

**An Interleukin-12-Expressing Oncolytic-Virus Infected Autologous Tumor Cell Vaccine  
Generates Potent Anti-Tumor Immune Responses**

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## **Abstract**

An IL-12-expressing oncolytic virus-infected cell vaccine (MG1-IL12-ICV) can prolong survival in murine models of peritoneal carcinomatosis in an NK and CD8<sup>+</sup> T-cell dependent manner. However, MG1-IL12-ICV enhances survival but does not provide durable cures in aggressive models of established disease suggesting the presence of immunosuppressive mechanisms. Here we show that MG1-IL12-ICV can generate specific anti-tumor T-cell responses and can delay tumor growth in prophylactic models. We further demonstrate that treatment of mice bearing tumors with MG1-IL12-ICV can recruit CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and CD11c<sup>+</sup> dendritic cells into the tumor microenvironment (TME) and can increase NK cell activity. Regulatory T-cells and myeloid derived suppressor cells do not appear to play a role in immunosuppression following therapy, but checkpoint molecules are upregulated. Overall, this thesis provides strong evidence for the favorable anti-tumor immune TME induced by MG1-IL12-ICV and provides avenues that can be explored to further improve outcomes with MG1-IL12-ICV.

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## List of Abbreviations

|             |   |                                                           |
|-------------|---|-----------------------------------------------------------|
| ACVS        | - | Animal Care and Veterinary Services                       |
| ADCC        | - | Antibody-dependent cellular cytotoxicity                  |
| ALL         | - | Acute lymphoblastic leukemia                              |
| APC         | - | Antigen presenting cells                                  |
| B-cells     | - | Bursa cells                                               |
| Bcl-2       | - | B-cell lymphoma 2                                         |
| CAR-T       | - | Chimeric antigen receptor T-cells                         |
| CCL         | - | Chemokine (C-C motif) ligand                              |
| CD          | - | Cluster of differentiation                                |
| CD40L       | - | CD40 ligand                                               |
| cDCs        | - | Conventional dendritic cells                              |
| CFSE        | - | Carboxyfluorescein succinimidyl ester                     |
| COX-2       | - | Cyclooxygenase-2                                          |
| CTL         | - | Cytotoxic T-lymphocyte                                    |
| CTLA-4      | - | Cytotoxic T-lymphocyte-associated protein-4               |
| CTV         | - | Cell trace violet                                         |
| D/PAMPs     | - | Damage/pathogen-associated molecular patterns             |
| DCs         | - | Dendritic cells                                           |
| DCT         | - | Dopachrome tautomerase                                    |
| DMEM        | - | Dulbecco's Modified Eagle's Medium                        |
| FBS         | - | Fetal bovine serum                                        |
| FFPE        | - | Formalin-fixed paraffin-embedded                          |
| Foxp3       | - | Forkhead box P3                                           |
| g/m-MDSCs   | - | Granulocytic/monocytic myeloid derived suppressor cells   |
| GITR        | - | Glucocorticoid-induced TNF receptor                       |
| GM-CSF      | - | Granulocyte-macrophage stimulating factor                 |
| H&E         | - | Hematoxylin and Eosin                                     |
| HMGB-1      | - | High mobility group box-1                                 |
| HSV         | - | Herpes simplex virus                                      |
| HVEM        | - | Herpesvirus entry mediator                                |
| <i>i.p.</i> | - | <i>intraperitoneal</i>                                    |
| <i>i.v.</i> | - | <i>intravenous</i>                                        |
| ICV         | - | Infected Cell Vaccine                                     |
| IDO         | - | Indoleamine 2,3-dioxygenase                               |
| IFN         | - | Interferon                                                |
| IL          | - | Interleukin                                               |
| <i>irr</i>  | - | Irradiated                                                |
| ITAM/ITIMs  | - | Immunoreceptor tyrosine-based activation/inhibitor motifs |

|                  |   |                                                   |
|------------------|---|---------------------------------------------------|
| JAK              | - | Janus Kinase                                      |
| KIRs             | - | Killer cell immunoglobulin-like receptors         |
| LAG3             | - | Lymphocyte-activation gene 3                      |
| MDSC             | - | Myeloid derived suppressor cell                   |
| MHC              | - | Major histocompatibility complex                  |
| MOI              | - | Multiplicity of infection                         |
| MYD88            | - | Myeloid differentiation primary response 88       |
| NCR              | - | Natural cytotoxicity receptors                    |
| NK               | - | Natural killer                                    |
| NKT              | - | Natural killer T-cells                            |
| NOs              | - | Nitric oxides                                     |
| NSCLC            | - | Non-small cell lung cancer                        |
| OV               | - | Oncolytic virus                                   |
| OVA              | - | Ovalbumin                                         |
| PBS              | - | Phosphate buffered saline                         |
| PC               | - | Peritoneal carcinomatosis                         |
| PD-1             | - | Programmed death-1                                |
| PD-L1/2          | - | Programmed death ligand 1/2                       |
| pfu              | - | plaque forming unit                               |
| PGE <sub>2</sub> | - | Prostaglandin E2                                  |
| PRR              | - | Pattern recognition receptor                      |
| ROS              | - | Reactive oxygen species                           |
| RPMI             | - | Roswell Park Memorial Institute                   |
| sc.              | - | <i>subcutaneously</i>                             |
| SD               | - | Standard deviation                                |
| STAT             | - | Signal transducer and activator of transcription  |
| STING            | - | Stimulator of interferon genes                    |
| TAM              | - | Tumor-associated macrophages                      |
| TAN              | - | Tumor associated neutrophils                      |
| TCR              | - | T-cell receptors                                  |
| TGF- $\beta$     | - | Transforming growth factor- $\beta$               |
| TIM-3            | - | T-cell immunoglobulin and mucin-domain containing |
| TLR              | - | Toll-like receptor                                |
| TME              | - | Tumor microenvironment                            |
| TNF- $\alpha$    | - | Tumor necrosis factor- $\alpha$                   |
| TRAIL            | - | TNF-related apoptosis-inducing ligand             |
| T-regs           | - | Regulatory T-cells                                |
| VSV              | - | Vesicular stomatitis viurs                        |
| wt               | - | <i>wildtype</i>                                   |

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# 1. Introduction

## 1.1 Cancer

The second leading cause of death in the world, cancer affects half of all Canadians, and accounts for nearly 30% of all Canadian deaths<sup>1,2</sup>. In addition to the personal trauma of being diagnosed with and treated for cancer, it is a significant financial burden to the health care system<sup>2</sup>. With the average age of the Canadian population continuing to rise, cancer-related health costs are expected to follow<sup>2</sup>. Hence, newer therapies that prevent recurrence after treatment and reduce duration of treatment and follow-up are being sought after to reduce both cancer-associated deaths and the burden on the healthcare system.

With over a hundred different types identified, cancer is a broad term encompassing diseases that share certain characteristics (or hallmarks of cancer) including sustained proliferative signaling, resistance to cell death, evasion of growth suppressors, replicative immortality, ability to induce angiogenesis, invasion and metastasis<sup>3,4</sup>. As such, cancers can originate in a variety of organs and can result in localized or disseminated disease and may lead to secondary complications through metastasis<sup>1</sup>. Similarly, treatment for each type of cancer is different and requires emphasis on the immune niche present in the specific microenvironment of each tumor.

### 1.1.1 Peritoneal Carcinomatosis

Peritoneal carcinomatosis (PC) results from metastasis of abdominal cancers (including gastrointestinal and gynecological malignancies) throughout the lining of the peritoneal cavity, and is one of the major causes of terminal complications in these malignancies<sup>5-7</sup>. Locoregional spread of tumors throughout the abdominal cavity (without metastasis to other organs) has been shown to be one of the most common causes of death in recurrent gastric, colorectal and epithelial ovarian cancer<sup>5</sup>. Once tumor spreads throughout the peritoneal cavity, complete cytoreduction is

often not possible. Despite treatment with extensive chemotherapy and surgical debulking, presentation with PC is often considered terminal<sup>5</sup>. Therefore, novel treatments are being explored for the treatment of PC, including immunotherapies that have shown promise in the treatment of several cancers.

In the treatment of PC, adoptive transfer of T-cells, chimeric antigen receptor T-cell (CAR T-cell) therapy, dendritic cell (DC) vaccines, cytokine (e.g., interleukin (IL)-15 and 18) and antibody (e.g., catumaxomab) infusions have all demonstrated promise in pre-clinical models and early phase clinical trials<sup>7-11</sup>. They demonstrate a role for natural killer (NK) cells and Cluster of Differentiation (CD)3<sup>+</sup> T-cells in controlling PC, but also identify immunosuppressive mechanisms that hinder the efficacy of treatment<sup>10-12</sup>. For example, Katz *et al.* demonstrated that intraperitoneal (*i.p.*) infusion of CAR T-cells was more effective in controlling carcinoembryonic antigen-positive peritoneal tumors when used in combination with checkpoint inhibitors or antagonistic antibodies to regulatory T-cells (CD4<sup>+</sup> forkhead box p3 (Foxp3)<sup>+</sup>; T-reg) and myeloid derived suppressor cells (MDSCs)<sup>11</sup>. Similarly, in a CT26 peritoneal tumor model, Ma *et al.* demonstrated that IL-18 synergized with checkpoint inhibitors to enhance survival of mice bearing tumors through the accumulation of CD8<sup>+</sup> T-cells and reduction of T-regs<sup>12</sup>. By contrast, monotherapy with an oncolytic reovirus in an ovarian model of PC was itself sufficient to reduce frequency of T-regs and MDSCs within the tumor microenvironment (TME)<sup>10</sup>. These and other studies demonstrate that immunotherapy is a promising tool for the treatment of PC; but a thorough understanding of the immune TME of individual tumors over the course of therapy is required to appropriately counter any immunosuppressive mechanisms that may arise. The different components of the TME that are relevant to this project are detailed in the following section.

## 1.2 The Immune Response to Tumors

The interplay between tumors and immune cells crafts the tumor as it grows and dictates the TME, which is a delicate balance of anti and pro-tumor factors<sup>13,14</sup>. This milieu involves pro-inflammatory immune cells like NK cells, Th1 CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> T-cells, Th1 Macrophages and Bursa cells (B-cells), as well as cytokines such as interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and IL-12 and 18<sup>13-16</sup>. Pro-tumor or immunosuppressive subsets include CD4<sup>+</sup> T-regs, MDSCs, Th2 CD4<sup>+</sup> T-cells and macrophages and cytokines such as IL-4, IL-10 and transforming growth factor (TGF)- $\beta$ , as well as expression of checkpoint molecules like Programmed Death-1 (PD-1), Programmed Death Ligand 1(PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).<sup>13,14</sup>.

As the tumor grows, immune-mediated editing of the tumor landscape occurs in three stages: elimination, equilibrium, and escape. During elimination, immune-surveillance by NK and CD8<sup>+</sup> T-cells will detect (through Major Histocompatibility Complex (MHC)-I/II, Type-1 IFN response, Damage-Associated Molecular Patterns (DAMPs) or stress ligands (like MIC A/B) on tumor cells) and clear (via perforin and granzyme-B release or Fas-FasL engagement) nascent or growing tumors<sup>15,17-23</sup>.

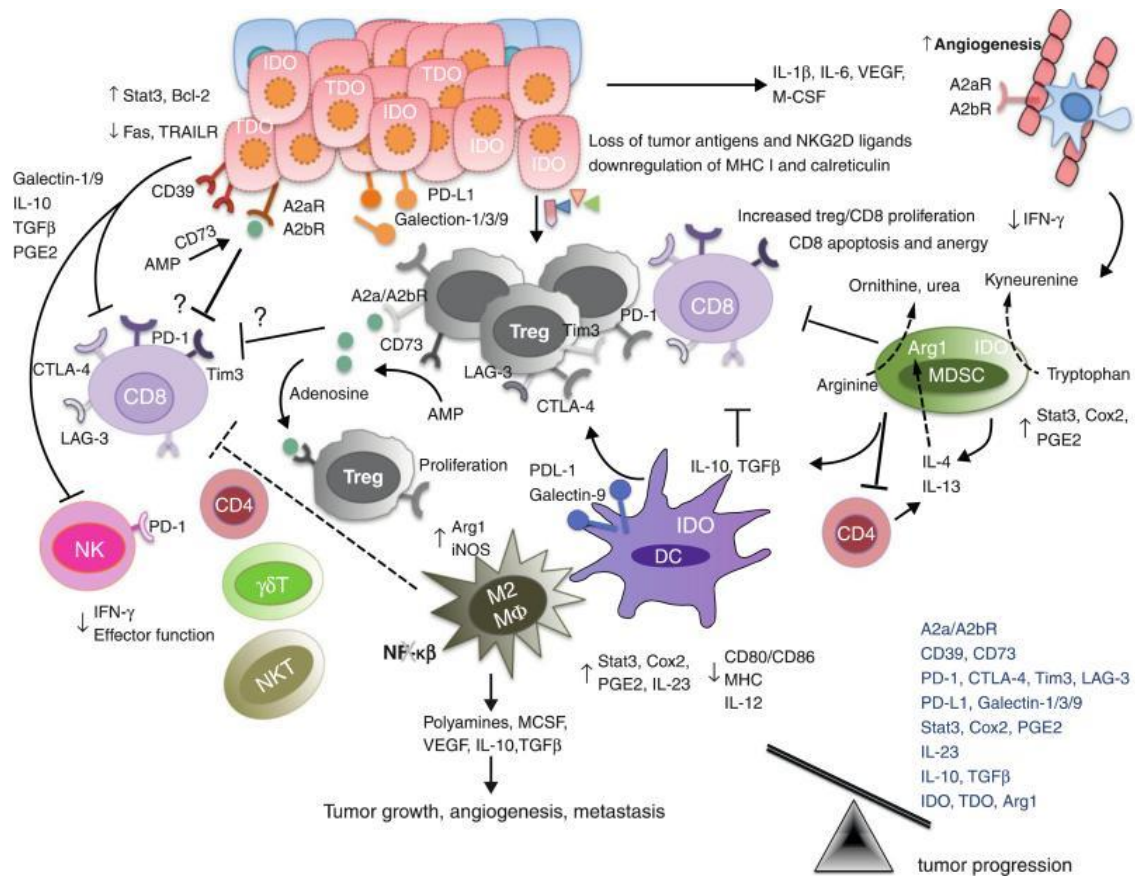
However, at this stage, some variant tumor cells may escape elimination and the tumor enters a state of immune-mediated dormancy. An adaptive immune response is critical for maintaining these tumor cells at bay, as well as IL-12 and IFN- $\gamma$  production, while immunosuppressive elements such as IL-10 and 23 prevent complete eradication of these cells<sup>15,17,18,24,25</sup>. Interestingly, NK cells and type-1 IFNs have been shown to be dispensable for maintenance of equilibrium<sup>15,25</sup>. Eventually, tumor cells will employ several mechanisms to finally escape the immune response resulting in apparent and established disease<sup>16,17</sup>. Mechanisms such as loss of MHC-I expression

(or downregulation of immunodominant epitopes), upregulation of pro-survival molecules (like B-cell Lymphoma (Bcl)-2 and Signal Transducer and Activator of Transcription (STAT)-5) as well as increase of immunosuppressive molecules (like PD-L1 and indoleamine 2,3-dioxygenase (IDO)) are employed to escape the immune response (Figure 1)<sup>15,26–29</sup>. Indirectly, the tumor may release factors (IL10, IL-23) that promote the expansion of immunosuppressive subsets that will in-turn tone down the immune response and allow tumor escape<sup>17</sup>. Most importantly, however, these same mechanisms can be and are upregulated to escape immunotherapeutic interventions<sup>18</sup>. Hence, understanding the immune interplay at this stage of tumor editing either during a normal or an engineered immune response against the tumor is important for the design of successful therapeutics.

### *1.2.1 Natural Killer Cells*

NK cells are sentinels of the innate immune system that are primarily responsible for immune-surveillance<sup>30</sup>. A plethora of germline-encoded activating and inhibitory receptors on NK cells, attached to immunoreceptor tyrosine-based activation/inhibitor motifs (ITAM/ITIMs) allow NK cells to gauge targets before triggering a cytotoxic response without any prior sensitization to their targets<sup>30–33</sup>. NK cells can recognize “self” by detecting MHC-I through Killer Cell Immunoglobulin-like receptors (KIRs) on humans and Ly49 in mice preventing harm to the host’s cells<sup>32,34–38</sup>. Interactions of activating receptors such as NKG2D and natural cytotoxicity receptors (NCRs) with their cognate ligands (for example, H60, Mult-1) as well as CD27-CD70 interactions are also involved in triggering NK cells<sup>30,39–41</sup>. NK cells are additionally involved in antibody-dependent cellular cytotoxicity (ADCC), where antibody-coated cells are recognized through FcγRIIIa (CD16), which recognizes the Fc portion of IgG1 allowing it to be involved in adaptive responses in conjunction with B or plasma cells<sup>42</sup>. Mechanistically, NK cells mediate cellular





### **Figure 1. The escape phase of immunoediting.**

Mechanisms that allow tumors to escape the immune response are detailed. These include evasion of recognition (through loss of expression of immunodominant antigens, MHC-I, calreticulin (phagocytosis signal) and stress-induced ligands), expression of molecules that increase resistance (STAT3), survival (BCL-2), immunosuppression (IDO, PD-L1, CTLA-4, CD39, etc.) and angiogenesis (IL-6, M-CSF, VEGF). Tumors will also induce recruitment and expansion of immunosuppressive cells such as MDSCs, M2 Macrophages and regulatory T-cells, which will themselves express more suppressive molecules like IDO, iNOS, Arg1, IL-10 and TGF-  $\beta$ . Overall, DC, NK and CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation, maturation and activity will be suppressed allowing tumor escape. This can be mediated through engagement of inhibitory receptors (PD-1, LAG-3, CTLA-4), expression of immunosuppressive cytokines (IL-10, TGF-  $\beta$ ), alteration of metabolic pathways (arginine metabolism) or direct apoptosis of these pro-inflammatory cells. *Adapted with copyright permission from Mittal et al. 2014.*

cytotoxicity through controlled degranulation of perforin and granzyme-B as well as the release of pro-inflammatory cytokines IFN- $\gamma$  and TNF-related apoptosis-inducing ligand (TRAIL)<sup>30,43–47</sup>.

NK cells play an important role in combating transformed cells<sup>30,42,48,49</sup>. A landmark study published in Lancet correlated inherent cytotoxicity of lymphocytes with reduced incidence of cancers in a cohort followed over 11 years<sup>50</sup>. Depletion and correlation studies have further confirmed the role of NK cells in protecting against malignant cells. Loss of MHC-I on growing tumor cells or the expression of stress-induced ligands can trigger NK cells into action<sup>44,49,51,52</sup>. Additionally, expression of p53 in early senescent tumors induces the release of NK-cell recruiting and activating chemo/cytokines (chemokine (C-C motif) ligand (CCL)-2, IL-12) facilitating NK cell-mediated killing of tumors in the early (elimination) phase of cancer growth<sup>15,53</sup>. Furthermore, release of IL-12 and IL-18 by DCs and IL-2 by T-cells facilitate NK cell maturation and activity<sup>30,54–58</sup>. However, a switch to an immunosuppressive environment through the recruitment of MDSCs, T-regs, increased expression of checkpoint molecules (PD-1, T-cell immunoglobulin and mucin-domain containing (TIM-3)) and release of suppressive molecules like TGF- $\beta$  and IDO can lead to failure of NK cell responses and allow cancer escape<sup>31,32,59–64</sup>.

Their intrinsic capacity to combat tumor cells without the necessity for prior sensitization have made NK cells a very lucrative target for immunotherapy. Additionally, an efficient innate response from NK cells has been shown to be necessary for the generation and activity of DCs that eventually prime an efficient T-cell response, which is required for immunotherapeutic success<sup>56,65</sup>. In fact, NK cell activation has been shown to be involved in the success of several therapies including that of oncolytic virotherapy, and newer therapies to further NK cell function and relieve NK cell immunosuppression are being investigated in addition to therapies that promote adaptive immune memory<sup>44,49,66–68</sup>.

### 1.2.2 Dendritic Cells

DCs are the bridge between innate and adaptive immune responses. DCs are professional antigen presenting cells (APCs) responsible for generating primary adaptive responses that eventually lead to generation of memory<sup>69-71</sup>. DCs take up and process antigens upon encounter of damaged, infected, or transformed cells, and undergo maturation as they migrate to lymphoid structures. The uptake of antigen is promoted by NK-cell mediated killing of target cells that generate apoptotic blebs, which are taken up readily by infiltrating DCs<sup>30,72</sup>. Along with availability of antigens, presence of damage and pathogen-associated molecular patterns (D/PAMPs) and other adjuvanticity-increasing signals (like High Mobility Group Box-1 (HMGB-1)-mediated induction of Toll-Like Receptor (TLR)-4) is required for efficient recruitment of, phagocytosis by and activation of DCs<sup>73-76</sup>

At lymph nodes, mature DCs will present cognate antigens on MHC-I and MHC-II to T-cell receptors (TCR) on CD8<sup>+</sup> and CD4<sup>+</sup> T-cells respectively to induce clonal expansion of antigen-specific T-cells<sup>77-79</sup>. In addition to antigen presentation, engagement of co-stimulatory CD28 on T-cells by CD80/86 on DCs is required for generation and egress of protective anti-tumor T-cells from lymphoid structures<sup>70,78</sup>. Expression of co-stimulatory CD80/86 on DCs and other APCs is dependent on the signals received by the APC during priming or initial antigen encounter. For example, engagement of CD40 on the surface of DCs by CD40 ligand (CD40L) expressed on CD4<sup>+</sup> T-cells will induce increased expression of CD80/86 and release of copious amounts of immunostimulatory IL-12. This will in turn prime a robust NK and T-cell immune response<sup>69,70,78,80</sup>. Alternatively, an immunosuppressive environment may induce increased STAT3 signaling in DCs to induce tolerogenic DCs that will lead to expansion of regulatory rather than conventional T-cells<sup>81,82</sup>.

### 1.2.3 CD8<sup>+</sup> T-cells

Effector CD8<sup>+</sup> cytotoxic T-cells play a major role in the clearance of infected or transformed cells<sup>78,83</sup>. A major component of immunological memory, CD8<sup>+</sup> T-cells recognize foreign antigens in MHC-I complexes on target cells via the TCR and induce apoptosis<sup>48</sup>.

However, naïve T-cells reside in lymphoid organs, and to induce effector CD8<sup>+</sup> T-cell responses, presentation of cognate antigen to the TCR by DCs is required<sup>48,84–86</sup>. This induces clonal expansion of previously naïve CD8<sup>+</sup> T-cells, which will then migrate to sites of infection or the tumor to kill cells presenting the target antigen on their surface MHC-I<sup>48,78</sup>. During this expansion CD8<sup>+</sup> T-cells can proliferate every 4-6 hours (for up to 20 divisions during an acute infection)<sup>87,88</sup>. CD8<sup>+</sup> T-cells induce apoptosis via production and localized release of perforin and granzymes or via the Fas-FasL axis, eventually activating the caspase cascade in target cells<sup>43,48</sup>. Additionally, CD8<sup>+</sup> T-cells will produce cytokines to promote host defense, particularly IFN- $\gamma$ , which stimulates both innate and adaptive immune responses<sup>48,89</sup>. The extent of expansion and duration of a CD8<sup>+</sup> T-cell response is controlled by antigen exposure and usually subsides following pathogen clearance<sup>87,90</sup>. However, during chronic diseases continuous antigen-driven expansion is not observed mainly due to functional exhaustion and deletion<sup>87,91,92</sup>. After mounting a response, the CD8<sup>+</sup> T-cell population will undergo contraction, with 95% of cells being eliminated. The small portion of cells that survive then form the memory pool of T-cells and are maintained through homeostatic proliferation by IL-7 and IL-15<sup>84,93–95</sup>.

The generation of specific anti-tumor CD8<sup>+</sup> T-cell immune response is considered an important target for the efficacy of immunotherapies, and hence increased efforts to enhance DC activity and priming of tumor-specific T-cells have been made. In fact, several immunotherapies that have

shown promise in clinical trials facilitate the generation of such a response and a favorable T-cell infiltrate is correlated with improved outcomes<sup>96–100</sup>.

Overall, three major steps have been identified for effective cancer vaccination. First, uptake of immunogenic cancer-specific antigens by DCs must be facilitated by enhancing *in-vivo* uptake or by delivering antigens as part of a therapeutic vaccine such as antigen-pulsed DC vaccines<sup>78</sup>. This uptake must be accompanied with suitable co-stimulatory signals that may be delivered exogenously such as CD40 agonists or immunogenic cell death inducers<sup>78</sup>. This ensures that priming of CD8<sup>+</sup> T-cells in the lymph nodes generates effector T-cell responses and does not promote tolerance via regulatory T-cell induction<sup>78,82,101</sup>. And finally, entry of the generated T-cell response into the TME must be facilitated and approaches to overcome immunosuppressive factors in the TME must be employed<sup>78</sup>.

### **1.3 Immunosuppression in the TME**

Immunosuppression in the TME is orchestrated by a vast network of cells and cytokines. These include: i) regulatory CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> T-cells, ii) regulatory B-cells that induce T-reg proliferation, iii) natural killer T-cells (NKT), iv) tumor-associated macrophages (TAMs) and neutrophils (TANs), v) MDSCs, vi) release of factors like IL-1, IL-6, IL-10, IL-13, reactive oxygen species (ROS), nitric oxides (NOs) and TGF- $\beta$  and vii) upregulation of immune checkpoint molecules<sup>31,61,102–111</sup>. While an exhaustive review of all immunosuppressive subsets is beyond the scope of this project, the major factors explored in this study are detailed below.

#### ***1.3.1 Myeloid Derived Suppressor Cells***

MDSCs are a diverse set of immature myeloid cells that can be induced by emergency myelopoiesis, IL-4, IL-6, Granulocyte-macrophage stimulating factor (GM-CSF), prostaglandins (PGEs) and cyclooxygenase-2 (COX-2)<sup>31,112</sup>. While characterization of these subsets in humans is

still an evolving subject, in murine models, two major subsets have been identified: Granulocytic MDSCs and Monocytic MDSCs (g/m-MDSCs)<sup>113</sup>. While both subsets are CD11b<sup>+</sup> Gr-1<sup>hi</sup>, they are differentiated based on their expression levels of Ly6C/G<sup>113</sup>. Additionally, g-MDSCs primarily mediate immunosuppression through production of ROS, while m-MDSCs release arginase-1 and NOs<sup>102</sup>. MDSCs have also been shown to directly inhibit NK cell function through membrane-bound TGF- $\beta$ , and indirectly through the production of IL-10, which in turn downregulates IL-12 production by macrophages in a tumor niche<sup>106,110,114</sup>. MDSCs also play a role in suppressing T-cell responses and have been shown to induce T-regs<sup>112,115,116</sup>. Overall, increased MDSCs facilitate tumor escape in the final stage of tumor-editing by suppressing all cytolytic arms of the immune response<sup>15</sup>.

### *1.3.2 CD4<sup>+</sup> Regulatory T-cells*

Another subset involved in tumor escape is CD4<sup>+</sup> Foxp3<sup>+</sup> T-regs, presence of which has a negative impact on overall survival in patients<sup>117–120</sup>. Defined by their cardinal expression of the Foxp3 transcription factor, regulatory CD4<sup>+</sup> T-cells play a major role in suppressing autoreactive T-cells<sup>121</sup>. While natural regulatory T-cells develop in the thymus, immunosuppressive IL-10 and TGF- $\beta$  can also induce T-regs in the periphery or tumor niche<sup>122,123</sup>. In addition, expression of the high affinity IL-2 receptor (CD25) is considered an additional marker for T-regs, with constitutive activation of downstream STAT5 associated with functional fitness of these cells<sup>124</sup>. T-regs suppress all subsets in the TME including T-cells and DCs<sup>125</sup>. Inhibition of immune responses is mediated through expression of CTLA-4, glucocorticoid-induced TNF receptor (GITR), lymphocyte-activation gene 3 (LAG3), IL-10 and IL-35. T-regs downregulate activating co-stimulatory molecules on DCs through CTLA-4 interactions, directly induce death of

conventional T-cells through granzyme-B and perforin-mediated lysis, and suppress NK cells through TGF- $\beta$ <sup>61,126–128</sup>.

### *1.3.3 PD1-PDL1 and other Checkpoint Molecules*

In addition to TCR-MHC-I interactions, T-cell activation and priming is regulated by a secondary set of signals mediated by interactions of co-stimulatory and inhibitory ligands<sup>129</sup>. The latter consist of signaling through checkpoint molecules (like PD-1, PD-L1/2, CTLA-4, LAG-3, TIM-3) and are necessary for maintaining self-tolerance, preventing autoimmunity and damage to tissue from an over-activated immune response<sup>130</sup>. In the TME, however, checkpoint upregulation is a tumor escape mechanism and checkpoint blockade has proven to be a successful immunotherapeutic option<sup>15,130–133</sup>. While different checkpoint molecules induce immune suppression through different signaling pathways, an overall attenuation of the cytotoxic response is observed upon engagement of checkpoint receptors.

In particular, engagement of PD-1 on T-cells by its ligand PD-L1 recruits SHP2 phosphatases to attenuate CD28 signaling and also prevents TCR mediated Zap70 signaling inhibiting T-cell proliferation and activation<sup>130,134,135</sup>. Additionally, PD-L1 engagement with PD-1 on regulatory T-cells augments their suppressive capacity<sup>136</sup>. The alternate ligand for PD-1, PD-L2 plays a similar role in tumor escape by inducing suppression of T-cell activation<sup>137</sup>. PD-L1 is expressed constitutively on a variety of tumors, but can also be upregulated on tumors and immune cells in the TME (like Macrophages, DCs, MDSCs and T-cells) through IFN- $\gamma$ -induced Janus Kinase(JAK)-STAT signaling pathway<sup>130,138</sup>. In fact, correlation of PD-L1 expression with the extent of tumor infiltration by lymphocytes suggests a feedback loop, where a pro-inflammatory (IFN- $\gamma$ ) response induces upregulation of suppressive PD-L1 to attenuate the incoming immune response<sup>139</sup>.



## 1.4 Cancer Immunotherapy

The harnessing of the patient or host's own immune system to combat cancer through immunomodulating agents is termed "cancer immunotherapy"<sup>97,140</sup>. To overcome tumor-mediated immune escape, immunotherapies must either promote a robust boost to the faltering immune response or counteract the immunosuppression prevalent in the TME<sup>97</sup>. Successes of a myriad of immunotherapeutic approaches made cancer immunotherapy the scientific breakthrough of the year 2013<sup>141</sup>. Different approaches that have shown success in clinical trials include whole-cell based vaccines (Sipuleucel-T for the treatment of prostate cancer), checkpoint-blockade therapy (ipilimumab (anti-CTLA-4) and pembrolizumab (anti-PD1) for the treatment of melanoma and non-small cell lung cancer (NSCLC), oncolytic virotherapy (Herpes simplex virus (HSV)-1 expressing GM-CSF for the treatment of melanoma), adoptive cellular therapy (for melanoma) and CAR T-cells (for the treatment of acute lymphoblastic leukemia)<sup>97,140,142–146</sup>. Attempts to enhance the outcome of the above therapies and the possibility of combination therapies are actively being explored. Of relevance to this project are oncolytic virotherapy, cytokine therapy and whole cell tumor vaccines.

### 1.4.1 Oncolytic Virotherapy

Oncolytic viruses (OVs) are naturally found or engineered viruses that selectively replicate in and kill tumor cells without affecting otherwise healthy cells. Additionally, they induce a robust anti-viral immune response that may result in direct or indirect immune-mediated killing of cancer cells<sup>147–149</sup>. Following OV infection, enhanced inflammatory responses through engagement of TLRs and induction of immunogenic cell death (through release of HMGB-1 and ATP) synergizes with release of tumor-associated antigens upon viral cytolysis to activate and drive robust innate (NK and DC) and adaptive (T-cells) responses<sup>147,148,150–152</sup>.

OVs can specifically target cancer cells by utilizing surface receptors specifically or highly expressed on neoplastic cells and/or by exploiting defective anti-viral innate signaling in tumor cells<sup>147–149</sup>. Some viruses intrinsically utilize receptors that are highly expressed on tumor cells allowing them to specifically infect cancers, such as Measles virus which uses CD46, HSV-1, which uses herpesvirus entry mediator (HVEM) and poliovirus, which uses CD155<sup>147,150,153,154</sup>. Viruses can also be engineered or pseudo-typed to exploit receptors upregulated on cancer cells. Alternatively, some viruses exploit intracellular pathways for selectivity. For example, Newcastle Disease Virus specifically replicates in cells overexpressing the anti-apoptotic protein BCL-X<sub>L</sub>, and reovirus and vaccinia exploit enhanced Ras signaling<sup>147,155–157</sup>. Finally, defects in anti-viral type-I IFN signaling observed in many cancers provides a replicative advantage to certain OVs; while others can be engineered to become more susceptible to intact Type-I responses to enhance their safety profile in normal cells<sup>147,158</sup>.

One such virus that is attenuated to be susceptible to Type-I IFN responses, is the Maraba MG-1 virus, which is one of several viruses that have entered clinical trials following the success of Talimogene laherparepvec (HSV-1 expressing GM-CSF).

#### *1.4.1.1 Maraba MG1*

Originally isolated from Brazilian sandflies, the negative-stranded RNA rhabdovirus, Maraba has potent oncolytic properties<sup>159</sup>. Maraba is non-pathogenic in humans and serological response has been observed in less than 1% of humans tested<sup>160,161</sup>. In a comparison of several rhabdoviruses, Maraba demonstrated enhanced tumor cytotoxicity, rapid progeny production, and larger burst sizes: all properties that identified it as a potent oncolytic candidate<sup>159</sup>. Introducing mutations into the Maraba genome identified the MG1 variant consisting of mutations in the G (Glycoprotein) (Q242R) and M (Matrix) proteins (L123W) as a more potent oncolytic than wildtype (wt) Maraba.

The M protein of Maraba and other related rhabdoviruses are involved in blocking IFN- $\beta$  mRNA transport from the nucleus to the cytoplasm, thereby preventing the anti-viral IFN- $\beta$  cascade in infected cells<sup>159,162</sup>. The L123W mutation renders the M protein incapable of interfering with the IFN- $\beta$  response, making MG1 more susceptible to intact type-1 IFN responses found in healthy cells and consequently makes MG1 selective for type-1 IFN-deficient cancer cells. This was similar to effects previously observed with a related rhabdovirus Vesicular Stomatitis Virus (VSV), where introducing a deletion in the M protein at position 51 (VSV $\Delta$ 51) made the mutant strain sensitive to interferon signaling and therefore selective for interferon-signaling-deficient cancers. The Q242R mutation also attenuates the virus in healthy cells but the exact mechanisms are not fully understood. Interestingly, although these individual mutations did not enhance Maraba's ability to kill cancer cells, the combination of these mutations (as in MG1), made Maraba more cytolytic to cancer cells while maintaining its safety profile in healthy cells<sup>159</sup>. Similarly, *in-vivo* MG-1 has a higher maximum tolerable dose than *wt* Maraba, and treatment with MG1 can control tumor growth in athymic mice demonstrating its tumor-cytolytic properties<sup>159</sup>. In addition to its cytolytic potential, subsequent studies demonstrated the ability of MG1 to activate NK cells *in-vivo* through activation of conventional DCs, furthering its promise as a potent OV<sup>67</sup>. In this study, Zhang *et al.* showed that MG1 was effective in reducing lung metastasis in a B16lacZ model. They demonstrated that this was mediated through NK cell activation via conventional DCs (cDCs) and not through direct viral cytolysis, as an UV-inactivated replication-deficient MG1 was equally capable of reducing metastasis. Direct infection of cDC was shown to activate them to subsequently prime an NK cell response and depletion of DCs and NK cells abrogated the efficacy of MG1. They also demonstrated that structural integrity of the virus was necessary for this activation as longer UV inactivation of MG1 that resulted in structural damage did not provide

efficacy. This identified MG1 as a potent immune stimulator. Subsequently, in a prime-boost vaccination strategy, inclusion of tumor-peptide-encoding transgenes into MG1 demonstrated its ability to act as a potent boost to an adenovirus (encoding the same tumor peptide) prime, inducing robust specific CD8<sup>+</sup> T-cell responses that corresponded to tumor control<sup>163</sup>. Pol *et al.* demonstrated that MG1 encoding dopachrome tautomerase (DCT) by itself was insufficient to prime a T-cell response but was effective in expanding pre-existing DCT-responses generated by an adenovirus expressing DCT, and this was effective in controlling tumor growth. This demonstrated that MG1-based vectors maybe ideal for therapies requiring rapid expansion of pre-existing CD8<sup>+</sup> T-cell responses.

Overall, given its improved safety profile and potent oncolytic and immune-stimulating properties, the MG1 virus has already entered early phase clinical trials for solid tumors including melanoma and NSLC<sup>164,165</sup>. However, despite its apparent success, further avenues for improving the efficacy of MG1 are being explored. In particular, delivery in conjunction with whole cell tumor vaccines and the inclusion of an IL-12 expressing transgene has proven to enhance anti-tumor properties of MG1-based therapies<sup>66,166</sup>.

#### 1.4.2 Whole Cell Tumor Vaccines

One method of priming adaptive anti-tumor responses involves the use of tumor-derived peptides as target antigens, delivered alongside suitable adjuvants<sup>78,167</sup>. However, high mutation rates in tumors may lead to downregulation of these antigens or prevent presentation of these antigens on MHC-I<sup>78</sup>. Furthermore, the need to target more than one neoantigen for effective cancer therapy has been identified, and move towards more personalized cancer vaccines are being made<sup>78,167,168</sup>. Successful immunotherapy is often associated with T-cell response to mutated tumor epitopes in both murine and clinical settings<sup>169–172</sup>. While, advances in next-generation sequencing techniques

has allowed identification of these mutations, more than 95% of these mutations are unique to each individual, making it difficult to tailor therapy suitable for a broad range of patients<sup>172</sup>. To overcome this barrier, whole cell tumor vaccines or tumor lysates have been used to provide an increased repertoire of antigens, resulting in increased efficacy<sup>173</sup>. To achieve this, autologous tumor cells are treated to render them non-proliferative via rapid freeze-thaw cycles, heat-shock or irradiation or lysed to produce tumor lysates<sup>174,175</sup>. The use of whole tumor vaccines presents the entire repertoire of antigens (including mutated neoantigens) of the patient's tumors and precludes the need for identification or discovery of common antigens between cancer types<sup>176</sup>. An analysis of 173 published trials covering 3444 patients revealed that patients receiving whole tumor or tumor lysate-based vaccines had objective responses of 8.1% (138 of 1711), compared to only 3.6% (63 of 1733) in those receiving specific antigen-based therapy<sup>173</sup>. Interestingly, lack of difference between the use of autologous versus allogeneic cell lines suggested an increased role for epitope spreading in the success of these therapies over antigen-specific therapies (it is suggested that the chances of inducing epitope spreading may be higher with a larger initial multiclonal T-cell response)<sup>173,177</sup>. However, it is worth noting that this meta-analysis was conducted on a broad range of different therapeutics of varying levels of immunogenicity. Alternatively, the case against the use of allogeneic tumor cells may be made by the failure of the GM-CSF secreting allogeneic whole cell vaccine, GVAX, in obtaining clinical efficacy in phase III trials for prostate cancer, despite early promise<sup>178</sup>. Lack of sufficient relevant antigens in the allogeneic cell line may be attributed for this failure, but lack of sufficient adjuvanticity of the vaccine formulation itself or issues during commercial scale-up of vaccine production may also be alternative explanations<sup>78</sup>. To overcome issues with adjuvanticity, whole cell vaccines maybe combined with other immunotherapies. For example, treatment with a heterologous prime-boost

regimen consisting of GVAX (with chemotherapy to deplete T-regs) followed by boost with *Listeria monocytogenes* expressing a specific tumor antigen (mesothelin) enhanced overall survival of patients with pancreatic adenocarcinoma compared to patients who just received GVAX and chemotherapy alone<sup>179</sup>. The lack of efficacy of monotherapy with GVAX compared to when used in combination with other therapies suggests that more potent combinations (such as the prime-boost strategy) maybe required to fully avail the antigen repertoire presented by autologous whole cell vaccines (whether allogeneic or autologous).

One possibility is combination with OV therapy, which appears to further potentiate whole cell vaccine therapy, with viral oncolysis increasing presentation of antigens and eliciting a stronger immune response than either therapy alone<sup>66,166,180–182</sup>. In murine models, autologous whole cell vaccines infected with MG1 or VSVΔ51 has shown promise in treating acute lymphoblastic leukemia (ALL) and melanoma, and vaccines infected with an IL-12 expressing MG1 has shown promise in treating PC<sup>66,166</sup>.

#### 1.4.3 Infected Cell Vaccines

That immunogenicity of whole cell vaccines can be enhanced by *ex-vivo* infection with OVs was first observed in models involving oncolytic strains of the influenza A virus as early as 1967<sup>183</sup>. Since then several studies have demonstrated improved efficacy of whole cell vaccines infected with OVs in different models of cancer<sup>66,166,182,184</sup>. Particularly, our group has demonstrated the enhanced efficacy of rhabdovirus-infected cell vaccines (ICV). Briefly, ICVs are prepared by infecting whole tumor cells *ex-vivo* with an OV before or after irradiation prior to delivery as a therapy.

In a solid tumor model of B16F10 melanoma, Lemay *et al.* demonstrated that prophylactic vaccination with a VSVΔ51-ICV was effective in preventing tumor growth in 30% of subsequently

challenged mice compared to irradiated (*irr*) whole cells alone <sup>182</sup>. B16F10 tumors are refractory to VSVΔ51 treatment unlike other more permissive tumor cell lines due to poor *in-vivo* infectivity. However, upon *ex-vivo* infection, as during the preparation of an ICV, high levels of infection are observed. This *ex-vivo* infection was shown to be required for efficacy of the ICV but subsequent replication beyond that during the preparation of ICV was shown to be redundant (a replication deficient VSVΔ51 was equally effective). This suggested that the ICV platform maybe ideal for use in models where OV therapy alone maybe ineffective. Similarly, Conrad *et al.* demonstrated that in murine models of ALL, mice vaccinated with whole cell ALL vaccines infected *ex-vivo* with MG1 was effective in protecting over 90% of mice from subsequent tumor challenge, suggesting the generation of adaptive responses as observed by Lemay *et al.*<sup>166</sup>. They further demonstrated that *irr* tumor cells when delivered alone or in conjunction with MG1 (mixed with cells or separately) without *ex-vivo* infection was not effective, further delineating that *ex-vivo* infection is critical for the efficacy of ICVs. Both studies also demonstrated the need for cellular integrity as infected apoptotic or necrotic cells were ineffective in providing protection.

Interestingly, Lemay *et al.* observed enhanced efficacy when a VSVΔ51 expressing GM-CSF, an immune potentiating cytokine, was used to prepare the ICV (VSVgm-ICV), resulting in protection of over 95% of mice challenged with B16F10 tumors (compared to 30% without GM-CSF). Treatment with VSVgm-ICV lead to enhanced activation of DCs (CD86 expression) and NK and T-cells (CD69 expression) as early as 24 hours post-treatment and enhanced infiltration of activated NK and T-cells were observed when tumors were implanted in a Matrigel in vaccinated mice. This suggested that inclusion of immune-potentiating transgenes into the rhabdovirus may enhance the efficacy of ICVs in more difficult-to-treat models.

Similarly, Dr. Auer's laboratory has developed an MG1 virus expressing the immune stimulating IL-12 cytokine, which when used in an ICV platform has been shown to be highly effective in models of PC, conferring better protection and tumor control than an ICV prepared with the parental MG1 virus<sup>66</sup>.

#### *1.4.4 Interleukin-12*

The potent immune stimulating cytokine, IL-12 bridges the innate and adaptive arms of the immune response and is a key mediator of responses against infections or cancer<sup>185</sup>. In a normal response to infected or transformed cells, IL-12 is produced by APCs following engagement of TLRs<sup>186–188</sup>. Successful recognition of IL-12 via the IL-12R $\beta$ 1 and  $\beta$ 2 receptors will then activate NK and T-cells by triggering the canonical JAK-STAT-4 pathway<sup>185,189,190</sup>. Subsequent enhanced proliferation and upregulation of cytotoxic granules like perforin and granzyme-B in NK and T-cells is accompanied by increased IFN- $\gamma$  production, which further stimulates APCs to upregulate IL-12 production in a positive feedback loop<sup>186,191,192</sup>.

IL-12 belongs to a family of cytokines that also includes IL-23, 27 and 35<sup>193–195</sup>. The only heterodimeric cytokine family, all members of the IL-12 family consist of two covalently bound subunits: a helical  $\alpha$ -subunit, consisting of four helix bundles like those found in other type 1-cytokines, and a  $\beta$ -subunit that is structurally similar to the extracellular regions of type-1 cytokine receptors (such as the IL-6 receptor  $\alpha$ -chain and the ciliary neurotrophic factor receptor)<sup>186,193–199</sup>. In particular, IL-12 consists of IL-12p35 (35kDa) and IL-12p40 (40kDa) subunits, which are linked by a disulfide bond during production resulting in the release of the bioactive IL-12p70 cytokine<sup>190,194–196</sup>. Interestingly p35 subunit transcripts have been observed in several cell types, but the p40 subunit is only produced by monocytes, macrophages, neutrophils and DCs, and therefore bioactive IL-12p70 is only produced by these cell types<sup>193,197,200</sup>. While p35 cannot be



secreted alone, secreted p40 homodimers have been shown to antagonize IL-12p70 activity by competitively binding to IL-12R $\beta$ 1 subunit<sup>186,201</sup>. However, p40 (also a component of IL-23) itself can also act as a chemoattractant for macrophages and has been shown to be necessary for migration of activated DCs (for T-cell priming) during *Mycobacterium tuberculosis* infection<sup>202</sup>. IL-12 production by DCs/Macrophages is initiated by engagement of TLRs 4,7 or 8 and production can be amplified following secondary signals through cytokines such as IL1 $\beta$  or IFN- $\gamma$  and via direct cell-to-cell contact such as CD40-CD40L interactions<sup>186,190,203,204</sup>. On the other hand, IL-12 production can be suppressed by type I IFNs, IL-10, TGF- $\beta$ , PGE<sub>2</sub> as well as TIM-3 and CTLA-4 interactions<sup>59,186,190,205–207</sup>.

Once IL-12 is released, binding of p40 to IL-12R $\beta$ 1 initiates Tyrosine kinase-2 signaling and p35 to IL-12R $\beta$ 2 initiates JAK-2 signaling in target cells, both of which eventually lead to downstream phosphorylation and activation of STAT4<sup>189,193</sup>. As described before, this will then stimulate NK and T-cells, upregulating their cytotoxic potential and IFN- $\gamma$  production<sup>186,190,191</sup>. Additionally, IL-12 promotes differentiation of CD4<sup>+</sup> into a Th1 phenotype, promotes IgG production from B-cells and augments NK cell ADCC which can synergize to eradicate tumors<sup>186,190,208,209</sup>. Although IL-12 does not directly inhibit tumor growth, it induces release of IP-10, which has anti-angiogenic effects (as well as chemotactic properties). IL-12 also leads to tumor stroma destruction by inducing increased Fas expression on stromal cells, which can then be eliminated by infiltrating FasL-expressing T-cells<sup>186,210,211</sup>. Overall, IL-12 can induce a proinflammatory anti-tumor immune TME leading to immune-mediated control of tumor growth<sup>186</sup>.

However, despite the potential of IL-12 and successes in animal models, systemic administration in various clinical trials led to disappointing results due to significant toxicities<sup>186</sup>. In addition, systemic repeated administration was countered by enhanced IL-10 production and a highly

suppressive human TME<sup>186,212</sup>. Since then, a better understanding of IL-12 therapy has been made and newer clinical trials with IL-12 have been initiated. Focus has shifted towards controlled localized or intratumoral delivery of IL-12 or use of IL-12 as a low dose adjuvant to other more targeted therapies such as whole cell or DC vaccines<sup>186,213</sup>. Localized delivery can be achieved via genetic methods such as delivery of inducible plasmids coding for IL-12 or adoptive cell therapy with cells containing the IL-12 gene under inducible promoters<sup>186,214–216</sup>. Alternatively, IL-12 can be encoded in viral vectors to promote localized and controlled but prolonged expression of IL-12. In fact, adenovirus- and HSV-expressing IL-12 therapies are already in clinical trials<sup>180,186,217,218</sup>.

#### 1.4.5 MG1-IL12-ICV

MG1-IL12-ICV is a promising new immunotherapy developed by our group that has shown success in curing murine models of PC<sup>66</sup>. MG1-IL12-ICV combines some of the promising aspects of the above-discussed immunotherapeutic options into one cocktail. Briefly, the MG1-IL12-ICV platform utilizes *irr* autologous whole tumor cells infected with the IL-12 expressing MG-1 virus to stimulate immune mediated destruction of tumors<sup>66</sup>.

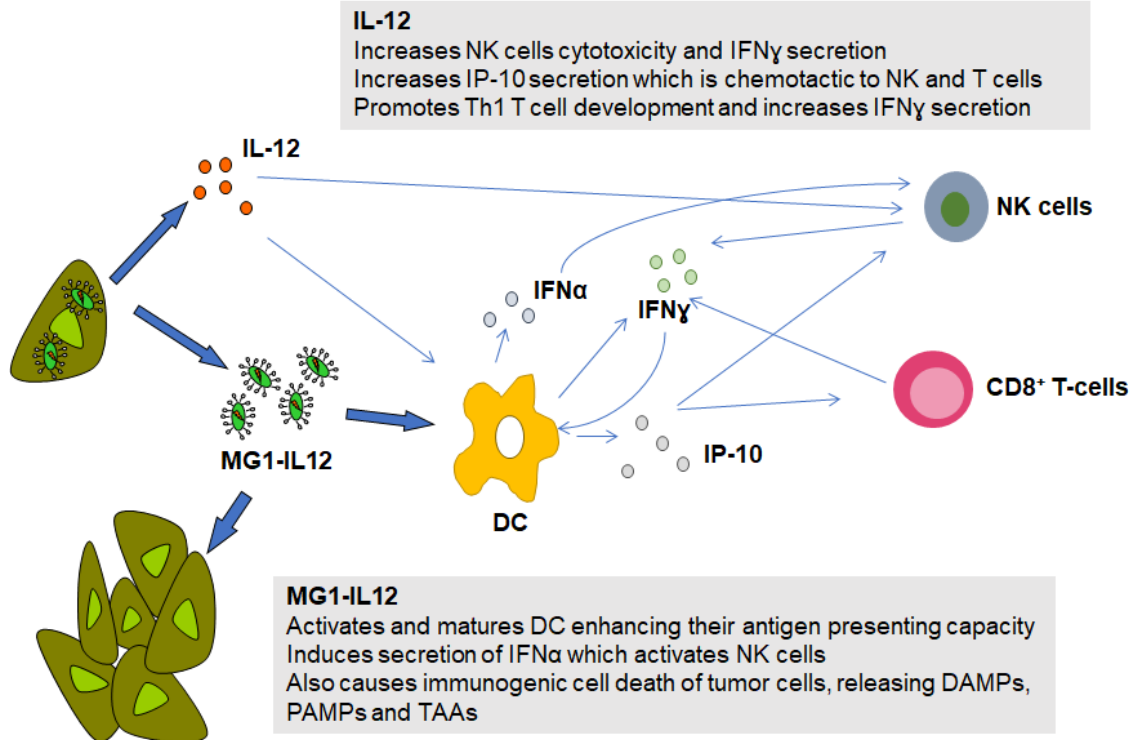
Previous reports from our group have described the design and validation of MG1-IL12<sup>66,219</sup>. Briefly, murine IL12 (p35 and p40 subunits connected by an elastin linker) was PCR amplified from pORF-mIL12 (IL12elasti (p35::p40); InvivoGen, SanDiego, CA) to introduce MluI (5') and (3') cloning sites, which was then inserted into the Maraba MG1 backbone between genes coding for the G and L proteins. After rescue, the recombinant MG1-IL12 virus was demonstrated to successfully infect and replicate in tumor cell lines and produced IL-12 upon replication.

In a colon cancer model of PC, Alkayyal *et al.* demonstrated that MG1-IL12-ICV treatment (delivered intraperitoneally) was able to completely eradicate established CT26lacZ peritoneal

tumors, resulting in complete cures in over 90% of mice. This was more effective than treatment with *irr* cells, MG1-ICV, MG-1 or MG1-IL12 alone, MG1-ICV delivered in conjunction with recombinant IL-12, or an MG1-IL12-ICV prepared with a replication deficient MG1-IL12. This suggested that delivery of a replication-competent MG1-IL12 in the ICV platform was necessary to cure PC models of established disease. Mice cured of peritoneal disease in the CT26lacZ model developed long-lasting adaptive immunity and rejected subsequent challenge with CT26lacZ tumors.

Mechanistically, Alkayyal *et al.* demonstrated that intravenous (*i.v.*) delivery of MG1-IL12-ICV increased IL-12 and IFN- $\gamma$  production in lung homogenates and enhanced recruitment of NK cells (but not T-cells) to the lung<sup>66</sup>. Splenic NK cells from the MG1-IL12-ICV treated mice were also more effective at killing target YAC-1 cells *ex-vivo*, suggesting that NK cells are primary mediators of MG1-IL12-ICV efficacy. Subsequently, in a B16F10 lung metastasis model, reduced lung metastases were observed in MG1-IL12-ICV vaccinated mice compared to mice receiving *irr* cells or MG1-ICV. Similarly, in a PC model of B16F10 tumors, enhanced recruitment of activated NK cells to the peritoneal cavity was observed, and these cells were able to preferentially reject RMA-S tumor cells in an RMA/RMA-S *in-vivo* cytotoxicity assay. Subsequent studies revealed that NK cell activation and recruitment was, in part, mediated by the release of IP-10 from DCs upon treatment with MG1-IL12-ICV. Finally, although these results indicated that NK cells maybe the primary drivers of ICV efficacy, depletion of CD8<sup>+</sup> T-cells also abrogated the efficacy of MG1-IL12-ICV, suggesting a crucial role for adaptive immunity that is yet to be fully elucidated.

A schematic of the known mechanisms of action of MG1-IL12-ICV is shown in Figure 2.



## **Figure 2 Proposed mechanisms of action of MG1-IL12-ICV.**

Upon delivery MG1-IL12-ICV produces MG1-IL12 viral particles, IL-12 and releases tumor-associated antigens. IL-12 increases NK cell activity and IFN- $\gamma$  production, and primes DCs to upregulate IP-10 production, which is a chemoattractant for both NK and T-cells. IL-12 may also induce a Th1 phenotype in CD4 T-cells. MG1-IL12 released from the ICV will also infect tumor cells and DCs *in-vivo*. This activates DCs and induces release of IFN $\alpha$ , which will further prime NK cells. Finally, viral infection of tumors will result in immunogenic cell death induced by viral cytolysis and immune-mediated destruction. Subsequent release of D/PAMPs and tumor-associated antigens will further drive the innate and adaptive immune responses. *IL12: Interleukin-12; ICV: Infected Cell Vaccine; NK: Natural Killer; IFN: Interferon; DC: Dendritic cells; D/PAMPs: Damage/Pathogen-associated molecular patterns; TAA: Tumor-associated antigens; IP-10: IFN- $\gamma$ -Inducible Protein 10.*

## 1.5 Rationale and Hypothesis

### 1.5.1 Rationale

In a CT26lacZ model of PC, treatment with MG1-IL12-ICV confers over 90% survival efficacy in mice bearing syngeneic peritoneal tumors<sup>66,219</sup>. However, in more difficult-to-treat models of established peritoneal disease (CT26 wt, B16F10 and MC38), MG1-IL12-ICV increases median survival but does not provide long-term durable cures, suggesting that further investigation into the barriers to efficacy of treatment with MG1-IL12-ICV is warranted.

While the first report on MG1-IL12-ICV detailed the necessity of NK-cell recruitment for efficacy of this vaccine, depletion of CD8<sup>+</sup> T-cells in the model also abrogated its efficacy, suggesting that the adaptive immune response is also critically involved. In fact, mice that are cured of tumors following MG1-IL12-ICV treatment demonstrate long-lived specific anti-tumor immunity and reject subsequent tumor challenges. We therefore investigated the generation of CD8<sup>+</sup> T-cell responses following vaccination with MG1-IL12-ICV, and whether this response can be enhanced using a prime-boost vaccination strategy (in our case, *irr* cells followed by MG1-IL12-ICV) that has been shown to generate strong target-specific T-cell responses in other models of pathogens and cancer including unpublished work done by our collaborators Oliver Varette and Dr. Jean-Simon Diallo in models of ICV<sup>179,180,220–222</sup>.

Furthermore, the lack of durable cures despite the generation of a strong anti-tumor immune response suggests the presence of immunosuppressive mechanisms in tumors of the peritoneal cavity that may develop over the course of MG1-IL12-ICV therapy. This also suggested that enhanced NK cell activity resulting from MG1-IL12-ICV treatment may become less effective as the tumor progresses or is insufficient to control tumor growth. Therefore, to better understand the effects of MG1-IL12-ICV and to improve its efficacy, we sought to investigate the nature of

immune responses generated upon treatment with MG1-IL12-ICV and any anti-tumor or immunosuppressive mechanisms that develop in the TME of established peritoneal tumors during ICV treatment.

#### *1.5.2 Hypothesis and Research Objective*

**Hypothesis:** Anti-tumor immune responses generated by MG1-IL12-ICV is hindered by immunosuppressive mechanisms established in the peritoneal TME over the course of therapy.

**Overall Research Objective:** To elucidate the nature of immune responses to MG1-IL12-ICV treatment generated in the peritoneal TME to identify an evidence-based approach for enhancing efficacy of MG1-IL12-ICV by utilizing combination therapies to augment anti-tumor immune responses or to limit immunosuppressive elements.

#### **Research Objectives**

- 1. Investigate the nature of anti-tumor CD8<sup>+</sup> T-cell responses generated upon vaccination with MG1-IL12-ICV.*
- 2. Examine the TME of progressing peritoneal tumors after treatment with MG1-IL12-ICV to identify anti-tumor and immunosuppressive mechanisms generated over the course of MG1-IL12-ICV therapy.*

## 2. Methods

### 2.1 Cells and Viruses

B16F10 (murine melanoma), CT26 (murine colon carcinoma), Vero (monkey kidney) and YAC-1 (murine lymphoblast) cell lines were obtained from American Type Culture Collection (Manassas, VA). MC38 (murine colon adenocarcinoma) and B16-OVA were gifts from Dr. John Bell and laboratory (Ottawa Hospital Research Institute, Ottawa, ON). Cell lines were maintained in HyClone™ Dulbecco's Modified Eagle's Media (DMEM; GE healthcare, Mississauga, ON, CA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO). Cell lines were regularly checked for mycoplasma contamination using Hoescht-staining and/or e-Myco™ VALiD Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Sangdaewon-Dong, South Korea).

Primary murine cells were harvested from spleens using mechanical dissociation or from tumors using Miltenyi's Murine Tumor Dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's protocol. Primary cells from spleen, peritoneal cavity wash and tumors were then treated with Ammonia Chloride Potassium (ACK)-lysis buffer to remove red blood cells and maintained in HyClone™ Roswell Park Memorial Institute (RPMI) medium (GE healthcare) supplemented with 10% FBS (Sigma-Aldrich). Cells from peripheral blood were treated twice with ACK-lysis buffer and then maintained in RPMI containing 10% FBS, 1% Non-Essential Amino Acids; 1% HEPES; and 0.5mM 2-β-mercaptoethanol) for *ex-vivo* analysis.

All cells, when cultured, were maintained at 37°C with 5% CO<sub>2</sub>.



Viruses (MG1, MG1-GFP and MG1-IL12) were propagated from seed stocks in Vero cells and viral titers were obtained via plaque assays using previously published methods<sup>223</sup>. The Maraba MG-1 platform is a patented product of Turnstone Biologics (New York, NY).

## **2.2 Animals**

Female C57Bl/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and were housed at the University of Ottawa Animal Care and Veterinary Service (ACVS) facilities (Ottawa, ON) in specific pathogen-free conditions. Animals were between 6-10 weeks of age at study initiation. Animal studies complied with the Canadian Council on Animal Care guidelines and were approved by the University of Ottawa Animal Research Ethics Board.

## **2.3 Infected Cell Vaccine**

Cell lines were harvested and washed twice with Phosphate Buffered Saline (PBS; Corning, Manassas, VA), and then resuspended in DMEM at  $5 \times 10^7$  cells/mL ( $\pm 10\%$ ). Cells were then irradiated (X-ray) for 60 gray before infection with MG-1 or MG1-IL12 at a Multiplicity of Infection (MOI) of 10, for 2 hours in rotation before *i.p.* delivery ( $5 \times 10^6$  cells;  $5 \times 10^7$  plaque forming units (pfu) of virus/ mouse) or subsequent *in-vitro* analysis. Irradiated cells (*irr*) were prepared similarly without the infection step.

## **2.4 Viral Cytotoxicity**

Cytotoxicity was measured by Alamar Blue (Resazurin sodium salt; Sigma-Aldrich, St Louis, MO) assay using a modified previously published protocol<sup>67</sup>. Briefly, cells were infected with different MOIs (0.1 -10) of MG1 or MG1-IL12 for 24 hours before addition of Alamar Blue. Viability of cells was measured by conversion of resazurin to resorufin over the next four hours, measured by change in absorbance at 570nm. Viability relative to mock-infected cells are reported.

## 2.5 Murine Models

For prophylactic models, C57Bl/6 mice were vaccinated with two doses of *irr* cells, MG1-ICV or MG1-IL12-ICV given one week apart. For heterologous prime-boost vaccination strategy, mice were given *irr* cells followed by MG1-IL12-ICV one week later. One week following second vaccination dose, animals were challenged with syngeneic  $1 \times 10^5$  cells subcutaneously (*sc.*) in the flank and monitored for tumor outgrowth. Mice were end-pointed when tumor volume ( $0.5 \times \text{length} \times \text{width}^2$ ) exceeded  $1625 \text{ mm}^3$ .

For therapeutic models, C57Bl/6 mice were implanted with  $1 \times 10^5$  cells *i.p.* and treated with six bi-weekly doses of *irr* cells, MG1-ICV, MG1-IL12-ICV starting 5 days post-tumor implantation. To test heterologous prime-boost vaccination strategy in a therapeutic model, mice were implanted with tumors and treated starting 3 days post-tumor implantation. Mice were monitored for respiratory distress and peritoneal distension and end-pointed when extensive respiratory distress or abdominal distension was observed as indicated by trained personnel or ACVS technicians.

To characterize immune subsets, C57Bl/6 mice were implanted with B16F10 or B16-OVA tumors ( $1 \times 10^5$ ) *i.p.* as described in the therapeutic model. A group of mice were treated with three doses of vaccination and sacrificed 15 days post tumor implantation, and the other group of mice were treated with five doses of vaccination and sacrificed 20 days post tumor implantation to assess early and late stages of treatment (Schedule A; refer to Figure 10). Alternatively, in another model, mice were sacrificed 18 hours after the first, third and sixth vaccination dose to study efficacy of successive vaccination doses (Schedule B, refer to Figures 20).

## 2.6 *In-vivo* cytotoxic T-lymphocyte (CTL) assay

*In-vivo* cytotoxic T-lymphocyte (CTL) assay was performed using a modified protocol as described elsewhere<sup>224</sup>. Briefly, splenocytes from naïve animals were labeled with Cell Trace Violet (CTV) or carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher Scientific, Waltham, MA), and then peptide-pulsed with a H2kb-restricted tumor antigen peptide *p15E* (KSPWFTTL; MBL International, Woburn, MA) or left unloaded, before *i.v.* delivery into vaccinated mice. Eighteen hours after injection, spleens from recipient mice were obtained and specific lysis of *p15E*-loaded splenocytes was calculated as described elsewhere<sup>224</sup>. First, ratio of non-peptide-loaded cells to peptide-loaded cells was calculated for each sample. Then, percent specific lysis was calculated =  $[1 - (\text{Average of Naïve Mice Ratio} / \text{Experimental Mice ratio})] \times 100$ . The same vaccination schedule was used as for prophylactic models; *in-vivo* CTL was performed on the same day as tumors would otherwise be implanted in vaccinated mice.

## 2.7 *Ex-vivo* Peptide Stimulation

Isolated cells were stimulated *ex-vivo* with H2kb-restricted peptides (10 µg/mL; VSV N protein peptide (VSV<sub>n</sub>): RGYVYQGL; ovalbumin (OVA) peptide: SIINFEKL) for 6 hours; 5 hours in the presence of BD GolgiPlug protein transport inhibitor containing brefeldin A (Becton Dickinson (BD), Franklin Lakes, NJ). Subsequently, cells were washed and stained using Fixable Viability Stain 510 (BD) and treated with Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (BD). Cells were then stained for extracellular markers (CD45, CD3 and/or CD8) as indicated for flow cytometry. For intracellular IFN-γ staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ (BD) following the manufacturer's protocol.

## **2.8 Immune Cell Profiling**

To characterize immune subsets, single-cell suspension of isolated cells from spleens, peritoneal cavity wash and tumors (where available) were obtained as described in Section 2.1 (Cells and Viruses) following treatment of animals with schedule A or B described under Section 2.5 (Murine Models). Cells were then stained with Fixable Viability Stain 510 (BD), treated with Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (BD), and then stained with extracellular markers. For intracellular IFN- $\gamma$  staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ (BD) following the manufacturer's protocol. Where required, cells were stained for intranuclear Foxp3 using eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher Scientific) following manufacturer's protocols. A detailed list of antibodies can be found in Appendix A.

### *2.8.1 NK Cell Responsiveness*

To study NK cell responsiveness to target YAC-1 cells, isolated cells were co-cultured with target YAC-1 cells at a 3:1 cell: YAC-1 ratio. Cells were co-cultured at 37°C 5% CO<sub>2</sub> in the presence of anti-CD107a antibody (BD) and BD GolgiStop™ Protein Transport Inhibitor (containing Monensin) for 4 hours before being stained for extracellular and intracellular (IFN- $\gamma$ ) markers, as described above.

### *2.8.2 Phagocytosis Assay*

To study phagocytosis and antigen processing, splenic cells were co-cultured with DQ-Ovalbumin (DQ-OVA; ThermoFisher Scientific) for 1 hour before staining for extracellular markers as described above.

### *2.8.3 Hematoxylin and Eosin Staining*

Harvested tumors were fixed in 10% buffered formalin for 24-72 hours and stored in 70% ethanol. Fixed tumors were then paraffin embedded by the University of Ottawa Histology Core Facility. Formalin-fixed paraffin-embedded (FFPE) tumors were then stained using standard hematoxylin and eosin (H&E) staining protocols.

## **2.9 NanoString Gene Expression Analysis of Formalin-fixed Paraffin-Embedded Tumors**

Tumor tissue from FFPE B16F10 peritoneal tumors from animals treated according to schedule A were micro-dissected using AVENIO Millisect System (Roche, Basel, Switzerland). RNA was then extracted using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (ThermoFisher Scientific). RNA was then analyzed using NanoString's nCounter PanCancer Immune Profiling Panel (Mouse) (NanoString Technologies, Seattle, WA) following manufacturer's protocols. Briefly, extracted RNA was hybridized with capture and reporter probe-sets for nCounter XT CodeSet Gene Expression Assay for PanCancer Immune Profiling Panel (Mouse) before analysis on an nCounter Dx Analysis System (NanoString Technologies) at the University of Ottawa Heart Institutes' Cardiometabolic microRNA Laboratory.

Data analysis, data quality checks and normalization were carried out using NanoString's nSolver Analysis Software (Version 3.0 or 4.0) following recommended guidelines as described elsewhere<sup>225</sup>. Heat maps, volcano plots, cell type scores and individual gene's fold up/downregulation was prepared using nSolver. Upregulated pathways were identified using Gene Ontology Consortium's Enrichment Analysis Tool by using genes that were at least 2 log2 fold upregulated with  $p < 0.05$  (Benjamin-Yukateli)<sup>226</sup>. Housekeeping genes used for this analysis is listed in Appendix B.

## 2.10 Data and Statistical Analysis

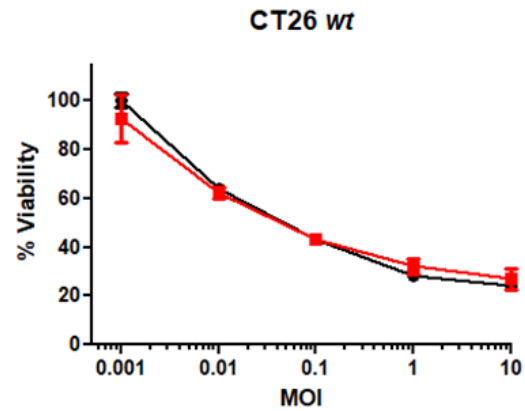
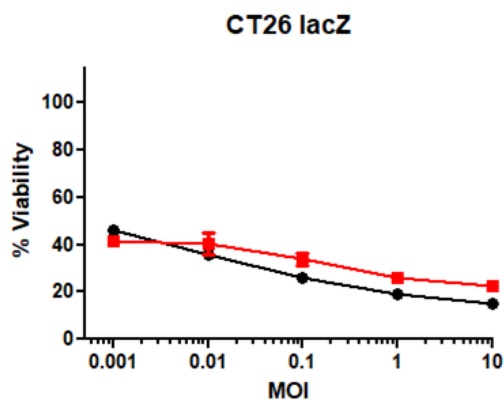
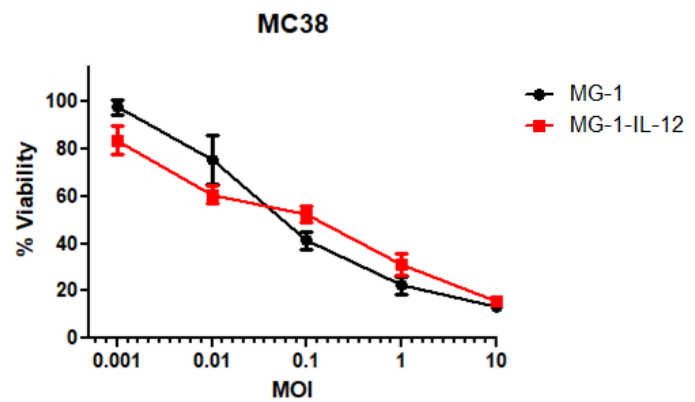
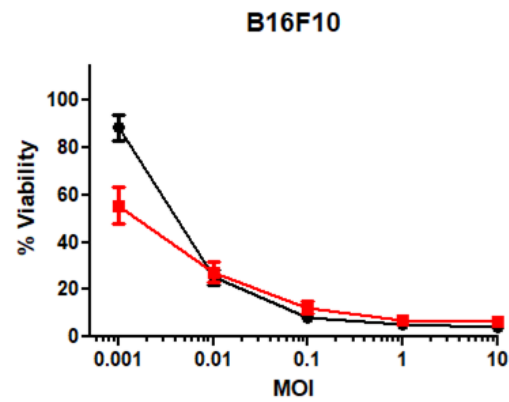
Data is presented as Mean  $\pm$  Standard Deviation (SD). All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Where samples were found to be non-parametric (using Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefors P-value) or D'Agostino and Pearson omnibus normality test), Kruskal Wallis with Dunn's Post-test was used. Otherwise, a one-way analysis of variance (ANOVA) with Bonferroni Post-test was conducted, with  $p < 0.05$  considered to be statistically significant. Alamar blue cytotoxicity and *in-vivo* CTL specific lysis values were calculated using Microsoft Excel (Microsoft, Redmond, WA).

Survival analyses were performed according to the Kaplan–Meier method using the Mantel-Cox log-rank test by Graph Pad Prism 5.0. Differences were found to be statistically significant when p-values were below the significance level of 0.05 after it was adjusted using Bonferroni correction for multiple comparisons (by dividing significance level (0.05) by the number of comparisons made in each survival curve; i.e., if five comparisons were made,  $p < 0.01$  was considered significant). Flow cytometry was performed at the University of Ottawa's Flow Cytometry Core Facility using the BD LSR Fortessa (BD) or BD Celesta (BD) Flow Cytometers. Analysis of flow data was conducted using FlowJo v10 (FlowJo, LLC, Ashland, OR).

### **3. Results**

#### **3.1 MG1 and MG1-IL12 demonstrate similar cytotoxicity in different solid tumor cell lines.**

The first step in characterizing the immune response to MG1-IL12-ICV was to establish that the inherent infectivity and cytotoxicity induced by the parental MG1 virus was similar to that of the engineered MG1-IL12 virus in different solid tumor cell lines. To this end, different cell lines (CT26lacZ, CT26 *wt*, B16F10 and MC38) were infected with different multiplicities of infection (0.001, 0.01, 0.1, 1 and 10) for 24 hours before addition of and fluorometric analyses of metabolized Alamar Blue. Cytotoxicity of both MG1 and MG1-IL12 was equivalent in all cell lines although CT26lacZ was more sensitive to viral oncolysis at lower MOIs (Figure 3). However, viral cytotoxicity was equivalent at the MOI (10) at which the ICV is prepared, similar to what we have previously reported<sup>66</sup>.





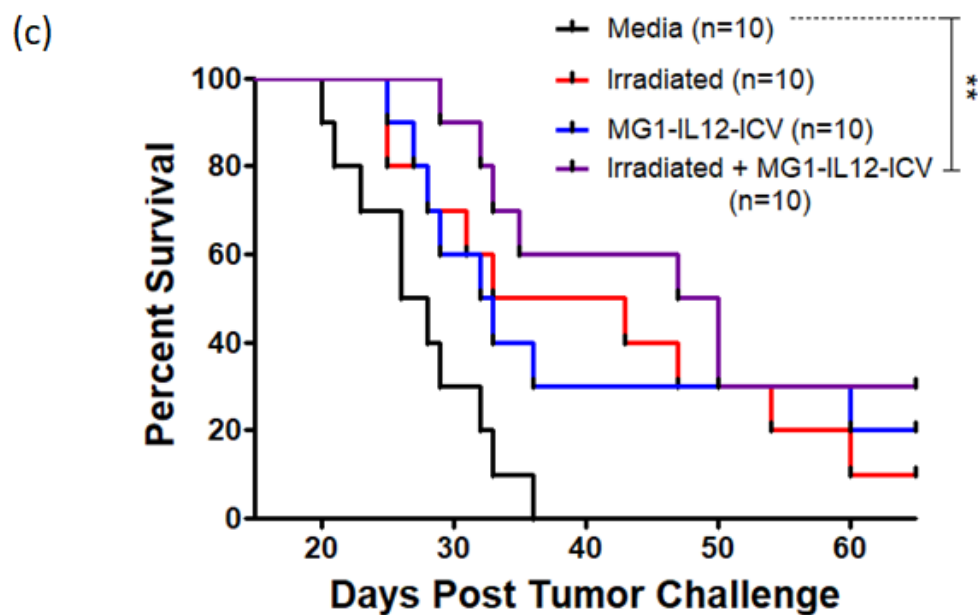
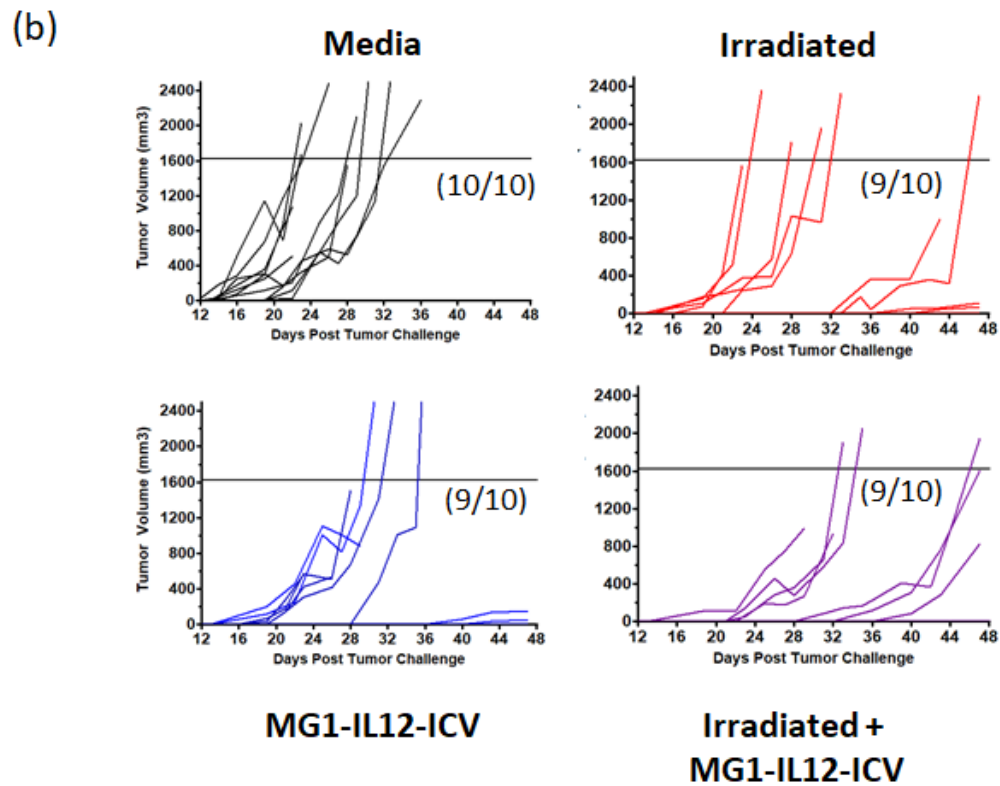
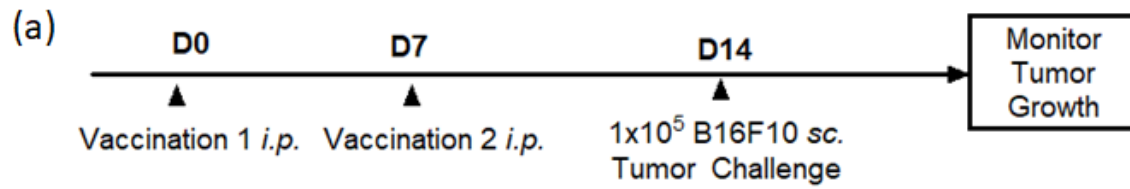
**Figure 3. MG1-IL12 exhibits similar viral cytotoxicity to MG1 in different solid tumor cell lines.**

Different solid tumor cell lines (CT26lacZ, CT26 wild type, B16F10 and MC38) were infected with different MOIs of MG1 or MG1-IL12 for 24 hours before addition of Alamar Blue. Fluorescence of metabolized Alamar Blue was monitored for up to 6 hours. Viability of infected cells relative to uninfected controls for each cell line is plotted. Data representative of three technical replicates. Mean  $\pm$  SD is presented. *wt*: *wildtype*; *IL*: *Interleukin*

### **3.2 A heterologous prime-boost vaccination regimen (irradiated cells followed by MG1-IL12-ICV) generates cell-mediated anti-tumor immunity and delays tumor-growth.**

#### *3.2.1 Vaccination with irradiated cells, MG1-IL12-ICV or a heterologous prime-boost delays tumor growth in a prophylactic subcutaneous model.*

Having established similar viral toxicity across different tumour models, I next utilized a prophylactic vaccination strategy similar to that utilized by Chantal *et al.* to determine if vaccination with MG1-IL12-ICV is able to generate anti-tumor memory responses. The prophylactic model allowed us to focus on the ICV's effects on the adaptive immune response while avoiding potential drawbacks of an immunosuppressive TME known to be present in established tumors. Briefly, mice were vaccinated *i.p.* with two doses of *irr* tumor cells or MG1-IL12-ICV 7 days apart; or were vaccinated with one dose of *irr* cells followed by vaccination with one dose of MG1-IL12-ICV (heterologous prime-boost) (Figure 4a). Vaccinated or untreated mice were then challenged with  $1 \times 10^5$  B16F10 wt cells *sc.* to monitor tumor outgrowth. Vaccination with two doses of *irr* cells (median survival: 38 days) and MG1-IL12-ICV (median survival: 32.5 days) modestly delayed tumor growth compared to non-vaccinated animals but this did not reach statistical significance (median survival: 27 days; Figures 4b and c). However, a heterologous prime-boost combination provided the best protection (median survival: 48.5 days, Media vs Prime-boost,  $p=0.0008$ , Mantel-Cox Log-Rank Test with Bonferroni correction for multiple comparisons). The three vaccination groups were, however, not found to be statistically significantly different than each other.



**Figure 4. Priming animals with irradiated cells prior to vaccination with MG1-IL12-ICV delays tumor growth and improves survival in a B16F10 model.**

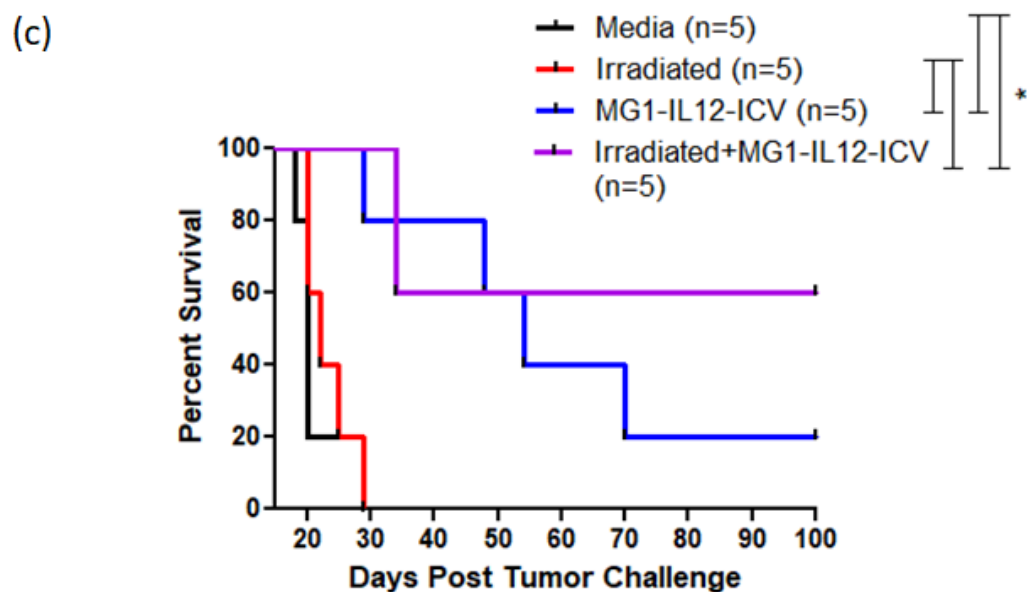
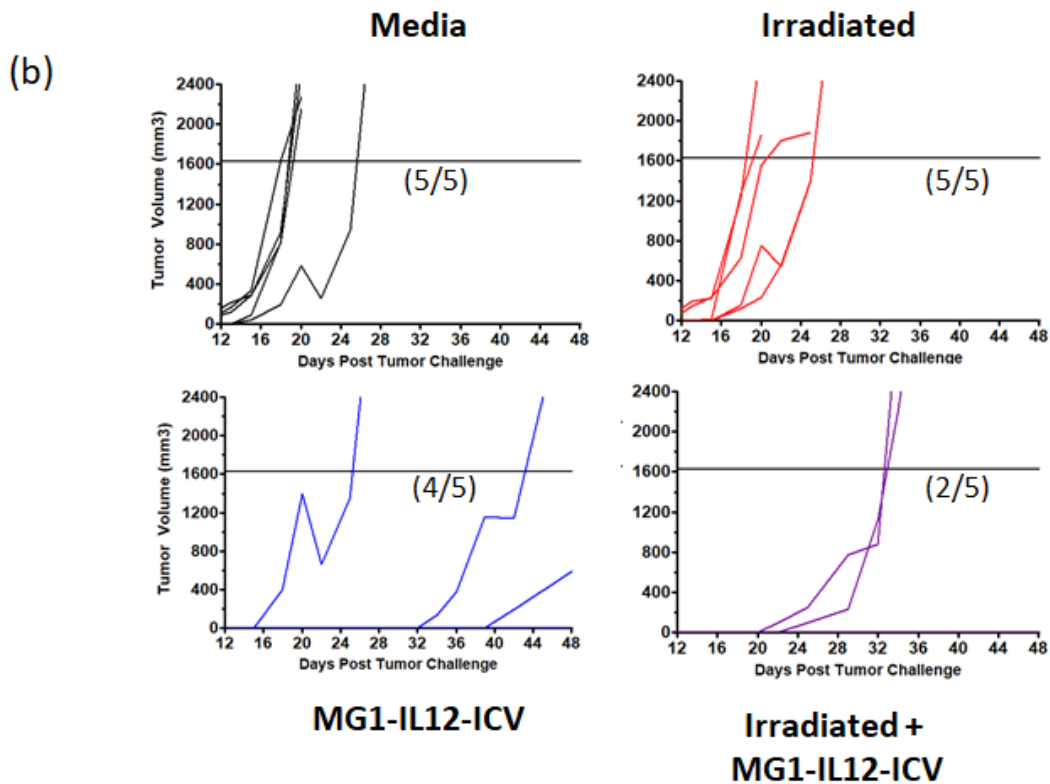
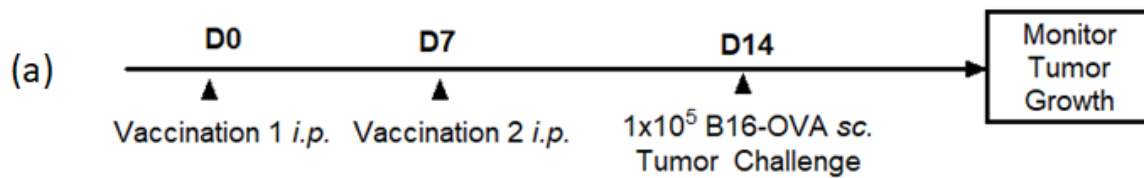
a) C57Bl/6 mice were vaccinated *i.p.* with two vaccinations one week apart as shown, before  $1 \times 10^5$  B16F10 subcutaneous challenge. b) Tumor volumes over time are shown. Number of mice that eventually grew tumors are indicated in parenthesis. c) Corresponding survival curves are shown. Statistical significance was calculated using Mantel Cox Log Rank Test with Bonferroni correction. Data is pooled from two separate experiments.  $**p < 0.002$ . *i.p.*: *intraperitoneal*; *sc.*: *subcutaneous*

### 3.2.2 A heterologous prime-boost in a B16-OVA model generates H2kb-SIINFEKL specific CD8<sup>+</sup> T-cells.

To allow correlation of CD8<sup>+</sup> T-cell responses to a delay in tumor growth, we utilized the B16-OVA model, which allows identification of SIINFEKL (an immunodominant OVA peptide)-specific CD8<sup>+</sup> T-cell responses (Figure 5a). Interestingly, in the B16-OVA model, vaccination with *irr* cells did not delay tumor growth (median survival: 22 vs 20 days). As previously observed, both MG1-IL12-ICV (median survival: 54 days) and heterologous combination (median survival not reached) provided a survival benefit compared to media-treated and *irr* cells ( $p < 0.008$ , Mantel-Cox Log-Rank Test with Bonferroni correction; Figure 5b and c).

To correlate the observed delays in tumor growth with the generation of a specific anti-tumor immune response, we performed saphenous bleeds on days 13 and 20 post-first vaccination and measured the CD8<sup>+</sup> T-cell response following *ex-vivo* stimulation with H2kb-restricted SIINFEKL or VSVn peptide (Figure 6a). No detectable anti-tumor CD8<sup>+</sup> immune response was detected 13 days following the first vaccination. However, a robust anti-viral immune response was detected in both MG1-IL12-ICV vaccinated and heterologous vaccinated mice ( $p < 0.05$ , one-way analysis of variance; Figure 6b). Similar results were obtained when saphenous bleeds were stimulated *ex-vivo* seven days post-first vaccination and 20 days post-first vaccination (six days post-challenge) (data not shown).

At the latter timepoint (20 days post-first vaccination), saphenous bleeds were also stained with H2kb-SIINFEKL tetramers alongside CD3 and CD4 surface stains (a CD8 antibody clone that did not interfere with tetramer staining was not available at the time). We observed the generation of



**Figure 5. Priming animals with irradiated cells prior to vaccination with MG1-IL12-ICV delays tumor growth and improves survival in a B16-OVA model.**

a) C57Bl/6 mice were vaccinated *i.p.* with two vaccinations one week apart as shown, before  $1 \times 10^5$  B16-OVA subcutaneous challenge. b) Tumor volumes over time are shown. Number of mice with growing tumors is indicated in parenthesis. b) Corresponding survival curves are presented. Statistical significance was calculated using Mantel Cox Log Rank Test with Bonferroni correction. \* $p < 0.01$ . *i.p.*: intraperitoneal; *sc.*: subcutaneous

(a)

The diagram illustrates the experimental timeline. A horizontal timeline starts at D0, marked with an upward arrow and labeled 'D0'. Below D0 is the text 'Vaccination 1 i.p.'. At D7, marked with an upward arrow and labeled 'D7', is 'Vaccination 2 i.p.'. At D14, marked with an upward arrow and labeled 'D14', is '1x10<sup>5</sup> B16-OVA sc. Tumor Challenge'. Above the timeline, 'Ex-vivo Analysis of Peripheral Blood' is written in red. Below this, 'D13' and 'D20' are written in red with downward arrows pointing to the timeline. At the end of the timeline is a box labeled 'Monitor Tumor Growth'.

Ex-vivo Analysis of Peripheral Blood

D13 D20

D0 D7 D14

Vaccination 1 i.p. Vaccination 2 i.p. 1x10<sup>5</sup> B16-OVA sc. Tumor Challenge

Monitor Tumor Growth

(b)

Dot plot showing the percentage of IFN- $\gamma$ <sup>+</sup> (of CD8<sup>+</sup>) cells across four conditions: Media, Irradiated, MG1-IL12-ICV, and Irradiated + MG1-IL12-ICV. Each condition has three treatment groups: DMSO (black), SIINFEKL (blue), and VSVn (red). The y-axis is labeled '% IFN- $\gamma$ <sup>+</sup> (of CD8<sup>+</sup>)' and ranges from 0 to 10. Statistical significance is indicated by asterisks (\*, \*\*, \*\*\*) and brackets.

| Condition                 | Treatment | % IFN- $\gamma$ <sup>+</sup> (of CD8 <sup>+</sup> ) | Significance |
|---------------------------|-----------|-----------------------------------------------------|--------------|
| Media                     | DMSO      | ~0.1                                                |              |
|                           | SIINFEKL  | ~0.1                                                |              |
|                           | VSVn      | ~0.1                                                |              |
| Irradiated                | DMSO      | ~0.1                                                |              |
|                           | SIINFEKL  | ~0.2                                                |              |
|                           | VSVn      | ~0.1                                                |              |
| MG1-IL12-ICV              | DMSO      | ~0.4                                                |              |
|                           | SIINFEKL  | ~0.5                                                |              |
|                           | VSVn      | ~2.5                                                | ***          |
| Irradiated + MG1-IL12-ICV | DMSO      | ~0.1                                                |              |
|                           | SIINFEKL  | ~0.2                                                |              |
|                           | VSVn      | ~2.8                                                | ***          |

(c)

%H2kb SIINFEKL<sup>+</sup>  
(of CD3<sup>+</sup> CD4<sup>-</sup>)

Media Irradiated MG1-IL12-ICV Irradiated + MG1-IL12-ICV

| Group                     | Mean (%) | Individual Data Points (%)   |
|---------------------------|----------|------------------------------|
| Media                     | ~1.0     | ~0.5, ~0.8, ~1.0, ~1.2, ~1.5 |
| Irradiated                | ~0.5     | ~0.4, ~0.5, ~0.6, ~0.7, ~0.8 |
| MG1-IL12-ICV              | ~0.5     | ~0.3, ~0.4, ~0.5, ~0.6, ~0.7 |
| Irradiated + MG1-IL12-ICV | ~1.7     | ~0.0, ~0.5, ~0.6, ~2.4, ~4.2 |



**Figure 6. A heterologous prime-boost generates SIINFEKL-reactive CD3<sup>+</sup> CD4<sup>-</sup> T-cells and a robust anti-viral T-cell response.**

a) C57Bl/6 mice were vaccinated *i.p.* with two vaccinations one week apart as shown, before  $1 \times 10^5$  B16-OVA subcutaneous challenge. Blood from the saphenous vein was then collected 13 and 20 days post-first vaccination. b) For day 13, saphenous blood cells were incubated *ex-vivo* with H2kb-restricted SIINFEKL or VSVn peptides and cells stained for intracellular IFN- $\gamma$  production. Percentage of CD8<sup>+</sup> T-cells positive for IFN- $\gamma$  upon *ex-vivo* stimulation is plotted. Significance was calculated using a one-way analysis of variance with Bonferroni post-test. c) For day 20, cells were stained with H2Kb-SIINFEKL specific tetramers. Percentage of CD3<sup>+</sup> CD4<sup>-</sup> T-cells that were SIINFEKL tetramer positive is plotted. *Mean  $\pm$  SD. DMSO: Dimethyl sulfoxide; IFN- $\gamma$ : Interferon- $\gamma$ .* \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

anti-tumor H2kb-SIINFEKL tetramer positive CD3<sup>+</sup> CD4<sup>-</sup> cells in two out of five mice that were vaccinated with the heterologous combination (1.7% ± 1.6%, not significant, Kruskal Wallis with Dunn's Post-test) but not in any of the other groups (Figure 6c). However, no correlation could be identified between mice that generated a T-cell response and those that delayed tumor outgrowth.

At this stage, my *ex-vivo* experiments suggested that MG1-IL12-ICV was able to generate a modest anti-tumor CD8<sup>+</sup> T-cell response when administered in a prime-boost model. However, this was in contradiction with the significant delay in tumor growth observed in our models. We therefore wanted to investigate whether a more robust T-cell response could be observed *in-vivo*, particularly if our *ex-vivo* assays were not sensitive enough to detect the T-cell response being generated. We therefore sought to investigate CD8<sup>+</sup> T-cell responses *in-vivo* using an *in-vivo* CTL assay.

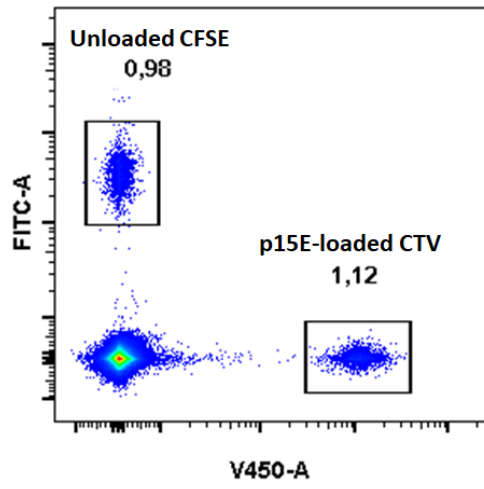
### *3.2.3 Heterologous prime-boost generates CD8<sup>+</sup> T-cell-mediated anti-tumor immunity as evidenced by an in-vivo cytotoxic T-cell assay.*

Unpublished work from our collaborators in the CT26 wt model had demonstrated that in prime-boost models of ICV, significant responses to a tumor-associated retroviral antigen, gp-70, can be observed using the *in-vivo* CTL assay in Balb/c mice. We therefore similarly chose to investigate CD8<sup>+</sup> T-cell responses to the p15E retroviral antigen. p15E, derived from the p15E transmembrane protein of the murine leukemia virus (MuLV), is an endogenous retroviral antigen constitutively expressed in C57Bl/6 mice (and overexpressed in H2b haplotype syngeneic tumors like B16 melanoma)<sup>227–230</sup>. CTLs against p15E have been shown to reduce pulmonary metastasis of B16 tumors and is often used to determine tumor-specific T-cell responses against several C57Bl/6 syngeneic tumors<sup>227–229</sup>. Briefly, spleens from naïve mice were labeled with fluorescent dyes and then pulsed with a tumor-associated H2kb-restricted p15E peptide or left unloaded. A 1:1 mixture

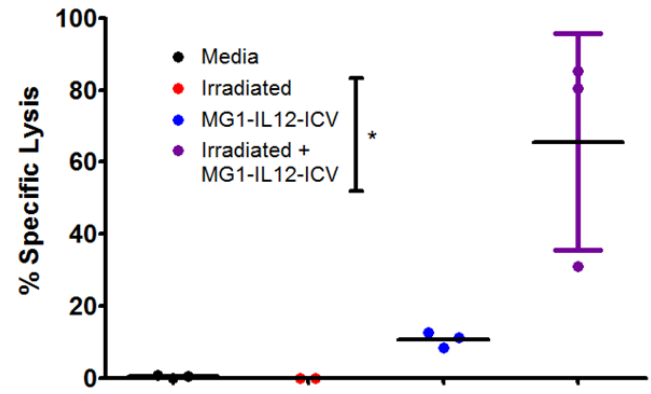
of tumor-peptide loaded and unloaded splenocytes were then delivered *i.v.* to vaccinated mice one-week post vaccination (following the same vaccination schedule as in Figure 5a). Spleens of vaccinated mice were then isolated 24 hours post injection, and preferential killing of tumor-peptide loaded splenocytes was analyzed by calculating percent specific lysis. A representative flow plot demonstrating the two labeled populations is shown in Figure 7a. Again, while all three vaccination strategies delayed tumor growth in the B16F10 model, only the heterologous combination was able to generate a robust specific anti-tumor CD8<sup>+</sup> T-cell response as measured by the CTL assay ( $65.6\% \pm 30.2\%$ ; compared to media:  $0.4 \pm 0.4\%$ , *irr*: 0%, and MG1-IL12-ICV:  $10.8 \pm 2.1\%$ ; Figure 7b). Heterologous vaccination was significantly better than vaccination with *irr* cells ( $p < 0.05$ , Kruskal Wallis with Dunn's Post-test) in generating a T-cell response, further corroborating *ex-vivo* findings that the prime-boost vaccination strategy was best at generating adaptive immune responses.

Overall, my investigations in Aim 1 revealed evidence from 2 models and 3 assays that vaccination with MG1-IL12-ICV is able to delay tumor growth and generates, albeit modest, specific anti-tumor CD8<sup>+</sup> T-cell responses, particularly when administered in a prime-boost vaccination regimen. I therefore began working on Aim 2 which sought to better understand the pro- and anti-tumor immune responses generated in the TME of progressing peritoneal tumors over the course of MG1-IL12-ICV therapy, to identify barriers to efficacy and avenues that can be explored to enhance outcomes in treatment-averse models.

(a)



(b)



**Figure 7. A heterologous prime-boost vaccination strategy generates strong anti-tumor responses.**

C57Bl/6 mice were vaccinated as described in Figure 4a before *i.v.* injection of labeled splenocytes from naïve mice loaded with (labeled with CTV) or without p15E (labeled with CFSE) tumor peptide. 24 hours post-injection of labeled splenocytes, spleens from recipient mice were analyzed for p15E specific killing. a) Representative flow plot of labeled cells from spleen of naïve mice and b) scatter plots of percent specific lysis are presented. Statistical significance was calculated using Kruskal Wallis with Dunn's Post-test. *Mean ± SD.*, CTV: *Cell Trace Violet*; CFSE: *carboxyfluorescein succinimidyl ester*. \* $p < 0.05$

### **3.3 MG1-IL-12-ICV increases survival in models of peritoneal carcinomatosis.**

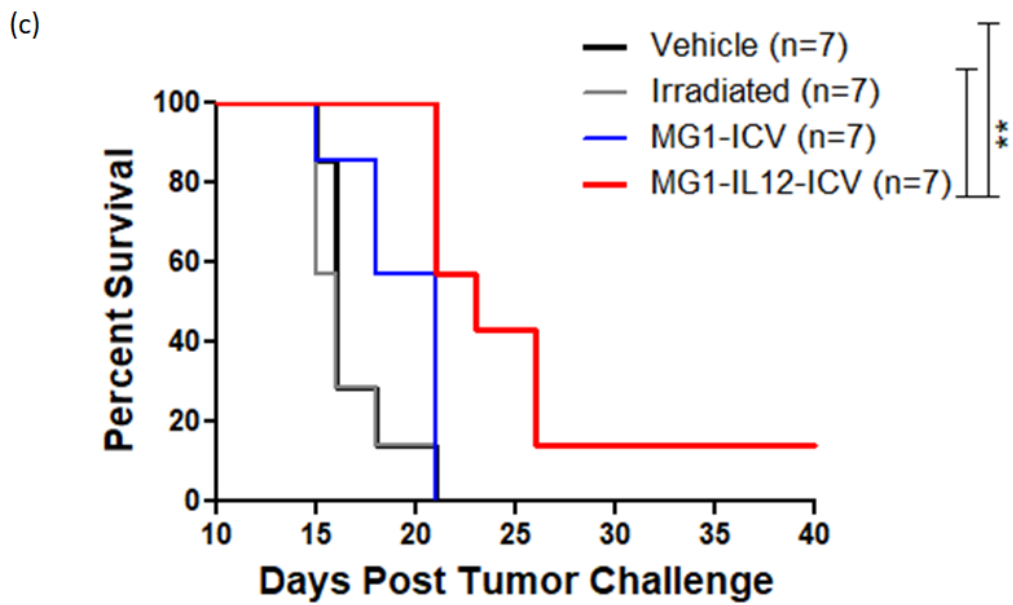
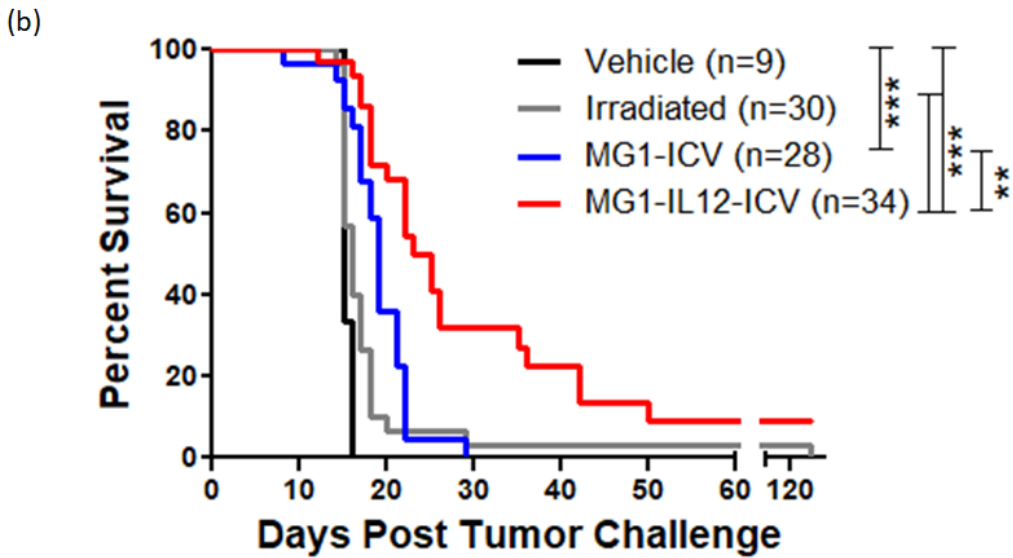
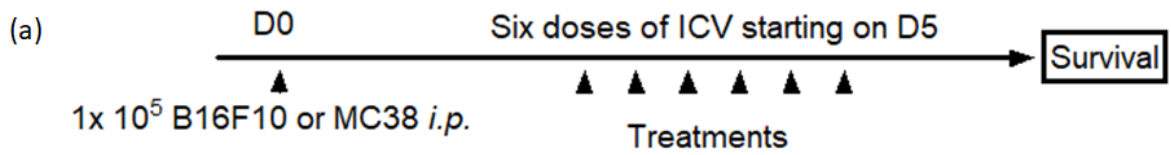
#### *3.3.1 Treatment with multiple doses of MG1-IL-12-ICV increases median survival in models of peritoneal carcinomatosis*

In a rapidly growing model of PC, where  $1 \times 10^5$  B16F10 cells are implanted intraperitoneally, treatment with six doses of MG1-IL12-ICV starting 5 days post-tumor implantation delays tumor growth and increases median survival of mice (25 days) compared to mice treated with vehicle (15 days ( $p < 0.0002$ , Mantel-Cox Log-Rank Test with Bonferroni correction)), *irr* cells (16 days ( $p < 0.0002$ )) or MG1-ICV (19 days ( $p < 0.002$ )) following the same dosing schedule (Figure 8a and b). Despite significant improvements in median survival, no treatment strategy provided durable cures. Data was pooled with B16F10 PC survival analysis experiments conducted previously in Dr. Rebecca Auer's laboratory by Dr. Almohanad Alkayyal.

In a similar model of PC, where  $1 \times 10^5$  MC38 cells are implanted intraperitoneally, treatment with MG1-IL12-ICV 5 days post-tumor implantation delays tumor growth and increases median survival of mice (23 days) compared to mice treated with vehicle (16 days ( $p < 0.002$ , Mantel-Cox Log-Rank Test with Bonferroni correction); Figure 8a and c) or *irr* cells (16 days ( $p < 0.002$ )). Similarly, no durable cures were obtained.

#### *3.3.2 A heterologous prime-boost strategy is not effective in treating peritoneal carcinomatosis.*

Given that a heterologous combination was most effective in generating a CD8<sup>+</sup> T cell response in non-tumor bearing mice and delaying tumour outgrowth upon subsequent challenge (indicating memory responses), we postulated that a heterologous combination may similarly stimulate immune response and provide protection in tumour bearing animals. To accommodate for the dosing schedule of a prime-boost vaccination strategy and given that mice receiving *irr* cells have



**Figure 8. Treatment with MG1-IL12-ICV increases median survival of mice bearing peritoneal tumors.**

a)  $1 \times 10^5$  B16F10 or MC38 cells were seeded in the peritoneal cavity of C57Bl/6 mice before treatment with six biweekly doses of the indicated vaccines. Survival curve of mice bearing b) B16F10 or c) MC38 tumors are shown. Statistical significance was calculated using Mantel Cox Log Rank Test with Bonferroni correction  $**p < 0.002$ ,  $***p < 0.0002$ . Data pooled from several experiments.



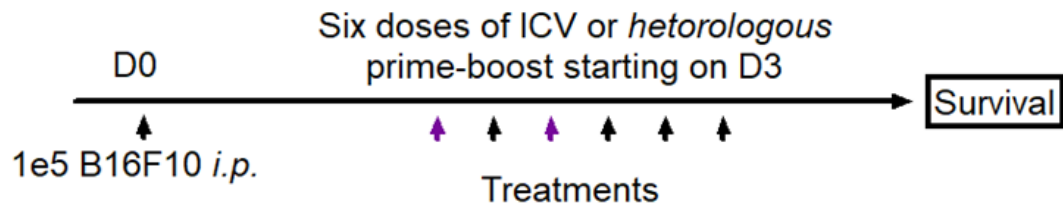
a median survival of 16 days, we began treatment earlier, 3 days post tumor-implantation (Figure 9a). Initiating treatment earlier in the B16F10 PC model with repeated dosing with MG1-IL12-ICV (median survival: 46 days,  $p < 0.002$ , Mantel-Cox Log-Rank test with Bonferroni correction; Figure 9b) provided survival benefit when compared to both the heterologous prime-boost (median survival: 18 days) and vehicle control (median survival: 17 days), and the latter two were not significantly different. Since my primary goal was to better understand the efficacy of the vaccine in the context of tumour bearing animals, subsequent studies were conducted using multi-dose strategy in the late treatment models of established disease.

### **3.4 Treatment with MG1-IL12-ICV increases immune infiltrates into the tumor**

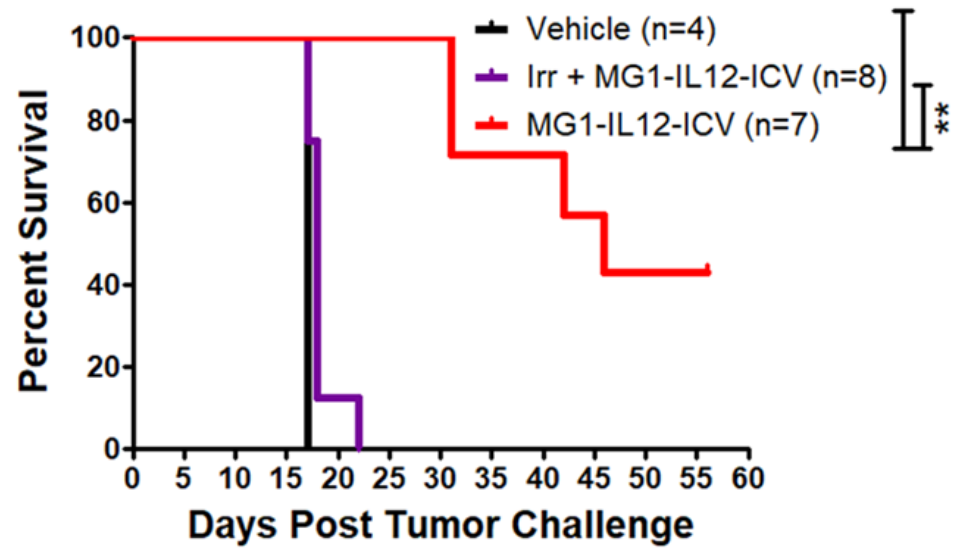
The previous studies suggested that despite an initial control of tumour growth, multi-dose MG1-IL12-ICV is ultimately unable to confer long term protection. To better understand this phenomenon and the relative immune response to the multi-dose strategy, I sought to characterize the immune infiltrates present in the peritoneal cavity and the tumour itself following administration of three (15 days post-tumor implantation) and five doses (20 days post-tumor implantation) of ICV respectively. These timepoints were chosen as they correspond to the median survival of vehicle-treated mice and MG1-ICV-treated mice respectively.

Additionally, tumors were also fixed in formalin and paraffin embedded for use in gene expression analysis and immunohistochemistry. Treatment schedule and H&E staining of representative tumors for each treatment groups are presented (Figure 10 a and b). H&E staining revealed increased infiltration of lymphocytes (or lymphocyte-like cellular structures) into the tumor of mice treated with MG1-IL12-ICV. Mice treated with MG1-IL12-ICV were also found to have reduced tumor burden when sacrificed 15 days and 20 days post tumor implantation (Appendix C). Tumors up to a maximum of 1 gram were collected for flow cytometry; some residual tumor

(a)



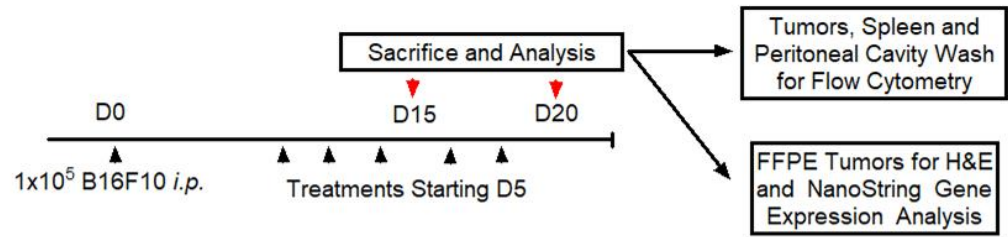
(b)



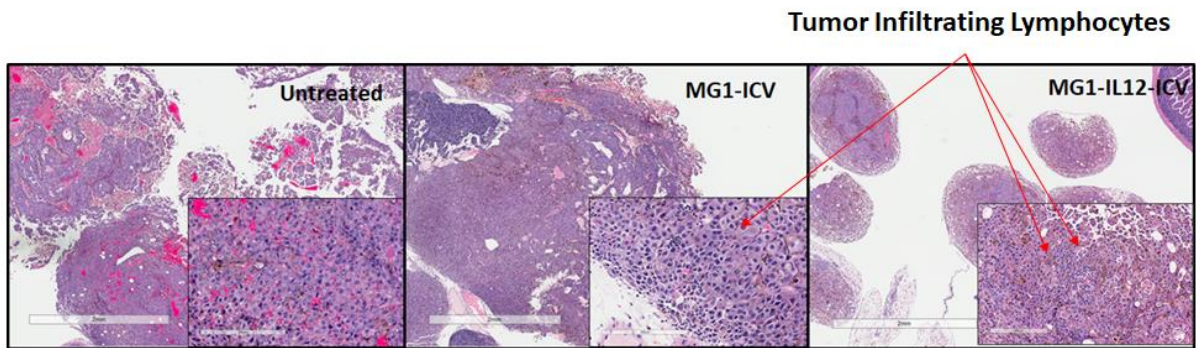
**Figure 9. Repeated dosing with MG1-IL12-ICV provides greater protection than heterologous prime-boost in peritoneal tumor bearing animals.**

a)  $1 \times 10^5$  B16F10 cells were seeded in the peritoneal cavity of C57Bl/6 mice before treatment with six biweekly doses of MG1-IL12-ICV or a heterologous prime-boost consisting of irradiated cells 3 days post-tumor implantation followed by MG1-IL12-ICV a week later (indicated in purple) as described in the schedule above. b) Survival curves for the different treatments are shown. Statistical significance was calculated using Mantel Cox Log Rank Test with Bonferroni correction.  $**p < 0.002$ .

a)



b)



**Figure 10. Treatment with MG1-IL12-ICV increases immune infiltrates into established B16F10 tumors.**

C57Bl/6 mice were injected *i.p.* with  $1 \times 10^5$  B16F10 cells before receiving three or five bi-weekly doses of infected cell vaccines or irradiated cells starting five days post-tumor implantation. **a)** Treatment schedule and timing of sacrifice and analysis is shown. Spleen, peritoneal cavity wash and tumors were collected and analyzed by flow cytometry. Tumors were also fixed in formalin and paraffin embedded for subsequent analysis. **b)** Hematoxylin and Eosin (H&E) staining of representative tumors harvested 15 days post-tumor implantation and treated with three doses of vehicle, MG1-ICV or MG1-IL12-ICV showing immune infiltrates is presented. Arrow points to cellular structures indicative of immune infiltration in MG1-ICV or MG1-IL12-ICV treated tumors. *FFPE: Formalin-fixed paraffin-embedded. i.p.: intraperitoneally*

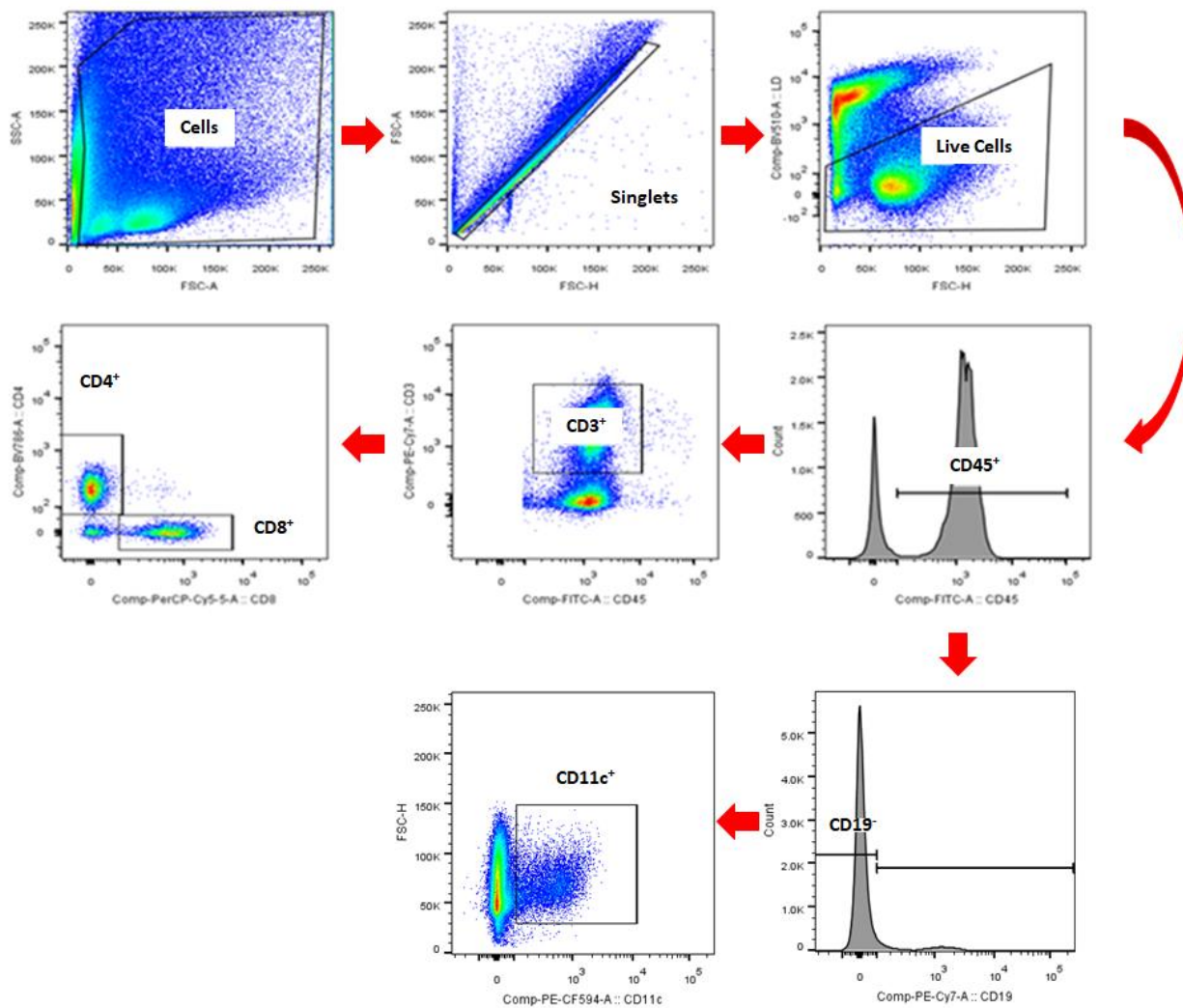
tissue could not be resected, and tumor tissue collected for fixing and paraffin-embedding was not weighed as matched normal tissue was collected in tandem.

#### *3.4.1 MG1-IL12-ICV treatment increases CD11c<sup>+</sup> dendritic cells, CD8<sup>+</sup> and CD4<sup>+</sup> T-cells in the tumor microenvironment*

We employed multi-parameter flow cytometric analysis to further investigate the nature of the immune infiltrates we observed by H&E. Using gating strategies similar to the one detailed in Figure 11, we characterized a number of immune cell subsets including DCs, T-cells, MDSCs and T-regs. These studies revealed an increased presence of immune infiltrates (CD45<sup>+</sup>) in the peritoneal cavity and tumors of mice treated with MG1-IL12-ICV, and the subsets present indicated the presence of a pro-inflammatory TME. One of my primary interests was in characterizing the T-cell response and the steps leading to the generation of a T-cell response; these analyses are outlined below beginning with recruitment of DCs (CD11c<sup>+</sup>).

##### *3.4.1.1 Dendritic Cells*

The recruitment of DCs represents a critical first step in the generation of an adaptive T-cell response. Flow cytometric analysis of CD45<sup>+</sup> cells revealed that there was an increased proportion of CD11c<sup>+</sup> cells as a percentage of CD45<sup>+</sup> cells in the peritoneal cavity wash at day 15 (11.7% ± 2.2%, p<0.05, Kruskal-Wallis with Dunn's Post Test) compared to vehicle-treated (8.2 ± 2.9%) mice (Figure 12a). Similarly, an increase in the total number of CD11c<sup>+</sup> cells in the peritoneal cavity was observed in MG1-IL12-ICV-treated mice at both day 15 (9.5x10<sup>5</sup> ± 4.7x10<sup>5</sup> cells) and day 20 (1.3x10<sup>6</sup> ± 1.0x10<sup>6</sup> cells) compared to vehicle-treated mice (3.9x10<sup>5</sup> ± 3.9x10<sup>5</sup>, p<0.01 Kruskal-Wallis with Dunn's Post Test Figure 13a). Although not statistically significant, DC infiltration into the tumor in MG1-IL12-ICV treated mice (14.2% ± 6.3% of CD45<sup>+</sup>) was higher

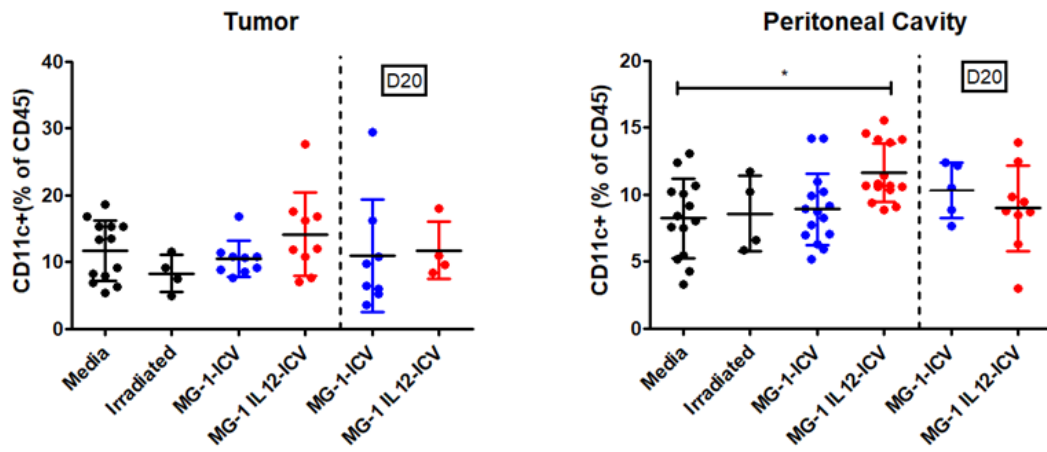


**Figure 11. Gating strategy for flow cytometric analysis of immune infiltrates in the peritoneal cavity and tumors**

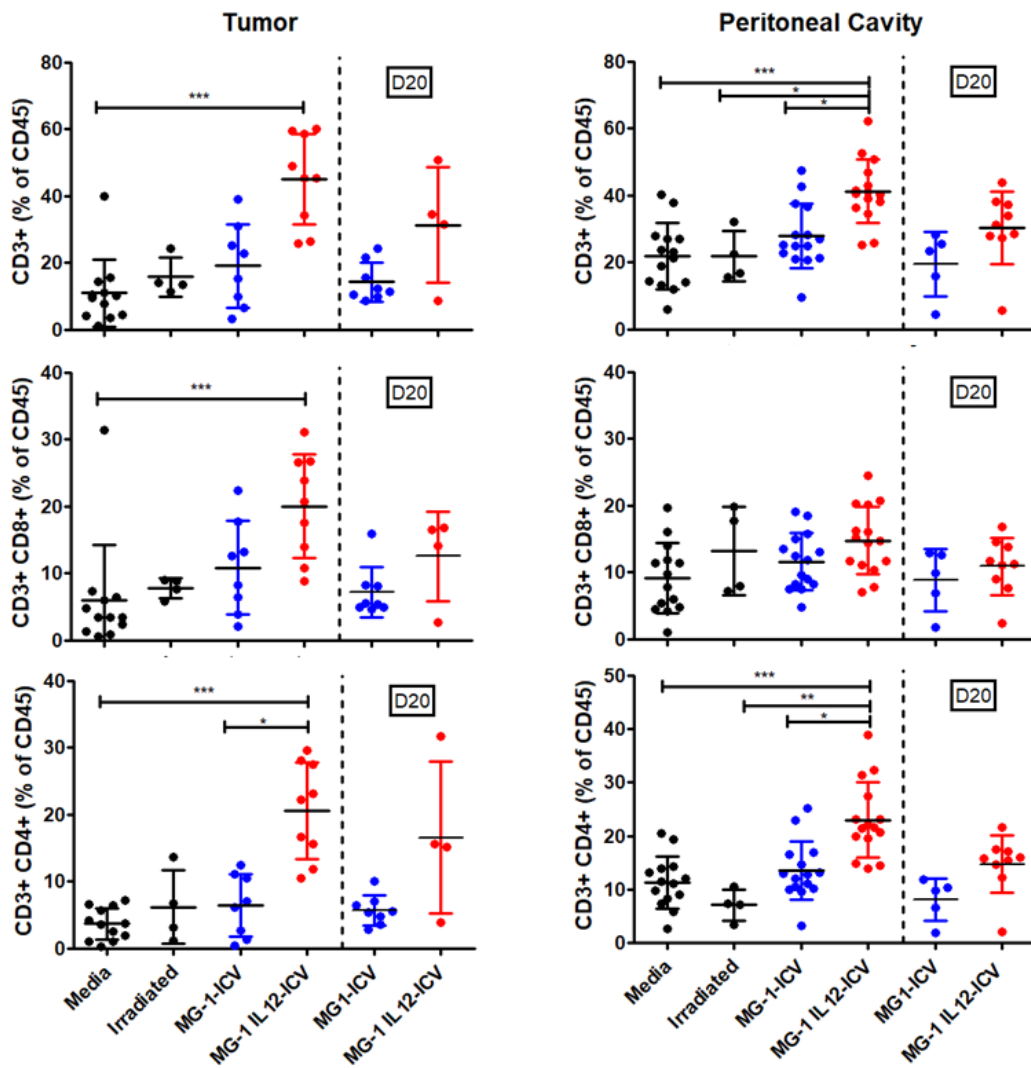
A representative gating strategy for flow cytometric analysis is presented. For all analysis, cells were initially identified by FSC-A and SSC-A, and then gated on singlets (FSC-H vs FSC-A), live cells (unstained by BV510 fluorescing fixable viability stain), and then on CD45<sup>+</sup> cells. Subsequent analysis depended on subsets being analyzed. Here we present analysis of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and CD11c<sup>+</sup> dendritic cells. *FSC: Forward Scatter; SSC: Side Scatter; A: area; H: height. BV: Brilliant Violet.*



## a) Dendritic Cells



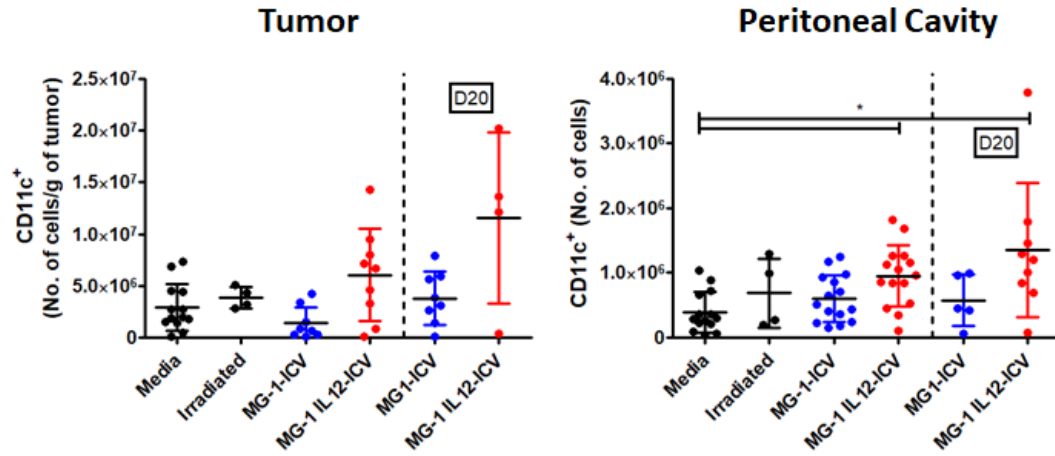
## b) T-cells



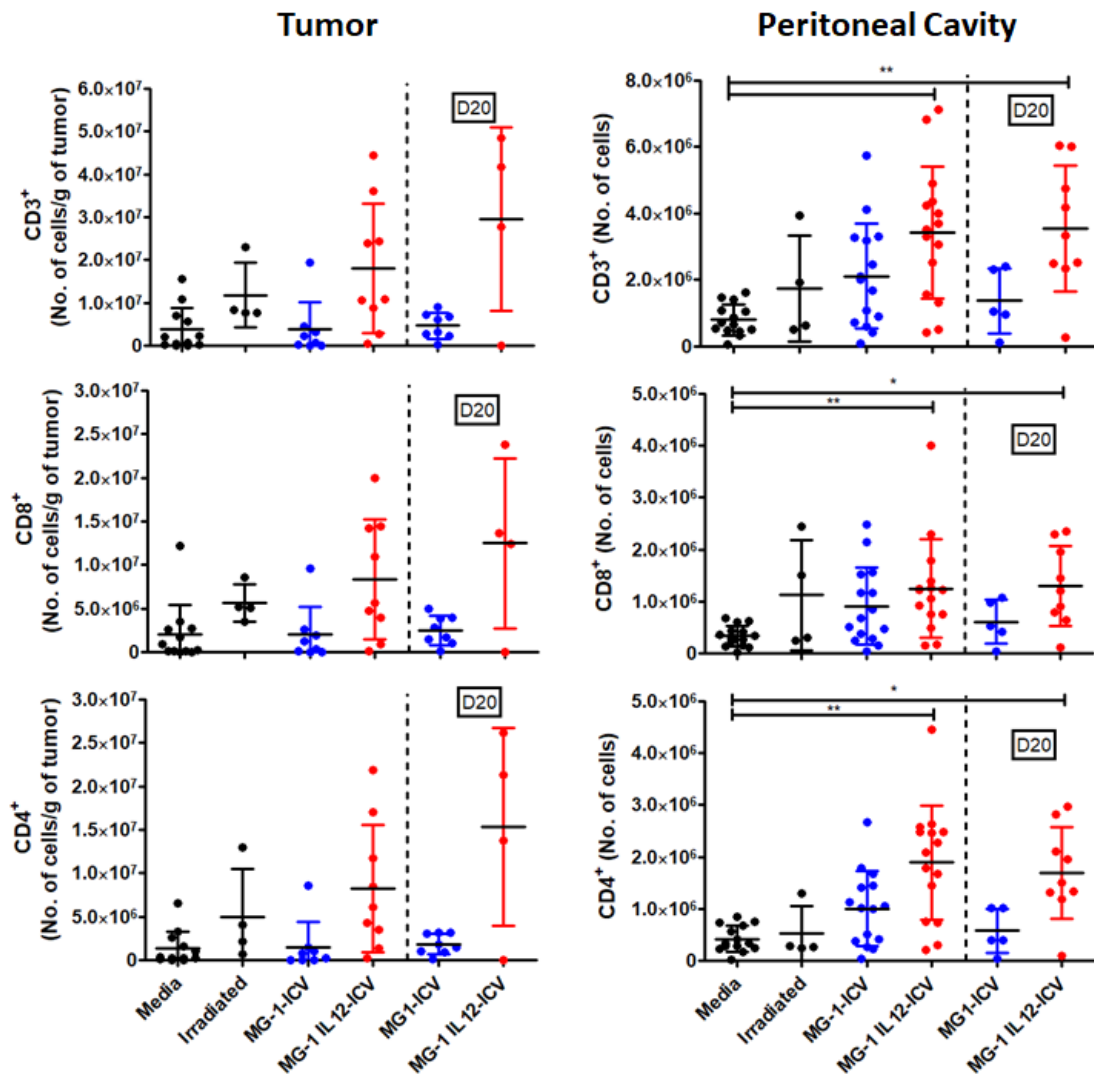
**Figure 12. MG1-IL12-ICV increases the proportion of CD11c<sup>+</sup> dendritic cells and CD8<sup>+</sup> and CD4<sup>+</sup> T-cells in the TME.**

Scatter plots depicting proportion of a) CD11c<sup>+</sup> dendritic cells and b) CD3<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup> T-cells as a percentage of CD45<sup>+</sup> cells in the tumor and peritoneal cavity wash as determined by flow cytometry is presented. Mice were treated according to the schedule in figure 10, and peritoneal cavity wash and tumor were analyzed by flow cytometry after 15 and 20 days post-tumor implantation. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. Results from three pooled experiments. *Mean ± SD* \*p<0.05 \*\*p<0.01 \*\*\*p<0.001. *D20 refers to end-point on day 20.*

## a) Dendritic Cells



## b) T-cells



**Figure 13 MG1-IL12-ICV increases total number of CD11c<sup>+</sup> Dendritic Cells and CD8<sup>+</sup> and CD4<sup>+</sup> T-cells in the TME.**

Scatter plots depicting total number of a) CD11c<sup>+</sup> Dendritic Cells and b) CD3<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup> T-cells in the peritoneal cavity wash and number of cells per gram of tumor are presented. Mice were treated according to the schedule in figure 10, and peritoneal cavity wash and tumor were analyzed by flow cytometry after 15 and 20 days post-tumor implantation. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. Results from three pooled experiments. *Mean ± SD \*p<0.05 \*\*p<0.01*

than in other groups at day 15, and total number of CD11c<sup>+</sup> cells per gram of tumor was also higher at both timepoints in MG1-IL12-ICV-treated mice.

#### 3.4.1.2 T-Cells

Upon investigation, I observed that the increase in DCs within the peritoneal cavity and tumour following vaccination was associated with a significant infiltration of CD3<sup>+</sup> T-cells into the TME of MG1-IL12-ICV-treated mice, comprising on average  $45.0 \pm 13.5\%$  of the CD45<sup>+</sup> cells in the tumor compared to only  $11.1 \pm 10.1\%$  in untreated tumors ( $p < 0.001$ , Kruskal-Wallis with Dunn's Post-test, Figure 12b). In contrast, treatment with *irr* cells or MG1-ICV alone had less than 20% T-cells as a proportion of CD45<sup>+</sup> cells. The relative increase in T-cell infiltration ( $31.4 \pm 17.3\%$ ) was maintained at day 20 compared to MG1-ICV. Similarly, although not statistically significant, the total number of T-cells per gram of tumor tissue was higher in MG1-IL12-ICV treated animals at both time points (Figure 13b). Increased T-cell infiltration into the peritoneal cavity of MG1-IL12-ICV treated mice was also observed at both day 15 ( $3.4 \times 10^6 \pm 2.0 \times 10^6$  cells) and day 20 ( $3.5 \times 10^6 \pm 1.9 \times 10^6$  cells) ( $p < 0.01$ , Kruskal-Wallis with Dunn's Post-Test, Figure 13b). The proportion of CD45<sup>+</sup> cells at day 15 that were CD3<sup>+</sup> was also higher ( $41.3 \pm 9.5\%$ ) in the peritoneal cavity of MG1-IL12-ICV-treated mice compared to mice in all other treatment groups.

Examination of the relative proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltrates revealed an enhanced infiltration of both subsets. In particular, there was a significant increase in the proportion of CD8<sup>+</sup> CD3<sup>+</sup> T-cells in the tumor ( $20.1 \pm 7.7\%$ ) following vaccination with MG1-IL12-ICV when compared to vehicle-treated tumors ( $6.0 \pm 8.3\%$ ,  $p < 0.001$  Kruskal-Wallis with Dunn's Post-Test). Interestingly, we did not observe an increase in the proportion of this subset in the peritoneal cavity in any group at any time point examined, but the total number of CD3<sup>+</sup> CD8<sup>+</sup> T-cells was higher in MG1-IL12-ICV treated-mice at both timepoints compared to vehicle-treated mice (day 15:

$1.3 \times 10^6 \pm 9.5 \times 10^5$  ( $p < 0.01$ ); day 20:  $1.3 \times 10^6 \pm 7.7 \times 10^5$  ( $p < 0.05$ ) compared to vehicle-treated mice ( $3.3 \times 10^5 \pm 2.0 \times 10^5$ ; Kruskal-Wallis with Dunn's Post-test).

CD4<sup>+</sup> T-cell infiltration was similarly increased albeit more pronounced in both the peritoneal cavity and in the tumors, with proportion of CD4<sup>+</sup> infiltrates into the tumor of MG1-IL12-ICV-treated mice ( $20.6 \pm 7.2\%$ ) being significantly higher than both vehicle-treated ( $3.7 \pm 2.4$ ;  $p < 0.001$ ) and MG1-ICV-treated ( $6.5 \pm 4.7\%$ ;  $p < 0.05$ ) mice (Kruskal-Wallis with Dunn's Post-test) at day 15. In contrast to our observation of the CD3<sup>+</sup> CD8<sup>+</sup> subset, proportion of infiltrating CD45<sup>+</sup> cells that were CD4<sup>+</sup> in the peritoneal cavity following MG1-IL12-ICV treatment was significantly higher than in all other groups. The total number of CD4<sup>+</sup> T-cells were also subsequently higher in MG1-IL12-ICV-treated mice at both timepoints (day 15:  $1.9 \times 10^6 \pm 1.1 \times 10^6$  ( $p < 0.01$ ); day 20:  $1.7 \times 10^6 \pm 8.8 \times 10^5$ ,  $p < 0.05$ ) compared to vehicle-treated mice ( $4.2 \times 10^5 \pm 2.5 \times 10^5$ ; Kruskal-Wallis with Dunn's Post-test).

Importantly, although the total number of both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells at day 20 were similar to those observed at day 15, the proportion of CD45<sup>+</sup> cells that were CD8<sup>+</sup> or CD4<sup>+</sup> was lower at the later timepoint suggesting the infiltration of other subsets, such as immunosuppressive cells that may infiltrate the TME at later timepoints to counter the proinflammatory environment.

#### *3.4.2 MG1-IL12-ICV decreases the presence of MDSCs and does not alter the CD8:T-reg ratio.*

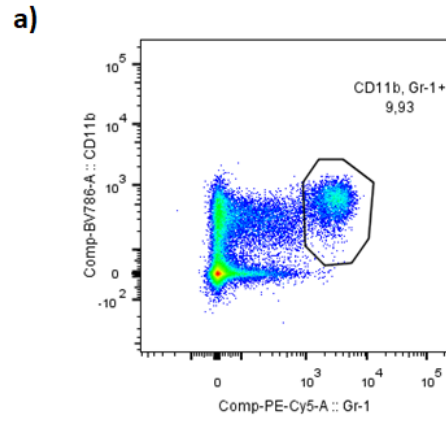
Recruitment of immunosuppressive cells may be one potential mechanism responsible for dampening the initial immune response, therefore I sought to identify whether known immunosuppressive subsets were being recruited in response to MG1-IL12-ICV vaccination. Unexpectedly, MG1-IL12-ICV treatment resulted in a significant reduction in the proportion of MDSCs (CD11b<sup>+</sup> Gr1<sup>hi</sup>) present in the TME ( $7.1 \pm 6.2\%$ ) compared to vehicle-treated tumors ( $24.1 \pm 9.8\%$ ), and the reduction was maintained later in therapy ( $p < 0.05$ , Kruskal-Wallis with Dunn's

Post-test, Figure 14a and b). Although not significant similar trends were also observed in the peritoneal cavity (Figure 14b). The total number of MDSCs were also not significantly different between the groups, although a higher number of MDSCs per gram of tumor was observed in MG1-ICV-treated mice, suggesting that these subsets may play a role in suppressing the efficacy of MG1-ICV (Figure 14c).

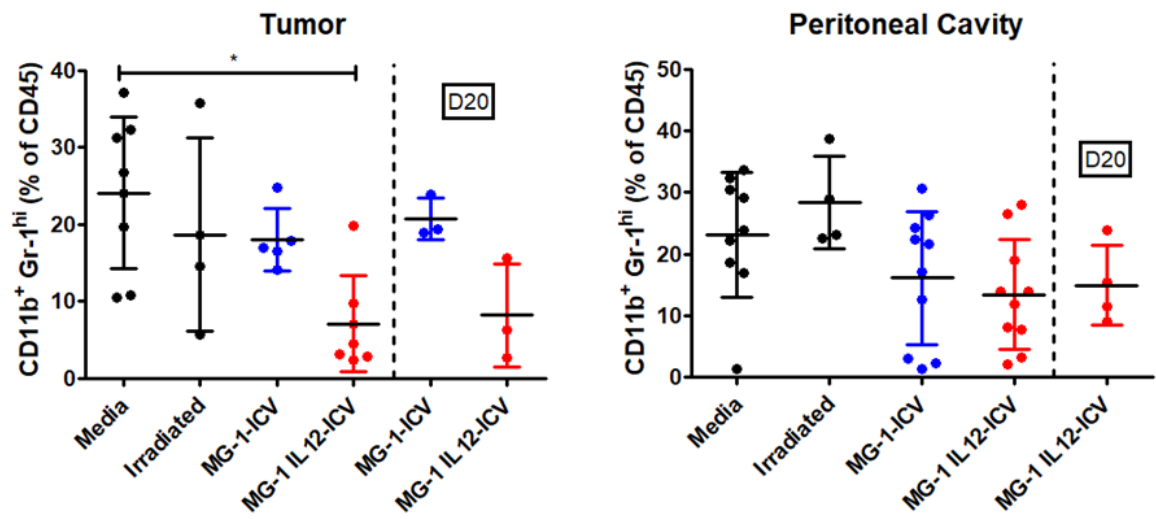
I also observed that despite modest increases in presence of regulatory  $CD4^+ Foxp3^+$  T-cells in the tumour following vaccination with MG1-IL12-ICV, there was no significant change in the CD8:T-reg ratio, a more reliable indicator of immune suppression. In MG1-IL12-ICV treated tumors there were  $1.6 \pm 0.6\%$  T-regs compared to  $0.5 \pm 0.4\%$  in vehicle-treated tumors ( $p < 0.05$ , Kruskal Wallis with Dunn's Post-test, Figure 15). Similarly, no significant change in the CD8:T-reg ratio was observed in the peritoneal cavity despite increases in the number of T-regs in MG1-IL12-ICV-treated mice at the later timepoint. These results suggest that the efficacy of MG1-IL12-ICV is not being abrogated due to the increased presence of these suppressive cells in the TME.

### **3.5 Gene expression profile of formalin-fixed paraffin-embedded tumors from MG1-IL12-ICV treated mice reveal an inflamed immune signature**

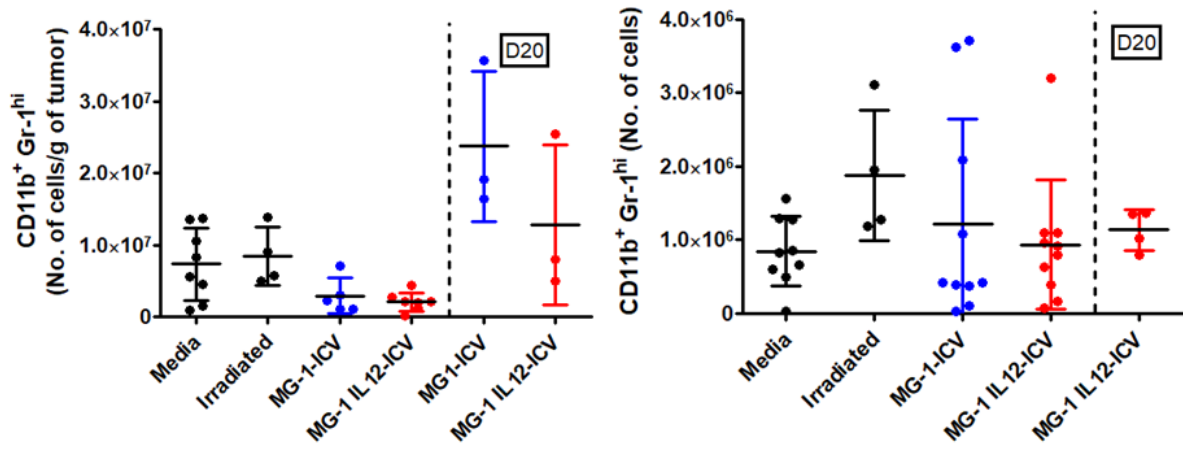
To enable a greater level of immune characterization and to identify relevant immunological changes, RNA was obtained from micro-dissected FFPE tumors and analyzed using the NanoString Immune Profiling Gene Expression Panel as described in Section 2.9 (Figure 16). This approach allows us to quantitate the relative differences in immune related transcripts present across time points and treatment methods in an unbiased manner. Therefore, selected MG1-IL12-ICV-treated tumors resected at early and late timepoints (15 and 20 days post tumor-implantation) and vehicle-treated tumors resected at day 15 were analyzed to identify additional factors that may



b)



c)

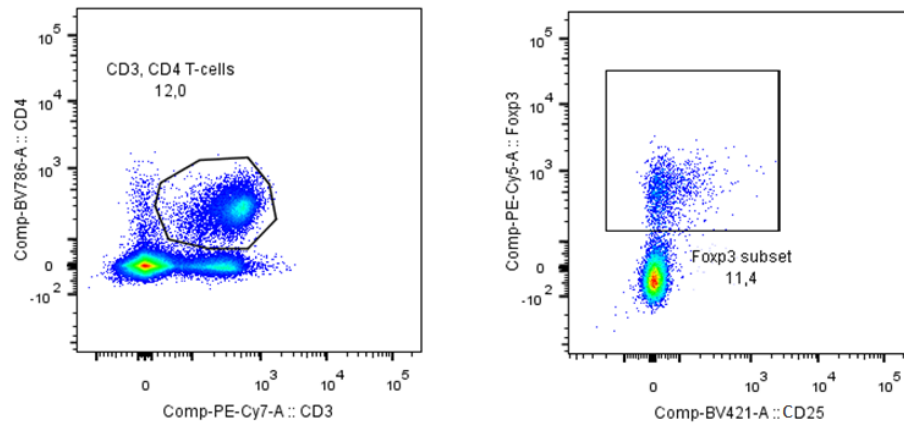




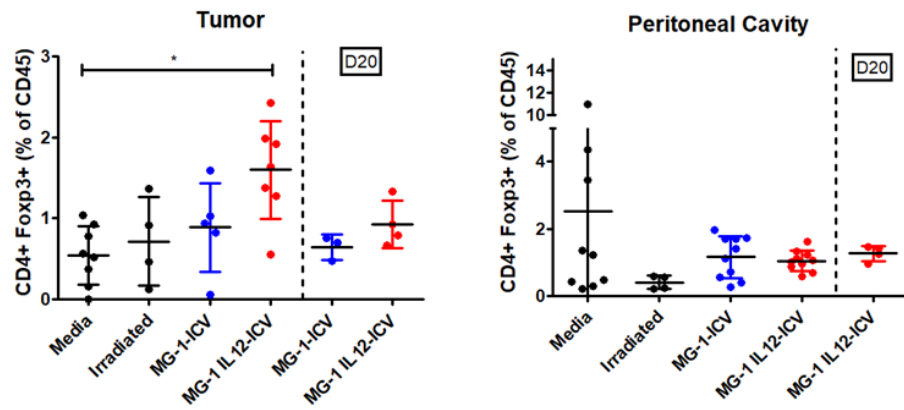
**Figure 14. MG1-IL12-ICV decreases presence of CD11b<sup>+</sup> Gr-1<sup>hi</sup> MDSCs in the TME**

a) Representative dot plot showing gating for CD11b<sup>+</sup> Gr-1<sup>hi</sup> cells. b) Scatter plots depicting proportion of CD11b<sup>+</sup> Gr-1<sup>hi</sup> cells as a percentage of CD45<sup>+</sup> cells in the tumor and peritoneal cavity wash as determined by flow cytometry. c) Scatter plots depicting total number of CD11b<sup>+</sup> Gr-1<sup>hi</sup> cells in the peritoneal cavity and per gram of tumor are presented. Mice were treated according to the schedule in figure 10, and peritoneal cavity wash and tumor were analyzed by flow cytometry after 15 and 20 days post-tumor implantation. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. Results from two pooled experiments. *Mean ± SD* \*p<0.05 *MDSC: Myeloid Derived Suppressor Cells*

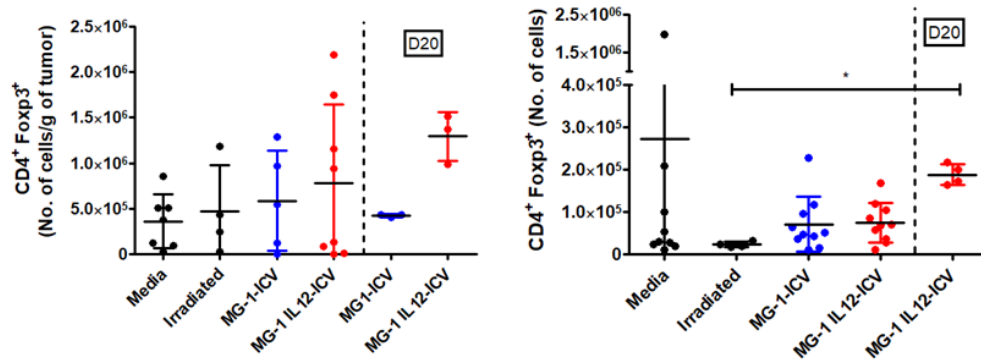
(a)



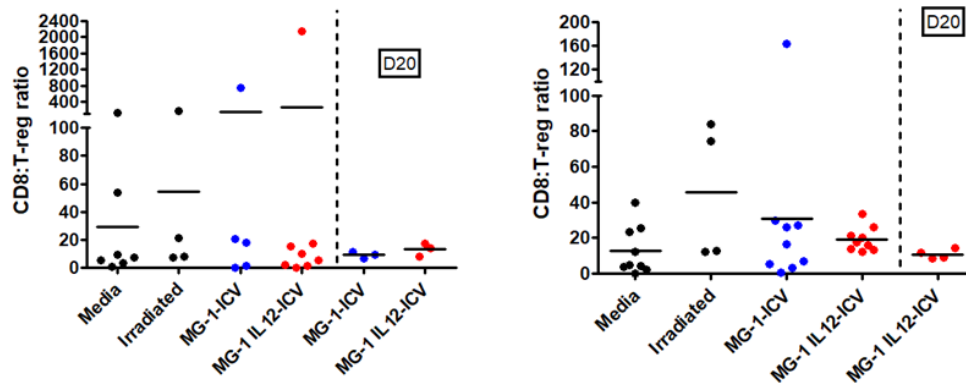
(b)



(c)

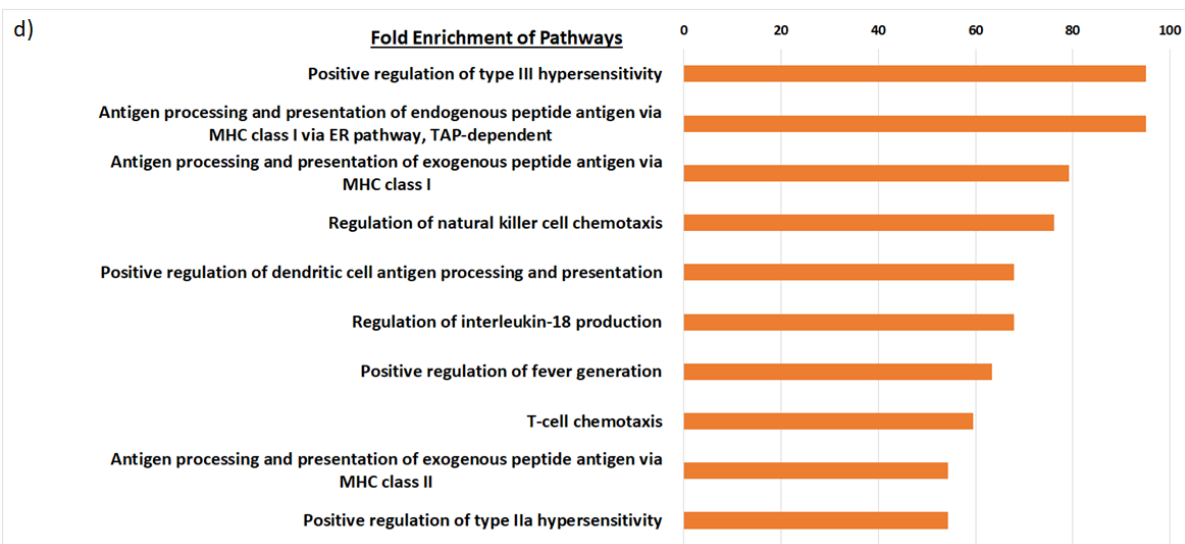
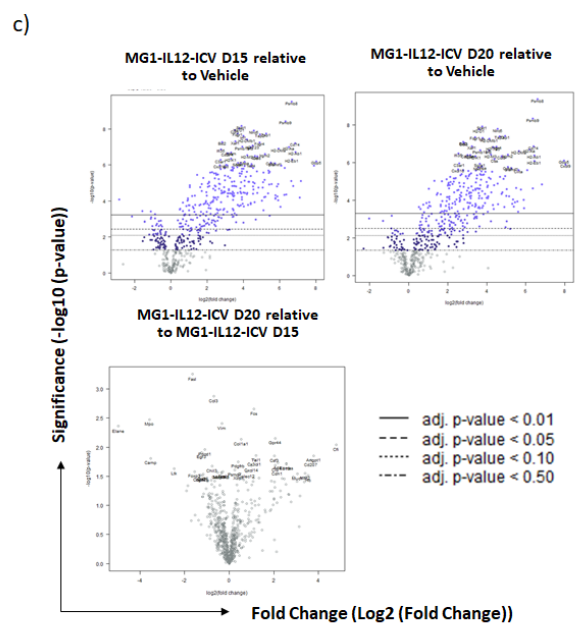
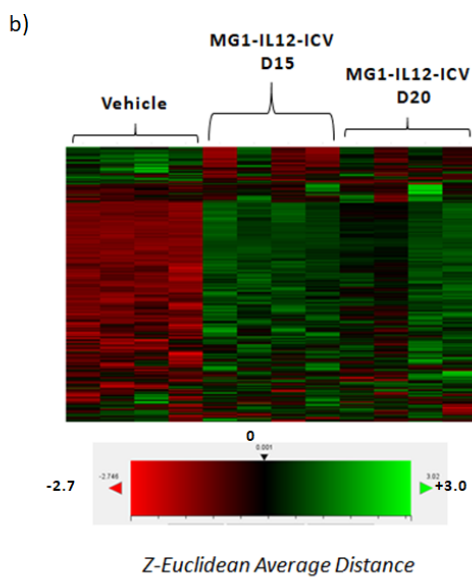
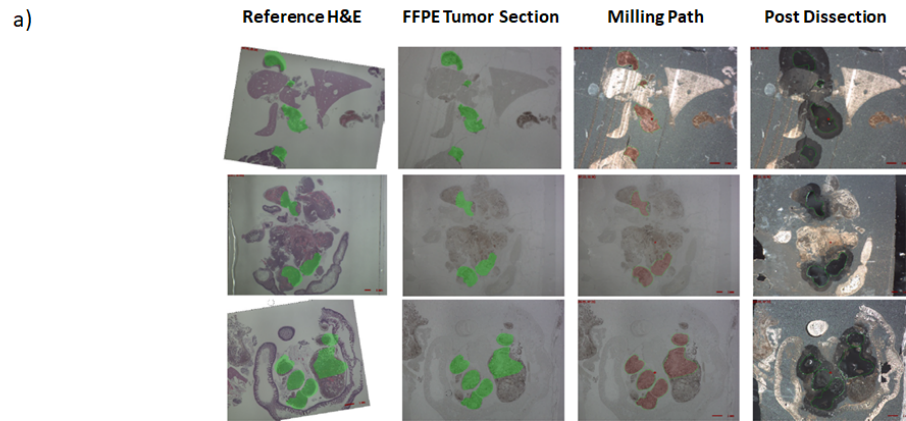


(d)



**Figure 15. MG1-IL12-ICV increases the presence of CD4<sup>+</sup> Foxp3<sup>+</sup> T-regs in the tumor but does not alter the CD8:T-reg ratio.**

a) Representative gating strategy for CD4<sup>+</sup> Foxp3<sup>+</sup> T-regs is shown. Scatter plots depicting b) proportion of CD4<sup>+</sup> Foxp3<sup>+</sup> cells as a percentage of CD45<sup>+</sup> cells and c) total number of CD4<sup>+</sup> Foxp3<sup>+</sup> cells in the tumor and peritoneal cavity wash as determined by flow cytometry. d) Scatter plots depicting the ratio of CD8<sup>+</sup> T-cell: CD4<sup>+</sup> Foxp3<sup>+</sup>. Mice were treated according to the schedule in figure 10, and peritoneal cavity wash and tumor were analyzed by flow cytometry after 15 and 20 days post-tumor implantation. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. Results from two pooled experiments. *Mean ± SD* \*p<0.05



**Figure 16. MG1-IL12-ICV treatment results in an increase in transcripts involved in antigen processing and presentation and NK and T-cell responses.**

- a) Representative pictures of FFPE sections stained with H&E used as reference to identify tumor tissue and corresponding unstained serial section used for microdissection (and subsequent RNA isolation) are shown. Selected tumor area is highlighted in green. Milling path of micro-dissector corresponding to the selected area is shown in red. Non-tumor tissue left on FFPE serial section after microdissection is also shown.
- b) Heatmap of gene expression of micro-dissected FFPE tumors from mice treated with MG1-IL12-ICV (or vehicle) and sacrificed 15 or 20 days after tumor implantation reveals increased presence of immune-related genes in MG1-IL12-ICV-treated tumors. Z-Euclidean average distance was used to calculate fold change.
- c) Volcano plots demonstrate that MG1-IL12-ICV-treated tumors extracted 15 days or 20 days post-tumor implantation have similar gene expression profiles to each other but different to that of vehicle-treated tumors.  $-\log_{10}(\text{p-value})$  vs  $\log_2(\text{fold change})$  is plotted.
- d) Gene Ontology (GO) analysis of genes upregulated in MG1-IL12-ICV-treated tumors extracted 15 days post-tumor implantation revealed upregulation of pathways involved in antigen processing and presentation and NK and T-cell chemotaxis. Fold enrichment of the top 10 highly upregulated pathways identified via GO analysis are shown.

be contributing to the efficacy of treatment, and those that may be playing a role in suppressing the induced immune response. Representative pictures of FFPE serial sections micro-dissected for tumor tissue for subsequent RNA extraction is shown in Figure 16a. Consistent with our flow cytometric phenotyping, this approach revealed significant enrichment of pro-inflammatory immune associated genes in the MG1-IL12-ICV-treated tumors at both early and late time-points compared to vehicle treated tumors (Figure 16b and d), suggesting that MG1-IL12-ICV can heat up the TME and break immune privilege of established tumors. We also expected to identify transcriptional differences between tumors resected at early and late time-points, but no significant differences were observed between these two time-points in MG1-IL12-ICV-treated tumors (Figure 16c).

#### *3.5.1 Genes involved in antigen processing and presentation and NK and T-cell chemotaxis were upregulated in MG1-IL12-ICV-treated tumors.*

Gene ontology analysis of genes that were significantly upregulated (2 log<sub>2</sub> fold change, Benjamin Yekutieli,  $p < 0.05$ ) in MG1-IL12-ICV treated tumors resected at day 15 relative to vehicle-treated tumors were analyzed against the *Mus musculus* genome to identify pathways that were highly upregulated. The top 10 pathways with the highest fold change include pathways involved in antigen processing and presentation of both exogenous and endogenous antigens, as well as those involved in T-cell and NK cell chemotaxis, and regulation of IL-18, which is known to synergize with IL-12 in regulating efficacy (Figure 16d).

#### *3.5.2 Gene correlation analysis identifies T-cells and cytotoxic cells as significantly upregulated in MG1-IL12-ICV-treated tumors.*

NanoString “Cell Scores” are obtained by correlating expression of genes that are specific to certain cell types to predict and identify the presence of these cells within the tumor<sup>231</sup>. Gene

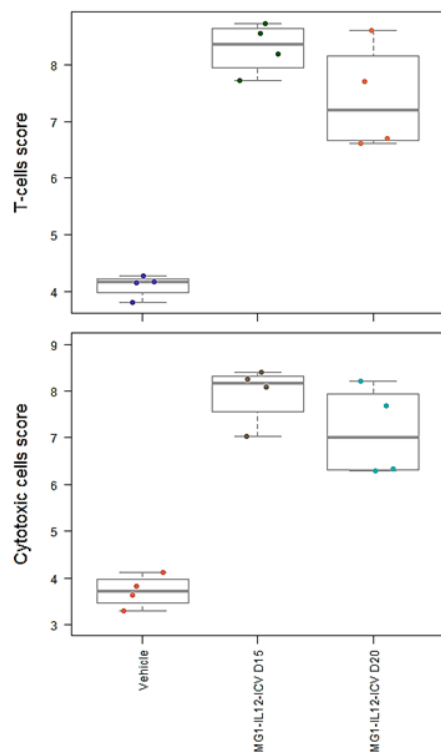
expression levels of two (or more) genes that can serve as specific markers for a cell type should theoretically be perfectly correlated (i.e. a slope of 1 is obtained when gene expression levels are plotted). By evaluating gene expression of cell type-specific marker genes within experimental samples, we can evaluate how significant these correlations are and whether cell type abundance can be reliably predicted using solely gene expression data. In our data set, T-cell and cytotoxic cell-specific gene markers were significantly correlated, and both subsets were found to be more abundant in MG1-IL12-ICV-treated tumors than in vehicle-treated tumors (Figure 17a). Sample gene expression correlation data for genes associated with cytotoxic cells, such as genes coding for granzyme-A/B (*Gzma/b*) and perforin (*Prf1*) is shown in Figure 17b, demonstrating that correlations were close to 1, and that these markers can reliably predict presence of cytotoxic cells. Taken together, the flow phenotyping, gene expression analysis and presence of tumour specific CD8<sup>+</sup> T cell responses in vaccinated animals suggest that MG1-IL12-ICV generates a robust anti-tumour response. However, the survival experiments suggest that this response is ultimately ineffective in controlling tumour growth, a finding which cannot be attributed to changes in major immunosuppressive cell populations.

### **3.6 Immune checkpoint regulators are upregulated following MG1-IL12-ICV therapy**

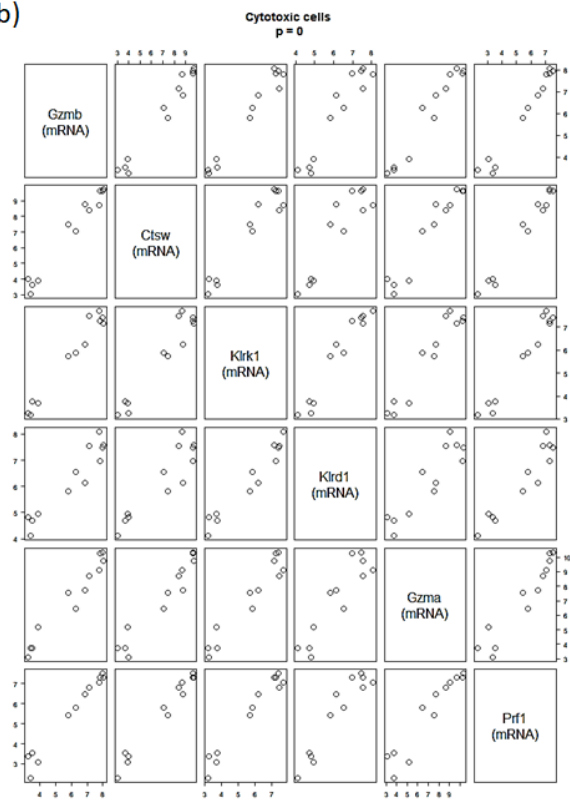
#### *3.6.1 PD-L1 expression is upregulated on CD45<sup>+</sup> immune infiltrates in the peritoneal cavity.*

Given that MG1-IL12-ICV creates a favorable pro-inflammatory immune response but is unable to control tumour growth, I next sought to examine whether the T-cells themselves were intrinsically impaired by investigating changes in the expression of checkpoint molecules. Mice were treated as in Figure 10, and peritoneal cavity wash and tumors were analyzed for PD-L1 and PD-L2 expression. PD-L1 expression on CD45<sup>+</sup> subsets in the peritoneal cavity and in tumors were upregulated in MG1-IL12-ICV-treated animals at day 20 (Figure 18). On average,  $43.4 \pm$

a)



b)

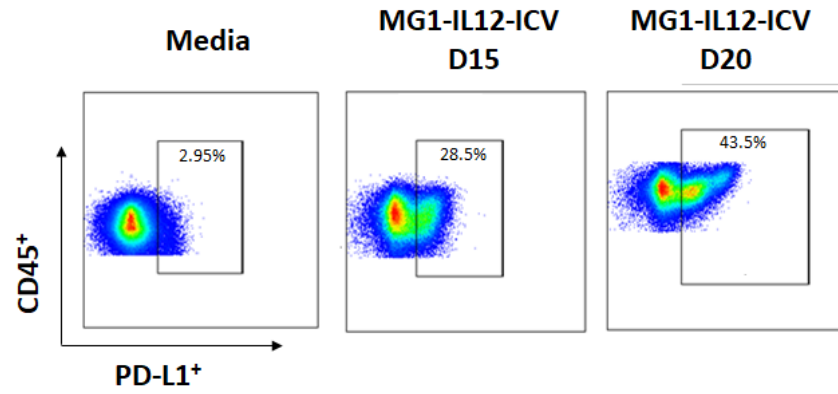




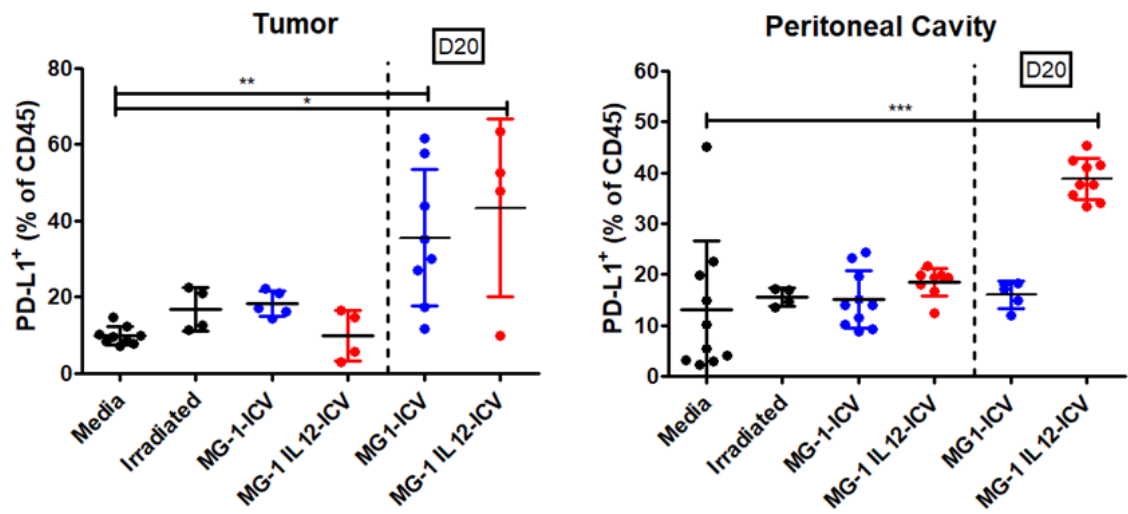
**Figure 17. Gene expression data reveals increased abundance of T-cells and cytotoxic cells in MG1-IL12-ICV-treated tumors.**

a) Box and Whisker plots of NanoString Cell Scores for T-cells and cytotoxic cells are presented. Median is represented by the horizontal line in the middle of the box; the edges of the box represent the first and third quartiles and the tails represent 1.5 times the interquartile range. b) Correlation of normalized gene expression data for genes specific to cytotoxic cells is presented. A strong correlation with a slope close to 1 indicates that candidate genes are specific to a cell type and is stably expressed.

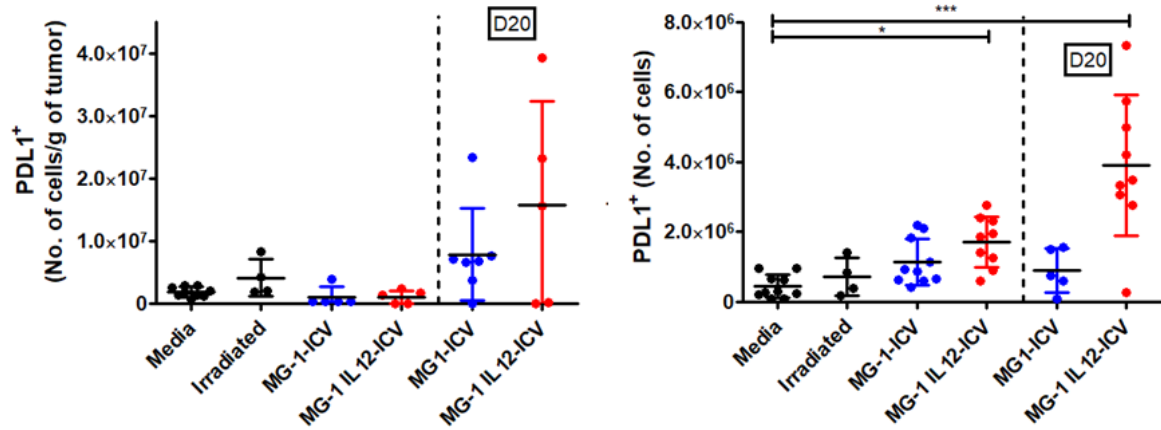
a)



b)



c)



**Figure 18. Temporal increases in the expression of PD-L1 on CD45<sup>+</sup> cells in the TME is observed with repeated treatment with MG1-IL12-ICV**

a) Representative dot plots showing PD-L1 expression on CD45<sup>+</sup> subsets in the peritoneal cavity are presented. Scatter dot plots of b) percentage of and c) total number of cells of CD45<sup>+</sup> cells that are PD-L1<sup>+</sup> is shown. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. *Mean ± SD*. \* $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$ . *PD-L1: Programmed Death Ligand 1*.

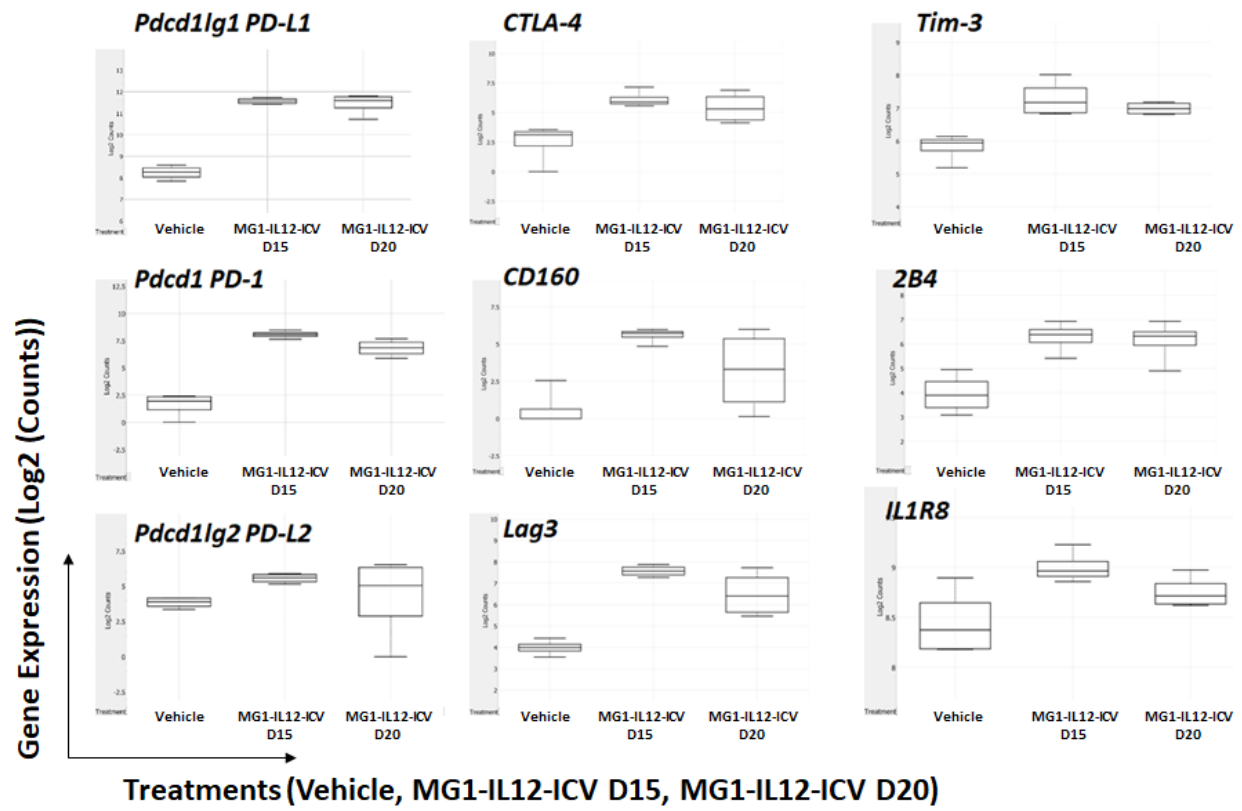
23.4% of CD45<sup>+</sup> subsets was PD-L1<sup>+</sup> in MG1-IL12-ICV treated tumors at day 20 compared to only 10.0 ± 6.6% in MG1-IL12-ICV treated tumors at day 15 (Figure 18b). Similarly, increased number of PD-L1<sup>+</sup> CD45<sup>+</sup> cells per gram of tumor were observed at day 20 in MG1-IL12-ICV-treated mice, although this was not statistically significant (Figure 18c). In the peritoneal cavity, 38.7 ± 4.1% of CD45<sup>+</sup> cells were PD-L1<sup>+</sup> at day 20, while only 18.5 ± 2.7% were PD-L1<sup>+</sup> in the peritoneal at day 15 (Figure 18b). However, the total number of CD45<sup>+</sup> cells in the peritoneal cavity that were PD-L1<sup>+</sup> was higher in MG1-IL12-ICV treated mice at both day 15 ( $1.7 \times 10^6 \pm 7.3 \times 10^5$ ,  $p < 0.05$ ) and day 20 ( $3.9 \times 10^6 \pm 2.0 \times 10^6$ ,  $p < 0.001$ ) compared to vehicle-treated mice ( $4.4 \times 10^5 \pm 3.4 \times 10^5$ ) suggesting that these mechanisms may have been initiated early on during treatment (Kruskal-Wallis with Dunn's Post-test). No such changes in PD-L2 expression was observed (data not shown).

### *3.6.2 Gene expression of other checkpoint molecules are also upregulated in MG1-IL12-ICV-treated tumors.*

While flow analysis pointed to PD-L1 as a viable target, gene expression analysis of other known checkpoints including (PD-L1, PD-1, CTLA-4, Tim-3, Lag3, 2B5, CD160 and IL1R8) revealed that these were also upregulated in MG1-IL12-ICV-treated tumors compared to untreated tumors (Figure 19). Interestingly, PD-L2 gene expression was only modestly upregulated (no change was observed in flow data; not shown). This suggests that single checkpoint blockade may not enhance efficacy of MG1-IL12-ICV, as they may be compensated for by other checkpoints.

### **3.7 Enhanced NK cell activation is reduced following successive ICV treatment**

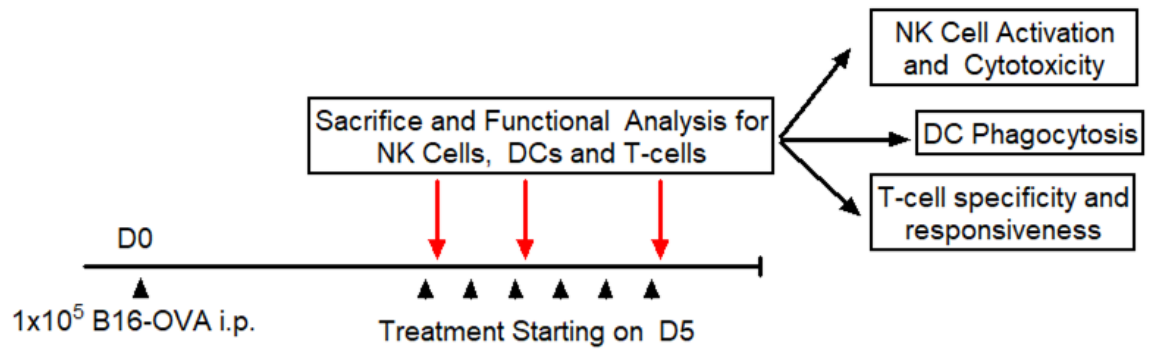
Given that efficacy of MG1-IL12-ICV appears to disappear later in therapy, we began to investigate cellular responses within 18 hrs of vaccination following the first, third and sixth dose of ICV (Figure 20a). Although, my project has focused on the adaptive response, I now sought to



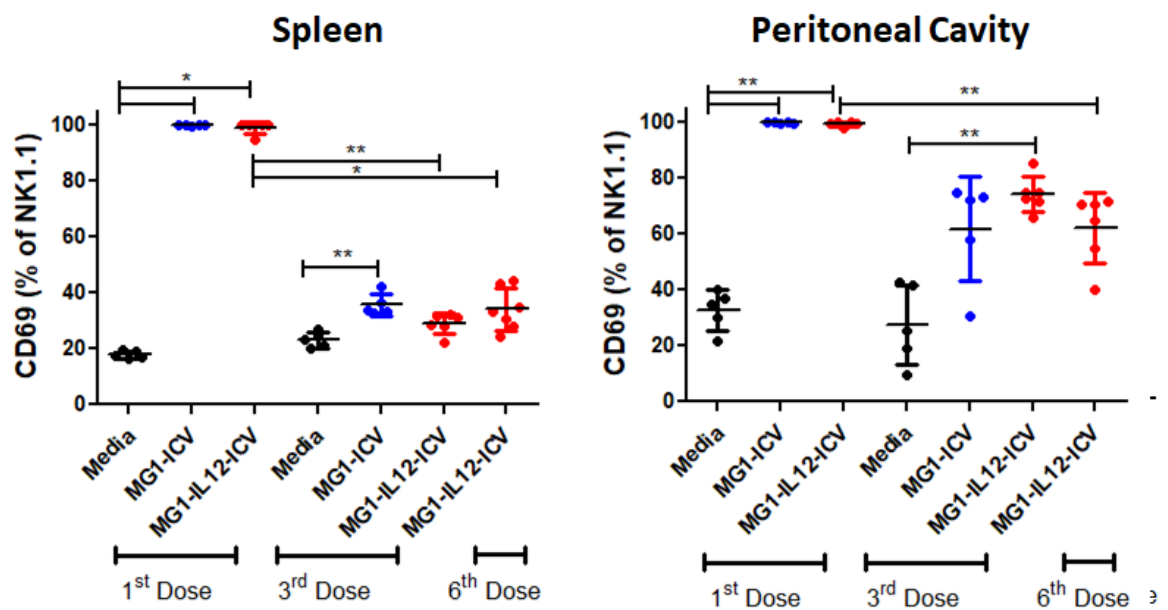
**Figure 19. Gene expression of several checkpoint molecules are upregulated in MG1-IL12-ICV treated tumors.**

Box and Whisker plots of gene expression of PD-L1, PD-1, PD-L2, CTLA-4, Tim-3, Lag 3, CD160, 2B4 and IL1R8 in MG1-IL12-ICV-treated (isolated 15 or 20 days post-tumor implantation) or untreated tumors are presented. Median is represented by the horizontal line in the middle of the box; the edges of the box represent the first and third quartiles and the tails represent 1.5 times the interquartile range.

a)



b)



**Figure 20. MG1-IL12-ICV increases CD69 expression on NK cells but this activation diminishes after multiple doses of ICV**

C57Bl/6 mice were implanted with B16-OVA tumors before receiving 1, 3 or 6 doses of vehicle, MG1-ICV or MG1-IL12-ICV before sacrifice and analysis of cellular responses. a) Treatment regimen is shown. b) CD69 expression on CD45<sup>+</sup> NK1.1<sup>+</sup> cells were examined in spleen and peritoneal cavity wash cells 18hrs post each dose of ICV by flow cytometry. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. *Mean ± SD. DC: dendritic cell; NK: natural killer cells. i.p. intraperitoneal* \*p<0.05, \*\*p<0.01.



investigate NK cell responses, which have previously been shown to drive ICV efficacy, and determine if NK cell responses are similarly diminished over the course of therapy. Additionally, we utilized the B16-OVA cell line to enable identification of tumor-specific T-cell responses and investigated splenic DC activation as well.

### *3.7.1 Increased CD69 expression on NK cells induced by MG1-IL12-ICV is reduced following successive doses.*

Cells from spleen or peritoneal cavity wash of B16-OVA tumor-bearing mice receiving MG1-IL12-ICV, MG1-ICV or vehicle were isolated after the first, third and sixth dose and stained for NK cell phenotype markers and CD69 (Figure 20a). Both MG1-ICV and MG1-IL12-ICV induced CD69 expression on nearly 100% of NK cells found in the spleen ( $p < 0.05$ ) and peritoneal cavity wash ( $p < 0.01$ ) after the first dose (Kruskal-Wallis, with Dunn's Post-Test, Figure 20b). However, this activation was completely abrogated after the third ( $28.9 \pm 3.7\%$ ,  $p < 0.01$ ) and sixth dose ( $34.0 \pm 7.4\%$ ,  $p < 0.05$ ) in the spleen in MG1-IL12-ICV treated mice (Kruskal-Wallis, with Dunn's Post-Test). CD69 downregulation was also observed in MG1-ICV treated mice after dose 3. Since MG1-ICV treated mice and vehicle-treated mice do not survive to the sixth dose, only MG1-IL12-ICV treated data is available for the sixth dose.

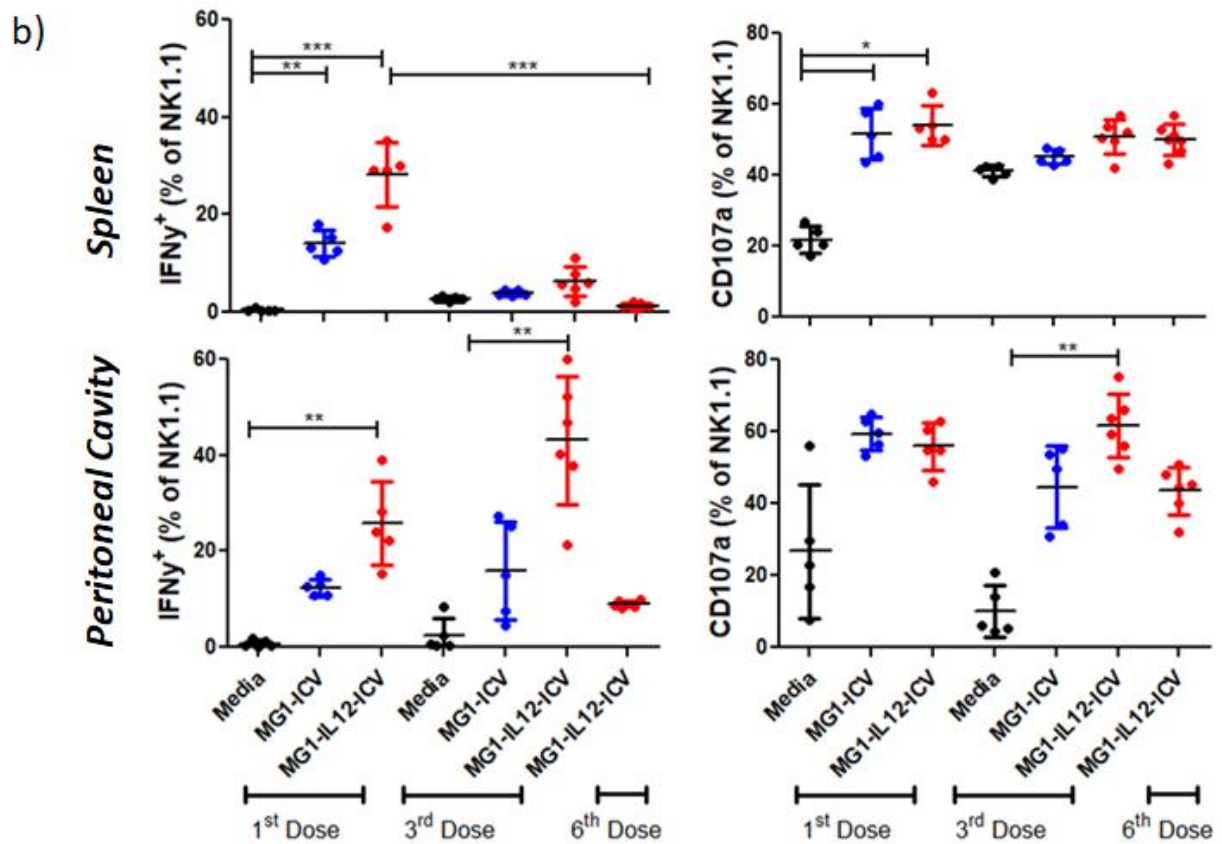
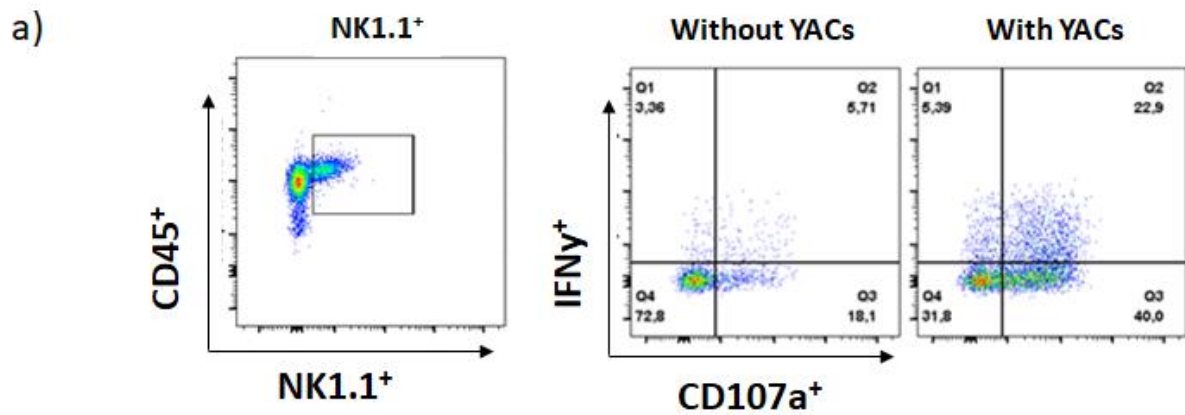
Downregulation of NK cell CD69 expression is also observed in the peritoneal cavity, with  $74.0 \pm 6.3\%$  of NK cells that were CD69<sup>+</sup> after the third dose, and  $62.0 \pm 12.5\%$  were CD69<sup>+</sup> after the sixth dose. Activation after the sixth dose of MG1-IL12-ICV was significantly lower than after the first dose of MG1-IL12-ICV in the peritoneal cavity ( $p < 0.01$ , Kruskal-Wallis with Dunn's Post-test), suggesting that NK cell responses are diminished over the course of therapy. Since CD69 is a surrogate marker for activation, we then investigated responsiveness of NK cells by co-culturing with target YAC-1 cells to better understand the nature of NK cell dysfunction following therapy.

### *3.7.2 Enhanced responsiveness of NK cells to target YAC-1 cells is reduced following successive doses of ICV.*

Cells were harvested as above and then co-cultured with target YAC-1 cells at a 3:1 effector: target ratio, and IFN- $\gamma$  production and degranulation by NK cells was measured (Figure 21a). IFN- $\gamma$  production from NK cells was increased in cells isolated from MG1-IL12-ICV-treated mice after the first dose in both the spleen ( $28.3 \pm 6.5\%$ ) and peritoneal cavity ( $25.8 \pm 8.7\%$ ) compared to cells from vehicle-treated mice (spleen,  $0.5 \pm 0.3$  ( $p < 0.001$ ); peritoneal cavity wash,  $0.7\% \pm 0.8$  ( $p < 0.01$ )) (Kruskal-Wallis with Dunn's Post-test, Figure 21b). Similar to CD69 expression, IFN- $\gamma$  expression was abrogated in the spleen after the first dose. In the peritoneal cavity, IFN- $\gamma$  production was increased after the third dose of MG1-IL12-ICV in response to YAC-1 cells ( $43.1 \pm 13.4\%$ ), but this was abrogated after the sixth dose. Interestingly, increased degranulation (CD107a expression), upon YAC-1 co-culture, was maintained in cells obtained from the spleen and peritoneal cavity after each dose of MG1-IL12-ICV. In the peritoneal cavity, degranulation of NK cells from MG1-IL12-ICV-treated mice was higher than those from the peritoneal cavity of vehicle-treated mice, particularly after the third dose (MG1-IL12-ICV:  $61.7 \pm 8.9\%$  vs Vehicle:  $10.1 \pm 7.7\%$ ,  $p < 0.01$ , Kruskal-Wallis with Dunn's Post-test). Overall, this suggests that MG1-IL12-ICV is more effective at activating NK cells and maintaining this activation than MG1-ICV, as we have previously reported. But, more importantly, this reveals that subsequent doses of ICVs are not as effective as early doses in activating NK cell responses and may identify why MG1-IL12-ICV does not provide durable cures despite early tumor control.

### **3.8 Early splenic dendritic cell activation is abrogated after the first dose of ICV**

Given that activation of innate responses maybe abrogated after successive doses, I utilized a similar treatment schedule to investigate DCs from spleens of treated mice after each dose of ICV.



**Figure 21. Early doses of MG1-IL12-ICV increases responsiveness of NK Cells to target YAC-1 cells.**

a) Representative flow plots for gating of NK cells and IFN- $\gamma$  production and CD107a surface expression with and without YAC-1 cells are shown. b) Scatter plots of NK cell responsiveness (CD107a and IFN- $\gamma$  expression) after multiple doses of MG1-IL12-ICV in the spleen and peritoneal wash after co-culture with target YAC-1 cells (*Target: Effector ratio of 1:3*) for 4 hrs in the presence of Golgi Transport Inhibitor (Monensin) and anti-CD107a antibody before staining for surface markers and intracellular IFN- $\gamma$  is shown. Statistical analysis was conducted using Kruskal Wallis with Dunn's Post-test. *Mean  $\pm$  SD. IFN: Inteferon* \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\* $p < 0.001$ .

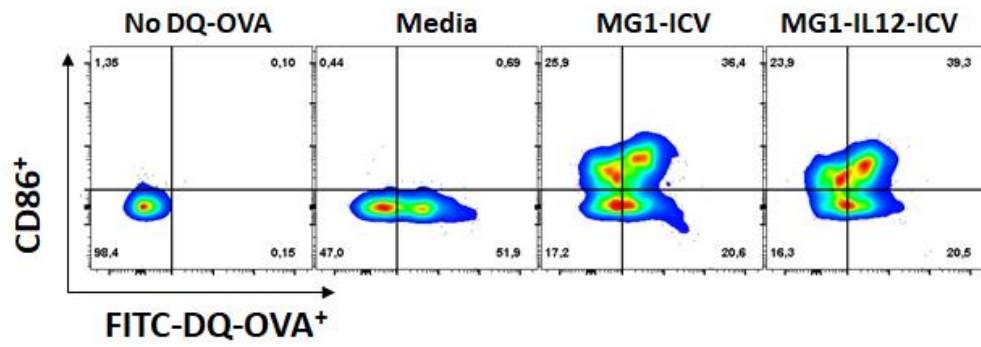
Splenocytes were harvested from B16-OVA tumor bearing mice receiving vehicle, MG1-ICV or MG1-IL12-ICV after the first, third and sixth dose of ICV and incubated for 1 hr with DQ-OVA. Fluorescence of the normally quenched fluorophore increases following phagocytosis and processing enabling direct quantification of the antigen processing capacity by flow cytometry. Despite the increase in DCs found in the TME, no significant difference in DQ-OVA phagocytosis was observed between the groups after different doses of ICV suggesting that their intrinsic phagocytic capabilities may not be enhanced by MG1- or MG1-IL12-ICV. However, DQ-OVA phagocytosis and processing was lower in MG1-IL12-ICV groups after the third ( $31.0 \pm 2.2\%$ ,  $p < 0.01$ ) and sixth ( $42.9 \pm 6.4\%$ ,  $p < 0.05$ ) doses of ICV, compared to the first dose of MG1-IL12-ICV ( $62.2 \pm 3.5\%$ ) (Kruskal-Wallis with Dunn's Post-test, Figure 22). In addition to antigen uptake and presentation by DCs activation of T cells requires engagement of co-stimulatory molecules such as CD86. Interestingly, CD86 expression on CD11c<sup>+</sup> dendritic cells from MG1-IL12-ICV-treated mice ( $59.0 \pm 4.9\%$ ,  $p < 0.01$ ) and MG1-ICV-treated mice ( $56.1 \pm 4.4$ ,  $p < 0.05$ ) was significantly higher than in vehicle-treated mice ( $1.0 \pm 0.2\%$ ; Kruskal-Wallis with Dunn's Post-test; Figure 22). However, activation was completely abrogated when cells were harvested after the third and sixth doses. These findings suggest that activated DCs are present in the spleen at least at early time-points and may be involved in generating specific T-cell responses.

### **3.9 MG1-IL12-ICV induces anti-tumor and anti-viral CD8<sup>+</sup> T-cell responses**

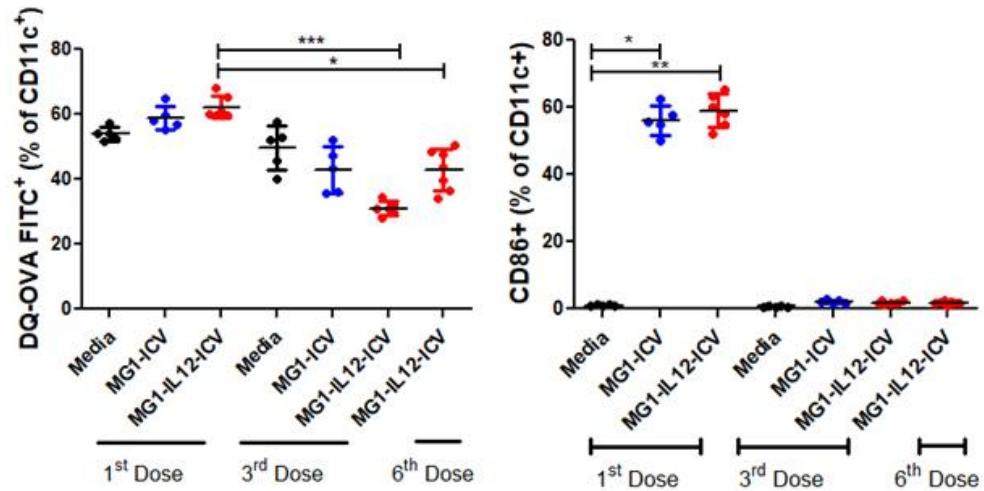
#### *3.9.1 MG1-IL12-ICV generates H2kb-SIINFEKL Tetramer<sup>+</sup> CD8<sup>+</sup> T-cells*

Provided that we could detect an increase in DC infiltration, antigen uptake and activation, I next sought to determine whether this response was associated with an anti-tumour T-cell response in MG1-IL12-ICV vaccinated animals. Using a tetramer containing the immunodominant epitope of ovalbumin (H2kb-SIINFEKL tetramer) we assessed the presence of tumour targeting CD8<sup>+</sup> T cells

a)



b)



**Figure 22. Enhanced activation of splenic DCs by ICV is abrogated after the first dose.**

CD86 expression and DQ-OVA phagocytosis and processing after 1 hr of co-culture with DQ-OVA was measured by flow cytometry. a) Representative flow plots and b) scatter plots depicting CD86 expression or DQ-OVA expression in CD11c<sup>+</sup> cells is shown. Kruskal Wallis with Dunn's Post-test. *Mean ± SD*. \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$

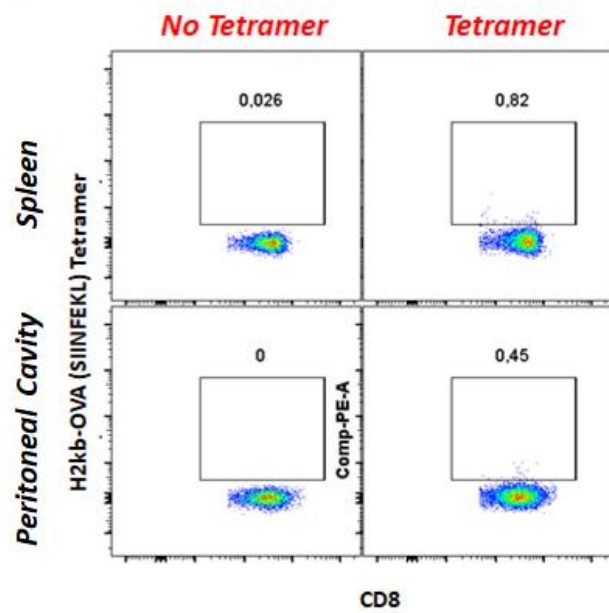
present in the spleen and peritoneal cavity after the third dose of treatment (13 days following tumour implantation) as shown in Figure 23. Notably, MG1-IL12-ICV vaccination resulted in  $0.41 \pm 0.21$  % (spleen) and  $0.26 \pm 0.26$ % (peritoneal cavity) H2kb- SIINFEKL tetramer<sup>+</sup> compared to only  $0.08 \pm 0.09$ % (spleen) and  $0.13 \pm 0.18$ % (peritoneal cavity) in vehicle-treated mice ( $p < 0.05$ , Kruskal-Wallis with Dunn's Post-test, Figure 23b) indicating the presence of specific anti-tumour response. However, since tetramer staining does not capture the functionality of these tumor reactive cells we next sought to address this.

### *3.9.2 Ex-vivo stimulation with a viral peptide induces robust IFN- $\gamma$ production from CD8<sup>+</sup> T-cells from mice treated with MG1-IL12-ICV*

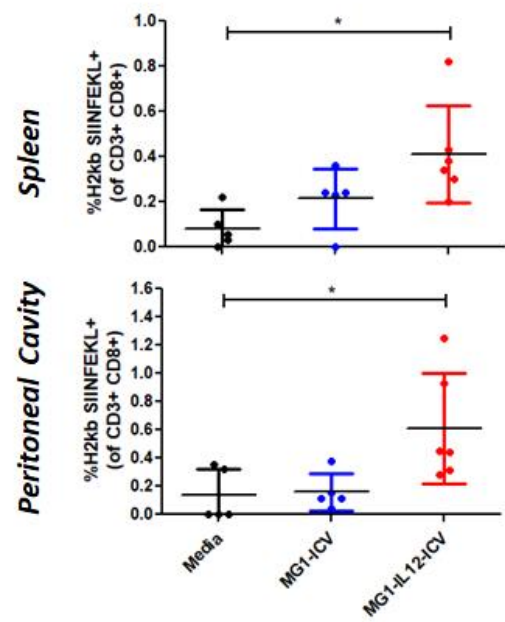
To test functionality, we directly stimulated cells *ex-vivo* with H2kb- restricted SIINFEKL or VSVn peptides before analysis of IFN- $\gamma$  production (Figure 24 a). As expected, a robust anti-viral VSVn response was detected in cells obtained from the spleen and peritoneal cavity of mice vaccinated with either MG1-ICV or MG1-IL12-ICV. In particular, in the peritoneal cavity, MG1-ICV induced  $34.4 \pm 8.6$  % and MG1-IL12-ICV induced  $46.6 \pm 4.1$ % IFN- $\gamma$  production from CD8<sup>+</sup> T-cells when stimulated *ex-vivo* with VSVn (Figure 24b). Interestingly, no detectable response to SIINFEKL was observed when stimulated *ex-vivo* suggesting that the tetramer positive cells may likely be non-functional and therefore ineffective in clearing tumour at this time point.



a)

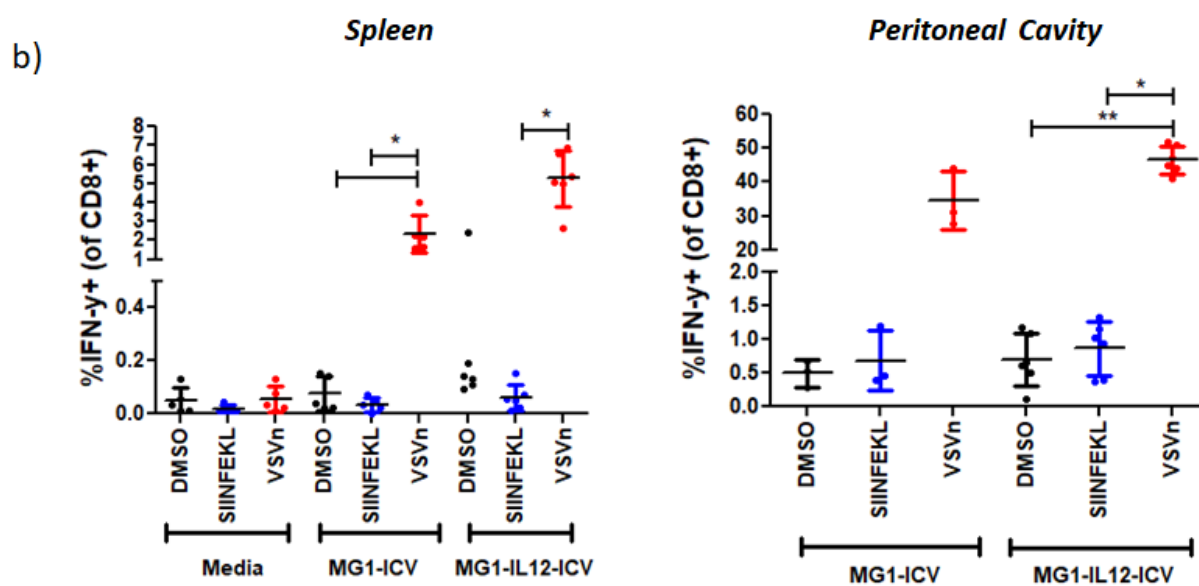
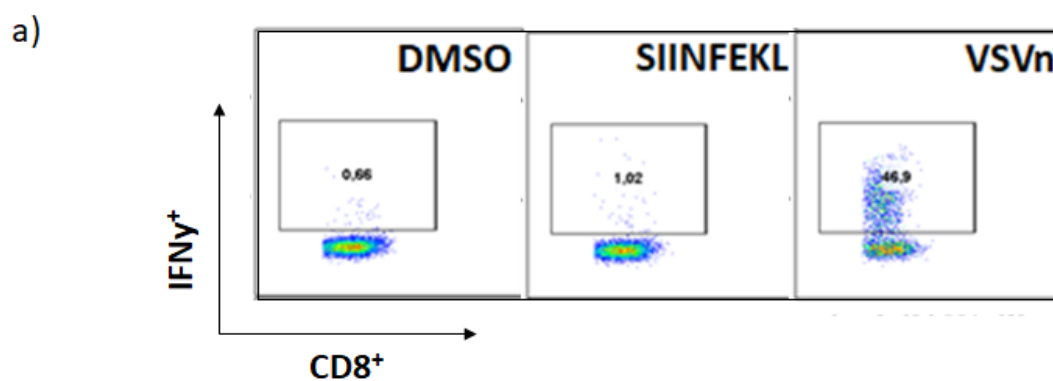


b)



**Figure 23. Repeated dosing with MG1-IL12-ICV generates tumor specific CD8<sup>+</sup> T-cells**

a) Representative scatter plot for tetramer staining is shown. b) Scatter plot showing H2Kb-OVA (SIINFEKL) tetramer positive cells as a percentage of CD8<sup>+</sup> T-cells in spleen and peritoneal cavity of mice bearing B16-OVA tumors and treated with 3 doses of ICV (13 days post infection). Kruskal Wallis with Dunn's Post-test. *Mean ± SD* \* $p < 0.05$



**Figure 24. Treatment with MG1-IL12-ICV generates a robust anti-viral CD8<sup>+</sup> T-cell response**

Cells from spleen and peritoneal cavity of treated mice were stimulated with H2kb-restricted peptides (SIINFEKL, VSVn or DMSO) for 6 hrs with Brefeldin A (for 5 hr) before staining for IFN- $\gamma$ . a) Representative flow plots of IFN- $\gamma$  expression in CD8<sup>+</sup> T-cells upon *ex-vivo* stimulation with the indicated stimuli is shown. b) Scatter plot showing percentage of CD8<sup>+</sup> T-cells positive for IFN- $\gamma$ . Kruskal Wallis with Dunn's Post-test. *Mean  $\pm$  SD. IFN: Inteferon* \*  $p < 0.05$ , \*\* $p < 0.01$

#### 4. Discussion

Oncolytic virotherapy, whole cell tumor vaccines and cytokine-therapy are three promising therapeutic options for the treatment of cancer. All three approaches have proven to be effective in pre-clinical models and several have been shown to have efficacy in clinical trials. However, as discussed before, these therapies have limitations when delivered as single therapy. In contrast, MG1-IL12-ICV combines the potent oncolytic properties of the Maraba MG-1 oncolytic virus with a personalized antigen repertoire of autologous whole cell vaccines and the immune-stimulating properties of IL-12 all in a single therapy<sup>66</sup>. Previously, our group has shown MG1-IL12-ICV to be a promising approach for the treatment of PC and provides better tumor control when compared to treatment with MG1 or MG1-IL12 virus alone, *irr* autologous tumor cells alone, MG1-ICV alone or MG1-ICV delivered simultaneously with recombinant IL-12.

MG-1 can preferentially infect and kill cancer cells leaving normal tissue unaffected, while inducing a robust anti-viral and anti-tumor immune response<sup>67,159</sup>. However, the degree to which MG-1 can infect and replicate in different tumor types is variable, and infectivity of tumors *in-vivo* is significantly lower than observed *in-vitro*<sup>182</sup>. *Ex-vivo* infection of tumors, as during the preparation of ICVs, can overcome this barrier by enhancing infection and replication of viruses in tumors and allowing *in-vivo* replication and transgene expression upon delivery<sup>182</sup>. In fact, efficacy of MG1-IL12-ICV is abrogated when a replication deficient MG1-IL-12 virus is used<sup>219</sup>. Rhabdovirus (MG-1 and the closely related VSVΔ51) based-ICVs have proven to be effective in leukemic and solid tumor models (such as B16F10) but limitations exist<sup>166,182</sup>. The inclusion of the potent IL-12 cytokine transgene into the MG-1 backbone further potentiates the efficacy of the ICV platform, with complete cures observed in the CT26lacZ model of PC<sup>66</sup>. However, limited

efficacy in more resistant tumor models, suggested that a more thorough investigation into the nature of this vaccine was warranted.

Our initial investigation of MG1-IL12-ICV revealed an essential role for the enhanced activation of innate compartments of the immune system (including NK and DCs). This finding was not surprising given the known role of IL-12 as a regulator of NK cell activity<sup>186</sup>. However, during these investigations we observed that depletion of CD8<sup>+</sup> T-cells impaired vaccine efficacy, suggesting an involvement for the adaptive arms of the immune response<sup>66,219</sup>. However, the nature of this response was not elucidated. Additionally, although the IL-12 expressing vaccine can control tumor growth at early stages in treatment-refractory models (such as B16F10), long-term durable cures have rarely been achieved. This suggests that immunosuppressive mechanisms in the TME of MG1-IL12-ICV-treated animals may arise over the course of therapy. Alternatively, mechanisms that render the vaccine ineffective may also be playing a role.

My work undertaken during this thesis has contributed significantly to our understanding of the nature of CD8<sup>+</sup> T-cell responses generated in response to MG1-IL12-ICV therapy, and their role in providing protection in mice bearing established peritoneal tumors. Furthermore, an exhaustive analysis of the TME revealed that MG1-IL12-ICV was indeed effective in creating a robust pro-inflammatory environment within the TME, while preventing the expansion of immunosuppressive cell types such as MDSCs. However, increased presence of checkpoint molecules was observed in the TME. This suggested a potential avenue to explore for additional combination therapies that could synergize with MG1-IL12-ICV. In addition, these studies determined that successive doses with MG1-IL12-ICV also results in attenuated innate responses which may also contribute to reduced vaccine efficacy.

#### **4.1 Delayed tumor growth following prime-boost vaccination is not dependent on anti-tumor CD8<sup>+</sup> T-cell responses.**

Generation of a robust adaptive immune response, particularly a CD8<sup>+</sup> T-cell response, has been shown to be required for the efficacy of cancer therapies in both pre-clinical and clinical studies<sup>96–100</sup>. Previously, depletion of CD8<sup>+</sup> T-cells in mice bearing peritoneal B16F10 tumors rendered treatment with MG1-IL12-ICV ineffective. However, the nature of the role of CD8<sup>+</sup> T-cells in the efficacy of MG1-IL12-ICV was not elucidated.

To avoid the potential impact of the known immunosuppressive TME present in established tumors, we elected to utilize a tumor naïve (or prophylactic treatment model) to better understand how MG1-IL12-ICV can prime adaptive immune responses on its own. To this end, I vaccinated mice with two doses of *irr* B16F10 cells or MG1-IL12-ICV or a heterologous combination of *irr* cells followed by MG1-IL12-ICV (prime-boost) before implanting syngeneic B16F10 tumors *sc.* one week post-second vaccination (Figure 4). In this context, we observed that whole cell autologous tumor vaccines could delay tumor growth regardless of adjuvanticity. However, the prime-boost vaccination strategy was the best at delaying tumor growth in a subset of mice, suggesting greater potency in generating an adaptive immune response. Interestingly, vaccination controlled tumor growth in only a subset of mice and most mice eventually grew out tumors. Although the delayed tumor growth may be attributed to the cancer cells undergoing the three stages of cancer immunoediting (elimination, equilibrium, and escape) at different rates, eventually evading all anti-tumor mechanisms, it is unclear which mechanisms are at play<sup>15</sup>. This also suggests that protective immune responses generated by these vaccines can be improved as they are currently unable to completely eradicate tumor growth.

Confirming the generation of an adaptive response in prime-boost animals, an *in-vivo* CTL assay showed that H2kb-restricted p15E tumor antigen loaded splenocytes were preferentially killed in mice receiving the heterologous regimen and somewhat in mice treated with two doses of MG1-IL12-ICV. Since the splenocytes were loaded with H2kb-restricted peptides, it suggests that the killing would primarily be mediated by CD8<sup>+</sup> T-cells that can recognize the p15E peptide being presented on MHC-I on the loaded splenocytes. This insinuated that perhaps delayed tumor outgrowth is not directly co-related with the generation of a CD8<sup>+</sup> T-cell response, as both MG1-IL12-ICV and *irr* cells-treated mice also delayed tumor outgrowth.

In a B16-OVA model, where MG1-IL12-ICV and heterologous prime-boost provide similar protection against tumor growth in naïve animals, the presence of modest levels of tumor-reactive CD8<sup>+</sup> T-cells was observed in peripheral blood of heterologous-treated mice. However, no correlation could be observed with mice that delayed or rejected tumor growth and those that exhibited a measurable anti-tumor CD8<sup>+</sup> T-cell response. This suggested that other adaptive mechanisms such as a CD4<sup>+</sup> T-cell response may be playing a role in generating protective responses.

In fact, several studies have demonstrated that CD4<sup>+</sup> T-cells can reject tumor growth in a CD8<sup>+</sup> T-cell independent manner<sup>232-234</sup>. Using transgenic MataHari and Marilyn murine models, each containing pure populations of antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T-cell, respectively, Perez-diez *et al.* demonstrated that CD4<sup>+</sup> T-cells were more effective at controlling tumors *in-vivo* than corresponding CD8<sup>+</sup> T-cells despite limited indication of *in-vitro* cytotoxicity<sup>232</sup>. CD8<sup>+</sup> T-cell independent CD4<sup>+</sup> T-cell mediated tumor control has been posited to synergize with NK cells, NKT cells or macrophages in different studies in both MHC-II-dependent and independent manners<sup>232,233,235</sup>. While the role of CD4<sup>+</sup> T-cells in our solid tumor prophylactic models have not



been elucidated, more studies in this regard is warranted considering findings suggesting that a CD8<sup>+</sup> T-cell response may aid but does not directly correlate with tumor growth delays when treated with ICVs. In fact, a recent unpublished study on ICVs in prophylactic leukemia models has demonstrated that depletion of CD4<sup>+</sup> T-cells significantly abrogates the efficacy of vaccines (more than the depletion of CD8<sup>+</sup> T-cells), and depletion of both T-cell subsets completely abrogated the efficacy<sup>236</sup>. Similarly, Labbe *et al.* demonstrated that in an IL-12 expressing leukemia vaccine, efficacy was primarily mediated by CD4<sup>+</sup> T-cells, and that depletion of CD8<sup>+</sup> T-cells alone did not abrogate efficacy<sup>237</sup>.

Alternatively, persisting activated NK cells at time of tumor challenge or innate cells exhibiting memory-like features or synergizing with adaptive responses may play a role in controlling tumor growth in these models. It has been previously demonstrated that cytokine-induced memory-like NK cells can persist *in-vivo* for extended periods and maintain their enhanced cytotoxic potential<sup>238–240</sup>. Jing *et al.* demonstrated that IL-12/15/18-preactivated NK cells underwent rapid proliferation at sites of tumor challenge utilizing IL-2 produced by CD4<sup>+</sup> T-cells<sup>240</sup>. Since, our tumor challenge is only 7 days post-vaccination, it can be posited that a pre-activated NK cell pool may contribute to vaccine efficacy. Furthermore, Bouwer *et al.* showed that efficacy of their DC vaccination strategy was dependent on NK cell IFN- $\gamma$  production during challenge, and our vaccination may similarly be dependent on heightened NK cell responses. Another study demonstrated that after vaccination, host DCs were able to continuously generate tumor-reactive NK cells leading to long term NK-cell mediated protection against tumor challenge without the generation of a memory pool of NK cells<sup>68,241</sup>. Whether similar mechanisms maybe present in our models is not known and future studies to evaluate these possibilities are required.

However, it must be noted that our assays are targeted at detecting CD8<sup>+</sup> T-cell responses to a specific antigenic epitope in both the *in-vivo* CTL and *ex-vivo* peptide stimulation assays. While T-cell assays targeting these epitopes may be of high predictive value in therapeutic strategies encoding these specific TAAs, it may not be the ideal platform or have the sensitivity to detect anti-tumor immune responses generated by a whole cell tumor vaccine. This is particularly important, given the target of whole cell vaccines to elicit a T-cell response to a variety of neoantigens, which may or may not contain the epitope being tested for. To overcome this issue, *ex-vivo* stimulation with IFN- $\gamma$ -treated whole tumor cells may allow detection of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in peripheral blood of vaccinated mice. However, depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets during vaccination and after tumor implantation may be the ideal way to quantify the contribution of different adaptive subsets to the delay of tumor growth in these models. While this model provides an exciting tool for the exploration of adaptive responses generated by ICV platforms (allowing us to avoid an established immunosuppressive TME and the engagement of an enhanced innate response), it became gradually clear that treatment successes observed in the prophylactic model does not translate to successes in a therapeutic model. In particular, while *irr* cells alone were able to protect in a prophylactic subcutaneous tumor model, it shows no efficacy in a therapeutic model with mice containing established peritoneal tumors.

This was further established when the heterologous prime-boost vaccination strategy was used to treat a therapeutic B16F10 model of PC. Despite the ability to generate enhanced CD8<sup>+</sup> T-cell responses and to delay tumor growth in prophylactic models, the heterologous prime-boost strategy performed significantly worse than repeated dosing with MG1-IL12-ICV. This also suggested that a heightened CD8<sup>+</sup> T-cell response may not be sufficient to improve outcomes in our models of established disease. Further corroborating this, MG1-IL12-ICV was shown to have

better efficacy in models of established peritoneal disease when compared to a prime-boost vaccination strategy previously described by Pol *et al.*, consisting of a DCT-encoded adenovirus prime followed by a DCT-encoded MG1 boost (a therapeutic strategy currently in clinical trials)<sup>163,219</sup>.

Therefore, we then sought to investigate the TME of established peritoneal tumors over the course of MG1-IL12-ICV therapy to garner a better understanding of the immune responses generated upon treatment and to rationally identify targets to enhance efficacy of MG1-IL12-ICV in difficult-to-treat models.

#### **4.2 MG1-IL12-ICV induces a pro-inflammatory tumor microenvironment in a model of peritoneal carcinomatosis**

We have established that in PC models of B16F10 and MC38 PC tumors, MG1-IL12-ICV can control tumor growth and increase survival of mice bearing established peritoneal tumors; however long term durable cures are not obtained. Given that NK and CD8<sup>+</sup> T-cells are required for efficacy of MG1-IL12-ICV, we sought to investigate the complex interplay between pro and anti-tumor immune subsets in mice bearing peritoneal tumors<sup>66</sup>. Since an analysis of MG1-IL12-ICV's ability to engage NK cells has previously been reported, here we sought to investigate the contribution of other immune subsets with a particular emphasis on the generation of a T-cell response including the role of DCs, further our understanding of NK cell responses over the course of therapy, and elucidate how immunosuppressive elements (MDSCs, T-regs and checkpoints) may eventually allow tumor escape<sup>66,219</sup>.

*4.2.1 MG1-IL12-ICV increases presence of DCs in the peritoneal cavity and upregulates pathways involved in antigen processing and presentation.*

The importance of phenotypically active DC subsets in promoting both adaptive and innate responses to mediate tumor control has been previously discussed<sup>70,77,242–245</sup>. In addition to their oncolytic properties, successful OV's are potent activators of DCs, increasing their ability to process and present antigens to T-cells and to activate other immune cells in the TME<sup>9,246,247</sup>. In fact, efficacy of some OV-based vaccines have been entirely attributed to their ability to activate DCs irrespective of oncolytic properties, such as the Modified Vaccinia Ankara, the inactivated form of which can promote anti-tumor immunity through activation of CD103<sup>+</sup> DCs when delivered intratumorally<sup>248,249</sup>. Both MG-1 and the related VSV-Δ51 have been previously demonstrated to augment adaptive and innate responses through DC-dependent pathways<sup>67,250</sup>. Similarly, we previously reported that NK cell activation and recruitment following MG1-IL12-ICV treatment was mediated in part by release of IP-10 from DCs<sup>66</sup>.

Here, I have shown that MG1-IL12-ICV therapy can recruit and maintains an increased presence of DCs in the peritoneal cavity and tumors of B16F10 tumor-bearing mice and that the APC repertoire within MG1-IL12-ICV-treated tumors are actively involved in antigen-processing pathways. Additionally, although not statistically significant, there were more CD103<sup>+</sup> CD11c<sup>+</sup> cells (DCs involved in cross-presentation and priming of CD8<sup>+</sup> T-cell responses; data not shown) in the tumors of MG1-IL12-ICV or MG1-ICV-treated tumors compared to *irr* cells-treated or untreated tumors.

Overall, these findings suggest an increased presence of APCs in the TME of MG1-IL12-ICV-treated animals. However, this does not indicate whether these DCs or APCs that take up antigen in the peritoneal cavity can prime anti-tumor T-cells in secondary lymphoid organs. DCs that take

up antigens must mature as they make their way to lymph nodes or the spleen, increasing their surface expression of activation molecules CD80 and CD86, antigen presenting MHC-I and MHC-II and downregulating phagocytic receptors<sup>70,78,251</sup>. This maturation allows DCs to present cognate antigen to T-cells alongside necessary co-stimulation (CD80/86) that is recognized by CD28 on T-cells to promote conventionally activated T-cells<sup>70,78</sup>. To this end, we investigated phagocytic capability of and CD86 expression on splenic DCs of MG1- or MG1-IL12-ICV treated animals after successive doses of ICV. Nearly 100% of splenic DCs were activated (CD86<sup>+</sup>) after the first dose of MG1 or MG1-IL12-ICV treatment, but this activation returned to basal levels after the third and sixth dose of ICV. This suggested that priming of T-cell responses may take place early on during therapy, and that successive vaccinations may not play a role in priming T-cell responses. Since IFN- $\gamma$ -producing anti-viral T-cell responses could be detected at later time-points, it can be posited that the activated splenic DCs were priming conventional (mostly against viral epitopes) and not regulatory T-cells (as CD86 may also engage CTLA-4 on T-cells to induce tolerance)<sup>252,253</sup>.

Whether the ongoing presence and activity of DC/APCs in the peritoneal cavity at later timepoints has any role in generating a T-cell response in draining lymph nodes to neoantigens that may arise as the tumor evolves was not addressed. However, given the lack of co-stimulatory molecule CD86 in splenic DCs, it can be posited that similar activation levels are present in DCs in the lymph nodes, and clonal expansion of T-cells reactive to neoepitopes may not take place later in therapy. In an alternate interpretation of the data, the lack of CD86 on DCs later in therapy may play a positive role in maintaining a pro-inflammatory environment by preventing tolerogenic DCs from inducing regulatory T-cells later in therapy. Activation of DCs in the spleen early on in therapy maybe attributed to the increased recognition of DAMPs and PAMPs (viral RNA, apoptotic blebs,

etc.). Transgene expression of IL-12 and subsequent upregulation of IFN- $\gamma$  on NK and T-cells may also play a role in activating DCs. The lack of activation after later doses may be attributed to rapid neutralization of virus leading to loss of efficacy of ICVs after multiple doses or a reduced sensitivity to IL-12 after prolonged exposure to the cytokine. This is addressed in more details in section 4.2.4.

#### *4.2.2 MG1-IL12-ICV breaks immune privilege of B16F10 tumors and recruits tumor and virus-reactive T-cells into the TME.*

A primary goal of OV-mediated cancer therapy is to generate anti-tumor adaptive responses<sup>249,254</sup>. This includes priming of antigen-specific T-cell responses by DCs, followed by appropriate chemotaxis to sites of tumor and metastasis, and finally tumor infiltration by these T-cells to drive adaptive control of tumors<sup>254</sup>. A recent update on *talimogene laherparepvec* (HSV expressing GM-CSF) demonstrated that patients responding to intratumoral OV treatment in synergy with checkpoint blockade had increased CD8<sup>+</sup> T-cell responses, and that clinical response rates were not associated with baseline CD8<sup>+</sup> T-cell levels (indicating that a pre-existing immunity was not required)<sup>255</sup>. MG1 and VSV $\Delta$ 51-based pre-clinical studies have shown similar results, where increased specific adaptive responses correlated with tumor control<sup>163,250</sup>.

With MG1-IL12-ICV therapy, we observed increased presence of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in tumors of treated-mice after ICV-treatment, suggesting that MG1-IL12-ICV can break immune privilege of B16F10 tumors, effectively making them immunologically “hot” tumors. Increased presence of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in the peritoneal cavity was also observed. Visually, when H&E stained sections of MG1-IL12-ICV treated tumors are analyzed, lymphocyte-like cells are observed within the tumor boundary of MG1-IL12-ICV-treated tumors (Figure 10) as well as the formation of tertiary lymphoid structures surrounding the tumor bed (not shown). A similar profile

was not observed in untreated or MG1-ICV-treated tumors. In effect, this showed that MG1-IL12-ICV treatment primed and recruited both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell arms of the immune response. MG1-IL12-ICV treatment can also activate T-cells (indicated by CD69 expression) in tumor-naïve animals (data not shown), suggesting that T-cells may also be more activated following therapy. Furthermore, gene expression analysis of MG1-IL12-ICV-treated tumors at early and late stages of treatment revealed upregulation of genes involved in T-cell and NK-cell chemotaxis, further confirming that MG1-IL12-ICV can induce and recruit an adaptive response to the TME. Further corroborating flow cytometry data, NanoString Cell Score analysis (correlating cell-specific expression of genes to predict cell presence) revealed an increase in T-cell and cytotoxic cell (T-cell and NK cell) subsets within the tumors. At later timepoints, although the proportion of CD3<sup>+</sup> T-cells (as a percentage of CD45<sup>+</sup>) in the tumor and peritoneal cavity was higher in MG1-IL12-ICV-treated tumors than MG1-ICV-treated tumors, proportions were lower than those observed at early timepoints. However, total number of T-cells present in the TME of MG1-IL12-ICV treated mice was maintained at later timepoints suggesting an ongoing T-cell response.

However, despite this robust influx of allegedly-specific T-cells into the tumor and peritoneal cavity, mice treated with MG1-IL12-ICV still succumbed to disease. Given our earlier findings suggesting that MG1-IL12-ICV therapy maybe more potent at generating anti-viral and not anti-tumor immune responses, we investigated specificity of the CD8<sup>+</sup> T-cells generated upon ICV therapy. Utilizing the B16-OVA model we showed that MG1-IL12-ICV treatment generated tumor-specific CD8<sup>+</sup> T-cells using SIINFELK-tetramer staining. *Ex-vivo* peptide stimulation, however, indicated a robust response to viral peptides and minimal responses to SIINFELK peptide. Since MG1-IL12-ICV is a whole cell vaccine, the generation of a modest anti-tumor reactive T-cell repertoire is not wholly unexpected, as it is more likely to contribute to the

generation of a wide array of tumor antigen-specific T-cells as opposed to a large number of T-cells to one antigen. Anti-tumor activity of these cells is expected to in turn induce epitope-spreading to increase the CD8<sup>+</sup> T-cell arsenal specific to the tumor. Assays that use whole tumor cells as *ex-vivo* stimulation may serve as a better indicator of T-cell responses (for both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells) and may give us a more accurate representation of the adaptive repertoire upon ICV therapy.

It has been previously posited that the balance between anti-viral and anti-tumor adaptive responses is key to the success of OV therapy, so as to allow sufficient replication of viruses without skewing the immune response away from the tumor<sup>249</sup>. It is well established that detection by pattern recognition receptors (PRR) of virus-associated PAMPs is key for the activation of inflammasomes through stimulator of interferon genes (STING), myeloid differentiation primary response 88 (MYD88), etc<sup>256–258</sup>. The signaling cascade eventually leads to the release of cytokines including IFN- $\gamma$ , IL-6 and IL-12 that create a pro-inflammatory microenvironment at the site of OV infection. Viral oncolysis and subsequent release of DAMPs and immunogenic cell death markers further drive APC maturation and the induction of cellular immune responses, including adaptive responses to the virus and the tumor<sup>246,256,259</sup>. As viral infection overtakes the cellular machinery of tumors, increased presentation of OV epitopes on tumor MHC-I and MHC-II will further allow even anti-viral T-cells to efficiently kill tumor cells<sup>249,256</sup>. To this end, we checked for viral remnants in our FFPE tumors after three doses of vaccination but did not observe any viral signature through IHC or PCR using MG1-specific primers (data not shown). This may suggest limited viral infection of OVs later in therapy, or that any virally infected tumors have already been eliminated providing another explanation for the reduced tumor burden in MG1-IL12-ICV treated animals. Furthermore, it has recently been posited that anti-viral CD4<sup>+</sup> T-cells



(as may be present in the peritoneal cavity of ICV-treated animals) can promote cross-presentation of tumor antigens to CD8<sup>+</sup> T-cells by activating DCs through CD40-CD40L interactions and can also provide T-cell help to both anti-viral and anti-tumor CD8<sup>+</sup> T-cells through the release of IFN- $\gamma$ , TNF- $\alpha$  and IL-2<sup>256,260</sup>. We have previously demonstrated that CD4<sup>+</sup> T-cell depletion does not abrogate the efficacy of ICVs; but it is possible that loss of Th1 help may be compensated for by depletion of regulatory CD4<sup>+</sup> T-cells, and a more thorough investigation into the nature of CD4<sup>+</sup> T-cell responses in MG1-IL12-ICV therapy is warranted.

Here, our experiments suggest that MG1-IL12-ICV can generate tumor-specific CD8<sup>+</sup> T-cell responses and breaks tumor immune privilege by inducing infiltration of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. A robust anti-viral response was also observed and the contribution of the balance between anti-tumor and anti-viral adaptive responses in the efficacy of this promising vaccine may require further study. Given that we identified a robust pro-inflammatory environment, we then sought to identify mechanisms by which these responses are attenuated by immunosuppressive elements.

#### *4.2.3 Increased expression of checkpoint molecules and not MDSCs or regulatory T-cells contribute to immunosuppression.*

The final hurdle to OV-based therapies is overcoming immunosuppression in the TME. MDSCs, regulatory T-cells or TAMs have all been implicated in immunosuppression through direct cell-mediated effects or through the release of immunosuppressive cytokines like IL-10, TGF- $\beta$  or PGE<sub>2</sub><sup>102,111,254,261</sup>. Finally, checkpoint molecules on both tumor and immune cells can dampen the anti-tumor responses generated after OV therapy and may induce an immunosuppressed or exhaustive phenotype. OVs have been tailored to directly deplete MDSCs, convert M2 to M1 macrophages, and have been shown to synergize with checkpoint inhibitors to potentiate tumor control<sup>249,254,262–264</sup>.

Here, we have showed that in the TME of MG1-IL12-ICV-treated mice there is a significant decrease in the proportion of MDSCs and this is maintained later in therapy. It is worth noting that the levels of MDSCs in tumors of MG1-ICV treated mice was higher than that in MG1-IL12-ICV treated mice and these levels were similar at later timepoints. While the reduced proportion of MDSCs may be a result of increased influx of other pro-inflammatory subsets, previous studies have shown a direct effect of IL-12 in regulating MDSC activity. Steding *et al.* demonstrated that IL-12 therapy induced maturation of MDSCs and reduced their ability to suppress T-cell responses<sup>265</sup>. They also demonstrated that MDSCs expressed IL-12R $\beta$ 1 and 2, and that direct engagement of IL-12 was responsible for this effect<sup>265</sup>. Although, we have not directly assessed the suppressive nature of these subsets in our model, it can be hypothesized that due to their reduced proportions and the ability of IL-12 to reduce MDSC suppression, these subsets are not major immunosuppressive players in our model. Investigating regulatory T-cells, we found modest increases in the presence of these subsets in tumors of MG1-IL12-ICV treated animals, but the CD8: T-reg ratio was not altered between the different groups or over the course of therapy. Cao *et al.* demonstrated that IL-12 inhibited tumor-induced regulatory T-cell proliferation via indirect mechanisms, in part, mediated by the IFN- $\gamma$  signaling axis<sup>266</sup>. Overall, this suggested that these suppressive cell types may not be major players in driving immunosuppression in response to MG1-IL12-ICV therapy.

We then investigated immune checkpoints in the TME and observed increased expression of PD-L1 but not PD-L2 on immune subsets later on during therapy. Preliminary investigations revealed increased presence of CD11b<sup>+</sup> F4/80<sup>+</sup> macrophages that were PD-L1<sup>+</sup> in the peritoneal cavity of MG1-IL12-ICV-treated mice (data not shown) suggesting that suppressive macrophages may be playing a role in mediating checkpoint inhibition of immune responses. Increased expression of

PD-L1 later in therapy indicated that PD1-PD-L1 axis maybe increasingly engaged later during ICV therapy to mediate immunosuppression. However, it is widely known that checkpoint molecules, including PD-1 and PD-L1, are upregulated in response to stimulatory signals (and hence associated with activation) in addition to being driven by oncogenic pathways<sup>130,138,267–269</sup>. Since a proinflammatory TME is observed in MG1-IL12-ICV treated tumors, we checked gene expression of several widely reported checkpoint molecules in MG1-IL12-ICV-treated FFPE tumors at early and late timepoints during therapy, and observed increases in PD-L1, PD-1, CTLA-4, Tim-3, 2B4, CD160, Lag3 and IL-1R8. Similar to our flow data, we did not observe significant increases PD-L2 gene expression. It is worth noting that our flow data was specific to surface expression of these molecules on immune cells, but our gene expression data is based on all cells present in the tumors. Increased expression of checkpoint-related genes in tumors at day 15 suggests that at the transcriptional level, tumors and tumor-infiltrating cells may already have entered an immunosuppressive state early during treatment.

This also suggested that although use of checkpoint inhibitors have been shown to synergize with OV's and other immunotherapies in mediating tumor control, single checkpoint blockade may not be sufficient to enhance efficacy of our immune-stimulating ICV therapy, as other checkpoints may compensate for this loss of inhibition. In fact, Shim *et al.* demonstrated a similar phenomenon, where monoclonal antibodies against PD-1 and TIM-3 (both of which were upregulated upon therapy) did not enhance survival of mice bearing B16 melanoma tumors when treated with VSV<sup>270</sup>. However, this contrasts with others who have demonstrated increased synergy of OV's with checkpoint blockade. Whether checkpoint blockade can enhance therapeutic efficacy of ICV's can only be confirmed when these therapies are compared to head-to-head in our models. In fact, previous unpublished studies in our laboratory revealed that PD-1 blockade modestly enhanced

survival of B16F10 bearing mice treated with MG1-IL12-ICV but durable cures were again not obtained, suggesting that our model maybe refractory to single checkpoint blockade as observed by Shin *et al.*

Overall, our investigations revealed that lack of durable cures in MG1-IL12-ICV treated mice may not be mediated by MDSCs or regulatory T-cells. There is evidence showing increased presence of checkpoint molecules but whether they play a role in attenuating the pro-inflammatory responses induced by MG1-IL12-ICV and whether they are valid combination therapy targets is not known.

#### *4.2.4 Reduction in NK cell activity and responsiveness after multiple doses of ICV may identify a possible mechanism for loss of ICV efficacy.*

The efficacy of MG1-IL12-ICV has been shown to be dependent on the recruitment and activation of NK cells. Given our improved understanding of the evolving immune TME in response to MG1-IL12-ICV therapy, we then sought to determine if the enhanced NK cell responses previously reported were maintained later during therapy and whether subsequent loss of NK cell activity could contribute to a lack of durable cures.

We observed that both MG1- and MG1-IL12-ICV therapy upregulated CD69 expression on NK cells in the spleen and peritoneal cavity after the first dose. CD69 is an early marker of NK and T-cell activation and can be upregulated following a variety of signals including the recognition of viral particles and through cytokine-dependent mechanisms<sup>271–273</sup>. In the peritoneal cavity, after the third dose MG1-IL12-ICV induced higher NK cell activation than MG1-ICV, but activation declined over the course of therapy. This suggested that both therapies were able to induce NK cell activation but MG1-IL12-ICV is better at maintaining this response, at least in the site of tumor progression. Although not as potent as IFN- $\alpha$ , IL-15 or 18, IL-12 can induce significant CD69

expression on NK cells; *in-vivo* IL-12 may further synergize with other cytokines in enhancing and maintaining CD69 expression on NK cells<sup>273–275</sup>. Return of CD69 expression to basal levels in the spleen after the first dose suggested that systemic activation is lost after the first exposure to therapy. Since CD69 is an early marker of NK cell activation, we expected that each successive dose of ICV should be able to equally activate NK cells inducing high levels of CD69. Contrary to expectations, subsequent doses were not as effective at inducing NK cell activation as the first dose. Whether this is because an activated (but CD69<sup>-</sup>) NK cell repertoire is already present, or if NK cells have entered a non-responsive state is not known. Investigating NK cell maturity or other NK cell activation markers that persist longer after activation (like killer cell lectin-like receptor G1(KLRG-1)) may inform the dynamics of NK cell activation following ICV therapy<sup>276</sup>.

Alternatively, assessment of NK cell function may be more informative. To this end, we quantified IFN- $\gamma$  production and degranulation of NK cells following *ex-vivo* co-culture with target YAC-1 cells. We observed increased IFN- $\gamma$  production from NK cells isolated from spleen or peritoneal cavity of mice treated with MG1-IL12-ICV compared to cells from MG1-ICV-treated mice. The canonical function of IL-12 is to induce rapid IFN- $\gamma$  release from NK cells, and this data suggests that inclusion of IL-12 transgene into MG1 potentiates NK cell activity *in-vivo*. However, cells isolated from the spleen after the third and sixth dose and cells from the peritoneal cavity after the sixth dose showed no IFN- $\gamma$  production following co-culture, suggesting a similar downregulation of NK activity as observed with CD69 expression. This suggests a functional anergy present at later stages of treatment and indicates a lack of efficacy of latter doses of ICV. Contrastingly, however, enhanced degranulation of NK cells isolated from MG1- or MG1-IL12-ICV treated mice was maintained over the course of therapy. Vahlne *et al.* previously demonstrated that upon YAC co-culture, NK cells exhibiting a mature phenotype (CD11b<sup>hi</sup> CD27<sup>lo</sup>) were more likely to produce

IFN- $\gamma$  and immature cells (CD11b<sup>lo</sup> CD27<sup>hi</sup>) were more likely to degranulate, and those exhibiting intermediate phenotypes performing both functions equally effectively<sup>277</sup>. If this can be taken as an indication of what may be happening *in-vivo*, it would suggest that activated and mature NK cells are no longer present at later stages of treatment and may provide an explanation for why MG1-IL12-ICV does not provide durable cures.

Overall, our findings suggest that NK cells from MG1-IL12-ICV-treated animals are more active and more responsive than those from MG1-ICV treated animals, further confirming previously reported findings. The complete abrogation of IFN- $\gamma$  production after later doses and gradual decline in activation over the course of therapy suggests that NK cells may have entered an exhausted or immunosuppressed phenotype or that ICVs are no longer able to induce activation of immature NK cells. Alternatively, this suggests that humoral or cellular anti-viral responses generated after early vaccination doses may render subsequent doses of ICV ineffective. This may be mediated by immediate neutralization of the virus after ICV delivery before replication, transgene expression or infection of tumors can take place, effectively eliminating any possibility for innate activation as may be observed with early doses. Future experiments designed to test whether NK cells, after repeated dosing with MG1-IL12-ICV, can be activated with an unrelated NK cell-stimulant may shed light as to whether NK cells have entered a suppressed phenotype or MG1-IL12-ICV is no longer engaging the immune response due to rapid clearance.

### **4.3 Conclusion**

This project provides a robust understanding of the immune responses generated upon treatment with MG1-IL12-ICV. A better understanding of the complex immune interplay that leads to generation of anti-tumor responses and those present in the TME of established tumors during MG1-IL12-ICV therapy will allow us to rationally identify potential changes to dosing regimens,

consider avenues for combination therapies and explore additional opportunities to enhance efficacy of MG1-IL12-ICV as a standalone therapy.

Briefly, we have showed that while MG1-IL12-ICV can delay tumor growth when used as a prophylactic vaccination, a heterologous combination of irradiated cells and MG1-IL12-ICV is most effective in generating anti-tumor CTL responses that contributes to delayed tumor growth. We have also identified that generation of specific anti-tumor CD8<sup>+</sup> T-cells does not solely predict delayed tumor growth in prophylactic models and identified the need to explore other components of the adaptive response that may be engaged during treatment with MG1-IL12-ICV.

We have shown that MG1-IL12-ICV can enhance survival of mice bearing aggressive peritoneal tumors but does not provide long term durable cures. This is mediated by an enhance pro-inflammatory TME generated upon MG1-IL12-ICV therapy that includes recruitment of DCs, generation of tumor and virus reactive T-cells and enhanced activation of NK cells. We have also identified that MG1-IL12-ICV treatment is not hindered by MDSCs and CD4<sup>+</sup> T-regulatory cells, but maybe affected by increased expression of checkpoint molecules at later stages of therapy. And finally, we found that early doses of MG1-IL12-ICV effectively engages and activates innate cells, but subsequent doses are ineffective in this regard.

Overall, we demonstrate that MG1-IL12-ICV is a promising new therapeutic avenue for treatment of PC, and maybe effective in other models of cancer. We have furthered our understanding of this immune-stimulating therapy taking it one step closer to the clinic.

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## Appendix

### Appendix A: List of antibodies and tetramers

| Fluorochrome-conjugated antibody    | Company        |
|-------------------------------------|----------------|
| AF647 CD8 Clone KTI5                | MBL            |
| APC IFN gamma Clone XMG1.2          | eBioscience    |
| BV421 CD103 Clone M290              | BD Biosciences |
| BV421 CD107a Clone ID4B             | BD Biosciences |
| BV421 CD11c Clone N418              | BD Biosciences |
| BV421 CD25 Clone 3C7                | BD Biosciences |
| BV421 CD273 Clone TY25              | BD Biosciences |
| BV421 CD279 Clone J43               | BD Biosciences |
| BV421 CD69 Clone H1.2F3             | BD Biosciences |
| BV421 F4/80 Clone T45-2342          | BD Biosciences |
| BV421 IL-10 Clone JES5-IGE3         | BD Biosciences |
| BV510 CD3e Clone 145-2c11           | BD Biosciences |
| BV650 CD11b Clone M1/70             | BD Biosciences |
| BV650 CD69 Clone H1.2F3             | BD Biosciences |
| BV711 CD86 Clone GL1                | BD Biosciences |
| BV711 F4/80 Clone T45-2342          | BD Biosciences |
| BV786 CD11b Clone M1/70             | BD Biosciences |
| BV786 CD274 Clone M1H5              | BD Biosciences |
| BV786 CD4 Clone RM4-5               | BD Biosciences |
| FITC CD45.2 Clone 104               | BD Biosciences |
| PE CD152 (CTLA4) Clone UC10-4F10-11 | BD Biosciences |
| PE CD273 Clone TY25                 | BD Biosciences |
| PE CD4 Clone RM4-5                  | BD Biosciences |
| PE CD40 Clone 3123                  | BD Biosciences |
| PE OVA tetramer                     | MBL            |
| PE Tim3 Clone SDi2/Tim-3            | BD Biosciences |
| PeCF594 CD11c Clone HL3             | BD Biosciences |
| PeCF594 NK1.1 Clone PK136           | BD Biosciences |
| Pe-Cy5 FoxP3 Clone FJK-165          | eBioscience    |
| Pe-Cy5 Ly-6G (Gr-1) Clone RB6-8C5   | eBioscience    |
| Pe-Cy7 CD19 Clone ID3               | BD Biosciences |
| Pe-Cy7 CD27 Clone LG.3A6            | BD Biosciences |
| PE-Cy7 CD3e Clone 145-2C11          | eBioscience    |
| PE-Cy7 Granzyme B Clone NGZB        | eBioscience    |



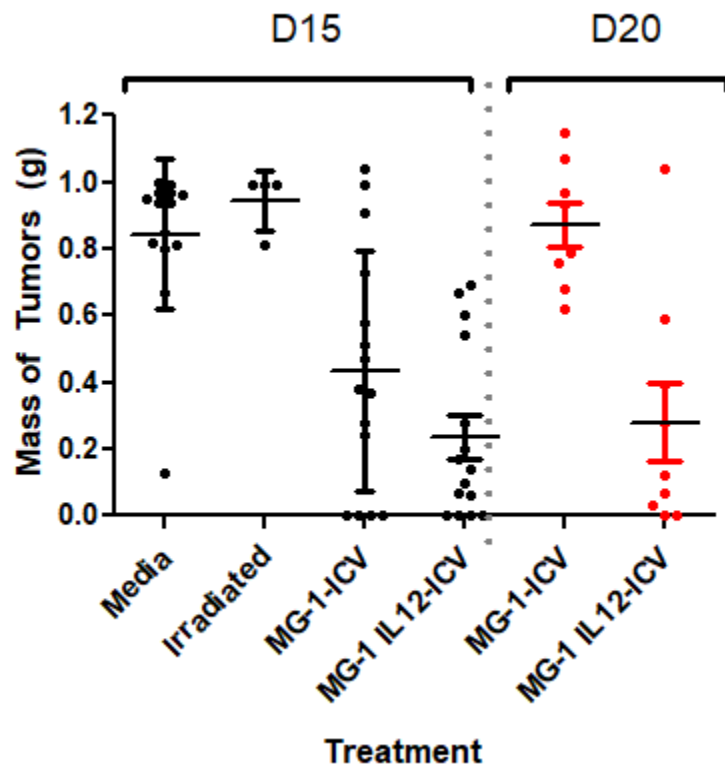
|                                             |                |
|---------------------------------------------|----------------|
| PerCP-Cy5.5 CD3 Clone UCHT1                 | BD Biosciences |
| PerCP-Cy5.5 CD45.2 Clone 104                | BD Biosciences |
| PerCP-Cy5.5 CD8 alpha Clone 53-6.7          | BD Biosciences |
| PerCP-Cy5.5 MHCII (IA/IE) Clone M5/114.15.2 | BD Biosciences |

## Appendix B: List of Housekeeping Genes used for Normalization of NanoString Data

### Gene Name

1. *Eif2b4*
2. *Tbp*
3. *Eef1g*
4. *Nubp1*
5. *Sdha*
6. *Sap130*
7. *Edc3*
8. *Hdac3*
9. *Rpl19*
10. *Oaz1*
11. *Abcf1*
12. *Ppia*
13. *Gusb*
14. *G6pdx*
15. *Polr1b*
16. *Polr2a*
17. *Sf3a3*

Appendix C: Weight of B16F10 tumors collected for flow cytometry



B16F10 tumors collected for immune profiling were weighed (maximum collected 1.0 gram).

Data from three separate experiments.

# Curriculum Vitae

## Education and Honours

### **MSc Microbiology and Immunology**, University of Ottawa, Ottawa, ON (2016-18)

Ontario Graduate Scholarship (2017-18); International Admission Scholarship (2016-18); Dept. of BMI Syed Sattar Award for Research Excellence (2018); Faculty of Medicine Award of Excellence in Graduate Studies (2017); Dept. of BMI Poster Day 1<sup>st</sup> prize in MSc Microbiology and Immunology (2017).

### **HBSc Biochemistry (Microbiology and Immunology) (Co-op) with Minor in Economics**, University of Ottawa, Ottawa, ON (2011-16)

Chancellor's Scholarship for International Students (2011-16); Undergraduate Research Excellence Award (2016); CanHepC Summer Student Scholarship (2015; 2016); Science Student's Association Research Excellence Award (2016); International Student Scholarship (2013-14); Undergraduate Research Opportunity Program (2014); Science Student's Association International Student Scholarship (2012-13); Dean's Honour List (2011-16).

## Work Experience

### **Graduate Student**

*Dr. Rebecca Auer, The Ottawa Hospital Research Institute (2016-current)*

- Characterizing innate and adaptive immune responses to novel oncolytic therapeutics to overcome tumor mediated immune suppression.
- Flow cytometry, RNA/DNA extraction, q/PCR, ELISAs and other fluorometric assays, tissue culture, animal work, student supervision.

### **Research Assistant/Honours Student**

*Drs. Angela Crawley and Curtis Cooper, The Ottawa Hospital Research Institute (2015-16)*

- Studied the effects of Hepatitis C virus core protein on CD8<sup>+</sup> T-cells and on the use of liver enzyme normalization as a predictive marker for successful viral responses upon therapy.
- Flow cytometry, primary cell culture, blood processing, fluorometric assays, clinical research experience, data analysis.

### **Molecular Biology Research Assistant**

*Levesque Lab, Agriculture and Agri-Food Canada (2015)*

- Maintenance of oomycete culture collection and development of protocols for identification of oomycetes from environmental samples.

### **Scientific and Office Support**

*Food Safety Division, Canadian Food Inspection Agency (CFIA) (2013- 2014)*

- Data analysis and preparation of associated guidelines and reports on surveillance of the major foodborne pathogens being monitored in Canada.

### **UROP Student Researcher**

*Dr. Christopher Boddy, University of Ottawa (2014)*

- Oxytetracycline production under the RpoN sigma factor in *Escherichia coli*.

## Publications

**Hepatitis C virus core protein reduces CD8<sup>+</sup> T-cell proliferation, degranulation and perforin production but increases STAT5 activation** *Sarwat T Khan, Winston Karges, Curtis L Cooper, Angela M. Crawley (Published Jan 2018, Immunology)*

**Dysfunctional Natural Killer Cells in the Aftermath of Cancer Surgery** *Leonard Angka, Sarwat T Khan, Marisa Kilgour, Rebecca Xu, Michael Kennedy, Rebecca Auer (Published Aug 2017, International Journal of Molecular Sciences)*

**Liver enzyme normalization predicts success of Hepatitis C oral direct-acting antiviral treatment** *Sarwat T Khan, Micheline McGuinty, Daniel J Corsi, Curtis L Cooper (Published Apr 2017, Clinical and Investigative Medicine)*

## Service/Leadership Roles

- **Managing Editor**, University of Ottawa Journal of Medicine (2017-current);
- **Coordinator**, Molecular Journal Club, Cancer Therapeutics Program (2017-current);
- **Volunteer**, Let's Talk Science (2017-current);
- **Reviewer**, University of Ottawa Journal of Medicine (2016-2017);
- **President of the University of Ottawa Co-op Student's Association** (2015-2016);
- **President of AIS/D Habitat for Humanity Club** (2010-2011);
- **Vice President of Nooraloy School** - A school for underprivileged children (2010-11).

## First Author Posters Presented at Conferences

**Keystone Symposia Cancer Immunotherapy: Combinations** (Montreal, QC, 2018).

**2<sup>nd</sup> Summit for Cancer Immunotherapy** (Gatineau, QC, 2017).

**6<sup>th</sup> Canadian Symposium on HCV** (Banff, AB, 2017)

**The Canadian Association for HIV Research** (Winnipeg, MB, 2016)

**5<sup>th</sup> Canadian Symposium on HCV** (Montreal, QC, 2016)

## Additional Work Experience

### **Teaching Assistant (2015-18)**

Molecular Biology, Faculty of Science

General Intermediary Metabolism, Faculty of Science

Undergraduate Chemistry Teaching Labs, Faculty of Science

Proctor, Medical Education, Faculty of Medicine, Univ. of Ottawa

**Lab Volunteer (2013)**, Dr. Christopher Boddy, Univ. of Ottawa

**Telefundraiser (2011-2014)**, Development Office, University of Ottawa.

Languages: Native/Bilingual Proficiency in English and Bengali; intermediate knowledge of Spanish; fluent in spoken Hindi/Urdu.