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Review

Co-operative radio-immune-stimulating cancer therapy

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ABSTRACT

Radiation therapy for cancer treatment is delivered more or less in the same mode during the past 100 years. Low dose (2 Gy) fractions are given daily until a high target dose (60-70 Gy) is achieved. This treatment regime aims at eradicating the tumour by radiation induced cancer cell death. But traditional fractionated radiation therapy also decreases the number of radiation sensitive T-cells (CD3+, CD4+, and CD8+) in the tumour and thus prohibits immunogenic cell death. Several pre-clinical studies show that radiation therapy given by hypo-fractionation dramatically enhances the effect of otherwise non-effective immune-therapy. This opens up the possibility for an alternate cancer therapy regime using radiation in co-operation with immune therapy, instead of counteracting as in conventional fractionated radiation therapy regimes. This review summarizes the effects of various fractionation modes of radiation on the tumour and various immune cells: CD4+ and CD8+ T-cells, T_{reg}, natural killer (NK) cells and dendritic cells (DCs). A number of pre-clinical studies which demonstrate the enhanced therapeutic response of malignant tumours to various combinations of immunotherapy (IMU) with single fraction or hypo-fractionated radiation therapy (RT) are reviewed. The clinical trials of combining immune therapy and radiation therapy carried out so far have been performed by using conventional radiation therapy with sparse effect. Clinical studies of combining established IMU regimes with a single 8 Gy fraction RT could open up the possibility for a deeper co-operation between biology and physics. This therapeutic co-operative regime may also reduce the probability of relapse, and if relapse occurs the treatment can be repeated.

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INTRODUCTION

Since Roentgen's discovery of the X-rays 1895, radiation therapy (RT) has been one of the most successful modalities used to treat cancer [1]. The traditional treatment regimes for anticancer therapies are believed to work by acting on cancer cells in the target area through direct induction of tumoural, stromal and endothelial cell death by apoptosis, necrosis or by inducing cell cycle arrest. But despite substantial technical improvements in the current RT treatment modalities, and despite the developments in chemotherapy (CT), the results of cancer treatment are not always successful. More recently, the importance of the tumour microenvironment has been recognized and new therapy regimes have been developed that function by modulating tumour cell-extrinsic pathways [2]. The strong infiltration of human tumours by activated CD8+ cytotoxic T lymphocytes (CTL) and CD4+ helper T (Th) cells is a hallmark for improved survival after therapy. But the infiltration of immune suppressive cells such as regulatory T-cells (T_{reg}), M2 macrophages and myeloid-derived suppressor cells (MDSC) counteract the immune tumour cell death [3].

Immunotherapy is a therapy regime which utilizes the fact that the immune system has a potential to

react against tumour antigens, which could result in immunological control of the tumour. There is an increasing body of evidence that the activation of CTL has a positive effect on the long-term survival of cancer patients receiving traditional therapies such as surgery, CT or RT [4-7]. It has been clearly demonstrated that tumour immune reactivity is of importance in the treatment of several types of tumours [8].

Traditional fractionated radiation therapy, however, decreases the number of radiation sensitive T-cells (CD3+, CD4+, and CD8+) in the target area and thus dampens the effect of immunotherapy on the irradiated tumour [9]. It has been reported that CD8+ T-cells that escape from the target area and rush to the cancer cells outside the target region (metastases) may give rise to an abscopal effect [10-12]. Currently, there is a growing trend in cancer research to treat a broad range of malignancies by combining conventional fractionated radiation with immunotherapy, but with sparse effect [13-20].

Recent findings, however, show that hypo-fractionated or single-fraction regimes dramatically enhance the effect of immune therapy, which otherwise have none or limited effect [21-32]. The main reason for this effect, we believe, is that a single fraction of irradiation destroys the action of immune suppressive cells such as T_{reg}, M2 macrophages and MDSC, which cause the tumour's immune-suppression to be repealed. This gives the CD8+ CTL and CD4+ Th cells a possibility to act and additional immune therapy get an opportunity to effectively attack the tumour. A single fraction of high absorbed dose (≈8 Gy) also results in an increased release of tumour antigens. This enhances the production of killer CD8+ T-cells, which will attack the tumour cells, if not eradicated by the succeeding radiation fractions. Preclinical studies using this approach are showing high fractions of complete remissions of tumours in animal models by using the combination of radiation and IFN-gamma-transfected tumour cell based immune therapy [25]. Most cell-based immune therapies, however, require advanced laboratory facilities and need long time for preparations. Pre-manufactured vaccine is, however, in the pipeline and preclinical results show that the combination of RT and Listeria PSA vaccine causes significant tumour regression by augmenting PSA-specific immune response, and that it could serve as a potential clinical treatment regimen for prostate cancer [33].

This review will summarize the present understanding on how irradiation of tumours affects the immunogenic tumour cell death, and how to use various fractionation regimes of radiation therapy for a smart clinical co-operation with immune therapy.

Radiation modulation effects on immune cell activity

The proteasome in tumour cells is a sensitive target for radiation that increase the presentation of tumour antigens by enhanced accumulation of antigen/MHC (major histocompatability complex) class I complexes on the cell surface [34].

Chakraborty *et al.* [35] found that radiotherapy induces unique biologic alterations in cancer cells affecting Fas-gene expression, which, consequently, may influence the overall lytic efficiency of CTL. In a mouse adeno-carcinoma cell model, they found that the *in vitro* treatment of carcino-embryonic antigen (CEA) expressing MC38 adeno-carcinoma cells with a sub-lethal absorbed dose of 20 Gy enhanced Fas expression at molecular, phenotypic and functional levels. Furthermore, irradiation sensitized these targets to antigen-specific CTL via the Fas/Fas-ligand pathway [35].

They also examined the *in vivo* effect of localized irradiation of s.c. growing tumours, in combination with CTL adoptive immunotherapy which caused the up-regulation of Fas in the tumour cells, based on immune-histochemistry. Moreover, localized irradiation of the tumour significantly potentiated tumour rejection by these carcinoma-embryonic antigen-specific CTL. Their results showed that regulation of the Fas-pathway in tumour cells by irradiation plays an important role in their sensitization to antigen-specific CTL [35].

Liao [36] demonstrated that local single fraction radiotherapy stimulates the immune response by enhancing the antigen presentation of MHC class I [36]. The mechanism underlying these effects is probably at the level of the proteasomes in the cytoplasm of the tumour cell, which are essential for the production of antigenic peptides for loading onto MHC class I molecules. Paulos *et al.* [37] showed that preconditioning of tumour bearing C57BL/6 mice with 5 Gy total body irradiation

activated the innate immune system, as demonstrated by a significant increase in the absolute number of host CD11c+CD86 high DCs and serum levels of IL-12 [37].

The DCs either initiate an effective cytotoxic response against antigen-bearing cells, or produce tolerance, depending on the context in which those antigens are presented [38]. It has been shown that cell death caused by radiation therapy release tumour antigens, which facilitates an effective response of the dendritic cells [39]. It has been demonstrated that antigen presentation by MHC class I is increased for many days by single fraction radiation therapy. The most pronounced effect was recorded at 7 days after irradiation with an absorbed dose of 8 Gy. This might be one of the reasons why the efficacy of tumour immunotherapy is most effective in combination with single fraction radiation therapy [17].

Maximum loading of the tumour micro-environment with cancer antigen occurred 2 days after radiation therapy and coincided with the optimal time for CD8+ T-cell transfer [40]. Radiation therapy activation of dendritic cells induces the secretion of interleukin-1 beta (IL-1_{β}), which is required for the adequate polarization of IFN_{γ} producing CD8+ T-cells [41-42].

The different cells of the immune system are affected to various degrees by ionizing radiation. Even irradiation with low absorbed dose in the order of 0.5-1 Gy functionally modulates various immunological processes [43]. Bogdandi *et al.* [44] studied the effects of acute exposure to low- and high-dose radiation on the quantitative and functional parameters of the immune system [44]. They irradiated C57BL/6 mice with different absorbed dose (0.01, 0.05, 0.1, 0.5 and 2 Gy) of gamma radiation and isolated splenocytes at various times. Alterations in the distribution and surviving fraction of splenocytes such as CD4+ and CD8+ T-cells, T_{reg}, natural killer cells (NK), DCs and B lymphocytes were analyzed by flow cytometry. A single radiation exposure, with an absorbed dose of 2 Gy, increased apoptosis in all subpopulations of splenocytes. CD8+ CTL and B-cells were rather resistant to low (<2 Gy) absorbed dose. They were, however, very sensitive to irradiation at absorbed dose above 2 Gy. Expression of the CD4 T-helper 1 (Th1)- and T-helper 2 (Th2)-type cytokines decreased after low absorbed dose but increased after high absorbed doses. Interleukin 6 (IL-6) reacted at the early times and IL-10 at the later times. IL-5 levels were consistently elevated. NK cells and DCs were the least sensitive immune cells [44].

The expression profiles of CD4+ and CD8+ T-cells and T_{reg} from patients with newly diagnosed *glioblastoma multiforme* are quite different when compared with normal healthy volunteers [45]. But how various absorbed doses or various fractionation patterns or methods of radiation delivery can affect T-cell populations and alternative regulatory molecules in glioma patients is still under debate [46-48].

The immune response to tumour cells is primarily a result of the release of tumour antigens and various molecular species from dying tumour cells, which activate CD8+ CTL [49]. A single fraction of irradiation (8 Gy) given to a tumour, kills some tumour cells which causes a release of tumour antigen to the microenvironment. The irradiation also speeds up the maturation of immature DCs, present in the microenvironment, which then phagocytise the tumour antigens. The antigen processing is also favoured by the release of various molecules from the dying tumour cells (i.e. HSP, HMGB1, ATP) that bind to receptors (Toll like TLR4, P2RX7) on the DCs. This promotes the priming of lymphocytes in the lymph-nodes and secretion of TL-1β which polarize CD8+ T-cells to produce IFNγ and increase their proliferation [49]. The CD8+ T-cells migrate towards the tumour cells. On its migration towards the tumour they are normally pacified or suppressed by the presence of virgin MDSC which, however, are destroyed by irradiation. Thus, a single fraction of radiation promotes the proliferation of CD8+ T-cells and its infiltration, and eradication of the tumour by immunogenic cell death (ICD). By multiple fractions the CD8+ T-cells are successively killed and no ICD occur.

In Figure 1 a schematic view of the various steps in the process of co-operative radio-immune tumour cell killing is given.

Other components affecting the outcome of radiotherapy are the effects on endothelial cells, circulation and infiltrating immune cells within the tumour microenvironment. A balance between favourable tumour-infiltrating immune cells, including cytotoxic T-cells, NK cells and DCs, and unfavourable

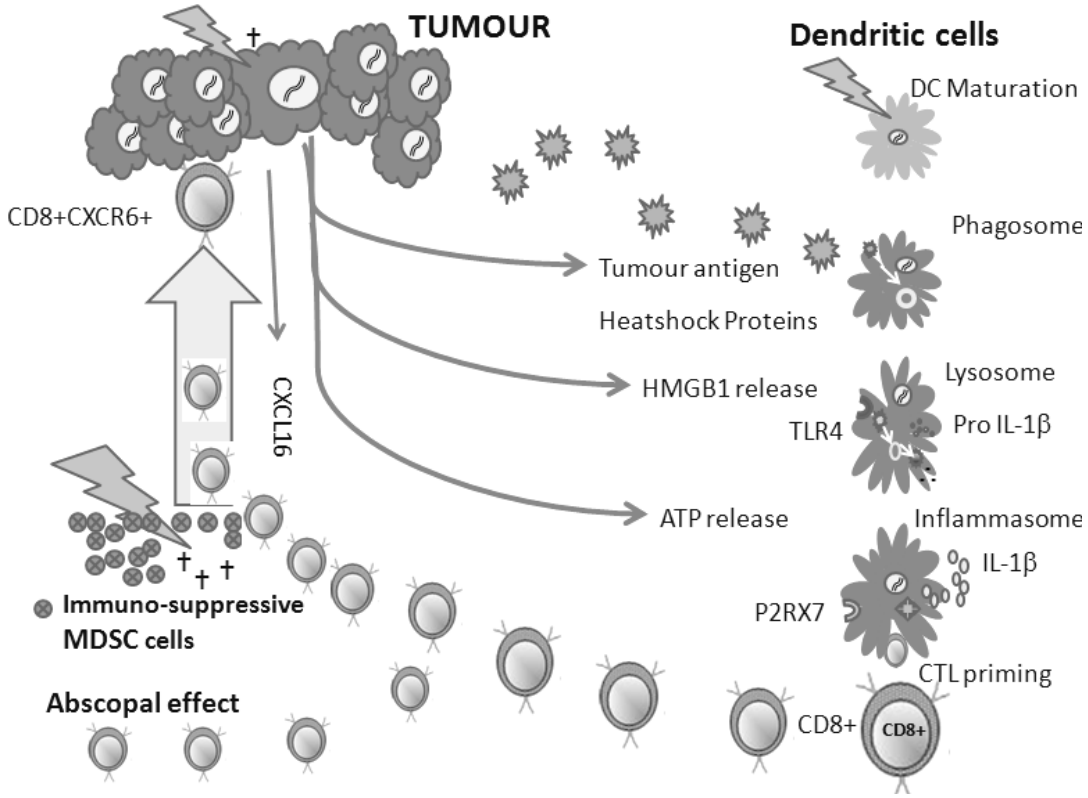

Figure 1. Schematic view of the various steps in the process of radio-immune tumour cell killing. A single fraction of radiation therapy causes release of antigen from dying cells which are phagocytised by DCs which also become activated by the irradiation. Molecules like HMGB1, also released from dying cells, binds to TLR4 on DCs which favour antigen processing and up-regulation of pro-IL-1β. Dying cells also release ATP which binds to the receptor P2RX7 on DCs. This activates NLRP3 inflammasome which secrete IL-1β polarizing CD8+ T-cells to produce IFNγ and proliferate. The CD8+ T-cells then infiltrate and eradicate the tumour. Distant microscopic metastases might also be affected by escaping and migrating CD8+ T-cells (Abscopal effect) [49]. Immunosuppressive MDSC cells are destroyed by irradiation and open the way for CTL to act upon the tumour cells.

immune suppressing cells such as tumour-associated macrophages and regulatory T-cells, determines the final tumour-control probability [50].

A vaccination regimen may augment the effector functions of CTL as well as increase the number of lymphoid cells within the tumour. But even though it has been documented that large populations of lymphocytes enter the tumours, lyses of all tumour cells and total eradication of the neoplasm do not occur. This is partly due to the immunosuppressive factors produced by the tumour cells resulting in non-functioning CTLs [51-55].

The normal process of myelopoiesis, which takes place in the bone marrow, generates immature myeloid cells (IMCs). These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells. Factors produced in the tumour microenvironment promote the accumulation of MDSCs at the tumour site [56]. Immunoregulatory MDSC present in tumours can suppress the function of tumour-infiltrating activated T-cells, and therefore play a role in tumour associated immunosuppression [55]. The inadvertent generation of MDSC in clinical trials involving vaccine-based strategies may represent a significant obstacle to successful tumour immunotherapy as mentioned above. As will be shown in this review a single fraction of radiotherapy with an absorbed dose in the order of 8 Gy, however, seems to temporarily eliminate the MDSC present in the target and open a window for effective immune therapy.

In conclusion, this review urges for a new cancer treatment concept based on the co-operation of an 8 Gy single fraction external RT combined with an effective established immune therapy regime. In Figure 2 a schematic view of the concept of co-operative radio-immune-stimulating therapy is displayed.

Modulating immune-response by radiation fractionation

Low dose hyper (ultra)-fractionation

One attempt to modulate the immune response has been to use low-dose ultra-fractionation (UF) radiotherapy. Krause *et al.* [57] investigated ultra-fractionation in radio-resistant murine DDL1 T-cell Lymphoma in mice. Irradiation was performed during 2, 4 and 6 weeks, either with ultra fraction (UF) with 0.4 Gy per fraction, 3 fractions per day at 7 days per week, or conventional fractionation (CF) with 1.68 Gy per fraction, one fraction per day at 5 days per week. Tumour growth delay was evaluated as the time that tumours needed to reach fivefold the starting volume (GDV_5). The results showed that tumour growth delay (GDV_5) was not significantly different between UF and CF groups. Previous experiments on human A7 glioblastoma showed a negative effect of UF on local tumour control. Thus the results obtained by those preclinical studies do not support any benefit for the use of ultra-fractionated schedules in radiotherapy [57].

Beauchesne [58], however, demonstrated high efficiency of ultra-fractionated radiotherapy (RT < 0.75 Gy per fraction) on glioma xenografts. In a phase II clinical trial he studied the safety, tolerability, and efficacy as well as overall survival (OS) and progression-free survival (PFS), in patients with newly diagnosed, inoperable glioblastoma (GBM). Thirty-one patients with histologically proven, newly diagnosed, and unresectable supratentorial GBM (WHO grade IV) were enrolled. No acute

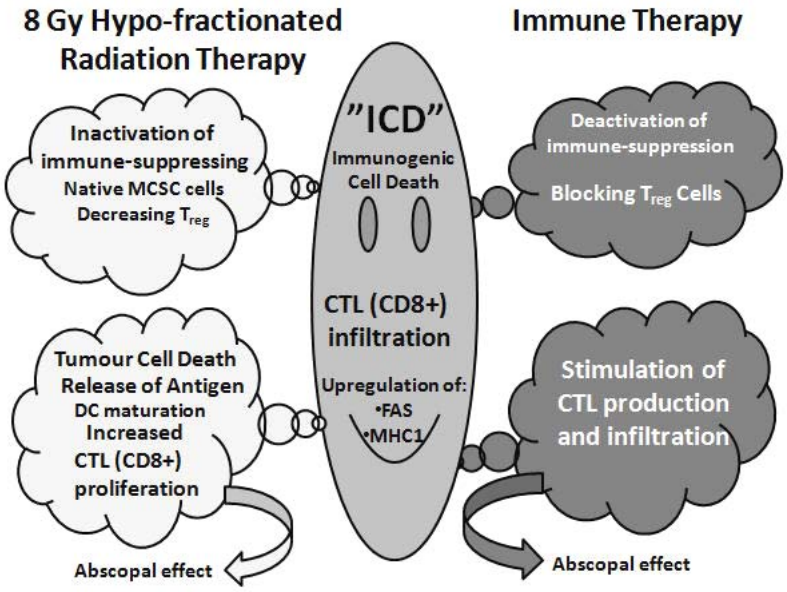

Figure 2. Schematic view of the concept of Co-operative Radio-Immune-Stimulating Therapy.

1. Immune suppressing MDSC cells surrounding the tumour are deactivated by RT.
2. RT also down regulates generation of regulatory CD4+ T-cells (T_{reg}) secreting immune suppressive IL10.
3. RT up-regulates tumour antigens, co-stimulatory molecules MHC-1 complex and FAS, which makes tumours more susceptible to immune mediated attack.
4. RT up-regulates chemokine CXCL16 that promote CD8+ T-cell migration and infiltration, which promote immune cell death (IDC) of the tumour.
5. These processes can be further promoted by various immune therapies.

Grade III tentorial GBM (WHO grade IV) were enrolled. The radiation therapy regimen consisted of ultra-fractionated focal irradiation with 3 daily doses of 0.75 Gy delivered at least 4 hours apart. Irradiation of the tumours was performed 5 days a week (Monday through Friday), for 6–7 consecutive weeks, 90 fractions for a total of 67.5 Gy absorbed dose and/or until IV CNS toxicity was observed. Median progression-free survival (PFS) and overall survival (OS) from initial diagnosis were 5.1 and 9.5 months, respectively. When comparing the results with the EORTC/NCIC trial, in both PFS and OS multivariate analysis, ultra-fractionation showed superiority over conventional RT alone, but not over RT and TMZ. Thus the ultra-fractionation regimen is safe and may prolong the survival of patients with GBM [58].

Conventional moderate dose (2 Gy) fractionated radiation therapy

Uh *et al.* [9] measured the number of lymphocytes in peripheral blood of 19 patients with squamous cell lung cancer before and after 50-60 Gy fractionated radiation therapy. As shown in Figure 3 they found that the total numbers of lymphocytes, CD3+, CD4+, and CD8+ lymphocytes, and NK-cells, B-cells, and CD25+IL2 cells in peripheral blood was significantly decreased [9].

Dovsak *et al.* [59] studied the influence of surgical treatment and radiotherapy of the advanced intraoral cancers on complete blood count, body mass index, liver enzymes and leukocyte CD64 expression. They found that surgery caused lymphopenia, which was worsened by radiotherapy. Expression of CD64 on monocytes and neutrophils was, however, elevated after radiotherapy [59].

High dose ablative (15-25 Gy × 1) or hypo-fractionation (8 Gy) radiation therapy

The use of sterotactic techniques with single radiation exposure or hypo-fractionated radiation therapy primes the immune response and increases the therapeutic outcome [60-64].

Lee *et al.* [62] reported that reduction of tumour burden after ablative RT (15-25 Gy × 1) depends largely on T-cell responses [62]. They found that ablative RT dramatically increases T-cell priming in draining lymphoid tissues, leading to reduction/ eradication of the primary tumour or distant metastasis in a CD8+ T cell-dependent fashion. The ablative RT-initiated immune responses and tumour reduction are abrogated by conventional fractionated RT or adjuvant chemotherapy in agreement with the previous paragraph. But ablative RT-initiated immune responses are greatly amplified by local immunotherapy which is in agreement with the experience of others [25, 65].

Schaue *et al.* [67] studied the tumour immunity with fractionated radiation in mice bearing B16-OVA murine melanoma treated with an absorbed-dose up to 15 Gy, given in various-size fractions. The contribution of T_{reg} was determined by the proportion of CD4+CD25(hi)Foxp3+ T-cells. After single radiation fraction, however, tumour control increased with the absorbed dose, as did the number of tumour-reactive T-cells. This was offset at the highest dose by an increase in T_{reg} representation in agreement with the observation of other authors [66]. Fractionated treatment with medium-size radiation doses of 7.5 Gy/fraction gave the best tumour control and tumour immunity while maintaining low T_{reg} numbers [67].

Henke *et al.* [68] found that re-treatment of recurrent high-grade glioma with hypo-fractionated (5 Gy per fraction) radiation therapy with a median total absorbed dose of 20 Gy seems to be feasible even after a previous complete course of radiotherapy [68]. Thus it should be feasible to consider hypo-fractionated radiotherapy, with about 8 Gy given as one or two fractions to recurrent glioma in combination with immune therapy, in re-treatment of recurrent high-grade glioma.

Preclinical experience of co-operative radition and immune therapy

Combination of 5 Gy × 4 radiation therapy, Pulsed Electric Fields (PEF) and immunization with syngeneic Interferon-gamma secreting tumour cells

Persson *et al.* [32] presented a summary of results of tumour treatment experiments performed during 1998-2002 by using Pulsed Electric Fields (PEF) combined with radiation therapy (RT) and immunization with syngeneic Interferon-gamma (IFNγ) secreting tumour cells [32]. Fischer-344 rats with N29 glioma tumours implanted on both flanks were treated with radiation therapy (RT)

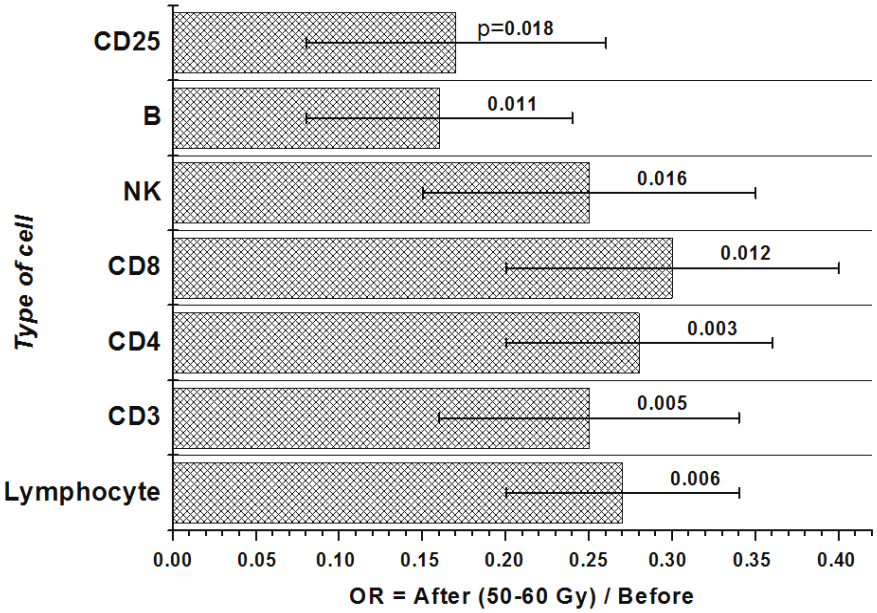

Figure 3. The effect on observed ratio of lymphocyte subsets in peripheral blood after and before local fractionated radiation therapy (50-60 Gy) [9].

and Pulsed Electric Fields (PEF) followed by injections of IFNγ secreting syngeneic cells. The left tumour only was treated once with PEF (16 pulses at an electric field strength of 1400 V/cm, and 1.0 ms time constant) followed by radiation therapy (RT) with Co-60 gamma radiation (5 Gy × 4). Once a week during a period of three weeks, the animals were given intraperitoneal (i.p.) injections of N29 syngeneic tumour cells (modified to secrete interferon-gamma, and sterilized with 100 Gy ^{137}Cs gamma radiation).

Tumour Growth Rate (TGR) was estimated from tumour volume measurement data of each individual tumour, fitted to a model of exponential growth from the day of radiation treatment. In order to be able to compare the tumour growth rate recorded in all the different treatment modalities, the difference in tumour growth rate between the exposed rat and the corresponding control was normalized to the growth rate of the control of the series in question.

Thus we defined the quantity specific therapeutic effect (STE) as the difference in tumour growth rate between the control and exposed tumour divided by tumour growth rate of the controls.

$$\text{STE} = \frac{\overline{TGR_C} - \overline{TGR_E}}{\overline{TGR_C}};$$

where $\overline{TGR_E}$ The average of the individual tumour growth rate constant in the group of exposed rats (day^{-1})

$\overline{TGR_C}$ The average of the individual tumour growth rate constant in the group of control rats (day^{-1})

- The STE is equal to 0 when the average of tumour growth rate of the exposed group, TGR_E, is equal to the average of the tumour growth rate of the controls, TGR_C.
- The STE is equal to 1 when the average tumour growth rate of the exposed group is equal to 0, ($TGR_E = 0$), which means arrested tumour growth.
- The STE is larger than 1 when the average tumour growth rate of the exposed group is negative ($TGR_E < 0$), which means a declining tumour volume.
- The STE is < 0 when the average of tumour growth rate TGR_E of the exposed group is larger than the average of the tumour growth rate of the controls, TGR_C.

The "Therapeutic Enhancement Ratio" (TER) of the combined treatments of radiation therapy (RT) and vaccination (Vac) is defined as the ratio of the specific therapeutic effect of the experimental

combination (STE_{RT+Vac}) and the specific therapeutic effect the hypothetical combination of independently applied radiation therapy and vaccination ($STE_{RT} + STE_{Vac}$).

$$TER = \frac{STE_{RT+Vac}}{STE_{RT} + STE_{Vac}}$$

The STE is normalized to the growth rate of the control of each individual experiment and thus the STE values of various experimental series can be combined. The following Figure 4 displays TGR and STE of all the experiments performed during 1998-2002 (981112 PEF; 010529 PEF + RT + IFNγ, 010827 PEF + IFNγ, 020102 PEF + IFNγ, and 020404 PEF + RT + IFNγ).

Radiation therapy (RT) was the most effective independent treatment that showed significantly ($p < 0.001$) decreased tumour growth rate. But the tumour growth in animals independently treated with pulsed electric fields (PEF) also showed highly significant ($p < 0.001$) decreased growth rate of the treated tumour. The combination of radiation therapy and pulsed electric fields (RT + PEF) was as effective as the independent treatments with PEF or RT. This is, however, in contrast with the results of previous experiments that showed a large enhanced effect of these combined treatments [69].

There was a large variation in the growth rate of tumours in animals treated only with IFNγ syngeneic tumour cells, and no significant change in tumour growth rate compared to the controls was found. This is in agreement with the results by Graf *et al.* [21] who found that vaccination only resulted in a marked enhancement of tumour growth [21].

The tumour growth rate of tumours in animals treated with both pulsed electric fields PEF and IFNγ syngeneic cells showed a slight but not significant decrease in growth rate of the treated tumour. But the combination of radiation therapy and immunization with IFNγ syngeneic tumour cells (RT + IFNγ) surprisingly showed a significant decrease ($p < 0.01$) in growth rate. The combination of pulsed electric fields, radiation therapy and immunization with IFNγ syngeneic tumour was the most effective treatment that showed a highly significant decrease ($p < 0.001$) in the growth rate of the directly exposed tumour. The specific therapeutic effect (STE) was most enhanced for treatment with the combination of all the 3 treatments PEF, RT, and immunization with IFNγ syngeneic cells (PEF + RT + IFNγ), while the specific therapeutic effect (STE) was only slightly enhanced for treatment with the combination of PEF + RT, compared to STE of radiation treatment only.

The Therapeutic Enhancement Ratio (*TER*) of the combined treatment with vaccination and RT, is the ratio of the specific therapeutic effect of the experimental combination (STE_{Vac+RT}) and the sum of the specific therapeutic effects of the individual treatments ($STE_{Vac} + STE_{RT}$) [70]. This parameter was evaluated from the STE data and displayed in Figure 4. All treatments with RT and PEF combined with IFNγ resulted in TER values >1 which indicate synergistic effects. IFNγ + PEF treatments resulted in the highest TER values: $TER_{PEF+IFN\gamma} = 2$; $TER_{PEF+RT+IFN\gamma} = 1.6$, while TER value for RT + IFN_{γ} was slightly lower, $TER_{RT+IFN\gamma} = 1.3$.

Proliferation assay performed with non-adherent spleen cells from controls and treated rats, demonstrated decreased immunological response of separate treatments with PEF or radiation therapy. But the combined treatment indicated an increased immunological T-cell response. Thus the combination of Pulsed Electric Fields and radiation therapy seems to be a promising new modality in tumour treatment. In the present study on rats, N29 glioma tumours were implanted on both flanks and only the results of the tumours exposed to PEF and RT have been considered. The results of the growth of the untreated contra lateral tumours, however, also indicate that a systemic response on the unexposed tumour was achieved with immunization using syngeneic tumour cells, when the contra lateral tumour was exposed to Pulsed Electric Fields and radiation therapy combined [32].

Combination of 15 Gy × 1 RT and immunization with syngeneic cellular tumour vaccine

Graf *et al.* [21] treated rats bearing a 5-day intracranial (i.c.) syngeneic glioma with a subcutaneous (s.c.) vaccination consisting of irradiated glioma cells or a multimodality approach composed of radiotherapy plus s.c. vaccination. Vaccination of rats harbouring a T9 glioma with 5×10^6 irradiated T9.17 glioma cells (a clone derived from the T9 glioblastoma cell line) resulted in a marked enhancement of i.c. glioma growth and a significant

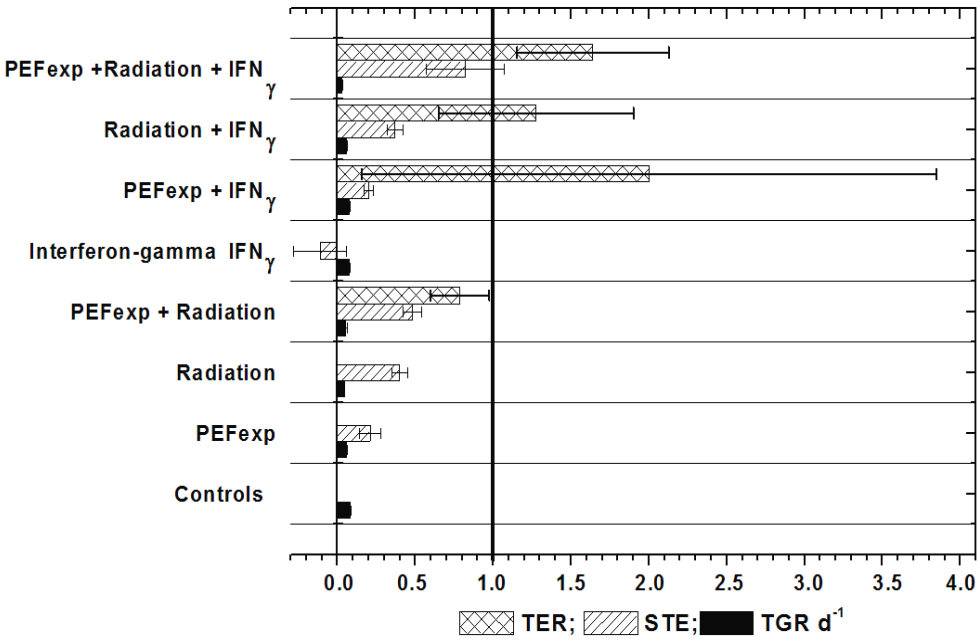

Figure 4. Tumour growth rate (TGR) and specific therapeutic effect (STE) of all the experiments performed (981112 PEF; 010529 PEF + RT + IFNγ, 010827 PEF + IFNγ, 020102 PEF + IFNγ, and 020404 PEF + RT + IFNγ), and Tumour enhancement ratios (TER) of the combined treatments [32].

decrease in survival. Histopathology of the tumours from vaccinated rats revealed a massive glioma composed of healthy tumour tissue lacking any marked inflammation, oedema or haemorrhage. Analysis of the tumour-infiltrating mononuclear cells indicated that glioma tumours from vaccinated rats contained a 10-fold greater lymphoid infiltrate per milligram of tumour as compared to tumours from non-vaccinated rats, suggesting that the vaccination had induced immune cells to localize to the i.c. glioma. Combined treatment consisting of 15 Gy of whole head irradiation of the 5-day glioma followed by vaccination with T9.17 cells resulted in a significant increase in survival compared to that of non-treated rats, 45% of which remained tumour-free. Microscopic evaluation of the tumour implantation site in survivors revealed the presence of hemosiderin-laden macrophages and other mononuclear cells, with the absence of tumour cells within the residual lesion. When survivors were challenged s.c. with viable T9.F glioma cells, a delayed-type hypersensitivity (DTH) reaction appeared at the challenge site. T-cells purified from these rats proliferated and secreted Th-l-associated cytokines when stimulated with irradiated T9.17 glioma cells, and lysed T9.F target cells. In contrast, when these rats were challenged s.c. with the unrelated MadB106 adeno-carcinoma, tumour formation was observed.

These findings indicate that the treatment of an established i.c. glioma with a cellular vaccination alone may induce enhanced tumour growth. But, when the vaccination is combined with radiation therapy, the results are beneficial in terms of increased survival time or complete remission that is accompanied by the development of tumour-specific cellular immunity [21]. Their findings are in close agreement with the survival rate of 75% ($p < 0.05$) achieved later in a study of intracranial (i.c.) syngeneic N29 tumours in Fisher-344 rats treated with IFNγ secreting vaccine combined with 5 Gy single fraction RT of the brain [25].

Combination of 6 × 1 Gy RT and immunization with (GM-CSF, IL-4, IL-12)

Lumniczky *et al.* [30] in Hungary performed a study in a mouse glioma (Gl261) brain tumour model with single fraction radiotherapy combined with administration of cytokine-producing cancer cell vaccines. Their brain tumour bearing mice were treated with various cytokine producing vaccines made by *in vitro* transduction of Gl261 tumour

cells with different genes such as: IL-4, IL-6, IL-7, GM-CSF, TNFα. Immunotherapy alone with vaccines producing either IL-4 or GM-CSF resulted in complete remission in 20–40% of the mice. By combining immunotherapy using GM-CSF, IL-4, or IL-12 producing vaccines with local tumour radiotherapy (single fraction 6 Gy X-ray radiations) about 80–100% of the glioma-bearing mice were cured. The high efficiency of the combined treatment was maintained even under suboptimal conditions while neither of the individual modalities alone cured any of the mice [30]. Their results are in good agreement with the survival rate of 75% ($p < 0.05$) achieved in the Lund study of N29 tumours in rats treated with IFNγ secreting vaccine combined with 5 Gy single fraction RT [25].

Combination of 5 Gy × 4 RT and immunization with IFNγ secreting tumour cells

Persson *et al.* [11] studied the tumour growth rate response of N29 rat glioma tumour cells subcutaneously implanted on both hind legs of Fischer-344 rats. At around 30 days after inoculation, RT was given with ^{60}Co gamma radiation with 4 daily fractions of 5 Gy only to the right-lateral tumours. At days 26, 42, and 54 after inoculation, immunization was performed with irradiated syngeneic IFNγ-gene transfected cells. *Tumour growth rate* (TGR fraction per day) of the right-lateral irradiated tumour was significantly decreased ($p < 0.01$) after RT alone and with the combination of RT and immunization. But immunization alone gave no significant decrease of the TGR but significantly increased the time of survival [11]. Figure 5 presents the results of "Tumour Growth Rate" of the right-lateral irradiated tumour, the "Specific Therapeutic Effect", and the "Therapeutic Enhancement Ratio" of the combined treatment IFNγ-cell vaccination and RT [70].

Combination of 8 Gy × 1 RT and immunization with CEA vaccine

Chakraborty *et al.* [15] studied how external beam radiation of tumours alters phenotype of tumour cells to render them susceptible to vaccine-mediated T-cell killing. The model they used consisted of mice transgenic for human carcino-embryonic antigen (CEA) and a murine carcinoma cell line transfected with CEA. The vaccine regimen consisted of a prime and boost strategy using vaccine and avipox recombinants expressing CEA and three T-cell co-stimulatory molecules. A single 8 Gy fraction of radiation given to tumour, induced up-regulation of the death receptor Fas *in situ* for up to 11 days. Neither radiation at this absorbed dose, nor vaccine therapy, was capable of inhibiting growth of the established tumour. But when vaccine therapy and local radiation of tumour were used in combination, dramatic and significant cures were achieved. This was mediated by the engagement of the Fas/Fas ligand pathway because antigen-bearing tumour cells expressing dominant-negative Fas were not susceptible to this combination therapy. Following the combination of vaccine and local radiation, tumours demonstrated a massive infiltration of T-cells not seen with either modality alone. Mice cured of tumours demonstrated CD4+ and CD8+ T-cell responses specific for CEA but also revealed the induction of high levels of T-cell responses to two other antigens (gp70 and p53) over-expressed in tumour, indicating the presence of a consequential antigen cascade [15].

Combination of 40 Gy × 1 RT and immunization with DC vaccine

Chen *et al.* [71] found that combined single fraction radiation therapy with an absorbed dose of 40 Gy and dendritic cell vaccine resulted in a significant decrease in the rate of local tumour relapse and the numbers of liver metastases. All local tumours became regressed after irradiation with 40 Gy. But, 3 out of 8 (38%) mice with irradiation treatment had local tumour recurrence, whereas only 1 out of 8 (13%) mice treated with both irradiation and DC vaccine injection had tumour recurrence and 87% mice survived ($p < 0.05$). This indicates that there is a significant synergistic effect of combined single fraction RT and DC-vaccine administration in treatment of local solid tumours. The related mechanisms for this strong antitumour immunity of the combined therapy might be associated with the production of apoptotic and necrotic tumour antigens and heat shock proteins by irradiation. This results in phagocytosis, migration and maturation of DCs which with the action of the DC-vaccine induce more efficient tumour-specific cytotoxic T lymphocyte activity through a cross-presentation pathway [71].

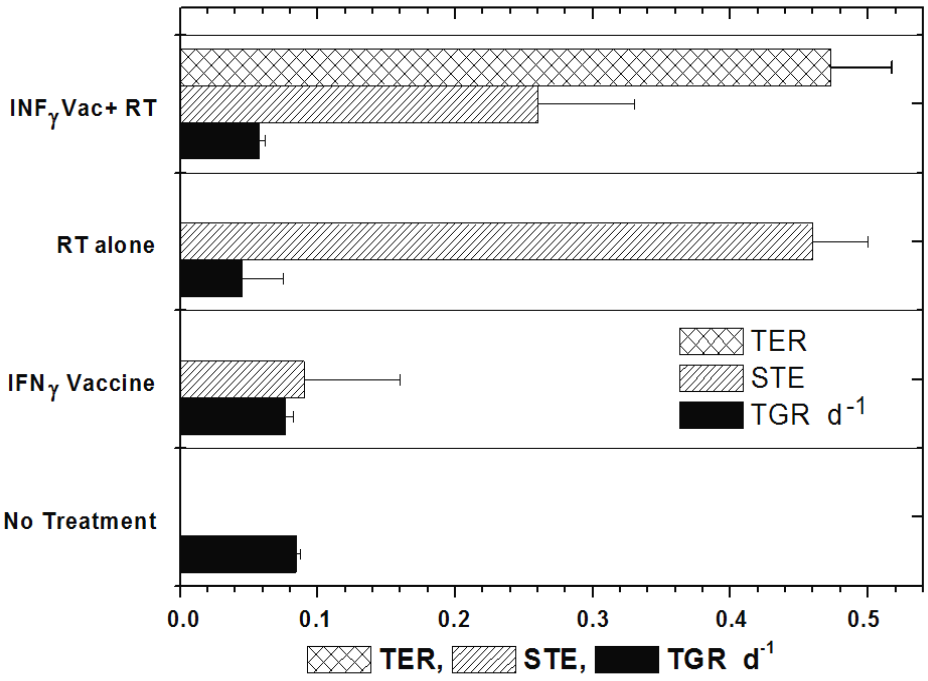

Figure 5. Results of Tumour Growth Rate of the right-lateral irradiated tumour of the study [11]. The specific therapeutic effect (*STE*) is defined as the tumour growth rate difference between the control and exposed tumour, divided by tumour growth rate of the controls for the same time period, and the Therapeutic Enhancement Ratio (*TER*) of the combined treatment with IFNγ-cell vaccination and RT, which is the ratio of the specific therapeutic effect of the experimental combination (STE_{RT+Vac}) and the specific therapeutic effect with the hypothetical combination of the two independently applied agents (STE_{RT} + STE_{Vac}) [70].

Combination of 4 Gy × 2 RT and immunization with (GM-CSF)

Newcomb *et al.* [23] performed a study combining radiation therapy and vaccination by using modified autologous tumour cells in mice with intracerebrally established invasive GL261 glioma. The animals were treated with two fractions of radiation therapy (2 × 4 Gy) to the whole brain after which peripheral vaccination with cells transfected to secrete *granulocyte-macrophage colony-stimulating factor* (GM-CSF) was performed. Antitumour immunity was associated with an increased number of tumour-infiltrating lymphocytes (TIL) in brain tumours and increased tumour-specific production of IFNγ. In mice given radiation therapy or vaccination alone, less than 10% increase in survival time was observed. But by combining radiation therapy and vaccination a highly significant increase in the survival time, of about 40-80%, was observed. Five out of the 6 surviving animals (≈83%) acquired antitumour immunity, which was observed by the rejection of tumour challenge [23]. These results are in good agreement with the results of (75%) long term survivals and acquired antitumour immunity in N29 rats treated with the combination of radiation and immune therapy with cells secreting IFNγ [25].

Combination of (5 or 15 Gy) × 1 RT and immunization with IFN-γ secreting tumour cells

Persson *et al.* presented a study of single-fraction radiation therapy with 5 or 15 Gy Co-60 gamma radiation, combined with intra-peritoneal injections of syngeneic interferon gamma (IFNγ)-transfected cells in rats with intra-cerebral N29 or N32 glioma tumours at days 7, 21 and 35 after inoculation [25]. For intra-cerebral N29 tumours, single-fraction radiation therapy with 5 or 15 Gy had no significant effect on the survival time. However, a single fraction radiotherapy session of 5 or 15 Gy combined with immunization by i.p. injection of irradiated syngeneic tumour cells induced a significant anti-tumour response to intra cranial implanted glioblastoma tumours in Fischer-344 rats. In the

group inoculated with N29 tumour cells and treated with 5 Gy RT combined with immunization, the median survival time was significantly increased by 87% ($p = 0.003$), and 75% of the animals survived for more than 170 days. But in those treated with 15 Gy the median survival time was increased by 45% ($p = 0.03$) and 50% of the animals survived for more than 170 days. The surprisingly worse results with 15 Gy might be due to an increased level of immune suppressive T_{reg} cells present at higher absorbed dose [66-67].

In the rats that were inoculated with N32 tumour cells, the combination of single fraction irradiation of 5 Gy with immunization of IFNγ secreting syngeneic cells resulted in no increase in survival time. Irradiation with 15 Gy resulted in an increased median survival time of about 40% ($p < 0.001$). But none of these rats survived longer than 30 days. The difference in response of N29 and N32 cell lines indicate that there are differences in immune response in the different clones of glioma [25].

Combination of 4 Gy × 2 RT and anti-CD137 antibodies

CD137 is a member of the tumour necrosis factor (TNF) receptor family and a potent T cell co-stimulatory molecule [72]. The immune response induced by CD137 monoclonal antibodies (BMS-469492, Bristol-Meyer Squibb) directed to the co-stimulatory molecule CD137 has shown to generate effective antitumor responses in several animal models and in clinical trials [73-75]. Treatment of murine lung (M109) and breast (EMT6) carcinoma with CD137 monoclonal antibodies (BMS-469492) generates tumour growth retardation of 3 days in M109 tumours and of 12.5 days in EMT6 tumours. In combination with radiation therapy, however, the tumour responses were enhanced in both tumour models [76].

A recent study in mice with intracerebrally established invasive GL261glioma has applied the combination of radiotherapy with anti-CD137 antibody directed to the co-stimulatory molecule CD137 [77]. The mice were treated with two fractions (2 x 4 Gy) of radiation therapy to the whole brain. Non-specific rat IgG or anti-CD137 mAb was administered either alone or in combination with RT.

The results summarized in Table 1 show that the combination of radiation (4 Gy × 2) with anti-CD137 therapy resulted in complete tumour eradication and prolonged survival in six out of nine (67%) mice with established brain tumours ($p < 0.001$). Five of the six long-term survivors in the combination group demonstrated acquired antitumour immunity by rejecting challenge tumours. Antitumour immunity was associated with an increased number of tumour-infiltrating lymphocytes (TILs) in brain tumours and increased tumour-specific production of IFNγ.

Newcomb *et al.* [24] also tested the combination of radiotherapy (2 × 4 Gy) with immunotherapy by using a rat IgG2a monoclonal antibody against mouse CD137 (BMS-469492, clone 1D8 produced and purified by Bristol-Myers Squibb, Princeton, NJ). The antibody is directed to the co-stimulatory molecule CD137 that showed effective anti-tumour responses generated in various animal models of cancer [78]. The combination of radiation and anti-CD137 therapy resulted in complete tumour eradication and prolonged survival in six out of nine (67%) mice with established GL261 glioma brain tumours ($p < 0.001$). Five out of the six (83%) long-term survivors in the RT combination group demonstrated acquired antitumor immunity which was observed by rejection of challenge tumours in the mice. Antitumor immunity was associated with an increased number of tumour-infiltrating lymphocytes (TILs) in brain tumours and increased tumour-specific production of IFNγ. In view of the finding that radiation enhanced the antitumor effect of anti-CD137 therapy and since anti-CD137 therapy is already used in clinical trials, the combination with local hypo-fractionated (2 x 4 Gy) radiation seems to be a good approach for clinical translation [24, 77].

Combination of 12 Gy × 1 RT with blockade of the CTLA-4 pathway

The cytotoxic T lymphocyte-associated protein CTLA-4 is involved in the immune regulatory mechanisms that have key roles in the negative regulation of T-cell activation and anti-tumour immune response [79]. It has previously been demonstrated that blockade of the CTLA-4 protein enhances anti-tumour responses both in experimental

Table 1. Median survival time of rats, with 9 animals in each group, after the different types of treatments [77].

Type of treatment	Survival time (days)	Number of animals surviving > 120 days
IgG	31	0
Anti-CD137	42	0
RT (4 Gy×2) alone	No data	No data
IgG + RT (4 Gy×2)	37	2
Anti-CD137 + RT (4 Gy×2)	114	6 (67%)

systems and in clinical trials [80-81]. Another investigation on the effects of systemic CTLA-4 blockade with monoclonal antibody (9H10) to CTLA-4 employed in a mice model with well-established glioma showed that CTLA-4 blockade confers long-term survival in 80% of treated mice [82].

In a mouse model of the poorly immunogenic metastatic mouse mammary carcinoma 4T1, however, neither anti-tumour response nor survival-time was affected by using an anti-CTLA-4 monoclonal antibody for blocking the CTLA-4 protein. But in combination, a single fraction of radiation therapy (12 Gy) with the anti-CTLA-4 monoclonal antibody administration 1, 4, and 7 days after RT, inhibition of the growth of the primary irradiated tumour was observed. Also the survival-time of the mice was significantly increased from 40 to 49 days ($p < 0.0005$) by this combined treatment. The elicited antitumor immune response by the combined treatment was also effective in the inhibition of lung metastases [28, 83-84]. Thus the combination of local RT with CTLA-4 blockade might be applied as radio-immune-modulating therapeutic strategy.

Combination of 10 Gy × 1 single fraction RT combined with immunotherapy with PSA vaccine

Listeria monocytogenes (Lm)-based PSA vaccines (ADXS31-142) have been shown previously to be highly efficient in stimulating anti-tumour responses to impact on the growth of established tumours in different tumour models [85]. A randomized phase II clinical trial has shown that the Lm-based vaccine can be safely given in patients undergoing fractionated radiation therapy for localized prostate cancer, with the majority of patients generating a PSA-specific cellular immune response to the vaccine [13].

Hannan *et al.* [33] combined immunotherapy with Lm-based PSA vaccine (ADXS31-142), with a single fraction of 10 Gy radiation therapy in a mouse model of prostate cancer [33]. Mice bearing PSA-expressing TPSA23 tumour were divided into 5 groups receiving: no treatment, ADXS31-142, single fraction RT (10 Gy), control Listeria vector, and combination of ADXS31-142 and single fraction RT (10 Gy). A tumour growth curve was generated by measuring the tumour volume biweekly. Tumour tissue, spleen, and sera were harvested from each group for IFNγ ELISpot, intracellular cytokine assay, tetramer analysis, and immune-fluorescence staining. There was a significant ($p < 0.0001$) tumour growth delay in mice that received combined ADXS31-142 and RT treatment as compared with mice of other cohorts, and this combined treatment caused complete regression of their established tumours in 60% of the mice. By extracting tumour volume data from the original publication [33] the tumour growth of the different groups is displayed in Figure 6.

Tumour Growth Rate is estimated from the tumour volume measurement data of each individual tumour, fitted to a model of exponential growth from the day of radiation treatment. The Specific Therapeutic Effect (*STE*) is defined as the tumour growth rate difference between the control and exposed tumour, divided by tumour growth rate of the controls for the same time period. The therapeutic enhancement ratio (*TER*) of the combined treatment Vaccination and RT is the ratio of the specific therapeutic effect of the experimental combination (STE_{Vac+RT}) and the sum of the individual specific therapeutic effects ($STE_{Vac} + STE_{RT}$) [70]. Those parameters have been derived from fitting of the experimental values of Figure 6 and are displayed in Figure 7.

MHC tetramer analysis [86] indicated large increase in PSA-specific CTLs in animals receiving Listeria PSA vaccine ADXS31-142 alone. But the therapeutic effect was limited (STE = 0.15 ± 0.01), probably due to the immunosuppressive effect by the tumour. The therapeutic effect of RT alone was somewhat larger but still rather low (STE = 0.29 ± 0.01). The combination of RT + Listeria PSA vaccine, however, gives a largely enhanced therapeutic effect (STE = 0.96 ± 0.07). The reason is probably that the single 10 Gy fraction of RT mute the immune-suppressive effect of the tumour and enhance the proliferation and infiltration of tumour specific CTL. The Therapeutic Enhancement Ratio of the combined treatment is TER = 2.2 ± 0.2, which indicates a strong synergistic effect. Thus the combination therapy with RT and Listeria PSA vaccine causes significant tumour regression by augmenting PSA-specific immune response and it could serve as a potential clinical treatment regimen for prostate cancer [33].

Clinical studies of combining radiation with immune therapy

Combination of conventional RT and TNFerade therapy

TNFerade is a biologic adeno-vector with a radiation-inducible promoter, carrying the human tumour necrosis factor-alpha gene, which has been studied in patients with solid tumours [87-88]. The goal of this first study was to determine the safety and toxicity of TNFerade in combination with radiation therapy. TNFerade was administered weekly by intratumoural administration for 6 weeks with concomitant radiation (30 to 70 Gy). Overall, 21 out of 30 patients (70%) demonstrated objective tumour response (five complete responses, nine partial responses, and seven minimal responses). In four of the five patients with synchronous lesions, a differential response between lesions treated with TNFerade + radiation compared with radiation only was observed [87-88].

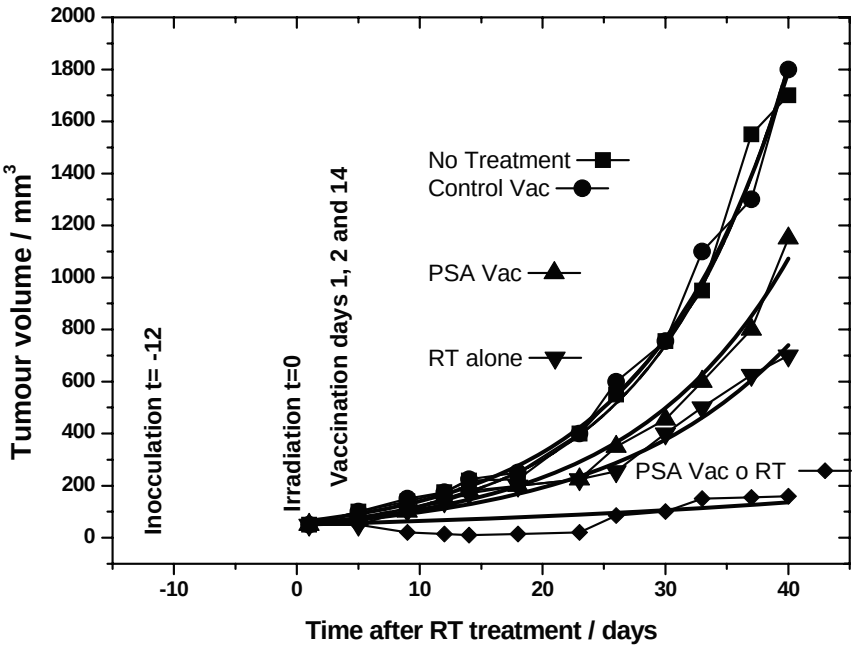

Figure 6. Tumour regressions upon combined RT and PSA vaccine treatment. C57/B6 mice (n = 50) bearing palpable TPSA23 tumours (5 mm in diameter) were randomized to one of the five treatment arms: No treatment, Control Lm vaccine, PSA vaccine (PSA Vac), RT alone, and PSA vaccination + RT. A single fraction RT (10 Gy) was given on day 0, and vaccines (CFU) were administrated on days 1, 7, and 14. Tumour volume was measured until the study endpoint (20 mm in diameter) was reached. The mean tumour volume from each group estimated from Figure 1 in Hannan *et al.* [33] is shown in the figure. The solid lines show the results of fitting the tumour volume data (TV) to the equation: $TV = 50 \cdot \exp(TGR \cdot t)$ where TGR is the tumour growth rate day^{-1}.

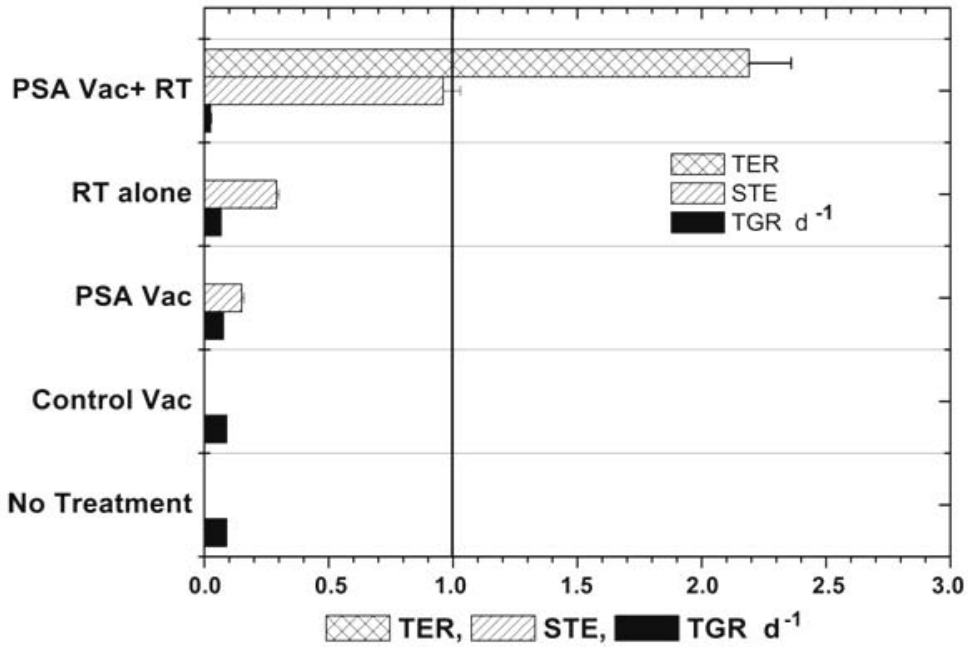

Figure 7. Tumour Growth Rate is estimated from the tumour volume measurement data of Hannan *et al.* [33] fitted to a model of exponential growth (Figure 6) from the day of radiation treatment [33]. The Specific Therapeutic Effect (*STE*) is defined as the tumour growth rate difference between the control and exposed tumour, divided by tumour growth rate of the controls for the same time period. The Therapeutic Enhancement Ratio (*TER*) of the combined treatment of two therapeutic agents Vaccination and RT is defined as the ratio $STE_{RT+Vac}/(STE_{RT} + STE_{Vac})$ [70].

Another study was to assess the tolerance of combining TNFerade and radiation therapy in patients with soft tissue sarcomas of the extremity [89]. TNFerade was administered in combination with single-daily fractionated radiation therapy in 14 patients with soft tissue sarcoma of the extremities. Eleven patients (85%) showed objective or pathological tumour responses (2 complete and 9 partial), and one had stable disease. Partial responses were achieved despite some of these tumours being very large (up to 675 cm^2). Of the 11 patients who underwent surgery, 10 (91%) showed a pathological complete response/partial response. TNFerade + radiation therapy was well tolerated in the treatment of patients with soft-tissue sarcoma of the extremity. The high number of objective responses observed warrants additional studies of this approach in a larger controlled prospective trial [89].

A further study has been performed to evaluate the feasibility and tolerability of weekly intratumoral TNFerade (TM) injections combined with concurrent chemotherapy with *Capecitabine* and radiotherapy in the treatment of patients with locally advanced rectal cancer. The result was complete pathologic response observed in 2 out of 9 patients [90].

Combination of conventional RT (RT/TMZ) and WT1-immunotherapy

Like many other solid tumours, glioma has been found to express a protein characteristic for Wilms' tumour 1 (WT1) [91]. A peptide based immunotherapy targeting the WT1 gene has successfully been used in patients with recurrent glioma. The clinical response indicates that CD8+ CTL are the main effectors of this WT1 vaccination [92]. A phase II clinical trial of the WT1 vaccination for patients with recurrent malignant glioma resulted in a partial response rate of 9.5% but no complete response. The median length of period with progression-free survival was 20 weeks [93].

In planning for a clinical trial of WT1 vaccination involving patients with newly diagnosed malignant glioma, the goal was to combine concurrent radiation/TMZ therapy with WT1 immunotherapy. The critical question is, however, if the depletion

of lymphocytes caused by the current standard radiation/TMZ treatment is a drawback for a combination with WT1 immunotherapy. Therefore a clinical study was performed in order to determine how the concomitant radiation/TMZ therapy affects the WT1-specific T-cells and other T-cells in terms of their frequencies and total numbers. This study concluded that, even after the decrease of the absolute numbers of lymphocytes, the fraction of WT1 specific T-cells was stable. They concluded that it may be possible to apply WT1 immunotherapy after the end of 6 weeks of radiation/TMZ therapy [46].

In another clinical study of 8 patients with primary glioma it was found that concomitant radiation/ TMZ therapy integrated with autologous dendritic cell-based immunotherapy was feasible and well tolerated. The median progression-free survival (PFS) was 75% at 6 months and 50% at 18 months. The median time of survival for all patients was 24 months. One patient was still free from progression or recurrence at 34 months [94].

Combination of conventional RT 2 Gy × 30 with AFTV vaccination therapy

Autologous formalin-fixed tumour vaccine (AFTV) was prepared from formalin fixed and/or paraffin embedded glioma tumour tissue obtained on surgery and premixed with original adjuvant. In a clinical pilot study of 12 patients, the autologous tumour vaccine (AFTV) was inoculated at least 4 weeks after the primary conventional glioma treatments were concluded. Of these 12 patients, four responded to the AFTV therapy, one showed a complete response, one showed a partial response, two showed minor responses, and one had stabilization of disease. The median survival period was about 11 months from the initiation of the AFTV treatment. But three of these patients survived for 20 months or more after AFTV inoculation [95].

In a subsequent phase I/IIa clinical trial, the AFTV was inoculated in 24 patients with newly diagnosed glioblastoma multiforme, in combination with conventional fractionated radiotherapy. The treatment protocol in that study included aggressive tumour resection, fractionated radiotherapy, 2 Gy per fraction up to a total dose of 60 Gy, and 3 concomitant courses of AFTV administered with an interval of one week during the last 3 weeks of irradiation. The median duration of overall survival was 21.4 months (95% CI 13.8–31.3 months). The actuarial 2-year survival rate was 40%. These results demonstrate that vaccine treatment in combination with fractionated radiotherapy may be effective in patients with newly diagnosed glioblastoma [96].

The outcome of the phase I/IIa clinical trial might have been more successful if it had been combined with hypo-fractionated radiation therapy (8 Gy).

Combination of IMRT 30-60 Gy with DC-T-cell immune therapy

Hasumi *et al.* [97] treated 26 patients, who had recurrent or stage IV malignancies that failed prior standard surgical and/or adjuvant therapy, with *intensity modulated radiotherapy* (IMRT) combined with dendritic cell-based immunotherapy. They hypothesized that radiation would lower the tumour burdens, decrease the number and function of regulatory cells in the tumour environment, and release products of tumour cells that could be acquired by intratumoral injected immature dendritic cells (iDC). Three days after injection (day 0) with autologous iDC combined with a cytokine-based adjuvant and KLH (keyhole limpet hemocyanin), followed 24 h later by i.v.-infused activate T-cells (expanded with anti-CD3 and IL-2) of the injected lesions, the tumour was treated during days 8-12 with IMRT up to 30-60 Gy fractionated radiation with absorbed dose fractions in the range of 4-14 Gy. On day 19 and 29, IMRT was followed by another injection of intratumoral iDC and i.v.-infused activated T-cells. No toxicity was observed with cell infusion while radiation-related toxicity was observed in seven patients. Five patients had progressive disease, eight demonstrated complete resolution at treated sites but developed recurrent disease at other sites, and 13 showed complete response at various follow-up times with an overall estimated Kaplan-Meier disease-free survival of 345 days. Most patients developed KLH (keyhole limpet hemocyanin) antibodies supporting their hypothesis that the co-injected iDC are functional with the capacity to acquire antigens from their environment and generate an adaptive immune response [97].

Combining a recombinant cancer vaccine with standard definitive radiotherapy

Gulley *et al.* [13] presented a randomized phase II clinical trial designed to determine if a poxviral vaccine encoding prostate-specific antigen (PSA) can induce a PSA-specific T-cell response when combined with radiotherapy in patients with clinically localized prostate cancer. Thirty patients were randomized in a 2:1 ratio into vaccine plus radiotherapy or radiotherapy-only arms. Those patients in the combination arm received a "priming" vaccine with recombinant viral (rV) PSA plus rV containing the T-cell co-stimulatory molecule B7.1 (rV-B7.1) followed by monthly booster vaccines with recombinant fowl pox PSA. The rV-PSA was constructed by insertion of the entire human PSA gene into the viral genome, whereas the rV-B7.1 was constructed by insertion of the entire human B7.1 co-stimulatory molecule gene into the viral genome. The vaccines were given with local granulocyte-macrophage colony-stimulating factor and low-dose systemic interleukin-2. Standard fractionated external beam radiation therapy, with 2.0 Gy per fraction to 70 Gy, was given between the fourth and the sixth vaccinations.

Seventeen of the 19 patients in the combination arm completed all eight vaccinations and 13 of these 17 patients had at least 3-fold increases in PSA-specific T-cells versus no detectable increases in the radiotherapy-only arm ($p < 0.0005$). The vaccine regimen was given to patients undergoing radiation therapy for localized prostate cancer, and the majority (76%) of patients generated a PSA-specific cellular immune response to vaccine although with no complete tumour regression [13]. Long-term follow-up of these prostate cancer patients treated with vaccine and definitive radiation therapy demonstrated that vaccine combined with RT does not appear to have significant differences with regard to PSA control or late-term toxicity compared with standard RT treatment. They also found limited evidence of a long-term immune response following vaccine therapy [98].

This study clearly demonstrates the detrimental effect of conventional fractionated RT upon the immunogenic tumour cell death. The outcome of this clinical phase II trial might have been more successful if it had been combined with hypo-fractionated radiation therapy (8 Gy).

Other clinical trials of combining radiation therapy and immune-therapy with vaccine are in progress. One study evaluates the safety and effects of vaccine treatment plus radiation to the liver in patients with solid tumours that have spread to the liver [99-100]. Another study aims to determine if combined treatment with PSA/TRICOM vaccine and 153Sm-EDTMP radiation can delay progression of prostate cancer better than radiation alone [100].

CONCLUSION

The above reviewed preclinical studies of co-operative radio-immune-stimulating therapy clearly demonstrate that this is a very challenging new cancer therapy regime. Ongoing clinical trials of immunotherapy combined with conventional radiation therapy are giving promising preliminary results [20, 101-102]. The clinical study of combining IMRT 30-60 Gy with DC-T-cell Immunotherapy supports the hypothesis that hypo-fractionated radiation therapy co-operates with co-injected iDC to acquire antigens from tumour environment and generate an adaptive immune response [97].

But still there seems to be no clinical study in progress fully adopting the co-operative concept of an 8 Gy single fraction external RT combined with an effective established immune therapy regime, although several preclinical studies urge for this [23-25, 31, 33, 102-103]. The most realistic approach for starting clinical testing might be giving IMRT in gradually decreasing number of fractions (from 7 to 1) in combination with immune therapy.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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