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Manipulation of NKG2D ligands by cytomegaloviruses: impact on innate and adaptive immune response

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NKG2D is a potent activating receptor expressed on NK cells, NKT cells, γδ T cells, and CD8 T cells. NKG2D recognizes cell surface molecules structurally related to MHC class I proteins induced by infection or other type of cellular stress. The engagement of NKG2D leads to NK cell cytotoxicity and cytokine secretion or to a co-stimulation of CD8 T cells. Both human and mouse cytomegalovirus (CMV) have evolved numerous mechanisms to evade NKG2D-mediated immune response. This review describes the mechanisms used by CMV to inhibit NKG2D ligand expression and the recent advances in exploiting the NKG2D recognition pathway for mounting efficient and long-lasting immune response.

Keywords: NKG2D, cytomegalovirus, immune evasion

NK cells are lymphocytes of innate immunity which target virally infected and tumor cells. Their activity is determined by a delicate balance of signals from a sophisticated repertoire of inhibitory and activating receptors expressed on their cell surface (reviewed in Diefenbach and Raulet, 2001; Lanier, 2005). Inhibitory receptors gauge the expression of MHC class I molecules, which are constitutively expressed by healthy cells but may be downregulated under conditions of cellular stress. Therefore, inhibitory receptors provide a mean for NK cells to exhibit cytotoxicity toward infected or transformed cells via the "missing-self" axis. In contrast, "induced-self" recognition involves the engagement of various activating NK cell receptors by proteins expressed under cellular stress, thus allowing the immune response to proceed. One of the most potent activating receptors which mediate the "induced-self" axis is NKG2D, a receptor shared by both the cells of innate and adaptive immunity.

NKG2D RECEPTOR

NKG2D is a type-2 transmembrane glycoprotein expressed as a disulfide linked homodimer on the cell surface (Diefenbach et al., 2002; Jamieson et al., 2002). It is encoded by *KLRK1* (killer cell lectin-like receptor subfamily K, member 1), a single gene with little polymorphism located on the mouse chromosome 6 and in the syntenic position on human chromosome 12 (Houchins et al., 1991). In mice, alternative RNA splicing results in two NKG2D isoforms – long (NKG2D-L) and short (NKG2D-S) – that differ in 13 amino acids (Diefenbach et al., 2002; Gilfillan et al., 2002). Resting mouse NK cells express very little NKG2D-S, but its expression is induced after NK cell activation. Neither isoform can be detected in resting mouse CD8 T cells but after T cell receptor (TCR) stimulation the expression of both isoforms is upregulated. Within its intracellular domain NKG2D has no signaling motif but it associates with signal-transducing proteins through charged residues in the transmembrane region. The NKG2D-L isoform pairs with the DAP10 signaling molecule, while NKG2D-S associates with either DAP10 or DAP12. However, because CD8 T cells do not express DAP12, the two NKG2D isoforms that are expressed by activated T cells can interact with DAP10 only, whereas activated NK cells can transmit signals through both DAP10 and DAP12 (**Figure 1**). The only isoform expressed in humans corresponds to the long form in mouse and it interacts with DAP10 (Bauer et al., 1999; Wu et al., 1999; Rosen et al., 2004).

NKG2D is expressed by all NK cells, most NKT cells, a subset of γδ T cells, all human CD8 T cells, activated mouse CD8 T cells and a subset of CD4 T cells (Bauer et al., 1999; Diefenbach et al., 2000, 2002; Girardi et al., 2001; Jamieson et al., 2002; Ehrlich et al., 2005). On NK cells NKG2D serves as a primary activating receptor, meaning that the engagement of NKG2D can override inhibitory signals in the absence of the "missing-self" recognition (Cerwenka et al., 2001; **Figure 1**). In addition to cytotoxicity, activation of NK cells via NKG2D triggers the production of different cytokines, including IFN-γ, TNF-α, and MIP-1β. Via this mechanism NK cells are involved in the regulation of adaptive immunity. The engagement of the NKG2D receptor on CD8 T cells is insufficient to generate a T cell response (Bauer et al., 1999; Ehrlich et al., 2005). Instead, NKG2D acts as a co-stimulatory receptor which augments TCR-induced responses (Groh et al., 2001; Maasho et al., 2005; Markiewicz et al., 2005). The ability of NKG2D to co-stimulate T cells may be determined by additional factors, such as the activation state of the T cells or the cellular environment (Roberts et al., 2001; Meresse et al., 2004; Verneris et al., 2004).

NKG2D LIGANDS

NKG2D ligands are distantly related homologs of the MHC-I proteins and are characterized by a striking structural diversity, different expression patterns, and regulation mechanisms. Human NKG2D ligands are MHC class-I-related protein A (MICA), MICB, and UL16-binding proteins (ULBP1-6). MICA and MICB,

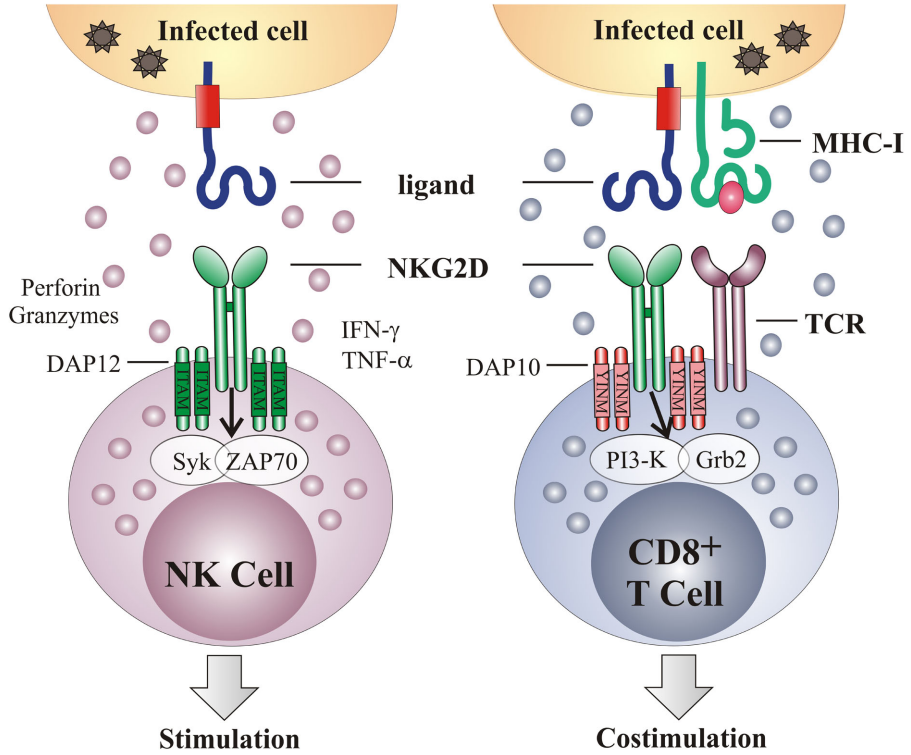

FIGURE 1 | NKG2D acts as an activating and a co-stimulatory receptor. NKG2D is a type-2 transmembrane homodimer that signals via association with adapter molecules DAP10 or DAP12. Association with DAP12 leads to phosphorylation of the ITAM and triggering of the Syk and/or Zap70 signaling cascade. Association with DAP10 results in tyrosine phosphorylation on the YINM motif and recruitment of the PI3K and Grb2 cascade. On NK cells, NKG2D serves as an activation receptor, such that NKG2D engagement is sufficient to trigger NK cell-mediated cytotoxicity and cytokine production. In contrast, NKG2D acts as a co-stimulatory receptor on CD8 T cells, requiring TCR-mediated signaling for their full activation. Grb2, growth factor receptor-bound protein 2; IFN-γ, interferon γ; ITAM, immunoreceptor tyrosine-based activating motif; PI3K, phosphoinositide 3-kinase; Syk, spleen tyrosine kinase; TCR, T cell receptor; TNF-α, tumor necrosis factor α; Zap70, zeta-chain associated protein kinase 70.

encoded by the genes within human MHC (Groh et al., 1996; Bauer et al., 1999), are the only NKG2D ligands containing three immunoglobulin-like domains (α1, α2, and α3), but unlike MHC molecules, they do not associate with β2 microglobulin nor bind antigenic peptides. All other NKG2D ligands are related to MHC-I molecules but contain only α1 and α2 domains. Although named by their ability to bind human CMV (HCMV) protein UL16, only the first two identified ULBP proteins ULBP1, ULBP2, and the subsequently described ULBP6 bind this viral protein (Cosman et al., 2001; Radosavljevic et al., 2002; Bacon et al., 2004; Eagle et al., 2009). Like MIC proteins, ULBP5 and ULBP6 are transmembrane proteins, while proteins ULBP1-3 are anchored to the membrane via glycosylphosphatidylinositol (GPI; Eleme et al., 2004). ULBP family is also known as the retinoic acid early transcript 1 (RAET-1) family, since they show sequence homology to the mouse retinoic acid early 1 (RAE-1) proteins (Nomura et al., 1994; Cerwenka et al., 2000; Diefenbach et al., 2001). Five members of the RAE-1 family (RAE-1α–ε) are highly related to each other (>85% amino acid homology) and differentially expressed in various mouse strains. A second family of mouse NKG2D ligands is the H60 family that comprises three members H60 (a, b, and c) of which H60a is a minor histocompatibility antigen (Malarkannan et al., 1998; Takada et al., 2008; Whang et al., 2009). Finally, murine UL16 protein-like transcript 1 (MULT-1) is the sole member of the third family of mouse NKG2D ligands (Carayannopoulos et al., 2002; Diefenbach et al., 2003).

NKG2D ligands are highly polymorphic, in particular MICA and MICB for which 70 and 31 alleles with different affinities for NKG2D have been identified, respectively (Steinle et al., 2001). There is also some degree of polymorphism in RAET-1 genes and mouse H60 and RAE-1 genes (Eagle et al., 2006; Romphruk et al., 2009). The reason for such a high degree of polymorphism is unknown at the present but it may have evolved to counter the selective pressure formed by pathogens and their evasion strategies, in order to maintain a proper recognition of infected cells by NK cells.

MOLECULAR MECHANISMS IN CONTROL OF NKG2D LIGAND EXPRESSION

NKG2D ligand expression is induced by various stress conditions, including cellular stress, malignant transformation or infection. The exact mechanism by which this occurs is, however, mostly unknown. NKG2D ligands are constitutively expressed on many tumor cell lines and actively growing epithelial and hematological

tumors (reviewed in Nausch and Cerwenka, 2008). The expression of NKG2D ligands makes tumors susceptible to killing by NK cells *in vitro* and is associated with a decreased tumor progression and a decreased capacity to form metastases *in vivo* (Bauer et al., 1999; Cerwenka et al., 2000, 2001; Diefenbach et al., 2000, 2001). NKG2D ligands are also induced on cells infected with bacteria and viruses, including cells infected with cytomegalovirus (CMV; Tieng et al., 2002; Siren et al., 2004; Vankayalapati et al., 2005; Draghi et al., 2007; Ward et al., 2007, 2009; Fang et al., 2008; Walsh et al., 2008). The control of NKG2D ligands expression includes both transcriptional and posttranscriptional mechanisms. The promoter regions of MIC genes contain heat shock elements similar to those found in the HSP70 gene, which bind heat shock factor-1 and induce MIC transcription in stressed cells (Jinushi et al., 2003; Venkataraman et al., 2007). Similarly, the retinoic acid-inducible element is mapped in the RAE-1γ promoter thus suggesting that gene expression is directly regulated by the retinoic acid (Nomura et al., 1994, 1996). Both human and mouse cells upregulate NKG2D ligands following treatment with DNA-damaging agents (Gasser et al., 2005) that activate a major DNA damage pathway initiated by ATM (Ataxia telangiectasia, mutated) or ATR (ATM- and Rad3-related) protein kinases (Bartkova et al., 2005; Gorgoulis et al., 2005). Oncogenes, such as the adenovirus E1A, induce transcription NKG2D ligands (Routes et al., 2005). These findings are the proof that there is a link between the constitutive activity of the DNA damage pathway and oncogenes in tumors, and a frequent upregulation of NKG2D ligands by transformed cells. Recent findings suggest that activation of the phosphatidylinositol-3-kinase pathway induced by murine CMV (MCMV) infection or tumor transformation is required for the induction of the RAE-1 family of mouse NKG2D ligands (Tokuyama et al., 2011). Finally, the expression of HCMV *immediate early gene* (*IE1* and *IE2*) displaces histone deacetylases and induces transcription of MIC proteins in infected cells (Venkataraman et al., 2007).

Cytokines and toll-like receptor (TLR) signaling differentially influence NKG2D ligand expression in different cell types and environments. TGF-β downmodulates the expression of RAE-1 proteins, which might be one of the immune suppressive mechanisms mediated by this cytokine (Routes et al., 2005; Eisele et al., 2006). IFN-γ decreases the expression of MICA and ULBP2 on melanoma and glioma cells, while IFN-γ and IFN-α selectively downregulate H60a on sarcoma cell lines (Jinushi et al., 2003; Schwinn et al., 2009). The downregulation of NKG2D ligands by the proinflammatory cytokine IFN-γ may be unexpected, but, on the other hand, it may represent a mechanism to prevent sustained triggering of the NKG2D receptor or, in the context of tumor cells, a strategy to escape from immune surveillance. Furthermore, IFN-γ-inducible small non-coding microRNAs (miRNAs) mediate downregulation of MICA (Yadav et al., 2009). On the other hand, the treatment of peritoneal macrophages or dendritic cells (DC) with TLR agonists upregulates the transcription of ULBP genes that may participate in the activation of NK and T cells (Hamerman et al., 2004; Draghi et al., 2007; Ebihara et al., 2007; Nedvetzki et al., 2007; Kloss et al., 2008).

In addition, various posttranscriptional mechanisms prevent the expression of NKG2D ligands on healthy cells. Although MICA and MICB genes are continuously transcribed, proteins are not expressed on the cell surface because their translation is held in check by cellular miRNAs. Cellular stress, such as a heat shock or viral infection, induces MICA and MICB transcription to levels that exceed the repressive ability of cellular miRNAs and allow MIC proteins to be quickly expressed on the cell surface (Stern-Ginossar et al., 2008b; Yadav et al., 2009). In healthy cells, MULT-1 protein undergoes ubiquitination and subsequent lysosomal degradation preventing its surface expression. In response to cellular stress, such as ultraviolet irradiation or heat shock, ubiquitin-dependent degradation of MULT-1 is reduced, leading to expression of MULT-1 protein at the cell surface (Nice et al., 2009). Among other NKG2D ligands, MICA, MICB, and ULBP5 are transmembrane proteins containing lysine residues within their cytoplasmic tails and therefore might be regulated by ubiquitination as well. Collectively, multiple checkpoints operating at different levels of NKG2D ligand synthesis facilitate the expression of NKG2D ligands to a broad range of cellular emergencies.

MANIPULATION OF NKG2D LIGAND EXPRESSION BY CYTOMEGALOVIRUS

Infection with both HCMV and MCMV upregulates the transcription of NKG2D ligands, which can result in NKG2D-mediated lysis of infected cells by NK cells. Viruses have developed different evasive mechanisms to prevent the expression of NKG2D ligands on the cell surface (Wilkinson et al., 2008; **Figure 2**). The HCMV protein UL16 binds MICB, ULBP1, ULBP2, and ULBP6 in the endoplasmatic reticulum (ER) and retains these ligands inside the cell (Cosman et al., 2001; Dunn et al., 2003; Rolle et al., 2003; Vales-Gomez et al., 2003; Welte et al., 2003; Wu et al., 2003). The structure of UL16 revealed that this viral protein mimics a central binding motif of otherwise structurally unrelated NKG2D, thus enabling the virus to evade several diverse NKG2D ligands (Muller et al., 2010). Another HCMV immunoevasin, UL142, retains newly synthesized full-length MICA in the *cis*-Golgi compartment (Chalupny et al., 2006; Ashiru et al., 2009). Interestingly, the *MICA*008* allele, which lacks the cytoplasmic domain, is resistant to the action of UL142. Since *MICA*008* is frequently found in humans, this suggests that during co-evolution HCMV exerts selective pressure to the host and thus drives diversity and polymorphism of NKG2D ligands. In addition to targeting already synthesized proteins, HCMV employs miRNAs to prevent the translation of ligands for NKG2D. HCMV encodes miRNA–UL112 that competes with endogenous miRNA for binding to the 3′-UTR (untranslated region) of the MICA transcript, thus repressing the translation of this NKG2D ligand (Stern-Ginossar et al., 2007, 2008a).

Most wild-derived and laboratory mouse strains fail to control MCMV during the first days post infection in spite of intact NK cells expressing the NKG2D receptor. The explanation for this phenomenon came with the discovery of MCMV inhibitors that prevent the expression of NKG2D ligands on the surface of infected cells (**Figure 2**). The deletion of one or more of these viral immunoevasins results in an attenuated MCMV replication *in vivo* during early days post infection (Lenac et al., 2008; **Figure 3**). However, if mice are depleted of NK cells or an NKG2D blocking

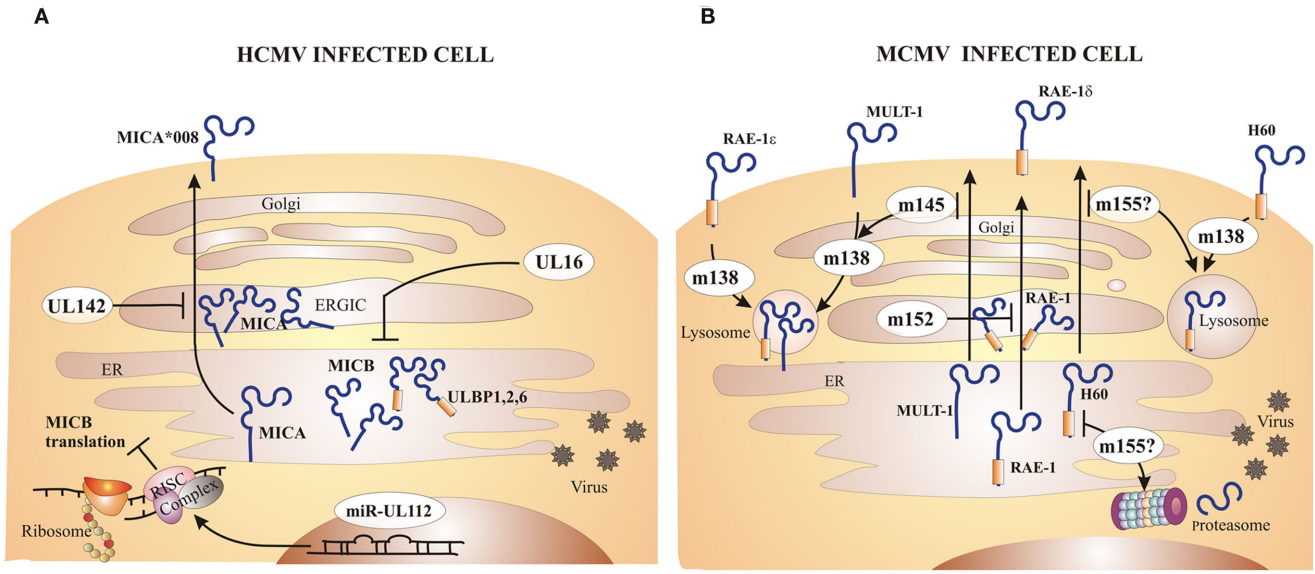

FIGURE 2 | Major CMV inhibitors of NKG2D ligands. Both HCMV and MCMV encode immunoevasins that inhibit expression of NKG2D ligands on the surface of infected cells. **(A)** The HCMV protein UL16 inhibits MICB, ULBP1, ULBP2, and ULBP6 expression by retaining them inside the cell. UL142 prevents MICA expression via retention in the *cis*-Golgi compartment. Certain alleles of *MICA*, such as *MICA*008*, are resistant to inhibition by HCMV due to truncation of the cytoplasmic domain. HCMV microRNA (miR)–UL112 forms RISC complex that binds to the MICB transcript and represses its translation. **(B)** MCMV encodes four immunoevasins that redundantly downmodulate all mouse NKG2D ligands. The *m152* product retains RAE-1 family of proteins in ER/*cis*-Golgi compartment. Not all RAE-1 proteins are equally susceptible to inhibition by MCMV, namely RAE-1δ is more resistant than other RAE-1 proteins. The *m145* product interferes with MULT-1 expression by making it more susceptible to the inhibition by the other immunoevasin, *m138*. The *m155* product redirects H60 for lysosomal and/or proteasomal degradation. Surface RAE-1ε, MULT-1, and H60 are additionally downmodulated by *m138* which interferes with their recycling.

antibody is administered prior to the viral challenge, the replication of MCMV can be restored. gp40, encoded by *m152* gene, was the first MCMV protein found to inhibit NKG2D. This finding was unexpected, because m152 was known to be involved in the maturation of MHC-I molecules and thus expected to enhance, rather than inhibit, the NK cell response (Ziegler et al., 1997, 2000; Krmpotic et al., 1999). The m152 protein targets RAE-1 ligands for downregulation, thus promoting viral escape from NK cell control (Lodoen et al., 2003; Arapovic et al., 2009). In this case, a single viral gene product acts in evasion of both NK cell and T cell antiviral immune responses. The m152 retains RAE-1 proteins in the ERGIC/*cis*-Golgi compartment (Krmpotic et al., 2002; Lodoen et al., 2003; Arapovic et al., 2009). However, not all RAE-1 isoforms are equally susceptible to MCMV regulation. RAE-1δ is more resistant to MCMV than other RAE-1 isoforms which is, at least in part, due to the absence of the PLWY motif in the RAE-1δ intracellular domain (Arapovic et al., 2009). Subsequent studies have identified three more MCMV proteins involved in the regulation of NKG2D ligands. The product of *m155* gene downmodulates the expression of H60 (Lodoen et al., 2004; Hasan et al., 2005), although the underlying molecular mechanism is not entirely understood. Lodoen et al. (2004) reported that H60 is degraded in MCMV-infected cells in a proteasome-dependent pathway. Our results, however, show that the acquisition of endo-b-*N*-acetylglucosaminodase H resistance and the half-life of the protein are not affected by the virus, indicating that *m155* affects H60 after it leaves the ER (Hasan et al., 2005). The product of the *m145* gene binds MULT-1 after it leaves ER (Krmpotic et al., 2005) and makes it more susceptible to another viral inhibitor. Namely, MCMV proteins involved in downregulation of NKG2D ligands are not restricted to m145 family; *m138* gene product also downmodulates MULT-1, as well as H60 (Lenac et al., 2006). The m138 was originally described as a viral protein that binds the Fc portion of immunoglobulin G and was designated as fcr-1 (Thale et al., 1994). Since the deletion of this gene results in viral attenuation *in vivo*, it was assumed that the attenuation is caused by enhanced susceptibility of the virus to antibody dependant cellular cytotoxicity. However, *m138* deletion mutant virus was also attenuated in B cell-deficient mice, suggesting additional function for this protein (Crnkovic-Mertens et al., 1998). A subsequent study demonstrated that *m138*/fcr-1 targets MULT-1 and H60 for internalization and thus contributes to viral resistance to NK cell-mediated attack *in vivo* (Lenac et al., 2006). Likely, additional virus-encoded proteins or miRNAs that inhibit expression of human and mouse NKG2D ligands will be discovered in the future.

Despite so many immunoevasion strategies targeting NKG2D, immunocompetent hosts succeed in the containment of CMV infection. This raises the question of why would the virus evolve such a robust immunosubversive potential if the host is able to cope with primary infection. One of the possible explanations is that NKG2D inhibitors, by facilitating virus escape from immune surveillance, may ensure virus dissemination to various organs, including the salivary glands, and aid in the horizontal virus

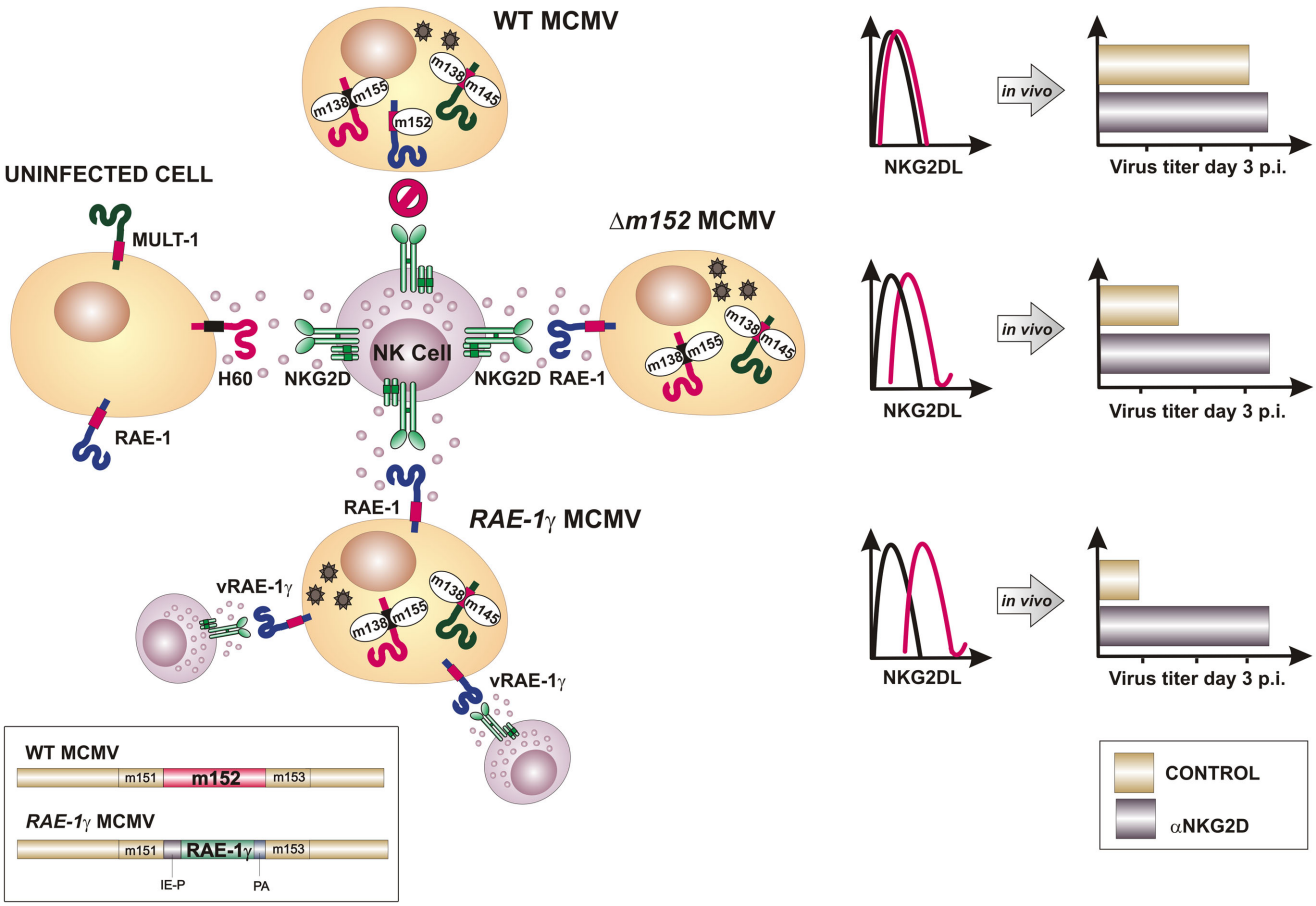

FIGURE 3 | Insertion of NKG2D ligand RAE-1γ into the MCMV genome overcomes viral immunoevasins of NK cells. To avoid lysis of infected cell by the NK cells, MCMV encodes four immunoevasins (m138, m145, m152, and m155) that downmodulate cell surface expression of mouse NKG2D ligands. Consequently, blocking of NKG2D has little or no effect on virus growth *in vivo* (upper part). Deletion of either of NKG2D immunoevasins from the MCMV genome (e.g., *m152*) results in the reconstitution of the surface expression of its respective target and attenuated phenotype *in vivo* that can be restored by NKG2D blocking (in the middle). Recombinant virus *RAE-1*γMCMV was engineered by replacing *m152* gene, whose product inhibits RAE-1 expression, with a cassette expressing RAE-1γ under HCMV immediate early 1 promoter (IE-P). Infection with *RAE-1*γMCMV results in high surface expression of cellular and virally encoded RAE-1γ (vRAE-1γ) and profoundly attenuated phenotype *in vivo* (bottom part). PA, polyadenylation sequence.

spread. Furthermore, by increasing the titer of the replicating virus, the load of the latent viral genome could be increased, thus representing an advantage for the virus to reactivate from latency and spread.

THE ROLE OF NKG2D IN SHAPING VIRUS SPECIFIC CD8 T CELL RESPONSE

In striking contrast to the well-defined role of NK cells in control of early MCMV infection, very little is known regarding the contribution of these innate effector cells in the regulation of a specific immune response and long-term immunosurveillance of viral infection. We previously investigated the contribution of specific lymphocyte subsets in the control of MCMV latency (Polic et al., 1998). Recurrent infection was measured upon ablation of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells individually and in combination. These studies revealed that control of CMV latency and prevention of recurrent infection is organized in a redundant and hierarchical fashion. Importantly, recurrence did not occur, or occurred only rarely upon depletion of any individual subset. However, depletion of NK cells led to a low (0–25%) or high frequency (80–87%) of recurrence when performed in conjunction with depletion of $CD4^+$ or $CD8^+$ T cells, respectively. This study provides strong evidence demonstrating that innate immune responses mediated by NK cells also operate in control of latent MCMV infection, but the ultimate mechanism(s) involved remained unsettled. Related to this, the role of viral evasion of NK cells with regard to their role in control of chronic/latent infection remains largely undefined. In addition, since some NK cell receptors are also expressed on cells of specific immune responses, the outcome of viral regulation of these receptors may also have a direct role in shaping the quality of virus specific immune response. For instance, NKG2D acts as a co-stimulatory receptor on CD8 T cells (Groh et al., 2001; Maasho et al., 2005; Markiewicz et al., 2005), which are primary effector cells in the resolution

of a productive MCMV infection and establishment of latency (Krmpotic et al., 2003). It is therefore feasible that viral inhibitors of NKG2D ligands may have a direct impact on antiviral T cell priming and effector functions.

To modify MCMV in a way that it overrides inhibitory signals delivered to NK cells by self MHC-I molecules and potentiated by viral immunoevasins, we have introduced an NKG2D ligand to the MCMV genome (Slavuljica et al., 2010). The ligand employed was a member of the RAE-1 family, RAE-1γ, which was inserted in place of its viral inhibitor *m152* (Lodoen et al., 2003; Krmpotic et al., 2005; **Figure 3**). The insertion of RAE-1γ into the MCMV genome resulted in a recombinant virus with profoundly attenuated phenotype *in vivo*, even in immunologically immature newborn mice and transgenic mice lacking type I interferon receptor, as well as in mice immunologically suppressed by total body γ-irradiation. Despite an attenuated phenotype, the MCMV expressing the NKG2D ligand efficiently primed and maintained an adaptive immune response, including the CD8 T cell response that was comparable to the one induced by the wild-type MCMV (**Figure 4**). In addition, the adoptive transfer of $CD8^+$ T cells from mice vaccinated with MCMV expressing *RAE-1γ* to immunodepleted and infected host mice resulted in the protection against CMV disease. Intriguingly, in a challenge infection, mice immunized with MCMV expressing *RAE-1γ*

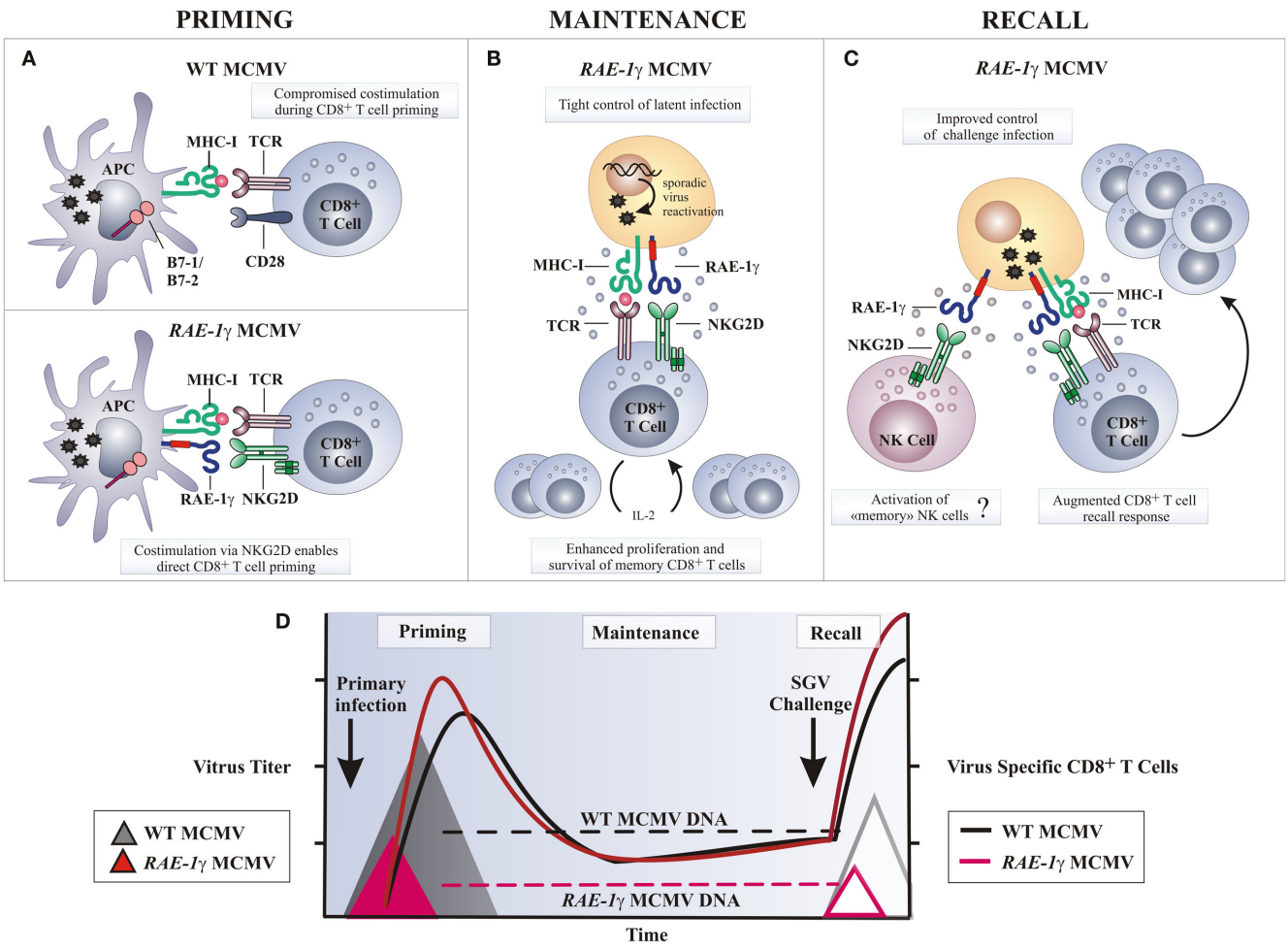

FIGURE 4 | Potential mechanisms of amplified CD8 T cell response following RAE-1γMCMV infection. (A) Naive CD8 T cells require engagement of the TCR by MHC-I molecules presenting antigen along with co-stimulatory signals, such as CD28 ligation by molecules of B7 family, to be fully activated. Wild-type MCMV (WT MCMV) dowmodulates B7.1 and B7.2 from the surface of infected APCs resulting in compromised T cell priming. Following *RAE-1*γMCMV infection co-stimulation via RAE-1γ–NKG2D interaction may replace B7-CD28 interaction and allow optimal CD8 T cell priming. **(B)** Stochastic MCMV reactivation during latency enables memory CD8 T cells co-stimulation *via* NKG2D–RAE-1γ interaction. Consequently, memory CD8 T cells are maintained or even inflated over time despite significantly reduced viral DNA load. **(C)** Challenge infection or any other serious stress leads to reactivation of latent *RAE-1*γMCMV and enhanced virus specific T cell response as a consequence of NKG2D ligand expression on cells undergoing virus reactivation. **(D)** Despite profoundly attenuated replication *in vivo* and reduced latent viral DNA load as compared to the WT MCMV, *RAE-1*γMCMV efficiently primes and maintains virus specific CD8 T cell response. In a challenge infection, mice immunized with the *RAE-1*γMCMV (red line triangle) resist salivary gland virus (SGV) challenge better as compared to mice initially infected with the WT MCMV (gray line triangle). APC, antigen presenting cell.

resisted challenge with virulent salivary gland-derived MCMV, with improved survival compared with mice initially infected with wild-type MCMV, suggesting that expression of RAE-1γ in the context of the MCMV infection provides an immune stimulus that results in enhanced effectiveness of the adaptive immune response.

What could be the mechanism behind a more robust CD8 T cell response after the infection with a recombinant MCMV expressing the NKG2D ligand RAE-1γ instead of its viral inhibitor *m152*? DCs can prime the CD8 T cell response in two ways; by direct presentation of endogenous peptides, or alternatively, exogenously acquired antigens may be presented, this function known as cross-presentation (Bevan, 2006). Although MCMV can infect DCs (Andrews et al., 2001), thus providing viral peptides for direct presentation, the majority of MCMV-specific CD8 T cells are not directly primed (Snyder et al., 2010). It is a current belief that viral immune evasion genes, which inhibit antigen presentation and modulate the maturation and function of infected DCs, profoundly impair direct priming of CD8 T cells (Andrews et al., 2001; Basta et al., 2002; Loewendorf et al., 2004; Munks et al., 2007; Benedict et al., 2008). MCMV encodes three immunoevasion proteins m04, m06, and m152 that interfere with MHC-I expression and severely impair CD8 T cells recognition of infected cells (Pinto and Hill, 2005; Lemmermann et al., 2011). Since *m152* gene is absent from the genome of MCMV expressing *RAE-1γ*, the density of antigen-MHC-I complexes on the surface of infected DCs may be higher and therefore strengthen antigenic signals delivered to T cells. In T cells, co-stimulation is largely provided by the interaction of CD28 and CD40L on the CD8 T cells with B7 molecules and CD40 on the DCs, respectively. MCMV targets the co-stimulation signals by altering the ratio of positive and negative co-signaling molecules on the DC surface. The MCMV encoded m138, m147.5, and m155 downmodulate expression of B7.1 (Loewendorf et al., 2004), B7.2 (Mintern et al., 2006), and CD40 (Loewendorf et al., 2011), respectively, and therefore compromise T cell priming (**Figure 4**). Moreover, MCMV selectively upregulates and retains inhibitory ligand PD-L1 on the surface of infected DCs to ensure the induction of T cell anergy, tolerance, or deletion (Benedict et al., 2008). Since co-stimulation of CD8 T cells by the NKG2D is in many ways similar to co-stimulation by the CD28 molecule and leads to increased proliferation, cytotoxicity, and cytokine response (Markiewicz et al., 2005), MCMV expressing *RAE-1γ* may tip the signaling balance of infected DCs toward positive ones and thus promote T cell priming. It might be possible that the engagement of NKG2D on CD8 T cells by RAE-1γ on infected DCs, and perhaps on other antigen presenting cells, provides a potent co-stimulatory signal during T cell priming able to override viral immunoevasins and allow direct CD8 T cell priming to take place (**Figure 4**).

Cytokines, such as IL-2, IL-12, and IFN-α/β, stimulate CD8 T expansion and differentiation and are required for optimal T cell responses. Early in the course of an MCMV infection, IFN-α/β is mainly produced by the activated plasmacytoid DCs (pDCs; Dalod et al., 2002, 2003) and is critical for host survival (Presti et al., 1998; Strobl et al., 2005). It mediates a number of immunoregulatory functions that vary depending upon the amount produced, with high IFN-α/β levels promoting immune suppression (Biron, 2001; Theofilopoulos et al., 2005). MCMV infection of BALB/c mice induces a very high systemic production of IFN-α/β resulting in the ablation of CD8α DCs and delayed CD8 T cell responses (Andrews et al., 2003; Robbins et al., 2007). In contrast, an efficient virus control by NK cells after infection with MCMV expressing *RAE-1γ* decreases the intensity and the duration of pDC activation and limits the production of IFN-α/β (Slavuljica et al., 2010). In this manner NK cells prevent cytokine-mediated loss of splenic CD8α DCs and potentially promote T cell priming.

RAE-1γ–NKG2D co-signals may also positively regulate the CD8 T cell response at later times after priming. The expansion of the antiviral CD8 T cells in a latently infected host, also known as "memory inflation," is a feature of the immune response to MCMV (Holtappels et al., 2002; Karrer et al., 2003). Based on the detection of viral transcripts in tissue during latency, it has been proposed that it is driven by the stochastic episodes of reactivation of a limited number of viral genes (Reddehase et al., 2008). In support of this, inflationary CD8 T cells display an effector memory phenotype suggestive of repeated antigen stimulation (Sierro et al., 2005; Munks et al., 2006). The accelerated clearance of productive infection with MCMV expressing *RAE-1γ* leading to a reduced latent viral genome load may also reduce latency-associated viral transcription and compromise "memory inflation" (Reddehase et al., 1994). However, the number of antiviral CD8 T cells increases, rather than fades over time, suggesting that the NKG2D co-stimulation may augment TCR signal during latency and substitute for a reduced latent viral genome load (**Figure 4**). Recently, it has been recognized that NK cells can be long-lived and mount an antigen-specific recall response (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009; Paust et al., 2010). Whether or not infection with MCMV expressing *RAE-1γ* induces memory NK cells to mount responses during a subsequent encounter with MCMV remains merely a speculation.

There is an urgent need to develop new therapeutic and preventive antiviral regimens, because at present no effective vaccine for CMV is available. In this regard, the mouse model of infection is currently the best system to experimentally define critical parameters affecting recurrent herpes virus infections. Both HCMV and MCMV employed numerous strategies to downregulate the expression of NKG2D ligands, pointing to the importance of NKG2D signaling in virus surveillance. The MCMV engineered to express an NKG2D ligand shows an outstanding safety profile, due to improved innate immune clearance following inoculation, but also because of being equally or even more immunogenic than the wild-type MCMV, leading to a more efficient adaptive immune response. Therefore, it is possible that an approach like the one described here might be used to generate an attenuated vaccine or a CMV-based vaccine vector.

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