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Immunological Effects of Proton Radiotherapy: New Opportunities and Challenges in Cancer Therapy

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ABSTRACT

Radiation therapy can be categorised by particle type into photon, proton and heavy ion therapies. Proton radiotherapy is highlighted due to its unique physical properties, such as the Bragg peak and minimal exit dose, which offer superior dose distribution. This makes proton radiotherapy especially advantageous for treating tumours near vital organs with complex structures, such as gliomas near the brain, nasopharyngeal carcinoma near the brainstem and mediastinal tumours near the heart. Proton irradiation can induce distant effects through immunogenicity within the target area. The reduced low-dose zone outside the target provides better lymphatic system protection and immune benefits. Additionally, combining proton radiotherapy with immunotherapy may offer further biological advantages. These features make proton radiotherapy a promising option in cancer treatment. This article may aid in the understanding of proton radiotherapy and its immune effects and lead to new effective options for tumour treatment.

1 | Background

Radiotherapy (RT) is a cancer treatment that uses photons or charged particles to accumulate energy to kill malignant cells. Although RT is a relatively well-established treatment modality, there is still some need for optimisation. RT has traditionally been used to control tumour growth, but recent studies have shown that it also has immunomodulatory properties and can be used in combination with immunotherapeutic agents. Immunotherapy, such as immune checkpoint inhibitors used in combination with photon RT, is currently in clinical use and has shown good efficacy. In view of the differences in the biological effects that occur as a result of different ray energies and dose rates, the effect of immunotherapy combined with proton RT is worth further study. Proton therapy has been increasingly used for the treatment of common malignant tumours because of its physical dosimetry advantage, which minimises irradiation to distal target organs. Studies on the molecular mechanisms of RT in combination with immunotherapy still need to be further explored.

Abbreviations: CIRT, carbon ion radiotherapy; CRT, calreticulin; CSF-1, colony-stimulating factor 1; CTL, cytotoxic T-lymphocyte; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; DDR, DNA damage response; FLASH, Flash radiotherapy; HMGB1, high-mobility group protein B1; ICD, immunogenic cell death; IFN, interferon; LET, linear energy transfer; MDSCs, myeloid-derived suppressor cells; MHC-I, major histocompatibility complex class I; PD-L1, programmed cell death ligand 1; RBE, relative biological effect; RT, radiotherapy; SOBP, spread-out Bragg peak; TAA, tumour-associated antigen; TAMs, tumour-associated macrophages.

This article provides a comprehensive overview of recent advances in the immunological effects of proton RT obtained from cellular, animal and clinical studies. We also discuss studies on the characteristics of proton RT and the radiobiological mechanisms, aiming to inspire innovative concepts for integrating proton RT with immunotherapy.

2 | Cell-Based Experiments

Previous studies have demonstrated that proton RT affects the activity of DNA damage response (DDR) pathways and the induction of immunogenic regulation in tumour cells [1]. Proton RT may help induce the release of a number of signalling molecules that are beneficial for tumour-specific immune responses. These signalling molecules may guide the immune system to identify and target cancer cells more efficiently.

2.1 | Proton Radiation Enhances the Anti-Tumour Activity of Immune Checkpoint Inhibitors and Shows Higher Immunogenicity Than Photons

Several studies have compared the immune effects of charged particles, including protons and carbon ions, and those of photons. Gameiro et al. [2] examined the effect of proton radiation on the activity of tumour cell growth and the induction of immunogenic modulation of tumour cells using flow cytometry and immunofluorescence analysis. Both proton and photon radiation induced the upregulation of the expression of surface molecules related to immune recognition. Proton radiation–mediated cell surface expression of calreticulin (CRT), which led to the release of high-mobility group protein B1 (HMGB1) and ATP, increased the sensitivity of cytotoxic T-lymphocytes (CTLs) to kill tumour cells [3]. Furthermore, these molecules critical for T-cell recognition were upregulated to a similar extent as observed after exposure to photon radiation. Proton radiation exposure increased the number of CTLs specific to a given tumour-associated antigen (TAA). Blocking surface CRT eliminated the enhanced CTL-killing ability induced by proton radiation. In various tumour cell lines, protons significantly downregulated programmed cell death ligand 1 (PD-L1) and induced higher levels of CRT on the surface of tumour cells compared to photons. This result supports the notion that proton radiation therapy enhances T-cell-mediated anti-tumour activity, suggesting a novel role for proton therapy in promoting antitumour responses when used in combination with T-cell-mediated immunotherapy.

Durante and Formenti considered proton radiation more effective than photons when combined with immunotherapy [4]. Compared to photons, protons have the advantage of reducing damage to blood lymphocytes, which are necessary for an effective immune response [5]. Additionally, in cell culture, the immunogenicity of radiation may be related to radiation density. Higher linear energy transfer (LET), which causes dense ionising radiation, is more effective than low LET [6]. Compared to low LET photons, proton radiation appears to improve the ceramide pathway more effectively [7].

2.2 | Similarities and Differences in the Immune Pathways of Tumour Cells in Response to Different Types of Radiation

Du et al. [8] compared the immune responses and potential mechanisms in oesophageal cancer cell lines after photon, proton and carbon ion irradiation. Oesophageal cancer cells were irradiated with a single 15 Gy photon, proton or carbon ion beam, and the cells were subjected to RNA sequencing and gene enrichment analyses. The RNA sequencing data showed different gene expression profiles and biological processes at 6–24 h after irradiation with photons, protons and carbon ion beam. However, 3 days after irradiation, the same gene expression patterns were detected in all groups, with upregulation of interferon-stimulated gene (*OAS1*) and major histocompatibility complex class I (MHC-I) gene (*HLA-B*) expression. Moreover, all three irradiation modalities induced the expression of PD-L1 and PD-L2. One difference is that the expression of PD-L1 and PD-L2 after proton and carbon ion irradiation lasted longer compared to photon irradiation. Post-irradiation, a high enrichment for the regulation of interferon signalling and increased cytokine production, which is essential for attracting T-cells and dendritic cells (DCs) into cancerous tissues, was observed. All three groups of treated cells showed enrichment in the activation pathway of STING, which establishes an effective natural immune response by inducing the expression and secretion of type I interferon and interferon-stimulated genes.

Lupu-Plesu et al. [9] evaluated the mRNA levels of angiogenic, inflammatory and antitumor immune-related genes by RNA-seq in multiply irradiated surviving head and neck squamous cell carcinoma cells. They found significant changes in gene expression, indicating the potential impacts of irradiation on the tumour microenvironment (TME). Both proton and photon RT-stimulated vascular endothelial growth factor activity, but the activity was induced to a lesser extent after proton RT compared to photon RT. Moreover, photon RT increased tumour lymphangiogenesis in patients with head and neck squamous cell carcinoma, tending towards a more aggressive phenotype. Proton RT also downregulated the expression of the genes implicated in (lymphatic) angiogenesis, inflammation and immunological tolerance compared to photon RT [10]. Miszczyk et al. [11] showed that protons may be more efficient in cell killing because they may also induce necrosis in addition to apoptosis. Moreover, tumour cell necrosis is critical to eliciting inflammatory responses and possible local immune activation by spillover of cell lysates into the circulation. The differences in the patterns of photon- and proton-induced cell killing suggest that cell necrosis caused by proton RT may trigger stronger inflammation and immune modulation compared to programmed cell death induced by photon RT. Although the results are preliminary, these findings point to the exploration of gene expression markers associated with necrosis or apoptosis, which may allow the development of immune activation biomarkers and contribute to improved proton RT.

3 | Animal Model Experiments

Although cellular experiments provide evidence in an ex vivo setting, the effects of RT on immunity need to be studied in vivo to explore these pathways in a real physiological context.

Through the use of mouse or other animal models, researchers can analyse the effects of proton RT on immune cells, including measuring the number, activity and functional status of immune cells.

3.1 | T-Lymphocytes (T-Cells): Proton RT Both Damages T-Cells and Can Lead to Positive Immunity

The effects of proton RT on T-cells are complex and contradictory. For example, RT can damage these lymphocytes, leading to adverse reactions [12]. Pecaut et al. irradiated the heads of Sprague-Dawley rats with protons at total doses of 1.5, 3 and 4 Gy. A significant dose-dependent reduction in thymus mass was observed, and considerable decreases in the amounts of lymphocytes and platelets in the blood were detected. Flow cytometry analysis showed low numbers and significantly altered proportions of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells in the blood and spleen of mice after proton RT. Radiation significantly increased the spontaneous proliferation of blood and spleen leucocytes. Proton RT of the head can have a profound effect on the systemic distribution and composition of lymphocyte populations in relation to the radiation dose. Therefore, proton-induced immunomodulation should be considered when evaluating adjuvant immunotherapy in patients undergoing RT [13].

Proton RT also leads to positive immunological effects. One study showed that proton RT induced the expression of adhesion molecules in endothelial cells, which increases T-cell translocation within the tumour, and increased the synthesis of interferon-γ (IFN-γ) in the primary tumour [14]. Proton RT led to an increase in the expression of the CXCR3 chemokine in tumour-associated myeloid cells through a type I IFN-dependent mechanism, which attracts CTLs to the TME. Proton RT also induces the release of DAMPs and TAAs to facilitate antigen presentation by DCs. These events are necessary for the start of T-cells that are antigen-specific. This can induce tumour cell killing by increasing T-cell activation, cytokine release and cytolytic activity [15, 16]. The susceptibility of CTLs to kill tumour cells is increased by proton radiation–mediated cell surface expression of CRT [2]. Additionally, $CD8^+$ cytotoxic T-cells bind antigens on MHC-I, enabling T-cells to target tumour cells. Proton RT has been demonstrated to increase and modify antigen presentation by cancer cells [17, 18]. Previously reported studies showed that T-cell infiltration of the tumour core is a positive predictor of the response to RT. In one study, the distribution of T-cells in tumours was examined using immunostaining for CD3 (a marker for T-lymphocytes) [2]. In a mouse model of lung adenocarcinoma, T-cells were located at the edges of untreated tumours and were absent from the tumour interior, a feature of tumour ‘immune rejection’.

3.2 | Tumour-Associated Macrophages (TAMs): Proton Radiation Promotes Macrophage Polarisation to M1 Macrophages

TAMs are the most abundant immune cells in the TME, and they can be classified into two types: M1 and M2. During tumour growth, M2 macrophages perform pro-tumour functions. In contrast, M1 macrophages are linked to anti-tumour activities. Macrophages confer strong radiation resistance in humans [19]. RT has little effect on macrophage viability and primarily induces a macrophage phenotype switch [20]. Genard et al. [21] showed that low-dose irradiation (below 1 Gy) and high-dose irradiation (above 10 Gy) tended to polarise TAMs towards an M2-like phenotype, whereas medium-dose irradiation (1–10 Gy) deflected TAMs towards an M1-like phenotype. Irradiation induces the recruitment of TAMs through CSF1 and CCL2 pathways. Radiation also induces the recruitment and infiltration of TAMs to hypoxic sites via CXCL12/CXCR4-dependent signalling pathways [22–24]. CSF-1 mainly activates the PI3K/AKT, JAK/STAT and RAS/RAF/MEK/ERK signalling pathways. These events ultimately lead to migration, proliferation and cytokine expression in TAMs [25, 26]. Genard et al. discovered that proton-irradiated tumours may exhibit a preferential selection for M1 macrophages because of their increased radioresistance. Furthermore, the authors demonstrated that exposure to 10 Gy of proton irradiation reprogrammed M0 unpolarised macrophages into the M1 phenotype, boosted the M1 phenotype in existing M1 macrophages and triggered the development of the M1 phenotype in M2 macrophages. Higher amounts of phosphorylated H2AX were linked to the resistance of M1 macrophages to proton irradiation. This increase may be indicative of increased DNA damage repair activity or double-strand breaks [27]. Furthermore, research has demonstrated that macrophages play a role in regulating the infiltration of $CD8^+$ CTLs into lung tumours, whereas proton radiation therapy significantly decreases the number of macrophages present in these tumours.

3.3 | DCs: Proton Radiation Maintains the Balance of the Immune State

DCs are significant immune response regulators that are crucial in bridging the gap between innate and adaptive immunity by triggering T-cell-specific immune responses [28, 29]. RT induces immunogenic cell death (ICD), releasing DAMPs and TAAs. HMGB1, CRT and ATP in DAMPs activate DCs via specific receptors, induce DC maturation and promote CTL activation, which contributes to the emergence of a systemic anti-tumour immune response [30, 31]. ATP binds to the P2X7 purinergic receptor (P2X7R) to generate signals that enhance phagocytosis recruitment. The CRT-CD91 receptor interaction sends signals that promote APC recognition of TAAs released by tumour cells and enhance phagocytosis. HMGB1-Toll-like receptor 4 signalling promotes DC maturation and pro-inflammatory cytokine release, inducing a higher rate of DC processing and presentation of TAAs [32, 33]. DCs secrete IL-12 in response to IFN-γ produced by activated $CD8^+$ T cells through an atypical NF-κB-dependent mechanism [34]. This leads to a positive feedback loop between DCs and T-cells. RT significantly upregulates the major co-stimulatory molecules CD80 and CD86 on T-cells, which bind to the T-cell receptor molecule CD28, leading to T-cell activation, proliferation and differentiation [35, 36]. Yu et al. [37] showed that irradiation of DCs with 0.2 Gy X-rays induced stimulatory effects in DCs, including upregulation of CC-chemokine receptor 7 through activation of the ATM/NF-κB pathway and increased IL-12 production. However, König et al. [38] found no negative effects on phagocytosis, migration or IL-12 secretion in immature

DCs in response to different doses of photon RT, proton RT or carbon ion RT. The migratory ability of immature DCs was increased by proton RT; the dose and the type of radiation may be the cause of this effect [22].

Preclinical studies combining immunotherapy and charged particle irradiation have shown promising results. Carbon ion irradiation combined with DC injection was shown to exhibit anti-metastatic effects in preclinical research data [39]. Moreover, the combination of DC injection with CIRT in vivo enhances the surface exposure of CRT on cells, promotes DC maturation and boosts the immunogenicity of tumour cells [40]. However, studies in animal models have used C-ion irradiation, and more experiments are still needed to prove the therapeutic effect of proton RT in combination with DCs [41, 42]. In contrast to these findings, Merrick et al. discovered that DCs were very resistant to radiation-induced apoptosis and retained migration and phagocytosis ability after radiation. Nevertheless, irradiated DCs were less effective inactivating lymphocytes and also less capable of producing immune-activated IL-12 at maturity. Proton irradiation may also reduce immune activation and suppress immune responses in DCs [43]. DC irradiation may change the equilibrium from tumour regression to one of tumour expansion and escape [44, 45].

3.4 | Natural Killer (NK) Cells: Proton Radiation Makes NK Cells More Cytotoxic

NK cells directly recognise activating ligands on the surface of target cells and play an important role in the immunosurveillance and immune homoeostasis of malignant cells [46]. NK cells are crucial for the production of IFN-γ and exhibit cytotoxic properties [47]. Through antibody-dependent cell-mediated cytotoxicity, NK cells also kill antibody-coated tumour cells [48, 49]. Proton RT enhances NK cell function by stimulating tumour cells to upregulate the receptors NKG2D and NKG2D ligand (NKG2DL) and through granzyme B, perforin and antibody-dependent cell-mediated cytotoxicity; the inhibitory molecules TGF-β, MHC and PD-L1 were also upregulated [26]. Gridley et al. [50] compared photon and proton whole-body irradiated mice and found that electron-irradiated mice had higher leucocyte and lymphocyte counts and greater NK cell cytotoxicity in their spleens compared to proton-irradiated mice. Wang et al. [51] performed single-cell RNA-seq and found that carbon ion RT induced Klrk1 gene expression and significantly activated the NKG2D/NKG2D-ls pathway in lung cancer. Single-cell RNA sequencing revealed that irradiated tumours exhibited activation of NK cells and different TAMs [1].

3.5 | Myeloid-Derived Suppressor Cells (MDSCs): Proton Radiation May Alter the Immunosuppressive, Recruitment and Differentiation Characteristics of MDSCs

The immunosuppressive function of MDSCs is a significant characteristic of these cells [52]. Myeloid cells from the bone marrow are typically unable to differentiate into MDSCs under physiological circumstances. However, in the TME, myeloid cells can proliferate and differentiate into MDSCs, which exhibit tumorigenic activities [26, 53]. The cGAS/STING signalling pathway and CCL2/CCR2 are activated in MDSCs by RT-induced DNA damage. Low-dose RT upregulates the expression of CSF1, which is associated with differentiation, recruitment and immunosuppressive properties of TAMs and MDSCs [54]. CSF-1/CSF-1R and CCL2/CCR2 activate the JAK/STAT signalling pathway and PI3K/AKT signalling pathway in MDSCs [55]. However, a single high dose of RT caused a considerable reduction in MDSCs. One study showed that the downregulation of ring finger protein 20 (RNF20) enhanced TNF-α responses in mouse colonocytes and innate immune cells, and RNF20-deficient mice have more MDSCs than wild-type mice. Thus, increased RNF20 upon cluster DNA damage may enable anti-tumour immune responses by reducing MDSC activation [56, 57]. MDSCs further suppress immune function and reduce the activation of other leucocyte populations. MDSCs release the inflammatory cytokine prostaglandin E2 (PGE2), which supports tumour growth and tumour regeneration while protecting tumour cells from apoptosis. PGE2 is increased after irradiation, and its release correlates with the LET of the radiation and oxygen concentration [58, 59]. Chen et al. found that tumours in a mouse liver tumour model were significantly smaller 12 days after proton irradiation. The authors also observed increased DNA damage, upregulation of IL-6 levels and modulation of the immune TME. Proton RT increased the level of PD-L1 expressed in tumour cells and MDSCs; the increase in PD-L1 positively correlated with the irradiation dose. In a Hepa1-6 homozygous mouse model, the combination of proton RT with anti-PD-L1 delayed tumour growth to a greater extent compared to proton RT alone; this was associated with an increase in tumour-infiltrating T-cells and attenuated MDSC recruitment in the microenvironment [60].

Finally, there are many different kinds of immunologic responses to proton RT, which have complicated effects on the immune system. Figure 1 illustrates the impact of proton RT on various immune cell types. On the basis of this evidence showing the effects of proton RT on the differentiation and functional status of immune cells, the combination of proton RT and immunotherapy has great potential and prospects.

4 | Clinical Research

Whether proton RT has a clinical advantage over conventional photon RT in enhancing tumour immunogenicity remains to be explored. On the one hand, radiation causes tumour cell mutations, induces T-cell activation, increases the immunogenicity of tumour cells and produces neoantigens. On the other hand, proton RT damages fewer immune cells as a result of less damage to normal tissues. Therefore, proton RT can stimulate a stronger immune response [61]. Lymphangiogenesis and metastasis are low after proton RT compared to conventional RT. The expression of regulatory pro-inflammatory genes in cells after proton and photon irradiation is different [9]. For most cells, apoptosis sensitivity is the main factor of radiosensitivity. The higher radiosensitivity of lymphocytes is a result of their tendency to undergo apoptosis. Lymphocytes are involved in a number of key mechanistic roles following tumour RT, including the enhancement of anti-tumour intrinsic and adaptive immune responses, enhancement of tumour recognition and killing through upregulation of antigen-presenting mechanisms and

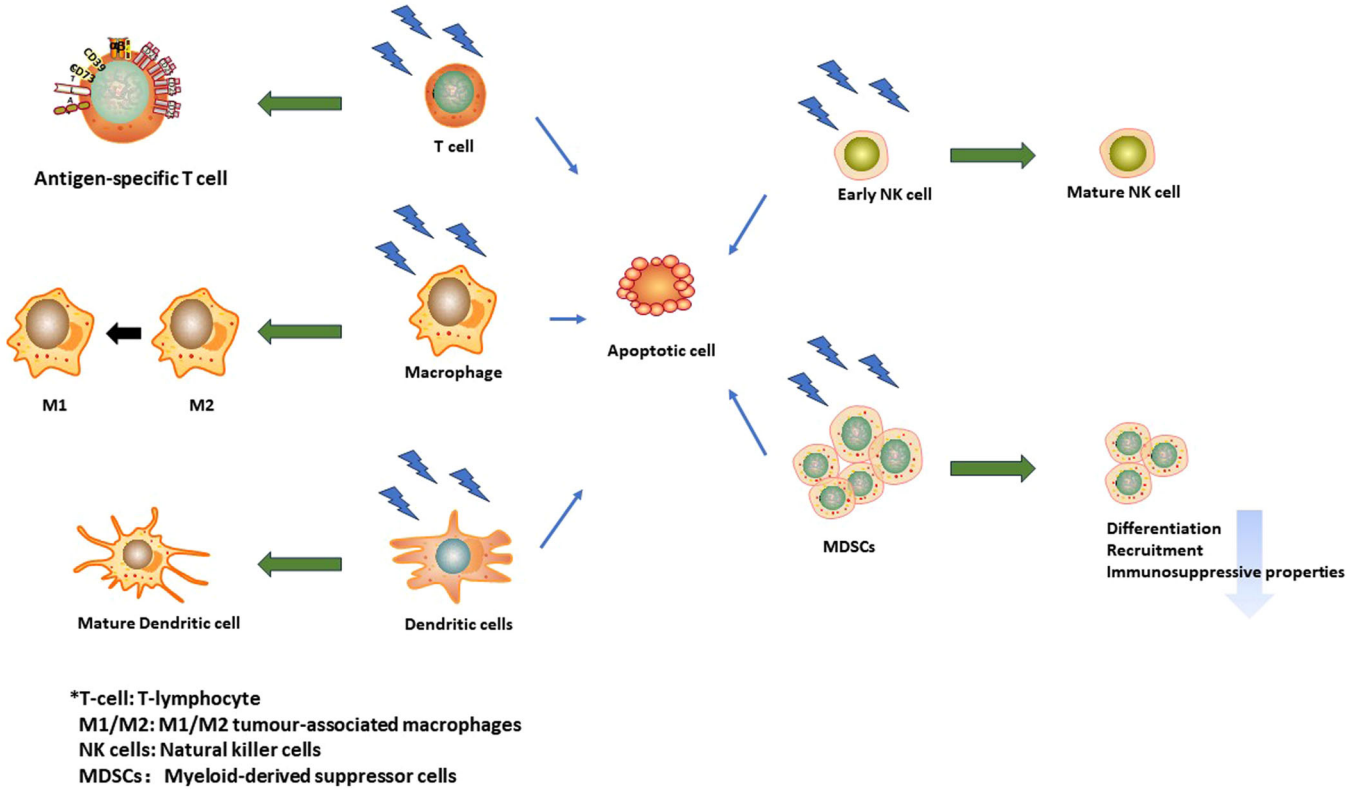

FIGURE 1 | The effect of proton radiotherapy on different immune cells.

induction of lymphocytes into the TME by positive immunomodulatory pathways. As proton therapy minimises exposure to normal tissues, it may induce fewer immunogenic effects than photon irradiation, particularly in terms of its impact on circulating lymphocytes. For example, the incidence of severe radiation-induced lymphopenia in glioblastoma patients treated with proton RT was only 14% in a published survey compared to 39% in patients who received photon RT [62]. However, this study has not reported data from randomised controlled trials.

In vivo and clinical data on the systemic immune responses caused by proton RT are scarce. However, preliminary in vivo studies involving carbon ions showed a considerable decrease in the number of lung metastases in mouse models even without synchronous immunotherapy [63]. Immunotherapy was shown by some studies to be a viable strategy for anti-tumour immune responses when it was linked to CIRT [64]. The relationship between immunotherapy and proton RT is currently under investigation, with future research focusing on how protons can induce higher levels of ICD in tumour cell [65].

When searching for clinical trials related to proton RT on the official clinical trial website (https://clinicaltrials.gov/), only a few clinical trials combining proton RT and immunotherapy were found. Table 1 lists the clinical trials related to proton RT combined with immunotherapy: two of these trials are still in the state of recruitment, and two have no study results. The clinical research of immunotherapy and proton RT is still at a relatively preliminary stage, and results may vary depending on study conditions, patient populations and treatment protocols. Thus, while there are indications that proton RT may have some positive effects on the immune system, more research is required to completely elucidate the mechanism and determine how proton RT might be used in cancer treatment.

5 | Proton Flash RT (FLASH) Combined With Immunotherapy

FLASH is an RT technique that uses ultra-high doses of radiation to widen the treatment window. FLASH has been observed to provide greater preservation of normal tissue at high dose rates without compromising tumour control [60]. Considering the limitations of photons (lack of conformability and superficial treatment depth) and the physical dose distribution of the photon beam stream, the unique depth-dose properties of proton beams make them the most promising FLASH modality for clinical translation.

The effect of proton FLASH on immune cells differs from that of conventional protons. Shukla et al. [66] compared conventional proton therapy (CPT) to flash proton therapy (FPT) in a mouse model of non-small cell lung cancer. Mice bearing in situ lung tumours were treated with chest RT using CPT (< 0.05 Gy/s) and FPT (> 60 Gy/s) dose rates. FPT was more effective in increasing the infiltration of cytotoxic $CD8^+$ T-lymphocytes within the tumour while decreasing the percentage of immunosuppressive regulatory T-cells among T-cells. $CD3^+$ T-cell infiltration was higher in tumours treated with FPT compared to tumours treated with CPT. The FPT-treated group exhibited a significant increase in T-cell counts compared to both the CPT and untreated groups. FPT irradiation increased the infiltration distance of $CD8^+$ T-cells compared to CPT irradiation, suggesting increased $CD8^+$ T-cell motility from the

TABLE 1 | Clinical trial[a] of proton radiotherapy combined with immunotherapy.

NCT number	Study title	Conditions	Radiotherapy	Immunotherapy	Enrollment	Date
NCT06133062[b]	Atezolizumab and bevacizumab with proton radiotherapy for unresectable hepatocellular carcinoma	Hepatocellular carcinoma	Proton radiotherapy	Atezolizumab Bevacizumab	45	2023/11/16–2030/9/30
NCT03267836[c]	Neoadjuvant avelumab and hypofractionated proton radiation therapy followed by surgery for recurrent radiation-refractory meningioma	Meningioma	Proton radiotherapy	Avelumab	9	2018/1/10–2023/4/6
NCT05625893[b]	Proton beam radiotherapy followed by tecentriq and avastin for primary liver cancer with Vp2-4 portal vein invasion	Hepatocellular carcinoma	Proton beam therapy	Atezolizumab Bevacizumab	63	2022/9/16–2025/12/31
NCT03539198[c]	Study of proton SBRT and immunotherapy for recurrent/progressive locoregional or metastatic head and neck cancer	Head and neck cancer	Proton stereotactic body radiation therapy (SBRT)	Nivolumab	19	2018/7/3–2020/1/6

[a]https://clinicaltrials.gov/.
[b]Recruiting status.
[c]Completed status (Results not yet available).

infiltration margins into the tumour core. FPT treatment was more effective than CPT irradiation in recruiting cytotoxic $CD8^+$ T-cells into the tumour and reducing the recruitment of immunosuppressive regulatory T-cells. Additionally, FPT was more effective in reducing tumour-promoting M2-like macrophages in lung tumours while increasing the infiltration of anti-tumour M1-like macrophages compared to CPT. Finally, FPT treatment reduced checkpoint inhibitor expression in lung tumours, suggesting reduced immune tolerance.

Iturri et al. investigated the potential immune response generated by FPT in high-dose proton therapy in a rat model of glioma in situ. FPT did not result in the memory impairment observed with conventional high-dose proton therapy and induced tumour-infiltrating lymphocyte recruitment. Moreover, FPT did not cause severe neuroinflammatory side effects [67]. However, in FPT, dose partitioning and dose rate also affect peripheral blood lymphocytes. Pen-beam scanning FLASH reduced lymphocyte depletion by 69.2% compared to conventional graded intensity-modulated proton RT. FLASH single treatment fraction offers superior sparing of circulating blood and lymphocytes compared to hypofractionated FLASH and conventional IMPT, supporting the assumptions of reducing the risks of lymphopenia compared to proton therapy at conventional dose rates. Faster conformal FLASH delivery, such as passive patient-specific energy modulation, may further enhance the sparing of the immune system [68]. These studies indicate that FPT modulates the immune system to improve tumour control and may therefore be a promising new treatment option.

6 | DDR Closely Correlated With Successful Combination of RT and Immunotherapy

DNA damage leads to cell death through different pathways and the subsequent release of small molecules, such as *ATP*, calpain and HMGB1, which can trigger an immune response [69]. Proton radiation increases the sensitivity of CTLs to kill tumour cells and was shown to induce the cell surface expression of CRT in various human tumour cell lines in vitro [2]. The release of HMGB1 positively correlated with particle LET, and the extracellular concentrations of HMGB1 were higher in human cancer cells after proton irradiation [70]. Radiation damage leads to double-stranded DNA fragments that extrude from the nucleus and accumulate in the cytoplasm; this results in activation of the double-stranded DNA sensor cGAS/STING, which leads to expression of the type I interferon gene and initiates the immune response [71]. Interferons recruit and activate DCs capable of inducing radiation-generated immune responses [72]. Patients with metastatic non-small cell lung cancer showed higher serum interferon levels after RT and CTLA4 blockage relative to baseline. These results suggest that interferon activation may be an important pathway for the effects of the RT and immunotherapy combination [73].

Considering that variable proton RBE may be caused by double-strand break repair capacity, Choi et al. investigated DDR signalling in four hepatocellular carcinoma cell lines with different RBE values in response to X-ray or proton beam irradiation. Time-course analysis of phosphorylation of DDR markers

(H2AX, ATM, DNA-PKcs and CHK2) showed slight differences in DDR signalling between the two groups. Phosphorylation of DDR markers increased uniformly 30 min after photon and proton irradiation and returned to basal levels thereafter. In cell lines with high RBE, proton irradiation prolongs the activation duration of the DDR. Compared to cells irradiated with X-rays, the activated DDR signals persist longer in cells exposed to proton irradiation [74].

7 | Various Combinations of Radiation Therapy and Immunotherapy Are Under Exploration

There is a complex interplay between proton RT and immunotherapy. In addition to directly causing tumour cell death, RT generates a strong anti-tumour immune response via several mechanisms, including enhanced tumour antigen presentation and upregulation of MHC-I expression [75]. While radiation combined with immune checkpoint blockade holds the promise of a significant synergistic effect that may extend beyond the radiation target, several studies have shown that the immunogenicity of this combination may be limited by the immunosuppressive mechanisms associated with photon RT [76]. Proton RT not only enhances the immunoadjuvant effect of RT but also limits the immunosuppressive mechanisms. In cancer patients undergoing RT, proton radiation significantly reduced grade 4 lymphopenia compared to photon radiation [77]. Proton therapy has good dose uniformity and shields surrounding normal tissues from the effects of radiation. This is thought to be the main reason for the reduction of the incidence of lymphocytopenia. Whether immunotherapy combined with proton radiation is superior to photon radiation therapy needs to be further investigated.

Photon therapy in combination with immunotherapy is widely used in the treatment of a variety of tumours, but it may have limitations in controlling distant metastases compared to proton therapy [78, 79]. Local lymph node irradiation in lymph node-positive disease enhances local control but may also have an effect on immune-specific T-cells. Precision irradiation with proton beams effectively minimises damage to lymph node function [11]. Additionally, the optimal radiation dose (low vs. large split), timing and sequencing of immune-conjugated proton RT, and the search for biomarkers to predict response to combination therapy need to be clarified in future research [80].

Research on the synergistic interactions between RT and immunotherapy should also focus on the mechanisms by which RT enhances the effects of immunotherapy. However, whether immunotherapy itself can induce radiosensitisation of tumour cells is unclear and has not been studied in depth. The discovery of several regulators of immune checkpoints and radiosensitivity (e.g., p53 and PARP inhibitors) has made the relationship between radiation therapy and immunotherapy more complex than previously thought and warrants further study [81].

Research to enhance proton radiation-mediated antitumour immunity through immunomodulation is ongoing. Several drugs that stimulate components of the immune response are being studied and produced. These include immune adjuvants, tumour vaccines and cytokines. Such immunostimulants promote the activity of DCs and/or T-cells. Moreover, several drugs have been identified that block the anti-tumour immune response. These include drugs that inhibit the function of or deplete immunosuppressive cells and that inhibit the function of immune checkpoint molecules (CTLA-4, PD-1 and PD-L1) [5].

Despite the advantages of proton RT in terms of tumour killing and immune activation, not all tumours and patients can benefit from this treatment, and proton RT is only indicated for some tumour types. Additionally, proton RT equipment is expensive to build and maintain, so not all healthcare facilities offer this treatment. Proton radiation therapy is usually much more expensive than conventional radiation therapy, largely because of the high cost of proton radiation therapy equipment and the complexity and specialisation involved in the treatment process. The high cost of treatment may make it unaffordable for many patients, especially if they do not have health insurance or are not fully covered by insurance. While the initial cost of proton therapy is higher, it reduces the risk of adverse effects and second primary tumours. Therefore, in terms of a complete treatment cycle, proton therapy can save a considerable amount of treatment cost per patient and effectively prolong patient survival. Physicians should consider various aspects when administering a combination of radiation therapy and immunotherapy to choose the optimal option for their patients.

8 | Conclusion and Prospects

Proton RT combined with immunotherapy shows great efficacy, in part from the ability to protect normal tissues more effectively during RT compared to photon RT, particularly by reducing the exposure of circulating T-lymphocytes and other immune cells. Peripheral blood lymphocytes are very sensitive to radiation, and lymphopenia may occur during RT, which is usually associated with a poor prognosis [4]. In lymphocytes in vitro, protons induce more chromosomal aberrations than X-rays at the same dose, producing aggregated DNA damage that is difficult to repair and triggering different DDR signals [82]. DNA repair pathways are closely linked to the immune response [83].

In this article, we provided an overview of the damage mechanisms and immune effects of proton RT, with the aim of providing guidance for proton RT and immunotherapy combination protocols. As an innovative paradigm in cancer treatment, proton RT combined with immunotherapy will require further research, especially concerning patient selection and biomarkers for predicting treatment response. Efforts should be directed towards the discovery and validation of biomarkers that accurately predict patient response to treatment and reflect the state of the patient's immune system and tumour characteristics. The use of biomarkers to stratify patients will effectively screen patients who may benefit from proton RT combined with immunotherapy while reducing unnecessary treatment risks and costs. It may aid in increasing the cure rate of cancer patients and the patient's survival rate.

In conclusion, most research on proton RT combined with immunotherapy is at the stage of preclinical studies, with the search for the optimal combination of proton RT and immunotherapy ongoing. Proton FLASH, which modulates the

immune system to improve tumour control, may become a new alternative to tumour RT combined with immunotherapy.

Author Contributions

Anhang Zhang: conceptualisation (lead), data curation (lead), formal analysis (lead), investigation (equal), methodology (equal), project administration (equal), resources (equal), software (equal), writing – original draft (lead). **Liyuan Fan:** conceptualisation (equal), formal analysis (equal), project administration (equal), software (equal), supervision (equal), writing – review and editing (equal). **Qi Liu:** data curation (equal), investigation (equal), methodology (equal), software (equal), validation (equal), visualisation (equal). **Xiaoxin Zuo:** data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), resources (equal). **Jian Zhu:** funding acquisition (supporting), project administration (lead), supervision (lead), writing – review and editing (lead).

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The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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