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 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 single beam techniques. Here we report the development 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-CMVluxLuc). 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-lux) was implanted subcutaneously into BALB/c mice (cOlAhsd; Harlan UK). Tumour growth was monitored by caliper 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 6 Gy) using the SARRP and/or 10 mg/kg anti-mCTLA-4 (p. clone 10C11; Bioxcell, UK). For irradiation mice were anasthetised and CBIT images were acquired using the small animal research platform (SARRP; Xtraxi, USA). Mantoux software was used to identify the isocentre of the tumour and fractionated irradiation administered (225 kv peak X-rays beam; dose rate of 0.5 Gy/min using a 45 cm Gyn beam aperture 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.

**Methods 2**

- **FACS analyses:** 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 passage through 70um 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 (Chometec).

- At least 100,000 viable cells per test were stained using multi-colour protocols. Staining conjugated antibodies to: cell surface CD45, CD3, CD4, CD8, CD8a, 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 the various immune cell populations in each sample. In all cases, the gating strategy was performed relative to the FMO (Fluorescence Minus One) control. Data was acquired and analysed on spleen samples. Acquisition used a FACS Canto System II flow cytometer (BD Biosciences) and analysis was performed by Biovsoft software (version 3.1, Biorad Inc.)

**Combination RT and anti-mCTLA4 therapy**

The effect of monotherapy/combotherapy with 10mg/kg anti-mCTLA-4 p. qd. (1) and radiotherapy 6Gy qd. (4) on mouse body weight and tumour growth is detailed below.

The effect of monotherapy/combotherapy with 10mg/kg anti-mCTLA-4 treatment was well tolerated; although there was a measurable drop in body weight in groups 2 and 4 (RT, and RT + 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 at the 6Gy qd. dose resulted in a statistically significant reduction in tumour growth (p<0.005 Two-way ANOVA) versus both vehicle and RT alone.

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

**FACS analysis of lung tumours**

No statistically significant impact was noted in either the ratio of CD8/CD4+FoxP3+ cells or %CD8+ T-cells, although there was a trend in treatment response for both measures. Tumour cell isolates from the lungs of 4T1-lux bearing mice were normalised to age-matched lungs of non-tumour bearing mice to give an indication of tumour burden.

As 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-CTLA-4 were tolerised at a more accurate dose 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.

- FACS analyses of lung tumours and lungs, showed no significant changes in TL. A trend in response was seen in the lungs of mice which were irradiated, with whole lung cell isolates observed with 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 strategies suitable for testing subsequently in clinical trials for breast cancer.

- Although the 4T1-lux model is poorly immunogenic and resistant to standard therapies, those protocols make it an ideal model for advanced breast cancer and for further exploring combination strategies involving immunotherapy.