Exploiting The Antitumor Immune Response Using Il-12 Armed Oncolytic Mg1 Virus In An Infected Cell Vaccine

By

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Abstract:

Despite improvements in chemotherapy and radical surgical debulking, peritoneal carcinomatosis (PC) remains among the most common causes of death for abdominal cancers. Immunotherapies have demonstrated efficacy in selected solid malignancies but their potential in PC is poorly explored. Here I report that intraperitoneal injection of an infected cell vaccine (ICV), consisting of autologous tumor cells infected *ex-vivo* with an oncolytic Maraba MG1 virus expressing interleukin-12 (IL-12), promotes the migration of activated natural killer (NK) cells to the peritoneal cavity in response to the secretion of interferon gamma-induced protein-10 (IP-10) from dendritic cells. This recruitment of cytotoxic, IFNγ-secreting NK cells is associated with a dramatic reduction in tumor burden and improved survival in a colon cancer model of PC. Even in mice with bulky PC (tumors >8 mm), a complete radiological response was demonstrated within 8-14 weeks, associated with 100% long-term survival. Importantly, these results were recapitulated in human lymphocytes exposed to human tumor cell lines infected with MG1-IL12. Finally, I demonstrate that MG1-IL12-ICV generates an effective CD4 and CD8 T cell response in mice following prophylactic immunization associated with the maturation of peritoneal dendritic cells and enrichment of tumor-specific peritoneal T cells. The research presented in this thesis suggests that an MG1-IL12-ICV is a promising therapy that could provide benefit to the thousands of patients diagnosed with PC each year.

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List of Abbreviations

Ab = Antibody

ACT = Adoptive cell transfer

ADCC = Antibody dependent cytotoxicity

APC = Antigen presenting cell

BM = Bone marrow

BM-DC = Bone marrow-derived dendritic cell

CD = Cluster of differentiation

CFSE = Carboxyfluorescein succinimidyl ester

CTLA4 = Cytotoxic T lymphocyte antigen 4

DAMPs = damage-associated molecular patterns

DC = Dendritic cell

DCT = dopachrome tautomerase

ECM = extracellular matrix

GFP = Green fluorescent protein

GM-CSF = Granulocyte-monocyte colony stimulating factor

HLA = Human leukocyte antigen

ICV = Infected cell vaccine

IFN = Interferon

IL = Interleukin

IP = Intraperitoneal

irrB16 = gamma-irradiated B16-F10 cells

irrCT26lacZ = gamma-irradiated CT26lacZ cells

irrSW620 = gamma-irradiated SW620 cells

irrK562= gamma-irradiated K562 cells

IV = Intravenous

KIR = killer inhibitory receptor

MDSCs = Meyloid-derived suppressor cells

MHC = Major histocompatibility complex

MOI = Multiplicity of infection

NDV = Newcastle disease virus

NK = Natural killer

OV = Oncolytic Virus

PAMP = pathogen associate molecular pattern

PBMC = Peripheral blood mononuclear cells

PBS = Phosphate-buffered saline

PCR = Polymerase chain reaction

Pfu = Plaque forming units

TAA = Tumor associated antigen

TGFβ = Transforming growth factor β

TIL = Tumor infiltrating lymphocyte

TLR = Toll-like receptor

TNF = Tumor necrosis family

TRAIL = TNF-related apoptosis inducing ligand

Treg = Regulatory T cell

VEGF = Vascular endothelial growth factor

VSV = Vesicular stomatitis virus

WT or wt = Wild type

β-gal = β-galactosidase

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1 INTRODUCTION

1.1 Cancer

Cancer is the leading cause of death in Canada in 2015. Almost half of Canada's population (42%-45%) will develop cancer in their lifetime, and about 25% of Canadians will die of cancer [1]. While traditional cytotoxic therapies—such as chemotherapy and radiation—form the backbone of cancer treatments, immunotherapy is emerging as a promising and highly effective therapeutic strategy. In fact, cancer immunotherapy was named the scientific breakthrough of 2013 by Science magazine [2].

Cancer is a group of related diseases characterized by abnormal cell growth and tissue invasion, causing significant morbidity and/or death if left untreated [3]. More than 100 types of cancer have been recognized, affecting organs like lungs, breast, skin, colon, prostate, and lymphoid organs. Based on the type of originating cell, cancers are classified as carcinomas (epithelial), leukemias (blood and lymphatic system), or sarcomas (connective tissue) [4].

Two fundamental publications by Hanahan and Weinberg described eight biological capabilities, referred to as "Hallmarks of cancer", that are acquired during cancer development [5, 6]. These hallmarks are: self-sufficient proliferative signalling, insensitivity to growth suppressors, resistance to cell death, replicative immortality, stimulation of angiogenesis, active invasion and metastasis, reprogramming of energy metabolism, and avoidance of immune destruction [5, 6]. Since their description, many therapeutic strategies targeting these hallmarks have proven to be effective. Moreover, research breakthroughs have fine-tuned these hallmarks, enabling for a further in-depth understanding of the processes that are involved in cancer initiation and progression [5].

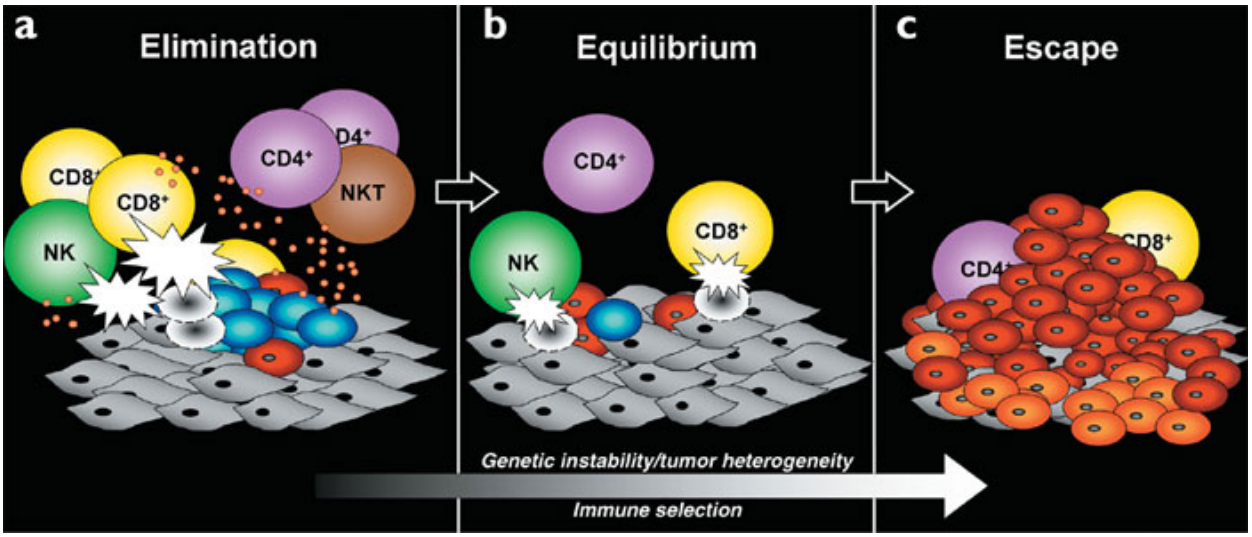
1.2 Cancer immunoediting

Beyond regulating the tumor, the immune system can also shape tumor immunogenicity and even assist cellular transformation [7]. The term cancer immunoediting refers to the immune system's ability to both protect the host and sculpt the tumor [8]. During this process, the immune system can completely eliminate some tumors, but can produce a non-protective, tumor promoting immune state in others. Cancer immunoediting has established the basis for individualized immunotherapies [7], and has revolutionized the idea of cancer immunosurveillance to include the immune system at all stages of disease formation and progression [9].

An important aspect of cancer immunoediting is that antigens expressed by nascent tumor cells can induce an adaptive, T cell-mediated anti-tumor immune response, and this interaction with the immune system can alter these antigens' expression [7]. Dunn *et al.* proposed a model of cancer inmmunoediting based on three processes, called the three Es of cancer immunoediting: elimination, equilibrium, and escape (Figure 1.1) [9]. This model proposes that the immune response has the capability of eliminating most tumor cells [9]. Equilibrium is then established between the surviving tumor cells and the anti-tumor immune response [9]. The cells that can still proliferate under this immune pressure have reduced immunogenicity and can evade the immune response (escape), forming a detectable tumor that impairs the normal functions of organs [10].

Figure 1.1. The three Es of cancer immunoediting.

(a) Elimination process: immunosurvelliance, where both innate and adaptive immune systems are at work to eliminate cancer cells. (b) Equilibrium process: selection of tumor cell variants that survive the immune attack; this is the longer process where cells can be in dormancy. (c) Escape: development and expansion of the tumor. Blue cells correspond to developing tumor cells, red cells to tumor cell variants, and grey cells to underlying stroma and non-transformed cells. Small orange circles represent cytokines and cytotoxic activities of lymphocytes against tumor cells are represented by white flashes [9].


a
Elimination
CD8+
CD8+
CD4+
NKT
NK
b
Equilibrium
CD4+
CD8+
NK
c
Escape
CD8+
Genetic instability/tumor heterogeneity
Immune selection

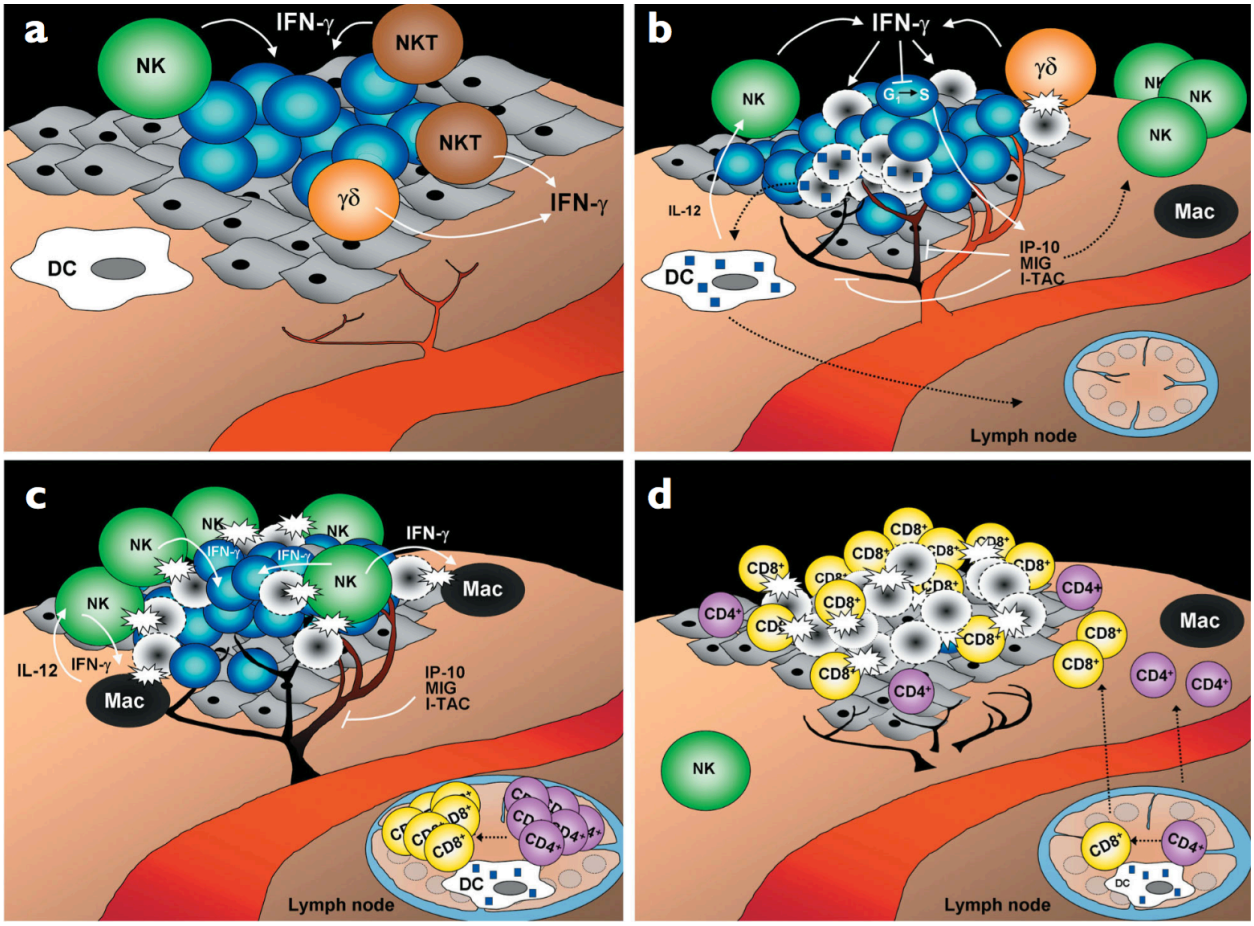
In the process of elimination, both the innate and the adaptive immune systems work together to detect and destroy tumor cells before the formation of tumor masses. In the first step of this process, interferon gamma (IFN-γ) is released from innate immune cells, including natural killer cells (NK cells), following the downregulation of major histocompatibility complex (MHC class I) expression on tumor cells [11]. This decrease in surface MHC-I expression—a ligand for NK cell inhibitory receptors—activates NK cells due to the lack of inhibitory receptor signalling. [11]. Both types of interferons (IFN-α/β and IFN-γ) play important roles in the anti-tumor immune response [12]. IFN-α/β acts on several host cells, including dendritic cells (DCs), to present tumor antigen to $CD8^+$ T cells. IFN-γ acts on hematopoietic and tumor cells to increase antigen presentation and cytotoxic activity [7]. As tumor elimination progresses, IFN-γ induces anti-proliferative and apoptotic mechanisms to encourage tumor death through the JAK/STAT pathway [13]. Other components, like the chemokines CXCL9 (Monokine induced by gamma interferon), CXCL10/IP-10 (interferon inducible protein -10) and CXCL11 (interferon inducible T cell chemoattractant), are induced from the tumor cells and from the surrounding tissue [14]. Some of these chemokines also kill tumor cells by blocking the formation of new blood vessels within the tumor (anti-angiogenic capacity) and by recruiting NK cells and macrophages to the inflammatory site [9].

In the final step of the elimination process, NK cells infiltrate the tumor. Resident macrophages promote their activation by producing IFN-γ and interleukin-12 (IL-12), resulting in massive killing of tumor cells [9, 15]. This process uses different mechanisms, including apoptosis-inducing ligand, tumor necrosis factor-related (TRAIL), perforin, and intermediates of reactive oxygen and nitrogen. Tumor-specific $CD4^+$ T helper cells expressing IFN-γ (T_H1 cells) and $CD8^+$ T cells home to the tumor site, destroying more tumor cells [9]. If the elimination

process is successful in destroying the tumor, cancer as a disease is prevented and there is no progression to the following phases **(Figure 1.2)**.

Figure 1.2. Model for the elimination phase of the cancer immunoediting process.

(a) Initiation of the immune response: transformed cells are recognized by lymphocytes, stimulating the production of IFN-γ. (b) IFN-γ initiates a cascade of innate immune reactions by the induction of chemokines, anti-proliferative signals, and the activation of macrophage and NK cytolytic activity. (c) Tumor growth is kept in check by NK cells. (d) Tumor-specific $CD4^+$ and $CD8^+$ T cells recognize and destroy tumor cells. Tumor cells (blue), non-transformed cells (gray), dead tumor cells (white to gray dashed lines) [9].


a
IFN-γ
NK
NKT
NKT
γδ
IFN-γ
DC
b
IFN-γ
NK
γδ
NK
NK
NK
Mac
IL-12
DC
IP-10
MIG
I-TAC
Lymph node
c
NK
NK
NK
NK
NK
IFN-γ
IFN-γ
IFN-γ
Mac
IL-12
IFN-γ
Mac
IP-10
MIG
I-TAC
CD8+
CD4+
DC
Lymph node
d
CD8+
CD4+
Mac
NK
CD4+
CD4+
DC
Lymph node

The second phase of cancer immunoediting is equilibrium **(Figure 1.1b)**. In this phase, the tumor is held in functional dormancy by the immune system, which selects for immune-resistant tumor cell variants [9]. IFN-γ and lymphocytes apply an important selection pressure on tumor cells that are genetically unstable and mutate easily [16]. In that way, many tumor cell variants are killed, but new ones appear, increasing the resistance to immune attack. This equilibrium state can occur over many years, and is the longest of the three cancer immunoediting phases [9]. This process has been linked to a balance between IL-12 and IL-23, promoting elimination and persistence, respectively [17]. Equilibrium between immunosuppressive cells and immunoactive cells with antitumor functions in the tumor microenvironment also contributes to tumor dormancy [9].

The last phase is the escape phase **(Figure 1.1c)**, where variants within the tumor that survive the immunoediting process are insensitive to immunological detection, allowing the tumor to expand uncontrollably. Different mechanisms contribute to tumor cell escape including the reduction of immune recognition (e.g. prevention of antigen presentation), increased resistance (e.g. Programmed death-ligand 1 (PDL-1) up regulation), and the immunosuppressive tumor microenvironment (e.g. myeloid derived suppressor cells, regulatory MDSCs, T cells, M2 macrophages etc..) [7]. Genetic changes take place in an uncontrolled manner and malignant disease is clinically observable. As a result, early detection is an important factor for outcome since the tumor keeps evolving [9].

1.3 Generating an antitumor immune response

The tumor microenvironment is composed of several different cell types, including immune cells, endothelial cells, fibroblasts, adipocytes, and nerves. Additionally, an extracellular matrix (ECM) containing collagen and peptidoglycans confers a structured interaction between

non-transformed cells and malignant cells [18]. Non-malignant cells of the tumor microenvironment can have tumor-promoting functions, and can be highly immunosuppressive [19]. Depending on the activation state and phenotype of the immune cells present in the tumor, promotion or inhibition of different aspects of tumor development can result [20].

Immune surveillance initiates when a growing tumor triggers inflammatory signals such as necrotic products and damage-associated molecular patterns (DAMPs) [9]. This process induces inflammation and stimulates antigen-presenting cells (APCs) such as DCs. Tumor-derived antigens are engulfed and the APCs, which migrate to the draining lymph nodes where they stimulate effector immune cells like T and B lymphocytes to induce the adaptive response [21].

Antitumor immune responses can be either at the local or the systemic level. An effective immune response should be specific, durable and generate immunological memory. Different cells are involved in the antitumor immune response, specifically APCs, T cells and NK cells [22].

NK cells survey "self" cells through MHC-I (HLA) [23]. The killer-cell immunoglobulin-like receptors (KIR) on NK cells bind to HLA molecules, signalling the activation or inhibition of the NK cells [24]. NK cell activation is usually controlled by a balance of signals originating from activating and inhibitory receptors [25]. Inhibitory receptors such as NKG2A play a critical role in NK tolerance by binding to MHC-I, which is expressed on all healthy, nucleated cells [25]. When NK cells interact with MHC-I, the inhibitory receptor initiates a negative signal, which abrogates any stimulatory signals from activating receptors interacting with the potential target cell [26]. Many tumors and virus-infected cells frequently lose surface expression of

MHC-I to evade recognition by the CD8+ specific cytotoxic T cell. However, these cells become vulnerable to NK cell-mediated cell lysis due to the absence of the inhibitory MHC-I signal [27].

On the other hand, NK cell activating receptors, including NKG2D, NKp30, NKp44, and NKp46, are important for eliciting NK cell responses toward tumors or virally infected cells [26]. The NKG2D receptor has been shown to specifically recognize non-classical MHC molecules, which are HLA-related receptors lacking peptide loading capacity. These HLA receptors are up-regulated on stressed cells, such as tumors, but not expressed on normal cells [28]. This killing mechanism does not rely on antibody-antigen reactions, but instead on a loss of inhibitory ligands on the target cell [29]. NK cells can also lyse antibody-coated target cells through a process known as antibody-dependent cellular cytotoxicity (ADCC), which activates NK cells through the FcγRIII surface receptor [30, 31].

T cell-mediated immune responses are also important in tumor regression [32]. Tumor antigens are normally presented to the TCR of $CD8^+$ T cells by the MHC-I on the tumors. Upon activation, $CD8^+$ T cells expand and kill tumor cells directly [33]. The cytotoxicity in both NK cells and T cells is mainly initiated through the secretion of cytotoxic proteins including pore-forming proteins (Perforin), granzyme B, IFN-γ, TRAIL, or FasL dependent mechanisms [20, 34].

$CD4^+$ T cells also participate in the antitumor immune response, recognizing tumor antigens presented on MHC class II molecules. Studies performed using murine models of myeloma demonstrated the important role of tumor-specific CD4+ T cells in avoiding early tumorigenesis [35]. These cells secrete IFN-γ, which enhances antigen presentation and induces nitric oxide synthesis in macrophages, thereby inhibiting tumor cell growth [36]. In some cases, $CD4^+$ T cells act by producing TNF-α, suppressing the function of $CD8^+$ T cells and impairing

antitumor immunity [37]. In this case, TNF-producing cells are attracted by tumor cells via MHC-II expression [37]. In addition, suppressive immune populations such as T_{reg} and MDSCs secrete cytokines like transforming growth factor-β (TGF-β), IL-4, IL-6, and IL-10 to further suppress the cytotoxic functions of effector lymphocytes [38].

B cells play a complementary role via the production of antibodies that mediate ADCC of tumor cells via Fc receptors on NK cells [39]. In addition, they improve the ability to recruit tumor-infiltrating lymphocytes (TILs), and act as an additional stimulatory signal to trigger the T cell response [40].

Different immune phenotypes have been described in patients having solid tumors. T cell infiltration is observed in some solid tumors, including colorectal cancer, breast cancer, melanoma, and gastrointestinal stroma tumors [34, 41]. The presence of infiltrating cells is generally associated with a satisfactory clinical outcome and could be more important for prognosis than the cancer stage [34]. However, an accumulation of T cells in the tumor microenvironment can induce different immunosuppressive activities and oncogenic pathways, leading to a poorer clinical outcome [34]. For instance, the RET/PTC3 (RP3) fusion protein, a tyrosine kinase, is known to activate NF-kappaB transcriptional programs and results in secretion of proinflammatory mediators [42]. This oncogenic protein, however, was found to induce the recruitment of the $CD11b^{+}Gr1^{+}$ myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment, leading leads to a tumor promoting, innate response [42, 43].

Anti-cancer immunity contributes to cancer control after radiotherapy and chemotherapy, with the specific immune response related to the anti-cancer agents used [44, 45]. These immune responses can be associated with the induction of immunogenic cell death (ICD), a type I programmed cell death (PCD) that alters the cell surface composition and induces the release of

soluble proteins that activate antigen presentation to T cells [46]. This can lead to the elimination of residual cancer cells or keep the micrometastases in an equilibrium or dormancy stage, as discussed above [47-49].

1.4 Cancer Immunotherapy

Cancer immunotherapy is a treatment that treats tumors with the immune system. The importance of the immune system is made evident by the increased cancer incidence in immunocompromised hosts, and the ability of immunocompetent individuals to display spontaneous cancer regression [50]. Immunity against tumors has alsobeen demonstrated in many animal models. For instance, defects in the IFN-γ receptor (IFNGR1) have been shown to induce spontaneous tumors [51], while conversely the presence of tumor-infiltrating lymphocytes (TILs) are associated with an improved prognosis [52]. Therefore, cancer immunotherapy has the potential to accomplish complete remission and a durable cure. Targeting tumors with T cells, NK cells, and DCs are possible choices for cancer immunotherapy [53]. Particularly, antitumor strategies for $CD4^+$ and $CD8^+$ cells, and some tumor-suppressive strategies that up-regulate DCs and NKs have been employed [53].

Human cancers bear antigens that are tumor specific or have increased expression compared to normal cells. The first tumor antigen cloned was MAGEA1 by Bruggen and colleagues in 1991 [54, 55]. Tumor antigens are classified into three general groups: tumor-specific antigens (TSAs), tumor-associated antigens (TAAs), and cancer-germline/cancer testis antigens (CTAs) [56]. The TSAs are neoantigens, produced by somatic mutation; they are not encoded in non-malignant host genomes, thus representing abnormal cellular proteins and are ideal targets for specific immune recognition [57]. TAAs are antigens encoded in the normal

genome but that are expressed differently in the tumor [58]. Some of these participate in oncogenic processes, since they possess growth-promoting functions. TAAs' antigenicity is related to their abnormal expression and are more immune tolerant than TSAs. CTAs can be expressed in cancer cells and normal cells, such as fetal ovaries and trophoblasts [59]. They are encoded in normal genomes but have highly restricted tissue expression, making them attractive targets for immunotherapy [59].

To take advantage of these tumor antigens, T cells extracted from TILs are used for adoptive cell transfer (ACT). Their ease of isolation, culture, and expansion ex vivo show a great potential. More recently, the genetic manipulation of TILs enables redirection of target specificity towards specific antigens expressed in the tumor [60]. T cells can now be manipulated to express modified receptors or CARs (protein-fusion-derived chimeric antigen receptors) with higher tumor specificity, or to select for natural TCR chains with increased tumor specificity [60, 61]. Human NK cells lack CD3/TCR molecules and are characterized by the expression of CD16 and CD56 [62]. They can mediate cytotoxicity in response to target cell stimulation and release cytokines through the engagement of activating and inhibitory receptors [63-66]. They are also regulated by cytokines, including IL-1, IL-15, IL-12 and IL-18, and by interactions macrophages, DCs, and mesenchymal stromal cells [64, 65]. Activated allogeneic NK cells such as the NK-92 cell line have been proposed as an immunotherapeutic strategy for cancer due to their lack ofI KIRs [67-69]. Even though this cell line has been considered an "off-the-shelf" product due to its ease of manipulation and expansion, it has potential limitations such as the risk of alloimmune responses generated by T and B cells, which prevent its persistence and limit multiple infusions [67].

DCs are derived from bone marrow and are present in all tissues. They are the most important APCs for priming naïve T cells [70]. DCs have been used extensively in cancer immunotherapy for vaccination by ex vivo manipulation [71]. In methods using antigen-specificity, the tumor antigen is loaded onto APC or incorporated at the protein or DNA level [72]. DCs generated in vitro are used in vaccination strategies since they have a high capacity to induce T cell responses [73]. This is useful for poorly immunogenic antigens, such as tumor-associated self-antigens [74]. Recently, allogeneic DCs have been used as vaccine vehicles, allowing large, clinical-grade vaccine batches [75]. Vaccination can also be achieved by injecting components directly in the tumor. These include (1) modifying dead tumor cells to secrete immune attracting factors like granulocyte-macrophage-colony stimulating factor (GM-CSF), promoting local accumulation of DCs; (2) adding DC activators with the antigen, such as Toll-like receptor (TLR) ligands or mAb to stimulate CD40; or (3) using recombinant vectors with the antigen and an innate stimulus, such as plasmid DNA having the antigen and immunostimulatory CpG sequences [76-78]. More trials exploring increasing APC activation and antigen presentation are necessary to improve currently weak T cell responses to vaccines. Another way to improve antigen presentation is by reducing negative checkpoint signals that regulate the T cell response. For example, blocking CTLA-4 (cytotoxic T lymphocyte antigen-4), which is negative regulator of T cell activation, has a significant influence in antitumor response when used in murine models [21].

1.4.1 Personalized cancer vaccine

Most cancer vaccines use a specific cancer antigen expressed by a group of diseases. The antigen specific vaccine is delivered in a viral DNA or cellular vector, joined to an adjuvant in

order to activate the innate immune system and initiate an adaptive immune response [79]. Recombinant, adjuvanted proteins or peptides based on tumor antigens can also be used [80]. This anti-tumor response usually is durable and improves survival [80]. The primary goal of these vaccines is to generate a T cell response against an existing cancer instead of preventing the disease [81].

Personalized DC vaccines are made from mutated proteins expressed by tumors from individual patients. Mutated peptides, called neoantigens, were shown to activate melanoma patients' cytotoxic T cells [82]. This finding was confirmed by Carreno *et al.,* where safe vaccination of three patients led to the proposed inclusion of neoantigens into therapeutic strategy [82].

Since most cancer mutations are patient-specific, a personalized approach is required to develop a potent vaccine, including the identification of the mutated or neoantigens that should be included in the vaccine formulation [83]. This is a challenge, as only a fraction of mutated peptides are immunogenic [84]. Prediction algorithms and biochemical assays comparing MHC binding affinity between mutant and wild type peptides are used to predict potential neoantigens to a limited extent [85]. Following prediction, mRNA expression level and antigen immunogenicity is assessed by determining T cell stimulation [83]. For strongly immunogenic antigens, immunization is achieved with a long synthetic peptide in combination with RNA-based-vaccines or adjuvants. As stated before, $CD4^+$ and $CD8^+$ T cells are involved in cancer control, so a personalized vaccine may require cell-specific neoantigens from both CD4 and CD8 T cells to harness robust antitumor immune responses [83, 86].

1.4.1.1 Whole cell vaccine

Whole cells vaccines are prepared with weakened or dead tumor cells collected during a biopsy or surgery. Cells are killed with low dose radiation to inhibit tumor proliferation and formation. However, intact antigens representing the patient's specific mutanome are still present on the surface of the cell and can stimulate immune responses [87-89]. To enhance the immune response to these antigens, the whole tumor cell is injected with immune stimulating compounds such as proteins and cytokines. Three categories are proposed for whole cell vaccines: autologous vaccines, allogeneic vaccines, and gene-modified vaccines. Autologous vaccines are generated using tumor cells from the patient to be vaccinated. This vaccine contains the unique tumor cell antigens, creating a highly personalized tumor immune response. Allogeneic vaccines are prepared from tumor cells retrieved from different people withthe same cancer type. Using different cell lines from different tumors (allogenic) in vaccine production increases the possibility of the patient having tumor antigens that match some of the vaccine cells [88]. They can be manufactured in large quantities and can be stored. Several clinical trials have demonstrated the safety, feasibility and efficacy of utilizing allogeneic whole cell vaccines [90-94]. An example of this is the allogeneic prostate GVAX vaccine, obtained from two tumor cell lines—LNCaP and PC-3—which show a favourable overall survival in phase I/II trial compared to those observed in phase II Taxane chemotherapy trials [89, 92, 95]. Another type of whole cell vaccine is the gene-modified vaccine, which genetically modifies isolated and cultured patient tumor cells. These can be modified to express immune-signalling/stimulating molecules or natural tumor-specific antigens [87-89].

Whole cancer cell vaccines use multiple tumor antigens present in these damaged cells in order to activate the immune response, inducing antigen specific T cells [88]. Since whole tumor cells are used as a vaccine, it presents a broad spectrum of all possible antigens, in contrast to

other vaccines. It is possible to target multiple tumor antigens simultaneously using whole cell vaccines [88]. Comparing immune responses pre- and post-vaccination can help identify novel tumor antigens using immunized lymphocytes and serologic responses [88]. There are several factors influencing the immune response to whole tumor antigens, such as the type of antigens and the cancer patients' immune status. For instance, individuals who participate in clinical trials are usually elderly, having a lower number of peripheral T cells and reduced TCR levels [87]. Memory T cells in this patient population are high, but the diversity and functional ability of $CD4^+$ and $CD8^+$ T cells are reduced, leading to inadequate antitumor immune responses [87].

1.4.2 IL-12 cancer therapy

Interleukin 12 (IL-12) is a pro-inflammatory heterodimeric cytokine secreted by monocytes, macrophages, DCs, and neutrophils in response to viral or bacterial infections [96]. It is a highly pleiotropic type I cytokine interconnecting innate and adaptive immunity by inducing the secretion of IFN-γ, cell growth, and cytotoxicity of NK cells and T cells; it also supports the differentiation of naïve CD4+ T cells toward Th1 helper effector cells [31, 96]. The development of adaptive T cell responses can lead to memory responses and a durable cure [97, 98]. Several murine tumor models were used in preclinical studies where IL-12 showed strong antitumor activities [99-101]. IL-12 does not have the direct ability to inhibit cancer growth. Instead, it is a very important component of the Th1-type immune response against cancer, especially because it is secreted locally in the tumor [96, 102-104]. IL-12 lead to tumor regression and increased survival when administered to tumor-bearing mice [101]. IL-12 causes an increase in the cytolytic activity and growth of lymphocytes ex vivo and in vivo [105]; however, the promising effectiveness of this cytokine is compromised by an extremely cytotoxic profile. Organ toxicity and haemolytic anemia were observed in trials that used

multiple IL-12 doses [102-104]. Indeed, two deaths was recorded in the early 90s in patients that received multiple doses of IL-12, resulting in liver toxicity [106]. Even though the index of circulating CD8+ T cells was enhanced after IL-12 treatment, its efficacy was minimal, ranging from 0% to 11% [105, 107]. Regardless of the robust tumor shrinking that was observed after IL-12 administration in melanoma patients, subcutaneous and hepatic metastases were also observed following this shrinkage, indicating that IL-12 is also effective for tumor debulking [105].

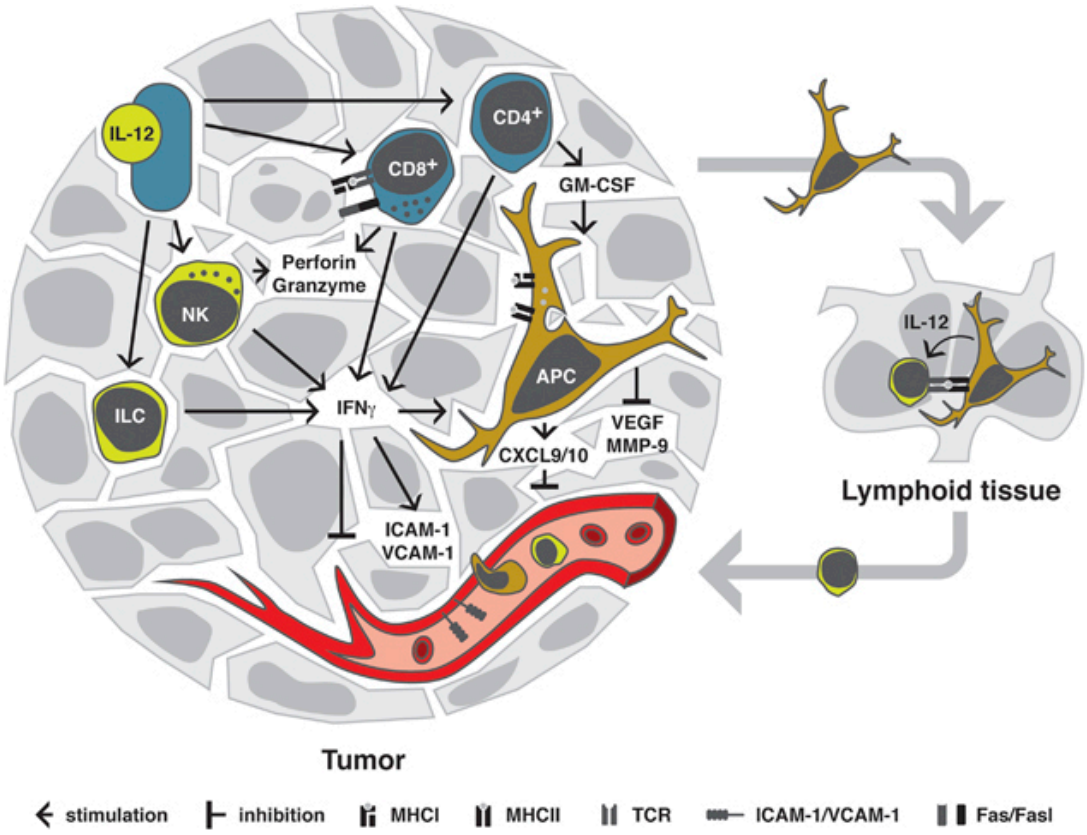
IL-12 performs many functions in anti-tumor immunity. It acts by increasing the production of IFN-γ from NK cells, ILCs and T cells, which in turn upregulates antigen presentation by MHC-I and II [108]. This upregulation is mainly due to the increase in expression of MHC-I and II molecules and the enhanced expression and activity of the proteasomes, leading to more processing and presentation of tumor antigens (non-self), thus promoting the engagement of $CD8^+$and $CD4^+$ T cells [108-110]. Simultaneously, IL-12 stimulates the expansion and cytotoxicity of NK cells, $CD8^+$ and $CD4^+$ T cells via perforin, granzyme and Fas ligand (FasI) [111]. It also induces Th1 polarization, increasing ADCC against tumor cells, inducing IgG and suppressing IgE production [111-113]. Other antitumor effects of IL-12 are related to its ability to inhibit angiogenesis, remodel the extracellular matrix and tumor stroma, influence myeloid-derived suppressor cells, and up regulate MHC-I molecule expression **(Figure 1.3)** [103].

The development of IL-12 studies in clinical trials can be separated into phases. From 1996 to 2005, intensive studies were done where maximal tolerated doses, optimal treatment schedules, and the susceptibility of tumors was studied [102]. This discovered low immune responses and high patient toxicity that terminated several studies. In the second phase (2006-2010), new interest in IL-12 appeared and the employed strategies can be divided into three

groups: (1) the application of IL-12 alone or in combination with chemotherapy or monoclonal antibodies for active non-specific immunotherapy (innate immune responses); (2) the use of IL-12 as an adjuvant with tumor cells or antigens for vaccine production (stimulate adaptive antitumor responses); and (3) gene therapy, which is the focus of recent studies [103].

Figure 1.3. Cellular responses to IL-12.

IL-12 exerts its functions directly on lymphoid cells such as NK cells, T cells and ILCs. These immune cell populations secrete IFN-γ, which in turn induces tumor suppressive pathways. In addition, IL-12 enhances antigen presentation as well as the cytotoxic function of NK cells and $CD8^+$ T cells through the induction of perforin, granzyme, and Fas ligand (Fasl). IFN-γ has suppressive effects on vascularization and angiogenesis by down regulating VEGF and MMP-9. Furthermore, IFN-γ induces chemotactic proteins such as CXCL9 and CXCL10 to facilitate immune cell recruitment to the tumor [102].


IL-12
CD8+
CD4+
GM-CSF
Perforin
Granzyme
NK
APC
ILC
IFNγ
VEGF
MMP-9
CXCL9/10
ICAM-1
VCAM-1
IL-12
Lymphoid tissue
Tumor
stimulation
inhibition
MHCI
MHCII
TCR
ICAM-1/VCAM-1
Fas/FasI

The potent effect of IL-12 in preclinical studies has lead to continued investigations to determine clinical settings, particularly those to minimize cytokine toxicity. Studies suggest that the best treatment regimen to administer IL-12 appears to be cycles for five consecutive days or injections for two consecutive weeks [102]. Strikingly, administration of a small preconditioning dose of IL-12 prior to treatment attenuates IL-12-induced toxicity [106, 114]. Research efforts on IL-12 has revealed that intratumoral/local treatment results in reduced toxicity compared to systemic administration [103]. Clearly, IL-12 is a promising therapeutic strategy but a more tumor-directed delivery may reduce toxicity while maintaining therapeutic benefit. Gene delivery vectors such as oncolytic viruses are considered very effective in intratumoral directed delivery of IL-2 due to their oncotropic characteristics.

1.4.3 Oncolytic Viruses

Oncolytic viruses (OVs) are emerging as promising highly effective immunotherapeutic agents. These viruses selectively replicate in cancer cells and can kill them without harming normal tissue. They can be DNA or RNA viruses, found naturally or genetically engineered. The use of OVs started in the mid-1950s with the use of NDV, which was reported to have oncolytic activity [115]. The effectiveness and safety of NDV has been corroborated in clinical settings and is based on its tumor selective replication, oncolytic ability, and capacity to activate the host antitumor immunity [115]. OVs have been used in immunotherapeutic strategies such as *in situ* vaccines, immunomodulatory transgenes, or in combination with other immunotherapies [116, 117]. Immune responses induced by virotherapy can be positive, inducing the antitumor immune reaction, and negative, limiting virus replication and spread. They can mediate antitumor activity by selectively replicating in neoplastic cells, with a lytic effect on tumor cells, and by inducing a systemic antitumor immune response via induction of

innate and adaptive immune cells. In vesicular stomatitis virus (VSV)-infected tumor cells, for instance, the viral infection induces type III IFN interleukin-28 (IL-28) that sensitizes tumor cells to be targeted by NK cells via upregulation of RAE, H60, and MULT-1 [118].

Cancer abnormalities—including altered stress responses, cell signalling, and homeostasis—allow OVs to replicate in cancer cells [119]. OV-permissive signalling pathways include RB/E2F/p16, p53, PKR, EGFR, Ras, and others [120] [119]. These altered signalling pathways render the cellular environment favourable for the OVs replication, leading to the death of cells in a sometimes immunogenic fashion [121]. The cellular antiviral machinery, used for the detection and elimination of viruses, may also be impaired in cancer cells [120]. For example, normal cells sense virus infection and secrete type I IFN to block virus replication by inducing cellular antiviral signaling pathways. Cancer cells often contain downregulated and defective genes associated with the type I IFN response, which allow them to escape interferon-mediated growth control programs, but simultaneously compromise their innate antiviral response [122, 123]. Another example of this is a defect of the protein kinase R (PKR) in some of these malignant cells. PKR is a critical factor in controlling viral infections; when it is absent, viral replication increases [124]. In addition, many of the traits proposed by Hanahan and Weinberg, which describe the conversion of normal cells into cancer cells, provide a permissive environment for OVs, including continuous proliferation, resisting cell death, growth suppression avoidance, genome instability, DNA damage induced stress and the avoidance of immune destruction [116].

Two classes of OVs are reported. One of them naturally replicates in cancer cells and does not harm humans. This group includes autonomous parvovirus, Newcastle disease virus (NDV), myxoma virus (MYXV; poxvirus), Seneca valley virus (SVV; picornavirus), and

reovirus. The other group includes genetically manipulated attenuated viruses, which include measles virus (MV; paramyxovirus), vaccinia virus (VV; poxvirus), and poliovirus (PV; picornavirus). This group also includes some viruses with mutations/deletions induced in viral genes required for replication in normal cells, including adenovirus (Ad), VSV, and herpes simplex virus (HSV) [116]. Some OVs are also used as vectors for immunotherapy, such as adenoviruses, HSV-1, poliovirus, MV, poxviruses and NDV. Importantly, the FDA has recently approved an oncolytic HSV, Imlygic (T-VEC), for use in melanoma patients, and several OVs are currently being investigated in clinical trials [124].

Genetic manipulation has enabled the rapid diversification of OVs used for cancer treatment. The amplification of the virus during replication increases virus dose in the tumor, enhancing its activity. Safety features can be built in, such as drug and immune sensitivity [116].

OVs used as cancer vaccines provide several advantages over other cancer therapies. OVs are tumor-selective, and thus could be used for safe, specific *in situ* cancer vaccination. OVs can also induce different immunogenic/inflammatory types of cells death, including the immunogenic cell death (ICD) of cancer and stromal cells [121]. This provides a natural group of TAAs, which, in combination with DAMPs, OV-derived pathogen-associated molecular patterns (PAMPs), and inflammatory cytokines, can all initiate anti-tumor immunity [121].

Genetic engineering has further improved the utility of OVs in inducing oncolysis and antitumor immunity. Numerous IL-12 expressing OVs have been investigated for their anti-cancer properties, and have been promising in preclinical studies. These viruses have been reviewed in “Interleukin-12-expressing oncolytic virus: A promising strategy for cancer immunotherapy” and include Sindbis virus (SINV), Vesicular stomatitis virus (VSV), Semliki Forest Virus (SFV), Newcastle disease virus (NDV), Herpes simplex virus (HSV), and

Adenovirus [125]. Unlike systemic administration of recombinant IL-12, OV-directed intratumoral IL-12 expression demonstrated minimal organ toxicity. Additionally, a significant antitumor effect of these viruses partially or completely regressedestablished tumors and increased survival in murine tumor models [126-132]. These studies have suggested a mode of action where IL-12 expressing OVs have multimodal effects, directly on immune cells such as cytotoxic and regulatory T cells, NK cells, and DCs; and indirectly through chemokine induction CXCL9 and IP-10 which robustly interfere with tumor angiogenesis [125, 133]. Therefore, an ultimate OV candidate expressing IL-12 should have broad range of oncotropism to maximize the efficiency of IL-12 expression within the tumor microenvironment.

1.4.3.1 Maraba MG1 virus

Maraba is a rhabdovirus with a small genome that is easy to manipulate, and stable enough to incorporate transgenes and to apply directed mutagenesis. It was isolated from sandflies in Brazil [134]. Maraba MG1 is a variant that was engineered with two point mutations, M protein (L123W) and G protein (Q242R) [135]. The L123W mutation attenuates the virus at the level of the interferon response, allowing IFN-β mRNA to be transported from the nucleus to the cytoplasm (a process that is blocked by the wtMRB M protein), while the Q242R mutation improves virus replication in malignant cells while attenuating replication in normal cells [135]. It displays strong oncolytic activities, demonstrating rapid virus production, good cytolytic activity against several different tumor lines, and a large burst size [135]. It has a broad oncotropism *in vitro* with a high selectivity and reduced virulence in normal cells. Oncolytic MG1 virus can be used as an oncolytic vaccine platform with a potent ability to increase T cell immunity, including tumor-specific $CD4^+$ and $CD8^+$ T cells [136]. In murine melanoma tumor

models, antigen-encoding MG1 virus mediated recall immunization, with an increase in the median survival and remission in approximately 20% of animals [136].

MG1 virus activates NK cells via direct infection and maturation of dendritic cells [137]. Both NKs and DCs are required for MG1 efficacy and their cellular activation requires intact viral particles [137]. Production of type I interferon and other cytokines are required for the NK cell response induced by DCs following MG1 administration [137]. Therapeutic benefits have increase if MG1 is used in association with Ad in a vaccine setting using a heterologous prime-boost treatment regimen, generating strong antigen-specific T cell immune responses [136]. This prime-boost strategy had been extensively studied in the murine B16F10 melanoma tumor model by utilizing two different viruses, MG1 and Ad, which both express the melanoma TAA dopachrome tautomerase (DCT), skewing the generated CD8 T cell response toward DCT protein leading to enhanced efficacy [136]. Even though this strategy is very effective in generating anti-tumor immune responses, it requires predetermination of the immune dominant TAA in order to construct the prime-boost viruses. An alternative strategy that includes a rich source of TAAs, such as whole tumor cell vaccine, is warranted to overcome this limitation.

1.4.4 Virus-Infected Tumor Cell Vaccine

OV-infected tumor cell vaccines were first reported by Lindenmann and Klein in 1967 to be an effective platform to generate an anti-tumor immune response [138]. Tumor immunogenicity can be increased using naturally occurring viruses. Local production of interferon type I (α, β) acts as an adjuvant, increasing the intensity of the response, bystander T cell proliferation, and the survival of memory cells [139]. The adjuvant characteristic of type I IFN is associated with the strong immune response produced by live viruses, in contrast to viral peptides, which are poorly immunogenic by themselves [140].

Vaccines used in cancer treatment can be preventive, as is the case for the human papilloma virus (HPV) vaccine, or therapeutic, such as those which treat cancer after it appears. One of the main barriers in cellular stimulation is the poor immunogenicity of most tumor antigens. Therapeutic vaccines can help solve this problem by presenting antigens (Ags) in a different way, particularly by using activated DCs. One way to present the antigen, as explained earlier, is by isolating DCs, introducing the Ag ex vivo to the DC, and re-infusing the cells to the host.

Viral vectors are most commonly used as cancer vaccines because they can be easily produced. These vectors also demonstrate a good ability to stimulate cytotoxic T cell responses. [79]. The ability of a virus to activate host-antitumor immunity is crucial in cancer treatment, since many tumors are immunoevasive or immunosuppresive, or are otherwise unable to support an immune response [115].

We and others have developed an oncolytic vaccine platform using tumor cells that have been infected with a mutated vesicular stomatitis virus (VSV-Δ51) [141]. This infected cell vaccine (ICV) showed strong protection from subsequent tumor challenge. Efficacy was further increased by viral expression of GM-CSF (VSVgm-ICV) [141]. This ICV stimulates both innate and adaptive immune cells and has a significant impact in aggressive and immunosuppressive tumors [141]. If autologous tumors are used in this immunotherapy, a personalized vaccine could potentiate the immune response against a patient's unique tumor antigens, making the therapy more specific [141].

Personalized cancer vaccination has been improved by the advances in nucleic acid technologies, particularly in the field of genomics, with the possibility of targeting the mutanome, which is considered an optimal target for cancer immunotherapy [142]. An example

of a described tumor mutanome comes from the B16F10 murine melanoma cell, which has 563 mutations in expressed genes and is widely used for studies in immunotherapy [110]. The use of next generation sequencing (NGS) to analyze mutanomes showed that human cancers contain 10s to 100s of non-synonymous mutations [143, 144]. The vast majority of these mutations are patient-specific, limiting the use of this approach for broadly applicable drugs [110, 143, 144]. The mutanome study of B16F10 melanoma demonstrated that many of these mutations in tumors are immunogenic and can confer antitumoral vaccine activity [86]. Software analysis of mutanome sequences could help predict immunogenic sequences, aiding in cancer vaccine production with high specificity and immune activity.

1.5 Peritoneal carcinomatosis

Peritoneal carcinomatosis (PC) is one of the most common and feared sites of metastases for abdominal malignancies, including gastrointestinal and ovarian malignancies [145]. Its presentation is associated with a significantly reduced quality of life and a very poor prognosis, with median survival of 6-12 months. Chemotherapy is less effective in patients with peritoneal carcinomatosis and treatment cannot be administered once patients develop complications associated with the disease, such as a bowel obstruction [146].

Direct intraperitoneal perfusion of chemotherapy has consistently been shown to be superior to systemic (intravenous) administration [147-151]. This is because direct intraperitoneal administration overcomes the barriers between the peritoneal compartment and the blood compartment, maintaining high regional drug concentration while keeping systemic drug levels at the minimal [152-154]. Hyperthermic Intraperitoneal Chemotherapy (HIPEC) is the most common form of chemotherapy administration and is usually given following a cytoreductive surgery [150]. Hyperthermia improves the treatment outcomes by enhancing the

drug penetration and inhibits the cellular repair mechanisms [155, 156]. Despite this direct administration leading to higher survival rates than systemic chemotherapy, the results are still disappointing and treatment is rarely curative [157]. However, several independent groups have reported that immune modulating agents can provide a significant therapeutic benefit in preclinical models of PC [158-161]. These promising findings suggest that strategies designed to boost the anti-tumor immune response within the peritoneal cavity should be pursued in the treatment of PC.

2 HYPOTHESIS

I hypothesize that an MG1-IL12 based infected cell vaccine will lead to a profound NK cell response and subsequently shape a better T cell response that will improve vaccine outcome. This vaccine will lead to robust tumor regression following treatment in a therapeutic setting. In addition, due to the chemotactic properties of IL-12, local intratumoral delivery of the vaccine will improve its therapeutic index.

3 RESULTS

3.1 In vitro cytotoxicity, growth kinetics, and IL-12 expression from MG1 oncolytic virus encoding murine IL12 (MG1-IL12)

A murine IL12 transgene (p70), which is composed of p35 and p40 subunits, was incorporated into the backbone of the oncolytic Maraba virus variant MG1 to create MG1-IL12. This replication competent OV was found to infect both murine (B16 and CT26lacZ) and human (SW620 and HCT-15) tumor cell lines. Murine melanoma B16 and colon cancer CT26lacZ showed replication behaviours of MG1 that were very similar at all assessed MOIs. Interestingly, unlike normal B16 cell viability at lower MOIs (0.001 and 0.01), CT26lacZ exhibited very high cytotoxicity from both viruses MG1 and MG1-IL12 (mean ~70 and 80 percent respectively). This observation can be attributed to CT26lacZ being generally more susceptible to viral infection compared to B16 cells **(Figure 3.1)**. The insertion of the IL12 gene did not impact the fitness of MG1, as replication and spreading kinetics in single and multi-step growth curves on Vero cells were similar to parental MG1 virus **(Figure 3.2)**. Moreover, I quantified culture supernatants from MG1-IL12 and parental MG1-infected Vero cells by ELISA for mIL12. Supernatants from MG1-IL12 infected Vero cells demonstrated a high level of mIL12 expression, compared to undetectable levels with parental MG1 **(Figure 3.3.a)**. Furthermore, IL12 was detected in the culture media of irradiated B16 (22 pg/cell) and irradiated CT26lacZ (180 pg/cell) cells infected with MG1-IL12 **(Figure 3.3.b and c)**. This large variation of IL-12 expression between B16 and CT26lacZ cell lines can be explained by the higher susceptibility of CT26lacZ to viral infection, which should correlate with more IL-12 being translated and secreted.

Together, these results demonstrate that MG1-IL12 can successfully infect murine tumor

cells resulting in viral replication and IL-12 secretion, allowing for an MG1-IL12 infected cell vaccine (ICV).

Figure 3.1. Viral potency in infecting tumor cell lines.

Cytotoxicity of MG1 and MG1-IL12 assessed in murine B16F10 and CT26lacZ cell lines and human HCT15 and SW720 cell lines at the indicated MOIs 48 hours post-infection using Alamar Blue staining.

Viral cytotoxicity

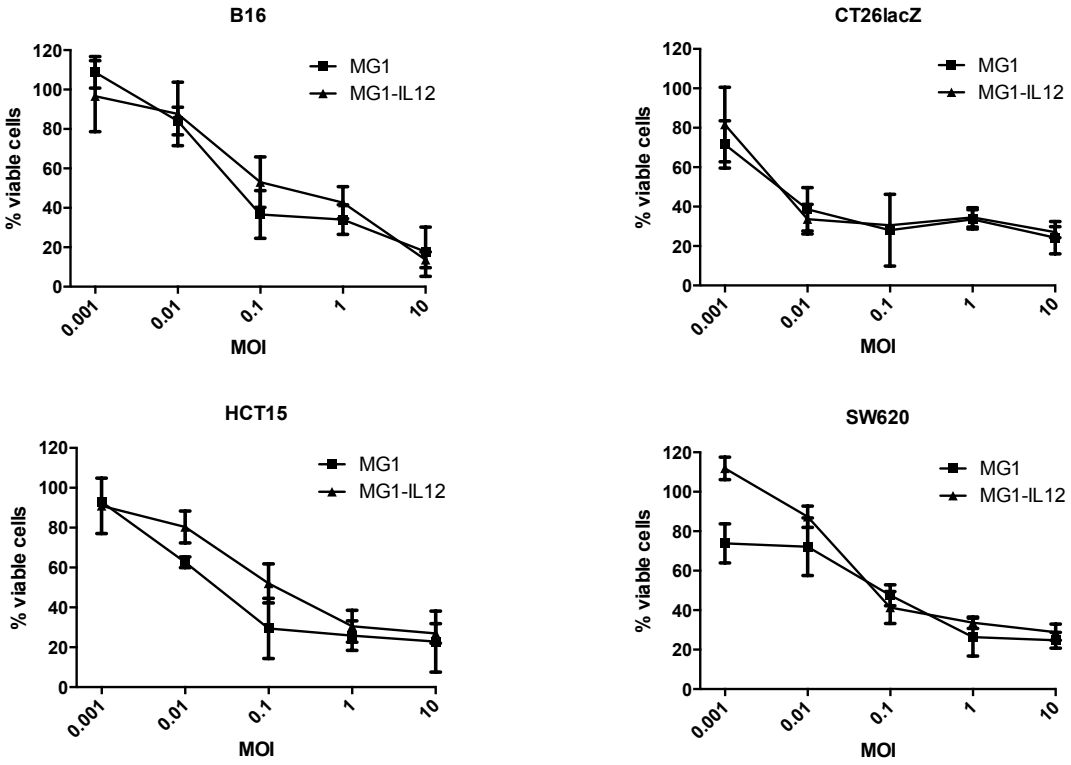
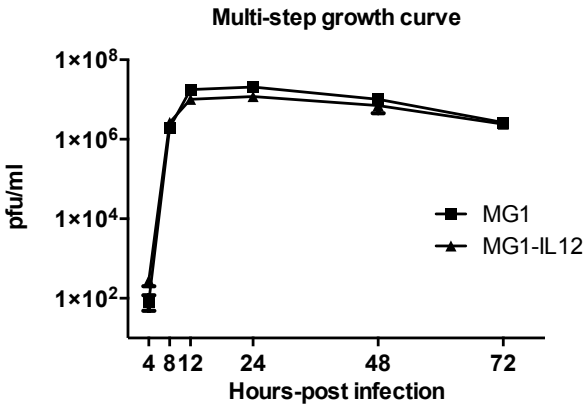


Figure 3.2. Viral growth kinetics of MG1 and MG1-IL12 viruses.

(a) Single-step and (b) multi-step growth curves were performed with MG1 and MG1-IL12 viruses. For single-step, Vero cells were infected at an MOI of 10 whereas for multi-step growth curves an MOI of 0.001 was used. Supernatants were collected at the indicated time points post-infection and titered accordingly.


Multi-step growth curve
1×10^8
1×10^6
1×10^4
1×10^2
pfu/ml
MG1
MG1-IL12
4 8 12 24 48 72
Hours-post infection

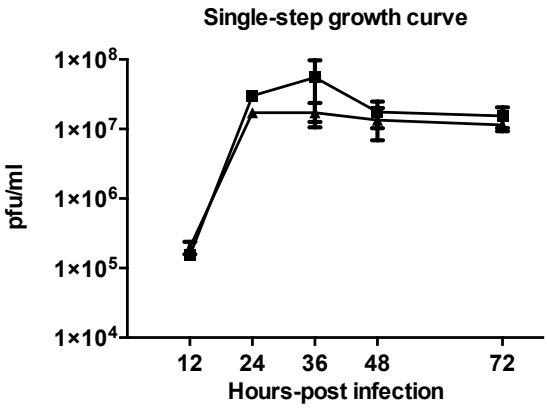
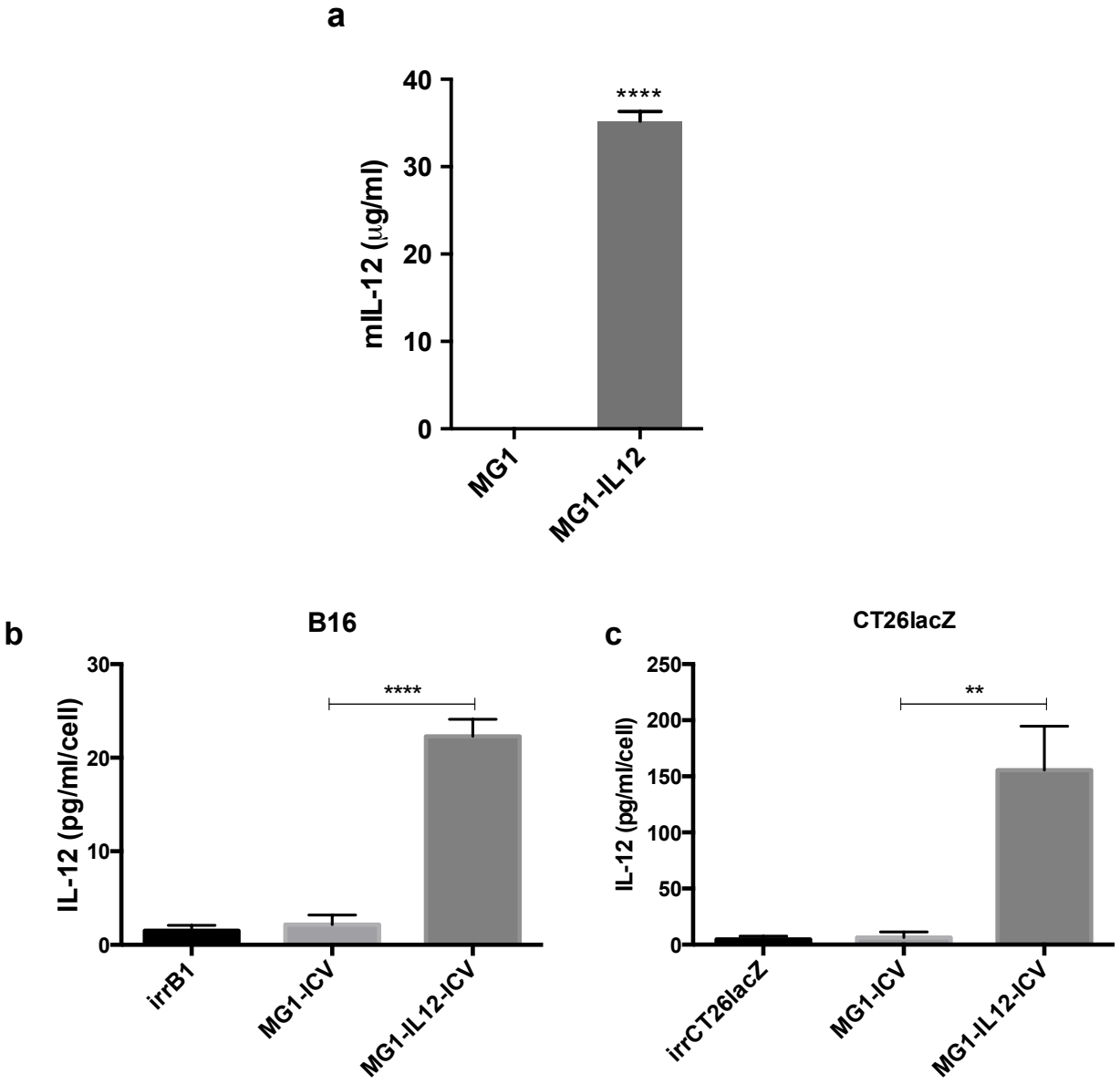

Single-step growth curve
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
pfu/ml
12 24 36 48 72
Hours-post infection

Figure 3.3. Interleukin-12 (IL-12) expression levels from cell monolayers and from the infected cell vaccine.

Production of mIL-12 from (**a**) Vero monolayer, **(b)** B16F10 ICV and **(c)** CT26lacZ ICV infected with MG1 or MG1-IL12 at MOIs of 5, 10 and 10, respectively, was examined 6 hours post infection by ELISA. All data are presented as mean of triplicate wells. Error bars =SD (** $p < 0.005$, **** $p \leq 0.00005$).


a
40
30
20
10
0
mIL-12 (μg/ml)
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MG1
MG1-IL12
b
B16
30
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10
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IL-12 (pg/ml/cell)
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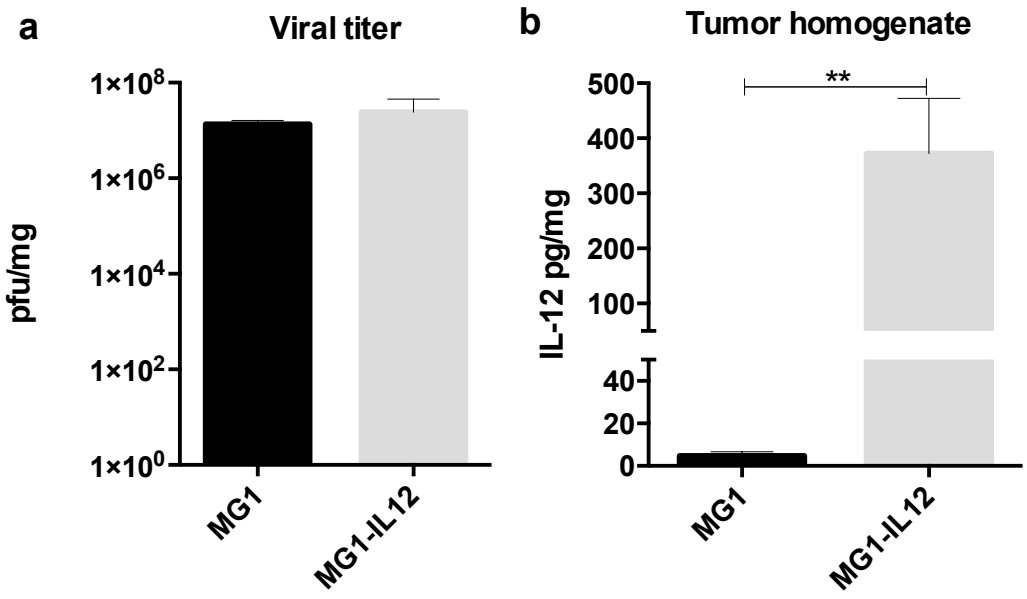
irrB1
MG1-ICV
MG1-IL12-ICV
c
CT26lacZ
250
200
150
100
50
0
IL-12 (pg/ml/cell)
**
irrCT26lacZ
MG1-ICV
MG1-IL12-ICV

3.2 In vivo viral delivery and IL-12 expression within CT26lacZ subcutaneous tumors

To confirm *in vivo* expression of mIL-12, mice bearing eight day old subcutaneous (sc) CT26lacZ tumors were treated intravenously (i.v.) with MG1-IL12 or parental MG1. Viral titres obtained from subcutaneous tumor homogenates showed similar levels of viral infection indicating that both viruses were successfully delivered and replicated within the tumors **(Figure 3.4a)**. Importantly, I detected significantly higher levels of IL-12 in tumor homogenates after 24 hours from mice treated with MG1-IL-12 compared to parental MG1 **(Figure 3.4b)**.

Figure 3.4. Localized IL-12 expression from the tumor following virus injection.

(a) Viral titres and (b) IL12 expression was assessed in vivo in a subcutaneous CT26lacZ tumor model. Tumor-bearing BALB/c mice were treated with 5×10^8 plaque-forming units (PFU) of MG1 or MG1-IL12. 24 h after treatment, tumors were removed and IL12 was detected by ELISA in homogenates from MG1-IL-12- treated tumors. All data are presented as mean of n = 3 mice per group. Error bars = SD ** $p < 0.005$).

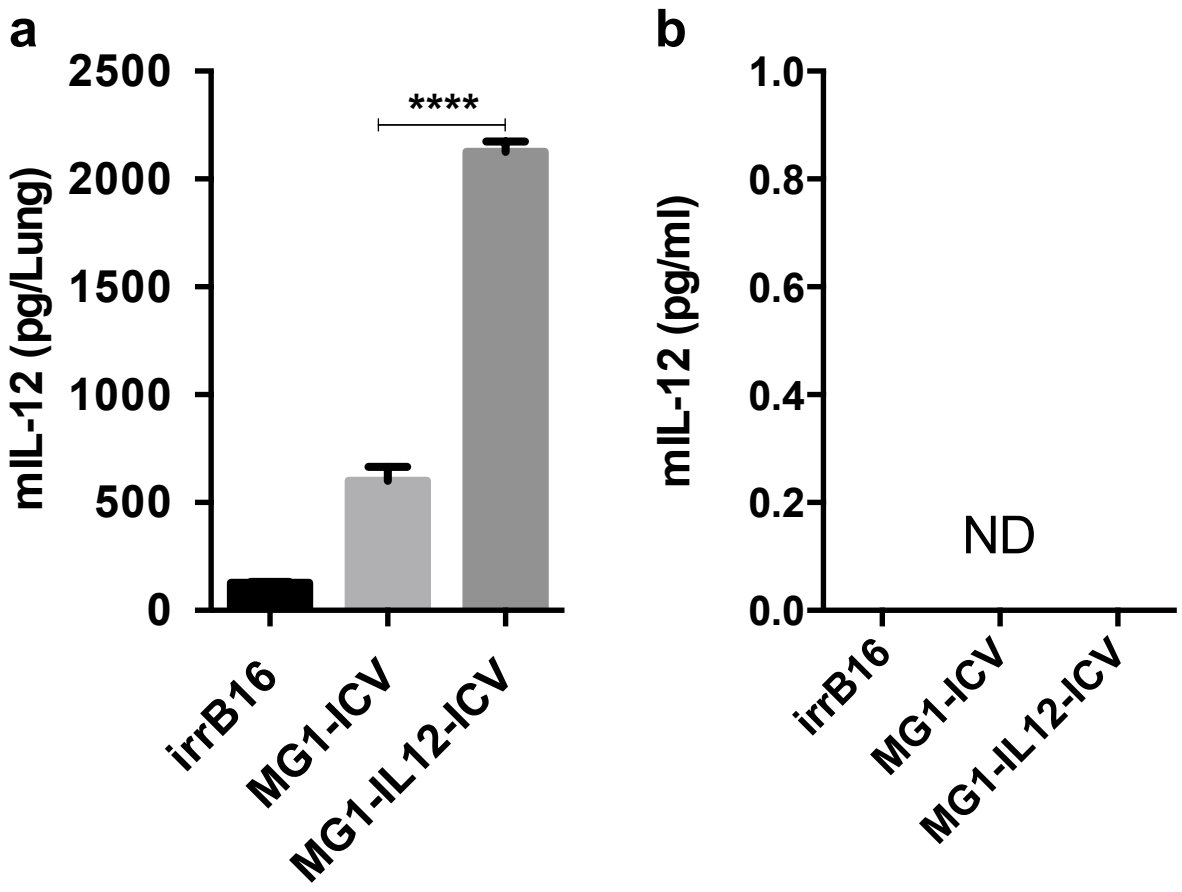

a
Viral titer
pfu/mg
1×10^8
1×10^6
1×10^4
1×10^2
1×10^0
MG1
MG1-IL12
b
Tumor homogenate
IL-12 pg/mg
**
500
400
300
200
100
40
20
0
MG1
MG1-IL12

3.3 Intravenous MG1-IL12-ICV injection restricts IL-12 expression to the lungs

To determine whether MG1-IL12-ICV is well tolerated and expresses IL-12 following intravenous injection, I intravenously (i.v.) injected 5×10^5 γ-irradiated B16F10 cells either mock infected or infected with MG1 or MG1-IL12. We have previously shown that iv administration of ICVs is associated with a rapid and dose-dependent accumulation of injected cells which persist in the lungs for up to 1 day in tumor-free animals [162]. As expected, following ICV delivery, I detected significantly higher levels of IL-12 (>2000 pg/lung) in lung homogenates from mice receiving MGI-IL12-ICV in comparison to animals receiving cells alone (~100pg/lung) or MG1-ICV (~500 pg/lung) (**Figure 3.5a**, t = 24hr). Even though injecting irradiated B16F10 cells alone induced a basal level of IL-12, the incorporation of mIL-12 into MG1 had evidently increased the accumulation of IL-12 cytokine four-fold in the murine lungs compared to the level found in the parental MG1 used in the ICV. Interestingly, I was not able to detect any IL-12 levels from the serum following MG1-IL12-ICV injection (**Figure 3.5b**). This finding indicates the safety of MG1-IL12-ICV, which does not leak IL-12 into the circulation system and suggests a localized ICV delivery to harness the advantage of IL-12 expression.

Figure 3.5. Barriers in the lungs prevent IL-12 leakage into the bloodstream.

(a) IL-12 was detected in the lungs 18 hours after intravenous treatment with 5×10^5 ICV. (b) Undetectable levels of IL-12 from mice's serum following ICV injection. All data are presented as mean of n = 3 mice per group. Error bars = SD (**** $p \leq 0.00005$).


a
2500
2000
1500
1000
500
0
mIL-12 (pg/Lung)
****
irrB16
MG1-ICV
MG1-IL12-ICV
b
1.0
0.8
0.6
0.4
0.2
0.0
mIL-12 (pg/ml)
ND
irrB16
MG1-ICV
MG1-IL12-ICV

3.4 Intravenous injection of MG1-IL12-ICV elicits strong NK cell functions.

We have previously demonstrated that infecting autologous tumor cells *ex vivo* with OVs can elicit a robust immune response against established, non-permissive tumors *in vivo* [141]. These results, however, are inadequate to establish their differential effectiveness as an infected cell vaccine (ICV). In addition, our previous study has shown that iv administration of ICVs is associated with a rapid and dose-dependent accumulation of injected cells which persist in the lung for up to 1 day in tumor free animals [162].

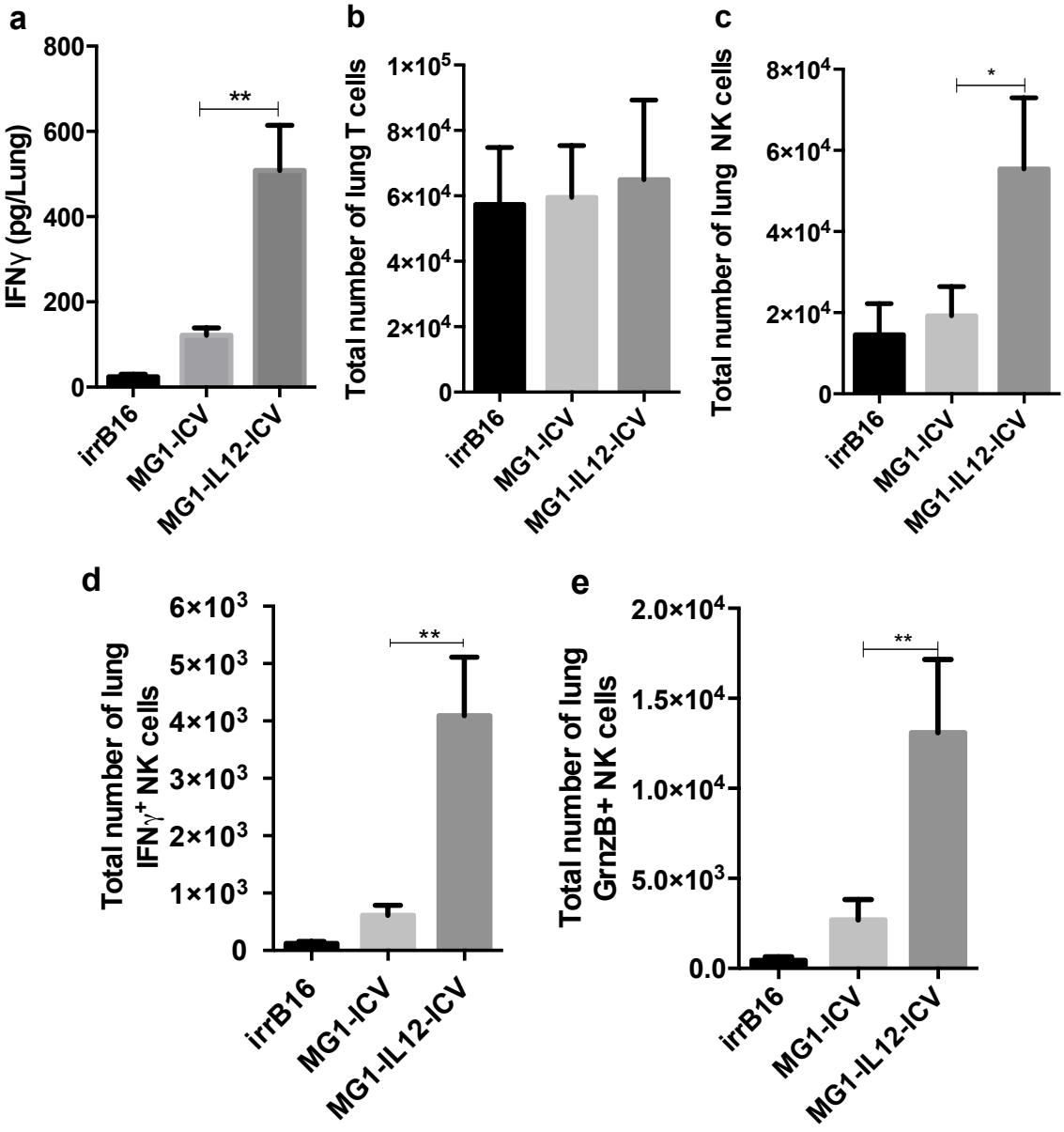
To determine whether the increased concentrations of IL-12 had any functional effect in increasing the synthesis of IL-12 responsive cytokines, I measured the levels of IFN-γ 18 hours following ICV injection. In agreement with the increase in IL-12, levels of IFN-γ were also elevated (~500 pg/lung vs. ~100 pg/lung or ~10 pg/lung) in the lungs of mice treated with MG1-IL12-ICV compared to mice receiving MG1-ICV or irradiated cells, respectively (**Figure 3.6a**). The functional boost of IFN-γ observed in the MG1-IL12-ICV increased approximately five times more than that observed in the MG1-ICV, an added antitumor potential that can be attributed to the mIL-12 transgene.

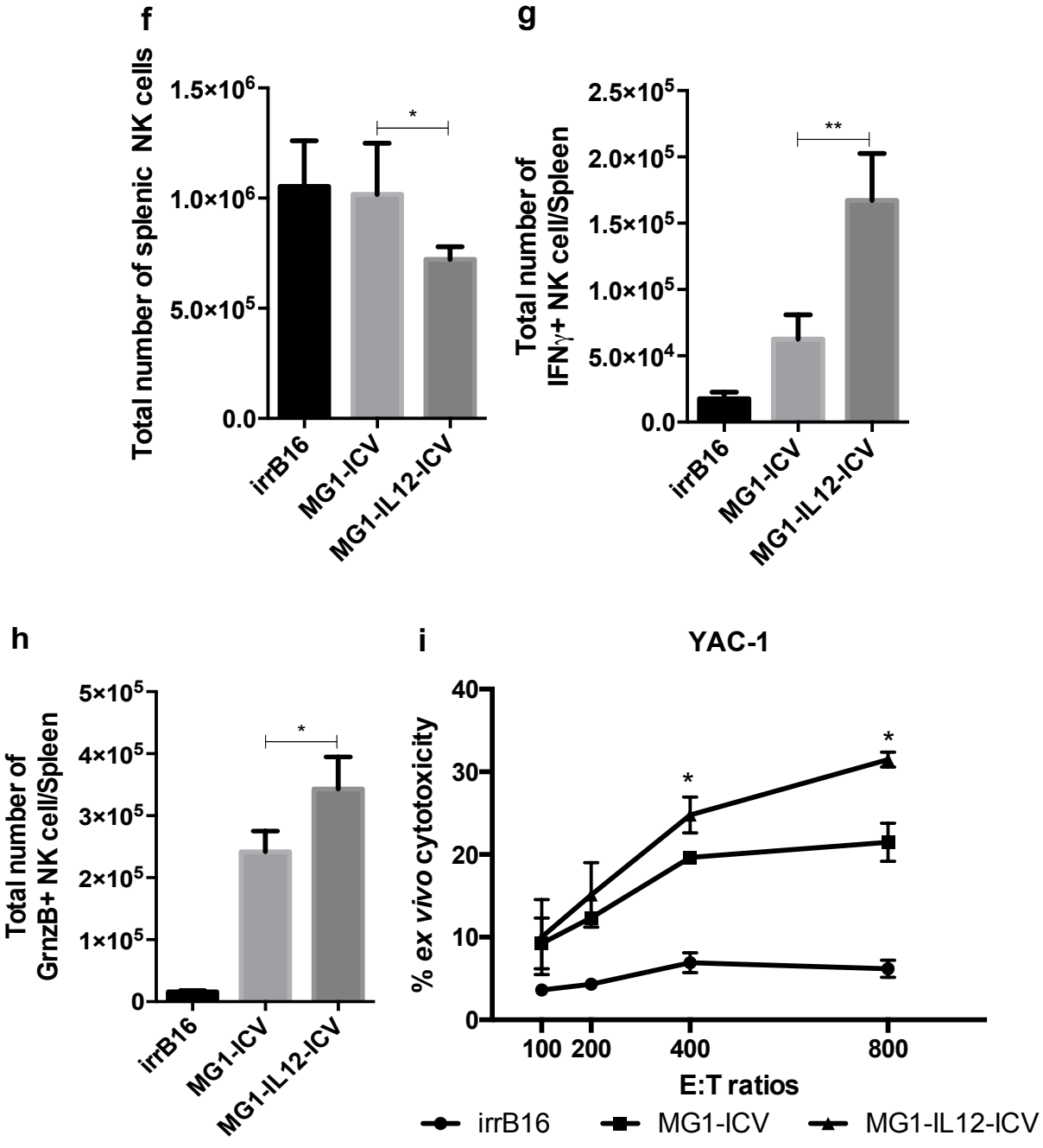
Since IL-12 targets both NK and T cells to promote IFN-γ secretion [96], I next sought to determine which cell types were responding to treatment with our MG1-IL12-ICV. The impact of IL-12 and consequently IFN-γ on lung T cell counts was insignificant (**Figure 3.6b**). However, the outcomes in the NK cell count as well as in the $IFN\gamma^+$ and Granzyme B^+ NK cell levels showed statistically significant differences. The mean lung NK cell count reached an average of ~5.5 $\times 10^4$ cells (up to ~7.5 $\times 10^4$ cells) in 24 hours (**Figure 3.6c**). Meanwhile, the IFN-γ and Granzyme B levels increased on average more than 7-fold (up to 10-fold) and 5-fold (up to 7-fold) in MG1-IL12-ICV over those in MG1-ICV, respectively (**Figure 3.6d and e**),

representing a 60% better response of NK cells to the recombinant MG1-IL12-ICV than the parental MG1-ICV. Once again, this improved vaccine performance can be attributed to the incorporation of mIL-12 into the MG1 genome. Interestingly, vaccination with MG1-IL12-ICV was not found to impact the total number of T cells in the lungs or T cell cytolytic proteins. However, a 3-fold increase in the total number of NK cells present in the lungs was observed, concomitant with a significant decrease in the total number of splenic NK cells, suggesting that MG1-IL12-ICV improves NK cell influx and recruitment to the lungs (**Figure 3.6f**). To further examine the effect, I measured the *ex vivo* cytotoxic activity of NK cells against YAC-1 target cells, which are mouse lymphoma cells that are sensitive to NK cell mediated lysis [38]. I found that splenocytes isolated from MG1-IL12-ICV treated mice exhibited a significantly higher level of YAC-1 killing (**Figure 3.6i**), which indicates strong tumoricidal action. The NK cells stimulated by the recombinant MG1-IL12-ICV demonstrated an *ex vivo* toxicity rate of 30 percent at an effector-to-target cell ratio (E:T ratio) of 800, which was 10 percent higher than that with the MG1-ICV. The higher NK cell killing of MG1-IL12-ICV was also observed at the 200 E:T ratio. These data support a role for MG1-IL12-ICV in promoting NK cell recruitment and activation at the site of vaccine delivery and a concomitant systemic activation of splenic NK cells, which were on average approximately 5-fold (up to 6-fold) higher among $IFN\gamma^+$ NK cells and approximately 3-fold (up to 4-fold) higher among $GranzymeB^+$ NK cells (**Figure 3.6g and h**).

Figure 3.6. MG1-IL12-ICV enhances NK cell cytokine secretion and cytotoxicity.

(a) IFN-γ concentrations were measured in lung homogenates of tumor naïve mice 18 hours after i.v. delivery of 5×10^5 irradiated cells infected with MG1 or MG1-IL12 (MOI = 10). (b - e) Total number of CD3+ T cells, NK1.1+ cells, NK1.1+ IFNγ+ cells and NK1.1+ GranzymeB+ cells present in the lungs were quantified 18 hrs after i.v. treatment with 5×10^5 irradiated B16F10 cells alone (irrB16) or MG1-or MG1-IL-12 infected cells (MOI=10) in tumor naïve mice. (f – h) The quantification of total splenic NK1.1+ cells, NK1.1+ IFNγ+ cells and NK1.1+ GranzymeB+ cells. (i) Ex vivo chromium release cytotoxicity assay of splenocytes (effector,E) isolated from tumor naive C57BL/6 18 hours following treatments with 1×10^6 irradiated B16F10 cells alone or infected with either MG1 or MG1-IL12 (MOI=10) towards target YAC1 cells at the indicated effector to target (E:T) ratios. All data are presented as mean of n = 3 mice per group. Error bars = SD (* $p < 0.05$, ** $p < 0.005$).


a
IFNγ (pg/Lung)
800
600
400
200
0
**
irrB16
MG1-ICV
MG1-IL12-ICV
b
Total number of lung T cells
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
irrB16
MG1-ICV
MG1-IL12-ICV
c
Total number of lung NK cells
8×10^4
6×10^4
4×10^4
2×10^4
0
*
irrB16
MG1-ICV
MG1-IL12-ICV
d
Total number of lung IFNγ+ NK cells
6×10^3
5×10^3
4×10^3
3×10^3
2×10^3
1×10^3
0
**
irrB16
MG1-ICV
MG1-IL12-ICV
e
Total number of lung GrnzB+ NK cells
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0.0
**
irrB16
MG1-ICV
MG1-IL12-ICV


f
Total number of splenic NK cells
1.5×10^6
1.0×10^6
5.0×10^5
0.0
*
irrB16
MG1-ICV
MG1-IL12-ICV
g
Total number of IFNγ+ NK cell/Spleen
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
**
irrB16
MG1-ICV
MG1-IL12-ICV
h
Total number of GrnzB+ NK cell/Spleen
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
*
irrB16
MG1-ICV
MG1-IL12-ICV
i
YAC-1
% ex vivo cytotoxicity
40
30
20
10
0
*
*
100 200 400 800
E:T ratios
irrB16
MG1-ICV
MG1-IL12-ICV

3.5 MG1-IL12-ICV attenuates B16F10 lung micro-metastases

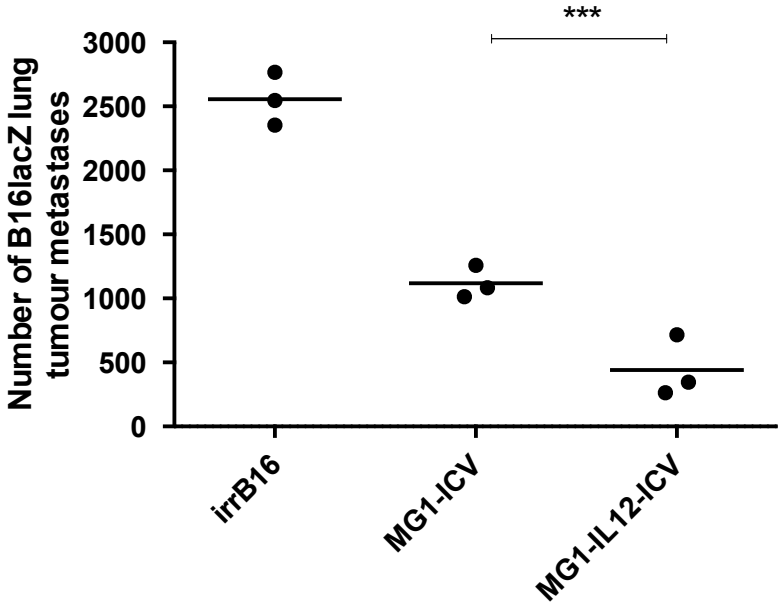
In order to determine whether the enhanced NK cell function and cytotoxicity translates into improved tumor clearance, I treated B16F10 lung metastases with irradiated cells, MG1-IL12-ICV, or MG1-ICV alone by i.v. delivery (**Figure 3.7**). Systemic delivery of MG1-IL12-ICV was sufficient to significantly attenuate the number of detectable lung metastases (mean = <500 tumor metastases count vs. >1000 or >2500, respectively) in comparison to treatment with MG1-ICV or irradiated cells. These results suggest that MG1-IL12-ICV can stimulate NK cell recruitment and effector functions. This supports the enhanced NK cell killing of Yac-1 target cells as described above (**Figure 3.6i**).

Unfortunately, when doses higher than 1×10^6 infected cells were administered i.v., mice began to experience immediate respiratory distress. This is likely due to pulmonary embolism (data not shown). Alveolar ventilation/pulmonary perfusion (V/Q) mismatch, which is characteristic of acute respiratory distress syndrome (ARDS) [40, 41], or the presence of pulmonary emboli [40, 41, 42], can cause hypoxia that may lead to death through Type I respiratory failure [40]. However, literature involving experimental animal or human models has limited data on the involvement of MG1 oncolytic virus in incidents of acute pulmonary distress after intravenous administration. Moreover, i.v. administration of cytokine recombinant vaccines was well documented to cause a systemic or inflammatory cytokine storm, resulting in a condition called 'cytokine release syndrome' (CRS). This massive release of cytokines, which results from a barrage of activated lymphocytes (e.g. B cells, T cells and NK cells) or myeloid cells (e.g. macrophages, dendritic cells, and monocytes) [39], causes a systemic inflammatory response that can lead to death [69].

Figure 3.7. The attenuation of B16F10 lung metastases following MG1-IL12-ICV treatment

B16F10 micro-metastases were observed in the lungs four days after i.v. vaccination with MG1-ICV or MG1-IL12-ICV. Data are presented as mean of n = 3 mice per group. Error bars = SD

*** $p < 0.0005$.


***
Number of B16lacZ lung tumour metastases
3000
2500
2000
1500
1000
500
0
irrB16
MG1-ICV
MG1-IL12-ICV

Classes of cytokines implicated in this potentially lethal non-specific toxicity are interleukins, such as IL-1 [70] and IL-6 [39, 69], and interferons such as IFN-γ [39].

Given the potential risks of intravenous delivery, including the risk of pulmonary embolism [40, 41, 42], stroke, and systemic cytokine storm [39, 69-70], I hypothesized that intraperitoneal (i.p.) delivery of the ICV [43, 45] in the setting of peritoneal carcinomatosis [44, 45] would take advantage of NK cell recruitment to the site of ICV delivery while maintaining safety. However, intraperitoneal delivery is not without problems. The perioperative approach to intraperitoneal chemotherapy, for instance, has been documented to cause relatively high rates of morbidity [45].

3.6 MG1-IL12-ICV enhances NK cell activation and improves survival in a model of peritoneal carcinomatosis.

My initial findings suggest that the improved anti-tumor response elicited by MG1-IL12-ICV in comparison to MG1-ICV is in part due to potent chemotactic properties of IL-12, which contribute to the enhanced recruitment of cytotoxic NK cells to the site of delivery (**Figure 3.6c**). Therefore, I next sought to assess whether vaccinating mice i.p. with MG1-IL12-ICV could improve tumor clearance within the peritoneal cavity and promote improved survival. Similar to my previous observations with i.v. vaccination, I observed an increased proportion of NK cells (19% vs. 49%, $p = 0.0073$) 24 hours after i.p. vaccination, as compared to MG1-ICV (**Figure 3.8b**). Moreover, the survival rates with MG1-IL12-ICV showed longer persistency at 100 percent rate (~18 d) and at endpoint (~42 d). Comparatively, with MG1-ICV, the mice started to die at approximately 15 days and reached final end-point at approximately day 22 (**Figure 3.8a**). This survival difference of 7 d was likely due to the presence of mIL-12 in the vaccine. Moreover, the effectiveness of the IL-12 protein in significantly inhibiting metastatic growth of

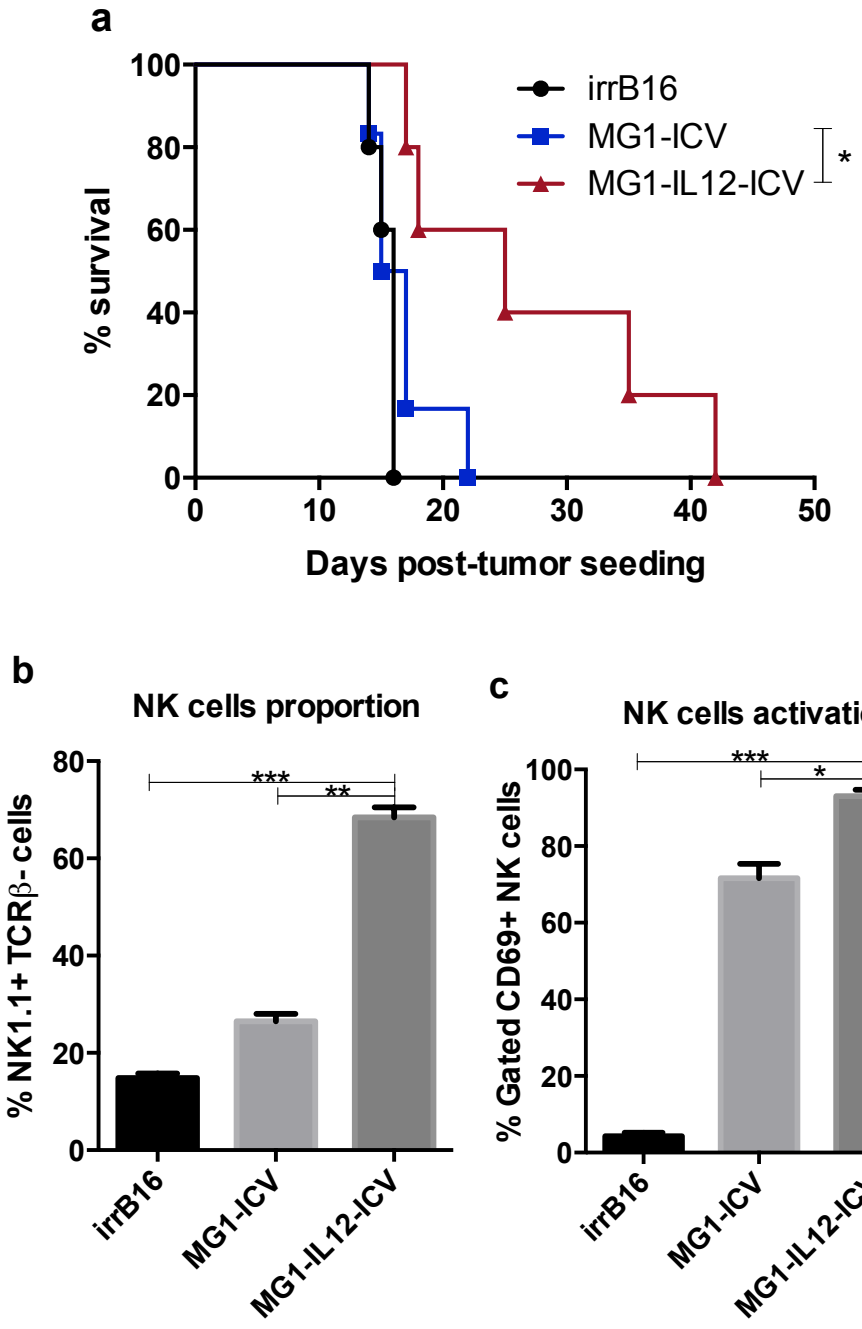
B16F10 melanoma cells reinforces the findings of previous studies [63].

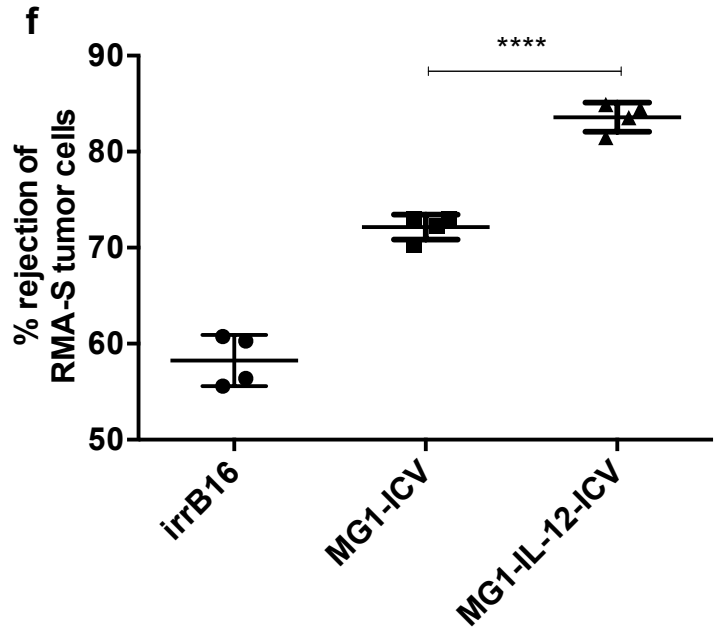
Notwithstanding, NK cells had accumulated at higher rates (mean = ~50% vs. ~20%, respectively) following MG1-IL12-ICV than those with MG1-ICV (**Figure 3.8b**), indicating the superior chemotactic strength of IL-12. In addition to its chemotactic properties, MG1-ICV maximized NK cell activation from ~75% activation to ~95% activation. The infiltrating NK cells also displayed higher expression of the activation marker CD69 (**Figure 3.8c**) [163, 164].

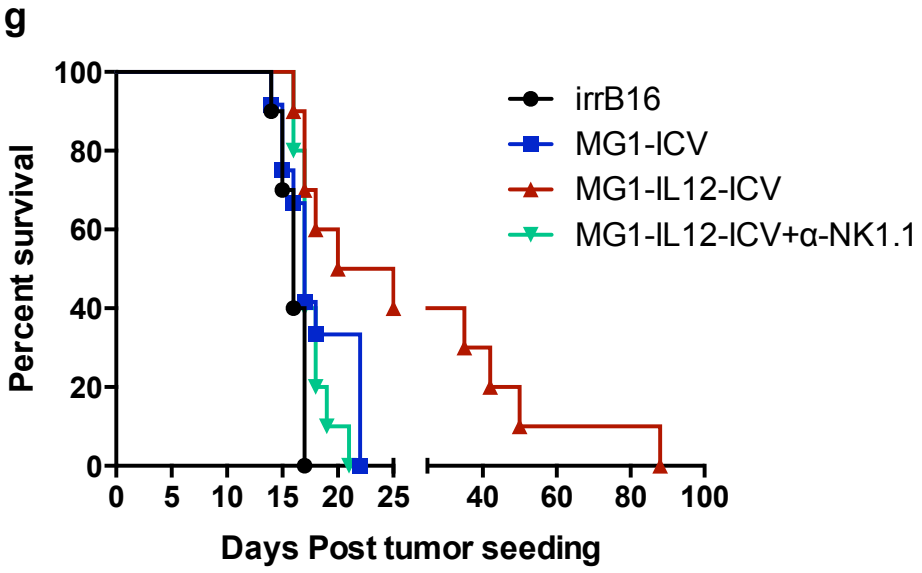
Furthermore, DC accumulation in the peritoneum was also significantly increased and associated with a high expression profile of CD80/CD86, which are DC activation and maturation markers. These findings shed light on the role of DCs in the mode of action of MG1-IL12-ICV and possibly provide the mediating link between the vaccine and NK cells (**Figure 3.8d and e**). To complement these findings, I performed an *in-vivo* NK cell cytotoxicity assay by challenging vaccinated mice with the NK-sensitive RMA-S and NK-resistant parental RMA tumor cell lines to investigate whether the activated NK cells that migrated into the peritoneal cavity were tumoricidal. Vaccination with MG1-IL12-ICV significantly improved tumor cell clearance (**Figure 3.8f**). The rejection rates of the RMA-S murine lymphoma cells [47, 48] were highest with MG1-IL12-ICV, which was approximately 10 percent higher than that of MG1 ICV (mean = >80% vs. >70%, respectively) (**Figure 3.8f**).

Figure 3.8. MG1-IL12-ICV-dependent NK cell activation enhances tumor cell clearance and improves host survival.

(a) Mice were treated once with 1×10^4 cells of the indicated vaccines on day 3. n = 10 mice per group. $p < 0.02$, log rank test. (b) Mice vaccinated i.p. with 1×10^4 irrB16, MG1-ICV, or MG1-IL12-ICV were subsequently challenged with a mixture of 1×10^6 each of RMA and RMA-S cells at a 1:1 ratio. The percentage of RMA-S cells rejected was calculated as described in materials and methods. (c-f) The effect of irrB16, MG1-ICV, or MG1-IL12-ICV on the recruitment and activation status ($CD69^+$) of NK cells ($NK1.1^+$ $CD3^-$) and ($CD80/86^+$) of DCs to the peritoneal cavity respectively was examined by flow cytometry 24 hours after-i.p. vaccination with 1×10^4 cells. (g) Kaplan–Meier survival analysis of C57BL/6 mice-bearing i.p. B16F10 tumors seeded at 5×10^5. 1 day following tumor seeding, NK cells were depleted using anti-NK1.1 antibody and continued every other day for a total of seven doses.


a
100
80
60
40
20
0
% survival
0
10
20
30
40
50
Days post-tumor seeding
irrB16
MG1-ICV
MG1-IL12-ICV
*
b
NK cells proportion
% NK1.1+ TCRβ- cells
80
60
40
20
0
***
**
irrB16
MG1-ICV
MG1-IL12-ICV
c
NK cells activation
% Gated CD69+ NK cells
100
80
60
40
20
0
***
*
irrB16
MG1-ICV
MG1-IL12-ICV


f
****
90
80
70
60
50
% rejection of RMA-S tumor cells
irrB16
MG1-ICV
MG1-IL-12-ICV


g
100
80
60
40
20
0
Percent survival
0
5
10
15
20
25
40
60
80
100
Days Post tumor seeding
irrB16
MG1-ICV
MG1-IL12-ICV
MG1-IL12-ICV+α-NK1.1

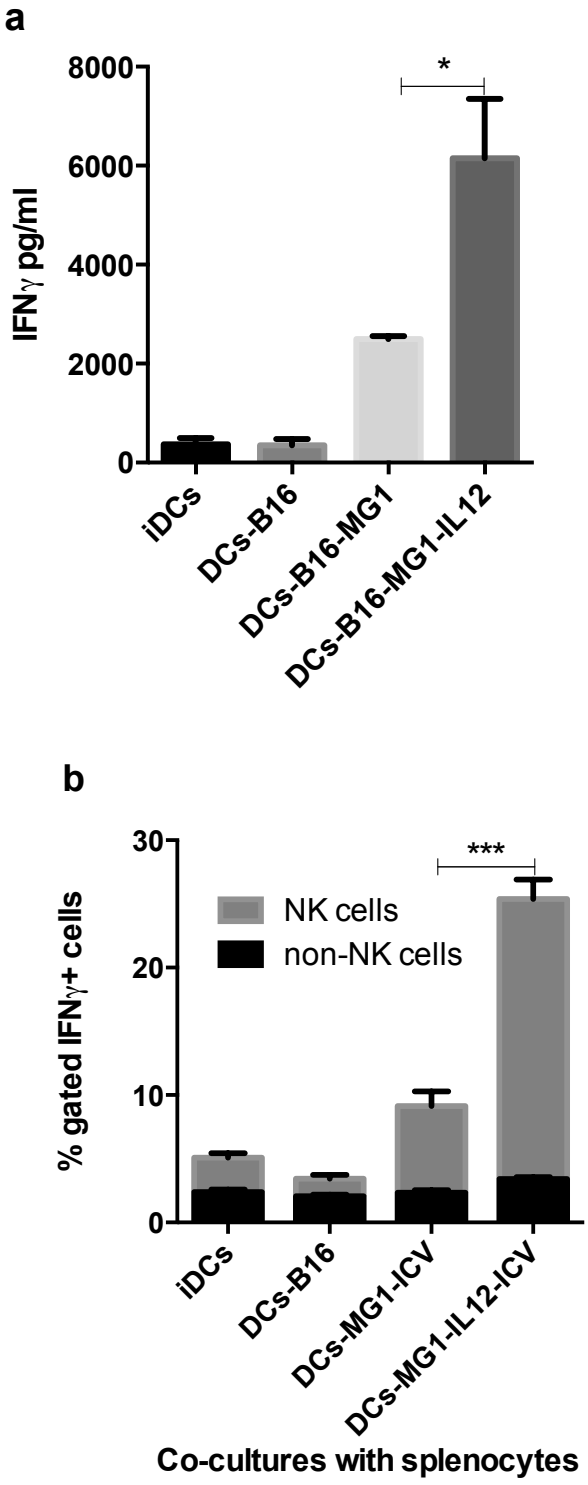
In support of this conclusion, the protective effect of vaccinating mice bearing B16F10 peritoneal tumors with MG1-IL12-ICV was completely abrogated by depleting NK cells, further suggesting that the therapeutic benefit of this treatment strategy is dependent upon NK cell recruitment and activation **(Figure 3.8g)**.

3.7 Bone-marrow derived DCs pulsed with MG1-IL12-ICV improve IFN-γ production from NK cells

My data clearly establish the ability of MG1-IL12-ICV to promote NK cell activation, migration and function; however, it was unclear whether DCs, which are key mediators of NK cell function *in vivo,* were involved in this process. To understand the interaction between NK cells and DCs in the presence of MG1-IL12-ICV, I quantified IFN-γ production from splenocytes cultured in the presence of bone marrow derived DCs, which were either untreated or cultured with mock, MG1, or MG1-IL12 infected B16F10 cells. Previous studies [50, 52-53] have demonstrated that the interactions between DCs and NK cells result in DC maturation and activation of NK cells. Notably, I found that splenocytes cultured with DCs previously exposed to MG1-IL12-ICV resulted in a significant increase in NK cell-specific IFN-γ secretion, suggesting that DCs promote NK cell cytokine secretion (**Figure 3.9a and 3.9b**). These data demonstrate two important IL-12 stimulatory functions when integrated to the MG1 backbone in MG1-IL12-ICV and in the presence of DCs. First, it stimulated significantly higher levels of IFN-γ (mean = 6000 pg/ml vs. ~2000 pg/ml) than MG1-ICV (**Figure 3.9a**). Second, it also significantly stimulated the increased availability of IFNγ-activated NK cells far higher than parental MG1 (mean = 25% vs. ~10%) (**Figure 3.9b**).

Figure 3.9. MG1-IL12-ICV enhances IFN-γ secretion from NK cells through DCs.

(a) IFN-γ secretion and (b) population of $IFN\gamma^+$ cells present in naïve splenocytes (2×10^5) was measured by ELISA and flow cytometry, respectively, following co-culture (5:1) with isolated $CD11c^+$ cells previously stimulated with irrB16, MG1-ICV or MG1-IL12-ICV (t = 18 hrs). All data are presented as mean of triplicate wells. Error bars =SD (* $p < 0.05$, *** $p \leq 0.0005$).

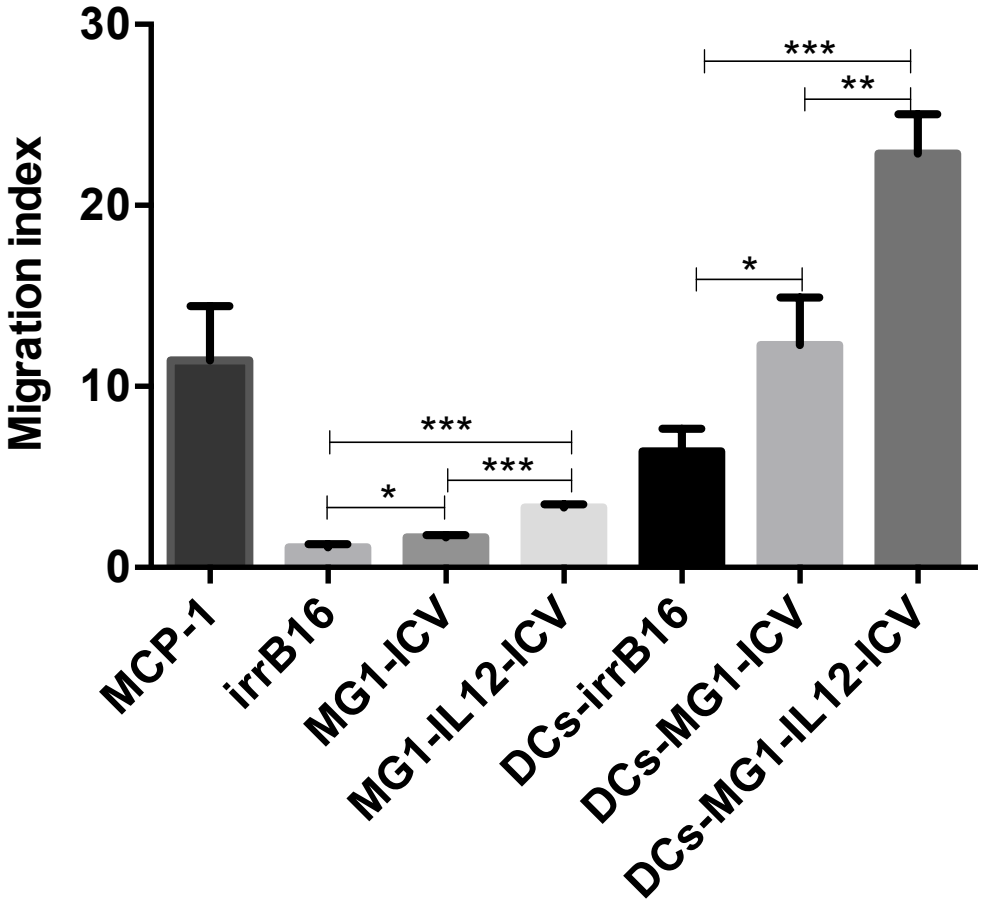

a
8000
6000
4000
2000
0
IFNγ pg/ml
*
iDCs
DCs-B16
DCs-B16-MG1
DCs-B16-MG1-IL12
b
30
20
10
0
% gated IFNγ+ cells
***
NK cells
non-NK cells
iDCs
DCs-B16
DCs-MG1-ICV
DCs-MG1-IL12-ICV
Co-cultures with splenocytes

There is also clear evidence of the significant positive impact of parental MG1 in stimulating IFN-γ expression (**Figure 3.9a**), though at reduced levels when compared to MG1-IL12-ICV. Evidently, DCs alone have no significant influence on IFN-γ secretion in B16F10 culture medium or in NK cell activation. The latter observation indicates that there exists an optimal level of DC stimulatory function for IL-12 [50] (e.g. the level of IL-12 produced), which determines the effectiveness of DCs in activating NK cells.

3.8 DCs-MG1-IL12-ICV co-culture induces a massive NK cell migration.

I next investigated the ability of DCs to promote NK cell migration using an *in vitro* transwell chemotaxis assay. The migration index (MI) of NK cells across a 5 μm membrane was found to be significantly increased by either MG1-ICV (mean = 2.0) or MG1-IL-12-ICV (mean = 4.0) at MI below 5 (mean <5.0), indicating a doubled migration difference attributable to the presence of IL-12 (**Figure 3.10**). Meanwhile, NK cell migration was further increased by media conditioned in the presence of DCs, suggesting that DCs provide the stimulus for increased NK cell activation and migration. While DCs alone increased NK cell migration (mean ~5.0), the presence of MG1-ICV and of the mIL-12 (MG1-IL12-ICV) almost doubled (mean ~5.0) or quadrupled it (mean ~20.0), respectively.

Figure 3.10. MG1-IL12-ICV enhances NK cell migratory behaviour *in vitro* through DCs. Migration of isolated, naïve NK cells across a transwell membrane (pore size = 5 um) into cell free media conditioned was measured following a 3 hr incubation as described in materials and methods. All data are presented as mean of triplicate wells. Error bars =SD (* $p < 0.05$, ** $p < 0.005$, *** $p \leq 0.0005$).


Migration index
30
20
10
0
***
**
*
***
***
*
MCP-1
irrB16
MG1-ICV
MG1-IL12-ICV
DCs-irrB16
DCs-MG1-ICV
DCs-MG1-IL12-ICV

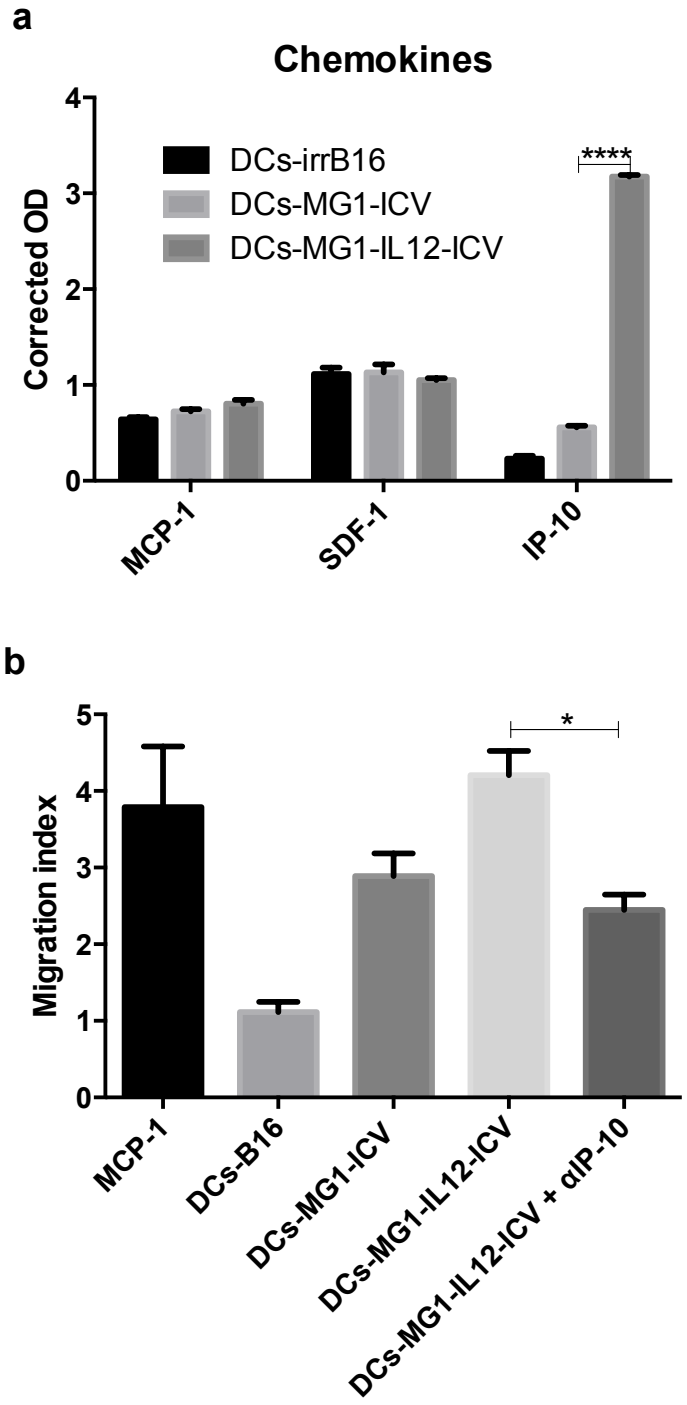
3.9 NK cell migration in response to MG1-IL12-ICV is partly dependent upon the secretion of IP-10 from DCs.

Next, I sought to identify which chemokines commonly secreted by DCs were mediating the observed effects. Although the exact chemokines regulating the transit of DC precursors into peripheral tissues remains largely unknown, three chemokines had been associated with this function: CCL2 (MPC-1), CXCL12 (SDF-1), and IP-10 [55]. While I was unable to any effect on MCP-1 and SDF-1 (stromal cell-derived factor-1) secretion, MG1-IL12-ICV induced a significant increase in IP-10 (IFN-inducible Protein-10) (**Figure 3.11a**). The insignificant performance of MCP-1 disputes its previous association with DCs, despite studies attesting to its chemotactic influence on NK cells [60]. Meanwhile, SDF-1 (CXCL12) has been observed in high levels with NK cells in malignant ascites [57], potentially indicating a suppressive effect of the IL-12 cytokine on SDF-1. The IP-10 performance confirms the NK migratory relationship with this chemokine, which is dependent upon IFN-γ [55, 58-59].

However, the migration index revealed relatively interesting outcomes. Both MG1-ICV (mean = 3 vs. 1) and MG1-IL12-ICV (mean >4 vs. 1) performed effectively in increasing NK migration, far more than DCs alone (**Figure 3.11b**). Interestingly, the neutralization of IP-10 in conditioned media derived from DCs cultured with MG1-IL12-ICV significantly inhibited the migratory capacity of NK cells *in vitro,* confirming its central role (**Figure 3.11b**).

Figure 3.11. MG1-IL12-ICV enhances NK cell migratory behaviour and activation in vitro by stimulating secretion of IP10 from DCs.

(a) The release of MCP-1, SDF-1 and IP-10 into the culture media following co-culture of DCs ($CD11c^+$) with the indicated treatments was determined by ELISA. (b) Quantification of the effects of IP-10 neutralization on NK cell chemotaxis following the addition of IP-10 neutralizing antibodies (100ng/ml) in conditioned media with DCs-(MG1-IL12-ICV) co-cultures.n=3, Error bars=SD, p values (*$p<0.05$, **** ≤ 0.00005).


a
Chemokines
Corrected OD
4
3
2
1
0
DCs-irrB16
DCs-MG1-ICV
DCs-MG1-IL12-ICV
****
MCP-1
SDF-1
IP-10
b
Migration index
5
4
3
2
1
0
*
MCP-1
DCs-B16
DCs-MG1-ICV
DCs-MG1-IL12-ICV
DCs-MG1-IL12-ICV + αIP-10

3.10 MG1-IL12-ICV is able to eradicate CT26lacZ colon cancer peritoneal metastatic disease in mice.

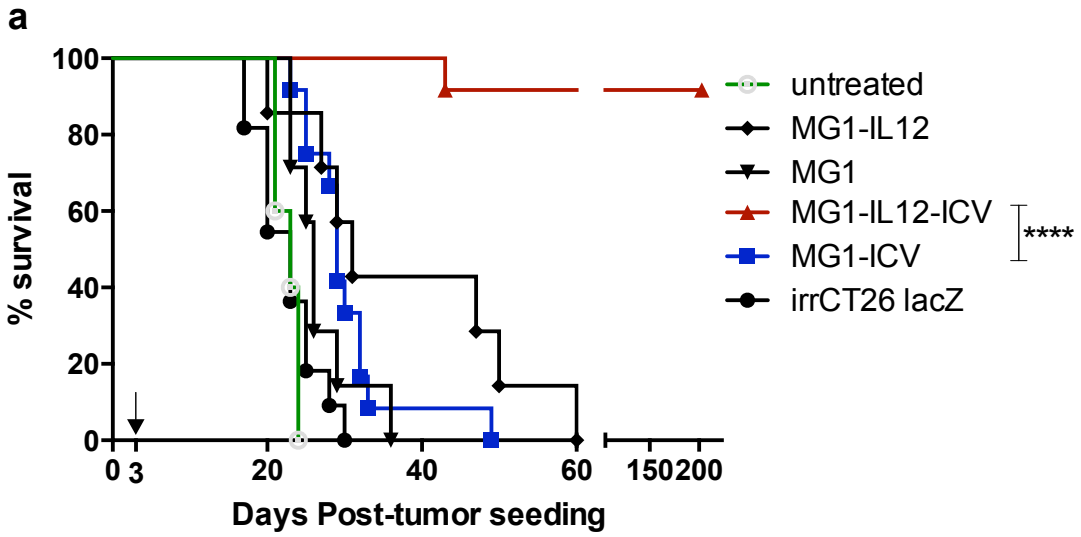
Together our findings suggest that the MG1-IL12-ICV can significantly slow the outgrowth of B16F10 tumors within the peritoneal compartment by stimulating the recruitment of activated NK cells. Next, I wanted to assess the efficacy of MG1-IL12-ICV in a more clinically relevant tumor model by using CT26, a colon cancer cell line, in a peritoneal tumor model.

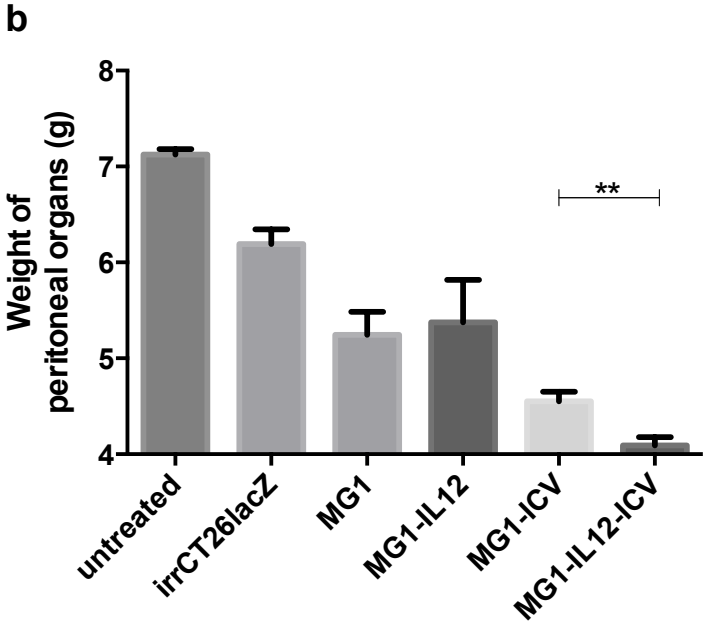
To accomplish this, BALB/c mice were seeded i.p. with 5×10^5 CT26lacZ tumor cells. Three days later mice were treated with a single dose of irradiated cells alone, virus alone or the infected cell vaccines (**Figure 3.12a**). Mice treated with irradiated CT26lacZ cells, MG1, MG1-IL12 and MG1-ICV all had significantly increased peritoneal tumor burden (mean= >6g, >5g, ~5.5g, and ~4.5g, respectively) and lower median survival times (20 to 30 days) in comparison to mice receiving MG1-IL12-ICV (>90% 26/28 mice survived > 200 days) (**Figure 3.12a** and **b**). Moreover, the weight of peritoneal organs including tumor burden in the MG1-IL12-ICV treatment group was significantly lower than the rest of the samples at slightly above four grams **(Figure 3.12b)**. The ICV approach demonstrated significant reduction in peritoneal tumor burden and was enhanced with the incorporation of IL-12 (mean ~4.5 g vs. ~4.0 g, respectively), indicating a relatively better treatment against peritoneal carcinoma using the MG1-IL12-ICV.

Interestingly, the cured mice developed a long lasting immunity such that when the surviving mice were re-challenged with 5×10^5 CT26lacZ cells on the flank 148 days after treatment, they rejected the tumors (5/5 mice). However, this anti-tumor memory was specific to CT26lacZ tumors and all mice developed tumors (5/5 mice) when challenged with syngeneic 4T1 tumor cells on the opposite flank.

Figure 3.12. MG1-IL-12-ICV improves survival of CT26lacZ tumor-bearing mice.

(a) BALB/c mice received a single dose of either virus alone (MG1 or MG1-IL12, MOI = 1×10^5 pfu/ml), an infected cell vaccine (MG1-ICV or MG1-IL12-ICV, 1×10^4 infected cells), irradiated cells alone (irrCT26lacZ) or left untreated. 3 days following i.p. tumor seeding (5×10^5), n = 10-12 mice per group, $p < 0.0005$, log rank test. (b) Weight of peritoneal organs including any associated tumor burden was measured 16 days post tumor seeding.


a
100
80
60
40
20
0
% survival
0 3
20
40
60
150200
Days Post-tumor seeding
untreated
MG1-IL12
MG1
MG1-IL12-ICV
MG1-ICV
irrCT26 lacZ
****


b
8
7
6
5
4
Weight of peritoneal organs (g)
**
untreated
irrCT26lacZ
MG1
MG1-IL12
MG1-ICV
MG1-IL12-ICV

3.11 Direct intraperitoneal (intratumoral) vaccination exploits the optimum benefits of MG1-IL12-ICV.

Having established the chemotactic capabilities of MG1-IL12-ICV on NK cells to the vaccination site, I sought to assess the vaccine efficacy following different routes of vaccination. Comparatively, parallel administrations of the recombinant MG1-IL12-ICV for peritoneal carcinomatosis were conducted via three different delivery routes: intraperitoneally (i.p), intravenously (i.v.), and subcutaneously (s.c.) (**Figure 3.13)**. After CT26lacZ tumor seeding at day 0, mouse survival revealed a highly interesting inter-administration route performance. Mice survival rates under the i.p. route persisted at 100 percent at and beyond 100 days, while survival under i.v. and s.c. routes (20 days and 40 days, respectively) demonstrated significant limitations (>80% and >60% less effective in conferring survival than the i.p. route). Thus, surprisingly, the route of vaccination plays an import role in MG1-IL12-ICV conferred efficacy.

3.12 MG1-IL12-ICV confers toxicity at a very high dose

Since high IL-12 cytokine administration has been reported to induce haemolytic anemia and organ failure mainly due to the massive IFN-γ response [103], I investigated the tolerability of MG1-IL12-ICV in naïve mice in order to establish the maximum tolerable dose (MTD) for further per-clinical testing and development. Doses of 1×10^8 and 1×10^9 of MG1-ICV or MG1-IL12-ICV were tested. The changes in the body weights and survival were monitored following a single administration of ICVs at the indicated doses. Mice lost ~10% and ~20% of their weight within 48 hours following MG1-ICV vaccination at doses of 1×10^8 and 1×10^9

Figure 3.13. Intra-tumoral vaccination is essential to maximize the benefit of MG1-IL12-ICV vaccine.

BALB/c mice received a single dose intraperitoneally (IP), subcutaneously (SC), or intravenously (IV) of 1×10^4 MG1-IL12-ICV 3 days following i.p. tumor seeding (5×10^5), n = 5 mice per group, $p < 0.005$, log rank test.

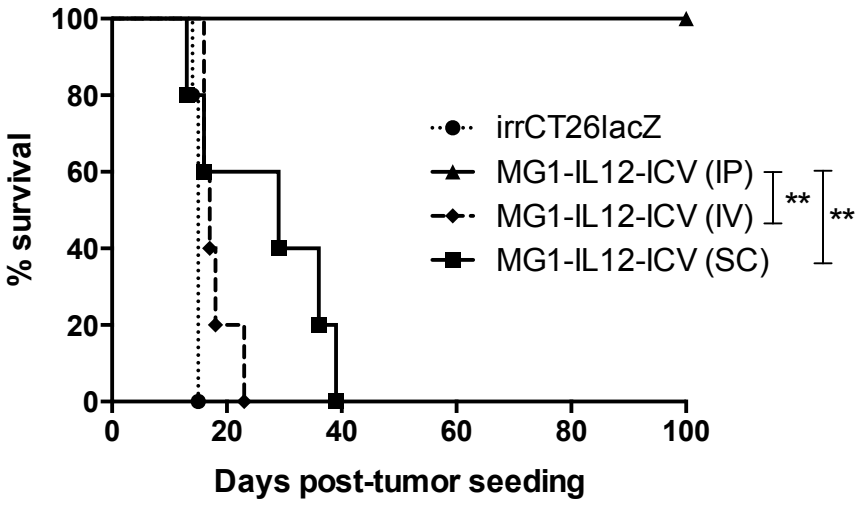
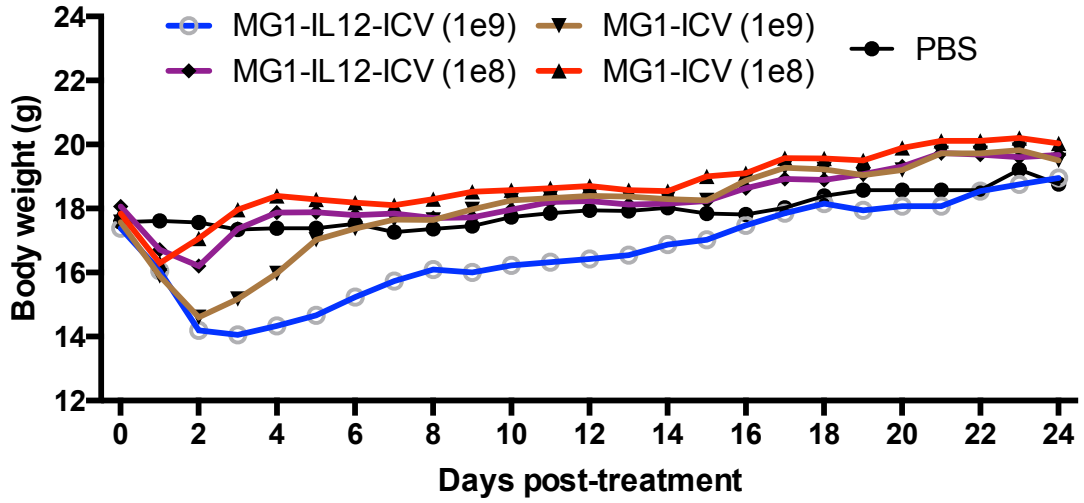

100
80
60
40
20
0
% survival
0
20
40
60
80
100
Days post-tumor seeding
irrCT26lacZ
MG1-IL12-ICV (IP)
MG1-IL12-ICV (IV)
MG1-IL12-ICV (SC)
**
**

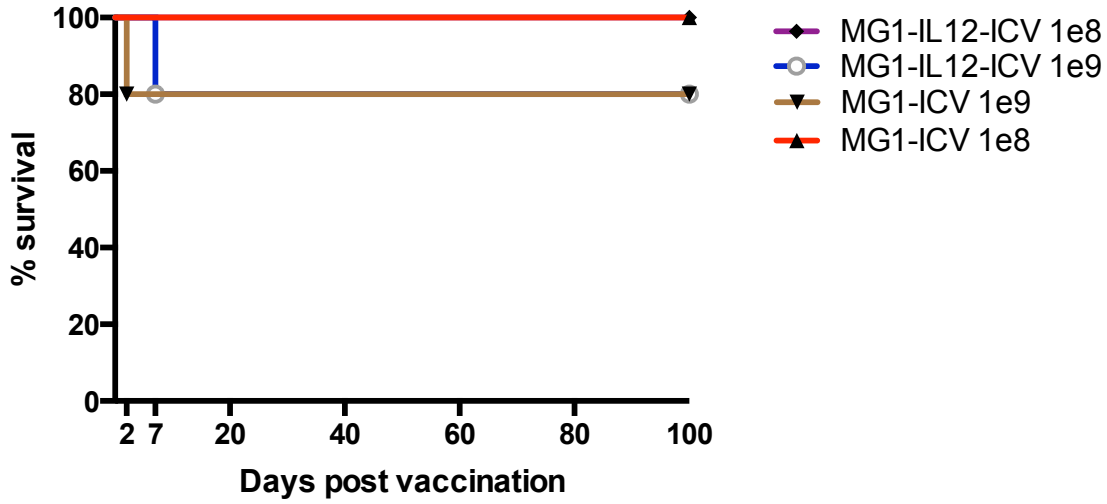
Figure 3.14. Tolerability and toxicity assessment of high doses of ICV administration.

(a) The average of body weights and (b) survival of mice vaccinated intraperitoneal with MG1-ICV or MG1-IL12-ICV at doses of 1×10^8 and 1×10^9. n = 5 mice per group, $p < 0.005$, log rank test.

a


MG1-IL12-ICV (1e9)
MG1-ICV (1e9)
PBS
MG1-IL12-ICV (1e8)
MG1-ICV (1e8)
Body weight (g)
24
22
20
18
16
14
12
0
2
4
6
8
10
12
14
16
18
20
22
24
Days post-treatment

b


MG1-IL12-ICV 1e8
MG1-IL12-ICV 1e9
MG1-ICV 1e9
MG1-ICV 1e8
% survival
100
80
60
40
20
0
2
7
20
40
60
80
100
Days post vaccination

respectively (**Figure 3.14a)**. However, the weight loss was recovered quickly and mice retained their normal weight by day 5 following vaccination. Comparatively, vaccination with 1×10^8 MG1-IL12-ICV resulted in the same trend of body weight loss and recovery as compared 1×10^8 dose of MG1-ICV. Interestingly, at a higher dose, 1×10^9, MG1-IL12-ICV induced a relatively similar weight loss as compared to 1×10^9 dose of MG1-ICV. However, the weight loss was persistent such that mice required ~15 days to fully recover their. Unexpectedly, in both MG1-ICV and MG1-IL12-ICV, a dose of 1×10^9 infected cells was very toxic to the mice such that 20% of the tested mice were found dead **(Figure 3.14b)**. Taking these data together, along with the labour, time, cells, and virus consumption for the preparation of a 1×10^8 dose, a suboptimal 5×10^6 ICV dose was used for subsequent therapeutic experiments.

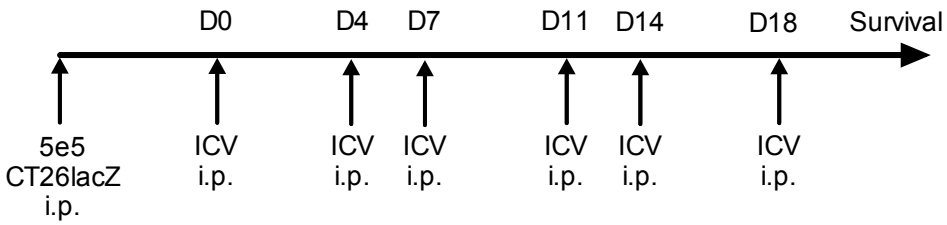
3.13 MG1-IL12-ICV cures mice with lethal, bulky, measurable peritoneal tumors

Since peritoneal carcinomatosis is a common presentation for late stage gastrointestinal and gynecological malignancies, I sought to determine whether the MG1-IL12-ICV could provide therapeutic benefit in a clinically relevant model of colon cancer (CT26) with peritoneal disease at time of treatment. Therefore, I sought to measure the effects of treatment in established bulky tumors. Between days 10 and 17 following implantation, tumors were visualized by MRI and mice bearing significant tumor masses (Class 1 > 8 mm and Class 2 > 3 mm) were randomly allocated into a treatment group prior to treatment with 6 doses of irradiated cells, MG1-ICV or MG1-IL12-ICV administered six times (D0, D4, D7, D11, D14, and D18) over a three week period (**Figure 3.15a**). Despite the lethal tumor burden, evident by the loss of all animals treated with irradiated cells by day 15, MG1-IL12-ICV provided complete

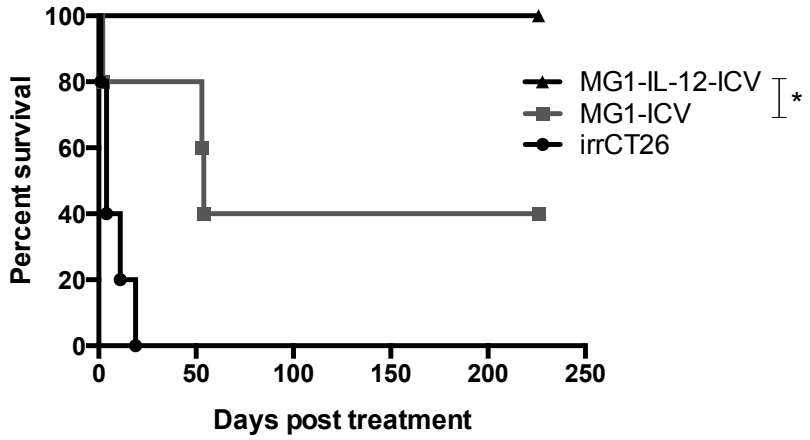
Figure 3.15. MG1-IL-12-ICV improves survival and clearance of CT26lacZ tumors.

(a) Experimental timeline of tumor seeding and ICV vaccinations. (b) Kaplan–Meier survival analysis for BALB/c mice-bearing tumors greater than 3 mm that received two doses of either ICV (MG1-ICV or MG1-IL12-ICV, 5×10^6 infected cells) or irradiated cells (irrCT26lacZ) each week over a three week period (total number of intended doses per animal = 6). $n = 7$, $p < 0.05$, log rank test. (c) Coronal T2-weighted FSE MR images (TR/TE 1500/42ms, 256x256 matrix, 35mm FOV, 1mm slice thickness) for one representative mouse bearing class I tumor (>8mm). (1) Day 10, bulky tumor mass with intermediate T2 signal evident prior to treatment. (2) Day 17, an increase in tumor size is evident, (3 and 4) are two different views of the same tumor on week six demonstrating a marked tumor size reduction six weeks post-treatment initiation, and (5 and 6) at thirteen weeks post-treatment initiation with no residual tumor signal at site of the previous large peritoneal tumor deposit.

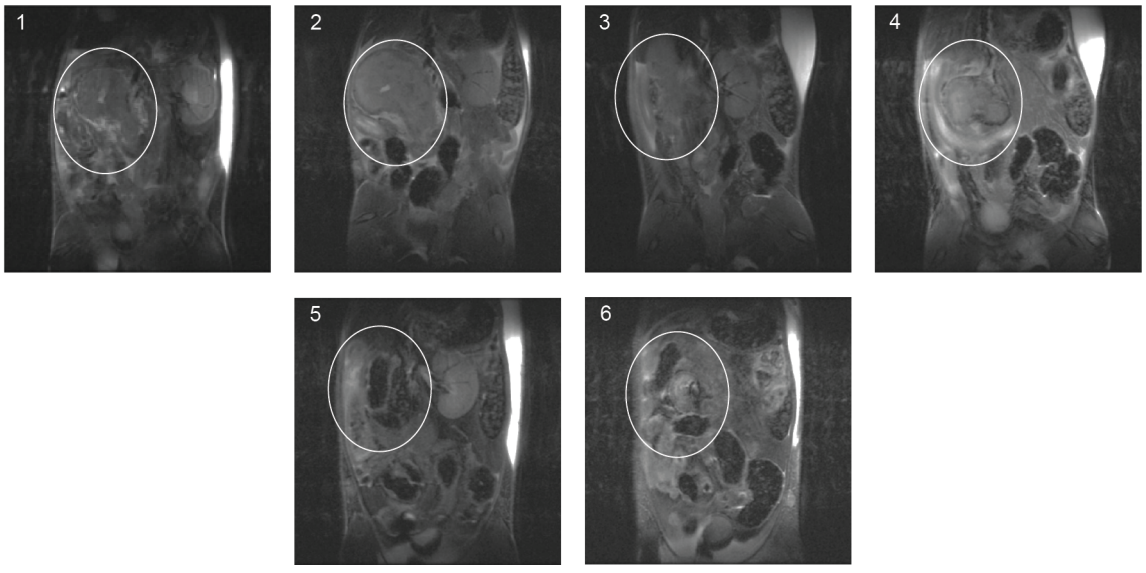
a


D0
D4
D7
D11
D14
D18
Survival
5e5
CT26lacZ
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.

b


Percent survival
100
80
60
40
20
0
0
50
100
150
200
250
Days post treatment
MG1-IL-12-ICV
MG1-ICV
irrCT26
*

c


1
2
3
4
5
6

protection (21/21 survived > 200 days) (**Figure 3.15b**). Strikingly, follow-up MRI scans confirmed that large tumor masses present at the early stages of treatment were dramatically reduced at later time points **(Figure 3.15c)**.

Collectively, these results demonstrate that MG1-IL12-ICV is an effective therapeutic approach for promoting the clearance of large, established tumors within the peritoneum in a murine model of peritoneal carcinomatosis.

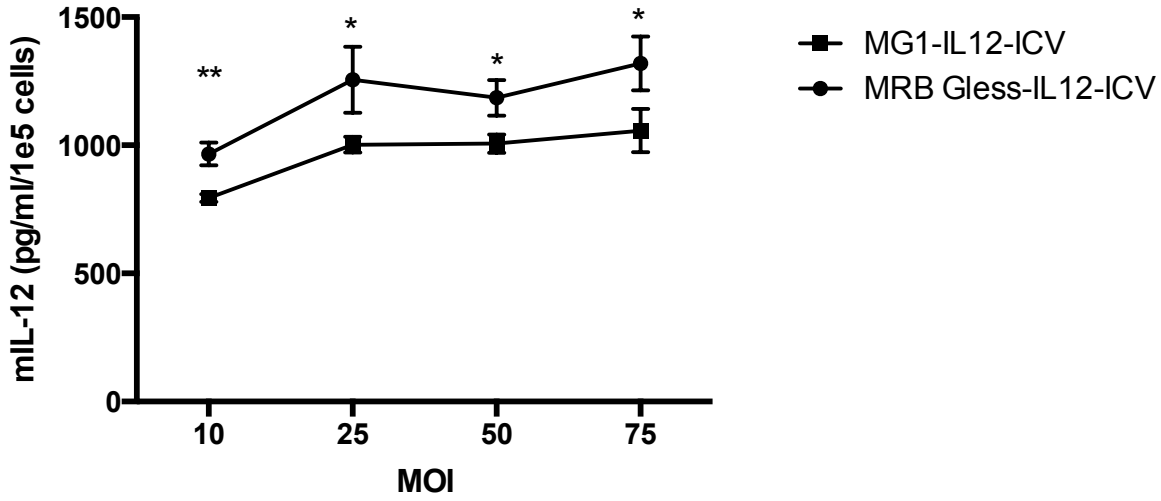
3.14 A replication-competent virus expressing IL-12 is required to achieve best overall survival

To better understand the requirements of MG1-IL12-ICV induced efficacy and develop a safer vaccine, I created a non-replicating G-less MG1-IL12 virus. This virus is able to infect cells for one cycle during the vaccine preparation, but cannot replicate and spread beyond the ICV to the tumor cells. This way the ICV can secrete IL-12 but with a controlled expression level to avoid any IL-12 associated toxicity. In addition, I assessed the injection of recombinant IL-12 along with MG1-ICV treatments to further understand the importance of IL-12 transgene expression in the ICV. Interestingly, G-less MG1-IL12-ICV secreted a significantly higher level of IL-12 *in vitro* compared to the level secreted by the replicating virus (**Figure 3.16a**). This observation suggested an added benefit of using a non-replicating virus. The non-replicating virus leads to prolonged cellular integrity of the ICV, which may contribute to an increase in the level of IL-12. However, despite the heightened levels of IL-12 that were detected from the G-less MG1-IL12-ICV, this vaccine did not have any therapeutic efficacy in CT26lacZ peritoneal carcinomatosis model when compared to MG1-IL12-ICV (**Figure 3.16b**). Indeed, the therapeutic treatment of peritoneal carcinomatosis with Gless MG1-IL12-ICV resulted in 0% survival levels comparable to treatment with irradiated CT26lacZ survival. Collectively, these data indicate that viral replication is an absolute requirement to exploit maximum ICV efficacy.

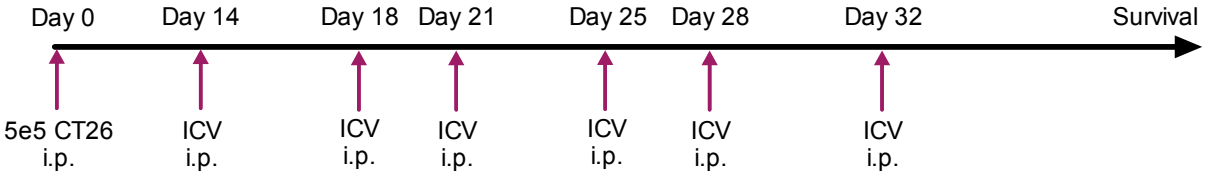
Figure 3.16. Viral replication is absolutely required in order to obtain an optimum MG1-IL12-ICV efficacy.

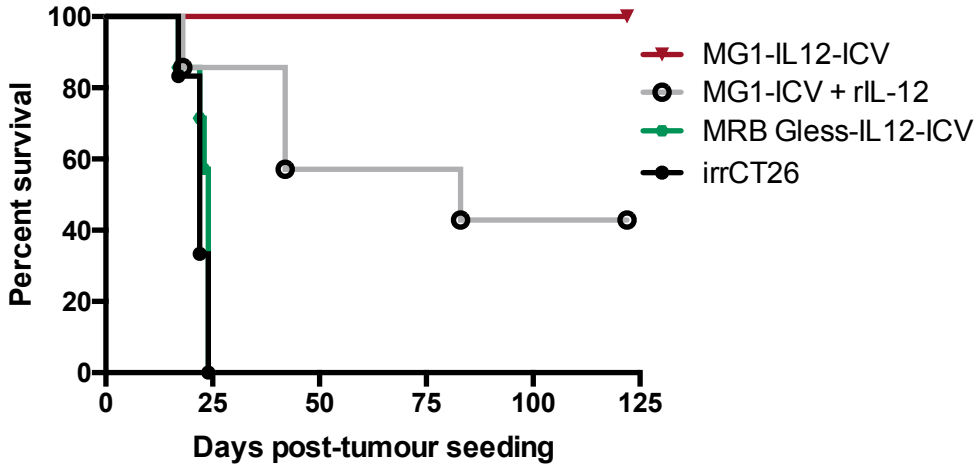
(**a**) In vitro IL-12 expression levels from MRB G-less-IL12-ICV an MG1-IL12-ICV at different MOIs. Data represent means of triplicate wells Error bars =SD. * $p < 0.05$ and ** $p < 0.005$. (**b**) The Kaplan-Meier survival curve of mice bearing CT26lacZ peritoneal tumors, treated with MG1-IL12-ICV, MRB G-less-IL12-ICV, MG1-ICV + rIL12 and irrCT26. n = 7 mice per group, $p < 0.005$, log rank test.

a


mIL-12 (pg/ml/1e5 cells)
1500
1000
500
0
10
25
50
75
MOI
**
*
*
*
MG1-IL12-ICV
MRB Gless-IL12-ICV

b


Day 0
Day 14
Day 18
Day 21
Day 25
Day 28
Day 32
Survival
5e5 CT26 i.p.
ICV i.p.
ICV i.p.
ICV i.p.
ICV i.p.
ICV i.p.
ICV i.p.

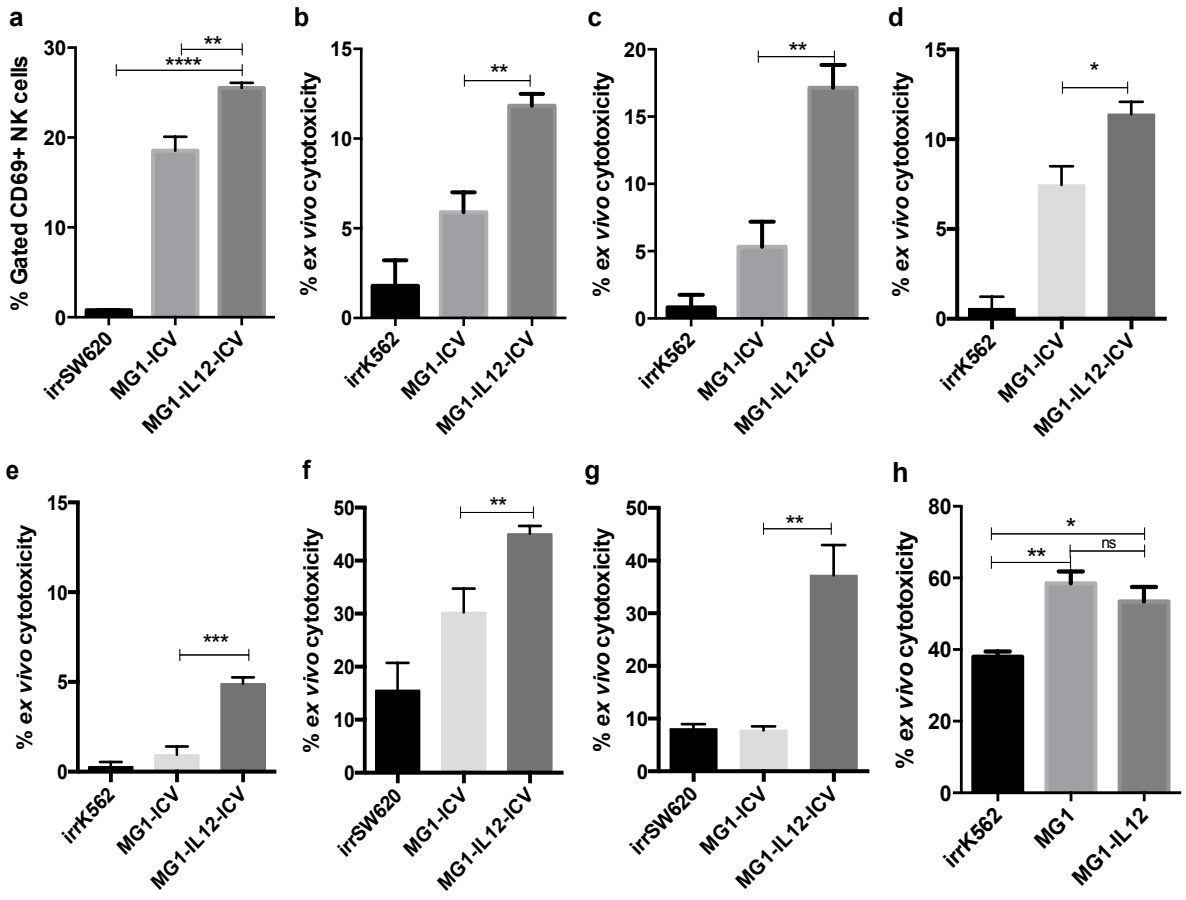

Percent survival
100
80
60
40
20
0
0
25
50
75
100
125
Days post-tumour seeding
MG1-IL12-ICV
MG1-ICV + rIL-12
MRB Gless-IL12-ICV
irrCT26

3.15 MG1-IL12-ICV enhances ex vivo human NK cell cytotoxicity.

Given the fact that murine p40 and p35 subunits of IL-12 share 70% and 60% homology with their human counterparts, respectively, they are able to functionally activate human NK and T cells [165]. I next sought to confirm that the vaccine could elicit a similar effect on human NK cells *ex vivo*. To accomplish this, irradiated SW620 colon cancer cells and K562 myelogenous leukemia cells were infected with MG1 or MG1-IL12 and cultured with peripheral blood mononuclear cells (PBMCs) isolated from healthy donors or colorectal cancer patients (as part of the Perioperative Blood Collection Protocol approved by the Ottawa Health Science Network Research Ethics Board #2011884). In agreement with our previous findings, MG1-IL12-ICV resulted in a significant increase (mean difference >5%; <20% for MG1-ICV vs. ~25% for MG1-IL12-ICV) in NK activation measured by CD69 expression (**Figure 3.17a**). Furthermore, increased *ex vivo* NK cell cytotoxicity of MG1-IL12-ICV was observed consistently in all tested PBMCs from healthy donors (**Figure 3.17b and c**) and cancer patients (**Figure 3.17d-g)**. In addition, as expected, incubating PBMCs with MG1 or MG1-IL12 viruses (non-cell associated) resulted in comparable cytotoxicity levels (**Figure 3.17h**). Arguably, this observation could be due to the fact that both viruses had minimal or no replication within the PBMCs and hence IL-12 was not efficiently expressed, indicating that the ICV is required to accommodate the viral replication and facilitate transgene expression and translation.

Figure 3.17. MG1-IL12-ICV enhances human NK cell activation *in vitro*.

(a) Percentage of $CD56^{+}/CD3^{-}/CD69^{+}$ NK cells in PBMCs cultured with irradiated SW620 cells alone or cells infected with MG1 or MG1-IL12 (MOI – 10) was quantified by flow cytometry (t=18 hr). *Ex vivo* cytotoxicity of human NK cells from (b and c) two healthy individuals, (d-g) four cancer patients. NK cells from two individual cancer patients were isolated and cultured with irradiated cells, MG1-ICV, or MG1-IL12-ICV infected cells (MOI – 10) or (h) straight viruses (t = 18 hr). Cytotoxic potential of NK cells against K562 tumor cells was quantified by ^{51}Cr release as described in materials and methods. All data are represented as means of triplicate wells. Error bars =SD. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$).


a
% Gated CD69+ NK cells
30
20
10
0
**
****
irrSW620
MG1-ICV
MG1-IL12-ICV
b
% ex vivo cytotoxicity
15
10
5
0
**
irrK562
MG1-ICV
MG1-IL12-ICV
c
% ex vivo cytotoxicity
20
15
10
5
0
**
irrK562
MG1-ICV
MG1-IL12-ICV
d
% ex vivo cytotoxicity
15
10
5
0
*
irrK562
MG1-ICV
MG1-IL12-ICV
e
% ex vivo cytotoxicity
15
10
5
0
***
irrK562
MG1-ICV
MG1-IL12-ICV
f
% ex vivo cytotoxicity
50
40
30
20
10
0
**
irrSW620
MG1-ICV
MG1-IL12-ICV
g
% ex vivo cytotoxicity
50
40
30
20
10
0
**
irrSW620
MG1-ICV
MG1-IL12-ICV
h
% ex vivo cytotoxicity
80
60
40
20
0
*
**
ns
irrK562
MG1
MG1-IL12

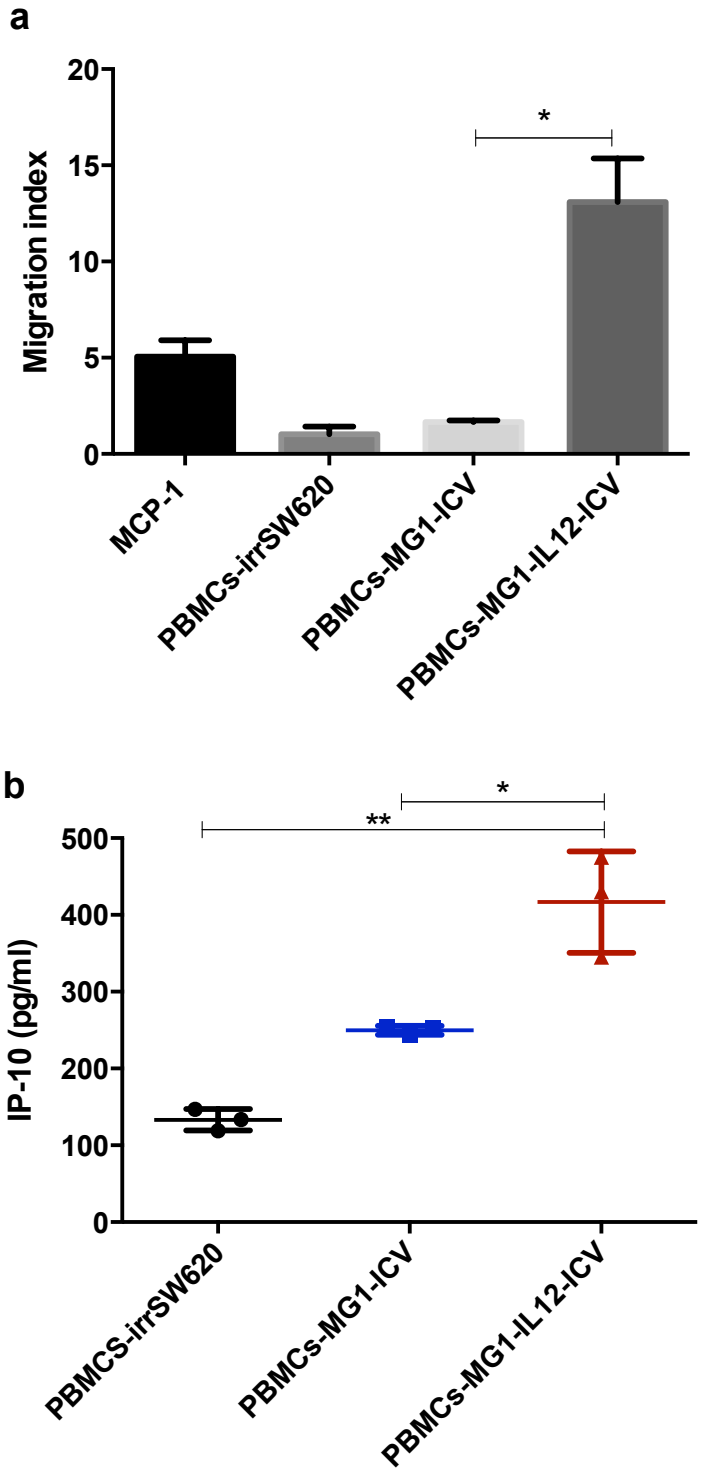
3.16 The migratory capacity of human NK cells is improved upon MG1-IL12-ICV stimulation.

To further evaluate the chemotactic effect of MG1-IL12-ICV on NK cells and support the increased migration capability of NK cells observed in our murine studies, I performed an *ex vivo* migration assay utilizing the conditioned media of PBMC-MG1-IL12-ICV co-cultures. As expected, the NK cell migration outcomes reflected earlier results in mice: MG1-IL12-ICV demonstrated a superior recruitment effect for NK cells at an average Migration Index of 13 (up to 15) compared to MG1-ICV (mean = 2). This represents a significantly higher (6-fold) chemotactic power of MG1-IL12-ICV over MG1-ICV in recruiting human NK cells (**Figure 3.18a**). Moreover, the increased NK cell chemotaxis was coupled with an increased secretion of IP-10 chemokine after MG1-IL12-ICV stimulation (mean ~410 pg/ml) compared to MG1-ICV stimulation (mean ~250 pg/ml) (**Figure 3.18b**).

Together, these results suggest that the activation and enhanced migratory capacity of human NK cells cultured in the presence of MG1-IL12-ICV is associated with an increased ability of NK cells to eradicate tumor cells. This provides support for our hypothesis that autologous infected tumor cell vaccines may provide a therapeutic benefit in the treatment of patients with PC.

Figure 3.18. MG1-IL12-ICV induces human NK cell migration and IP-10 secretion.

(a) The stimulatory effects of cell free conditioned media prepared from the indicated groups on human NK cell migration was assessed by flow cytometry at 3 hrs, as described in materials and methods. (b) IP-10 secretion was measured by ELISA following culture of PBMC medium conditioned by the indicated groups. Data represents means of triplicate wells. Error bars =SD. (* $p < 0.05$, ** $p < 0.005$).


a
Migration index
20
15
10
5
0
*
MCP-1
PBMCs-irrSW620
PBMCs-MG1-ICV
PBMCs-MG1-IL12-ICV
b
IP-10 (pg/ml)
500
400
300
200
100
0
*
**
PBMCS-irrSW620
PBMCs-MG1-ICV
PBMCs-MG1-IL12-ICV

3.17 MG1-IL12-ICV generates B16F10 specific T cell responses

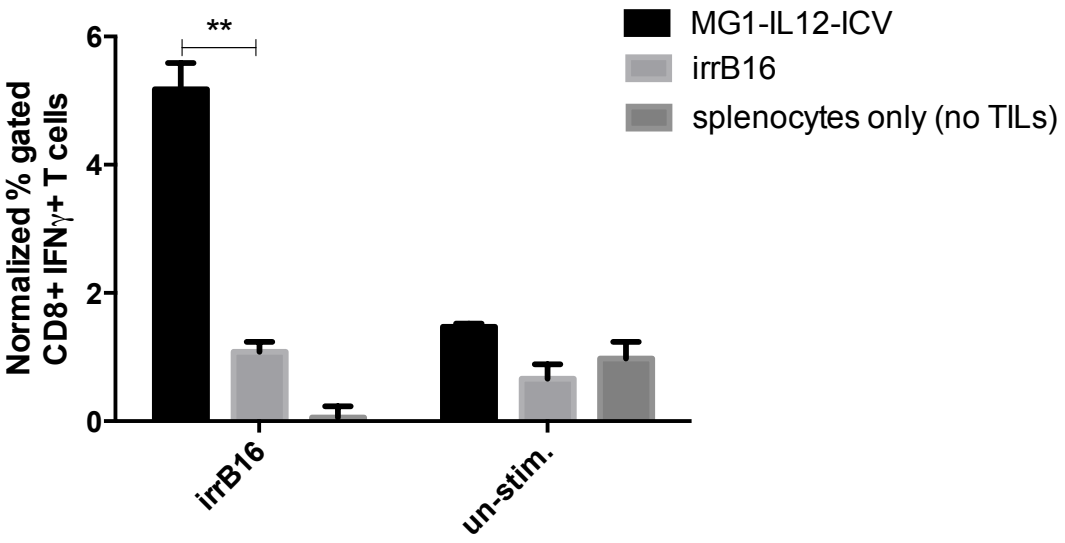
To characterize antitumor T cell responses following MG1-IL12-ICV vaccination in the prophylactic B16F10 model, I injected B16F10 cells implanted in matrigel in the flank of C57Bl/6 mice. The use of matrigel allowed me to easily isolate tumor infiltrating lymphocytes (TILs) for subsequent *ex vivo* stimulation with B16F10 cells. Interestingly, CD8 T cells isolated from MG1-IL12-ICV vaccinated mice responded better to *ex vivo* stimulation with B16F10 cells by up-regulating IFN-γ expression as compared to CD8 T cells extracted from irrB16-injected mice. Indeed, there was a five-fold increase of B16F10 specific CD8 T cells in TILs of the MG1-IL12-ICV treatment group as compared to mice treated with irrB16 alone (**Figure 3.19**). This indicates that tumor specific T cells were efficiently generated following MG1-IL12-ICV vaccination, providing the rationale to further characterize T cells in this ICV model.

3.18 Diversified antitumor immunity is elicited following MG1-IL12-ICV vaccination

Intraperitoneal prophylactic vaccination using MG1-ICV and MG1-IL12-ICV was performed to determine its effect in recruiting tumor specific CD8 T cells. To further investigate antigenic T cell responses and recruitment, I stimulated splenocytes from mice that were prophylactically vaccinated twice and seven days apart with irrB16, MG1-ICV and MG1-IL12-ICV according to the timeline in (**Figure 3.20a**), followed by intraperitoneal B16F10 tumor cell challenge. As expected, CD8 T cells from MG1-ICV and MG1-IL12-ICV were able to induce IFN-γ expression in response to *ex vivo* VSVn peptide stimulation, a VSV immunogenic peptide that shares the same amino acid sequence with Maraba MG1 virus. However, I was not able to detect any other tumor specific peptide-induced T cell stimulation in response to DCT peptide, a melanoma specific antigen.

Figure 3.19. Prophylactic treatment with the MG1-IL12-ICV leads to generation of B16F10 specific T cells.

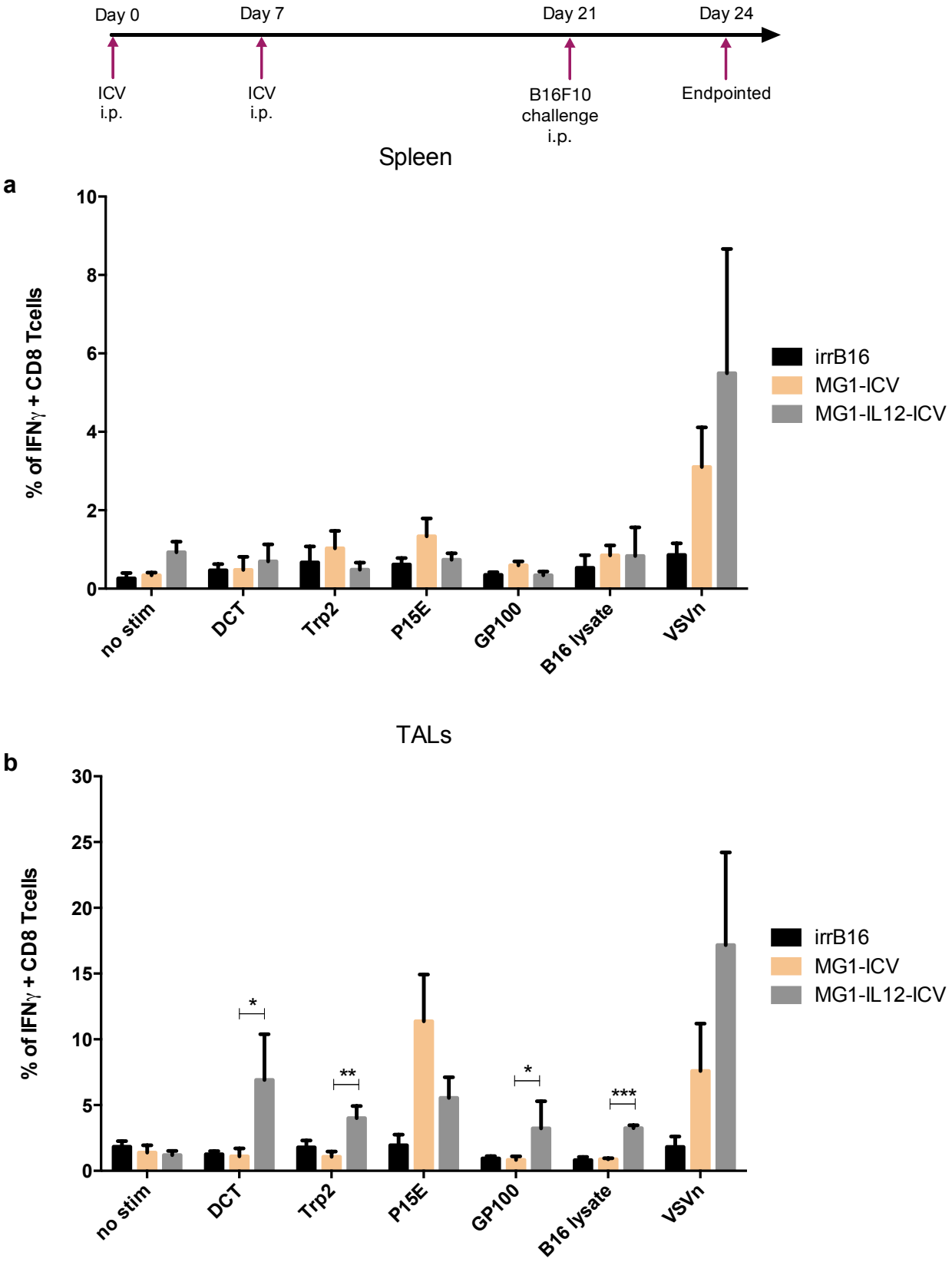
C57BL/6 mice were prophylactically immunized on days 0 and 7 with MG1-IL12-ICV or irrB16 control, re-challenged on day 21 with B16F10 cells in matrigel and euthanized on day 24. TILs were isolated from the matrigel plugs and stimulated ex vivo with B16F10 in the presence of naïve splenocytes as a source of antigen presenting cells (APCs). FACS analysis was carried out on IFNγ^+ CD8$^+$ T cells and the means were calculated based on n=3 mice per group. Error bars =SD. ** $p < 0.005$.

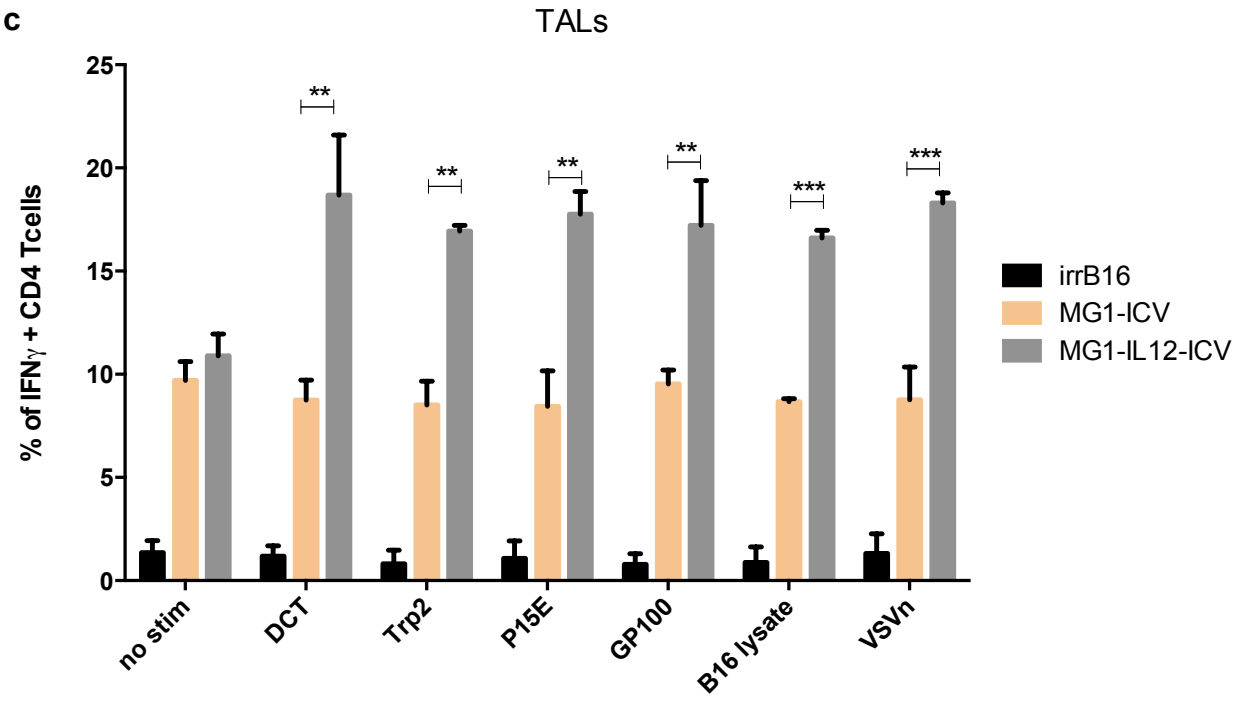

MG1-IL12-ICV
irrB16
splenocytes only (no TILs)
Normalized % gated CD8+ IFNγ+ T cells
6
4
2
0
**
irrB16
un-stim.

Surprisingly, I found that, unlike splenocytes, tumor associated lymphocytes (TALs) contained a diversified profile of tumor specific T cells, as evidenced by IFN-γ secretion from CD8 T cells in response to DCT, Trp2, and GP100 B16F10 melanoma antigens in mice treated with MG1-IL12-ICV (**Figure 3.20b**). Interestingly, responses against P15E peptide, which is an endogenous TAA in many tumor cell lines, were detected in both MG1-ICV and MG1-IL12-ICV vaccinations. MG1-ICV elicited a higher response compared to MG1-ICV-IL12 (12% vs. 7%, respectively). This finding indicates a stronger stimulatory effect of MG1-ICV on harnessing P15E specific CD8+ T cells in the absence of IL-12. Moreover, weaker responses against several peptides were also detected after MG1-ICV. These results suggest that an immune response to a broad spectrum of B16F10 tumor antigens might have developed as a consequence of MG1-IL12-ICV therapy, a concept known as epitope spreading or antigen cascade. By contrast, TALs isolated from MG1-ICV treated mice only responded to P15E peptide, but not to any other tested melanoma associated antigens. In addition, MG1-IL12-ICV resulted in the generation of robust and similarly broad-spectrum CD4 T cell responses; this was not observed with MG1-ICV vaccination (**Figure 3.20c**). Collectively, these results demonstrate a pronounced effect of the IL-12 based vaccine that led to the generation of polyclonal tumor specific T cell responses, which might contribute to a better treatment efficacy by minimizing the selective pressure on tumor antigen(s) escape.

Figure 3.20. MG1-IL12-ICV induces T cell responses to multiple tumor epitopes.

Mice were vaccinated intraperitoneally with 5×10^6 cells on days 0 and 7, and then challenged with B16F10 cells i.p. on day 21 and euthanized three days later. (a) Splenocytes, (b and c) $CD8^+$ and $CD4^+$ peritoneal lymphocytes, respectively, were isolated and restimulated *ex vivo* with the indicated peptides, stained for extracellular and intracellular markers, and examined by flow cytometry for IFN-γ expression. Data are presented as means +/- SD with 3-5 mice per group. P values; (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).


Day 0
Day 7
Day 21
Day 24
ICV i.p.
ICV i.p.
B16F10 challenge i.p.
Endpointed
Spleen
a
% of IFNγ + CD8 Tcells
irrB16
MG1-ICV
MG1-IL12-ICV
no stim
DCT
Trp2
P15E
GP100
B16 lysate
VSVn
TALs
b
*
**
*
***

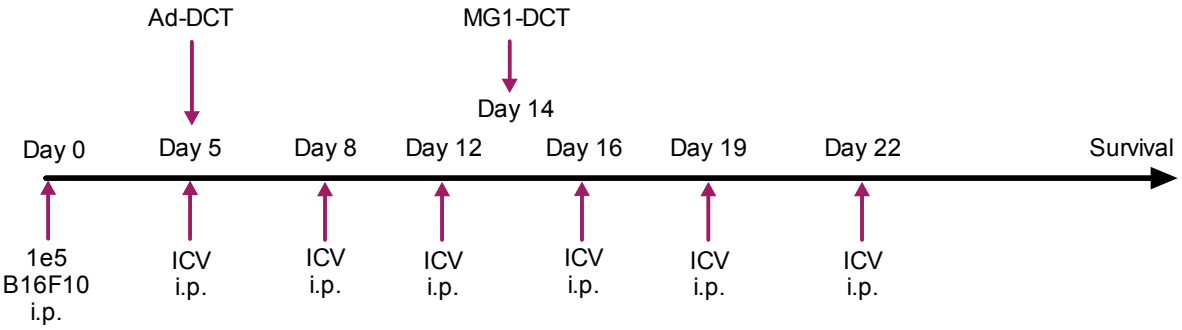

c
TALs
% of IFNγ + CD4 Tcells
25
20
15
10
5
0
**
**
**
**
***
***
no stim
DCT
Trp2
P15E
GP100
B16 lysate
VSVn
irrB16
MG1-ICV
MG1-IL12-ICV

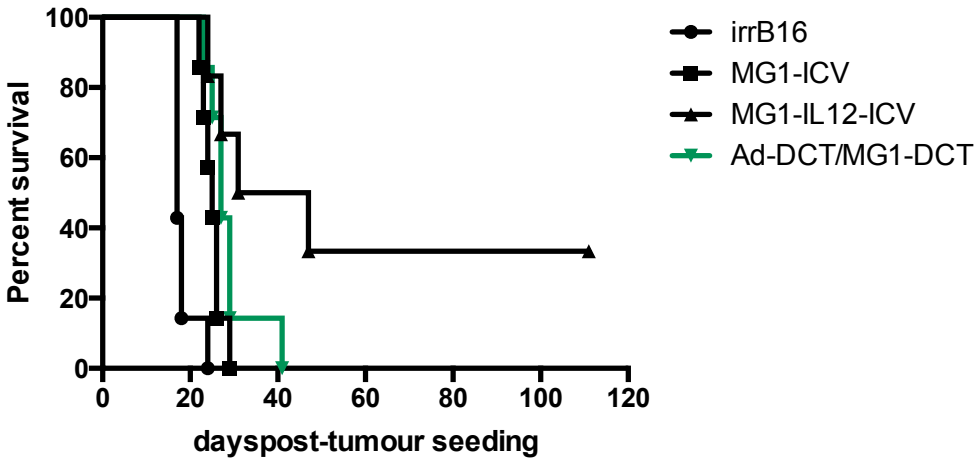
3.19 Multi-dose treatment improves survival in the B16F10 peritoneal model

Having established that the MG1-IL12-ICV induces strong tumor epitope spreading that potentially minimizes tumor escape, I sought to assess the efficacy of this vaccine in the B16F10 peritoneal carcinomatosis model. In addition, I wanted to compare this vaccination strategy to a previously published vaccination strategy (Ad-DCT prime/MG1-DCT boost, 9-days apart) that was found to be very effective in generating a robust DCT specific T cell response leading to better survival outcomes in the B16F10 lung metastasis model [136]. Both the Ad-DCT/MG1-DCT and the MG1-IL12-ICV treatments demonstrated longer median survival times compared to MG1-ICV or irrB16 (27 d, 39 d vs. 25 d and 17 d, respectively) (**Figure 3.21**). Surprisingly, MG1-IL12-ICV showed a much longer median survival time (12 days longer) than Ad-DCT/MG1-DCT treatments. Indeed, MG1-IL12-ICV effectively preserved the lives of more than 30 percent of the B16F10 tumor bearing mice beyond 100 days, while all animals in the Ad-DCT/MG1-DCT treatment group succumbed by day 41. Moreover, the living mice from the MG1-IL12-ICV treated group did not develop vitiligo, an autoimmune disease that had been observed previously in long-term survivors following Ad-DCT/MG1-DCT treatments. This reinforces the potential of MG1-IL12-ICV for eliciting a profound and diversified T cell response that does not compromise safety [136]. These data indicate that the recombinant MG1-IL12-ICV can sustain its antitumor effect far longer than both MG1-ICV and Ad-DCT/MG1-DCT, with a substantial survival rate that translated into complete tumor regression in some cases (>30%).

Figure 3.21. Durable cures of B16F10 in the peritoneal compartment following multi-dose treatment with MG1-IL12-ICV.

Kaplan–Meier survival analysis for the assessment of a six-dose treatment regimen given to mice bearing 5-day old peritoneal B16F10 tumors. A control group was used to compare the efficacy of MG1-IL12-ICV to Ad-DCT prime and MG1-DCT boost treatment.


Ad-DCT
MG1-DCT
Day 14
Day 0
Day 5
Day 8
Day 12
Day 16
Day 19
Day 22
Survival
1e5
B16F10
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.
ICV
i.p.


Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
120
dayspost-tumour seeding
irrB16
MG1-ICV
MG1-IL12-ICV
Ad-DCT/MG1-DCT

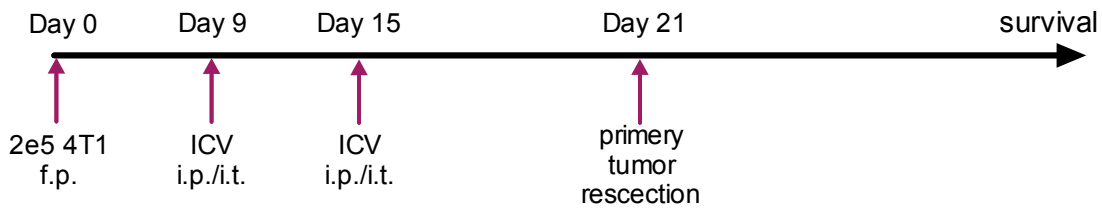
3.20 Direct intratumoral vaccination with MG1-IL12-ICV improves survival over intraperitoneal vaccination in 4T1 breast cancer model

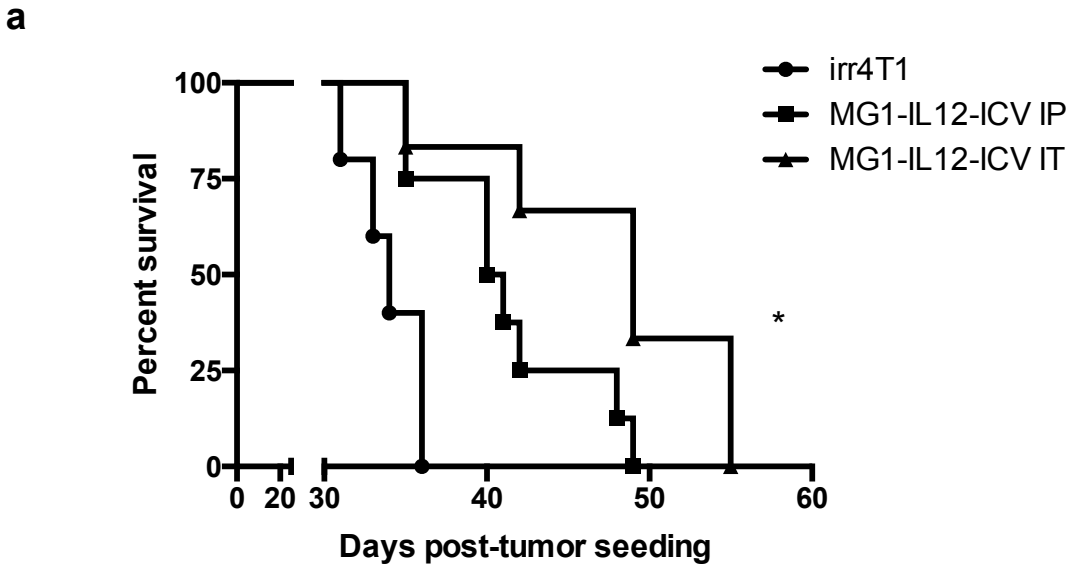
To further extend the observation of the MG1-IL12-ICV efficacy, I tested the therapeutic benefit of the vaccine in a very challenging orthotopic triple negative 4T1 breast cancer model. In this model, the tumors are seeded in the mammary fat pad. Following orthotopic tumor injection, the route of vaccination efficacy (either IP or IT) was then tested at day 9 and 15. Resection of the primary tumor took place on day 21 followed by the monitoring of survival (**Figure 3.22a**). Although the survival differential is not large, a statistically significant difference of approximately six days (49 d for IP vs. 55 d for IP) was observed, suggesting a therapeutic advantage of administering the vaccine by the i.t. route in this breast tumor model.

To characterize the mechanism whereby i.t. vaccination outperforms i.p. vaccination, resected primary tumor weight and tumor infiltrating lymphocytes (primary tumor) were quantified. Tumor volumes from i.t. injected animals corroborated the survival benefit of i.t. injection (mean = 255 mm^3 i.t. vs. mean =506 mm^3 ip) **(Figure 3.22b)**. Additionally, total T cell count was also congruent with i.t. injected mice having 2 fold greater total T cell counts/tumor volume compared to i.p. mice **(Figure 3.22c)**. These results suggest that i.t. administration of MG1-IL12-ICV vaccine leads to improved survival in the 4T1 model through recruitment of T cells at the vaccination site, leading to reduced tumor progression.

Figure 3.22. A comparison of intratumoral versus intraperitoneal route of vaccination with MG1-IL12-ICV in 4T1 orthotopic model.

(a) A Kaplan–Meier survival analysis of MG1-IL12-ICV comparing two different sites of delivery, n = 5-8 mice per group, $p < 0.05$, log rank test. (b) Tumor volumes and (c) Average T cell counts of resected 4T1 tumors on day 21 post-tumor seeding. Data represent means of 5-8 mice per group. Error bars =SD. (*** $p < 0.0005$).


Day 0
Day 9
Day 15
Day 21
survival
2e5 4T1
f.p.
ICV
i.p./i.t.
ICV
i.p./i.t.
primery
tumor
rescection


a
irr4T1
MG1-IL12-ICV IP
MG1-IL12-ICV IT
Percent survival
100
75
50
25
0
0
20
30
40
50
60
*
Days post-tumor seeding

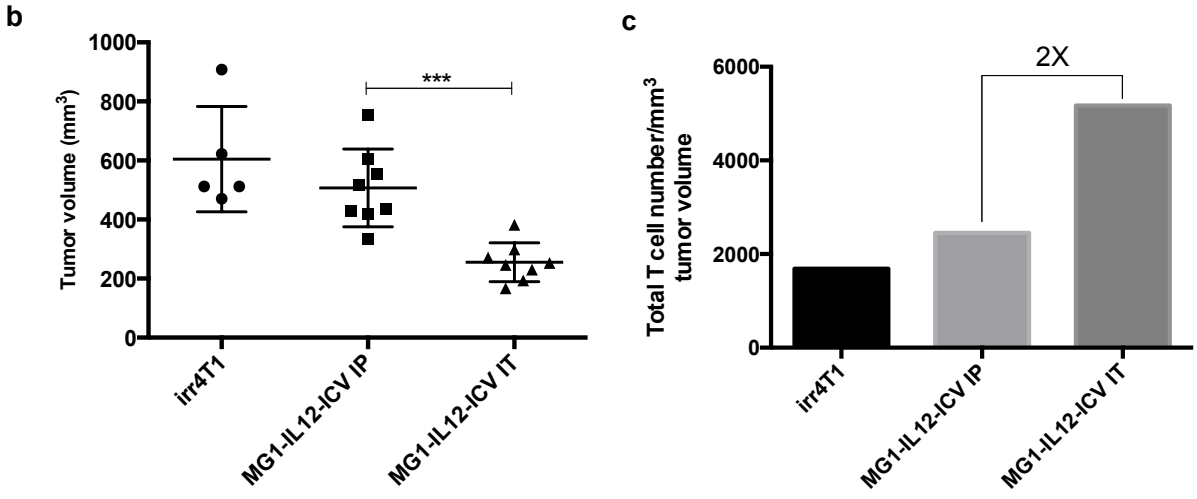
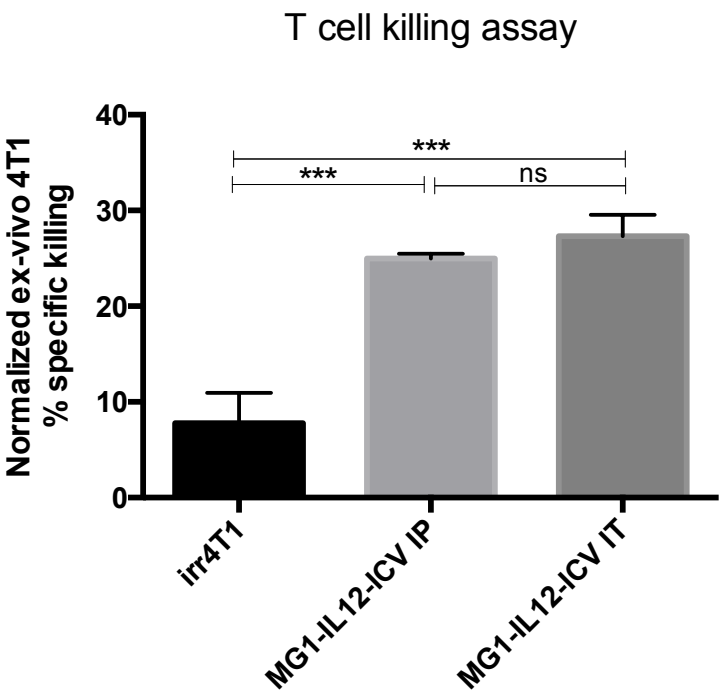

b
Tumor volume (mm3)
1000
800
600
400
200
0
***
irr4T1
MG1-IL12-ICV IP
MG1-IL12-ICV IT
c
Total T cell number/mm3
tumor volume
6000
4000
2000
0
2X
irr4T1
MG1-IL12-ICV IP
MG1-IL12-ICV IT

Figure 3.23. The route of MG1-IL12-ICV vaccination does not influence the induction of tumor specific T cells.

Tumor infiltrating T cells from both i.t. and i.v. vaccinated animals retained similar ex vivo antitumoral cytotoxicity against 4T1 cells. Data represent means of triplicate wells. Error bars =SD, *** $p < 0.0005$.


T cell killing assay
Normalized ex-vivo 4T1
% specific killing
40
30
20
10
0
***
***
ns
irr4T1
MG1-IL12-ICV IP
MG1-IL12-ICV IT

3.21 MG1-IL12-ICV induces a comparable quality of tumor specific T cells regardless the route of vaccination.

To determine differences in the tumor specificity and cytotoxicity of T cells recruited into the MG1-IL12-ICV vaccination sites using the two different administration routes (i.p. and i.t.), the specific tumoricidal performance of the respective T cells was measured *ex vivo* using 4T1 tumor cells normalized to the syngeneic CT26lacZ cell line. The i.t. administered vaccine demonstrated a tumoricidal rate of 28 percent (up to 30%) while the i.p. administered vaccine showed a specific killing rate of 25 percent. Differences between these values are not statistically significant (**Figure 3.23**). However, T cell tumoricidal rates from MG1-IL12-ICV vaccinated mice from both routes of administration were significantly higher compared to T cells from irr4T1 treated mice (8 – 11%). Thus, the different routes of ICV administration seem to induce comparable antitumor T cell responses, indicating the APCs at both sites are able to process and present tumor antigens for T cell development. Nevertheless, enhanced T cell infiltration in i.t. vaccinated mice and associated survival benefit suggests that further optimization of the route of vaccination is warranted.

3.22 Intravenous delivery of MG1-IL12-ICV improves survival over intraperitoneal vaccination

The orthotopic 4T1 model leads to spontaneous lung tumor metastasis. Mice usually succumb 1 month following tumor implantation due to respiratory distress arising from large lung tumor burden. Our previous observations indicate that local intratumoral vaccination with MG1-IL12-ICV is a key factor to improve vaccine efficacy. In this experiment, I vaccinated mice with MG1-IL12-ICV twice (6 days in between), either i.p. or i.t. (**Figure 3.24a**). The primary tumors were resected and followed by another four doses of the vaccine (in varying gaps

(3 – 4 d): Day 28, Day 31, Day 35, and then Day 39). However, the i.t. vaccinated group continued the vaccination via the intravenous route. I reasoned that i.v. vaccination is a better way of delivering the vaccine directly to the lungs of mice, which is the site of tumor metastasis in the 4T1 orthotopic model. Interestingly, this vaccination strategy appeared to be effective in prolonging the lifespan of the treated mice.

In i.p. vaccinated mice, all mice survived 40 days, but succumbed by day 50 (**Figure 3.24b**). Meanwhile, full survival of the mice reached 44 days with the MG1-IL12-ICV i.t.-i.v. administered groups, which did not fully succumb until day 63. The i.t-i.v. route improved the overall survival moderately. Noticeably, i.t-i.v. treated mice had decreased lung tumor burden as compared to the i.p treated group (data not shown). Though mice in the i.t-i.v. treatment group were called for endpoint due to their severe respiratory distress status, it was mainly due to large pleural tumor burden. These results suggest that i.v injection of MG1-IL12-ICV is effective in controlling tumor progression within the lungs; however, other sites of metastasis (pleural cavity) were not affected by the treatment. In this case, there may be a role for direct intrathoracic injection of MG1-IL12-ICV.

3.23 Adoptive transfer of MG1-IL12-ICV-induced TILs improves efficacy in 4T1 model

Having established that MG1-IL12-ICV is very effective in generating tumor specific T cells, I reasoned that the combination of adoptive T cell therapy and vaccination could further improve survival (**Figure 3.25a**). To harvest a relatively pure tumor specific T cell population from the tumors with a minimal virus specific T cell contaminant, I expanded TILs prior to adoptive T cell transfer. In accordance with my previous observations, i.p. administration of MG1-IL12-ICV did not impact the tumor volume as compared to irr4T1 treatments (**Figure 3.22b and Figure 3.25b**). Moreover, immunohistochemistry staining for $CD3^+$ cells within the

resected primary tumors showed that MG1-ICV vaccination is associated with comparable counts of $CD3^+$ cells to irr4T1 vaccination (mean = 13 cells/mm^2 vs 11 cells/mm^2, respectively). However, MG1-IL12-ICV treatment induced significantly more $CD3^+$ cell infiltration into the tumors compared to the other treatment groups (mean= 29 cells/mm^2) (**Figure 3.25c**).

Following primary tumor resection on day 21 post-tumor seeding, T cells were isolated from the resected tumors and expanded in vitro for 10 days. T cells were then infused back and only mice infused with MG1-IL12-ICV-generated T cells had a significant survival improvement. Mice receiving the MG1-ICV exhibited a survival rate similar to mice vaccinated with irr4T1 (**Figure 3.25d**). However, mice that received MG1-IL12-ICV treatments had a longer survival rate (100 % at ~42 d post-tumor seeding). Strikingly, ~30 percent of the mice treated with MG1-IL12-ICV and infused with T cells recovered and demonstrated durable cures, indicating that MG1-IL12-ICV potentiates better T cells to improve adoptive T cell therapy.

Figure 3.24. Intravenous administration of MG1-IL12-ICV improves the survival of spontaneous mammary 4T1 tumor model.

(a) Experimental timeline. (b) A Kaplan–Meier survival analysis of MG1-IL12-ICV efficacy assessing the benefits of intravenous vaccination. N = 5-8 mice per group, $p < 0.05$, log rank test.

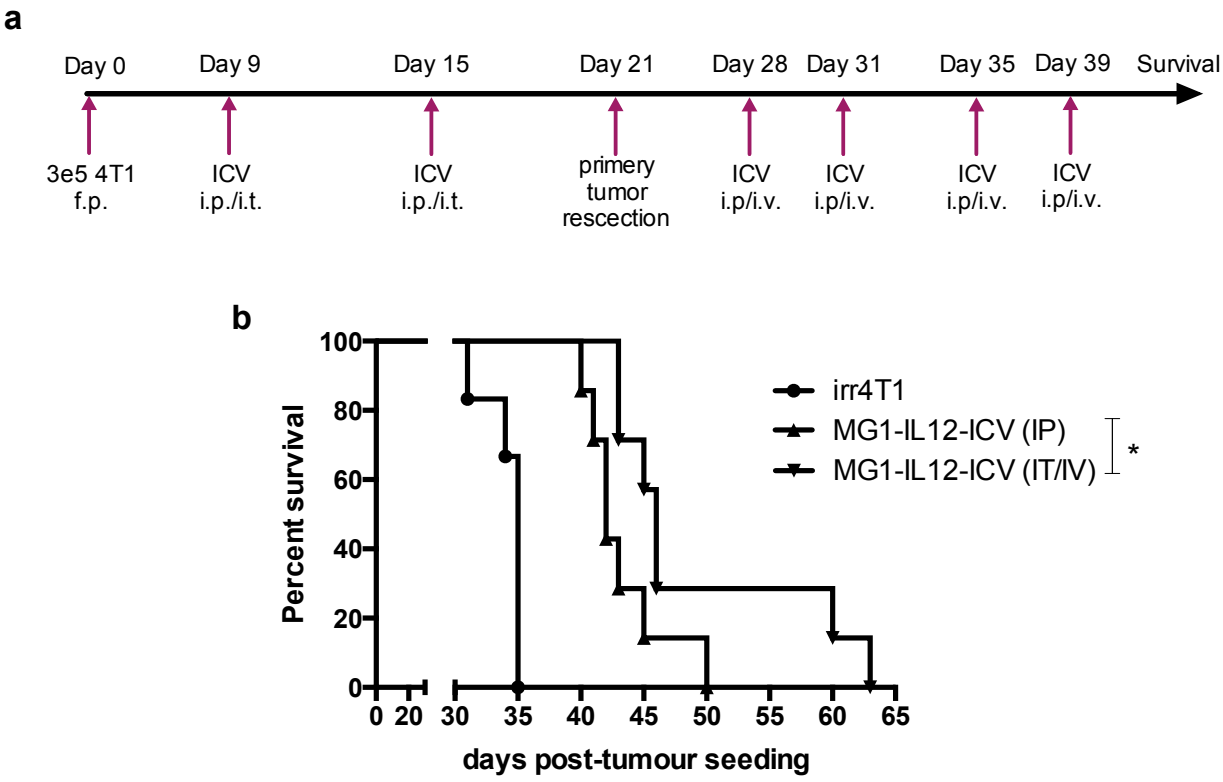
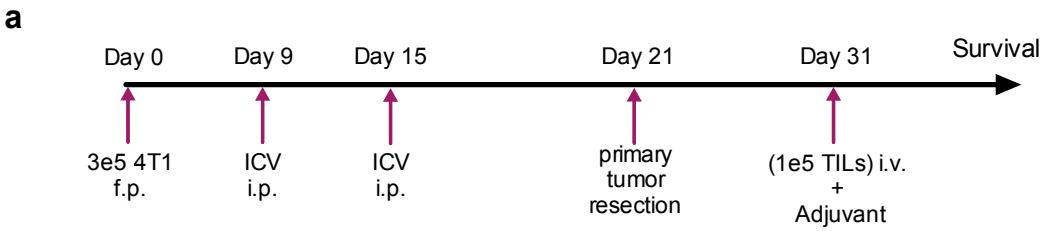
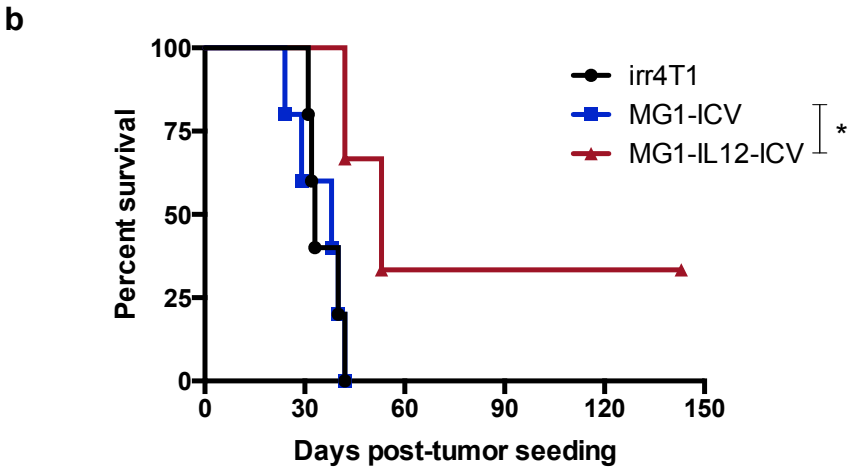
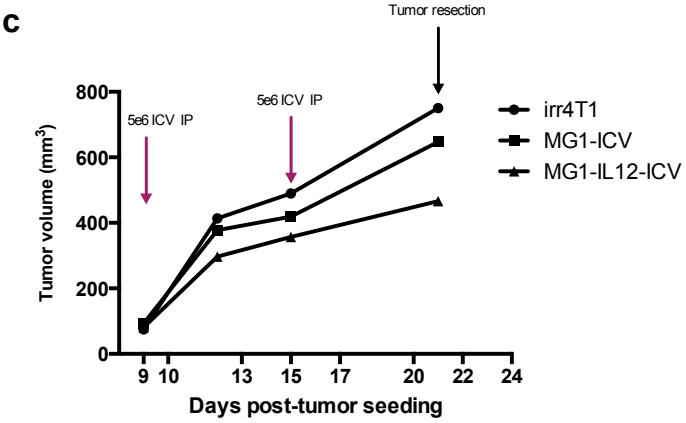

a
Day 0
Day 9
Day 15
Day 21
Day 28
Day 31
Day 35
Day 39
Survival
3e5 4T1 f.p.
ICV i.p./i.t.
ICV i.p./i.t.
primery tumor rescection
ICV i.p/i.v.
ICV i.p/i.v.
ICV i.p/i.v.
ICV i.p/i.v.
b
Percent survival
100
80
60
40
20
0
0 20 30 35 40 45 50 55 60 65
days post-tumour seeding
irr4T1
MG1-IL12-ICV (IP)
MG1-IL12-ICV (IT/IV)
*

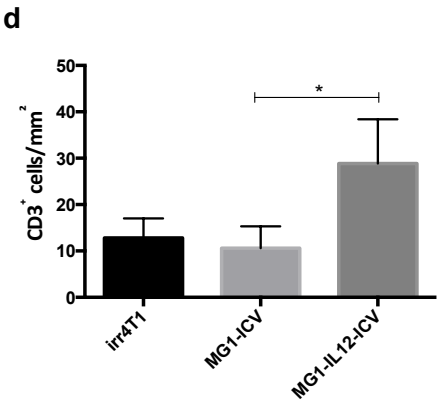
Figure 3.25. MG1-IL12-ICV synergises with adoptive T cell transfer.

(a) Experimental timeline of ICV vaccination and adoptive T cell infusion. (b) Average of the 4T1 primary tumor outgrowth following treatment initiation and until tumor resection. (c) The number of CD3+ (spots) cells/ mm^2 of primary tumor sections. The numbers of $CD3^+$ cells were averaged from two tumor sections per mouse and data are presented as mean of n = 5 mice per group. Error bars = SD * $p < 0.05$ and (d) A Kaplan–Meier survival analysis evaluating the synergistic effects of MG1-IL12-ICV with adoptive T cell transfer therapy.


a
Day 0
Day 9
Day 15
Day 21
Day 31
Survival
3e5 4T1
f.p.
ICV
i.p.
ICV
i.p.
primary
tumor
resection
(1e5 TILs) i.v.
+
Adjuvant


b
Percent survival
100
75
50
25
0
0
30
60
90
120
150
Days post-tumor seeding
irr4T1
MG1-ICV
MG1-IL12-ICV
*


c
Tumor resection
5e6 ICV IP
5e6 ICV IP
Tumor volume (mm3)
800
600
400
200
0
9
10
13
15
17
20
22
24
Days post-tumor seeding
irr4T1
MG1-ICV
MG1-IL12-ICV


d
CD3+ cells/mm2
50
40
30
20
10
0
*
irr4T1
MG1-ICV
MG1-IL12-ICV

4 DISCUSSION

Using oncolytic viruses as therapeutic agents involves converting pathogenic microorganisms into non-pathogenic vectors for therapeutic purposes [166], such as cytokine gene expression. The use of viral vectors in vaccine development is not new; however, its adaptation to using OVs harnesses their own intrinsic antitumor activities to bolster the vaccine. Any virus, virulent to humans or not, has two conditions before use as a vector. First, the viral replication capability in normal tissues must be eliminated (i.e. its ability to cause disease). Thus, it is often necessary to attenuate OVs before using them in patients. Second, the virus must be amenable to genetic engineering [167]. This condition is crucial in ensuring that the resulting vaccines possess a dual capacity of direct toxicity to the tumor cells and of immunogenicity, which is necessary in recruiting innate immune elements, such as NK cells and DCs, to invade the tumor tissue and lyse the cancer cells. These elements constitute the scientific rationale and mechanism behind my use of an infected cell vaccine in treating cancer.

OVs are one of the growing groups of viruses that can efficaciously act as vectors for therapeutic genes against cancer. However, the majority of OVs under clinical development are DNA viruses, which replicate slower and consequentially have more time to develop immune evasive strategies [168]. This evasion is beneficial, as immune clearance of the OV would impair its effectiveness. In addition, oncolytic virotherapy is considered a non-traditional form of immunotherapy. Unlike cytokine immunostimulatory methods, OVs can induce a powerfully immunogenic cell death that leads to a subsequent cytokine storm and immune cell influx to the site of viral infection [169].

4.1 MG1 virus, a clinical candidate for IL-12 transgene expression.

The Maraba virus variant MG1, like other OVs, has been known in existing literature [137] for its capability to selectively infect tumor cells, replicate within these cancer cells, and initiate systematic pro-inflammatory reactions innate immune recruitment leading to tumor lysis [135]. In this study, the invasive capability of MG1-IL12 was demonstrated together with its capacity to directly cause and further initiate the lytic destruction of the tumor cells. Its retained infectious selectively and attenuated virulence to normal tissues, making it an ideal candidate for cancer immunotherapy.

The Maraba MG1 backbone has been genetically modified with mutations in both the G and M proteins that make it hyper virulent in cancer cells but attenuated in normal cells [135]. The MG1 variant has a 100-fold higher maximum tolerated dose compared to wild-type Maraba [135]. When compared to other OVs, MG1 has an extremely wide tropism for infecting cancer cells, a very rapid life cycle, a large burst size, and rapid and robust transgene expression [135, 170]. The rapid life cycle of the MG1 virus is a crucial characteristic that enables it to effectively establish a productive infection in cancer cells and to recruit immune elements [137]. The combination of the oncolytic Maraba virus variant MG1 with the mIL-12 transgene (MG1-IL12) constitutes a strong antitumor agent that combines oncolytic viral immunotherapy, gene therapy, and cytokine immunostimulatory therapy in a single recombinant vaccine [171].

Additionally, the lack of pre-existing neutralizing antibodies in the human population—a major hurdle associated with many other OVs—warrants further development of the Maraba oncolytic virus for clinical applications [135, 172-175]. This deficiency might be beneficial in preventing its antigenic suppression, allowing it to freely recruit more non-specific immune elements to target tumor cells. Moreover, this window prior to the development of neutralizing

antibodies allows the vector to express any antitumor agents engineered into its genome, such as the mIL12 transgene in the structure of the MG1-IL12 variant.

4.2 The potency of utilizing MG1-IL12 in ICV

ICVs are a powerful, personalized cancer therapy platform that exploits the efficacy of OVs, autologous cell vaccines, and immunostimulatory cytokines (e.g. interleukin-12 and interferon-γ), while overcoming key barriers. The presence of IL-12 in MG1-IL12-ICV harnessed the inherent capacity of the immune system, innate and adaptive, to establish a fast and efficient antitumor immune response (**Figure 3.8f, and Figure 3.22c)** [166, 167]. Thus, the present studyincreasingly demonstrates that OVs can direct an innate and adaptive immune response against the cancer at both the site of administration and at distant sites. However, in order for this to occur, the OV must infect and express its immunogenic proteins within a cancer cell. Only when the viral glycoprotein is expressed does the antitumor action becomes effective in destroying cancer cells **(Figure 3.16b)**. This finding has been supported by a variety of studies highlighting the adjuvanticity of VSV-G, a rhabdovirus glycoprotein, in inducing potent immune responses [176-180].

As a solitary oncolytic viral therapy, the MG1-ICV had been found in this study to be less efficacious in the CT26lacZ colorectal cancer model than MG1-IL12-ICV (**Figure 3.12**). Interestingly, the current study revealed the capability of the MG1-ICV vaccine to indirectly induce low—and ultimately ineffective—IL-12 cytokine expression in animals (**Figure 3.5**). Moreover, the MG1-ICV had been found to have a far weaker chemotactic ability for murine and human NK cells (**Figure 3.10 and Figure 3.18**). Nevertheless, with the insertion of the mIL-12 transgene into its genomic backbone, the MG1 virus gains these immune-stimulating abilities while remaining an oncolytic antitumor agent against multiple types of carcinoma.

Vaccinating patients with their own cancer cells (autologous cell vaccine) has been tried in the past with variable success [87, 89]. Most have mixed the whole cell vaccine with non-specific adjuvants, such as the Bacillus Calmette-Guerin (BCG) tuberculosis vaccine, but difficulties in overcoming immune suppression within the tumor microenvironment have limited their results [181]. In fact, the innate immune system has several established signalling mechanisms to suppress antitumor immunity, whether innate or adaptive, such as up-regulation of inhibitory receptors [182], myeloid-derived suppressor cells [183], or lymphoid immune suppressor cells (e.g. regulatory T cells or Th17 cells) [183].. For instance, myeloid-derived suppressor cells (MDSCs), which are immature myeloid progenitor cells that suppresses natural killer T (NKT) cells, natural killer (NK) cells, and effector T cells, act through several immune evasion mechanisms including production of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and arginine (ARG), promoting tumor development and growth as well as metastasis [183-186]. Furthermore, cancer cells can significantly reduce the expression of MHC molecules (e.g. MHC I or MHC II), making it highly difficult, if not impossible, for T cells to detect malignant cells [182]

Tumor cell infection and resulting apoptosis are classic antitumor mechanisms attributed not just to OVs, [166, 167] but also to NK cells [187]. NK cells perform innate immunosurveillance of the human body to detect malignant cells and either directly destroy them or release cytokines to prime the adaptive immune response [187]. Recruitment of NK cells into the tumor microenvironment has been demonstrated in the present study. The MG1 virus can activate NK cell cytotoxicity through direct infection and maturation of conventional DCs [137, 187]. In this study, the heightened expression of IL-12 cytokine played a single overwhelming influence in the recruitment of NK cells into the tumor. The MG1 virus, like any virus, expresses

proteins that initiate an immune response designed to remove this foreign protein from its specific microenvironment. Equally, the massive secretion of IFN-γ by NK cells also serves to activate NK cells (which has been described above).

4.3 Therapeutic efficacy and antitumor immune response achieved with MG1-IL12-ICV

Despite the lack of clinical success with whole cell vaccines, clinical trials have consistently shown that survival is significantly better in those patients that are able to mount an immune response to the whole cell vaccine [188]. The greater the immune response elicited, the greater the invasion of immune cells into the tumor, leading to an improved prognosis. IL-12 has been used previously to direct anti-tumor immune responses, but the short half-life of this cytokine when administered as a recombinant protein—and the dose-limiting toxicities encountered following systemic administration—have diminished its clinical effectiveness [102]. Nevertheless, I have demonstrated a far more optimistic potential for the IL-12 cytokine when expressed from the MG1-IL12-ICV. At a minimum, IL-12 had very long survival rates in treated mice when administered in intervals of 3-4 days (**Figure 3.21, Figure 3.22a and Figure 3.24b**). A stable source of IL-12 allows this continuous production within the tumor microenvironment, effectively allowing it to perform its chemotactic function more persistently than when administered as a recombinant protein **(Figure 3.16b)**.

That said, the strong immunological rationale for cytokine-based vaccines continues to drive the development of novel experimental approaches in numerous laboratories worldwide. Indeed, more than 20 active anti-cancer clinical trials are listed on clinicaltrials.gov covering just IL-12 and IL-15 alone. This approach to immunotherapy is not without problems and dangers. Intravenous administration of the IL-12 protein, particularly in large doses for acute effects, has been established as effective but toxic both in animal and human studies [106, 189, 190]. Large

doses are often necessary due to the short half-life of IL-12 following systemic intravenous delivery. While low doses address the concerns over systemic toxicity, its effectiveness will be largely limited to IL-12-sensitive cancer cells. Thus, it is clear that the short half-life problem must be resolved and this may be accomplished using a carrier virus or vaccine, bearing a gene that can express IL-12 within the cancer cells. This innovative form of gene therapy has been possible with the successful utilization of MG1-IL12 in the infected cell vaccine.

IL-12 gene therapy shows greater efficacy and less toxicity than recombinant IL-12 therapy (e.g. in renal carcinoma) [171, 191], which offers an alternative direction for future investigation with IL-12 based therapies. However, systemic IL-12 gene therapy alone has not been found effective in eradicating aggressive tumors or metastatic cells [192, 193], which can either be due to the inherent limits of the gene therapy itself, its manner of administration, or both. Thus, there may be significant factors involved with the treatment delivery method used to achieve specific treatment objectives. These efficacy issues indicate future developments in the direct-to-tumor (i.e. in situ) delivery routes that may go beyond the current use of intratumoral injection or, to a lesser extent, intraperitoneal carcinomatosis, which were both employed in this study.

A review of the IL-12 cytokine literature reveals a complex network of roles for IL-12 in activating innate immune cellular elements [194, 195]. Activated antigen-presenting cells (APC), such as DCs and monocytes/macrophages, directly secrete IL-12 [96, 195], as do pathogen-associated adaptive responses [194]. However, the present study demonstrated that the IL-12 levels secreted through the MG1-IL12-ICV are far higher than those produced through adaptive immune responses (**Figure 3.4b and Figure 3.5**). Moreover, the IL-12 cytokine induced-antitumor activity was stronger against CT26lacZ colon cancer than B1F10 melanoma, as

observed in the murine peritoneal carcinomatosis models (**Figure 3.15 and Figure 3.21**). These findings could be explained by the fact that CT26lacZ tumors are more permissive to MG1-IL12 virus infection as compared to B16F10 tumors. As a result, a productive infection beyond the ICV (i.e. viral spreading from the ICV to the tumor) is taking place, which is believed to be accompanied by an enormous expression of IL-12 in the tumor microenvironment and beyond, in contrast to a low level of IL-12 expression in B16F10 model that is limited to the ICV.

In this study, the induction of IFN-γ [196], from NK cells was also observed to be highly active against DC co-cultured B16F10 murine melanoma cells in the presence of the MG1-IL12-ICV. This led to heightened invasion of NK cells within a period of 24 h (**Figure 3.9 and Figure 3.10**). This interferon has been known to play a role in rejecting many tumor cell lines and hepatocellular carcinoma in a NK cell-dependent manner [196-198], making NK cell effects on the tumor cells more consistent and highly reliable. Moreover, it has been recognized for its role in rejecting hepatocellular carcinoma cells [196, 199]. Furthermore, IFN-γ was secreted by T cells in this study and others [96].

Another chemotactic protein, the chemokine IP-10 secreted by the APCs [200], is dependent upon IFN-γ and in turn, depends upon IL-12. In this study, the secretion of IP-10 tripled in the presence of IL-12 over MCP-1 and SDF-1 (**Figure 3.11a**). Meanwhile, NK cells also secrete the necessary IFN-γ levels. This demonstrates two important findings: (1) IP-10 responds strongly to the stimulatory effect of the IL-12 cytokine in MG1-IL12-ICV acting through IFN-γ [201]; (2) The NK cells responding to the chemotactic signals of DCs also secret IFN-γ, which in turn secrete IP-10 [201-203]. Although IP-10 flows into the tumor microenvironment through DC secretion events that induce immune cell chemotaxis, it also has been reported to exert a strong anti-angiogenic function that suppresses tumor outgrowth and

metastasis [201]. The extent to which attenuation of tumor outgrowth is due to the angiostatic effects of MG1-IL12-ICV in relation to IP-10 induction is yet to be elucidated.

4.4 Contribution of the IL-12 cytokine to MG1-ICV

Utilizing IL-12 in OV therapy has been shown previously to improve their therapeutic index in murine models [125], but this is the first study to clearly define the role of IP-10, secreted from DCs, in NK cell chemotaxis and activation. This study confirmed previous findings that IL-12 mediates the function of IFN-γ in inducing DCs to secrete IP-10 [200, 201, 204]. This recruitment synergizes with the IL-12 mediated direct effect on cytotoxicity and activation of NK cells, leading to a robust targeting and killing of tumor cells in the peritoneal carcinomatosis model. While not investigated in our model, it is possible that i.p. delivery allows for antigen presentation in the multitude of intra-abdominal and retroperitoneal lymph nodes, which has been previously documented to be essential for efficacy [205].

While NK cells appear paramount for the efficacy of the MG1-IL12-ICV, clearly an adaptive immune response is mounted following curative treatment, as illustrated by rejection of implanted CT26lacZ tumors, but not of syngeneic implanted 4T1 tumors. Moreover, the pattern of tumor response by MRI, including the pseudo-progression seen at 1 week and the ongoing tumor response beyond 6 weeks, is suggestive of an adaptive T cell response [206, 207]. These essential responses may not be mediated by the chemotactic IL-12 cytokine. OVs have exhibited a potent ability to generate an anti-tumor T cell mediated immune response, but, in general, it is thought to require viral infection of cancer cells [119, 121]. The ICV platform optimizes tumor cell infection by incubating tumor cells ex-vivo at a high multiplicity of infection. This may explain, in part, the improved efficacy of the MG1-IL12 ICV over viral administration alone **(Figure 3.12a)**.

4.5 Delivery methods for the MG1-IL12-ICV

Effective delivery of OVs has been a challenging task, which has stimulated a growing body of literature on OV targeting in the contemporary immunotherapy research. Ensuring viral delivery and robust infection of cancer cells can pose a significant barrier to efficacy [208], whether performed i.v., i.p., or i.t. In fact, direct intratumoral delivery (in situ vaccination) provides an intuitive assurance on the enhanced effectiveness against cancer cells, while other delivery mechanisms have been shown to have inadequately resolved the expected barriers. The recently approved GM-CSF expressing herpes virus (talmogene laherparepvec) is directly injected into an accessible tumor to avoid the problems of viral delivery, effectively turning the OV into an in-situ ICV [209]. The current study demonstrated a six to seven-fold increase in the recruitment of NK cells to the lungs (**Figure 3.6c**) and a three to four-fold decrease in splenic NK cells when using the systemic (i.v.) delivery method (**Figure 3.6f**), thereby decreasing the lung tumor metastasis by half compared to the MG1-ICV and five-fold among lung cancer cells exposed to irradiated cells (**Figure 3.7**). This result demonstrates that even the perceived inefficiency (e.g. fast plasma clearance [210]) of the i.v. administration method cannot seriously obscure the chemotactic effect of the MG1-IL12-ICV.

However, in the context of ICV, intravenous injection had been associated with a mismatch in the alveolar ventilation/pulmonary perfusion ratio, which leads to an acute episode of respiratory distress syndrome [211]. Often, this syndrome has been preceded with the formation of pulmonary emboli, which leads to respiratory failure [211], or systemic cytokine storm [212]. This uncertainty in relation to intravenous delivery of endothelial cells via infected cell vaccination, however, does not remove the risk of pulmonary embolism and acute

respiratory distress in the use of the MG1-IL12-ICV in clinical application. At this point, the uncertainty may only be properly addressed in future studies.

Conversely, the intraperitoneal delivery method, a form of a regional or targeted delivery [213], has demonstrated several important features in this study. In the two dose regimen, four distinct characteristics have been observed. First, it resulted in slightly longer protective action in orthotropic breast cancer compared to irr4T1 treatment (**Figure 3.22a**). However, its deterioration rate is largely comparable with irradiated cells treatment once its protective efficacy starts to break down. Second, the resultant breast tumor volume is only slightly better than that in irr4T1 treatments (**Figure 3.22b**). Third, the chemotactic outcome for T cell trafficking with the MG1-IL12-ICV is also only slightly better than irr4T1 therapy in breast tumors (**Figure 3.22c**). Fourth, the tumoricidal effect of MG1-IL12-ICV far surpasses that of irr4T1 treatments. However, this behaviour appeared to be largely vaccine dependent rather than route of administration related.

Meanwhile, in the multiple dose (i.e. six-dose) regimen, two features of the i.p. route appeared distinct. First, it has only slightly diverging protective periods with irradiated cells treatments (**Figure 3.24b**). However, it demonstrated persistently higher survival rates with a moderately high survival level compared to zero survival in irradiated cells treatments after 25 days from treatment initiation. Nevertheless, this protective behaviour appeared to be route of administration dependent. Compared to these results with i.p. vaccination, i.t.-i.v. vaccination displayed a far longer protective period and survival persistence but no survival beyond 63 days post tumor seeding. This difference between the two vaccination strategies highlights the importance of localizing the MG1-IL12-ICV vaccine at the tumor site to elicit the most chemotactic potency. Perhaps combining MG1-IL12-ICV with the current standard of care

therapy, such as early perioperative intraperitoneal chemotherapy (EPIC), could be effective, although a safer method such as intraoperative intraperitoneal chemotherapy (IIC) may be preferable. Intraoperative extensive peritoneal lavage has also been suggested to increase the chances of removing residual tumor cells from the peritoneal cavity. Moreover, performing a cytoreductive surgery is a common practice to remove all macroscopic tumors before any chemotherapy or immunotherapy is performed [210]. This approach is highly logical in increasing the odds of successful therapy and to avoid tumor cell proliferation that can overwhelm the innate immune cellular elements stimulated by the IL-12 recombinant MG1 infected cell vaccine or chemotherapy.

A comparative experiment performed in this study between the curative characteristics of MG1-IL12-ICV and its consequently associated survival rate has revealed interesting features dependent on treatment frequency. The two-dose treatment regimen demonstrated similar protection within 30 days between i.p. and i.t. delivery, but faster deterioration of the i.p. protection In effect, although this conferred protection can be attributed with reasonable certainty to MG1-IL12-ICV, the greater persistency in survival rate can be largely attributed to the i.t. delivery method, which apparently provided the immune system greater advantage over i.p. Meanwhile, the multiple dose (six-dose) of i.p. regimen with MG1-IL12-ICV in the 4T1 model demonstrates no added benefits in comparison to the two-dose regimen, as mice did not survive beyond 50 days. Interestingly, employing the proposed tumor co-localized vaccine delivery (i.t. and i.v), mice achieved longer protection with approximately two-weeks prolonged survival rate.

In many ways, as demonstrated in this study, MG1-IL12-ICV may be delivered through direct injection into accessible tumors with far better efficacy than systemic injection with all its accompanying barriers, which often take the form of acute respiratory distress secondary to

pulmonary embolism or systemic cytokine storm. In the case of peritoneal carcinomatosis, direct intratumoral delivery (i.p. injection) of MG1-IL12-ICV has superior efficacy and this is most likely explained by the chemotactic properties of IL-12. As previously indicated, the IL-12 expression attracts the presence of NK cells and their invasion into the tumor microenvironment where the vaccine was directly delivered.

4.6 Enhanced epitope spreading with the MG1-IL12-ICV

The adaptive T cell responses in this study were characterized and revealed important information in the behaviour of MG1-IL12-ICV. The experiment involving ex vivo peptide stimulation of splenic T cells demonstrated a viral specific T cell response to VSVn with no such responses to any tested TAAs. Strikingly, the tumor specificity profile following ex vivo stimulation of T cells isolated from the peritoneum (TALs), the site of vaccination and the site of tumor challenge, revealed a significant IFN-γ expression, highlighting the immunogenicity of MG1-IL12-ICV to induce a polyclonal T cell response that correlates with a broad spectrum of TAAs **(Figure 3.20)**. This finding aligns well with the purpose of using the autologous infected cell vaccine platform to provide potentially thousands of proteins and tumor epitopes to target. Although DCT, Trp2, and gp100 are common antigens examined in B16F10 tumor models, they may not be the most immunogenic epitopes targeted after the vaccination with MG1-IL12-ICV. Castle *et al*, have reported that the B16F10 tumor cell line has acquired over 550 somatic mutations that potentially encode novel immunogenic epitopes [110]. Therefore, more experiments need to be carried out to assess the generation of B16F10 mutanome-specific T cell responses, which could unleash the potential of MG1-IL12-ICV as a personalized vaccine.

Meanwhile, the generated CD4 T cell response following MG1-IL12-ICV vaccination presented a different response profile compared to MG1-ICV treatment. It appears that IL-12

induced a generalized CD4 T cells response that is dominant across all tested tumor epitopes. These results propose a potential cytotoxic role of these CD4 T cells in eliminating B16F10 tumors and perhaps employ previously described mechanisms [214-216]. The role of CD4 T cells has been investigated following Ad-DCT prime-MG1-DCT boost treatment regimen and was found to be minimal [136]. This might explain the potency of MG1-IL1-ICV treatments in B16F10 peritoneal carcinomatosis over Ad-DCT/MG1-DCT treatments. However, more experiments are warranted to investigate the relative contributions of CD4 and CD8 T cells to the improved treatment outcomes in the setting of MG1-IL12-ICV **(Figure 3.21)**.

Meanwhile, our multimodal approach utilizing the engineered oncolytic MG1-IL12 virus as an infected cell vaccine has a combined effect on tumors by 1) infecting and directly lysing the tumor tissues as an initial tumor debulking process, 2) recruiting the innate (NK cell) immune system to the tumor microenvironment by overexpressing IL-12, 3) activating and maturing DCs by expressed viral proteins and IFN-γ secreted by activated NK cells, 4) the activated DC in turn secreting IP-10, which further recruits NK cells to the vaccination site, 5) Local DCs efficiently processing and presenting TAA to T cells for priming, and 6) memory T cells exerting their cytolytic functions on tumor cells.

4.7 Conclusion

In this era of emerging immunotherapies, including immune checkpoint inhibitors [217], combining treatments with an ICV holds significant promise [180]. Moreover, recombinant vaccine engineering offers an enhanced capability to creatively infiltrate aggressive tumors with powerful chemotactic agents that drive innate immune responses to hyper-activate. Gene therapy, immunotherapy, and virotherapy have not been previously shown to be integrated into a single therapeutic or prophylactic treatment to better induce tumoricidal apoptosis within the

tumor microenvironment or prolong protective cover against oncogenesis when exposed to oncogenic conditions or agents. The present study has demonstrated that all these can be achieved using current biomedical technology, particularly in the MG1-IL12-ICV.

However, as with the addition of IL-12 to MG1, it is important to consider how immune activation might hinder viral replication, oncolysis, and transgene expression [218]. In fact, immune suppression, through innate immune elements as negative regulatory receptors [182], myeloid-derived suppressor cells [183], and lymphoid immune suppressor cells (e.g. regulatory T cells or Th17 cells) [183], is a phenomenon that immunologists must contend with in order to avoid the frustrations of neutralizing therapeutic objectives. In various contexts, immunotherapies such as anti-CTLA-4 antibody can be both complementary and inhibitory towards OV therapy [219, 220]. Moreover, there is a real need to re-evaluate the current delivery systems and therapeutic dosing and timing in order to address the problem presented by respiratory distress syndrome [211], systemic cytokine storm [212], and pulmonary embolism [211]. In this study, the use of different vaccine delivery methods, depending on the tumor location, potentially provides a viable option to minimize these life-threatening incidents. However, current literature remains limited in exploring proven solutions for these technical and clinical challenges. Further studies must be conducted to explore the role of the MG1-IL12-ICV in high-risk phenomena and to explore alternate methods that may be used to either minimize or eliminate these threats.

In the present research, I have clearly shown that the addition of IL-12 to the oncolytic rhabdovirus MG1 improves efficacy by recruiting and activating NK and T cells to the site of tumor burden. Further studies exploring the relative contributions of viral oncolysis and innate and adaptive immunity will allow us to harness the immunotherapeutic potential of the ICV

platform. Moreover, within the IL-12 cytokine literature, further studies are also necessary to explore a model of IL-12 therapy that will present less toxicity than the existing IL-12 gene therapy [171, 191]. It is also necessary to explore in depth the divergent reactions of MG1-IL12-ICV to the various immunogenic epitope-bearing stimulants to enhance OV therapy through an adjuvant therapeutic model. Finally, it would be of particular interest to explore the role of TILs/TALs in enhancing the therapeutic effect of the MG1-IL12-ICV.

5 MATERIAL AND METHODS:

5.1 Cell lines and mice:

Murine CT26lacZ colon carcinoma, murine B16F10 F10 melanoma, human SW620 colorectal adenocarcinoma, human HCT15 colorectal adenocarcinoma, human A549 lung carcinoma, murine YAC-1 lymphoma, and human K562 leukemic cell lines (all from American Type Tissue Collection) were propagated in Dulbecco's modified Eagle's medium (Hyclone) for the adherent cell lines, or Roswell Park Memorial Institutes Media (Hyclone) for non-adherent cell lines, supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada). Rauscher murine leukemia virus–induced T cell lymphoma (RMA) and RMA-S (MHC-deficient variant of RMA) were obtained from Dr. A. Veillette (Institut de Recherches Clinique, Montreal, Quebec, Canada). Female Balb/C and C57BL/6 mice 6- to 8 weeks old were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in pathogen-free conditions and all experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

5.2 MG1-IL12 construction:

Murine IL-12 was PCR amplified from pORF-mIL-12 (IL-12elasti(p35::p40)) (InvivoGen, San Diego, CA, USA) to add MluI (5') and (3') cloning sites to facilitate cloning into Maraba MG1 [221]. The recombinant MG1-IL12 virus was rescued as described previously [3]. Briefly, A549 cells were infected with vaccinia virus expressing T7 polymerase and subsequently transfected using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) with 2 mg of MG1-IL-12 DNA plasmid together with pCI-Neo plasmids encoding for Maraba N, P and L (1, 1.25, 0.25 mg,

respectively). The rescued virus was passaged on SNB19 cells, then plaque purified, amplified and titered on Vero cells.

5.3 Viability assays:

B16lacZ, CT26lacZ, SW620, and HCT15 cell lines were seeded into 96-well plates (2×10^4 cells/well). 24 hours later, cells were infected with MG1 or MG1-IL-12 viruses at a multiplicity of infection (MOI) of 0.001–10 pfu/cell. Alamar Blue (Sigma-Aldrich, St Louis, MO) was added following 48 hours of incubation to a final concentration of 20 μg/ml. The absorbance was read at 570 nm after 6-hour incubation.

5.4 Antibodies and FACS analysis:

For splenic and lung lymphocyte population analyses, organs were harvested from mice and red blood cells lysed using ammonium chloride–potassium lysis (ACK) buffer. The following monoclonal antibodies were used: anti-TCR-β (H57-597), anti-NK1.1 (PK136), both from eBiosciences. Spleen and lung NK cell IFN-γ and Granzyme B secretion were analysed following a 1 hour GolgiPlug (BD Biosciences) incubation using: anti-CD3 (17A2), anti-NK1.1 (PK136), anti-IFN-γ (XMG1.2), and anti-Granzyme B (16G6) all from BD Biosciences. The monoclonal antibodies used for human NK cell migration and activation are anti-CD56 (HCD56) from Biolegend, and anti-CD3 (UCHT1) and anti-CD69 (FN50) from eBiosciences. For ex vivo T cell restimulation assay, the intracellular staining of IFN-γ was performed by stimulating one to two million lymphocytes with designated antigens for an initial 1.5 hours at 37°C followed by another 3.5 hours at 37°C after GolgiPlug (BD Biosciences) addition to the cells. The cells were then surface stained with antibodies: CD4-PerCP (clone GK1.5, BD Bioscience, 1/200 dilution) and CD8-PE (clone 53-6.7, BD Bioscience, 1/200 dilution). Subsequently, lymphocytes were

permeabilized and fixed using the BD Cytofix/Cytoperm kit (BD Bioscience, Cat# 554714) according to the manufacturer protocol. Intracellular IFN-γ was stained with IFNγ-APC (clone XMG1.2, eBioscience, 1/100 dilution). Fluorescence-activated cell sorting (FACS) acquisitions were conducted on a CyAn-ADP using Summit software (Beckman Coulter, Mississauga, Canada) and data were analyzed with Kaluza software (Beckman Coulter).

5.5 Ex vivo splenocyte cytotoxicity assay

The ^{51}Cr-release assay was performed as previously described [12]. Briefly, splenocytes were harvested from treated and control mice two days after treatment. ACK buffer treated splenocytes were resuspended and mixed with chromium labelled YAC-1 cells at specified effector-to-target (E:T) ratios.

5.6 In vivo tumor rejection assay

The *in vivo* rejection assay was performed as described previously [12]. Briefly, RMA and RMA-S were labeled with 5 and 0.5 mmol/L CFSE, respectively. 1×10^6 cells containing a 1:1 mixture of each cell type was injected i.p. into C57BL/6 mice 24 hrs following ICV treatment. Peritoneal cells were collected the following day (24 hr) by washing the peritoneum with 5 mL of PBS containing 2 mmol/L EDTA. Collected cells were analysed by flow cytometry for the presence of CFSE-labeling.

5.7 Virus infection of B16F10 cells and co-culture with bone marrow-derived DCs for chemotaxis and chemokines analysis

B16F10 cells infected with MG1 or MG1-IL12 (MOI = 0.1 pfu/cell) were harvested 18 hrs after infection and cultured with bone marrow-derived dendritic cells (DCs) as described elsewhere at a 3:1 ratio in DC medium (1% FBS) (complete RPMI supplemented with 2-Mercapoethanol (cat

#21985-023, Gibco, life technologies)) in 96-wells plates [222]. Media was collected after 24 hours and stored at -80°C until further analysis.

5.8 Cytokine and chemokine analyses

Murine IFN-γ from DC co-culture supernatant was detected by FlowCytomix (eBioscience) kit as per manufacturer's instructions. For lung IL-12 and IFN-γ expression, lungs from C57BL/6 mice treated with irrB16, MG1-ICV, or MG1-IL12-ICV at 5×10^5 cells/100ul/mouse i.v. were resected and homogenized in 1ml PBS 24-hours after treatment. Murine MCP-1, SDF-1, and IP-10 chemokines were assayed 18 hours post ICV treatment from the peritoneal fluids of C57Bl/6 mice (*in vivo*) or from tissue culture supernatant using ELISArray kits (SABiosciences) as per manufacturer's instructions.

5.9 Murine transwell chemotaxis assay

Tissue culture supernatants for assessment of chemokines or chemotaxis were generated in DC media. Chemotaxis of NK cells was assessed using a Transwell system as described previously [8]. Briefly, 500 μl of conditioned media from DC cultures was added to the lower chamber of Transwell plates with 5-μm pores (Costar, Corning). 3×10^5 of $DX5^+$ sorted NK cells were added to the upper chamber, and plates were incubated for 3 hours at 37°C. Cells in the lower chambers were harvested, stained with trypan blue and counted. A migration percentage was calculated as (total number NK cells in bottom chamber / total number NK cell input) x 100. To calculate NK cell index: (NK cell migration percentage / NK cell migration percentage from media alone group).

5.10 Human transwell chemotaxis assay

Conditioned media were generated in DC media through direct ICV-PBMCs co-culture at 3:1

ratio for 18 hours. 1×10^6 PBMCs were added to the upper chamber, and plates were incubated for 3 hours at 37°C. Cells in the lower chambers were harvested, stained with anti-CD56 (HCD56) and anti-CD3 (UCHT1), and quantified by FACS. A migration percentage was calculated as (total number NK cells in bottom chamber / total number NK cell input) x 100. To calculate the NK cell index: (NK cell migration percentage / NK cell migration percentage from media alone group).

5.11 DC-MG1-IL-12-ICV/splenocyte co-cultures

DC-MG1-IL-12-ICV were isolated by MACS $CD11c^+$ selection (Miltenyi Biotec) and co-cultured with naïve splenocytes at 1:5 ratio in DC medium, at 2×10^5 splenocytes/well in 96-well plate format. Twenty-four hours later, cell-free supernatant was stored at -80°C for measurement of IFN-γ. Intracellular IFN-γ staining on splenocytes by intracellular FACS was also performed as described above.

5.12 Mouse models:

Therapeutic treatment model. For one dose treatments, CT26lacZ and B16F10 peritoneal carcinomatosis tumors in BALB/c and C57Bl/6 mice, respectively, were treated with 1×10^4 ICV on day 3 after seeding 5×10^5 tumor cells intraperitonealy. For the B16F10 six dose treatment regimen, 1×10^5 B16F10 tumor cells were seeded within the peritoneum and the treatments were started on day 5 and given twice a week for three consecutive weeks at 5×10^6 ICV dose. For the CT26lacZ bulky tumor model, 5×10^5 tumor cells were seeded within the peritoneum and the treatment regimen of six doses of ICV was initiated following magnetic resonance (MR) scan confirmation of a tumor with a size of >3 mm. Animals were sedated with isoflurane gas and MR scanning was performed with a 7 Tesla GE/Agilent MR 901 (GE Healthcare, Chicago,

USA). For each mouse, three MR pulse sequences were used: one localizer and two fast spin echo (FSE) sequences in the coronal and axial planes. The parameters for the FSE sequences were: echo train length 8, bandwidth=16 kHz, echo time=42ms, repetition time=1500ms, field of view=35mm, matrix 256x256, slice thickness=1mm. The total MR scan time per mouse was approximately 15 minutes. Follow-up MR scans were performed one week, six weeks and thirteen weeks post-treatment start using the same MR scan parameters.

Prophylactic treatment model. C57BL/6 mice were vaccinated with a single dose of 1×10^3 irrB16, MG1-ICV, or MG1-IL-12-ICV i.p. The following day, mice were challenged with 3×10^5 B16F10-LacZ cells i.v. and sacrificed 4 days after tumor cells injection followed by staining and quantification of lung metastases with β-gal (Bioshop, Burlington, Canada) as described previously [13]. The total number of surface lung metastases was determined on all lobes of the lungs using a stereomicroscope (Leica Microsystems, Concord, Canada).

5.13 Viral plaque assay

MG1/MG1-IL12 viruses were titered in serum free DMEM at 10-fold increments, and 250 μL of the serial dilutions were added to each well of a twelve-well plate in duplicate. The titers of infectious viral particles were determined by standard plaque assay on Vero monolayer cells with 1% agarose overlays mixed with 2x DMEM (Gibco) containing 20% FBS at 1:1 ratio. The viral concentrations (plaque forming unit) (PFU/mL) were quantified following 24 hours of incubation at 37^{o}C after infection by counting the number of plaques per well. The viral concentration was calculated based on the following formula: (the averaged # of plaques in duplicate wells) x (dilution factor)/(initial volume of infection).

5.14 Tumor infiltrating/associated lymphocyte extraction

At the indicated time-points, tumor infiltrating/associated lymphocytes **(**TILs/TALs) were collected from the peritoneum by washing the peritoneal cavity with 5 mL of PBS containing 2 mmol/L EDTA. The peritoneal wash was then applied and mashed through a 40 μm nylon strainer. Cell suspensions were centrifuged and re-suspended with 6 ml of 40% Percoll (Sigma-Aldrich, St Louis, MO) and overlaid on 6 ml of 80% Percoll followed by a 23 minute centrifugation at 400g at RT (ascending rate: 2; descending rate: 1). Interphase containing lymphocytes were then collected and washed twice with complete RPMI and re-suspended to the desired final concentrations to be used for the ex vivo T cell stimulation assays.

5.15 Adoptive transfer of the expanded T cells from 4T1 TILs

The orthotopic 4T1 spontaneous metastasis model was conducted by seeding 2×10^5 4T1 breast tumor cells into the mammary fat pad of BALB/c mice. Mice were then vaccinated with the ICV i.p. at a 5×10^6 dose on days 9 and 15 followed by a complete resection of the primary tumor on day 21. The resected 4T1 tumors were dissociated with a mouse tumor dissociation kit (Miltenyi Biotec). CD90.2 beads (Miltenyi Biotec) were then used to isolate T cells from the dissociated tumors and plated at 1×10^6 cells in 3 ml volume of Aim-V media supplemented with %2.5 human serum in a T25 flask (media was supplemented with 25 ng/ml of IL-2 and 25ng/ml of IL-17) (Sigma-Aldrich, St Louis, MO). Three days after the initial culture and every other day afterword for a total of 10 days, 1.5 ml of the supernatant was replaced with fresh media containing IL-2. On day 10, T cells were harvested, washed, and resuspended with PBS to a final concentration of 1×10^5 cells and infused intravenously to the mice from the same experiment (which was day 31 after 4T1 tumor seeding).

5.16 Statistical analysis

All statistical analyses were determined using GraphPad Prism 6.0 software. Statistical significance, where applicable, was determined by the Student t test or one-way ANOVA with a cut off $P = 0.05$. Data are presented as mean ±SD. Survival analysis were performed according to Kaplan-Meier method using log-rank test and the difference between groups were significant only when $P<0.05$.

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Appendices

Appendix I: Interleukin-12-expressing oncolytic virus: A promising strategy for cancer immunotherapy

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Review Article

Interleukin-12-expressing oncolytic virus: A promising strategy for cancer immunotherapy

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الملخص

الفيروسات القاتلة للأورام السرطانية؛ هي من أنواع العلاجات المبتكرة المضادة للسرطان، التي تستهدف وتدمر الأنسجة السرطانية بانتقائية دون المساس بالخلايا السليمة. اكتسب علاج الأورام بالفيروسات القاتلة المزيد من الاهتمام للمزيد من التطور كنوع جديد من العلاج المناعي بعد موافقة إدارة الغذاء والدواء الأمريكية مؤخرا على فيروس الهربس لعلاج الورم الملانيني المتقدم. ويقوم النهج العملي لتحقيق أقصى قدر من الفاعلية للفيروسات القاتلة للأورام السرطانية على تسليح الفيروسات بالسايتوكاينز المعززة للمناعة، لتكون قادرة على تعزيز القدرة المناعية للهجوم على الخلايا السرطانية بفاعلية. وقد تم تصنيف انترليوكن -١٢ كاسايتوكاينز قوي له أنشطة مضادة للأورام فاعلة، تقوم بتنشيط كلا من الجهاز المناعي الفطري والتَكَيُفي المضاد للأورام. وأظهرت العديد من الدراسات أن انترليوكن -١٢ ذي خصائص الفيروسات القاتلة للأورام السرطانية، يحسن مؤشر العلاج لعينات الورم قبل السريرية بواسطة تنشيط وتوظيف الخلايا الجذعية، والخلايا السامة الطبيعية القاتلة والخلايا "ت" السامة للخلايا التي بدورها تحسن إزالة الورم. في هذا العرض، نناقش آلية المناعة لعمل الفيروسات القاتلة التي تحوي انترليوكن -١٢.

الكلمات المفتاحية: العلاج المناعي؛ انترليوكن -١٢؛ العلاج بالفيروسات؛ السرطان؛ الفيروس القاتل للورم السرطاني

Abstract

Oncolytic viruses (OVs) are an emerging class of novel anti-cancer therapeutic agents that selectively infect and destroy cancerous tissues without damaging normal cells. With the recent US Food and Drug Administration (FDA) approval of Herpes Virus (T-VEC) for the treatment of advanced melanoma, oncolytic virotherapy has gained more attention for further development as a novel form of immunotherapy. A viable approach to maximize the efficacy of OVs involves arming them with immune-enhancing cytokines that are capable of boosting the host's immune response to effectively attack tumour cells. Interleukin-12 (IL-12) is a powerful cytokine with potent antitumour activities that activates both innate and adaptive anti-tumour responses. Several studies have demonstrated that IL-12-expressing OVs improve the therapeutic index in pre-clinical tumour models by activating and recruiting dendritic cells (DCs), cytotoxic natural killer (NK) cells and cytotoxic T cells, which subsequently improve tumour clearance. In this review, the immunological mechanisms of IL-12–expressing viruses are discussed.

Keywords: Cancer; Immunotherapy; Interleukin-12; Oncolytic virus; Virotherapy

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Introduction

One of the most potent alternative cancer treatments is gene therapy using various agents, such as oncolytic viruses (OVs), which are viruses that can selectively infect, replicate, and generate a greater immune response against cancer and directly kill tumour cells.[1] OVs have been used in clinical trials for the treatment of various cancers, such as pancreatic cancer, ovarian cancer, colorectal cancer and glioma.[2] OVs are designed to target and hijack cancerous cell machinery including: (a) pro-apoptotic targeting where the viruses delay apoptosis of infected cells to promote their replication for the synthesis and assembly of a large number of new viruses before killing the infected cancer cell; (b) transcriptional targeting where an essential viral gene is placed under the regulation of tumour specific promoters; (c) transductional targeting where the ability of the tumour cells to up regulate their tumour-specific receptors is exploited for specific targeting of cancer cells by OVs, and (d) targeting strategies based on the tumour microenvironment where modified microenvironments, such as angiogenesis, hypoxia and activation of certain proteases are exploited.[3] The infected cancer cells eventually produce more infectious particles that infect neighbouring cancer cells; thus, the anti-cancer "infection" spreads.

Direct tumour cytotoxicity appears to be only one of the key mechanisms mediating the anti- efficacy of OV.[4] Interestingly, immune-mediated tumour suppression for overall OV efficacy is likely influenced by the initial period of vigorous OV replication and lytic activity, which optimally set the stage for subsequent antitumour immune response.[4,5] Studies have shown that T and NK cell recruitment into tumours associated with increased survival and enhance antitumour efficacy, subsequently, genetically modified viruses coding for immunomodulatory agents, such as cytokines or chemokines, have come into focus. Such engineered viruses can promote an efficient anti-tumour immune response using several mechanisms, including the induction of intrinsic cellular stress pathways that activate innate immunity, expression of stress-induced self-ligands rendering this cell susceptible to natural killer (NK) cell-mediated lysis and the enhanced presentation of tumour-specific antigens to cytotoxic $CD8^+$ T cells.[6] A milestone has been achieved in the usage of cytokine-armed oncolytic viruses when the FDA recently approved talimogene laherparepvec (T-VEC), a herpes simplex virus (HSV) expressing the immunostimulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), in the treatment of patients with metastatic melanoma.[7] A number of OVs that harbour IL-12 have been demonstrated, in which IL-12 significantly enhances the anticancer immune response. Interleukin-12 (IL-12) is a heterodimeric cytokine produced primarily by phagocytic cells and antigen-presenting cells in response to bacteria, bacterial products, and intracellular parasites. The major target cells of IL-12 action include NK and T cells, which induce IFN-gamma (IFN-γ) production, cytotoxic activity, increased proliferation in cooperation with other costimulatory signals, and differentiation of T helper type 1 (Th1) cells.[6] IL-12 is one of the most potent anti-cancer cytokines. Its antitumoural effect is mediated by the activity of T, NK, and NKT cells and by an anti-angiogenic effect. Given its ability to activate both NK and cytotoxic T cells, IL-12 represents the ideal candidate for tumour immunotherapy in humans.

Unfortunately, systemic administration of IL-12 to cancer patients causes excessive clinical toxicity and severe side effects.[8] To avoid such severe side effects, scientists have used novel methods to deliver this cytokine directly to the tumour site, and one of these methods involves the use of OVs. Taking advantage of OV oncotropism, OVs appear to be a promising platform to effectively deliver IL-12 and restrict it is expression within the tumour microenvironment. The present review summarizes the most promising IL-12-expressing OVs as shown in preclinical models and patients.

Adenovirus

Adenoviruses belong to the family of *Adenoviridae*. Their name is derived from the human adenoid tissues from which they were first isolated.[9] Adenoviruses are non-enveloped viruses with a genome of double stranded linear DNA 26–48 kb in size and a capsid of 65–80 nm in diameter.[9] The capsid contains penton, hexon and fibre proteins that are important for the infection of cells by the virus. The virus uses receptor-mediated endocytosis to enter cells.[10] The first vector systems developed for gene delivery and expression were derived from adenoviruses.[11] Subsequently, adenoviruses have emerged as one of the most frequently used viral vectors for gene therapies, including cancer viral therapy.[11] This emergence is related to their ability to affect the metabolic activity of various cells (replicating and non-replicating cells), host large inserted genes, and code for proteins without incorporating into the cell genome of the host.[11]

Various adenovirus mutants, which are capable of killing tumour cells, have been engineered to utilize tumour-associated promoters to regulate the expression of essential viral genes, thus restricting viral replication and spreading to tumour cells.[12] A common strategy in designing oncolytic adenoviruses is to genetically engineer the adenoviral early region 1A (E1A).[13] This process involves a bondage of the CR2 region of adenoviral E1A to the retinoblastoma protein (RB) and other related proteins that regulate the E2F family of transcription factors, which plays a pivotal role in the regulation of cellular proliferation and stimulates the entrance of quiescent cells into the S phase.[13] Given that cancerous cells often exhibit a dysfunctional RB and uncontrolled cell cycle, deletion of the CR2 region allows the mutant adenovirus to replicate selectively in tumour cells.[14,15]

Several adenovirus mutants have been generated to maximize virus selectivity and efficacy, including mutant dl1520 (ONYX-015/CI-1042), mutant dl 922–947 and mutant AdA-24. Mutant dl1520 (ONYX-015/CI-1042) was the first engineered replication-selective virus used in humans and has been demonstrated to be an anti-cancer agent in pre-clinical studies and clinical trials.[16] Various studies have shown that arming oncolytic adenovirus with immune modulatory cytokines, such as IL-2 and IL-12, induce enhanced antitumour activity. Expression of the genes associated with the pre-cited interleukins induces the production of cytokines that activate the type-1 immune response, thus resulting in the regression of the development

of malignant tumours.[17] Huang et al. demonstrated that the co-expression of IL-12 and 4-1BBL via an oncolytic adenovirus significantly enhanced the antitumour and anti-metastatic effects compared with the expression of the individual genes coding for either cytokine in a preclinical study.[18] Moreover, their study revealed a synergistic enhancement in interferon (IFN)-γ levels in mice treated with adenovirus simultaneously expressing IL-12 and 4-1BBL. The latter study also reported that the increased antitumour immune response is potentially mediated via the enhanced cytolytic activity of cytotoxic T lymphocytes (CTLs) and IFN-γ-releasing immune cells. Another study investigated the antitumoural activity of a recombinant adenovirus expressing murine IL-12 (AdmIL-12) in a murine model of prostate cancer and revealed that the tumour growth was reduced by greater than 50%, whereas the survival time increased from 23.4 to 28.9 days.[19]

Herpes simplex virus (HSV)

HSV is an enveloped double-stranded DNA virus belonging to the family of *Herpesviridae*.[20] The virus infects human cells by fusion of the viral envelope proteins with the host cell plasma membrane. There are two types of HSV: HSV1 and HSV2. In the case of HSV-1, three major gene regions, alpha, beta, and gamma, are present in the genome and act simultaneously to regulate the entry, replication and spreading of the virus in host cells.[3]

To allow a replication of HSV in malignant tumour cells specifically, a variety of mutants, such as R3616 and G207, have been designed by inactivating the function of the viral genes that encode for ribonucleotide reductase, thymidine kinase and infected cell protein 34.5 (ICP34.5).[21–23] These mutants can replicate preferentially in proliferating cancer cells that exhibit a high endogenous ribonucleotide reductase activity. Phase I clinical trials using the latter mutants have revealed an effective therapy in the treatment of malignant brain tumours.[21]

The advantage of using oncolytic HSVs as agents for localized IL-12 delivery, expression, and immunomodulatory activity relies on the fact that they are tumour specific and trigger a limited antitumour adaptive immune response.[24] Tooba et al. reported the efficiency of G47Δ-mIL12, a genetically engineered IL-12-expressing oncolytic HSV, in the treatment of glioblastoma, an aggressive and fatal adult brain tumour. Using a murine glioblastoma stem cell (GSC) model they demonstrated that G47Δ-mIL12 infects and replicates similar to its unarmed oncolytic HSV counterpart *in vitro*, whereas G47Δ-mIL12 significantly enhances survival of mice with intracerebral 005 tumours *in vivo*. The study also revealed that in addition to targeting GSCs, G47Δ-mIL12 increases IFN-γ release, hinders angiogenesis by inducing IP-10 protein, and decreases the number of regulatory T cells in the tumour. G47 armed with immunomodulatory molecules, such as IL-12, significantly increases the efficacy of oncolytic HSV-1 therapy in glioma treatment.[24]

Newcastle disease virus (NDV)

NDV, also known as avian paramyxovirus-1 (APMV-1) belongs to the family of *Paramyxoviridae*. The genome is a single-stranded negative-sense RNA that is15 kb long and contains six genes encoding at least eight proteins, including six structural (NP, P, M, F, HN, L) and two non-structural (V and W) proteins.[25] NDV accesses the host by first binding to the cells via the HN protein and acid-containing host cell receptors. Further fusion of the viral and cell-surface membranes occurs followed by a release and replication of the viral RNA into the cytoplasm.[26]

NDV possesses various properties that make it an excellent anti-tumour agent, including good cell binding, entrance into the cell cytoplasm via receptor-mediated endocytosis, replication in tumour cell cytoplasm selectively and independently of cell proliferation, proven safety with no serious side effects, and possibility of used as an adjuvant.[27–29]

The oncolytic properties of NDV have been studied both in animal models and humans. Partial to complete regression of tumours was observed in both cases, even in patients with advanced tumours who were unresponsive to standard therapy.[30] The occurrence of acute or chronic side effects was negligible.[30,31] In patients suffering from breast or ovarian cancer, an enhanced survival rate was also noticed.[32]

Various studies have reported the oncolytic activity of IL-12-expressing NDVs.[33–37] Ren and his colleagues investigated and compared the antitumoural effect of genetically engineered NDV strains expressing both IL-12 and/or IL-2 (rClone30—interleukin-2, rClone30—interleukin-12, and rClone30—interleukin-12—interleukin-2).[38] Their study revealed that rClone30—interleukin-12—interleukin-2 was more efficiently inhibited hepatoma in mice compared with either rClone30—interleukin-12 or rClone30—interleukin-2 alone. Another study investigated the immunomodulatory activity of low titres of NDV AF2240 on human peripheral blood mononuclear cells (PBMC).[38]

Semliki forest virus (SFV)

SFV is an alphavirus belonging to the family of *Togaviridae*. SFV contains a single positive-strand RNA genome that replicates in the cytoplasm of infected cells.[39] The genome encodes only nine functional proteins, including four non-structural proteins that are involved in viral RNA synthesis, four structural proteins that form the capsid (the C protein) and the envelope (the E1, E2 and E3 proteins) and a small 6-kDa protein encoded by the structural region of the genome that is not incorporated into virions.[40,41] As with many other viruses, the pathogenic characteristics of SFV have been manipulated to treat diseases rather than cause them.[41] Interestingly, SFV is considered an efficient vector system to deliver and express transgenes due to its ability to replicate and grow to a high titre in cultured cells, such as baby hamster kidney (BHK) cells and chick embryo cells.[41]

Various studies have been performed to screen the oncolytic properties of SFV.[42–44] VA7-SFV, which encodes the enhanced green fluorescent protein gene (EGFP), is novel virotherapy candidate against unresectable osteosarcoma, an aggressive malignant tumour primarily noted in children and young adults that involves a proliferation of malignant mesenchymal cells producing immature bone or osteoid.[42] Their study demonstrated a profound regression in tumour size in mice treated with the vector.[42] Using a poorly

immunogenic MC38 colon adenocarcinoma model to evaluate the therapeutic potential of SFV vectors, Rodriguez-Madoz et al. demonstrated that a single intratumoural injection of two IL-12-expressing SFV vectors (SFV-IL-12 or SFV-enh-IL-12) induced greater than 80% complete tumour regression with long-term tumour-free survival.[45] However, lower doses of SFV-enhIL-12 were more effective than SFV-IL-12 in inducing antitumoural responses, demonstrating a positive correlation between the IL-12 expression level and the therapeutic effect.[45] Moreover, repeated intratumoural injections of suboptimal doses of SFV-enhIL-12 increased the antitumoural response.[45] Quetglas et al. also reported that SFV-IL-12 is effective in the treatment of liver cancer in a murine study, but the efficiency is dependent on a long-term immune response.[46]

Vesicular stomatitis virus (VSV)

VSVs are members of the family *Rhabdoviridae* and the prototypes of the *Vesiculovirus* genus. VSVs are bullet shaped with a genomic structure involving a single strand negative-sense RNA composed of five genes (N, P, M, G, and L) representing the nucleocapsid protein, phosphoprotein, matrix protein, glycoprotein, and the large protein, which is a component of the viral RNA polymerase, respectively.[47] The oncolytic activity of VSV toward various cancers, such as glioma, hepatocellular carcinoma, breast carcinoma, melanoma and lung cancer, has been demonstrated in several preclinical tumour models.[48–50] The oncolytic strategy of VSV is primarily based on a defect in the type I IFN response of numerous malignant tissues.[51,52] A disadvantage of the clinical application of VSV is associated with the potential for neurotoxicity.[53,54] This deleterious effect has been observed in many preclinical studies. However, to overcome this issue, strategies involving IL-12 combinational therapy are beneficial from a safety prospective. Derek et al., reported that IL-12 is essential to recover from VSV infection of the murine central nervous system (CNS).[55] This antiviral effect is mainly mediated by the induction of the neuronal isoform of nitric oxide synthase (NOS-1) and is independent of the proinflammatory cytokines IFN-γ and TNF-α.[55]

Shin et al. demonstrated that recombinant VSV vectors incorporating genes coding for viral fusion protein (rVSV-F) and interleukin 12 (rVSV-IL12) have significant antitumour effects against squamous cell carcinoma (SCC) in an orthotopic murine model.[51] Cultured human and murine SCC cells are good platforms for efficient replication of rVSV-F, whereas normal human and mouse keratinocytes were not suitable for the vector.[51] Injection of multiple doses of the vectors in a single SCC nodule in mice revealed that recombinants significantly contribute to the regression of the tumour and a prolongation of the animal survival.[51,52] Using an orthotopic murine SCC model, Shung et al. assessed the antitumoural activity of a combination of rVSV-F or rVSV-IL12 with systemic cisplatin (a chemotherapy agent), The combination of cisplatin with either VSV type has a positive effect on the elimination of tumour cells *in vitro*.[56] *In vivo*, the addition of cisplatin increased the antitumoural effect of rVSV-F but hindered the activity of rVSV-IL12.[56]

Sindbis virus (SINV)

Similar to SFV, SINV is a virus that belongs to the *Togaviridae* family.[57] SINV is an alphavirus with a genome composed of a positive single-stranded RNA of approximately 12 kb that encodes four non-structural and two envelope proteins.[58] SINV is transmitted by mosquitoes and is not typically responsible for severe disease in humans.[59] Thus, SINV serves as a potential platform for investigations of cancer treatment through gene therapy due to the absence of pre-existing neutralizing antibodies. Another factor that makes SINV a suitable oncolytic agent is related to the fact that its lifecycle does not involve a DNA phase, thereby eliminating any risk of genomic integration.[60] The mechanism by which SINV targets tumour cells is thought to involve interactions with the high-affinity laminin receptor, a receptor for SINV in mammalian cells.[61] This receptor is overexpressed in many cancers and makes tumour cells easy targets for the virus.[61] Another advantage of using SINV in the treatment of cancer is associated with the fact that the virus is a blood-borne pathogen and can be administered steadily to target metastatic tumours.[60]

The oncolytic properties of SINV have been demonstrated using various cells line and animal models. Using BHK tumours in SCID/beige mice, it was demonstrated that injections of a SinRep/LacZ vector decreased the size of the tumour significantly in mice.[60] Moreover, the same study revealed that the efficiency of the vector in destroying tumour cells was enhanced in the presence of NK cells. In addition, a therapeutic effect of administering IL-12-expressing SINV vectors has been demonstrated for the treatment of human ovarian tumours in SCID mice.[60] In addition, Sin/IL12, an IL-12-carrying SINV vector, has a beneficial effect on the regression of malignant tumours in mice. This vector results in an increase in the survival time, which is primarily due to IFN-γ-induced NK cell recruitment.[60]

Conclusion

A revolution in molecular and cancer biology has occurred in recent years that has allowed a better understanding of the interactions between viruses and host cells. This new understanding has also provided an impetus for the development of cancer treatment by gene therapy that involves the delivery of specific genes by OVs to efficiently target tumour cells. Numerous viruses have been investigated for their anti-cancer properties and appear to be promising in different phases of clinical trials in humans. Unlike the systemic administration of recombinant IL-12, IL-12-expressing OVs exhibited no IL-12-associated organ toxicity. Extensive animal studies on the use of IL-12-expressing OVs for cancer treatment have clearly demonstrated a significant antitumour effect of such viruses that led to partial or complete regression of cancer concomitant with an increased survival rate in murine tumour models. These studies have suggested a model of action wherein IL-12-expressing OV has multimodal effects: (a) directly on immune cells, such as cytotoxic T cells, NK cells, and DCs, and (b) indirectly through IP-10 chemokine induction, which robustly interferes with tumour angiogenesis (Figure 1). These studies have clearly demonstrated multiple mechanisms by which IL-12-expressing OVs mediates

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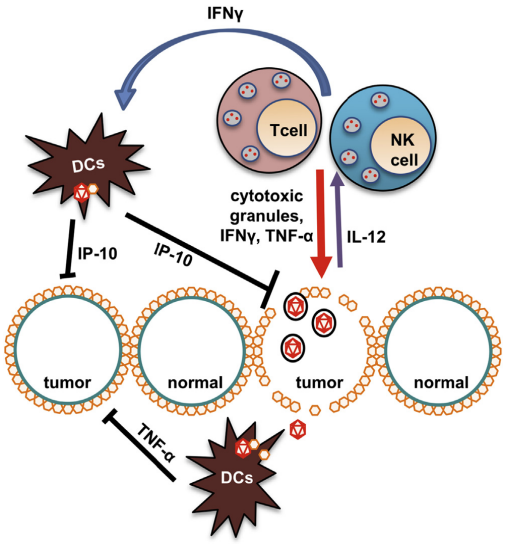

Figure 1: Interleukin-12-expressing OVs (OV-IL12) exert multimodal antitumoural activity. OVIL12 infects and spreads within tumour cells, selectively lysing tumour cells and sparing normal cells. During the viral replication, OV-IL12 releases IL-12 protein, leading to a direct and indirect cascade of events that modulate the tumour microenvironment. Direct induction typically occurs in all immune cells that express IL-12 receptors are noted on the cell surface of a variety of cells, including NK cells, cytotoxic T cells (mainly CD8 T cells) and DCs. These three subsets play major roles in either tumour cell lysis (NK and T cells) or antigen processing and presentation (DCs), which further supports the development of long-term T cell memory. An indirect impact on the tumour milieu is mainly regulated by IFNγ-induced mechanisms that act via several methods on both host and tumour cells to enhance tumour regression. Effects on the host include the upregulation of IP-10 chemokine that has a robust antiangiogenic effect on tumour cells. All together, these multifaceted antineoplastic advantages of OV-IL12 viruses induce tumour regression and ultimately tumour eradication.

tumour protection in several pre-clinical models. Therefore, an ideal IL-12-expressing OV candidate should have the potential to utilize all of the mechanisms that have been illustrated. Therefore, the research focus should be geared toward the investigation of more combinative therapies, including immunomodulatory agents, chemotherapy and radiotherapy, to achieve the optimal benefit.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

AAA and ABM contributed equally to this review.

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Appendix II: Preventing postoperative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells.

Tai LH, de Souza CT, Bélanger S, Ly L, **Alkayyal AA**, Zhang J, Rintoul JL, Ananth AA, Lam T, Breitbach CJ, Falls TJ, Kirn DH, Bell JC, Makrigiannis AP, Auer RA.

Cancer Res. 2013 Jan 1;73(1):97-107. Epub 2012 Oct22.

Contribution of author: Alkayyal AA performed experiments in figure 4a and 4b and had helped in developing methodology, Analysis and interpretation of data

Appendix III: Perioperative influenza vaccination reduces postoperative metastatic disease by reversing surgery-induced dysfunction in natural killer cells.

Tai LH, Zhang J, Scott KJ, de Souza CT, **Alkayyal AA**, Ananth AA, Sahi S, Adair RA, Mahmoud AB, Sad S, Bell JC, Makrigiannis AP, Melcher AA, Auer RC.

Clin Cancer Res. 2013 Sep 15;19(18):5104-15. Epub 2013 Jul 23.

Contribution of author: Alkayyal AA performed experiments in figure 4a and had helped in spleens and lung processing, flowcytometry, developing methodology, Analysis and interpretation of data.

Appendix IV: A mouse tumor model of surgical stress to explore the mechanisms of postoperative immunosuppression and evaluate novel perioperative immunotherapies.

Tai LH, Tanese de Souza C, Sahi S, Zhang J, **Alkayyal AA**, Ananth AA, Auer RA.

J Vis Exp. 2014 Mar 12;(85). doi: 10.3791/51253.

Contribution of author: Alkayyal AA had helped in developing the 4T1 orthotopic breast cancer model, Analysis and interpretation of data.

Appendix V: Maraba MG1 virus enhances natural killer cell function via conventional dendritic cells to reduce postoperative metastatic disease.

Zhang J, Tai LH, Ilkow CS, **Alkayyal AA**, Ananth AA, de Souza CT, Wang J, Sahi S, Ly L, Lefebvre C, Falls TJ, Stephenson KB, Mahmoud AB, Makrigiannis AP, Lichty BD, Bell JC, Stojdl DF, Auer RC.

Mol Ther. 2014 Jul;22(7):1320-32. Epub 2014 Apr 3.

Contribution of author: Alkayyal AA performed experiments in figure 4e, figure 4f, X-gal staining for the lungs in figure 5d, figure 6b and figure 6c, and had helped in spleens and lung processing, flowcytometry, developing methodology, Analysis and interpretation of data.

Appendix VI: Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity.

Ilkow CS, Marguerie M, Batenchuk C, Mayer J, Ben Neriah D, Cousineau S, Falls T, Jennings VA, Boileau M, Bellamy D, Bastin D, de Souza CT, **Alkayyal AA**, Zhang J, Le Boeuf F, Arulanandam R, Stubbert L, Sampath P, Thorne SH, Paramanthan P, Chatterjee A, Strieter RM, Burdick M, Addison CL, Stojdl DF, Atkins HL, Auer RC, Diallo JS, Lichty BD, Bell JC.

Nat Med. 2015 May;21(5):530-6. Epub 2015 Apr 20.

Contribution of author: Alkayyal AA engineered, rescued and manufactured MG1-FGF2 virus that was used in figure 4.

Appendix VII: Surgical Stress Abrogates Pre-Existing Protective T Cell Mediated Anti-Tumor Immunity Leading to Postoperative Cancer Recurrence.

Ananth AA, Tai LH, Lansdell C, **Alkayyal AA**, Baxter KE, Angka L, Zhang J, Tanese de Souza C, Stephenson KB, Parato K, Bramson JL, Bell JC, Lichty BD, Auer RC.

PLoS One. 2016 May 19;11(5):e0155947. Erratum in: PLoS One. 2016;11(7):e0159471.

Contribution of author: Alkayyal AA had helped in spleens and lung processings, flowcytometry,

Analysis and interpretation of data.

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Curriculum Vitae

Languages

English
Arabic

Employment History

Faculty member candidate Jun 2008 – Present
Faculty of Applied Medical Science, University of Tabuk, Tabuk, KSA.

Clinical lab intern. Jul 2007 - Jun 2008

KAUH, Jeddah, KSA and King Faisal Specialist Hospital and Research Centre, Jeddah, KSA.
Internship Clinical Rotation: Microbiology (6 months), Assisted Reproductive Technology (IVF) (3 months), Virology (3 months).

Education

Ph.D. in Microbiology and Immunology. Jan 2012 – present
Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada. Thesis title "Exploiting Antitumor Immune Response Using IL-12 Armed Oncolytic MH1 Virus in an Infected Cell Vaccine"

Post-Graduate Certificate in Cancer Biology and Therapeutics Program Sep 2016 – present
Harvard Medical School, Harvard University, Boston, USA

Post-Graduate Certificate in Clinical Trials Training Program Jun 2015 – Jun 2016
Harvard Medical School, Harvard University, Boston, USA

M.Sc. in Laboratory Medicine. GPA: 3.8 out of 4 Mar 2010 – Nov 2011
Department of Applied Medical science, Faculty of Science, Royal Melbourne Institute of Technology, Melbourne, VIC Australia

B.Sc. in Medical Laboratory Technology. GPA: 3.9 out of 5 Sep 2003 – Apr 2007
Department of Medical Technology, Faculty of Applied Health Sciences, KAU, Jeddah, KSA.

Skills

Clinical lab skills.
Conduct diagnostic immunological assays including ELISA, Flowcytometery etc…
Isolation and identification of bacteria and parasites from various clinical specimens, performing antibiotic sensitivity tests, and operation of blood culture machines such as Bact Alert.

Performing laboratory diagnostic tests for viral detection and identification.
Preparation of viable sperms for intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI).

Research lab skills

Performing molecular techniques such as metagenomic DNA and RNA extraction, PCR, qPCR and qRT-

PCR.
Immunological assays such as but not limited to flowcytometery, Tumour infiltration lymphocytes (TILs) isolation and purification, lymphocytes expansion and stimulation, Lymphocytes tracking and migration etc…
Cloning, generation of recombinant RNA and DNA viruses, viral manufacturing for vaccine purposes.
Tissue culturing and viral titration and quantification.
Cellular protein extraction and analysis using Immunohistochemistry (IHC) and ELISA, surface protein localization assays such as immunoflorescence.
Animal handling and animal studies.

Awards

Harvard Medical School (DHFMR) scholarship, Harvard Universit 2016
A scholarship to cover the tuition fees of Cancer Biology & Therapeutics Program

Terrey Fox Research Institute Travel Award 2016
A travel award to cover travel and hotel expenses during Terrey Fox Annual meeting, Vancouver, BC, Canada.

Harvard Medical School (DHFMR) scholarship, Harvard University 2015
A scholarship to cover the tuition fees of GCSRT Clinical Trials training program

Terrey Fox Research Institute Travel Award 2014
A travel award to cover travel and hotel expenses during Terrey Fox Annual meeting, Montreal, QC, Canada.

University of Tabuk graduate studies scholarship recipient. 2012-2016
A granted full scholarship to pursue Ph.D studies in North America.

University of Tabuk graduate studies scholarship recipient. 2009-2011
A granted full scholarship to pursue M.Sc. studies in Australia.

Publications and Patents

1. **Alkayyal, A. A,** John C. Bell, and Rebecca C. Auer. Oncolytic Rhabdovirus Expressing IL12 (PAT 103736P-2**).**
2. **Alkayyal, A. A.**, Tai, L. H., Zhang, J., de Souza, C. T., Charles Lefebvre, Ananth, A. A., John C. Bell, David F. Stojdl and Rebecca C. Auer. IL-12 Mediated NK Cell Recruitment Improves Anti-tumour Efficacy in an Oncolytic Infected Cell Vaccine. (Under review in **Cancer Immunology Research** Jul 2016.).
3. Tai, L. H., **Alkayyal, A. A.**, Tanese de Souza, C., Sahi, S., John C. Bell, David F. Stojdl and Rebecca C. Auer. Phosphodiesterase-5 inhibition reduces postoperative metastatic disease by targeting surgery-induced myeloid derived suppressor cells. (Manuscript under preparation).
4. **Alkayyal AA**, Mahmoud AB, Auer RC. Interleukin-12-expressing oncolytic virus: A promising strategy for cancer immunotherapy. **Journal of Taibah University Medical Sciences**. 2016;11(3):187-93.
5. Ananth AA, Tai LH, Lansdell C, **Alkayyal AA**, Baxter KE, Angka L, et al. Surgical Stress Abrogates Pre-Existing Protective T Cell Mediated Anti-Tumor Immunity Leading to Postoperative Cancer Recurrence. **PLoS One**. 2016;11(5):e0155947.
6. Carolina S. Ilkow, Monique Marguerie, Cory Batenchuk, Daniela Ben Neriah, Sophie Cousineau, Theresa Falls, Christiano Tanese de Souza, Fabrice LeBoeuf, Rozanne Arulanandam, Lawton Stubbert, Steve Thorne, Piriya Paramanthan, **Alkayyal, A. A.**, Avijit

Chatterjee, Christina Addison, David Stojdl, Jean-Simon Diallo, Brian Lichty, and John C. Bell. Reciprocal cellular cross- talk within the tumor microenvironment promotes oncolytic virus activity. **Nature Medicine**. 2015;21(5):530-6. (Impact factor 28.054)

7. Zhang, J., Tai, L. H., Ilkow, C. S., **Alkayyal, A. A.**, Ananth, A. A., de Souza, C. T., et al. (2014). Maraba MG1 virus enhances natural killer cell function via conventional dendritic cells to reduce postoperative metastatic disease. **Molecular Therapy**, 22(7), 1320-1332. (Impact factor 7.825)

8. Tai, L. H., Tanese de Souza, C., Sahi, S., Zhang, J., **Alkayyal, A. A.**, Ananth, A. A., et al. (2014). A mouse tumor model of surgical stress to explore the mechanisms of postoperative immunosuppression and evaluate novel perioperative immunotherapies. ***J Vis Exp***(85). (Impact factor 1.19)

9. Tai, L. H., Zhang, J., Scott, K. J., de Souza, C. T., **Alkayyal, A. A.**, Ananth, A. A., et al. (2013). Perioperative influenza vaccination reduces postoperative metastatic disease by reversing surgery-induced dysfunction in natural killer cells. **Clinical Cancer Research**, *19*(18), 5104-5115. (Impact factor 8.2)

10. Tai, L. H., de Souza, C. T., Belanger, S., Ly, L., **Alkayyal, A. A.**, Zhang, J., et al. (2013). Preventing postoperative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. **Cancer Research**, *73*(1), 97-107. (Impact factor 9.2).