Establishment of an Orthotopic Murine Lung Cancer Model For The Testing of Coxsackievirus A21 (CVA21) Virotherapy

VIRALYTICS
Targeted Cancer Treatment

THE UNIVERSITY OF NEWCASTLE
AUSTRALIA

Yvonne Wong[1], Eric (Su Han) Chan[1], Min Yuan Quah[1], A/Prof Darren Shafren[1] and Dr Gough G. Au[1]

1. The Picornaviral Research Unit, The School of Biomedical Sciences, Faculty of Health, University of Newcastle, Newcastle, NSW, 2300, Australia.

Abstract

Oncolytic virotherapy is a novel targeted therapeutic approach that induces direct tumour lysis and generation of secondary anti-tumour immune responses. We have previously demonstrated the oncolytic effects of the human enterovirus Coxsackievirus A21 (CVA21) against lung cancer in both *in vitro* studies and a subcutaneous xenograft mouse model. However, the use of subcutaneous xenograft models does not optimally represent the invasive and metastatic properties of lung cancer. Our goal was to develop an orthotopic model that would reflect these properties. To do this, we established a murine lung cancer cell line that was susceptible to CVA21 infection.

Methods

Cells:
Lewis Lung Carcinoma (3LL) cells
All cells were maintained in RPMI media with 10% FBS at 37ºC with 5% CO_2.

Transfection of full human ICAM-1 receptor DNA into 3LL murine lung cancer cells:
3LL cells were stably transfected using the pEF-BOS-ICAM-1 plasmid with Lipofectamine plus reagant and Lipofectamine LTX [Invitrogen].

Fluorescence-activated cell sorting (FACS) of 3LL-ICAM-1 cells:
3LL cells transfected with the human ICAM-1 receptor DNA were pre-stained with an anti-ICAM-1 FITC antibody [Abcam, Sapphire Bioscience] prior to being sorted using the FACS Aria machine [Becton Dickinson]. The distinct population of ICAM-1 positive cells were collected and cultured.

Flow cytometric evaluation of ICAM-1 receptor levels:
Quantitative flow cytometry for ICAM-1 receptor levels was performed using QuantiBRITE™PE beads (BD #340495) as a calibrator to determine the number of antibodies bound per cell. 3LL-ICAM-1 murine lung cancer cells were harvested before staining with FITC-conjugated antibodies against ICAM-1 [Abcam, Sapphire Bioscience].

Evaluation of virus induced cytopathic effect (CPE):
Each cell line was incubated with serially diluted CVA21 inocula for 72 hr and cell viability was examined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cell proliferation assay.

3LL-ICAM-1-luc orthotopic lung cancer mouse model:
3LL-ICAM-1 cells were transfected with the luciferase gene (3LL-ICAM-1-luc cells), allowing bioluminescent monitoring of tumour burden. Two days prior to tumour inoculation, mice were injected with 200 μL of TM-β1 antibody. Female Athymic nude mice (n=8), were inoculated intravenously with 3LL-ICAM-1-luc cells (5×10^6 cells in a volume of 200 μL) via the tail vein. Tumour development was monitored on a weekly basis using the Xenogen IVIS100 bioluminescent live imaging system. Ten days post-tumour inoculation, mice were sacrificed to confirm tumour establishment.

Results

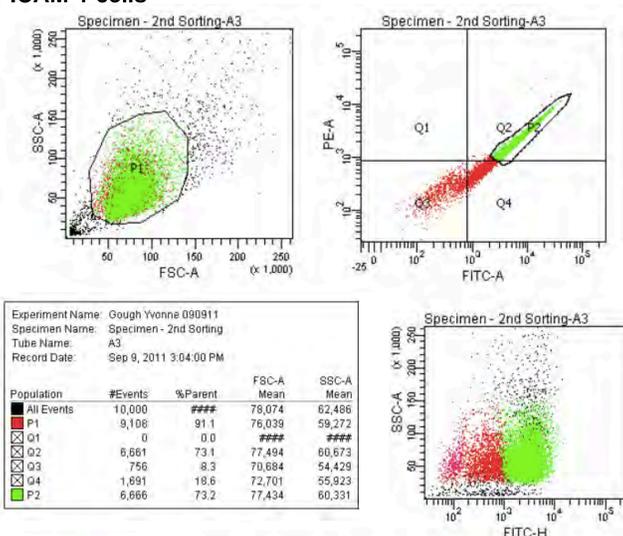### 1. Fluorescence-activated cell sorting (FACS) of 3LL-ICAM-1 cells

Figure 1. Fluorescence-activated cell sorting (FACS) of 3LL-ICAM-1 cells pre-stained with anti-ICAM-1 FITC antibody. 3LL murine cancer cells transfected with the full human ICAM-1 DNA were sorted based on the anti-ICAM-1 FITC staining for the population of 3LL cells expressing ICAM-1 (green). The fluorescence data confirmed the successful transfection of 3LL murine cancer cells with the human ICAM-1 receptor (3LL-ICAM-1).

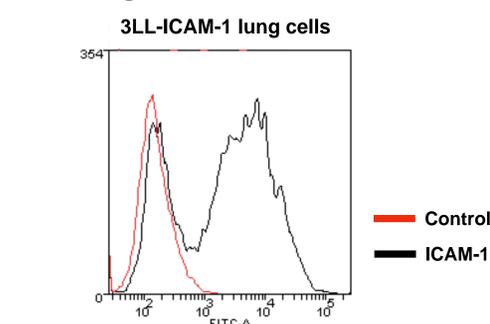### 2. ICAM-1 surface-expression on 3LL and 3LL-ICAM-1 murine lung cancer cell lines

Figure 2. Expression of cellular receptors used by CVA21 on the surface of 3LL and 3LL-ICAM-1 murine lung cancer cell lines. Three weeks after the second cell sorting, the 3LL and 3LL-ICAM-1 murine lung cancer cell lines were examined by flow cytometry for stable expression of ICAM-1 (black histogram). Two distinct populations of 3LL-ICAM-1 murine lung cancer cells were seen. Due to this mixed population of 3LL-ICAM-1 cells, the cells were subjected to four rounds of further sorting to isolate the 3LL-ICAM-1 cells.

3. Oncolytic activity of CVA21 on 3LL and 3LL-ICAM-1 murine lung cancer cell lines
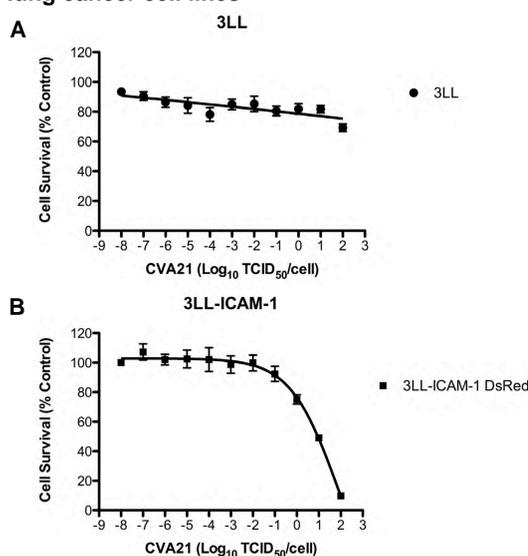

Figure 3. Dose response curves of CVA21 on 3LL and 3LL-ICAM-1 murine lung cancer cell lines. (A) Oncolytic effect of CVA21 in 3LL murine lung cancer cell line. 3LL murine lung cancer cells were resistant to CVA21, as expected due to the lack of the ICAM-1 receptor. (B) Oncolytic effect of CVA21 in 3LL-ICAM-1 murine lung cancer cell line. 3LL-ICAM-1 cells were more sensitive than 3LL cells following CVA21 treatment. This is consistent with the high expression of ICAM-1 on the surface of these cells. CVA21 dose is defined as multiplicity of infection (MOI) expressed as tissue culture infectious dose per cell ($TCID_{50}$/cell)

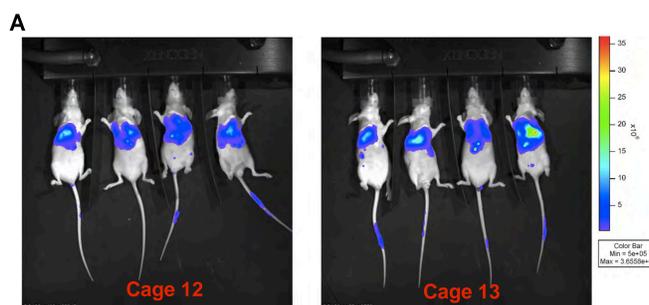### 4. 3LL-ICAM-1-luc orthotropic lung mouse model in athymic nude mice

Figure 4A. Bioluminescent imaging of 3LL-ICAM-1 luciferase expressing lung tumours (3LL-ICAM-1-luc cells) established orthotopicaly in athymic nude mice. Mice were imaged at Day 7 post-tumour inoculation to observe tumour growth. All eight mice showed high levels of bioluminescent intensity indicating metastatic spread in the lung/thoracic cavity.
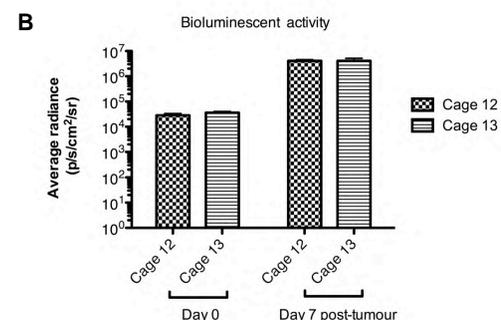

Figure 4B. Average radiance of 3LL-ICAM-1-luc orthotopic lung tumour, as measured by Xenogen bioluminescent imaging. At Day 7 post-tumour inoculation, mice were imaged and the lung tumours were seen to have increased in bioluminescent intensity by an approximate of 2-fold. The data illustrate that the 3LL-ICAM-1-luc lung tumours possess an acceptable take rate and fast growth progress in the athymic nude mice.

5. Necropsy of athymic nude mice bearing 3LL-ICAM-1-luc orthotopic lung tumours
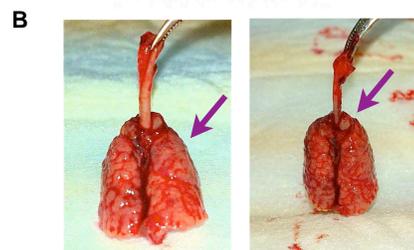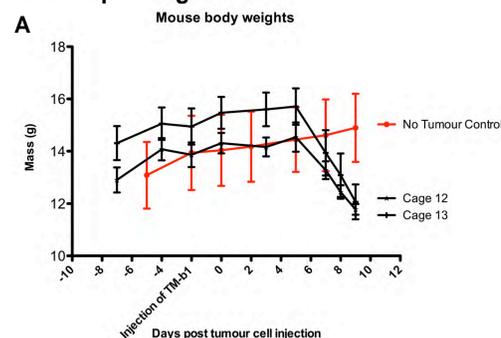

Figure 5. (A) Athymic nude mice body weights. Huge decrease in body weights in mice bearing the 3LL-ICAM-1-luc lung tumour were observed (~20% weight loss) as compared to the no tumour control mice (in red). **(B) 3LL-ICAM-1-luc lung tumour necropsy images.** At Day 10 post-tumour inoculation, mice were culled due to extreme weight loss and tumour burden in the lungs. Upon necropsy, lungs were retrieved and images were taken of the lungs. Tumour nodules were clearly seen on the surface of the lungs. This is consistent with the bioluminescent imaging taken on the mice.

Conclusion

- 3LL murine lung cancer cells were successfully traxsfected with the full human ICAM-1 receptor required for viral entry and infection by CVA21.

- The distinct population of ICAM-1 positive 3LL murine lung cancer cells were selected by FACS for *in vitro* and *in vivo* studies.

- 3LL-ICAM-1 murine lung cancer cells were sensitive to Coxsackievirus A21 (CVA21) lytic infections as compared to the non-transfected 3LL murine lung cancer cells, which were resistant to CVA21 infection.

- Well-spread tumour distribution on the lungs and in the lung/thoracic cavity based on the bioluminescent imaging were observed in the 3LL-ICAM-1-luc orthotopic lung mouse model.

- We demonstrated that the 3LL-ICAM-1-luc lung tumours grew rapidly in an *in vivo* setting with the mice bearing tumours having to be culled 10 days post-tumour inoculation due to extreme weight loss and tumour burden.

- Upon the successful inoculation of 3LL-ICAM-1-luc tumour cells orthotopically in the lung, we will investigate the use of CVA21 as a treatment against these lung tumours.

Acknowledgement