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Understanding the Mechanism of Stimulatory NK-cell Ligand Expression

Author
Tokuyama, Maria

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Understanding the Mechanism of Stimulatory NK-cell Ligand Expression

By

Maria Tokuyama

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Committee in charge:

Professor Laurent Coscoy, Chair
Professor David H. Raulet
Professor Nilabh Shastri
Professor Lee W. Riley

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Abstract

Understanding the Mechanism of Stimulatory NK-cell Ligand Expression

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Maria Tokuyama

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Natural killer (NK) cells are lymphocytes that play a major role in the elimination of virally-infected cells and tumor cells. NK cells recognize and target abnormal cells through activation of stimulatory receptors such as NKG2D. NKG2D ligands are self-proteins, which are absent or expressed at low levels on healthy cells but are induced upon cellular stress, transformation, or viral infection. The exact molecular mechanisms driving expression of these ligands remain poorly understood.

Using mouse cytomegalovirus (MCMV) as a model, the present dissertation describes three distinct factors that contribute to the induction of the RAE-1 family of NKG2D ligands during MCMV infection. First, we show that activation of the phosphatidylinositol-3-kinase (PI3K) pathway, which is often dysregulated during infection and tumorigenesis, is required for RAE-1 expression in MCMV-infected cells as well as in transformed cell lines. Second, genetic manipulation of the MCMV genome has revealed a novel viral protein encoded by the *m18* gene that is both necessary and sufficient to induce RAE-1 expression. Finally, studies using mouse strains that are deficient in various innate signaling molecules demonstrate that innate signaling through the MyD88 adaptor protein and the IRF3 transcription factor are involved in RAE-1 induction.

Collectively, we have shown that RAE-1 expression during MCMV infection involves a combination of signals to ensure that its expression is limited to truly diseased cells.

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Chapter 1: Introduction

The immune system keeps us alive and most of us healthy, despite the presence of over a quadrillion bacteria in our intestines, 10 times more than the number of cells we possess. How does it achieve such an extraordinary task? The immune system is highly regulated with many checks and balances. Many years of research has revealed that the essence is in their ability to discriminate healthy cells from unhealthy cells.

Most cells are equipped with innate "sensors" called pattern recognition receptors (PRRs) that directly recognize pathogen-associated molecular patterns (PAMPs) of invading microbes and viruses. PRRs are thought to be critical sentinels of the innate immune system that serve to discriminate self from non-self (Medzhitov, Janeway 2000). Although new PRRs and PAMPs are continuing to be discovered, many PAMPs have been identified for specific PRRs. Initiation of signaling cascades through these sensors results in the activation of key inflammatory molecules such as type-I IFN, IL-12, IL-6, and TNFα, to name a few (Kumar, Kawai & Akira 2011). This system is extremely rapid and efficient such that exposure of cells to a PAMP such as LPS is sufficient to induce a primary response within six hours post-stimulation. Importantly, invasion by bacterial or viral species that possess these PAMPs activates PRR signaling and results in a rapid production of inflammatory molecules that alert the rest of the immune system of the infection.

In a similar but distinct manner, natural killer (NK) cells utilize an array of cell surface receptors to discriminate healthy cells from diseased cells. NK cells possess receptors that are inhibitory and those that are stimulatory. Most of the ligands for NK cell receptors are self-proteins with an exception of a few, which are viral proteins that are expressed on the surface of infected cells (Raulet, Vance 2006). Major Histocompatibility Complex I (MHC-I) proteins are the main ligands for inhibitory receptors. MHC-I is normally expressed on healthy cells, and a loss of MHC-I expression leads to NK cell activation in a process called "missing-self." On the contrary, ligands for stimulatory receptors, which are mainly self, although not exclusively, are normally absent or expressed at low levels on healthy cells, and their induction upon cellular stress or infection activates NK cells in a process called "induced self-recognition." The net balance of inhibitory and stimulatory signals determines whether NK cells become activated to release cytotoxic effectors and pro-inflammatory molecules (Raulet 2003). Therefore, similar to PRRs, NK cell receptors survey the environment for abnormality.

NK cells possess multiple stimulatory receptors including NKG2D, DNAM1, 2B4 and a family of natural cytotoxicity receptors (NCRs), which include NKp30, NKp44, NKp46, and NKp80. The cognate ligands for these receptors are as follows: NKG2D ligands (NKG2D), PVR and PVRL2 (DNAM-1), and CD48 (2B4). Ligands for NCRs are less defined, and they include heparan sulphate proteoglycans, viral haemagglutinin, nuclear factor HLA-B-associated transcript 3, and C-type lectin (Raulet, Guerra 2009). The best characterized stimulatory NK cell receptor is NKG2D. Unlike inhibitory receptors, which recognize a loss of MHC-I expression, NKG2D depends on the emergence of NKG2D ligands, which are normally absent on healthy cells. Both in vitro and in vivo studies to date have shown that once NKG2D ligands are expressed at the surface of diseased cells, NK cells become activated via NKG2D and secrete pro-inflammatory molecules or cytotoxic effectors (Diefenbach et al. 2000, Cerwenka et al. 2000, Diefenbach et al. 2001, Cerwenka, Baron & Lanier 2001). Thus, expression of NKG2D ligand serves as a molecular pattern that implicates abnormality. Much less is known about how the ligands for these receptors are regulated such that their expression is limited to cells that have

endured cellular stress. Moreover, it is yet defined how a cell "senses" infection, cellular stress or transformation to know when NKG2D ligand expression is required. The goal of this thesis was to identify novel molecular cues that evoke expression of NKG2D ligands using mouse cytomegalovirus (MCMV) infection as a model.

Cytomegalovirus

Cytomegalovirus (CMV) is a member of the beta-herpesvirus family. It is a large, double-stranded linear DNA virus that is approximately 230kbp in size. CMV is composed of a genomic DNA, capsid proteins, tegument proteins, and envelope proteins (Knipe, Howley 2001). HCMV has a high rate of seroprevalence, but in most healthy individuals, it does not cause any disease. However, HCMV infection of an immunocompromised host or a fetus leads to severe complications and fatality in some cases (Crough, Khanna 2009). Vertical transmission of HCMV from pregnant mothers to fetuses can cause congenital infection in up to 50% of the cases (Boppana et al. 2005, Ross et al. 2006). Infants born with congenital HCMV infection are at a very high risk for developing sensorineural hearing loss (SNHL), and in fact, congenital HCMV infection is the leading cause of SNHL in children (Boppana et al. 2005, Andrei, De Clercq & Snoeck 2009, Cheeran, Lokensgard & Schleiss 2009, Damato, Winnen 2002). Mouse CMV (MCMV) is a homologue of HCMV that has a similar genomic composition as HCMV and encode many proteins that are orthologues of HCMV proteins (Rawlinson, Farrell & Barrell 1996). MCMV is a relevant mouse model for HCMV because mice are the natural hosts of MCMV, thus the virus has evolved true mechanisms to evade the host and the host has evolved mechanisms to sense infection.

The replication cycle of CMV is approximately 24 hours and the following events occur within this cycle: 1) receptor-mediated endocytosis, 2) circularization of viral DNA and entry into the nucleus, 3) expression of immediate early (IE) genes that are essential for subsequent expression of early (E) and late (L) genes, and 4) packaging and budding of the virus. Expression of immediate early genes occurs within two hours of infection, and proteins encoded by these genes activate cellular pathways that are necessary for viral replication and cell survival (Knipe, Howley 2001). In MCMV, there are three immediate early proteins, IE1, IE2, and IE3. IE2 is dispensable, but IE3 is essential for viral replication (Manning, Mocarski 1988, Angulo, Ghazal & Messerle 2000). Multiple reports have suggested different cellular receptors for HCMV entry including integrins, platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) (Feire, Koss & Compton 2004, Soroceanu, Akhavan & Cobbs 2008, Chan, Nogalski & Yurochko 2009). Whether these receptors are also utilized by MCMV for cellular entry has not been shown. MHC-I molecules and heparin sulfate contribute to cellular entry of MCMV, but the exact receptor to which MCMV binds has not been conclusively shown (Price et al. 1995).

Due to the high seroprevalence of HCMV in the human population and the absence of disease in healthy people, HCMV has recently been considered as a potential vehicle for vaccines (Mohr et al. 2008). Several groups have tested the efficacy of CMV vaccines using MCMV as a model. One group inserted an Ebola nucleoprotein (NP) epitope into MCMV and observed high levels of NP-specific CD8 T cells within eight weeks after a single dose immunization (Tsuda et al. 2011). Additionally, two sequential injections were sufficient to protect animals against lethal challenge by a mouse-adapted Ebola Zaire virus. Another group demonstrated that a single-dose immunization with MCMV encoding tetanus toxin fragment C was sufficient to induce sustained levels of anti-tetanus antibodies in mice (Tierney et al. 2012).

The use of CMV as a vaccine vector may be promising because BAC recombination technology has been widely used to successfully manipulate the genome of the virus. Furthermore, using existing knowledge of the virus, a virus that is attenuated *in vivo* but is capable of replicating *in vitro* can serve as good platforms for vaccine development. However, because CMV is such a large DNA virus encoding over 170 genes, understanding the immunomodulatory function of as many of these genes as possible would be extremely beneficial for optimal vaccine design.

NKG2D receptor

NKG2D is a type II transmembrane protein that is part of the C-type lectin family of proteins. NKG2D was originally discovered along with the other NKG2 proteins, NKG2-A, -B, and -C (Houchins et al. 1991). NKG2D gene is located within the NK gene complex (NKC) on chromosome 6 in mice and chromosome 12 in humans along with other NK cell receptors such as CD94 and Ly49 family of receptors. In humans, NKG2D is expressed in NK cells, CD8 T cells, and γδ T cells in the periphery and in the intestine. In mice, NKG2D is expressed in NK cells, activated CD8 T cells, NKT cells, and a fraction of γδ T cells (Jamieson et al. 2002). In mice, two isoforms of NKG2D are expressed, a long and a short isoform. The long isoform associates with DAP10 and the short isoform associates with both DAP10 and DAP12 (Diefenbach et al. 2002). In humans, NKG2D associates with the adaptor molecule DAP10 because the long form of NKG2D is expressed. Signaling through the DAP10-associated NKG2D results in recruitment and activation of PI3K and Grb2, while signaling through the DAP12-associated NKG2D results in a classical ITAM-mediated activation of Syk and Zap70 (Wu et al. 1999, Lanier et al. 1998). The presence of different NKG2D-adaptor complexes on different cell types seems to confer differential outcomes of NKG2D signaling (Raulet 2003).

Activation of NKG2D receptor occurs through recognition of NKG2D ligands, which are a family of proteins that are closely related to MHC-I. NKG2D ligands contain α1 and α2 domains similar to MHC-I, but they do not present peptides or associate with other molecules. These ligands are expressed at low to undetectable levels on normal healthy cells but are induced upon cellular stress, transformation or infection. The impact of NKG2D and NKG2D ligand interaction during tumorigenesis and infection has been studied for many years. In vivo expression of mouse NKG2D ligands on tumors is sufficient for efficient clearance, and in vitro stimulation of NKG2D with any one of the NKG2D ligands activates NK cells (Diefenbach et al. 2000, Cerwenka et al. 2000, Diefenbach et al. 2001, Cerwenka, Baron & Lanier 2001).

More recently, NKG2D deficient (*Klrk1*-/-) mice were generated in the Raulet lab, and the effect of NKG2D deficiency on tumorigenesis was investigated *in vivo*. The study illustrated an increased rate and frequency of tumor growth in Eμ-myc model of lymphoma and TRAMP model of prostate tumors, respectively (Guerra et al. 2008). The same study also suggested a role of NKG2D in immunoediting of TRAMP tumors such that NKG2D plays a role in preferentially selecting for tumors that do not express or have lost expression of NKG2D ligands. Although this study showed normal NK cell development in NKG2D deficient mice, subsequent generation of *Klrk1*-/- mice by the Polic lab showed an altered development of NK cells (Zafirova et al. 2009). In this study, NKG2D deficiency also resulted in increased resistance to MCMV infection. They attributed this phenotype to dysfunctional NK cells that arose due to the lack of NKG2D signaling during development. Several other studies have used *Klrk1*-/- mice to illustrate the importance of NKG2D signaling in various contexts. One study showed that NKG2D stimulation during influenza infection in combination with long-term cigarette smoke exposure results in NK cell hyperresponsiveness (Wortham et al. 2012). Another study showed

that NKG2D signaling in conditions of metabolic dysfunction can promote atherosclerosis (Xia et al. 2011).

NKG2D ligand

To date, there are eight human NKG2D ligands (MICA, MICB, ULBP1-4, RAET1G, and RAET1L) and nine mouse NKG2D ligands (MULT-1, H60a-c, and RAE-1α-ε). Human NKG2D ligands are encoded on chromosome 6, while mouse NKG2D ligands are encoded on chromosome 10. For human ligands, MICA, B, ULBP4, and RAET1G are transmembrane proteins, while ULBP1-3 and RAET1L are GPI-anchored proteins. For mouse ligands, MULT-1, H60a, and b are transmembrane proteins, while H60c and RAE-1α-ε are GPI-anchored proteins (Champsaur, Lanier 2010). All of the NKG2D ligands bind to NKG2D receptor, albeit at different affinities. Analysis of the sequences of genes encoding NKG2D ligands suggests that they originated through diversification and multiple rounds of gene duplication, especially for the RAE-1 family of genes (Samarakoon, Chu & Malarkannan 2009). The presence of multiple NKG2D ligands for one receptor seems to account for expression of at least one of the ligands during cellular stress or infection. Accordingly, not all of the NKG2D ligands are induced upon any given trigger, whether it is viral infection or tumorigenesis. Furthermore, different modes of NKG2D ligand regulation have been illustrated including transcriptional regulation for MICA and B, post-translational regulation by miRNAs for MICA and B, ubiquitin-mediated post-translational regulation of MULT-1, and shedding of cell surface MICA and RAE-1ε (Groh et al. 1996, Stern-Ginossar et al. 2008, Nachmani et al. 2009, Nice, Coscoy & Raulet 2009, Salih, Rammensee & Steinle 2002).

Many viral infections induce expression of NKG2D ligands at the transcript level. However, viruses encode viral proteins that actively block surface expression of the ligands or downregulate surface-resident NKG2D ligands (Table 1.1). Although only a handful of viral evasins have been identified, other viruses listed here most likely possess mechanisms to evade NKG2D recognition as well. The fact that viruses encode means of preventing surface expression of these ligands suggests that ligand expression upon viral infection is inevitable. Therefore, NKG2D ligand induction upon viral infection is most likely a consequence of cells sensing invasion by a replicating virus.

The role of NK cells during MCMV infection

NK cells play a critical role in the clearance of MCMV-infected cells and serve as one of the major effectors of the innate immune response. Ly49H is a stimulatory NK-cell receptor that recognizes a MCMV protein, m157. Because m157 is expressed at the cell surface of MCMV-infected cells, encounter with NK cells expressing Ly49H leads to activation of NK cells and efficient clearance of infected cells. In Balb/c mice, *ly49h* gene is absent, which makes them highly susceptible to infection compared to C57BL/6 mice that encode a functional *ly49h* gene (Arase et al. 2002, Smith et al. 2002). Although NK cell activation depends on the balance of signaling through its stimulatory and inhibitory receptors, Ly49H interaction with m157 dominates the response in C57BL/6 mice. In the absence of Ly49H signaling, as in the case for Balb/c mice, MHC-I expression and signaling through NK inhibitory receptors seem to determine the outcome (Babic et al. 2010). Furthermore, in MA/My strain of mice, a distinct NK stimulatory receptor Ly49P, which recognizes H-2D^k MHC-I molecule in conjunction with MCMV viral protein m04, dictates NK cell response and host susceptibility during infection (Kielczewska et al. 2009).

The main function of NK cells is to participate in the innate immune response. This is largely because NK cells rapidly respond to infected cells or transformed cells to secrete key effectors, express germline encoded receptors that do not rearrange upon stimulation, and do not retain memory of past infections. However, recent advancements in the field have demonstrated that NK cells possess a "memory" phenotype. In particular, Ly49H-expressing NK cells that expand during the acute phase of MCMV infection have the capacity to remain elevated for two to three weeks post-infection, and transfer of these long-lived NK cells into a new host results in rapid expansion upon MCMV infection and a rescue of mice from a lethal challenge by MCMV (Sun, Beilke & Lanier 2009). Additionally, generation of long-lived "memory-like" NK cells during MCMV infection requires IL-12 (Sun et al. 2012).

NK cell activation can also affect generation of an effective anti-viral T cell response. Early NK cell activation through Ly49H promotes recruitment and expansion of MCMV-specific CD8 T cells by limiting the cytokine storm that is associated with infection in the absence of early viral control (Robbins et al. 2007). However, NK cell activation through Ly49H could also trigger killing of essential antigen presenting cells, such as cDCs, which are important for generation of anti-viral T cell responses (Robbins et al. 2007). Altogether, data to date have revealed that the significance of NK cells during MCMV infection may reach beyond its role in the innate immune response.

Thesis goals and major findings

Despite some evidence showing the role of certain pathways and effector molecules in the expression of NKG2D ligands, much remains to be learned about the process, and uncovering the molecular mechanisms that drive expression of each of the NKG2D ligands remains an active area of research in the field. In particular, very little is known concerning the mechanisms of NKG2D ligand induction in virus-infected cells. CMV infection results in the induction of RAE-1, MULT-1, and H60a in the mouse. However, both human and mouse CMV encode proteins that specifically inhibit expression of each of the NKG2D ligands at the protein level, suggesting that NK cell recognition of CMV-infected cells has put evolutionary pressure on the virus to evade this arm of the immune system. The inducibility of RAE-1 in MCMV-infected cells prompted us to use this well characterized virus to investigate the molecular mechanism of RAE-1 induction.

Many viruses utilize host cellular machineries to promote their survival and replication. Thus, sensing abnormal activation of a cellular pathway may be an effective way for cells to know when it is under stress. We hypothesized that RAE-1 induction during MCMV infection may be a result of overactivation of cellular pathways that normally carry out essential cellular functions. Strikingly, our studies showed that virus-induced activation of phosphatidylinositol-3-kinase (PI3K) is essential for the induction of the RAE-1 family of mouse NKG2D ligands. Further studies demonstrated that PI3K is also important for the maintenance of RAE-1 and MULT-1 expression on transformed cells, showing the breadth of our findings (Tokuyama et al. 2011). These results suggest that activation of the PI3K pathway, which occurs in cells infected with numerous viruses and in cancer cells, represents a common signal for regulating RAE-1 expression. Finally, the effect of PI3K inhibition on MULT-1 expression also reveals the possibility that PI3K activation may play a role in regulating expression of other NKG2D ligands in cells infected with other viruses and other pathologic states such as inflammatory diseases.

NKG2D ligand induction at the transcript level is a consequence of many viral infections (Table 1.1). Although multiple factors could be involved in the induction of NKG2D ligands, we

searched for a region within the MCMV genome that may be required to trigger this response. Using BAC-recombination, we generated a series of deletion mutant MCMV and identified a single viral protein encoded by the *m18* gene that is both necessary and sufficient for RAE-1 induction. The mechanism of m18-mediated induction of RAE-1 is still under investigation. Current data suggest that m18-mediated induction of RAE-1 requires activation of the PI3K pathway and that m18 can activate the RAE-1ε promoter, but it does not require E2F transcription factors to do so. Finally, gene expression profile of m18-expressing cells suggests that m18 activates a differentiation program involved in development and morphogenesis.

Previous studies have shown that TLR ligands can induce expression of RAE-1 in a MyD88-dependent manner (Hamerman, Ogasawara & Lanier 2004). However, the extent to which MyD88 signaling or other innate signaling is involved in the induction of NKG2D ligands during a viral infection has not been studied. In an ongoing study, we have demonstrated that RAE-1 induction occurs in MyD88-dependent manner, and the downstream transcription factor IRF3 also seems to be involved. However, our attempts to identify a particular receptor responsible for the MyD88-dependent signaling have not yielded a conclusive result.

Table 1.1.

Virus	Induced NKG2D Ligands	Viral Evasins	References
HIV	ULBP1-3	Nef	Ward, J. et al. 2009; Cerboni, C. et al. 2007
Influenza A	MICB, ULBP1-3, RAE1TG,	?	Ebihara, T. et al. 2007
Measles	MICB, ULBP1-2, RAET1G	?	Ebihara, T. et al. 2007
Respiratory syncytial virus (RSV)	MICB, ULBP1-4, RAET1G	?	Ebihara, T. et al. 2007
Vesicular stomatitis virus (VSV)	MICA	?	Jensen, H. et al. 2011
Polyoma virus	ULBP3	miR-J1-3p, J1-5p	Bauman, Y. et al. 2011
Adenovirus serotype 5 (Ad5)	MICA, B, RAE-1	E3/19K	Routes, J.M. et al. 2005; McSharry, B.P. et al. 2008
Mouse hepatitis virus (MHV)	MULT-1, H60, RAE-1	?	Walsh, K.B. et al. 2008
Ectromelia Virus (ECTV)	RAE-1	?	Fang, M. et al. 2008
Human cytomegalovirus (HCMV)	MICA, B, ULBP1, 2, 6	UL16, UL142, miR-UL112	Dunn, C. et al. 2003; Ashiru, O. et al. 2009; Stern-Ginossar, N. et al. 2007
Murine cytomegalovirus (MCMV)	MULT-1, H60A, RAE-1	m138, m145, m152, m155	Jonjic, S. et al. 2008
Epstein-Barr Virus (EBV)	ULBP1, 4	miR-BART2-5p*	Pappworth, I.Y., et al. 2007; Kong, Y. et al. 2009
Kaposi's sarcoma-associated virus (KSHV)	?	K5, miR-K12-7†	Thomas, M. et al. 2008; Nachmani, D. et al. 2009

*miR-BART2-5p targets MICA and MICB, but these ligands have not been shown to be induced during infection
†K5 downregulates MICA/B and miR-K12-7 targets MICB, but these ligands have not been shown to be induced during infection

Chapter 2: Expression of the RAE-1 family of stimulatory NK-cell ligands requires activation of the PI3K pathway during viral infection and transformation

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Maria Tokuyama, Clarisse Lorin, Frederic Delebecque, Heiyoun Jung, David H. Raulet and Laurent Coscoy

Background

Natural killer (NK) cells are specialized lymphocytes of the innate immune system that target both tumor cells and virally-infected cells. NK-cell cytotoxicity is regulated by a balance of signaling through inhibitory and stimulatory receptors (Raulet, Vance 2006, Raulet, Guerra 2009). Most of the inhibitory receptors generally recognize major histocompatibility complex I (MHC-I) molecules, a set of proteins often downregulated during viral infection or tumorigenesis. Stimulatory receptors recognize a wide variety of self-proteins that are induced upon viral infection or cellular transformation. Together, a net positive signal activates NK cells to secrete proinflammatory molecules TNF-α and IFN-γ, as well as effectors of lysis, granzymes and perforin (Lodoen, Lanier 2006).

NKG2D is a well-studied and potent NK-stimulatory receptor that is expressed on the surface of NK cells, activated CD8 T cells, and subsets of γδ T cells and NKT cells (Raulet 2003). NKG2D can also function as a co-stimulatory receptor to enhance T-cell activation (Diefenbach et al. 2001, Groh et al. 2001). The human genome encodes at least seven NKG2D ligands (MICA, MICB, ULBP1-4, and RAET1G), and the mouse genome encodes at least nine NKG2D ligands (MULT-1, H60, H60b, H60c, and RAE-1α-ε). Although the ligands bind NKG2D with varying affinities, they all trigger NK cell killing of target cells similarly. NKG2D ligand transcripts can be detected in certain cell types or during specific phases of development, but in general, ligand expression is low or absent in healthy cells (Raulet 2003). However, ligands are induced during various stress conditions including transformation, DNA damage, and viral infection. Accordingly, NKG2D ligands are constitutively expressed on many tumor cell lines and on a large array of tumors including melanomas, leukemias, various carcinomas, and neuroblastomas (Diefenbach et al. 2000, Groh et al. 1999). NKG2D ligands are also upregulated in cells infected with viruses such as cytomegalovirus (CMV), measles, Influenza A, and respiratory syncytial virus (Welte et al. 2003, Ebihara et al. 2007). To counteract this NK recognition, tumors and viruses have evolved ways to shed or block surface expression of NKG2D ligands (Salih, Rammensee & Steinle 2002, Jonjic et al. 2008). In particular, studies using mouse CMV (MCMV) with deletion mutations in genes encoding proteins that block ligand expression have shown that the ability of the virus to evade NKG2D recognition has a significant advantage on viral fitness *in vivo* (Lodoen et al. 2003, Hasan et al. 2005, Krmpotic et al. 2005). Furthermore, aberrant expression of NKG2D ligands can lead to unwanted NKG2D signaling, which has been implicated in autoimmune diseases, such as rheumatoid arthritis and

type 1 diabetes (Van Belle, von Herrath 2009). Therefore, regulation of ligand expression under different conditions is critical to prevent targeting of healthy cells.

Several modes of regulation have been shown for NKG2D ligand expression. At the transcriptional level, expression of human NKG2D ligands MICA and MICB seems to be controlled by heat shock elements in their promoters (Groh et al. 1996). Damage of genomic DNA also leads to increased expression of RAE-1, MULT-1, ULBP1-3 and MICA, and RAE-1 induction occurs through the action of ataxia telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3-related (ATR), as well as checkpoint effector kinase1 (Chk1) (Gasser et al. 2005). Additionally, it was reported that c-Myc regulates RAE-1ε at the transcriptional level (Unni, Bondar & Medzhitov 2008). At the post-transcriptional level, the expression of MICA and MICB can be inhibited by cellular microRNAs, and MICB expression can also be inhibited by viral microRNAs (Stern-Ginossar et al. 2008, Nachmani et al. 2009). Finally, the expression of MULT-1 is regulated post-translationally through ubiquitination (Nice, Coscoy & Raulet 2009).

The effect of NKG2D ligand expression on NK cell activity, both *in vitro* and *in vivo*, has been best characterized with the RAE-1 family of mouse NKG2D ligands. Cells that normally do not express NKG2D ligands become highly susceptible to NK cell-mediated lysis *in vitro* when transduced with RAE-1 (Diefenbach et al. 2000, Cerwenka et al. 2000). Ectopic expression of RAE-1 in tumor cells also results in efficient clearance of tumor cells after subcutaneous transfer *in vivo*. Clearance *in vivo* is mediated by NK cells and in some cases CD8 T cells, despite expression of inhibitory MHC-I molecules in some tumor cells (Diefenbach et al. 2001, Cerwenka, Baron & Lanier 2001). Together these data demonstrate that RAE-1 expression results in NK-cell susceptibility both *in vitro* and *in vivo*, and highlight the importance of understanding the molecular mechanism of RAE-1 expression.

Despite some evidence showing the role of certain pathways and effector molecules in the expression of NKG2D ligands, much remains to be learned about the process, and uncovering the molecular mechanism that drive expression of each of the NKG2D ligands remains an active area of research in the field. In particular, very little is known concerning the mechanisms of RAE-1 induction in virus-infected cells. CMV infection results in the induction of transcripts encoding numerous NKG2D ligands, including RAE-1, MULT-1, and H60a in the mouse. However, both human and mouse CMV encode proteins that specifically inhibit expression of each of the NKG2D ligands at the protein level, suggesting that NK cell recognition of CMV-infected cells has put evolutionary pressure on the virus to evade this arm of the immune system. The inducibility of RAE-1 in MCMV-infected cells prompted us to use this well characterized virus to investigate the molecular mechanism of RAE-1 induction. Strikingly, our studies showed that virus-induced activation of phosphatidylinositol-3-kinase (PI3K) is essential for the induction of the RAE-1 family of mouse NKG2D ligands. Further studies demonstrated that PI3K is also important for the maintenance of RAE-1 and MULT-1 expression on transformed cells, showing the breadth of our findings. These results suggest that activation of the PI3K pathway, which occurs in cells infected with numerous viruses and in cancer cells, represents a common signal for regulating RAE-1 expression. Finally, the effect of PI3K inhibition on MULT-1 expression also reveals the possibility that PI3K activation may play a role in regulating expression of other NKG2D ligands in cells infected with other viruses and other pathologic states such as inflammatory diseases.

Materials and Methods

Cells. Established tail-derived fibroblasts were prepared as described previously (Gasser et al. 2005). Established fibroblasts, BALB/c 3T3 (ATCC, CCL-163), NIH 3T3 (ATCC, CRL-1658), and BOSC (ATCC, CRL-11270) cells were maintained in DMEM with 5% FBS and 1% penicillin and streptomycin. YAC-1s (ATCC, TIB-160) and A20s were maintained in RPMI. Peritoneal macrophages obtained from C57BL/6 mice were cultured overnight in RPMI with 10% MCSF provided by Dr. Portnoy (UC Berkeley), 10% FBS, and 1% penicillin and streptomycin.

Virus propagation, infection and titering. MCMVΔ152 and Δ152-rev viruses were generously provided by Dr. Hill (Oregon Health and Science University, Oregon). MCMVΔ152-GFP virus was kindly provided by Dr. Jonjic (University of Rijeka, Croatia). MCMVWT (Smith strain) and MCMVΔm04+m06+m152 viruses were generously provided by Dr. Koszinowski (Max von Pettenkofer-Institute, Munich, Germany). All viruses were propagated in NIH 3T3 cells and titered in BALB/c 3T3 cells. For all infection experiments, fibroblasts were infected at MOI of 5, input virus removed at 2 hrs pi, and infection was allowed to take place for a total of 24 hrs. Supernatants were collected at the time of harvest at 24 hrs pi and used for titering in BALB/c 3T3s. For UV-inactivation of the virus, viral supernatant was placed directly under the UV light in a sterile tissue culture hood for 30 minutes. To confirm successful UV-inactivation, MCMV e1 gene was PCR amplified from viral genomic DNA isolated from equal volumes of untreated or UV-treated virus stock. Briefly, viral DNA was extracted from viral supernatants by adding an equal volume of phenol/chloroform followed by another round of chloroform extraction, and isopropanol was used to precipitate the DNA. UV-inactivation was further confirmed by performing a plaque assay on supernatants obtained from cells infected with either untreated or UV-treated virus.

Flow cytometry. Fibroblasts and 3T3 cells were harvested in 2mM EDTA PBS and stained with monoclonal anti-mouse pan-specific RAE-1, RAE-1α/β/γ, RAE-1β/δ, RAE-1ε, MULT-1, H60A or Rat IgG_{2A} isotype control (all purchased from R&D) followed by PE-conjugated goat anti-rat IgG (Southern Biotech). YAC-1s, A20s, and peritoneal macrophages were first incubated with an anti-mouse CD16/CD32 FcBlock (BD), followed by pan RAE-1 antibody and FITC or PE-conjugated anti-rat IgG_{2A} antibody (BD). All samples were co-stained with 7-AAD (BD). MCMV m157-specific monoclonal antibody (6H1.2.1) was generously provided by Dr. Wayne Yokoyama (Washington University School of Medicine, MO).

Reverse transcription quantitative real-time PCR. RNA from fibroblasts and macrophages were extracted in Trizol (Invitrogen), treated with RQ1 DNase (Promega), and total RNA was reverse transcribed using oligo$(dT)_{15}$ primer (Promega) and SuperScriptII (Invitrogen) at 42ºC for 50 minutes. cDNAs were analyzed using ABI7300 Real Time PCR System. RAE-1 isoform specific primers were described previously (Ogasawara et al. 2003). Primers for RAE-1 and ISG15 are described in the Supplementary Table. cDNAs from uninfected tail fibroblasts were used to amplify regions within the catalytic and regulatory domains of class I PI3K using primers described in Table S2.1.

Inhibitor treatments. Inhibitors for all infection experiments were added to the media 2 hrs pi to first allow viral attachment and entry, and they were left in the culture media for the remainder

of the 24 hr infection. Phosphonoacetic acid (PAA) was purchased from Sigma Aldrich and used at a final concentration of 100 ug/ml, pH 7.4. YAC-1s were cultured in the presence of PI3K inhibitors for 18 hrs. U0126, SB203580, SB600125, SB218078, UCN-01, LY294002, Rapamycin, and NU7026 were purchased from Calbiochem. PI3Kαi2, PI-103, TGX-221, and AS252424 were purchased from Cayman Chemicals. Caffeine was purchased from Sigma, and IC87114 was kindly provided by Dr. Okkenhaug (Babraham Institute, Cambridge, UK). The final concentrations of all of these inhibitors are stated in the figure legends of the corresponding figures.

Constructs and transduction. The coding region of RAE-1α or γ isoforms was cloned into a retroviral vector, pBMN-IN. p110α H1047R was cloned into a retroviral vector pMG-hygro. Retroviral supernatants were obtained as described previously (Jarousse, Coscoy 2008). MCMV ie1, 2, and 3 fused to GFP was cloned into pEGFP.N1 (Clontech) and transiently transfected using Lipofectamine 2000 (Invitrogen).

Western blotting. Mouse fibroblasts or peritoneal macrophages were serum-starved overnight and infected with MCMV in the presence of DMSO or LY294002 or treated with PDGF (Sigma) for 24 hrs. Fibroblasts transduced with empty vector or transduced with p110α H1047R were serum-starved overnight. Cell lysates were analyzed by western blotting for phospho-Akt S473 and Akt according to manufacturer's instructions (Cell Signaling). The relative ratio of P-Akt to total Akt was determined using ImageJ.

Statistical analysis. A two-tailed, paired student t-test was performed on all samples where statistical significance is indicated.

Results

RAE-1 mRNA and protein are induced upon MCMV infection.

Most cell lines constitutively express varying levels of RAE-1 at the cell surface (Diefenbach et al. 2000). Because most cells *in vivo* generally express very low levels of NKG2D ligands, if any at all, we utilized established mouse-tail fibroblasts that do not express RAE-1 at the surface to investigate the mechanism of RAE-1 induction upon MCMV infection. These fibroblasts have previously been used to demonstrate RAE-1 induction upon activation of the DNA damage response (Gasser et al. 2005). Upon infection of fibroblasts with MCMV for 24 hours, there was a significant induction of RAE-1 expression at the RNA level (Fig. 2.1A). In order to further observe RAE-1 induction at the cell surface, we utilized a MCMV mutant (MCMVΔ152) lacking the m152 gene, which encodes an immune evasin that downregulates RAE-1 protein. Using this virus, we observed RAE-1 surface expression starting 18 hours post-infection with an even higher expression at 24 hours post-infection (Fig. 2.1B). Importantly, RAE-1 surface expression was not observed upon infection with the revertant virus (MCMVΔ152-rev) at 24 hours post-infection, despite no significant difference in the levels of RAE-1 mRNA induction (Fig. 2.1C).

Although previous studies have demonstrated the ability of MCMV to induce RAE-1 expression, it was not determined whether induction occurs specifically in infected cells or also in neighboring uninfected cells by an indirect mechanism. To address this question, we distinguished infected versus uninfected cells by staining cells with an antibody specific for an MCMV protein, m157, that is expressed at the cell surface of infected cells (Tripathy et al.

2006). Co-staining experiments demonstrated that RAE-1 induction occurs only in infected cells, suggesting that RAE-1 induction is a direct consequence of infection (Fig. 2.1D). The m157-positive cells that express RAE-1 at low levels are most likely cells that were recently infected in the cultures and have not had sufficient time to upregulate RAE-1. In subsequent experiments, we determined which events associated with the viral life cycle are necessary for RAE-1 induction.

Viral gene expression is necessary for RAE-1 induction.

Upon entry, MCMV initiates a sequence of well characterized events including transcription of immediate early and early genes, which are essential for viral replication and for the activation of cellular pathways aimed at priming the cell for efficient viral replication (Isaacson, Juckem & Compton 2008). Expression of these early genes is also required for the expression of late genes and subsequent packaging and budding of the virus (Gibson 2008).

To investigate whether expression of viral genes is necessary for RAE-1 induction, fibroblasts were infected with either MCMVΔ152 or UV-inactivated MCMVΔ152 for 24 hours. UV inactivation significantly impaired the ability of MCMVΔ152 to induce expression of RAE-1 both at the RNA and protein levels throughout the course of the infection (Fig. 2.2A and B). Interferon-Stimulated Gene 15 (ISG15) expression was significantly induced upon infection by both MCMVΔ152 (Δ152) and UV-inactivated virus (UV) (Fig. 2.2C), indicating that neither viral entry nor activation of the interferon response was affected by the UV treatment. As a control, MCMV early gene 1 (e1) product was PCR amplified using viral genomic DNA extracted from either MCMVΔ152 or UV-inactivated MCMVΔ152, and no amplification was observed from the UV-inactivated viral genomic DNA (data not shown). UV-inactivation was also confirmed by the lack of plaque forming units in the supernatant of cells infected with UV-inactivated virus for 24 hours (data not shown).

We next determined whether viral DNA replication is required for the induction of RAE-1 using phosphonoacetic acid (PAA), a chemical inhibitor that binds to the viral DNA polymerase and blocks CMV viral replication (Huang 1975). Infection of fibroblasts with MCMVΔ152 for 24 hours in the presence of PAA did not inhibit RAE-1 induction, indicating that viral DNA replication and late gene expression are dispensable for RAE-1 induction (Fig. 2.2D). Altogether, our results suggest that expression of viral genes at an early stage upon infection prior to viral replication is necessary for the induction of RAE-1.

The DNA damage response is not required for RAE-1 induction by MCMV.

Stress-induced activation of the DNA damage response, through the action of ATM or ATR and Chk1, has been implicated in the induction of RAE-1 and other NKG2D ligands (Gasser et al. 2005, Ward et al. 2009, Richard et al. 2010). Additionally, CMV has been shown to manipulate the DNA damage response (Shen et al. 2004, Gaspar, Shenk 2006). Therefore, we tested the role of the DNA damage response in the induction of RAE-1 in MCMV-infected cells by infecting fibroblasts for 24 hours in the presence or absence of specific inhibitors of the DNA damage response pathway. Inhibition of Chk1 using SB218078 and UCN-01, or inhibition of ATM/ATR using caffeine did not affect RAE-1 induction upon MCMV infection, indicating that activation of the DNA damage response is not required for MCMV-induced RAE-1 expression (Fig. S2.1).

RAE-1 induction requires activation of the PI3K pathway.

Many cellular pathways are activated early on upon viral infection to achieve a state of pro-survival and increased cellular proliferation for optimal replication and production of progeny virus (Yurochko 2008). Common cellular pathways activated upon viral infections include the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K) pathways (Cooray 2004, Buchkovich et al. 2008). The PI3K pathway, in particular, is crucial in controlling cell growth and survival and is a key pathway in promoting cellular transformation, another condition known to trigger NKG2D ligand expression (Cully et al. 2006). Because our data suggested that early events upon viral infection are necessary to induce RAE-1, we hypothesized that manipulation of some of these cellular pathways are involved in the induction. To test this hypothesis, fibroblasts were infected with MCMVΔ152 for 24 hours in the presence of known inhibitors of these pathways, and RAE-1 surface expression was analyzed. MCMV-induced RAE-1 induction was not affected by the presence of MAPK inhibitors (Fig. S2.2). Remarkably however, surface expression of RAE-1 was completely abrogated in the presence of LY294002, a global inhibitor of PI3K that binds to the catalytic domain of the kinase (Walker et al. 2000) (Fig. 2.3A). Viral titers in the supernatant collected from cells infected for 24 hours in the presence or absence of LY294002 were not significantly different, indicating that the absence of RAE-1 surface expression was not due to a lack of viral entry and replication (Fig. 2.3B). Of note, input virus was removed two hours post-infection, and therefore, virus present in the supernatant at 24 hours post-infection is a measure of progeny virus produced as a result of infection and replication. Furthermore, inhibitors were added at two hours post-infection to fresh culture media to prevent possible blockage of viral attachment or entry.

The requirement for active PI3K to induce RAE-1 suggests that MCMV infection activates the PI3K pathway. To determine whether the PI3K pathway is activated upon MCMV infection in our system, cellular lysates from MCMV-infected fibroblasts were analyzed by western blotting with an antibody specific for Akt phosphorylated at Serine 473. As positive controls, whole cell lysates were obtained from cells treated with 20% fetal bovine serum (FBS) or cells stably expressing the catalytic subunit of PI3K (p110α) with an H1047R mutation that renders PI3K constitutively active (Zunder et al. 2008). Very little Akt phosphorylation was observed in uninfected cells. By contrast, Akt phosphorylation was readily detectable in MCMV-infected cells as well as in cells treated with FBS and cells stably expressing p110α H1047R (Fig. 2.3C). When cells were infected in the presence of LY294002, the phosphorylated form of Akt was no longer detectable. Altogether, our data indicate that MCMV infection activates the PI3K pathway, in accordance with data obtained with HCMV (Johnson et al. 2001), and that this activation is required for the induction of RAE-1.

Signaling through p110α PI3K is essential for RAE-1 induction in MCMV-infected cells.

There are three classes of enzymes in the PI3K superfamily, class I, II, and III. Akt activation occurs mainly through class I PI3K, and this is critical in regulating cell survival, metabolism, apoptosis, and cell cycle. Class I PI3Ks are heterodimeric molecules composed of a catalytic and a regulatory subunit and are classified into class IA or class IB PI3K. The catalytic subunits of class IA PI3K are p110α, β or δ, whereas class IB PI3K contains p110γ (Paez, Sellers 2003). It is becoming increasingly appreciated that PI3K catalytic subunits play non-redundant roles in regulating the biology of the cell (Vanhaesebroeck, Vogt & Rommel 2010). Thus, we hypothesized that RAE-1 induction upon MCMV infection occurs through a particular PI3K isoform such that a specific signal is required for its expression.

In order to determine which of the PI3K isoforms are involved in RAE-1 induction, we first determined the expression patterns of each of the PI3K catalytic and regulatory subunits in our fibroblasts at the steady state level by RT-PCR analysis. Our analysis showed that all of the catalytic and regulatory subunits were detected to varying degrees in these cells (Fig. 2.4A). We then employed isoform-specific inhibitors to test the role of each of the class I PI3Ks on MCMV-induced RAE-1 expression. RAE-1 surface expression was greatly diminished when cells were infected in the presence of inhibitors for p110α (PI3Kαi2 and PI-103), but not in the presence of inhibitors for p110β (TGX-221), p110δ (IC87114) or p110γ (AS252424) (Fig. 2.4B). Similar to treatment with LY294002, treatment with either PI3Kαi2 or PI-103 did not result in a significant change in the viral titer in the supernatant, indicating that the loss of RAE-1 expression in these cells was not due to the lack of viral entry or replication (Fig. 2.4C).

Compared to LY294002, PI-103 is much more selective for p110α, but at higher concentrations it is still able to inhibit other targets in the pathway, namely DNA-PK and mTORC1 (Κνιγητ ετ αλ. 2006). Therefore, to rule out possible contributions from these molecules on RAE-1 induction, selective inhibitors of DNA-PK and mTORC1 (NU7026 and rapamycin, respectively) were tested in the same assay. Neither NU7026 nor rapamycin treatment inhibited RAE-1 induction, suggesting that indeed RAE-1 induction upon MCMV infection involves signaling specifically through the p110α-containing PI3K (Fig. 2.4D). These results were further confirmed using a wide range of inhibitor concentrations in MCMV-infected cells (Fig. S2.3).

p110α PI3K is also important for the maintenance of RAE-1 and MULT-1 on transformed cells.

The gene encoding p110α is an oncogene that is commonly mutated in human cancers (Samuels et al. 2004, Bachman et al. 2004). These mutations in p110α cause the PI3K pathway to be constitutively active, resulting in cellular transformation and oncogenesis (Kang, Bader & Vogt 2005, Ikenoue et al. 2005, Samuels et al. 2005). Because RAE-1 molecules, along with other mouse and human NKG2D ligands, are frequently expressed on the surface of transformed cell lines as well as in some tumors *in vivo* (Diefenbach et al. 2000, Groh et al. 1999), we hypothesized that RAE-1 expression in transformed cells is also dictated by p110α PI3K signaling. To test this hypothesis, we first tested the effect of LY294002 treatment on three different types of transformed cell lines that all constitutively express RAE-1 at the cell surface: A20 (a B lymphoma cell line), NIH 3T3 (adherent fibroblast cell line) and YAC-1 (a T lymphoma cell line). Cell surface RAE-1 expression was susceptible to inhibition by LY294002 treatment in all three cell lines tested (Fig. 2.5A), suggesting that RAE-1 expression in these cells also depends on active PI3K.

To test the role of specific PI3K isoforms on RAE-1 expression in transformed cells, YAC-1 cells were treated with the same isoform-specific inhibitors used in Figure 2.4B and Figure S2.3, at a wide range of concentrations. RAE-1 surface expression was measured at 24 hours post-treatment. Similar to the effect observed in MCMV-infected cells, PI-103 treatment led to a significant decrease in the expression of RAE-1, whereas treatment with selective inhibitors of the other isoforms of PI3K (p110β, γ, and δ) or an inhibitor of DNA-PK had no significant effect on RAE-1 expression, even at high concentrations (Fig. 2.5B). In NIH 3T3 cells (Fig. 2.5C) and A20 cells (data not shown), we also observed inhibition of RAE-1 expression by PI-103, but not by inhibitors of other PI3K isoforms. The lack of response to inhibitors that target the p110β, γ, and δ isoforms of PI3K strongly indicates that signaling

through p110α is specifically involved in the expression of RAE-1 in transformed cells as well. We also tested whether RAE-1 expression was impacted by rapamycin, an inhibitor of mTORC1, a downstream effector of PI3K. Whereas rapamycin did not inhibit RAE-1 expression in infected cells (Fig. 2.4D), 3T3 cells (Fig. 2.5C) or A20 cells (data not shown), it did block RAE-1 expression in YAC-1 cells (Fig. 2.5B). These data suggest that mTORC1 plays a role in supporting RAE-1 expression in some transformed cells but not in others or in MCMV-infected fibroblasts.

In order to address whether the PI3K pathway regulates other mouse NKG2D ligands, we determined the effect of PI3K inhibition on expression of MULT-1 and H60a in NIH 3T3 cells, which constitutively express all three types of mouse NKG2D ligands. Similar to the results with RAE-1, MULT-1 expression was suppressed in 3T3 cells treated with LY294002 or PI-103, but not with the other inhibitors (Fig. 2.5C). In contrast, H60a expression was not significantly affected by the inhibition of PI3K. Altogether the data illustrate a specific role of p110α PI3K in regulating RAE-1 and MULT-1 mouse NKG2D ligands in transformed cells.

Discussion

Activation of NK cells through the interaction between NKG2D and NKG2D ligand is critical in the clearance of tumor cells, virally-infected cells, and stressed cells (Raulet, Vance 2006). Although reports have implicated various effector molecules in regulating NKG2D ligand expression, the mechanism of ligand induction is still poorly understood. Here, we show that dysregulation of the PI3K pathway is a key signal required for expression of the RAE-1 family of NKG2D ligands during both infection and transformation and that it is further involved in the expression of another mouse NKG2D ligand, MULT-1.

In this study, we used both mouse fibroblasts and primary peritoneal macrophages to study the expression of the RAE-1 family members (Fig. 2.1 and Fig. S2.4). Because both macrophages and fibroblasts are infected by MCMV *in vivo*, the use of both of these cell types *in vitro* is informative (Hsu et al. 2009). We chose fibroblasts to perform all of the experiments because MCMVΔ152-infected macrophages do not express RAE-1 protein at the cell surface (Fig. S2.4C). The absence of surface expression occurred despite efficient infection of these cells, activation of the PI3K pathway, and a strong induction of RAE-1 mRNA, suggesting a cell type-specific block in a post-transcriptional step of RAE-1 biogenesis that does not prevent PI3K activation (Fig. S2.4A, B, and D).

RAE-1 expression requires sensing of "patterns of pathogenesis."

Using fibroblasts, we observed that RAE-1 induction occurs only in infected cells (Fig. 2.1D) and that this induction requires active viral gene expression (Fig. 2.2). Because viral infections are often accompanied by production of defective viral particles that do not contain the entire viral genome, such specificity will presumably spare cells infected with defective viral particles from NK-cell mediated killing. This discrimination may be beneficial for the host because cells exposed to defective viral particles contain nucleic acids that function as pathogen associated molecular patterns (PAMPs) and activate innate immune sensors that induce production of type-I IFN and other proinflammatory molecules. Indeed, UV-inactivated MCMV is a potent activator of ISG15 (Fig. 2.2C). NK-mediated killing of these cells could potentially curtail inflammatory cytokine production. Additionally, cells infected with defective particles may also serve as good sources of antigens for cross-presentation by antigen presenting cells.

Recently, Vance et al. described the principle that signals associated with active bacterial infection and manipulation of the host cell machinery, termed "patterns of pathogenesis," can serve to activate the innate immune response (Vance, Isberg & Portnoy 2009). Here, we illustrated that UV-inactivated viral particles were sufficient to stimulate the IFN response, but expression of RAE-1 proteins was absolutely dependent on active infection and manipulation of the host cell machinery; activation of PI3K signaling being one example of such manipulation. Hence, PI3K activation appears to function as a "pattern of pathogenesis" for induction of RAE-1 expression. However, additional considerations, described below, indicate that PI3K activation is not sufficient for Rae1 induction, suggesting that several signals may function cooperatively to induce RAE-1 expression.

Multiple signals are required for the expression of RAE-1.

We observed that RAE-1 expression requires viral genes that are expressed prior to viral replication, as demonstrated by the use of UV-inactivated virus and PAA (Fig. 2.2). This may suggest that viral early proteins are mediating the induction of RAE-1. Because immediate early proteins are the first to be expressed upon MCMV infection, we tested their role in the induction of RAE-1. Overexpression of GFP-fused MCMV ie1, ie2, and ie3 proteins alone or in combination did not lead to the induction of RAE-1 (Fig. S2.5), suggesting that although these proteins may play a role, they are not sufficient for RAE-1 expression.

In this manuscript, we focused on the involvement of cellular pathways that are activated during viral infection in the expression of RAE-1. The PI3K pathway regulates cellular functions including metabolism, cell cycle progression, proliferation, and apoptosis, and it is often dysregulated in infected and transformed cells (Cooray 2004, Engelman 2009). Class IA PI3K is generally activated through receptor tyrosine kinases (RTKs), which are receptors for growth factors, cytokines, and hormones. If class IA PI3K activation alone is sufficient to induce RAE-1 induction, it should occur in response to many cellular stimuli independently of infection. To test this, we stimulated cells for 24 hours with PDGF, which activates the well-studied RTK, PDGF receptor. Despite robust activation of the PI3K pathway, as illustrated by phosphorylation of Akt, RAE-1 induction did not occur (data not shown and Fig. S2.6A). Interestingly, PDGF-R has been shown to be a cellular receptor for HCMV (Soroceanu, Akhavan & Cobbs 2008). Thus, the lack of RAE-1 induction with PDGF treatment is consistent with our finding that viral gene expression is absolutely necessary. Additionally, overexpression of a constitutively active form of p110α, p110α H1047R, by itself was insufficient to induce RAE-1 expression, again despite robust activation of the PI3K pathway (Fig. S2.6B and 2.3C).

Together these results strongly suggest that RAE-1 induction is tightly regulated such that expression of the main viral immediate early proteins (ie1, ie2, and ie3) or activation of class IA PI3K in the absence of infection are not sufficient to induce ligand induction and that additional signals are likely required for the induction. Future investigations are necessary to identify these additional pathways that are necessary for RAE-1 induction.

The role of PI3K activation in regulating RAE-1 expression.

To determine whether PI3K activation is involved in regulating RAE-1 at the post-translational level, we stably expressed the coding region of RAE-1α or RAE-1γ isoform from an exogenous promoter. The choice of these isoforms came from our observation that they were induced upon MCMV infection at the RNA level (Fig. S2.8A) and at the protein level, confirmed by the use of two different mutant viruses lacking m152 (Fig. S2.8B and C). RAE-1 expression

in these cells was not affected by LY294002 treatment, arguing that trafficking of RAE-1 and sustained expression of mature RAE-1 proteins at the cell surface is not dependent on PI3K activation (Fig. S2.7B).

Notably, although LY294002 treatment of infected cells resulted in a complete loss of RAE-1 expression at the cell surface (Fig. 2.3A), RAE-1 mRNA was still induced five fold compared to uninfected cells (Fig. S2.7A). Therefore, it is possible that the PI3K pathway plays a role in regulating RAE-1 at least in part at the post-transcriptional level, prior to trafficking to the surface. Activation of PI3K can enhance cellular translation via formation of the translation initiation complex containing eIF4E (Gingras, Raught & Sonenberg 1999), raising the possibility that RAE-1 is regulated at the translational level upon PI3K activation. Nevertheless, LY294002 treatment reduced the amount of RAE-1 mRNA in infected cells by three fold, suggesting a possible role of PI3K in either transcription of the RAE-1 gene or stabilization of RAE-1 mRNA as well. It has recently been shown that transcripts of two human NKG2D ligands, MICA and MICB, are regulated by several cellular microRNAs (Stern-Ginossar et al. 2008), raising an additional possibility that PI3K regulates microRNAs that target RAE-1.

The importance of PI3K signaling in transformed cells and cancer cells.

The gene encoding the p110α catalytic subunit of PI3K is often mutated in mouse and human tumors so as to render PI3K constitutively active, and thus functions as an oncogene (Zhao, Vogt 2008). Despite the involvement of p110β, γ, and δ isoforms in cancer, p110α is the only catalytic subunit of PI3K found to be mutated in tumors, suggesting a unique role of p110α in cellular transformation (Vanhaesebroeck, Vogt & Rommel 2010). We observed that p110α PI3K is important for the expression of RAE-1 in multiple transformed cell lines (Fig. 2.5). The mechanism by which the different PI3K heterodimers mediate their non-redundant functions is poorly understood (Vogt et al. 2010). It is possible that the specificity of p110α is achieved at the cell surface receptor level (i.e. RTKs or GPCRs) or by one of the downstream effectors of PI3K. Nonetheless, the role of p110α PI3K in regulating RAE-1 expression during both viral infection and transformation is an intriguing finding that deserves further investigation.

In this study, we also observed an effect of PI3K inhibition on MULT-1 expression, but not H60a. The observed difference in the requirement for PI3K signaling for expression of different mouse NKG2D ligands is interesting and may have several explanations. One possibility is that the difference reflects a specialization in NKG2D ligands, in which RAE-1 and MULT-1 respond to activated PI3K, whereas H60a responds to distinct cellular cues or stress pathway mediators. The notion that NKG2D ligands respond to different stress pathways was already suggested by the finding that MULT-1 is unique among the mouse NKG2D ligands in being regulated by stress associated with heat shock or UV irradiation (Nice, Coscoy & Raulet 2009). The possibility that H60a has unique regulatory properties is also suggested by the sequence of its 3' untranslated region (UTR), which is unusually long (3kb) in comparison to those of RAE-1 and MULT-1 (400bp and 700bp, respectively), suggesting that it may contain a unique set of regulatory elements.

PI3K and its downstream mediators such as Akt and mTORC1 have been key targets in the development of cancer therapies (Engelman 2009). In particular, chemical analogues of the inhibitors used in this study are used in clinical studies as therapeutics for cancer. As cancer drug development progresses, it will be important to take into consideration the potential for these PI3K inhibitors to greatly diminish NK-cell recognition and cytolysis of targets; especially

because NK cells are important for recognition and clearance of tumor cells (Raulet, Guerra 2009).

Here, we identified a common pathway between infected and transformed cells that is required for expression of the RAE-1 family of mouse NKG2D ligands. The results of this study are the first to demonstrate the role of the PI3K pathway in the expression of NKG2D ligands or other events that sensitize cells for immune recognition. Our data suggest that PI3K dysregulation in the context of disease is a key signal sensed by cells for expression of RAE-1. This study provides an important direction for future investigations designed to elucidate how NKG2D ligand expression is regulated and how it is restricted to diseased cells.

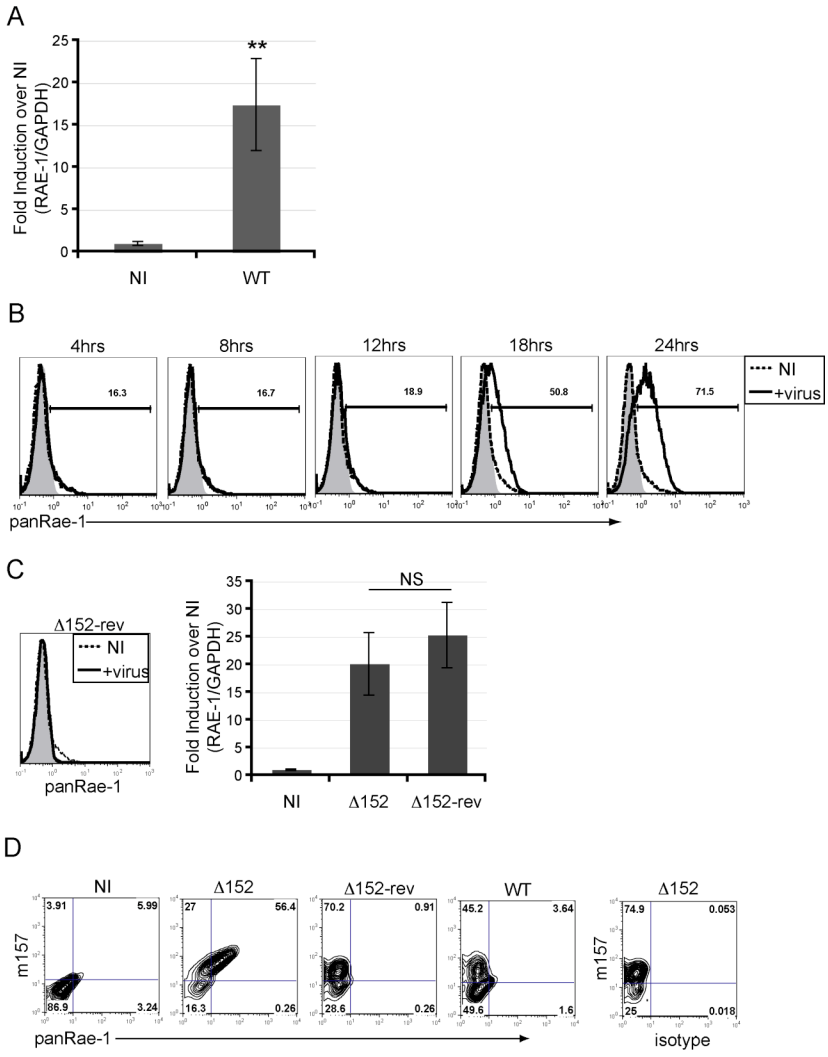


Figure 2.1. RAE-1 mRNA and protein are induced upon infection of mouse fibroblasts. A) The level of RAE-1 mRNA induction was determined in MCMV-infected fibroblasts (WT) at 24 hrs post-infection (pi) using RT-qPCR. Fold induction was determined by normalizing the infected samples to uninfected samples (NI). SD and statistical significance were determined from five independent samples. **p<0.01 compared to NI. B) RAE-1 expression on the surface of fibroblasts infected with MCMVΔ152 for 4, 8, 12, 18 and 24 hrs pi was determined using a pan RAE-1 antibody. The data shown are gated on live, 7AAD negative cells. Histograms show isotype control (shaded gray), uninfected (dashed black), and infected cells (solid black). C) RAE-1 surface expression on fibroblasts infected with MCMVΔ152 revertant (Δ152-rev) was analyzed using a pan RAE-1 antibody at 24 hrs pi. The level of RAE-1 mRNA in cells infected with Δ152 and Δ152-rev was quantified by RT-qPCR. Fold induction was determined by normalizing the infected samples to uninfected samples (NI). The data in the figure are representatives of three independent experiments. NS, not statistically significant. D) RAE-1 expression was determined in infected versus uninfected cells. Cells were infected with Δ152, Δ152-rev or WT virus and co-stained for MCMV m157 and RAE-1 at 24 hrs pi.

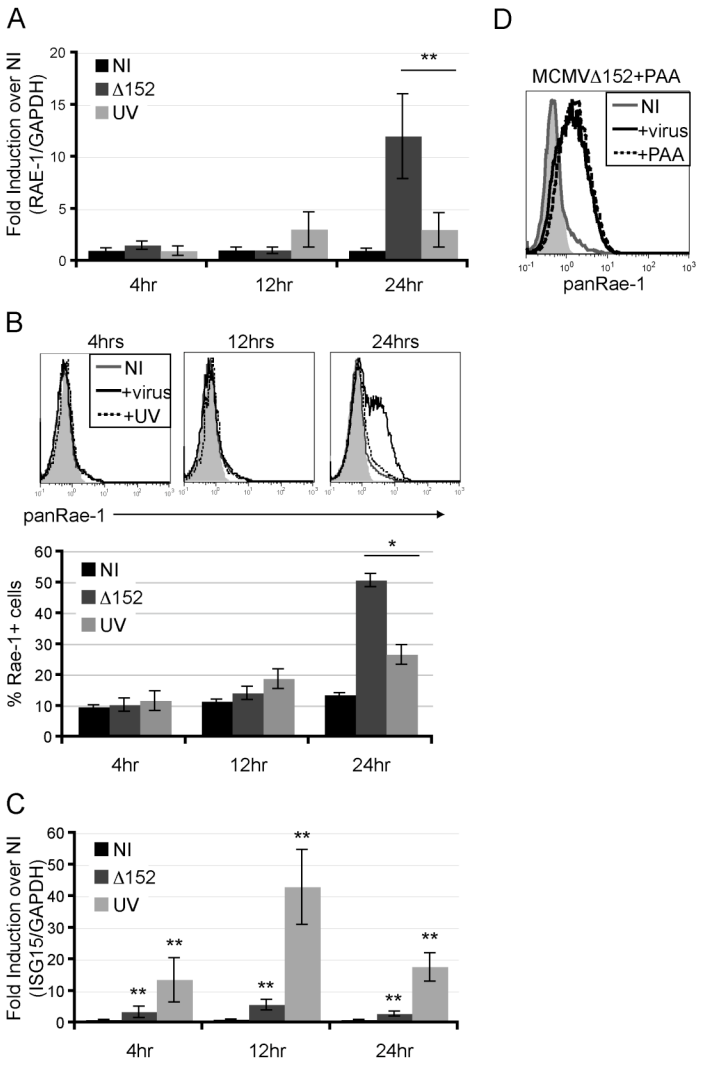


Figure 2.2. Viral early gene expression is necessary for the induction of RAE-1. Fibroblasts were infected with either MCMVΔ152 (Δ152) or UV-inactivated MCMVΔ152 (UV) for 4, 12, and 24 hrs, and RAE-1 mRNA expression (A), RAE-1 cell surface expression (B), and ISG15 mRNA expression (C) were determined using RT-qPCR and flow cytometry. Fold induction was determined by normalizing the infected samples to uninfected samples (NI). SD and statistical significance were determined from three independent samples. *$p<0.05$ and **$p<0.01$. D) Fibroblasts were infected with MCMVΔ152 in the presence of 100ug/ml PAA and stained with a pan RAE-1 antibody. Histograms show isotype control (shaded gray), uninfected (solid gray), MCMVΔ152-infected (solid black), and cells infected with MCMVΔ152 in the presence of PAA (dashed black). The histogram is a representative figure of three independent experiments.

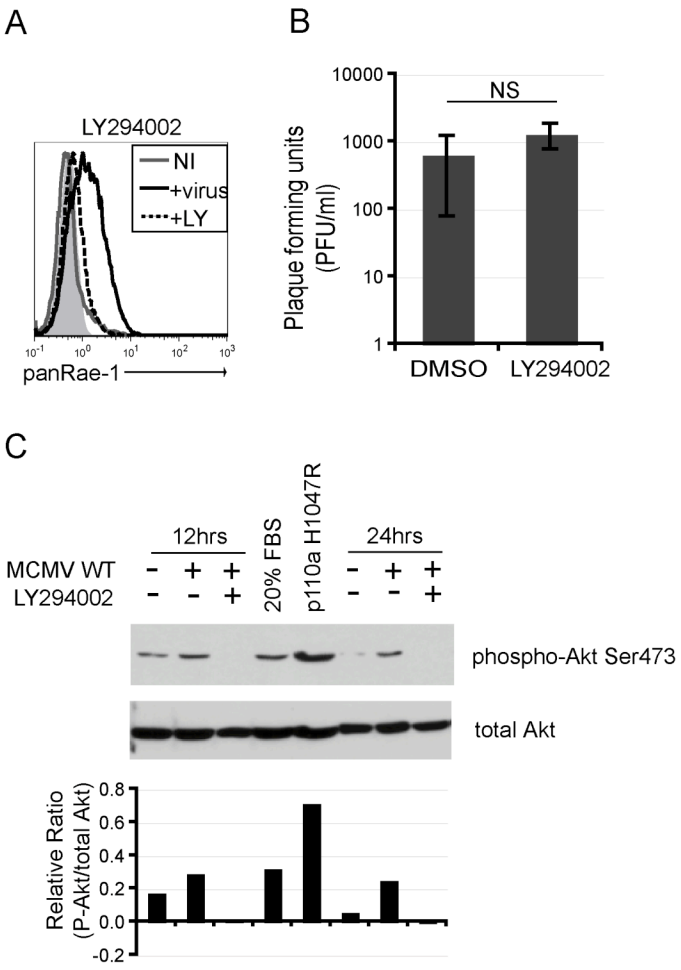


Figure 2.3. PI3K pathway is required for RAE-1 induction in MCMV-infected cells through the activation of Akt. A) Fibroblasts infected with MCMVΔ152 in the presence of 20uM LY294002 were stained for RAE-1 at 24 hrs pi. Histograms show isotype control (shaded gray), uninfected (solid gray), MCMVΔ152-infected (solid black), and MCMVΔ152-infected cells in the presence of LY294002 (dashed black). The histogram is a representative figure from three independent experiments. B) Plaque forming units (PFU)/ml were determined in supernatants of infected cells in the presence of DMSO or LY294002. SD and statistical significance were determined from three independent experiments. NS, not statistically significant. C) Whole cell lysates from fibroblasts infected with MCMV WT in the presence of DMSO or LY294002 were obtained at 12 and 24 hrs pi. Lysates were also obtained from cells treated with 20% FBS or cells overexpressing p110α H1047R. All samples were blotted for phospho-Akt S473 and total Akt. The relative ratio of P-Akt to total Akt from the blot was determined. The data is a representative experiment from ten independent experiments.

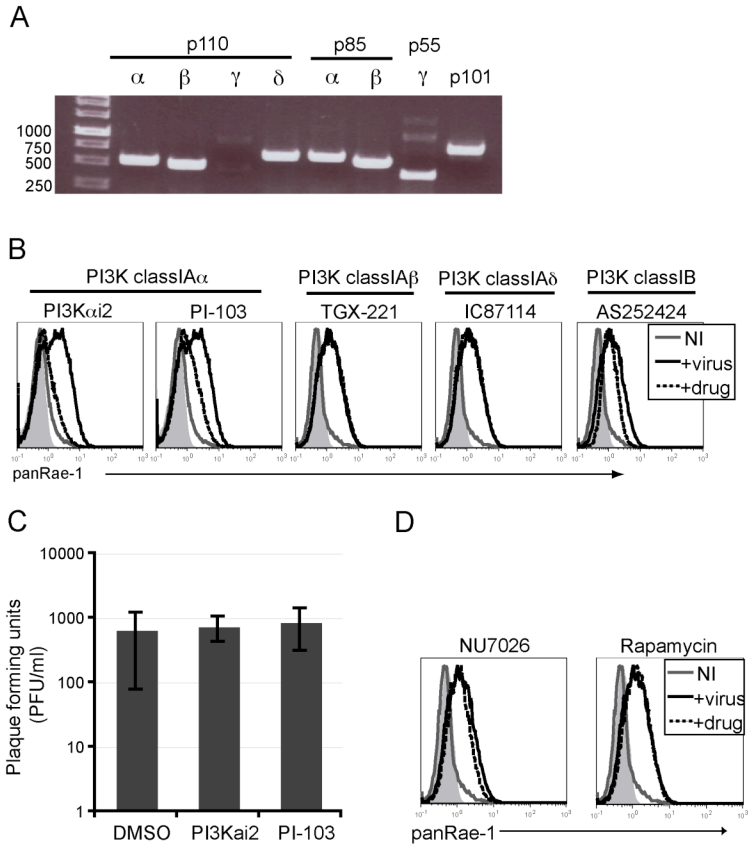


Figure 2.4. p110α PI3K is specifically involved in the induction of RAE-1 in fibroblasts infected with MCMV. A) Expression of PI3K catalytic and regulatory domains of class IA and IB PI3K in mouse fibroblasts at the steady state level was determined by RT-PCR. Top gel shows amplification of fibroblast cDNA and the bottom gel shows amplification of no RT controls. Size of the ladder is indicated in base pairs. B) Fibroblasts infected with MCMVΔ152 in the presence 25uM PI3Kαi2, 1.25uM PI-103, 5uM TGX-221, 10uM IC87114 or 10uM AS252424 were surface stained for RAE-1 at 24 hrs pi. C) PFU/ml was determined in supernatants from cells infected in the presence of DMSO, PI3Kαi2 or PI-103. SD and statistical significance were calculated from three independent experiments. NS, not statistically significant. D) Fibroblasts infected with MCMVΔ152 in the presence of 10uM NU7026 or 100nM rapamycin were stained for RAE-1 at 24 hrs pi. All histograms show isotype control (shaded gray), uninfected (solid gray), MCMVΔ152-infected cells in the presence of DMSO (solid black) or inhibitors (dashed black). All histograms are representative figures from three independent experiments.

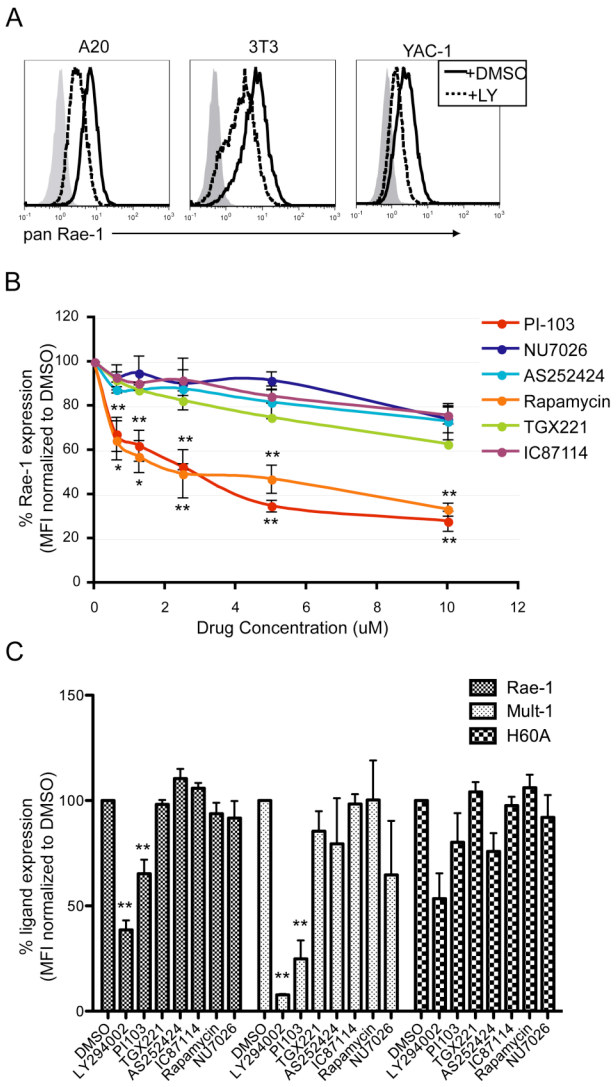


Figure 2.5. p110α PI3K is involved in the maintenance of RAE-1 and MULT-1 expression on transformed cells. A) A20, BALB/c 3T3 and YAC-1 tumor cells were treated with 20uM LY294002 for 24 hrs and stained for RAE-1 at the cell surface. Histograms show isotype control (shaded gray), untreated (solid black) and treated cells (dashed black). The histogram is a representative figure from multiple independent experiments. B) YAC-1 cells were treated with the indicated inhibitors at 10uM, 5uM, 2.5uM, 1.25uM, and 0.625uM and surface stained for RAE-1. SD and statistical significance were determined from three independent experiments. Statistical significance (** or *) on the bottom corresponds to PI-103 and the ones on top correspond to rapamycin treatments. C) NIH 3T3 cells expressing RAE-1, MULT-1, and H60a were treated with the indicated inhibitors for 24 hrs and stained for RAE-1, MULT-1 or H60a (20uM LY294002, 1uM PI-103, 1uM TGX-221, 100nM Rapamycin, 10uM AS252424, 10uM IC87114, and 10uM NU7026). Percent RAE-1 and ligand expression were determined by normalizing the MFI of RAE-1 or other ligands in inhibitor-treated cells to the MFI of RAE-1 or other ligands in DMSO-treated cells. SD and statistical analyses were calculated from three independent experiments. **p<0.01 and *p<0.05.

Supplementary Figures

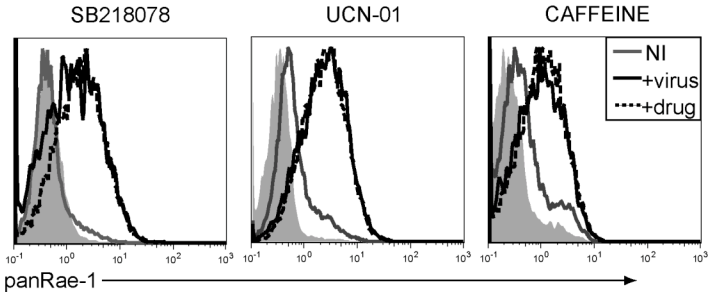


Figure S2.1. MCMVΔm04+m06+m152-mediated induction of RAE-1 is independent of the DNA damage pathway. RAE-1 surface expression was determined in fibroblasts infected with MCMV lacking m04, m06, and m152 (MCMVΔm04+m06+m152) in the presence of 150nM SB218078, 70nM UCN-01 or 5mM Caffeine. The data shown are gated on live, 7AAD negative cells. Histograms show isotype control (shaded gray), uninfected (solid gray), MCMVΔm04+m06+m152-infected (solid black), and MCMVΔm04+m06+m152-infected cells in the presence of inhibitors (dashed black).
Chapter 2: Expression of the RAE-1 family of stimulatory NK-cell ligands requires activation of the PI3K pathway during viral infection and transformation

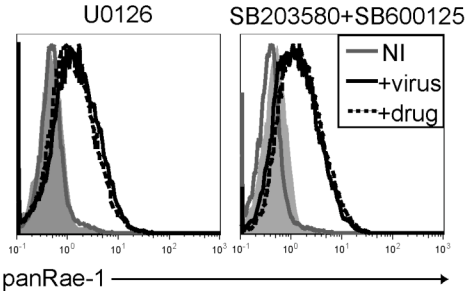


Figure S2.2. Erk and p38/Jnk activation is not required for MCMV-mediated induction of RAE-1. Fibroblasts infected with MCMVΔm04+m06+m152 in the presence of 2uM U0126 or 1uM SB203580 and SB600125 were stained with the pan RAE-1 antibody. The data shown are gated on live, 7AAD negative cells. Histograms show isotype control (shaded gray), uninfected (solid gray), MCMVΔ3-infected (solid black), and MCMVΔm04+m06+m152-infected cells in the presence of inhibitors (dashed black).

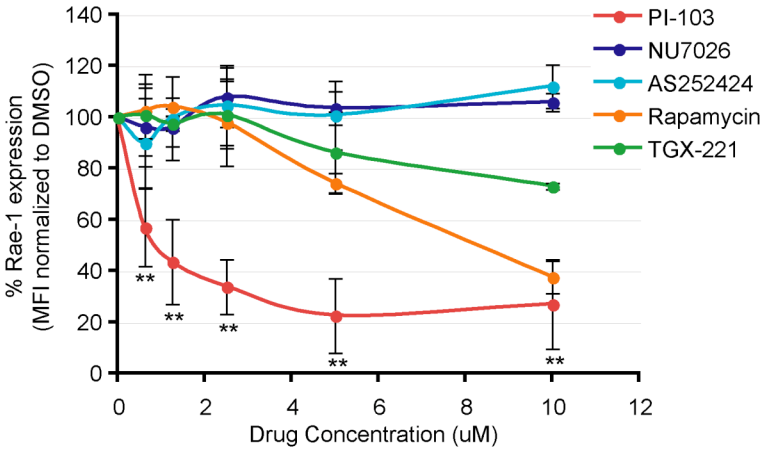


Figure S2.3. p110α PI3K is specifically involved in the induction of RAE-1 in fibroblasts infected with MCMV. Fibroblasts infected with MCMVΔm04+m06+m152 in the presence of the indicated inhibitors at 10uM, 5uM, 2.5uM, 1.25uM, and 0.625uM were stained for RAE-1. The percent RAE-1 expression was determined by normalizing the MFI of RAE-1 in cells that were treated with the inhibitors to the MFI of RAE-1 in cells that were treated with DMSO. SD and statistical significance were determined from three independent experiments. **p<0.01.

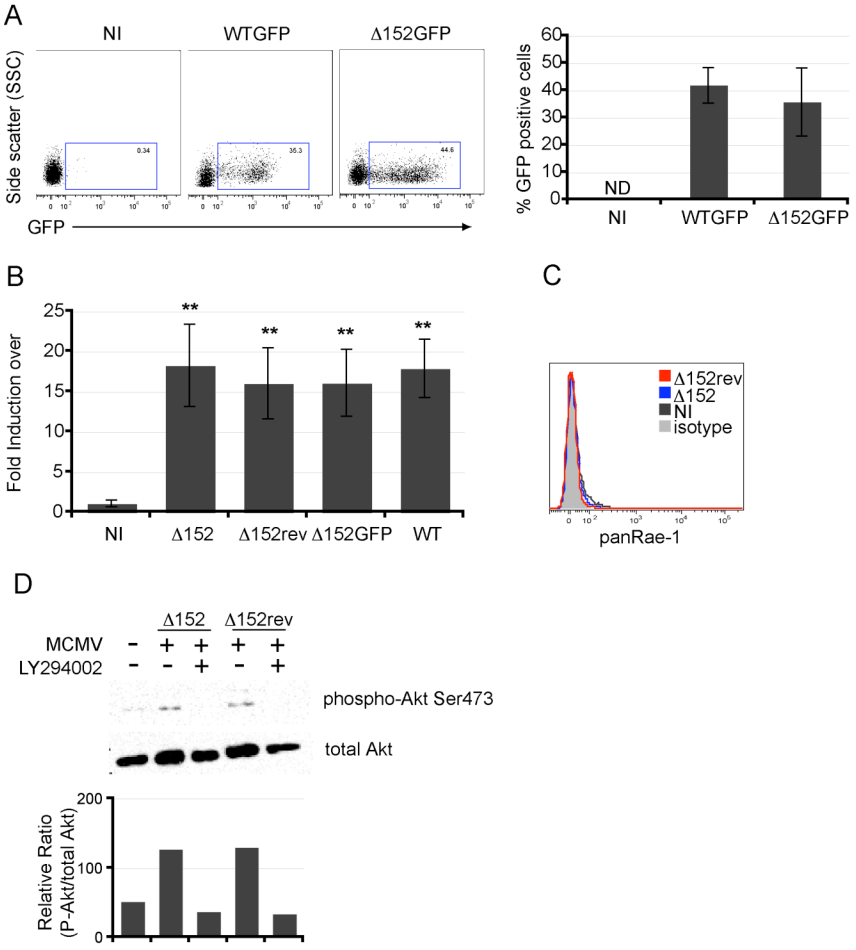


Figure S2.4. MCMV infection of peritoneal macrophages does not induce RAE-1 surface expression. A) Peritoneal macrophages from C57BL/6 mice were infected with GFP expressing MCMVWT (WT-GFP) or MCMVΔ152 (Δ152GFP) at moi 1 for 24 hrs. The level of GFP expression was determined by flow cytometry. B) RAE-1 mRNA level in peritoneal macrophages infected with MCMVΔ152 (Δ152), MCMVΔ152-revertant (Δ152-rev), MCMVΔ152-GFP (Δ152-GFP) or MCMVWT (WT) was quantified by RT-qPCR and was normalized to the level of HPRT for each sample. The data represent fold induction over uninfected cells (NI). SD and statistical significance were determined based on three independent experiments. **p<0.01. C) RAE-1 surface expression in peritoneal macrophages infected with Δ152 or Δ152-rev was determined by flow cytometry using a pan RAE-1 antibody. D) Cellular lysates were obtained from peritoneal macrophages infected with Δ152 or Δ152-rev for 24 hrs in the presence of DMSO or LY294002 and used to probe for Akt phosphorylation at serine 473 or total Akt. The blot is a representative figure of three independent experiments.

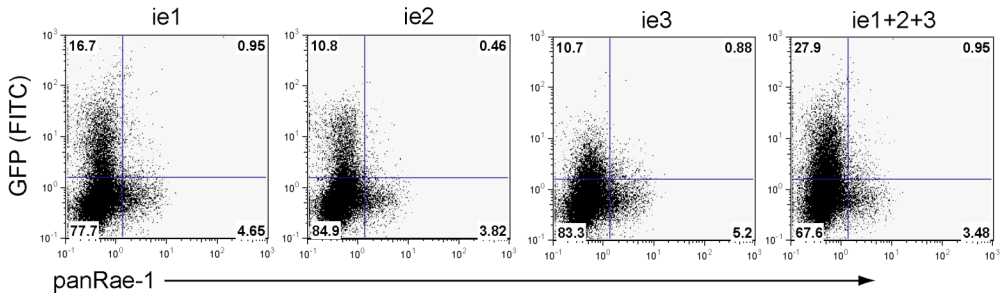


Figure S2.5. Expression MCMV immediate early genes is not sufficient to induce RAE-1 induction. Fibroblasts were transiently transfected with GFP-fused MCMV ie1, 2, and 3 alone or in combination (ie1+2+3) for 24 hrs and stained for RAE-1. The data shown were gated on live, 7AAD negative cells.

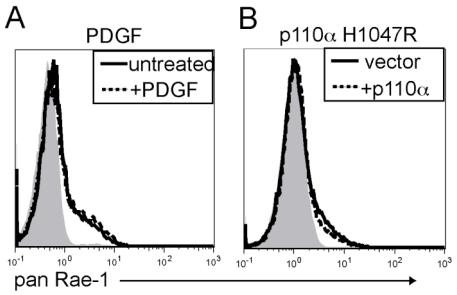


Figure S2.6. Activation of PI3K by PDGF or overexpression of a constitutively active form of p110α does not induce RAE-1 expression. Fibroblasts treated with (A) 20ng/ml of PDGF for 24 hrs or (B) stably expressing p110α H1047R (constitutively active) were stained with the pan RAE-1 antibody. The data shown are gated on live, 7AAD negative cells. Histograms show isotype control (shaded gray), untreated (solid black), and treated or empty vector (dashed black).

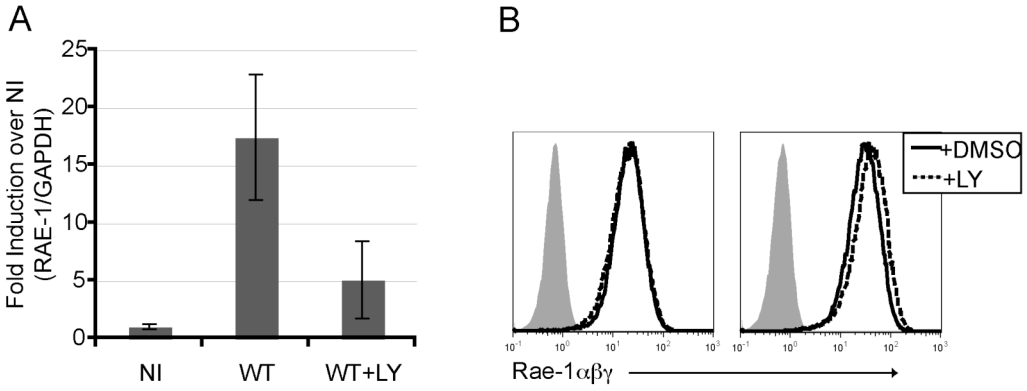


Figure S2.7. PI3K pathway is involved in post-transcriptional regulation of RAE-1 upon MCMV infection. A) The level of RAE-1 mRNA was determined in fibroblasts infected with wildtype MCMV in the presence (WT+LY) or absence (WT) of LY294002 using RT-qPCR at 24 hrs pi. Fold induction was determined by normalizing the infected samples to uninfected samples (NI). SD was determined from three independent experiments. B) Fibroblasts stably transduced with RAE-1α or γ were treated with LY294002 for 24 hrs and stained with the RAE-1αβγ-specific antibody. Histograms show isotype control (shaded gray), DMSO-treated (solid black), and LY294002-treated cells (dashed black).

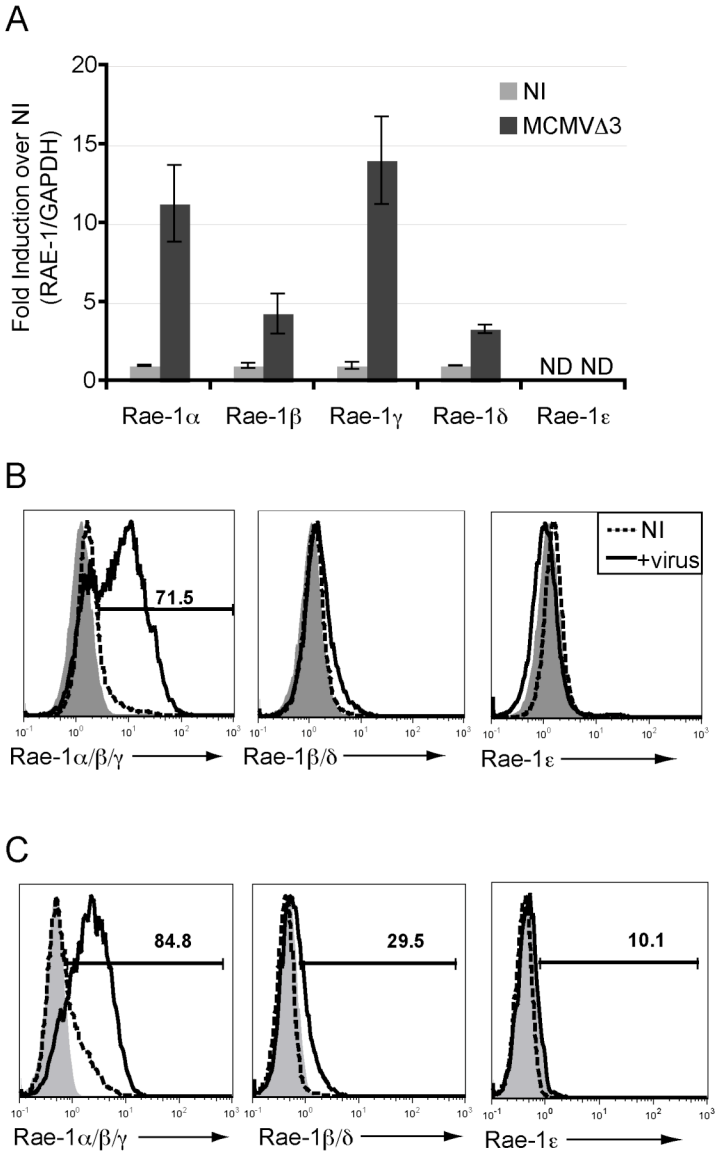


Figure S2.8. Expression of RAE-1 isoforms is differentially controlled in mouse fibroblasts infected with MCMV. (A) RNA extracted from uninfected fibroblasts (NI) or fibroblasts infected with MCMVΔm04+m06+m152 (MCMVΔ3) were used to quantify the expression levels of the RAE-1 isoforms using RT-qPCR. Fold induction was determined by normalizing the infected samples to uninfected samples (NI). SD was determined from two independent experiments. ND; undetectable. Fibroblasts infected with MCMVΔ3 (B) or MCMVΔ152 (C) were surfaced stained with RAE-1 α/β/γ, β/δ or ε antibody at 24 hrs pi. The data shown were gated on live, 7AAD negative cells. Histograms show isotype control (shaded gray), uninfected (dashed black), and infected cells (solid black). The values represent the percent of live RAE-1 positive cells.

Table S2.1.

Target gene	Sequence (5'-3')	Product Size (bp)
Rae-1	**F:**ACCCGAATGCAGACAGGAAGTTGA	72
	R:GGACCTTGAGGTTGATCTTGGCTT	
GAPDH	**F:**GAAGGTCGGTGTGAACGGA	240
	R: GTTAGTGGGGTCTCGCTCCT	
HPRT	**F:**CTGGTGAAAAGGACCTCTCG	108
	R:TGAAGTACTCATTATAGTCAAGGGCA	
ISG15	**F:**TCCATGACGGTGTCAGAACT	123
	R:GACCCAGACTGGAAAGGGTA	
p110α	**F:**AAAGGCCACTGTGGTTGAATTGGG	536
	R:CAAATGGCACACGTTCCCGCTTAT	
p110β	**F:**TGCTTTCCAAACAGGTGGAAGCAC	494
	R:TGGCAGCCACGTTACTACTGTTCA	
p110γ	**F:**AGCCAGCGCATCAGCAAGA	529
	R:CGATGAAGATGCAGTTGTTGGCAATCTTT	
p110δ	**F:**ACTGTGTCAAGCTCGGAGGTGAAT	618
	R:TCTGGGCCACATCCTCGTGTTTAT	
p85α/p55α/ p50α	**F:**AAGAACAATGCCAAACCCAGGAGC	630
	R:TGCACGAGGGAGGTGTGTTGATAA	
p85β	**F:**ATCAAAGTCTTCCACCGGGATGGT	570
	R:TGAGGTCAGGTTTGAGGCTGTTCA	
p55γ	**F:**GACAAATTGCGGGACATGCCAGAT	426
	R:AGGCTTCAATGGCAGTCCTCTTCA	
p101	**F:**TCCACACTACGGGTTGTGGTCTTT	799
	R:ACCACTTCTGTCAGGGTTAGCGTT	
NheI-Rae1-KpnI	**F:**ATCGGCTAGCATGGCCAAGG	*
	R:CGATGGTACCTTTCCTCTTCACATTGCA	
MCMV e1	**F:** TCGAAGAGGAATGTTCTCCACGCA	175
	R: GGACAGCGAACTAGCAGCATAACT	

F: forward primer sequence

R: reverse primer sequence

*The product size for this primer depends on the different Rae-1 isoforms. For each isoform, the final product would be an additional 20 nucleotides to account for the restriction sites and extra flanking sequences.

Chapter 3: Identification of a MCMV protein, m18, as an inducer of RAE-1 family of NKG2D ligands

Background

NKG2D is a potent stimulatory immunoreceptor that is expressed on the surface of NK cells but is also expressed on activated CD8 T cells, some subsets of NKT cells and γδ T cells. There are at least eight human NKG2D ligands (MICA, MICB, ULBP1-4, and RAET1G and L) and at least nine mouse NKG2D ligands (MULT-1, H60a-c, and RAE-1α-ε). NKG2D ligands are normally absent or detected at low levels in adult tissues but can be induced upon various cellular stress conditions or viral infections (Raulet 2003). Despite having varying affinities for NKG2D, expression of any one of these ligands can activate NK cells to secrete pro-inflammatory molecules TNFα and IFNγ as well as cytotoxic effectors granzymes and perforin.

Multiple cellular pathways and effectors contribute to the induction of NKG2D ligands during conditions of stress or infection: DNA damage response through Chk1 (Gasser et al. 2005), c-myc overexpression in conjunction with a loss of expression in tumor suppressor genes (Unni, Bondar & Medzhitov 2008), activation of MyD88 signaling by microbial ligands (Hamerman, Ogasawara & Lanier 2004), AP-1 activation upon stimulation by mitogens (Nausch et al. 2006), and PI3K activation during viral infection and transformation (Tokuyama et al. 2011). Growing evidence suggests that NKG2D ligand expression is a marker for cells that have sensed a disturbance or dysregulation in their normal cellular function. For the most part, expression of a single effector is not sufficient for NKG2D ligand expression at the cell surface, pointing to the presence of a certain threshold of cellular disturbance that cells can tolerate, above which cells induce expression of stress response proteins such as NKG2D ligands. Uncovering the various requirements for induction of NKG2D ligands will further our understanding on how these ligands are regulated. Moreover, it will elucidate intrinsic sensing mechanisms that are in place to detect dysregulation of cellular pathways, and perhaps gives rise to a molecular ruler that gages the "stress level" of cells at any given time.

Many viruses and microbial products induce expression of NKG2D ligands to varying degrees. However, to reduce detection by NK cells, viruses have evolved methods to prevent cell surface expression of NKG2D ligands. Human immunodeficiency virus (HIV) and human and mouse cytomegaloviruses (CMV) are two well-studied examples of viruses that evade NKG2D-mediated recognition by NK cells. HIV-encoded Vpr protein is sufficient to induce expression of ULBP1-3, but another viral protein Nef counteracts this induction (Ward et al. 2009, Cerboni et al. 2007). Both HCMV and MCMV induce NKG2D ligand expression and encode multiple proteins that specifically inhibit surface expression of NKG2D ligands (Jonjic et al. 2008).

Our current understanding of NKG2D ligand expression has revealed common cellar pathways that are involved in the induction of NKG2D ligands during cellular stress, transformation, and viral infection. As such, identification of viral proteins that induce expression of NKG2D ligands, and the mechanism by which they do so, can support existing knowledge on NKG2D ligand regulation or reveal novel mechanisms of regulation. Here, we have identified a mouse CMV (MCMV) protein encoded by the *m18* gene that is necessary and sufficient to induce expression of the RAE-1 family of mouse NKG2D ligands. In support of our previous finding on the role of PI3K activation in RAE-1 expression, we show that m18-mediated induction of RAE-1 also occurs through PI3K activation and inhibition of this activation effectively prevents RAE-1 induction. Furthermore, we show that m18 protein acts on the promoter of RAE-1 to induce its expression but is not mediated by the E2F transcription

factors. This study complements existing knowledge on the regulation of NKG2D ligand expression, and further studies on m18 may reveal novel mechanisms of ligand regulation. Furthermore, elucidation of cellular factors that are activated by m18 may be good indicators of various signals that are sensed by the cells to induce expression of stress ligands.

Materials and Methods

Cells. Established tail-derived fibroblasts were prepared as described previously (Gasser et al. 2005). Established fibroblasts, BALB/c 3T3 (ATCC, CCL-163) and NIH 3T3 (ATCC, CRL-1658) were maintained in DMEM with 5% FBS and 1% penicillin and streptomycin. RAW264.7 cells (ATCC, TIB-71) were maintained in RPMI containing 10% FBS and 1% penicillin. Peritoneal macrophages obtained from C57BL/6 mice were cultured overnight in RPMI with 10% MCSF provided by Dr. Portnoy (UC Berkeley), 10% FBS, and 1% penicillin and streptomycin.

Plasmids, constructs, and luciferase assay. MCMV m18 and m19 gene sequences were obtained from the genomic sequence of the Smith strain of MCMV (NC_004065.1). These genes were cloned into pCDNA3.1(+) (Invitrogen), pIRES2GFP (Clontech), or pQCXIN (Clontech). For generation of N- or C-terminal GFP fusion m18, the m18 gene was cloned into either pEGFP.C1 or pEGFP.N1 (Clontech), respectively. pGL3 Rae-1E promoter firefly luciferase construct was generated as described previously (Jung H. et al, submitted). pGL3-Basic Firefly and pRL-SV40 Renilla constructs were obtained from Promega. Briefly, pGL3-Firefly luciferase constructs (either Basic or Rae-1E) were co-transfected with m18 pCDNA3.1 or empty vector and pRL-SV40 at a ratio of 33:66:1 using Lipofectamine 2000 at a DNA to lipid ratio of 1:5 (ug to ul). Cells were lysed using Passive Lysis Buffer (Promega) at 24 hours post-transfection and lysates were analyzed upon addition of either Firefly luciferase substrate (Thermo Fischer) or Renilla luciferase substrate (Biotium) using an LMAXII^{384} luminometer (Molecular Devices).

Transduction and selection. The coding region of m18 was cloned into a retroviral vector, pQCX-IN, which is a neomycin-modified version of pQCXIX (Clontech). Retroviral supernatants were obtained two days after transfection of Pheonix cells, a packaging cell line, with m18-pQCXIN. Fibroblasts were infected with the supernatants by centrifugation for two hours at room temperature in the presence of 4ug/ml of polybrene (Santa Cruz Biotechnology). Fibroblasts were selected with 4mg/ml of G418 (Fisher Scientific) for five to seven days before analysis.

Flow cytometry. Fibroblasts, 3T3, TrampC2, and RAW cells were harvested in 2mM EDTA PBS and stained with monoclonal anti-mouse pan-specific RAE-1, RAE-1$\alpha/\beta/\gamma$, or Rat IgG_{2A} isotype control (all purchased from R&D) followed by PE-conjugated goat anti-rat IgG (Jackson ImmunoResearch). RAW cells were first incubated with an anti-mouse CD16/CD32 FcBlock (BD), followed by RAE-1 antibody and PE-conjugated anti-rat IgG_{2A} antibody (BD). All samples were co-stained with 7-AAD (BD).

Reverse transcription quantitative real-time PCR. RNA from fibroblasts and macrophages were extracted in Trizol (Invitrogen), treated with RQ1 DNase (Promega), and total RNA was reverse transcribed using oligo$(dT)_{15}$ primer (iDT DNA Technologies) and SuperScriptII (Invitrogen) at 42ºC for 50 minutes. cDNAs were analyzed using ABI7300 Real Time PCR

System. RAE-1 isoform specific primers were described previously (Ogasawara et al. 2003). Primers for RAE-1 and MCMVe1 are described in Table S2.1.

Virus production, propagation, and infection. The original mutant MCMV lacking genes m01 through m22 (MCMVΔ1-22) was a kind gift from Dr. Hidde Ploegh (Whitehead Institute, MIT, MA). To generate further deletion mutants of MCMV, *E. coli* strain DH10B transformed with MCMV BAC pSM3fr and a plasmid encoding arabinose-inducible Red recombination genes, pkD46, was obtained from Dr. Martin Messerle (Hannover Medical School, Germany). In order to use a kanamycin selectable system, a PCR product consisting of a kanamycin cassette and 50bp flanking arms on both ends that are homologous to the region of interest in pSM3fr was generated using pACYC177 (NEB) as a template. Transformation of the PCR product and induction of recombination was performed as described (Borst, Benkartek & Messerle 2007). The resulting BAC product was analyzed for correct insertion of the kanamycin cassette by PCR and agarose gel electrophoresis of EcoRI-digested BAC DNA. For production of mutant viruses, NIH 3T3 cells were transfected with the BAC DNA of interest and supernatant was collected about a week later, once the cells began displaying cytopathic effects. The supernatants were passaged several times in NIH 3T3 cells until we obtained enough virus for infection experiments. In order to confirm that the deletion of interest is still maintained in the viral particles, viral DNA was extracted from the supernatant as described previously (Tokuyama et al. 2011), and the presence of kanamycin in the correction region was detected by PCR. All infection experiments were performed at a MOI of 1.

Western blotting. Mouse fibroblasts were serum-starved overnight, and control cells were treated with 20% FBS for 15 minutes. Cells were lysed in lysis buffer according to the Cell Signaling protocol and lysates were analyzed by western blotting for phospho-Akt (P-Akt) S473 and Akt (Cell Signaling) or beta-actin (Abcam). For P-Akt and Akt blots were detected by HRP-cojugated anti-rabbit IgG (Cell Signaling) and beta-actin blots were detected by HRP-conjugated anti-mouse IgG (GE Healthcare). The relative ratio of P-Akt to total Akt was determined using ImageJ.

Inhibitor treatments. PI3K inhibitors were added to fresh media at 6 hrs post-transfection and were maintained in the culture media until cells were harvested for analysis. LY294002, Rapamycin, and NU7026 were purchased from Calbiochem. PI3Kαi2, PI-103, TGX-221, and AS252424 were purchased from Cayman Chemicals. IC87114 was kindly provided by Dr. Okkenhaug (Babraham Institute, Cambridge, UK). The final concentrations of all of these inhibitors are stated in the figure legends of the corresponding figures.

Microarray analysis and gene ontology analysis. The platform used for gene expression analysis was GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Genomics core at Gladstone Institute (San Francisco) performed the hybridization and detection. Raw CEL files were obtained from Gladstone, and each value was RMA normalized using GenePattern software (Broad Institute). Gene symbol associated with each probe was matched in Excel using annotation information obtained from NetAffx Analysis Batch Query. The annotated dataset was collapsed to the mean value of overlapping probe sets using GenePattern. From the collapsed dataset, the bottom 7% of expression values as well as samples with standard deviation of less than 0.1 (7%) were filtered from the dataset using GeWorkbench program. Only datasets that had

a p-value of less than 0.01 between empty vector-expressing cells and m18-expressing cells were further analyzed. In Excel, samples that had a 2.5-fold or bigger difference in all three independently-derived cells were determined. Standard deviation between the three sets were calculated for this dataset and ranked in the order of least to most different between the sets. Gene ontology analysis was performed using a free online program called, GOrilla. The dataset containing the upregulated genes, listed in Table 4.1, were compared to the original dataset before filtering to look for enrichment in certain molecular functions.

Statistical analysis. A two-tailed student t-test was performed on all samples where statistical significance is indicated.

Results:

Genes m01 through m22 are necessary for MCMV-mediated induction of RAE-1. Studies on the induction of NKG2D ligands are somewhat limited because most common cell lines constitutively express ligands at high levels, and a further increase in ligand expression is difficult to show. Here, we utilized established fibroblast cells that were originally derived from mouse tails. These cells lack basal expression of RAE-1 at the cell surface but rapidly induce expression upon cellular stress and MCMV infection. The Raulet lab has used these cells to discover the role of DNA damage response in the induction of mouse NKG2D ligands upon cellular stress (Gasser et al. 2005). More recently, our group showed the role of the PI3K pathway in ligand expression during MCMV infection using these cells (Tokuyama et al. 2011). Primary peritoneal macrophages also induce expression of NKG2D ligands upon stimulation by microbial ligands as well as upon MCMV infection and serve as good physiological models of ligand expression.

In order to determine whether MCMV, which is a good inducer of NK2D ligands, also possesses protein(s) that would be sufficient to induce NKG2D ligand expression, several deletion mutants of MCMV that lack various non-essential regions of the viral genome were tested for their ability to induce NKG2D ligand expression in both fibroblasts and primary peritoneal macrophages. Surprisingly, one of the deletion mutants that lacks the first 22 open reading frames (ORFs), MCMVΔm01-m22, showed a significant defect in its ability to induce RAE-1 at the mRNA level in both fibroblasts and peritoneal macrophages when compared to wildtype MCMV (Fig. 3.1A and B). In order to account for variability in infectivity between Δm01-m22 and WT virus, we compared the expression level of MCMV early gene 1(e1) and detected similar levels of MCMV e1 expressed by both viruses. Furthermore, a previous study has observed no replication defect of Δm01-m22 in vitro and no defect in propagation of Δm01-m22 virus was observed in our hands (Mohr et al. 2008, and data not shown). These observations suggest that the lack of RAE-1 induction observed in Δm01-m22-infected cells is not because the virus is replication incompetent. Thus, we identified a region within the first 22 genes at the 5' end of the viral genome that is required for RAE-1 induction during MCMV infection. Because modulation of the CMV genome can be performed with relative ease, identification of a specific viral protein(s) within this region was pursued.

A MCMV protein encoded by gene m18 is necessary for RAE-1 induction.

Genetic manipulation of cytomegalovirus was performed using Bacterial Artificial Chromosome (BAC) containing the entire genome of HCMV or MCMV. Multiple groups have

manipulated the genome of CMV by inducing homologous recombination between the CMV BAC and a target cassette in recombination competent *E. coli*.

There are multiple selection methods to manipulate a BAC. Here, a BAC containing the genome of Smith strain MCMV, pSM3fr, was used as a template for deletions of genes between m01 and m22. Using DH10B *E. coli* that is capable of homologous recombination, kanamycin resistance cassette was first introduced between the following regions: m01-m06, m06-m12, m12-m18, m18-m22, or m01-22 (Fig. 3.2). Proper insertion of the kanamycin cassette and simultaneous deletion of the genes of interest were confirmed by agarose gel electrophoresis of EcoRI-digested recombinant BAC (data not shown). In order to ensure that progeny virus also maintained the recombination product of interest, viral DNA extracted from viral supernatants were also analyzed by PCR for the presence of kanamycin cassette in the desired region (data not shown). BAC DNA of interest obtained through this process was transfected into NIH 3T3 cells for propagation of viral particles. The newly generated viruses were titered and used to infect fibroblasts at MOI of 1. Interestingly, when RAE-1 expression in these cells was analyzed, we observed a lack of RAE-1 induction by MCMVΔm12-18 and Δm18-m22 (Fig. 3.3A), suggesting that there are genes between m12 and m22 that are responsible for the induction of RAE-1 expression by WT virus.

In order to identify individual genes that mediate this induction, further single gene deletions of each gene within the m12 to m22 interval were made using the same technique. Most of the genes within this region are not required for the induction of RAE-1. However, induction was abrogated when fibroblasts were infected with MCMVΔ18 and MCMVΔ19, indicating that these two genes are required for the induction (Fig. 3.3B and C). Viral proteins encoded by m18 and m19 are largely uncharacterized. One group has identified m18 as an early gene that contains a CTL epitope (Holtappels et al. 2002), and nothing is known about m19. Unlike many of the other genes within m01 to m22, which have been characterized as part of the m02 glycoprotein family, m18 and m19 do not seem to belong to in any particular family of proteins (Rawlinson, Farrell & Barrell 1996). Furthermore, based on their sequence, they do not seem to have orthologues in the HCMV genome.

Expression of m18 in the absence of infection is sufficient to induce expression of RAE-1.

Due to the uncharacterized nature of the proteins encoded by m18 and m19, we wondered whether expression of these proteins in trans during infection by MCMVΔ18 or Δ19 would rescue RAE-1 induction. To this end, fibroblasts were infected with MCMVΔ18, Δ19 or Δ1-22 at MOI of 1, followed by overexpression of either m18 or m19. RAE-1 expression was then quantified at 24 hours post-infection. For all three of the deletion viruses, expression in trans of m18, but not m19, was sufficient to rescue RAE-1 induction to a similar, if not higher, level as infection by WT MCMV (Fig. 3.4A). The *m18* gene is encoded in the anti-sense strand, and it is separated by a short 144bp sequence from the adjacent *m19* gene. Thus, it is possible that deletion of the *m19* gene disrupts the promoter for m18 and abrogates its expression, which could account for the failure of this virus to induce RAE-1.

To determine whether m18 is sufficient for RAE-1 induction, or instead cooperates with other MCMV-induced components, m18 was overexpressed by itself in fibroblasts. Surprisingly, m18 overexpression was sufficient to induce both RAE-1 mRNA and cell surface RAE-1 protein (Fig. 3.4B and C). The induced cell surface RAE-1 protein was identified as RAE-1α, β, or γ, as determined by positive staining with the RAE-1αβγ antibody. Using isoform-specific RT-qPCR primers, induction of RAE-1α and γ isoforms was observed, which is in accordance with our

previous finding that MCMV infection in these fibroblasts results in a predominant induction of RAE-1α and γ (Figure S3.1, and (Tokuyama et al. 2011)). Taken together, we have identified a novel effector of RAE-1 induction and have attributed RAE-1 induction during MCMV infection as a function to a previously uncharacterized viral protein, m18.

M18-mediated induction of RAE-1 requires activation of the PI3K pathway.

Vpr-mediated induction of NKG2D ligands seems to occur through activation of the DNA damage response. However, in our previous study, we reported that MCMV-mediated induction of RAE-1 is independent of the DNA damage response and inhibition of this pathway does not abrogate ligand induction, arguing against the possibility that MCMV protein m18 induces RAE-1 through the DNA damage response. We did, however, observe that the PI3K pathway was required for the induction of RAE-1 during MCMV infection (Chapter 2). Therefore, the role of PI3K activation in m18-mediated induction of RAE-1 was tested. Inhibition of PI3K using a global chemical inhibitor of PI3K, LY294002, during m18 expression completely abrogated RAE-1 surface expression (Fig. 3.5A). The nature of the PI3K isoform that is involved in this process was also investigated using isoform-specific small molecule inhibitors of PI3K. In support of our previous finding that p110α PI3K plays a major role in MCMV-mediated RAE-1 induction, we observed that treatment of m18-expressing cells with PI-103 diminished RAE-1 induction (Fig. 3.5A). Because PI-103 can also inhibit mTORC1 (Knight et al. 2006), we separately tested whether rapamycin, an inhibitor of mTORC1, inhibited RAE-1 expression. Rapamycin-treated cells still induced RAE-1 to a similar extent as DMSO treated cells, implying a role of p110α PI3K activation in m18-mediated ligand induction.

Finally, m18 was tested for its ability to activate the PI3K pathway. Fibroblasts were transduced with m18-expressing retrovirus to generate cells stably expressing m18. Consistent with our transient transfection assay, stable expression of m18 resulted in constitutive cell surface expression of RAE-1 (Fig. 3.5B). Cell lysates from m18 stables, compared to untransduced cells or cells stably expressing the empty vector, showed increased phosphorylation of Akt at Serine 473, which suggests that m18 is activating the PI3K pathway (Fig. 3.5C). Many viruses activate the PI3K pathway to allow cell survival, prevent apoptosis, and enhance the translational machinery (Cooray 2004). Similarly, CMV infection also activates the PI3K pathway. These data suggest that m18 expression alone is sufficient for PI3K activation, but it does not exclude the possibility that other viral proteins also activate this pathway. Studies comparing the phosphorylation status of Akt in cells infected with WT MCMV versus MCMVΔ18 should clarify whether m18 is the sole activator of PI3K or not.

M18 acts on the promoter of RAE-1 to induce its expression.

Overexpression of m18 results in an induction of RAE-1 both at the mRNA level and at the protein level, suggesting that m18 may induce RAE-1 transcription. Using a luciferase reporter that is fused to the minimal promoter for RAE-1ε, which was originally constructed by Ben Hsiung in the Raulet lab, the effect of m18 expression on the RAE-1ε promoter was analyzed in fibroblasts. Expression of m18 compared to expression of empty vector significantly induced RAE-1 promoter activity as measured by firefly-luciferase activity, and this induction was not observed using a firefly-luciferase reporter that only contains a basic promoter (Fig. 3.6A). A similar trend was observed with the control SV40 promoter, but this difference was not statistically significant. Importantly, the effect of m18 on RAE-1ε promoter was recapitulated in

two other cell lines, NIH 3T3 cells and TrampC2 cells (Fig. 3.6B and D), suggesting that this is not an artifact of the cells we are using.

The Raulet lab previously showed that RAE-1ε transcription is induced during proliferation and that the minimal promoter of RAE-1ε is activated by E2F transcription factors, which are well-characterized regulators of cell proliferation (Jung H. et al, submitted). Within this minimal RAE-1 ε promoter, there are two E2F binding sites with a core sequence of CCCG. These two sites were mutated to ATAT and tested for the ability of m18 to induce RAE-1 ε transcription, as done previously (Fig. 3.7A and Jung et al. submitted). Using constructs that lack the first E2F binding site, the second E2F binding site, or both sites together, we did not observe any defect in m18-mediated induction of RAE-1ε transcription in either fibroblasts or NIH 3T3 cells (Fig. 3.7B and C). The data therefore suggest that m18 induces a distinct mode of transcriptional regulation of RAE-1.

Gene expression profile of cells stably expressing m18.

In order to determine the gene expression profile of m18 overexpression, a microarray analysis was performed on three independently derived cells stably expressing m18 and was compared to cells stably expressing an empty vector (Fig. 3.8A). The quality of the RNA was analyzed by a bioanalyzer, and the analysis showed a nice distribution of 28s, 18s and 5s ribosomal RNA (Fig. 3.8B, UC Berkeley Functional Genomics Laboratory). An Affymetrix Mouse Gene 1.0ST chips were used to analyze three biological replicates of cells either expressing m18 or empty vector. The resulting genes listed in Table 3.1 are at least 2.5-fold different between m18 and empty vector- expressing cells and listed in order of lowest to highest standard deviation between the three samples. Importantly, RAE-1 was one of upregulated genes on the list confirming our observation that m18 expression induces expression of RAE-1.

Gene ontology analysis on the functions of the upregulated genes shows a significant enrichment for genes involved in DNA binding and transcription factor activity (Fig. S3.2). Gene ontology analysis on the biological processes of these genes shows a significant enrichment for genes involved in developmental process, anatomical structure morphogenesis, cell differentiation, and cell proliferation (data not shown). The functional role of these genes may contribute to the transcriptional activity of m18 on the promoter of RAE-1ε (Fig. 3.6A). The role of these genes in development and differentiation strongly correlates with the original discovery of RAE-1 transcripts in early embryos during development and expression of RAE-1 transcripts upon retinoic acid induction of differentiation (Nomura et al. 1996, Zou et al. 1996).

According to a prediction program for deciphering transcription factor binding elements (SABiosciences DECODE), many of the genes that are upregulated in m18-stably expressing cells contain common transcription factor binding sites upstream of their transcriptional start site. Some common transcription factors that bind and may regulate expression of these genes include Ecotropic Virus Integration Site 1 (Evi1), Peroxisome Proliferator-Activated Receptor (PPAR) gamma, and POU class 3 homeobox (POU3) proteins. One conceivable hypothesis is that RAE-1 transcription upon m18 expression is regulated by common factors that regulate expression of genes that are upregulated by m18 expression. However, consensus binding sites for Evi1, PPAR, or POU3 are not found in the minimal RAE-1ε promoter, which is efficiently induced by m18 expression.

Furthermore, the minimal RAE-1ε promoter that is activated by m18 does not contain consensus binding sites for any of the transcriptional regulators that are upregulated by m18 (Table 3.1). If these transcriptional regulators do play a role in RAE-1 regulation, they may do so

indirectly, or perhaps by binding non-consensus sites within the promoter. Alternatively, these transcription factors may be members of a set of genes that are induced by a common stress signal represented by m18, and RAE-1 is another member of this set of genes.

Discussion:

NKG2D ligands are induced upon infection by many viruses. However, the mechanisms by which these ligands are induced are unclear for the most part. Studies to date have only revealed two viral proteins, Ad5 E1A and HIV Vpr, to be sufficient to induce NKG2D ligand expression (Ward et al. 2009, Routes et al. 2005). The Ad5-encoded E1A protein interacts with the transcriptional coadaptor p300 to induce expression of RAE-1 in mouse fibrosarcoma cell line, while HIV Vpr activates the DNA damage response involving the ATM and ATR pathways (Norman et al. 2011). HCMV and MCMV also induce expression of NKG2D ligands, but the mechanism by which they do so is undefined.

In our study, we observed that RAE-1 induction is abrogated in fibroblasts infected with MCMV lacking the *m18* gene, and overexpression of m18 alone, in the absence of viral infection, is sufficient to induce RAE-1 expression. The data show that m18 expression and activation of cellular factors by m18 is an essential part of RAE-1 induction during MCMV infection. However, the data does not completely exclude the possibility that during an infection, other pathways or factors cooperate with the m18-signaling cascade to promote expression of RAE-1. Future studies on the role of various cellular pathways in RAE-1 induction may reveal a network of signals that all contribute to various aspects of RAE-1 biogenesis.

PI3K and m18: how do they regulate RAE-1 expression?

Previously, we observed that RAE-1 induction upon MCMV infection of mouse fibroblasts requires activation of the PI3K pathway. In support of this study, m18-mediated induction of RAE-1 also requires activation of the PI3K pathway through specific involvement of the p110α catalytic subunit of PI3K. However, our previous work has indicated that activation of the PI3K pathway alone, through expression of constitutive active version of p110α or treatment of cells with serum, is not sufficient to induce expression of RAE-1 (Figure S2.6). Therefore, m18-mediated induction of RAE-1 must require another factor(s). Because PI3K activation may be regulating RAE-1 at a post-transcriptional level (Fig. S2.7), an attractive hypothesis is that m18 activates a transcriptional regulator that promotes transcription of RAE-1 de novo, which then requires PI3K activation for enhanced translation, a process that is promoted by downstream effectors of PI3K. Another possibility is that m18 relieves suppression of RAE-1 by miRNAs that may normally help maintain low levels of RAE-1. Alternatively, inhibition of PI3K may block a key interaction between m18 and its cellular partner that is required for the induction of RAE-1. Thus, in the absence of m18, activation of PI3K alone is not sufficient to induce RAE-1 expression. It will be important to test all of these possibilities to understand exactly how PI3K activation and m18-mediated induction of RAE-1 are linked.

Transcriptional regulation of RAE-1 by m18

Using the minimal promoter of RAE-1ε fused to a firefly-luciferase reporter, we observed that m18 expression is sufficient to turn on this promoter in fibroblasts, NIH 3T3 cells, and TrampC2 cells. Furthermore, E2F binding sites within the RAE-1ε promoter were not necessary for m18 to activate RAE-1 transcription in the cell lines tested. It is possible that the reason why E2Fs are not required in this system is because these fibroblasts are already in a

highly proliferative state and grown in serum-containing media such that the contribution of E2F in RAE-1 induction is minimal; this is in comparison to observations made in the Raulet lab indicating that RAE-1 induction during proliferation of primary fibroblasts requires E2F to bind to the promoter of RAE-1ε. Further studies in less cultured cells or in primary cells may reveal a role for E2F in m18-mediated induction of RAE-1.

The observation that m18 activates the minimal RAE-1ε promoter suggests that transcriptional regulators of RAE-1 that act on the 185bp promoter sequence are activated by m18. In order to identify potential regulators in this system, it would be worthwhile to systematically generate truncations of this promoter to determine the region within this promoter that is required for activation by m18. Characterization of the minimal region necessary for m18-mediated induction may reveal novel transcriptional regulators of RAE-1, which will greatly contribute to our understanding of RAE-1 expression.

The role of RAE-1 in differentiation and development

RAE-1 transcripts were originally discovered as a potential regulator of embryogenesis and were highly expressed in various organs including brain and heart as well as in limbs of a mouse fetus (Nomura et al. 1996). Additionally, they were induced upon treatment by retinoic acid in mouse embryonal carcinoma F9 cells, which have pluripotent capacity and can differentiate into endoderm-like cells upon retinoic acid treatment (Zou et al. 1996). More recently, RAE-1 expression was observed in the subventricular zone (SVZ) of the adult mouse brain and this expression was important for proliferation of neurospheres (Popa et al. 2011). Thus, RAE-1 may have a role during development that is independent of its role as a NKG2D ligand.

Enrichment for genes that are involved in differentiation and developmental processes in cells that induce RAE-1 upon m18 expression may suggest an overlapping effect of m18 expression with initiation of a cell differentiation program; similar to differentiation of F9 cells upon retinoic acid treatment. If this is the case, expression of m18 in cells like F9 cells may also cause differentiation. Further analysis of the expression profile of cells stably expressing m18 may reveal novel regulators of development and differentiation and may be an important avenue to pursue.

The importance of m18 for the virus

Viruses are well known for their ability to rapidly evolve and adapt to pressures, especially if these pressures are deleterious for viral replication and survival. Viruses are also thought to only encode for information that is absolutely necessary and are notorious for their ability to hijack cellular machinery whenever possible to minimize their genomic content. Thus, it is puzzling why MCMV has maintained m18 in its genome if expression of this protein potentially results in recognition and elimination of its resident host cells by NK-cells. In fact, the *m18* gene is present in all five strains of MCMV that have been sequenced, three of which are MCMV from wild mice. The safeguard mechanism to evade detection by NK cells is possession of proteins that specifically downregulate surface expression of NKG2D ligands. For the RAE-1 family of proteins, m152- and m138-encoded viral proteins block trafficking of RAE-1 to the surface and downregulate surface resident RAE-1, respectively (Jonjic et al. 2008). One likely explanation for why MCMV has maintained genes that functionally counteract each other is that m18 has alternative functions that are essential for the viral life cycle and elimination of m18 was not an option for the virus. Together with the possible role of m18 in cellular

differentiation and development, characterization of MCMVΔ18 pathogenesis in vivo will be of great interest in future studies.

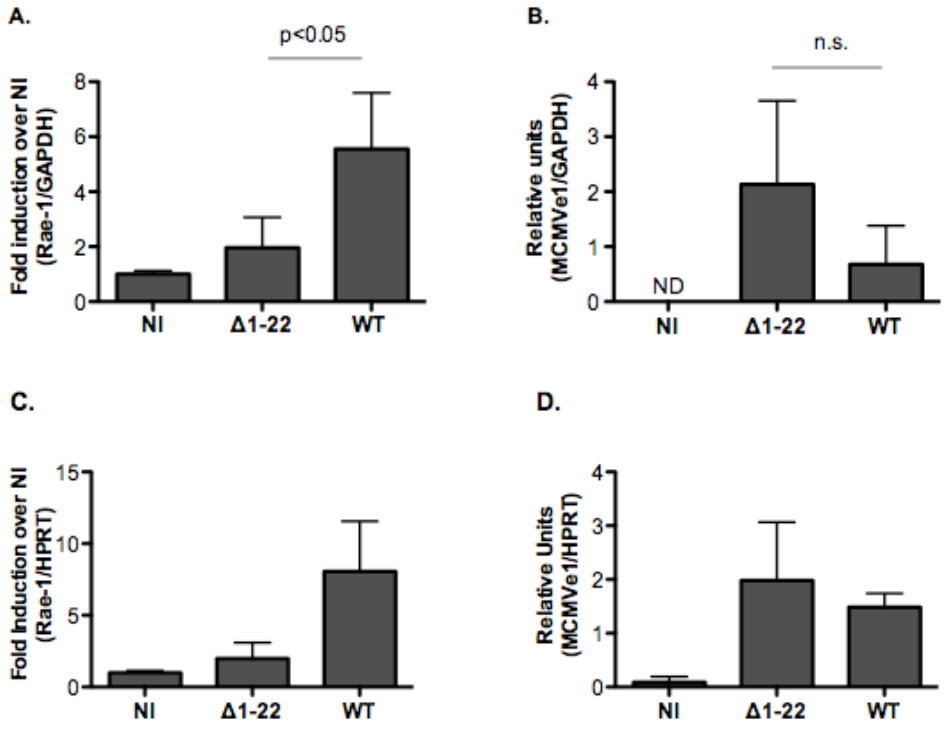


Figure 3.1 MCMV lacking genes m01 through m22 cannot induce RAE-1 expression. Fibroblasts (A and B) or primary peritoneal macrophages from C57BL/6 mice (C and D) were infected with either wildtype (WT) MCMV or MCMV lacking m01-m22 (Δ1-22). RNA was harvested at 24 hours post-infection and analyzed for expression of RAE-1 (A and C) or MCMVe1 (B and D) by RT-qPCR. All data are normalized to HPRT expression. For RAE-1 expression, data was normalized to uninfected (NI) samples to show fold induction. For MCMVe1 expression, the graph represents the relative units after normalization to HPRT. Fibroblast data is a combination of four independent experiments and peritoneal macrophage data is a combination of data from two mice.

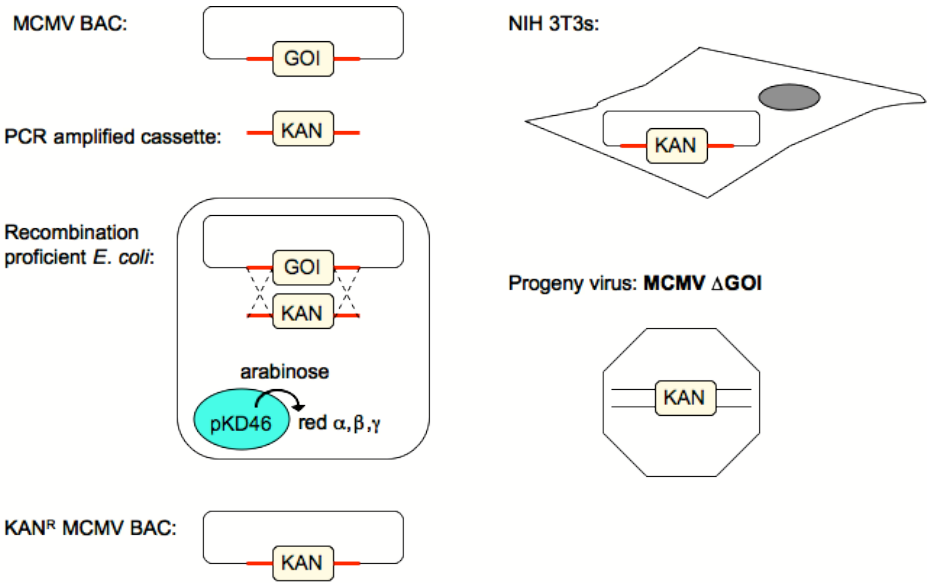


Figure 3.2. A diagram depicting the method of BAC recombination. Selected deletions of genes within the genome of MCMV were achieved through BAC recombination. The entire MCMV genome inserted into a BAC (pSM3fr) was transformed into an *E.coli* that also contains a plasmid encoding Redαβγ from prophage lambda (pKD46) under the control of an arabinose inducible promoter. A kanamycin cassette (KAN) was PCR amplified such that it is flanked by 50bp sequences homologous to the region in MCMV that is of interest (GOI). Upon purification of BAC DNA containing the KAN cassette, NIH3T3s were transfected with the DNA to produce progeny virus that has a deletion in the GOI (MCMV ΔGOI).

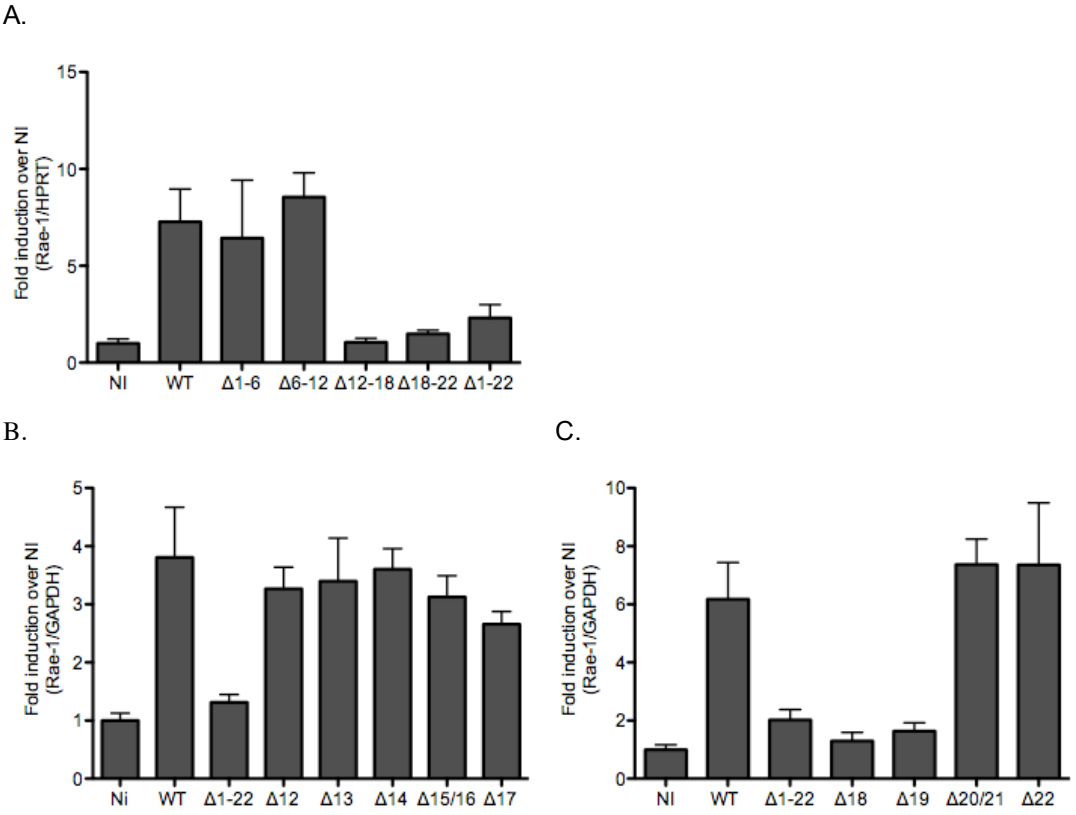


Figure 3.3. MCMV lacking genes m18 and m19 are necessary for the induction of RAE-1. Fibroblasts were infected with MCMV BAC-derived virus containing deletions in multiple genes (A) or single genes (B and C) as indicated in each of the graphs. In each experiment, BAC-derived wildtype (WT) virus and Δ1-22 virus, which were recreated from the same BAC, were included as controls. For all experiments, fibroblasts were infected for 24 hours at MOI of 1, RNA was harvested, and RAE-1 expression was analyzed by RT-qPCR. RAE-1 expression levels were normalized to expression levels of HPRT (A) or GAPDH (B and C). The graphs indicate fold induction over uninfected samples (NI) and are representatives of at least two data sets.

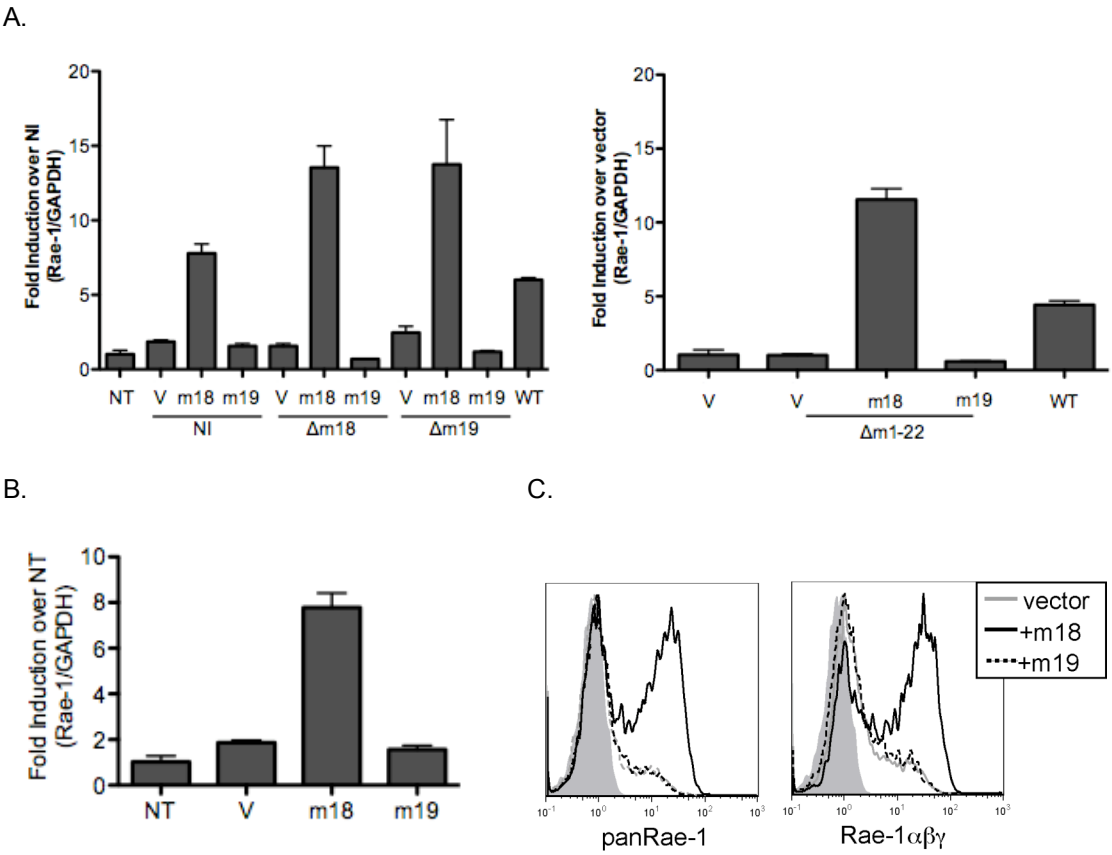


Figure 3.4. Overexpression of m18, but not m19, alone is sufficient to induce RAE-1 expression. Fibroblasts that were first infected with MCMVΔ18, Δ19 or Δ1-22 (A) or left uninfected (B) were transfected with empty vector (V), m18 pCDNA3.1 (m18) or m19 pCDNA3.1 (m19) as indicated. For infection experiments, cells were transfected four to six hours post-infection. In all samples, cells were harvested at 24 hours post-infection, and RAE-1 mRNA expression was quantified by RT-qPCR. As a control, fibroblasts were also infected with wildtype (WT) virus in the same experiment. RAE-1 expression was normalized to GAPDH, and fold induction over untransfected (NT) or empty vector (V) was graphed. C) Fibroblasts were co-transfected with the indicated constructs and pIRES2GFP at a ratio of 3 to 1. At 24 hours post-transfection, RAE-1 cell surface expression was analyzed by flow cytometry using either panRAE-1 or RAE-1αβγ-specific antibody. Shaded histogram shows isotype staining, solid gray line shows empty vector-transfected cells, solid black line shows m18-transfected cells, and dashed black line shows m19-transfected cells. All cells are gated on 7-AAD negative live, GFP positive cells.

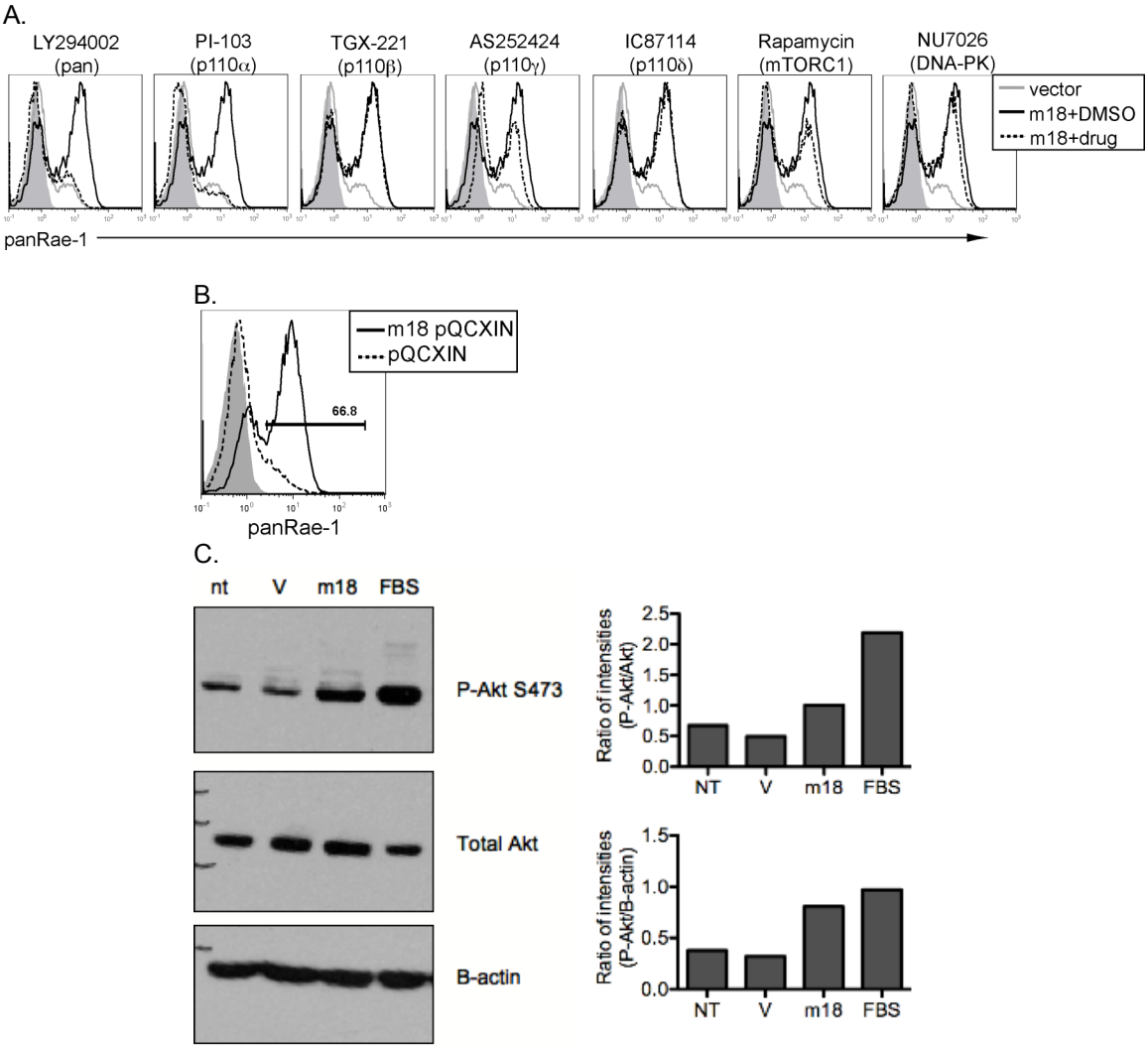


Figure 3.5. PI3K activation is involved in the induction of RAE-1 by m18. A) Fibroblasts were co-transfected with m18 pCDNA3.1 and pIRES2GFP at a ratio of 3 to 1, and either DMSO (solid black line) or the indicated inhibitors (dashed black line) were added at six hours post-transfection for the remainder of the experiment. The drug concentrations used are as follows: 1.25uM PI-103, 5uM TGX-221, 10uM AS252424, 10uM IC87114, 100nM rapamycin, and 10uM NU7026. At 24 hours post-transfection, cells were harvested, and RAE-1 expression was analyzed by flow cytometry. Shaded histogram shows isotype staining. All samples are gated on 7-AAD negative live, GFP positive cells. B) Fibroblasts stably transduced with m18 pQCXIN were selected for 7 days with neomycin, and RAE-1 expression was analyzed by flow cytometry. The value indicates percent RAE-1 positive cells in m18-transduced cells. C) Fibroblasts stably expressing m18 were serum-starved overnight and analyzed for phospho-Akt by western blotting. Not transduced (nt), empty vector (V), m18 pQCXIN (m18), and 20% FBS (FBS). The graphs show the ratio of P-Akt to Akt (top) or P-Akt to b-actin (bottom).

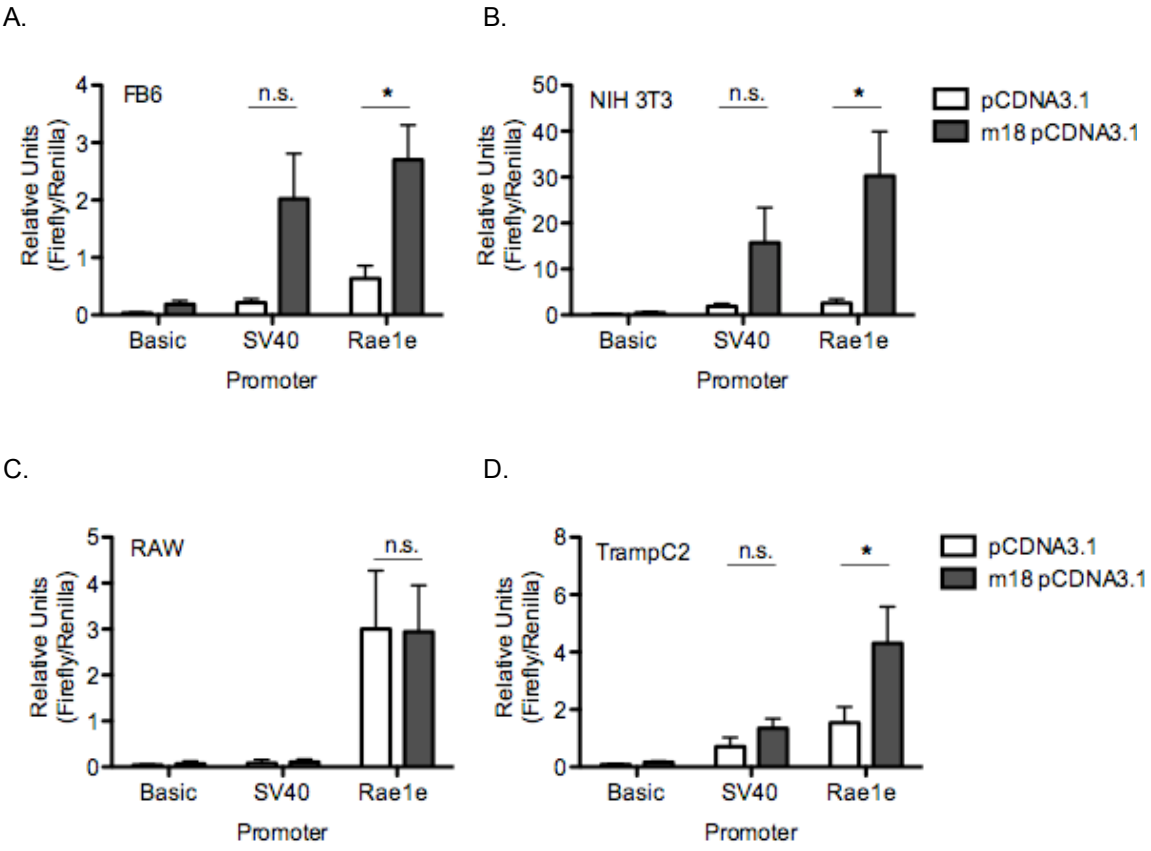


Figure 3.6. The minimal RAE-1ε promoter can be induced by m18. Fibroblasts (A), NIH 3T3 cells (B), RAW cells (C), and TrampC2 cells (D) were co-transfected with a renilla-luciferase reporter and firefly-luciferase reporter containing a basic promoter, SV40 promoter or RAE-1e promoter. Additionally, these cells were transfected with either pCDNA3.1 or m18 pCDNA3.1. At 24 hours post-transfection, cells were lysed, and renilla and firefly activities were measured independently. Each graph shows a relative unit obtained by taking the ratio of firefly signal over renilla signal. Three independent experiments were combined into each graph and statistical significance was calculated using a student t-test. * $p<0.05$, n.s., not significant.

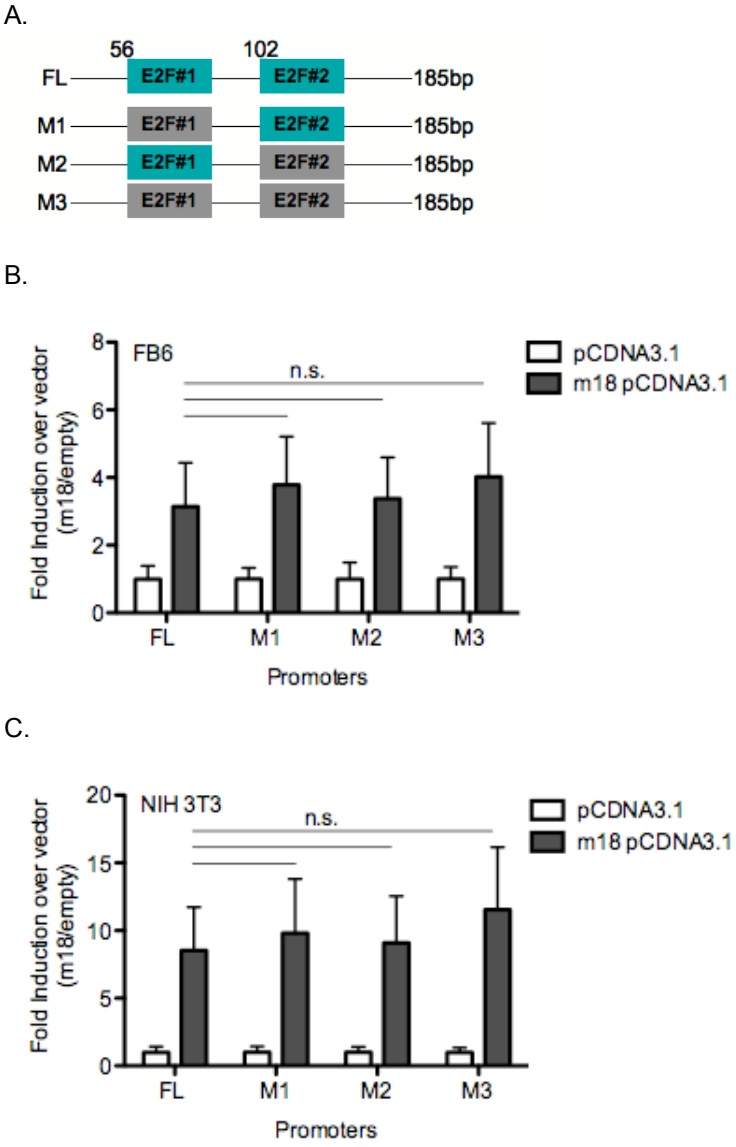


Figure 3.7. E2F binding sites within the RAE-1ε minimal promoter is not required for m18-mediated induction of RAE-1. A) A diagram depicting full-length (FL) RAE-1ε promoter, promoter with a mutated version of the first E2F binding site (M1), the second E2F binding site (M2) or both sites (M3). Fibroblasts (B) or NIH 3T3 cells (C) were co-transfected with renilla-luciferase reporter, the indicated RAE-1ε promoter firefly-luciferase reporter, and either pCDNA3.1 or m18 pCDNA3.1. At 24 hours post-transfection, cells were lysed, and renilla and firefly activities were measured independently. Each graph shows fold induction of m18-transfected signal over empty vector-transfected signal. Three independent experiments were combined into each graph and statistical significance was calculated using a student t-test. n.s., not significant.

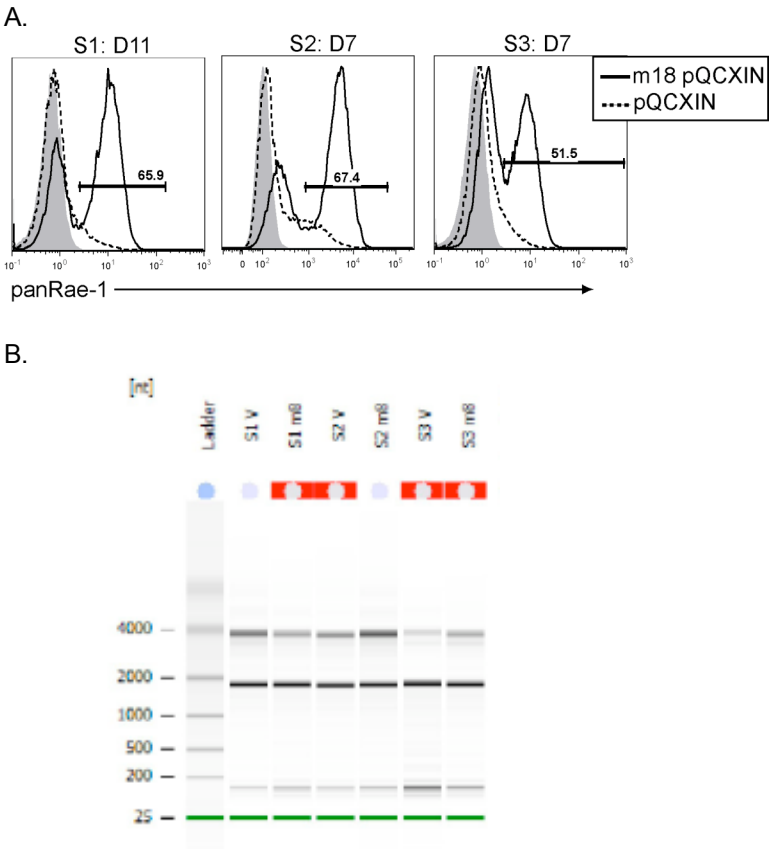


Figure 3.8. Fibroblasts stably expressing m18 were used for gene expression analysis. A) Three independently-derived fibroblasts transduced with either empty pQCXIN vector or m18 pQCXIN were selected with neomycin for the indicated number of days (D11, D7, and D7), and RAE-1 expression was analyzed for each set. The values represent percent RAE-1 positive cells, which were gated on 7-AAD negative live cells. B) RNA extracted from each sample was analyzed for quality control using a bioanalyzer. The bands from the top represent 28s, 18s, and 5s ribosomal RNA.

Table 3.1.

Gene Symbol	Gene Name	Fold Induction (log2)	Fold Induction	Std. Dev.
Upregulated Genes				
Armcx2†	armadillo repeat containing, X-linked 2	1.655	3.149	0.123
Mt2	metallothionein 2	1.349	2.548	0.127
Igfbp2	insulin-like growth factor binding protein 2	2.091	4.262	0.157
Mgp	matrix Gla protein	1.909	3.757	0.163
Gpc3†	glypican 3	2.402	5.286	0.188
Rragd	Ras-related GTP binding D	1.474	2.778	0.188
Csrp2†	cysteine and glycine-rich protein 2	1.544	2.916	0.199
Sulf2	sulfatase 2	2.047	4.134	0.214
Gm1673	predicted gene 1673	1.324	2.504	0.217
Cbln1	cerebellin 1 precursor protein	2.948	7.717	0.234
Lef1*	lymphoid enhancer binding factor 1	2.054	4.153	0.253
Il13ra1	interleukin 13 receptor, alpha 1	1.332	2.517	0.277
Gstm7	glutathione S-transferase, mu 7	1.797	3.474	0.278
Asphd2	aspartate beta-hydroxylase domain containing 2	1.903	3.739	0.283
Cd24a	CD24a antigen	2.375	5.186	0.287
Igf2†	insulin-like growth factor 2	1.712	3.277	0.287
Prox1*†	prospero-related homeobox 1	1.412	2.661	0.296
Sqrdl	sulfide quinone reductase-like (yeast)	1.584	2.998	0.336
Fam49a	family with sequence similarity 49, member A	1.991	3.976	0.343
Ppp1r1a	protein phosphatase 1, regulatory (inhibitor) subunit 1A	1.767	3.403	0.355
Rgl1	ral guanine nucleotide dissociation stimulator,-like 1	1.612	3.056	0.358
Clu	clusterin	1.485	2.799	0.365
Sgk3	serum/glucocorticoid regulated kinase 3	1.503	2.835	0.372
Gm15107 /// Ott /// Luzp4 /// Gm15085 /// Gm15127 /// Gm15128 /// Gm15080 /// Gm10439 /// Gm15097 /// Gm15091	ovary testis transcribed/leucine zipper protein 4	5.148	35.462	0.384
BC028528	cDNA sequence BC028528	1.902	3.739	0.393
Bmyc†	brain expressed myelocytomatosis oncogene	1.386	2.614	0.404
Alcam†	activated leukocyte cell adhesion molecule	1.685	3.215	0.413
Ass1 /// Gm5424	argininosuccinate synthase 1	2.909	7.511	0.417
Cxcl12	chemokine (C-X-C motif) ligand 12	1.519	2.866	0.422
Hoxb7 /// Hoxb8*	homeobox B7/B8	1.331	2.517	0.440
Tspan13†	tetraspanin 13	3.735	13.320	0.445
Akr1c13	aldo-keto reductase family 1, member C13	1.366	2.578	0.453
Phlda2	pleckstrin homology-like domain, family A, member 2	2.508	5.688	0.455
Eif4e3	eukaryotic translation initiation factor 4E member 3	1.858	3.626	0.468
Mgat4a	mannoside acetylglucosaminyltransferase 4, isoenzyme A	1.782	3.438	0.474
Raet1d /// Raet1b /// Raet1e	retinoic acid early transcript 1d/b/e	1.625	3.085	0.489
Hey1	hairy/enhancer-of-split related with YRPW motif 1	2.112	4.324	0.493
Foxf1a*†	forkhead box F1a	1.843	3.587	0.509
Bex2*	brain expressed X-linked 2	1.650	3.138	0.518
Cited1*	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	1.926	3.799	0.518
Cd59a /// Cd59b	CD59a antigen/CD59b antigen	1.446	2.725	0.521
Foxc2*†	forkhead box C2	1.701	3.251	0.523
Pcp4	Purkinje cell protein 4	3.918	15.112	0.561
Ifitm1 /// Gm7676	interferon induced transmembrane protein 1	3.373	10.362	0.570
Ifitm1	interferon induced transmembrane protein 1	2.796	6.943	0.601
Pde9a	phosphodiesterase 9A	1.800	3.482	0.619
Npl	N-acetylneuraminate pyruvate lyase	2.280	4.856	0.625
Krtdap†	keratinocyte differentiation associated protein	3.004	8.023	0.657
Serpine2†	serine (or cysteine) peptidase inhibitor, clade E, member 2	2.990	7.944	0.672
2610017I09Rik	RIKEN cDNA 2610017I09 gene	3.041	8.233	0.765
Tspan7†	tetraspanin 7	2.836	7.142	0.845
Fzd3†	frizzled homolog 3 (Drosophila)	2.340	5.064	0.846
Gm5458 /// Gm6337 /// Gm3149 /// Gm5797 /// 4930555G01Rik /// Gm2888 /// Gm3047 /// Gm8159 /// Gm3187 /// Gm3453 /// Gm6337 /// Gm3476 /// Gm10413 /// Gm3685 /// Gm3099 /// Gm3149 /// Gm10338	unkonwn	2.501	5.662	0.866
Six2*†	sine oculis-related homeobox 2 homolog (Drosophila)	1.836	3.570	0.887

Table 3.1. cont.

Gene Symbol	Gene Name	Fold Induction (log2)	Fold Induction	Std. Dev.
Downregualted Genes				
Plekho2	pleckstrin homology domain containing, family O member 2	-0.989	1.985	0.602
Tmem62	transmembrane protein 62	-1.034	2.047	0.458
Lekr1	leucine, glutamate and lysine rich 1	-1.053	2.074	0.826
Herc6	hect domain and RLD 6	-1.068	2.097	0.456
Ifi204 /// Mnda	interferon activated gene 204/myeloid cell nuclear differentiation antigen	-1.115	2.166	0.641
Itga3	integrin alpha 3	-1.148	2.215	0.631
Lpcat4	lysophosphatidylcholine acyltransferase 4	-1.187	2.276	0.330
Ifi202b	interferon activated gene 202B	-1.198	2.295	1.104
Gm10473	predicted gene 10473	-1.358	2.564	0.554
Myd88	myeloid differentiation primary response gene 88	-1.220	2.329	0.567
Suox	sulfite oxidase	-1.526	2.879	0.242
Srsy	serine-rich, secreted, Y-linked	-1.577	2.983	1.260
Gmcl1l /// Gm2863 /// Gm2799 /// Gm2825 /// Gm14346	unkonwn	-2.416	5.338	1.303

† Genes associated with differentiation and development

* Genes encoding transcriptional regulators

Supplementary Figures

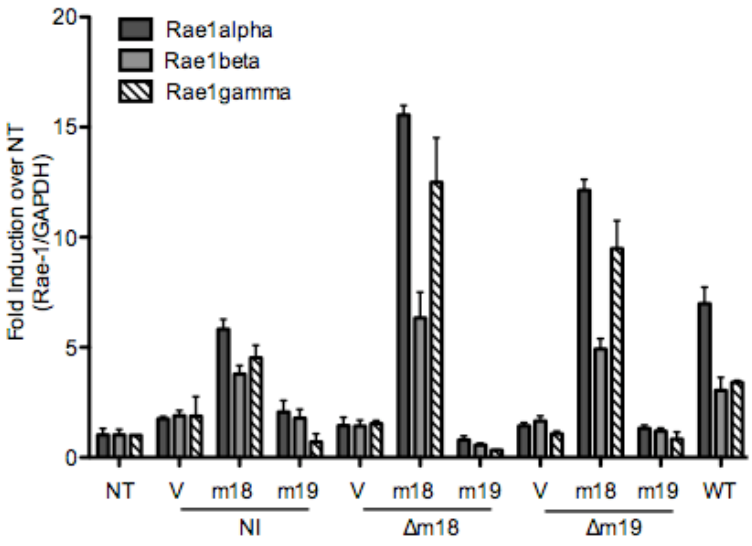


Figure S3.1. RAE-1α and γ are preferentially induced upon m18 expression. Fibroblasts that were first infected with MCMVΔ18, Δ19 or Δ1-22 were transfected with empty vector (V), m18 pCDNA3.1 (m18) or m19 pCDNA3.1 (m19) as indicated. Cells were transfected four to six hours post-infection. In all samples, cells were harvested at 24 hours post-infection, and RAE-1 mRNA expression was quantified by RT-qPCR. As a control, fibroblasts were also infected with wildtype (WT) virus in the same experiment. RAE-1 expression was normalized to GAPDH, and fold induction over untransfected (NT) was graphed. This experiment was performed using the same samples that were used in Fig. 3.4A.

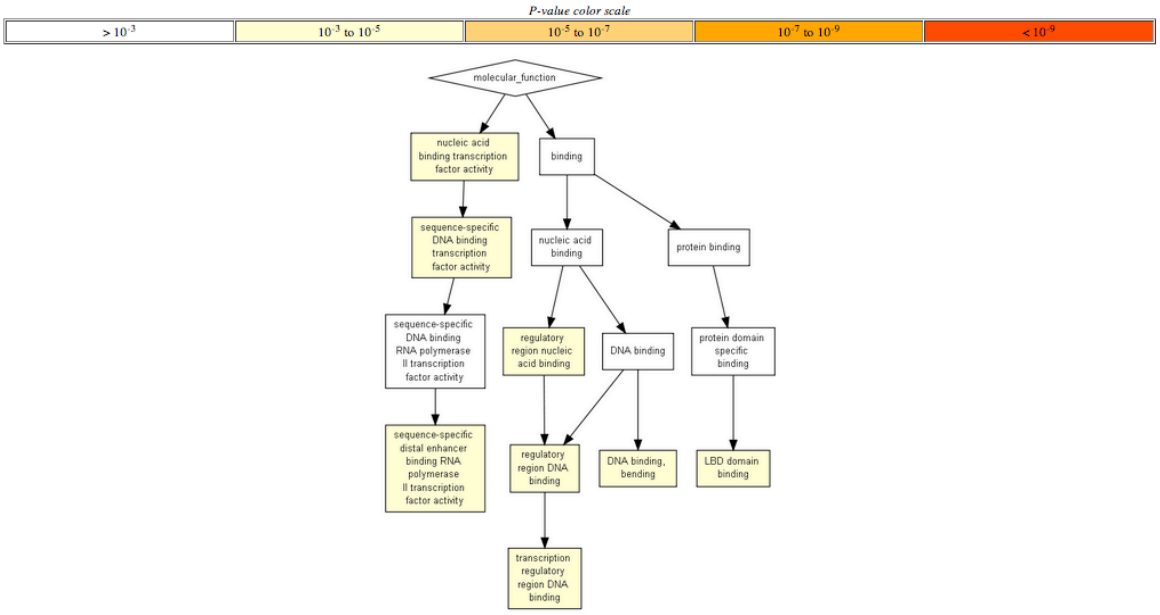


Figure S3.2. Gene ontology analysis of genes that are upregulated by m18 expression. Using a free online gene ontology analysis program called GOrilla, molecular functions that are enriched in the set of genes that are upregulated above 2.5-fold in m18-expressing cells were determined. P-values indicate significant enrichment from the entire original gene set.

Chapter 4: The role of innate signaling pathways in the induction of RAE-1 family of NGK2D ligands

Background

Natural Killer (NK) cells are an important component of innate defense. NK cells detect abnormality within the host, whether it is due to invasion by a foreign pathogen or dysregulation of normal homeostasis that results in tumorigenesis (Raulet 2004). NK cells express stimulatory and inhibitory receptors that are expressed at the cell surface and the balance of signaling through both stimulatory and inhibitory receptors dictates activation of NK cells, whereby NK cells become activated when positive signaling through stimulatory receptors outweigh negative signals through inhibitory receptors (Raulet, Vance 2006). Unlike receptors on T cells and B cells that undergo rearrangement and selection for high affinity to antigens, NK cell receptors are germline encoded. NK cell activation causes these cells to secrete pro-inflammatory molecules such as TNFα and IFNγ as well as cytotoxic effectors, perforin and granzyme. Together these effectors are important to recruit other cells of the immune system to sites of infection or transformation as well as to directly lyse target cells.

NKG2D is a potent stimulatory NK cell receptor that can stimulate NK cell activation upon binding to its cognate ligand. NKG2D ligands are self-proteins that can be induced upon infection and cellular stress. There are multiple NKG2D ligands that all potentiate NK cell activation. Humans encode up to eight NKG2D ligands (MICA, MICB, ULBP1-4, RAET1G, and RAET1L) and mice encode up to nine NKG2D ligands (MULT-1, H60a-c, and RAE-1α-ε). All of these proteins serve as ligands for NKG2D, and interaction between NKG2D and NKG2D ligands results in NK cell activation. Although many stimuli of cellular stress and viral infections evoke expression of NKG2D ligands, the mechanism by which each stimulus induces its expression is ill defined (Raulet 2003).

Pattern recognition receptors (PRRs) are germline encoded innate signaling receptors that are critical in the recognition of pathogen-associated molecular patterns (PAMPs), which are components of microbial pathogens. There are many types of PRRs, but they are broadly categorized into either the family of Toll-like receptors (TLRs), which reside at the cell surface or surface of endosomal compartments, or cytosolic sensors such as NOD-like receptors (NLRs) and Retinoic-Acid-Inducible gene I (RIG-I) that reside in the cytoplasm of cells (Kumar, Kawai & Akira 2011). These sensors recognize conserved microbial molecules as well as nucleic acids. Binding of these innate sensors to their cognate ligands activates a cascade of signaling events that eventually result in the expression of type-I Interferon (type-I IFN) and pro-inflammatory molecules that mediate protective mechanisms and also help bridge the innate immune response to the adaptive immune response (Kumar, Kawai & Akira 2011).

In our study, mouse cytomegalovirus (MCMV) was used as a model to understand how expression of the RAE-1 family of stimulatory NK cell ligands is regulated. MCMV infection, similar to other viral infections, activates signaling through PRRs, and infected cells secrete high levels of type-I IFN as well as pro-inflammatory molecules such as IL-12, IL-6, and TNFα (Ruzek et al. 1997). The RAE-1 family of proteins has been previously shown to be upregulated upon stimulation by microbial ligands such as LPS (Hamerman, Ogasawara & Lanier 2004). According to this study, stimulation of primary peritoneal macrophages with LPS, a TLR4 ligand, causes RAE-1 induction in a manner dependent on the adaptor molecule, MyD88. This was the first study to implicate the involvement of innate signaling pathways in the induction of NKG2D ligands. Several other studies have also shown induction of human NKG2D ligands

upon treatment of human cells with TLR ligands (Ebihara et al. 2007, Kloss et al. 2008). However, no study has shown a link between innate sensing of viral infection and NKG2D ligand expression, and furthermore, there is no data elucidating the downstream effector of MyD88 that may be involved in this process. This chapter describes data obtained so far in an ongoing study on the role of innate signaling on RAE-1 induction during MCMV infection. Our attempts to identify a PRR that senses MCMV to induce expression of RAE-1 revealed that MyD88 adaptor protein is involved in RAE-1 induction, but the specific PRR responsible for this signaling has not been determined. Additionally, the IRF3 transcription factor, which is a downstream effector of MyD88, was found to play a role in this process, but IRF3 overexpression is not sufficient to induce expression of RAE-1 suggesting that other signals are required for this process.

Materials and Methods

Mice. The following mice were provided by Dr. Gregory Barton's lab (UC Berkeley) as a collaboration: MyD88/Trif KO, MyD88 KO, TLR9 KO, Unc93b KO, TLR2/4 KO NrampR, WT NrampR, TLR2/4/9 KO NrampR, and IRF3 KO. The following mice were provided by Dr. Russell Vance's lab (UC Berkeley): STING Gt/Gt and IL-1R KO mice. IFNR KO mice were originally obtained from Dr. Daniel Portnoy's lab (UC Berkeley) and subsequently bred in our lab. The age of mice varied between 5 to 13 weeks. Both female and male mice were used. Within each experiment, the age and sex of the wildtype and KO mice were similarly matched.

Cells. Established tail-derived fibroblasts were prepared as described previously and were maintained in DMEM with 5% FBS and 1% penicillin and streptomycin (Gasser et al. 2005). In order to obtain peritoneal macrophages, mice were sacrificed and skin was removed to reveal the peritoneum. Using a 25G5/8 size needle, 5ml of PBS was injected into the peritoneum. Mice were physically shaken to allow even dispersion of peritoneal cells, and maximal volume of cells was harvested using the same needle. Cells were then counted and cultured overnight in RPMI containing 10-15% MCSF provided by Dr. Portnoy (UC Berkeley), 10% FBS, and 1% penicillin and streptomycin.

Stimulation. Peritoneal macrophages were treated with the following concentrations of control TLR ligands: 1uM of CpG oligonucleotide 5'-TCCATGACGTTCCTGACGTT-3' with phosphorothioate linkages (IDT), 100ng/ml of lipopolysaccaride (LPS, Sigma), 1ug/ml of Pam3CK4.

Reverse transcription quantitative real-time PCR. RNA from macrophages and fibroblasts were extracted in Trizol (Invitrogen), treated with RQ1 DNase (Promega), and total RNA was reverse transcribed using oligo$(dT)_{15}$ primer (iDT DNA Technologies) and SuperScriptII (Invitrogen) at 42ºC for 50 minutes. cDNAs were analyzed using ABI7300 Real Time PCR System. Primers for RAE-1 and ISG15 are described in Table S2.1.

Virus propagation, infection and titering. MCMV WT (Smith strain) was generously provided by Dr. Koszinowski (Max von Pettenkofer-Institute, Munich, Germany). All viruses were propagated in NIH 3T3 cells and titered in BALB/c 3T3 cells. Fibroblasts were infected at MOI of 5 and macrophages were infected at either MOI of 1 or 2.5, as indicated in the figure legends. Input virus was removed at 2 hrs pi, and infection was allowed to take place for a total of 24 hrs.

Cells were directly lysed in TRIZOL, transferred to eppendorf tubes, and maintained at -20ºC until use.

Statistical analysis. A two-tailed student t-test was performed on all samples where statistical significance is indicated.

Results

MyD88 signaling plays a substantial role in RAE-1 induction during MCMV infection.

MCMV is a dsDNA virus that can stimulate both TLR9 and cytosolic DNA sensors to induce expression of IFNβ. The level of contribution by these sensors depends on the cell type. For instance, pDCs depend largely on TLR9 for IFNβ production because they express high levels of this receptor, while other cells such as cDCs or MEFs depend on the cytosolic DNA sensors for expression of IFNβ (Yanai et al. 2009). The adaptor molecule MyD88 associates with TLR9 to transduce further signaling. Additionally, the cytosolic molecule STING, which was recently shown to directly bind cyclic dinucleotides, has been implicated as an adaptor molecule for a cytosolic DNA sensor (Burdette et al. 2011, Ishikawa, Barber 2011). Because there are potentially multiple receptors that can recognize MCMV, any of which could play a role in RAE-1 induction, the role of these two main molecules, MyD88 and STING, in MCMV-mediated induction of RAE-1 was investigated to distinguish signaling through TLRs versus cytosolic sensors.

Primary peritoneal macrophages were first obtained from MyD88/Trif double knockout (KO) mice and infected with MCMV at MOI of 1 for 24 hours. When compared to wildtype macrophages, MyD88/Trif KO macrophages induced significantly less RAE-1 in response to viral infection (Fig. 4.1A), strongly indicating the involvement of MyD88 or Trif signaling in the induction of RAE-1 upon MCMV infection. In order to test whether macrophages lacking just MyD88 signaling would also reveal a similar phenotype as MyD88/Trif double KO macrophages, primary peritoneal macrophages from mice deficient in MyD88 alone were obtained and infected with MCMV. Although statistical significance could not be calculated due to a small sample size, the dependency of RAE-1 induction on MyD88 signaling seems to recapitulate the data obtained with MyD88/Trif double KO macrophages (Fig. 4.1B). The difference in ISG15 induction between MyD88/Trif KO and MyD88 KO is unexpected and requires further studies to clarify these differences. As controls, MyD88 KO or wildtype macrophages were stimulated with known TLR ligands, and RAE-1 and ISG15 expression in these samples were also determined. In support of a previous study, RAE-1 was efficiently induced upon treatment with a TLR4 ligand LPS in a MyD88-dependent manner (Fig. S4.1) (Hamerman, Ogasawara & Lanier 2004). However, none of the other ligands induced expression of RAE-1. The level of ISG15 expression was variable depending on the ligand, but in all cases was MyD88-dependent, as expected.

Because RAE-1 induction may also depend on activation of innate signaling through a cytosolic sensor, the role of STING was tested in the same system. To this end, primary peritoneal macrophages were obtained from mice containing a T596A mutation in STING (STING Gt/Gt), which renders the protein incapable of upregulating IFNβ expression upon stimulation by microbial ligands such as *L. monocytogenes* (Sauer et al. 2011). Induction of both RAE-1 and ISG15 was consistently reduced in STING Gt/Gt macrophages compared to wildtype macrophages, but neither sample set was statistically significant (Fig. 4.1C). The consistent trend in lower RAE-1 and ISG15 expression in STING Gt/Gt cells may suggest that signaling through

STING has a minor role in RAE-1 induction; with increased sample size, the difference may prove to be significant. However, the data overall suggests a predominant role of MyD88/Trif signaling, most likely through MyD88, in the induction of RAE-1 in peritoneal macrophages infected with MCMV.

In this system, expression of ISG15 transcript was used to measure the IFN response. The reason for measuring ISG15 instead of secreted forms of type-I IFN by a common IFN bioassay is that MCMV present in the supernatant of infected cells can infect the L929 indicator cells used for the bioassay and cause false positives. Furthermore, IFNβ mRNA expression upon MCMV infection peaks at six to eight hours post-infection, and it is this time point that accurately represents activation of IFNβ through the initial sensing of the virus. Thus, the time point at which RAE-1 induction is detected, 24 hours post-infection, is too late to observe the first burst of IFNβ activation.

None of the candidate TLRs detectably contribute to MCMV-mediated induction of RAE-1.

The adaptor molecule MyD88 associates with multiple receptors to promote expression of type-I IFNs and pro-inflammatory molecules including TLR2, 4, 7, 9, IL-1R, and IL-18R. In particular, mice deficient in TLR9 are highly susceptible to infection by MCMV, suggesting that TLR9 plays a key role in the control of viral infection (Delale et al. 2005). Moreover, deficiency in TLR7, despite its role in recognition of RNA species, can increase susceptibility to MCMV when TLR7/9-deficient mice are infected with sublethal dose of the virus (Zucchini et al. 2008). Together, TLR7 and 9 seem to contribute to the anti-viral response to MCMV. Not surprisingly, mice deficient in MyD88, which is the sole adaptor protein for TLR7 and 9, are just as susceptible to MCMV as TLR9-deficient animals (Delale et al. 2005). Therefore, the role of TLR7 and 9 in MCMV-mediated induction of RAE-1 was investigated.

Primary peritoneal macrophages were obtained from mice deficient in TLR9 or Unc93b, which is a molecule required for trafficking of TLR3, 7, and 9 to the endosomal compartments. These cells were infected with MCMV for 24 hours, and RAE-1 induction was quantified by RT-qPCR. MCMV infection of macrophages from TLR9 or Unc93b KO mice induced RAE-1 to comparable levels as infection of WT macrophages (Fig. 4.2A and B), suggesting that these nucleic acid sensing endosomal TLRs are not required for RAE-1 induction. Similar to MyD88 single KO macrophages, TLR9 and Unc93b showed lower levels of ISG15 induction compared to WT macrophages. Together, the data points to a role for TLR9 via MyD88 in the induction of ISG15, but the MyD88-dependent induction of RAE-1 does not seem to require sensing through TLR9.

TLR2, which is displayed at the cell surface, has recently been shown to play a role in IFNβ expression in inflammatory monocytes infected with MCMV (Barbalat et al. 2009). Although the role of TLR2 in response to MCMV infection in other cell types is unknown, we investigated its involvement in MCMV-mediated induction of RAE-1. Due to a lack of mice deficient in TLR2 alone, primary peritoneal macrophages from TLR2, 4 double KO mice were infected with MCMV, and RAE-1 mRNA was quantified at 24 hours post-infection. Because these mice were generated on a Nramp-resistant (NrampR) background, peritoneal macrophages from NrampR wildtype mice were obtained for comparison. In the limited number of samples analyzed, RAE-1 induction did not seem to depend on signaling through either TLR2 or 4 (Fig. 4.2C). However, these receptors were required for the induction of ISG15, suggesting that in addition to TLR9, TLR2 and/or 4 are important to activate the interferon response in MCMV-

infected peritoneal macrophages. As controls, TLR2/4 KO and wildtype macrophages were stimulated with TLR2 or 4 ligands, Pam3CSK4 (Pam) or LPS, respectively, or a TLR9 ligand CpG, which should not be affected by the loss of either TLR2 or 4. Unlike data obtained in the MyD88 KO experiments, wildtype NrampR macrophages did not induce RAE-1 upon stimulation with LPS (Fig. S4.2), indicating a possible role of Nramp-sensitivity in the induction of RAE-1 upon LPS stimulation. As expected, ISG15 expression upon stimulation with LPS and Pam was dependent on TLR2 and 4 signaling. ISG15 expression upon CpG stimulation was also dependent on TLR2 or 4 signaling in this case, even though CpG is a TLR9 ligand. Further experiments are necessary to explain these discrepancies.

Several studies have demonstrated redundancy in TLR signaling during an infection. In the absence of signaling through one TLR, another one can compensate for the lack of signaling. Therefore, the possibility that MCMV-mediated induction of RAE-1 occurs by signaling through more than one TLR was tested in peritoneal macrophages. To this end, primary peritoneal macrophages were obtained from mice lacking TLR2, 4, and 9 on a NrampR background. These cells were infected with MCMV and RAE-1 expression was compared to NrampR wildtype macrophages. TLR2, 4, 9 KO macrophages did not have a defect in ISG15 expression upon MCMV infection, and furthermore, there was no difference in the level of RAE-1 induction between WT and KO macrophages (Fig. 4.2D). The data suggests that RAE-1 induction is most likely not dependent on redundant signaling through TLR2, 4, or 9. It remains possible that signaling through these TLRs is redundant with some other signal. Further studies are necessary to explain differences in ISG15 expression upon MCMV infection.

RAE-1 induction does not depend on IFN receptor signaling or IL-1 receptor signaling.

MCMV infection results in the production and secretion of type-I IFNs both *in vitro* and *in vivo*. Type-I IFN production is stimulated by activation of TLRs and cytosolic sensors. Once secreted, it binds to the IFN receptor (IFNR) and activates a JAK/STAT cascade that results in the expression of many IFN-stimulated genes (ISGs) (Takaoka, Yanai 2006). Because none of the tested TLRs seem to contribute to RAE-1 induction, it is plausible that RAE-1 induction is a secondary effect to the initial sensing of the virus and that IFNR signaling is required for its induction. To test this hypothesis, primary peritoneal macrophages were obtained from IFNR KO or wildtype mice. Macrophages were then infected with MCMV at MOI 2.5 and RAE-1 or ISG15 expression was quantified. RAE-1 induction in MCMV-infected IFNR KO macrophages was comparable to wildtype macrophages (Fig. 4.3A), and a similar trend was observed at MOI of 1 as well (data not shown). The level of ISG15 was much lower in IFNR KO macrophages in accordance with ISG15 being one of the secondary effectors, although not statistically significant. Thus, RAE-1 induction upon MCMV infection does not seem to require signaling through the IFNR.

As none of the MyD88-associating TLRs seemed to be required for the induction of RAE-1, we next tested the role of IL-1R signaling in this process. Primary peritoneal macrophages were obtained from mice deficient in the IL-1R and infected with MCMV. RAE-1 quantification by RT-qPCR revealed that IL-1R KO macrophages also do not have a defect in RAE-1 induction upon MCMV infection (Fig. 4.3B). Therefore, MyD88-dependent upregulation of RAE-1 occurs neither through IL-1R nor TLRs that sense MCMV.

The IRF3 transcription factor is involved in the induction of RAE-1 during MCMV infection.

The main transcription factors that become activated upon MyD88 signaling include NF-kB, AP-1, IRF3, and IRF7. These transcription factors are responsible for the expression of pro-inflammatory molecules and type-I IFN. MCMV infection has been shown to cause phosphorylation of IRF3 and subsequent nuclear translocation (Le et al. 2008). Previous work done in the lab showed a modest decrease in the ability of MCMV to induce cell surface expression of RAE-1 when IRF3 was knocked down in fibroblasts (Clarisse Lorin, data not shown). Furthermore, IRF3 seems to be involved in the induction of RAE-1 during DNA damage activation (Stefan Gasser and David Raulet, submitted). Therefore, the role of IRF3 in the induction of RAE-1 during MCMV infection was further probed in macrophages.

Primary peritoneal macrophages were obtained from mice deficient in IRF3 from Genghong Cheng's lab at UCLA. Upon infection with MCMV, RAE-1 induction was significantly inhibited in IRF3 KO macrophages, and this corresponded with an absence of ISG15 induction, which is dependent on IRF3 activation (Fig. 4.4). The difference in the induction between wildtype and IRF3 KO macrophages were less dramatic when mice from the Barton lab at UC Berkeley were used (Fig. S4.3). However, experiments in these macrophages should be repeated because these IRF3 KO macrophages induced expression of ISG15, which is not expected. Altogether, the data suggest that IRF3 is required for optimal induction of RAE-1 in MCMV-infected primary peritoneal macrophages, but it is not absolutely required.

Overexpression of IRF3 is not sufficient to induce expression of RAE-1.

IRF3 is a transcription factor that becomes activated upon phosphorylation by TBK-1. Once activated, IRF3 translocates from the cytoplasm to the nucleus where it forms either a homodimer or a heterodimer with IRF7 to bind to IFN stimulated response elements (ISRE) and induce expression of type-I IFN (Taniguchi et al. 2001). Because of its role as a transcription factor and its involvement in the induction of RAE-1 upon MCMV infection, its potential to promote transcription of RAE-1 in the absence of viral infection was determined. To this end, fibroblasts were transfected with a plasmid encoding either wildtype IRF3, an inactive form of IRF3 (IRF3KD), or IRF7 and the level of RAE-1 transcript was quantified at 24 hours post-transfection. As a control for the expression of IRFs, the level of ISG15 expression was also measured in the same samples. Overexpression of IRF3 was not sufficient to induce expression of RAE-1, whereas significant RAE-1 induction occurred in fibroblasts infected with MCMV at an MOI of 5 for 24 hours (Fig. 4.5A and B). In contrast, IRF3 overexpression and MCMV infection resulted in similar induction of ISG15 expression (Fig. 4.5A and B). Overexpression of IRF7 was not sufficient to induce ISG15, supporting previous findings on the requirement of IRF7 to dimerize with IRF3 for binding to ISRE. Thus, IRF3 seems to be involved in the induction of RAE-1 during MCMV infection of macrophages, but it is clearly not the sole regulator of RAE-1 expression.

Discussion

Innate signaling through PRRs is critical to respond immediately to invasion by microbes and viruses. Accordingly, PRRs can detect a wide range of ligands including proteins, lipids and nucleic acids to produce inflammatory molecules (Medzhitov, Janeway 2000). A hallmark of PRR stimulation is the production of type-I IFNs, which in turn activates IFNR signaling and subsequent expression of many ISGs (Takaoka, Yanai 2006). Together with pro-inflammatory molecules, these targets of innate signaling are required to effectively bridge the innate immune response with the adaptive immune response. PRRs can be expressed on a variety of cell types,

but dendritic cells and macrophages play prominent roles in the production of type-I IFNs and pro-inflammatory molecules upon PRR signaling.

Use of specific PRR ligands outside of the context of a true pathogen has greatly facilitated the characterization of an array of adaptor molecules, kinases and transcription factors, which are involved in innate signaling through both TLR family of receptors or cytosolic receptors. These studies have revealed that common effector molecules can be utilized by distinct receptors and activation of distinct receptors can cause expression of common inflammatory genes. Relatively more recently, various strains of mice deficient in specific PRRs or downstream signaling molecules have been generated, and these mice have been used to understand innate signaling in the context of a whole pathogen, not just the ligand. These studies have revealed that PRRs can have redundant roles to compensate for each other when in need. Furthermore, our group as well as others have suggested that cells are able to discriminate between invasion by a replicating, infectious pathogen versus engulfment of non-infectious pathogen particles (Tokuyama et al. 2011, Vance, Isberg & Portnoy 2009). Understanding the entire network of innate signals that is activated upon infection by any given pathogen is a challenge and remains to be an active area of research.

In this study, MCMV was used to dissect the role of innate signaling pathways in the induction of RAE-1 family of NKG2D ligands. RAE-1 is not generally considered to be a pro-inflammatory molecule, but expression of RAE-1 at the cell surface of diseased cells results in activation of NK cells, which in turn secretes pro-inflammatory molecules TNFα and IFNγ; thus RAE-1 is tightly linked with activation of the innate immune response. We and others have shown that MCMV infection of cells *in vitro* leads to RAE-1 expression within 24 to 48 hours post-infection, suggesting that signals that are activated early on during infection are responsible for its expression (Tokuyama et al. 2011, Lodoen et al. 2003). Because a previous study showed that LPS stimulation of macrophages induces RAE-1 expression in a MyD88-dependent manner, we asked whether MCMV-mediated induction of RAE-1 is also MyD88-dependent, and if so, which PRR initiates the signal and which downstream effectors are required for this expression.

The data contained in this chapter indicates that signaling through MyD88 plays a substantial role in the induction of RAE-1 in peritoneal macrophages infected with MCMV. However, we have yet to identify a receptor associated with MyD88 that mediates this expression. Our data suggests that the IRF3 transcription factor plays an important role in RAE-1 induction upon MCMV infection, but IRF3 is not the sole regulator of RAE-1 expression. Together, the data so far suggests several possibilities for how MyD88 regulates RAE-1 induction: 1) MyD88 signaling is required to activate other transcription factors besides IRF3, which are necessary for RAE-1 induction, 2) signaling through IL-18R, which also associates with MyD88, leads to RAE-1 induction, or 3) MyD88 signaling is required for the expression of an unknown gene that in turn regulates RAE-1 expression at the transcript level. Future studies that test these hypotheses may reveal novel regulator(s) of RAE-1, and perhaps also reveal a new axis of innate immune signaling during viral infection.

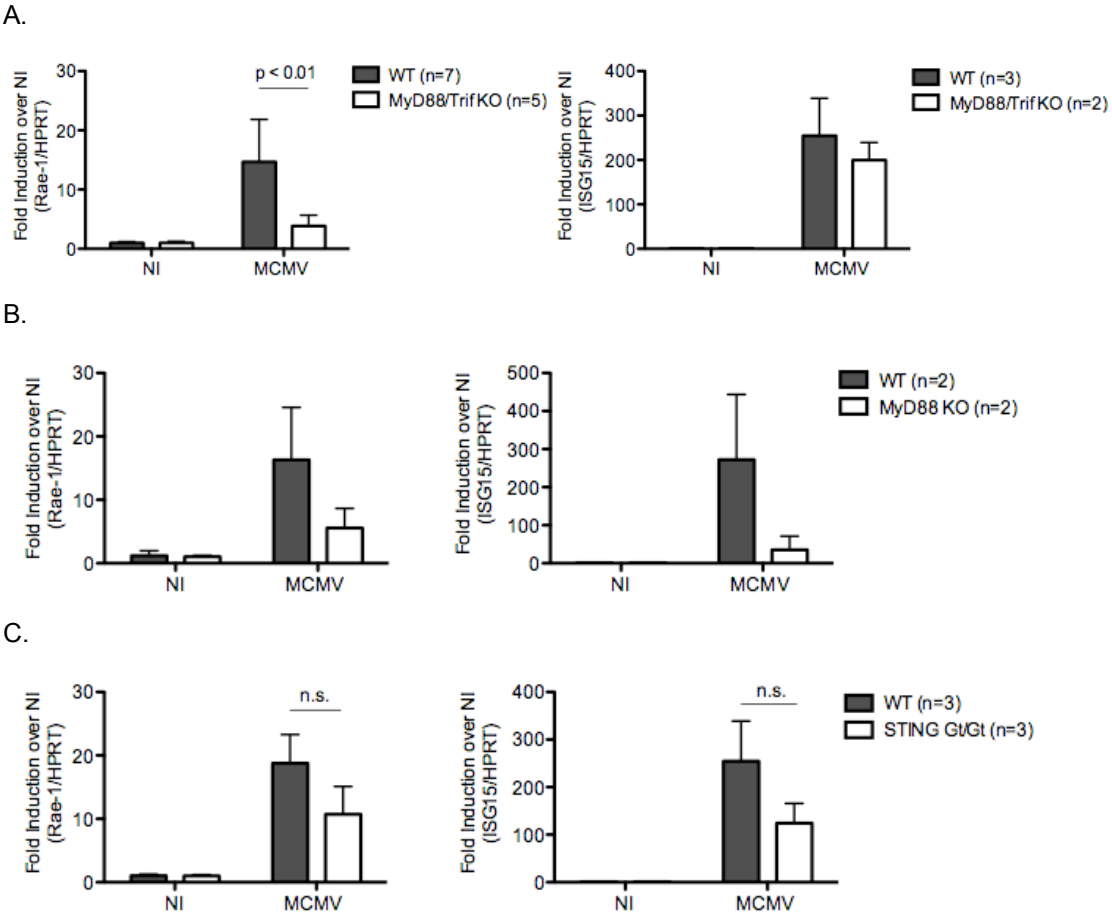


Figure 4.1. MCMV-mediated induction of RAE-1 in peritoneal macrophages depends largely on MyD88 signaling. Primary macrophages were obtained from MyD88/Trif double KO (A), MyD88 single KO (B) or STING Gt mutant (C) mice. All samples were compared to wildtype (WT) C57BL/6 mice. For all samples, macrophages were infected at MOI of 1.0 for 24 hours, and expression of either RAE-1 (left column) or ISG15 (right column) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples, number of mice per group (n), and statistical values for groups consisting of at least three mice.

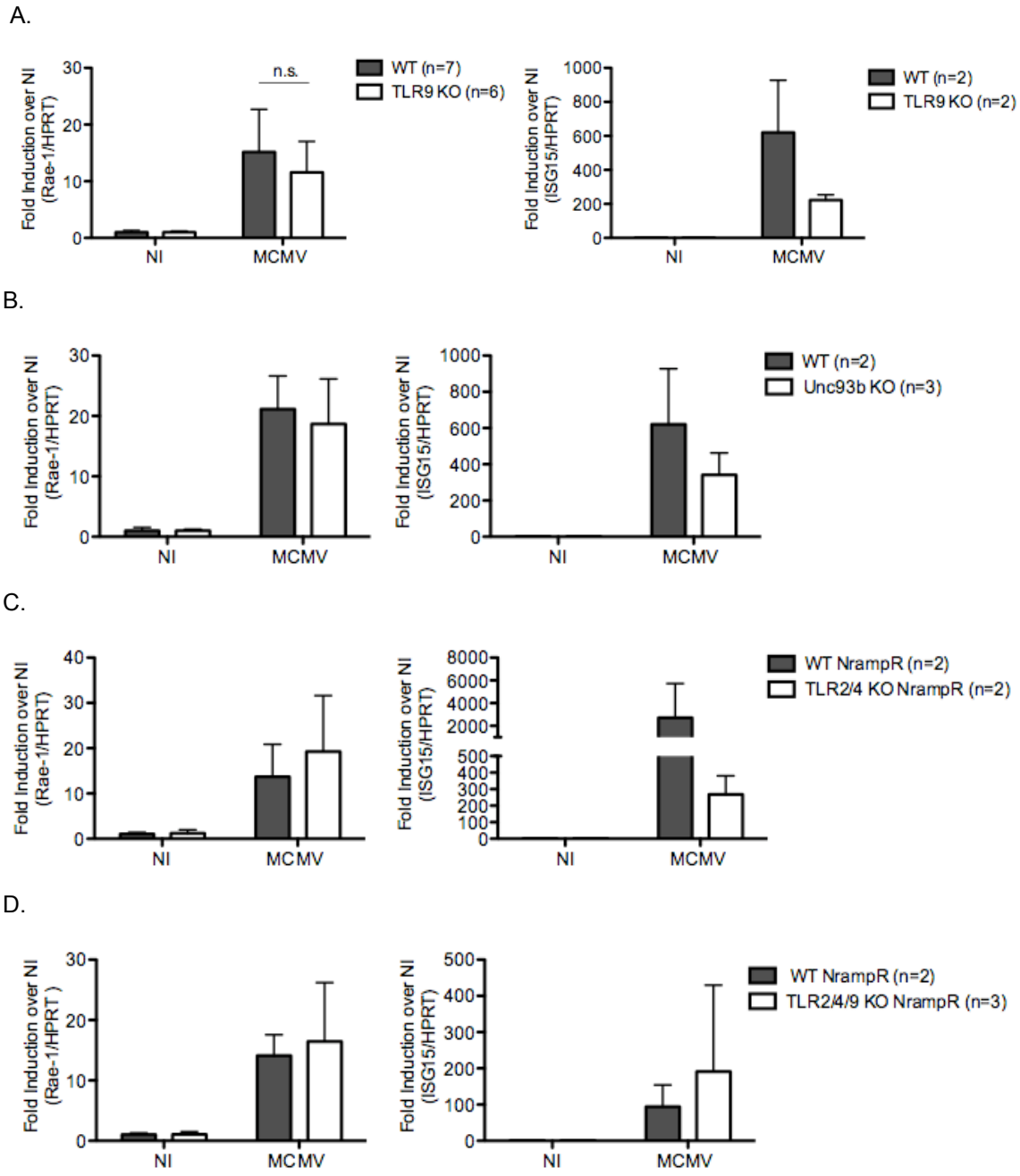
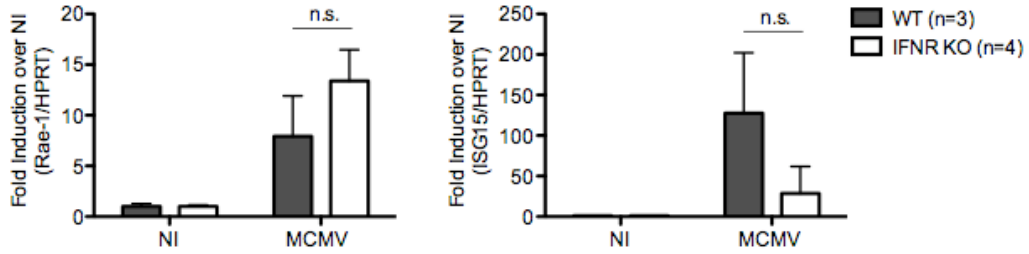


Figure 4.2. MCMV-mediated induction of RAE-1 in peritoneal macrophages is not dependent on predicted TLRs. Primary macrophages were obtained from TLR9 KO (A), Unc93b KO (B), TLR2/4 KO (C), or TLR2/4/9 KO (D) mice. Samples from TLR9 and Unc93b KO mice were compared to C57/BL6 (WT) mice. TLR2/4 and TLR2/4/9 KO are on a Nramp-resistant (NrampR) background, thus compared to WT NrampR mice. For all samples, macrophages were infected at MOI of 1.0 for 24 hours, and expression of either RAE-1 (left column) or ISG15 (right column) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples, number of mice per group (n), and statistical values in groups consisting of at least three mice.

A.



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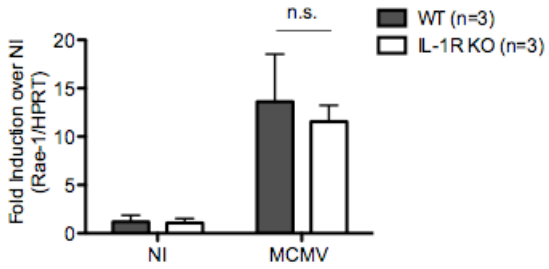

Figure 4.3. MCMV-mediated induction of RAE-1 in peritoneal macrophages does not require IFNR or IL-1R signaling. Primary macrophages were obtained from either IFNR KO (A) or IL-1R KO (B) mice and wildtype (WT) C57BL/6 macrophages were obtained for comparison. For all samples, macrophages were infected at MOI of 2.5 for 24 hours, and expression of either RAE-1 (left column) or ISG15 (right column) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples, number of mice per group (n), and statistical values determined by student t-test.

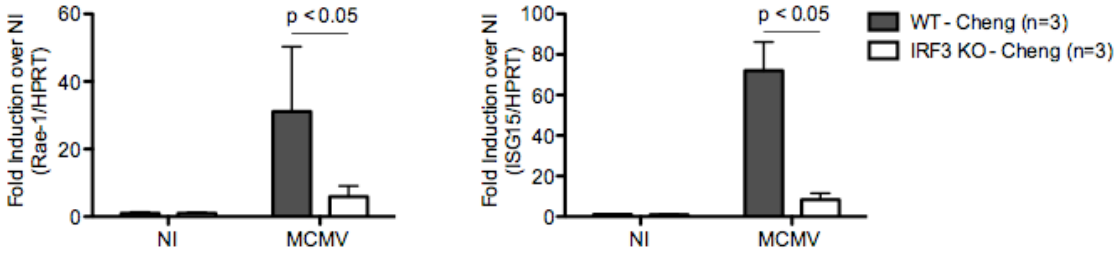

Figure 4.4. IRF3 is involved in MCMV-mediated induction of RAE-1 and ISG15. Primary macrophages were obtained from IRF3 KO mice from Genghong Cheng's lab at UCLA. Macrophages were infected at MOI of 1.0 for 24 hours, and expression of either RAE-1 (left) or ISG15 (right) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples, number of mice per group (n), and statistical values were calculated by student t-test.

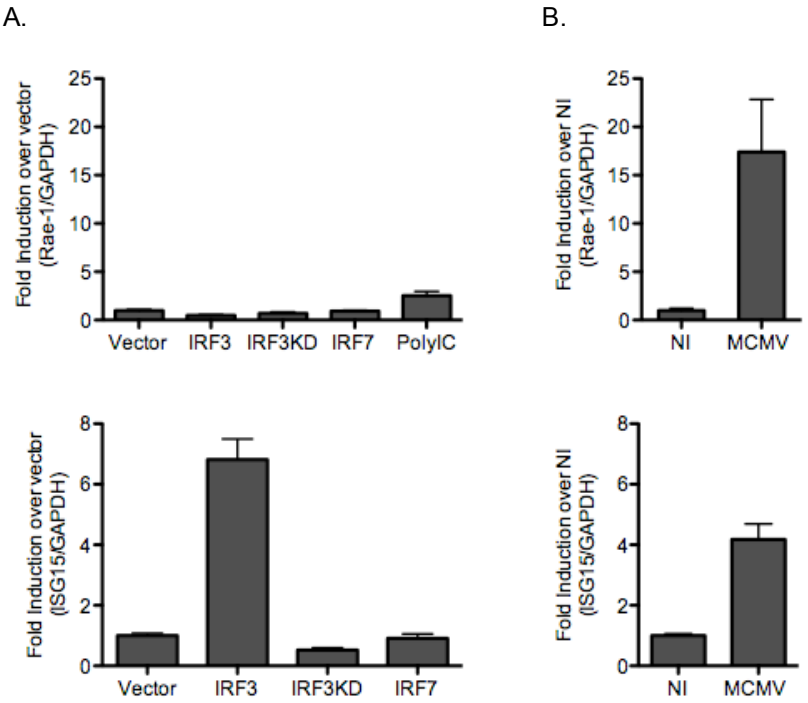

Figure 4.5. Overexpression of IRF3 transcription factor is not sufficient to induce RAE-1 in mouse fibroblasts. Mouse tail fibroblasts were transiently transfected with plasmids encoding IRF3, a kinase dead version of IRF3 (IRF3KD), IRF7, or empty vector (A) or infected with MCMV at MOI of 5 (B). At 24 hours post-treatment, cells were harvested and analyzed for expression of either RAE-1 (top row) or ISG15 (bottom row) by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. The graph depicts fold induction over either empty vector or uninfected controls, and standard deviation is calculated from two to four experiments.

Supplementary Figures

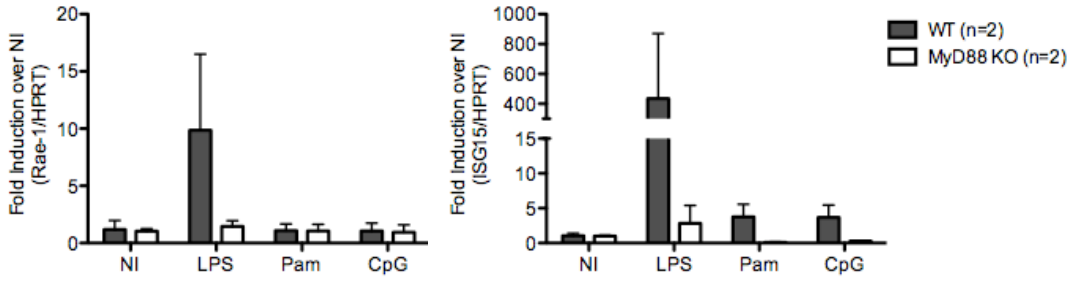

Figure S4.1. MyD88 KO mice respond to TLR ligands as expected. Primary macrophages were obtained from MyD88 single KO mice. Cells were treated the either lipopolysaccaride (LPS, 100ng/ml), Pam3CSK4 (Pam, 1ug/ml), or CpG-B (1uM) for 24 hours, and expression of either RAE-1 (left column) or ISG15 (right column) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples and number of mice per group (n).

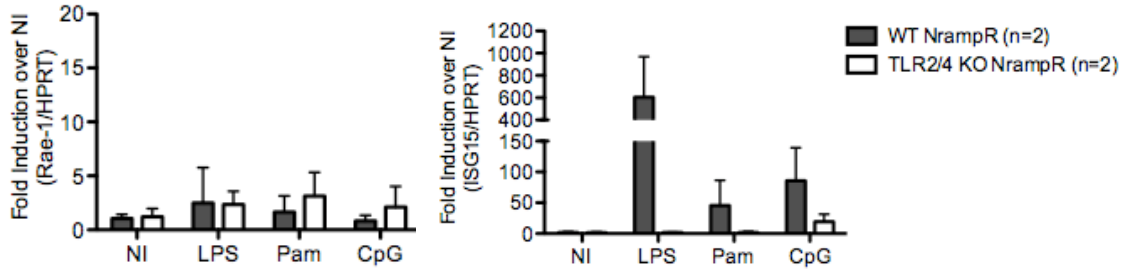

Figure S4.2. NrampR wildtype mice cannot induce RAE-1 with LPS stimulation and ISG15 induction by CpG shows a TLR2/4 dependency. Primary macrophages were obtained from TLR2/4 KO NrampR mice. Cells were treated the either lipopolysaccaride (LPS, 100ng/ml), Pam3CSK4 (Pam, 1ug/ml), or CpG-B (1uM) for 24 hours, and expression of either RAE-1 (left column) or ISG15 (right column) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples and number of mice per group (n).

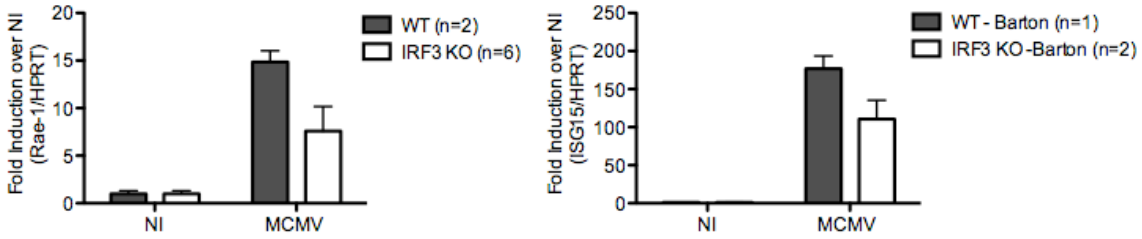

Figure S4.3. The role of IRF3 in MCMV-mediated induction of RAE-1. Primary macrophages were obtained from IRF3 KO mice from Gregory Barton's lab at UC Berkeley. Macrophages were infected at MOI of 1.0 for 24 hours, and expression of either RAE-1 (left) or ISG15 (right) was quantified by RT-qPCR. Both RAE-1 and ISG15 expression was normalized to expression of HPRT. Each graph depicts fold induction over uninfected (NI) samples, and number of mice per group (n).

Chapter 5: Concluding Remarks and Future Directions

In this thesis, MCMV was used as a tool to probe the mechanism by which cells "sense" an infection and to understand what signals are necessary for the commitment to express stress ligands that alert the immune system of abnormality. This thesis describes two cellular players that contribute to this commitment process and one novel viral protein that triggers this response on its own.

Most pathogens take advantage of existing host cellular machinery for their own survival and replication. For instance, viruses are capable of utilizing much of the host's translational machinery to translate their own mRNAs, while depriving the host of being able to translate these mRNAs. Similarly, many key cellular pathways are dysregulated during a viral infection for the benefit of the virus. Once such example is the PI3K pathway. Because PI3K pathway controls many cellular functions including proliferation, translation, and metabolism, it is no surprise that many viruses activate this pathway via either direct interaction with PI3K or by an indirect mechanism. Furthermore, in transformed cells or in tumors, the PI3K pathway is often constitutively activated to promote growth. In the studies summarized in Chapter 2 of this thesis, we have illustrated that PI3K activation during MCMV infection and in transformed cells is a major contributor of RAE-1 expression. The results suggest that perhaps cells sense dysregulation of a cellular pathway as a cellular pattern to trigger expression of stress ligands. Future studies should explore the exact mechanism by which PI3K activation triggers RAE-1 by focusing on its contribution to different aspects of RAE-1 biogenesis. Additionally, because overexpression of PI3K is not sufficient to induce RAE-1 expression, other cellular pathways and factors must be involved in this process.

The main focus of the second chapter is on how the host senses MCMV infection to induce expression of RAE-1. The third chapter complements the second chapter by revealing a novel viral protein that triggers this expression. We observed that MCMV encodes a single protein m18 that is necessary and sufficient to induce expression of RAE-1. In other words, from the host's perspective, cells are able to discriminate a virus that contains this viral protein from a virus that does not, and this difference alone triggers a stress response that results in the expression of RAE-1. The data also implied that m18-mediated induction of RAE-1 is dependent on activation of the PI3K pathway suggesting that m18 alone must be modulating the PI3K pathway as well as other cellular factors that are required for RAE-1 expression. The viral protein encoded by m18 is a large 1040 amino acid protein. Therefore, it would not be surprising if m18 interacts with multiple factors that together contribute to the stress response. Future studies on characterizing interacting partners of m18 may lead to the identification of cellular factors that set the "activation level" of cells to a point of stress response commitment. It is often the case that viral proteins are capable of carrying out multiple functions to be as efficient as possible. Studies to date on the role of RAE-1 as a NKG2D ligand would suggest that MCMV-mediated induction of RAE-1 is disadvantageous to the virus. In support of this point, MCMV encodes for m138 and m152 proteins that specifically prevent cell surface expression of RAE-1. The fact that MCMV has not eliminated m18 from its genome, despite having to counteract its effect, suggests that the maintenance of m18 must be advantageous for the virus. Because of its ability to activate the PI3K pathway, it is possible that m18 gives virally-infected host cells an advantage under conditions where nutrients are limited. It is also possible that a virus with m18 has a competition advantage over a virus that had lost this gene. In vivo studies comparing wildtype and virus lacking m18 should reveal the true function of m18.

PRRs are innate sensors that immediately sense a pathogen once it has invaded the host. MCMV contains double-stranded DNA, which is a source for signaling through endosomal and cytosolic DNA sensors and subsequent production of pro-inflammatory molecules. RAE-1 proteins are stress ligands for NKG2D receptor on NK cells and are expressed within 24 hours post-infection. Thus, the trigger for expression of RAE-1 must occur relatively early upon infection. To test whether any of the PRRs and downstream signaling through PRRs contributes to the induction of RAE-1 during MCMV infection, peritoneal macrophages from a number of mouse strains lacking single or combinations of PRRs or downstream effector molecules were tested for their ability to induce RAE-1. The results summarized in Chapter 4 of this thesis reveal that a signaling axis involving the adaptor molecule MyD88 plays a significant role in MCMV-mediated induction of RAE-1. Furthermore, the data implicate a significant role of the transcription factor IRF3 in mediating this response. Surprisingly, none of the MyD88-dependent TLRs predicted to play a role in MCMV infected cells were required. Because MyD88 by itself does not recognize PAMPs, it will be important in future studies to clarify how MyD88 is involved in the induction of RAE-1. These studies may also reveal viral components that initiate the response and contribute to our understanding of how cells sense viral infections.

In conclusion, the thesis project has identified several key effectors of RAE-1 induction during MCMV infection. The most important message from the collective data presented here is that RAE-1 expression requires multiple signals. Because RAE-1 expression may result in the destruction of the cell, it is reasonable for its expression to be tightly regulated. A combination of signals through innate signaling, perturbation in cellular pathways, and other cues is how cells know when enough is enough. Further studies in the area may allow for manipulation of the system to induce NK cell killing of any target cell. More broadly, innate sensing of dysregulated cellular pathways may contribute to expression of genes beyond RAE-1 and could be an important area to understand how an individual cell knows it is under attack.

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Appendix Section 1: In vivo infection with MCMV

Materials and Methods

Mice. All animal experiments were approved by the Animal Care and Use Committee of UC Berkeley (#R292-0512BCR). For all infection experiments, C57BL/6 mice that were maintained at UC Berkeley were used.

Viruses and propagation. MCMVΔ152 was generously provided by Dr. Hill (Oregon Health and Science University, Oregon). MCMV WT (Smith strain) and MCMV WTGFP viruses were generously provided by Dr. Koszinowski (Max von Pettenkofer-Institute, Munich, Germany). All viruses were propagated in NIH 3T3 cells and titered in BALB/c 3T3 cells.

In vivo infection. Subcutaneous injections of virus in the earflap of mice were performed following the Robey lab protocol. Briefly, mice were injected intraperitoneally with 2.5% Avertin (volume equal to (total weight of the mouse (g) X 10) + 40ul), and injected subcutaneously in each earflap with 2.5X10^6 pfu of MCMV concentrated in 30ul total volume. The draining dorsal cervical lymph node was harvested at 24 hours post-infection and used for two-photon analysis or prepared for freezing and sectioning (Chtanova et al. 2008).

For intraperitoneal infection of mice, 2X10^6 pfu of MCMV and 2X10^6 pfu of MHV68, and 100ug of CpG oligonucleotide 5'-TCCATGACGTTCCTGACGTT-3' with phosphorothioate linkages (IDT) were resuspended in 200ul of PBS and injected into the peritoneal cavity of mice (performed in collaboration with Andrea Pezda from our lab). At 72 hours post-infection, mice were sacrificed and skin was removed to reveal the peritoneum. Using a 25G5/8 size needle, 5ml of PBS was injected into the peritoneum. Mice were physically shaken to allow even dispersion of peritoneal cells, and maximal volume of cells was harvested using the same needle.

Two photon and immunofluorescent microscopy. Two photon microscopy was performed by Seong-ji Han in the Robey lab according to previously published protocols (Chtanova et al. 2008). Frozen sections were prepared by Janine Coombes in the Robey lab according to previously published protocols (Chtanova et al. 2008). For staining of the frozen sections, slides were fixed either in 4% PFA or cold acetone for 10mins at room temperature (RT) for RAE-1ε staining and panRAE-1 staining, respectively. Sections were blocked in 10% non-specific donkey serum (Jackson ImmunoResearch) for 90 mins at RT. RAE-1ε staining was performed using an unconjugated RAE-1ε antibody (R&D systems) at 1:50d incubated overnight at 4°C, followed by addition of a secondary anti-rat IgG Cy5 at 1:100d incubated for 2 hrs at RT. PanRAE-1 staining was performed using a panRAE-1 antibody (R&D Systems) that was biotinylated (Raulet lab) at 1:50d incubated overnight at 4°C, followed by Streptavidin Cy5 at 1:100d incubated for an hour at RT. CD169 staining was performed using a purified rat anti-mouse CD169 antibody (AbD Serotec) at 1:25d incubated overnight at 4°C, followed by addition of a secondary anti-rat Alex647 at 1:100d incubated for 2 hrs at RT. Coverslips were applied to the sections with VectaShield (Vector Laboratories) mounting medium containing DAPI. Each section was imaged with the following criteria: Bin 2X2, z-stack 1uicron stepwise, 20x magnification. At least five sequential images were taken in the z-axis for deconvolution analysis. All images were compiled using Imaris Bitplane Software.

Reverse transcription quantitative real-time PCR. RNA from peritoneal cells were extracted in Trizol (Invitrogen), treated with RQ1 DNase (Promega), and total RNA was reverse transcribed using oligo$(dT)_{15}$ primer (Promega) and SuperScriptII (Invitrogen) at 42ºC for 50 minutes. cDNAs were analyzed using ABI7300 Real Time PCR System. Primers for RAE-1 and HPRT are described in Table S2.1.

Results

Previous studies have shown that in vivo infection with MCMV results in infection of various cell types. Within the first 24 hours post-infection, MCMV infects subcapsular sinus macrophages in the lymph node and reticular fibroblasts in the spleen and hepatocytes (Hsu et al. 2009). By 72 hours, many cell types are infected in the spleen including dendritic cells, granulocytes, NK cells, macrophages, and a small population of T cells, and B cells (Benedict et al. 2006). Systemic infection upon intravenous infection leads to infection of the lung, heart, kidney, liver, and spleen (Sacher et al. 2012). Studies to date have used the GFP-reporter virus to determine various cell types that are infected in vivo, but characterization of the interaction between infected cells and immune cells in vivo is limited.

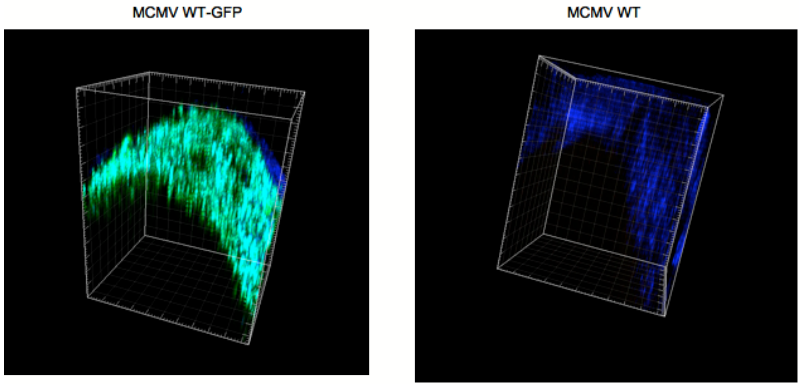
MCMV encodes multiple viral evasins of NKG2D ligand expression (Jonjic et al. 2008). The lack of viral evasins negatively impacts viral fitness in a NKG2D-dependent manner (Lodoen et al. 2003, Hasan et al. 2005, Krmpotic et al. 2005). These studies have implied that infection with viruses lacking viral evasins results in the expression of NKG2D ligands in vivo and render them susceptible to NK cell lysis. Whether this is the case or not has not been shown and many open questions remain in the field. Some of these questions include 1) are NKG2D ligands ever expressed upon infection by WT MCMV or are viral evasins sufficient to completely block ligand expression? 2) in the absence of viral evasins, are NKG2D ligands induced only on infected cells or do neighboring cells also express NKG2D ligands? 3) does expression of NKG2D ligands render cells susceptible to NK-cell lysis by direct interaction? Thus, characterization of MCMV-infected cells and its interaction with NK cells in vivo is much needed in the field. Two-photon microscopy is a powerful technique that allows for live-imaging of cells in an intact lymph node and the dynamic interactions between fluorescently-labeled cells (Miller et al. 2002, Bousso, Robey 2003). Here, we performed preliminary studies on MCMV-infected lymph nodes to determine whether we would be able to image MCMV-infected cells by two-photon microscopy using a GFP-reporter virus and determined whether NKG2D ligands can be detected by fluorescent imaging.

The Robey lab at UC Berkeley has utilized two-photon microscopy to visualize cellular dynamics of *Toxoplasma gondii*-infected lymph nodes (Chtanova et al. 2008). In their system, parasites are injected subcutaneously in the earflap of mice, which leads to synchronous infection of cells in the draining lymph node. In collaboration with Janine Coombes and Seong-ji Han in the Robey lab, we infected C57BL/6 mice with a GFP-reporter MCMV in a similar fashion. At 24 hours post-infection, the draining dorsal cervical lymph node was harvested and analyzed by two-photon microscopy. MCMV-infected cells were readily detected as seen by still images of the lymph node (Appendix 1.1A and B). At this time point, MCMV-infected cells are most abundant near the capsule of the lymph node. As a control, lymph node from a mouse infected with a non-GFP version of MCMV was monitored for GFP to determine background auto-fluorescence in this channel. Because MCMV-infected cells can be visualized by two-photon microscopy, this technique has the potential to allow for visualization of interactions between infected cells and other immune cells in the lymphoid organ.

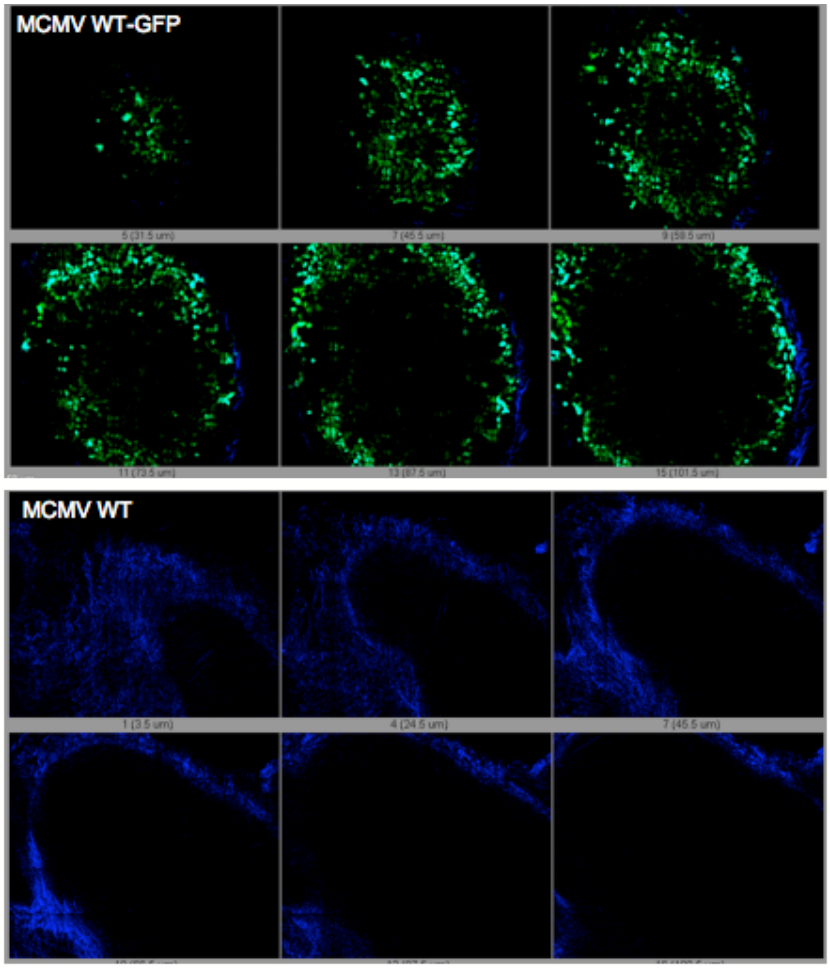
Next, we determined whether in vivo MCMV infection results in the expression of RAE-1 proteins. Similar to the data obtained using two-photon microscopy, cells infected with WTGFP virus were detected near the capsule of the lymph node when sections of the lymph node were analyzed using a fluorescent microscope (Appendix 1.2A and 1.3). MCMV encodes viral evasins m152 and m138 that downregulate cell surface expression of all isoforms of RAE-1 and RAE-1ε, respectively (Lodoen et al. 2003, Arapovic et al. 2009a). Thus, cells infected with WT virus that encodes viral evasins do not express RAE-1 at the cell surface in vitro. Accordingly, sections of lymph nodes obtained from mice infected with WT MCMV and stained with either a RAE-1ε or a panRAE-1 antibody showed minimal expression of RAE-1, supporting previous in vitro findings (Appendix 1.2 and 1.3). Furthermore, RAE-1 expression was detectable using both RAE-1ε and panRAE-1 antibody when mice were infected with MCMVΔ152. The specificity was confirmed by using an isotype antibody as a control. Further experiments are necessary to determine whether RAE-1 expression in the lymph nodes is restricted to cells that are infected or whether non-infected neighboring cells can also induce RAE-1 expression. We also observed a dramatic loss of subcapsular macrophages in sections obtained from mice infected with MCMV compared to uninfected lymph node sections, as determined by a loss in CD169 staining (Appendix 1.4). This implies that these cells are susceptible to infection by MCMV and that infection and activation of the immune response cause destruction of macrophages. This is in accordance with a previous study showing disruption of the splenic architecture upon MCMV infection (Benedict et al. 2006). Future studies should confirm these results and determine what cell types are infected, which cells express RAE-1, and whether effectors that are important for RAE-1 induction *in vitro* are also involved *in vivo*.

MCMV can efficiently infect primary peritoneal macrophages ex vivo, and induction of the RAE-1 transcript is readily detectable by RT-qPCR (Chapter 4). Thus, we asked whether MCMV infection of peritoneal resident cells in vivo could also induce expression of RAE-1. In collaboration with Andrea Pezda from our lab, mice were infected with MCMV intraperitoneally, total peritoneal-resident cells were harvested via peritoneal lavage, and RAE-1 expression in these cells were quantified by RT-qPCR at 72 hours post-infection. In the same experiment, separate mice were infected with a mouse gamma herpesvirus, MHV-68, or injected with a TLR ligand, CpG. In vivo MCMV infection of peritoneal cells resulted in a robust induction of RAE-1 at the mRNA level, and surprisingly, infection with MHV-68, which has not been shown to induce RAE-1, also resulted in RAE-1 induction (Appendix 1.5). Because cells extracted via peritoneal lavage is composed of heterogeneous mixture of cell types, the experiment does not reveal which cells are inducing RAE-1. However, it is an effective in vivo assay for detection of RAE-1 expression and should facilitate future studies on virus-mediated induction of RAE-1 *in vivo*.

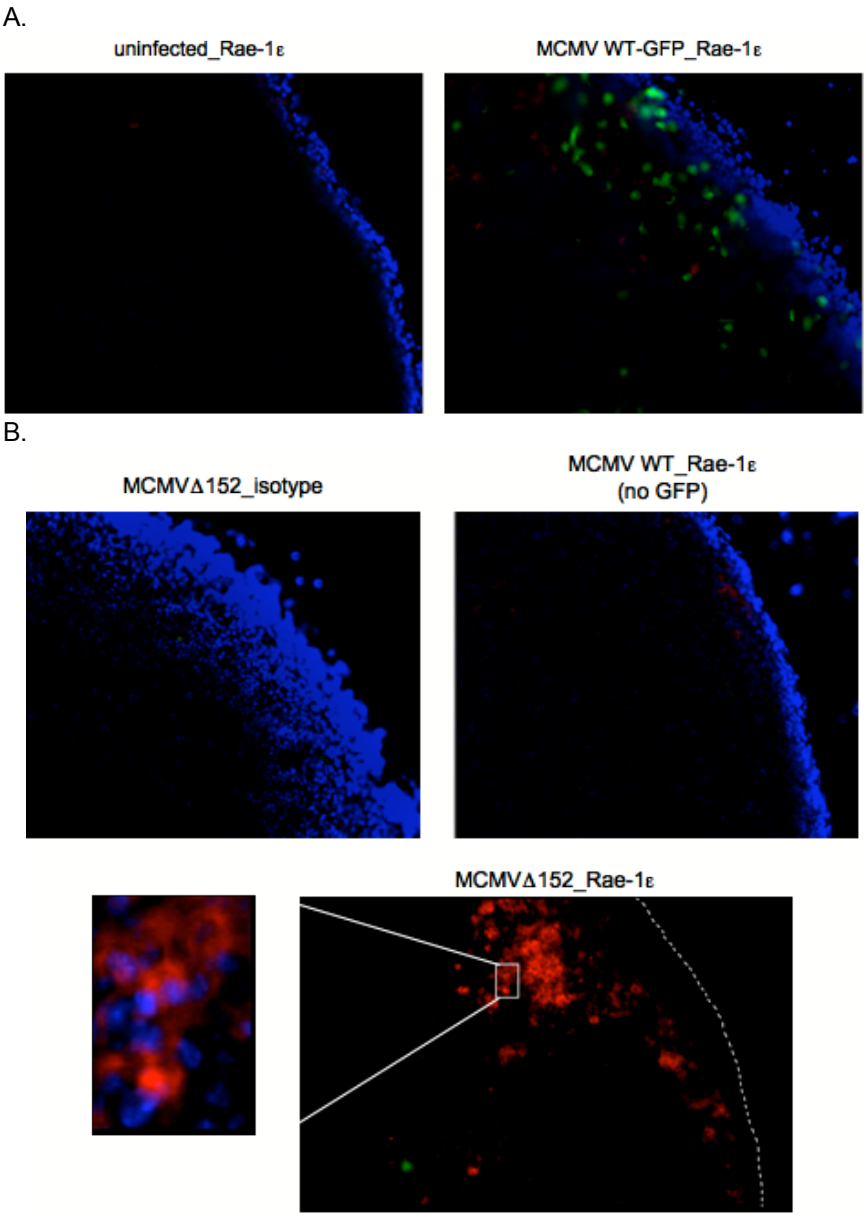
A.



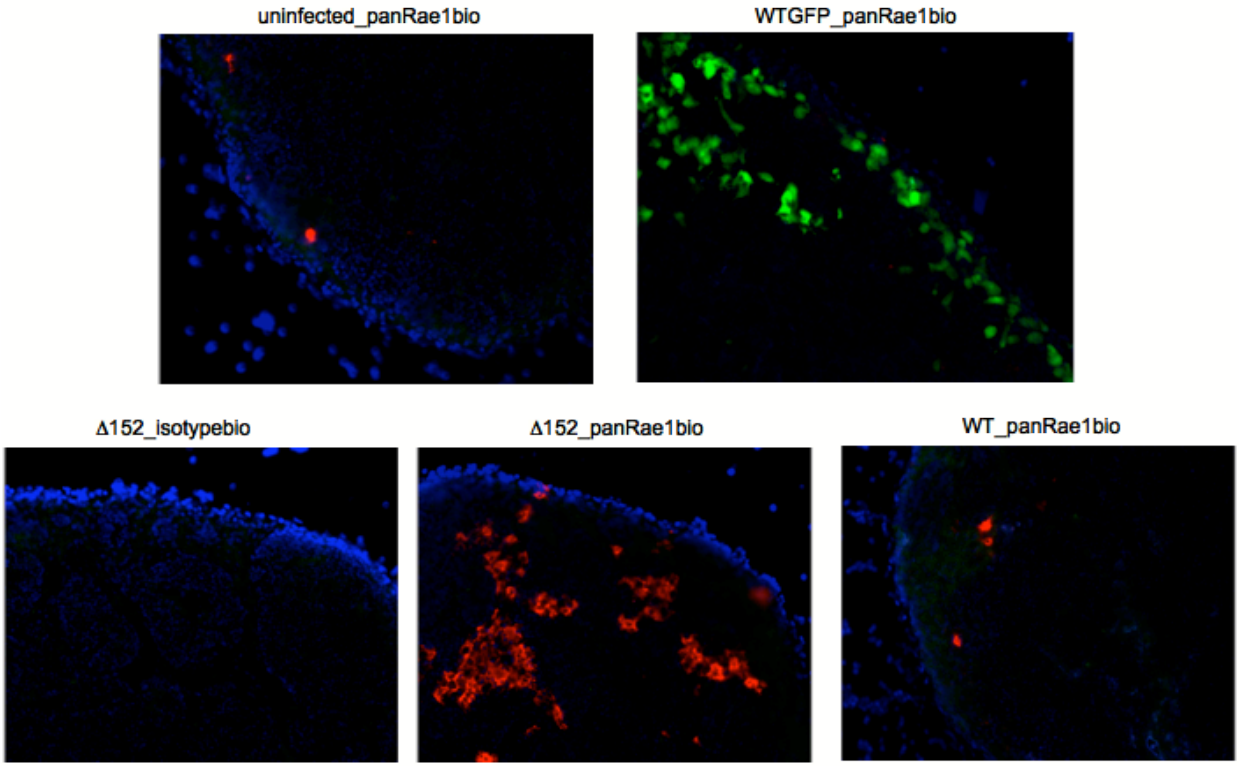
B.



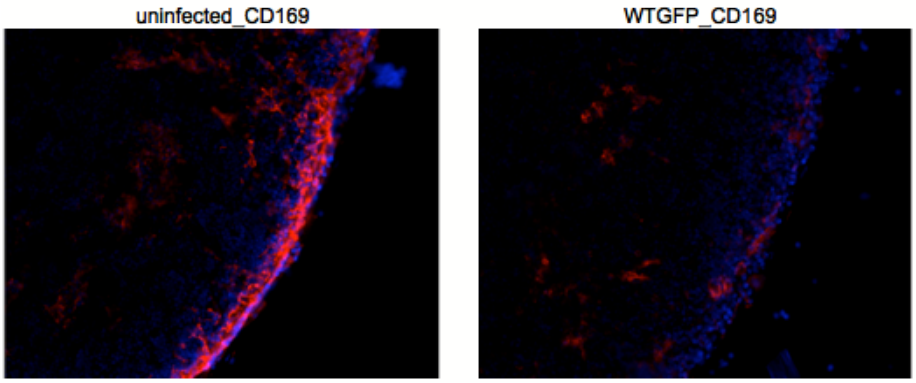
Appendix 1.1. Two-photon imaging of MCMV-infected lymph node. A) C57BL/6 mouse was infected in the earflap with 2.5X10^6 pfu of MCMV and the dorsal cervical draining lymph node (LN) was harvested at 24 hour post-infection for analysis by two-photon imaging. A) Three-dimensional image of the LN, a side view. B) Two-dimensional image of the LN from the top and progressively moving deeper into the center of the LN (left to right). Images show cells infected with WT-GFP virus (green) and the LN capsule detected by second harmonic signal (blue).



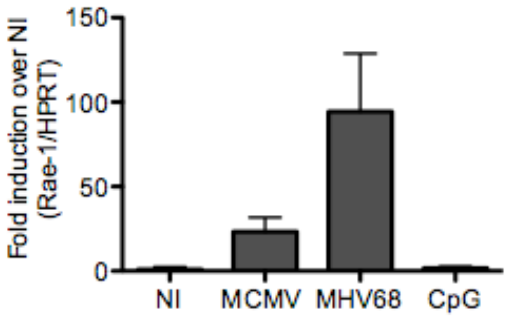
Appendix 1.2. MCMV-infected lymph nodes express RAE-1ε. A C57BL/6 mouse was infected with MCMV WT-GFP (A), MCMV WT (B), or MCMVΔ152 (B), and dorsal cervical draining lymph node was harvested at 24 hours post-infection. 20um cryosections of the lymph node were stained with either an isotype or RAE-1ε-specific antibody. A) A representative section showing WT-GFP infected cells (green) and trace amounts of RAE-1e staining (red). B) Representative sections showing RAE-1ε-expressing cells (red). In all images, DAPI staining is represented in blue or dotted line.



Appendix 1.3. MCMV-infected lymph nodes express RAE-1. A C57BL/6 mouse was infected with MCMV WT-GFP, MCMV WT, or MCMVΔ152, and dorsal cervical draining lymph node was harvested at 24 hours post-infection. Cryosections of the lymph node were left unstained, or stained with either an isotype or panRAE-1-specific antibody. Images show cells infected with WT-GFP (green), cells expressing RAE-1 (red) and DAPI-stained nuclei (blue).



Appendix 1.4. CD169-expressing macrophages are lost in MCMV-infected lymph nodes. A C57BL/6 mouse was infected with MCMV WT-GFP or left uninfected, and dorsal cervical draining lymph node was harvested at 24 hours post-infection. Cryosections of the lymph node were stained for CD169. Images show CD169+ macrophages (red) and DAPI-staining nuclei (blue). The GFP channel for these images was turned off.



Appendix 1.5. Peritoneal cells induce RAE-1 expression upon in vivo infection with MCMV or MHV-68. C57BL/6 mice were infected intraperitoneally with MCMV, MHV68 or injected with CpG. Peritoneal-resident cells were harvested at 72 hours post-infection by peritoneal lavage. RNA was extracted from the cells and reverse transcribed for quantification of RAE-1 mRNA level by RT-qPCR. The data represents combined data from five mice per viral infection and three uninfected and three CpG-injected mice. RAE-1 expression was normalized to expression of HPRT and graphed as fold induction over uninfected (NI).

Appendix Section 2: MCMV encodes a viral protein that preferentially downregulates RAE-1ε

Materials and Methods

Cells. Tail fibroblasts were derived from a tail of a C57BL/6 mouse from Genghong Cheng's lab (UCLA) as previously described (Gasser et al. 2005). Briefly, cells were obtained from a single tail by incubating the tail with Pronase and Collagenase IV (Sigma) in the presence of Amphotericin B for two hours at 37°C. Cells were maintained in culture until post-crisis (~30 days) and continued to be cultured. Cells were maintained in DMEM with 5% FBS and 1% penicillin and streptomycin.

Viruses, propagation, and infection. MCMVΔ6 lacking genes m145 through m158, originally generated by Dr. Koszinowski (Max von Pettenkofer-Institute, Munich, Germany) was generously provided by Dr. Hill (Oregon Health and Science University, Oregon). MCMVΔ3 lacking m04+m06+m152 and MCMV WT (Smith strain) viruses were generously provided by Dr. Koszinowski (Max von Pettenkofer-Institute, Munich, Germany). The mutant MCMV lacking genes m01-22 (Δ1-22), m128-139 (Δ128-139), m01-17 and m144-158 (Δ1-17+144-158), m128-133 (Δ128-133) were kind gifts from Dr. Hidde Ploegh (Whitehead Institute, MIT, MA). MCMV lacking m138 (Δ138) was provided by Dr. Fenyong Liu (UC Berkeley, CA) as a collaboration. All viruses were propagated in NIH 3T3 cells and titered in BALB/c 3T3 cells. For all infection experiments, fibroblasts were infected at MOI of 5, input virus removed at 2 hrs post-infection, and infection was allowed to take place for a total of 24 hrs.

Flow cytometry. Fibroblasts were harvested in 2mM EDTA PBS and stained with monoclonal anti-mouse pan-specific RAE-1, RAE-1α/β/γ, RAE-1β/δ, RAE-1ε, or Rat IgG_{2A} isotype control (all purchased from R&D) followed by PE-conjugated goat anti-rat IgG (Southern Biotech). All samples were co-stained with 7-AAD (BD).

Constructs. All of the constructs that were used in this section were cloned by Brian Sullivan in our lab. The constructs for m138 and HA-tagged m138 were made in pIRES2GFP. The RAE-1ε/δ chimeras were cloned into pBMN.IN. All of the primers used for cloning and amplification of m138 are listed in Appendix Table 2.1.

Results

To date, MCMV has been shown to encode four viral evasins that specifically block cell surface expression of NKG2D ligands: m138 (RAE-1, MULT-1, and H60), m145 (MULT-1), m152 (RAE-1), and m155 (H60) (Lodoen et al. 2003, Hasan et al. 2005, Krmpotic et al. 2005, Arapovic et al. 2009a, Lenac et al. 2006). In vitro studies have determined that m138 downregulates RAE-1 surface expression by dynamin-dependent endocytosis and degradation, MULT-1 by lysosomal degradation, and H60 by a mechanism that is yet defined (Arapovic et al. 2009a, Lenac et al. 2006). The viral protein m145 prevents MULT-1 surface expression by preventing maturation of the protein past the ER/Golgi compartment (Krmpotic et al. 2005). The viral protein m152 is capable of directly interacting with RAE-1, and similar to its effect on MHC-I, m152 seems to block RAE-1 in the ER/cis-Golgi compartment and prevent further maturation (Arapovic et al. 2009a, Ziegler et al. 1997, Zhi et al. 2010). The viral protein m152

was originally discovered to downmodulate surface expression of all RAE-1 isoforms equally (Lodoen et al. 2003).

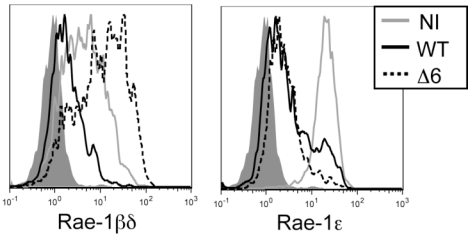
While the data presented in this section was being generated, the Jonjic group published the paper showing that m138 is a viral evasin for RAE-1ε isoform in addition to its effect on MULT-1 and H60, and another paper showing that RAE-1δ isoform is resistant to downmodulation by MCMV (Arapovic et al. 2009a, Arapovic et al. 2009b). Despite some overlaps in our studies, some of our results contradict Jonjic's published data, and further studies are necessary to clarify these differences.

In our system, fibroblasts were derived from tail of C57BL/6 mice and maintained in culture until post-crisis. These established tail fibroblasts constitutively express high levels of RAE-1δ and RAE-1ε, but not RAE-1αβγ (Appendix 2.1 and data not shown). As expected, both RAE-1δ and ε isoforms were downregulated when cells were infected with WT MCMV (Appendix 2.1). Additionally, in support of previous findings, RAE-1δ downregulation was prevented upon infection with a mutant MCMV lacking genes m145-158 (MCMVΔ6), within which m152 is contained (Appendix 2.1, and Lodoen et al. 2003). In contrast, RAE-1ε isoform was downregulated to the same extent as infection with WT MCMV. The data suggested that in addition to m152, another viral protein was capable of downmodulating the RAE-1ε isoform, specifically. In order to determine the region within MCMV that is responsible for downregulation of RAE-1ε, a series of mutant MCMV that lack various non-essential genes were tested for its ability to downregulate RAE-1ε. In most samples, RAE-1ε was downregulated to a similar extent as infection with WT virus by 24 hours post-infection (Appendix 2.2). However, infection with MCMV lacking genes m128 through m139 (Δ128-139) showed a severe defect in this downmodulation. Because infection by MCMV lacking genes m128 to m133 did not show this defect, the data suggested that a viral element within m133 to m139 was responsible for downregulation of RAE-1ε. In two of the samples, WT and Δ128-133, two peaks in RAE-1ε expression were observed. This is most likely because efficiency of infection was not 100%.

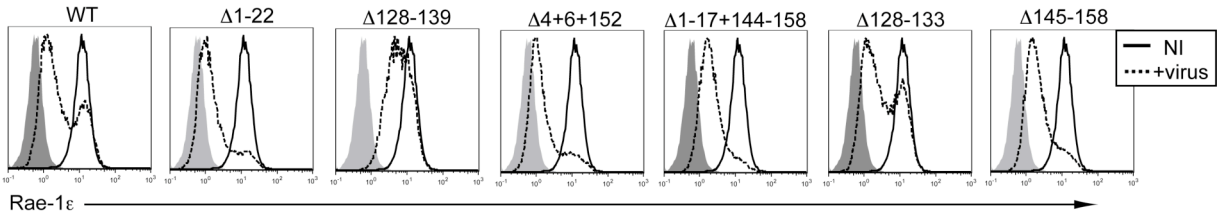
Because Jonjic's group showed that m138, which is contained between m133 and 139, can specifically downregulate expression of RAE-1ε, we transfected fibroblasts with either m138 or HA-tagged m138 plasmids containing a GFP gene under the control of an IRES. Both RAE-1δ and RAE-1ε expression were determined in the same cells at 24 hours post-transfection. In support of results published by Jonjic's group, the RAE-1ε isoform was susceptible to downregulation by m138, but not the RAE-1δ isoform (Appendix 2.3). Furthermore, the downregulation occurred specifically in GFP-positive cells indicating that RAE-1ε expression is only affected in cells that express m138. To further confirm this phenotype, MCMV with a deletion in m138 (MCMVΔ138) was obtained from Fenyoung Liu's lab at UC Berkeley as a collaboration. The absence of m138 in the viral genomic DNA was confirmed by PCR using primers that amplified m138 (Appendix 2.4A and Appendix Table2.1). Surprisingly, when fibroblasts were infected with MCMVΔ138, RAE-1ε expression was downregulated to the same extent as MCMV lacking genes m145 through m155 (MCMVΔ6), which cannot downregulate RAE-1δ (Appendix 2.4B). As expected, MCMVΔ138 downregulated RAE-1δ because m152 is present in this virus. The discrepancy between our data on MCMVΔ138 and Jonjic's data on MCMVΔ138 should be reconciled in future studies.

The RAE-1 family of proteins are highly similar in sequences (Raulet 2003). There are several amino acids that differ between the isoforms, but the biggest difference is in the PLWY motif, which is present in all of the isoforms except for the RAE-1δ isoform. Jonjic's group

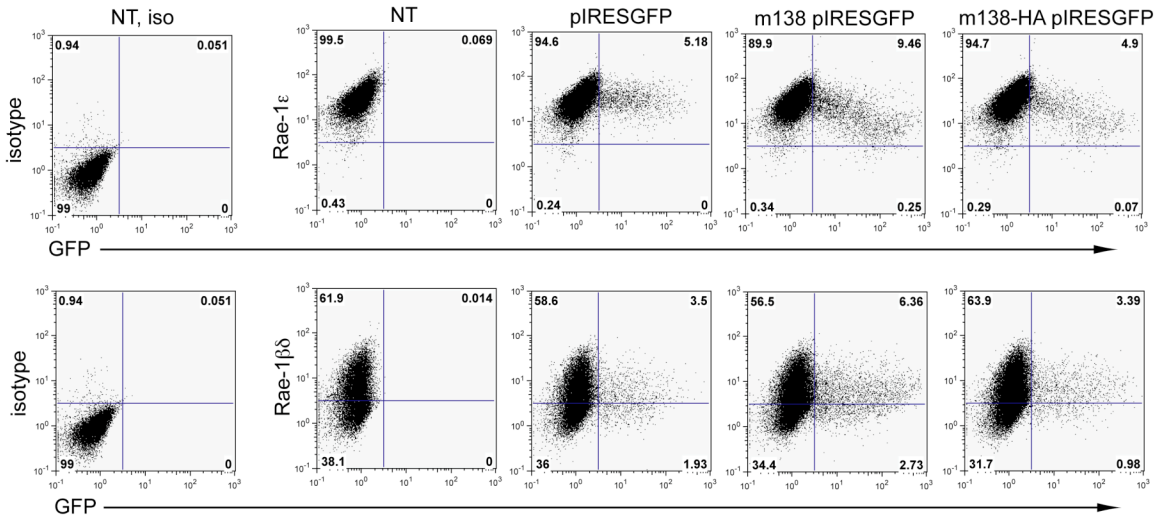
showed that the lack of this motif makes RAE-1d resistant to downregulation by MCMV, and susceptibility can be rescued if this motif is inserted into the RAE-1δ sequence (Arapovic et al. 2009b). In order to determine whether differential susceptibility of RAE-1ε to MCMV in our system can be attributed to differences between the RAE-1δ and RAE-1ε sequences, RAE-1ε chimeras were generated in collaboration with Brian Sullivan from our lab. The chimeras contained amino acid changes at various positions within the RAE-1ε protein sequence that correspond to the sequence from RAE-1δ (Appendix 2.5). In total, nine chimeras were generated, but only eight chimeras have been tested so far. To observe expression of the chimeric molecules, a different set of established tail fibroblasts, which do not express any RAE-1 at the basal level and used in Chapters 2 through 4 of this thesis, were used. Stable expression of all eight chimeras, except for RAE-1ε E99K, was detectable by the RAE-1ε antibody (Appendix 2.6). RAE-1ε E99K could not be detected by the RAE-1ε antibody but was detectable with a panRAE-1 antibody, suggesting that this amino acid is part of an epitope that is recognized by the RAE-1ε antibody. Finally, all of the chimeras were susceptible to downregulation by WT MCMV as expected. The effect of infection by MCMV lacking m145 to m155 (Δ6) was also determined, but we did not observe any significant rescue in RAE-1ε expression by any of the amino acid changes that were tested (data not shown). There are several other positions within RAE-1ε that could be changed to the corresponding RAE-1δ sequence (Appendix 2.5). Future studies may benefit from creating additional RAE-1ε chimeras at these sites to determine how RAE-1δ and RAE-1ε are differentially regulated by MCMV.



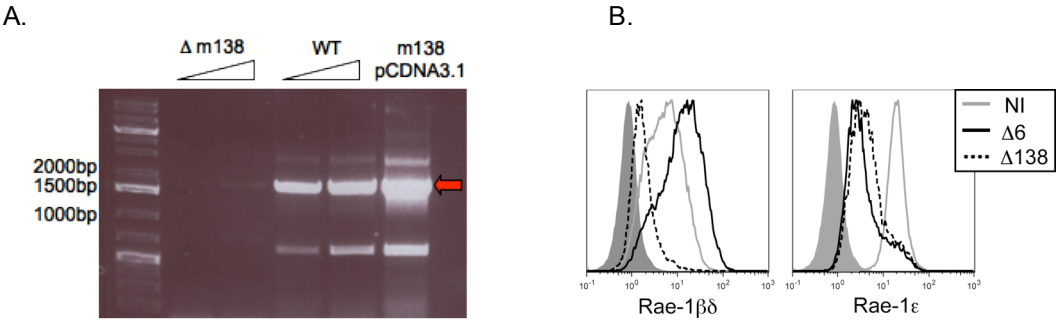
Appendix 2.1. RAE-1δ and ε are differentially regulated by MCMV. Fibroblasts that constitutively express RAE-1δ and ε were infected with either MCMV WT (solid black line) or MCMVΔ145-158 (Δ6, dashed black line) or left uninfected (NI, solid grey line). At 24 hours post-infection, RAE-1 surface expression was determined by staining with RAE-1βδ or RAE-1ε-specific antibody or isotype antibody (shaded grey). All samples are gated on 7AAD-negative live cells.



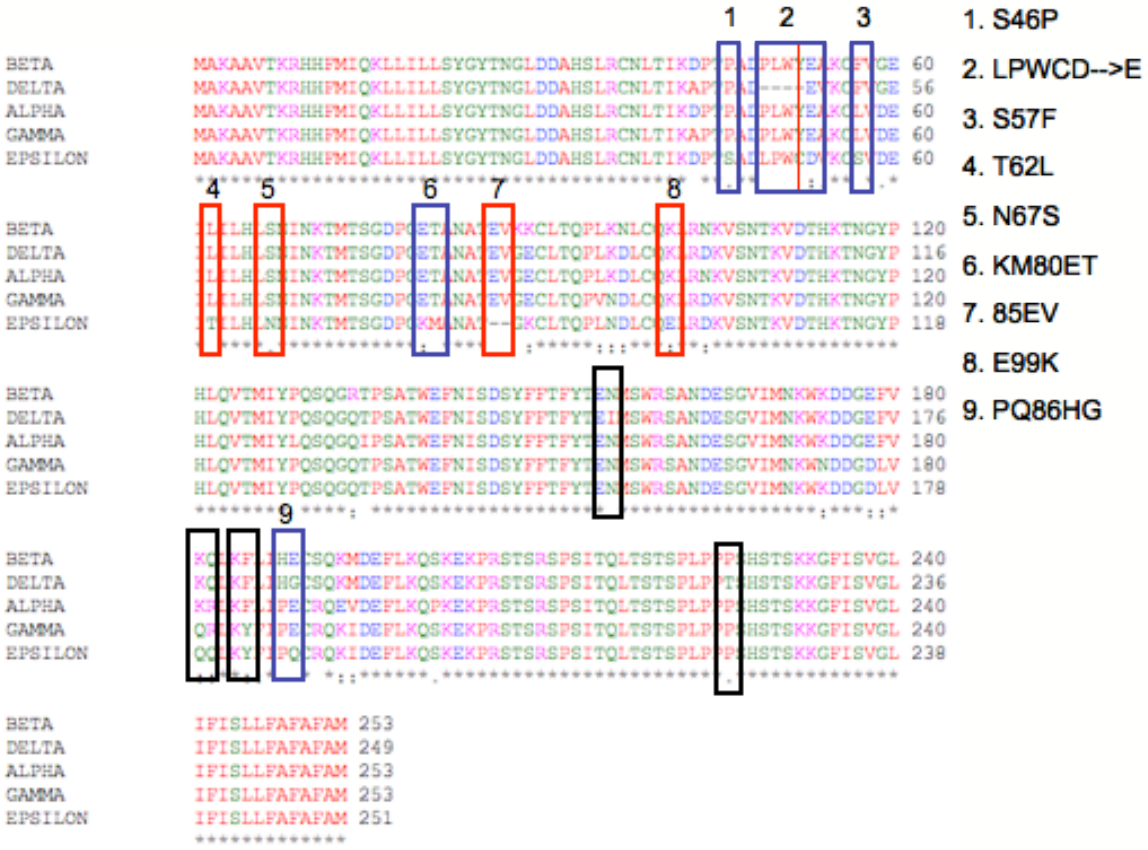
Appendix 2.2. A gene between m133 and m139 is responsible for downregulating RAE-1ε. Fibroblasts that constitutively express RAE-1δ and ε were infected with either MCMV WT (solid black line) or MCMVΔ145-158 (Δ6, dashed black line) or left uninfected (NI, solid grey line). At 24 hours post-infection, RAE-1 surface expression was determined by staining with RAE-1βδ or RAE-1ε-specific antibody or isotype antibody (shaded grey). All samples are gated on 7AAD-negative live cells.



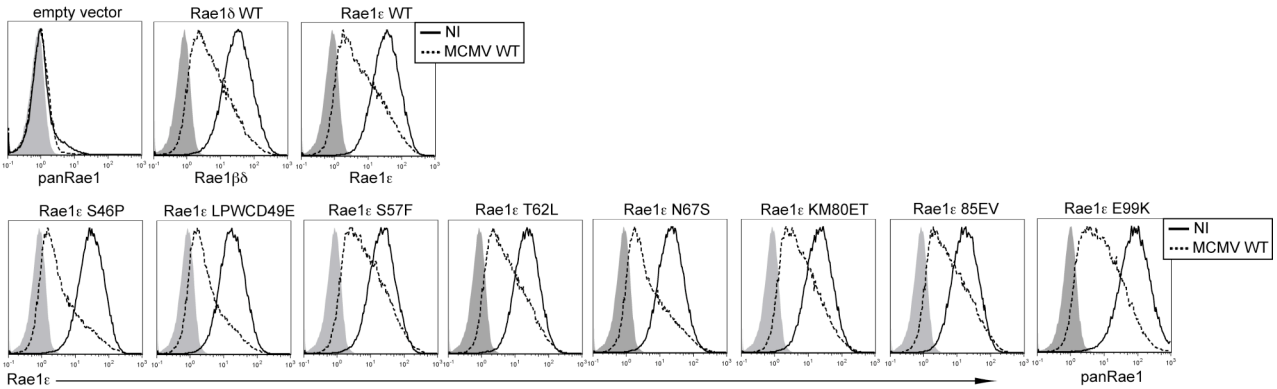
Appendix 2.3. MCMV protein encoded by the *m138* gene can downregulate RAE-1ε but not RAE-1δ. Fibroblasts that constitutively express RAE-1δ and ε were transiently transfected with empty vector (pIRESGFP), m138 pIRESGFP, HA-tagged m138 (m138-HA pIRESGFP) or left untransfected. At 24 hours post-infection, RAE-1 surface expression was determined by staining with antibodies specific for RAE-1βδ or RAE-1ε, or an isotype antibody, as indicated. All samples are gated on 7AAD-negative live cells.



Appendix 2.4. MCMVΔ138 is not capable of rescuing RAE-1ε downregulation. A) Viral DNA was extracted from MCMVΔ138 or MCMV WT, and m138 was amplified by PCR using m138-specific primers. As a positive control, m138-containing plasmid (m138 pCDNA3.1) was also amplified. The expected size of m138 is 1700bp (red arrow). B) Fibroblasts that constitutively express RAE-1δ and ε were infected with MCMVΔ145-158 (Δ6, solid black line), MCMVΔ138 (dashed black line) or left uninfected (NI, solid grey line). At 24 hours post-infection, cells were harvested and stained with isotype antibody or antibodies against RAE-1βδ or RAE-1ε. All samples are gated on 7AAD-negative live cells.



Appendix 2.5. An alignment of RAE-1α through ε isoforms indicating RAE-1ε/δ chimeras that were generated. The protein sequences of RAE-1α through ε isoforms were aligned using ClustalW2 (EMBL-EBI). Boxed regions indicate differences between RAE-1ε and δ sequences. The numbers above the boxes represent RAE-1ε to δ chimeras that were generated and specific amino acid changes are indicated on right hand side.



Appendix 2.6. All of the RAE-1ε/δ chimeras tested are expressed and can be downregulated by WT MCMV. Established tail fibroblasts were transduced with the indicated chimeric constructs or empty vector (PMBN.IN). After neomycin selection for stable transductants, cells were infected with WT MCMV (dashed black line) at MOI of 5 for 24 hours or left uninfected (solid black line) and stained with RAE-1βδ, RAE-1ε, or panRAE-1 antibody. Shaded grey histogram represents isotype staining. All samples were co-stained with 7AAD, and the histograms show live cells that are 7AAD negative.

Appendix Table 2.1.

Rae-1δ	**F:** atcgACGCGTctagatgatgcacactctcttaggtgcaac **R:** atcgCCGCGGtcacattgcaaatgcaaatgcaaataataaagatatgaagatg
Rae-1ε	**F:** atcgACGCGTctggatgatgcacactctcttaggtgc **R:** atcgCCGCGGtcacattgcaaatgcaaatgcaaataataaagatatgaagatg
Rae-1ε S46P	**F:** ggatcctaccCcagcagaccttccctggtgtg **R:** ggtctgctgGggtaggatccttgatggtcaagttgcac
Rae-1ε LPWCD49E	**F:** cagcagacGAAgtgaagtgctcagtggatgaaataactatcctcc **R:** ctgagcacttcacTTCgtctgctgaggtaggatccttgatgg
Rae-1ε S57F	**F:** gaagtgctTCgtggatgaaataactatcctccatttaaataacataaacaagacc **R:** ggaggatagttatttcatccacGAagcacttcacgtcacaccaggg
Rae-1ε T62L	**F:** cagtggatgaaataCTtatcctccatttaaataacataaacaagaccatgacttcag **R:** cttgtttatgttatttaaatggaggataAGtatttcatccactgagcacttcacgtcac
Rae-1ε N67S	**F:** ggatgaaataactatcctccatttaaGtaacataaacaagaccatgacttcaggtgacc **R:** ggtcttgtttatgttaCttaaatggaggatagttatttcatccactgagcac
Rae-1ε KM80ET	**F:** ccagggGAGACAgcaaatgccactggcaaatgtttgac **R:** ggcatttgcTGTCTCccctgggtcacctgaagtcatg
Rae-1ε 85EV	**F:** gcaaatgccactGAAGTGggcaaatgtttgacacaacctctgaacg **R:** gccCACTTCagtggcatttgccatcttccctg
Rae-1ε E99K	**F:** cgatttgtgccagAagttgagggacaaggtgtctaacacc **R:** gtccctcaactTctggcacaaatcgttcagaggttgtg
Rae-1ε PQ86HG	**F:** gaaatacttcatacACGGatgcagacagaaaattgatgaattcttaaagcagtcc **R:** atcgCCGCGGtcacatcgcaaatgcaaatgcaaataataaagatatgaagatg
M138	**F:** atcgGGATCCgccgccaccATGgcgccttcgacgctg **R:** atcgGGATCCttacgtgtgacgtacgcaacccg
HA-m138	**F1:**gctTACCCATACGATGTTCCAGATTACGCTAGCCTCcatcaattacct gcgtgccagtgg **F2:**gatcctagctcttgttggagctgcagttgctTACCCATACGATGTTCCAGATTA CGC **F3:** atcgGGATCCgccgccaccatgtctgcacttctgatcctagctcttgttggagc **R:** atcgGGATCCttacgtgtgacgtacgcaacccg

F: forward primer sequence
R: reverse primer sequence