CHARACTERIZATION OF THE ANTI-TUMOUR IMMUNE RESPONSE FOLLOWING TREATMENT WITH AN INFECTED LEUKEMIA CELL VACCINE

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ABSTRACT

Current treatment methods for Acute Leukemia (AL) only provide temporary therapeutic efficacy as most patients will experience relapse within 2 years following first remission. Our lab has determined that vaccination with autologous cells infected with oncolytic virus MG1 can provide durable cures in a pre-clinical mouse model of AL. However, the mechanism(s) by which the infected cell vaccine (ICV) stimulates T cell dependent anti-tumour immunity and provides protection against tumour growth is unknown. This thesis was aimed to determine 1) what antigen presenting cell populations are activated post ICV immunization and 2) what T cell subsets are important in developing anti-tumour immunity during ICV immunization. My thesis has demonstrated that ICV immunization is more effective at inducing *in vivo* dendritic cell activation compared to irradiated L1210 cells alone and this activation may be a reason as to why we see improved anti-tumour efficacy in our ICV model. In addition, we have determined that CD4 T cells play an essential anti-leukemic role during ICV immunization and that neutralizing antibody production is a CD4 T cell dependent mechanism. Our data also demonstrates that both CD4 and CD8 T cell populations from ICV immunized mice provide a leukemia-specific anti-tumour immune response. Taken together, this data suggests that CD4 T cells may be acting as helper T cells to aid in the robust activation of leukemia-specific anti-tumour CD8 T cells. Our pre-clinical data characterizing the immune response has improved our understanding of the mechanism(s) which contribute to the efficacy of the ICV and will help provide a rationale framework with which to begin translating this treatment to clinical trials.

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LIST OF ABBREVATIONS

AL	Acute Leukemia
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APC	Antigen Presenting Cell
ATP	Adenosine Tri-Phosphate
BMDC	Bone Marrow Derived Dendritic Cell
CAR	Chimeric Antigen Receptor
CLL	Chronic Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
CR1	Complete Remission 1
CR2	Complete Remission 2
CRS	Cytokine Release Syndrome
CRT	Calreticulin
CTLA-4	Cytotoxic T-lymphocyte Associated Protein-4
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DFS	Disease Free Survival
DRR	Durable Response Rate
ELISA	Enzyme Linked Immunosorbent Assay
ELISpot	Enzyme Linked Immunospot Assay
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group Box Protein-1
ICD	Immunogenic Cell Death
ICV	Infected Cell Vaccine

IFN	Interferon
IL	Interleukin
Irr	Irradiated
MG1	Maraba MG1 Virus
MHCI/II	Major Histocompatibility Complex Class I/II
MLL	Mixed Lineage Leukemia
MOI	Multiplicity of Infection
NDV	New Castle Disease Virus
NK cell	Natural Killer Cell
NS	Not Significant
OS	Overall Survival
OV	Oncolytic Virus
PD-1	Programmed Cell Death Receptor-1
PD-L1	Programmed Cell Death Ligand-1
Ph	Philadelphia Chromosome
RANTES	Regulated upon Activation, Normal T Expressed, and Presumably Secreted
RBC	Red Blood Cell
SCT	Stem Cell Transplantation
SFC	Spot Frequency Count
TAA	Tumour Associated Antigen
TCR	T Cell Receptor
TIL	Tumour Infiltrating Lymphocyte
TKI	Tyrosine Kinase Inhibitors
TLR-4	Toll Like Receptor-4
TNF	Tumour Necrosis Factor
T-VEC	Talimogene laherparepvec
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stromatitis Virus

1 INTRODUCTION

1.1 Acute leukemia

1.1.1 Classification

Each year roughly 2000 Canadians are diagnosed with Acute leukemia (AL), a haematological malignancy characterized by the accumulation of immature and abnormal blood cells in the bone marrow and peripheral blood. Common symptoms of leukemia include extreme fatigue, bleeding, anemia, and being prone to infectious diseases.[1] Patients are diagnosed with either Acute Lymphoblastic Leukemia (ALL) or Acute Myelocytic Leukemia (AML) depending on the cell type effected. T cells, B cells, and Natural Killer (NK) cells are the origin of ALL, the most common being mature B cells. Non-lymphoid cells such as Red Blood Cells (RBCs) and megakaryocytes are the main origins of AML.[2] AML and Chronic Lymphoblastic Leukemia (CLL) are the most common type of leukemias observed in adults, while ALL is the most common leukemia diagnosed in children.[3]

1.1.2 Molecular basis of disease

The Philadelphia chromosome (Ph) is a common molecular marker of ALL. Ph is a translocation between chromosome 9 and 22 t(9;22) that is observed in 20-30% of adult ALL patients. Ph causes enhanced tyrosine kinase activity by downstream signalling pathways. Tyrosine kinase inhibitors (TKIs) have been incorporated into treatment methods for Ph+ ALL patients. Ph+ ALL patients have a worse prognosis than Ph- patients with a dismal long-term disease-free survival (DFS) rate of < 10%. [1,4]

30% of cancers diagnosed in children under the age of 15 are leukemia; the most common type of childhood cancer.[5] It has been reported that ionizing radiation is the main significant

environmental risk factor associated with childhood leukemia.[6] Genetic risk factors can include gene fusions in the mixed lineage leukemia (*MLL*) gene *in utero* as well as genetic polymorphisms that cause deficiencies in the ability to properly metabolize xenobiotic substances. For example, children with variants in cytochrome P4501A1 (CYP1A1) have worse leukemia prognosis than children who do not have these variants.[7–9] The overall five-year survival rate of both children and adolescents with leukemia is an astonishing 90.4%.[10] This impressive survival rate dampens when looking at adult populations with leukemia as increasing age at diagnosis is correlated with decreased survival. For example, the overall five-year survival rate of ALL patients age 40-64 is 30% and if the diagnosis occurs at 65 years of age or older it drops to 15%.[3]

1.1.3 Prognosis and current standard of care

Standard treatment methods for leukemia are: chemotherapy, radiotherapy, and allogenic stem cell transplantations (SCT). Founded in the 1960s, the typical treatment for leukemia, follows regimen-specific chemotherapy combining the drugs cytarabine and anthracycline. The overall goal of chemotherapy is to restore normal hematopoiesis, kill cancer cells, and prevent resistant cancer cells from growing; leading to complete remission. However, minimal residual disease (MRD) is one possible outcome observed in leukemia patients after therapy. MRD, also referred to as "incomplete remission" is when a low level of leukemia is detected after therapy in patients. MRD is detected in the laboratory by flow cytometry and polymerase chain reactions (PCR) as these techniques are more precise than morphology analysis.[11] If MRD is present but not detected in patients this can lead to serious consequences as it is associated with a higher risk of relapse in patients.[11] Many patients will receive either consolidation chemotherapy or a SCT post-chemotherapy if MRD is detected or if relapse occurs.[12]

1.2 The dilemma of AL

While these current treatment methods are effective at inducing complete remission 1 (CR1) in 80-90% of adult AL patients, disease will unfortunately recur in roughly 40% of adult patients.[2] Complete remission 2 (CR2) is very rare to obtain once a patient has relapsed.[13] For example, Gökbuget et al. observed that 100% of AL patients that received a non-SCT treatment for their cancer relapse died within 1 year of cancer recurrence.[14] Fielding et al. published that the overall five-year survival rate of a study done on 609 ALL patients after their first relapse is a dismal 7%.[15] Although, SCT seems to be the best treatment option for relapsed leukemia patients the clinical barriers include: elderly patients being unable to receive SCT because it is an invasive procedure and associated with complications. Difficulties in finding a human leukocyte antigen (HLA)-matched sibling or unrelated donor required for SCT is another limitation to the widespread adoption of this approach. Due to the high rate of AL relapse, it is a very difficult disease to cure with therapies such as chemotherapy and SCT. We are desperately in need of novel therapies and strategies for AL. Combining different types of cancer immunotherapies may be the answer to increase the therapeutic efficacy of AL.

1.3 Cancer immunotherapy

Cancer immunotherapy has demonstrated recent success in oncology research; activating the immune system of cancer patients to fight against their disease. There are many novel and promising cancer immunotherapies in the field of oncology. In 2013, *Science* even stated that cancer immunotherapies were the "Breakthrough of the Year."[16] Cancer immunotherapies can be distinguished based on their target/mechanism of action. In particular, immune checkpoint inhibitors, chimeric antigen receptor (CAR) T cells, whole tumour cell vaccines, oncolytic viruses (OVs), and infected cell vaccines are discussed below.

1.3.1 Immune checkpoint inhibitors

Immune checkpoint receptors and ligands are essential for ensuring an appropriate immune response by balancing immune co-stimulatory and inhibitory signals. For example, immune checkpoint receptors/ligands protect against auto-immunity and prevent damage caused by excessive inflammation. However, immune checkpoint receptors can also be exploited by the tumour tissue to escape immune surveillance and allow tumour outgrowth.[17] Therefore, checkpoint inhibitors were developed to prevent immune interactions between checkpoint receptors on immune and/or cancer cells to overcome immunosuppression leading to enhancement of the anti-tumour immune response (Figure #1).[18] Common checkpoint inhibitors are cytotoxic T lymphocyte antigen-4 receptor (CTLA-4) and programmed cell death-1 receptor (PD-1) antibodies that target T cells and programmed cell death receptor ligand-1 (PD-L1) on tumour cells. For example, pre-clinical studies have demonstrated that *in vivo* CTLA-4 blockade resulted in tumour regression in murine models of colon, prostate and ovarian cancer.[17,19] Currently, three checkpoint inhibitors have received approval from the United States Food and Drug Administration (FDA) for unresectable and metastatic melanoma: Ipilimumab (anti-CTLA-4), Pembrolizumab (anti-PD-1), and Nivolumab (anti-PD-1).[18] Overall, these inhibitors have shown success in the clinic as both Ipilimumab and Nivolumab have an overall survival advantage in melanoma patients compared to standard chemotherapy.[20,21] Xerri et al. has demonstrated that CTLA-4 is upregulated in T-cell lymphoma patients suggesting that anti-CTLA-4 therapy may be an effective treatment option for hematological malignancies.[22] Phase I and II clinical trials have been done using checkpoint inhibitors for hematological malignancies showing promising responses, but have yet to be tested on AL patients.[17,23,24] In 2015, Ansell et al. demonstrated an 87% response rate to Nivolumab in patients with relapsed classic Hodgkin's

lymphoma.[25] Overall, checkpoint inhibitors have become a standard part of solid tumour therapy suggesting they will soon get more involved with hematological malignancy treatment.

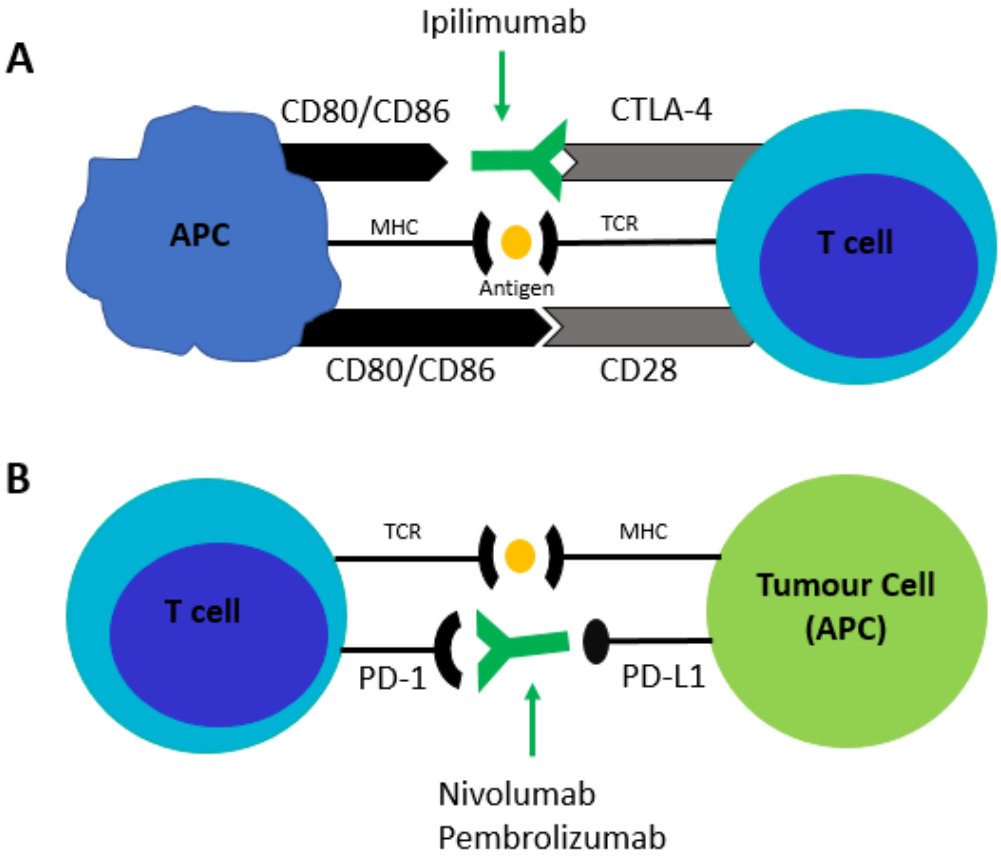

A
Ipilimumab
CD80/CD86
CTLA-4
APC
MHC
TCR
T cell
Antigen
CD80/CD86
CD28
B
TCR
MHC
T cell
Tumour Cell
(APC)
PD-1
PD-L1
Nivolumab
Pembrolizumab

Figure #1. The mechanism of immune checkpoint inhibitors for cancer treatment.

A: CTLA-4 out-competes CD28 binding for the receptor CD80 on antigen presenting cells (APCs) leading to APC inhibition. Ipilimumab inhibits the CTLA-4 receptor on T cells subsequently allowing CD28 to bind to CD80 allowing APC activation. **B:** PD-1 is a co-inhibitory receptor on T cells. Nivolumab and Permbrolizumab bind to PD-1 on T cells increasing T cell activation and cancer cell death. Figure adapted from reference #18.

1.3.2 Chimeric Antigen Receptor (CAR) T cells

T cells cans be enabled to target specific cells and/or antigens by engineering a chimeric antigen receptor (CAR) on their cell surface. CAR T cells are composed of an antigen binding domain that is specific for a tumour associated antigen (TAA) and an intracellular T cell activation domain. These domains make is possible for activated CAR T cells to directly target cancer cells and provide anti-tumour immunity. Many CAR T cells have been engineered against different TAAs such as: human epidermal growth factor-2 (HER2), mesothelin (MSLN) and CD19.[26] However, the most promising clinical outcomes have been demonstrated in B cell leukemias using CAR T cells against CD19. [27–29] CD19 is a specific antigen on both healthy B cell lymphocytes and malignant B cells. The first phase I clinical trial for CD19 CAR T cells was targeting B cell ALL. This clinical trial achieved a complete remission (CR) rate of 88%.[28] Other clinical trials have also been performed for B cell ALL and impressive CR rates were demonstrated – 67% and 90%.[27,29] These CR rates are very encouraging; however, high relapse rates are still seen in patients receiving CD19 CAR T cell therapy. A common complication is the existence of a small CD19-negative B cell leukemia cell population that are not targeted during therapy.[26] Ruella et al. showed that approximately 30% of patients that relapsed from CD19 CAR T cell therapy was due to expansion of CD19-negative cancer cells.[30] Serious side effects have also been reported such as: cytokine release syndrome (CRS) and neural toxicities.[26] CRS is an immune mediated disorder that is caused by an excess of pro-inflammatory cytokines being secreted by activated T cells. Common cytokines released during CRS are IFN-gamma, TNF-alpha, IL-10, IL-6, and IL-2.[31] Several complications can occur due to the excess secretion of these cytokines. For example, the inflammatory response can cause endothelial cell damage which can lead to heart failure. Respiratory failure is other serious side effect. Unfortunately,

Morgan et al. and Turtle et al. have reported patients passing away from CRS in clinical trials for CD19 CAR T cells.[32,33] Research is being done on how to inhibit CRS is patients receiving CAR T cells. Aplenc et al. reported administering anti-cytokine therapy (anti-IL-6 antibody) to a patient that developed CRS post CD19 CAR T cell treatment. Anti-IL-6 therapy was effective at inhibiting CRS and this patient achieved CR.[27] CAR T cells are an up and coming cancer immunotherapy that have impressive potential, especially for blood cancers. There are many serious side effects of CAR T cell therapy that many researchers are learning how to manage for future patients.

1.3.3 Whole tumour cell vaccines

Whole tumour cell vaccines are a therapeutic strategy for cancer that can induce a robust anti-tumor immune response. There are two main types of whole tumour cell vaccines: autologous and allogenic.[34] Autologous tumour cell vaccines are created when tumour cells from a cancer patient are isolated and manufactured *in vitro* into a tumour vaccine. The tumour cells are then administered back into the cancer patient and are known to produce strong and long-term anti-tumour immune responses. This strategy is unique as it creates a personalized approach to cancer treatment by using the patient's own cells as the vaccine.[34] Autologous tumour cell vaccines have an advantage as they induce a polyclonal anti-tumour immune attack. Since the whole heterologous tumour cell is the vaccine, multiple tumour associated antigens (TAA) are targeted. An issue with autologous cell vaccines is that this treatment can be restricted to tumour type and stage as an adequate amount of tumour cells need to collected from the patient.[35] Allogenic tumour cell vaccines are very similar to autologous vaccines but the tumour cells used as the vaccine are not from the patient.[34] The cells used in allogenic vaccines are derived from

laboratory grown cancer cell lines; this means that they do not contain patient specific TAAs but there is an abundance of these cells.

There are different ways to manipulate tumour cells *in vitro* to engineer a potent cancer vaccine. For example, patient's tumour cells can be manipulated *in vitro* by irradiating the cancer cells and then immunizing the patient with irradiated cancer cells. Irradiated cancer cells present TAAs to antigen presenting cells (APCs) which should in turn stimulate tumour specific T cells creating a long-term anti-tumour immune response. Another strategy is to transfect patient's cancer cells with immune stimulatory cytokines – likely to cause an increase in APC activation and anti-tumour immunity.[36] For example, the tumour cell vaccine GVAX, has been genetically engineered to secrete granulocyte macrophage-colony stimulating factor (GM-CSF).[37] GM-CSF is a cytokine that attracts dendritic cells (DCs) and macrophages to the vaccination site and is able to induce tumour infiltrating lymphocytes (TILs). DCs are able to play a critical role in T cell mediated anti-tumour immunity, making GM-CSF aid in the efficacy of the vaccine. GVAX has been reported to be used in both autologous and allogenic tumour cell vaccines.[37] GVAX has been shown to promote anti-tumour immunity in murine models of cancer by the mechanism of DC activation which in turn induced CD4 and CD8 T cell priming leading to anti-tumour immunity.[38] Most research on whole cell vaccines has been demonstrated in solid tumours, however, GVAX has been used in clinical trial for chronic myeloid leukemia (CML) patients. Smith et al. used an allogenic leukemia cell line (K562) that expressed GM-CSF as the vaccine. This study found that GVAX reduced tumour burden in CML patients that had residual disease while undergoing chemotherapy.[39]

Overall, whole cell vaccines are a promising avenue for cancer immunotherapy but many researchers are demonstrating that whole cell vaccines need to be combined with another immunotherapy to induce a robust and long-term anti-tumour immune response.

1.3.4 Oncolytic viruses

Oncolytic viruses (OVs) are a promising therapeutic strategy as they are able to target and kill cancer cells while leaving healthy cells unharmed (Figure #2). Cancer cells generally have mutations in their anti-viral interferon (IFN) signalling pathways. For example, these mutations make cancer cells able to inhibit apoptosis and enhance angiogenesis, therefore, making them resilient tumour cells.[40] OVs exploit the mutations that cancer cells have which leads to viral replication solely in tumour tissue.

OVs have been developed in the lab for about 20 years but just recently the first OV was approved by the FDA.[41] In 2015, talimogene laherparepvec (T-VEC) was approved for the treatment of melanoma. T-VEC is a herpes simplex virus type 1 that is able to replicate in cancer cells and produce GM-CSF to induce a robust anti-tumour immune response. In a phase III clinical trial, melanoma patients treated with T-VEC had a significantly better durable response rate (DRR) and longer overall survival (OS) compared to patients that only received GM-CSF.[41] T-VEC has been genetically engineered in the laboratory by the deletion of the genes *ICP34.5* and *ICP37.* These deletions are able to help attenuate its virulence and increase its tumour selective replication. Many OVs are genetically engineered by creating mutations in non-essential viral genes to make them more oncolytic and enhance their safety profile.

For example, MG1 is a genetically engineered Maraba virus from the rhadbovirus family. Wild-type (WT) Maraba virus is composed of a single stranded RNA genome and 5 proteins as such:

3'N-P-M-G-L-5'. MG1 has mutations in the matrix "M" (L123W) and glycoprotein "G" (Q242R) protein. Brun et al. demonstrated using viability assays and plaque assays that MG1 versus WT Maraba had enhanced ability to infect malignant cells and decreased ability to harm healthy cells. These results are due to both IFN-dependent and independent mechanisms.[42] Brun et al. also demonstrated that systemic delivery of MG1-GFP specifically replicated in CT26 colon cancer tumours using bioluminescent imaging. Durable survival and tumour regression was also observed in MG1 treated mice that had been previously injected with CT26. Whereas, 100% of untreated control mice died of tumour burden.[42] This data shows the promise of using OVs, specifically MG1, for cancer therapy.

OVs are known to work in 3 main ways to provide anti-tumour immunity. Firstly, OVs are able to infect and kill tumour vascular endothelial cells, leading to tumour cell exhaustion and death.[43] Arulanandam et al. has shown that vascular endothelial growth factor (VEGF) signalling in tumor blood vessels inhibits the type 1 IFN pathway by enhancing the repressing transcription factors PRD1/BF1/Blimp1 therefore, sensitizing tumour vascularization to OV infection.[44] Also, the cytolytic ability of OVs can cause tumour cell lysis and viral spread. Due to this mechanism, the OV is able to migrate to other areas of the tumour microenvironment resulting in cancer cell death. Lastly, during cancer cell lysis tumour antigens are subsequently exposed and able to induce a robust anti-tumour immune response by uptake from APCs. This response has been regarded as one of the most important mechanisms as to how OVs provide systemic anti-tumour immunity because APCs are then able to become activated and stimulate the long-term adaptive immune response.[40]

1.3.4.1 The role of antigen presenting cells (APCs) in mediating T cell activation

APC activation is necessary for stimulating T cell activation which mediates the long-term adaptive immune response. The three main APC populations are: B cells, macrophages, and DCs. However, literature has shown that DCs have a superior ability to process antigens and further cross present antigens to CD8 T cells.[45] Once APCs are activated by foreign antigens, they will present antigen fragments on the Major Histocompatibility Complex (MHC) peptide. T cell receptors (TCR) on the surface of T cells will bind to the MHC peptide presenting the antigen. CD8 T cells bind to the MHCI peptide while CD4 T cells bind to the MHCII peptide on APCs. For activation to occur, T cells also need to bind to co-stimulatory molecules such as CD80/86 and CD40 on the surface of the APC.[45] Therefore, APCs play a critical role in the initial steps of the anti-tumour immune response.

OVs are known to activate APC populations after administration *in vivo*. Norbury et al. demonstrated that within 6 hours of vaccinia virus administration in mice, a robust APC activation phenotype was observed. Notably, DCs were the only activated APCs that were able to stimulate naïve CD8 T cells resulting in virus-specific T cells.[46] Zhang et al. reported MG1 infection of bone marrow derived dendritic cells (BMDCs) induced a robust increase in CD80/CD86 activation markers on CD11c+ cells. Also, when DCs were ablated *in vivo* MG1 was not effective at inhibiting tumour metastases.[47] This demonstrates that importance of DC activation after OV infection to aid in anti-tumour immunity.

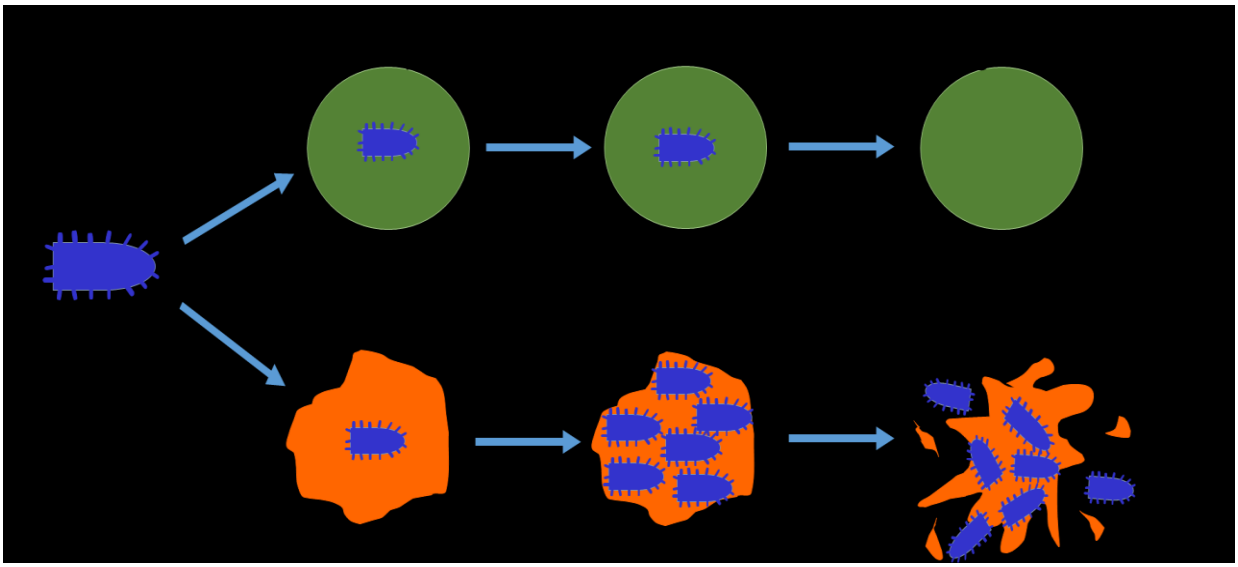


Figure #2. Oncolytic Virotherapy.

Oncolytic viruses (OVs) directly target and kill cancer cells. OVs can infect both healthy and cancer cells. Healthy cells will activate anti-viral pathways when infected so that the virus is unable to replicate. Cancer cells do not have intact anti-viral pathways which leads to viral replication and cancer cell lysis.

1.3.4.2 RANTES secretion and anti-tumour immunity

Immune-stimulatory cytokines can also be inserted into recombinant viral vectors to induce robust DC activation. RANTES (regulated upon activation, normally T expressed, and presumably secreted), also known as CCL-5, is a known pro-inflammatory chemokine that binds to CCR1, CCR3 and CCR5 receptors.[48] RANTES interaction with its receptors allows for DC maturation and, infiltration of CD4/CD8 T cells and NK to the tumour site.[49] The oncolytic adenovirus expressing RANTES (Ad-RANTES) has demonstrated successful pre-clinical cancer data. Lapteva et al. demonstrated that Ad-RANTES aided in the recruitment of DCs to the tumour site and subsequent DC activation compared to the adenovirus alone. Overall, intra-tumoural vaccination with Ad-RANTES significantly stimulated tumour regression in an EG.7 murine lymphoma model.[50] Lavergne et al. also showed that intra-tumoural injection of RANTES-encoding-DNA induced delayed tumour growth in mice with established lymphoma. Anti-tumour immunity was associated with an increase of tumour CD4/CD8 T cells, DCs, and NK infiltrating cells.[51] This data implies that RANTES secretion can be utilized to help provide anti-tumour immunity in cancer immunotherapy vaccination strategies, mainly by aiding in DC activation.

1.3.5 Infected Cell Vaccines (ICVs)

Infected cell vaccines (ICVs) are a type of cancer immunotherapy combining OVs and whole tumour cell vaccines. In theory, ICVs are able to present a wide range of TAAs with the aid of a robust OV infection. The concept of the ICV was first introduced more than 50 years ago by researchers Murray and Cassel. This team used melanoma cells infected with the New Castle Disease Virus (NDV) to produce an ICV for the treatment of melanoma. Phase II clinical studies were done using NDV-ICV for metastatic melanoma and the 10-year DFS rate was 60% -

significantly better than surgery alone.[52] This study also reported that CD8 T cells played a critical role for the NDV-ICV to provide long-term therapeutic efficacy.[52]

Currently, ICVs are produced in the laboratory by infecting tumour cells with an OV followed by irradiation to inactivate the tumour cells. Lemay et al. has demonstrated that prophylactic immunization of irradiated-vesicular stromatitis virus (VSV) infected tumour cells (ICV) protect 30% of mice from colon cancer challenge. When the ICV was engineered to secrete GM-CSF (ICV-GM), the vaccine protected 95% of mice from tumour challenge. Interestingly, mice that received the ICV-GM compared to controls had a significant increase of activated DCs in their spleen within 24 hours post immunization as demonstrated by flow cytometry.[53] This shows the importance of the activating the innate immune response post immunization.

1.3.5.1 The Role of the T Cell Mediated Anti-Tumour Immune Response

There are two main subtypes of T cells: CD4 and CD8. CD8 T cells are known as Cytotoxic T-Lymphocytes (CTLs) that are effector immune cells that target and kill cancer/infected cells. In comparison, CD4 T cells are known as helper cells that assist in immune cell activation and proliferation. For example, CD4 T cells are known to help activate cytotoxic CD8 T cells and aid in B cell maturation and antibody production.[54] In the presence of IL-2, van Den Broeke et al. demonstrated that CD4 T cells are required for NK cells to provide cytotoxic activity against tumour cells.[55] Contrary to the helper T cell role, literature has also demonstrated that there are CD4 T cells with direct cytotoxic function.[56–60] The main killing mechanisms used are similar to cytotoxic CD8 T cells; the production of IFN-gamma, perforin, and granzyme-B.[56–58]

Lemay et al. has demonstrated that mice immunized with ICV-GM compared to controls had an increase in CD3 T cells secreting IFN-gamma.[53] Alkayyal et al. has also demonstrated anti-

tumour immunity using an ICV platform, autologous tumour cells engineered to secrete IL-12 infected with MG1 virus (MG1-IL12-ICV). B16 melanoma cancer cells were used in this model. Mice immunized with MG1-IL12-ICV compared to mice that either received MG1-ICV or irradiated B16 cells had a significant survival advantage when challenged with B16 tumours. Alkayyal et al. demonstrated that CD3 and CD8 T cells were required for MG1-IL12-ICV to provide anti-tumour immunity as immunized mice depleted of these T cell subsets died of tumour burden.[61]

1.4 Development of an ICV for AL

Our lab has developed of an ICV for AL by using autologous MG1 infected AL cells to create a personalized leukemia cell vaccine. By combining both the OV and whole tumour cell vaccine, the ICV treatment should induce a robust anti-tumour immune response in AL patients, ideally preventing relapse. The ICV is produced in the laboratory by infecting AL (L1210) cells with MG1 virus followed by irradiation. The ICV is the administered in the tail-vein of mice every 7 days for a total of 3 doses. To determine if the ICV provides anti-tumour immunity immunized mice are challenged with viable L1210 cells and survival is monitored (Figure #3). Conrad et al. has shown promising murine pre-clinical data demonstrating that administration of the ICV protects murine recipients from leukemia with over 95% of ICV immunized mice achieving long-term protection against leukemia cells compared to 100% mortality in unimmunized mice, a truly remarkable outcome (Figure #4). Notably, mice that are immunized with irradiated L1210 cells all succumb to leukemia challenge, demonstrating that MG1 virus is necessary for the anti-tumour immune response.[62] The development of an ICV for the clinic could be the future of effective therapy for AL patients.

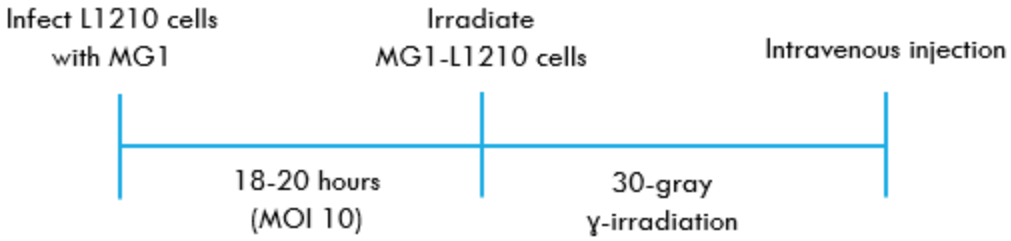
Our lab has shown that the protective effects of the ICV in part depend upon T cell mediated immunity.[62] ICV immunized athymic (lacking T cells) mice were not protected against challenge with leukemia cells, and showed similar survival outcomes as unimmunized mice. Based on this data from our lab, the adaptive immune response is essential for the protective effects of the ICV, but it is unknown as to what T cell subsets are critical.

Conrad et al. as also demonstrated that mice first vaccinated with MG1 virus followed by ICV immunization showed an increase in survival to leukemia challenge compared to mice that only receive the ICV. Pre-treatment with MG1 induces anti-viral antibodies against MG1 in the serum of mice.[62] Somehow, these pre-existing anti-viral antibodies increase the efficacy of the ICV to leukemia challenge. It would be informative to understand the mechanism behind this phenomenon.

Overall, the development of the ICV for the clinic is a priority but more needs to be known about the mechanism(s) by which the ICV provides anti-tumour immunity.

L1210: Murine Acute Lymphoblastic Leukemia

ICV Generation



ICV Timeline

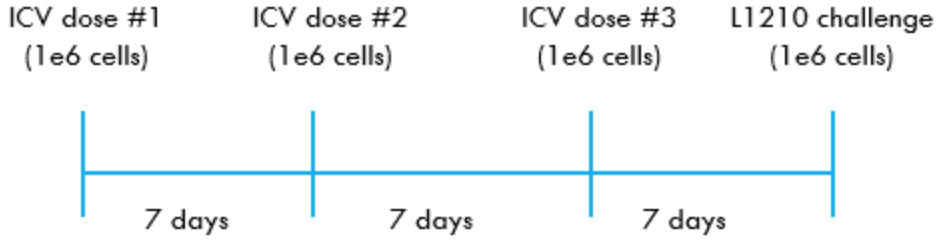


Figure #3. The infected leukemia cell vaccine (ICV) preparation, immunization, and L1210 challenge model.

Acute lymphoblastic leukemia cells (L1210) are infected with MG1 virus at an MOI 10 for 18 hours. Post infection the ICV is harvested and re-suspended in PBS followed by 30-gray ɣ-irradiation. DBA/2 mice then receive tail veil injections of $1x10^6$ cells of the ICV, once weekly for three weeks, followed one week later by $1x10^6$ viable L1210 cells.

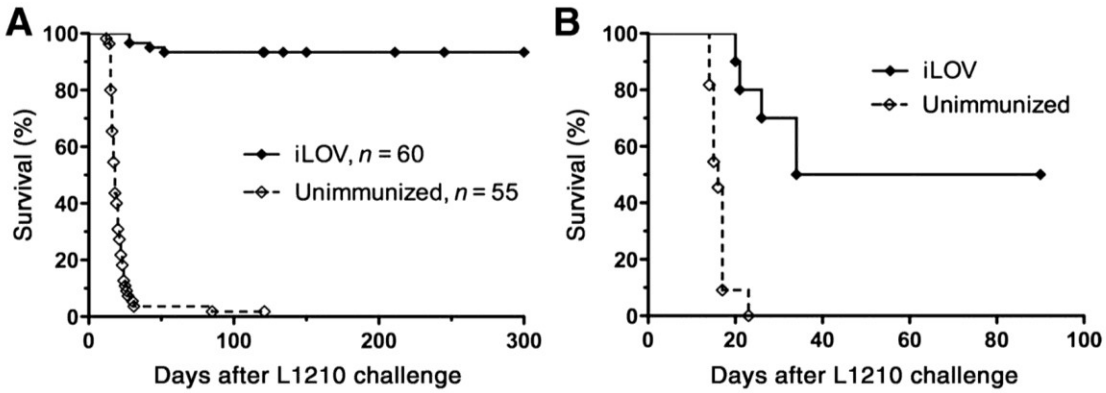

A
Survival (%)
100
80
60
40
20
0
0
100
200
300
Days after L1210 challenge
iLOV, n = 60
Unimmunized, n = 55
B
Survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
Days after L1210 challenge
iLOV
Unimmunized

Figure #4. The infected cell vaccine provides protection against leukemia by generating anti-tumour immunity.

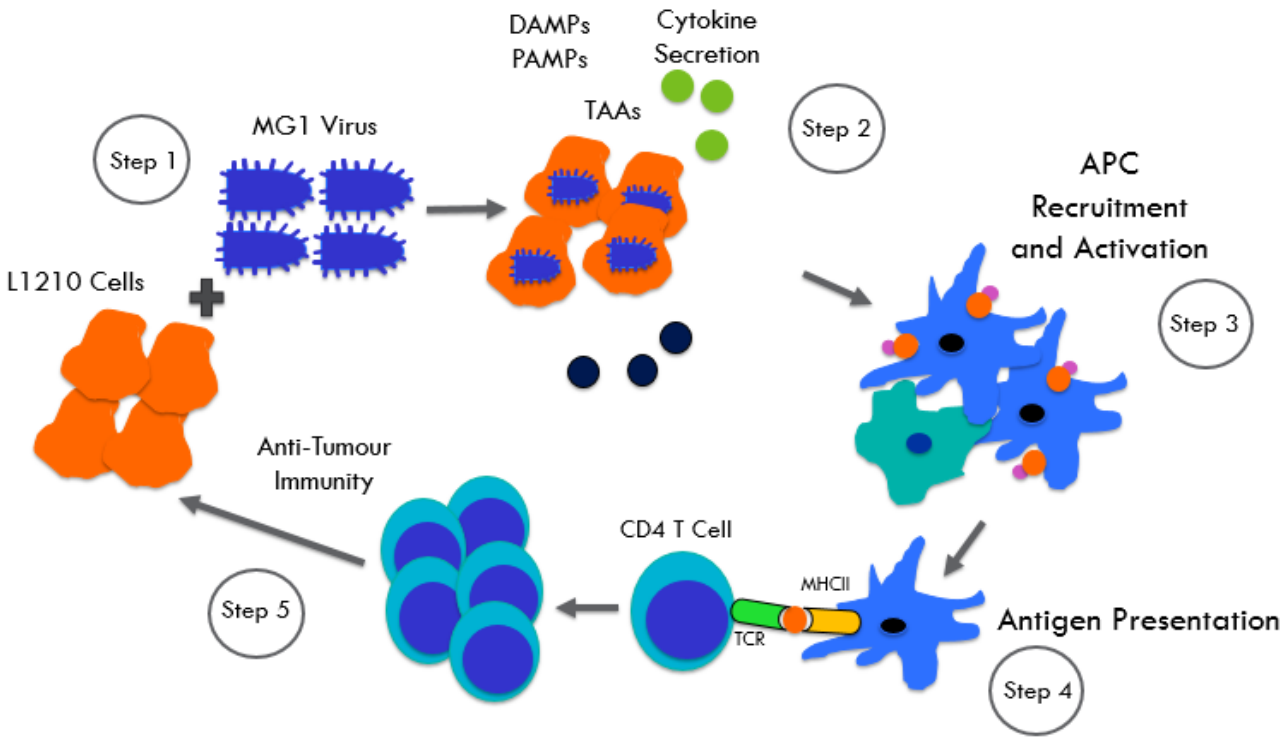
A: DBA/2 mice were administered the ICV, once weekly for three weeks, followed one week later by $1x10^6$ viable L1210 cells. 95% of immunized mice achieved long-term protection against L1210 cells compared to unimmunized mice. **B:** DBA/2 mice received the ICV 24 hours after administration of viable L1210 cells. In these figures the ICV is referred to as iLOV (immunotherapy by leukemia-oncotropic virus).[62]

2 HYPOTHESIS & OBJECTIVES

2.1 Hypothesis

MG1 infection and irradiation activates antigen presenting cells, specifically dendritic cells, by the production of pro-inflammatory cytokines, the presentation of TAAs, and the presentation of Pattern Associated Molecular Patterns (PAMPs) and Danger Associated Molecular Patterns (DAMPs). This will in turn stimulate tumour specific CD4 T cells to mediate the protective effects of the ICV.

2.1.1 The model



2.2 Objectives

The overall research objective of my thesis was to identify the mechanisms required to generate an anti-tumour immune response following vaccination with autologous leukemia cells infected with MG1 (ICV). My thesis was aimed to answer the two following questions:

1. What antigen presenting cell (APC) populations are activated post ICV immunization?
2. What T cell subsets are important in developing anti-tumour immunity during ICV immunization?

3 MATERIAL & METHODS

3.1 Reagents

Rat anti-mouse *in vivo* antibodies CD4 (Clone GK1.5 – catalog #: BE0003-1) and CD8α (Clone 53-6.72 – catalog #:BE0004-1) were obtained from Bio X Cell. Flow cytometry antibodies: CD3 (Pe-Cy7), CD4 (PE), CD8 (FITC), CD19 (FITC), CD11c (BV421), F4/80 (BV711), CD40 (PE-CF594), CD80 (BV605), MHCII (PerCP-Cy5.5), and fixable viability stain (FVS510) were obtained from BD Biosciences. A complete list of the fluorescently labeled antibodies used can be found in Table 1. CD3 (catalog #: 19851), CD4(catalog #:19852) and CD8 (catalog #:19853) isolation kits were obtained from StemCell Technologies. Celltrace Violet was obtained from ThermoFisher Scientific and CFSE was obtained from Biolegend. VSVn peptide (Balb/c background) was a kind gift from the Diallo lab. Phorbol myristate acetate (PMA) and ionomycin were obtained from Sigma-Aldrich.

Table 1. Antibody List

Species	Reactivity	Target	Fluorophore	Company	Cat #
Rat	Mouse	CD3	Pe-Cy7	BD Biosciences	560591
Rat	Mouse	CD4	PE	BD Biosciences	553730
Rat	Mouse	CD8	FITC	BD Biosciences	553031
Rat	Mouse	CD19	FITC	BD Biosciences	561740
Rat	Mouse	F4/80	BV711	BD Biosciences	565612
Rat	Mouse	CD40	PE-CF594	BD Biosciences	562847
Rat	Mouse	MHCII	PerCP-Cy5.5	BD Biosciences	562363
Hamster	Mouse	CD80	BV605	BD Biosciences	563052

Armenian Hamster	Mouse	CD11c	BV421	BD Biosciences	565451

3.2 Cell lines

Murine B cell acute lymphoblastic leukemia cell line (L1210) was obtained from American Type Culture Collection (ATCC). Cells were maintained in suspension culture in Dulbeccos's modified Eagle Medium (DMEM)-high glucose (HyClone), with 10% newborn calf serum:fetal calf serum (NCS:FBS) at a 3:1 ratio at 37°C/5% CO_2. Cells were split every 2 to 4 days to maintain a concentration between $5x10^5$ to $1x10^6$ cells/ml. Vero cells (ATCC) were maintained in adherent cell culture conditions in DMEM and 10% NCS:FBS. Vero cells were used for virus propagation, viral titering, and neutralizing antibody assays.

3.3 Mice

6-week-old female DBA/2 mice were purchased from Charles River Laboratories and housed in a contaminant level 2 (CL2) biosafety unit at the University of Ottawa accredited by the Canadian Counsel of Animal Care (CCAC). Institutional guidelines and review board for animal care, The Animal Care and Veterinary Service of the University of Ottawa, approved all animal studies.

3.4 MG1 virus propagation and titering

The rhabdovirus, MG1[42] was propagated in Vero cells by infecting cells at an MOI 0.01 for 72 hours. Supernatants were harvested post infection and spun down at 1500 x g for 10 minutes to remove the cell pellet. Cell debris was removed by passing the supernatant through a 0.22-micron filter and the virus was concentrated by centrifugation in the Avanti JXN high speed centrifuge (Beckman Coulter) at 14000 x g for 90 minutes at 4°C. The viral pellet was

resuspended in PBS, and stored at -80°C. MG1 virus was titered by plaque assay. Serial dilutions of virus were performed from -1x10^2 to -1x10^{10} in serum free-DMEM. 800 µl of viral dilutions were plated onto confluent Vero cells and incubated for 1 hour. Viral overlay (50% 6% carboxymethyl cellulose, 40% 2X DMEM, 10% FBS) was added to each well post infection and let to incubate for 72 hours until plaques were formed. After incubation, wells were stained with Crystal Violet, let to dry, and then plaques were manually counted to obtain viral concentration. Calculation used to obtain viral titer was:

$$\frac{pfu}{ml} = \frac{(\#\ of\ viral\ plaques\ in\ well)}{(dilution\ of\ input\ virus)(volume\ of\ input\ virus)}$$

3.5 Vaccine preparations

The Infected Cell Vaccine (ICV) was prepared as follows: L1210 (1x10^6 cell/ml) cells in a total volume of 20 ml 10% DMEM were infected with MG1 virus at MOI of 10 for 18-20 hours at 37°C and 5% CO_2. Following infection cells were counted and viability by trypan blue exclusion was determined using the Vi-Cell cell counter (Beckman Coulter). Cells were washed twice in PBS and resuspended at a concentration of 1x10^7 cells/ml in PBS and subsequently received 30-Gray ɣ-irradiation (HF-320, Pantak).

3.6 Vaccine administration and leukemia challenge

100 µl of the freshly prepared vaccine was administered intra-venously (i.v.) to DBA/2 mice once every 7 days for 3 weeks. Seven days following the last vaccination, mice were challenged i.v. with 1x10^6 L1210 cells. For L1210 challenge, cells were pelleted by centrifugation and washed once in PBS. Cells were counted on the Vi-cell and resuspended at 1x10^7 cells/ml in

PBS. Mice were end pointed upon development of signs of leukemia such as: lethargy and respiratory distress, hind limb paralysis, and anal masses.

3.7 *In vivo* T cell depletion

DBA/2 mice were administered 100 µg of CD4, CD8, or both CD4/CD8 depleting antibodies (BioXCell) intra-peritoneally (i.p.) on days 0 and 1 (2 days prior to and 1 day prior to vaccination). Mice were then vaccinated with the ICV once a week for 3 weeks and then challenged one week later with L1210 cells to monitor survival. The respective depleting antibodies were given every 3-4 days (100 µg) to maintain depletion of the T cell populations. Antibody injections were stopped the day prior to L1210 challenge. For the neutralizing antibody assay, mice were not challenged with L1210 cells. Blood was collected from mice throughout antibody injections to confirm T cell depletion by flow cytometry.

3.8 Adoptive T cell transfer

6-week-old DBA/2 mice were either unimmunized (naïve donors) or ICV immunized once a week for 3 weeks (ICV immunized donors). One week post the last immunization, spleens were collected from all donors in sterile conditions. Naïve spleens were pooled together while immunized spleens were pooled together. Two donor mice were needed for every recipient to obtain enough cells. CD3 T cells were isolated from spleens using a negative T cell isolation kit (Stemcell Technologies). Pre-and post isolated cells were set aside to assess CD3 T cell purity by flow cytometry. $1.5x10^7$ CD3 T cells were injected into the tail vein of naïve DBA/2 recipient mice. Eight days post adoptive transfer recipient mice were challenged with $1x10^6$ L1210 cells and survival was monitored.

3.9 RANTES ELISA

L1210 cells were plated at a density of $3.75x10^5$ cells/plate in 25 cm^2 flasks in a total volume of 7.5 ml media. 18-24 hours after plating, cells were infected with MG1 virus at a MOI of 10 for 18 hours or cells received 30-gray irradiation. MG1-ICV cells were infected and then irradiated. Post infection/irradiation cells were centrifuged at 1500 x rpm for 5 minutes and supernatants were harvested and stored at -80°C. RANTES ELISA kit (Abcam – Catalog #:ab100739) was performed to assess RANTES secretion. Assay was performed as instructed by the manufacturer's protocol. Briefly, 100 µl of a 1:1 dilution of the supernatant: media was plated into each well and incubated at room temperature for 2 hours. After incubation, biotinylated RANTES detection antibody was added to each well and left to incubate for 1 hour at room temperature. Streptavidin solution was added to each well and incubated at room temperature for 45 minutes. Lastly, TMB One-Step development solution was added to each well for 30 minutes followed by stop solution. The ELISA plate absorbance was read at 450 nm immediately after adding the stop solution.

3.10 Flow cytometry

Cells were collected from the spleen, blood, or lymph nodes at appropriate time points and red blood cells were lysed using ammonium-chloride-potassium buffer. Cells were washed with PBS and counted on the Vi-cell or hemocytometer. $1x10^6$ cells/well were plated in a V-bottom 96-well plate (Corning). Cells were spun down at 500 x g for 5 minutes and subsequently stained with FVS510 viability dye (BD Biosciences) for 30 minutes at 4°C. Viability dye was neutralized with Flow Buffer (0.5% Bovine Serum Albumin in PBS) and cells were centrifuged. Anti-mouse CD16/32 (BD Biosciences) was added to all wells for 5 minutes at 4°C to block Fc receptors. Cells were then stained with appropriate antibodies such as: anti-mouse CD40, CD80, MHCII,

CD11c, CD19, F4/80, CD3, CD4, CD8, IgG (BD Biosciences) for 25-30 minutes at 4°C. Cells were then washed with PBS and fixed using 1% Paraformaldehyde (PFA) and later read on the BD Celesta or BD Fortessa at the University of Ottawa. Data was analyzed using FlowJo Software. Representative gating strategies are shown in (Figure #6A and Appendix Figure #22) but include: gating out cell debris and then gating on single viable cells. Fluorescence Minus One (FMOs) samples were used determine the appropriate gates for all fluorophores. To assess T cell depletion, cells were gated on CD3+ T cells and then CD4 and CD8 T cell subsets were analyzed. (CD19-,CD11c+) cells are referred to as DCs and (CD11c-, CD19+) cells are referred to as B cells.

3.11 Neutralizing antibody assay

6-week old DBA/2 mice were depleted of either CD4, CD8 or both CD4/CD8 T cells while receiving ICV immunization once of week for 3 weeks (previously described in methods). Control cohorts were both unimmunized mice or ICV immunized. One week post the last immunization blood was collected from the saphenous vein of mice. Serum was isolated from the blood by centrifugation at 2000 x g for 10 minutes and heat-inactivated for 30 minutes at 56°C to degrade complement. Serial dilutions of serum were made in serum-free DMEM and then incubated with MG1-GFP virus for 45 minutes at 37°C 5% CO_2 in a 96-well plate. The viral serum mixture was then plated onto confluent Vero cells for 72 hours at 37°C 5% CO_2 in a 96-well plate. Samples were performed in technical triplicate. 24 hours post infection, GFP expression was assessed on the EVOS cell imaging microscope (ThermoFisher). 72 hours post infection, cells were fixed with a 3:1 ratio of methanol acetate for at least 15 minutes and then rinsed extensively with tap water. Cells were stained with Coomassie Blue for 20-30 minutes and then let to dry. After wells had dried, 1% Sodium Lauryl Sulfate (SLS) was added to wells

overnight. In a deep well dish, 1:8 dilution of cells post-SLS and tap water was made and the absorbance was read at 570 nm on the Multiskan (ThermoFisher Scientific).

3.12 *In vivo* killing assay

This assay was performed essentially as described previously [63]. Briefly, L1210 cells and/or donor splenocytes were labelled with fluorescent dyes such as: Carboxyfluorescein Succinimidyl Ester (CFSE) and/or Celltrace Violet (CTV). CFSE low cells was stained with 0.1 µM of dye and CFSE high cells at 10 µM. In other experiments, cells were stained with 5 µM CFSE and 5 µM of CTV. Cells were washed in PBS and stained with the individual dyes at a concentration of $5x10^7$ cells/ml in pre-warmed PBS for 15 minutes or 20 minutes for CFSE or CTV respectively at 37˚C/5% CO_2. After labelling, cells were spun down at 300 x g for 10 minutes and supernatant was discarded. Cell pellets were resuspended in 10% RPMI to neutralize the dyes. Cell staining was confirmed by flow cytometry. In certain experiments, CFSE stained cells and CTV stained cells were mixed together at a 1:1 ratio. Subsequently, $4x10^7$ cells total was injected into naïve or ICV immunized recipient mice and cells were collected 18 hours later. For experiments involving tail vein injections, splenocytes were collected whereas a peritoneal wash was performed from mice that received i.p. injections. Collected cells were counted and flow cytometry was done to assess the ratio of donor splenocytes to L1210 cells. *In vivo* killing was determined using the formula:

$$\%\ specific\ lysis = 1 - \frac{(\%\ CTV\ ICV)/(\%\ CFSE\ ICV)}{(\%\ CTV\ naive)/(\%\ CFSE\ naive)}\ x\ 100$$

3.13 IFN-Gamma ELISpot

Mice were either unimmunized, immunized with $1x10^6$ irradiated L1210 cells or the ICV once a week for 3 weeks. Spleens were harvested 45 days after immunization and pooled together

according to their appropriate experimental groups. CD3, CD4 or CD8 T cells were isolated from splenocytes using negative isolation kits (StemCell Technologies). A fraction of the input and isolated populations was put aside to assess T cell purity by flow cytometry. Effector T cells were prepared at a 4:1 ratio of $4x10^6$ T cells/ml to $1x10^6$ naïve donor splenocytes/ml in serum-free RPMI. Re-stimulants consisted of: serum-free RPMI, 1 µg of VSVn peptide, and 1% PFA fixed L1210 cells, and PMA/Ionomycin. All re-stimulants were prepared in serum-free RPMI and L1210 cell re-stimulation was prepared at $1x10^6$ cells/ml.

Serum-Free RPMI was added to murine IFN-gamma ELISpot (MabTech – cat #:3321-2A) wells for 30 minutes at RT (room temperature), as instructed in manufacturer's protocol. Media was removed for wells and 100 µl of each re-stimulant was added to the wells. 100 µl of the 4:1 mixture of effector cells:naïve donor splenocytes was added to each well after the re-stimulants. Overall, each well contained $5x10^5$ cells/well and $1x10^5$ re-stimulant cells/well (5:1 ratio). The Elispot was incubated for 18 hours at 37˚C and 5% CO_2 in light sensitive conditions.

Spots on the IFN-gamma ELISpot plate were developed according to the manufacturer's protocol. Briefly, the plates were emptied and washed with PBS. IFN-gamma secreted by effector cells was captured by addition of a biotinylated anti-mouse IFN-gamma antibody to wells for 45 minutes. Streptavidin-alkaline phosphatase (ALP) was added to each well followed by the addition of a detection solution that precipitated when reacted with ALP. When spots started to emerge, the plates were rinsed in tap water and let to dry. The number of IFN-gamma secreting cells/well was quantified by an Immunospot© ELISpot plate reader at Cellular Technology Ltd (Ohio, USA). Graphs are represented as spot frequency counts (SFC)/ # of effectors cells added to each well.

3.14 Statistical analysis

Statistical analyses were calculated using GraphPad Prism software. Statistical tests used were Mantel-cox and a two-way ANOVA with a Bonferroni post-test. Mean and standard error of the mean (SEM) are shown. P values are represented as ns (not significant), * < 0.05, ** < 0.01 ,*** < 0.001, and **** < 0.0001.

4 RESULTS

4.1 What antigen presenting cell populations (APCs) are activated post ICV immunization?

An effective anti-tumour T cell mediated immune response requires APCs to first uptake, process and present tumour antigen on MHCII to naïve T cells while engaging CD28 through expression of a co-stimulatory molecules such as CD80 and CD40. The surface expression of MHCII, CD80, and CD40 have been used extensively to assess the level of APC activation by flow cytometry. We first sought to determine if (1) specific APC subsets were activated post immunization and (2) if the irradiated L1210 ICV immunization mice differentially activated APCs.

4.1.1 Flow cytometry for antigen presenting cell (APC) populations

We sought to determine if APCs were being activated post immunization by flow cytometry. Naïve mice were either unimmunized or received one dose of irradiated L1210 or ICV i.v.. Spleen, cardiac blood, and pooled lymph nodes were collected 4, 24 and 72 hours post immunization and stained for APC markers including: CD11c (dendritic cells) CD19 (B cells) and F4/80 (macrophage) and activation markers such as: CD40, MHCII, and CD80. An outline of the experimental timeline is shown in Figure #5 and a representative gating strategy in Figure #6A.

4.1.1.1 Dendritic cell activation

Briefly, DCs were identified as single, viable, CD19-, CD11c+ lymphocytes. Although a number of analyses were performed I focused on the relative impact of the vaccination strategies on the MHCII+/CD40+ double positive population as the simultaneous upregulation of these two markers would allow both antigen presentation and co-stimulation of T cells. Our results demonstrate that although the percentage of MHCII positive DCs present in the spleen did not

increase there was a temporal increase in the percentage of the MHCII+ cells with increased CD40 expression with ICV immunization but not irradiated cells alone. The representative flow cytometry plots and the quantitative results for n=5 are shown in Figure #6B and 6C. Although the percentage of the MHCII cells did not differ significantly between the groups there was a detectable increase in the mean fluorescence intensity (MFI), suggesting that the per cell expression of MHCII was increased following vaccination with the ICV (Figure #6D). A detectable increase in the MFI of CD40 was also noticed following vaccination with the ICV (Figure #6E). Notably, both the increase in the percentage of the MHCII+/CD40+ population as well as the MHCII and CD40 MFI returned to baseline levels by 72 hours. No changes were observed in CD80 expression levels and frequency despite reliable detection (data not shown). Taken together, this data demonstrates that the ICV induces DC activation post immunization and this could potentially be aiding in the anti-tumour immune response.

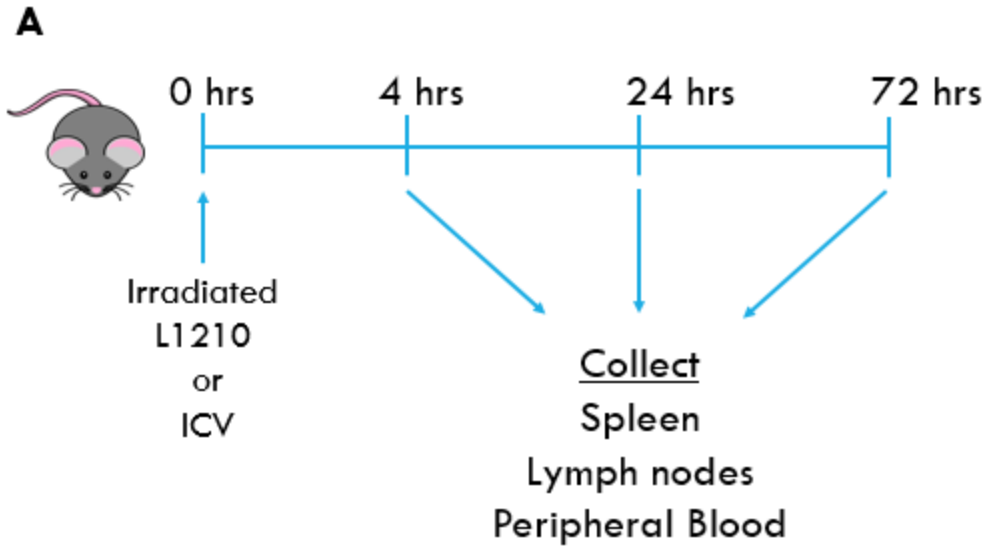
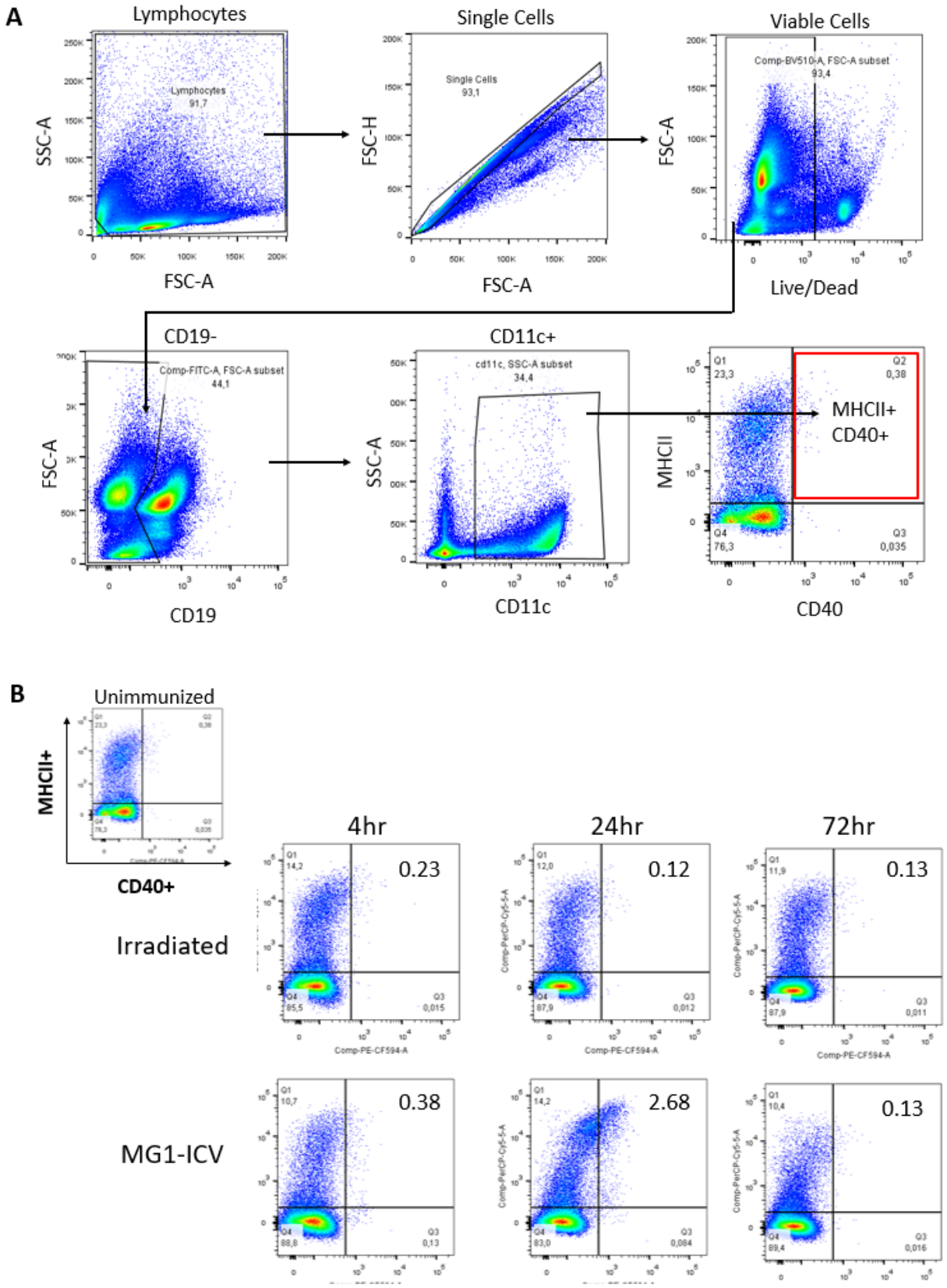
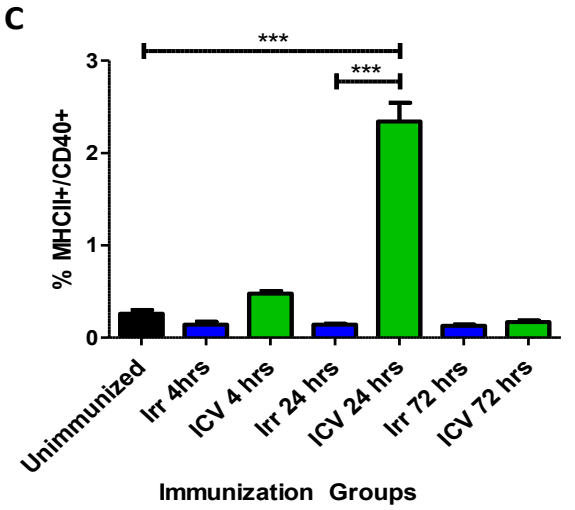
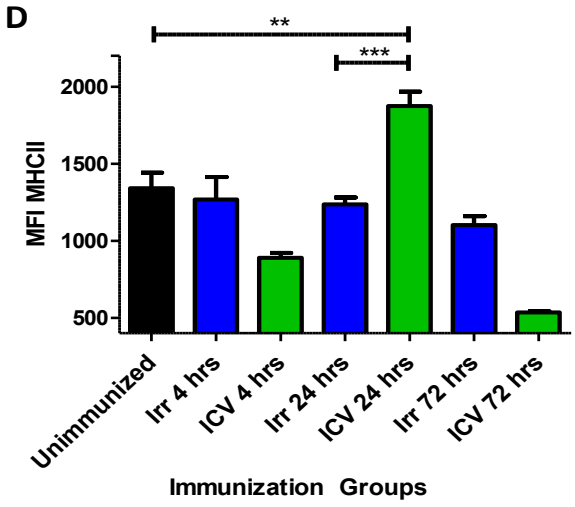

A
0 hrs
4 hrs
24 hrs
72 hrs
Irradiated
L1210
or
ICV
Collect
Spleen
Lymph nodes
Peripheral Blood

Figure #5. Experimental timeline and procedure for APC activation.

A: Mice were either unimmunized, immunized with the ICV or irradiated L1210 cells. Spleens, cardiac blood, and pooled lymph nodes were collected 4, 24 and 72 hours post injections. Red blood cells were lysed with ACK buffer, washed with PBS, followed by viability staining and then stained with CD40, CD80, MHCII, CD19, CD11c, and F4/80 antibodies for 30 minutes at 4°C and analyzed by flow cytometry.


A
Lymphocytes
SSC-A
FSC-A
Lymphocytes
91,7
Single Cells
FSC-H
FSC-A
Single Cells
93,1
Viable Cells
FSC-A
Live/Dead
Comp-BV510-A, FSC-A subset
93,4
CD19-
FSC-A
CD19
Comp-FITC-A, FSC-A subset
44,1
CD11c+
SSC-A
CD11c
cd11c, SSC-A subset
34,4
MHCII
CD40
MHCII+
CD40+
Q1
23,3
Q2
0,38
Q4
76,3
Q3
0,035
B
Unimmunized
MHCII+
CD40+
4hr
24hr
72hr
Irradiated
0.23
0.12
0.13
MG1-ICV
0.38
2.68
0.13
Comp-PE-CF594-A
Comp-PerCP-Cy5-5-A


C
***
***
% MHCII+/CD40+
3
2
1
0
Unimmunized
Irr 4hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups


D
**
***
MFI MHCII
2000
1500
1000
500
Unimmunized
Irr 4 hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups

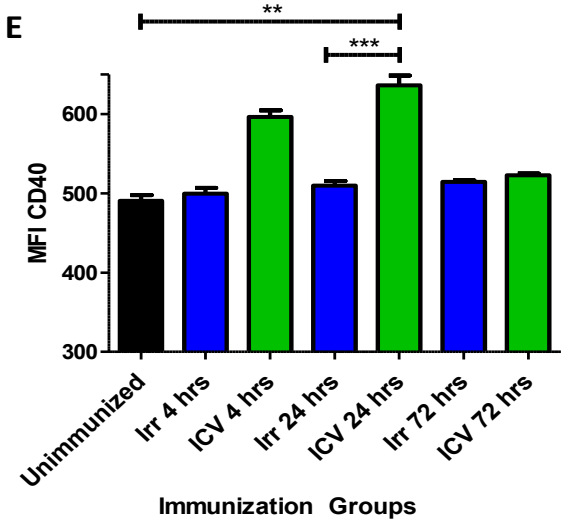

E
**
***
MFI CD40
600
500
400
300
Unimmunized
Irr 4 hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups

Figure #6. ICV immunization increases MHCII+/CD40+ population on DCs in the spleen.

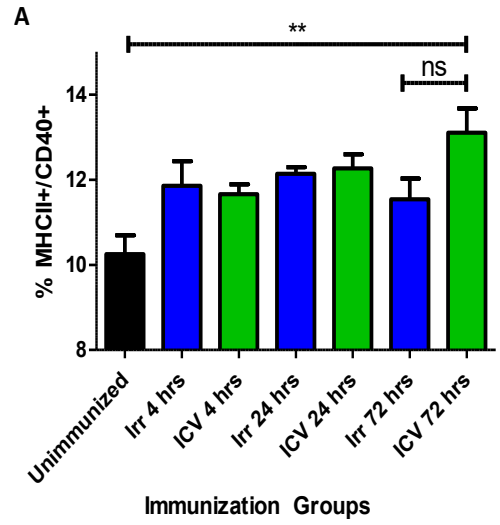
A: Example of the gating strategy used to assess DC population. Cells are gated on lymphocytes, single cells, viable cells, CD19-, CD11c+ and then the MHCII+/CD40+ population was analyzed. **B:** Representative flow cytometry plots of the MHCII+/CD40+ population from splenocytes. Unimmunized plot is shown as a control and demonstrates the axes. Plots show irradiated L1210 and ICV immunized spleen samples at 4, 24 and 72 hours post immunization. **C:** Quantitative representation of flow cytometry plots showing the % of MHCII+/CD40+ splenocytes. **D:** Mean fluorescence intensity (MFI) of MHCII from MHCII+ cells. **E:** MFI of CD40 from CD40+ cells. (N=5 for each group). Statistical analysis determined by a two-way ANOVA with a Bonferroni post test done on GraphPad Prism.

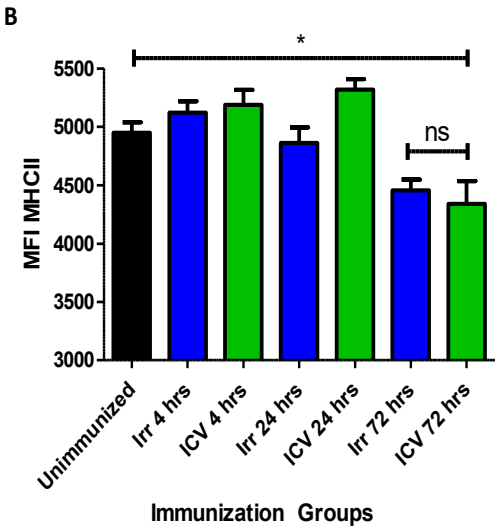
4.1.1.2 B cell activation

CD19+ cells (B cells) were also analyzed to determine if they had a role in acting as APCs post ICV immunization. B cells (CD11c-/CD19+) were analyzed by the same methods as DCs, (Appendix – Figure #22). Unlike DCs, there were no significant differences in the MHCII+/CD40+ population at 24 hours (Figure #7A). However, 72 hours post vaccination with either irradiated L1210s or the ICV resulted in a significant increase in the percentage of the MHCII+/CD40+ cells. The MFI of MHCII stays quite consistent throughout the time course, but significant changes are seen in level of expression of CD40 (Figure #7B and C).

The lymph nodes of all mice were also collected to examine the activation of the APC populations. The lymph nodes are a secondary lymphoid organ, like the spleen, where activated APCs migrate to interact with T cells. The cervical, mesenteric, and inguinal lymph nodes were collected and pooled together. Surprisingly, we did not see any changes in the lymph nodes of mice post ICV immunization compared to the control (data not shown). This could be due to pooling the lymph nodes. Blood was collected from mice post ICV immunization and no significant changes in activation marker were seen. Overall, this suggests that the spleen may be the main secondary lymphoid organ aiding in the activation of the anti-tumour T cell response in our ICV model.

To summarize, we have determined that DCs are activated post ICV immunization. DCs in the spleen, increase the level of expression of MHCII/CD40 and frequency within 24 hours post ICV immunization. Also, this activation is a trait of the ICV and is not seen is mice that received irradiated L1210 cells.


A
**
ns
% MHCII+/CD40+
14
12
10
8
Unimmunized
Irr 4 hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups


B
*
ns
MFI MHCII
5500
5000
4500
4000
3500
3000
Unimmunized
Irr 4 hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups

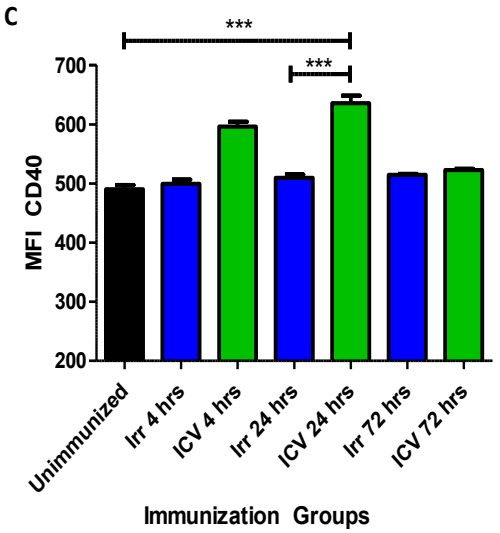

C
***
***
MFI CD40
700
600
500
400
300
200
Unimmunized
Irr 4 hrs
ICV 4 hrs
Irr 24 hrs
ICV 24 hrs
Irr 72 hrs
ICV 72 hrs
Immunization Groups

Figure #7. The changes of B cell activation markers post vaccination.

Cells are gated on lymphocytes, single cells, viable cells, CD11c- and CD19+ cells. Analysis was done on the double positive MHCII+/CD40+ subset. **A:** The % of MHCII+/CD40+ cells within the spleen. **B:** The MFI of MHCII on MHCII+ cells in the spleen. **C:** The MFI of CD40 on CD40+ cells in the spleen.

4.2 Does MG1 infection and irradiation increase RANTES secretion?

RANTES is a chemokine involved in aiding in DC activation.[50,51] We wanted to determine if one of the mechanisms of activating DCs post ICV immunization could be through RANTES chemokine secretion. L1210 cells were either irradiated, infected with MG1, or MG1 infected and irradiated (MG1-ICV). Samples were compared to control L1210 cells. Supernatants were collected and RANTES secretion was determined. Our results show that the *in vitro* MG1 infection and irradiation (MG1-ICV) does significantly increase L1210 cell RANTES secretion compared to control and irradiated L1210 cells (Figure #8). Therefore, RANTES secretion could possibly be aiding in the activation of DCs post ICV immunization, but more research will still need to be done to determine this link.

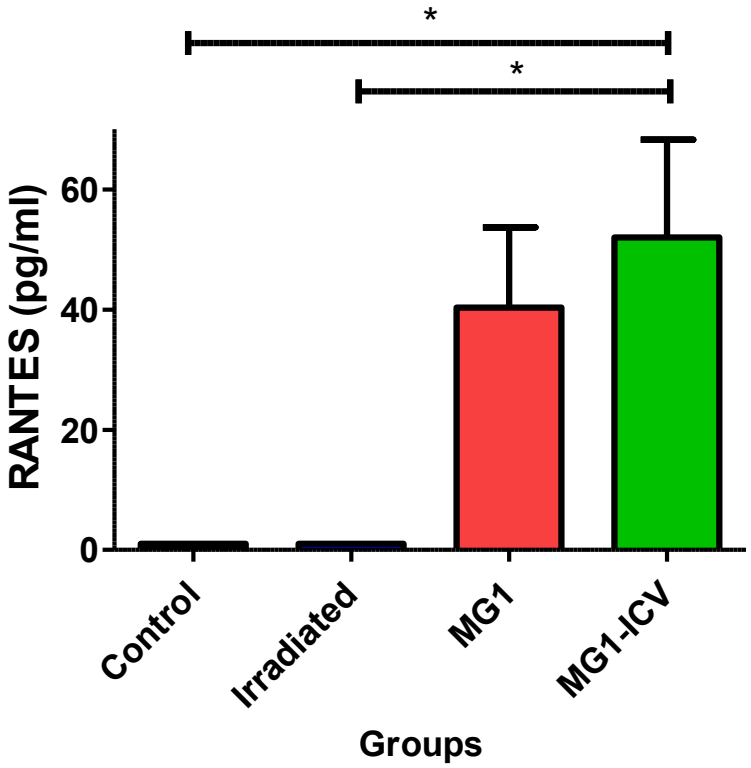

*
*
RANTES (pg/ml)
60
40
20
0
Control
Irradiated
MG1
MG1-ICV
Groups

Figure #8. RANTES secretion is induced by MG1 infection and irradiation.

L1210 cells were plated at a density of $3.75x10^5$ cells/plate in a total volume of 7.5 ml. L1210 cells were either irradiated (30-gray), infected with MG1 virus at an MOI of 10 for 18 hours, or MG1 infected and irradiated (MG1-ICV). Zero hours post irradiation and/or infection, cells were centrifuged and supernatants were collected. 100 µl of the supernatants were analyzed for RANTES secretion using a RANTES ELISA. N=3 for all groups. Statistical analysis determined by a two-way ANOVA with a Bonferroni post test on GraphPad Prism. ELISA performed by summer student, Leah Monette.

4.3 What T cell subsets are important in developing anti-tumour immunity during ICV immunization?

Previous work in our laboratory by Conrad et al. has demonstrated that T cells are required for an effective anti-tumour immune response against leukemia cells following vaccination with the ICV. In particular, athymic mice immunized with the ICV were not protected from leukemia challenge.[62] However, these studies did not reveal which T cell subsets are critical for generating anti-tumour immunity during the vaccination period. We have determined that CD4 T cells are essential for the protective anti-tumour effects of the ICV. We have also demonstrated that CD4 T cells are required for the anti-viral immune response against MG1 which could be related to the anti-tumour immune response. Notably, CD4 T cells also produce IFN-gamma in response to L1210 re-stimulation demonstrating that these cells could be providing a direct or indirect anti-tumour immune response. Lastly, we have shown that CD8 T cells produce a robust amount of IFN-gamma in response to L1210 cell re-stimulation, demonstrating that CTLs provide a very significant leukemia specific anti-tumour immune response.

4.3.1 In vivo T cell depletion

Individual T cell subsets were depleted to determine whether CD4 or CD8 T cells are required for developing an anti-tumour immune response following ICV immunization. Naïve mice depleted of either CD4, CD8, or both CD4/CD8 were either unimmunized of administered the ICV as shown in Figure #9. Prior to performing this assay, I optimized the concentration of the CD4 and CD8 antibodies by administering the appropriate antibody on day 0 and 1 and collecting the spleen and/or blood 24 hours and 4 days post injections. Assessment of the T cell population by flow cytometry revealed that either antibody delivered i.p. was sufficient to deplete respective T cell populations at concentrations as low as 100 μg (Appendix – Figure #23)

and was used for further experiments. This dosing strategy was sufficient to maintain depletion of T cell populations throughout the vaccination and at the time of challenge (Figure #10 and #11) Notably, it was also found that depleted T cell populations reconstitute within 21 days post L1210 challenge (Appendix -Figure #24).

In agreement with our previous findings[62], I have found that depletion of both CD4 and CD8 T cells results in 100% of mice succumbing to leukemia challenge within 28 days – confirming that the ICV requires T cells to effectively provide anti-tumour immunity (Figure #12). However, while CD4 T cell depletion resulted in 75% of ICV immunized succumbing to leukemia challenge with a median survival of 73.5 days post challenge – only 26.7% of ICV immunized mice in the CD8 T cell depleted group succumb to leukemia challenge (Figure #12). This data demonstrates the importance both CD4 and CD8 T cells during ICV immunization and that CD4 T cells are playing an essential anti-leukemic role.

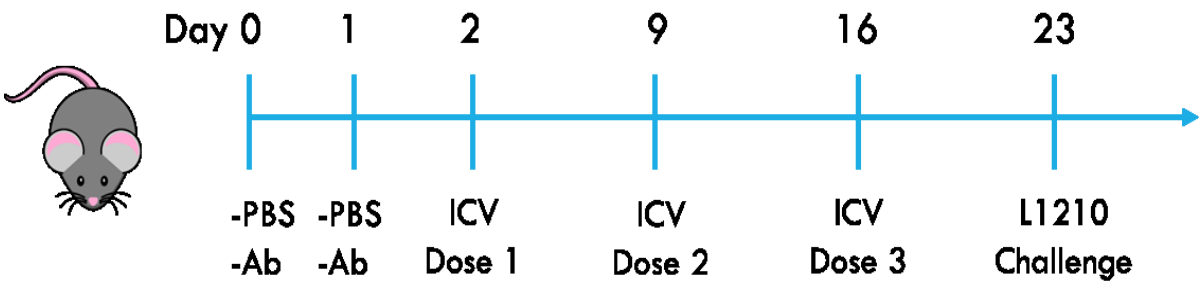

Day 0
1
2
9
16
23
-PBS
-Ab
-PBS
-Ab
ICV
Dose 1
ICV
Dose 2
ICV
Dose 3
L1210
Challenge

Figure #9. Experimental timeline for *In Vivo* T cell depletion.

6-week-old DBA/2 mice were injected with PBS, CD4, CD8 or both antibodies (100 μg) on days 0 and 1, followed by administration of the ICV on day 2, 9 and 16, and L1210 challenge on day 23. Antibody injections were administered every 3-4 days until the day of L1210 challenge (day 23) to sustain depletion. Survival was monitored post L1210 challenge.

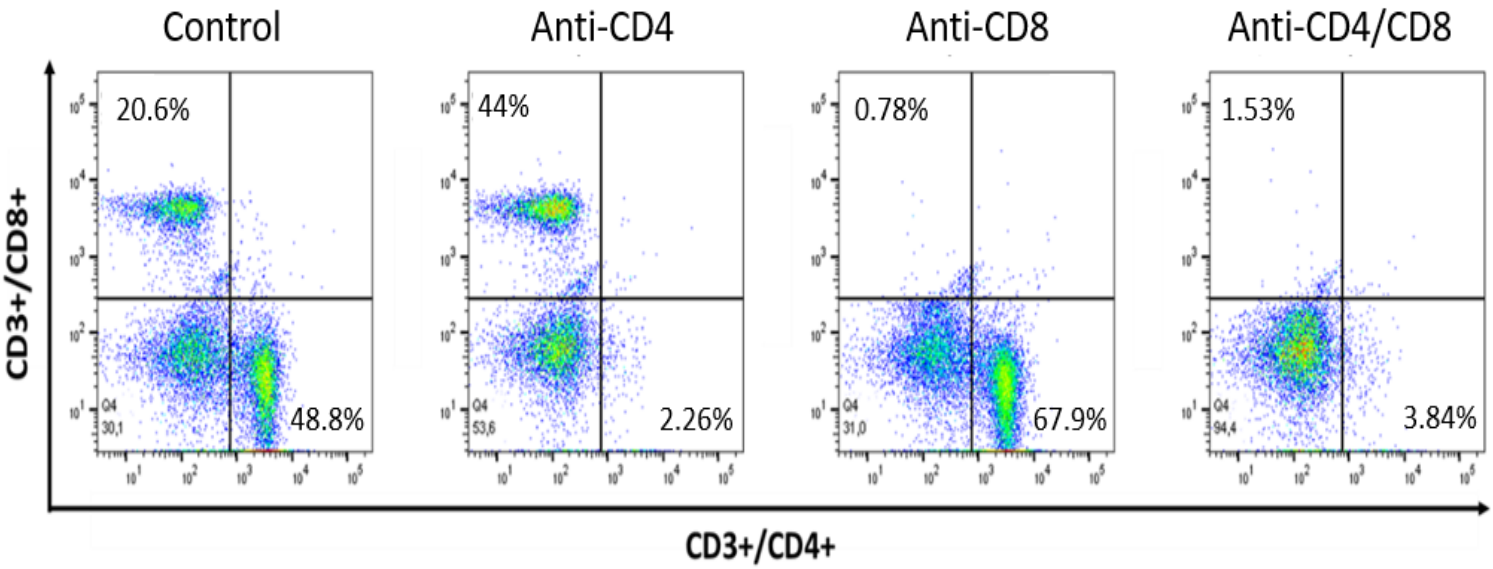

Control
Anti-CD4
Anti-CD8
Anti-CD4/CD8
CD3+/CD8+
CD3+/CD4+
20.6%
48.8%
Q4
30,1
44%
2.26%
Q4
53,6
0.78%
67.9%
Q4
31,0
1.53%
3.84%
Q4
94,4

Figure #10. Confirmation of T Cell Depletion.

6-week-old DBA/2 mice were injected with PBS, and/or both 100 µg CD4 or 100 µg CD8 antibody on days 0, 1 and 4. Peripheral blood from the saphenous vein was collected on day 8 to confirm T cell depletion 4 days post antibody injection. Blood was stained with CD3, CD4, and CD8 antibodies to perform flow cytometry.

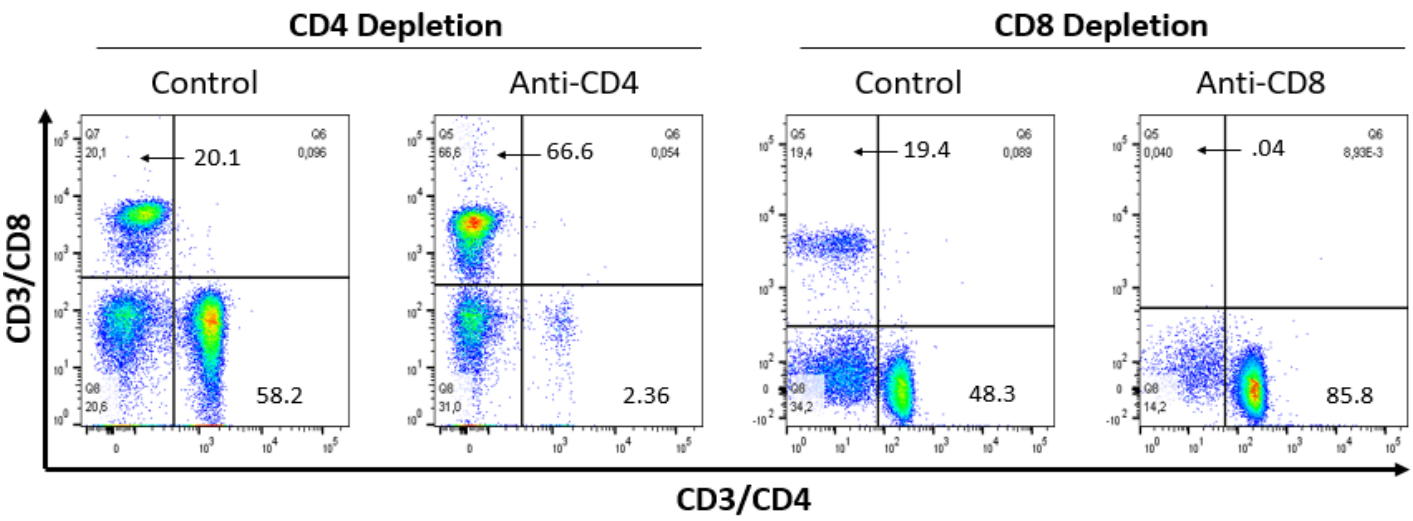

CD4 Depletion
CD8 Depletion
Control
Anti-CD4
Control
Anti-CD8
20.1
66.6
19.4
.04
58.2
2.36
48.3
85.8
CD3/CD8
CD3/CD4

Figure #11. T cell depletion is maintained at the day of L1210 challenge.

DBA/2 mice were administered intra-peritoneally either PBS, 100 µg CD4 or 100 µg CD8 antibody on day 0 and day 1. The antibody was administered every 3-4 days until L1210 challenge. The ICV was tail vein injected on days 2, 9, and 16. Blood was collected from the saphenous vein of mice on the day of the L1210 challenge, stained with CD3, CD4, and CD8 antibody and analyzed by flow cytometry.

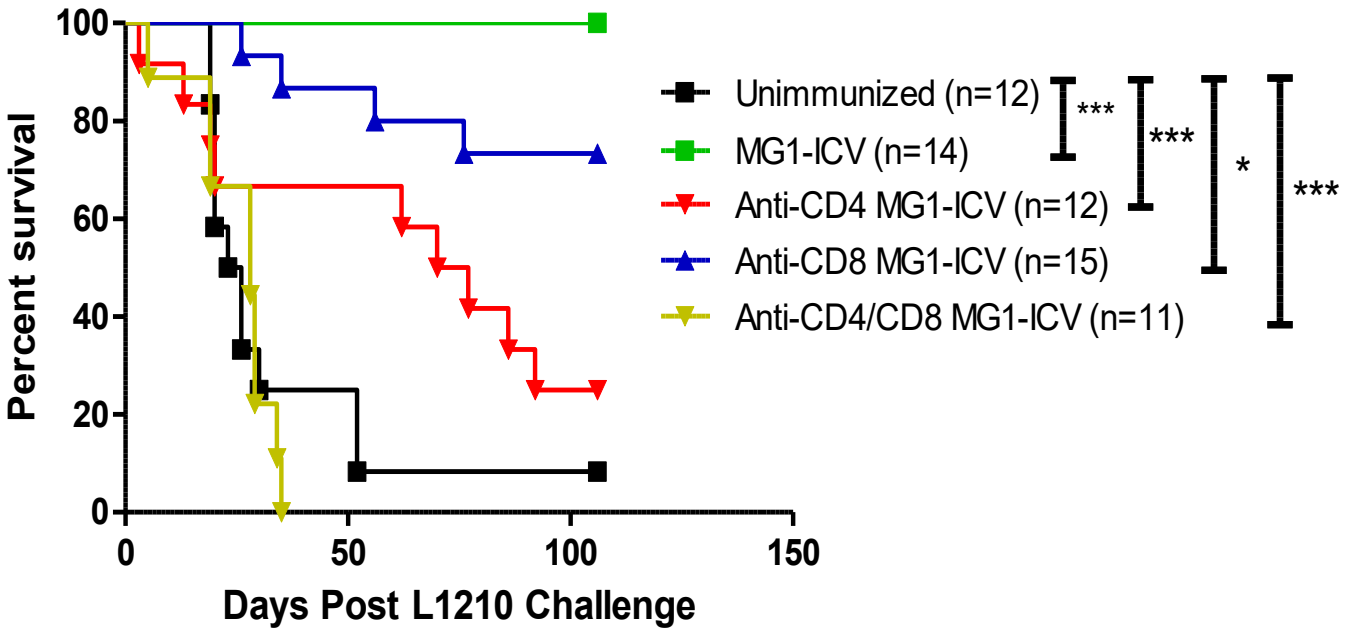
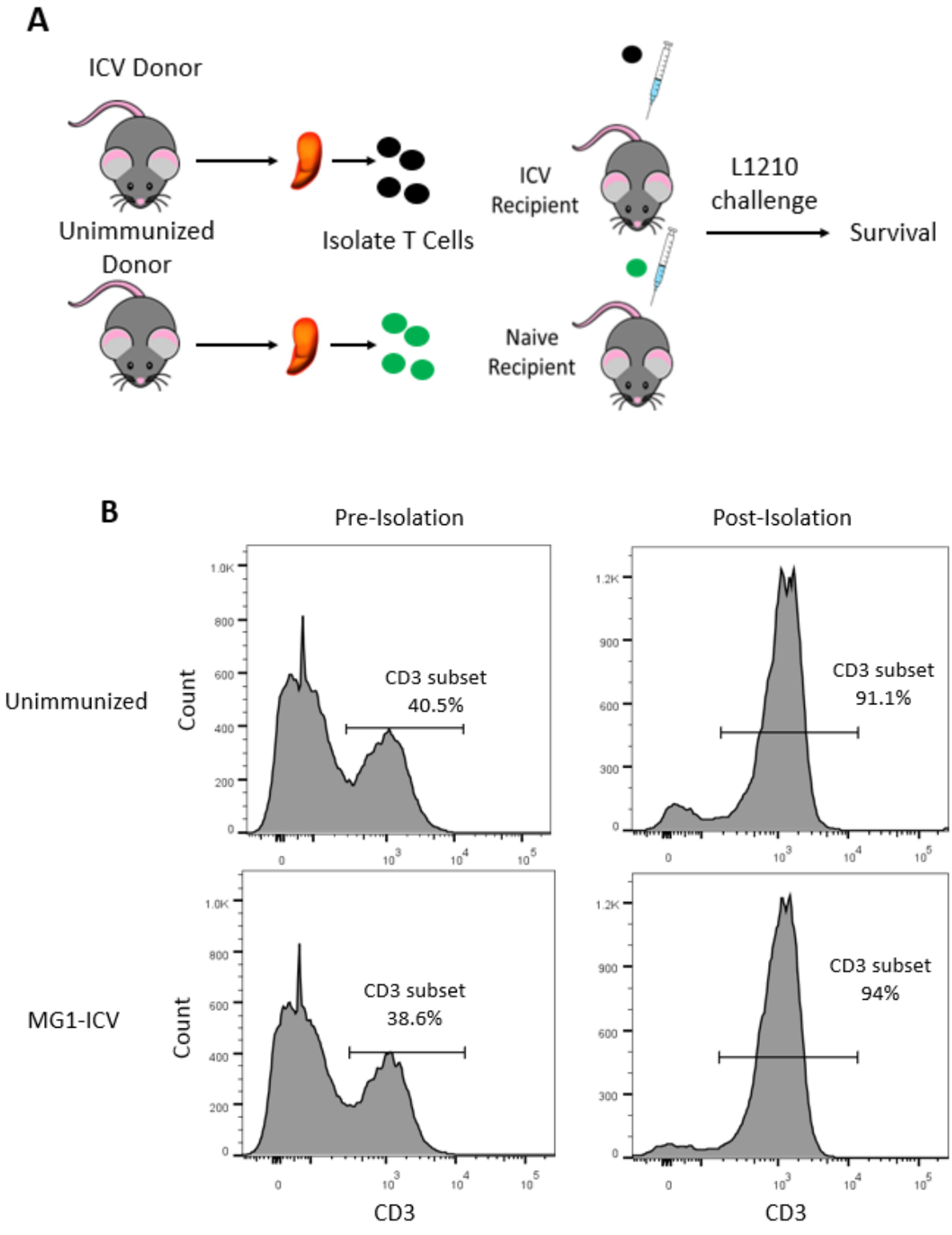

Percent survival
100
80
60
40
20
0
0
50
100
150
Days Post L1210 Challenge
Unimmunized (n=12)
MG1-ICV (n=14)
Anti-CD4 MG1-ICV (n=12)
Anti-CD8 MG1-ICV (n=15)
Anti-CD4/CD8 MG1-ICV (n=11)
***
***
*
***

Figure #12. T cells are required for the protective effects of the ICV.

6-week old DBA/2 mice were either administered PBS, anti-CD4, anti-CD8 antibodies or both on days 0 and 1, followed by administration of the ICV on day 2, 9 and 16, and L1210 challenge on day 23. Antibody injections were administered every 3-4 days until the day of L1210. Survival was monitored. Statistical significance was calculated by the Log-Rank (Mantel-Cox) Test on GraphPad Prism.

4.3.2 Adoptive T cell transfer

To further validate the findings of the *in vivo* T cell depletion I attempted to adoptively transfer CD3 T cells from vaccinated animals to naïve mice as outline in Figure #13A. Post-immunization the spleens of mice that received the indicated vaccinations were collected and CD3 T cells were isolated using a negative selection kit. CD3 T cell purity was assessed by flow cytometry of pre- and post-isolated samples. CD3 T cell purity of >90% was achieved (Figure #13B). CD3 T cells from unimmunized or ICV immunized mice were injected i.v. into wild-type DBA/2 recipient mice. 8 days following the adoptive transfer recipient mice were challenged with L1210 cells and survival was monitored. Surprisingly, adoptive transfer of ICV primed CD3 T cells did not provide long term, durable protection and 100% of recipients succumbed to leukemia challenge (Figure #13C). Although this experiment could be interpreted as an inability of the ICV primed CD3 T cells to provide protection against leukemia challenge it was unclear from this experiment whether the transferred T cells were present in sufficient numbers or able to expand following challenge. This assay should be optimized in the future to ensure that these parameters can be monitored to see if we can demonstrate the protective effects of CD3 T cells in this model. Further potential areas of optimization will be mentioned in the discussion.


A
ICV Donor
Unimmunized Donor
Isolate T Cells
ICV Recipient
Naive Recipient
L1210 challenge
Survival
B
Pre-Isolation
Post-Isolation
Unimmunized
MG1-ICV
Count
CD3 subset 40.5%
CD3 subset 91.1%
CD3 subset 38.6%
CD3 subset 94%
CD3

C

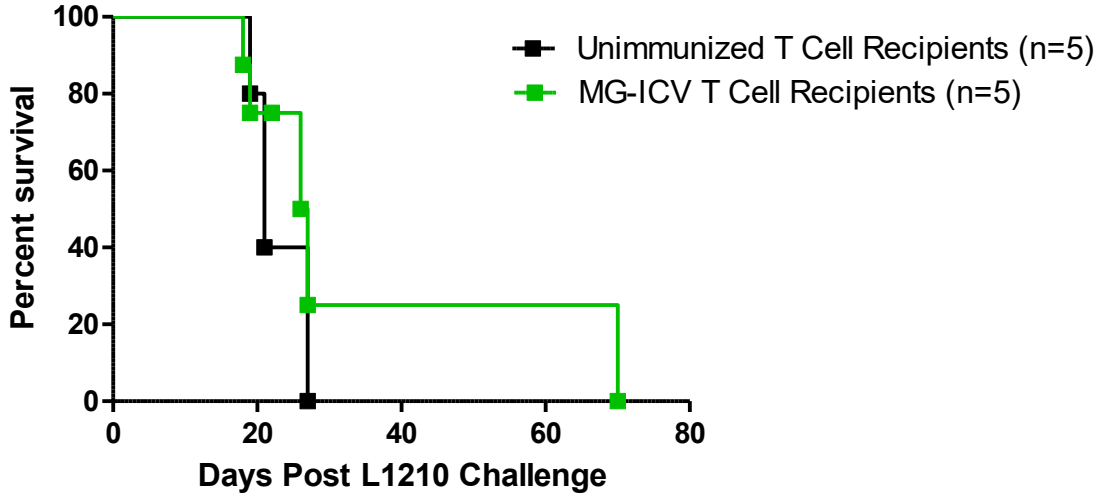

Unimmunized T Cell Recipients (n=5)
MG-ICV T Cell Recipients (n=5)
Percent survival
100
80
60
40
20
0
0
20
40
60
80
Days Post L1210 Challenge

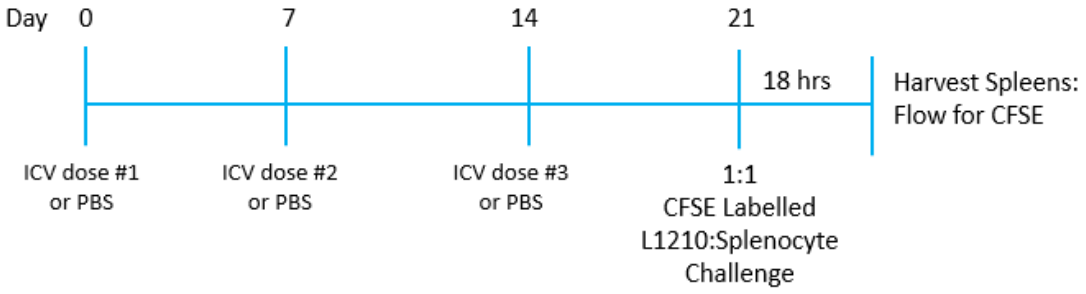
Figure #13. Adoptive transfer of ICV-Primed CD3 T cells does not protect mice from leukemia.

A: Donor mice were either unimmunized or ICV immunized once a week for three weeks. 7 days post immunization donor spleens were collected and CD3 T cells were isolated using a negative selection kit. $1.5x10^7$ T cells/ mouse were injected into the tail vein of wild-type recipient mice. 8 days post adoptive T cell transfer all mice were challenged with $1x10^6$ L1210 cells. **B:** Isolated T cells were stained with a CD3 antibody and flow cytometry was performed. CD3 T cell isolation was > 90% pure. **C:** Survival curve of adoptive transfer of non-primed or ICV-primed T cells results in recipient mice succumbing to L1210 challenge. N= 5 mice/per group.

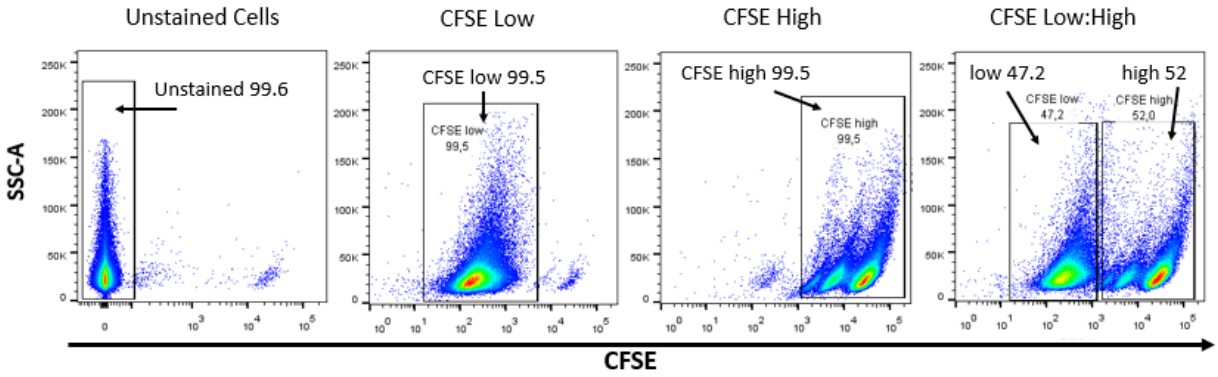
4.3.4 In vivo killing assay

Provided the direct tumour cytolytic effect of T cells we next sought to determine whether the anti-tumour immunity provided by the ICV was associated with increased T cell mediated killing. A number of different approaches with relative strengths and weaknesses have been developed for measuring T cell mediated killing including an *in vivo* approach that utilizes target and non-specific cell populations differentially labelled with fluorescent dyes.[63,64] However, this approach has generally been performed with the knowledge of a tumour specific antigen that is pulsed onto syngeneic splenocytes prior to injection. Flow cytometry of harvested splenocytes at a later time point can be performed to assess if the tumour specific peptide-pulsed cells have been preferentially targeted *in vivo*. However, no such antigen is currently known for the L1210 model, so I attempted to use labelled L1210 cells and whole splenocytes. In this experiment, control whole splenocytes were labelled with a low concentration of CFSE and L1210 cells were labelled with a high concentration of CFSE. Experimental timeline is shown in Figure #14A. Cell labelling was optimized to shown that L1210 cells could be effectively labelled with CFSE at a high (10 μm) and low dose (1 μm) and clearly visualized (Figure #14B). However, 18 hours following injections of $9x10^6$ labelled cells mixed together at a 1:1 ratio and administered into the tail-vein of unimmunized or ICV immunized mice a very few CFSE positive cells could be observed and only those with a high concentration of dye could be visualized (Figure #14C). I could no longer distinguish the difference between the CFSE low and high populations. This could have been due to the number of labelled cells that we injected, the proliferation of the CFSE high cells, or time point of spleen collection.

A


Day 0
7
14
21
18 hrs
Harvest Spleens:
Flow for CFSE
ICV dose #1
or PBS
ICV dose #2
or PBS
ICV dose #3
or PBS
1:1
CFSE Labelled
L1210:Splenocyte
Challenge

B


Unstained Cells
CFSE Low
CFSE High
CFSE Low:High
Unstained 99.6
CFSE low 99.5
CFSE high 99.5
low 47.2
high 52
SSC-A
CFSE

C

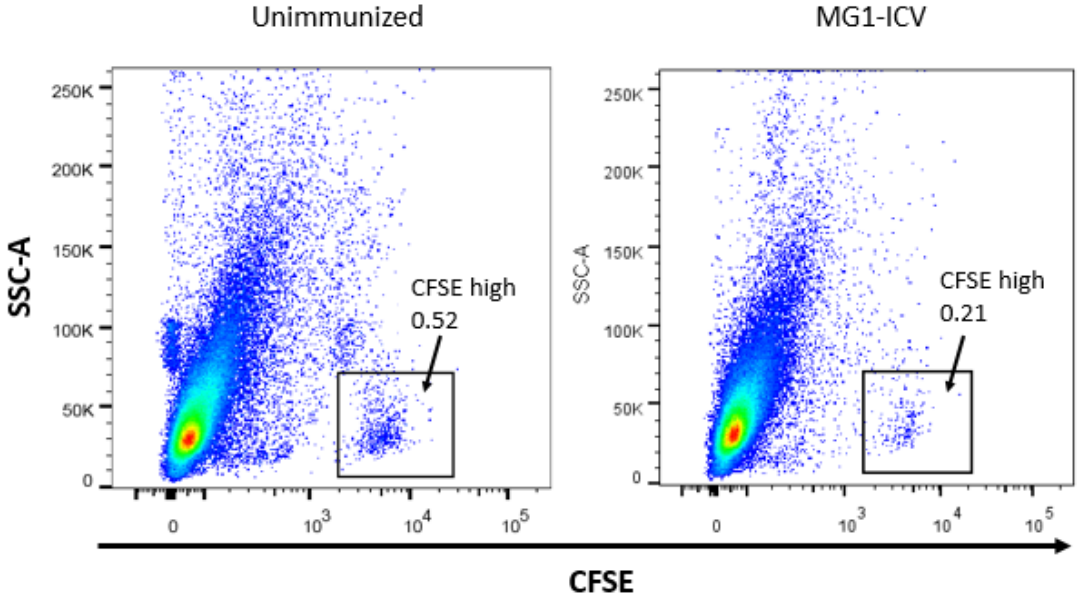
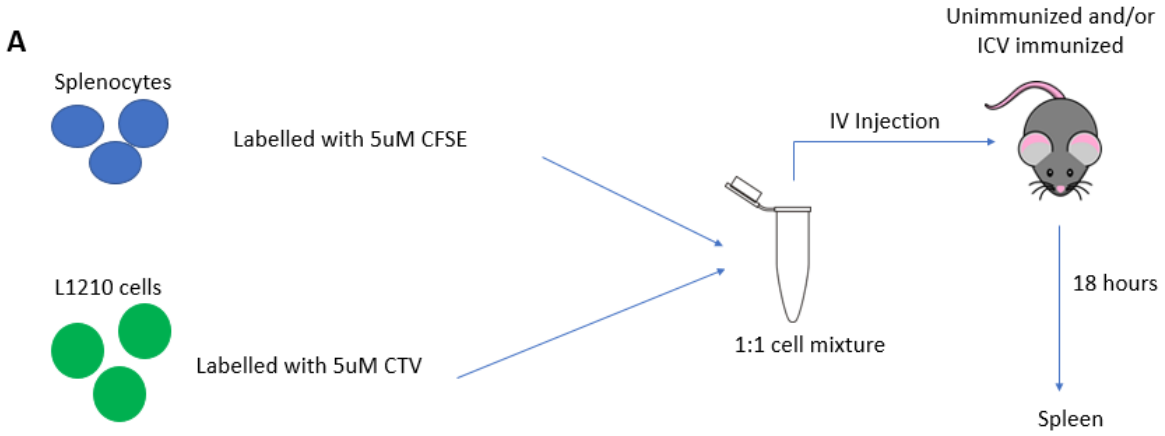

Unimmunized
MG1-ICV
CFSE high
0.52
CFSE high
0.21
SSC-A
CFSE

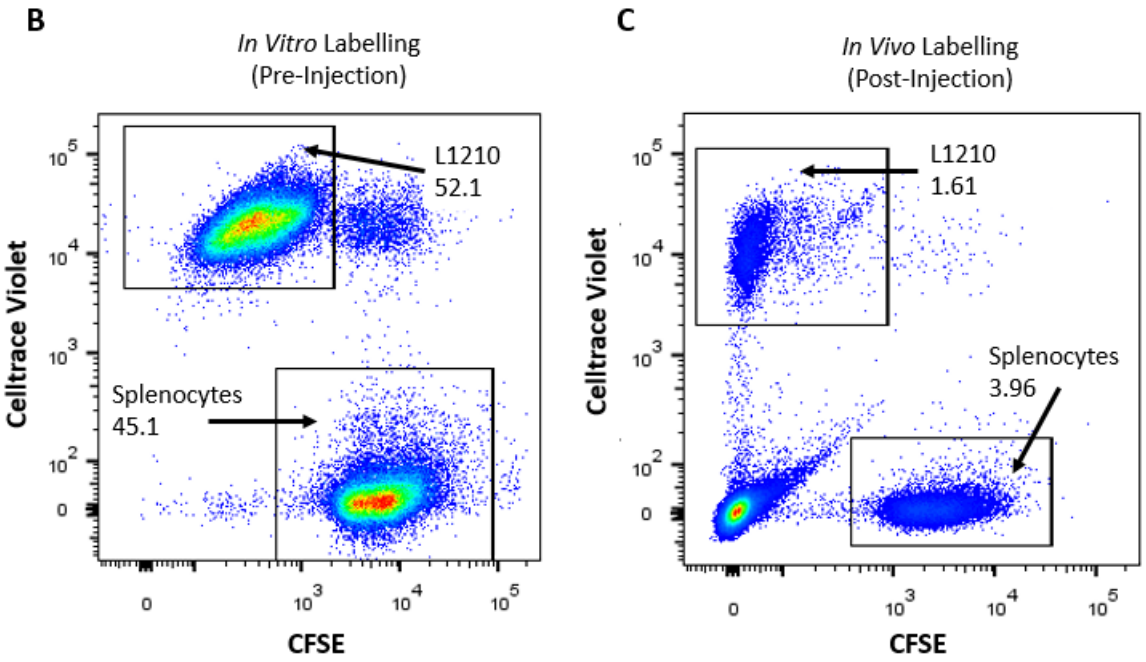
Figure #14. Low and high concentrations of CFSE is insufficient at demonstrating anti-tumour immunity.

A: DBA/2 mice were either unimmunized (PBS) or vaccinated with the ICV. On day 21, mice received a 1:1 mix of CFSE low donor splenocytes: CFSE high L1210 intra-venously. $9x10^6$ cells total was injected. 18 hours post injections, spleens were harvested, and flow cytometry was performed to assess CFSE labelled populations. **B:** L1210 were labelled with CFSE low (1 μm) or CFSE high (10 μm) fluorescent dye. Samples of both unmixed and equally mixed cells are represented. **C:** Flow cytometry plots of splenocytes collected from unimmunized and ICV immunized mice after being injected with 1:1 mix of CFSE low control cells:CFSE high L1210 cells. (n=1).

After determining that we could not see anti-tumour immunity using the CFSE low:CFSE high labelled cells for the *in vivo* killing assay we decided to try a different approach. Since we were unable to detect the CFSE low from the CFSE high population it could be due to the CFSE high population proliferating and incorporating in with the CFSE low population. To address these issues, I next attempted to label the L1210 and splenocyte populations with two different fluorescent dyes, Celltrace violet (CTV) and CFSE respectively. In addition, I injected more cells compared to the previous experiment to determine if the cell number was the issue as outlined in Figure #15A. Prior to injections, it was first determined that 5 μM of CTV was sufficient to label L1210 cells. Successful *in vitro* labelling of the 1:1 mixture of CTV labelled L1210 cells and CFSE labelled splenocytes was achieved (Figure #15B). Labelled cells were injected into an unimmunized mouse as previously done and the spleen from the recipient mouse was collected after 18 hours for flow cytometry (Figure #15C). The data was encouraging and demonstrated that there was successful detection of both the control splenocytes and L1210 cells from the recipient spleen. However, the ratio of the injected cells was 2.46:1 not 1:1. This discrepancy could arise from either innate killing of the L1210 cells or differential cell homing. Due to the hypothesis that the L1210 cells could be migrating to other organs, blood, bone marrow, lungs, liver and brain of a recipient mouse were all collected. Flow cytometry was performed on all organs and the labelled L1210 cells were not detected in any of these sites (Appendix – Figure #25). Overall, based on this preliminary experiment we thought it would be feasible to assess if ICV immunized mice could reject the L1210 cells more effectively compared to unimmunized mice. A cohort of naïve and ICV immunized recipients were injected with a 1:1 mixture of labelled cells and representative flow cytometry plots are shown in Figure #15D. Unexpectedly, only 1 out of 4 unimmunized replicates had visual L1210 cell labelling in the spleen while all

replicates showed the control splenocyte population with no-labelled L1210 cells present in ICV immunized mice despite the presence of a control splenocyte population. Together these findings suggest that L1210 cells are homing to a different organ that was not directly assessed in our preliminary experiments or that there is a strong innate response.


A
Splenocytes
Labelled with 5uM CFSE
L1210 cells
Labelled with 5uM CTV
1:1 cell mixture
IV Injection
Unimmunized and/or
ICV immunized
18 hours
Spleen


B
In Vitro Labelling
(Pre-Injection)
L1210
52.1
Splenocytes
45.1
Celltrace Violet
CFSE
C
In Vivo Labelling
(Post-Injection)
L1210
1.61
Splenocytes
3.96
Celltrace Violet
CFSE

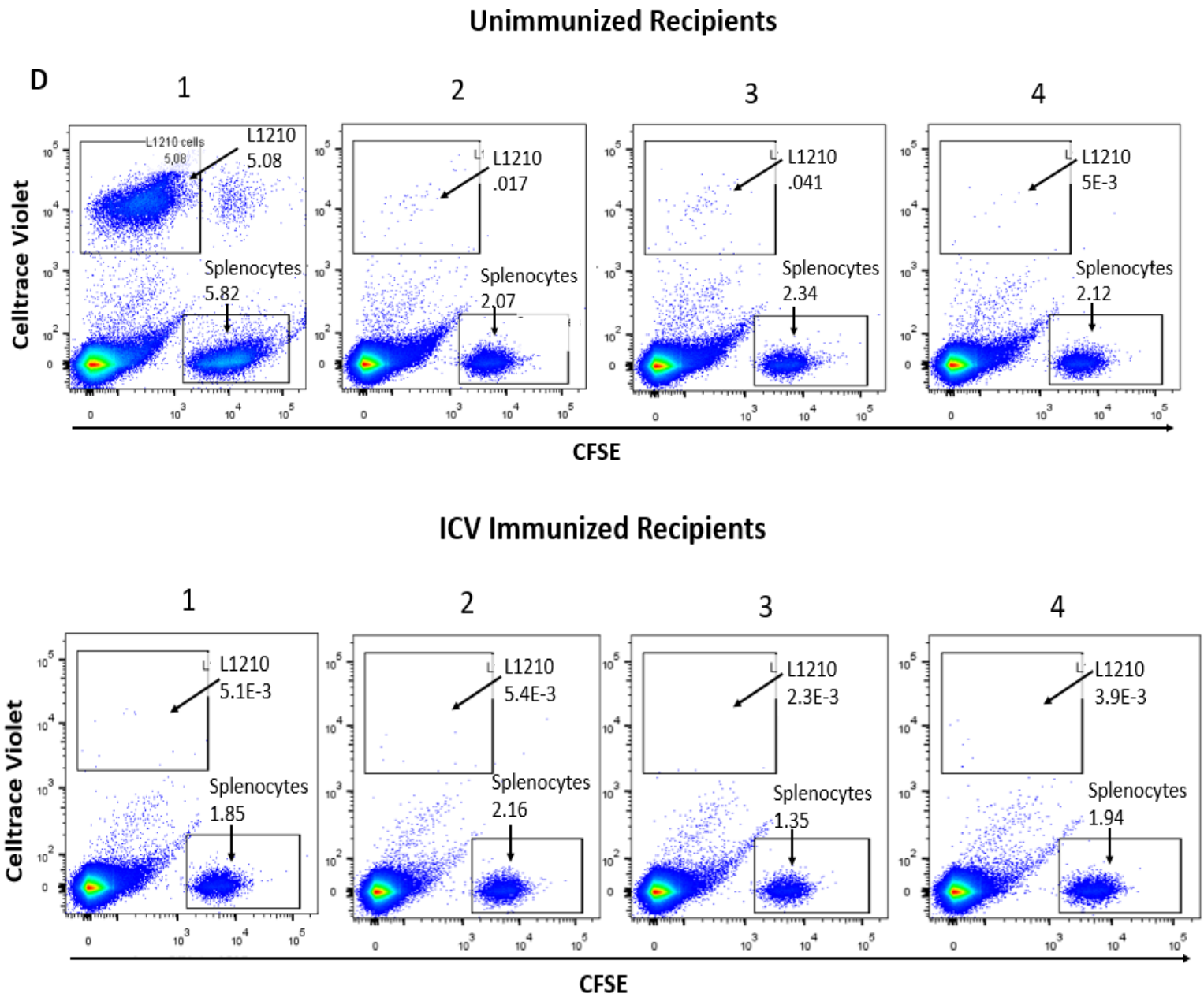

Unimmunized Recipients
D
1
2
3
4
Celltrace Violet
L1210 cells
5.08
L1210
5.08
Splenocytes
5.82
L1210
.017
Splenocytes
2.07
L1210
.041
Splenocytes
2.34
L1210
5E-3
Splenocytes
2.12
CFSE
ICV Immunized Recipients
1
2
3
4
Celltrace Violet
L1210
5.1E-3
Splenocytes
1.85
L1210
5.4E-3
Splenocytes
2.16
L1210
2.3E-3
Splenocytes
1.35
L1210
3.9E-3
Splenocytes
1.94
CFSE

Figure #15. Inconsistent results are obtained using the *in vivo* killing assay to detect anti-tumour immunity.

A: Donor splenocytes were labelled with 5 μm of CFSE and L1210 with CTV. $2x10^7$ cells/cell type were injected into the tail vein of either unimmunized of ICV immunized mice. 18 hours post injections, spleens were harvested and flow cytometry was performed. **B:** Flow cytometry plot of the mixture of L1210 cells and splenocytes labelled with respective dyes prior to injections. **C:** Unimmunized recipient spleen was harvested 18 hours post injection and flow cytometry was performed to assess the labelled cell types. **D:** Unimmunized and ICV immunized spleens were harvested post injections and flow plots are shown. (N=4 for each group). Each number represents a different replicate.

Lastly, we sought to investigate whether directly injecting cells into the peritoneal cavity could be used for the *in vivo* killing assay. A 1:1 ratio of labelled splenocytes and L1210 was injected intra-peritoneally and 18 hours post injections a peritoneal wash was performed on recipient mice (Figure #16). Cells collected from the peritoneal wash were gated on lymphocytes, single cells, and then unstained cells were excluded from the analysis. Although both L1210 cells and control splenocytes were observed in the peritoneal wash of mice and it was determined that there was 27.8% specific lysis of L1210 cells in the ICV immunized compared to the control. The ratio of the splenocytes to the L1210 cells was compromised in the unimmunized animal, suggesting that the specific lysis calculation may be skewed. Unfortunately, the splenocytes may not have been staying within the peritoneal cavity and that could be why we saw a low percentage of them compared to the L1210 cells after the peritoneal wash.

Although these optimization steps demonstrate a possibly utility of this approach I sought to move onto a well documented and reliable method, the IFN-gamma ELISpot assay, for detecting anti-tumour immunity that could be transferable to a clinical setting.

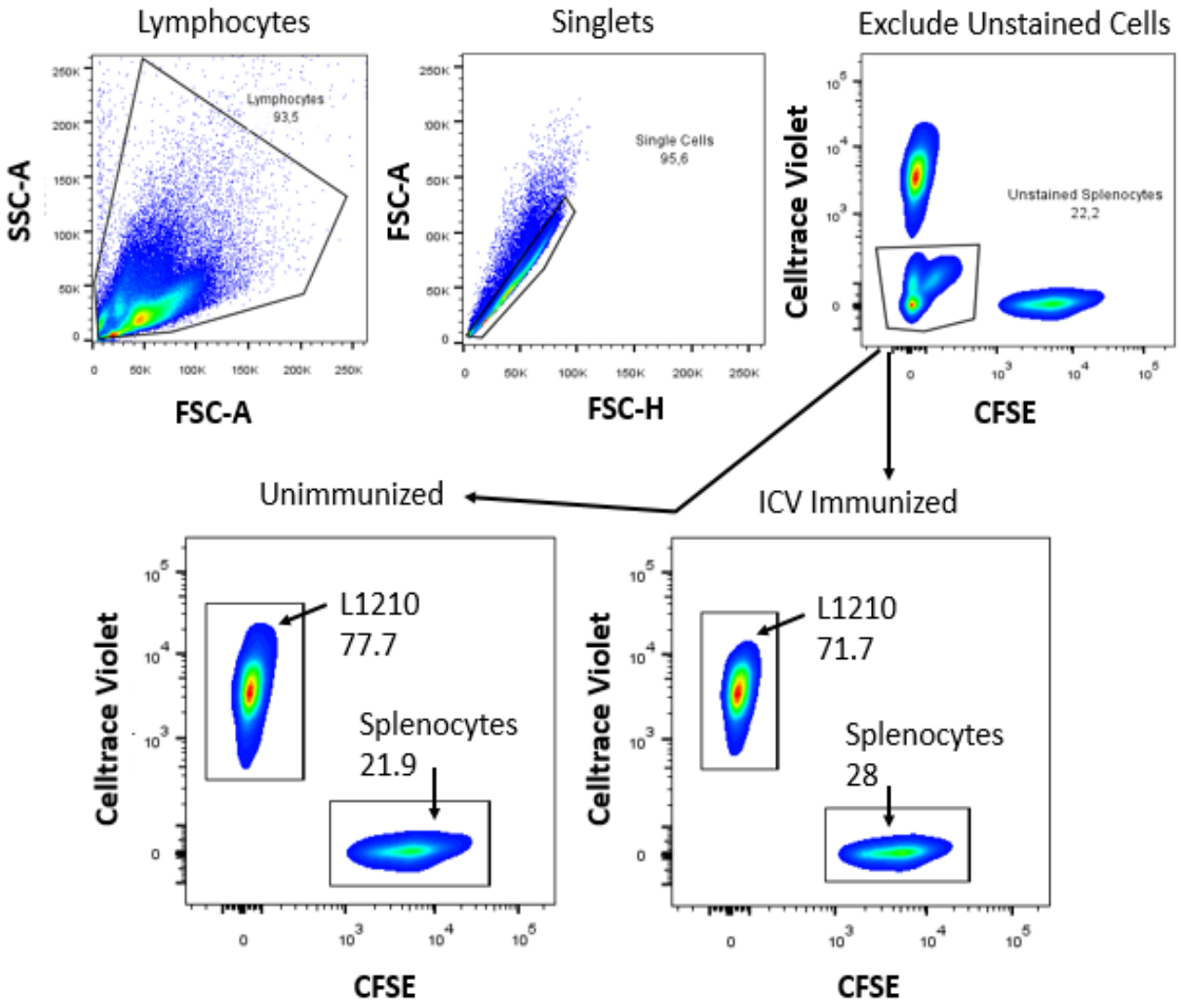

Lymphocytes
Singlets
Exclude Unstained Cells
SSC-A
FSC-A
Lymphocytes
93,5
FSC-A
FSC-H
Single Cells
95,6
Celltrace Violet
CFSE
Unstained Splenocytes
22,2
Unimmunized
ICV Immunized
Celltrace Violet
CFSE
L1210
77.7
Splenocytes
21.9
Celltrace Violet
CFSE
L1210
71.7
Splenocytes
28

Figure #16. Peritoneal wash used for *in vivo* killing assay results in a skewed ratio of L1210 cells to control splenocytes.

Donor splenocytes were labelled with 5 μm of CFSE and L1210 with CTV. $2x10^7$ cells/cell type were injected intra-peritoneally of either an unimmunized of ICV immunized mouse (n=1). 18 hours post injections, a peritoneal wash was performed and flow cytometry was done to assess L1210 cell killing. Cells were gated on lymphocytes, singlets, exclusion of unstained cells, and then stained cell populations were analyzed.

4.3.5 IFN-gamma ELISpot

An ELISpot is sensitive assay for detecting the number of tumour specific immune cells as determined by quantifying cytokine secreting cells in either a mixed of purified population presented with target cells or antigens. To further characterize the immune response of the T cell subsets in ICV immunized mice an IFN-gamma ELISpot was performed. The IFN-gamma ELISpot would also be able to quantify the # of CD4 and CD8 T cells from ICV immunized mice that have an L1210 -specific anti-tumour immune response. Firstly, a preliminary ELISpot was done to ensure adequate sensitivity and technical replication. CD3 T cells were isolated from unimmunized and ICV immunized mice (>87% purity as shown in Appendix – Figure #26). Effector T cells were mixed at a 4:1 ratio with whole splenocytes and plated into wells. Effector cells were re-stimulated with either media, L1210 cells and either, anti-CD28 magnetic beads or and PMA/Ionomycin as a positive control. The plate was then incubated for 18 hours and spot frequency counts (SFC) were quantified. Notably, CD3 T cells from unimmunized mice produced IFN-gamma in response to anti-CD28 and PMA/Ionomycin as expected. However, PMA/Ionomycin produced spots that were easier to read and the wells looked less hazy and was selected as a positive control for the future assays (Figure #17A). Quantification of the SFCs also revealed that CD3 T cells from ICV immunized mice produced more IFN-gamma in response to L1210 cells than unimmunized mice suggesting that this approach could be used to effectively measure tumour specific T cell responses (Figure #17B).

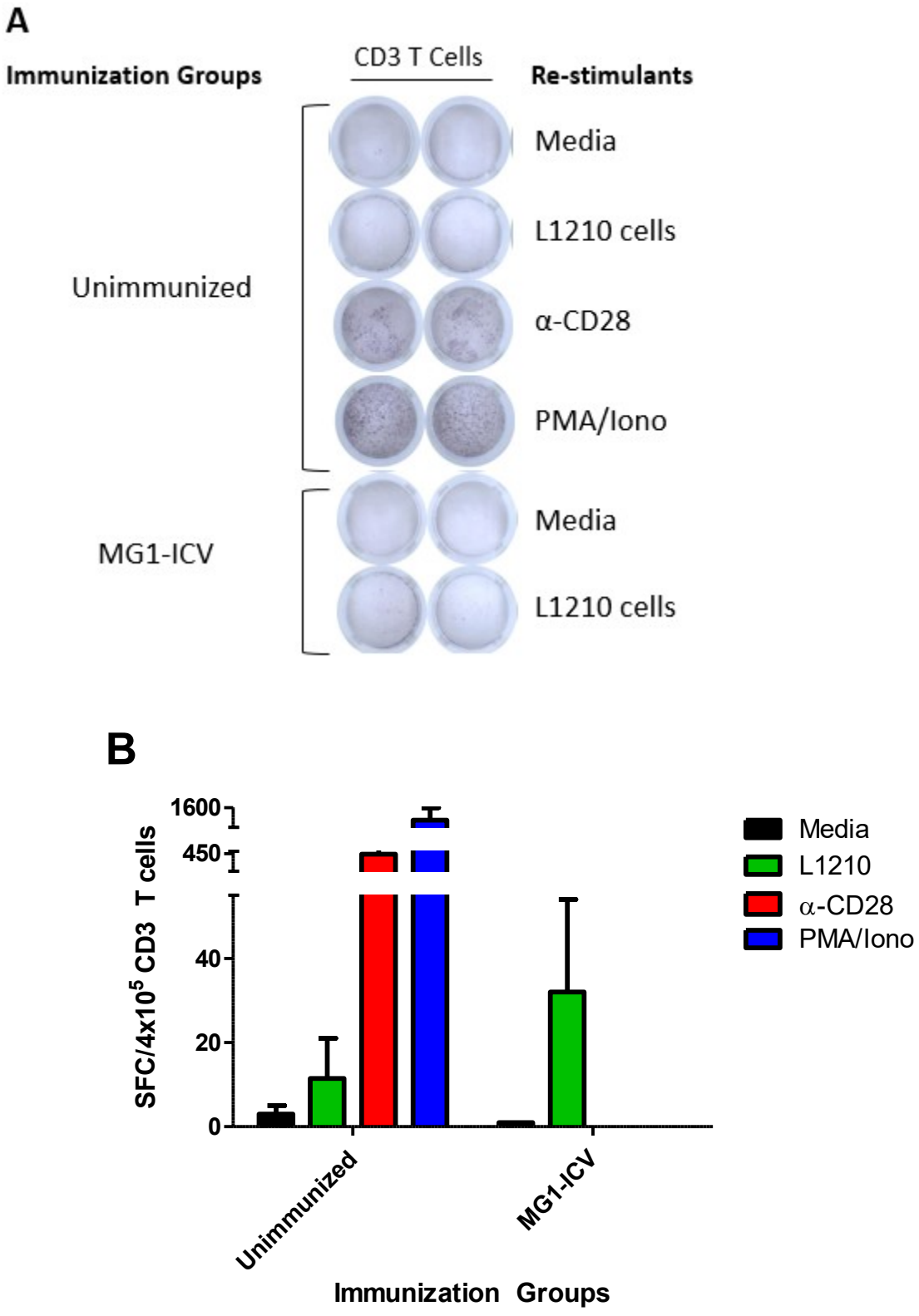
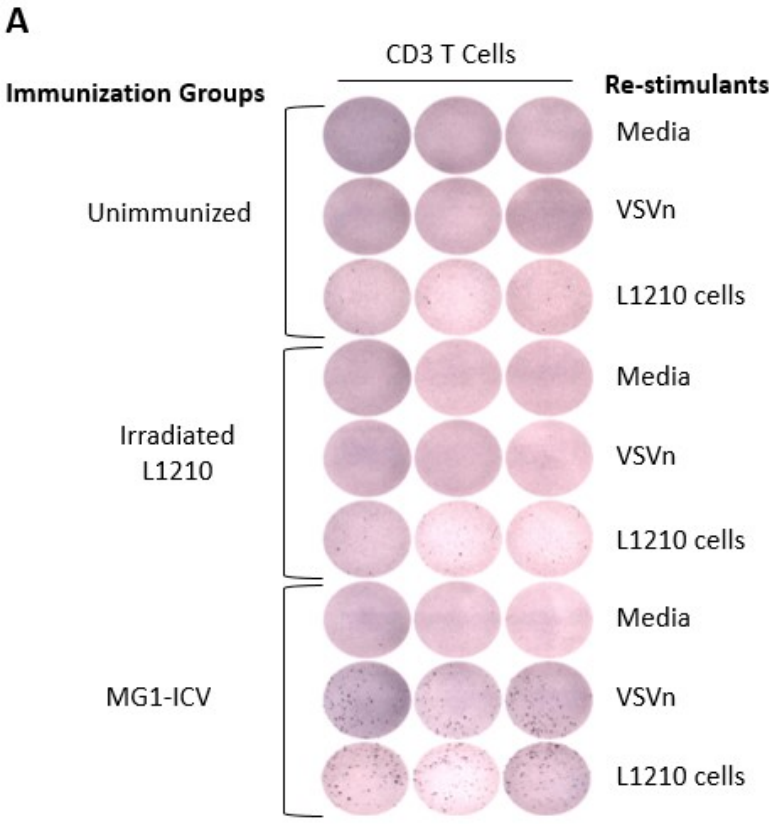

A
Immunization Groups
CD3 T Cells
Re-stimulants
Media
L1210 cells
α-CD28
PMA/Iono
Unimmunized
Media
L1210 cells
MG1-ICV
B
SFC/4x10^5 CD3 T cells
1600
450
40
20
0
Media
L1210
α-CD28
PMA/Iono
Unimmunized
MG1-ICV
Immunization Groups

Figure #17. Preliminary ELISpot determines that PMA/Ionomycin is an effective positive control.

Mice were either unimmunized or administered the ICV. Two months post immunization spleens were collected from mice. CD3 T cells were negatively isolated from spleens. T cells were mixed with naïve splenocytes at a 4:1 ratio. The effector cells were then co-cultured with re-stimulants at a 5:1 ratio (media, 1% PFA fixed L1210 cells, anti-CD28 magnetic beads, PMA/Ionomycin) for 18 hours. **A:** ELISpot plate visually demonstrating the spot frequency counts (SFC)/well. **B:** SFC/well quantified. Samples were all performed in technical duplicate. N=1 mouse/immunization group. Mean is shown and error bars represent the standard deviation of the technical duplicates.

Next, having optimized this approach a complete experiment was performed to compare IFN-gamma secretion in CD3, CD4, CD8 T cells from unimmunized, irradiated L1210 immunized, and ICV immunized mice. CD3, CD4 and CD8 T cells were isolated from pooled splenocytes of the respective groups using a negative selection kit. Purity of samples post-isolation is shown by flow cytometry (Appendix – Figure #27) and was >90% for every sample. T cells were mixed at a 4:1 ratio with whole splenocytes and plated into wells. T cells were re-stimulated with either media, VSVn peptide, and L1210 cells. Figure #18A shows the visual representation of the SFC/per well. The number of CD3 T cells from ICV immunized mice that produced IFN-gamma in response to L1210 cells was significantly higher than irradiated L1210 immunized and unimmunized mice. This data was quantified in Figure #18B. This demonstrated the IFN-gamma ELISpot was detecting a CD3 T cell anti-tumour immune response from the ICV.


A
CD3 T Cells
Immunization Groups
Re-stimulants
Unimmunized
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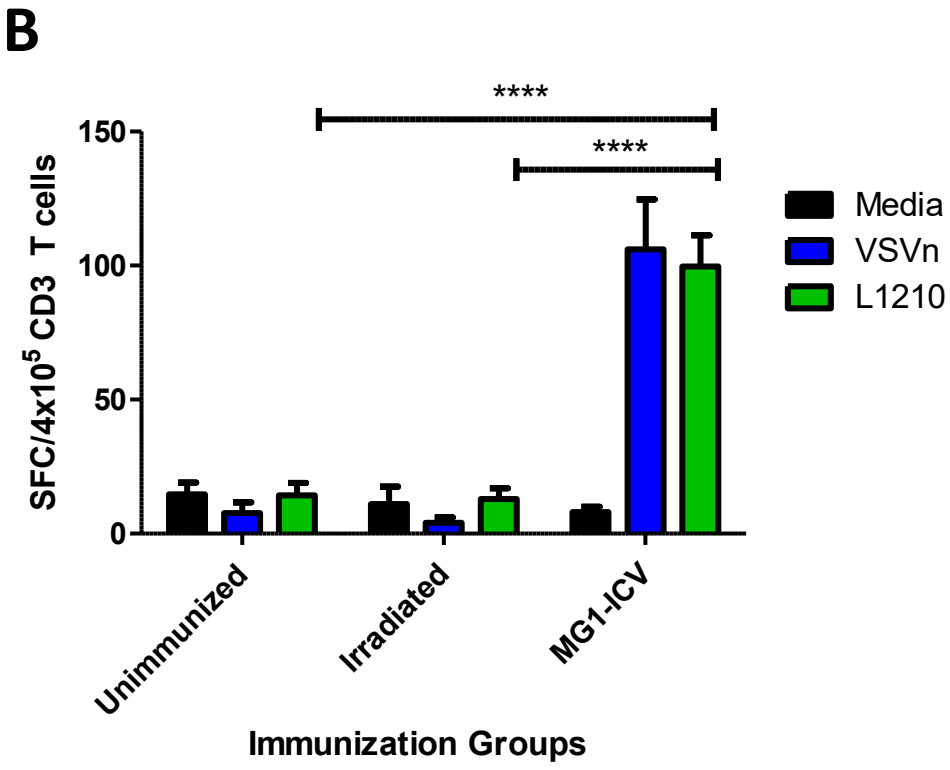
VSVn
L1210 cells
Irradiated L1210
Media
VSVn
L1210 cells
MG1-ICV
Media
VSVn
L1210 cells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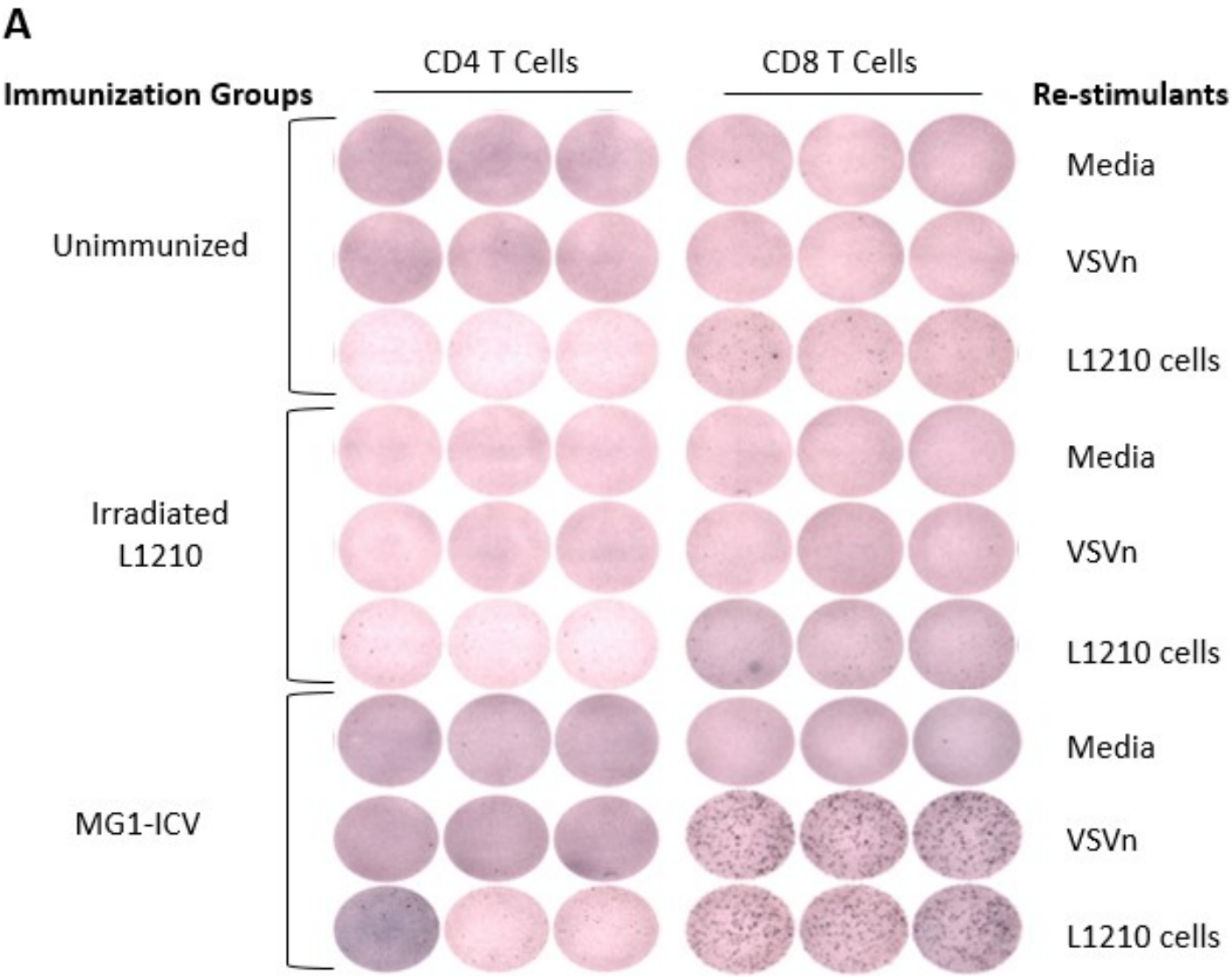
Immunization Groups

Figure #18. CD3 T cells from ICV immunized mice secrete IFN-gamma in response to L1210 re-stimulation.

Mice were either unimmunized, immunized with irradiated L1210 cells or the ICV. 45 days post immunization spleens were collected from all mice. CD3 T cells were negatively isolated from spleens and the pooled together based on respective cohorts. Pooled T cells were mixed with naïve splenocytes at a 4:1 ratio. The effector cells were then co-cultured with re-stimulants at a 5:1 ratio (media, VSVn peptide, 1% PFA fixed L1210 cells) for 18 hours. **A:** ELISpot plate visually demonstrating the spot frequency counts (SFC)/well. **B:** SFC/well quantified. PMA/Ionomycin (PMA/Iono) was used as a positive control. Samples were all performed in technical triplicate. N=4 for unimmunized samples and N=5 other groups. Mean and SEM are shown and a two-way ANOVA was performed on GraphPad Prism for statistical analysis.

After determining that CD3 T cells from ICV immunized mice produced IFN-gamma upon L1210 re-stimulation we sought to compare the CD4 and CD8 T cell response. Figure #19A shows the visual representation of IFN-gamma secreting spots per well. The spots were further quantified and the results demonstrated that the number of SFCs of CD4 T cells from ICV immunized mice was significantly higher than irradiated L1210 and unimmunized mice (Figure #19B). This demonstrates that CD4 T cells are producing IFN-gamma in an L1210-specific anti-tumour immune response. In comparison, CD8 T cells from ICV immunized mice produced an extremely significant number of IFN-gamma SFCs in response to L1210 re-stimulation compared to irradiated L1210 and unimmunized mice (Figure #19C). These results show that CD3, CD4, and CD8 T cells from ICV immunized mice all produce L1210-specific IFN-gamma. However, when the three T cell populations are graphed together it is interesting to note how many more SFCs are present within the CD8 T cell subset (Figure #19D). This shows that CD8 T cells are providing a strong tumour-specific anti-tumour immune response.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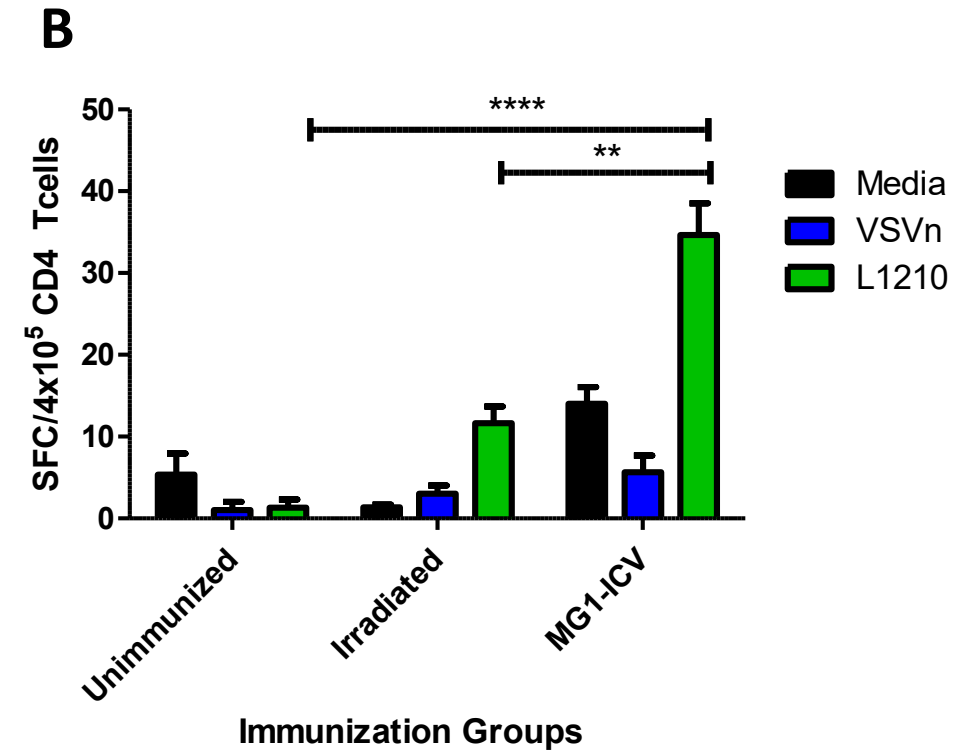
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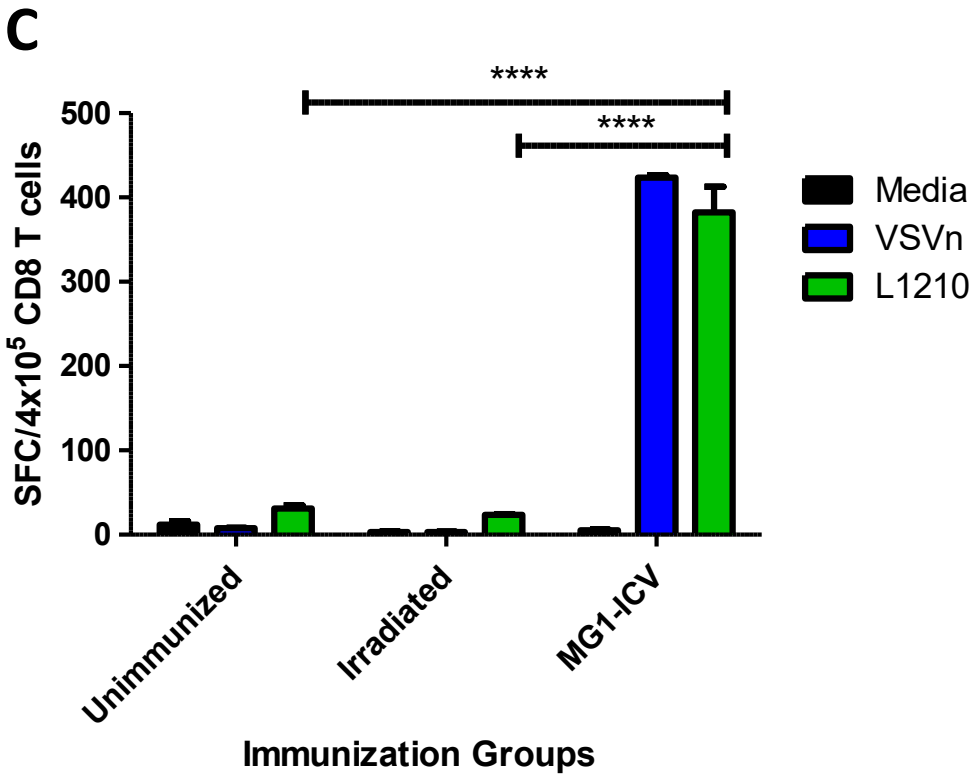
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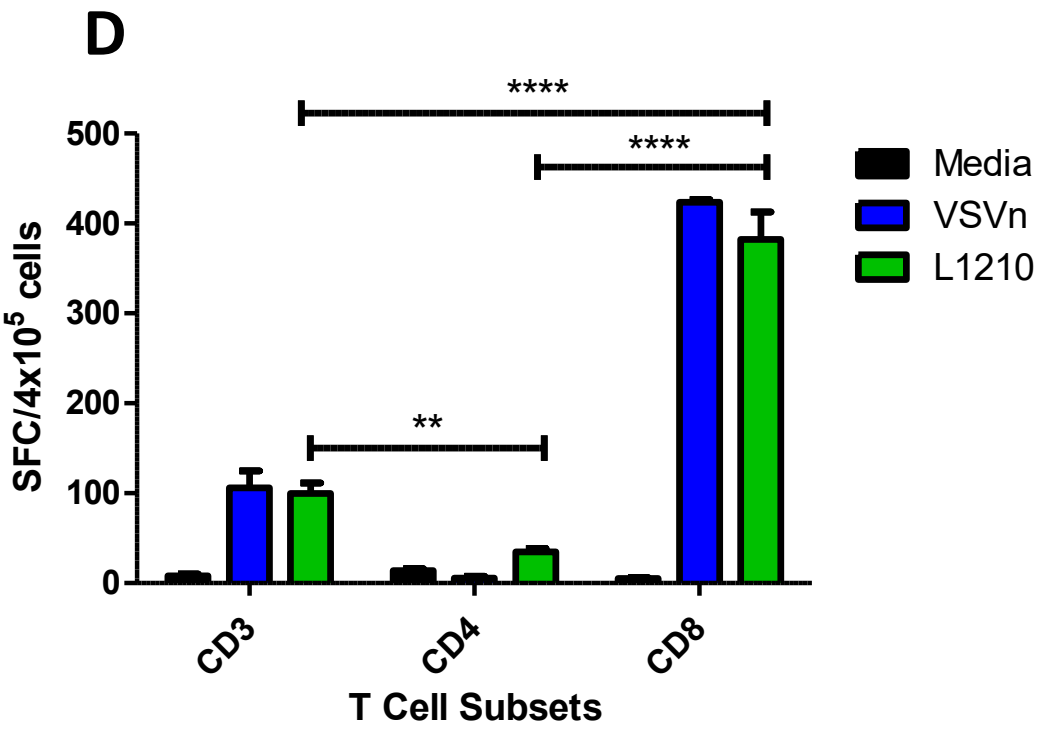
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T Cell Subsets
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L1210

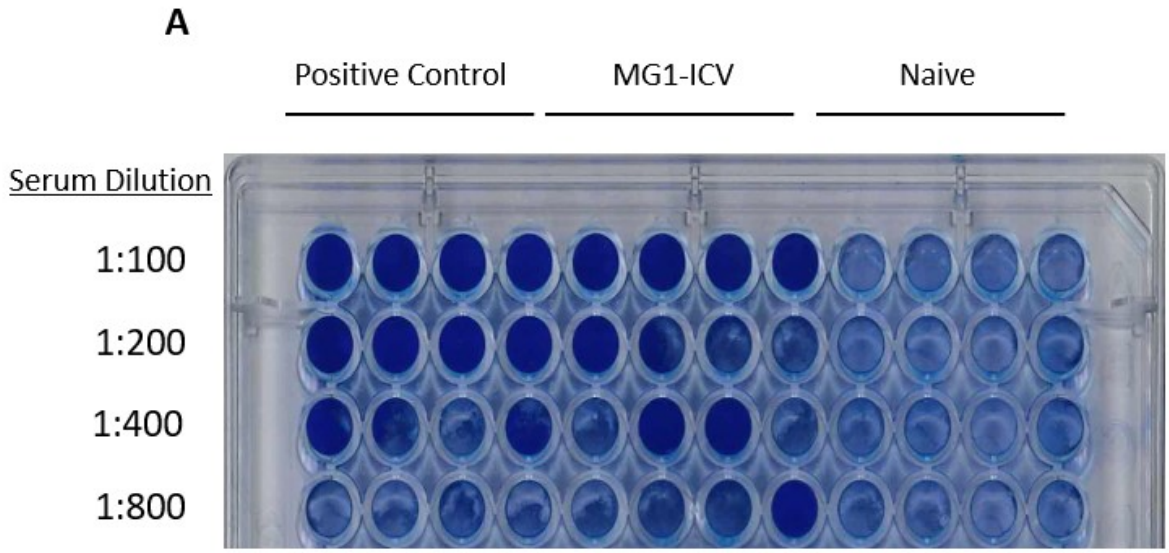
Figure #19. Comparing IFN-gamma secretion upon L1210 re-stimulation in different T cell subsets.

Mice were either unimmunized, immunized with irradiated L1210 cells or the ICV. 45 days post immunization spleens were collected from all mice. Splenocytes were pooled from the appropriate cohorts and then CD4 and CD8 T cells were negatively isolated. Pooled T cells were mixed with naïve splenocytes at a 4:1 ratio. The effector cells were then co-cultured with re-stimulants at a 5:1 ratio (media, VSVn peptide, 1% PFA fixed L1210 cells) for 18 hours. **A:** ELISpot plate visually demonstrating the SFC. **B:** IFN-gamma secretion of CD4 T cells. **C:** IFN-gamma secretion of CD8 T cells. **D:** Comparing IFN-gamma secretion of CD3, CD4, and CD8 T cell subsets of ICV immunized mice in one graph. Samples were all performed in technical triplicate. N=4 for unimmunized samples and N=5 other groups. Mean and SEM are shown and a two-way ANOVA was performed on GraphPad Prism for statistical analysis.

4.4 Identifying T cell functions in response to the ICV

4.4.1 Neutralizing antibodies against MG1

Interestingly, Conrad et al. previously reported that prior exposure to MG1 and the presence of neutralizing antibodies increased the ability of the ICV to provide protection against greater tumour challenges.[62] These findings suggest that generation of an effective anti-viral antibody response may improve the efficacy of the vaccine and may partly explain how the relative T cell subsets contribute to vaccine efficacy. To investigate this possibility, we collected serum from unimmunized and ICV immunized mice 7 days post ICV to confirm that ICV immunized mice produce MG1 neutralizing antibodies. As expected, a neutralizing antibody assay, clearly revealed that ICV immunized mice produced a relatively high titre of MG1 neutralizing antibodies which were absent in unimmunized controls (Figure #20A). To determine whether either T cell subsets were required for this response serum from T cell depleted groups was also assessed for the presence of neutralizing antibodies. Comparable to the *in vivo* T cell depletion assay, ICV immunized mice were depleted of either CD4, CD8 and CD4/CD8 T cell subsets. Seven days post ICV immunization, blood was collected via cardiac puncture and serum was isolated. A neutralizing antibody was performed to determine the amount of MG1 neutralizing antibodies from each serum sample. As demonstrated before, ICV immunized mice produce MG1 neutralizing antibodies and when mice were depleted of CD8 T cells this response was unaltered. However, depletion of CD4 T cells inhibited the production of MG1 neutralizing antibodies (Figure #20B). Although further work will be required, this data suggests that CD4 T cells are required for the anti-viral immune response generated during ICV immunization.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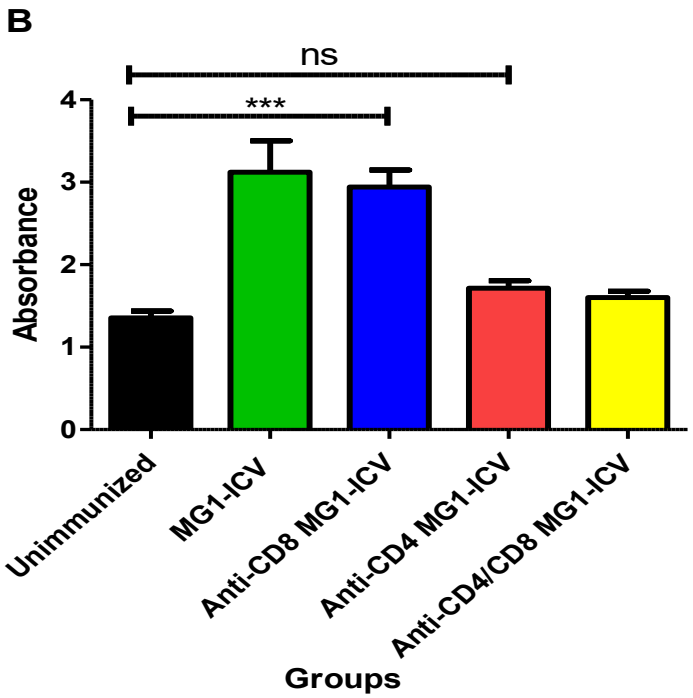
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Anti-CD8 MG1-ICV
Anti-CD4 MG1-ICV
Anti-CD4/CD8 MG1-ICV
Groups

Figure #20. MG1 neutralizing antibody production is a CD4 T cell dependent mechanism.

A: 6-old old DBA/2 mice were either unimmunized (naïve) or ICV immunized once a week for three weeks. Serum was collected from mice. Serum was incubated with MG1-GFP virus for 45 minutes and then plated onto Vero cells for 72 hours. Vero cells were stained with Coomassie blue to assess the production of neutralizing anti-viral antibodies. Positive control was serum collected from a C57/BL6 mouse that had been administered MG1 virus (donated by Dominic Roy). Serum was plated in technical quadruplicate. (N=1/group). **B:** 6-week-old DBA/2 mice were depleted of either CD4, CD8, or both T cell populations. ICV immunization was administered once a week for three weeks. 7 days post immunization serum was isolated from mice and same protocol was followed as A. Plates were read on the Multiskan to measure absorbance at 570 nm. Absorbance measurements at 1:100 serum dilution. N=4 per/group. Statistical significance was calculated by a two-way ANOVA with a Bonferroni post-test on GraphPad Prism.

5 DISCUSSION

Currently, there are no effective treatment options for AL patients as approximately 40% of patients will relapse within 2 years following therapy. We have developed an ICV therapy for AL patients that has shown promising murine pre-clinical data and could potentially be the solution to overcome leukemia relapse. Before progressing the ICV to clinical trials, more pre-clinical data needs to be demonstrated and an *ex vivo* potency assay to monitor the patient's anti-tumour immunity from the ICV needs to be proposed. The research conducted within my thesis has demonstrated plenty of pre-clinical data characterizing the anti-tumour immune response following treatment with an ICV for leukemia. The two main questions that we were interested in answering were 1) What APC populations are activated post ICV immunization and 2) What T cell subsets are important in developing the anti-tumour immune response during ICV immunization? My thesis has also determined that an IFN-gamma ELISpot can be used to detect L1210-specific anti-tumour immunity and could be used as an *ex vivo* potency assay for future clinical trials.

5.1 Activation of APC populations post ICV immunization

Generating a robust, durable adaptive immune response is contingent upon the uptake, processing and presenting of tumour antigens by professional APCs, including DCs, B cells and macrophages.[45] I first sought to determine what APC population were activated post ICV immunization.

Firstly, plenty of research represents CD11c+ cells as DCs[53,65], even though it is known to be a heterogenous marker also found on macrophages.[66] Due the heterogeneity of CD11c, the DC

activation that was represented in this thesis could also be a representation of macrophage activation. Before examining macrophages in the future, proper markers will need to be researched. For example, F4/80 was originally used in this experiment as a classic macrophage marker. However, it has been reported that freshly isolated DCs from the spleen of mice are both CD11c+ and F4/80+.[67] Macrophages were not able to be analyzed with this marker, as the majority of the CD11c+ cells were F4/80+ in my experiment. I suggest for future research to use these macrophage markers in combination: CD38, G-protein coupled receptor 18 (Gpr18), and Formyl peptide receptor 2 (Fpr2). Jablonksi et al. has shown that these specific markers can identify murine pro-inflammatory M1 macrophages by flow cytometry.[68] If future research determines that macrophages are activated post ICV immunization this information could be utilized to determine if they are essential for the anti-tumour immune response by performing an *in vivo* macrophage depletion similar to Cote's et al. research.[69] Due to marker selection, DCs and B cells were the APCs analyzed for my thesis.

My results demonstrated an increase in the frequency of a double positive MHCII+/CD40+ DCs 24 hours post ICV immunization suggesting that the ICV was better at promoting APC activation in comparison to irradiated L1210 cells alone (Figure #6). However, CD80, an activation marker that binds to the co-stimulatory molecule CD28 on T cells to aid in activation[70] was not altered post immunization. An increase in CD80 activation on DCs may not be necessary for T cell activation in our model of the ICV. It is possible that there is enough baseline expression of CD80 on DCs to bind to CD28 on T cells. It is also well known that CD80 activation generally follows CD40-CD40L interactions.[71] It is possible that 4, 24 and 72 hours were time points that did not discover sufficient CD80 activation; different time points could be assessed. Future

research should explore this experiment again possibly using a different antibody for CD80 or trying CD86 as an activation marker.

One of the limitations of my experiment is that it is unknown if DC activation is induced by MG1 alone. Lemay et al. showed that there was no difference in DC activation markers between VSV expressing GM-CSF and VSV-ICV expressing GM-CSF even though the latter provided enhanced anti-tumour immune responses.[53] Future research could compare MG1 versus and ICV administration in relation to APC activation.

The apparent lack of APC activation I have observed with irradiated cells alone is consistent with previous reports from our lab where 100% of mice immunized with irradiated L1210 cells alone succumb to tumour challenge.[62] However, my efforts have not investigated whether strategies aimed at further enhancing APC activation, such as the use of recombinant cytokines could be exploited to make the ICV more effective at providing anti-tumour immunity. For example, GVAX is a tumour cell vaccine that has been engineered to secrete GM-CSF and recruits DCs to the vaccination site. GVAX has been reported to provide enhanced anti-tumour effects in CML patients compared to tumour cell vaccine alone.[39] Alkayyal et al. has also reported that an MG1-ICV expressing IL-12, reduced colon cancer tumour burden in mice and it was more effective at providing anti-tumour immunity compared to MG1-ICV alone.[61] Exploring how to increase DC activation post immunization could be a mechanism to further optimize the ICV.

CD19+ B cells were also analyzed to assess if they were activated post ICV immunization. The level of expression of CD40 was increased post ICV immunization, however, unlike my findings with DCs, there were no changes in MHCII (Figure #7). Furthermore, I did not observe any

increase in CD80 expression following vaccination. These results suggest that B cells are unlikely to provide an effective activation signal to T cells post immunization.

In total, these findings are in general agreement with published research wherein CD11c+ activation is observed following delivery of rhabdovirus.[47,53] Also, our data demonstrating the preferential activation of DCs post immunization and without concomitant stimulation of B cells to stimulate T cell activation was previously unknown.

5.1.1 RANTES secretion

To elicit a detectable increase in APC activation which leads to the generation of a robust anti-tumour T cell response requires that the ICV provide immunogenic signals that are not generated by irradiated cells alone. The viral antigens and danger associated molecular patterns (DAMPs) generated by infected cells are potent inducers of APC activation. However, the ineffectiveness of a vaccine prepared with C1498 cells despite similar levels of infectivity and viral replication suggests that other cell intrinsic factors may be involved in promoting APC activation and an anti-tumour immune response. Previous work in our lab has shown that L1210 cells infected with MG1 results in the production of a number of chemoattracts and inflammatory cytokines. The secretion of RANTES, a stimulator of T cell activation, was notably increased to the greatest extent following infection with MG1. These results were confirmed during my thesis demonstrating that MG1 infection and irradiation upregulated RANTES secretion (Figure #8). I also determined that there was no RANTES expression in MG1-infected and irradiated C1498 cells (Appendix – Figure #28). Based on this data, it is tantalizing to speculate that RANTES or other cytokine secretion from the ICV could be aiding in the robust DC activation of T cells post ICV immunization that is observed *in vivo*. However, this data solely represents a positive correlation between DC activation and RANTES secretion so we cannot infer causality. Could

inhibiting RANTES decrease DC activation post ICV immunization? It would be worthwhile assessing if L1210 cells infected with MG1 and irradiation co-cultured with BMDCs induced DC activation. Following this, L1210 cells could be treated with a RANTES antagonist and then infected with MG1 and irradiated to determine if RANTES is necessary for DC activation. Both FLT-3 and GM-CSF are two other known chemokines known to induce DC activation.[72] It would be good to note if these cytokines are induced by MG1 infection and irradiation too.

5.1.2 Immunogenic Cell Death (ICD) and Anti-Tumour Immunity

Apoptosis and necrosis are two common ways that tumour cells are known to undergo cell death with cancer treatment. Tumour cell death by necrosis can lead to activation of the host's immune system by exposing tumour antigens and subsequently creating a pro-inflammatory environment.[45] However, immunogenic cell death (ICD) is another form of tumour cell death that leads to activation of the host's immune system against tumour cells.[73] Certain chemotherapy drugs are known to be either non-ICD or ICD inducers and this can correlate with the anti-tumour immune response. For example, Casares et al. reported that cell cancers treated with doxorubicin, a known ICD inducer, protects mice from subsequent tumour challenge whereas cancer cell treated with cisplatin, a non-ICD inducer does not protect mice from tumour challenge.[74] Irradiation is also known to cause ICD in tumour cell lines such as EL4 lymphoma and CT26 colon carcinoma.[73] Whole tumour cell vaccines take advantage of ICD as a way to stimulate the host's immune system against tumour antigens. Calreticulin (CRT), adenosine tri-phosphate (ATP), and high mobility group box protein 1 (HMGB1) are the three hallmark danger signals secreted by dying tumour cells that are associated with ICD.[73] When tumour cells undergo ICD they upregulate CRT on the surface on their membrane, secrete ATP and HMGB1. CRT binds to CD91 on DCs which in turn promotes phagocytosis of tumour cell antigens while

ATP and HMGB1 stimulate APC recruitment and activation. Specifically, HMGB1, a nuclear protein, binds to toll-like receptor 4 (TLR4) on DCs which is known to stimulate the optimal presentation of tumour antigens to T cells.[73] Apetoh et al. reported that small-interfering RNA (siRNA) deletion of HMGB1 in ICD-induced tumour cells inhibited cross presentation of tumour antigens to CD8 T cells *in vivo*.[75] This shows the importance of HMBG1 aiding in the anti-tumour T cell response. Overall, I think that future research should investigate if ICD is a prominent pathway that leads to activating DCs post ICV immunization. It could be possible that the ICV induces the hallmark markers of ICD, therefore, marking it such an immunogenic vaccine.

5.2 The Role of the T cell mediated anti-tumour immune response

5.2.1 In vivo T cell depletion

A primary goal of my thesis work was to investigate what T cell subsets were essential for generating an anti-tumour immune response following ICV treatment. As expected, depletion of both CD4 and CD8 T cells abolished the efficacy of the ICV as all immunized mice succumbed to tumour challenge (Figure #12). Other literature has also reported similar findings when CD4/CD8 T cells are depleted. For example, it has been shown that when CD4/CD8 T cells are depleted in effective cancer immunotherapies such as colon, glioblastoma and leukemia that 100% of mice succumb to the cancer.[60,61,76]

However, the impact of depleting the individual T cell subsets unexpectedly revealed an essential role for the CD4 T cells alone suggesting that CD8 T cells are not essential during the vaccination stage and during the first few weeks following tumour challenge. (Figure #12). This suggests that activated DCs are most likely interacting with CD4 T cells during immunization through MHCII and this interaction is leading to an anti-tumour immune response. However,

cross presentation is still most likely occurring as 25% of CD8 depleted mice die of leukemia challenge, showing the need for direct interaction with CD8 T cells. This data suggests that CD4 T cells are needed during the vaccination stage of the ICV while CD8 T cells are dispensable. During the vaccination stage, there are no tumour cells present meaning that CD8 T cells do not have a direct target. However, during vaccination, CD4 memory T cells are most likely being generated that are required to provide anti-tumour immunity at the time of tumour challenge. Both Janssen et al. and Bevan et al. have shown that CD8 T cells that differentiate following immunization in a CD4 depleted environment have impaired proliferative capacities and have decreased long term survival.[77,78] It is likely that the CD8 T cells that are present in the CD4 depleted animal cannot differentiate effectively into CTLs at the time of tumour challenge in our ICV model which leads to the mice succumbing to tumour challenge. Overall, my proposed mechanism is that CD8 T cells require the help and signals offered by the CD4 T cells to effectively provide long term anti-tumour immunity.

Based on this hypothesis, it is would informative to assess what would happen if we depleted the CD4 T cells at the time of tumour challenge but left them present during vaccination. I hypothesize that CD4 memory T cells would be generated during vaccination allowing for CD8 T cells to differentiate into effector CTLs that provide anti-tumour immunity. This experiment would demonstrate the potential role of CD4 helper T cells aiding in effective CD8 T cell differentiation.

Consistent with our results, Sharma et al. have also demonstrated the need for CD4 T cells during cancer immunotherapy in lung and cervical tumour models.[79] Sharma et al. demonstrated that depletion of CD4 T cells 1 day prior to tumour challenge resulted in a significant decrease in the efficacy of vaccine.[79] Dudley et al. have also shown that 20% of human melanoma biopsies

have tumour-specific CD4 T cells and that these CD4 T cells produce tumour-specific IFN-gamma.[80] These studies shows the role that CD4 T cells can be tumour-specific and that vaccine strategies require CD4 T cells to provide the most effective cancer immunotherapy – consistent with our model of the ICV. This research sheds light onto the critical role CD4 T cells play in cancer immunotherapy.

5.2.2 Adoptive T cell transfer

To confirm our findings of the *in vivo* T cell depletion an adoptive T cell transfer was performed. In contrast to our depletion results, 100% of mice that received CD3 T cells from ICV vaccinated animals succumbed to leukemia challenge (Figure #13C). Although this result at first glance is surprising, a number of possibilities could explain the discrepancy. In particular, the number of CD3 T cells to adoptively transfer is not standardized and differs from paper to paper.[81] The number of cells that we had transferred was lower comparably suggesting that we did not inject enough CD3 T cells. Also, in our experimental design the recipients of the adoptive transfer were naïve wildtype DBA/2 mice that possessed a full immunocompetent immune system, - whereas, many studies will condition recipients prior to adoptive transfer. For example, Wrzesinski et al. have shown that lymphodepleting recipients prior to adoptive transfer enhances the anti-tumour response by increasing the innate immunity as this depletes immunosuppressive immune cell populations such as regulatory T cells.[82] Other studies have used immunocompromised mouse models such as athymic mice or $RAG^{-/-}$ deficient mice.[53,83,84] Immunocompromised recipients allows the host to accommodate adoptively transferred cells which aids in the proliferation and growth of the injected cells.[85] Given these considerations, it would be worthwhile performing the adoptive T cell transfer experiment again using an athymic or lymphodepleted recipient in the future. Ideally, it would also be helpful to track the adoptively transferred cells in the recipient to

confirm cell transfer and proliferation Unfortunately, DBA/2 mice do not have CD45.1 and CD45.2 murine models. The CD45.1 and CD45.2 are convenient models to use to track cells from a donor to recipient. C57/BL6 mice do have CD45.1 and CD45.2 murine models for their genetic background. An ICV survival study has been performed using C57/BL6 mice using the syngeneic AML cell line C1498. This model is not as effective at providing anti-tumour immunity as 25% of ICV immunized mice survive leukemia challenge compared to 95% in the DBA/L1210 model. However, studying the C57/BL6 model would be easier as these advantageous murine models could be utilized.

5.2.3 In vivo killing assay

One objective of my thesis was to determine if we could correlate the increase of survival with the increase of T cell mediated killing. Development of an *ex vivo* potency assay would also allow us to test immune system alterations in CD4 and CD8 depleted mice. Also, prior to this work, our pre-clinical data demonstrating L1210 specific anti-tumour immunity has relied on survival curves.[62] Survival curves must be monitored up to 100 days post leukemia challenge, therefore, each pre-clinical study is very time-consuming. Hence, another added benefit of developing an *ex vivo* assay to detect L1210-specific anti-tumour immunity would be having an alterative method to detect anti-tumour immunity than survival curves. A similar approach has previously been used to evaluate tumour cell killing in other vaccination and tumour models. Fluorescently labelled splenocytes are normally pulsed with an appropriate tumour-specific peptide and control differentially labelled splenocytes are not pulsed with peptide. Unfortunately, no tumour specific antigen is currently known for the L1210 model and we attempted to modify the procedure to use whole labelled L1210 cells. The ICV provides anti-tumour immunity by presenting many potential antigens; a tumour specific peptide could be used if one were known.

This approach was plagued by several complications of the most important which was that the labelled L1210 cells were not consistently present in either unimmunized or ICV immunized spleens (Figure #14 and #15). One interpretation could be that the L1210 cells are inherently rejected in both groups at a very high rate, however, this seems unlikely given the very consistent and vastly different outcome in the survival of these treatment groups. Another, more likely issue with this approach is the differential homing properties of the control cells (splenocytes) and L1210 (leukemia) cells. In fact, it is well documented that L1210 cells do preferentially migrate to the bone marrow.[86] Given these barriers, we next decided to examine other methods for detecting L1210-specific anti-tumour immunity that could be translatable to the clinic. However, future ideas to optimize this assay would be to attempt pulsing splenocytes with L1210 cell lysate or using the DBA/2 syngeneic lymphoma cell line, P388, as a control.[87]

5.2.4 IFN-gamma ELISpot as a useful tool for evaluating anti-tumour immunity

IFN-gamma ELISpots are commonly used to measure the number of T cells reactive to a specific antigen or stimulus.[88] Advantages of ELISpots are that they are more sensitive than ELISAs and intracellular cytokine staining (ICS) and the results allow for direct quantification of tumour-reactive T cells.[89,90] Given that we do not have a confirmed antigenic peptide for L1210 cells we evaluated the presence of T cells reactive to whole cells. Comparison of IFN-gamma producing CD3, CD4, and CD8 T cells from unimmunized ICV immunized mice revealed that the presence of IFN-gamma producing T cells was significantly more in ICV immunized mice compared to controls. CD4 T cells produce more IFN-gamma SFCs when re-stimulated with L1210 cells suggesting that tumour-specific CD4 T cells are generated by the ICV (Figure #19B) Also, CD8 T cells from ICV immunized produce more IFN-gamma when SFCs when re-stimulated with L1210 cells compared to controls (Figure #19C). When comparing CD4 and CD8 T cells from

ICV immunized mice, there was 11x more CD8 T cells producing L1210-specific IFN-gamma than CD4 T cells (Figure #19D). Overall, both CD4 and CD8 T cells were producing L1210-specific IFN-gamma. Our data suggests that CD8 T cells are providing a robust tumour-specific anti-tumour immune response generated by the ICV. However, based on our T cell depletion survival curve we know that CD4 T cells are required to provide anti-tumour immunity. Therefore, it could be possible that CD4 T cells are acting as essential helper T cells to aid in the activation and differentiation of anti-tumour CD8 T cells. For example, plenty of literature has shown the development of cytotoxic CD8 T cells requires help from CD4 T cells during vaccination.[91–93] Gazzinelli et al. have demonstrated that mice administered with a *toxoplasma gondi*i vaccine develop resistance to infection and this is due to a CD8 T cell immune response. However, resistance to infection is completely abrogated when CD4 T cells are depleted during vaccination demonstrating the critical role CD4 T cells are playing to help develop a CD8 T cell response. This study even demonstrated that when CD4 T cells were depleted during vaccination, CD8 T cells produced less IFN-gamma when stimulated with the viral antigen.[93] Carvalho et al. also demonstrated that CD4 depletion during vaccination against malaria greatly reduced the number of virus specific- IFN-gamma secreting CD8 T cells in mice.[92] These studies outline the importance of CD4 T cells aiding in protective anti-viral CD8 T cell response. CD4 T cells could be depleted in ICV immunized mice during vaccination and CD8 T cells could be isolated and tested for tumour-specific IFN-gamma production. This experiment would help us answer if CD4 T cells are required during ICV immunization for the production of tumour-specific anti-tumour CD8 T cells. One mechanism could be that the cytokines produced by CD4 T cells is an essential signal to develop anti-tumour activated CD8 T cells. The classic model of CD4 T cells aiding in the activation of CD8 T cells is through CD4 T cell cytokine secretion.

Activated APCs will present antigens to CD4 T cells inducing CD4 T cell cytokine secretion. Following this, the same APC will present antigens to CD8 T cells via MHCI and the cytokines produced by CD4 T cells will act as chemo-attractants for the CD8 T cells.[94] For example, Green et al. showed that IFN-gamma secreting CD4 T cells were required for the production of virus-specific CD8 T cells.[91] This demonstrates the importance of CD4 T cell cytokine secretion. CD40-CD40L interactions is another published mechanism as to how CD4 T cells help activate CD8 T cells. CD4 T cells are able to recognize antigens presented by MHCII on APCs. Through the interaction of the CD40-CD40L, CD4 T cells are able to "super-activate" the APC by increasing the expression of CD80/CD86 on the APC. This then allows for the APC to efficiently activate CD8 T cells by MHCI antigen presenation.[94,95] There could be many different ways as to how CD4 T cells are possibly aiding in CD8 T cell activation during ICV immunization and future research should explore these mechanisms. My data helps explain the mechanisms of how the ICV in providing anti-tumour immunity *in vivo*. It also demonstrated that we were able to detect an L1210-specific anti-tumour immune response from the ICV. This is valuable for future clinical trials as a potency assay will be needed to quantify the anti-tumour immune response in patients immunized with the ICV. The IFN-gamma ELISpot could be the potency assay used for clinical applications.

5.3 Identifying T cell functions in response to the ICV

5.3.1 Neutralizing antibody production against MG1

After I determined that CD4 T cells were essential for generating anti-tumour immunity in our ICV model we were also interested in assessing their role in anti-viral immunity. It was evident that ICV immunized mice produce MG1 neutralizing antibodies post immunization (Figure #20) whereas unimmunized controls do not. When ICV immunized mice were depleted of CD4 T

cells MG1 neutralizing antibody production was abolished. In comparison, neutralizing antibody production was unaltered in CD8 depleted ICV immunized mice. These results are consistent with research demonstrating CD4 T cells are necessary for B cell maturation and antibody production.[96] Without CD4 T cells, B cells are unable to be activated, therefore, production of anti-viral and anti-tumour antibodies is inhibited.

It is curious how the ICV requires CD4 T cells for both anti-tumour and anti-viral immunity. Conrad et al. has demonstrated that pre-existing MG1 neutralizing antibodies prior to ICV administration increased the efficacy of the vaccine.[62] Similarly, Yang et al. has shown that pre-existing neutralizing antibodies against reovirus prior to immunization did not alter the efficacy of reovirus in animal models of glioblastoma.[97] This data does suggest that the anti-tumour and anti-viral immune response are intertwined; however, the mechanism is far from known. This research has highlighted that CD4 T cells could be a key immune cell involved in connecting these two immune responses. One hypothesis could be that the ICV activates CD4 T cells which in turn activates B cells to produce not only anti-viral antibodies, but also anti-tumour antibodies against leukemia cells. Another hypothesis could be that pre-existing neutralizing antibodies coat the ICV cells, making them more likely to be taken up by APCs, creating an ever more immunogenic vaccine. Future research should determine if the ICV promotes anti-tumour antibodies against leukemia specific cells.

6 CONCLUSIONS

AL is in need of a more effective treatment option as 40% of patients end up relapsing within 2 years post-standard therapy. The ICV is a promising and novel treatment method for AL that is in the process of progressing to future clinical trials. The goal of the ICV in the clinic would be

to collect AL patient's lymphoblasts at the time of cancer diagnosis. Patient's lymphoblasts would be sent to the lab, infected with MG1 and irradiated, to generate the personalized ICV. Prior to administering the ICV, patients would receive standard chemotherapy treatment to treat their AL. Post chemotherapy, the ICV would be administered to patients. The ICV should generate a patient-specific anti-leukemia response *in vivo* that should prevent AL relapse by generating immunological memory. (Figure #21). Throughout ICV treatment the patient's anti-leukemic immunity would be monitored with an *ex vivo* potency assay.

Before advancing the ICV to the clinic, an ample amount of pre-clinical data must be demonstrated as well as an *ex vivo* potency assay to track anti-leukemic immunity. This thesis has provided a significant amount of pre-clinical data demonstrating the integral requirements of the immune system utilized by the ICV to provide anti-tumour immunity. Our data has shown that an ICV vaccination strategy is more effective at promoting DC activation *in vivo* compared to irradiated L1210 cells alone and suggests that at least part of the improved efficacy of this strategy at stimulating an anti-tumour response is due to this difference. In addition, we have confirmed and expanded upon previous data and established an essential anti-leukemic role for CD4 T cells in tumour naïve recipients. However, the relative importance of the generation of MG1 neutralizing antibody production or IFN-gamma production by CD4 T cells will require further examination. This data also suggests that there is an critical relationship between CD4 and CD8 T cells during ICV immunization and that CD4 T cells are likely acting as helper T cells to aid in the robust anti-tumour activation of CD8 T cells.

This thesis has demonstrated that the IFN-gamma ELISpot assay detects L1210-specific anti-leukemic immunity for the ICV. Based on our results, the IFN-gamma ELISpot has potential to be used as the *ex vivo* potency assay to monitor patient's anti-tumour response during ICV

treatment. Detecting *ex vivo* anti-tumour responses is big leap to progress the ICV from bench to bedside.

Overall, the ICV is a promising therapeutic for AL patients that should provide long-term anti-tumour immunity. This thesis will help progress the ICV towards clinical trials by providing robust pre-clinical data and demonstrating a sufficient *ex vivo* potency assay to monitor anti-tumour immunity.

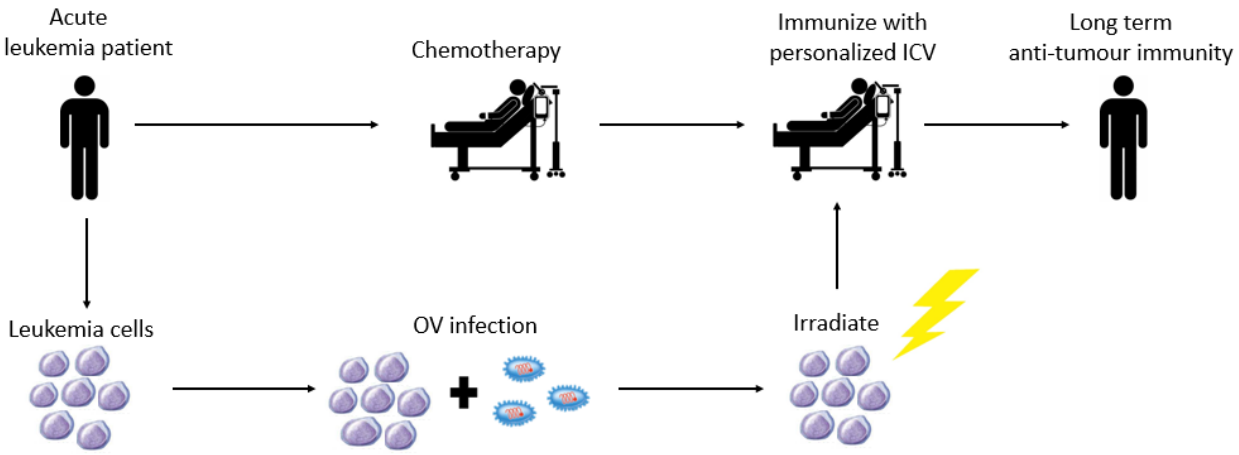
Acute
leukemia patient
Chemotherapy
Immunize with
personalized ICV
Long term
anti-tumour immunity
Leukemia cells
OV infection
Irradiate

Figure # 21. Bringing the ICV from bench to bedside

At the time of AL diagnosis, a patient's leukemic cells would be collected. Leukemia cells are infected with MG1 virus and irradiated in the laboratory to generate the personalized ICV. Post AL diagnosis, patients would undergo chemotherapy to treat their leukemia. After chemotherapy is complete and remission has been achieved patients would be given the ICV to prevent AL relapse and provide long-term anti-tumour immunity.

CONTRIBUTION OF COLLABORATORS

Julia Petryk, Bell lab senior animal technician, performed intra-venous injections for all experiments.

Christiano Tanese de Souza, Auer lab senior animal technician, harvested lymph nodes.

Leah Monette, summer student, helped collect supernatants for the RANTES ELISA. Leah helped perform preliminary ELISAs and performed the RANTES ELISA in my thesis.

Dr. Michael Kennedy aided in experimental design and research plan.

APPENDIX

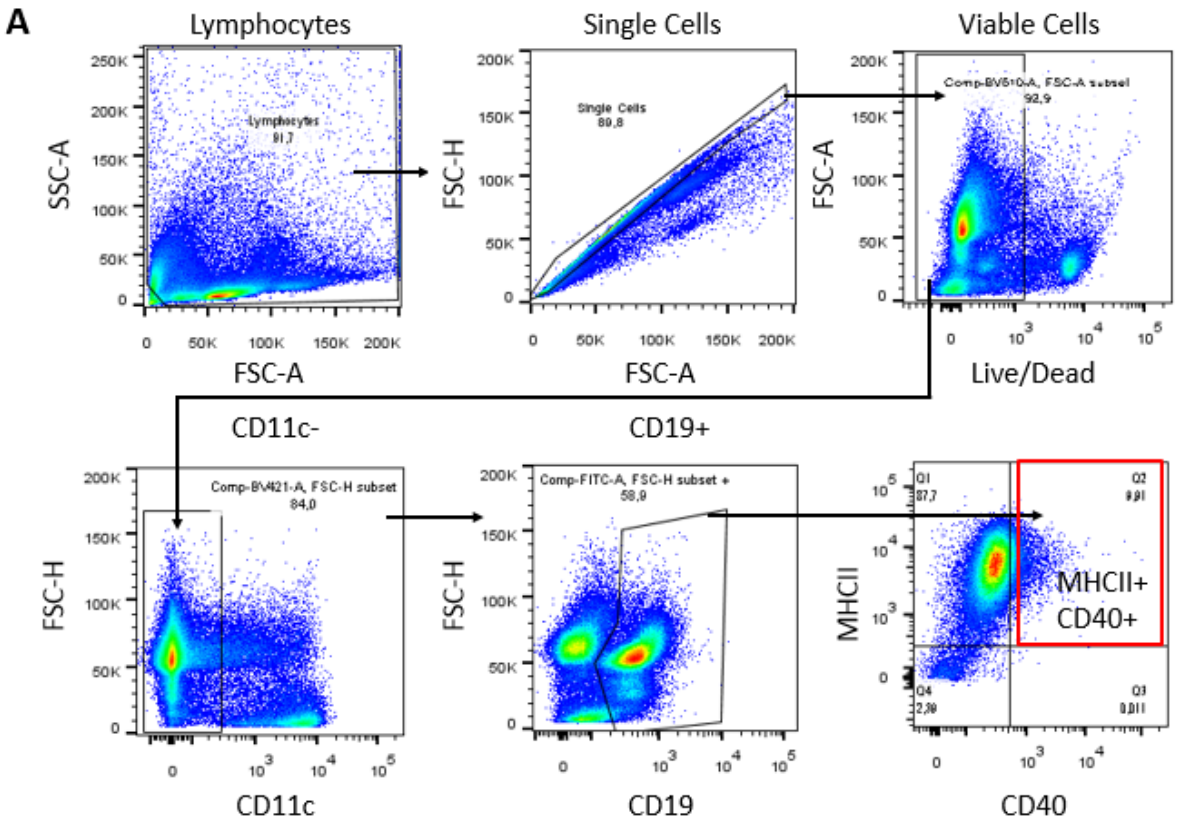
A
Lymphocytes
Single Cells
Viable Cells
SSC-A
FSC-A
FSC-H
FSC-A
FSC-A
Live/Dead
CD11c-
CD19+
FSC-H
CD11c
FSC-H
CD19
MHCII
CD40
MHCII+
CD40+

Figure #22. B cell gating strategy.

Spleens were collected from mice at 4, 24, and 72 hours post irradiated L1210 and ICV immunization. Samples were stained with antibodies for flow cytometry and data was analyzed. B cell gating strategy is shown. Cells are gated on lymphocytes, single cells, viable cells, CD11c- and CD19+ cells. Analysis was done on the double positive MHCII+/CD40+ subset.

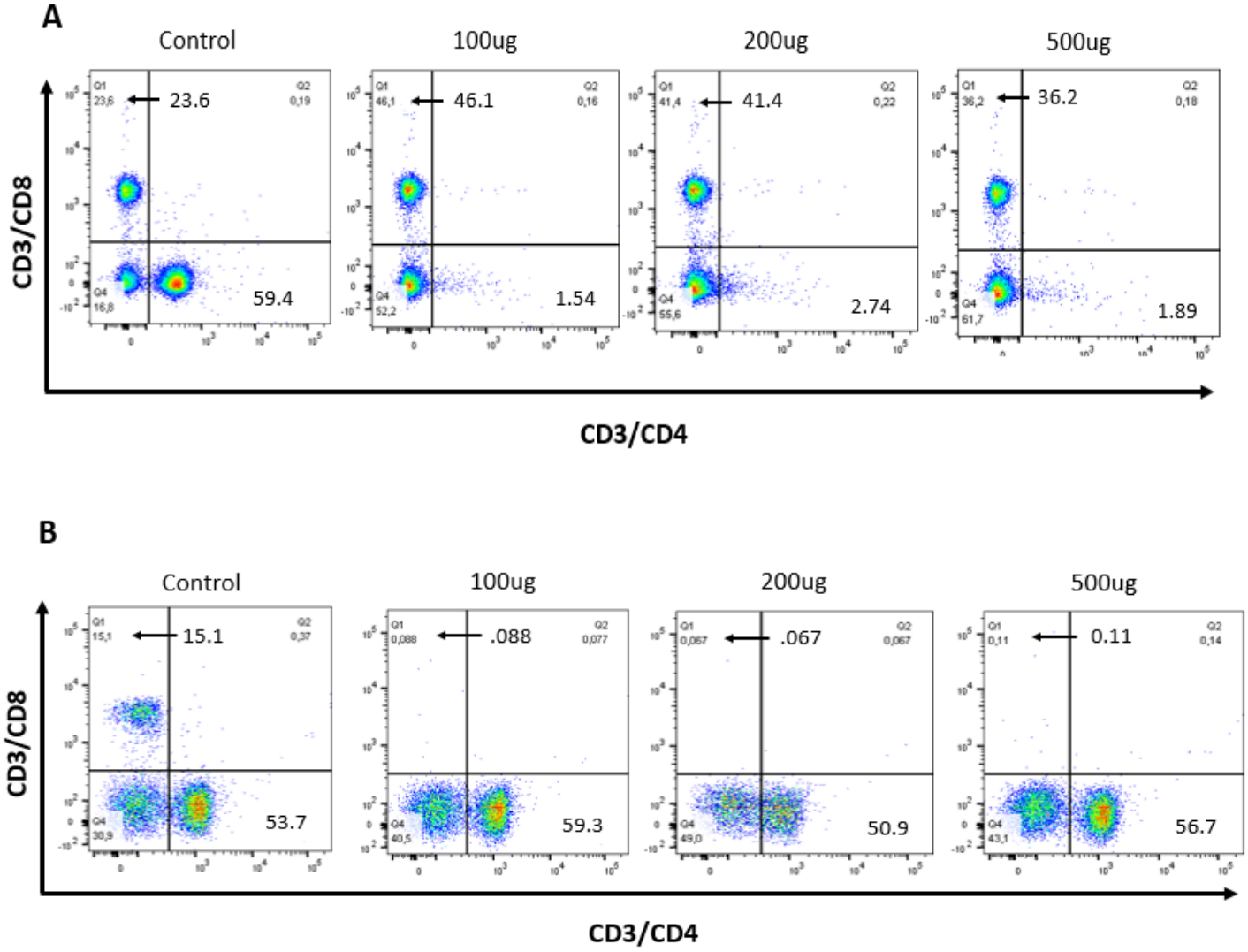
A
Control
100ug
200ug
500ug
23.6
46.1
41.4
36.2
59.4
1.54
2.74
1.89
CD3/CD8
CD3/CD4
B
Control
100ug
200ug
500ug
15.1
.088
.067
0.11
53.7
59.3
50.9
56.7
CD3/CD8
CD3/CD4

Figure #23. *In vivo* CD4 and CD8 antibody dose optimization.

DBA/2 mice were either injected intra-peritoneally with PBS (control), 100, 200, or 500 μg of CD4 or CD8 antibody on day 0 and 1. 24 hours following the last antibody injections spleens were collected. Splenocytes were stained with CD3, CD4, and CD8 antibodies and analyzed by flow cytometry. **A:** CD4 antibody dose response **B:** CD8 antibody does response.

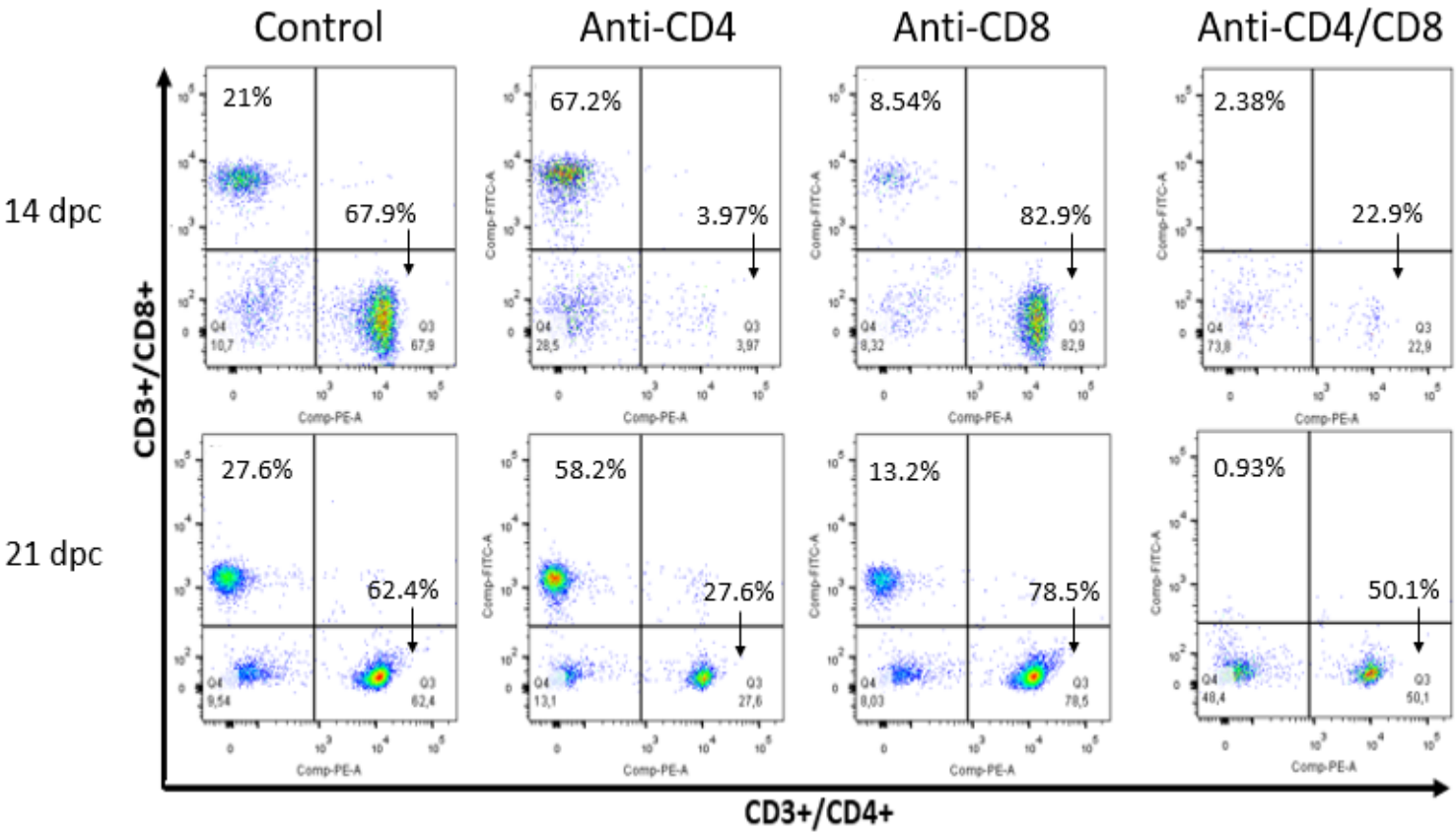
Control
Anti-CD4
Anti-CD8
Anti-CD4/CD8
14 dpc
21 dpc
21%
67.9%
67.2%
3.97%
8.54%
82.9%
2.38%
22.9%
27.6%
62.4%
58.2%
27.6%
13.2%
78.5%
0.93%
50.1%
CD3+/CD8+
CD3+/CD4+
Comp-PE-A
Comp-FITC-A

Figure #24. T cells reconstitute 21 Days post L1210 challenge.
Blood was collected from mice 14 days and 21 days post L1210 challenge (15 and 22 days post last antibody injection respectively) to determine if the T cells would repopulate after challenge. Blood was stained with CD3, CD4 and CD8 antibodies. T cells remain depleted 15 days post challenge and reconstitute by 21 dpc (days post challenge).

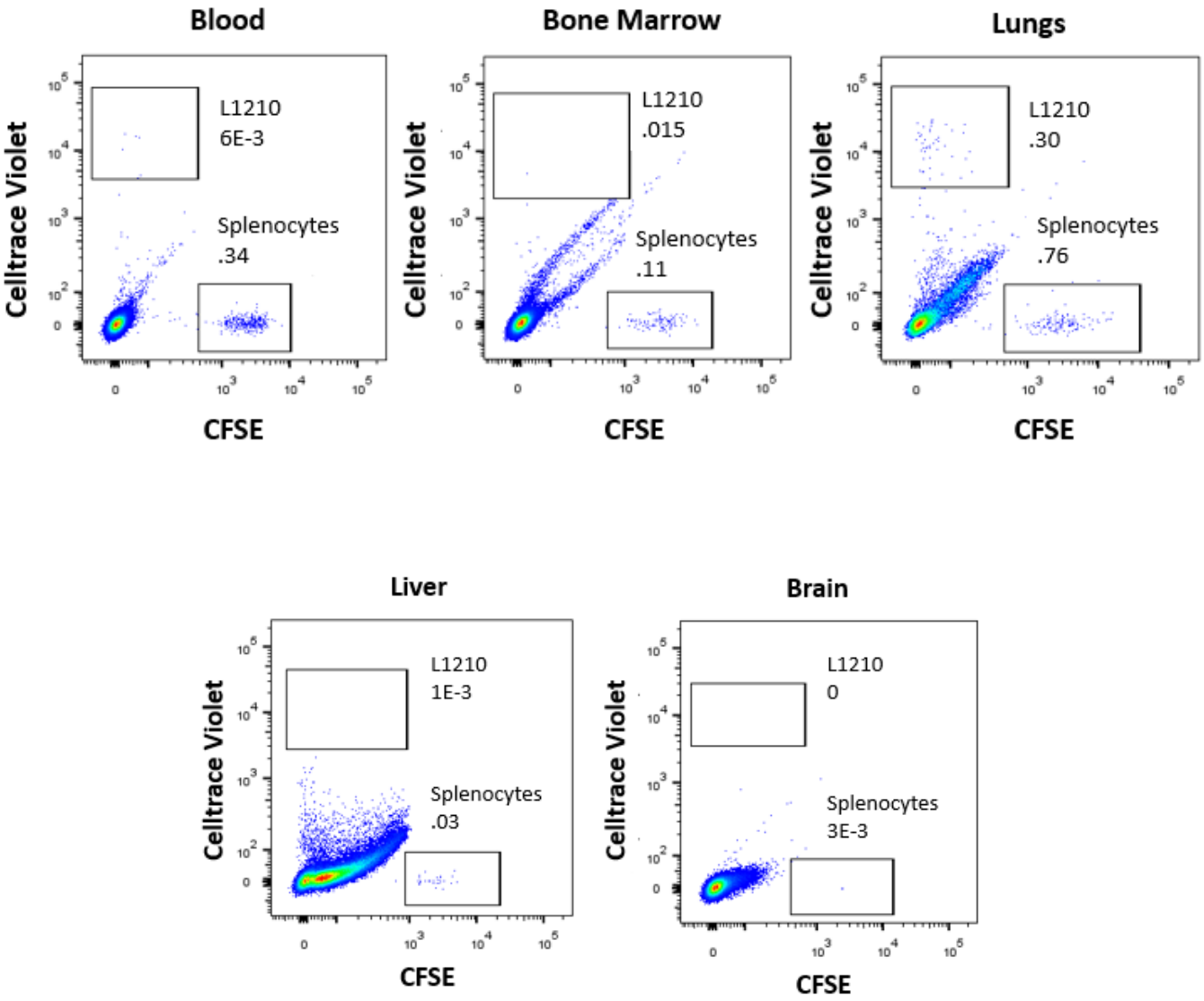
Blood
Bone Marrow
Lungs
Liver
Brain
Celltrace Violet
CFSE
L1210
6E-3
Splenocytes
.34
L1210
.015
Splenocytes
.11
L1210
.30
Splenocytes
.76
L1210
1E-3
Splenocytes
.03
L1210
0
Splenocytes
3E-3

Figure #25. Leukemia cell do not seem to migrate to other organs than the spleen.

$2x10^7$ CTV labelled L1210 cells and $2x10^7$ CFSE labelled splenocytes were mixed together and injected into the tail vein of an unimmunized mouse. 18 hours post injection, the spleen, blood, bone marrow, lungs, liver, and brain were collected. Flow cytometry was done on all organs to determine if the L1210 cells migrated or homed to different organs in the body compared to the control splenocytes.

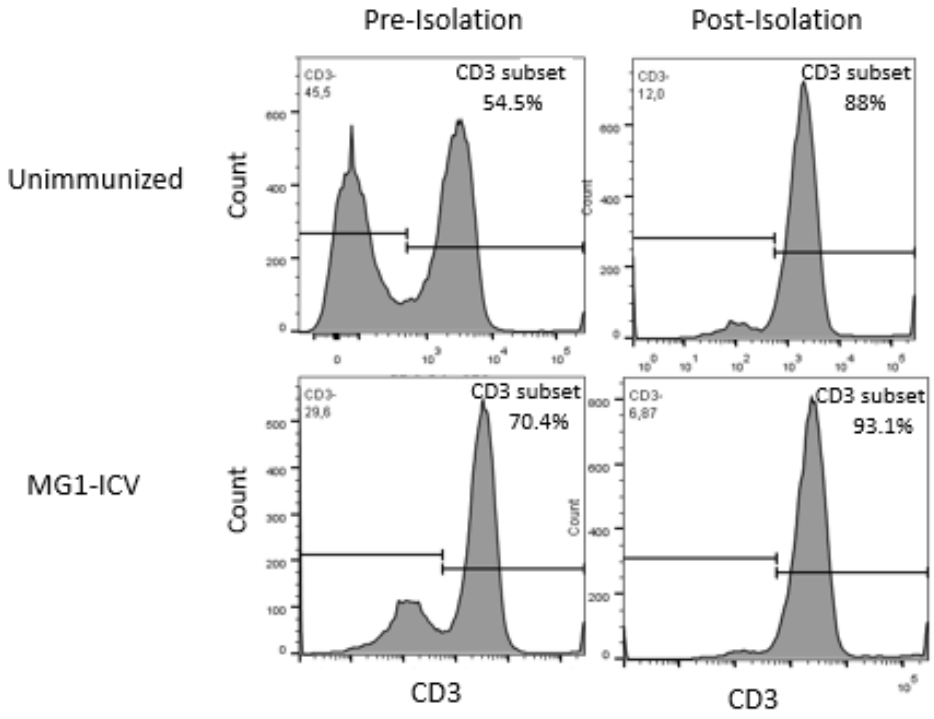
Pre-Isolation
Post-Isolation
Unimmunized
MG1-ICV
CD3 subset
54.5%
CD3 subset
88%
CD3 subset
70.4%
CD3 subset
93.1%
Count
CD3

Figure #26. Isolation of a pure population of CD3 T cells.

Spleens were collected from unimmunized or ICV immunized mice and CD3 T cells were isolated using a negative selection kit. Isolated T cells were stained with a CD3 antibody and flow cytometry was performed. CD3 T cell isolation was > 88% pure. These T cells were then used as effector cells for a preliminary IFN-gamma ELISpot assay.

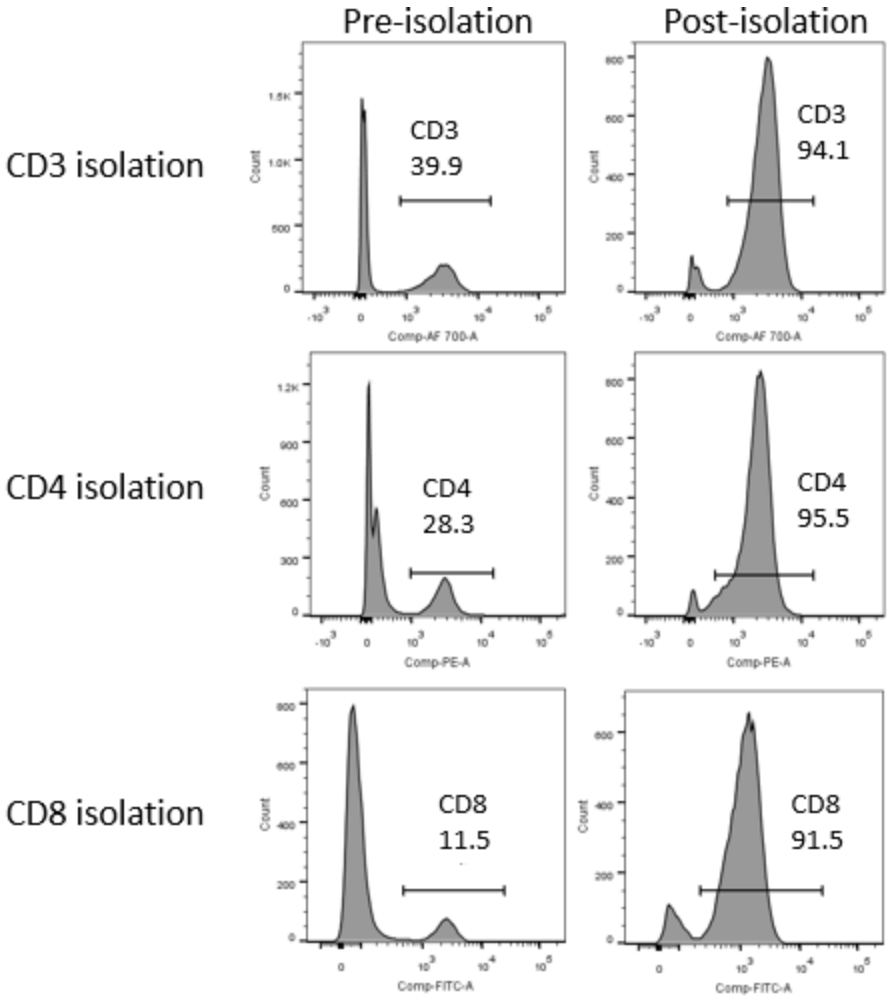
Pre-isolation
Post-isolation
CD3 isolation
CD4 isolation
CD8 isolation
CD3
39.9
CD3
94.1
CD4
28.3
CD4
95.5
CD8
11.5
CD8
91.5
Count
Comp-AF 700-A
Comp-PE-A
Comp-FITC-A

Figure #27. Isolation of CD3, CD4, and CD8 T cell populations.

Spleens were collected from unimmunized, irradiated L1210 immunized, and ICV immunized mice and CD3, CD4, and CD8 T cells were isolated using a negative selection kit. Isolated T cells were stained with a CD3, CD4 and CD8 antibody and flow cytometry was performed. All T cells isolations were > 90% pure. Data shown in a representative flow cytometry plot. These T cells were then used as effector cells for an IFN-gamma ELISpot assay.

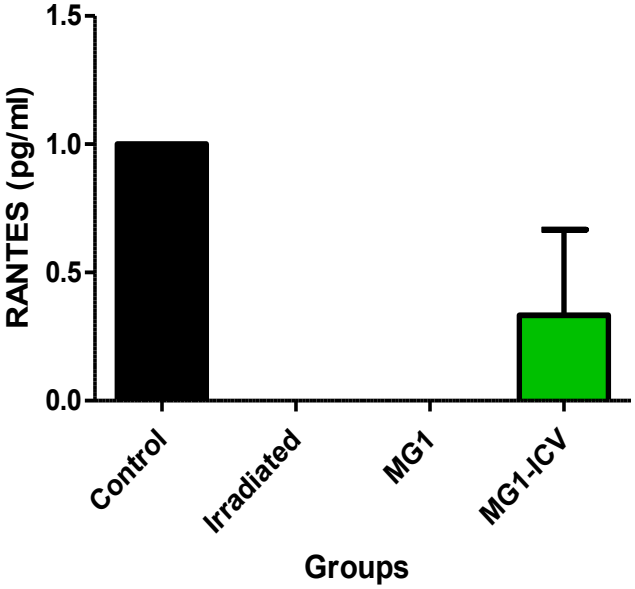
1.5
1.0
0.5
0.0
RANTES (pg/ml)
Control
Irradiated
MG1
MG1-ICV
Groups

Figure #28. C1498 cells do not secrete RANTES.

C1498 cells were plated at a density of $3.75x10^5$ cells/plate in a total volume of 7.5 ml. L1210 cells were either irradiated (30-gray), infected with MG1 virus at an MOI of 10 for 18 hours, or MG1 infected and irradiated (MG1-ICV). Zero hours post irradiation and/or infection, cells were centrifuged and supernatants were collected. 100 µl of the supernatants were analyzed for RANTES secretion using a RANTES ELISA. N=3 for all groups. ELISA performed by summer student, Leah Monette.

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