KANEMATSU GRANT REPORT

2017

Project Title: "A novel therapy for hepatic veno-occlusive disease with recombinant endothelial-targeted CD39".

Background: Veno-occlusive disease (VOD) of the liver is a common and devastating complication in patients undergoing blood cancer treatment (high-dose chemotherapy and bone marrow transplantation). Characterised by weight gain, fluid accumulation and organ failure, VOD occurs in up to 70% of patients undergoing this treatment. The novel drug '*anti-VCAM-CD39*' has the capacity to reduce inflammation and protect the cells lining the blood vessels in the liver.

Aims and progress:

1. Characterise mouse models of liver VOD and examine the anti-clotting and anti-inflammatory properties of the novel *anti-VCAM-CD39* therapeutic in these models.
   - **Progress:** We have established two mouse models of liver VOD. Data is shown below.
2. To compare the effects of *anti-VCAM-CD39* to that of the drug currently used to treat VOD in the same model (defibrotide).
   - **Progress:** Purification of anti-VCAM-CD39 from both mammalian and insect sources has been successful and these drugs are ready for trial in our now established animal models.
3. To learn how VOD occurs by studying the processes leading to activation of cells lining the liver, resulting clot formation and how promoting adenosine production can prevent these processes from occurring.
   - **Progress:** not yet commenced

Progress in Study Design and Methodology:

We have refined the proposed study methodology and have designed three mouse models of VOD.

1) **Administration of monocrotaline (MCT)**, a plant derived alkaloid: This is the most frequently published rodent model of VOD. Rats that are fed MCT display the clinical features of VOD with weight gain and fluid accumulation in the abdominal cavity.
   - **Progress:** We commenced a pilot study on one cohort of mice and were unable to see any signs of liver tissue damage when mice were fed MCT. We hypothesise that this is because mice cannot digest MCT to MCT-pyrrole (MCTP) which is the molecule responsible for liver toxicity. Therefore we purchased MCTP which we injected into mice and then assessed signs of VOD. We are continuing the titration of MCTP to thoroughly characterise the model.
2) **Bone marrow stem cell transplant without T-cells:** A more clinically relevant model in which mice are given chemotherapy agents busulphan and cyclophosphamide (Bu/Cy) for 7 days, and then injected intra-venously with stem cells from donor mice that are genetically different. Bu/Cy is given to humans to kill blood cells that are cancerous, and in mice it has the same effect, and the mice are rendered with no blood cells. When they receive the donor cells from a different mouse, it results in 'engraftment', whereby the new stem cells regenerate all the blood cells in the recipient. Liver VOD is a natural side effect of this process in humans and is reported to similarly manifest in mice. However, in humans this procedure often results in acute graft versus host disease (GVHD). This means that the donor white blood cells attack

the recipient's organs as they are 'foreign'. To prevent this from occurring, T-cells, a specific cell type from the blood, are first removed from the blood cell isolate of the donor.

- **Progress:** Our pilot study showed that a blood cell transplant excluding T-cells does not cause VOD in mice. This demonstrates that T-cells are important for VOD, and that a GVHD-like response underlies VOD, as the two diseases appear to have some overlapping pathological signs.

3) **Bone marrow stem cell transplant with T-cells:** Having shown that GVHD may be an important component of liver VOD we commenced a new set of experiments in which recipient mice are injected with donor stem cells together with a small dose of T-cells which are sourced either from the blood itself, or from the spleen, which is known to cause mild acute GVHD.
    - **Progress:** We found that when the T-cells are sourced from the blood there are no major signs of liver damage or overall disease in the mice. Because of this we next injected T-cells from the spleen and also observed the mice for up to 28 days. In this instance it was evident that mice had developed GVHD and there is evidence of onset of VOD in these mice.

Findings regarding establishment of a mouse model of VOD:
The attached figure displays the some data obtained from Aim 1 in which we established two mouse models of liver VOD. The toxin model using MCT or MCTP was the most reliable and reproducible model developed.

We studied a group of 3-6 mice each that were administered MCT or MCTP and killed at days 6-7 and 14. We monitored animal wellbeing as per animal ethics committee guidelines and did not record any adverse events. We analysed the change in the animals' weight, if any, as a result of these treatments and after the animals were killed, measured liver weights and processed the liver tissues for histology. We also conducted a full blood evaluation of the mice to determine whether the MCT/MCTP altered blood counts in these animals. As seen in Figure 1, both MCT and MCTP treatment leads to significant decrease in animal weight at day 6, and in both groups, animals continue to display significant weight loss at Day 14, albeit there is some recovery in body weight at day 14 in MCT but not in MCTP treated mice. Assessment of liver weight as a sign of liver enlargement shows that MCTP-treated mice at day 14 have higher liver/body weight ratios. We also measured the concentration of liver enzymes alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) in the blood after MCT and MCTP treatment. These enzymes are released into the blood by inflamed/damaged liver cells. We found that ALT levels are specifically increased at Day 14 in the MCTP-treated group consistent with signs of VOD in these animals. We then assessed the liver tissue with the conventional haematoxylin and eosin (H&E) staining protocol. The second half of Figure 1 shows representative H&E staining from 1 mouse at three different magnifications (10x, 20x, 40x); it is evident that the liver tissue is substantially damaged in MCTP treated mice from day 7 onward. Dying liver cells are visible, alongside dilated sinusoids as well as sinusoidal congestion.

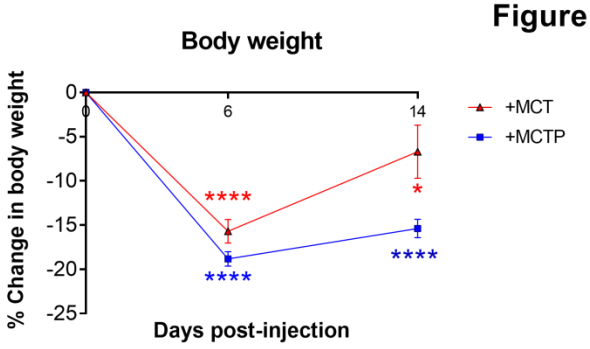
Anti-VCAM-CD39

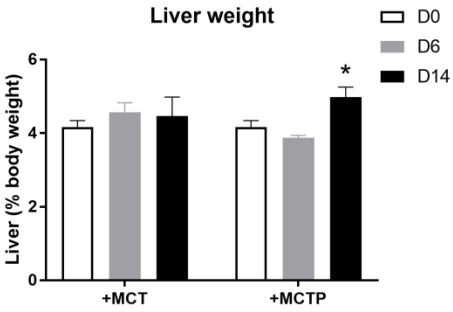
We have successfully purified anti-VCAM-CD39 from insect and mammalian cells. Figure 3 shows that structure of the recombinant protein that we are producing. We have further refined the protein production in insect cells and have been able to confirm that the drug specifically binds to its target i.e. VCAM-1 protein in endothelial cells in vitro using a flow cytometry approach (Fig 3). We also showed in an ELISA assay that the protein has a high affinity for VCAM-1.

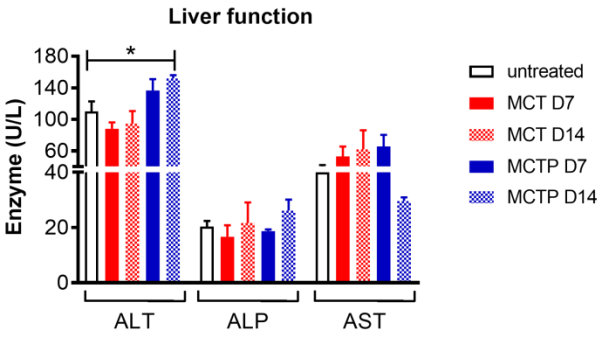
We are simultaneously studying the effects of a non-targeted CD39 protein ('scrambled'); from our earlier studies, we anticipate that targeted delivery of CD39 will enable us to use lower doses of this agent. Indeed, tail bleeding assays in mice have shown that doses under 1mg/kg do not prolong bleeding whereas the scrambled protein leads to intermittent bleeding in mice. Therefore, we have done all the confirmatory steps to ensure that anti-VCAM-CD39 at doses of up to 1mg/kg can be trialled in our VOD model without causing bleeding complications.

Having characterised the models, we will now proceed to testing the effect of anti-VCAM-CD39 and defibrotide in these mice as well as determine how these agents work to reduce the devastating effects of VOD (Aims 2 and 3). Anti-VCAM-CD39 has already showed promising results in our models of brain injury (stroke) and kidney injury (ischemia-reperfusion injury model) and we are confident that the same effects will be seen in this model of liver injury.

Figure 1


Body weight
% Change in body weight
0
-5
-10
-15
-20
-25
0
6
14
****
*
****
****
+MCT
+MCTP
Days post-injection


Liver weight
D0
D6
D14
Liver (% body weight)
6
4
2
0
*
+MCT
+MCTP


Liver function
Enzyme (U/L)
180
140
100
60
40
20
0
*
ALT
ALP
AST
untreated
MCT D7
MCT D14
MCTP D7
MCTP D14

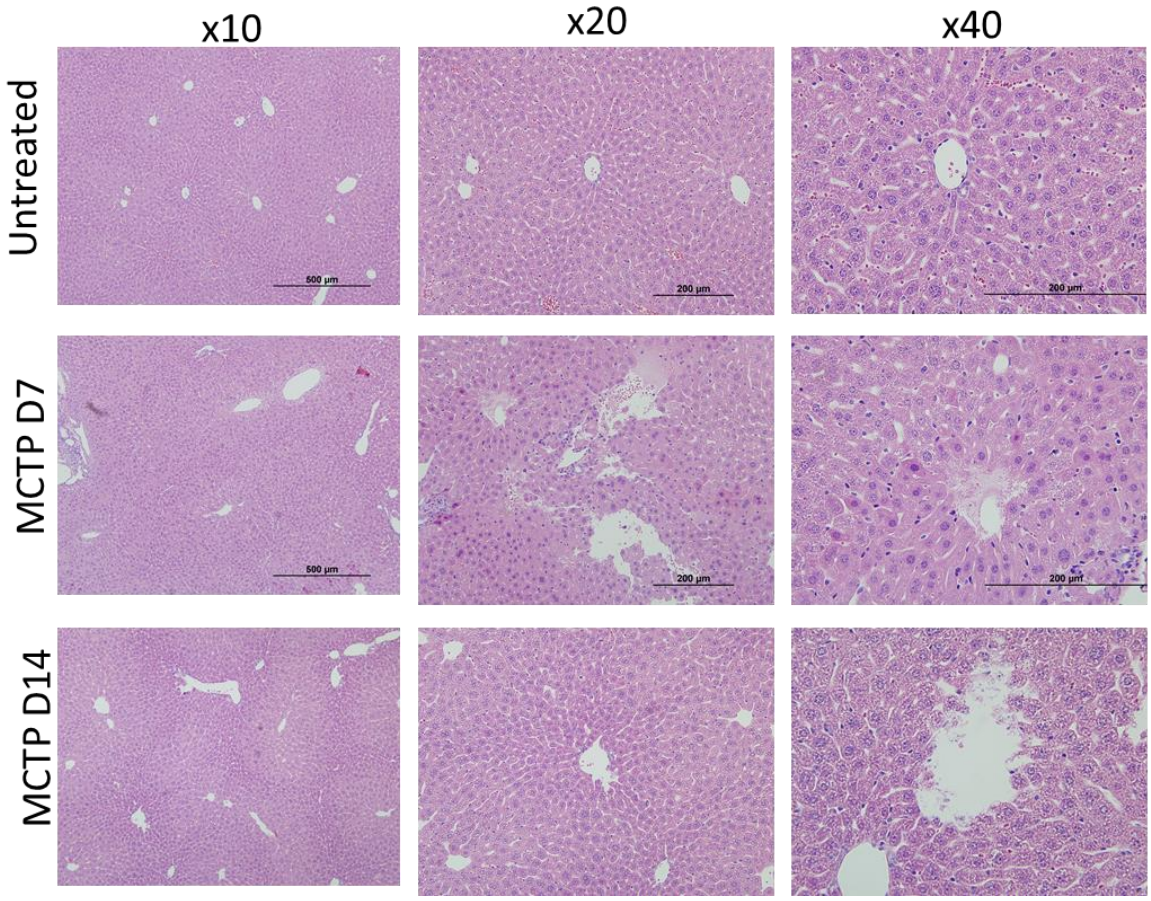
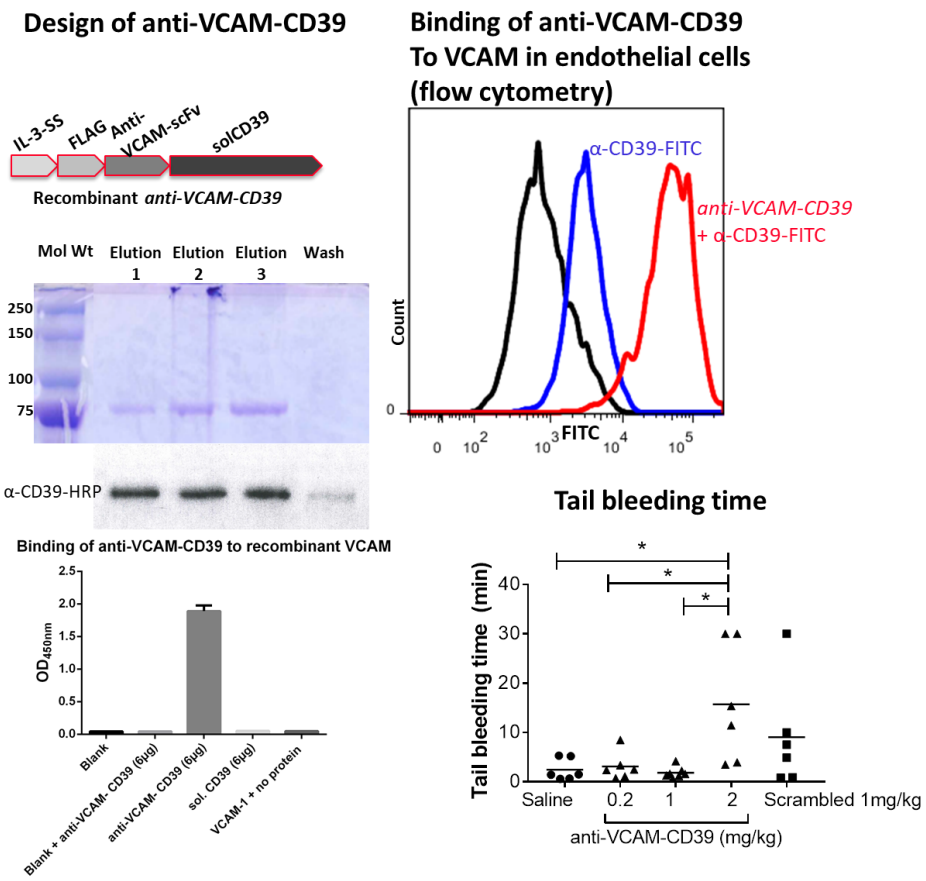

x10
x20
x40
Untreated
MCTP D7
MCTP D14
500 µm
200 µm

Figure 2


Design of anti-VCAM-CD39
IL-3-SS
FLAG
Anti-VCAM-scFv
solCD39
Recombinant anti-VCAM-CD39
Mol Wt
Elution 1
Elution 2
Elution 3
Wash
250
150
100
75
α-CD39-HRP
Binding of anti-VCAM-CD39 to recombinant VCAM
OD450nm
Blank
Blank + anti-VCAM- CD39 (6ug)
anti-VCAM- CD39 (6ug)
sol. CD39 (6ug)
VCAM-1 + no protein
Binding of anti-VCAM-CD39
To VCAM in endothelial cells
(flow cytometry)
α-CD39-FITC
anti-VCAM-CD39
+ α-CD39-FITC
Count
FITC
Tail bleeding time
Tail bleeding time (min)
Saline
0.2
1
2
Scrambled 1mg/kg
anti-VCAM-CD39 (mg/kg)