Systemic delivery of a TLR7 agonist in combination with radiation primes durable anti-tumor immune responses in mouse models of lymphoma

Simon J. Dovedi1, Monique H. M. Melis1, Robert W. Wilkinson3, Amy L. Adlard2, Ian J. Stratford2, Jamie Honeychurch+1, Timothy M. Illidge*1

+ J.H and T.M.I contributed equally to this work

1 Targeted Therapy Group, School of Cancer and Enabling Sciences, University of Manchester, Manchester Academic Health Sciences Centre, United Kingdom

2 Experimental Pharmacology Group, School of Pharmacy, University of Manchester, Manchester Academic Health Sciences Centre, United Kingdom

3 AstraZeneca, Alderley Park, Macclesfield, United Kingdom

* To whom correspondence may be addressed. Email: tmi@man.ac.uk

Prof. Tim Illidge
Professor of Targeted Therapy and Oncology
School of Cancer and Enabling Sciences
University of Manchester
Manchester Academic Health Science Centre
Manchester Cancer Research Centre
The Christie NHS Foundation Trust
Wilmslow Road, Manchester, M20 4BX
Direct line: 0161 446 8110
Academic PA: 0161 446 3473
Fax: 0161 446 3269
Abstract

Passive immunotherapy with monoclonal antibodies has improved outcome for patients with B cell malignancies although many still relapse and little progress has been made with T cell malignancies. Novel treatment approaches are clearly required in this disease setting. There has been much recent interest in developing therapeutic approaches to enhance anti-tumor immune responses by using novel immunomodulatory agents in combination with “standard” of care treatments. Here, we report that intravenous administration of the TOLL-like receptor (TLR)-7 agonist, R848 in combination with radiation therapy (RT) leads to the long standing clearance of tumor in T and B cell lymphoma bearing mice. In combination, TLR7 / RT therapy leads to the expansion of tumor antigen-specific CD8+ T cells and improved survival. Furthermore those mice that achieve long-term clearance of tumor following TLR7 / RT therapy are protected from subsequent tumor rechallenge by the generation of a tumor-specific memory immune response. Our findings demonstrate the potential for enhancing the efficacy of conventional cytotoxic anti-cancer therapy through combination with a systemically administered TLR7 agonist to improve anti-tumor immune responses and provide durable remissions.
Introduction

Passive immunotherapy with monoclonal antibodies have improved the outcome for patients in a wide range of B cell malignancies. Despite this success, little or no progress has been made with T cell lymphoma and furthermore many patients with B cell lymphoma continue to relapse and develop progressive disease following treatment. Clearly additional therapeutic approaches are urgently required. Recent approaches exploiting novel active immunotherapies have focused on developing therapeutic vaccines or immunomodulatory antibodies and small molecules that facilitate the generation of durable anti-tumor immune responses. To date several immunomodulatory approaches have demonstrated efficacy in preclinical and early phase clinical studies including anti-CTLA4 and anti-CD40 mAb, and small molecule agonists of the TOLL-like receptor (TLR) family member TLR9.

TLRs recognise a diverse repertoire of highly evolutionarily conserved pathogen-associated molecular patterns (PAMPs) present on foreign pathogens and are constitutively expressed by both professional antigen presenting cells (APCs) such as the dendritic cell (DC) and macrophage, and by effector B, T and natural killer (NK) cell populations. Signalling through individual TLRs can direct the phenotype of the ensuing immune response through modulation of cytokine production and polarisation of CD4⁺ helper, CD8⁺ effector and TReg cell responses. Agonism of TLR7, which is localised intracellularly to endosomal membranes by viral guanosine and/or uridine-rich single-stranded RNA, or by synthetic agonists, can engender strong T_H-1 biased immune responses through the activation of plasmacytoid DC and myeloid DC subsets. This phenotype of response is optimal for eliciting durable anti-tumor immune responses as it supports the activation and expansion of CD8⁺ T cells. Currently, Imiquimod (Aldara 5% cream, 3M) is the only
TLR7/8 agonist to receive FDA approval and is licensed in oncology for the topical treatment of basal-cell carcinoma and other dermatological malignancies (reviewed in 16). With the exception of therapeutic vaccination approaches, topical administration of TLR agonists are of limited utility in the treatment of the majority of solid tumors and in settings of non-dermatological disseminated disease.

In this study we have investigated intravenous administration of the TLR-7 agonist R848 in combination with radiation therapy (RT) in syngeneic models of T and B cell lymphoma. We hypothesized that the combination of a systemically administered TLR-7 agonist in combination with local RT would prime systemic anti-tumor immune responses. In this setting RT has the advantage that most non-Hodgkin’s lymphomas are radiosensitive and importantly has minimal deleterious systemic effect on immune effector cells in contrast to the immunosuppression seen with chemotherapy. Here we demonstrate that intravenous administration of R848 can enhance the therapeutic efficacy of RT by the generation of durable anti-tumor CD8+ T cell dependent immune responses leading to long-term survival and to the induction of protective immunological memory. This study provides proof of principle for translation to early phase clinical trial.
Methods

Mice and cell lines. C57Bl/6 and BALB/c mice were obtained from Harlan, U.K.. All animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. The B cell lymphoma line A20, the T cell lymphoma line EL4 and its ovalbumin expressing derivative EG7 were obtained from ATCC and maintained in RPMI-1640 medium supplemented with 10 % FCS, 1 % L-glutamine (Invitrogen, U.K.) and 50 μM 2-ME (Sigma, U.K.). EG7 media was also supplemented with 0.5 mg/ml G418 (Invitrogen, U.K.). All cell lines were routinely screened for Mycoplasma contamination.

Measurement of systemic immune activation by R848. Tumor bearing mice received a single i.v. dose of R848 (Alexis Biochemical, U.K.) at 3 mg/kg, 7 days after tumor implantation. At 2 and 4 hours post administration of R848, mice were euthanized and spleens harvested for profiling of CD69 expression on T and B cells (CD69, eBiosciences, U.K.; CD4, CD8 and CD19, BD Pharmingen, U.K.). DC activation was assessed by expression of CD40, class II MHC and CD80 (BD Pharmingen, U.K.). Plasma was isolated from peripheral blood and assayed for IL-1α, IL-2, IL-5, IL-6, IL-10, IL-12p70, IFNγ, TNFα, GM-CSF, IL-4 and IL-17 expression using a multiplex bead-based analyte detection system (Bender Medsystems, U.K.). Experimental groups contained 5 mice / group.

Tumor therapy. Mice were inoculated s.c. with either 3x10^6 EG7, 2x10^5 EL4 or 5x10^6 A20 cells. Irradiations were performed 7 days after inoculation (when tumors were approximately 100 mm^3) using a Pantak HF-320 320 kV x-ray unit (Gulmay Medical, U.K.). The machine was operated at 300 kV, 9.2 mA, with filtration fitted in
the x-ray beam to give a radiation quality of 2.3 mm Cu half-value layer. Mice were positioned at a distance of 350 mm from the x-ray focus, where the dose rate was 0.80 Gy/min. On day 7 after tumor inoculation, R848 was administered i.v. at a dose of 3 mg/kg in a dose volume of 50 μl/10 g in PBS and repeated q1w for up to 5 weeks. For B cell depletion, mice received 250 μg anti-CD20 mAb (IgG2a clone 18B12, a gift from Robert Dunn, Biogen-Idec, U.S.) as outlined in Supplemental Fig. S3A. For CD8 depletion experiments, mice were treated with a depleting antibody, YTS169; a gift from M. Glennie, Southampton University, as outlined in Supplemental Fig. S3B. For tumor rechallenge experiments, long-term surviving mice were implanted contralaterally with either EG7 or EL4 a minimum of 60 days after previous tumor implantation. Additional control mice were also implanted to confirm tumor growth. Experimental groups contained at least 5 mice / group and are representative of at least 2 independent experiments.

**Measurement of IFNγ production by CD8+ T cells isolated from long-term surviving mice.** For *in vitro* stimulation 3.5x10^6 splenocytes from either long-term surviving mice or control mice were cultured for 5 days in RPMI-1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 % L-glutamine, 50 μM 2-ME and 10 IU/ml human recombinant IL-2 in the presence of either 1x10^6 EG7 cells irradiated with 25 Gy or 1 μmol/ml SIINFEKL peptide (Anaspec, U.K.) in 12 well or 24 well plates respectively. Experimental groups contained at least 3 mice and are representative of 2 independent experiments. After 5 days in culture, cells were restimulated at a 1:1 ratio with 25 Gy irradiated EG7s for 16 hours in the presence of 3 μg/ml Brefeldin A (BD Pharmingen, U.K.) and 100 IU/ml human recombinant IL-2 (Chiron, NL). For FACS analysis, cells were washed and incubated with rat anti-
CD16/32 (eBioscience, U.K.) to block non-specific binding and then stained with a FITC conjugated anti-CD8α mAb. Cells were then fixed/permeabilized and stained for expression of IFNγ using an APC conjugated mAb (BD Pharmingen, U.K.).
Results

Systemic administration of R848 leads to activation of T and B lymphocytes, induction of cytokine expression and increased long-term survival in EG7 tumor-bearing mice.

In previous published studies, R848 has been administered locally either by subcutaneous/intra-tumoral injection or by transdermal absorption. To determine whether i.v dosing of R848 had the capacity to induce systemic immune activation in tumor-bearing mice, spleens and plasma were harvested 2 and 4 hours after a single i.v. treatment. R848 was well tolerated when dosed systemically at 3 mg/kg and induced the expression of the early activation marker CD69 on T (Fig. 1A) and B (Fig. 1B) lymphocytes at both 2 and 4 hours after dosing when compared to vehicle-treated controls ($P < 0.001$ two-tailed Student’s $t$ test). The systemic induction of cytokines in the serum of treated and control mice were also measured by multiplex bead array (Fig. 1C). We found that 2 hours after i.v. administration of R848, plasma concentrations of IL-5 increased by 7 fold, IL-6 by approximately 3000 fold and levels of TNFα, undetectable in untreated mice, also increased significantly ($P < 0.001$ two-tailed Student’s $t$ test). By 4 hours, levels of IL-6 had dropped by approximately 10 fold but remained 360 fold higher than in time-matched vehicle-treated mice. In addition, the expression of TNFα had dropped down to baseline levels and expression of the Th1 cytokine IFNγ was found to be significantly elevated ($P < 0.01$ two-tailed Student’s $t$ test).

To determine whether systemic delivery of a TLR7 agonist could lead to therapeutic anti-tumor responses, mice bearing EG7 tumors were treated with either a single or weekly i.v. dose of R848 as a monotherapy. Our data demonstrated that whilst ineffective at controlling tumor growth when administered as a single dose ($P$
> 0.05 Log-rank (Mantel-Cox) test), systemic dosing of R848 moderately improves survival over vehicle-treated control mice when dosed weekly (q1w) ($P < 0.05$ Log-rank (Mantel-Cox) test) (Fig. 1D). Using MTS and annexin V/PI assays we confirmed in vitro that R848 was not directly affecting the proliferation or viability of the tumor cells (Supplemental Fig. S1A and B), suggesting that the anti-tumor activity of systemic TLR7 therapy may have been immune mediated. These data demonstrate that i.v. administration of R848 leads to activation of T and B cells commensurate with the origination of a pro-inflammatory cytokine milieu in tumor bearing mice and that monotherapy with R848 provides a modest increase in the survival of EG7 tumor-bearing mice.

**R848 in combination with RT improves survival in a model of T cell lymphoma.**

Local RT delivered as a single 10 Gy dose to the tumor improves long-term survival in mice bearing sub-cutaneously implanted EG7 tumor (Fig. 2A) ($P < 0.05$ Log-rank (Mantel-Cox) test). The anti-tumor efficacy was however substantially improved with systemic administration of R848 in combination with RT (Fig. 2A and B). R848 when dosed q1w for 5 weeks but not as a single i.v. dose in combination with RT enhances the efficacy of local RT leading to a substantial increase in the frequency of long-term surviving (LTS) mice (defined as mice surviving greater than 60 days post therapy) when compared to both control mice (75 % of mice classified as LTS following treatment with RT and R848 q1w vs. 4% of control mice) ($P < 0.001$ Log-rank (Mantel-Cox) test) and to mice receiving monotherapy with either 10 Gy RT or R848 dosed q1w (75 % of mice classified as LTS following treatment with RT and R848 q1w vs. 25 % of mice treated with either monotherapy alone) ($P < 0.05$ Log-rank (Mantel-Cox) test) (Fig. 2A and B). This data demonstrates that the therapeutic
efficacy of RT and the ability to bring about long-term tumor control can be substantially augmented by combination with a systemically delivered TLR7 agonist.

**Long-term clearance of tumor with R848 and RT is CD8⁺ T cell dependent.**

We next investigated the mechanisms underlying this long-term tumor control observed following combination RT and R848 therapy. Initially colony forming assays were used to confirm that R848 was not acting as a radiation sensitizer directly through interaction with the tumor cells. We found that addition of 10 μM R848 to EG7 or EL4 cells irradiated at doses of up to 10 Gy *in vitro* did not effect clonogenic survival when compared to irradiation alone (Supplemental Fig. S2A and B). As the systemic administration of R848 leads to the potent activation of B lymphocytes (Fig. 1B) and induction of high levels of IL-6 expression (Fig. 1C) we first assessed the potential role for B lymphocytes in mediating the anti-tumor efficacy observed following combination therapy in the EG7 model (Fig. 3A). Depletion of B lymphocytes using an anti-CD20 mAb did not impact the therapeutic efficacy of this novel combination (*P* > 0.05 Mann Whitney test). Depletion of B lymphocytes was confirmed by flow cytometry on peripheral blood samples (Supplemental Fig. S3A).

Using pentamers specific for the immunodominant class I MHC restricted ovalbumin epitope SIINFEKL, we then determined the impact of combination therapy on the induction of tumor antigen-specific CTLs in mice bearing EG7 tumors (Fig. 3B). We observed that the frequency of SIINFEKL-restricted CTLs in the peripheral circulation was significantly higher (approximately 2 fold) 6 and 10 days post treatment with RT and R848 when compared to time-matched non-treated control mice (*P* < 0.05 two-tailed Student’s *t* test). By day 20, there was no significant difference in the frequency of SIINFEKL-restricted CTLs between mice treated with
RT and R848 and non-treated control mice ($P > 0.05$ two-tailed Student’s $t$ test).

Using depleting antibodies we explored the role of effector T cells in mediating the efficacy of combination RT and R848 therapy in the EG7 model. Whilst the depletion of CD4 T lymphocytes had no effect (data not shown), the depletion of CD8$^+$ T cells completely abrogated the therapeutic efficacy of combination RT and TLR7 agonist therapy (Fig. 3C). Depletion of CD8$^+$ T lymphocytes was confirmed by flow cytometry on peripheral blood samples (Supplemental Fig. S3B).

RT is one of a number of effective anti-cancer treatments that result in cellular stress and the expression of a plethora of damage-associated molecular patterns (DAMPs). Those DAMP’s recently identified include calreticulin, High Mobility Group Box 1 (HMGB1), and the extracellular release of ATP, which can lead to the activation of APCs such as the DC and engender tumor antigen-specific T cell responses $^{17-19}$. We therefore measured the release of the critical DAMPs that are known to be associated with immunogenic cell death and DC activation. The treatment of EG7 and EL4 cells with 10 Gy RT in vitro led to the release of the obligate immunogenic DAMP, HMGB1 but not to the expression of ecto-calreticulin (data not shown). To examine how the synthetic TLR7 agonist R848 may enhance the anti-tumor CTL response we next evaluated DC activation in response to co-culture with irradiated tumor cells in the presence and absence of agonist. Whilst the irradiated tumor cells were efficiently phagocytosed by bone marrow derived DCs following co-culture in vitro (Supplemental Fig. S4A), they were not able to induce DC maturation and no change was observed in the activation markers CD80 or CD86 ($P > 0.05$ two-tailed Student’s $t$ test) (Supplemental Fig. S4B). The addition of 1 $\mu$g/ml R848 to the co-culture medium led to potent activation of DCs; as evidenced by increased expression of CD80 and CD86, whilst maintaining a high degree of
phagocytosis of the irradiated tumor cells ($P < 0.05$ two-tailed Student’s $t$ test) (Supplemental Fig. S4A and B). Taken together these data demonstrate that the efficacy of combination therapy with RT and R848 is dependent on the activity of CD8$^+$ T cells and that in the absence of TLR7 agonism the outcome of DC interaction with irradiated tumor cells may not be sufficient to drive both their maturation and subsequent ability to prime therapeutic anti-tumor CTL responses.

**Treatment with R848 and radiotherapy generates long-lived memory T cells specific for multiple tumor-associated antigens.**

We next investigated whether immunological memory was engendered in mice treated with 10 Gy and R848 dosed q1w by harvesting splenocytes from LTS mice that had greater than 150 days of disease free survival (Fig. 4A) and then assessing the capacity of CD8$^+$ CTL to produce IFN$\gamma$ following co-culture with irradiated EG7 cells (Fig. 4B). We found that splenocytes from LTS mice had a significantly greater frequency of IFN$\gamma$-producing CD8$^+$ T lymphocytes following co-culture than that of tumor naïve controls (10.06 % ± 1.44 vs 3.02 % ± 0.48 respectively; $P < 0.01$, two-tailed Student’s $t$ test). To determine whether these memory immune responses were SIINFEKL-restricted, splenocytes were also co-cultured with SIINFEKL peptide for 5 days and then restimulated with irradiated EG7 cells for 16 hours (Fig. 4C). Although there was an increase in the frequency of SIINFEKL-restricted memory IFN$\gamma^+$ CD8$^+$ lymphocytes in mice treated with combination TLR7 and RT (3.54 % ± 1.47 (combination) vs 0.62 % ± 0.2 (control)) this was not significant ($P = 0.12$, two-tailed Student’s $t$ test). These data suggest that the immune response generated following combination RT and R848 therapy is not restricted to the usually immuno-dominant OVA epitope SIINFEKL, and may be specific for multiple tumor-associated antigens.
To test this hypothesis, LTS mice originally treated with RT in combination with R848; dosed q1w for 5 weeks, were rechallenged subcutaneously with either EG7 cells or the parental cell line EL4 (which lack expression of OVA). We found that all of the LTS mice were able to completely reject the implanted EG7 cells following contralateral rechallenge (data not shown). When LTS mice originally treated with combination RT and R848 were instead rechallenged with the parental cell line EL4, tumor growth was found to be significantly retarded ($P < 0.01$ Mann Whitney test) when compared to the naïve control mice (Fig. 4D). This data demonstrates that in addition to OVA-restricted T cell responses, memory T cells specific for an as yet undefined shared EL4/EG7 tumor-associated antigen(s) were also induced following TLR7 and RT.

**Radiation dose-fractionation further enhances the efficacy of combination RT and R848.**

Combination therapy with R848 dosed q1w and local RT delivered as a single 10 Gy fraction decreased tumor growth and increased long-term survival significantly when compared to non-treated control mice in both the EG7 (described above; Fig. 2B) and EL4 models (Fig. 5A and B) ($P < 0.01$, Log-rank (Mantel-Cox) test). However, unlike the therapeutic response observed in the EG7 model where 75% of mice were able to completely reject their tumor following treatment with local RT and R848 (dosed q1w), only 15% of EL4 tumor-bearing mice, which lack the expression of OVA were able to completely reject their tumors. There was a modest increase in survival in EL4 tumor-bearing mice treated with R848 and a single 10 Gy fraction versus monotherapy ($P = 0.058$, Log-rank (Mantel-Cox) test). Based on recent reports describing the greater immunogenicity of fractionated radiotherapy when compared to
single-fraction\(^3\) as well as reproducing the clinical delivery of RT more accurately we combined R848 (dosed q1w) with a fractionated radiation regimen comprising 10 Gy delivered in 5 fractions. Initially, we used colony forming assays to confirm \textit{in vitro} that the fractionated RT regimen was not more cytoreductive than a single 10 Gy dose of RT (data not shown). \textit{In vivo}, we observed significantly increased survival in mice receiving combination therapy with R848 and 10 Gy in 5 fractions when compared to both non-treated controls and to mice receiving either monotherapy alone in both the EG7 model (data not shown) and EL4 model (Fig. 5A and B) \((P < 0.01, \text{Log-rank (Mantel-Cox) test)}). Long-term surviving mice originally implanted with either EG7 (data not shown) or EL4 (Fig. 5C) cells and treated with a fractionated RT regimen in combination with R848 were also able to prime a memory immune response following tumor rechallenge leading to enhanced long-term survival. To expand our observations we also evaluated this combination in mice bearing established subcutaneously implanted A20 B cell lymphoma. We again determined that the combination of fractionated dose RT and weekly intravenous R848 administration significantly improved survival when compared to monotherapy with either RT or R848 \((100 \% \text{ of mice classified as LTS following treatment with fractionated RT and R848 q1w vs. 28.5 \% \text{ of mice that received RT alone vs. 16.6 \% that received R848 alone)} \) (Fig. 5D) \((P < 0.05, \text{Log-rank (Mantel-Cox) test)}). In all 3 lymphoma models, complete tumor rejection was observed in 100\% of mice following treatment with a combination of R848 (dosed q1w) and a fractionated RT regimen. This data demonstrates that the therapeutic efficacy of combination RT and TLR7 agonist therapy can be further enhanced by RT dose fractionation.
Discussion

In this study we have demonstrated that systemic administration of the imidazoquinolinamine analogue Resiquimod (R848) delivered in combination with RT leads to long-term clearance of tumor in models of T and B cell lymphoma. R848 is a TLR7-selective agonist which appears to be well tolerated when administered by i.v. injection in the mouse and can potently activate both T and B lymphocytes and induce the expression of the Th-1 cytokines IFNγ and TNFα as well as the Th-2 cytokines IL-5 and IL-6. Furthermore, our data demonstrate that weekly systemic administration of R848 in combination with RT leads to the induction of a tumor-specific CD8+ T cell response. In the EL4, EG7 and A20 models 100 % of mice treated with low dose fractionated RT (10 Gy in 5 fractions) in combination with R848 dosed q1w were able to completely reject the primary tumor. These long-term surviving mice are protected against subsequent tumor rechallenge by the induction of a tumor-specific memory immune response.

Preclinical studies have demonstrated that sub-cutaneous vaccination with tumor cells following in vitro irradiation can lead to DC activation in vivo and to the induction of CD8+ T cell responses which are dependent on the expression and/or release of DAMPs such as calreticulin and HMGB1 by tumor cells. Whilst we also detected the release of the obligate DAMP HMGB1 following irradiation of EG7 and EL4 tumor cells in vitro, co-culture of RT-treated tumor cells alone was unable to induce DC activation in our model system. Likewise we found that in situ vaccination using RT was rarely able to engender an immune response in vivo with the capacity to reject an established tumor (Fig. 2A). This latter observation is entirely in keeping with other published studies in a variety of tumor models and is presumably a consequence of tumor-derived immunosuppression; a characteristic of both clinical
and experimental malignancies, which may limit the origination and/or efficacy of a nascent anti-tumor immune response. Our data suggests that combining an effective anti-cancer treatment such as local RT with immunopotentiating agents, such as TLR agonists, may help overcome this suppressive milieu and elicit tumor-specific immune responses and improved tumor clearance.

Our data supports the hypothesis that additional manipulation of the immune system is necessary to elicit durable therapeutic anti-tumor immune responses following treatment with external beam ionising radiation. In the present study weekly systemic dosing of R848 for 5 weeks in combination with RT was associated with greater efficacy when compared to combination with a single i.v. dose. This enhancement of the anti-tumor response following treatment with an extended TLR-dosing schedule was also observed in a study that combined RT with the selective TLR9 agonist CpG oligodeoxynucleotide 1826. The precise mechanisms underlying this enhanced anti-tumor immune response are unknown but may reflect the need for regular priming of the anti-tumor immune response through repeated exposure to TLR-mediated ‘danger signals’. Although further investigation is required we speculate that this extended dosing schedule may prevent the re-establishment of an immunosuppressive tumor microenvironment and the induction of immunological anergy following an initial course of RT and TLR7 therapy.

As the systemic administration of R848 leads to the activation of both B and T lymphocytes we employed depleting monoclonal antibodies to CD20, CD4 and CD8 to delineate the role of these important immune effector cells. Importantly we observed that CD8 but not CD4 T cell or CD20 B cell depletion completely abrogated the therapeutic efficacy of the R848/RT combination. Tumor reactive, SIINFEKL-restricted CTLs could be detected in the circulation for at least 10 days after the
initiation of therapy in EG7 tumor bearing mice. This expansion of tumor-specific CD8+ T cells was found to quickly decline as the level of tumor load decreased. Interestingly, our data revealed that the immune response to irradiated EG7 cells was not restricted to ovalbumin. A large proportion of LTS mice originally implanted with EG7 cells and treated with combination RT and R848 therapy were resistant to rechallenge with EG7 cells. Moreover, a significant growth delay was observed in cohorts of LTS mice (originally bearing EG7 tumor) following rechallenge with parental EL4 cells when compared to tumor naïve control mice. Taken together, these data demonstrate the induction of a broad CD8+ T cell response to multiple tumor cell associated antigens following combination RT/R848 therapy. The generation of multiple CTL clones specific for diverse TAAs may prove beneficial for therapy. It has previously been shown that exposure of cells to external beam ionising radiation can enhance MHC class I expression and modulate the peptide repertoire available for presentation. Therefore, RT may enhance expression of multiple TAAs and provide novel TAAs leading to the generation of multiple CTL clones. The ‘danger signals’ provided by systemic TLR7 therapy may facilitate T cell priming to these antigens.

An important observation made during this study was the demonstration that the combination of R848 and fractionated RT resulted in significantly greater therapeutic efficacy in both the EG7 and EL4 models when compared to combination with a single dose RT, despite the higher radiobiological effect (RBE) and expected greater tumor cell lethality of the larger single dose. In the EL4 model, combination of a systemically administered TLR7 agonist with a fractionated course of 10 Gy RT delivered in 5 fractions of 2 Gy led to 100% of mice rejecting their primary tumor (versus 15% when systemic TLR7 therapy was combined with a single dose of 10 Gy). The mechanisms that underlie the differential efficacy observed following
combination of TLR7 therapy and either single dose or fractionated RT are currently unclear and form the basis of ongoing studies. Interestingly, a study that profiled gene-expression in breast, prostate and glioma tumors after both single dose (10 Gy) and fractionated (5 fractions of 2 Gy) RT revealed significant changes in the tumor microenvironment with several genes including multiple interferon (IFN)-related genes uniquely upregulated following fractionated therapy. Given that the induction of IFN is a major downstream effect of TLR7 agonism, combination with fractionated RT may lead to greater synergy and therapeutic response than with single-dose RT or monotherapy.

Although successful for the treatment of dermatological malignancy, the therapeutic efficacy of topically administered TLR7 agonists such as imiquimod (Aldara, Graceway Pharmaceuticals) is generally limited to the treatment site. Recently, two phase 1/2 clinical trials have evaluated intra-tumoral delivery of the TLR9 agonist PF-3512676 (Pfizer Inc.) in combination with RT in patients with B cell lymphoma and in patients with Mycosis Fungoides. Data from these two trials are highly encouraging with evidence of the induction of systemic anti-tumor immune responses following combination therapy. However, direct intratumoral administration of agents to non-cutaneous tumors is often challenging and frequently requires image-guided delivery such as ultrasound or computerised tomography. Systemic delivery of R848 was found to be well tolerated in a phase II trial of patients with chronic HCV infection. In the treatment of cancer recent clinical trials of an intravenously administered TLR7 selective agonist (852A, Pfizer) provides the proof of principle that systemic TLR7 therapy can be well tolerated and capable of inducing global immune activation further strengthening the possibility of translating the
findings from this study into early phase trials of a systemically administered TLR7 agonist in combination with RT.\textsuperscript{24,25}

In summary, this study demonstrates for the first time that the systemic delivery of a TLR7 agonist can enhance the immune response to radiation-induced tumor cell death in models of T and B cell lymphoma. The novel combination of R848 and RT leads to the generation of a tumor-specific CTL response with the capacity to both eradicate primary disease and induce long-term protective immunological memory. Our data suggest that this therapeutic combination may be a promising approach for the treatment of B and T cell malignancies and could be readily translated to early phase clinical trials.

**Acknowledgements:** We are grateful for the help of members of the Paterson Institute BRU and Flow Cytometry Core Facilities. We thank Prof. Martin Glennie and Alison Tutt for the supply of depleting antibodies. We also thank all members of the Targeted Therapy Group for helpful discussion and critical review of this manuscript. This work was funded with a grant from Leukaemia and Lymphoma Research (Ref: 08075).

**Authorship**

S.J.D. and J.H. designed the studies
S.J.D. performed research
S.J.D. wrote the paper
J.H. and T.M.I edited the manuscript

**Conflict of Interest Disclosure**

There are no conflicts of interest associated with this research.
References


Figure Legends

Figure 1. **Systemic activation of the immune system following i.v. administration of R848 in EG7 tumor bearing mice leads to increased survival.** A and B, splenocytes were harvested at 2 and 4 hours and T (A) and B (B) lymphocytes were analysed for CD69 expression by flow cytometry. C, induction of systemic cytokine responses were measured in the plasma using multiplex assays. Experimental groups contained 5 mice. *, P < 0.05, **, P < 0.01, ***, P < 0.001, two-tailed Student’s t test. D, survival curve for EG7 tumor bearing mice dosed i.v. with R848 either once or q1w. Experimental groups contained at least 7 mice and are representative of at least 2 independent experiments. *, denotes significance when compared to control mice. *, P < 0.05, Log-rank (Mantel-Cox) test.

Figure 2. **R848 enhances the therapeutic efficacy of radiation therapy.** A and B, combination of 10 Gy local RT with a single i.v. dose of R848 (A) or q1w dosing of R848 (B). Experimental groups contained at least 7 mice and are representative of at least 2 independent experiments. *, denotes significance when compared to control mice. *, P < 0.05, **, P < 0.01, ***, P < 0.001, Log-rank (Mantel-Cox) test.

Figure 3. **Therapeutic efficacy of RT and R848 combination is dependent on the activity of CD8+ T-lymphocytes.** A, depletion of CD20+ B cells does not inhibit the efficacy of 10 Gy local RT and R848 dosed q1w. *, P > 0.05 Mann-Whitney test. B, serial blood samples were taken 6, 10 and 20 days after the initiation of treatment and the frequency of SIINFEKL-restricted CD8+ T cells was determined using pentamers by flow cytometry. C, tumor bearing mice received 10 Gy local RT and R848 dosed
q1w in addition to a CD8-depleting antibody. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments. ***, $P < 0.001$, Log-rank (Mantel-Cox) test.

Figure 4. Long-term surviving mice treated with radiation and R848 are protected against subsequent tumor rechallenge by the induction of tumor-specific memory CD8+ cells. A, survival curve for EG7 tumor-bearing mice following combination therapy with 10 Gy local RT and R848 dosed q1w. ***, $P < 0.001$, Log-rank (Mantel-Cox) test. B and C, splenocytes were isolated from treatment naïve control mice and from long-term survivors originally treated with local RT and R848 dosed q1w and co-cultured with either 25 Gy irradiated EG7 cells (B) or SIINFEKL peptide (C) prior to being restimulated with fresh 25 Gy irradiated EG7 cells. **, $P < 0.01$, two-tailed Student’s $t$ test. Experimental groups contained at least 3 mice and are representative of 2 independent experiments. D, at greater than day 100 after initial tumor inoculation a cohort of long-term surviving mice were rechallenged contra-laterally with EL4 cells. **, $P < 0.01$ Mann-Whitney test. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

Figure 5. Combination of R848 dosed q1w with a RT regimen comprising 5 fractions of 2 Gy leads to complete eradication of EL4 and A20 tumors. A and B, EL4 tumor growth (A) and survival curves (B) following combination therapy with R848 dosed q1w and either a single 10 Gy dose or 5 fractions of 2 Gy local RT. C, at greater than day 60 after initial tumor inoculation a cohort of long-term surviving mice originally treated with 5 fractions of 2 Gy and R848 dosed q1w were
rechallenged contra-laterally with EL4 cells. $D$, survival curve of mice bearing established A20 tumors following combination therapy with R848 dosed q1w and 5 fractions of 2 Gy local RT. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments. *, denotes significance when compared to control mice. †, denotes significance when compared to monotherapy. */+, $P < 0.05$, **/++, $P < 0.01$, Log-rank (Mantel-Cox) test.
Figure 1

A. Expression of CD69 on T cells

B. Expression of CD69 on B cells

C. Concentration of cytokines (pg/ml)

D. Percent survival over time after tumor implantation

- Control
- R848 3mg/Kg single dose
- R848 3mg/Kg q1w
Figure 2

A

- Control
- 10Gy Local RT
- R848 3mg/Kg single dose
- 10Gy + 3mg/kg R848 single dose

B

- Control
- 10Gy Local RT
- R848 3mg/Kg q1w
- 10Gy + 3mg/kg R848 q1w

Legend:

* 0.03 > P > 0.01
++ 0.01 > P > 0.001
+++ P < 0.001
Figure 3

A

- Control
- αCD20 mAb
- 10Gy + 3mg/kg R848 q1w
- 10Gy + 3mg/kg R848 q1w + αCD20 mAb

B

- Control
- 10Gy RT + 3mg/kg R848 q1w

C

- Control
- 10Gy + 3mg/kg R848 q1w
- 10Gy + 3mg/kg R848 q1w + αCD8 mAb

*** P>0.05
Figure 4

A

![Graph showing percent survival over time after tumor implantation for Control and 100Gy + 3mg/kg R848 q1w groups.]

B

![Bar graph showing percentage of IFNγ expressing CD8+ T lymphocytes for Control and LT6 mice (R848+RT) groups.]

C

![Bar graph showing percentage of IFNγ expressing CD8+ T lymphocytes for Control and LT6 mice (R848+RT) groups.]

D

![Graph showing tumor volume over days post EL4 rechallenge for Control and 100Gy + R848 q1w groups.]

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Figure 5

A

No Treatment

Control

10Gy single dose RT

10Gy single dose RT + TLR7 agonist

5x2Gy fractionated RT

5x2Gy fractionated RT + 3mg/kg R848 q1w

B

No Treatment

10Gy RTx

3mg/kg R848 q1w

10Gy + 3mg/kg R848 q1w

C

Control

3mg/kg R848 q1w + 5x2Gy RT

D

NT

3 mg/kg R848 q1w

5x2Gy RT

5x2Gy RT + 3 mg/kg R848 q1w
Systemic delivery of a TLR7 agonist in combination with radiation primes durable anti-tumor immune responses in mouse models of lymphoma

Simon J. Dovedi, Monique H.M. Melis, Robert W. Wilkinson, Amy L. Adlard, Ian J. Stratford, Jamie Honeychurch and Timothy M. Illidge