that gamma irradiation not only influences the size and concentration of exosomes but also subtly shifts the macrophage phenotype. Specifically, the downregulation of iNOS in irradiated M1 macrophages and upregulation of Arg-1 in irradiated M2 macrophages imply a reprogramming towards a less inflammatory or resolving state. This aligns with the known plasticity of macrophages and the emerging understanding of how low-dose radiation can modulate immune cell functions beyond simple suppression, potentially by promoting wound-healing. Further detailed investigations into the morphological changes at higher resolution and specific molecular markers for each polarization state would provide a more comprehensive understanding.

Despite these promising findings, the study has several limitations. First, while the in vitro results provide an initial understanding of the exosome-mediated effects, in vivo validation is crucial to establish the translational relevance of these findings. Moreover, dose optimization and the cytokine modulation effects of gamma irradiation on other immune cell lines are needed to thoroughly ensure the efficacy of LDRT. By addressing these limitations, future research can build on our findings and contribute to the development of novel therapeutic strategies using gamma-irradiation for inflammatory diseases.

5. Conclusion

This study highlights that gamma irradiation can notably influence cytokine expression and amplify the anti-inflammatory effects of exosomes. The reduction in inflammatory cytokine levels points to the strong anti-inflammatory potential of low-dose gamma irradiation. These results emphasize the complex mechanisms by which LDRT modulates immune responses, primarily through cytokine regulation. This research not only enhances our understanding of LDRT's therapeutic potential but also lays the groundwork for future studies exploring its clinical applications.

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