Immunocompetent Syngeneic Models Demonstrate Additive Effects of Combination Strategies Using Checkpoint Immunotherapy and Inducers of Immunogenic Cell Death

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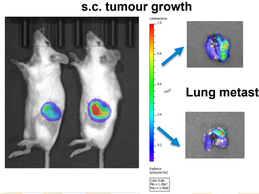
INTRODUCTION

Recent progress in the field of cancer immunotherapy (IT) has made it possible to translate several emerging immunostimulatory strategies to the clinic, resulting in promising clinical benefits. In addition, a number of treatment strategies such as radiotherapy (RT), oncolytic viruses, and chemotherapeutic agents such as oxaliplatin, doxorubicin, bortezomib, and mitoxantrone have been highlighted as potential inducers of immunogenic cell death (ICD) through a well defined mechanism resulting in the increased presentation of cell-associated antigens to CD4+ and CD8+ T lymphocytes by dendritic cells. Therefore, combination strategies of ICDs with IT could provide opportunities to harness the immune system to extend survival, even among metastatic and heavily pretreated cancer patients, and may increase the efficacy of IT in cancer types with low immunogenic status. RT is used across a number of cancers types and recent advances in stereotactic and image-guided micro-irradiation (IGMI) have resulted in an increase in tumor specific targeting with a corresponding reduction in associated side-effects. Here we report the application of IGMI and oxaliplatin in combination with IT (anti-CTLA-4) to examine its impact on tumor growth and immunity.

METHODS

- ***In vivo* modeling:** CT26 mouse colon carcinoma cells or bioluminescent mouse mammary carcinoma 4T1 cells (4T1-lux) were implanted subcutaneously into BALB/c mice (cOlaHsd; Envigo UK). Tumor growth was monitored by caliper measurements 3x weekly. Bioluminescent imaging for 4T1 was carried out to assess real-time tumor growth and metastatic lung tumors at end stage (Spectrum CT; PerkinElmer); Figure 1 shows imaging characterization.
- Mice were recruited to treatment groups when the mean tumor volume reached ~100–200mm^3; mice were pretreated with 10mg/kg anti-mCTLA-4 i.p. (clone 9d9; BioXcell, US) 24h prior to onset of hypofractionated RT (5 x 6Gy). Anti-mCTLA-4 therapy (b.i.w or q.2.d.) continued for 2 weeks in total. Oxaliplatin was dosed at 6mg/kg q.w.
- **IGMI**: Mice were anaesthetized and CBCT images were acquired using the small animal research platform (SARRP; Xstrahl, US). MuriSlice software was used to identify the isocenter of the tumor and fractionated RT was administered 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 primary tumors were excised, weighed, and assessed for immune cell infiltration by FACS.
- **FACS**: Cells were extracted from 4T1 tumors and spleen samples by mechanical methods, including passing through 70µm mesh. Cells were isolated from lungs by enzymatic digestion using Liberase (Sigma) and DNase (Roche). In the case of CT26 carcinoma tumors, digestion used Liberase TL (Roche) and DNase (Roche). For all sample types, the cell yield and viability was determined using a NucleoCounter (Chemometec). >100,000 viable cells per test were stained using multi-color flow cytometry and fluorescently conjugated antibodies to cell surface CD45, CD3, CD4, CD8, CCR4, CD49b and intracellular Foxp3. Acquisition used a FACS Canto System II flow cytometer (BD Biosciences) and analysis was by FlowJo software (version 8.8.7., Treestar Inc).

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



Lung metastasis

Figure 1: Generation and characterization of 4T1-lux
4T1 is a Stage IV mouse mammary carcinoma cell line that is highly tumorigenic, invasive and representative of TNBC (Tao *et al* 2008 BMC Cancer 8:228), as well as highly resistant to most therapeutic agents including immunotherapy (Pulaski *et al*, 1998 Cancer Res 58(7):1486). Bioluminescent 4T1 cells metastasize to the lung following subcutaneous implantation as previously reported for the parental line (Huang *et al* 2002 Cancer Res). Metastatic spread to the lungs was confirmed by terminal *ex vivo* bioluminescent imaging.

RESULTS

- Anti-mCTLA-4 monotherapy resulted in a statistically significant reduction in tumor growth for CT26 ($p<0.05$, Figure 3) but not 4T1 (Figure 2).
- RT monotherapy treatment resulted in a statistically significant reduction in tumor 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 tumor growth ($p<0.05$ Two-way ANOVA) versus both vehicle and RT alone (4T1 and CT26 Figures 2-3). The combination of anti-mCTLA-4 with RT results in an additive effect on TGI over single agent therapy alone.
- For RT & anti-CTLA-4 combination treated CT26 tumors, regression continued following cessation of treatment (80% CR) and a delay in tumor regrowth was observed (Figure 5).
- Oxaliplatin also induced a significant reduction in tumor growth ($p<0.01$) in the CT26 model and was additive with anti-mCTLA-4 (Figure 4).
- Tumor infiltrating lymphocytes (TIL) were assessed in primary tumors at endpoint; the % of viable lymphocytes/ratio of CD4+, CD8+ and CD4+/FoxP3+ T-cells are summarized in Figure 6. No statistically significant impact was noted in either the ratio of CD8+:CD4+/FoxP3+ cells or %CD8+ T-cells in the 4T1 model; however, a significant increase in CD3+CD8+ cells was observed following IR treatment and in combination (Figure 6B). Oxaliplatin in combination with anti-CTLA-4 induced a statistically significant increase in CD8+ TILs in comparison to both monotherapies (Figure 6C, G4vG3 or G4vG2, $p<0.05$).
- Whole lungs from 4T1 tumor 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 summarized in Figure 7. 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.
- Total cell isolates from the lungs of 4T1 tumor bearing mice were normalized to age-matched lungs of non-tumor bearing mice to give an indication of tumor burden. Although there is a clear reduction in the normalized 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 tumor burden more directly.

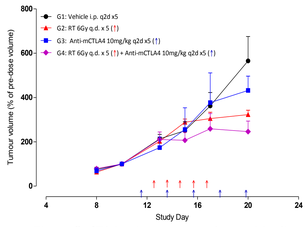


Figure 2: 4T1 *in vivo* response to combination dosing of anti-mCTLA-4 + RT

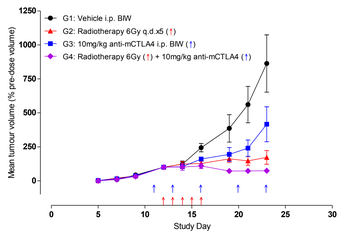


Figure 3: CT26 *in vivo* response to combination dosing of anti-mCTLA-4 + RT

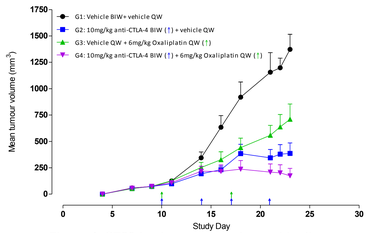


Figure 4: CT26 *in vivo* response to combination dosing of anti-mCTLA-4 + RT

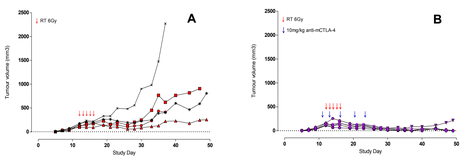


Figure 5: CT26 *in vivo* outgrowth following A. RT treatment and B. anti-mCTLA-4 + RT in study summarized in Figure 3.

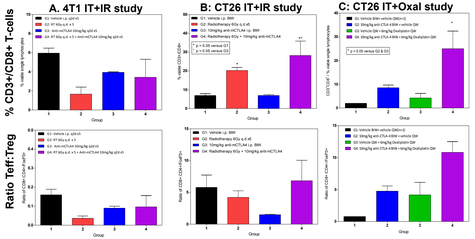


Figure 6: Flow cytometry analysis of subcutaneous tumors from 3 studies summarized in Figures 2-4.

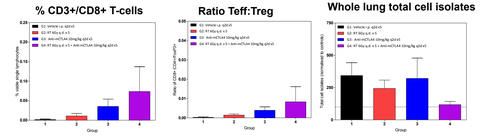


Figure 7: Flow cytometry analysis of 4T1 metastatic lung tumors from study summarized in Figure 2

SUMMARY

- **CT26 but not 4T1 tumors exhibited a moderate response to anti-CTLA-4 IT, whilst ICD inducers oxaliplatin and RT alone resulted in a statistically significant tumor growth inhibition.**
- **Combination of either ICD+IT resulted in an additive TGI over one or both monotherapies: RT for 4T1; RT and IT for CT26. Additionally for CT26, tumor response in the IT+RT, but not the RT group, continued following the cessation of dosing resulting in complete regression in 80% of remaining tumors.**
- **FAC analysis of TILs in 4T1 tumors 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 which may point to an abscopal response.**
- **For CT26 tumors, a significant increase in CD8+ T-cells was observed for both RT and IT+RT compared with vehicle control and versus IT alone for the IT+RT group. There was a trend in increasing CD8+ T-cells for the IT+RT versus RT alone which may be driving the increased efficacy seen with the treatment. With oxaliplatin a combination effect mediated by CD8+ cells was also observed.**
- **Combination of anti-mCTLA-4 immunotherapy with an ICD inducers IGMI/oxaliplatin resulted in an additive TGI in syngeneic models CT26 and 4T1 models and effectively demonstrates their applicability for further exploring combination strategies involving immunotherapy.**