Evolutionary struggles between NK cells and viruses

Lewis L. Lanier

Abstract | Natural killer (NK) cells are well recognized for their ability to provide a first line of defence against viral pathogens and they are increasingly being implicated in immune responses against certain bacterial and parasitic infections. Reciprocally, viruses have devised numerous strategies to evade the activation of NK cells and have influenced the evolution of NK-cell receptors and their ligands. NK cells contribute to host defence by their ability to rapidly secrete cytokines and chemokines, as well as to directly kill infected host cells. In addition to their participation in the immediate innate immune response against infection, interactions between NK cells and dendritic cells shape the nature of the subsequent adaptive immune response to pathogens.

Granzymes
Proteolytic enzymes that are present in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells. Granzymes activate caspases in the target cells, and this causes apoptosis.

Perforin
A protein with similarity to the ninth component of complement. It is present in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells. Perforin subunits assemble into a pore-forming structure that causes membrane damage in the target cell.

Department of Microbiology and Immunology, and the Cancer Research Institute, University of California San Francisco, San Francisco, California 94143-0414, USA.
e-mail: lewis.lanier@ucsf.edu
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Natural killer (NK) cells contribute to the innate immune defence against tumours and microbial pathogens. They sense their environment by using a sophisticated repertoire of receptors that allows them to distinguish between normal cells and transformed cells or cells infected with intracellular pathogens[1] (BOX 1). NK cells are most abundant in the blood, liver and spleen, but they are also present in lymph nodes, and they can migrate into inflamed or tumour-bearing tissues and organs. 'Resting' mature NK cells constitutively express transcripts for certain cytokines, such as interferon-γ (IFNγ)[2], and they contain pre-formed cytolytic mediators (granzymes and perforin), stored in intracellular granules[3]. Despite already being armed for attack, NK cells require activation by type I interferons (that is, IFNα or IFNβ) or pro-inflammatory cytokines, such as interleukin-15 (IL-15), IL-12 and IL-18, before becoming fully functional effector cells that can provide optimal host defence against infections. In many situations, dendritic cells (DCs) are probably the main source of the type I IFN and IL-12 that is necessary for NK-cell activation, and in turn, IFNγ produced by NK cells can affect the maturation and effector functions of DCs, as well as other leukocytes, including macrophages, granulocytes and other lymphocytes that are responding to the infection. In some cases, NK cells can directly recognize and respond to a cell infected with a virus, but cognate recognition of the infected cell is not absolutely required for NK cells to participate in a response because they can be activated as bystander cells simply by exposure to IFNs and cytokines in their environment. For example, culture of NK cells with IL-12 and IL-18 (or other combinations of cytokines with IL-12) is sufficient to initiate secretion of IFNγ, without requiring any deliberate engagement of the activating receptors expressed by NK cells that detect alterations in the cell surface of the infected or transformed cells. In addition, because NK cells express CD16, an activating Fc receptor for IgG (FcγR), they can attack virus-infected cells that are coated with IgG, with specificity being contributed by the antibody. Therefore, the relative contribution of and effector mechanisms involved in an NK-cell response to a given pathogen can vary considerably. Finally, some viruses have devised means to evade detection by NK cells, which emphasizes the importance of NK cells in host defence.

Although NK cells were initially identified in the 1970s on the basis of their ability to kill tumour cells, soon thereafter, activated NK cells were also detected in virus-infected hosts. Studies from several laboratories showed that NK cells isolated after infection with any of several different viruses had increased cytolytic activity *in vitro* against tumour-cell targets, and that viral infection can result in NK-cell proliferation and recruitment to the infected tissues and organs (reviewed in REFS 4,5). In many cases, the increased NK-cell-mediated killing was attributed to activation of the NK cells by the production of type I IFNs induced in the host by viral infection, because it was known that NK cells could be directly stimulated by exposure to IFNα or IFNβ. Although type I IFNs are not mitogenic for NK cells, they do induce the production of IL-15 (REF. 6), which is a potent growth factor for NK cells, in addition to augmenting the cytotoxicity and cytokine production of NK cells. Hence, the induction of type I IFNs and IL-15 by viral infection could well account for

Box 1 | **NK cells in immunity to microbial pathogens other than viruses**

Although natural killer (NK) cells have been studied most extensively in terms of viral and tumour immunity, there is an increasing awareness that NK cells are activated, and in some situations potentially have a protective role, in immune responses to certain microorganisms, including *Legionella pneumophila*[109], *Mycobacterium tuberculosis*[110–112], *Shigella flexneri*[113], *Borrelia burgdorferi*[114], *Brugia malayi*[115], *Leishmania major*[116,117], *Toxoplasma gondii*[118,119], *Cryptococcus neoformans*[120], *Trypanosoma cruzi*[121], *Plasmodium yoelii*[122] and *Plasmodium falciparum* (reviewed in REF. 123). As yet, there is no clear evidence for direct, cognate recognition of bacteria, fungi or parasites by NK cells. However, because certain bacterial infections have been shown to induce the expression of NKG2D (NK group 2, member D) ligands, this provides a possible mechanism for NK-cell activation. In addition, the activation of myeloid cells by pathogen-encoded Toll-like-receptor ligands results in the production of pro-inflammatory cytokines, such as interleukin-12 (IL-12) and IL-18, which are potent inducers of interferon-γ (IFNγ) production by NK cells. The rapid secretion of IFNγ by NK cells at the site of infection, causing the activation of macrophages and dendritic cells, might be an important component of the immune response in many of these infections. Animal models and population-based studies of infected humans will be crucial to unveil how NK cells have evolved to respond to these diverse infections.

the presence of activated NK cells in virus-infected hosts. Perhaps the first clear evidence indicating cognate recognition of a viral pathogen by NK cells that is important to host protection emerged from studies of mouse cytomegalovirus (MCMV). The MCMV model system has greatly advanced our understanding at a molecular level of how NK cells respond directly, and indirectly, to viral infection and it has revealed a remarkable relationship between the evolution of the host and a persistent virus.

In this Review, I highlight recent advances in our understanding of the receptors and effector mechanisms used by NK cells to control certain viral infections in humans and mice, and the molecular basis for viral evasion of NK-cell-mediated protection.

NK cells in immunity to cytomegaloviruses

Ly49-receptor-dependent resistance to MCMV. CMVs are herpesviruses, which comprise a family of large double-stranded DNA viruses that commonly infect, among others, humans and mice. Early studies showed that infection with MCMV activates mouse NK cells *in vivo*[7]. But more importantly, NK cells are required for the efficient control of MCMV replication in C57BL/6 mice[8], and this resistance to infection is conferred by a single dominant genetic locus in the NK-cell complex (NKC) on mouse chromosome 6 (REF. 9). Subsequent studies showed that the activating DAP12-associated receptor Ly49H[10,11] is responsible for resistance to MCMV in C57BL/6 mice[12–15] and that Ly49H directly binds to a viral glycoprotein, m157, which is expressed on the surface of MCMV-infected cells soon after infection[16,17]. Although Ly49H$^+$ NK cells are required for efficient control of MCMV replication, essentially all NK cells (including Ly49H$^-$ NK cells from C57BL/6 mice and NK cells from MCMV-sensitive strains of mice lacking the *Ly49h* gene) become activated after infection with MCMV, probably as a consequence of the cytokine and type I IFN secretion that is induced by the viral infection. However, unlike the NK cells from MCMV-sensitive mouse strains or the Ly49H$^-$ NK cells from C57BL/6 mice, Ly49H$^+$ NK cells proliferate and have more persistent activation after infection[18]. Whether the proliferation of Ly49H$^+$ NK cells after MCMV infection is important for resolution of the disease has not been addressed. It is possible that the protection conferred by Ly49H$^+$ NK cells is mainly due to their more efficient cytolytic activity and cytokine production. Passage of MCMV through resistant C57BL/6 mice results in mutation of the gene encoding m157 (REFS 19,20), and sequencing of MCMV isolated from wild mice revealed frequent loss-of-function mutations in *m157* (REF. 19), which shows that there is a selective pressure against the expression of m157 in MCMV-infected cells.

These findings raised the question of why MCMV would possess a gene that confers a selective disadvantage to its survival. This might be explained, in part, by the finding that m157 binds not only to the activating Ly49H receptor, but also to inhibitory Ly49 receptors in certain mouse strains[16] (FIG. 1). For example, m157 binds the inhibitory receptor Ly49I in MCMV-susceptible 129/J mice, thereby potentially conferring a selective advantage to the virus at the population level. Although Ly49I is expressed by only a small subset of NK cells in 129/J mice, this receptor might be expressed by a greater proportion of NK cells, and thereby have a greater impact on MCMV infection, in other mouse strains. An important distinction is that Ly49I binds to MHC class I ligands, in addition to m157, whereas Ly49H does not bind to any host MHC class I molecules. The crystal structure of m157 has revealed that this viral protein has significant homology to MHC class I molecules[21], which indicates that the virus 'stole' a mouse MHC class I gene to engage inhibitory Ly49 receptors and dampen the NK-cell-mediated response against MCMV, thereby providing a selective advantage. The affinity of m157 for the inhibitory Ly49I receptor is higher than the affinity of its MHC class I ligand[21], which is consistent with the concept that the viral protein evolved to suppress NK-cell responses against MCMV-infected cells that had down-regulated the host MHC class I molecules (presumably to evade cytotoxic T cells)[22]. The extracellular domains of the inhibitory Ly49I and activating Ly49H receptors are similar, and based on mathematical models, it seems that the activating Ly49 receptors evolved more recently that the inhibitory Ly49 receptors[23]. Diversification of the Ly49 receptor family, with the creation of activating receptors, might well have been driven by selective pressures imposed by viral pathogens, such as MCMV.

Some mouse strains that lack the *Ly49h* gene also resist MCMV infection through NK-cell-mediated protection. For example, Ma/My mice, which lack Ly49H, have NK-cell-dependent resistance to MCMV infection[24]. Genetic analysis showed that genes in the NKC and the H2^k haplotype are necessary for resistance. Another DAP12-associated receptor, Ly49P, expressed by Ma/My mice was shown to respond only to MCMV-infected cells expressing H2-D^k, and recognition of these cells was blocked by an H2-D^k-specific monoclonal antibody. These findings surprisingly imply a form of H2-mediated restriction of NK-cell recognition, and indicate that Ly49P either recognizes a peptide induced by MCMV infection of the host cells or a viral protein that associates with H2-D^k. Additional genes in the NKC

Ma/My mice
A strain of inbred mice that is relatively resistant to infection with mouse cytomegalovirus (MCMV). These mice express the activating NK-cell receptor Ly49P, which recognizes MCMV-infected cells that express H2-D^k.

Plasmacytoid dendritic cells (pDCs). Also known as interferon-producing cells. A type of dendritic cell that is specialized for the production of type I interferons after stimulation by viruses.

confer NK-cell-mediated protection against MCMV in wild mice[25], and multiple genetic loci are involved in resistance to MCMV in NZW mice[26], although the receptors that are involved have not been identified.

Evasion of NKG2D-mediated host protection by MCMV. The selective pressures exerted on MCMV have also been shown by studies exploring the role of the activating NKG2D (NK group 2, member D) receptor in viral immunity. The NKG2D receptor, which is expressed by almost all mouse NK cells, recognizes a family of MHC-class-I-related proteins, namely RAE1 (retinoic acid early transcript 1), MULT1 (murine UL16-binding protein (ULBP)-like transcript 1) and H60 (REF. 1). Remarkably, MCMV has devoted considerable resources to blocking the expression of these NKG2D ligands on the surface of infected cells: the viral protein m152 inhibits expression of RAE1 (REF. 27); m155 and m138 downregulate expression of H60 (REFS 28,29); and MULT1 is targeted by m145 and m138 (REFS 29,30) (FIG. 2). In MCMV-infected cells, the genes encoding these NKG2D ligands are transcribed, but the viral proteins interact with and cause the degradation of the NKG2D ligand proteins, thereby circumventing detection by NKG2D on NK cells. MCMV has seemingly blocked all attempts by the NKG2D pathway to exert control on infection by this virus, although given the polymorphic nature of the genes encoding NKG2D ligands, additional alleles or loci might exist in some mouse strains that have not yet been evaded by the virus. Interestingly, some of these viral proteins are multi-functional: m152 downregulates both MHC class I molecules and RAE1 (REF. 27), whereas m138 inhibits the expression of both H60 and MULT1, as well as functioning as a viral Fc receptor[29].

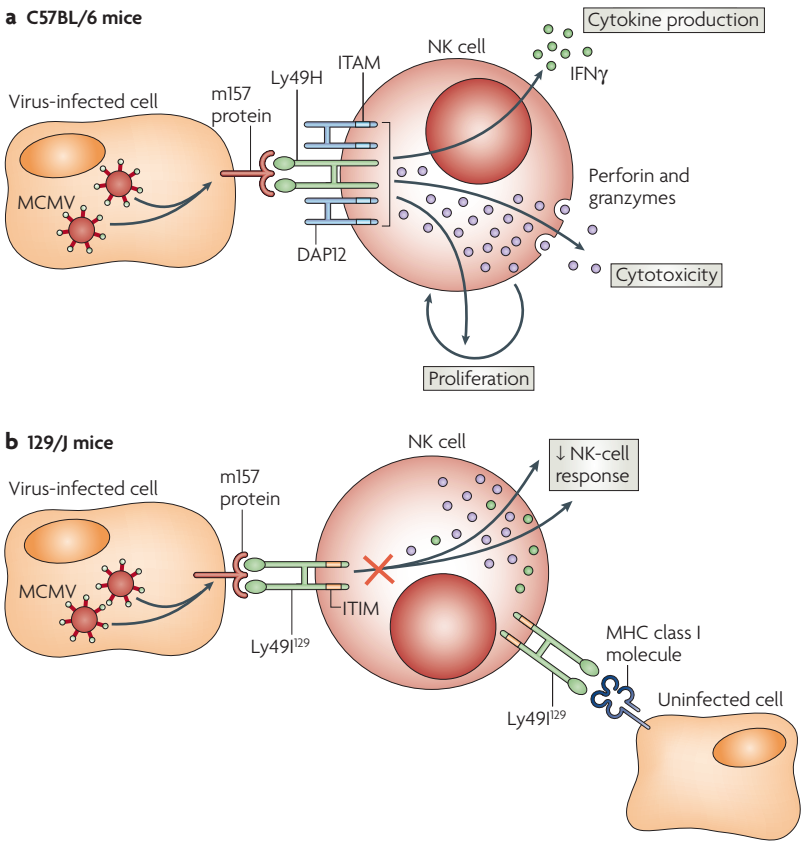

Figure 1 | **Activating and inhibitory NK-cell receptors for MCMV. a** | Natural killer (NK) cells from C57BL/6 mice express the DAP12-associated activating Ly49H receptor, which initiates cell-mediated cytotoxicity (through perforin and granzymes), cytokine production (such as interferon-γ (IFNγ)) and proliferation when these NK cells encounter mouse cytomegalovirus (MCMV)-infected cells that express the viral protein m157 on their cell surface. **b** | NK cells in certain other strains of mice, such as 129/J mice, have inhibitory receptors such as Ly49I^{129}, which binds to m157 on the cell surface of MCMV-infected cells and dampens the NK-cell response. Ly49I^{129} binds both host MHC class I ligands and MCMV m157, whereas Ly49H binds only m157 and not host MHC class I molecules. ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif.

NK-cell-mediated protection against MCMV. After infection with MCMV, NK cells are activated and are mobilized to sites of infection, such as the liver. This recruitment of NK cells in C57BL/6 mice requires virus-induced production of IFNα, which induces the expression of CC-chemokine ligand 2 (CCL2; also known as MCP1) by resident macrophages to recruit CC-chemokine receptor 2 (CCR2)-bearing macrophages into the liver, which in turn produce CCL3 (also known as MIP1α) that attracts NK cells to the site of infection[31,32]. The importance of type I IFN in resistance to MCMV infection has been shown by studies using mice deficient in Toll-like receptor 2 (TLR2), TLR3 or TLR9 or their downstream signalling adaptors TRIF (TIR (Toll/IL-1-receptor)-domain-containing adaptor protein inducing IFNβ) and MyD88, which are required for efficient IFN induction[33,34]. Plasmacytoid dendritic cells (pDCs; also known as interferon-producing cells (IPCs)) and DCs are the main source of the type I IFN that is required for NK-cell activation and recruitment[35] (FIG. 3). Type I IFNs induce DCs to produce IL-15 (REF. 36), an important NK-cell growth and differentiation factor that is required for NK-cell-mediated control of MCMV. Moreover, MCMV infection induces IL-12 and IL-18 secretion by DCs, and these cytokines augment the production of IFNγ by NK cells[37,38]. Reciprocally, activated Ly49H$^+$ NK cells in C57BL/6 mice protect DCs from infection and destruction by MCMV[37] (FIG. 3). Studies using perforin- or IFNγ-deficient NK cells have shown that both of these effector molecules are required for the efficient control of MCMV replication in infected mice[39,40]. NK cells themselves express certain TLRs — for example, activated NK cells express TLR3 and TLR9 (REFS 41–44) — but it is not known whether intrinsic TLR-dependent activation of NK cells during viral infection, rather than TLR-mediated DC activation, is important in disease control.

NK cells provide early control of MCMV replication, but they can also influence the ensuing virus-specific T-cell-mediated response. Robbins and colleagues[45] have reported that in congenic BALB/c mice with the protective C57BL/6 *Ly49h* gene, NK cells decrease the viral load, thereby decreasing the production of type I IFNs by pDCs, which is immunosuppressive to the host. As a result of the decreased type I IFN production by pDCs, cytotoxic T lymphocytes (CTLs) are generated more rapidly. However, consistent with prior

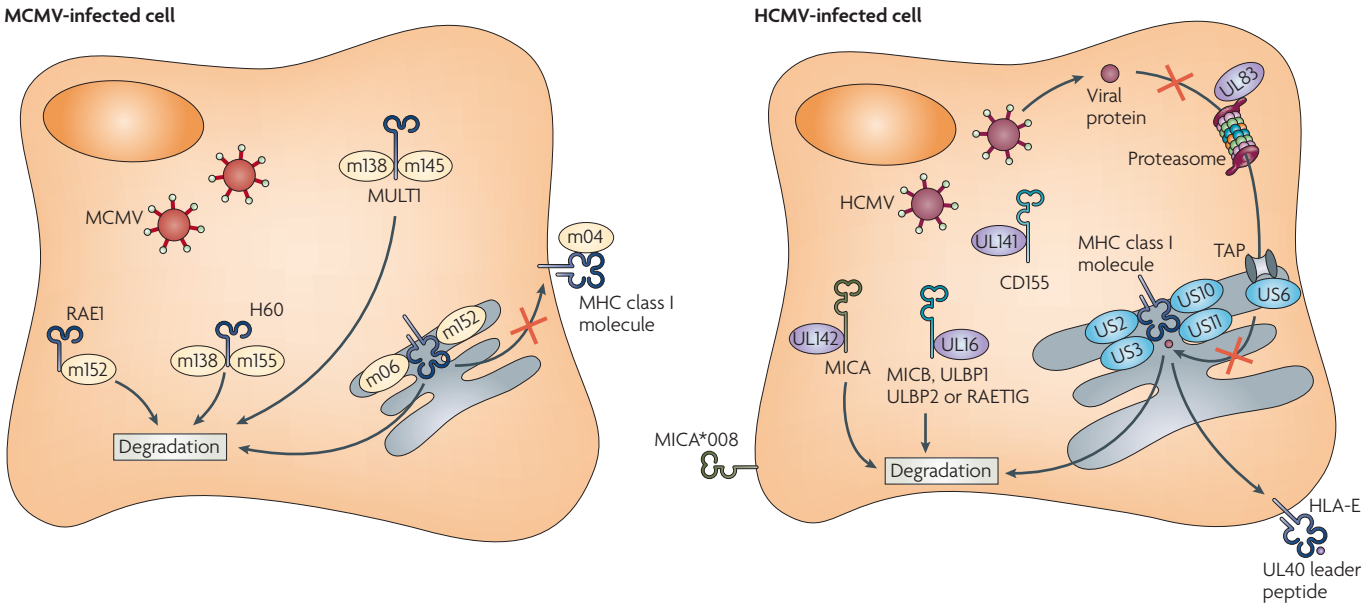

Figure 2 | **HCMV and MCMV proteins affecting NK-cell-mediated recognition of virus-infected cells.** The virus-encoded proteins m04, m06 and m152 inhibit MHC class I expression on the surface of mouse cytomegalovirus (MCMV)-infected cells by a complex process that differs depending on the MHC class I alleles present in the host[22]. m04 can be expressed on the cell surface of MCMV-infected cells in a complex with MHC class I molecules; however, how this influences the recognition of MHC class I molecules by receptors on natural killer (NK) cells or T cells is unknown. Human cytomegalovirus (HCMV) also blocks the expression of MHC class I molecules in infected cells in an allele-specific manner (reviewed in REF. 65). The HCMV proteins US2, US3, US10 and US11 interact with the MHC class I heavy chains on their own or with the heavy chains complexed with β_2-microglobulin, ultimately resulting in their degradation, whereas US6 blocks TAP (transporter associated with antigen processing) function and UL83 inhibits protein entry into the proteasome. UL40 provides a leader peptide that binds to HLA-E allowing its expression on the surface of HCMV-infected cells, presumably for interactions with the inhibitory CD94–NKG2A (NK group 2, member A) receptor on NK cells. Both MCMV and HCMV inhibit expression of the NKG2D ligands in infected cells. The MCMV-encoded m152 protein targets RAE1 (retinoic acid early transcript 1), as well as MHC class I molecules, for degradation; m145 and m138 cooperate to prevent MULT1 (murine UL16-binding protein (ULBP)-like transcript 1) expression; and m138 and m155 cause degradation of H60. In humans, the HCMV-encoded UL16 protein inhibits expression of MICB (MHC-class-I-polypeptide-related sequence B), ULBP1, ULBP2 and RAET1G (retinoic-acid early transcript 1G), whereas UL142 prevents expression of MICA. Certain alleles of MICA, such as the common allele *MICA*008*, are resistant to downregulation by HMCV because of a truncation of the cytoplasmic domain. CD155, a ligand for the activating NK-cell receptors DNAM1 (DNAX adhesion molecule 1) and CD96, is targeted by UL141.

observations[46], NK cells ultimately restrain the expansion and production of IFNγ by virus-specific $CD4^+$ and $CD8^+$ T cells in MCMV-infected C57BL/6 hosts. In other words, although NK cells seem to augment the generation of CTLs early during the immune response to MCMV infection, NK cells also, by as-yet-undefined mechanisms, ultimately restrain the clonal expansion of virus-specific $CD4^+$ and $CD8^+$ T cells later after infection. However, interpretation of these experiments involving the depletion of NK cells or type I IFNs is complicated because these treatments markedly alter the viral load, which itself might also significantly affect the T-cell response.

NK-cell recognition of human CMV. Functional Ly49 genes do not exist in humans, and so far there is no evidence that their counterparts, the KIR (killer-cell immunoglobulin-like receptor) genes, encode receptors analogous to Ly49H or Ly49P that can directly recognize human CMV-infected cells. Nonetheless, a human patient selectively lacking NK cells, but with normal B and T cells, was found to suffer life-threatening illness after infection with HCMV[47]. There are intriguing hints that NK cells that express the DAP12-associated CD94–NKG2C receptor complex preferentially proliferate after co-culture with HCMV-infected cells[48]. Both the activating CD94–NKG2C receptor complex and the highly related inhibitory CD94–NKG2A receptor complex recognize HLA-E as their ligand[49], although these functionally diverse receptors might discriminate between different peptides presented by HLA-E[50], potentially allowing the selective activation of CD94–NKG2C-bearing NK cells to mediate immunity against HCMV. The leader peptide derived from the HCMV protein UL40 can assemble into the peptide-binding groove of HLA-E and when this UL40 peptide is overexpressed with HLA-E it can engage the inhibitory CD94–NKG2A receptor complex and suppress NK-cell activation[51–53]. Further studies are required to determine how the CD94–NKG2A and CD94–NKG2C

HLA-E
A human MHC class I molecule that is composed of the HLA-E heavy chain, β_2-microglobulin, and a peptide that is often derived from the leader peptides of other MHC class I polypeptides or from certain microbial pathogens.