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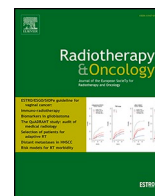
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Original Article

Interferon signaling is enhanced by ATR inhibition in glioblastoma cells irradiated with X-rays, protons or carbon ions

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A B S T R A C T

Background and purpose: Interferon (IFN) signaling plays an important role in antitumor immune responses. Inhibitors of the DNA damage response, such as ATR inhibitors, can increase IFN signaling upon conventional radiotherapy with X-rays. However, it is not known whether such inhibitors also enhance IFN signaling after irradiation with high linear energy transfer (LET) particles.

Materials and methods: Human glioblastoma U-251 and T98G cells were irradiated with X-rays, protons (LET: 4.8 and 41.9 keV/μm) and carbon ions (LET: 28 and 73 keV/μm), with and without ATR inhibitor (VE-822) or ATM inhibitor (AZD1390). DNA damage signaling and cell cycle distribution were analyzed by immunoblotting and flow cytometry, and radiosensitivity was assessed by clonogenic survival assay. IFN-β secretion was measured by ELISA, and STAT1 activation was examined by immunoblotting.

Results: High-LET protons and carbon ions caused stronger activation of the DNA damage response compared to low-LET protons and X-rays at similar radiation doses. G2 checkpoint arrest was abrogated by the ATR inhibitor and prolonged by the ATM inhibitor after all radiation types. The inhibitors increased radiosensitivity, as measured after X- and carbon ion irradiation. ATR inhibition increased IFN signaling following both low-LET and high-LET irradiation. ATM inhibition also increased IFN signaling, but to a lesser extent. Notably, both cell lines secreted significantly more IFN-β when the inhibitors were combined with high-LET compared to low-LET irradiation.

Conclusion: These findings indicate that DNA damage response inhibitors can enhance IFN signaling following X-, proton and carbon ion irradiation, with a strong positive dependency on LET.

Introduction

Glioblastoma (GBM) is the most common, and deadliest, type of brain cancer. Radiotherapy is part of the standard treatment, but treatment efficacy is limited by tumor radioresistance and damage to the normal brain [1]. Particle irradiation with carbon ions or protons may potentially improve GBM treatment [2,3]. The advantageous depth dose distribution of particle beams allows sparing of normal tissue [4,5]. Furthermore, carbon ions or protons with high linear energy transfer (LET) induce clustered DNA damage, which can be harder to repair and thus more potent in eliminating tumor cells [2]. To further enhance the tumor cell killing, radiotherapy may be combined with inhibitors of cell cycle checkpoints and DNA repair pathways [6,7]. It was previously thought that such inhibitors would not sensitize to high-LET irradiation,

based on an assumption that the clustered DNA damage would be mostly irreparable [8]. However, more recent studies indicate that DNA repair is also important upon high-LET irradiation [9–11]. Radiosensitizing effects have for example been observed with ATR and PARP inhibitors in combination with carbon ion irradiation [12–16].

Interestingly, combining radiotherapy with DNA damage response inhibitors may also trigger antitumor immune effects. For instance, abrogation of the radiation-induced G2 checkpoint by ATR inhibition results in increased micronucleus formation that can induce a type 1 interferon (IFN) response in various cell types [17–19]. The micronuclei are prone to rupture, and DNA from ruptured micronuclei can be recognized by the cytosolic DNA sensor cGAS, leading to type 1 IFN secretion via the cGAS-STING-TBK1-IRF3 pathway [20,21]. Alternatively, increased IFN production may be triggered by cytosolic RNA via

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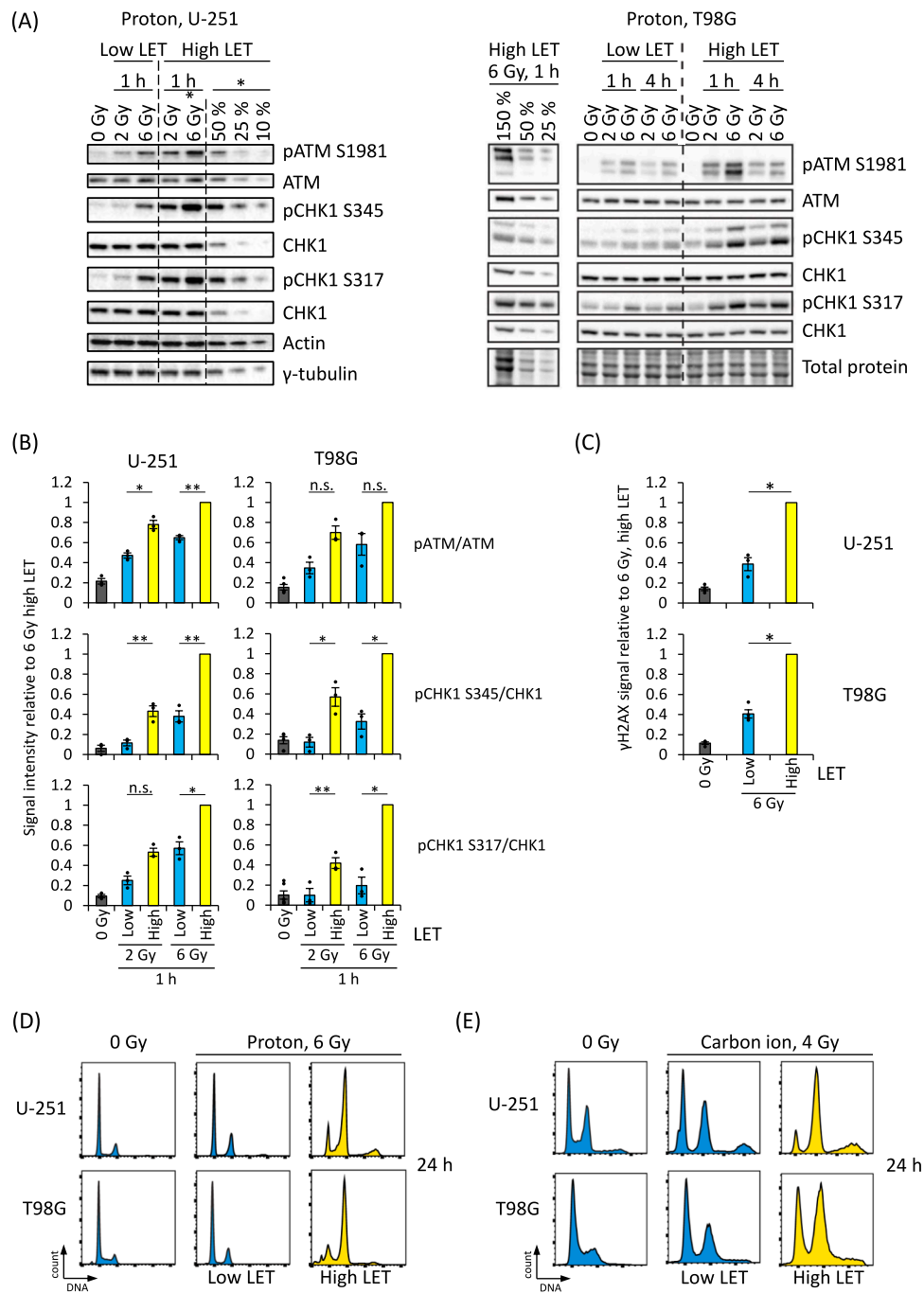


Fig. 1. Activation of the DNA damage response is stronger in GBM exposed to high-LET as compared to low-LET irradiation for the same radiation dose. (A) Representative immunoblots from U-251 and T98G cells, showing phosphorylated ATM and CHK1 after 2 and 6 Gy of proton irradiation. Asterisk indicates a dilution series of the sample treated with 6 Gy high-LET protons. (B) Quantification of signal intensity of the indicated markers from three experiments similar to that shown in A. Error bars: SEM ($n = 3$). (C) Quantification of γ H2AX intensity as measured by flow cytometry 0.5 h after proton irradiation. Error bars: SEM ($n = 4$). (D) DNA profiles obtained by flow cytometry analysis, showing cell cycle distribution at 24 h after exposure to 6 Gy of low- vs. high-LET protons. (E) Similar as in D with 4 Gy of carbon ions.

the RNA sensor RIG-1 [17,19]. Inhibiting ATR or ATM in combination with irradiation also promotes antitumor immune responses in mouse tumor models [22–25]. Moreover, ongoing clinical trials are investigating combinations of ATR inhibitors and immunotherapy, with and without radiotherapy [26].

While previous preclinical studies have shown enhanced antitumor immune signaling with DNA damage response inhibitors and X-irradiation, it remains unclear whether these inhibitors also boost immune signaling when combined with proton or carbon ion irradiation.

Furthermore, little is known about IFN signaling after irradiation and DNA damage response inhibition in GBM. Here we show that ATR inhibition can abrogate the G2 checkpoint and increase type 1 IFN signaling in two GBM cell lines after X-, proton and carbon ion irradiation. In addition, ATM inhibition increases IFN signaling, albeit to a lesser extent, despite not abrogating the G2 checkpoint. Our results suggest that DNA damage response inhibitors could be useful in combination with proton or carbon ion therapy to enhance tumor cell radiosensitivity and type 1 IFN responses.

Materials and methods

Cell culture

Human glioblastoma cell lines T98G and U-251 were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (Biowest) and 1 % penicillin/streptomycin solution (10000 U/mL) (ThermoFisher Scientific), at 37 °C in a humidified atmosphere with 5 % CO₂. T98G was kindly provided by Dr. Theodoros Theodorou and U-251 was purchased from CLS Cell Line Service (Eppelheim, Germany). Both cell lines were verified by short tandem repeat analysis (Eurofins).

Drug treatment

Inhibitors of ATM (AZD1390) and ATR (VE-822/berzosertib), both from Selleck Chemicals, were given to cells 15–30 min prior to irradiation for X-ray and carbon ion experiments, or immediately after irradiation for proton experiments. The inhibitors were removed after 24 h of exposure in clonogenic survival assays, but were otherwise present until the end of the experiment.

Cell irradiation

Proton irradiation was performed at Oslo cyclotron laboratory (University of Oslo) with an MC-35 cyclotron (Scanditronix) providing a beam energy of 15.5 MeV. The beamline and setup have been described previously [27,28]. The cells were irradiated in two positions; in front of the Bragg peak and at its distal end. The dose rate was the same for both positions. The medium was removed during irradiation and fresh medium was added immediately after irradiation. Carbon ion irradiation was performed using the IRABAT 95 MeV beam line at the GANIL facility (Caen, France), with further details described in [29]. X-rays (160 kV, 6.3 mA, Faxitron) were delivered at 1 Gy/min (filtration 0.8 mm Be + 0.5 mm Cu).

The LET values for carbon ions were estimated at 28 and 73 keV/μm and for protons 4.8 and 41.9 keV/μm. In our study we have termed the lowest LET value for each particle type "low-LET" with the purpose of separately comparing the two LET values within each radiation modality. The LET for 160 kVp X-rays is ~4 keV/μm [30].

Flow cytometry

Cells were fixed in 70 % ice cold ethanol. To eliminate sample-to-sample variation, we added an aliquot of barcoded reference cells stained with Alexa Fluor 647 Succinimidyl Ester (0.02 μg/μL) (ThermoFisher Scientific) to all samples. Samples were stained with mouse anti-γH2AX (Ser139), clone JBW301 (Millipore), conjugated to FITC (1:1000) or not (1:500), the latter followed by Alexa Fluor 488 anti-mouse IgG (1:500, Molecular Probes). DNA was stained with Hoechst 33258 (Sigma-Aldrich). Samples were analysed on an LSR II flow cytometer (BD Life Sciences) or on a CytoFLEX (V5-B4-R3) (Beckman Coulter). Results were analysed in FlowJo v.10.6.1 software (BD Life Sciences), using the Watson Pragmatic algorithm for cell cycle analysis. Instrument service on the LSR II was provided by the Flow Cytometry Core Facility (Oslo University Hospital).

Clonogenic survival assay

Cells were seeded in triplicate 6 cm dishes (500–6000 cells/dish) 18–24 h prior to treatment with X-rays and inhibitors. When colonies appeared (10–14 days), cells were fixed with 70 % ethanol and stained with methylene blue. Colonies with >50 cells were counted. For experiments with carbon ions, the same procedure was followed, except that cells were seeded in T-25 culture flasks at densities of 750–15000 cells/flask.

Western blotting

Cell lysis and immunoblotting was performed as previously described [18], with the exceptions that Criterion TGX Stain-free gels (Bio-Rad) were used and that protein concentration was not measured in samples shown in Fig. 1. The antibodies used are listed in Table S1. Protein bands were quantified in the ImageLab 4.1 software. A dilution series of one of the samples was included to measure the dynamic range and to create a standard curve for accurate quantification.

Enzyme-linked immunosorbent assay (ELISA) for secreted IFN-β

Growth medium supernatants were collected from the cell samples. Duplicates of 50 μl of concentrate were subjected to IFN-β measurement by ELISA (Human IFN-beta DuoSET ELISA, R&D Systems), after 20X upconcentration (Amicon Ultracel-10, Merck) of the supernatants as previously described [18]. The protein concentration of remaining adherent cells at time of harvest was measured as described [18], and used for normalization of ELISA IFN-β read-outs. (Normalization did not result in major alterations in the overall responses.)

Statistics

One- or two-sample, two-tailed paired Student's *t* tests were performed unless otherwise stated in the figure legend. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, n.s.: non-significant.

Results

To investigate effects of ATM and ATR inhibitors in combination with low- and high-LET irradiation, we first characterized DNA damage signaling after irradiation alone. Immunoblotting of samples harvested at 1 and 4 h after proton irradiation showed that high-LET protons (41.9 keV/μm) induced greater phosphorylation of ATM and the ATR target CHK1 compared to low-LET protons (4.8 keV/μm) at similar radiation doses (Fig. 1A and B). These effects were seen in both T98G and U-251 cells. Furthermore, flow cytometry analysis of the DNA damage marker γH2AX at 0.5 h after irradiation showed significantly stronger signals for high-LET protons than for low-LET protons (Fig. 1C). The γH2AX level in T98G cells exposed to 6 Gy of high-LET protons was comparable to that observed in cells exposed to 12 Gy of X-rays (Fig. S1A, S1B). To study potential differences in repair rates between cells exposed to low- and high-LET irradiation, we also assessed γH2AX at 24 h. The ratio of the signal at 24 h to that at 0.5 h was similar for cells irradiated with 6 Gy of low-LET protons and X-rays, whereas this ratio for cells irradiated with 6 Gy of high-LET protons was comparable to that observed for 15 Gy X-rays (Fig. S1B, right). Thus, high-LET protons likely induce DNA damage that is more difficult to repair. The high-LET carbon ions (73 keV/μm) induced levels of ATM and CHK1 phosphorylation that were approximately similar to those induced by low-LET carbon ions (29 keV/μm) at 1 h after irradiation (Fig. S1C). When comparing samples irradiated with 2 Gy of carbon ions and protons on the same immunoblot, the carbon ion-irradiated cells exhibited phosphorylation levels at 1 h that were similar to those of cells exposed to high-LET protons (Fig. S1C).

Since DNA damage signaling leads to activation of cell cycle checkpoints, we also analyzed cell cycle profiles 24 h after low- and high-LET proton and carbon ion irradiation. We found that G2 checkpoint activation was stronger following 6 Gy of high-LET compared to low-LET proton irradiation, as indicated by a higher proportion of cells in the G2/M phase in both cell lines (Fig. 1D). These results align with the increased CHK1 phosphorylation observed with high-LET proton irradiation (Fig. 1A and B), as the radiation-induced G2 checkpoint is generally dependent on ATR-CHK1 activation [6]. High-LET carbon ions also showed stronger G2 checkpoint activation than low-LET carbon ions (Fig. 1E), likely reflecting more prolonged CHK1 phosphorylation with high-LET carbon ions, despite similar phosphorylation levels

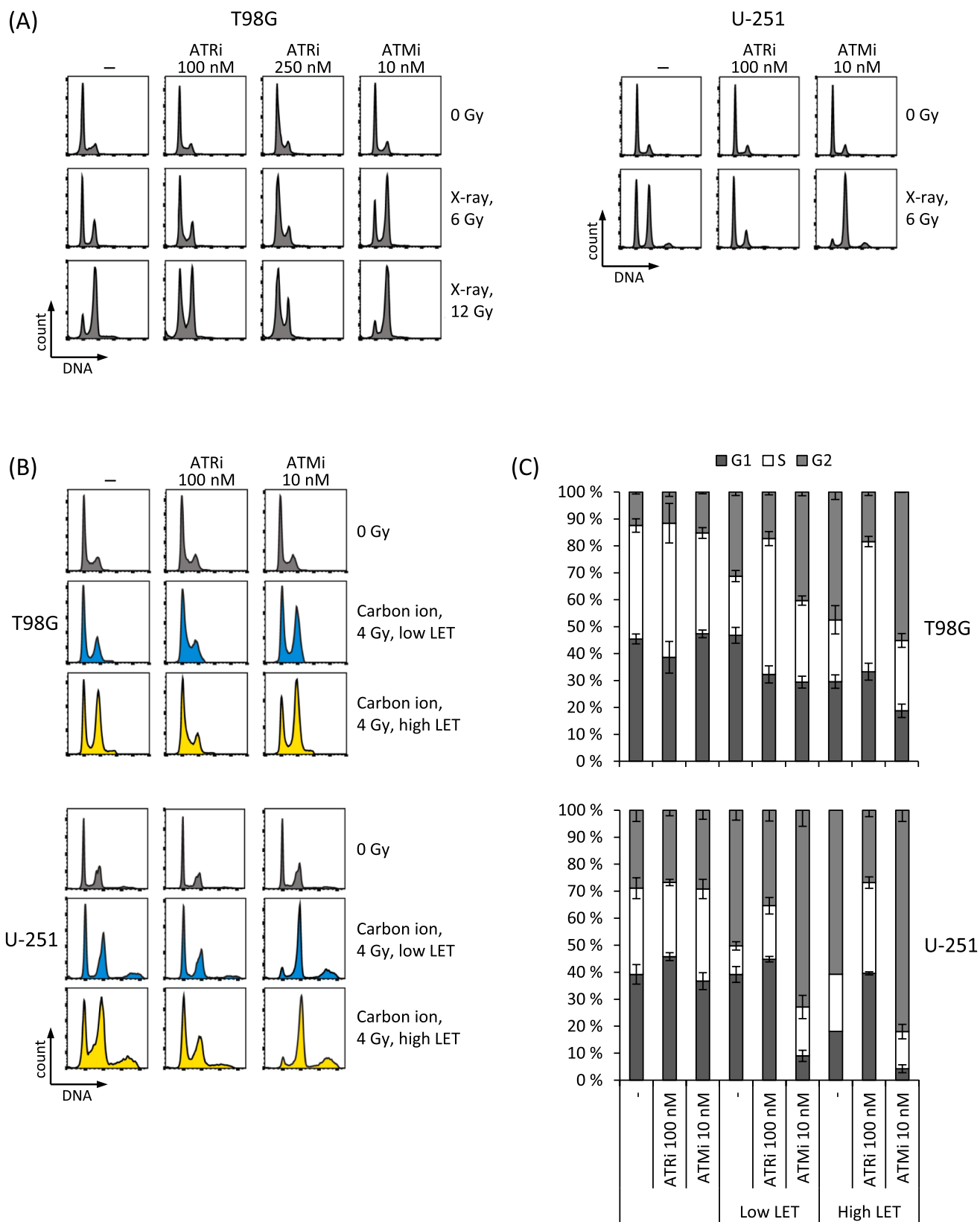


Fig. 2. The radiation-induced G2 checkpoint is abrogated by ATR inhibition and prolonged by ATM inhibition. (A) DNA profiles showing cell cycle distribution at 24 h after treatment with ATR and ATM inhibitors in combination with X-irradiation. Left: T98G, right: U-251. (B) DNA profiles at 24 h after treatment with the inhibitors and carbon ions, comparing low and high LET. (C) Quantification from cell cycle analysis performed on data as in B. Error bars: SEM ($n = 3$).

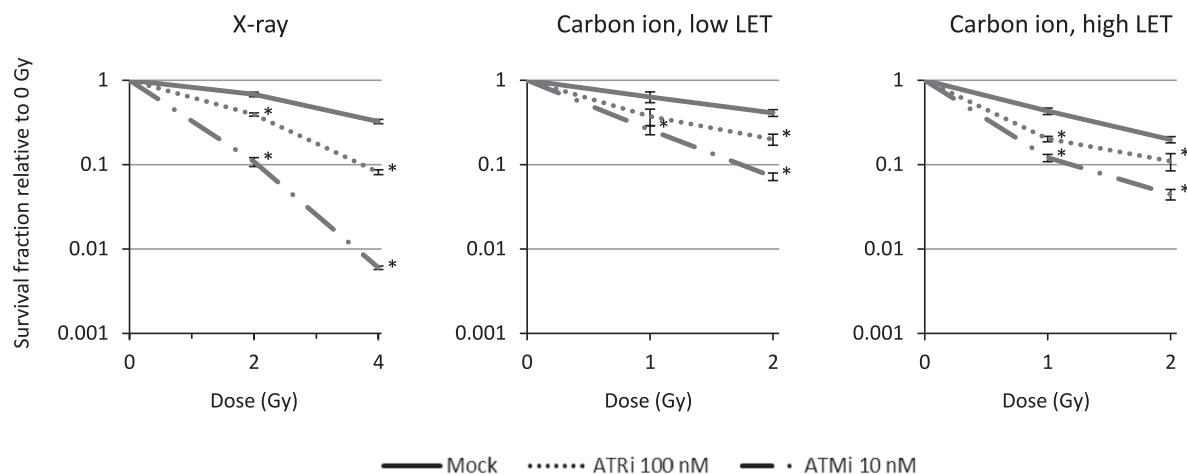


Fig. 3. ATM and ATR inhibitors enhance radiosensitivity after both low- and high-LET irradiation. Clonogenic survival of U-251 cells irradiated with 2 and 4 Gy of X-rays (left panel) or 1 and 2 Gy of carbon ions (middle and right panels) in combination with ATR and ATM inhibitors. Survival fractions relative to non-irradiated samples are plotted. The average survival fractions after treatment with the inhibitors alone were 0.6 (ATRi) and 0.8 (ATMi) for the experiments with X-rays, and 0.7 (ATRi) and 0.9 (ATMi) for the experiments with carbon ions. Error bars: SEM ($n \geq 3$).

between low- and high-LET carbon ions at 1 h (Fig. S1C).

We next investigated how radiation-induced G2 arrest was affected by co-treatment with inhibitors of the DNA damage response proteins ATR and ATM. Consistent with previous studies [18,31], the ATR inhibitor abrogated and the ATM inhibitor prolonged G2 arrest at 24 h after X-irradiation in both cell lines (Fig. 2A). These effects of the inhibitors were also seen with carbon ions and protons in both cell lines (Fig. 2B, C, S2A, and B), although G2 checkpoint abrogation in U-251 cells after 6 Gy of proton irradiation was minimal (Fig. S2B). Moreover, both inhibitors reduced clonogenic survival when combined with X-rays and both LETs of carbon ions, as measured in U-251 cells (Fig. 3). The sensitizing effect of the ATM inhibitor was smaller with carbon ions than with X-rays, whereas the effect of the ATR inhibitor was about the same with the different radiation modalities (Fig. 3 and Table S2). These findings support recent studies demonstrating that ATR and ATM inhibitors can sensitize cells to high-LET particle irradiation [13,16,32,33].

Since abrogation of the G2 checkpoint by ATR inhibition is known to induce a type 1 IFN response upon X-irradiation, we wondered whether similar responses would be induced after proton and carbon ion irradiation. We first examined phosphorylation of STAT1 (phospho-STAT1), a commonly used surrogate marker for extracellular type 1 IFN [20], although it may also be induced by other cytokines such as IL-6 or IL-22 [34,35]. Based on previous studies showing increased IFN signaling three days after ATR inhibition combined with X-irradiation in cancer cell lines [18,19], we conducted immunoblotting of U-251 cells at three days post treatment. Phospho-STAT1 levels were increased by ATR inhibition, but less so by ATM inhibition, after 4–6 Gy of X-irradiation (Fig. 4A). Similar results were obtained in cells irradiated with 4 Gy of carbon ions, with little difference between low- and high-LET irradiation (Fig. 4B). Next, we measured IFN- β levels in the growth medium via ELISA. Significantly higher IFN- β levels were detected for cells co-treated with inhibitors and high-LET carbon ions compared to those treated with inhibitors and X-rays (5–30 Gy) or low- and high-LET protons (Fig. 4C). Of note is that for carbon ions, only high-LET was included in these experiments. The highest IFN- β levels were observed with high-LET carbon ions in combination with the ATR inhibitor, although elevated levels were also seen with the ATM inhibitor. For U-251 cells treated with X-irradiation and inhibitors, the levels were close to the detection limit and varied between experiments, but the highest levels were detected with the ATR inhibitor (Fig. S3A).

Phospho-STAT1 levels were also detected by immunoblotting of T98G cells treated with combinations of inhibitors and X-, proton or

carbon ion irradiation. For all radiation types, except for high-LET protons, an increase in the phospho-STAT1 signal was observed for both inhibitors compared to irradiation alone (Fig. 5A, B, and S3B). The increase was not particularly strong and appeared similar for low- and high-LET particles. However, IFN- β levels in the growth medium of T98G cells exposed to inhibitors combined with either low- or high-LET carbon ions or high-LET protons were significantly higher than those in the growth medium of cells treated with inhibitors and low-LET protons or X-rays (Fig. 5C). This was found also when compared to very high doses of X-rays (30 Gy). The highest IFN- β levels were observed with ATR inhibition, although ATM inhibition combined with high-LET protons also led to increased IFN- β levels (Fig. 5C). Since our measurements of phospho-STAT1 levels did not reveal increased effects with high-LET irradiation, we hypothesized that this signal might be saturated at high extracellular IFN- β concentrations. Indeed, by addition of recombinant IFN- β , we found that the phospho-STAT1 signal became saturated approximately at IFN- β levels exceeding 500 pg/ml (Fig. S3C). This suggests that local IFN- β concentrations near the cells likely reached this threshold in our experiments.

Discussion

Radiotherapy with high energy X-rays is a cornerstone of cancer treatment. Recently, the interest in radiation-induced immune effects has escalated, with the goal to optimize combination treatments with immunotherapy. Inhibitors of DNA repair may counteract tumor radioresistance and may in addition enhance the antitumor immune effects. Particle radiotherapy with protons or carbon ions is currently being expanded in many countries, and increased immune signaling has also been observed in response to these radiation modalities [36,37]. However, knowledge about how DNA repair inhibitors modulate antitumor immune effects after particle irradiation has been lacking. To our knowledge, our study is the first to report effects on the IFN response after treatment with ATR and ATM inhibitors in combination with high-LET particle irradiation. Taken together, our results indicate that ATR inhibition increases IFN- β levels following irradiation with X-rays, protons or carbon ions in both cell lines, and that this effect is highly LET-dependent. Additionally, while ATM inhibition can increase IFN- β levels following irradiation, it does so to a lesser extent.

Notably, DNA repair inhibitors may be even more suitable for combinations with particles as opposed to conventional radiotherapy with X-rays. The radiosensitizing effects of the inhibitors on the surrounding normal tissue, including immune cells, will likely be reduced, due to the

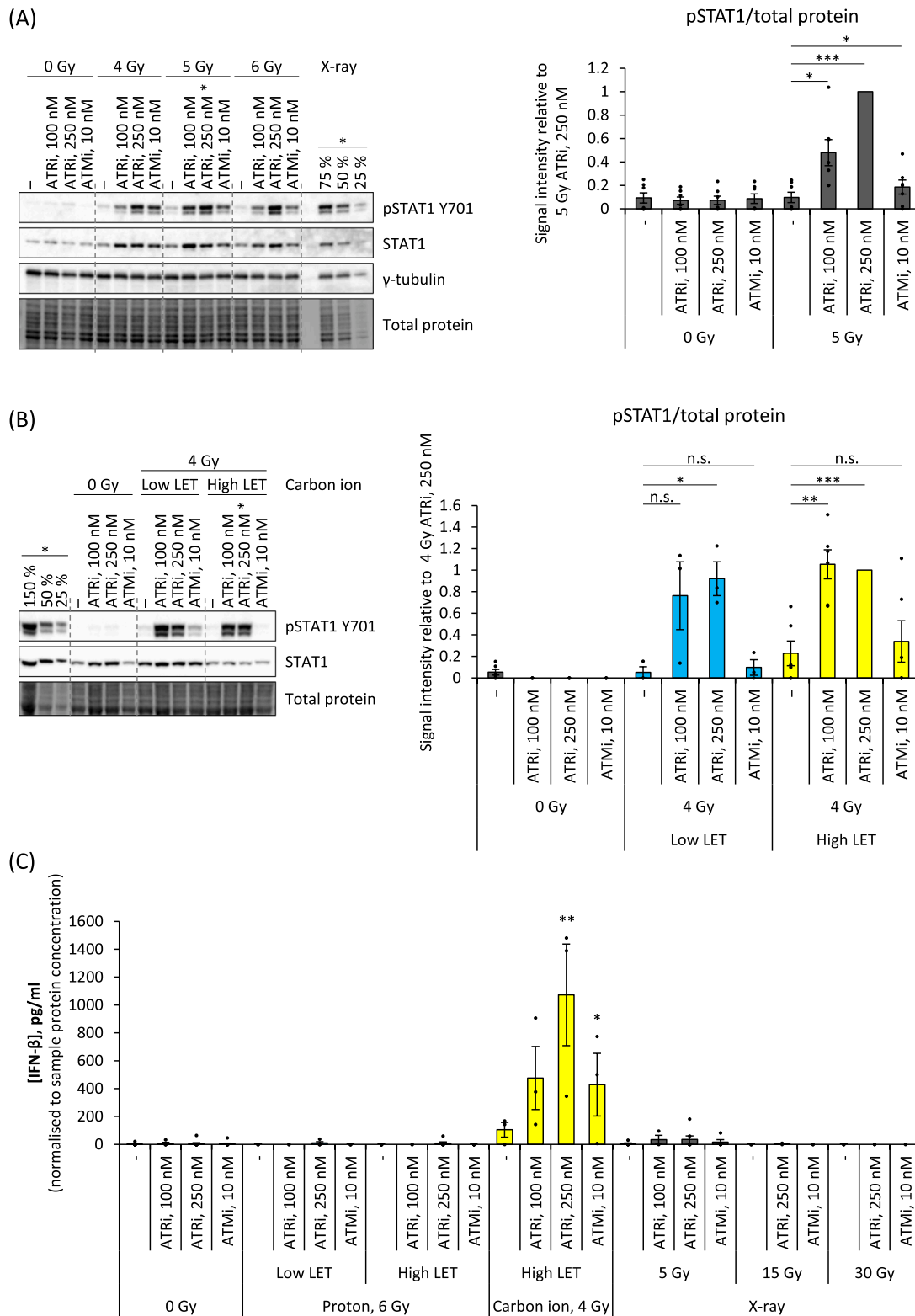


Fig. 4. ATR inhibition increases type 1 IFN signaling after both low- and high-LET irradiation in U-251 cells. (A) Left: Representative immunoblot showing phosphorylated STAT1 (pSTAT1) in U-251 cells at 72 h after treatment with 4, 5 and 6 Gy of X-rays in combination with indicated inhibitors. Asterisk indicates a dilution series of the sample treated with 5 Gy and 250 nM ATRi. Right: Quantification of pSTAT1 relative to total protein, normalized to the sample treated with 5 Gy in combination with 250 nM of ATRi. Error bars: SEM ($n = 7$). (B) Left: Similar as in A with 4 Gy of low- and high-LET carbon ion irradiation. Asterisk indicates a dilution of the sample treated with 4 Gy high-LET carbon ions and 250 nM ATRi. Right: Quantification of pSTAT1 relative to total protein. Error bars: SEM ($n = 3$ for low LET and $n = 6$ for high LET). (C) IFN- β levels measured by ELISA in 20X unconcentrated cell growth supernatants at 72 h after the indicated treatments. Unpaired two-tailed t test was used to address whether signals were significantly higher than for the respective inhibitor treatment combined with 5 Gy of X-rays.

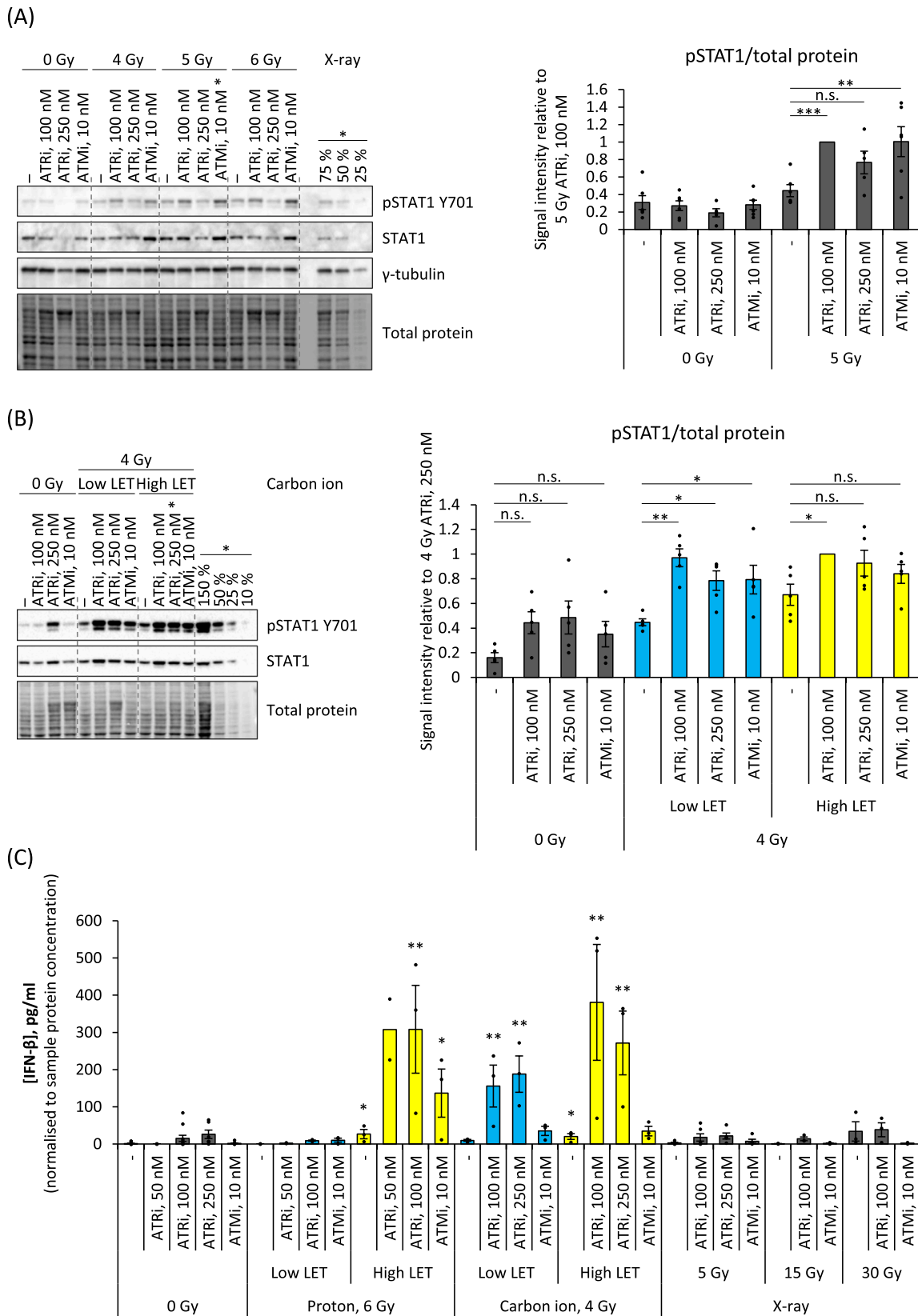


Fig. 5. IFN signaling is heavily induced in T98G cells treated with high-LET irradiation combined with ATR inhibitor. (A) Analysis of pSTAT1 in T98G cells, similarly as in Fig. 4A except that values are normalized to the sample treated with 5 Gy and 100 nM ATRi. Error bars: SEM ($n \geq 5$). (B) Left: Similar as in A with 4 Gy of low- and high-LET carbon ions. Right: Quantification of pSTAT1 relative to total protein. Error bars: SEM ($n \geq 4$). (C) IFN- β levels measured by ELISA in 20X upconcentrated cell growth supernatants similar as in Fig. 4C. Unpaired two-tailed t test was used to address whether signals were significantly higher than for the respective inhibitor treatment combined with 5 Gy of X-rays.

beneficial depth dose distribution of particle radiation. We thus expect improved tumor-specific radiosensitization and possibly improved antitumor immune response with particle irradiation. In support of the latter, recent clinical reports suggest that proton and carbon ion radiotherapy induce less lymphopenia compared to classical radiotherapy [38,39]. Our data suggest that a further benefit from combining ATR and ATM inhibitors with particle irradiation may be obtained through activation of the innate immune response within the tumor cells themselves.

In this study, ATR inhibition led to larger increases in IFN signaling compared to ATM inhibition. After ATR inhibition, the abrogation of G2 checkpoint arrest and cytosolic exposure of DNA from ruptured micronuclei likely contribute to the increased IFN signaling, as previously reported in other cell types following combination with X-irradiation [18,40]. Interestingly, the levels of secreted IFN- β were much higher when the inhibitors were combined with high-LET than low-LET irradiation. This effect was seen with carbon ions in U-251 cells and with both carbon ions and high-LET protons in T98G cells. The mechanism underlying this effect is not known and would be interesting to explore in future studies. Pointing towards possible mechanisms, studies have shown that more micronuclei are formed in cells exposed to high-LET than low-LET irradiation [13,41,42], likely due to induction of more complex DNA damage. On the other hand, ruptured micronuclei may not be sufficient to induce a strong IFN response [43]. Other sources of cytosolic DNA, such as mitochondria, may also be involved [21,44,45], particularly for ATM inhibition where the G2 checkpoint was not abrogated. Furthermore, IFN- β signaling may also be triggered by cytosolic RNA as described in previous studies with X-irradiation [17,19]. The RNA can be transcribed from cytosolic DNA or leak from mitochondria [46], but could also stem from reactivation of retroelements [47,48] through radiation-induced decompaction of chromatin. Notably, a recent study showed a more pronounced IFN- β signaling after proton as compared to X-irradiation, which was caused by proton-induced derepression of transposable elements [49].

The ATR and ATM inhibitors used in this study have been combined with classical radiotherapy in clinical trials for brain metastases (NCT02589522) and GBM (NCT03423628). GBM is typically highly radioresistant as well as invasive. Our results suggest that ATR or ATM inhibitors could increase tumor cell radiosensitivity and may also enhance radiation-induced immune signaling. The combination of these inhibitors with radiotherapy could thus likely facilitate eradication of the main tumor, as well as help eliminating invasive cells outside the irradiation field by promoting antitumor immune effects. Likely, triple combinations of radiotherapy, radiosensitizing drug and immune checkpoint blockade may be effective. In this approach the ATR/ATM inhibitor is used to increase tumor radiosensitivity and the antitumor immune effects of radiotherapy, while potential immunosuppressive effects, such as increased PD-L1 presentation, are counteracted by the immune checkpoint inhibitor.

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CRedit authorship contribution statement

Gro Elise Rødland: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Mihaela Temelie:** Writing – review & editing, Visualization, Methodology, Investigation, Conceptualization. **Adrian Eek Mariampillai:** Writing – review & editing, Visualization, Investigation, Formal analysis, Methodology. **Ana Maria Serban:** Writing – review & editing, Investigation. **Nina Frederike Jeppesen Edin:** Writing – review & editing, Methodology. **Eirik Malinen:** Writing – review & editing, Resources, Methodology. **Lilian Lindbergsengen:**

Writing – review & editing, Investigation. **Antoine Gilbert:** Writing – review & editing, Investigation. **François Chevalier:** Writing – review & editing, Supervision, Resources, Methodology. **Diana I. Savu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Randi G. Syljuåsen:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.radonc.2024.110669>.

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