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Original Article

Selective effects of radiotherapy on viability and function of invariant natural killer T cells *in vitro*

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ABSTRACT

Background and purpose: Immunotherapies involving the adoptive transfer of *ex vivo* expanded autologous invariant natural killer (iNKT) cells are a potential option for cancer patients and are under investigation in clinical trials. Most cancer patients receive radiotherapy at some point during their treatment. We investigated the effects of therapeutic doses of radiation on the viability and function of human primary cultures of iNKT cells *in vitro*.

Materials and methods: iNKT cell lines generated from 6 healthy donors were subjected to therapeutically-relevant doses of radiation. Cell cycle arrest and cell death were assessed by flow cytometry. Double strand DNA breaks were analysed by measuring phosphorylated histone H2AX expression by fluorescence microscopy. Cytolytic degranulation, cytokine production and cytotoxicity by antigen-stimulated iNKT cells were assessed by flow cytometry.

Results: Radiation inhibited viability of iNKT cells in a dose-dependent manner. Radiation caused double strand DNA breaks, which were rapidly repaired, and affected the cell cycle at high doses. Moderate doses of radiation did not inhibit degranulation or cytotoxicity by iNKT cells, but induced perforin expression and inhibited proliferation and interferon-γ production by surviving iNKT cells.

Discussion: Exposure of iNKT cell to radiation can negatively affect their viability and function.

Immunotherapies involving the adoptive transfer of *ex vivo* expanded autologous invariant natural killer (iNKT) cells and subsequent transfer back into the patients, is a potential option for cancer patients with limited responses to chemotherapy and radiotherapy. iNKT cells are rapid-acting cytotoxic T cells that co-express semi-invariant T cell receptor (TCR) α-chains (Vα24Jα18) and natural killer (NK) cell stimulatory receptors [1]. Whereas, conventional T cells recognise peptides presented by major histocompatibility complex (MHC) molecules, iNKT cells recognise glycolipids presented by CD1d, an MHC class I-like molecule on antigen presenting cells [1,2]. iNKT cell activation is followed by the rapid secretion of cytolytic molecules, such as granzyme B and perforin that induce apoptosis of tumour cells via caspase pathways [3], and by the rapid secretion of growth factors and cytokines, including a diverse array of T helper type 1 (Th1), Th2, Th17 and regulatory T (Treg) cytokines, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), IL-4, IL-5, IL-13, IL-10, IL-17 and IL-22 [4]. In addition to direct killing of tumour cells, iNKT cells play key roles in the activation and differentiation of T cells, NK cells, macrophages, dendritic cells (DC) and B cells via cytokine secretion and contact-dependent interactions, aiding in the indirect killing of tumour cells [5].

A number of glycolipid antigens have been shown to bind to CD1d and activate iNKT cells, of which α-galactosylceramide (α-GalCer) has been most extensively studied [6]. Therapeutic activation of iNKT cells with α-GalCer can promote tumour rejection in murine models [7,8], which has led to a number of clinical trials using intravenous injection of α-GalCer, adoptive transfer of α-GalCer-pulsed autologous DC, adoptive transfer of *ex vivo* expanded and activated autologous iNKT cells, or combinations of these [9–12]. However, despite the success in murine models, the clinical efficacies of iNKT cell-based therapies in humans have been modest. It is possible that the iNKT cell-based immunotherapy trials failed because they were carried out on patients with advanced disease, having failed conventional therapies, or because the cellular functions of iNKT cells were impaired by previous or ongoing radiotherapy, chemotherapy or surgery. We have previously observed that chemotherapies used for gastrointestinal cancers negatively affect iNKT cell function [Melo et al. unpublished]. The present study aimed to assess the effects of radiation on iNKT cell viability and function.

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Methods

Generation of iNKT cell lines

Primary lines of iNKT cells were generated from peripheral blood mononuclear cells (PBMC) from seven healthy adults prepared from anonymous buffy coat packs obtained from the Irish Blood Transfusion Service. $CD3^+$ $V\alpha24J\alpha18^+$ cells were sorted from the PBMC and expanded for at least 3 weeks with α-GalCer and IL-2 as described previously [13]. Purity of the iNKT cell lines was at least 92%. Ethical approval for the use of the buffy coat packs was obtained from the Research Ethics Committee of Trinity College Dublin.

OE33 and OE19 cell lines

Oesophageal adenocarcinoma (OE33) cells and oesophageal gastric junction cancer (OE19) cells were obtained from the European Collection of Authenticated Cell Cultures and maintained in compete RPMI (cRPMI) medium (RPMI 1640 containing 10% HyClone foetal calf serum (FCS), 1% penicillin–streptomycin, Gibco-BRL, Paisley) in T75 flasks in a humidified atmosphere with 5% CO_2 in air at 37 °C.

Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, Vα24Jα18 TCR used by iNKT cells (clone 6B11), IL-4, perforin, 7-aminoactinomycin D (7-AAD) granzyme B, (BioLegend), CD107a, (BD Biosciences), IFN-γ (Miltenyi Biotec), annexin V (Santa Cruz Biotechnologies), the fluorescent DNA intercalating agent propidium iodide (PI; Invitrogen), and the fixable viability dye efluor 506 (eBioscience) were used. Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences), and FlowJo Version 10 (Tree Star) software was used for analysis.

Radiotherapy

iNKT cells were irradiated with a biological X-ray irradiator RS 225 system (Gulmay Medical) or CIX2 X-Ray Cabinet (Xstrahl Life Science). Cells were mock irradiated (0 Gy), irradiated with single doses of 2 Gy or 10 Gy, or with five cumulative 2 Gy doses every 24 h. Mock irradiated cells were treated and transported in the same manner as the irradiated cells, except that they were not irradiated.

Cell death analysis

Cells were irradiated as described above and then stained for Annexin V and PI to measure cell death by flow cytometry. Cells were irradiated with single doses of 2 Gy or 10 Gy, or five cumulative 2 Gy doses every 24 h and compared with mock-irradiated control. Mock irradiated cells used as controls for cells treated with 5 daily doses of radiation were analysed after 5 days to control for other causes of cell death.

Cell cycle arrest analysis by PI staining

Cell cycle arrest was measured by PI staining and analysis by flow cytometry. iNKT cells were fixed with 70% ethanol in phosphate buffered saline (PBS) for 30 min at room temperature. 1 mg/mL PI (diluted 1:40) and 100 μg/mL of RNAse (Roche) were added for 30 min at 37 °C and cells were analysed immediately by flow cytometry.

Analysis of DNA damage by γH2AX detection

DNA damage in iNKT cells was detected by measuring phosphorylated H2A histone family member X (γH2AX) expression using fluorescence microscopy. iNKT cells were fixed with 4% paraformaldehyde and attached to poly-L-lysine (Sigma-Aldrich) coated slides, using a Shandon Cytospin 3 (ThermoFisher). Cells were treated for 2 h at room temperature with 5% FCS, 0.3% Triton X-100 to block non-specific binding sites. Cells were then incubated with 1:100 rabbit anti- γH2AX (ser139) antibody (Cell Signalling Technology) overnight at 4 °C. Cells were then incubated with 1:1000 dilution of Alexafluor 555-labelled donkey anti-rabbit secondary antibody (ThermoFisher) and placed onto labelled slides using Prolong gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Slides were sealed using a film-forming adhesive polymer and left at 4 °C until analysis. Slides were visualised using the IX51 Olympus inverted microscope, at 100× magnification. Foci of at least 25 cells were counted.

CD1d-dependent activation of iNKT cells and analysis of cytolytic degranulation and intracellular production of cytotoxic mediators and cytokines

Cytolytic degranulation of iNKT cells was tested by flow cytometric analysis of CD107a expression by Vα24Jα18 TCR positive cells after stimulation with CD1d transfected HeLa cells (HeLa-CD1d cells) presenting α-GalCer, as described [13]. HeLa-CD1d cells were a gift from Dr. Steven Porcelli (Albert Einstein College of Medicine, New York). Intracellular production of granzyme B and perforin, IL-4 and IFN-γ was analysed by flow cytometry as described [4]. Fixable viability dye eFluor-506 was used to exclude dead cells.

Total cytotoxicity assay

iNKT cell killing of CD1d transfected C1R cells (obtained from Dr. Steven Porcelli) presenting α-GalCer was tested using the Total Cytotoxicity and Apoptosis Detection Kit purchased from Immunochemistry Technologies as described previously [4].

Statistical analysis

Statistical analysis was done using GraphPad Prism Version 6.0. p values between groups were obtained using the paired t and Mann-Whitney U test where appropriate. P values of <0.05 (*) were considered statistically significant.

Results

To determine if radiotherapy induces cell death in iNKT cells, expanded iNKT cells, OE33 cells and OE19 cells were mock-irradiated (0 Gy), or irradiated with therapeutic doses of radiation relevant for oesophageal cancer patients in our facility (2 Gy, 10 Gy or 5 fractionated doses of 2 Gy). OE33 cells and OE19 cells were used as control cells to compare sensitivities between transformed epithelial cells and iNKT cells, since previous studies have shown that OE33 cells are more radiosensitive than OE19 cells [14]. Toxicity was examined by staining the cells with annexin V and PI and analysis by flow cytometry (Fig. 1A). Fig. 1B shows an increase in total iNKT cell death 48 h after 2 Gy and 10 Gy radiation, respectively, over the spontaneous cell death (0 Gy), which compares to OE19 cell death. OE33 cells showed a greater increase in cell death than OE19 cells when irradiated with 10 Gy at 24 and 48 h. To analyse the cytotoxic effect of cumulative doses, cells were irradiated with five cumulative doses of 2 Gy for 5 days. A dose dependent increase in cell death was observed (Fig. 1B). Up to 50% of

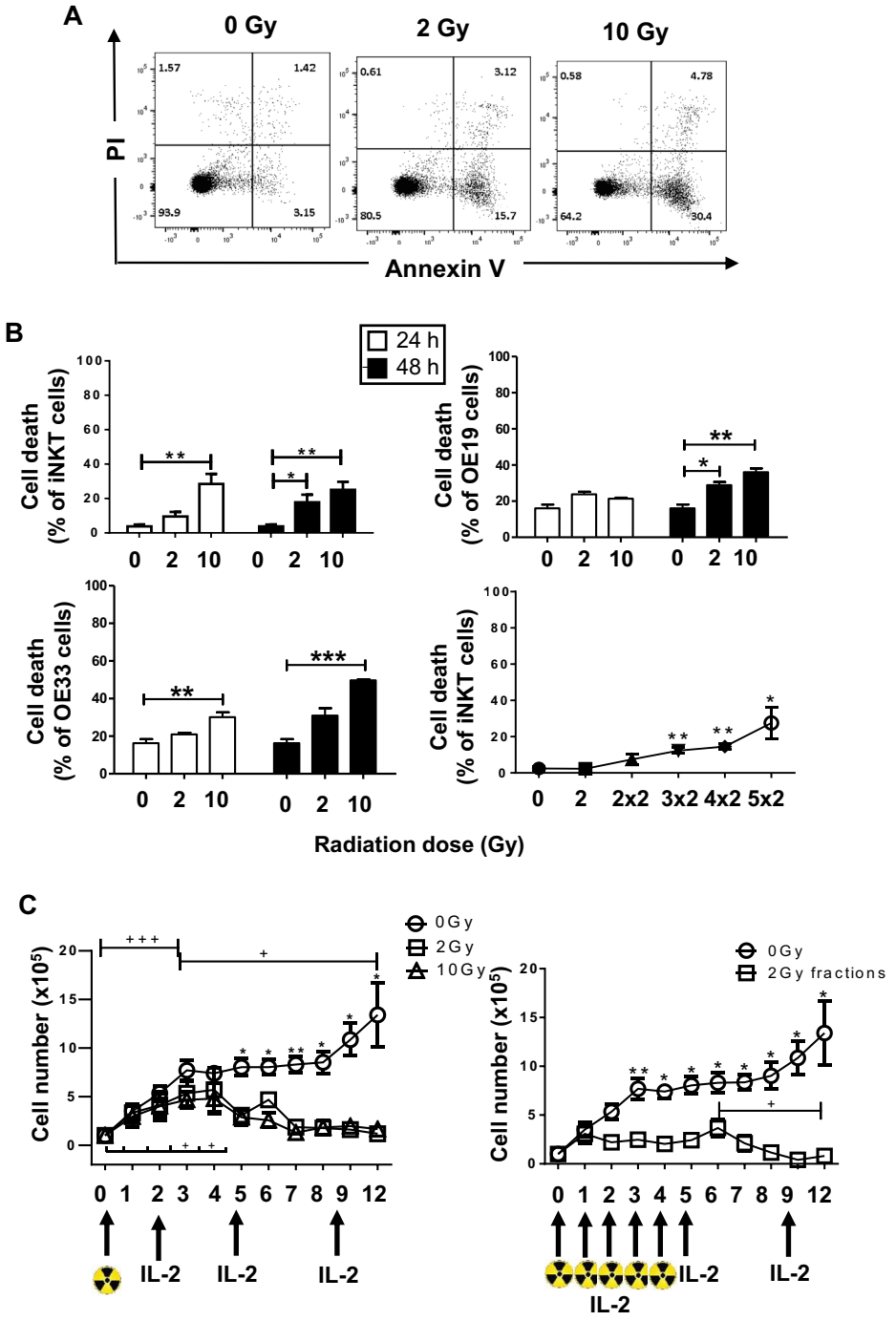

Fig. 1. Radiation induces human iNKT cell death. Lines of expanded iNKT cells from 6 healthy donors, OE33 cells and OE19 cells were mock irradiated (0 Gy), irradiated with single doses of 2 Gy, 10 Gy or five cumulative fractions of 2 Gy. iNKT cells, OE33 cells and O19 cells were then stained with Annexin V and PI and analysed by flow cytometry. (A) Representative flow plots of iNKT cells 48 h post irradiation with 0 Gy, 2 Gy and 10 Gy. (B) Graph shows mean percentages of dead iNKT cells (upper left panel), OE19 cells (upper right) and OE33 cells (lower left) 24 h or 48 h after radiation. The lower right graph shows the percentage of dead cells 24 h after cumulative doses of 2 Gy. Cell death in the mock-irradiated sample were measured after 5 days at the same time as when the cells treated with 5 daily doses of radiation were measured. (C) Cells were stimulated with α-GalCer and irradiated as indicated. Medium was replaced with fresh medium containing IL-2 on days 2, 6 and 9. Viable cells were counted every day using fluorescence microscopy. Results show means (±SEM) from 6 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ using the paired t test compared to 0 Gy.

iNKT cells were dead 24 h after the fifth dose of 2 Gy, indicating that the cells cannot effectively recover when irradiated for five consecutive days.

The effects of single 2 Gy, 10 Gy doses or five cumulative 2 Gy doses of radiation on proliferation of iNKT cells in response to activation with α-GalCer and culture in the presence of IL-2 was also tested by enumerating viable cells each day up to day 12. Fig. 1C shows that both single and cumulative doses significantly inhibited iNKT cell expansion.

We next investigated if iNKT cells can repair double strand breaks in DNA. Lines of expanded iNKT cells were mock irradiated (0 Gy) or irradiated with 2 Gy or 10 Gy. γ-H2AX was used as a marker for double strand breaks in the DNA and analysed by microscopy 20 min, 6 h and 24 h post radiation (Fig. 2A). We

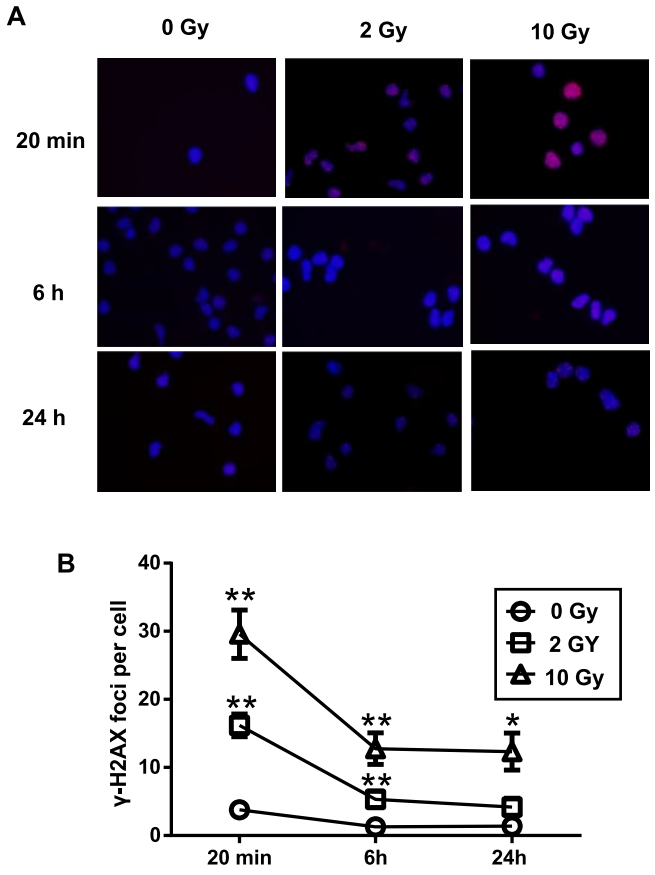

Fig. 2. Radiation induces double strand breaks in iNKT cell DNA *in vitro*. Lines of expanded iNKT cells were irradiated with mock radiation (0 Gy), or single doses of 2 Gy or 10 Gy. After 20 min, 6 h or 24 h, the cells were stained with γ-H2AX and DAPI and analysed by microscopy. (A) Micrographs show γ-H2AX (red) and nuclei (blue) at original magnification of 100×. (B) Graph shows mean numbers of γ-H2AX foci per cell 20 min, 6 h and 24 h post irradiation. Results are means of 25 cells from 5 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, using the paired *t* test compared to 0 Gy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed a significant increase in DNA double strand breaks 20 min post radiation, with an average of 16 foci of γ-H2AX per cell after 2 Gy radiation and 30 foci after 10 Gy. After 6 h the number of foci per cell was reduced, but still significantly higher compared to non-irradiated control cells. A further reduction in γ-H2AX foci was observed after 24 h. iNKT cells irradiated with 2 Gy were able to completely repair their DNA 24 h after radiation (Fig. 2B). However, γ-H2AX expression by iNKT cells was still significantly higher 24 h post radiation with 10 Gy compared to controls.

To determine if iNKT cells undergo cell cycle arrest after irradiation, iNKT cells were stained with PI and cell cycle distribution was analysed by flow cytometry (Fig. 3A). The intensity of the PI signal is directly proportional to DNA content. Cell cycle arrest of expanded iNKT cell lines was tested 6, 10 and 24 h after irradiation with 0, 2 and 10 Gy and 24 h after each dose of the five 2 Gy consecutive doses. Cell cycle was not altered after 6 h (Fig. 3B) or 10 h (Fig. 3C). In contrast, a decrease of 12.5 ± 3.5% iNKT cells in G0/G1 was observed 24 h after 10 Gy radiation (Fig. 3D). This is reflected by an increase in apoptotic cells in subG1. No difference was observed when cells were irradiated with five cumulative doses (Fig. 3F).

We also investigated the effects of radiation on the cytotoxic potential viable iNKT cells. Lines of iNKT cells were mock-irradiated (0 Gy), irradiated with single doses of 2 Gy, 10 Gy or five cumulative doses of 2 Gy. After 24 and 48 h, the numbers of iNKT cells were adjusted to account for cell death post radiation and equal numbers of viable iNKT cells were co-cultured for 4 h with CD1d-transfected HeLa cells previously pulsed with 100 ng/mL α-GalCer. Cytotoxic potential of iNKT cells was examined by measuring the expression of the degranulation marker CD107a by flow cytometry (Fig. 4A). A significant increase in CD107a expression was observed between unstimulated cells and cells stimulated with α-GalCer. No significant differences were observed in CD107a expression between stimulated non-irradiated and irradiated viable iNKT cells (Fig. 4B). A moderate but statistically-significant decrease in CD107a expression was observed after the fifth fractionated dose of 2 Gy when compared to control (Fig. 4C).

We also examined direct killing of CD1d-transfected C1R cells by iNKT cells after radiation (Fig. 4D). iNKT cells were mock-irradiated (0 Gy), irradiated with 2 Gy or 10 Gy, and then co-cultured in three different effector:target ratios (25:1, 10:1, 1:1) for 5 h at 37 °C. C1R-CD1d cell viability was reduced by 15% when co-cultured with iNKT cells prior to stimulation but were more significantly decreased when α-GalCer was present, with stimulated iNKT cells directly killing 73% of CD1d-transfected C1R cells. No significant difference in the specific lysis of target cells by iNKT cells after radiation was observed when compared to the untreated control (Fig. 4E), suggesting that cytotoxic function of the iNKT cells is still intact after irradiation.

In addition to measuring markers of degranulation and target cell killing by activated iNKT cells, we examined the expression of the cytotoxic mediators perforin and granzyme B. Lines of iNKT cells were mock-irradiated (0 Gy) or irradiated with single doses of 2 Gy or 10 Gy. After 24 or 48 h the iNKT cells were co-cultured for 4 h with equal numbers of HeLa-CD1d cells, which were previously loaded with medium α-GalCer. The production of granzyme B and perforin were measured by flow cytometry (Fig. 5A). The frequency of granzyme B expression by iNKT cells subjected to 2 Gy or 10 Gy radiation was similar to that of non-irradiated iNKT cells (Fig. 5B). Surprisingly, perforin was expressed by significantly higher proportions of iNKT cells 48 h after irradiation with 2 Gy and 10 Gy compared to non-irradiated iNKT cells (Fig. 5C). These results show that viable iNKT cells post radiation retain the potential to kill target cells.

Finally, we investigated if irradiation of iNKT cells affects cytokine production. iNKT cell lines were exposed to 0, 2 or 10 Gy doses of irradiation or five fractionated doses of 2 Gy. Cells were then co-cultured with CD1d transfected HeLa cells previously loaded with α-GalCer. The proportions of viable iNKT cells expressing intracellular IFN-γ and IL-4 as signature Th1 and Th2 cytokines were quantified by flow cytometry (Fig. 6A). CD1d-transfected HeLa cells pulsed with α-GalCer induced significant IFN-γ and IL-4 expression by iNKT cells. Irradiation with 2 Gy led to a significant decrease in the frequencies of viable iNKT cells that produced IFN-γ after 48 h (Fig. 6B). We also observed a significant inhibition of IFN-γ expression by stimulated iNKT cells after each cumulative dose of radiation, reaching the lowest point 24 h after the fifth fractionated dose (Fig. 6C). However, irradiation of iNKT cells with 2 or 10 Gy had no effect on IL-4 production after 24 or 48 h (Fig. 6D) or after the cumulative doses of 2 Gy (Fig. 6E).

Discussion

Radiation is an important component of cancer treatment [15]. It targets rapidly-dividing cells by generating free radicals that produce double strand breaks in DNA, leading to apoptosis of the cell [16]. Approximately two-thirds of all cancer patients will receive radiotherapy at some point during their treatment [17]. Patients generally receive 1.8–2.0 Gy daily fractions, applied over several

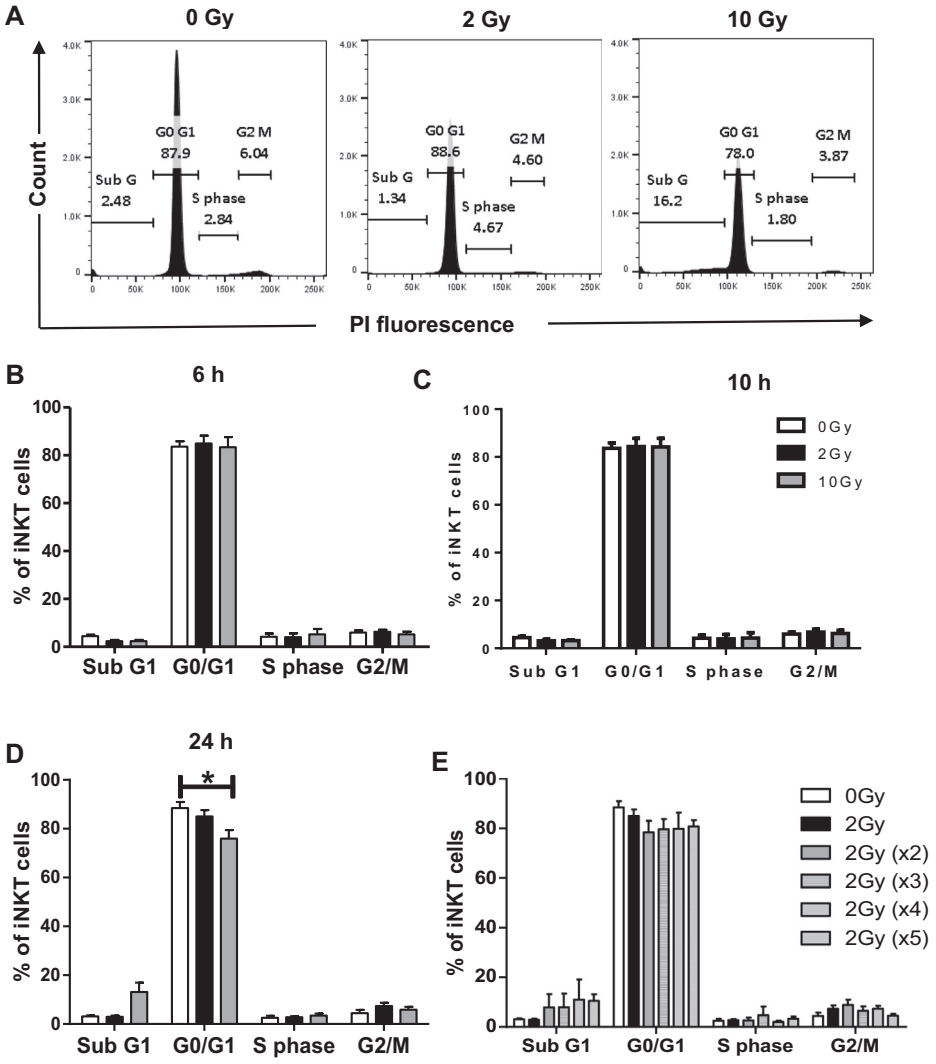

Fig. 3. Cell cycle of iNKT cells was altered 24 h post irradiation with 10 Gy. Lines of expanded iNKT cells from 6 healthy donors were mock irradiated (0 Gy), given single 2 Gy or 10 Gy doses, or five cumulative 2 Gy fractions. (A) Cells were stained with PI after irradiation and cell cycle arrest was assessed by flow cytometry. (B–E) Graphs show percentage of iNKT cells in different phases of the cell cycle 6 h (B), 10 h (C), 24 h (D) after single doses of 2 Gy or 10 Gy, or 24 h after five fractionated doses of 2 Gy (E). Results are means of 5 independent experiments. $^{*}p < 0.05$, using the paired t test compared to 0 Gy.

weeks to reduce toxicity against healthy host cells [18,19]. While radiation is intended to kill tumour cells, little is known about its toxicity for iNKT cells, which might be an important consideration if iNKT cell-based immunotherapies are used in patients who received or are receiving radiotherapy. It has been reported that murine iNKT cells are more radioresistant than T cells and B cells *in vivo* [20]. A few studies have compared circulating iNKT cell numbers in cancer patients who received radiation with patients who did not. Crough et al. demonstrated that the frequencies of iNKT cells and total T cells were lower in patients with a number of solid tumours, and by excluding patients who had received chemotherapy, they showed that prior radiation treatment contributed to the observed reduction in melanoma patients [21]. In a study of patients with advanced head and neck cancer, Kobayashi and co-workers found that patients who received radiation treatment had lower total T cell counts, but similar iNKT cell numbers to patients who did not receive radiation [22]. However, these studies did not investigate the direct effect of radiation on iNKT cells. In the present study we observed a dose dependent increase in iNKT cell death after radiation. Comparing the radiosensitivity of iNKT cells with those of two oesophageal/gastric cancer cell lines, which differ in their radiosensitivities with OE33 being more radiosensitive than OE19 [14], we found that iNKT cells have similar radiosensitivity to OE19 cells. Future studies are required to determine how the radiosensitivity of iNKT cells compares with those of other immune cells.

Double strand DNA breaks are thought to be the most lethal form of DNA damage, leading to apoptosis and death of cells that are unable to repair the damage. Double strand DNA breaks are always followed by phosphorylation of the histone, H2AX, denoted γ-H2AX, as a first step in recruiting and localizing DNA repair proteins. γ-H2AX foci quickly form and can be used as a marker for DNA damage [23]. Previous studies have shown that γ-H2AX expression in total lymphocytes reaches a maximum 30 min post-radiation and returns to baseline levels 24 h post-exposure [24–26]. Analysis of lymphocyte subtypes showed that $CD4^+$ and $CD8^+$ T cells and B cells display similar kinetics of γ-H2AX expression, but $CD4^+$ and $CD8^+$ T cells express more γ-H2AX than B cells [27]. We examined γ-H2AX expression by iNKT cell lines after radiation and found it to reach maximum expression 20 min post radiation, returning to baseline after 24 h. This suggest that the double strand DNA break repair capability of iNKT cells is intact.

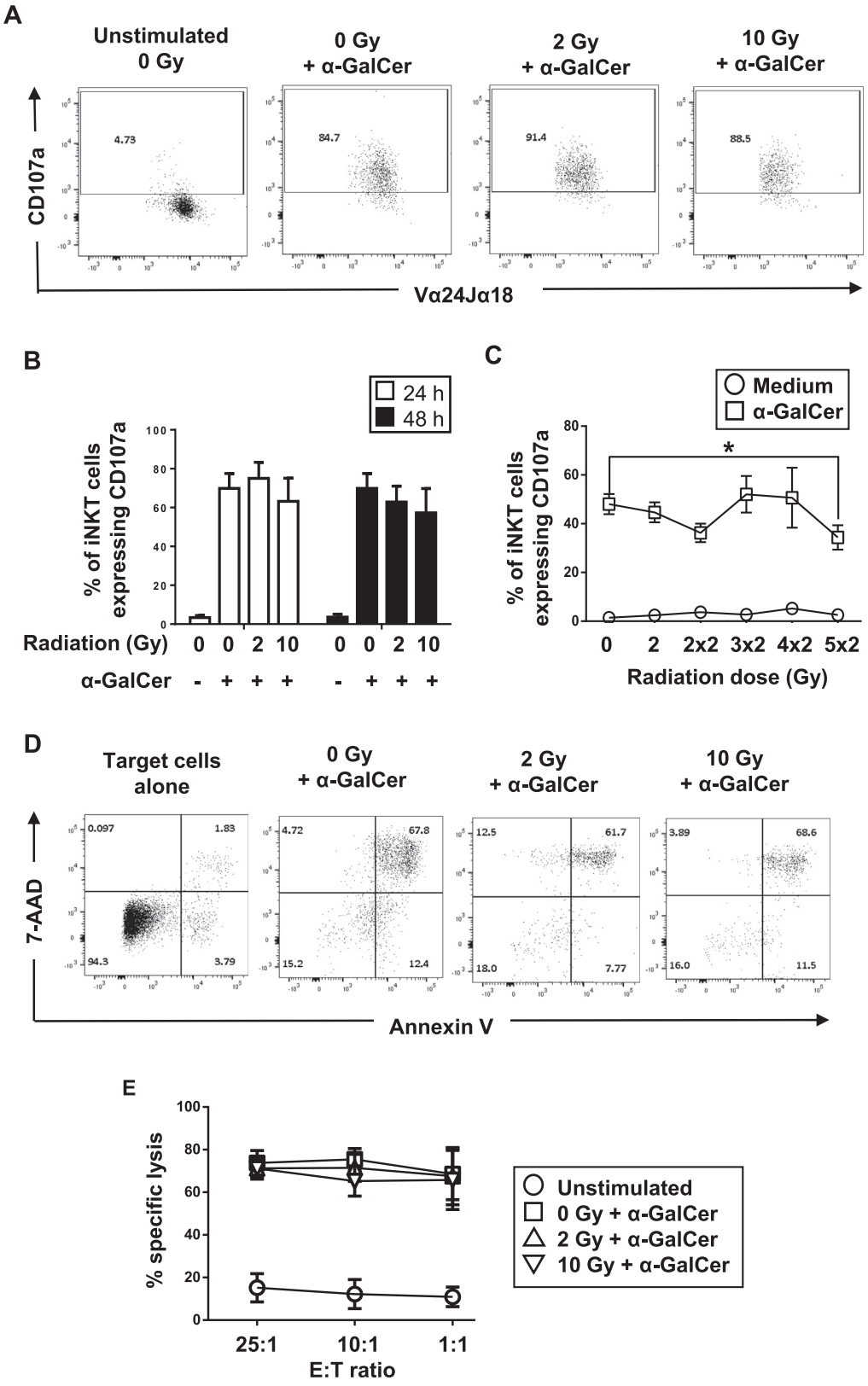

Fig. 4. Radiation does not inhibit cytotoxicity by viable iNKT cells in vitro. Lines of expanded iNKT cells from 6 donors were irradiated with 0 Gy, single 2 Gy or 10 Gy doses, or five cumulative 2 Gy fractions. 24 h and 48 h later the numbers of iNKT cells were adjusted to account for cell death post radiation and equal numbers of viable iNKT cells were co-cultured for 4 h with CD1d transfected HeLa cells, previously pulsed with α-GalCer. (A) Cells were then stained with dead cells stain and mAbs specific for cell-surface CD3 and Vα24Jα18 and CD107a and analysed by flow cytometry. (B and C) Graph shows mean (±SEM) percentages of viable iNKT cells that expressed CD107a 24 h and 48 h after single doses (B) or fractioned doses (C) of radiation. (D) Direct killing by iNKT cells post radiation was tested by co-culturing 0 Gy, 2 Gy or 10 Gy irradiated iNKT cells with CFSE-stained CD1d transfected C1R target cells for 5 h, previously pulsed with α-GalCer. Cells were then stained with annexin-V and 7-AAD to detect apoptotic and dead cells, respectively, by flow cytometry. **E,** Graph showing mean (±SEM) percentages of specific lysis of CD1d-transfected C1Rs by iNKT cells. Results are means of 6 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ using the paired t test compared to 0 Gy plus α-GalCer.

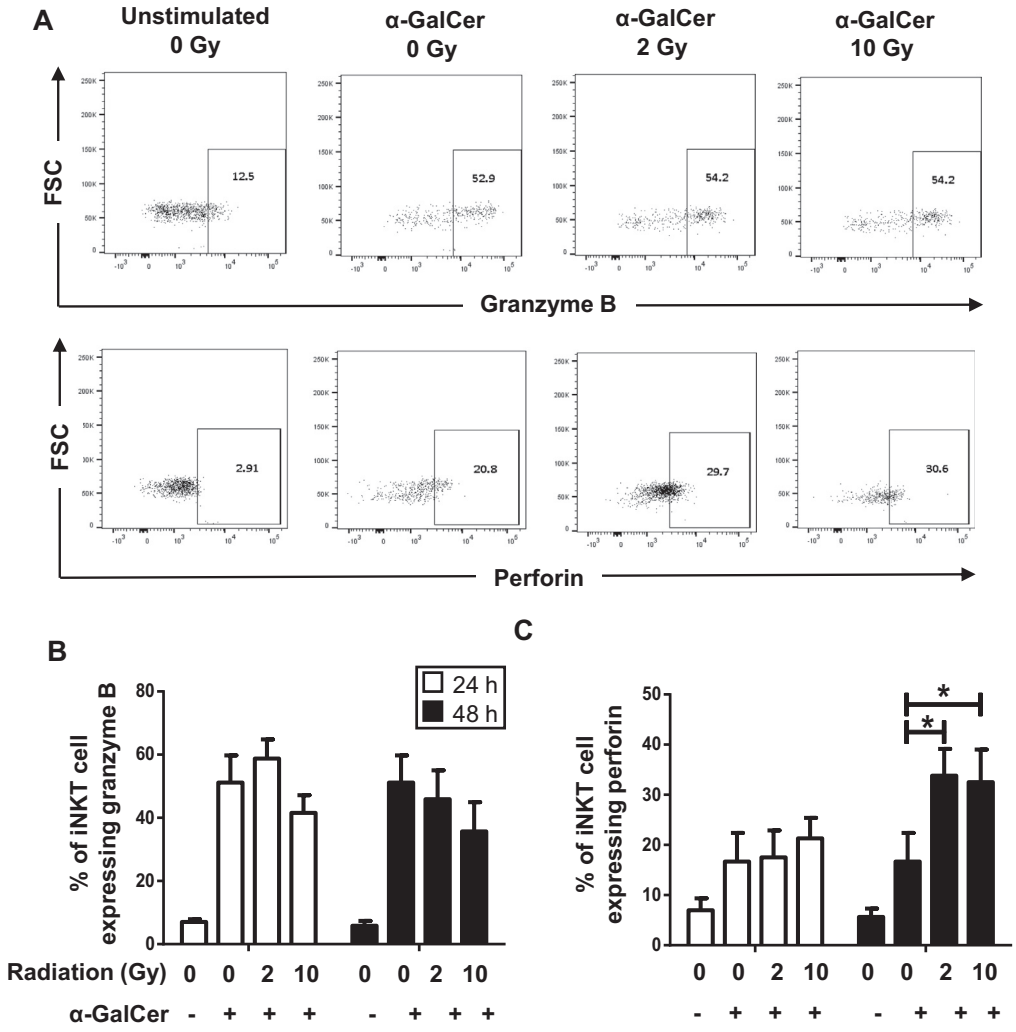

Fig. 5. Perforin expression by viable iNKT cells is induced by radiation. Lines of expanded iNKT cells from 6 healthy donors were cultured with 2 Gy, 10 Gy or mock radiation (0 Gy) for 24 h or 48 h. Equal numbers of viable iNKT cells were then co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer. Cells were then stained with a dead cell and mAb specific for granzyme B, perforin, CD3, and Vα24Jα18, and analysed by flow cytometry (A). (B and C) Graphs showing mean (±SEM) percentages of viable iNKT cells from 6 donors producing granzyme B (B) and perforin (C) 24 h or 48 h after irradiation. $^*p < 0.05$ using the paired t test compared to 0 Gy plus α-GalCer.

Radiosensitivity is predominantly determined by the ability of a cell to repair double stranded DNA breaks, therefore, our results suggest that significant proportions of iNKT cells can recover from damage caused by radiotherapy.

Sensitivity to radiation can also be determined by the cell cycle, with cells being most radiosensitive in the G2-M phase, less sensitive in the G1 phase, and least sensitive during the latter part of the S phase [28]. Cell cycle arrest was analysed by staining with PI at 6, 10 and 24 h after radiation. No significant differences were observed at 6 and 10 h, however, a decrease in the number of cells in G0/G1 was observed after 24 h. However, this might be caused by the reduction of viable cells rather than cells being arrested in another phase of the cycle.

iNKT cells direct anti-tumour immunity in part by the release of Th1 cytokines, which activate and modulate other immune cells such as cytotoxic T cells, NK cells and macrophages [5]. Low doses of radiation can enhance T cell responses, including proliferation and IFN-γ production [29]. On the contrary, IFN-γ production by iNKT cells has been reported to be lower in patients with advanced head and neck cancer after a cumulative dose of 50 Gy radiation compared to iNKT cells taken from the same patients prior to radiotherapy [22]. In the present study, IFN-γ but not IL-4 production by antigen-stimulated iNKT cells was not affected by single doses of radiation. Furthermore, the production of IFN-γ, but not IL-4, was significantly reduced when cells received cumulative doses of radiation. Thus, it is likely that radiotherapy will reduce the antitumour activity of iNKT cells. Since human iNKT cells can produce multiple cytokines upon activation [4], future studies should examine the effects of radiation on the production of other cytokines as well as to determine if the inhibition of cytokine production by radiation is short-lived or sustained.

iNKT cells can directly kill tumour cells via the release of perforin and granzyme B, which together lead to apoptosis of target cells [3]. We found no difference in cytolytic degranulation by iNKT cells or in direct killing of target cells by non-irradiated and irradiated iNKT cells. Interestingly, we observed a significant increase in the frequencies of iNKT cells expressing perforin, but not granzyme B, after treatment with both 2 Gy and 10 Gy irradiation, consistent with our findings that the ability of iNKT cells to kill target cells after radiation is not impaired. Perforin is a pore-forming protein that facilitates the entry of granzymes into the cytosol of the target cell, resulting in caspase-mediated cell apoptosis. Although granzyme B expression by iNKT cells was not increased by irradiation, it is possible that the expression of other granzymes may be affected. Further investigation is required to determine the causes

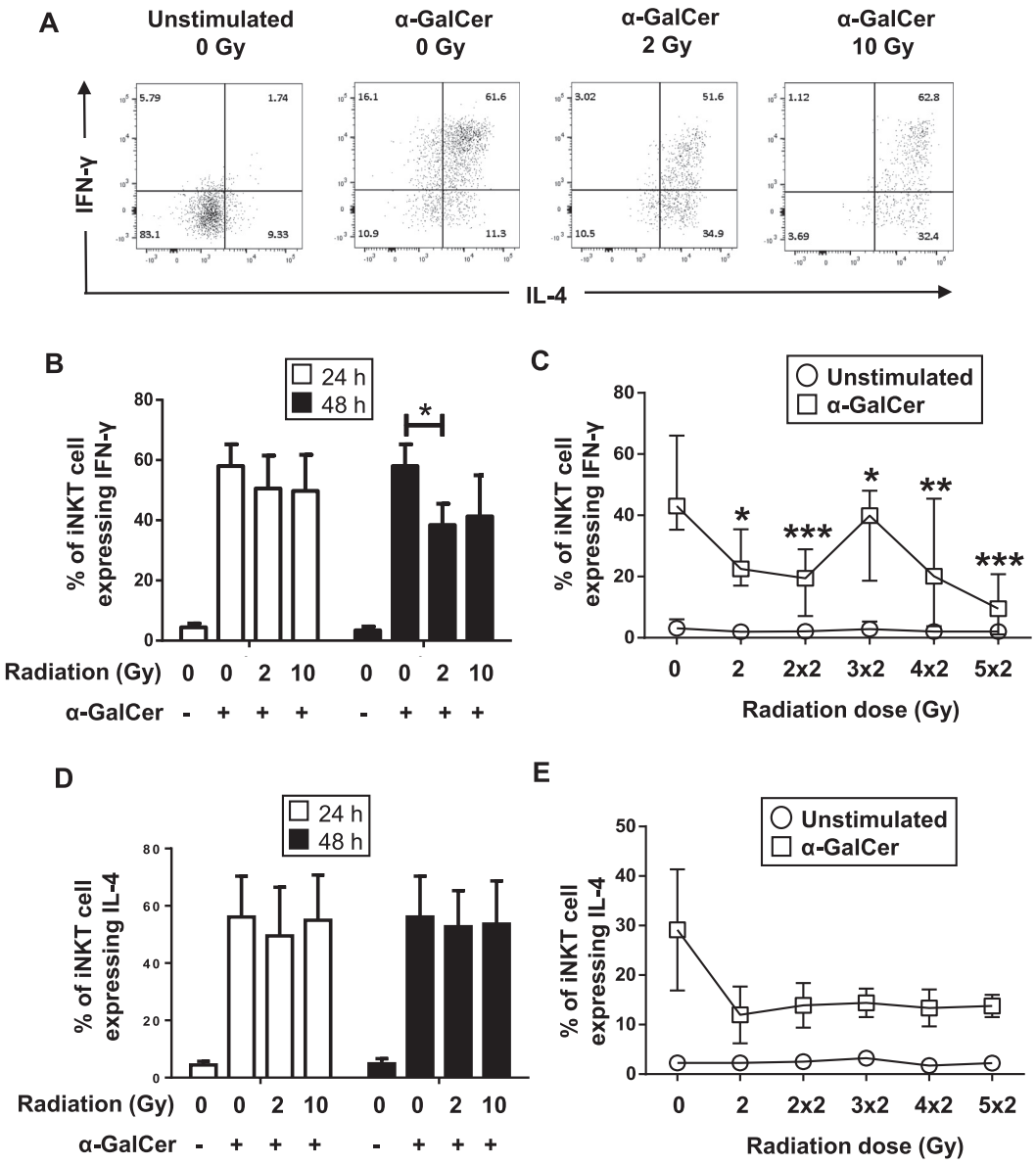

Fig. 6. Radiation inhibits IFN-γ, but not IL-4 production by viable iNKT cells. Lines of expanded iNKT cells were exposed to mock radiation (0 Gy), single doses of 2 Gy, 10 Gy or five cumulative doses of 2 Gy, and cultured for 24 h or 48 h. Equal numbers of viable iNKT cells were the co-cultured with CD1d transfected HeLa cells, previously pulsed with α-GalCer. Cells were then stained with dead cells stain and mAbs specific for cell surface CD3 and Vα24Jα18 and intracellular IFN-γ, IL-4, and analysed by flow cytometry (A). (B–E) Graphs show mean (±SEM) percentages of viable iNKT cells ($n = 6$) producing IFN-γ or IL-4 after single doses of 2 Gy or 10 Gy (B and D) or five cumulative doses of 2 Gy (C and E) after 24 h and 48 h radiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, using the paired t test compared to 0 Gy plus α-GalCer.

and consequences of the changes in the levels of perforin after radiation.

Several clinical trials combining radiotherapy and immunotherapy are currently ongoing [30,31]. The dose of radiotherapy, radiation volume, timing and the immune status of the patient are likely to contribute to the efficacy of this combination treatment and a number of studies have provided evidence that combined immunotherapy and radiotherapy resulted in enhanced antitumour immunity [32,33]. However, the results of the present study indicate that direct radiation induces cell death in some iNKT cells. This may partly be due to the generation of double strand DNA breaks, although iNKT cells are capable of rapidly repairing them. Remaining viable cells display normal cytolytic activity against CD1d-postitve target cells but show impaired IFN-γ, but not IL-4, production, which may compromise their antitumour activities. It should be noted that, while direct irradiation of *ex vivo* expanded iNKT cells can compromise their viability and function, these experiments may not fully recapitulate the functional consequences observed when mixtures of cells are irradiated *in situ* within a tumour. Future studies are required to determine the *in vivo* effects of radiotherapy on iNKT cells.

Conflict of interest statement

There are no financial or commercial conflicts of interests associated with the publication of this work.

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