Application of image-guided irradiation to a preclinical bioluminescent syngeneic metastatic breast cancer model in combination with immunotherapy to inform on combination strategies

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Background

Radiotherapy is a primary, adjuvant or neoadjuvant treatment for a number of different cancers including breast, lung and prostate.

Recent advances and the increased availability of image-guided micro-irradiation (IGMI) has resulted in more accurate targeting of patient tumours and sparing of normal tissue with an associated reduction in side-effects. This opens up the opportunity for multiple combination strategies prime amongst which, is the combination IGMI with immunotherapy; as such, clinically relevant models to interrogate immunotherapy and IGMI are required.

The image-guided small animal radiation research platform (SARRP) allows the treatment of animal models of cancer more accurately and importantly, with planned protocols similar to those utilised in the clinic.

The use of IGMI in the preclinical setting is less common; typically traditional irradiation studies utilise whole body irradiation with lead shielding to focus the radiation to a specific area on the animal or simple single beam techniques.

Here we report the application of the image-guided SARRP to a preclinical syngeneic model to demonstrate the combination outcome of irradiation and immunotherapy in a model of metastatic breast cancer.

Methods 1

- **Generation of 4T1-lux cell line:** 4T1 cells were generated to express firefly luciferase (4T1-lux); briefly, the cells were transduced utilising in-house packaged lentiviral particles containing a plasmid vector expressing firefly luciferase (pLVC-puro-CMVLucSh luciferase). The transduced cells were selected by puromycin and the resultant cell line (4T1-lux) was confirmed by STR Profiling (DDC Medical; UK).
- ***In vivo* modelling:** Bioluminescent mouse mammary carcinoma 4T1 cells (4T1-lux) were implanted subcutaneously in BALB/c mice (cOlaHsd; Harlan UK). Tumour growth was monitored by calliper measurements three times weekly, bioluminescent imaging was carried out to assess real-time tumour growth and metastatic lung tumours.
- Mice were recruited to treatment on day 12 and were treated with fractionated radiation (5 x 6Gy) using the SARRP, and/or 10mg/kg anti-mCTLA-4 i.p. (clone 9d9; BioXcell, US).
- For irradiation mice were anaesthetised and CBCT images were acquired using the small animal research platform (SARRP; Xstrahl, US). MuriSlice software was used to identify the isocenter of the tumour and fractionated irradiation administered (225 kV peak X-ray beams; dose rate of 2.5 Gy/min) using a multi beam approach in order to spare the surrounding normal tissue. Body weight and clinical condition of the mice were monitored daily. At termination the tumours were collected and assessed for immune cell infiltration by FACS.

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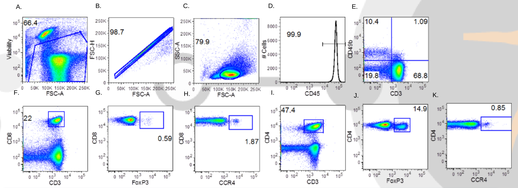
Methods 2

- **FACs analysis**: Cells were isolated from 4T1-lux tumours and lungs of tumour-bearing animals, as well as spleens and lungs from healthy animals. Cells were extracted from tumour and spleen samples by mechanical methods, including passing through 70µm mesh. Cells were isolated from lungs by enzymatic digestion using Liberase and DNase (Roche). For all sample types, the cell yield and viability was determined using a NucleoCounter (Chemometec).
- At least 100,000 viable cells per test were stained using multi-colour flow cytometry. Staining used fluorescently conjugated antibodies to: cell surface CD45, CD3, CD4, CD8, CCR4, CD49b and intracellular Foxp3; a viability dye was also included. Prior to antibody staining, non-specific binding was minimised by using an Fc block step.
- After gating on viable singlet events, the antibody staining patterns were used to distinguish between the various immune cell populations in each sample. In all cases, the gating strategy was performed relative to the FMO (Fluorescence Minus One) controls which were established on spleen samples. Acquisition used a FACS Canto System II flow cytometer (BD Biosciences) and analysis was by FlowJo software (version 8.8.7., Treestar Inc).

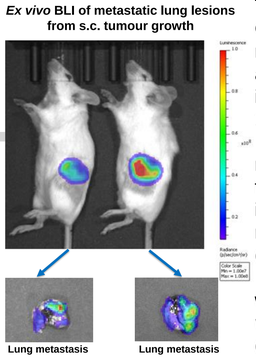
Representative flow cytometry plots on spleen sample. A-D: Selection of single, viable lymphocytes. **E**: NK cells are identified as CD3-CD49b+ cell population (top left corner of the plot). **F-H**: Subpopulations within CD8+ T lymphocytes, namely FoxP3+ (plot **G**) and CCR4+ (plot **H**). **I-K**: Subpopulations within CD4+ T lymphocytes, namely FoxP3+ (plot **J**) and CCR4+ (plot **K**).



Generation and characterisation of 4T1-lux

The 4T1 is a stage IV mouse mammary carcinoma cell line that is a highly tumorigenic and invasive a representative of TNBC (Tao et al 2008 BMC Cancer 8:228), and is highly resistant to most therapeutic agents including immunotherapy (Pulaski et al, 1998 Cancer Res 58(7):1486). Bioluminescent 4T1-lux cells metastasize to the lung following subcutaneous implantation as previously reported for the parental line (Huang et al 2002 Can Res). Metastatic spread to the lungs was confirmed by terminal *ex vivo* bioluminescent imaging (Spectrum CT; PerkinElmer).

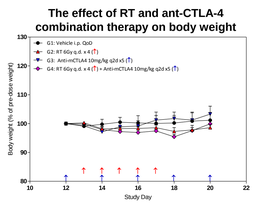
***Ex vivo* BLI of metastatic lung lesions from s.c. tumour growth**



Combination RT and anti-CTLA4 therapy

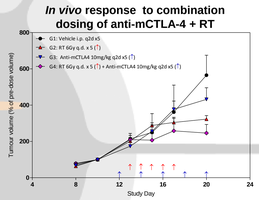
The effect of monotherapy/combination therapy with 10mg/kg anti-mCTLA-4 i.p. q2d. (↑) and radiotherapy 6Gy qdx5 (↑) on mouse body weight and tumour growth is detailed below.

The effect of monotherapy/combination therapy with 10mg/kg anti-mCTLA4 treatment was well tolerated; although there was a measureable drop in body weight in groups 2 and 4 (RT, and anti-mCTLA-4 + RT respectively), which peaked following the final RT fraction, there was no associated clinical signs and body weight recovered quickly.

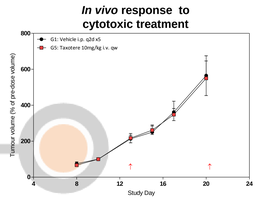


Anti-mCTLA-4 monotherapy had no statistically significant impact on tumour growth over the course of treatment. RT monotherapy treatment resulted in a statistically significant reduction in tumour growth ($p<0.05$ Two-way ANOVA) when compared with the vehicle control, and when combined with anti-mCTLA-4 therapy, the combination treatment resulted in a statistically significant reduction in tumour growth ($p<0.05$ Two-way ANOVA) versus both vehicle and RT alone.

***In vivo* response to combination dosing of anti-mCTLA-4 + RT**



***In vivo* response to cytotoxic treatment**

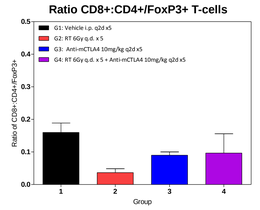


The addition of anti-mCTLA-4 to RT appears to exert an additive effect on TGI over single agent therapy alone. No response was seen to Taxotere therapy at the tested regimen.

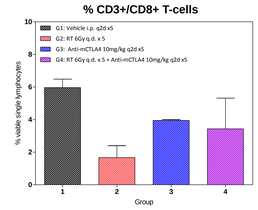
FACS analysis of primary s.c. tumours

Tumour infiltrating lymphocytes (TIL) were assessed in primary 4T1-lux tumours at endpoint; the % of viable lymphocytes/ratio of CD4+, CD8+ and CD4+/FoxP3+ T-cells is summarised below.

Ratio CD8+:CD4+/FoxP3+ T-cells



% CD3+/CD8+ T-cells

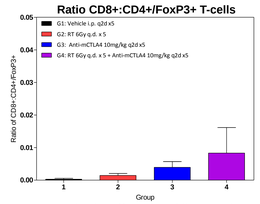


No statistically significant impact was noted in either the ratio of CD8+:CD4+/FoxP3+ cells or %CD8+ T-cells.

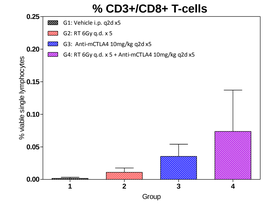
FACs analysis of lung tumours

Whole lungs from 4T1-lux tumour bearing mice were assessed at endpoint by FACS analysis for TILs; the % of viable lymphocytes/ratio of CD4+, CD8+ and CD4+/FoxP3+ T-cells is summarised below.

Ratio CD8+:CD4+/FoxP3+ T-cells



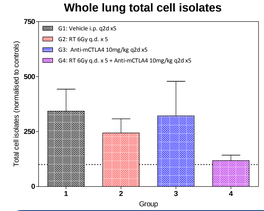
% CD3+/CD8+ T-cells



No statistically significant impact was noted in either the ratio of CD8+:CD4+/FoxP3+ cells or %CD3+/CD8+ T-cells, although there was a trend in treatment response for both measures.

Total cell isolates from the lungs of 4T1-lux tumour bearing mice were normalised to age-matched lungs of non-tumour bearing mice to give an indication of tumour burden.

Whole lung total cell isolates



Although there is a clear reduction in the normalised cell count derived from those mice on the combination therapy, this effect was not statistically significant. Future studies can employ *ex vivo* BLI to assess tumour burden more directly.

Summary

- A bioluminescent variant of 4T1 (4T1-lux) was generated by lentiviral transduction and characterised for s.c. and metastatic (lung) growth.
- Treatment of s.c. 4T1-lux tumours with RT (IGMI) and anti-mCTLA-4 were well tolerated as both monotherapy and in combination.
- Anti-mCTLA-4 treatment exerted no effect on s.c. tumour growth, whilst treatment with fractionated RT (IGMI) resulted in a significant TGI. Combination of both regimens resulted in an additive TGI over RT alone.
- FAC analysis of TILs in s.c tumours and lungs, showed no significant changes in TILs. A trend in response was seen in the lung samples which may be related to the decrease in whole lung cell isolates observed with combination treatment. Further investigation is warranted.

Conclusions

- IGMI of syngeneic metastatic breast cancer model using the SARRP platform was effectively demonstrated to have reduced side effects, improved safety and used to evaluate combinations with immunotherapy to derive treatment schedules suitable for testing subsequently in clinical trials for breast cancer.
- Although the 4T1-lux model is poorly immunogenic and resistant to standard therapies, these properties may make it an ideal model for advanced breast cancer and for further exploring combination strategies involving immunotherapy.