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Combination of radiotherapy with the immunocytokine L19-IL2: Additive effect in a NK cell dependent tumour model

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ABSTRACT

Background and purpose: Recently, we have shown that radiotherapy (RT) combined with the immunocytokine L19-IL2 can induce long-lasting antitumour effects, dependent on ED-B expression and infiltration of cytotoxic T cells. On the other hand, in certain tumours, IL2 treatment can trigger a natural killer cell (NK) immune response. The aim of this study is to investigate the therapeutic effect of our combination therapy in the ED-B positive F9 teratocarcinoma model, lacking MHCI expression and known to be dependent on NK immune responses.

Material and methods: In syngeneic F9 tumour bearing 129/FvHsd mice tumour growth delay was evaluated after local tumour irradiation (10 Gy) combined with systemic administration of L19-IL2. Immunological responses were investigated using flow cytometry.

Results: Tumour growth delay of L19-IL2 can be further improved by a single dose of RT administered before immunotherapy, but not during immunotherapy. Furthermore, treatment of L19-IL2 favours a NK response and lacks cytotoxic T cell tumour infiltrating immune cells, which may be explained by the absence of MHCI expression.

Conclusion: An additive effect can be detected when the NK dependent F9 tumour model is treated with radiotherapy and L19-IL2 and therefore this combination could be useful in the absence of tumoural MHCI expression.

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Radiotherapy (RT) affects both the tumour and its microenvironment, leading to the release of tumour-associated antigens, damage molecular patterns (DAMPs) and the upregulation of immunomodulatory cell surface molecules, thereby starting an antitumour immune response [3,4,14]. Recently, we have shown that the RT induced antitumour effect can be enhanced by administration of the immunocytokine L19-IL2 [18] which has been shown to have excellent tumour targeting properties and improved therapeutic outcome over IL2 treatment alone [8]. The IL2 moiety of this immunocytokine is able to stimulate the proliferation of cytotoxic T cells and natural killer (NK) cells and can therefore be used to strengthen a broad RT-induced immune response [2]. The L19 moiety binds to extra domain-B (ED-B), a part of the fibronectin present in tumour neovasculature and overexpressed in many solid tumours [11]. RT combined with L19-IL2 has therefore the potential to create powerful synergistic tumour eliminating effects, as confirmed by our group.

Recently, we reported that the success of this combination treatment is highly dependent on the presence of cytotoxic T cells [18]. Cytotoxic T cells are able to recognize and eliminate tumour cells that express tumour-associated peptides on their MHCI. In the high ED-B expressing C51 model, these immune cells were responsible for 75% of tumour cure and in the intermediate ED-B expressing LLC model an additive effect could be detected. However, in certain tumours, or in later stages of progression, tumour cells can downregulate their MHCI expression to escape from T cell mediated killing. As a consequence, tumour cells become more prone to be killed by NK cells, since MHCI expression of tumour cells inhibits the cytotoxic killing of NK cells [10]. As not

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all tumours demonstrate MHCI expression, it is unclear if the combination of RT with L19-IL2 is also beneficial in these kinds of tumours. To explore this clinically relevant question, we made use of the ED-B positive F9 teratocarcinoma model as it is independent of T cells and known to induce NK cell responses when treated with IL2-based therapeutics [2,9]. In this study we investigated whether the combination of RT with L19-IL2 can provoke a NK cell mediated immune response in the F9 tumour model and whether this immune response leads to a therapeutic effect.

Material and methods

In vivo experiments

All experiments were performed in accordance with local institutional guidelines for animal welfare and were approved by the Animal Ethical Committee of the University of Maastricht. Approximately 8 week old 129/SvHsd immunocompetent mice (Harlan Laboratories) were subcutaneously injected with 3×10^6 F9 teratocarcinoma cells resuspended in matrigel (MatrigelTM, BD Biosciences). F9 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 10% foetal calf serum in 0.1% gelatin coated dishes. Upon an average tumour volume of 200 mm^3, therapy was started using three different schedules: (I) a single dose of 10 Gy radiotherapy (RT) was locally given to tumours on day 0, combined with systemic therapy (vehicle PBS/L19 13.3 μg/IL2 6.7 μg/L19-IL2 20 μg) on day 1, 3 and 5 (Fig. 2A), (II) animals were treated as in I, however sacrificed on day 4 of the treatment schedule and used for flow cytometry analysis of tumours, spleen and lymph nodes and (III) systemic therapy was given on day 0, 2 and 4 with local radiotherapy on day 2 (Fig. 3A). For treatment schedule I and III, tumour growth and treatment toxicity (based on body weight) were monitored on a daily basis until the tumour volume reached 4 times the volume at the start of therapy (T4xSV). To calculate T4xSV volumes were normalized to the start of treatment (day 0). To correctly compare control groups with radiotherapy groups in treatment schedules I and III, data were normalized to the start of treatment.

Flow cytometry

Flow cytometric analysis was performed on single cell suspensions from tumours, spleen and lymph nodes excised at day 4 as previously described [18]. Analysis was performed with RBC lysis buffer (eBioscience, San Diego, CA), FC-block CD16/CD32 (BD Biosciences) and a combination of antibodies CD3-FITC, CD4-APC-H7, CD8-PE-CY7, CD19-PE, CD45-V500, CD45-Percp, CD45-PE, CD45-FITC, CD45-APC, CD45-PE-CY7, CD3e-eFLUO450, CD4-FITC, CD8a-V500 (BD Biosciences) and NKp46-APC (Miltenyi Biotec B.V.).

Immunofluorescence

7 μm frozen sections were fixed in acetone (4 °C) and stained for ED-B, CD31 and CD8 according to previous published methods [18] using L19-SIP and KSF-SIP biotinylated (Philochem), rat anti-CD31 (BD Biosciences) and rabbit anti-CD8 (clone 53.62.7, Department of Pathology, MUMC, Maastricht, The Netherlands), respectively. For MHCI staining, sections were blocked with 10% normal goat serum, incubated overnight at 4 °C with primary antibody (rat anti-MHCI, 1:50 in antibody diluent, Abcam). Visualization was done using streptavidin Alexa 488 antibody, goat anti-rabbit IgG Alexa 488 (Life Technologies), goat anti-rat IgG Alexa 488 or goat anti-rat IgG Alexa 594 (BD Bioscience). Fluorescent imaging was performed as described before [18].

Statistics

Statistical analyses were performed using GraphPad Prism Software (v5.03, San Diego, CA). For all parameters medians with 10–90th percentiles are reported. Non-parametric Mann–Whitney test was used to determine statistical differences. *p*-values smaller than 0.05 were considered statistically significant.

Results

F9 tumours are ED-B positive and MHCI negative

As the immunostimulatory effect of L19-IL2 is dependent on ED-B expression, we verified ED-B expression together with the vascular marker CD31 in the F9 teratocarcinoma model. Representative sections are shown in Fig. 1A. F9 tumours showed high ED-B expression associated with CD31 expression. To investigate a possible mechanism underlying RT + L19-IL2 induced NK cell versus cytotoxic T cell responses, we investigated MHCI expression of F9 tumours. F9 tumour cells were negative for MHCI expression, only other structures (i.e. endothelial cells and mouse stromal cells) stained positive for MHCI. The previously investigated tumour model C51, which was dependent on cytotoxic T cells [18] was included as positive control for MHCI staining (Fig. 1B).

Combination therapy provides an additive effect

No toxicities were observed based on body weight measurements and animal welfare monitoring. Combination treatment was performed using two schedules to optimize the best timing for irradiation when compared with L19-IL2 (Figs. 2A and 3A). According to the animal welfare regulations, we reduced the amount of animals by re-using the tumour growth data of single treatment control groups in schedules I and III.

Radiotherapy before immunotherapy

Compared to vehicle PBS treated animals (T4xSV = 3.2 [1.7–4.7] days), L19 treatment (3.3 [2.5–4.5] days $P = 0.9$) and IL2 (4.4 [2.5–5.6] days $P = 0.2$) treatment alone did not lead to an enhanced growth delay.

Both RT (4.3 [3.0–8.9] days $P < 0.05$) and L19-IL2 (7.7 [2.7–13.3] days $P < 0.001$) monotherapies significantly enhanced tumour growth delay compared to vehicle. Upon combination of RT with L19-IL2, a highly significant antitumour effect (11.5 [7.9–21.4] days $P < 0.0001$) was observed compared to RT + L19 (4.6 [3.0–7.4] days $P < 0.0001$) and RT + IL2 (4.7 [3.0–6.9] $P < 0.0001$) (Fig. 2B).

Flow cytometric analysis demonstrated that the percentage of baseline NK cells of all lymphocytes (i.e. $CD45^+$ population) in the tumour was low in vehicle (0.9% [0.3–1.2]) and RT + vehicle (0.9% [0.5–1.9]) treated animals. This number was significantly increased upon treatment with L19-IL2 (5.4% [2.1–11.5] $P < 0.001$) and RT + L19-IL2 (2.7% [1.0–11.7] $P < 0.01$). Furthermore, basal levels of CD8 + T cells inside F9 tumours were low and not increased upon treatment (Fig. 2D).

Radiotherapy during immunotherapy schedule

Local irradiation of F9 tumours with 10 Gy (day 2) significantly increased time to reach T4xSV in combination with IL2 (7.1 [5.6–8.9] days) compared to IL2 treatment alone (4.0 [2.7–6.4] days $P < 0.001$). However, RT did not show any additional effects on L19-IL2 treatment (10.1 [7.6–17.7] days) compared to the immunocytokine treatment alone (8.9 [3.2–14.9] days $P = 0.12$). Combining RT with L19-IL2 did significantly delay tumour growth compared to the IL2 combination treatment group ($P < 0.001$).

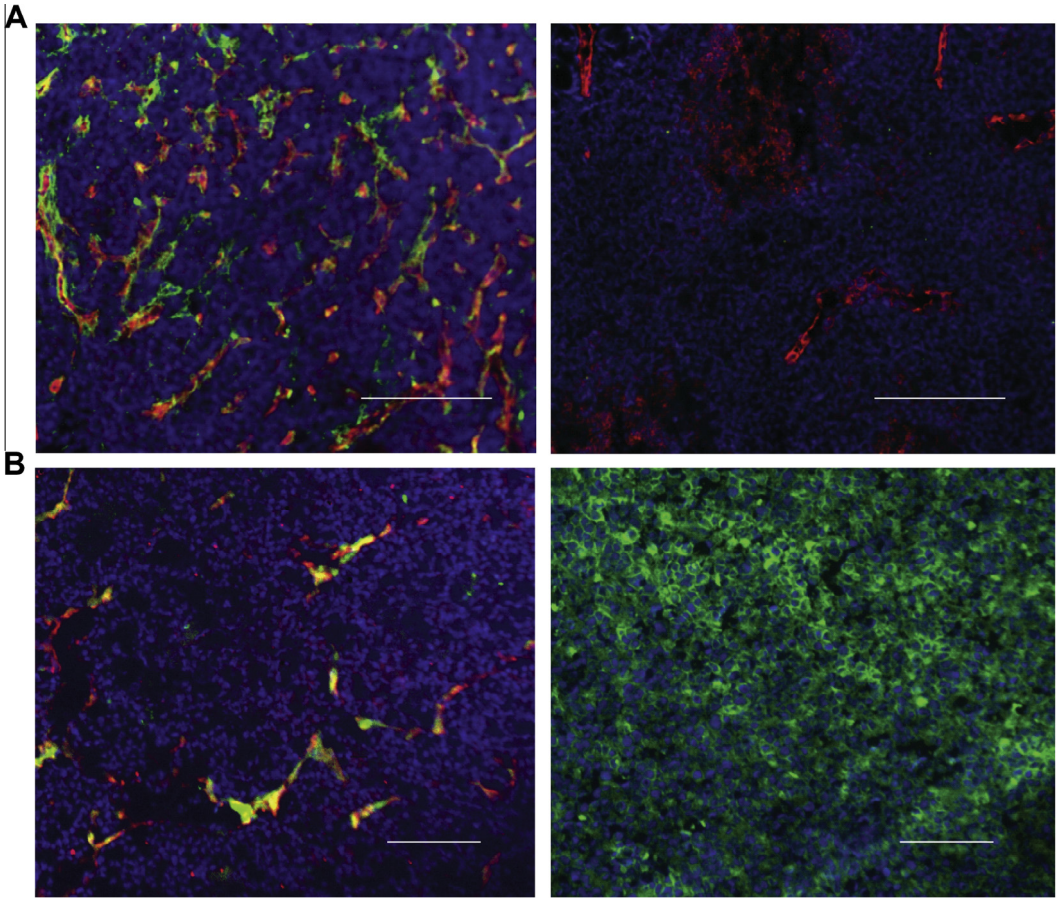

Fig. 1. (A) Representative images of ED-B expression in F9 teratocarcinoma. ED-B expression (left) or control (right) in green, vessels (red) and cell nuclei (blue). (B) MHCI expression (green) and vessels (red) in the F9 teratocarcinoma (left) and MHCI expression in the C51 colon carcinoma (green), cell nuclei in blue. Scale bar represents 100 μm.

Discussion

Radiotherapy causes immunogenic death of tumour cells, promotes antigen presentation and alters the tumour microenvironment [16]. Recently, we and others have shown that the selective delivery of IL2 to the tumour is a promising approach to enhance the therapeutic potential of RT [15,18]. In this study, we investigated the therapeutic potential and underlying immune response of the RT and L19-IL2 combination treatment in the ED-B positive but MHCI negative F9 teratocarinoma tumour model.

Combination of RT with L19-IL2 significantly retarded tumour growth compared to mice treated with RT plus IL2, regardless of RT timing. RT given before L19-IL2 treatment showed a therapeutic gain compared to the RT control groups and compared to single treatment arms. However, RT combined with L19-IL2 did not retard tumour growth compared with RT plus IL2 treatment group when the single RT dose was delivered during the immunotherapy treatment. Therefore the former effective treatment schedule was used to investigate underlying immunological mechanisms in more detail. Previously, we described that long-lasting antitumour effects of RT combined with L19-IL2 are highly dependent on expression of ED-B and the presence of cytotoxic T cells inside tumours [18]. In this study, we showed that in the ED-B positive F9 teratocarcinoma model the number of infiltrating NK cells, but not the number of cytotoxic T cells, are increased upon L19-IL2 treatment compared to vehicle or RT treatment arms. These results are in agreement with previous publications, where it was shown that, dependent on the tumour model, the efficacy of targeted IL2 treatment by F16-IL2, F8-IL2, L19-IL2 or NHS-IL2 can be based on NK cells [9,13], T-cells [1,5,15,17], or a combination of both [2,6]. A few possible explanations are available to clarify what favours an NK or T cell response after treatment with RT + L19-IL2. The low expression of MHCI may be an important one, since down-regulation or inactivation of tumour MHCI (which is involved in antigen processing and presentation) will prevent cytotoxic T cells from recognizing and eliminating tumours cells [16]. On the other hand, downregulation of MHC expression on their surface causes tumour cells to be more vulnerable for NK cell killing [16], which is in agreement with our recent findings. However, in clinical setting, tumours tend to have a heterogeneous expression of MHCI [7] and therefore a mixture of cytotoxic T cells and NK cells may become activated when patients are treated with stereotactic body radiation therapy (SBRT) and L19-IL2 in our clinical study (NCT02086721).

Furthermore, treatment with L19-IL2 alone delays tumour growth significantly, which is in line with previous studies [2], and was further enhanced by a single dose of 10 Gy applied prior to L19-IL2 treatment schedule (Fig. 2A) but not by a single dose of RT given during the immunotherapy treatment schedule (Fig. 3A). A possible explanation is that the immune response started after RT can be enhanced by L19-IL2 and therefore RT needs to be administrated before immunotherapy. Furthermore, RT might be able to kill infiltrating immune cells and therefore RT administered during the immunotherapy schedule may not have additional benefit.

However, for the systemic IL2 treatment, we detected a significant antitumour immune response when administered before RT (Fig. 3A). This effect was not detected in the other treatment

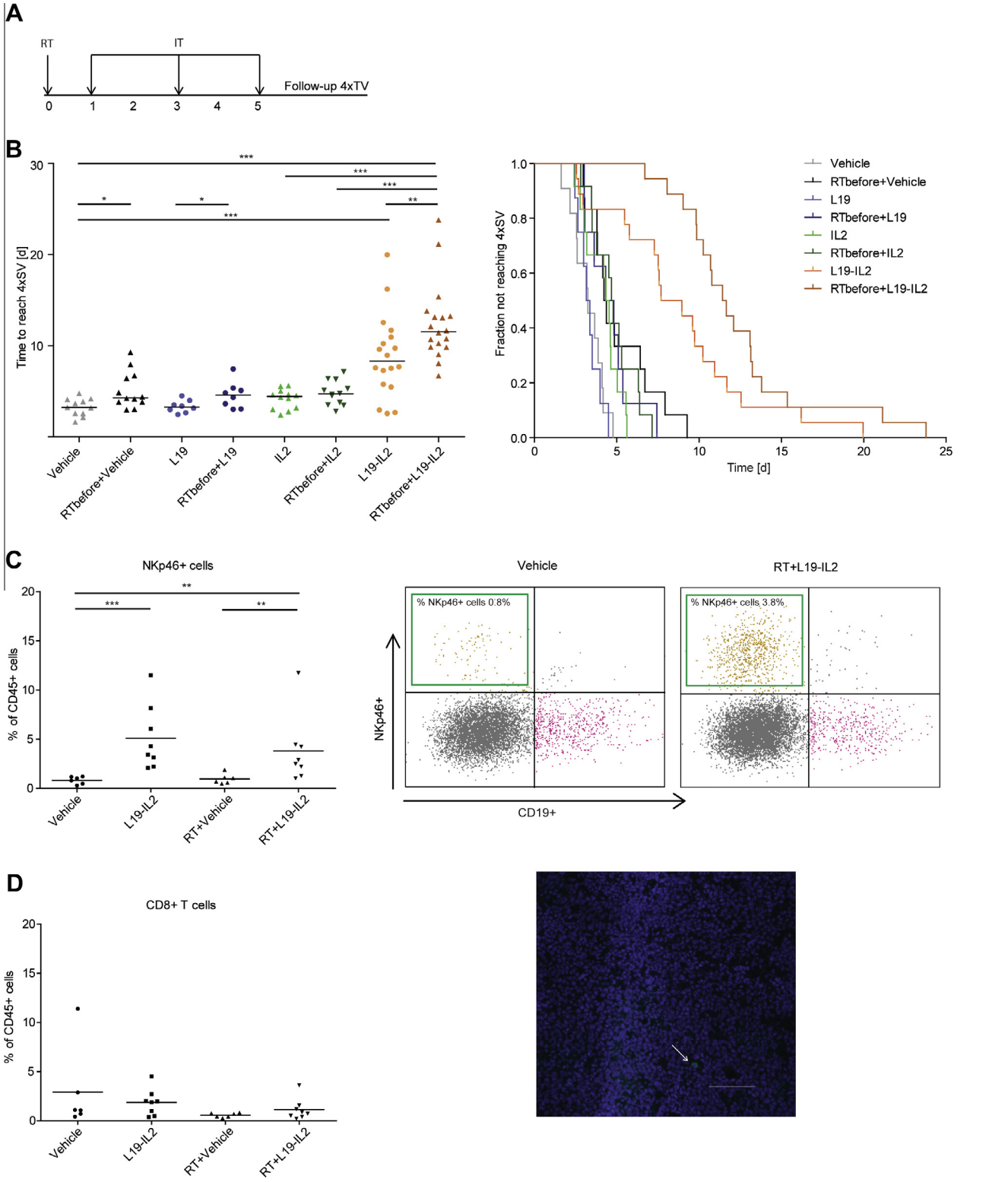

Fig. 2. (A) Treatment schedule. (B) Time to reach 4 times start volume (T4xSV) for the different treatment groups (left) and Kaplan–Meier curves showing fraction of tumours not reaching T4xSV (right). (C and D) Results of flow cytometry analysis, showing the percentage of NKp46$^+$ cells (C) and CD8 + cells (D, right) shows of all CD45$^+$ cells present in the tumour and representative image of a fluorescent CD8 staining. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

schedule, where IL2 was administered at day 1, 3 and 5 after RT (Fig. 2A). Similar to our findings, Milas et al. [12] showed that stimulation of the innate and adaptive immune system (by CpG oligodeoxynucleotides) prior to a single dose of local RT, is able to improve the outcome for RT treatment in mouse fibrosarcoma. Interestingly, the 'radiosensitizing effect' of systemic IL2 was not detected when IL2 was administered in the targeted form (L19-IL2) before RT. Since L19-IL2 binds to ED-B and therefore stimulates the immune response only locally and since intratumoural basal levels of NK cells are very low, systemic IL2 treatment

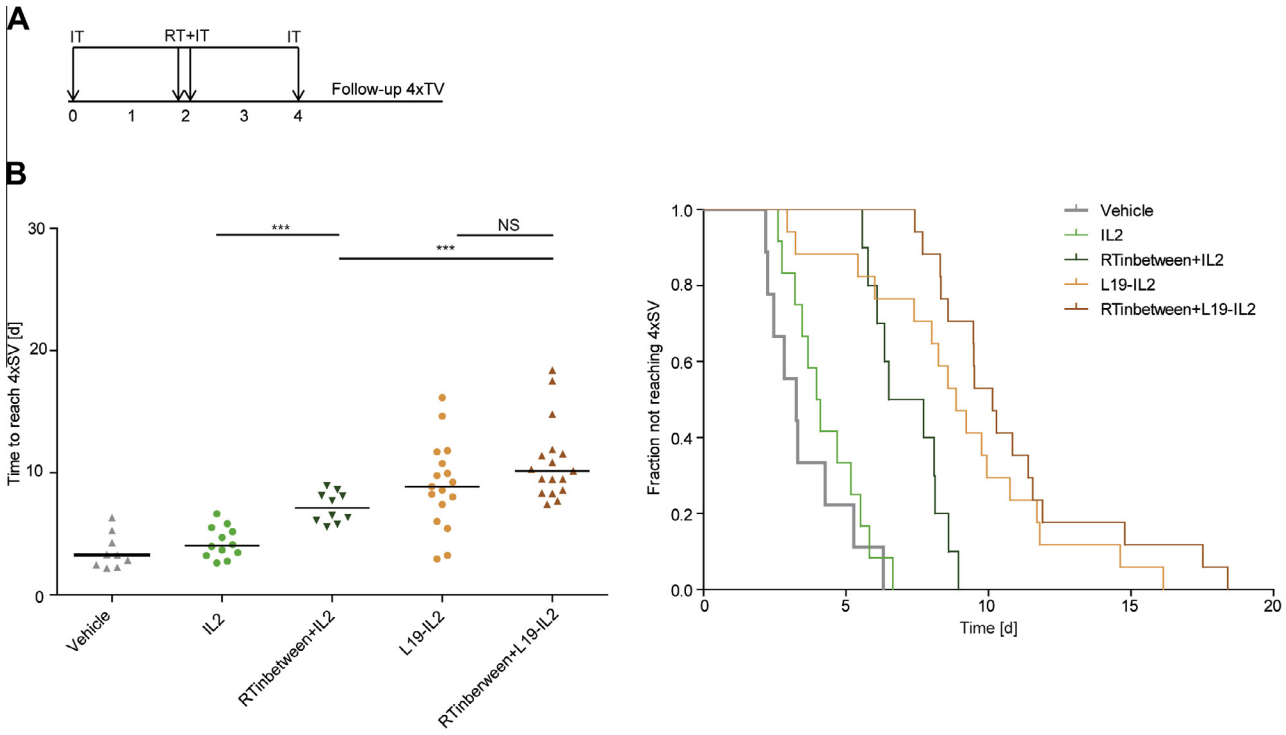

Fig. 3. (A) Treatment schedule. (B) Scatter-plot showing T4xSV (left) and Kaplan–Meier curves showing the fraction of tumours not reaching T4xSV (right). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

might have a higher impact compared to L19-IL2 in sensitizing tumours to irradiation. Taken together, administration of IL2 before but not after radiotherapy is able to delay tumour growth in this model and when L19-IL2 is administered before radiotherapy, this 'radiosensitizing effect' is not present.

In conclusion, combination treatment of RT with L19-IL2 is an efficient treatment combination in the MHCI negative but ED-B positive F9 tumour model and effects of L19-IL2 can be further improved by a single dose of RT before immunotherapy treatment. A single RT dose administered during immunotherapy treatment seems to be less efficient. Furthermore, the data suggest that treatment of L19-IL2 favours NK and lacks cytotoxic T cell tumour infiltrating immune cells, which may be explained by the absence of MHCI expression by these F9 tumours.

Conflicts of interest

D. Neri is a co-founder and shareholder of Philogen, the company which owns and develops L19-IL2.

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