

A140: Check point inhibitor modulation of tumour microenvironment at orthotopic and metastatic sites using bioluminescent syngeneic cell line models in immune competent mice.

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INTRODUCTION

Mouse allograft models are widely used to model the impact of cancer immunotherapy on tumour growth and tumour invading leucocytes (TILs), with the majority of such work typically carried out in the subcutaneous setting. However, orthotopic models are reported to better model cancer in patients as they form a single focal disease area as in the patient situation, facilitate metastatic spread via intra- and extra-thoracic lymph nodes and, in the case of syngeneic models, strain-specific tumour microenvironment interactions (immune and stromal components). Bioluminescent imaging (BLI) increases the usefulness of such models, as it allows for non-invasive longitudinal monitoring of tumour burden, allowing for optimal randomisation and reduction of false positives. Here we describe the generation of a bioluminescent panel of mouse cell lines commonly used for immunotherapy studies, assess the impact of luciferase expression on subcutaneous growth and the translation to orthotopic modelling.

METHODS

Generation of bioluminescent cell line panel: A panel of mouse cell lines were transduced utilising in-house packaged lentiviral particles containing a plasmid vector expressing firefly luciferase (pLVC-puro-CMV/LucSh luciferase). The transduced cells were selected by puromycin to establish stable cell lines. Bioluminescence was correlated to cell number against known standards *in vitro* to assess suitability for in-life, deep tissue imaging. The final cell panel was assessed for DNA changes from their respective parental cell lines by STR Profiling (DDC Medical; UK).

In vivo models: Wild type (WT) or bioluminescent cell variants (lux) were implanted into either BALB/c (EMT6; Envigo, UK) or C57BL/6J mice (B16 F10 and Pan02; CRL, UK). Tumour growth was monitored by calliper measurements 3x weekly and by weekly bioluminescent imaging (BLI) to confirm growth (Spectrum CT; PerkinElmer).

Direct pancreatic injection: Under anaesthesia a small transverse incision was made below the ribcage, the spleen gently exteriorised and the pancreatic tail located adjacent to the spleen. Pan02-lux cells suspended in Growth Factor Reduced Matrigel™ were injected into the pancreatic tail and the spleen and pancreas carefully returned to the abdominal cavity. The abdomen and the skin were closed using a suture line followed by application of Vetbond; peri- and post-operative analgesia was administered as required. Anti-CTLA-4 antibody (clone 9D9) sourced from BioXcell.

FACTS: Cells were isolated from tumours by enzymatic digestion using Liberase (Sigma) and DNase (Roche). Cells were then washed, counted and viability assessed (by Luna automated cell counter). Flow cytometry staining was used in order to distinguish immune cells (using CD45 marker) and subsequently determine the distribution of immune cell types present.

RESULTS

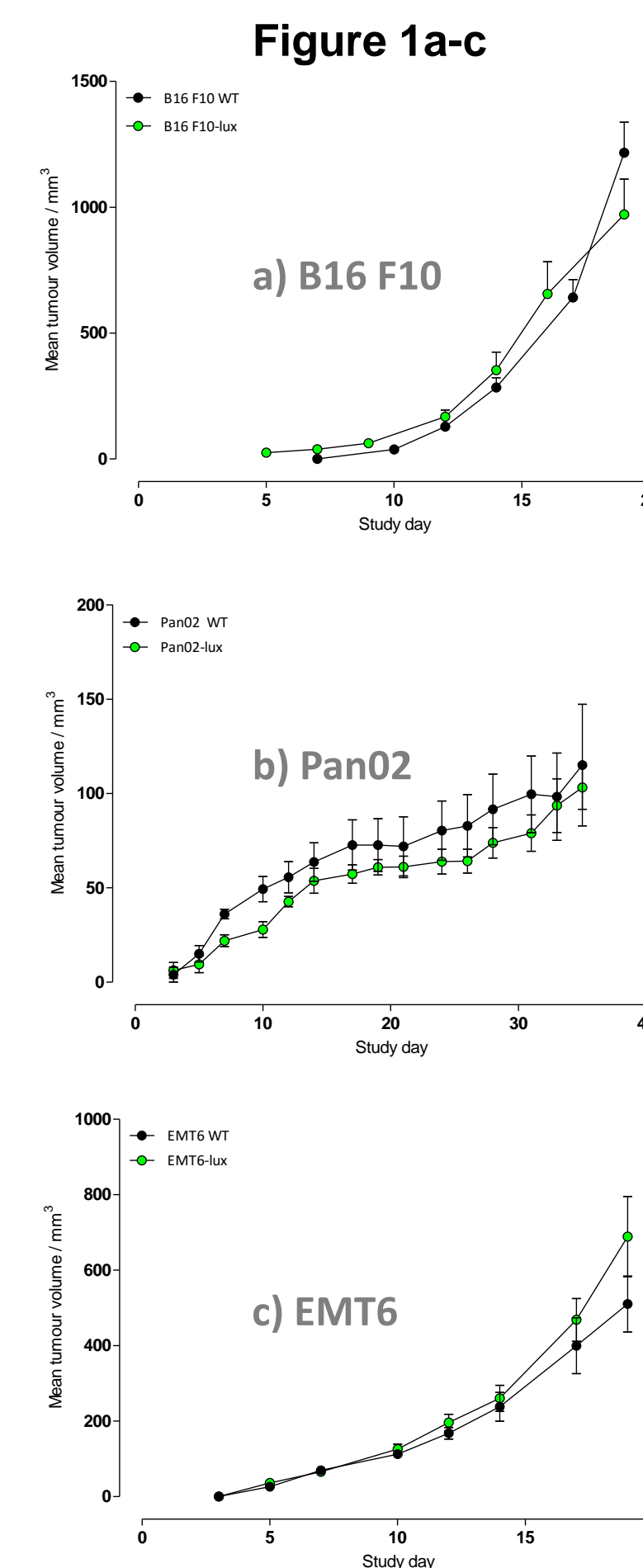
The panel of stably transduced bioluminescent mouse cancer cell lines is summarized in Table 1. Correlation of bioluminescent intensity to cell number was confirmed for all cell lines (data not shown) as well as confirmation of an STR profile consistent with the WT cell line. Figures 1a-c detail comparative plots for WT and lux variants of B16 F10, Pan02 (s.c.) and EMT6 (mammary fat pad); no significant differences in growth were evident for any of the models tested ($p < 0.05$).

Table 1: Summary of bioluminescent mouse cell lines

Cell Line	Disease	Mouse strain	BLI checked	DNA authentication (match to wildtype)
4T-1	Mammary carcinoma (stage IV)	BALB/c	Y	Y
B16-BL6	Melanoma	C57BL/6	Y	Pending
B16-F10	Melanoma	C57BL/6	Y	Y
CT26.WT	Colon carcinoma	BALB/c	Y	Y
EL4	Lymphoma	C57BL/6	Y	Pending
EMT6	Mammary carcinoma	BALB/c	Y	Y
H22	Hepatoma	BALB/c	Y	Y
Hepa 1-6	Hepatoma	C57L / C57BL/6	Y	Y
LL/2 (LLC1)	Lewis lung carcinoma	C57BL/6	Y	Pending
MBT-2	Bladder carcinoma	C3H/He	Y	Y
MC38	Colon adenocarcinoma	C57BL/6	Y	Y
P388D1	Lymphoma	DBA/2	Y	Y
P815	Mastocytoma	DBA/2	Y	Pending
Pan02	Pancreatic	C57BL/6	Y	Y
Renca	Renal adenocarcinoma	BALB/c	Y	Y
RM-1	Prostate carcinoma	C57BL/6	Y	Y

SUMMARY

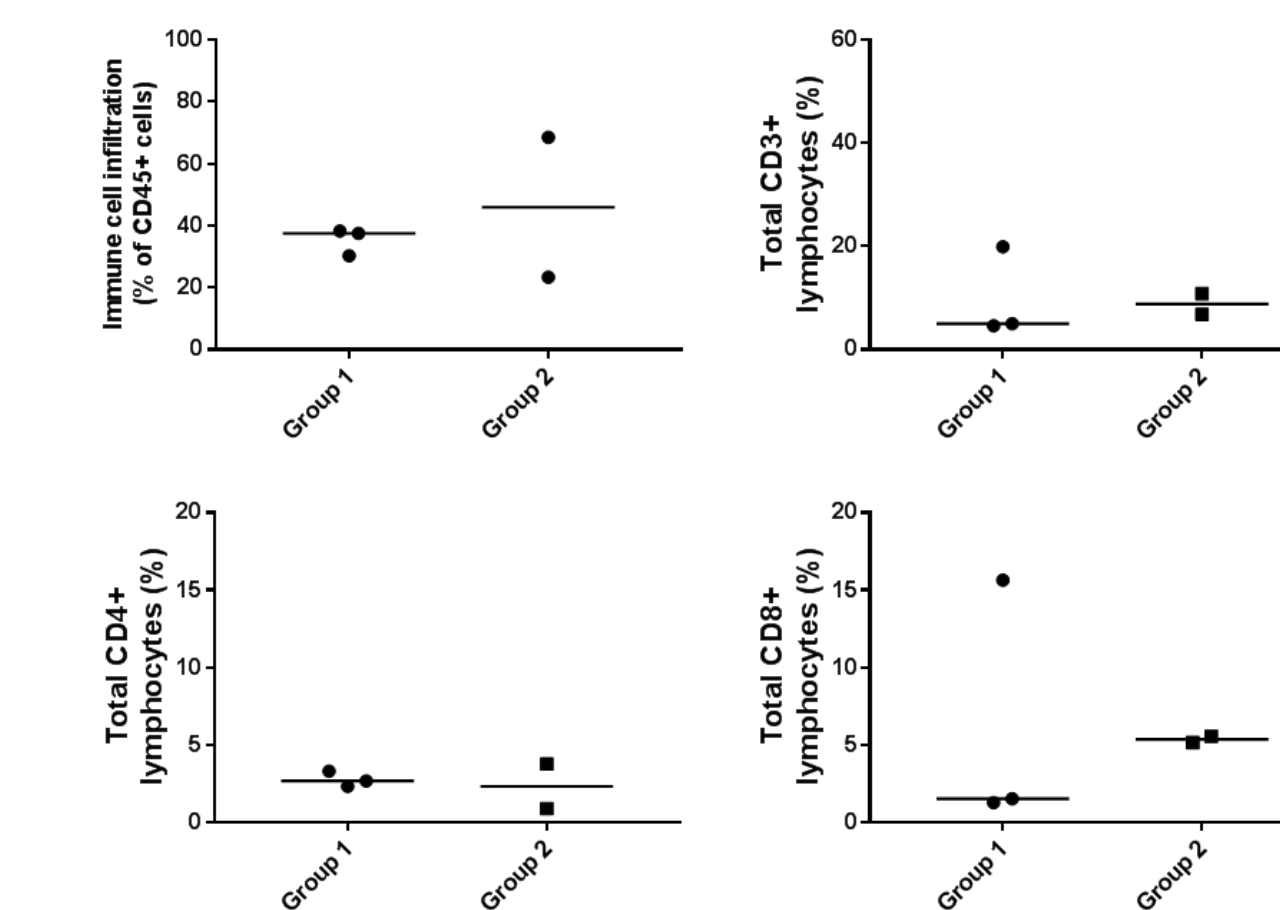
- A bioluminescent panel of mouse cancer cell lines suitable for further orthotopic development was generated by lentiviral transduction and following stable selection, characterised for bioluminescence and changes in STR profile from the respective parental lines.
- No significant differences in growth profile were exhibited when the bioluminescent variants of B16 F10 and Pan02 (subcutaneous) or EMT6 (mammary fat pad) were compared with the wild-type equivalents.
- When TILs were compared for the Pan02 WT and lux models, the data suggests that the models were comparable in most of the T-cell populations; however, small sample sizes and significant variability within groups prevent any firm conclusions to be made; further analysis and additional model assessment is required.
- Anti-CTLA-4 therapy resulted in a statistically significant impact on orthotopic Pan02 tumour growth (BLI) and final tumour weight; however, further characterisation by IHC and refinement of implantation conditions is required as the tumours exhibited low basal TIL levels, poor stromal and capillary infiltration. The use of non-growth factor reduced Matrigel and co-implantation of embryonic fibroblasts may improve stromal and blood vessel penetration.
- In summary, Crown Bioscience has developed a panel of bioluminescent mouse cell lines that are amenable to the development of orthotopic and metastatic models for further exploring strategies involving immunotherapy.



Comparison of Pan02 WT and lux TILs was carried out using the following marker panel:

Cell type	Markers
T cells	CD3, CD4, CD8, FoxP3

Figure 2: Plots presenting cell populations expressing lineage markers CD45, CD3, CD4 and CD8 as percentage of total isolated cells between the groups. Data are presented as Median, due to the spread of the data and small sample sizes:



Overall, the groups were reasonably comparable in majority of T cells. Due to the small sample set, statistical analysis was not performed. Group 1 – Pan02 WT, Group 2 – Pan02 lux.

Figures 3 and 4 summarize the effect of immune checkpoint therapy on the growth of orthotopic Pan02-lux tumours as measured by BLI and final tumour weight.

Figure 3: In-life tumour associated bioluminescence: orthotopic Pan02-lux

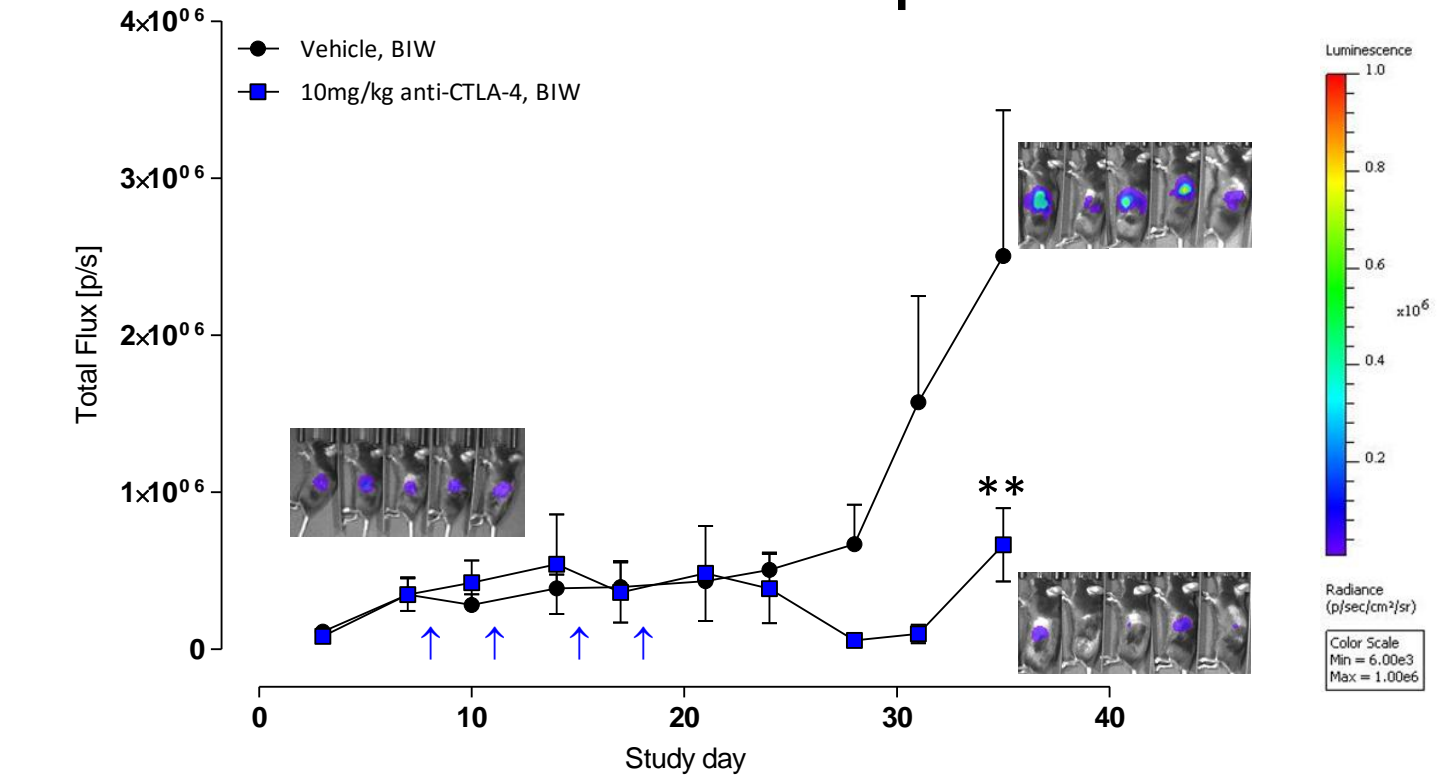
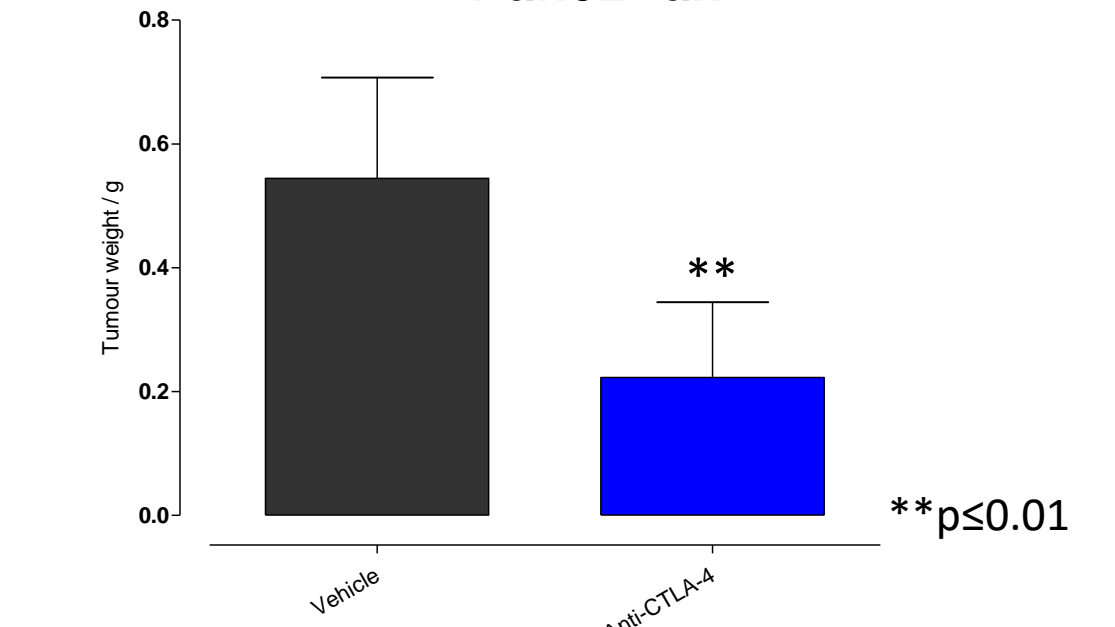


Figure 4: Final tumour weight orthotopic Pan02-lux



Although there is a clear and statistically significant impact of anti-CTLA-4 on the growth and final tumour weight of orthotopic Pan02-lux tumours, initial TIL characterisation of untreated primary tumours (data not shown) indicated the infiltration of immune cells in these tumours was very poor. Furthermore, the tumours exhibited poor stromal and capillary infiltration at odds with that one would expect for an orthotopic model, and this is reflected in the rather flat BLI growth curve. Further IHC/FACs will be carried out to assess TILs in the anti-CTLA-4 treated tumours. However, further refinements such as the use of supplemented Matrigel, and co-implantation of embryonic fibroblasts may significantly improve the stromal compartment.