

**Rhabdovirotherapy reduces the risk of metastatic disease after cancer  
surgery by enhancing natural killer cell function**

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## Abstract

In the present study, we characterized the ability of a novel oncolytic rhabdovirus - Maraba MG1 to boost Natural Killer (NK) cell activity. In tandem, we addressed the ability of this enhanced NK cell functionality to reduce the incidence of post-cancer surgery micrometastases. Due to the potential safety barriers associated with the use of a live virus immediately prior to surgery in cancer patients, we generated a single cycle replication virus (MG1-Gless) and UV-inactivated MG1 to stimulate NK cell function and reduce post-operative metastases. Our *in vivo* data demonstrate that significant NK cell activation and a similar level of reduction in postoperative tumor metastases was achieved with live MG1, MG1-Gless and UV-inactivated MG1, concluding that viral replication is important, but not necessary for NK cell activation. Mechanistically, we observed that dendritic cells (DCs) are necessary intermediates for MG1-induced NK cell activation. Finally, we characterized and compared a panel of UV-inactivated MG1 (2mins to 2hrs) to better understand the requirements for NK cell activation. Our results suggest that intact viral particle and cellular recognition and association are essential for NK cell mediated anti-tumor responses. These findings provide the preclinical rationale to develop safe and viable virotherapy-based interventional protocols that might reduce the risk of metastatic disease after cancer surgery.

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## **List of Abbreviations**

|                               |                                                  |
|-------------------------------|--------------------------------------------------|
| Ad                            | adenovirus                                       |
| APCs                          | antigen presenting cells                         |
| ATCC                          | American Type Cell Culture                       |
| B6                            | C57BL/6                                          |
| cAMP                          | cyclic adenosine monophosphate                   |
| CAs                           | catecholamines                                   |
| CCL2                          | C-C motif ligand 2                               |
| cDCs                          | conventional dendritic cells                     |
| CMI                           | cell-mediated immunity                           |
| CPE                           | cytopathic effects                               |
| CRC                           | colorectal cancer                                |
| CTLs                          | cytotoxic T lymphocytes                          |
| DCs                           | dendritic cells                                  |
| DMEM                          | Dulbecco's Modified Eagles Medium                |
| DTR                           | diphtheria toxin receptor                        |
| EM                            | Electron Microscopy                              |
| FBS                           | fetal bovine serum                               |
| GBM                           | glioblastoma multiforme                          |
| GFP                           | Green fluorescent protein                        |
| GM-CSF                        | granulocyte macrophage colony-stimulating factor |
| H&E                           | Hematoxylin & Eosin                              |
| H <sub>2</sub> O <sub>2</sub> | hydrogen peroxide                                |

|               |                                                |
|---------------|------------------------------------------------|
| HPA           | hypothalamo-pituitary-adrenal                  |
| HPV           | human papillomavirus                           |
| HSV           | herpes simplex virus                           |
| IFN- $\alpha$ | interferon- $\alpha$                           |
| IHC           | Immunohistochemistry                           |
| IL-1 $\beta$  | interleukin-1 $\beta$                          |
| IP-10         | Interferon gamma-induced protein 10            |
| IRAK4         | IL-1R-associated kinase 4                      |
| IRF           | interferon regulatory factor                   |
| ITIM          | immunoreceptor tyrosine-based inhibitory motif |
| ITAM          | Immunoreceptor tyrosine-based activation motif |
| i.v.          | intravenous                                    |
| L             | large protein                                  |
| M             | matrix protein                                 |
| mAbs          | monoclonal antibodies                          |
| MCMV          | murine cytomegalovirus                         |
| MG1           | Maraba MG1                                     |
| MHC           | major histocompatibility complex               |
| MIP-1,2       | Macrophage inflammatory protein-1,2            |
| MICA/B        | MHC-I related chain A and B                    |
| MMPs          | matrix metalloproteinases                      |
| MOI           | multiplicity of infection                      |
| MV            | Measles virus                                  |

|                             |                                                  |
|-----------------------------|--------------------------------------------------|
| MyD88                       | Myeloid differentiation primary response gene 88 |
| N                           | nucleoprotein                                    |
| NDV                         | Newcastle disease virus                          |
| NK                          | natural killer                                   |
| NKG2D                       | NK group 2, member D                             |
| NLR                         | NOD-like receptor                                |
| NO                          | nitric oxide                                     |
| NOD                         | nucleotide oligomerization domain                |
| O <sub>2</sub> <sup>-</sup> | superoxide anion                                 |
| OR                          | Odds ratios                                      |
| ORFV                        | Parapoxvirus Ovis                                |
| OV                          | oncolytic virus                                  |
| P                           | phosphoprotein                                   |
| PAMPs                       | pathogen-associated molecular patterns           |
| PBS                         | phosphate buffered saline                        |
| PCR                         | polymerase chain reaction                        |
| pDCs                        | plasmacytoid dendritic cells                     |
| PFS                         | progression free survival                        |
| PFU                         | plaque forming unit                              |
| PGs                         | Prostaglandins                                   |
| p.i.                        | post infection                                   |
| PI 3-kinase                 | phosphatidylinositol-3-kinase                    |
| PKR                         | protein kinase R                                 |

|               |                                                            |
|---------------|------------------------------------------------------------|
| PRR           | pattern recognition receptors                              |
| qRT-PCR       | quantitative reverse transcription-PCR                     |
| RAV           | rabies virus                                               |
| RIG- I        | retinoic acid-inducible gene I                             |
| RLR           | RIG- I-like receptor                                       |
| RNP           | ribonucleoprotein                                          |
| RPMI          | Roswell Park Memorial Institutes Media                     |
| RSV           | respiratory syncytial virus                                |
| SCID          | severe combined immunodeficiency                           |
| SD            | standard deviation                                         |
| SDS-PAGE      | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SHP-1         | Src homology phosphatase-1                                 |
| SNP           | single nucleotide polymorphism                             |
| ssRNA         | single strand RNA                                          |
| SV            | Sindbis virus                                              |
| T             | Thymine                                                    |
| TAA           | tumor associated antigens                                  |
| Tg            | transgenic                                                 |
| TGF $\beta$   | transforming growth factor $\beta$                         |
| Th            | helper T                                                   |
| TLR           | Toll-like receptors                                        |
| TNF- $\alpha$ | tumor necrosis factor- $\alpha$                            |
| TRAF6         | TNFR-associated factor 6                                   |

|       |                                                         |
|-------|---------------------------------------------------------|
| Trail | tumor necrosis factor-related apoptosis-inducing ligand |
| VSV   | vesicular stomatitis virus                              |
| U     | uracil                                                  |
| UV    | ultraviolet                                             |
| VSV   | Vesicular stomatitis virus                              |
| VV    | vaccinia virus                                          |
| WT    | wild type                                               |

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## **Chapter 1: Introduction**

### **1.1 Immune system**

#### **1.1.1 General overview**

The immune system can seek and destroy the pathogens (e.g. virus, bacteria, fungi, etc.) and nonliving substances (e.g. chemicals, toxins, etc.) around us to defend the body. The host defense mechanisms are made up of two general systems of immunity - innate immunity and adaptive immunity. Innate immunity, also called native immunity or natural immunity, is always present in the organism, can recognize general classes of pathogens and remove them immediately (Murphy et al., 2008). Innate immunity can't generate long-lasting immunological memories, but it is the critical first line of defense against foreign invaders. If pathogens overcome innate immunity, the adaptive immune response will follow with the generation of antigen-specific effector cells that destroy specific pathogens and development of long-term immunological memory (memory T cells and antibody) (Murphy et al., 2008). Innate immunity exists in all multicellular organisms, while adaptive immunity is only found in vertebrates (Thompson et al., 1995). For pathogen recognition, innate immune cells express pattern recognition receptors (PRR), which can detect repeating patterns of molecular structure on the surface of microorganisms, known as pathogen-associated molecular patterns (PAMPs). For example, three main families of PRRs, such as Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), can recognize viral PAMPs such as viral envelope proteins and genomes (Akira et al., 2006; O'Neill et al., 2007; Takeuchi et al., 2007). In adaptive immunity, B cells can detect specific antigens such as proteins, polysaccharides,



lipids, etc. via membrane bound antibodies (Fc receptors), while T cells receptors can only recognize small peptides displayed by major histocompatibility complex (MHC) molecules on the surfaces of antigen presenting cells (APCs) to eliminate the pathogens (Murphy et al., 2008).

### **1.1.2 The components of innate immunity**

Innate immunity consists of epithelial barriers, phagocytic leukocytes (e.g. macrophages, neutrophils), DCs, NK cells and circulating plasma proteins.

#### **1.1.2.1 Epithelial barriers**

The epithelium of the skin, gastrointestinal tract and respiratory tract as physical barriers constitute the first line of defense against pathogens. Tight junctions hold epithelial cells together to protect against infection from the outside environment (Yu et al., 2009). Mucosal epithelia can secrete mucus to prevent pathogens from attaching to the epithelium. In addition, surface epithelial can produce anti-microbial chemicals. For instance, lysozyme and phospholipase A in the tears and saliva have antibacterial properties (Lemansky et al., 2001). Low pH stomach acid and digestive enzymes can kill many ingested microbes. Furthermore, commensal bacteria can attach to epithelial cells and compete with pathogens, and it also secrete antimicrobial substances (Albrecht et al., 1996).

#### **1.1.2.2 Phagocytic leukocytes**

If pathogens cross the epithelial barriers, the phagocytic cells, such as macrophages and neutrophils, will detect and destroy many microorganisms prior to the development of the adaptive immune response (Murphy et al., 2008). PRRs on macrophages and neutrophils will recognize the PAMPs on the pathogen, which leads to phagocytosis and ends with the death of pathogens inside phagocytes. Upon phagocytosis, the phagocytes also produce a variety of toxic products such as nitric oxide (NO), the hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the superoxide anion ( $\text{O}_2^-$ ) that are toxic to engulfed microorganisms (Murphy et al., 2008). In addition, macrophages can secrete cytokines (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , IL-8, IL-12, etc) (Kim et al., 2004; Lucas et al., 2003; Chung et al., 2006) and chemokines (Interferon gamma-induced protein 10 (IP-10), Macrophage inflammatory protein-1,2 (MIP-1,2) etc.) (Kopydlowski et al., 1999) to stimulate inflammation and lymphocyte response. Also, macrophages as antigen-presenting cells form a link between innate and adaptive immunity (Murphy et al., 2008).

### **1.1.2.3 DCs**

DCs are also phagocytic cells which can engulf pathogens that break the epithelial barriers and secrete cytokines (e.g. IFN- $\alpha$ , IL-12, etc.) to stimulate other immune cells such as NK cells, macrophages, etc. (Murphy et al., 2008; Koyama et al., 2008). More importantly, DCs are specialized APCs, which can capture the antigens and present antigens to T cells, thereby initiating the adaptive immune response (Murphy et al., 2008). Therefore, DCs are important innate immune cells to bridge the innate immunity with the adaptive immunity. Based on the origin, localization and receptor expression, DCs can be

commonly divided into two types: plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs).

pDCs mainly accumulate in the blood and lymphoid tissues and are capable of producing massive amounts of type I IFN to respond to virus infection (Koyama et al., 2008; Merad et al. 2013). In human, pDCs are defined based on their expression of CD4, CD123, and BDCA2, while pDCs express CD11c, B220, mPDCA-1, and Ly6G/C in mouse (Colonna et al., 2004). pDCs play an important role in antiviral immune response because they are expert cells that produce type I IFNs upon recognition of viral nucleic acids. pDCs have high expression of TLR7 and TLR9, instead of TLR2, TLR3, TLR4 or TLR5 (Gilliet et al., 2008; Kawai et al., 2006). TLR7 has been shown to be crucial in the detection of the single strand RNA (ssRNA) viruses such as vesicular stomatitis virus (VSV), influenza virus, Sendai virus, and respiratory syncytial virus (RSV) for type I IFN production in pDCs (Gilliet et al., 2008). Similarly, TLR9 is critical for the recognition of DNA virus such as herpes simplex virus 1 (HSV1) and murine cytomegalovirus (MCMV) (Kadowaki et al., 2001; Asselin-Paturel et al., 2005; Villadangos et al., 2008). Production of type I IFNs by pDCs is strictly dependent on the TLR-MyD88 (Myeloid differentiation primary response gene 88) pathway because pDCs from MyD88<sup>-/-</sup> mice fail to produce type I IFNs in response to TLR7 or TLR9 ligands (Colonna et al., 2004). On stimulation, MyD88, IL-1R-associated kinase 4 (IRAK4), IRAK1 and TNFR-associated factor 6 (TRAF6) form a complex at the TLR, but this complex also needs interferon regulatory factor (IRF) family, in the case of pDCs, IRF7 translocates to the nucleus and starts type I IFN gene transcription (Gilliet et al., 2008; Kawai et al., 2006). After activation, pDCs increase the expression of co-stimulatory markers such as MHC molecules, CD80 and

CD86, but the expression level is much less than that of cDCs. Although pDCs are capable of presenting antigen for T cell activation, they are less efficient than cDCs (Villadangos et al., 2008). So pDCs are a unique subset of DCs against viral challenge.

cDCs are found both in lymphoid and peripheral tissues with several subtypes. The subset of cDCs that are  $CD11c^{+}CD8^{+}CD4^{-}CD205^{+}CD11b^{-}$  is the dominant cDC subtype in the thymus, also seen in the lymph nodes and spleen (Andoniou et al., 2005; Wu et al., 2007). The predominant subpopulations of cDCs in the lymph nodes are those that are characterized as  $CD11c^{+}CD8^{-}CD4^{-}CD205^{+/-}CD11b^{+}$ . There are also two subsets of cDCs that are  $CD11c^{+}CD8^{-}CD4^{+/-}CD205^{-}CD11b^{+}$  found in the spleen (Andoniou et al., 2005; Wu et al., 2007). Skin “Langerhans” DCs are characterized as  $CD11c^{+}CD4^{-}CD8^{+}CD205^{+}CD11b^{+}$  (Andoniou et al., 2005; Wu et al., 2007). Different cDCs TLRs include TLR1, TLR2, TLR3, TLR4, TLR5 and TLR8 (Iwasaki et al., 2004), which can detect a wide range of pathogens, and these TLR signals will activate cDCs to produce inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-15, IL-18, type I IFNs,  $TNF\alpha$ , and transforming growth factor  $\beta$  ( $TGF\beta$ ), which help activate other immune cells such as NK cells, T cells, and B cells to promote immune response against invading pathogens (Gilliet et al., 2008; Wu et al., 2007; Gilliet et al., 2002; Kuwajima et al., 2006). For example, cDCs secreted IL-12 and IL-18 is necessary for  $IFN\gamma$  production by NK cells and subsequent viral elimination (Jinushi et al., 2003). cDC derived IL-15 is required to modulate MHC-I related chain A and B (MICA/B), and the ligands for the activating receptor, NKG2D, leading to NK cell activation (Jinushi et al., 2003). In addition, cDCs are professional APCs, which recognize, uptake, process and display the antigens at the surface of cDCs in the context of MHC-I and MHC-II for presentation to  $CD8^{+}T$  cells

and CD4<sup>+</sup>T cells, respectively. Taken together, cDCs are an important subpopulation of the innate immune system and they influence both the innate and adaptive immune cells.

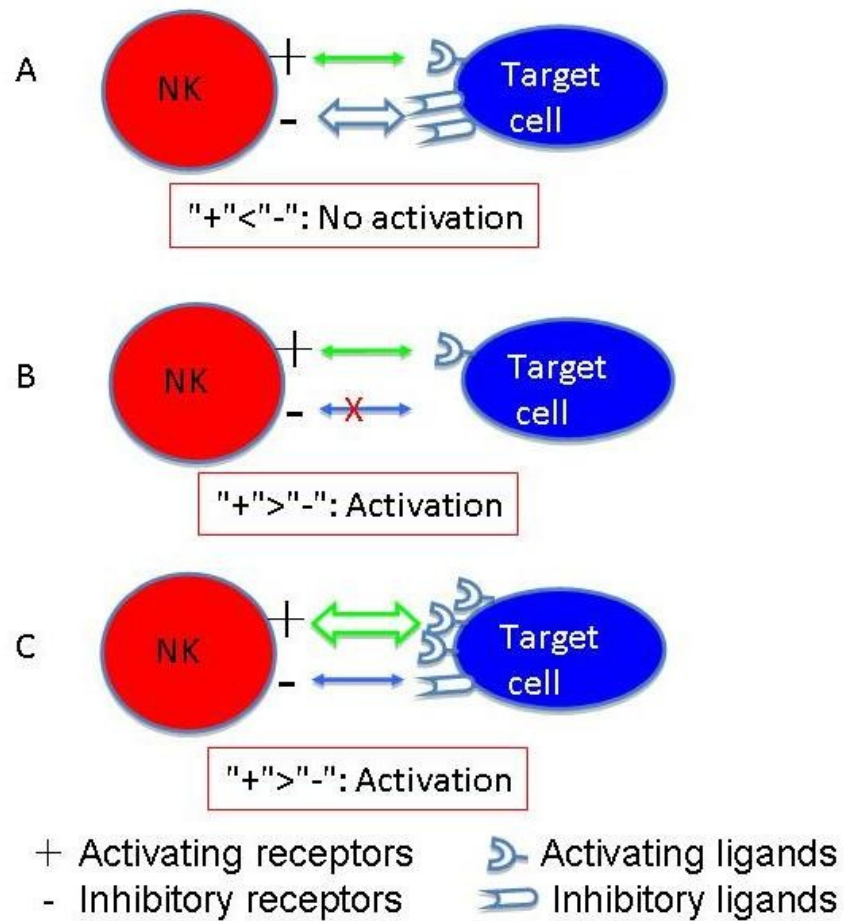
#### **1.1.2.4 Natural Killer cells**

NK cells are large granular, cytotoxic lymphocytes, that are involved in the direct killing of virally infected and malignant cells (Hamerman et al., 2005; Lanier et al., 2005). They originate from the common lymphoid progenitor cell in the bone marrow and are generally considered as an important component of the innate immune system (Eidenschenk et al., 2006). NK cells are able to kill target cells independent of antigen-specificity by increased levels of the cytotoxicity mediators granzyme B and perforin (Lanier et al., 2008). In addition, NK cells can initiate and amplify the inflammatory response and produce chemokines and cytokines such as IL-8, IFN $\gamma$  and granzyme B (Glaner et al., 2010; Vivier et al., 2008).

NK cells express an array of receptors that can either activate NK cell (activating receptors) or inhibit NK cell function (inhibitory receptors). They can recognize the target cells through these activating or inhibitory receptors expressed on the cell surface. NK cells preferentially kill the target cells with low or no expression of MHC-I (Ljunggren et al., 1985). There are three families of inhibitory receptors that recognize MHC-I molecules including KIR (killer cell immunoglobulin-like receptor, humans) (Jamil et al., 2011; Wagtmann et al., 1995), CD94/NKG2A (human and mice) (Carretero et al., 1997; Houchins et al., 1991; Petrie et al., 2008) and Ly49 (mice) (Norman et al., 2000; Yokoyama et al., 1989) receptor family. When an inhibitory receptor binds its ligand, the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of the receptor are

phosphorylated and recruit the Src homology phosphatase-1 (SHP-1) and SHP2, which dephosphorylate the protein substrates of tyrosine kinases related to NK cell activating receptors, resulting in the inhibition of cytotoxicity and cytokine production (Vivier et al., 2008). NK activating receptors include NKG2D (NK group 2, member D) receptor (human and mice), CD16 (human and mice), and the natural cytotoxicity receptors (NCR) such as NKp30 (human), NKp44 (human), and NKp46 (human and mice), etc. (Bauer et al., 1999; Biassoni et al., 2008; Bryceson et al., 2005; Sivori et al., 1997). Upon engagement with ligands (e.g. CD112, CD155), activating receptors recruit and interact with membrane-bound proteins via shared immunoreceptor tyrosine-based activation motifs (ITAMs), such as Fc $\epsilon$ RI- $\gamma$ , DAP12, and CD3- $\xi$ , to form functional signaling complexes associated with ZAP70 and/or syk kinases protein family, which leads to the activation of NK cells (Lanier et al., 2008), while NKG2D binds a different adaptor protein, DAP10, which activates the intracellular lipid kinase phosphatidylinositol-3-kinase (PI 3-kinase), initiating a different pathway to activate NK cells (Murphy et al., 2008). The tolerant or activation status of NK cells is dependent on the balance between stimulatory and inhibitory signals (Figure 1.1). Activated NK cells release perforin to form pores in the target cell membrane to allow granzymes to enter the cell and cause lysis through endonucleases, which is the most powerful way to destroy target cells (Bryceson et al., 2006). In addition, NK cells express TNF family ligands, TNF, CD178 (Fas ligand) and tumor-necrosis factor-related apoptosis-inducing ligand (Trail) to induce cell apoptosis, which is an alternative way for releasing granules (Zamai et al., 1998). Activated NK cells can secrete an important cytokine, IFN- $\gamma$ , which has been shown to increase NK cytotoxicity by sensitizing tumor cells to granule release or death

**Figure 1.1 The regulation of NK cell activation.**



NK cells receptors recognize ligands expressed on the surface of target cells. When signals of inhibition outweigh that of activation, NK cells will be inhibited (A). When signals of activation are greater than that of inhibition, NK cells will be activated (B, C).  
 (Adapted from Vivier et al., 2011)

receptor engaged cytotoxicity (Biron et al., 1999). NK cells also produce other cytokines such as TNF $\alpha$ , IL-3, GM-CSF (granulocyte macrophage colony-stimulating factor) which impact DC, macrophages, and neutrophils (Moretta et al., 2005). NK cells also produce some chemokines, including chemokine (C-C motif) ligand 2-5 (CCL2-5), lymphotactin, and IL-8, which are key to co-localization with other hematopoietic cells such as DC at the site of inflammation (Moretta et al., 2005).

There is an important crosstalk between DCs and NK cells. In response to viral infection, pDCs are expert cells to produce type I IFNs (Asselin-Paturel et al., 2005), but cDCs are also able to produce type I IFNs in response to viral challenges (Diebold et al., 2003; Lopez et al., 2003). Both cDCs and pDCs have been shown to contribute to virus elicited NK cell activation via type I IFN-dependent mechanisms (Gerosa et al., 2002). pDCs were shown to improve NK cell responses to MCMV infection due to IFN $\alpha$  secretion, and depletion of pDCs dampens NK response to MCMV infection (Delale et al., 2005). As mentioned earlier, cDCs secreted cytokines including IL-12, IL-15 and IL-18 are also important for NK cell activation (Jinushi et al., 2003). Some groups have reported that direct contact between DCs and NK cells was required for NK activation (Carbone et al., 1999; Wilson et al., 1999), however, reovirus matured DCs stimulated NK cells through soluble cytokines, independent of direct cell contact (Errington et al., 2008). The interaction between NK and DC is reciprocal. NK cells were reported to directly induce maturation of monocyte-derived DC or lysis of immature but not mature DCs (Gerosa et al., 2002; Piccioli et al., 2002).

NK cells play an important role in tumor control and metastasis. Smyth et al. found NK deficient mice were more prone to develop sarcomas induced by Methylcholanthrene



(Smyth et al., 2001, 2005). In another mouse model, local IFN- $\gamma$  treatment inhibiting the formation of lung metastasis is NK cell dependent (Ksienzyk et al., 2011). The role of NK cells in tumor immunosurveillance is also observed in humans. A prospective cohort study of a Japanese general population in 1986 began to look at cancer incidence (all sites) and death from all causes and their blood NK activities, and a 11-year follow-up survey showed that low NK cell activity is related with a higher cancer risk: both sexes with high and medium NK cytotoxic activity risk was 0.63 (0.43-0.92) and 0.59 (0.40-0.87), respectively, taking the risk of those with low cytotoxicity as reference (Imai et al., 2000). Also, impaired perforin-dependent NK cell cytotoxicity is observed in melanoma patients with metastasis (Jović et al., 2001). In humans, specific NKG2D gene polymorphisms have been associated with susceptibility to cancer (Hayashi et al., 2006). Hayashi et al. identified two major haplotype alleles: LNK1 and HNK1, which were constructed from five or three single nucleotide polymorphisms (SNPs) mostly located in the NKG2D gene region and closely associated with low and high natural cytotoxic activity of individuals. They also found that individuals who are genetically predisposed to have low natural cytotoxic activity had a consequent high risk of cancer development (Hayashi et al., 2006). Moreover, high expression of NKG2D ligand in colorectal cancers was associated with improved patient survival (Hayashi et al., 2006; McGilvray et al., 2009; Watson et al., 2006). The most direct evidence supporting the role for NKG2D in tumor surveillance comes from studies demonstrating that mice treated with NKG2D blocking Ab (Smyth et al., 2005) or genetically deficient in NKG2D (Guerra et al., 2008) have an increased susceptibility to tumor formation. Therefore, functional NK cells are important to prevent the host from the development of tumors and metastasis.

### **1.1.2.5 Circulating plasma proteins**

Many plasma proteins circulating in the blood are involved in the defense against microorganisms. Important among these are the complement proteins, which are activated by and bind to pathogens or antibody, thus complement proteins play a role in innate and adaptive immunity (Murphy et al., 2008). The complement system has three functions in host defense: (1) Microbes coated with complement proteins are opsonized by phagocytes. (2) Some proteolytic fragments of the complement system, C5a and C3a, can recruit neutrophils and monocytes to the site of complement activation to promote inflammation. (3) Complement activation causes the lysis or apoptosis of pathogens (Murphy et al., 2008).

### **1.1.3 The components of the adaptive immunity system**

B cell mediated humoral immunity and T cells mediated immunity are two powerful arms of the adaptive immune system.

B cell mediated adaptive immunity is called humoral immunity, which is good at dealing with extracellular microbes. Naïve B cells can detect antigens such as lipids, proteins, polysaccharides, and small chemicals, which are circulating in the body or attached to the surface of pathogens (Murphy et al., 2008). B cell responses are initiated once it encounters antigens and becomes activated. Next, clonal expansion of this population of antigen specific B cells begins to keep pace with the proliferation of pathogens. Activated B cells then divide and differentiate into plasma cells, which start to produce specific antibodies for the original antigen. Antibodies are able to mark antigens for destruction by phagocytes (like macrophages), neutralize antigens, stimulate

complement and initiate strong innate immune response (Murphy et al., 2008). When antigens were removed from the body, some plasma cells die due to apoptosis, and others will become memory B cells. When these memory B cells meet with the same antigen next time, they will respond much faster and more effectively to control the pathogens and defend the body. The goal of vaccination is to generate such memory B cells. (Murphy et al., 2008)

Dissimilar to B cells, T cells are only able to recognize small peptides presented on MHC molecules. The APCs such as DCs or macrophages will phagocytose the pathogens, then migrate to the lymphoid tissues and present peptide antigens from within vesicles to CD4<sup>+</sup>T cells via MHC II molecules or from within the cytosol to CD8<sup>+</sup>T cells via MHC I molecules (Murphy et al., 2008). Naïve CD4<sup>+</sup>T or CD8<sup>+</sup>T cells can be activated by specific antigens and undergo clonal expansion and differentiate into effector T cells (Murphy et al., 2008). For example, CD8<sup>+</sup> cells will become mature cytotoxic T cells, which directly destroy cells infected by pathogens by secreting enzymes to induce apoptosis. CD4<sup>+</sup> cells will differentiate into four main subsets of helper T (Th) cells: Th1 (CD4<sup>+</sup> T helper1) cells can secrete IFN $\gamma$  and stimulate macrophages and B cell antibody production. Th2 cells provide help for antibody production, especially switching to IgE. Th17 can enhance neutrophil response. CD4 regulatory T cells can suppress T cell response, which are important to prevent immune response from becoming uncontrolled and in preventing autoimmunity (Murphy et al., 2008). Similar to memory B cells, some activated T cells will become memory T cells that can respond to repeated exposure more rapidly and effectively. (Murphy et al., 2008)

#### **1.1.4 Immunity and cancer metastasis**

Metastasis is the dissemination of cancer cells to distant locations from the primary tumor in the body, which is the leading cause of cancer death. The immune system plays a significant role in controlling cancer metastasis. A recent study demonstrated that overexpression of IRF7 in 4T1.2 tumor cells inhibited metastasis to bone via type I IFN signaling pathway in a breast cancer spontaneous metastasis mouse model, and depletion of CD8<sup>+</sup> T cells together with NK cells significantly reduced metastasis-free survival without affecting the growth of primary tumor (Bidwell et al., 2012). In human studies, higher NK activity and the presence of CD8<sup>+</sup> T cell specific to tumor antigen are positive prognostic factors for metastasis free survival (Brittenden et al., 1996; Fujisawa et al., 1997). In addition, metastases happened less frequently if more infiltrated lymphocytes (e.g. NK cell, DC, and CD8<sup>+</sup> T cell) were found in the primary tumor (Dranoff et al., 2002), and tumor infiltrating CD8<sup>+</sup> T cells also predict good prognosis in many cancers, including melanoma (Haanen et al., 2006) and breast cancer (Kim et al., 2013). Studies also found that the recurrence rates were increased in patients with sarcomas, myelomas and melanomas due to the immunosuppressive therapy (Penn I et al., 1993). Furthermore, the clinical course in already immunosuppressed cancer patients is often accelerated, and more metastases occur in these patients (Detry et al., 2000; Martinez et al., 2003). Hence, cancer recurrence and patients' prognosis are highly associated with their functional innate and adaptive immune systems.

#### **1.2 Oncolytic virus (OVs)**

A virus is a small infectious agent that depends on host cells to replicate, and which can infect all kinds of organisms, from animals to plants to bacteria to archaea (Koonin et al., 2006). In relation to cancer, viruses are known as the cause 20% of human cancers such as human papillomavirus (HPV) causing cervical carcinoma and Hepatitis B virus causing hepatocellular carcinoma. Despite the oncogenic properties of viruses, viruses can also be used to treat cancer. These viruses are known as oncolytic viruses (Alemany et al., 2013). The idea of using viruses as anticancer treatment, which has existed well for over a century, is based on the occasional observation of tumor regression following virus infection (Kelly et al., 2007). But the tumor regressions were very rare, incomplete and lasted a few days or months (Alemany R., 2012). With the development of cell culture techniques in the 1950s, the propagation and purification of virus was significantly improved. Hence, the concept and practice of virotherapy started to grow. Many naturally occurring viruses such as Hepatitis virus, Mumps virus, Vaccinia virus and Adenovirus have been applied in clinical trials. However, the results were similar with that obtained 50 years earlier (Alemany R., 2013). Further development of OV was restricted by concerns of safety and efficacy. With the development of molecular biology in the 1990s, the recombinant DNA technology is available to modify virus genomes for improving the safety and efficacy of OV, thus a new wave of virotherapy has been initiated (Martuza et al., 1991).

### **1.2.1 OV- selectivity in cancer cells**

OVs are replicating therapeutics that are naturally selected or engineered to infect and grow in cancer cells without damaging normal cells (Russell et al., 2012; Stojdl et al.,

2003; Vaha-Koskela et al., 2007). Selectivity or targeting of malignant tumor cells is the first and most obvious mechanism of oncolytic virotherapy (Alemany R., 2013).

Some OV's have natural preferences to infect and replicate in cancer cells. Some tumor cells can express the surface receptors which are required for OV to bind/entry (e.g. CD46/CD150 for Measles virus (MV), high-affinity laminin receptor for Sindbis virus). (Shingai et al., 2005; Wang et al., 1992). In addition, some OV's' oncotropism is due to tumor intrinsic factors. For example, Newcastle disease virus (NDV) is susceptible to inhibition by interferon, while normal cells have intact interferon response which prevents them from virus infection and replication. Many types of cancers lack functional interferon response, so NDV can easily target these cancers without harming normal tissue (Norman et al., 2000). Activating mutations in Ras and mutations along the Ras pathway are observed in many human tumors. Tumors with an activated Ras pathway are unable to activate an anti-viral response mediated by the host protein kinase R (PKR). Reovirus replication is restricted by activation of the double-stranded RNA-activated PKR by early transcripts (Strong et al., 1998; Cornelis et al., 2004), hence reovirus can replicate and kill the host tumor cell with an activated Ras pathway.

In many cases, modification of virus genomes makes OV oncotropism possible. One often used method for virus retargeting is to fuse the virus attachment protein to a peptide or a single-chain antibody which is known to bind a tumor-related receptor. For example, tumor specific surface receptors, such as epidermal growth factor receptor, hypoxia-inducible factor 1, Her2-neu, androgen receptor and estrogen receptor, have been exploited for their use as receptors for virus entry or viral gene expression (Cattaneo et al., 2008; Dorer et al., 2009). Mutating viral genes that are crucial for virus survival in

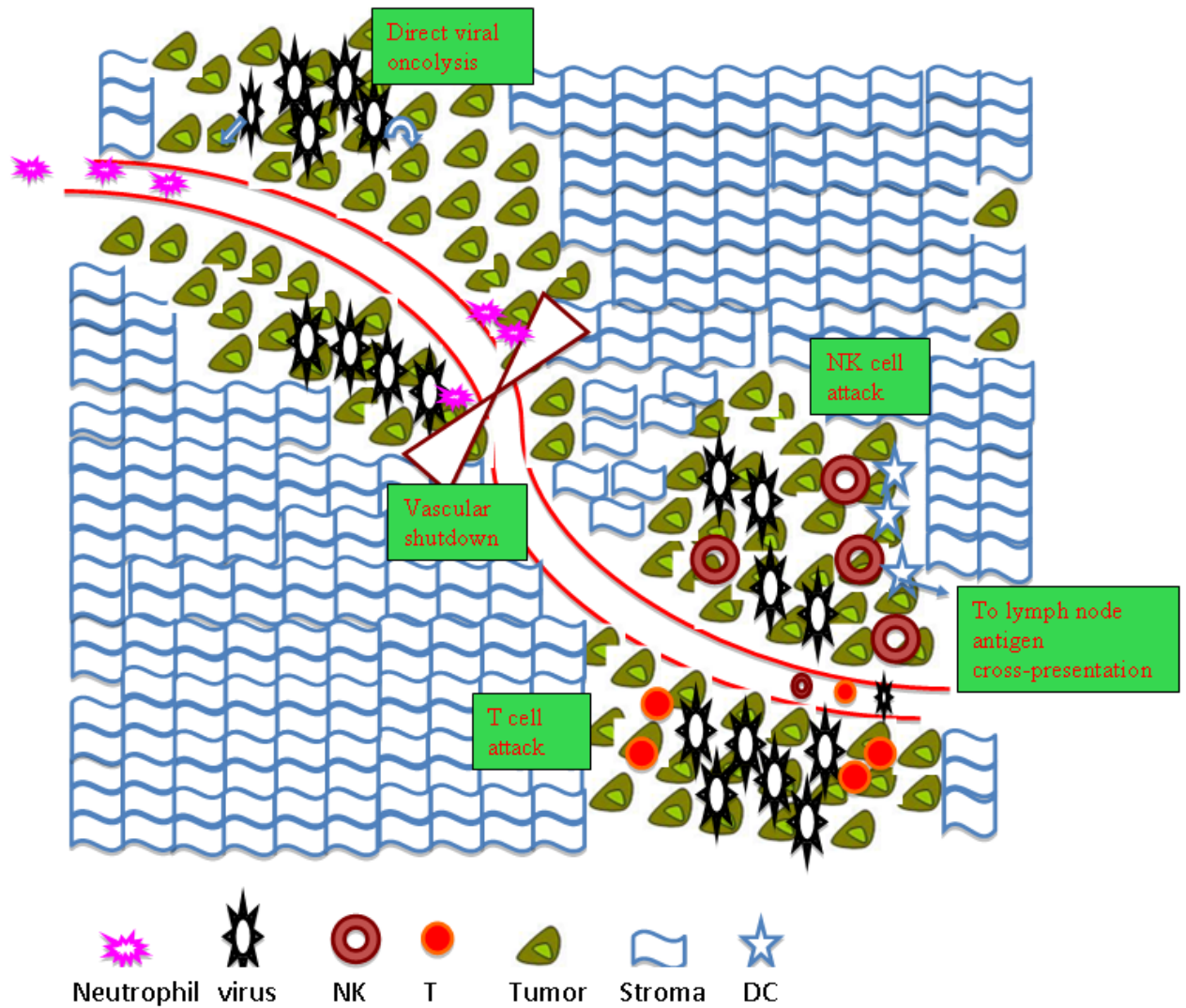
normal cells but are dispensable in tumor cells is another strategy to make OV's cancer specific. For instance, defects in Rb and p53-controlled tumor suppressor pathways were seen in more than 50% of tumor cells, and depletion of E1A and E1B genes leads to a restriction of adenovirus (Ad) replication to these tumors (Fukuda et al., 2003, 2009). Furthermore, defects in antiviral defense of tumor cells can be used to engineer OV. Normal cells respond to virus infection by producing of interferon and undergoing apoptosis to prevent virus spread. In response to these cells, viruses use various strategies to combat this innate immune response of infected cells. In contrast, researchers can engineer OV's to incapacitate their immune combat proteins, such as the C and V proteins of paramyxovirus family members, the NS1 protein of the influenza virus, and the matrix protein of VSV, and make them nontoxic to normal cells (Russell et al., 2012).

There are also other methods adopted to make oncotropic OV's. For example, the expression of one or more genes required for the viral cycle is controlled by the promoters functioning only in tumor cells, such as hypoxia-responsive promoters (Post et al., 2003). The insertion of tissue specific microRNA-binding elements in the 3'UTR of essential viral genes, prevents such microRNA expressing tissues from infection (Sugio et al., 2011; Kelly et al., 2010).

### **1.2.2 OV-multiple mechanisms of cancer killing**

OV therapy provides an alternative treatment for cancer and orchestrates several distinct mechanisms (Figure 1.2) to destroy malignant cells, including direct viral oncolysis, tumor blood vasculature shutdown, anti-tumor immunity, and sensitizing cancer cells to chemotherapy or radiotherapy.

**Figure 1.2 Oncolytic vesiculoviruses target tumor destruction in multiple ways**



Oncolytic vesiculoviruses selectively infect and kill malignant cells, break innate and adaptive immune tolerance towards tumors, and induce tumor-specific vascular shutdown.

(Adapted from Mahoney et al., 2013)



### **1.2.2.1 OV-Direct viral oncolysis**

The basis of OV therapy is their ability to selectively target tumor cells and go through progressive rounds of viral replication in order to achieve viral mediated oncolysis (e.g. Ad, HSV, etc) (Mullen et al., 2002). Some OVs can produce certain proteins during replication that are toxic to tumor cells. For instance, adenoviruses synthesize the E4ORF4 protein that induces cancer cell apoptosis (Shtrichman et al., 1998). Brain cancer-initiating cells infected with oncolytic Ad leads to autophagic cell death (Jiang et al., 2007). In addition, OVs can be genetically modified to enhance direct oncolysis. For example, oncolytic Ad was engineered to express tumor necrosis factor-related apoptosis-inducing ligand (Trail), which significantly enhances viral oncolysis in the GBM cell line and inhibits tumor growth *in vivo* (Wohlfahrt et al., 2007). Survivin has been shown to be an ideal target for cancer gene therapy due to its strong anti-apoptotic effect, so survivin shRNA was constructed into an oncolytic adenovirus to knockdown survivin expression, which induced cancer apoptosis and inhibited tumor growth both *in vitro* and *in vivo* significantly (Shen et al., 2009).

### **1.2.2.2 OV-Tumor vasculature shutdown**

In both preclinical (Breitbach et al., 2007; Kirn et al., 2007) and clinical settings (Liu et al., 2008), some OVs [e.g. Vsv and vaccinia virus (VV)] can cause vascular collapse and blood clotting at the tumor rim and subsequent wide-spread necrosis and apoptosis were seen throughout these tumors. Tumor vasculature shutdown is attributable to the recruitment of neutrophils to the tumor (Breitbach et al., 2007) and the infection of tumor-associated endothelial cells (Kirn et al., 2007). More importantly, vasculature

collapse was restricted to tumor beds without harming normal blood vasculature (Breitbach et al, 2011). Tumor blood vasculature shutdown caused by OV indicates that the therapeutic activity of OV can go far beyond simple viral oncolysis, which is very important when considering the barriers of OV intravenous delivery (e.g. neutralization, sequestration) and spread in the tumor (e.g. matrix barrier, acquired resistance) (Russell et al., 2012).

### **1.2.2.3 OV-Anti-tumor immunity**

The innate and adaptive anti-tumor immune response elicited by OVs constitutes an important component of OV therapy. The induction of anti-tumor immune response follows OV infection of tumor cells due to cell death, production of danger signals and cytokines, and release of tumor associated antigens (Benencia et al., 2005; Endo et al., 2008; Errington et al., 2008).

OV infection of tumor cells is suggested to be a source of immunogenic danger signals including viral proteins, nucleic acids, OV induced tumor cell necrosis and apoptosis, and released tumor associated antigens (TAAs) (Kepp et al., 2009; Tesniere et al., 2008). These immunogenic danger signals will activate innate immune cells responses (e.g. NK cell, DCs and macrophages). Studies have also demonstrated that proinflammatory cytokines (e.g. IL-6, IL-8, and IFN- $\beta$ ) and chemokines (e.g. CCL3-5, 11, IP-10) were secreted from a melanoma cell line infected with reovirus (Steele et al., 2011; Gujar et al., 2010; Errington et al., 2008). These cytokines and chemokines may amplify the innate immune response. Innate immune responses initiated by OV have the potential of mediating cytotoxicity directly against tumors while simultaneously mediating

downstream adaptive immune responses (Ghiringhelli et al., 2007; Lanier et al., 2005). One possible explanation is that virus infects and lyses tumor cells and spreads to some extent through the tumor bed. During this process, immune cells will be recruited into the tumor and become activated. Among them, DCs can uptake the TAAs and cross-present these antigens to T cells, resulting in the induction of tumor specific T cells (Mahoney et al., 2013). Among the innate immune cells that mediate this response, NK cells are a key component (Hamerman et al., 2005; Lanier et al., 2005). We and others have clearly implicated the innate immune response in the therapeutic efficacy of OV (Heinzerling et al., 2005; Kaufman et al., 2010; Lemay et al., 2012; Prestwich et al., 2009). Specifically, we have demonstrated that Parapoxvirus Ovis (ORFV) has a profound effect on NK cells following intravenous delivery and this NK cell activation is the main mechanism by which ORFV exerts its anti-tumor effect in a pulmonary lung metastasis model (Rintoul et al., 2012). In a B16ova subcutaneous tumor model, depletion of CD8<sup>+</sup> T cells or NK cells failed to prolong mice survival after intratumoral injection of VSV, which indicates that CD8<sup>+</sup> T cells and NK cells are required for the efficacy of intratumoral VSV therapy (Diaz et al., 2007). Similarly, both innate and adaptive immune responses are generated following viral oncolysis mediated by other OVs, such as HSV (Li et al., 2007), parvovirus (Bhat et al., 2011), and adenovirus (Endo et al., 2008).

OVs can be engineered with a cytokine or chemokine transgene to improve anti-tumor immunity through their local expression. In order to stimulate APCs, many cytokines (e.g. GM-CSF, Flt3L) or chemokines (e.g. CCL3, CCL5) have been inserted into OVs (Lee et al., 2010; Vigil et al., 2007; Cerullo et al., 2010; Grote et al., 2003; Liu et al., 2003; Senzer et al., 2009; Bergman et al., 2007; Li et al., 2011; Ramakrishna et al.,

2009). For example, GM-CSF has been added into many OV, including VV(Lee et al., 2010), NDV(Vigil et al., 2007), Ad (Cerullo et al. 2010), MV(Grote et al., 2003), HSV(Liu et al., 2003; Senzer et al., 2009 ) and VSV(Bergman et al., 2007). Talimogene laherparepvec (formerly known as OnVEX<sup>GM-CSF</sup>) is an oncolytic HSV deleted for ICP34.5 and ICP47 and expressing human GM-CSF. GM-CSF is a cytokine engaged in the maturation of APCs such as DCs, and stimulate CMI (Eager et al.,2005). In a mouse of model A20 bilateral flank tumor, Talimogene laherparepvec was able to induce tumor regression in both injected tumors and noninjected tumors on the contralateral flank while its attenuated HSV counterpart without GM-CSF only induced regression of the injected lesion (Liu et al.,2003). In a phase II clinical trial, Talimogene laherparepvec treatment showed a 26% response (complete response 8/50, and partial response 5/50) rate with durability in both injected and noninjected lesions together with survival rates (52% at 24months) (Senzer et al.,2009). In order to activate lymphocytes, genes encoding other cytokines (e.g. IL-2, IL-12, IFN $\alpha/\beta$ , IFN- $\gamma$ , etc) have been added into OVs. Similarly, introduction of such transgenes into OVs have shown improved efficacy of OV therapy (Shin et al., 2007; Zhao et al., 2008; Shashkova et al., 2007; Su et al., 2006).

OVs have also been used as a form of cancer vaccine to elicit anti-tumor immunity against various cancers. Our group previously reported that OV therapy can combine with a tumor cell based vaccine. After tumor cells were lethally irradiated and infected with OV (e.g. VSV<sub>GM-CSF</sub>) ex vivo, infected tumor cells were injected into C57BL/6 mice for immunization and found that VSV<sub>GM-CSF</sub>-infected cell vaccine leads to stronger NK cell and T cell activation than OV or irradiated cells alone. More importantly, in both B16 subcutaneous tumor model and B16 pulmonary metastasis model, VSV<sub>GM-CSF</sub>-

infected cell vaccine treatment significantly impacted tumor growth than OV or irradiated B16 cell alone (Lemay et al., 2012). In addition, OVs can be engineered with a TAA transgene to infect and debulk the tumors, while the expression of the TAA can induce a tumor specific immune response. One strategy used to induce strong TAA specific immune response is through the use of heterologous prime-boost vaccine strategies where the priming and boosting vectors are different viruses (Pol et al., 2011). Pol et al. reported that in a B16F10 lung metastasis model, they used two different OVs, Ad and Maraba MG1 expressing melanoma antigens hDCT as “Ad-hDCT priming and MG1-hDCT boosting” strategy to treat B16F10 lung metastases, and found that this strategy can induce the most robust hDCT specific immune response among all different combination strategies and significantly prolong the survival with complete remission in more than 20% of the mice treated (Pol et al., 2013).

#### **1.2.2.4 OV-Sensitizing tumor cells to chemotherapy and radiotherapy**

OV has been shown to sensitize cancer cells to chemotherapy and radiation therapy. For example, the E1A gene product of adenovirus can induce high p53 expression in some cancer cells and make them susceptible to DNA damage from chemotherapy and radiation (Lowe et al., 1994). In a phase II clinical trial, intratumoral adenovirus (ONYX-015) in combination with 5-FU and cisplatin in head and neck cancer patients produced higher complete responses compared with either ONYX-015 or chemotherapeutic agents alone (Khuri et al., 2000). Also, modified VSV and Ad, which express the sodium iodide symporter (NIS), allowed tumor-targeted radioiodide uptake in prostate cancer and

multiple myeloma, respectively, which significantly improved the efficacy of OV therapy (Goel et al., 2007; Hakkarainen et al., 2009).

### **1.2.3 OV- Clinical translation**

In the past two decades, great progress has been achieved in the development of OVs for cancer treatment and many of them have entered into the various stages of clinical trials. Reovirus, Ad, VV, MV and HSV are leading in the ranking of frequency in clinical trials, followed by Coxsackie virus, Seneca valley virus, Retrovirus, VSV, Poliovirus and Parvovirus (Alemany et al., 2013). The majority of these studies were Phase I/II trials. Overall, OVs have demonstrated excellent clinical tolerability and no transmission were found from treated patients to contacts and caregivers (Senzer et al., 2009). However, safety concerns for viral therapies still exist as clinically effective doses of viruses may produce serious toxicity and virus spreading. In addition, OV mutation may regain pathogenicity through conversion to its wild type counterpart (Zeyaulah et al., 2012). Clinical efficacy of single-agent oncolytic virotherapy has gained support in recent phase I/II clinical trials. In one trial, an oncolytic vaccinia virus engineered to express GM-CSF, named JX594, was administered by intratumoral injection to nonresectable hepatocellular cancer patients and objective responses were observed in 3 of 10 patients (Park et al., 2008). In the second trial, an oncolytic HSV encoding GM-CSF (talimogene laherparepvec, formerly known as OncoVEX<sup>GM-CSF</sup>) was intratumorally injected into patients with metastatic melanoma and resulted in complete response in 8 of 50 treated patients (Senzer et al., 2009). Recently, a phase II b study of JX594 to treat advanced liver cancer patients who failed Sorafenib was used to determine whether JX594 plus best

supportive care (BSC) is more effective in improving survival than BSC. However, this study did not meet its primary endpoint of overall survival for JX594 plus BSC compared to BSC alone. JX594 is also being investigated in phase II studies in colorectal, kidney and ovarian cancer, and continues to hold great promise for the patients. (Press release of Jenerex Biotherapeutics, September 3<sup>rd</sup>, 2013)

So far, there are 4 phase III OV clinical trials which have been designed. First, an oncolytic adenovirus with E1B-55KD and partial E3 deleted developed by Shanghai Sunwaybio, named H101, was approved in China after a Phase III trial, which showed that H101 was well tolerated and has good efficacy when combined with chemotherapy. In this trial they found that advanced head and neck cancer patients treated with H101 and chemotherapy had a higher objective response rate (79%) compared with chemotherapy alone (40%) (Xia et al., 2004). The second Phase III trial was to evaluate the safety and efficacy of talimogene laherparepvec compared to a control therapy with GM-CSF in over 400 patients with unresected stage IIIB, IIIC or IV melanoma (Andtbacka et al., 2013). Recently Andtbacka RHI et al. reported a planned interim analysis of this trial at the 2013 ASCO (American Society of Clinical Oncology) annual meeting. There was a significant difference in durable response rate between talimogene laherparepvec treated patients (16%) and GM-CSF treated patients (2%),  $p < 0.0001$ . Interim overall survival showed a trend in favor of talimogene laherparepvec (HR=0.79, 95 percent CI 0.61-1.02). Fatigue, chills and pyrexia were the most observed adverse events. Serious adverse events were found in 26% of talimogene laherparepvec patients and 13% of GM-CSF patients (Andtbacka et al., 2013). The third phase III clinical trial (NCT01166542) is ongoing to evaluate the efficacy of REOLYSIN (Reovirus Serotype 3

Dearing) in combination with paclitaxel and carboplatin in platinum-refractory head and neck cancers. The fourth one (NCT01438112) is not yet opened, which is an integrated phase II/III and designed to test the safety and efficacy of recombinant Ad for non muscle invasive bladder cancer. These clinical results seem very encouraging, and talimogene laherparepvec appears to be on the verge of FDA approval for melanoma patients.

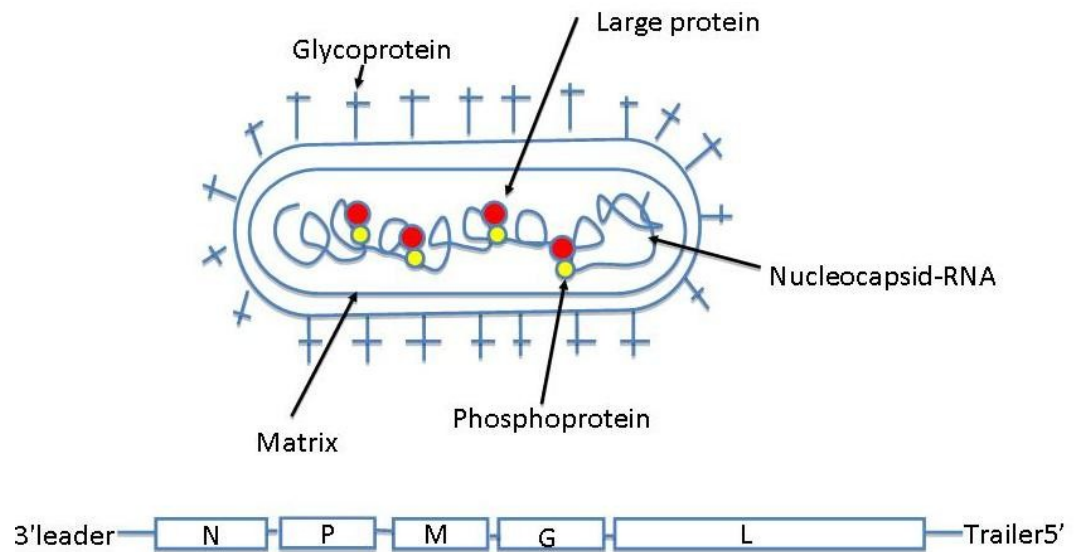
#### **1.2.4 Rhabdovirus OV– Maraba MG1 virus**

Rhabdoviruses belong to the family Rhabdoviridae, which is a family of enveloped viruses. They contain a non-segmented genome made of a single stranded negative-sense RNA molecule. Rhabdoviruses are uniquely bullet-shaped and currently grouped into six genera that have been shown to infect a wide range of organisms from animals, plants to fungi with the exception of bacteria (Fauquet et al., 2005). The rabies virus (RAV, Lyssavirus) and VSV are the two best-studied rhabdoviruses. RAV is known to cause deadly human diseases (Dietzschold et al., 2008) and VSV infects insects and livestock, causing self-limiting illness (Fauquet et al., 2005).

VSV particles are made of a ribonucleoprotein (RNP) core and a lipoprotein envelope surrounding that core (Wagner et al., 1997). The VSV virus genome has five genes (Figure 1.3) that encode (from 5' terminus), the large subunit of the RNA-dependent RNA polymerase (L), the glycoprotein (G), the matrix protein (M), the phosphoprotein (P) and the nucleoprotein (N) (Wagner et al., 1997). The RNP core is the infectious unit of all rhabdoviruses and includes genomic RNA tightly associated with abundant N protein. The RNP core also contains less P and L protein. The G and M proteins are the major components of the VSV virus envelope (Wagner et al., 1997). The rhabdovirus life cycle



**Figure 1.3 VSV virion structure and genome organization**



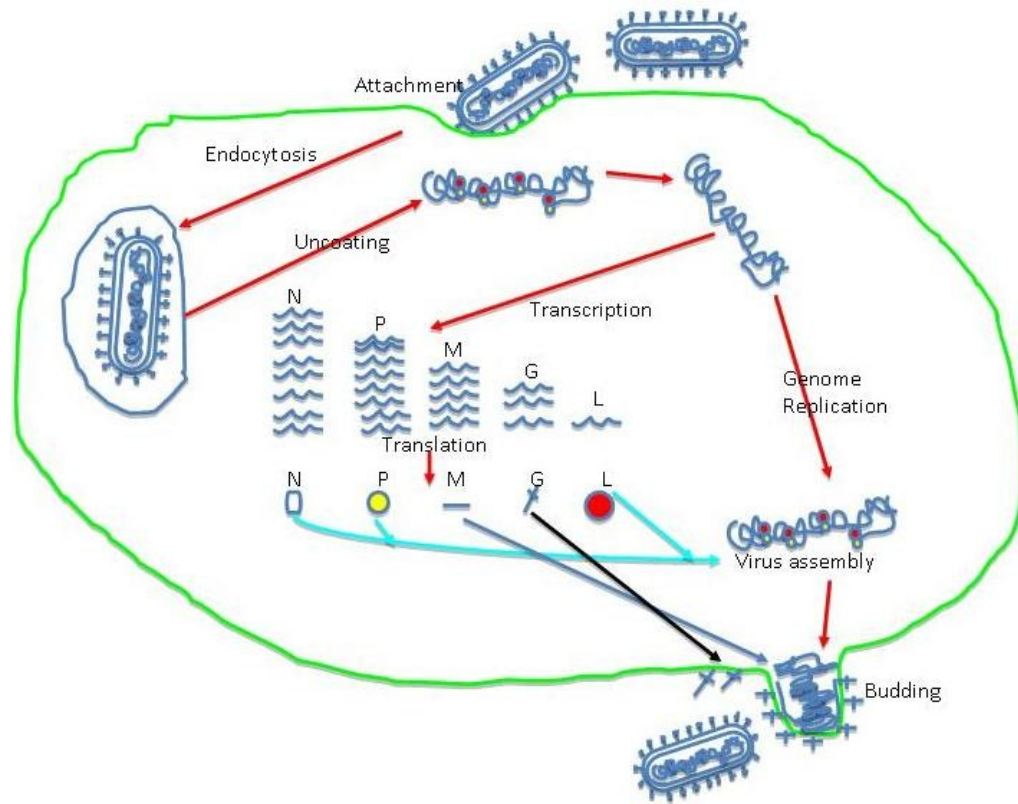
VSV encodes five structural proteins: nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and large (L) proteins. The VSV genome is arranged in the order 3'-(leader), N, P, M, G, L, (trailer)-5'. (Adapted from Li et al., 2012)

(Figure 1.4) occurs only in the cytoplasm of infected cells and different viral proteins play different roles in this process. The N protein functions to promote RNA encapsidation and allow genome replication; L and P proteins mainly function as RNA-directed RNA polymerase; G and M proteins functions include viral entry into the host cell, virus particles assembly and budding out of the host cells (Wagner et al., 1997; Ivanov et al., 2011).

VSV is very vulnerable to type I IFN (Muller et al., 1994), thus type I IFN defective tumors are susceptible to virus infection and replication (Stojdl et al., 2003). The M protein of VSV can turn off the antiviral gene expression of the host cell by blocking the transportation of mRNA from the nucleus to the cytoplasm (Her et al., 1997). VSV also possesses another immune escape mechanism associated with the down-regulation of NKG2D-ligand surface expression. It therefore escapes recognition and lysis by NK cells bearing NKG2D receptors (Helle et al., 2011). The oncolytic VSV $\Delta$ 51 has a mutation in M protein, which can't shut off the nucleoplasmic transportation of mRNA, and thus this virus retains its oncolytic ability by infecting only tumor cells which are IFN-deficient (Stojdl et al., 2003). Currently, VSV and its recombinants (VSV $\Delta$ 51, VSV-IFN $\beta$ ) have been extensively studied as OV in preclinical and clinical settings. VSV attacks cancer cells via multiple mechanisms, including the direct killing of cancer cells (Gaddy et al., 2007), blocking the blood supply to tumors (Breitbach et al., 2007) and stimulating innate and adaptive anti-tumor immunity (Diaz et al., 2007). A phase I clinical trial of VSV-IFN $\beta$  organized by the Mayo Clinic is ongoing to evaluate its safety profile in patients with liver cancer (NCT01628640).

WT Maraba virus was isolated from phlebotomine sand flies and has been categorized

**Figure 1.4 Overview diagram of rhabdovirus life cycle**



Schematic of rhabdovirus life cycle: attachment, endocytosis, uncoating, genome replication, transcription, translation, viral assembly, and budding steps. (Adapted from Li et al., 2012)

to be a member of the Vesiculoviruses group in the family Rhabdoviridae (Travassos da Rosa et al., 1984). It is antigenically closely related to VSV, which has been shown to cause vesicular disease in livestock (Koppers-Lalic et al., 2011). Maraba MG1 is a mutant strain derived from WT Maraba virus with double mutations in the G protein (Q242R) and M protein (L123w) (Brun et al., 2010). It has been established as the most potent OV of screened wild type rhabdoviruses (Brun et al., 2010). Maraba MG1 is an attenuated strain which has a 100-fold greater maximum tolerable dose than WT Maraba *in vivo*, but still retains high killing ability in cancer cells. Furthermore, systemic administration of MG1 durably cures mice in syngeneic and xenograft models, which demonstrates MG1 as a superior OV compared to VSV $\Delta$ 51 (Brun et al., 2010). Systemic administration of MG1 will be entering into Phase I clinical trial this year. Although MG1 is a potent OV, its effect on anti-tumor immunity is poorly understood.

### **1.3 Replication incompetent virus**

#### **1.3.1 Single replication-cycle VSV virus**

VSV G protein is able to attach to and fuse with host cell membranes (Ivanov et al., 2011). Following viral attachment, the virus enters into the host cell and uncoats itself to release the viral genome followed by gene expression. Dr. Rose's group has previously constructed a single-cycle VSV vector by depleting the G gene from its genome. Its progeny has no G protein on its envelope, so it can't bind to any target cells. Thus this recombinant virus is capable of a single round of replication and can only be grown in cell lines expressing G proteins (Schnell et al., 1997).

### **1.3.2 Ultraviolet (UV) inactivated virus**

UV at a wavelength of 254nm (UVC) is a commonly used method for virus inactivation. Low dose UV radiation causes covalent dimers in nucleic acids between adjacent thymine (T) or uracil (U) residues to prevent virus replication (Michalke et al., 1969; Miller et al., 1974). UVC radiation is more effective at inactivating viruses with single-stranded nucleic acid because they are unable to repair DNA damage without the complementary strand (Rauth et al., 1965). In addition, UV-irradiation induced RNA-protein and protein-protein crosslinks has been reported at high doses (Smirnov et al., 1990).

### **1.3.3 Replication incompetent virus and anti-tumor immunity**

Compared with replication competent viruses, replication incompetent viruses are much safer with lower toxicity due to the loss of its replication ability. They are often used as vaccines and vaccine vectors (Tim et al., 2006). However, recently researchers have used them to treat cancers in animal models and have achieved significant efficacy via stimulation of anti-tumor immune responses. Galivo *et al.* found that a single replication cycle VSV achieved comparable therapeutic efficacy compared to a fully replication competent VSV in animal tumor models. This was achieved through initiating anti-tumor immunity in place of viral oncolysis (Galivo et al., 2010). In another study, a replication deficient Sindbis virus (SV) was used to treat severe combined immunodeficiency (SCID) mice bearing ES-2 ovarian carcinoma xenografts. The researchers found that NK cell activation was largely responsible for SV anticancer efficacy. Furthermore, Sin/IL-12 achieved a better efficacy than SV alone in the same

model, which is also NK cell dependent through a separate IFN- $\gamma$  mechanism (Granot et al., 2011).

Although UV-inactivated virus can not replicate at all, some of them are still able to elicit the host immune response. Hidmark et al. reported that UV-inactivated Semliki Forest virus induced IFN $\alpha/\beta$  production in myeloid dendritic cells. This response is dependent on viral entry and IRF-3 but not MyD88 (Hidmark et al., 2005). UV-inactivated Sendai virus [hemagglutinating virus of Japan envelope (HVJ-E)] has also shown anti-tumor effects in mice bearing CT26 colon carcinoma by enhancing adaptive immune response through blocking regulatory T cells (Masayuki et al., 2007).

Therefore, these valuable attributes of replication incompetent viruses make them possible candidates for further clinical development.

## **1.4 Cancer surgery**

### **1.4.1 The role of surgery in cancer therapy**

Cancer surgery is a critical and standard therapy for most solid malignancies. The reason behind this assertion is that a cure can likely be reached by eradicating the cancer from the body. It not only provides the best chance of curing many types of cancer but also plays a role in the prevention, diagnosis, and staging of cancers. For example, preventive surgery may be conducted to remove the polyps in the colon which are considered precancerous tissue (Itah et al., 2009). A tissue sample might be removed for testing to help diagnose cancer or determine the extent of cancer (McCahill et al., 2002). Surgery might be performed to relieve the discomfort due to the advanced cancer or debulk the cancerous tumor when it is not possible to remove an entire tumor (McCahill

et al., 2002). In essence, what kind of surgery chosen to provide the best care for the patients is dependent on the type of cancer and patients' health.

#### **1.4.2 Cancer surgery and metastases**

Although we discussed many options of surgery for cancer patients, it is worth mentioning that surgery in our study is intended for curative cancer treatment, which is often a major, open surgery, capable of inducing stress response, instead of a minor, minimally invasive surgical procedure. Surgery has been compared to a double-edged sword: it not only removes the primary tumor but also brings some unwanted consequences. Surgery, the cornerstone of solid cancer treatment, has been long suspected to promote metastatic disease formation (Raven et al., 1990). Although no clinical study has been performed to directly test this hypothesis due to the ethical and methodological constraints, clinical support for it does exist indirectly. One human study found that cancer surgery could improve the survival of women with breast cancer dramatically in the early years. However, they also noticed that there was a spike in the risk of death at 8 years in women with surgery, and this was not seen in women without surgery. They reasoned that surgical resection of primary tumor might lead to promotion of the metastases formation (Demicheli et al., 2001). In addition, more complicated surgeries and postoperative complications are associated with a worse cancer patients' outcome (Eberhardt et al., 2009; Lerut et al., 2009). In cancer patients with large bowel obstruction, surgeons occasionally performed a staged procedure: (1) a colostomy to relieve the obstruction and (2) a colectomy to remove the tumor. This dual insult led to a higher cancer recurrence (Fielding et al., 1974). A randomized trial reported that a

minimally invasive technique, video-assisted thoracoscopic lobectomy, decreased the incidence of metastasis at 5 years to 4% compared with 14% of the conventional lobectomy group (Sugi et al., 2000). Many studies including work from this lab have clearly shown that cancer surgery promotes metastasis formation in implanted and spontaneous animal tumor model (Glasner et al., 2010; Tai et al., 2013; Tsuchiya et al., 2003).

Our laboratory (Seth et al., 2013), using a reproducible model of surgical stress, has confirmed a significant (two to four-fold) increase in the formation of pulmonary metastases at 3 days following surgery. This prometastatic effect of surgery can be replicated in 2 different mouse strains (C57BL/6 and Balb/c mouse), using 2 different cell types (B16F10lacZ melanoma cells and CT26lacZ colon cells), and following three different surgical procedures (laparotomy, left nephrectomy and left partial hepatectomy). Moreover, it is not induced when mice are subjected to anaesthesia alone. These findings confirm that this phenomenon is neither host and cancer, nor surgery specific and that results from our surgery model have broad applicability (Seth et al., 2013). Furthermore, we used the 4T1 breast cancer cell line to establish a mouse model of spontaneous metastasis and surgery and found that left nephrectomy promoted lung metastasis compared to the no surgery control groups (Tai et al., 2013). In another study, Tsuchiya et al. found that in the colon 26-L5 pulmonary metastases model followed by 5 different forms of surgical stress (untreated control, anesthesia only, anesthesia and laparotomy, anesthesia and laparotomy and appendectomy, anesthesia and laparotomy and appendectomy and left hepatic lobectomy), increased incidence of metastatic disease was proportional to the magnitude of surgical stress (Tsuchiya et al., 2003).



### **1.4.3 Proposed mechanisms for surgery induced metastases**

Several possible mechanisms have been suggested to characterize the promotion of surgery-induced metastatic disease formation. First, surgery has been shown to increase dissemination of tumor cells into the blood and lymphatic circulations due to the manipulation of the tumor and its vasculature (Yamaguchi et al., 2000). Tumor cells were transiently detected in the blood of many cancer patients after surgery through polymerase chain reaction (PCR) (Weitz et al., 2001). Second, surgery can increase cancer cell proliferation (Kirman et al., 2002) and potentiate the invasive ability and motility of free cancer cells by releasing matrix metalloproteinases (MMP) (Kerman et al., 2006), and by increasing adhesion-molecule expression on tumor cells (Reviewed in van der Bij et al., 2009). Third, primary tumors were reported to secrete anti-angiogenic factors such as angiostatin and endostatin (O'Reilly et al., 1997, 1994). Therefore, surgical resection of primary tumor might eliminate these factors against angiogenesis. At the same time, surgery will elevate the levels of growth factors and cytokines (e.g. epidermal growth factor and transforming growth factor- $\beta$ ) that promote tumor proliferation (Hofer et al., 1999).

There are also some other perioperative factors (e.g. blood transfusion, hypothermia) that might play a role in metastatic progression. A meta-analysis, including 36 studies on colorectal cancer patients, demonstrated a moderate association between perioperative blood transfusion and cancer recurrence, with an Odds ratios (OR) of 1.42 (95% CI, 1.20 to 1.67) (Amato et al., 2006). In addition, hypothermia is seen in more than 50% of surgical procedures. However, studies found that milder hypothermia didn't increase

cancer recurrence in cancer patients (Yuce et al., 2005), and only severe hypothermia was found to increase susceptibility to metastasis in animal studies (Ben-Eliyahu et al., 1999).

Last but importantly, perioperative suppression of CMI is regarded as a significant risk factor for metastasis. Suppression of CMI may be a critical factor when present in the perioperative period in combination with other adverse mechanisms known to enhance metastases (Goldfarb et al., 2006-2007). After major surgery, a significant increase was seen in plasma levels of Interleukin-10 (IL-10), IL-1rA (IL-1 receptor antagonist), sTNF $\alpha$  (soluble TNF $\alpha$  receptor) (Krohn et al., 1999) and sIL-2r (soluble IL-2 receptor) (Khan et al., 1999), which are known to decrease CMI, while proinflammatory cytokines such as TNF $\alpha$  and IL-1 were relatively low (Khan et al., 1999). Also, surgery can activate the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system due to perioperative factors including psychological stress to release stress hormones (e.g. glucocorticoids, catecholamines (CAs), opioids), which are related with suppression of CMI (Seok et al, 2010). For example, glucocorticoids, known as immunosuppressants, are increased for several days after major surgery, and their levels usually reflect the extent of tissue damage and immunosuppression (Hogevold et al., 2000). Studies have demonstrated that the inhibition of glucocorticoids decreases NK cell suppression, T cell apoptosis and tumor metastasis after surgery in animal models (Shakhar et al., 2003; Deguchi et al.1998) and improve immune function by reducing lymphopenia and by enhancing secretion of IL-6 (Jameson et al., 1997). Prostaglandins (PGs) are synthesized in surgery damaged tissue and remain at high levels after surgery (Brinkmann et al., 1998). CAs and PGs were shown to directly downregulate the activity of NK cells and CTLs (cytotoxic T lymphocytes) through increased intracellular cAMP

levels (Elenkov et al., 2000). In addition, CAs and PGs were found to reduce Th1 cytokines including IL-2, IL-12 and IFN $\gamma$  (Specht et al., 2001; Elenkov, et al., 2002) and increase the release of immunosuppressive cytokine IL-10 (Platzer et al., 2000). More importantly, combined administration of  $\beta$ -adrenergic antagonist and a cyclooxygenase-2 inhibitor reduced postoperative NK cell suppression and improved survival rates in spontaneous postoperative metastasis in mice (Glasner et al., 2010). Furthermore, opioids are secreted from the pituitary and the adrenal medulla into the circulation in response to stress and pain perioperatively, which has been shown to suppress CMI (Panerai et al., 1997), and the opiate antagonist naltrexone dramatically reduces postoperative immunity suppression in rats, as demonstrated by increased levels of NK cytotoxicity, lymphocyte proliferation and IFN $\gamma$  production (Nelson et al., 2000). Studies also indicate that general anesthesia and opiate analgesia are immunosuppressive (Galley et al. 2000), which are associated with an increase in cancer recurrence rates as shown in rectal cancer (Gupta et al., 2011), breast (Exadaktylos et al., 2006) and ovarian (Lin et al., 2011), when compared to regional or local blockade of nerve conduction.

Surgery induced NK cell dysfunction as measured in a standard [ $^{51}\text{Cr}$ ]-release assay, which is an important aspect of suppression of CMI and has been documented in both patients (Espi et al., 1996; Lukomska et al., 1983; Pollock et al., 1991, 1992) and in animal models (Ben-Eliyahu et al., 1999; Da Costa et al., 1998; Page et al., 2001). Researchers found that colon cancer patients with decreased NK cell activity prior to surgery had a 3.5 fold increase in the development of metastasis in the following 31 months (Kodak et al., 1997). In our surgical stress model, a reduction in the expression of NKG2D is observed, which indicates NK cells are suppressed in surgical stressed mice

(Tai et al., 2013). We and other groups found that NK cell suppression following surgery is largely responsible for increased metastases in animal models (Benish et al., 2008; Glasner et al., 2010; Goldfarb et al., 2011; Tai et al., 2013). Specifically, in our intravenous pulmonary metastasis and surgery (left nephrectomy) model, we showed that surgery increased lung metastasis at 3 days after intravenous cell injection. In addition, in mice depleted of NK cells, both surgery group and non-surgery groups developed more lung metastasis burden, however, surgery induced metastasis was completely abrogated by the depletion of NK cells. This phenomenon was confirmed by reproducing the same result in NK deficient transgenic mice (Tai et al., 2013). In severe combined immunodeficiency (SCID) mice (without T and B cells but with NK cells intact), we repeated our surgical stress experiment and still observed surgery induced metastasis (Seth et al., 2013). Taken together, surgery enhanced metastasis formation via impairing NK cells in our B16lacZ pulmonary metastasis and surgery model.

#### **1.4.4 The perioperative period: a therapeutic window of opportunity for immune modulation**

Although the immediate perioperative period appears to have an impact on long-term cancer recurrence, it also represents a window of opportunity to intervene in the metastatic process (Neeman et al., 2013; Tai et al., 2013). Clinical trials of preoperative non-specific immune stimulation with low dose IFN $\alpha$  (Oosterling et al., 2006) or IL-2 (Bohm et al., 2002; Brivio et al., 1996, 2006) have demonstrated less NK and T cell suppression following surgery. In two randomized studies of patients undergoing resection of colorectal cancer (CRC) primary tumors (Brivio et al., 2006) and hepatic

metastases (Brivio 1996), preoperative low dose subcutaneous (s.c.) IL-2 was associated with an improved prognosis. In the first study, 86 CRC patients with stage II or III disease were randomized to receive low dose IL-2 twice a day for 3 consecutive days prior to surgery or no preoperative treatment. At a median follow-up of 54 months there were significantly fewer recurrences in the IL-2 group (21.4% vs 43.1%,  $p=0.03$ ) and a trend toward improved overall survival. In the second study, 50 CRC patients with Stage IV disease, undergoing curative or palliative surgery, were randomized to the same two treatment arms. The median progression-free survival (PFS) and overall survival were significantly longer in the IL-2 group. However, dose associated toxicity has put a limitation on further development. High dose IL-2 induces hypotension and a capillary leak syndrome (Smith et al., 1997). In addition, IFN $\alpha$  is associated with thrombocytopenia and leucopenia (Fioravanti et al., 2011). Thus, new and safe perioperative therapy to prevent metastatic disease is urgently needed.

Since OV $\alpha$ s are good NK stimulants, we applied this into the perioperative setting to boost NK cell function and prevent surgery induced NK dysfunction. In animal studies, we recently established that perioperative NK cell stimulation with several OV $\alpha$ s, including ORFV and VV, can rescue surgery-induced NK cell suppression and effectively attenuate lung metastases (Tai et al., 2013). In human cancer patients, we demonstrated for the first time that oncolytic VV markedly increases NK cell activity (Tai et al., 2013). While these data are encouraging, there are several potential barriers to the administration of a live, replicating virus immediately before surgery, including the potential for an overwhelming systemic inflammatory response and the risk of infection

of operating room staff. New therapeutic strategies are under development to resolve the safety concerns associated with administering live OV in the perioperative period.

## **1.5 Rationale**

The perioperative period is a uniquely susceptible time for the formation of metastases. Surgery enhances the development of metastatic disease. NK cell suppression has been linked to the development of metastatic disease in animal models and in cancer patients. Preclinical work from our laboratory and others has demonstrated that a profound but reversible suppression of NK cell function in the postoperative period plays a critical role in the enhanced development of metastases following surgery. Considering the dose related toxicity of recombinant cytokine therapy, we propose OV as a potential candidate to stimulate the innate immune system and eradicate surgery-induced micrometastatic disease. The current project evaluates the innate immune response, especially NK cell stimulation, upon MG1 administration. Concurrently, we will describe the first novel use of MG1, single replication cycle MG1 (MG1-Gless) and replication incompetent UV inactivated MG1 (MG1-UV<sup>2min</sup>) as perioperative immune therapy in the preclinical setting. This panel of related viruses will permit further characterization of the mechanism of innate immune activation and anti-tumor efficacy of MG1 in the context of cancer surgery. These results will provide the preclinical rationale to propose a clinical trial of safe perioperative OV to improve cancer outcomes in cancer surgery patients by eradicating metastatic disease.

## **1.6 Hypothesis**

Perioperative use of non-replicating MG1-UV<sup>2min</sup> is a safe and effective approach to rescue surgery induced NK dysfunction and to attenuate the subsequent formation of metastases.

## **1.7 Objectives**

- (1) Characterize the ability of intravenous delivered MG1 to stimulate NK cells of the innate immune system in a murine model of metastases.
- (2) Investigate the mechanism of innate immune activation by exploring MG1 interaction with NK cells and DCs.
- (3) Create replication defective MG1 viruses by (a) design, cloning and rescue of a G-less MG1 virus (MG1-Gless) capable of a single cycle of replication and (b) ultraviolet (UV) inactivation of MG1(MG1-UV).
- (4) Compare the ability of this panel of viruses, intravenously delivered, to (a) undergo viral entry, transcription and translation, (b) attenuate the formation of metastases in two murine models, (c) activate NK cell cytokine secretion and cytotoxicity, and (d) interact with DCs.

## **Chapter 2: Materials and Methods**

### **2.1 Cell lines**

B16F10LacZ melanoma cell line was obtained from Dr. K. Graham (London Regional Cancer Program, Ontario) and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and grown at 37°C and 5% CO<sub>2</sub>. Cells were resuspended in DMEM without serum for intravenous (i.v.) injection through the lateral tail vein.  $3 \times 10^5$  cells at more than 95% viability were injected in a 100µl volume per mouse. 4T1 mammary carcinoma and YAC-1 cell lines were purchased from American Type Cell Culture (ATCC) and maintained in Roswell Park Memorial Institutes Media (RPMI) supplemented with 10% fetal bovine serum. All cell lines were confirmed to be mycoplasma free and show appropriate microscopic morphology at time of use.

### **2.2 Viruses**

#### **2.2.1 Maraba MG1 and MG1-UV**

Maraba MG1 expressing green fluorescent protein (GFP) was kindly provided by Dr. David Stojdl (CHEO, Ottawa) and it was prepared and titred as previously described (Brun et al., 2010). Inactivation of virus was performed on MG1-GFP preparations at a concentration of  $1 \times 10^9$  plaque forming unit (PFU) in 1ml PBS using UVC irradiation in the Spectrolinker XL-1000 UV crosslinker (Spectronics, Westbury, NY) at 120mJ/cm<sup>2</sup> for 2 minutes, 5 minutes, 30 minutes, 1 hour and 2 hours respectively. Confirmation of virus inactivation was measured by plaque assay and GFP expression prior to use. MG1 in this study refers to MG1-GFP.



### **2.2.2 MG1-Gless**

MG1-Gless expressing GFP was cloned based on wild type G-deficient Maraba-GFP virus. Briefly, PCR was carried out with 100 ng of mutagenic primer and 100 ng DNA template (wild type G-deficient Maraba virus) with Hot Start addition of enzyme and typical PCR setup (98°C-10 seconds, 60°C-30 seconds, 72 °C for 7.5 minutes for 33 cycles). The parental plasmid was digested with DpnI (NEB) (37 °C for 1 hour) and 10 µl of the 25 µl DpnI-digested PCR mixture was used to transform TOP-10 competent cells (Invitrogen, Carlsbad, CA). Positive clones were screened by introduction of non-coding change restriction site changes followed by sequencing. The mutant described here is Leu-123 to Trp in the M protein (L123W).

MG1-Gless in this study refers to MG1-Gless-GFP.

### **2.2.3 MG1-Gless virus rescue**

At 24 hours post A549 cell seeding ( $3.0 \times 10^5$  cells/well in 6-well plates), VV (multiplicity of infection (MOI) 10) expressing the T7 RNA polymerase in OptiMEM medium was used for 1.5 hours of infection. Following removal of vaccinia virus, each well was transfected with LC-KAN (low copy kanamycin resistant vector) Maraba (2µg) together with pCI-Neo constructs encoding for Maraba N (1µg), P (1.25µg), L (0.25µg) and G (0.25µg) with lipofectamine 2000 (5µl/well) according to the manufacturer's instructions. The transfection reagent was removed 5 hours later and replaced with DMEM supplemented with 10% FBS. At 48 hours post transfection, medium was filtered (0.2µm) to remove contaminating VV, and 1 ml was used to infect 293FT (pre-transfected with G) cells in each well of a 6-well plate. Cytopathic effects (CPE) and GFP

visible 24-48 hours later were indicative of a successful rescue, which was confirmed by purifying viral RNA and reverse transcriptase (RT)-PCR with Maraba-specific primers. T-REx™-293 (Invitrogen, Burlington, ON) (G gene was cloned into this cell line to conditionally supply G protein) was used to manufacture MG1-Gless virus. Lastly, purification on OptiPrep™ Density Gradient Medium was performed (Diallo et al., 2012).

#### **2.2.4 Titration of MG1-Gless**

Vero cells ( $2 \times 10^4$ ) were seeded in 96 well plates and incubated at 37°C in 5% CO<sub>2</sub> for 24 hours. Cells were infected with 50µl of 10-fold serial dilutions of MG1-Gless for 2 hours, then replaced with 100µl DMEM supplemented with 10% FBS. 24 hours later, GFP<sup>+</sup> cells were counted with a fluorescent microscope.

Unless otherwise specified,  $1 \times 10^8$  PFU/100µl of MG1 or virus variant was administered into mice intravenously.

#### **2.3 Mice**

Female 6- to 8-week-old C57BL/6 (B6) and BALB/c mice were purchased from Charles Rivers Labs (St Constant, Canada). CD11c.diphtheria toxin receptor (DTR) transgenic (Tg) mice were bred in the Central Animal Facility at McMaster University. Animals were housed in pathogen-free conditions and all studies performed were in accordance with institutional guidelines at the Animal Care Veterinary Service facility of the University of Ottawa and McMaster University.

#### **2.4 Experimental metastasis models**

#### **2.4.1 Therapeutic treatment model**

B16F10-LacZ cells ( $3 \times 10^5$ ) were injected i.v. into B6 mice. Mice were then treated intravenously with 3 doses of  $1 \times 10^8$  PFU MG1 in 100  $\mu$ l of PBS on days 1, 3, and 8, or control treated with 100 $\mu$ l PBS. At 14 days after cell injection, mice were euthanized, lungs were harvested and stained with X-gal (Bioshop, Mississauga, Canada) as described previously (Kirstein et al., 2009). The total number of lung surface visible metastases was determined on all 5 lobes using a stereomicroscope (Leica Microsystems, Concord, Canada).

#### **2.4.2 Prophylactic treatment model**

Mice were i.v. administered with a single dose of  $1 \times 10^8$  PFU MG1 or MG1-UV or MG1-Gless in 100 $\mu$ l PBS at 1 day before  $3 \times 10^5$  B16F10-LacZ cells were injected intravenously into B6 mice, mice were sacrificed at 3 days after tumor cells injection, and lungs were harvested and processed as mentioned above.

#### **2.5 Surgery and experimental metastasis model**

The surgery and experimental metastasis model was conducted as previously described (Tai et al., 2013). Briefly, mice received an i.v. challenge of  $3 \times 10^5$  B16lacZ cells to establish pulmonary metastases. Surgery (abdominal laparotomy and left nephrectomy) started 2 hours following tumor inoculation. Animals were euthanized at 3 days following tumor inoculation and their lungs were stained and total number of surface visible metastases was determined on all lung lobes as described above. For rescue of tumor cell clearance assays and survival study,  $1 \times 10^8$  PFU of MG1 or MG1-Gless or UV

inactivated MG1 virus was injected into mice 24h before surgery and  $3 \times 10^5$  B16lacZ cell were injected 2h before surgery.

## **2.6 Surgery and spontaneous metastasis model**

The surgery and spontaneous metastasis model was conducted as previously described (Tai et al., 2013). Briefly,  $1 \times 10^5$  4T1 breast tumor cells in 50 $\mu$ l of sterile PBS were injected orthotopically into the mammary fat pad of BALB/c mice at day 0. At 14 days post-tumor cell implantation, a complete resection of the mammary primary tumor along with abdominal nephrectomy was performed. For groups receiving OV treatment,  $1 \times 10^8$  PFU of OV was given 24 hours prior to surgery at 13 days. At 28 days post tumor implantation, lungs were isolated and weighed and photographs were taken and prepared for H&E staining.

## **2.7 Viability assay**

The B16F10lacZ cells were seeded into 96-well plates ( $1 \times 10^4$  cells/well). The next day cells were infected with the indicated viruses at various MOIs (0.01–1 PFU/cell). Following a 48-hour incubation, the cytopathic effects (CPE) were measured by pictures of cells under microscopy and metabolic changes by Alamar Blue. Alamar Blue (Resazurin sodium salt; Sigma-Aldrich, St Louis, MO) was added to a final concentration of 20 $\mu$ g/ml. After a 6 -hour incubation, the absorbance was read at a wavelength of 573 nm.

## **2.8 One-step growth curve**

Vero cells ( $2 \times 10^5$  cells/well of a 6 well plate) were infected with the indicated viruses at an MOI of 3 for 1 hour. Cells were then washed with PBS and incubated at 37°C. 100 µl aliquots were taken at time 0, 4, 8, 12, 24, and 48 hours post infection and titers assessed with a standard plaque assay.

## **2.9 Antibodies and Flow cytometry analysis**

To analyze splenic lymphocyte populations, spleens were removed from mice and RBCs lysed using ammonium chloride-potassium chloride lysis buffer . To analyze lung immune-infiltrating cells, lungs were removed from mice and digested with extraction buffer and lymphocytes were isolated using gradient Percoll (Sigma-Aldrich Canada Co., Oakville, ON). The following monoclonal antibodies (mAbs) were used: anti-TCRβ (H57-597), anti-CD122(TM-beta1) anti-CD69 (H1.2F3), anti-CD11c (N418), anti-CD19 (eBio1D3), anti-B220 (RA3-6B2), anti-CD11b (M1/70) and anti-F4/80 (BM8) were purchased from eBioscience. Isotype controls were purchased from BD Biosciences. Spleen NK cell IFNγ and GranzymeB secretion were examined following a 60min GolgiPlug (BD Bioscience) incubation using: anti-TCRβ (H57-597), anti-CD122(TM-beta1), anti-GranzymeB (16G6) and anti-IFNγ (XMG1.2) all from BD Bioscience. Flow cytometry acquisitions were performed on a CyAN-ADP using Summit software (Beckman Coulter). Data was analyzed with Kaluza software (Beckman Coulter).

## **2.10 NK cell depletion in experimental metastasis model**

NK cells were depleted using an optimized dose and schedule of anti-NK1.1 antibody kindly provided by Dr. Andrew Makrigiannis. Briefly, 200µg were injected i.p. on days

-4, -1 and +1 (Tai et al., 2013).  $3 \times 10^5$  B16lacZ cells were intravenously injected into B6 mice at day 0 and  $1 \times 10^8$  PFU MG1 or MG1-UV<sup>2min</sup> virus was given intravenously at day -1. The lung tumor burden was quantified at 3 days post-tumor cell injection. NK cell depletion was confirmed at day 0 prior to cell injection.

### **2.11 *Ex vivo* NK cell cytotoxicity assay**

The chromium release assay was performed as previously described (Patel et al., 2010). Briefly, splenocytes were isolated from treated and control mice at the indicated time. Pooled and DX5<sup>+</sup> sorted NK cells were resuspended at a concentration of  $1.5 \times 10^6$  cells/ml and then mixed with chromium labelled target cells (YAC-1, B16lacZ and 4T1), which were re-suspended at a concentration of  $3 \times 10^4$  cells/ml at different E:T (50:1 or 25:1, 12:1, 6:1 or 3:1). For rescue of NK cell impairment assays,  $1 \times 10^8$  PFU MG1 or MG1-Gless or UV inactivated MG1 were injected into mice 1 day before surgery. For assessment of NK cell cytotoxicity of CD11c.DTR mice, Diphtheria Toxin (DT) (4ng/g body weight) or PBS were i.p. injected at 1 day prior to  $1 \times 10^8$  PFU MG1 i.v. injection, and NK cells were isolated from spleen at 2 days after MG1 injection for assay. For NK-DC co-culture cytotoxicity assay, isolated NK cells or cultured bone marrow-derived dendritic cells (BMDCs) (Lutz et al., 1999) were infected with MG1 at MOI of 3 for 1 hour, then washed for 3 times. Then non-infected NK cells and BMDCs or infected BMDCs (1:1 ratio) co-culture at 37 °C incubator for overnight, the other groups (NK, DC+MG1, and NK+MG1) also were incubated at the same condition for overnight. The second day, NK cytotoxicity assay was performed on Yac-1 cells.

## 2.12 Western blotting

B16lacZ cells ( $2 \times 10^5$ ) were infected with MG1 or UV inactivated MG1 at 3 MOI as described above. After 18h, cells were washed with PBS for 3 times and lysed. For *in vivo* MG1 infection of lung with or without tumor tissue,  $1 \times 10^8$  PFU MG1 were i.v. injected into C57BL/6 mice at day -1, mice received intravenous PBS or  $3 \times 10^5$  B16lacZ cells at day 0, and total proteins were extracted from lung tissues at day+1. Protein concentrations of cell lysates were measured using the BCA<sup>TM</sup> protein assay (Thermo Scientific).  $1 \times 10^6$  PFU of naked virus was also used directly in the western blot. Protein samples were separated by SDS-PAGE (Bio-rad) and transferred to polyvinylidene difluoride membranes. Blots were probed with anti-Rhabdovirus antibody recognizing the viral proteins. Immunoreactive proteins were detected by enhanced chemiluminescence. Blots were probed with an anti-GAPDH antibody (Sigma) to confirm equal protein loading.

## 2.13 Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from either virus infected cells or lung tissue using RNeasy<sup>®</sup> Mini Kit (Qiagen, Canada) or straight virus using the QIAamp<sup>®</sup> Viral RNA Mini Kit (Qiagen, Canada), following the manufacturer's suggested protocols. Quantitative RT-PCR of Maraba MG1 genome was performed using the Taqman One-Step RTPCR Master Mix kit (Applied Biosystems, Alameda, CA, USA) with the following primers:

Maraba-forward: 5'-GGTGATGGGCAGACTATGAAA-3';

Maraba-reverse: 5'-CCTAAGGCCAAGAAACAAAAGAG-3';

Maraba-probe:

5'-/56-FAM/CCTCGATCA/ZEN/AGAGTGTTTGAACCCTGT/3IABkFQ/-3';

Mouse-Gapdh-forward: 5'-GTGGAGTCATACTGGAACATGTAG-3';

Mouse-Gapdh-reverse: 5'-AATGGTGAAGGTCGGTGTG-3';

Mouse-Gapdh-probe: 5'-/5/HEX/TGCAAATGG/ZEN/CAGCCCTGGTG/3IABkFQ/-3'.

#### **2.14 Hematoxylin & Eosin (H&E) staining**

In 4T1 spontaneous metastasis experiments, mouse lungs were harvested and fixed in 10% neutral buffered formalin for 24h. All tissues were paraffin embedded and sectioned at the Department of Pathology and Laboratory Medicine, University of Ottawa. H&E staining was also performed at the same histology facility.

#### **2.15 Immunohistochemistry (IHC) analysis of lung tumor tissue**

IHC was performed on formalin-fixed, paraffin-embedded 5µm mouse lung tissue sections. The rabbit anti-Rhabdovirus antibody recognizing viral proteins was used at a dilution of 1:250 for 1 hour. Microwave heating in a solution of sodium citrate (pH 6) was performed prior to incubation with the primary antibody. The goat anti-rabbit antibody conjugated with horseradish peroxidase was applied as the secondary antibody. 3, 3'-Diaminobenzidine as enzyme substrate was used to develop brown staining wherever the enzymes were bound. Negative control slides were prepared with lung tissue from uninfected B16lacZ tumor bearing mice. Positive control slides were prepared from pancreatic tumor bearing mice receiving intratumoral injections of MG1 virus.

#### **2.16 Electron Microscopy (EM)**



*Negative staining of straight MG1 virus* 20ul of  $1 \times 10^9$  PFU/ml virus in PBS was prepared. EM forceps were used to remove a pre-made carbon-coated copper EM grid from storage Petri dish and flat was placed on the surface of the agar well. A 10  $\mu$ L MLA pipette was used to place 1 drop of suspension onto EM grid. The grid was air dried for approximately 3-5 minutes and then 1 drop of 2% Phosphotungstic Acid stain was placed on the grid. The grid was allowed to dry approximately 30 seconds and removed from agar and placed on corresponding filter paper in small Petri dish. The grid was screened under the Hitachi H7100 Transmission electron microscope.

*For synchronized infection*,  $1 \times 10^6$  B16F10lacZ cells or BMDCs were incubated in 500  $\mu$ l 10% FBS DMEM and MG1 or MG1-UV<sup>2min</sup> virus at a MOI of  $5 \times 10^2$  PFU/cell for 20min at 4°C. This permitted viral binding to the cell surface. The cells were then incubated for 20minutes at 37°C, followed by a wash in 1ml of PBS. Then cells were fixed in 2.5% cacodylate buffered glutaraldehyde for a minimum of 2 hours. Cells were centrifuged and the pellet resuspended in sodium cacodylate buffer pH7.2 and through subsequent pelleting and resuspension, post-fixed in 2% Osmium Tetroxide for 2 hours, rinsed in water, and dehydrated through graded Ethanol up to absolute. A final dehydration in pure acetone was followed by three changes in Spurr's resin, and a final embedding at 65 °C. Thin (80nm) sections were cut using a Leica Ultracut R ultramicrotome, and stained with uranyl acetate, and lead citrate. The grids were screened on a Hitachi 7100 transmission EM and the images digitally captured.

## **2.17 Statistical analysis**

Statistical significance was determined by student t test (unpaired) or one-way ANOVA and post Tukey's test with a cutoff P value of 0.05. Data is presented as +/- SD (standard deviation). Survival curve were represented according to the KaPlan-Meier method, and compared using the log-rank test using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). Difference between groups were considered significant when  $P < 0.05$ .

## Chapter 3: Results

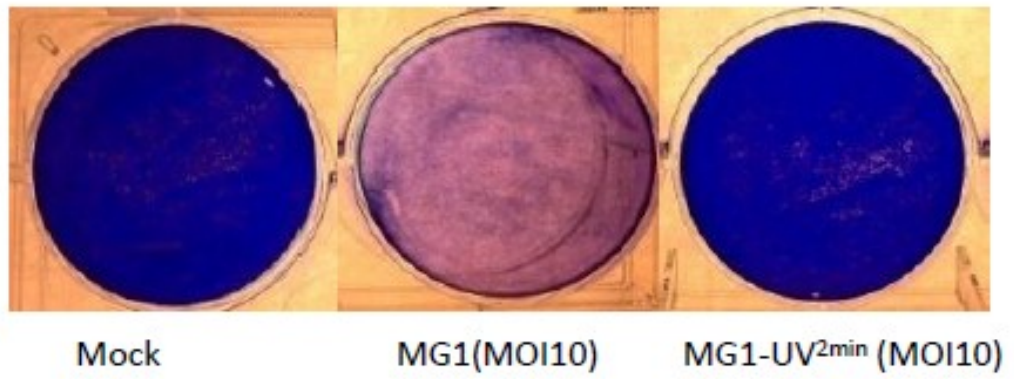
The first objective was to characterize the ability of intravenous delivered MG1 to stimulate NK cells of the innate immune system in a murine model of metastases. Towards this end, we sought to determine the extent to which oncolysis by MG1 was critical for its efficacy.

### **3.1 Live MG1 but not UV inactivated MG1 can infect, replicate in and kill B16lacZ cells *in vitro***

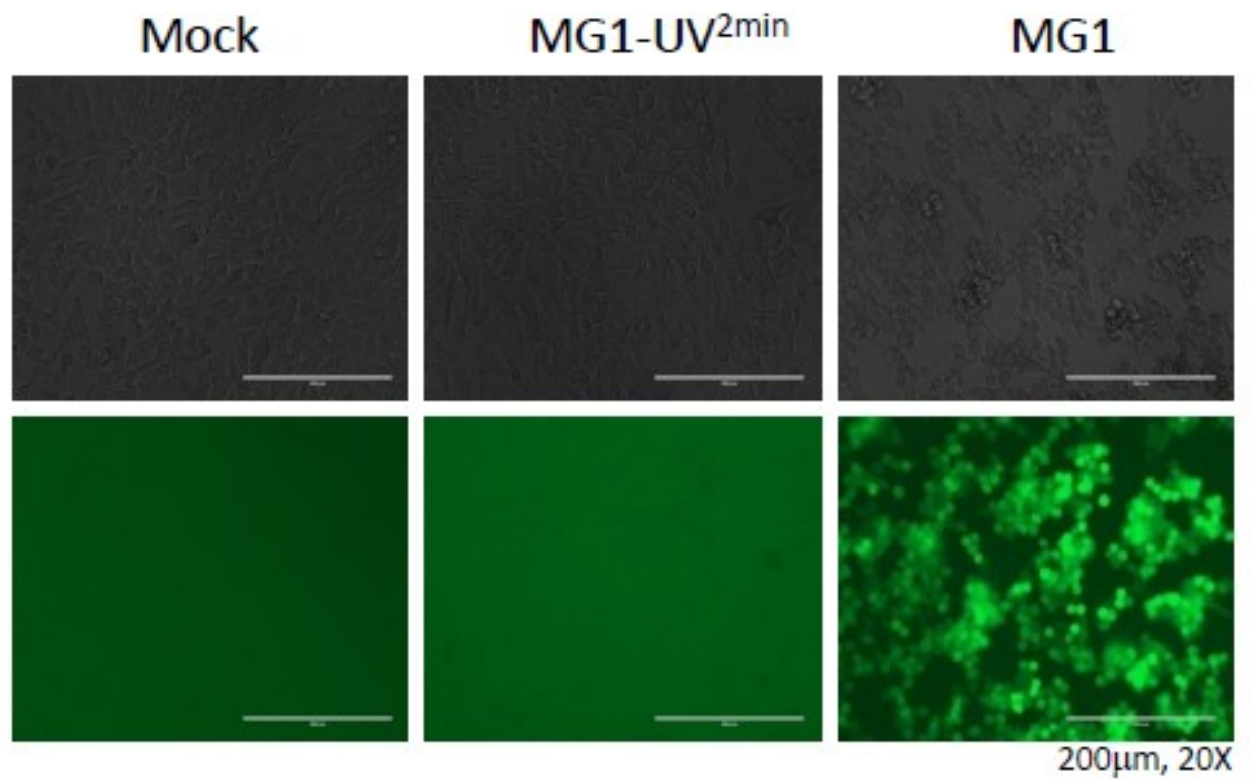
Maraba MG1 has been established as a potent oncolytic virus, however, when we used UV to inactivate MG1 virus for 2 minutes, we found that 2-minutes of UV inactivation abrogated plaque formation (Figure 3.1a), CPE and virus gene expression (Figure 3.1b) in B16lacZ cells. Live MG1 was able to effectively infect and kill melanoma B16lacZ cells as measured by CPE (Figure 3.1c) and cell viability assay (Figure 3.1d), while MG1-UV<sup>2min</sup> could not kill B16lacZ cells (Figure 1c&d) *in vitro*. These data indicate that B16lacZ cells are very susceptible to MG1 infection *in vitro*. As mentioned in the Aim 3b, we successfully generated a non-infectious and non-cytotoxic MG1, MG1-UV<sup>2min</sup>, by 2-minute UV irradiation.

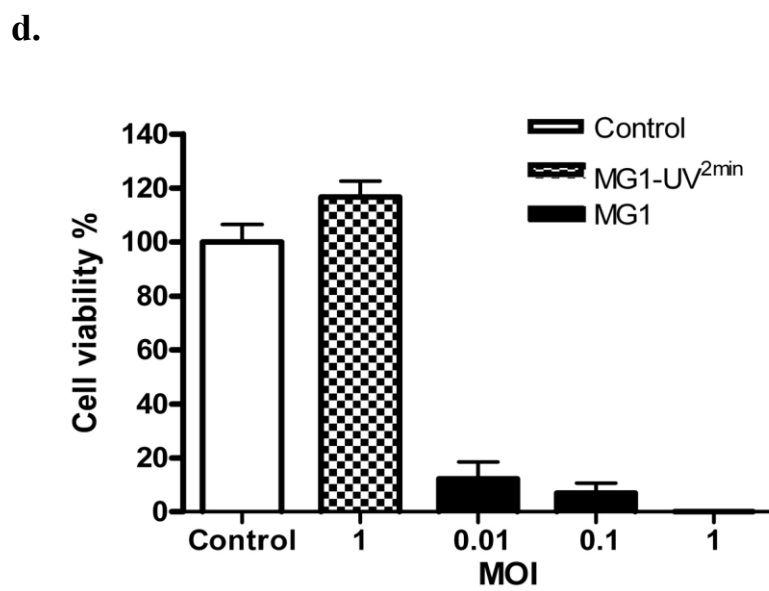
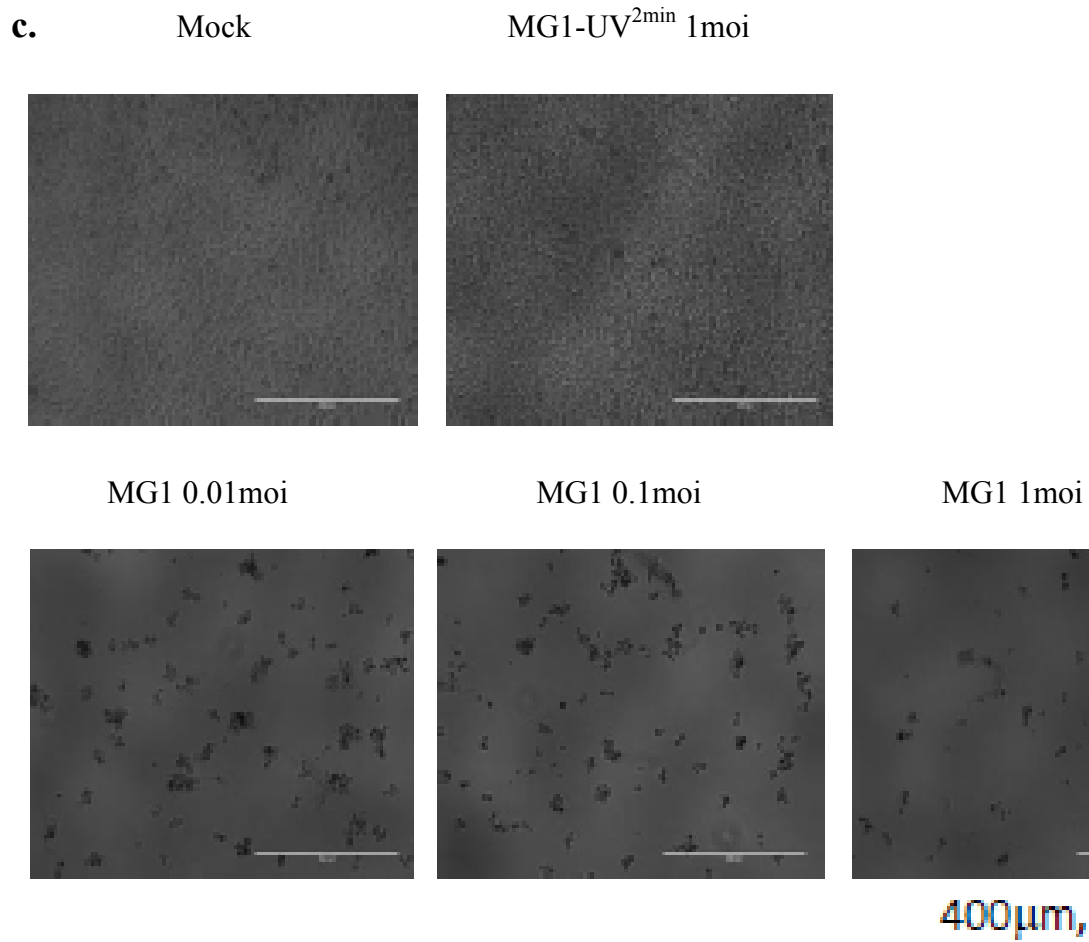
**Figure 3.1 MG1 infection of B16lacZ *in vitro* and confirmation of UV inactivated MG1**

**a.**



**b.**





**(a)** Plaque assay (infection at MOI of 10) and **(b)** CPE and GFP expression (infection at MOI of 1, 24h post infection (p.i) to confirm UV inactivated MG1 (MG1-UV<sup>2min</sup>). (Scale bar: 1cm=200um) **(c)** CPE on B16lacZ cell lines of live MG1 and MG1-UV<sup>2min</sup> at indicated MOI, 48hours after infection (magnification: x10, scale bar: 1cm=400um). **(d)** B16lacZ cells were infected with indicated virus at different MOI. 48h p.i., cell viability was assessed by Alamar Blue (control- no infection). Data are representative of 2 similar experiments.

### **3.2 The *in vivo* efficacy of MG1 in the B16lacZ tumor model is not dependent on viral oncolysis**

MG1 has been reported to be an efficacious OV that resulted in durable cures when administered in both the CT26 subcutaneous and ES2 ovarian xenograft models (Brun et al., 2010). Therefore, we simultaneously characterized both the oncolytic and anti-tumor effects of MG1 in the C57Bl/6 mouse strain and its syngeneic B16lacZ melanoma lung model. We have previously demonstrated that *in vitro* B16lacZ is very susceptible to MG1 virus infection and killing, but we wanted to determine if oncolytic activity was necessary for MG1 efficacy *in vivo*. Therefore, we tested for the *in vivo* efficacy of MG1 in both therapeutic and prophylactic B16lacZ lung tumor metastasis models.

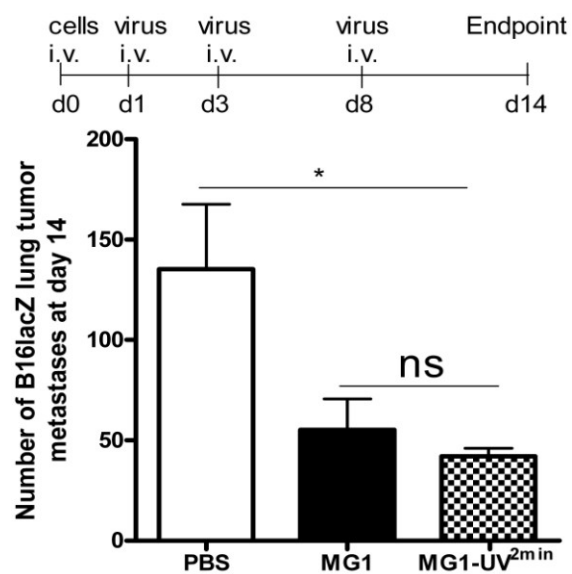
In the therapeutic treatment model, a triple dose of MG1 ( $1 \times 10^8$  PFU/mouse) treatment resulted in a significant reduction of lung metastases in tumor-bearing mice compared with PBS controls (Fig. 3.2a). Similarly, single prophylactic dose of MG1 ( $1 \times 10^8$  PFU/mouse) administered 1 day before tumor cell injection dramatically attenuated lung tumor metastases quantified at 3 days after tumor inoculation (Fig. 3.2b). To further investigate whether viral replication was necessary for the *in vivo* efficacy achieved in our B16lacZ lung metastasis models, treatment with MG1-UV<sup>2min</sup> was administered in parallel. Quantification of lung surface metastases in both models indicates that MG1-UV<sup>2min</sup> can significantly reduce lung metastases to equivalent levels as live MG1 (Figs 3.2a&b). In addition, we checked for the presence of virus in the lung at 2 days after MG1 injection. Not surprisingly, we detected neither virus protein by Western blot and IHC (Fig 3.2c&d) nor virus genomes by qRT-PCR (data not shown) in tumor bearing lungs, suggesting productive infection by MG1 is minimal in B16lacZ

metastases *in vivo*. Taken together, these data suggest that the *in vivo* efficacy of MG1 in our B16lacZ lung metastasis model is not dependent on viral oncolysis. We, therefore, hypothesized that MG1-elicited anti-tumor immunity might be responsible for the lung tumor clearance.

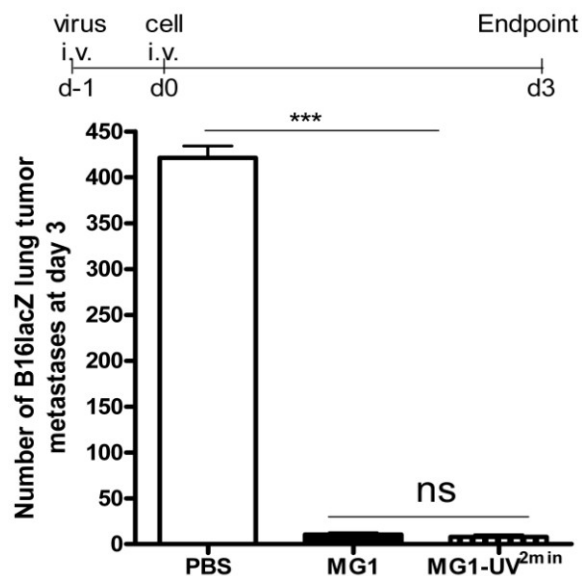


**Figure 3.2 The *in vivo* efficacy of MG1 and MG1-UV<sup>2min</sup> in B16lacZ lung metastasis model**

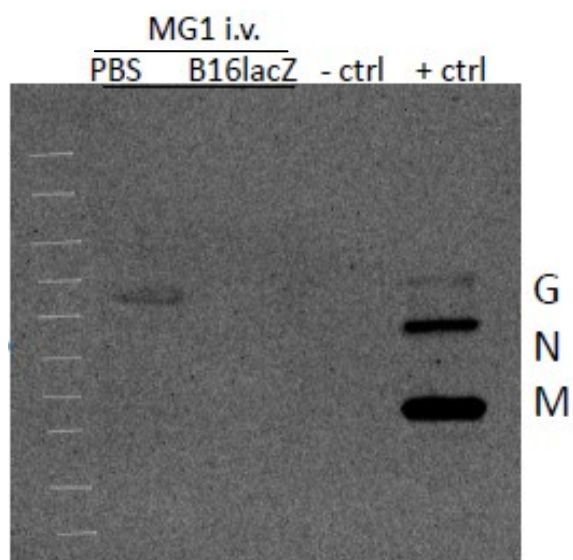
**a.**



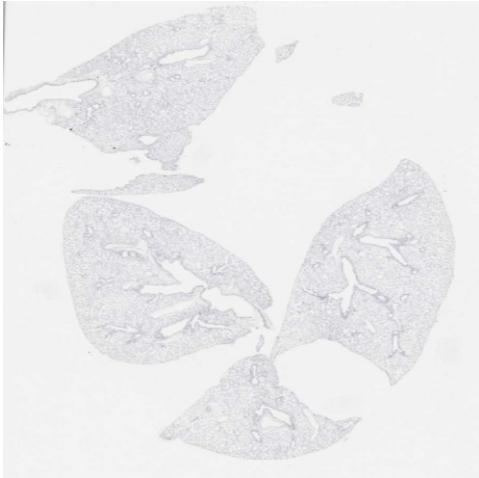
**b.**



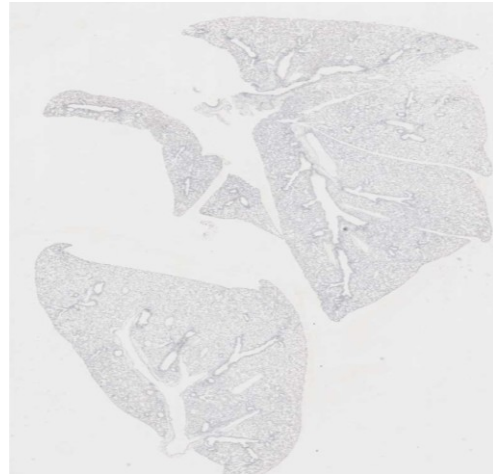
**c.**



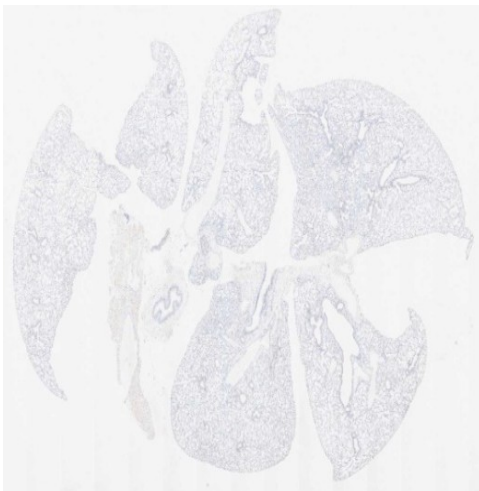
**d.** MG1 i.v. (naïve lung)



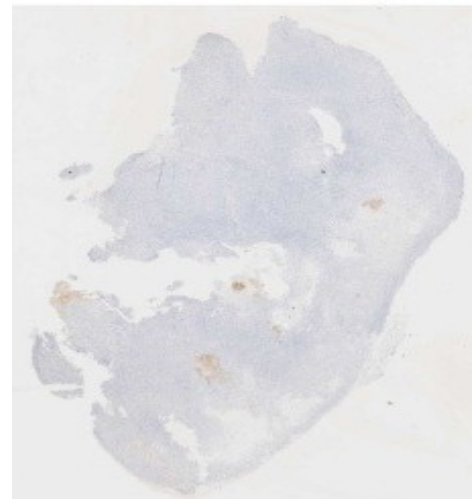
MG1 i.v. (B16lacZ tumor bearing lung)



B16lacZ tumor bearing lung (-)



MG1 i.t. Pancreatic tumor (+)



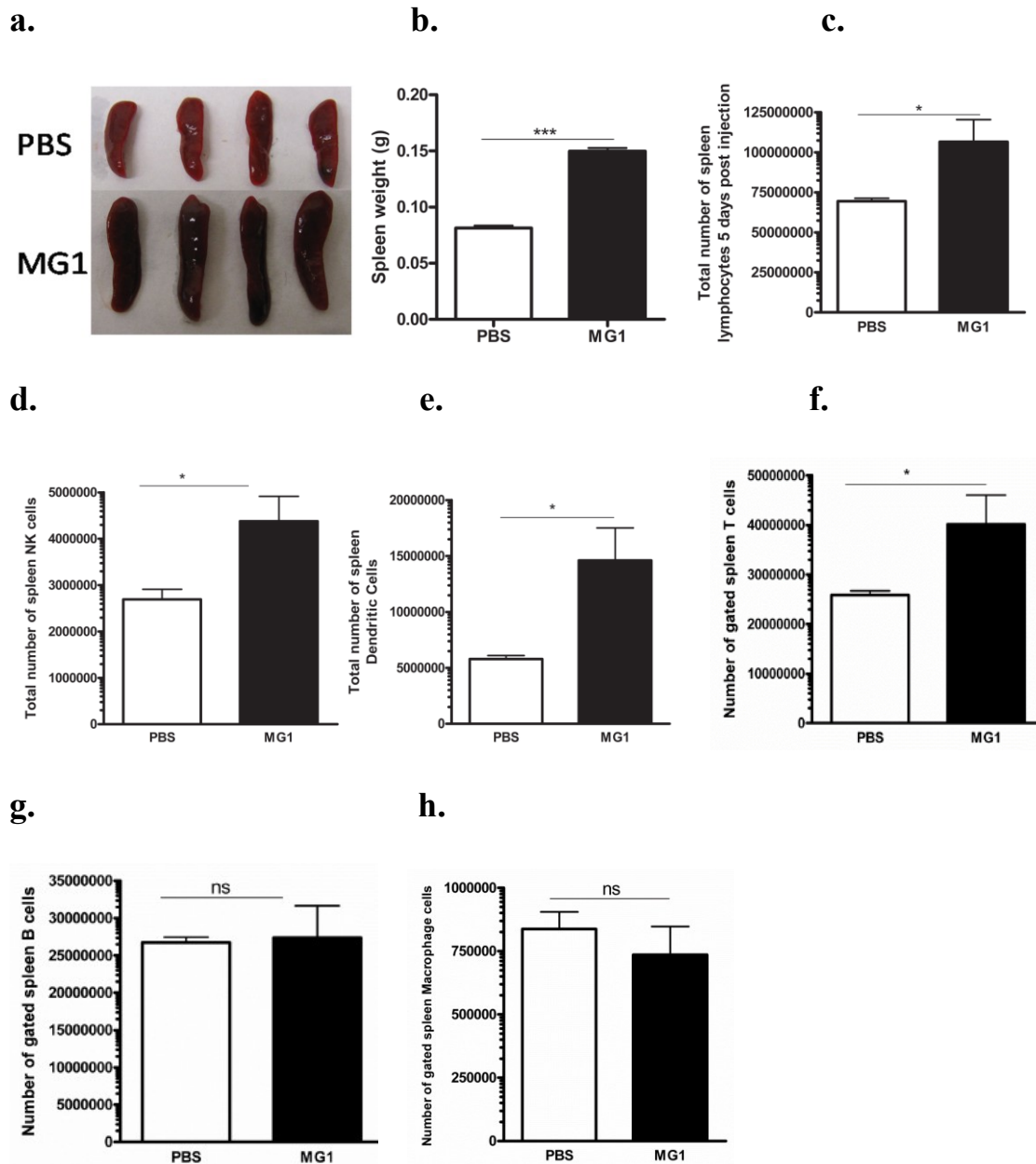
**(a)** B16lacZ cells were administered i.v. into B6 mice via tail vein at day 0. Virus treatment was given at day 1, 3 and 8 days. At day 14 lung metastases were stained and quantified. **(b)** Virus at  $1 \times 10^8$  PFU/ mouse were given at 1 day before cell injection. Lung metastases were quantified at 3 days after tumor inoculation. MG1 ( $1 \times 10^8$  PFU/mouse) was i.v. injected into B6 mice via tail vein at day 0 and on the next day mice received either PBS or  $3 \times 10^5$  B16lacZ cells i.v. injection. At day 2 lungs were assessed for presence of viral protein by **(c)** Western blot and **(d)** Immunohistochemistry. Data are

representative of 3 similar experiments with n=3-6/group (ns, not significant; \* $p<0.05$ ;  
\*\*\* $p<0.001$ ; -, negative control; +, positive control)

### **3.3 i.v. administration of MG1 leads to the expansion of immune cells in the spleen**

Given the ability of non-replicating MG1-UV<sup>2min</sup> to reduce lung metastases (Fig. 3.2a&b) and our inability to detect MG1 replication in tumor bearing lung (Fig. 3.2c&d), we wanted to evaluate the contribution of the immune system in the MG1-mediated efficacy in the lung metastasis model. Spleens isolated from B6 mice showed significant splenomegaly (Fig. 3.3a) and increased spleen weight (Figs. 3.3b) at 5 days after MG1 i.v. administration. The splenomegaly was associated with a dramatic increase in absolute numbers of lymphocytes in the mouse spleen (Fig. 3.3c). A significant expansion was observed in the number of immune lymphocytes (Figs 3.3d: NK cells, 3.3e: dendritic cells and 3.3f: T cells) as opposed to other immune populations (Fig. 3.3g: B cells and 3h: macrophage cells). Considering this early time point post virus administration and the significant expansion of innate immune cells, we reasoned that innate immune cells most likely play a significant role in MG1-mediated efficacy in the prophylactic treatment lung metastasis model.

**Figure 3.3 The early immune stimulation after MG1 i.v. administration in the spleen**



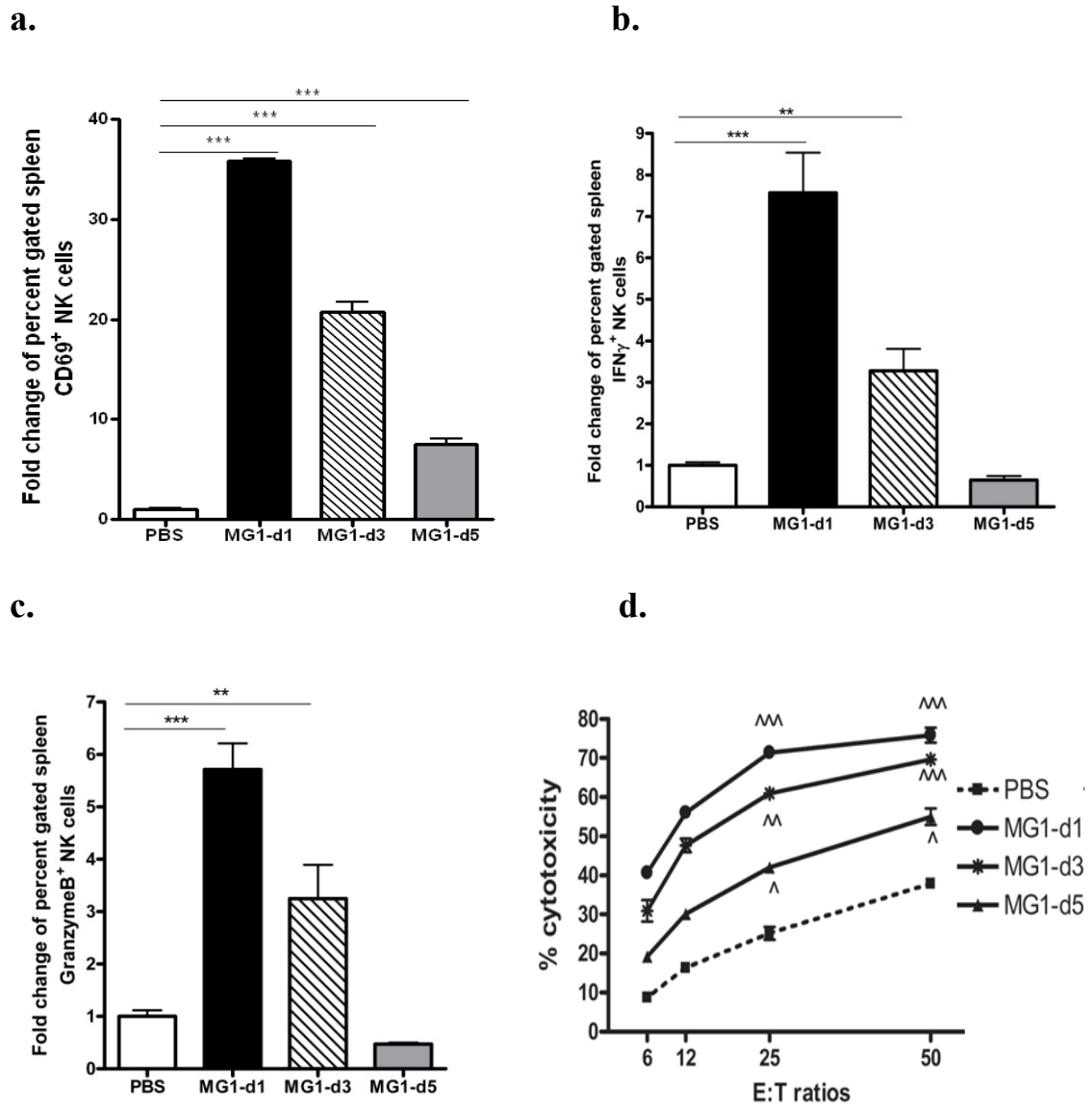
B6 mice were treated with MG1 or PBS i.v. and sacrificed at 5 days post virus treatment. Spleens were isolated and assessed as follows: **(a)** photographs, **(b)** weight, **(c-h)** total number of **(c)** lymphocytes, **(d)** NK cells ( $\text{TCR}\beta^-/\text{CD122}^+$ ), **(e)** DC ( $\text{CD11c}^+$ ), **(f)** T cells ( $\text{TCR}\beta^+\text{CD122}^-$ ), **(g)** B cells ( $\text{CD19}^+\text{B220}^+$ ) and **(h)** macrophages ( $\text{F4/80}^+\text{CD11b}^+$ ). Data

are the results of 3 similar experiments with  $n=4-5/\text{group}$  (ns, not significant; \*  
 $p<0.05$ ; \*\*\* $p<0.001$ )

### **3.4 Intravenous administration of MG1 demonstrates an immediate and dramatic activation of NK cells**

NK cells represent a major component of cellular innate immunity that control tumor growth and prevent metastases, we therefore examined NK cell functionality by flow cytometry and cytotoxicity assay. MG1 intravenous administration led to an immediate and strong activation of NK cells, as shown by significantly elevated expression of the early activation marker of NK cells, CD69 (Fig. 3.4a), intracellular cytokine expression of IFN $\gamma$  (Fig. 3.4b) and Granzyme B (Fig. 3.4c) at day 1 and day 3 post-infection. In parallel, *ex vivo* cytotoxicity of splenic NK cells isolated from MG1 infected mice at the same end points demonstrated dramatically enhanced tumor target killing compared to PBS controls (Fig. 3.4d). As set out in objective 1, our data clearly shows that spleen NK cells have been dramatically stimulated in both quantity and quality, which leads us to reason that NK cells might be mediator for the clearance of metastases.

**Figure 3.4 A significant NK cell activation in the spleen was observed after MG1 i.v. administration**



B6 mice were treated with MG1 ( $1 \times 10^8$  PFU) i.v or PBS and sacrificed at 1, 3 and 5 days post virus treatment. Spleens were harvested to assess NK cell (a) CD69 expression, (b) IFN- $\gamma$ , (c) Granzyme B secretion and (d) NK cytotoxicity (^,  $p < 0.05$ ; ^^,  $p < 0.01$ ; ^^,  $p < 0.001$  comparing treatment to PBS controls. The data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for the indicated E:T ratios).

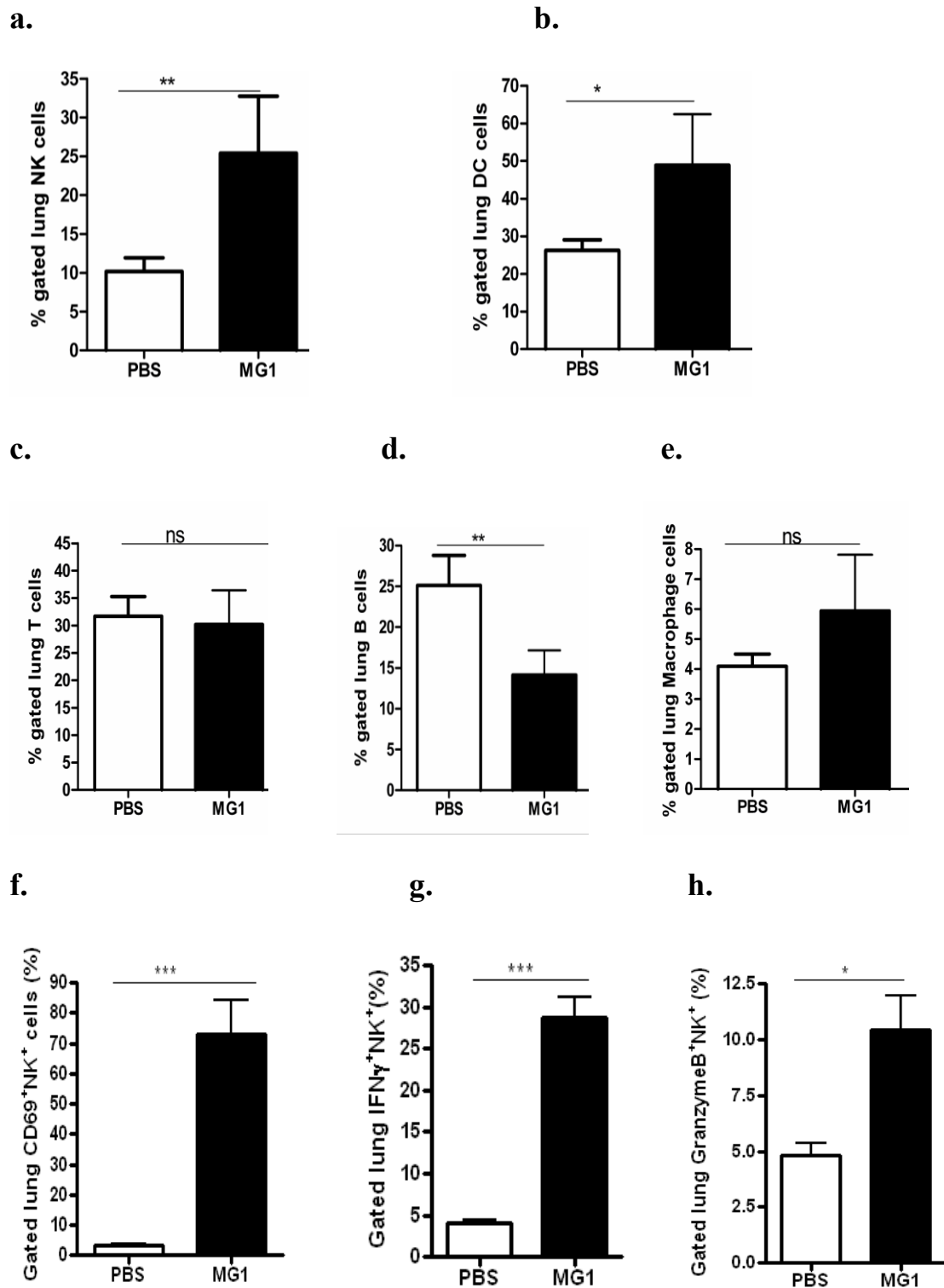


Data are representative of 3 similar experiments with n=4-6/group. (\*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ )

### **3.5 Lung innate immune cells, such as NK cells, are expanded and activated significantly in MG1 prophylactic treatment model of lung metastasis**

Spleen NK cells were activated by MG1 *in vivo* (Fig. 3.4a-d), however, the target organ is the lung in our prophylactic metastasis model. So we decided to examine the lung immune-infiltrating cells in our model. Similarly, we observed a significant increase in the proportion of lung NK cells (Fig. 3.5a) and DC (Fig. 3.5b) in mice treated with MG1 compared to the PBS group. However, we did not see the same trend in lung T cells (Fig. 3.5c), B cells (Fig. 3.5d) and macrophage cells (Fig. 3.5e). We also found a significant activation of lung NK cells as demonstrated by the increased expression level of CD69 (Fig. 3.5f), IFN $\gamma$  (Fig. 3.5g) and Granzyme B (Fig. 3.5h) at 1day post MG1 treatment compared to the PBS control. Hence, these data further suggest that activated innate immunity, specifically lung and splenic NK cells, might play a significant role in MG1-mediated efficacy *in vivo*.

**Figure 3.5 Examination of lung immune-infiltrating cells in the prophylactic treatment model**



B6 mice received a single dose of MG1 at 1 day before B16lacZ cells i.v. injection. At 3 days post tumor inoculation, the proportion of lung **(a)** NK cells ( $CD122^{+}TCR\beta^{-}$ ), **(b)** DCs ( $CD11c^{+}CD8\alpha^{+}$ ), **(c)** T cells ( $TCR\beta^{+}CD122^{-}$ ), **(d)** B cells ( $CD19^{+}B220^{+}$ ) and **(e)** Macrophage cells ( $F4/80^{+}CD11b^{+}$ ) were assessed. Analysis of the proportion of lung NK cell CD69 **(f)**,  $IFN\gamma$  **(g)** and Granzyme B **(h)** secretion was conducted at 1 day post MG1 administration.. Data are representative of 3 similar experiments with n=4-5/group. (ns, not significant; \*,  $p<0.05$ ; \*\*,  $p<0.01$ , \*\*\*,  $p<0.001$ )

### **3.6 DC-mediated NK cell activation is responsible for the *in vivo* efficacy of MG1 in the prophylactic treatment model**

To further confirm that MG1 stimulation of NK cells is responsible for the removal of tumor metastases, the prophylactic lung metastasis model was performed in the absence of NK cells through pharmacological depletion using anti-NK1.1 antibody. Prior to starting the experiment, NK cells depletion was confirmed by flow cytometry with less than 5% NK positive cells remaining in these mice. In mice depleted of NK cells, both PBS controls and MG1 treated mice developed increased lung tumor burden, suggesting a mediating role for NK cells in the clearance of metastases after MG1 administration (Fig. 3.6a). More importantly, we found that the ability of MG1 to attenuate lung metastases was completely abrogated in mice with NK cell depletion (Fig. 3.6a). Thus, our data demonstrate that NK cells play a dominant role in the reduction of metastases, which contributes to the *in vivo* efficacy of MG1 in this model.

As the interaction between OV and immune cells is critically important for the eradication of tumors, we decided to further characterize the interplay between NK cells and Maraba MG1. First, we wanted to determine if NK cells can be productively infected or activated by MG1 directly. We isolated and infected purified spleen NK cells with MG1 expressing GFP and measured cell viability (CPE) and productive infection (by GFP) at 24 hours. CPE pictures of NK cells infected with virus were the same as in the uninfected healthy NK cells and no GFP expression was detected following infection (Fig. 3.6b). In addition, we did not observe an increase in the early activation marker CD69 for NK cells (Fig. 3.6c), indicating that MG1 cannot directly infect or activate NK cells.

As MG1 is a single-stranded (ss) RNA virus, we reasoned that it might require other non-NK cells expressing different TLRs for interaction with immune cells. As mentioned previously, we observed expansion of DC both in the spleen (Fig. 3.3e) and in the lung (Fig. 3.5b) after MG1 treatment. We, therefore, hypothesized that DC might mediate MG1 recognition and uptake. Many reports have shown DC-mediated activation of NK cells, which results in increased NK cell functionality, such as cytolytic activity (Degli-Esposti et al., 2005). To address this inquiry, we used EM to analyze BMDCs infected with MG1 and observed different stages of MG1 virus entry into BMDCs, which suggest BMDCs can recognize and uptake MG1 virus (Fig. 3.6d). Then we co-cultured purified NK cells and BMDCs in the presence or absence of MG1 infection. At 24h following the NK-DC co-culture, we assessed NK cell cytotoxicity against YAC-1 target cells. We observed that MG1 infected DC-NK cell co-cultures displayed the highest amount of target cell lysis, while no cytotoxic activity was observed for NK cells or DC cultured alone in the presence or absence of MG1 infection (Fig. 3.6e). This data suggests that MG1 activation of NK cells requires DC.

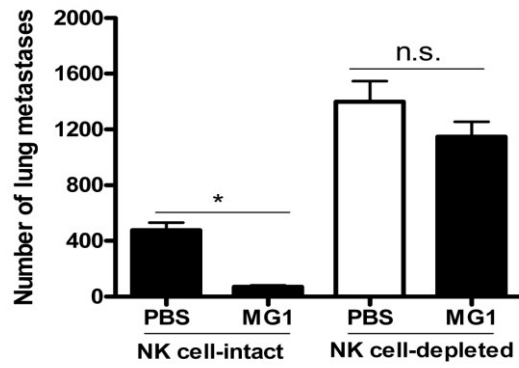
To further support our *ex vivo* data, we obtained CD11c.DTR Tg mice that allows for the inducible and specific ablation of DC with a single dose of DT (Jung et al., 2002). In the Tg mouse, a transgene was designed to place a simian DTR-Enhanced GFP (EGFP, Stratagene) fusion protein under the control of the CD11c/Itgax promoter, and transfer of a primate DTR into mice via transgenesis hence confers DT sensitivity to murine cells. Specificity and timing of cell ablation can be determined by cell type-restricted promoter / enhancer elements and by the regimen of the toxin administration, respectively (Jung et al., 2002). Both NK cell CD69 expression (Fig. 3.6f) and cytotoxicity (Fig. 3.6g) were

significantly reduced following MG1 administration in DT injected CD11c.DTR Tg mice compared to the MG1 control Tg mice. Also, NK cytotoxicity in MG1-DT group was lower than the PBS Tg mice (Fig. 3.6g), indicating that DC is required for the competence of NK cell function.

As set out in our Aim 2, these findings demonstrate the important mechanistic interplay between DC and NK cells following MG1 infection whereby MG1 activates NK cell function via DCs.

**Figure 3.6 DC-mediated NK activation following MG1 injection is responsible for the reduction of lung metastasis**

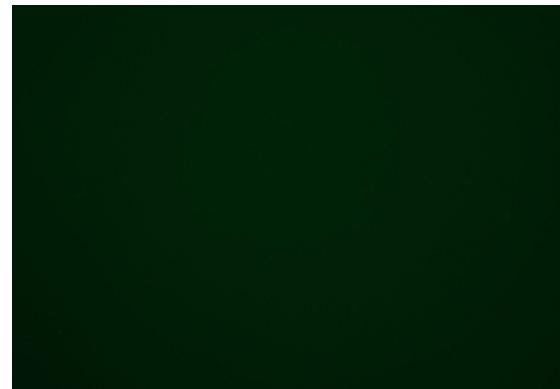
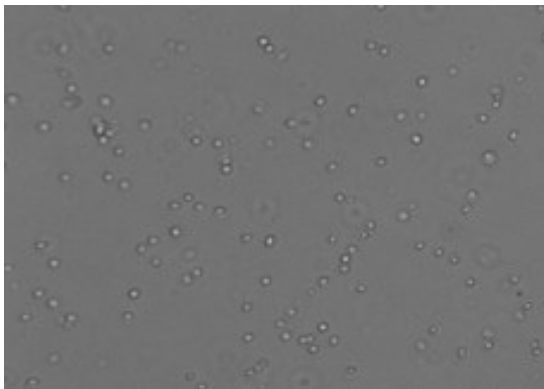
**a.**



**b.**

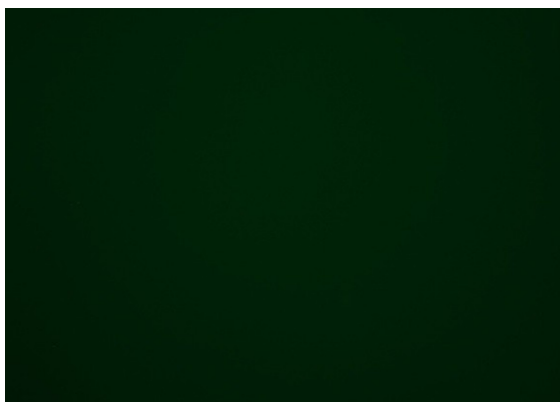
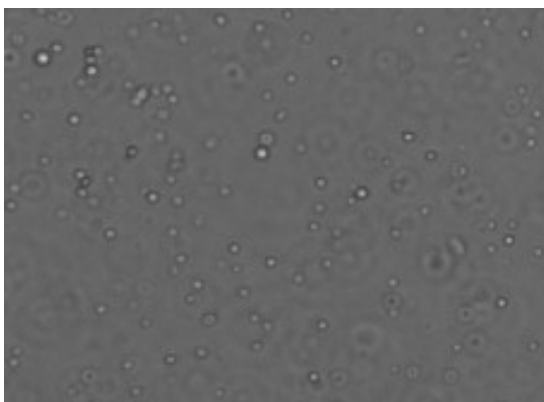
NK cell-CPE

NK cell- GFP



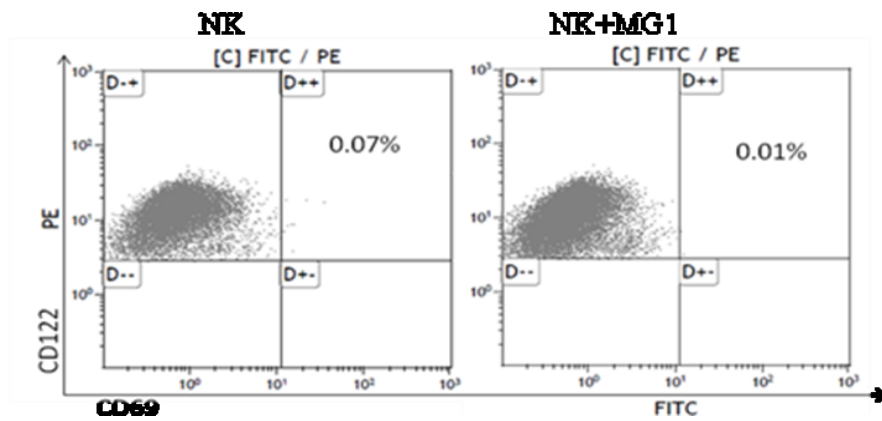
(NK cell + MG1)-CPE

(NK cell + MG1)-GFP

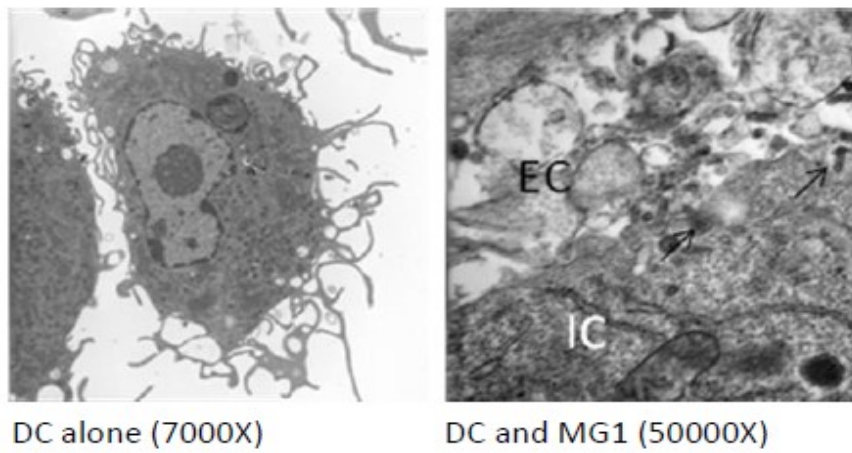




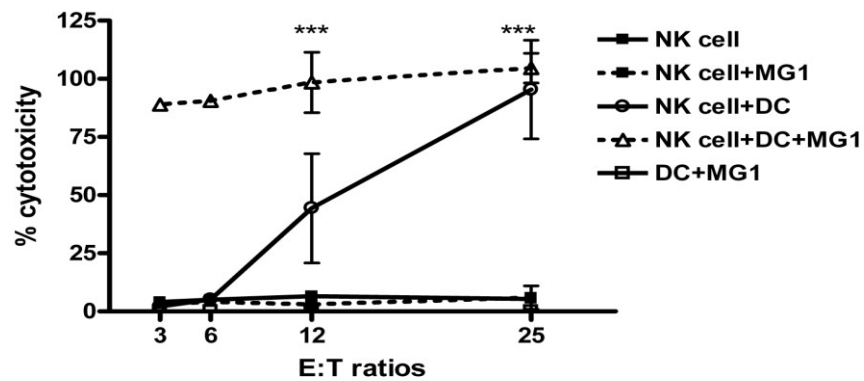
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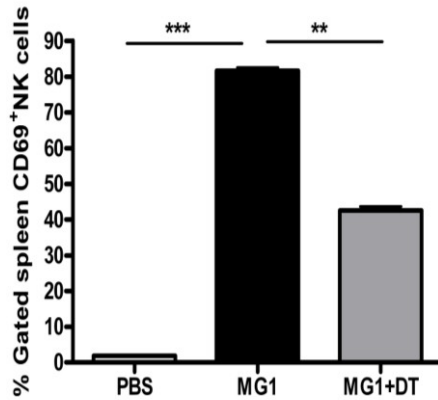
d.



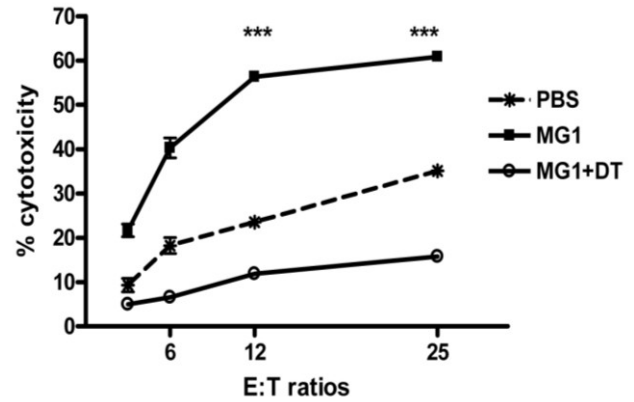
e.



f.



g.



(a) B6 mice were treated with MG1 i.v. or PBS at day -1. At day 0, all mice received  $3 \times 10^5$  B16lacZ tumor cells inoculation via tail vein. NK cells were depleted using anti-NK1.1 at days -4, -1 and +1. The number of lung tumor metastases was quantified at 3 days post cell injection. (b) Sorted NK cells from naïve B6 mice were infected with MG1-GFP at MOI of 3. At 24h post infection (p.i.), virus infectivity and cell killing were examined by assessing CPE and virus GFP expression; Shown are phase contrast (**left panel**) and fluorescent GFP (**right panel**). Then CD69 expression of NK cells with or without MG1 infection was performed by flow cytometry (c). (d) EM analysis of BMDCs and MG1 infected BMDCs. (EC=extracellular space; IC=intracellular space. Magnification:7000x and 50000x; arrow indicates the presence of virus). (e) *In vitro* NK cytotoxicity following NK cell-DC co-culture (\*\*\*,  $p < 0.001$  comparing NK cells+DC+MG1 to NK cells+MG1). (f, g) PBS or DT was injected i.p. into CD11c.DTR Tg mice. 24 hours following, MG1 and B16lacZ cells were injected i.v. Spleens were harvested at day 3 to assess (f) NK cell CD69 expression and (g) *ex vivo* NK cytotoxicity (\*\*\*,  $p < 0.001$  comparing MG1 treatment to PBS and MG1+DT controls; the data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for the

indicated E:T ratios). Data are representative of 3 similar experiments with n=5-6/group (\*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ).

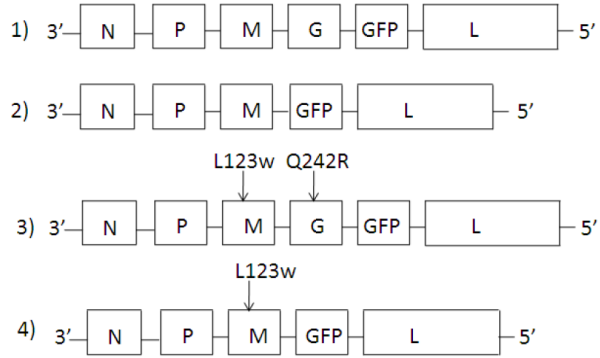
In Aim 3a, we sought to design, clone and rescue a G-less MG1 virus, which is capable of a single cycle of replication.

### **3.7 Generation of MG1-Gless: a single replication cycle virus**

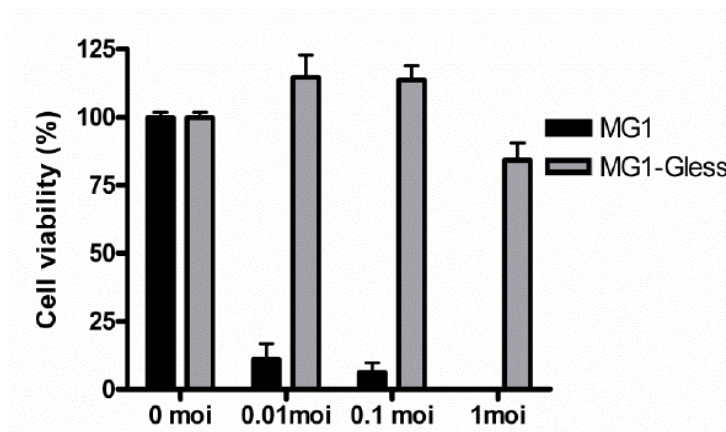
To further investigate the role of virus replication, including viral gene and protein expression and the production of live virus progeny on the activation of NK cell function by MG1 virus, we decided to generate a single-replication cycle virus-MG1-Gless, in which the gene encoding the MG1-G (glycoprotein) was removed (Fig. 3.7a). As we expected, MG1-Gless was much less cytotoxic during *in vitro* cell infection compared to MG1 (Fig. 3.7b), and no infectious virus particles were recovered from culture supernatants of B16lacZ cells during 48h following infection with MG1-Gless (MOI=3) (Fig. 3.7c); This is also corroborated by the following observation that upon exposure of fresh B16lacZ cells to supernatants collected from B16lacZ cells infected with MG1-Gless virus, neither GFP expression nor any cytotoxicity could be observed (Fig. 3.7d). Taken together, these data confirm that we generated a replication-defective, single cycle properties of the MG1-Gless virus.

**Figure 3.7 Single replication cycle MG1-Gless**

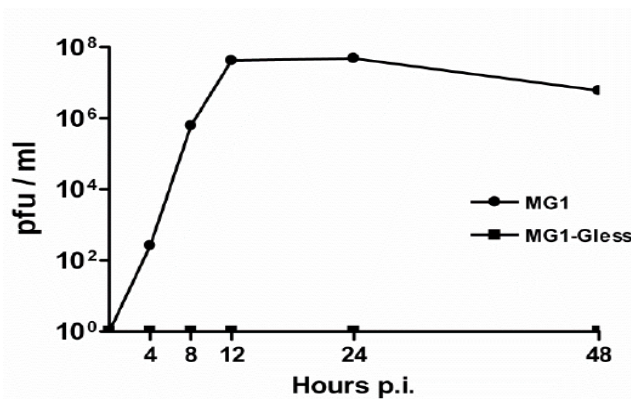
**a .**



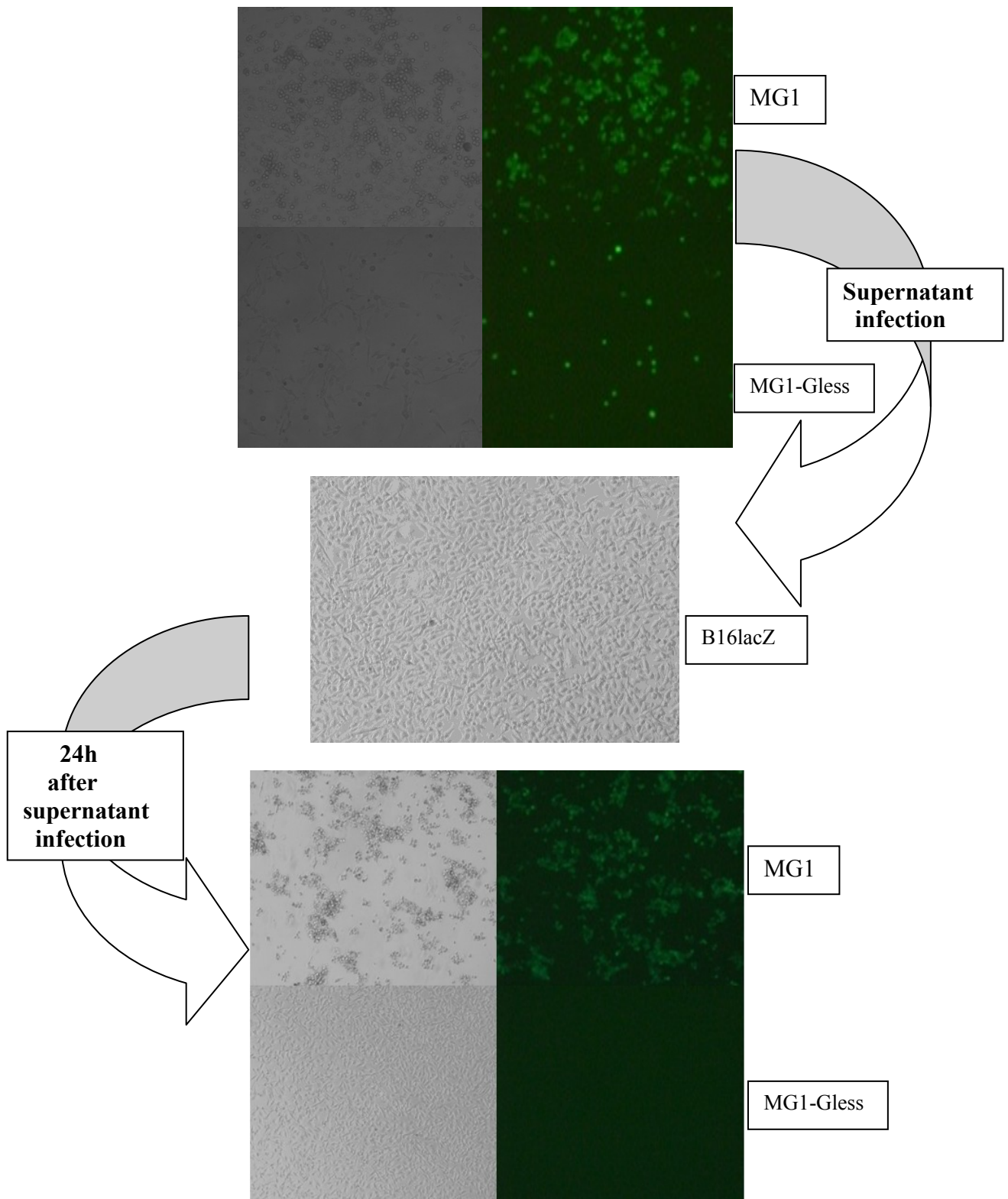
**b.**



**c.**



d.



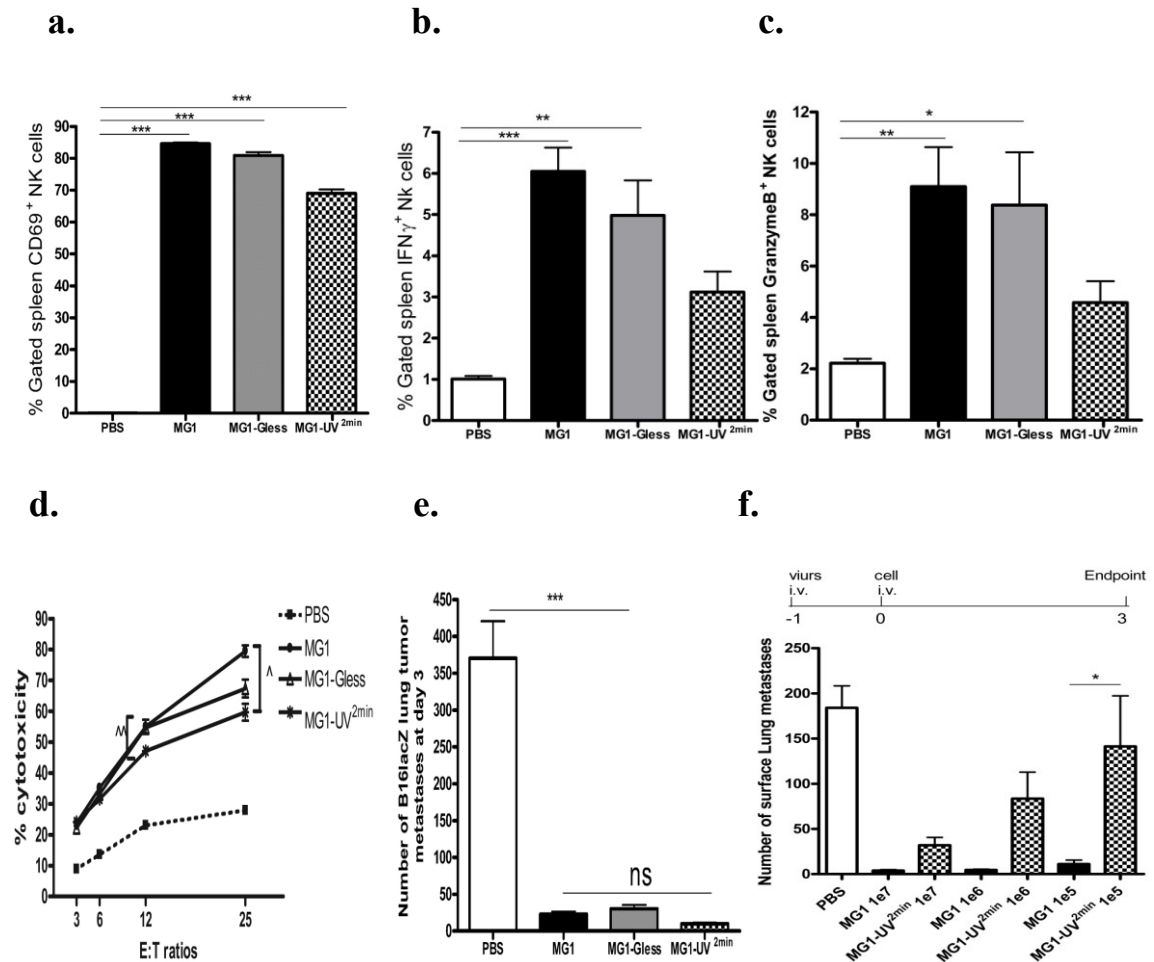
**(a)** The genome structure of 1) wild type Maraba-GFP virus; 2) wild type of Maraba-Gless-GFP virus; 3) MG1-GFP virus and 4)MG1-Gless-GFP virus. **(b)** B16lacZ cells were infected with MG1 or MG1-Gless at different MOI. At 48h p.i., cell viability was assessed by Alamar Blue. **(c)** One-step growth curve to assess the viral productivity of MG1, MG1-Gless in B16lacZ cells. **(d)** B16lacZ cells were infected with MG1-GFP or MG1-Gless-GFP virus at 0.1 MOI. At 24h p.i., 500  $\mu$ l filtered supernatant were transferred to new cell plates to do a second round infection. CPE and GFP pictures were taken at 24h p.i.. Data are representative of 2 similar experiments.

### **3.8 Virus replication is important, but not necessary for NK cell activation and attenuation of lung metastases**

In order to better understand the requirements for NK cell activation by MG1, we screened three variants of MG1: live MG1, MG1-Gless (capable of a single cycle of viral replication) and MG1-UV<sup>2min</sup> (replication-incompetent virus). All 3 MG1 variants demonstrated enhanced activation and function of NK cells (CD69, IFN $\gamma$ , Granzyme B, *ex vivo* cytotoxicity) over PBS controls (Figure 3.8a-d). However, live MG1 resulted in the highest NK cell activation and cytotoxicity, followed closely by MG1-Gless. While MG1-UV<sup>2min</sup> exhibited less amounts of NK cell function compared to its replicating counterparts (Figure 3.8a-d), it effectively attenuated *in vivo* B16lacZ lung metastases to near identical levels (Figure 3.8e). However, when we reduced the dose of virus administration in the prophylactic lung tumor metastasis model, we observed that live MG1 demonstrated a better efficacy than MG1-UV<sup>2min</sup> (Fig. 3.8f). In summary, as mentioned in Aim 4b&c, these data show that viral replication does indeed play a role in NK cell activation (higher NK activation with live MG1). More importantly, however, these data also demonstrates that a non-replicating OV (MG1-UV<sup>2min</sup>) at a high dose can reduce lung metastases as effectively as its live counterpart.



**Figure 3.8 Assessment of NK cell activation by i.v. administration of different MG1 variants**



PBS or indicated virus was injected i.v. into B6 mice. 24 hours post-virus injection, spleen NK cells were isolated to assess **(a)** CD69 expression, **(b)** IFN- $\gamma$ , **(c)** Granzyme B secretion via flow cytometry and **(d)** *ex vivo* NK cytotoxicity (^,  $p < 0.01$ ; ^^,  $p = 0.05$  comparing treatment to PBS controls; the data are displayed as the mean percent (+/-SD) of chromium release from triplicate wells for the indicated E:T ratios). **(e)** B6 mice treated with PBS or indicated virus at day 0. At day 1, all mice received B16lacZ tumor cell inoculation. Quantification of lung metastases at 3 days post cell injection. **(f)** Virus

at indicated dose were given at 1 day before cell injection. Lung metastases were quantified at 3 days after tumor inoculation. Data are representative of 3 similar experiments with n=4-6/group (\*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ; ns, not significant).

### **3.9 Viral particle structure, cellular association, viral proteins and viral genomic RNA is retained in minimally UV-inactivated virus (MG1-UV<sup>2min</sup>)**

The finding that MG1-UV<sup>2min</sup> could stimulate NK cells and reduce lung metastases as effectively as live MG1 was somewhat surprising, considering previous reports have indicated that a live, viable virus, which can express its genome, is required for the anti-tumor effects of oncolytic VSV (Galivo et al., 2010). One possible explanation for this discrepancy is the length of time the virus is exposure to UV. UV light is commonly used to deactivate viruses by creating nucleic acid dimers, which prevents further replication. However, the virus structure, viral capsids and protein components may still be intact and immunogenic. With a longer duration of UV exposure, however, proteins and other viral components may also be destroyed in addition to viral DNA/RNA (Smirnov et al., 1990). We, therefore, examined a panel of MG1 viruses that were UV inactivated at increasing time intervals ranging from 2 min (minimum amount of UV inactivation to abrogate plaque formation and GFP expression), 5 min, 30 min, 1 hour and 2 hours, and compared them to live MG1.

First, EM was used to visualize the morphology of this panel of UV inactivated viruses. EM examination of purified live MG1 revealed the classical characteristics of rhabdoviruses - individual bullet-shaped virus particles (Fig. 3.9a). After 2 min of irradiation, no significant morphological changes were observed and the virus particles closely resemble that of live MG1 (Fig. 3.9a). After 5 min of irradiation, a structural change of the virus envelope/particle was detected along with a greater proportion of clumped virus particles. During longer periods of UV irradiation (30 to 120 min), a progressively greater proportion of viral particles was aggregated and exhibited severe

viral structural change (Fig. 3.9a). To further study if virus-cell interaction was affected by UV irradiation of viruses, EM examination of B16lacZ cells infected with live MG1 was undertaken with specific attention to the presence of invaginated cellular membranes, coated pit formation containing bullet-shaped virus particles, and the presence of viral particles enclosed in vesicles within the cytoplasm of B16lacZ cells (Fig. 3.9b). MG1-UV<sup>2min</sup> infected B16lacZ cells demonstrated invagination of cellular membranes with viruses located within these forming coated pits. However, no intracellular viruses were observed (Fig. 3.9b). Cells infected with MG1-UV<sup>5min</sup> demonstrated coated pit formation, but no associated viruses. Infection of cells with UV-inactivated MG1 for longer time intervals did not reveal any membrane invagination and vesicle formation. In addition, no viruses were observed within cells (Fig. 3.9b). Given the finding that MG1 activates NK cells via direct interaction with DCs (Fig. 3.6c-h), we conducted our EM examination on BMDCs infected with MG1 and MG1-UV<sup>2min</sup>, and comparable results to B16lacZ target cells were observed with BMDCs infected with both MG1 and MG1-UV<sup>2min</sup> (Fig. 3.9c). Taken together, this data suggests that MG1-UV<sup>2min</sup> most closely resembles live MG1 in physical structure and target cell recognition/association.

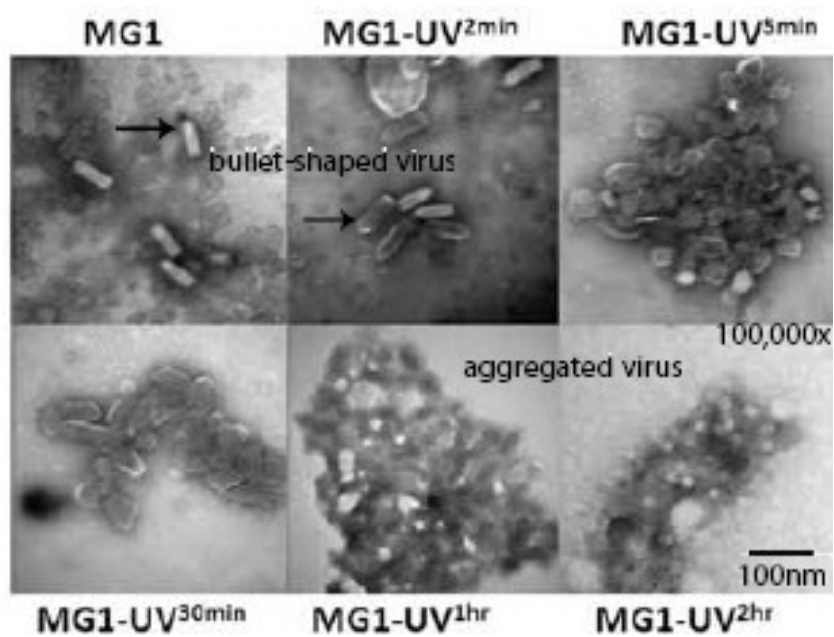
Next, we used western blot analysis to assess the integrity of MG1 viral proteins in pure virus preparations. Strong intensity bands, corresponding to the G, N and M rhabdoviral proteins were only detected for live MG1. MG1-UV<sup>2min</sup> demonstrated the same pattern of protein bands with less intensity as compared to live MG1 (Fig. 3.9d). MG1-UV<sup>5min</sup> showed a weak N protein band only. No recognizable proteins were observed for virus that was exposed to UV irradiation beyond 5min. Instead, protein aggregates at the top of the membrane were detected (Fig. 3.9d). For B16lacZ infected

MG1, we detected the presence of intense bands corresponding to G, N, P and M viral proteins for live MG1 only. No protein bands were detected for UV-inactivated viruses (Fig. 3.9e). These results suggest that cell-associated or intracellular viral proteins cannot be detected after UV treatment. In parallel, qRT-PCR was conducted to assess for viral genomic RNA in pure virus preparations. We observed viral genomic RNA above the limit of detection in live virus and the MG1-UV<sup>2min</sup> virus (Fig. 3.9f). qRT-PCR was repeated in B16lacZ cells infected with virus to assess for cell associated viral genomic RNA. Of the UV-inactivated MG1 variants, a low, but detectable amount of cell associated viral genomic RNA was observed for MG1-UV<sup>2min</sup> only (Fig. 3.9g). However, we did not observe an increase of genomic RNA during the time course for MG1-UV<sup>2min</sup>, and this corresponds with what we saw in EM analysis: virus attached to cell surface without entry (Fig. 3.9b&c) and no detection of viral proteins (Fig. 3.9e).

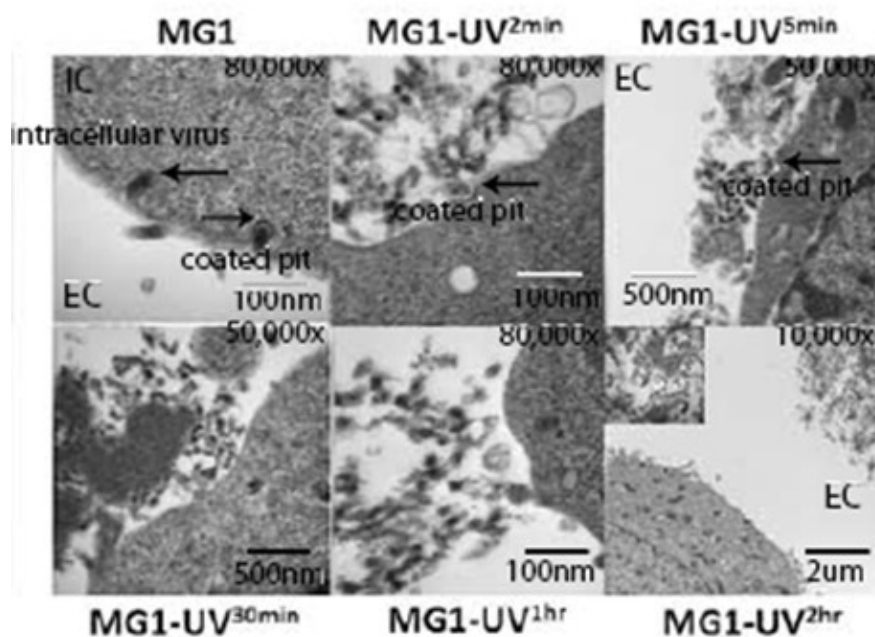
Taken together, this data suggests that MG1 UV exposure for 2min results in a non-replicating virus that still retains a measure of virus particle shape and integrity that permits target cell association. In addition, MG1-UV<sup>2min</sup> also retains viral proteins and both naked and associated viral genomic RNA. These characteristics of MG1-UV<sup>2min</sup> may provide a possible explanation for its ability to activate NK cells via DC and attenuate lung metastases. These data also meet our objectives 3b and 4a&d.

**Figure 3.9 Changes of virus morphology, cellular interaction, protein and genome after UV inactivation**

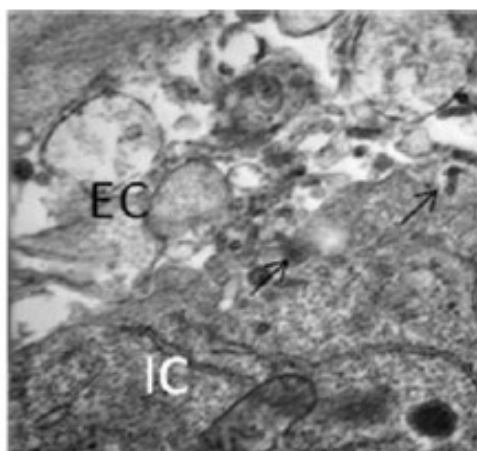
**a.**



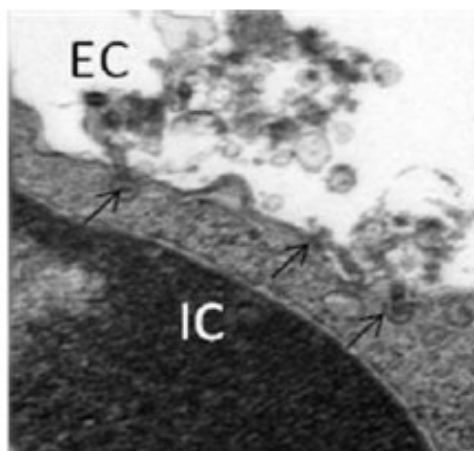
**b.**



c.

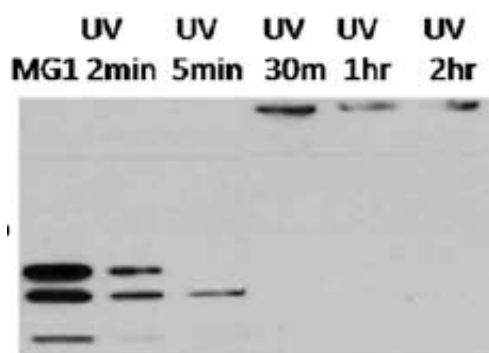


DC and MG1 (50000X)

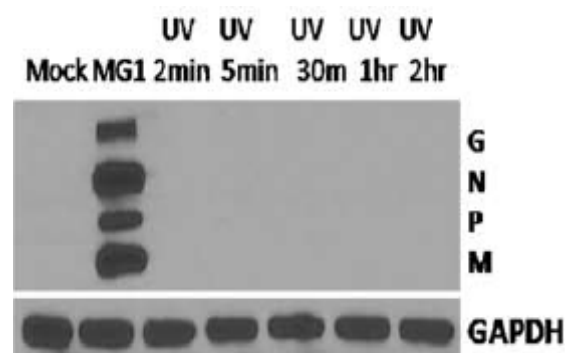


DC and MG1-UV<sup>2min</sup> (80000X)

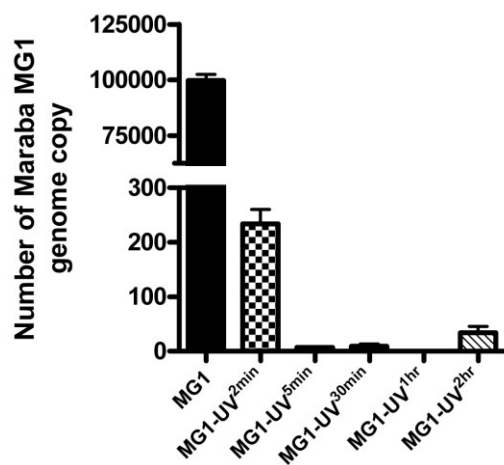
d.



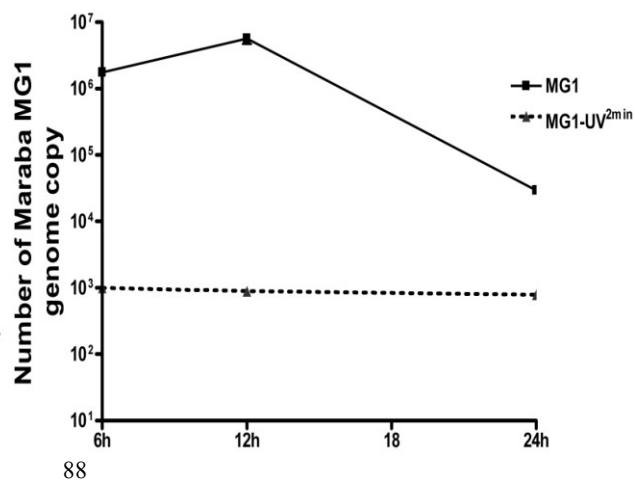
e.



f.



g.



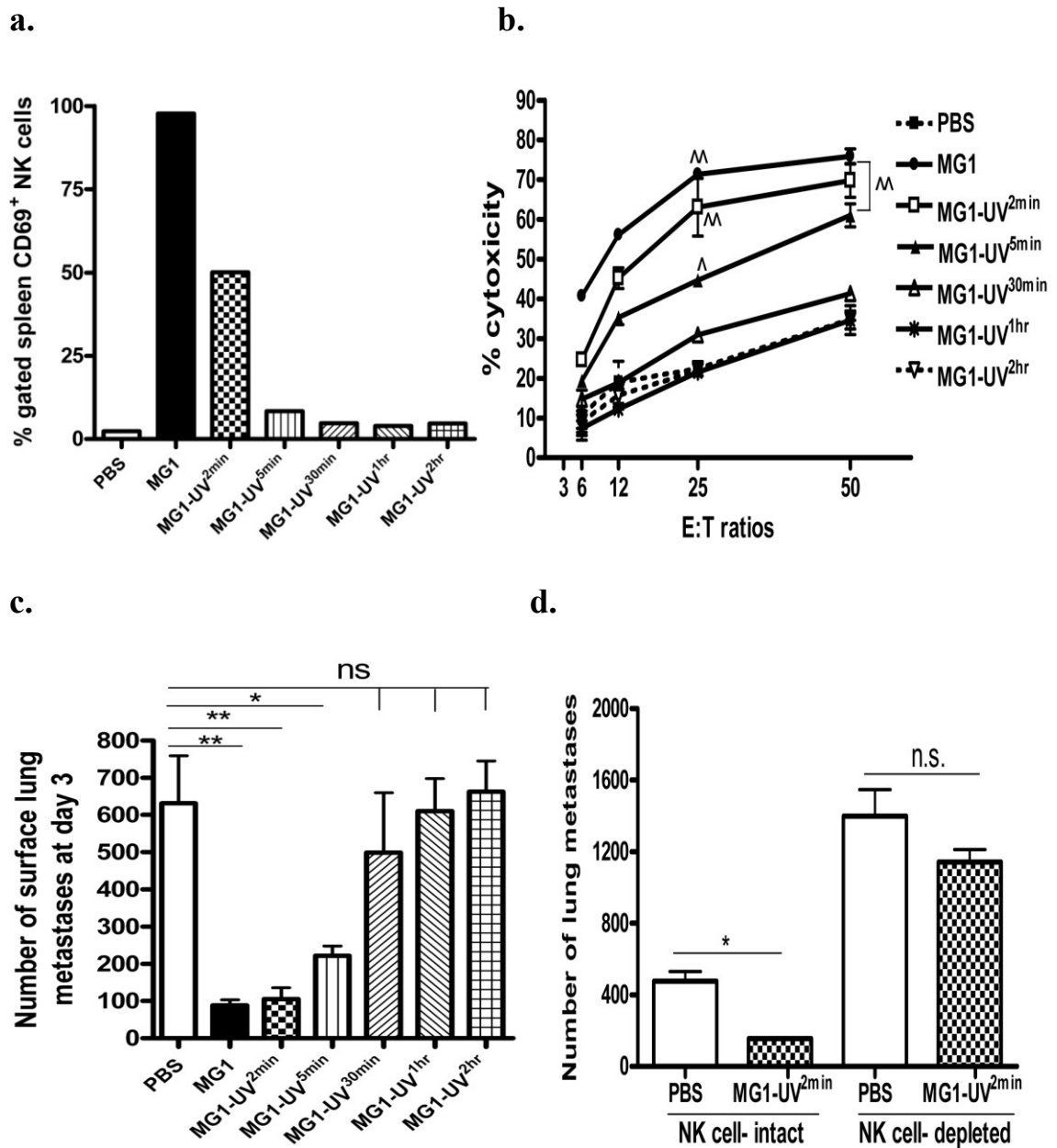
Electron Microscopy analysis of **(a)** purified virus, **(b)** B16lacZ cells infected with indicated virus (500 MOI), and **(c)** BMDCs infected with indicated virus. EC= extracellular space; IC = intracellular space. Western blot analysis of viral proteins from **(d)** purified virus preparations and **(e)** B16lacZ infected with indicated virus (18h p.i.). Anti-Rhabdovirus and anti-GAPDH antibodies were used. qRT-PCR analysis of extracted genomic RNA from **(f)** purified virus preparations and **(g)** B16lacZ cells infected with virus at indicated time point. Data are representative of 2 similar experiments.



### **3.10 A non-replicating, minimally UV inactivated MG1 (MG1-UV<sup>2min</sup>) is cable of activating NK cells and attenuates lung metastases with equivalent efficacy to live MG1**

We previously demonstrated that live MG1 does not directly infect and replicate in B16lacZ lung tumors in our model (Fig. 3.2), but can stimulate NK cells to clear lung metastases (Fig. 3.6). We have also clearly shown that non-replicating MG1-UV<sup>2min</sup> most closely resembles live MG1 (Fig. 3.9). We predict that MG1-UV<sup>2min</sup> might also be capable of activating NK cells and attenuate metastases accordingly. In addition, it might be the most potent candidate to activate NK cell function and remove B16lacZ lung metastases among this panel of UV-inactivated viruses. Thus, we proceeded to examine the capacity of different UV-inactivated viruses to promote NK cell activation. As anticipated, intravenous administration of MG1-UV<sup>2min</sup> induced significantly higher NK cell activation as measured by CD69 expression (Fig. 3.10a) and *ex vivo* cytotoxicity (Fig. 3.10b) than any other MG1-UV inactivated viruses. Furthermore, only MG1-UV<sup>2min</sup> and MG1-UV<sup>5min</sup> caused a significant reduction of lung metastases in the prophylactic treatment model among UV inactivated viruses. MG1-UV<sup>2min</sup>, but not MG1-UV<sup>5min</sup>, has an equivalent efficacy with MG1 (Fig. 3.10c). More importantly, we found that depletion of NK cells completely abrogated the ability of MG1-UV<sup>2min</sup> to attenuate lung metastases (Fig. 3.10d). Taken together, MG1-UV<sup>2min</sup> is the most potent candidate among these UV inactivated viruses to activate NK cells and reduce lung metastases. Furthermore, the anti-tumor ability of MG1-UV<sup>2min</sup> is entirely dependent on its ability to activate NK cell anti-tumor innate immunity.

**Figure 3.10 Characterization the ability of MG1-UVs to activate NK cell function and reduce lung metastases**



PBS or indicated virus was injected i.v. into B6 mice. 24 hours post-virus injection, spleens were harvested to assess **(a)** NK cell CD69 expression and **(b)** *ex vivo* NK cytotoxicity (^,  $p < 0.05$ , ^^,  $p < 0.01$  comparing treatment to PBS controls; the data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for the

indicated E:T ratios). **(c)** PBS or indicated virus was injected i.v. into B6 mice at day -1. At day 0, all mice received B16lacZ tumor cell inoculation. Quantification of lung tumor metastases at 3 days post cell injection. **(d)** B6 mice were treated with MG1-UV<sup>2min</sup> or PBS i.v. at day -1. At day 0, all mice received  $3 \times 10^5$  B16lacZ cell injections via tail vein. Quantification of lung metastases was performed at 3 days after cell inoculation in NK-intact and NK-depleted (anti-NK1.1 depletion) mice. Data are representative of 3 similar experiments with n=4-6/group. (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant).

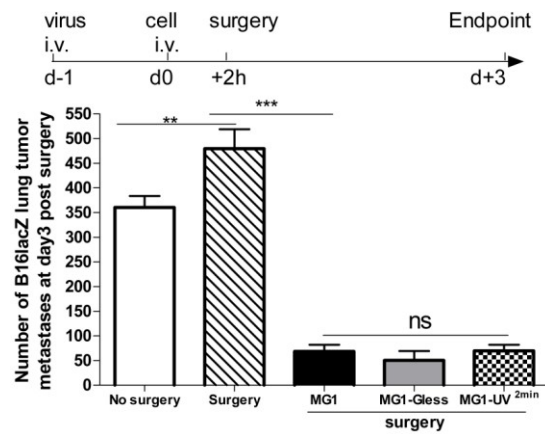
### **3.11 Reduction in postoperative lung metastases through NK cell stimulation using MG1-UV<sup>2min</sup>**

Previous work in our lab reported that surgery induced NK depression promoted postoperative metastatic disease formation. In the same study, we established that the perioperative use of replicating OV can rescue NK dysfunction and reduce surgery associated metastases (Tai et al., 2013). Given the finding that equal amounts ( $1 \times 10^8$  PFU/mouse) of live MG1 and non-replicating MG1-UV<sup>2min</sup> can reduce lung metastases to equivalent levels via NK activation (Fig. 3.2b, 3.8e, and 3.9f), we wanted to determine whether MG1-Gless and MG1-UV<sup>2min</sup> could reach the same efficacy in our surgical stress model, which would allow us to develop a perioperative OV with a safer, noninfectious *in vivo* profile. Using the B16lacZ surgical stress model, we found that perioperative use of live MG1, MG1-Gless and MG1-UV<sup>2min</sup> could all significantly attenuate metastatic disease following surgery. At 3d post-surgery, a striking decrease in tumor metastases was observed compared to surgery alone (Fig. 3.11a). Then, we tested NK cell function in the setting of perioperative OV administration. Suppressed NK cell cytotoxicity following surgery was recovered by perioperative use of all three MG1 variants (Fig. 3.11b). These findings were corroborated by reproducing the same results in a 4T1 spontaneous model of lung tumor metastases. The 4T1 murine breast carcinoma model is a highly aggressive tumor that spontaneously metastasizes from the primary mammary gland to multiple distant sites including the lungs. At 14d post-orthotopic tumor implantation, a complete resection of the primary mammary tumor along with abdominal nephrectomy (surgical stress) was performed. At 28d post-tumor inoculation, a significant decrease in lung tumor metastases was observed in surgically stressed mice

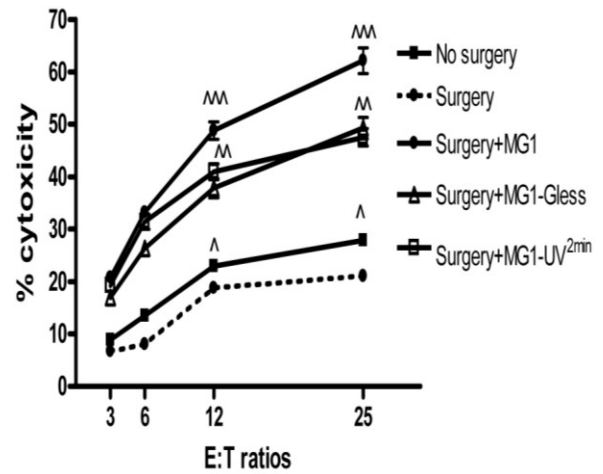
pretreated with all 3 variants of MG1 compared to surgery alone as shown by photographs and H&E staining of lung tissue (Fig. 3.11c), the number of lung tumor nodules (Fig. 3.7d) and lung weights (Fig. 3.7e). In addition to quantifying lung metastases, we conducted a survival experiment in this surgery and implanted B16lacZ lung metastasis model. Prophylactic treatments including MG1 and MG1-UV<sup>2min</sup> both improved overall survival compared to the surgery group (Fig. 3.11f). Therefore, all these three MG1 variants, including live MG1, MG1-Gless and MG1-UV<sup>2min</sup>, can attenuate surgery-induced metastases when used preoperatively in our surgical stress models. Importantly, these data establish that preoperative administration of a non-replicating OV, MG1-UV<sup>2min</sup>, is a viable perioperative therapeutic strategy. These data complete our objective 4.

**Figure 3.11 Reduction in postoperative lung metastases through NK cell stimulation using MG1-UV<sup>2min</sup>**

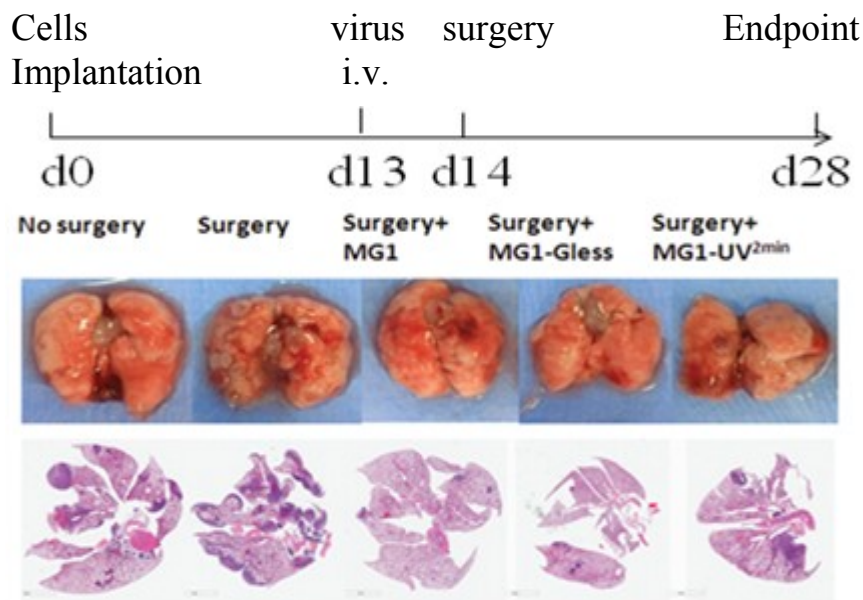
**a.**

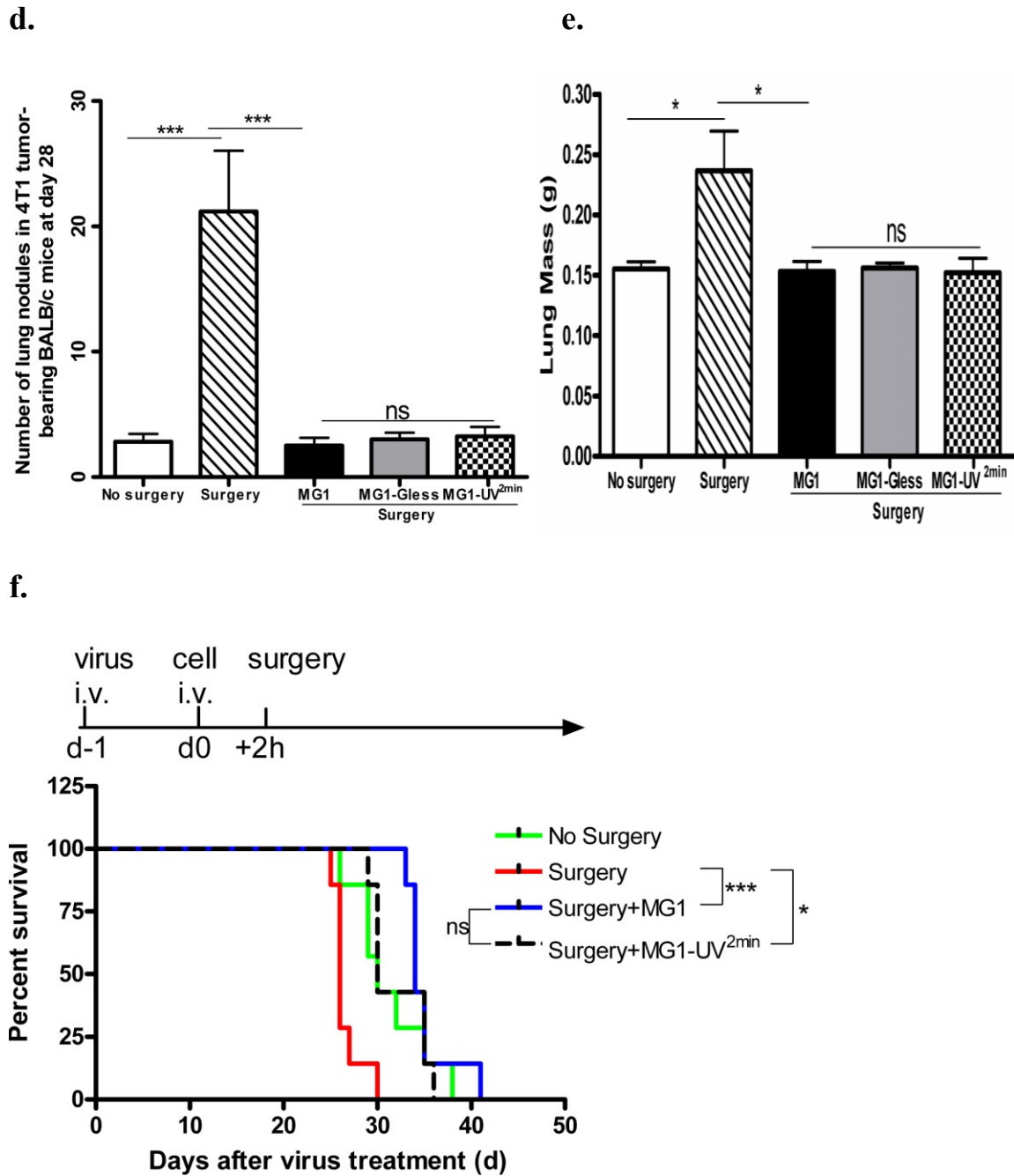


**b.**



**c.**





(a) Timeline of experiment design and quantification of B16lacZ lung tumor metastases at 3d in indicated treatment groups. (b) The ability of sorted NK cells to kill tumor targets from indicated treatment groups (^,  $p < 0.05$ ; ^^,  $p < 0.005$ ; ^^,  $p < 0.0001$ ; the data are displayed as the mean percent ( $\pm$  SD) of chromium release from triplicate wells for the

indicated E:T ratios). Assessment of 4T1 lung tumor metastases at 28d from indicated treatment groups by **(c)** representative lung photographs and H&E staining of lung tissues, **(d)** number of lung nodules and **(e)** lung weight. **(f)**  $3 \times 10^5$  B16lacZ cells were i.v. injected into B6 mice via tail vein,  $1 \times 10^8$  PFU MG1 or MG1-UV<sup>2min</sup> were given intravenously at 1 day before cells; surgery were performed at 2 hours after cells. Mouse overall survival was monitored. Data are representative of 3 similar experiments with n=5-10/group (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns: not significant).



## Chapter4: Discussion

Direct viral oncolysis has been the traditional hypothesis supporting the therapeutic efficacy of OV treatments *in vivo* (Russell et al., 2012; Stojdl et al., 2003; Vaha-Koskela et al., 2007). However, a growing body of evidence has highlighted the role of anti-cancer immunity in the underlying efficacy of OV therapies in recent years (Boisgerault et al., 2010; Melcher et al., 2011; Parato et al., 2009). In this study, we first characterized innate NK cell stimulation following intravenous administration of MG1 and found that MG1 activated NK cell function was responsible for the reduction of lung metastases in B16lacZ lung metastases model. Furthermore, we identified that a safe, non-replicating, non-infectious MG1-UV<sup>2min</sup> virus could significantly activate NK cell function and attenuate lung metastases as well as live MG1. Most importantly, perioperative administration of MG1, MG1-Gless, or MG1-UV<sup>2min</sup> could overcome surgery induced NK cell suppression and prevent the development of metastatic disease in the B16lacZ model of implanted lung metastases, as well as in the breast 4T1 model of spontaneous lung metastases to equivalent levels. Therefore, we propose the perioperative use of non-replicating MG1-UV<sup>2min</sup> as a more advantageous form of perioperative therapy compared to live MG1 because it carries no dose-related toxicities while maintaining equal efficacy.

Although B16lacZ was very susceptible to MG1 infection *in vitro* (Fig. 3.1c&d), the *in vivo* efficacy of live MG1 and non-replicating MG1-UV<sup>2min</sup> ( $1 \times 10^8$  PFU/mouse) in B16lacZ lung metastasis model was comparable (Fig. 3.2a&b). Previous work in our group reported that live ORFV replication was required for full treatment efficacy in B16lacZ lung metastases therapeutic treatment model (Rintoul et al., 2012). It is important to note the dose differences ( $1 \times 10^8$  PFU MG1-UV vs  $1 \times 10^7$  PFU ORFV-UV)

and UV exposure time differences (MG1-UV 2min vs ORFV-UV 5min) between these two studies. Given the *in vivo* efficacy of non-replicating MG1-UV<sup>2min</sup> (Fig. 3.2b) and an immediate and intense innate immune stimulation by MG1 in the spleen (Fig. 3.3&4) and lung (Fig.3.5), we reasoned that innate immunity might play a significant role in our prophylactic model. In addition, we found that depletion of NK cells completely abrogated the ability of MG1 and MG1-UV<sup>2min</sup> to attenuate lung metastases in our prophylactic treatment model (Fig.3.6a & Fig. 3.10d), which indicates that virus elicited innate NK anti-tumor immunity, instead of viral oncolysis (Fig. 3.2c&d), plays a dominant role in the *in vivo* efficacy of this model. Interestingly, when we decreased the dose of virus administration in the prophylactic treatment model, a significantly better efficacy was found in live MG1 treated mice than mice treated with MG1-UV<sup>2min</sup> (Fig. 3.8f). It is believed that at a high dose ( $1 \times 10^8$  PFU) of virus treatment in prophylactic model, although live MG1 elicits higher NK cell activation than MG1-UV<sup>2min</sup> (Fig.3.8a-d), this model is not robust enough to differentiate their efficacies. At a low dose ( $1 \times 10^7$  PFU or less), live MG1 retains NK activation ability, but MG1-UV<sup>2min</sup> decreases its ability of NK activation. Then this model becomes robust enough to allow MG1 to perform better than MG1-UV<sup>2min</sup> because live MG1 has higher NK cell activation.

DCs are another significantly expanded innate immune cell population, in addition to NK cells, in the mouse spleen (Fig. 3.3e) and lung (Fig. 3.5b) following MG1 injection. DCs are classical APCs, which directly sense the invading pathogens and initiate the immune response, resulting in production of inflammatory cytokines (e.g. IFNs, IL-2, IL-12, etc.) (Ferlazzo et al., 2004). DCs are involved in the activation of NK cells. In mice, it seems that both direct NK-DC cell contact and soluble factors are important for NK

activation. Separation of mouse NK cells and DCs in trans-well assays, which blocks direct cell-to-cell contact completely, inhibits NK cytotoxicity, which suggests that DC surface receptors are essential for NK cell activation (Fernandez et al., 1999). In addition, IFN- $\alpha/\beta$  and IL-12/18 produced by DCs may also play a critical role in murine NK cell activation (Andrews et al., 2003; Dalod et al., 2003; Orange et al., 1996). However, in humans, soluble factors such as DC-derived IL12/18 are crucial in NK activation instead of direct cell to cell contact (Nishioka et al., 2001; Yu et al., 2001). Some viruses, such as VV (Martinez et al., 2010) and HSV (Kim et al., 2012), have been reported to activate NK cells directly through TLR-2 signaling, however, we were not able to detect direct infection and activation of NK cells by MG1(Fig. 3.6b&c). Given the function of DCs and observed DCs expansion (Fig. 3.3e and Fig. 3.5b) after MG1 injection, we therefore reasoned that other TLR expressing innate immune cells such as DC might be responsible for the recognition and uptake of MG1 prior to NK cell activation. Results from our *in vitro* co-culture assay (Fig. 3.6e) and *in vivo* ablation of DC in CD11c-DTR Tg mice (Fig. 3.6f&g) suggest that MG1 activates NK cells through DC intermediates. In addition, our EM results depicting DC interaction and intake of live MG1 (Fig. 3.6d) suggests the presence of an intact viral capsid, viral proteins and viral genomic RNA that is capable of being recognized and processed by DC, are important for priming of an anti-tumor NK cell response. The induction of NK cell responses by DC following MG1 administration likely depends on the production of type I IFN and other proinflammatory cytokines (Janeeway et al., 2002; Medzhitov et al., 2002). Our current efforts involve identifying these cytokines DC expressed and downstream signalling molecules responsible for MG1 recognition and NK cell activation.

Although replicating MG1virus resulted in the highest NK cell activation, followed closely by MG1-Gless, we did observe that MG1-UV<sup>2min</sup> exhibited a significantly higher NK cell activation over PBS controls (Fig.3.8a-d). This suggests that virus replication is important, but not essential for NK cell activation. The activation of NK cells by MG1 appears to require viral capsid/particle integrity and cellular recognition/association capabilities, as evidenced by the abrogation of NK cell activity and tumor metastases clearance by MG1 inactivated for greater than 5min-UV because it loses their viral structure and is incapable of cell membrane association (Fig.3.9&10). The retention of virus particle structure in MG1-UV<sup>2min</sup> and its ability to invoke membrane invagination and association in B16lacZ cell (Fig. 3.9b) or BMDCs (Fig. 3.9c) suggests that its virus capsid proteins are likely intact, immunogenic and recognized by target cells. The detection of MG1-UV<sup>2min</sup> by anti- rhabdovirus antibody in western blot analysis further supports the intact immunogenicity of MG1-UV<sup>2min</sup> (Fig. 3.9d). These observed differences also explain the discrepancy between this study and the previous report (Diaz et al., 2007), which shows that viral replication is required for the anti-tumor effect of intratumoral VSV based on no efficacy in VSV-UV<sup>2hr</sup> (VSV exposed to UV irradiation for 2hours) treated group. We believe this is because VSV-UV<sup>2hr</sup> is similar to our MG1-UV<sup>2hr</sup>, which cannot activate innate immunity and reduce tumor metastases (Fig. 3.10a-c). In light of these findings, current work in the lab is focused on creating virus-like MG1 particles and purifying immunogenic MG1 proteins and genomic RNA to test their abilities to activate NK cell function and attenuate lung metastatic disease. We are also investigating how these MG1 components interact with DCs.

Virus inactivation by UV is mainly due to nucleic acid absorption of UV radiation.

The other components of the virus play minor roles in this inactivation process in most cases (Rauth, 1965), which provides inactivated virus the opportunity to preserve the integrity of the immunogenic epitopes on the viral envelope and lose its activity simultaneously. Our data depicts this process in great details: 2-min UV radiation is the minimum time to inactivate MG1 virus while preserving the maximal immunogenicity at the same time. UV exposure time longer than 2 minutes results in the gradual decrease of the viral immunogenicity (Fig. 3.10a&b) due to altered viral structure, damaged viral protein and RNA and/or decreased interplay between virus and cells (Fig.3.9).

When the primary tumor is removed, immune stimulation is most relevant therapy required to target micro-metastases. Clinically detectable efficacy requires high-dose of OV<sub>s</sub> (Heo et al., 2013), which raises the safety concerns of off-target effects, particularly in immunosuppressed patients (Bais et al., 2012). We believe that the unique attribute of non-replicating MG1-UV<sup>2min</sup> without dose associated toxicity is able to move OV therapy from bench to bedside even closer in the near future.

NK cell has been reported to play a significant role to prevent metastases (Ksienzyk et al., 2004; Zhou et al., 2012), and in perioperative setting we have previously shown that surgical stress promotes the formation of metastatic disease secondary to profound suppression of NK cells (Seth et al., 2013; Tai et al., 2013). In addition, our group has demonstrated that NK cells are highly activated following intravenous administration of several OV<sub>s</sub> (e.g. JX594, ORFV) and have further shown that OV administered immediately before cancer surgery can overcome surgery-induced NK cell suppression and prevent the prometastatic effect of surgery (Tai et al., 2013). However, these promising results were met with theoretical safety concerns associated with the use of a

live virus immediately prior to surgery in cancer patients. Particularly, concerns were raised about the potential for an overwhelming systemic inflammatory response, the risk of spread to members of the operating room team, and the acceptability of live virus from cancer patients themselves. Adair et al. recently published on the perioperative use of live reovirus prior to surgery in colorectal cancer patients (Adair et al., 2013). However, it is important to notice that OV infusion was done 6-28 days before surgery. Based on our results (Fig. 3.4 a-d), neoadjuvant OV administration at this time point before surgery will miss the peak of NK cell activation. Furthermore, NK cell suppression follows closely after surgery (Tai et al., 2013). Therefore, we suggest that OV use should be administered as close as possible to this susceptible perioperative period in order to boost the innate immune system and prevent the spread of micrometastases.

We demonstrated for the first time, a dramatic reduction in B16lacZ and 4T1 lung tumor metastases following perioperative administration of live MG1, MG1-Gless and MG1-UV<sup>2min</sup> compared to surgery alone (Fig. 3.11a,c-e). Similarly, we observed a significant recovery of NK cell cytotoxicity in perioperative OV treated groups compared to surgery alone (Fig. 3.11b). Most importantly, perioperative use of MG1 or MG1-UV<sup>2min</sup> not only reduces lung metastases but also significantly improves the overall survival of mice compared to surgery only (Fig. 3.11f). We are in favor of administering a non-replicating MG1-UV<sup>2min</sup> in the perioperative period, because it is a safe virus and eliminates the concerns associated with live MG1. In addition, MG1-UV<sup>2min</sup> also breaks the barrier of dose usage. On the other hand, although MG1-Gless is relatively safe, it is still a live virus. A G-less Maraba virus contains 1 attenuating point mutation that has the potential of reverting back to wild type Maraba virus and its associated potential

toxicities. In parallel, there are many complications involved in creating a G-less virus from a manufacturing standpoint, as a source of G protein producing cell line is required to rescue the virus.

The finding that a single intravenous injection of MG1-UV<sup>2min</sup> administered preoperatively significantly prolongs the overall survival in a surgical stress model compared to surgery alone (Fig. 3.11f) is encouraging because improvement in survival is the ultimate goal of cancer treatment. Since non-replicating MG1-UV<sup>2min</sup> is safe and without dose limitation, we have the opportunity to evaluate multiple doses of treatment in the perioperative period in the future study. In the current study, we used i.v. delivery as the route of virus administration; however, this might not represent the optimal route for virus delivery to stimulate innate immunity because different routes (s.c. vs i.v.) for immuno-stimulatory agents have different immune response (Wilson et al., 2007). Future research will involve the optimization of virus delivery routes to further improve immunotherapy. UV-OV therapy has some advantages over other methods such as cytokine therapy (e.g.IL-2, IFN $\alpha$ ), which is limited by toxicity (Fioravanti et al., 2011; Smith, 1997). In addition, a single cytokine administration only activates a single pathway to stimulate immunity. However, virotherapy can activate multiple pathways. Although OV therapy is limited by delivery, route and associated toxicity as well, UV-OV therapy circumvents these restrictions to mount powerful immunotherapy.

In the MG1/B16lacZ/C57BL/6 system, the host immunity will respond to both MG1 virus and B16lacZ cells, both anti-viral and anti-tumor cell immunity. Since each mouse got B16lacZ cells injection, it allows us to directly compare the anti-viral response over and above the anti-tumor cell immune response, which means the anti-tumor immunity

discussed in this study was strictly anti-viral immunity. Anti-viral immunity to OV in normal cells is an important safety component to prevent systemic toxicity. Since not all tumor cells have completely defective type I interferon (IFN) signaling, anti-viral immunity in these tumor cells will inhibit virus replication and spread in the tumor (Galivo et al., 2010). This also explains why some studies reported that inhibition of innate immunity enhanced OV replication and improve the efficacy of OV therapy (Alvarez-Breckenridge et al., 2012; Peng et al., 2013). In our model, anti-viral innate response acts as a major contributor to bystander killing of tumor cells, namely the anti-tumor effects. Therefore, when we design our strategy for OV therapy, special attention should be paid to the anti-viral immunity that plays multiple roles - safety against systemic virus toxicity, potential anti-tumor effects and inhibition of virus replication. In this current study, we established that a non-replicating MG1-UV<sup>2min</sup> is able to elicit anti-tumor immunity with reduced *in vivo* safety concerns.

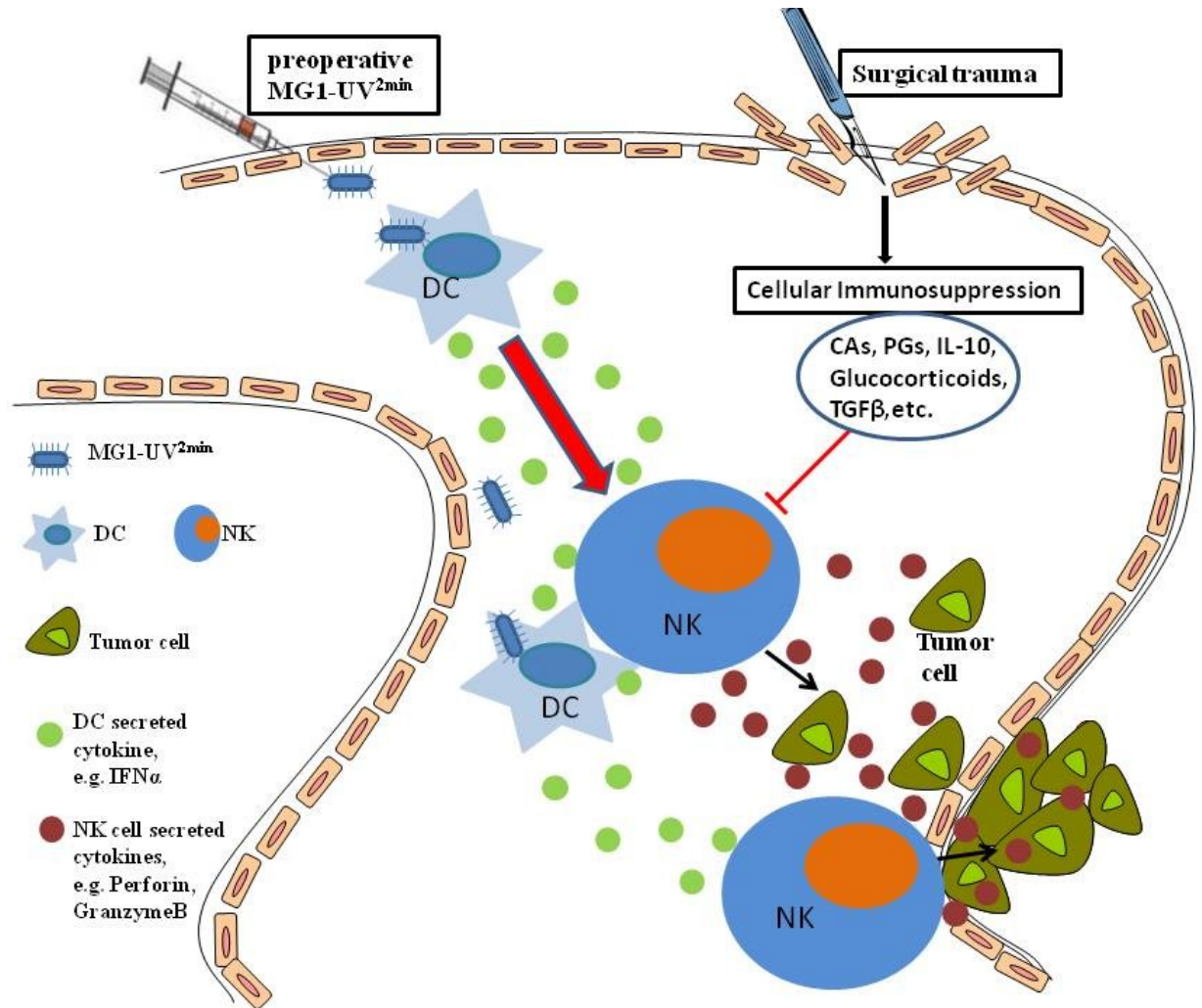
OV therapy has achieved remarkable progress in cancer treatment in the past two decades. More than ten different virus families have entered the clinical arena since the first application of engineered HSV (AU et al., 2005; Rudin et al., 2011; Tai et al., 2008). However, clinical trials haven't shown direct oncolysis as an essential mechanism for tumor destruction (Liu et al., 2007). The fundamental principle of OV therapy - a small initial OV inoculum will go through progressive rounds of viral replication in order to achieve viral mediated oncolysis (Granot et al., 2011; Russell et al., 2012; Vaha-Koskela et al., 2007), is modified by its oncolytic immunotherapy. In 2010, the GM-CSF-armed oncolytic adenovirus was the first OV to show the induction of anti-tumor immune response in human by recruiting NK cells and inducing tumor-specific cytotoxic T cells,



and these responses were frequently seen in injected and non-injected tumors (Cerullo et al., 2010). The anti-tumor immunity depends on the interaction between tumor and the host immunity. Tumors employ several mechanisms, such as the production of immunosuppressive cytokines and recruiting immune inhibitory cells (Melcher et al., 2011; Yang et al., 2010) to avoid anti-tumor immunity. OV therapy can break the immune tolerance towards tumors. OVs elicit initial antiviral innate immunity which leads to bystander killing of tumor cells. Tumor antigens are then released to further activate adaptive immunity. Our data shows that MG1 activated NK cells are responsible for the reduction of metastases (Fig.3.6b), which corroborates the concept of oncolytic immunovirotherapy.

In conclusion, the running model of this study is as follows: cancer surgery induces a series of physiological stress responses, such as secretion of glucocorticoids, CAs, PGs, increase of immune-suppression of cytokines (e.g. IL-10, TGF $\beta$ ) with decrease of proinflammatory cytokines (e.g. IL-2, IL-12), which results in NK cells suppression, consequently promoting the development of postoperative metastases. Administration of MG1 or MG1-UV<sup>2min</sup> at 1 day before surgery boosts NK cell function via DCs, thereby minimizing surgery induced NK cell suppression and preventing the development of postoperative lung metastases. This neoadjuvant therapy also significantly prolongs the survival of the surgical stressed mice (Fig. 4.1). The identification of MG1-UV<sup>2min</sup> as novel cancer neo-adjuvant therapy has the potential to impact the risk of recurrence in countless cancer patients who undergo surgical resection of their solid tumors in the future.

**Figure 4.1 Preoperative use of non-replicating MG1-UV<sup>2min</sup> prevents surgery induced postoperative metastasis via DC-mediated NK cells activation**



Surgical trauma results in a variety of physiological changes in the host, including profound immunosuppression. This state is characterized by the secretion of CAs, PGs, glucocorticoids, transforming growth factor  $\beta$  (TGF $\beta$ ), IL-10, etc, resulting in NK cell dysfunction following surgery. Dysfunctional NK cells are unable to clear malignant cells and disseminated micro-metastases become established in the postoperative period. The preoperative administration of non-replicating MG1-UV<sup>2min</sup> results in NK cell activation through either direct DC-NK contact or DC secreted cytokines such as IFN $\alpha$ . Activated

NK cells will secrete perforin and Granzyme B to kill tumor cells. Thus, preoperative use of MG1-UV<sup>2min</sup> overcomes surgery induced NK cell dysfunction and prevents postoperative metastases (Adapted from Tai et al., 2013).

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