Radiotherapy combined with the immunocytokine (L19-IL2) provides long-lasting anti-tumor effects

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Cancer cells have a poor immunogenicity; they are not recognized by the immune system and therefore have the opportunity to survive and proliferate. Radiotherapy causes immunogenic tumor cell death, thereby releasing tumor associated antigens which can be detected by the immune system, causing an anti-tumor immune response. Active immunotherapy can be used to further enhance the radiotherapy induced anti-tumor immune response. The combination of local radiotherapy to the primary tumor and systemic immunotherapy may therefore activate and stimulate a systemic anti-tumor response which provides the potential to treat patients with metastatic disease with a curative intent.
Abstract

Purpose: Radiotherapy (RT) modifies the tumor microenvironment and causes the release of tumor antigens, which can enhance the effect of immunotherapy (IT). L19 targets the extra domain B (ED-B) of fibronectin, a marker for tumor neo-angiogenesis, and can be used as immunocytokine when coupled to interleukin-2 (IL2). We hypothesize that RT in combination with L19-IL2 provides an enhanced antitumor effect, which is dependent on ED-B expression.

Experimental Design: Mice were injected with syngeneic C51 colon carcinoma, Lewis lung carcinoma (LLC), or 4T1 mammary carcinoma cells. Tumor growth delay, underlying immunological parameters and treatment toxicity were evaluated after single-dose local tumor irradiation and systemic administration of L19-IL2 or equimolar controls.

Results: ED-B expression was high, intermediate and low for C51, LLC and 4T1, respectively. The combination therapy showed (i) a long-lasting synergistic effect for the C51 model with 75% of tumors being cured, (ii) an additive effect for the LLC model, and (iii) no effect for the 4T1 model. The combination treatment resulted in a significantly increased cytotoxic (CD8+) T-cell population for both C51 and LLC. Depletion of CD8\(^+\) T-cells abolished the benefit of the combination therapy.

Conclusion: These data provide the first evidence for an increased therapeutic potential by combining RT with L19-IL2 in ED-B positive tumors. This new opportunity in cancer treatment will be investigated in a Phase I clinical study for patients with an oligometastatic solid tumor (NCT02086721).
Introduction

Radiotherapy (RT) causes cell cycle arrest or programmed cell death in rapidly proliferating cancer cells through the induction of DNA damage. Irradiated tumors stimulate the immune system by releasing tumor antigens, damage associated molecular patterns (DAMPs), and through upregulation of immunomodulatory cell surface and secretory molecules(1-4). This promotes the uptake of dying cells by antigen presenting cells, and provides cross-presentation of the tumor-derived antigens to T cells, thereby triggering a cytotoxic T-lymphocyte response, which might cause immunogenic cell death (ICD)(1, 5, 6). In some cases, tumor growth inhibition outside the field of radiation is observed, termed abscopal effect, which suggests the presence of a systemic radiation-induced anti-tumor immune response(7-10). However, in general, it is unlikely that radiotherapy alone provides a sufficient anti-tumor immune response. Therefore, the addition of active immunotherapy (IT) may increase the therapeutic potential(11-13).

Active immunotherapy is used to stimulate the immune system acting against tumor cells. Cytotoxic T-lymphocytes and Natural Killer (NK) cells play an important complementary role in the anti-tumor immune response since they release specialized lytic granules, which upon interaction with the tumor cell create pores in the lipid bilayer of the target cell resulting in cell death(14, 15). Interleukin-2 (IL2) is a cytokine with an essential role in the activation phase of the immune response; it stimulates the proliferation of cytotoxic T cells, NK cells and regulatory T cells, providing a balance between a pro- and anti-inflammatory immune response(16-18). Systemic administration of IL2 was introduced as immunotherapy for patients with metastatic melanoma and renal cell carcinoma, which resulted in a higher tumor response and survival(19). However, to reach an effective intra-tumoral dose of IL2 by systemic administration, high doses ought to be administered, which often leads to toxicity (e.g. capillary leakage syndrome, severe flu-like symptoms, and coma)(20). Currently intra-tumoral injections of IL2 are employed to reach a higher local concentration of IL2(21, 22), which shows promising results in combination with RT in a preclinical setting(23). However, these intra-tumoral injections are limited to accessible lesions.

An interesting alternative is the selective delivery of IL2 to the tumor by use of fusion proteins(16, 24). During tumor progression, synthesis of extracellular matrix components occurs, with in particular a modulation of vascular cell behavior and angiogenesis(16). Fibronectin of the tumor neovasculature expresses extradomain-B (ED-B), which is preserved in mice, humans and other mammals. ED-B expression can be used for targeted therapies because it is over-expressed in various solid tumors (e.g. melanoma, RCC, breast, colorectal, and non-small cell lung cancer), but absent in plasma and normal tissue fibronectin (except for regenerating tissues)(25-30). The small-immuno-protein (SIP) L19 was developed to specifically target the ED-B domain of fibronectin. In previous studies L19 was used for imaging and targeted (radio-)immunotherapy, proving that L19 actually targets the tumor(31-33). Moreover, in phase I clinical studies in patients with metastatic melanoma or RCC, administration of the immunocytokine L19-IL2 alone, or combined with chemotherapy (dacarbazine), was safe and showed clinical activity according to RECIST criteria or progression-free-survival(34, 35). Dacarbazine, however, does not have the
potential to induce an anti-tumor immune response, stimulate the exposure of DAMPs or activate ICD(36), which are all favorable characteristics induced by RT. Therefore, based on the known immunogenic effects of RT and the targeted immune stimulating potential of L19-IL2, we hypothesize that the combination of radiotherapy with L19-IL2 will cause an enhanced anti-tumor effect, which is dependent on the expression of ED-B.
Materials and Methods

Tumor cell lines.

Exponentially growing C51 colon carcinoma (kindly provided by Philogen S.p.A., Siena, Italy), Lewis lung carcinoma (LLC; kindly provided by G. Molema, UMCG, The Netherlands), and 4T1 mammary carcinoma (ATCC CRL-2539) cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Lonza) supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO₂ chamber at 37 °C. All cell lines were directly or indirectly purchased from a cell bank that performs cell line characterizations (short tandem repeat profiling) and were used within 6 months after resuscitation. In addition, cells were tested for mouse antibody production (MAP) and mycoplasma contamination.

Chemicals/reagents/antibodies.

The L19-IL2 immunocytokine and L19 (Philogen S.p.A.) were diluted with sterile phosphate buffered saline (PBS, Lonza) to concentrations of respectively 200µg/ml and 133µg/ml. Interleukin-2 (Proleukin, Novartis, Basel, Switzerland) was dissolved as described by the manufacturers’ guidelines and diluted with PBS to a concentration of 67µg/ml.

For the depletion experiments, the anti-CD8 antibody (Clone JTS169) and the isotype control (anti-Phyt IgG AFRC MAC 51), kindly provided by Bioceros Bv, Utrecht, The Netherlands, were diluted with PBS to a concentration of 2mg/ml.

Flow-cytometric analysis was performed on cells exposed to RBC lysis buffer (eBioscience, San Diego, CA), FC-block CD16/CD32 (BD Biosciences, San Jose, CA) and a combination of the 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., Leiden, The Netherlands).

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. To induce tumors, approximately 8-week-old immune competent mice were subcutaneously injected with syngeneic C51 (Balb/c; 1.5x10⁶), LLC (C57bl/6; 0.5x10⁶) or 4T1 (Balb/c; 1x10⁶) tumor cells, resuspended in Basement Membrane Matrix (MatrigelTM, BD Biosciences). Upon an average tumor volume of 200 mm³, tumors were irradiated with a single dose (10 Gy for all models, additional groups with 2 and 5 Gy for C51) on day 0, followed by systemic therapy (vehicle PBS / L19 13.3µg / IL2 6.7µg / L19-IL2 20µg) on day 1, 3 and 5. Tumor growth and treatment toxicity (based on body-weight) were monitored until reaching 4 times the volume at irradiation time (T4xSV). Flow cytometry was performed on tumors, spleen and lymph nodes excised at day 4 of the treatment schedule. Detailed treatment schedules are shown in supplementary figure 1.
To evaluate the causal relationship between the presence of cytotoxic T cells and tumor growth delay, an experimental set-up was designed to deplete cytotoxic-T cells in the Balb/c mice bearing C51 tumors. Similar to previous experiments, local irradiation was performed on day 0 (10 Gy) and systemic therapy (vehicle or L19-IL2) was administrated (day 1, 2, 5). In addition, CD8\(^+\) cells were depleted by intraperitoneal injection of 0.2 mg (100\(\mu\)L) anti-CD8 antibody or the negative control anti-Phyt IgG. The timing for the anti-CD8 injections was determined by blood withdrawal, via puncture of the saphenous vein (i) before, (ii) after tumor cell injection and (iii) 2, 3 or 5 days after injection with the blocking antibodies. The percentage CD8\(^+\) cells in the blood was determined as described below. At the end of the experiment the tumors were harvested for immunohistochemical analysis for CD8 positivity.

**Flow cytometry.**

The number of immune cell subpopulations present in tumor, spleen and lymph nodes during treatment was analyzed using FACSCanto II flow cytometry (FACS, BD Biosciences). Single cell suspensions of the tissues were obtained using the gentleMACS dissociator and the tumor dissociation kit (Miltenyi Biotec B.V.) according to manufacturer’s guidelines. Of the acquired single cell suspension, 1.0\(\times\)10\(^6\) cells were suspended in FACS buffer (PBS + 1% FCS) for analysis. Cells were incubated with FC-block to avoid non-specific binding, and staining was performed using the antibodies CD3-FITC, CD4-APC-H7, CD8-PE-CY7, CD19-PE, NKp46-APC and CD45-V500. The total CD45\(^+\) immune cells were selected from the viable population of cells (filtered for debris and doublets) for further sub-classification according to the strategy described in supplementary figure 2.

To determine the efficacy of anti-CD8 blocking antibody on the presence of specific immune subpopulations, collected blood was incubated with RBC lysis buffer and FC-block. Next cells were incubated with CD45-Percp, CD3e-eFLUO450, CD4-FITC, CD8a-V500, NKp46-APC and CD19-PE, and FACS and data analysis was performed (Supplementary Figure 3).

**Immunofluorescence.**

To investigate baseline ED-B expression, 7mm cryostat sections of C51, LLC and 4T1 tumors were fixed in acetone (4°C) and stained according to previous published methods(37). In brief, sections were incubated with the purified antibodies L19-SIP or KSF-SIP (2ug/ml; Philochem, Otelfingen, Switzerland), with rabbit anti-human-IgE (Dako, Glostrup, Denmark) and subsequently detected using goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies, Bleiswijk, The Netherlands). Blood vessels and cell nuclei were detected with rat anti-mouse CD31 (BD Biosciences) followed by donkey anti-rat Alexa 594 (Life Technologies) and DAPI (Life Technologies), respectively.
To quantify the ED-B expression, 3-12 photomicrographs (805.5 µ x 805.5 µ), depending on tumor size, from viable tumor regions in the largest tumor cross-section were acquired using an Olympus BX51WI (Center Valley, PA) fluorescence microscope equipped with a Hamamatsu EM-CCD C9100 digital camera, a motorized stage (Ludl Mac 2000) and a 10x objective. Micromanager 1.4 software was used for automated image acquisition (38). All image recordings were performed with the same settings and analyzed by an investigator blinded to the subject coding. Images were processed using ImageJ software v.1.49b (NIH, Bethesda, MD). The mean fluorescent intensity after correction for cutting and staining artefacts per image was averaged over all images per section to obtain ED-B intensity per tumor.

For the detection of CD8$^+$ T cells residing in tumors, sections were first incubated with anti-CD8 (clone 53.62.7, Department of Pathology, MUMC, Maastricht, The Netherlands) and visualized with goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies). DAPI was used as nuclear counterstain.

**Statistics.**

Statistical analyses were performed using GraphPad Prism Software (v5.03, San Diego, CA). For all parameters mean ± standard deviation (SD) are reported. The non-parametric Mann-Whitney test was used to determine the statistical differences between the different treatment groups. The log-rank (Mantel-Cox) test was used to compare the survival curves. We used a 2-way ANOVA to test the interaction (synergism) between radiotherapy and L19-IL2. A p-value smaller than 0.05 was considered statistically significant.
Results

Representative sections of the ED-B expression in the C51, LLC and 4T1 tumors and their respective fluorescent intensity, corrected for the intensity of the negative controls are shown in figure 1. We observed a high, intermediate and low ED-B expression for the C51 (451±99), LLC (326±70) and 4T1 model (157±143), which were significantly different from each other (all P<0.01). Based on body weight measurements and animal welfare monitoring, no toxicity was observed in any of the treatment combinations.

Combination therapy results in complete remission of 75% in the C51 model.

We evaluated the time to reach 4 times start volume (T4xSV) for all treatment groups in the C51 model with high ED-B expression. Experiments were started at an average tumor volume of 254±126 mm$^3$. L19, IL2 or L19-IL2 monotherapy increased the T4xSV to 6.1±0.9 (P<0.01), 6.3±1.2 (P<0.01) and 6.0±1.6 days (P<0.05), respectively, as compared to the vehicle (4.8±0.8 days) treated C51 tumor-bearing animals, but no significant differences between these three treatment groups were observed. Single-dose radiotherapy (10Gy) significantly enhanced tumor growth delay when preceding vehicle (P<0.001), L19 (P<0.001) or IL2 (P<0.001) treatment. Upon combination with L19-IL2 therapy, a highly significant (P<0.0001) synergistic anti-tumor effect was observed with 9/12 cures (Figure 2A, 2B). Reduction of the single-dose radiations to 5 or 2 Gy showed a dose-dependent treatment effect. For tumors treated with the combination of ionizing radiation and L19-IL2, a cure rate of 6/12 and 1/12 was observed for irradiation with 5 Gy (P<0.001) and 2 Gy (P=0.002), respectively, as compared to the combination with vehicle treatment (Figure 2A, 2B).

FACS analysis was performed to evaluate the underlying immunological parameters. The percentage of baseline cytotoxic T cells in the tumor was 22.2±9.2% of CD45$^+$ cells in vehicle treated animals. Radiotherapy slightly enhanced the cytotoxic T cell subpopulation (28.1±5.7%), without being significant (P=0.24). The percentage of cytotoxic T cells during combination treatment was significantly higher than in vehicle (38.6±10.8%, P<0.01) or L19-IL2 only (22.0±8.8%, P=0.01) treated animals. There was no significant difference in the CD45$^+$ population in the tumor between different treatment groups. In addition, no significant differences were observed in NKp46$^+$ NK cells, CD4$^+$ T cells or CD19$^+$ B-cells between the treatment groups (Figure 2C and Supplementary Table 1). Flow cytometry of the lymph node and spleen tissue showed no significant difference for any of the analyzed immune subpopulations (CD8$^+$, CD4$^+$, CD19$^+$ and NK; Supplementary Table 1).

Combination therapy results in increased growth delay in LLC model.

Next, we investigated the possible therapeutic effect of combined RT with L19-IL2 in the LLC model with intermediate ED-B expression. Experiments were started at an average tumor volume of 254±148 mm$^3$. There was no significant difference in tumor growth delay for L19 (4.3±1.2 days) or IL2 (5.1±1.1 days) compared to vehicle...
(4.4±1.1 days) treated animals. L19-IL2 monotherapy resulted in a significant tumor growth delay (P=0.02), increasing T4xSV to 6.0±1.4 days. Single-dose radiotherapy (10 Gy) only showed an increased growth delay (6.9±2.0 days; P=0.02), however, the combination of RT with L19-IL2 resulted in the largest growth delay (10.5±2.6). This was significantly longer than after RT or L19-IL2 only (P<0.01 and P<0.001, respectively; Figure 3A, 3B). There was no significant interaction between RT and L19-IL2 (2-way ANOVA; P=0.15), the effect of the combination therapy in LLC was additive.

The observed baseline percentage of cytotoxic CD8+ T cells in this LLC model was significantly lower than in the C51 model (P=0.002). The number of cytotoxic T cells as a percentage of CD45+ cells increased significantly upon L19-IL2 treatment: from 2.7±1.0% (vehicle) to 7.4±4.1% (L19-IL2, P=0.04), and from 1.9±0.8% (RT) to 4.4±2.6% (RT+L19-IL2, P=0.04; figure 3C). Radiation caused a significant decrease in the percentage of CD19+ and CD4+ cells in the tumor compared to vehicle treatment (Supplementary Table 1). No differences were observed in the percentage of NKp46+ cells in the tumor (Figure 3C). Analysis of the lymph nodes and spleen tissue showed no significant differences (Supplementary Table 1).

In the low/negative ED-B expressing 4T1 model, the addition of L19-IL2 to radiotherapy has no effect.

Next, we investigated if L19-IL2 had any off-target effects using the low ED-B expressing 4T1 model. Experiments were started at an average tumor volume of 152±48 mm3. For the 4T1 model, no statistically significant differences were observed between vehicle, IL2 and L19-IL2 treated animals, with an average T4xSV of 7.9±2.8, 8.7±1.6 and 9.2±2.4 days, respectively. Single-dose radiotherapy (10 Gy) increased growth delay significantly for all treatment groups: RT + vehicle (13.3±3.7 days, P=0.01), RT + IL2 (17.0±5.4 days, P<0.01) or RT + L19-IL2 (17.7±4.2 days, P<0.001), however, no statistically significant differences (P=0.47 and P=0.59) were observed between these irradiated groups (Figure 3A, 3B). There is no significant interaction between RT and L19-IL2 (2-way ANOVA; P=0.20).

Radiotherapy caused a significant increase in the presence of CD8+ T cells in the 4T1 tumor. The percentage of CD8+ T cells increased from 6.9±1.8 (vehicle) to 18.0±12.6 (RT + vehicle, P=0.04) and from 6.4±2.9 (L19-IL2) to 14.2±6.4 (RT + L19-IL2, P<0.01). Albeit, no significant differences (P=1.0) were observed for L19-IL2 treated animals compared to vehicle. No significant differences were observed for the percentage of NK cells in the tumor for any of the treatment groups (Figure 3D). The percentage of CD19+ cells were significantly higher for treatment with L19-IL2 alone (4.0±1.6) compared to vehicle (2.5±0.6, P=0.03), RT + vehicle (2.0±0.7, P=0.02) and RT + L19-IL2 (1.6±1.2, P=0.04; Supplementary Table 1). Analysis of the spleen and lymph nodes showed no significant difference for any of the analyzed immune cells.
Depletion of cytotoxic T cells prohibits complete remission.

Based on our observation that radiotherapy + L19-IL2 immunotherapy significantly increases the CD8⁺ T cell subpopulation, we assessed the causal relationship between the therapeutic effect and CD8⁺ T cells by depleting the CD8⁺ T cells in the C51 tumor model. Tumor cell injection did not result in changed immune subpopulations. Treatment with the CD8⁺ T cell depleting (JTS169) antibody abolished CD8⁺ T cells in the blood 2 days after injection (0.06±0.06%; >99% depletion). Cytotoxic CD8⁺ T cells were detectable again at day 3 (1.6±0.7%) after depletion and levels increased over time to 5.7±3.0% at day 5 (Supplementary Figure 4A). On the basis of these data we opted for 3-daily administrations of CD8⁺ depleting antibody in order to effect sustained ablation of CD8⁺ T cells (Figure 4A).

After depletion (3 days p.i.), the CD8⁺ T cell population was significantly reduced in blood (P<0.0001), whereas, the control groups receiving either isotype IgG (15.7±0.9%) or vehicle (18.1±3.1%) showed similar numbers of CD8⁺ cells as baseline (Figure 4B). CD8⁺ T cells were also depleted in the tumor (2.2±2.6%, vs 38.6±10.8% at baseline; Figure 4C), spleen and lymph nodes (Supplementary Figure 4B). Upon depletion of cytotoxic T cells, the combination of radiotherapy with L19-IL2 lost its therapeutic effect (T4xSV=11.25±3.0d) and was not superior (P=0.31) to radiotherapy only (T4xSV=10.0±3.0d). However, in agreement with previous results, the animals in the control groups (without CD8⁺ T cell depletion), still showed sustained anti-tumor effects (IgG: cure 5/8, vehicle: cure 3/8) after 10 Gy irradiation and L19-IL2 (Figure 4D).
Discussion

Radiation-induced cell death is an immunogenic process which can be used to initiate tumor-specific immune responses (39). The selective delivery of IL2 to tumor vascular components is promising in cancer immunotherapy (16, 40, 41) and may be used to enhance the therapeutic potential of RT. We hypothesized that the combination of RT with the targeted immunocytokine L19-IL2 may cause an enhanced anti-tumor effect dependent on the expression of ED-B. In this study, we assessed the therapeutic potential and underlying mechanisms of the combination therapy in three different tumor models with varying ED-B expression.

Based on growth delay experiments, the combination therapy showed a therapeutic gain compared with the single treatment arms, with an additive effect for the LLC model and a long-lasting highly synergistic effect for the C51 model for which a cure rate of 75% was observed. As expected no effect was observed for the 4T1 model, which has a low ED-B expression. The results show that ED-B expression is essential for the efficacy of combined irradiation and L19-IL2 administration. The C51 model showed the highest ED-B expression and the most promising results for the combination therapy suggesting that high ED-B expression may assure better L19-IL2 tumor targeting. Like our C51 model, ED-B is overexpressed in many solid tumors (25-27, 29, 41), which makes this combination therapy (RT + L19-IL2) potentially interesting for the majority of cancer types.

The highly synergistic effect observed in the C51 model upon radiotherapy and tumor-targeted L19-IL2 treatment is in agreement with previous results described by Yasuda et al. (23). They observed a complete eradication of a colon carcinoma cell line (Colon26) in Balb/c mice after the combination of radiotherapy with intra-tumoral injections with IL2. For the models presented in this study, no additional benefit was observed for the use of the single treatment with L19-IL2 in comparison to IL2 treatment. This is in contradiction with the results from previous studies, showing that L19-IL2 provides a stronger antitumor effect compared with equimolar dosing of untargeted IL-2 in an F9 teratocarcinoma or a human pancreatic carcinoma xenograft model (16, 42). This might be explained by the use of different mouse strains, tumor models and treatment schedules. However, in combination with radiotherapy, we did find a stronger anti-tumor effect when using L19-IL2 compared to IL2. This shows that, in agreement with previous results, L19-IL2 has an increased anti-tumor effect.

Upon combination therapy an increased number of cytotoxic T cells was observed in the tumor of the LLC and C51 model. A comparison between the used models shows that already at baseline, the number of cytotoxic T cells is higher for the C51 model than for the LLC and 4T1 models. Results are in agreement with previous publications, where it was already shown that, dependent on tumor model, the efficacy of IL2 treatment can be based on T cells (43, 44), or a combination of NK and T cells (16). In mice bearing C51 colon carcinoma, L19-IL2 as single treatment already showed an increased number of tumor-infiltrating cytotoxic T- and NK cells in
immunohistochemical analysis (16). This was confirmed in the clinical setting where both cell types were upregulated in the peripheral blood of patients as a result of L19-IL2 treatment (34). Johnson et al. (45) combined an alternative immunocytokine, KS-IL2, with radiofrequency ablation in a murine colon adenocarcinoma (CT26). The combination increased growth suppression, and a greater proportion of CD4^+ and CD8^+ cells was observed. Furthermore, the therapeutic effect of IL2 coupled to the human monoclonal antibodies F8 and F16 that recognize the ED-A and ED-B domains of fibronectin and the A1 domain of tenascin-C, respectively, was shown to be mediated by CD8^+ and NK cells in an in vivo AML model (46). Moreover, the antibody-based targeted delivery of IL4 and IL12 to tumor neovasculature has also been shown to eradicate tumors by both NK and CD8^+ T cells(47). In our study, we irradiated the tumors prior to administration of the immunocytokine L19-IL2. It is known that radiotherapy can promote a DC-mediated cytotoxic T-lymphocyte (CTL) response, the so-called immunogenic cell death (48). This form of cell death may be further enhanced by the targeted delivery of IL2 to the irradiated tumors. Our combination therapy may therefore favor the CTL response, because NK cells are able to detect and destroy malignant and virally infected cells directly (15). Indeed we have shown that depletion of the cytotoxic T cells in the C51 model inhibits the anti-tumor effect after combination therapy, providing evidence that the complete remission observed in the majority of C51 tumors, is attributed to the high number of cytotoxic T cells present in the tumor after combination therapy.

Evidence suggests that local radiation always elicits activation of the immune system, even though the proportion of tumor cells undergoing immunogenic cell death will vary (5, 7). Demaria et al. (7) showed that a single low dose of RT (2Gy) in combination with Flt3-Ligand (enhancing the number of available dendritic cells) could already trigger antitumor T cell responses, while Schaeue et al. (49) reported that only doses above 7.5 Gy were immunostimulatory. To test this in our study, the radiotherapy dose was reduced from 10Gy to 5Gy or 2Gy for the C51 model. The decrease in dose of irradiation resulted in a reduced number of tumor eradication, however in that in this model and experimental set-up the radiotherapy dose is an important parameter to generate cure. We therefore suggest that a minimal radiotherapy dose is necessary to provide sufficient immunogenic cell death to trigger the anti-tumor immune response. In our experiments, we only tested one single RT dose in combination with L19-IL2, showing excellent results. Therefore we expect that the use of a few high doses of radiotherapy (SBRT) is sufficient to release DAMPs and initiate the anti-tumor immune response, while limiting the damage to essential immunological (CD8^+) cells. In a previous clinical trial, Seung et al. (50) combined SBRT with systemic IL2 in patients with metastatic melanoma or RCC, which already provided a higher response rate compared to historical data. Based on our results the use of L19-IL2, instead of systemic IL2, will increase the potential and decrease toxicity. Therefore the clinical set-up combining SBRT with L19-IL2 seems very promising and will be investigated in a clinical trial (NCT02086721).

Since ED-B has an identical amino sequence in mice and humans, the human single chain Fv monoclonal antibody fragment L19 combined with IL2 can be directly used in clinical setting. In Phase I trials, L19-IL2 was already safely
administered in melanoma and renal cell carcinoma, even in combination with decarbazine, which is not an ICD inducer like RT(34-36). Based on our current results that ED-B expression is essential to obtain a therapeutic benefit, L19-SIP imaging should be included in a clinical trial set-up to evaluate the possibility to select patients for L19-IL2 treatment. However, the ultimate aim is to increase progression free survival by the irradiation of accessible, larger solid tumors/metastasis, initiating an anti-tumor immune response which will attack the solid lesions and its micro-metastasis.

In conclusion, the combination therapy of RT with L19-IL2 can enhance the immune response against diverse solid tumors, providing an additive or synergistic anti-tumor effect in the presence of ED-B. These findings can directly be translated to a Phase I clinical study in patients with an oligometastatic solid tumor, since the use of L19-IL2 is proven to be safe in patients. This promising new opportunity for cancer treatment is subject of clinical investigation.
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References


**Figure legends**

**Figure 1:** High, intermediate and low ED-B expressing tumor models. A: ED-B expression for C51 colon carcinoma, Lewis Lung Carcinoma (LLC) and 4T1 mammary carcinoma. ED-B expression is detected by L19(sip) biotinilated (green), vessels by anti-CD31 (red) and cell nuclei (DAPI staining) in blue. Scale bar 100 µm B: Quantification of the ED-B expression (intensity) is corrected for negative control (KSF(sip) biotinilated). ** P<0.01, ***P<0.001.

**Figure 2:** Combination therapy results in complete remission of 75% in the C51 model. A: Fraction of tumors not reaching 4 times start-volume (T4xSV). B: Time to reach 4 times start volume for the different treatment groups. C: Results of flow cytometry analysis, shown is the percentage of CD8+ and NKp46+ cells of all CD45+ cells present in the tumor. Data represent the mean of n = 6 - 12 tumors. *p<0.05, **p<0.01, ***p<0.001.

**Figure 3:** Combination therapy results in an additive effect in the LLC model (intermediate ED-B) and no effect in the 4T1 model (low ED-B). A: Survival curves LLC model and 4T1 model, showing the fraction of tumors not reaching 4 times start-volume (T4xSV). B: Scatter-plots of the LLC and 4T1 model, showing the time to reach 4 times start volume (T4xSV). Flow cytometric analyses of tumor leukocyte content in the LLC (C) and the 4T1 (D) model, showing the percentage of CD8+ and NKp46+ cells of all CD45+ cells in the tumor. *p<0.05, **p<0.01, ***p<0.001.

**Figure 4:** Depletion of cytotoxic T cells prohibits complete remission. CD8 depletion in the C51 model. A: Cartoon of treatment schedule. B: % CD8+ cells of CD45+ cells 3 days after i.p. anti-CD8, IgG or PBS (vehicle) and an example of the flow cytometry results showing the percentage of CD3+CD8+ cells present in the blood 3 days after anti-CD8 or IgG administration. C: % of CD8+ cells present in the tumor of CD8 depleted and non-depleted mice treated with RT and L19-IL2 analyzed by flow cytometry and an immunofluorescent CD8 staining (green), cell nuclei stained with DAPI (blue). D: Fraction of tumors not reacting 4x start volume (T4xSV) and time to reach T4xSV for the different treatment groups. *p<0.05, **p<0.01, ***p<0.001.
A
Combination therapy results in complete remission in 75% of the tumors

B
Cure (9/12)

C
CD8+ T cells

NKp46+ cells

Combination therapy treatment effect is dependent on radiation dose

Combination therapy results in complete remission in 75% of the tumors and the treatment effect is dependent on radiation dose.
Figure 3

(A) Lewis Lung Carcinoma

(B) 4T1 Mammary Carcinoma

(C) CD8+ T cells

(D) NKP46+ cells

Time [d]

Fraction not reaching T4xSV

T4xSV

% of CD45+ cells

% of CD45+ cells

% of CD45+ cells

% of CD45+ cells
Figure 4

A

Vehicle + anti-CD8
L19-IL2 + anti-CD8
RT + vehicle + anti-CD8
RT + L19-IL2 + anti-CD8
RT + L19-IL2 + IgG
RT + L19-IL2 + vehicle

-9 -4 -1 0 1 2 3 5 8 Time [d]

B

CD8+ T cells in blood

% of CD45+ cells

before anti-CD8
3d after anti-CD8
3d after IgG
3d after PBS

CD4+

CD8+

3d after anti-CD8
0.9%

3d after IgG
17.4%

C

CD8+ T cells in tumor

% of CD45+ cells

anti-CD8
no anti-CD8

***

D

Vehicle + anti-CD8
L19-IL2 + anti-CD8
RT + vehicle + anti-CD8
RT + L19-IL2 + anti-CD8
RT + L19-IL2 + IgG
RT + L19-IL2 + vehicle

% of CD3+CD8+ cells

% not reaching T4xSV

Time [d]

Cure rates:
5/8 3/8

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Radiotherapy combined with the immunocytokine (L19-IL2) provides long-lasting anti-tumor effects


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