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Resisting Viral Infection: the Gene by Gene Approach

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Abstract

This review focuses on genes required for resistance to mouse cytomegalovirus (MCMV), as identified through unbiased genetic screening. Components of the developmental, sensing, and effector pathways, functioning in multiple cell types, were detected by infecting 22,000 G3 mutant mice with MCMV at an inoculum easily contained by WT animals. Merging these findings with discoveries from hypothesis-based studies, we present a cohesive picture of the essential elements utilized by the mouse innate immune system to counter MCMV. We believe that many breakthrough discoveries will yet be made using a classical genetic approach.

Introduction

Certain viruses, administered at a given titer, tend to produce a binomial outcome: survival vs. death within a specified period of time. In a genetically diverse species like our own, it is reasonable to suspect that DNA sequence differences at certain loci in the host, most of them yet to be determined, may foretell who will live and who will die when an immunologically naïve individual becomes infected. There is therefore keen interest in the question: “what genes are essential for surviving infection by a virus?”

Because innate immunity utilizes a “one-size-fits-all” strategy that depends upon an unknown but certainly finite number of genes to cope with a much larger number of microbes, we expect that a core collection of genes is sufficient to establish resistance against most viruses. Some genes may afford protection specific to only a few viruses. Still others may be “private” in their ability to protect against only a single species of virus. As previously described [1], today’s “host resistance” mutation, conferring protection against a given infection, may come to reside in tomorrow’s “innate immunity” gene, provided the selective pressure exerted by microbes is strong enough.

A classical genetic approach has been extremely effective in identifying genes needed to resist viral infection. In one example of this approach, random germline mutations are chemically induced in mice of a uniformly homozygous genetic background that supports survival upon infection by a virus. After infecting these mice with a low inoculum of the virus, it is possible to find exceptional animals in which rapid death ensues. Through positional cloning, susceptibility can be attributed to a single mutation, be it dominant or

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recessive. If the procedure is repeated enough times, insight into the nature of the immune response to the virus begins to emerge.

We have taken this approach with mouse cytomegalovirus (MCMV), a β -herpesvirus of mice known to be contained for a period of weeks by innate immune mechanisms alone (NK cells in particular) [2,3]. Coevolution of cytomegaloviruses with their host species has resulted in multiple viral functions that counter innate immune defenses, and if adaptive immunity is eliminated, mutations in the viral genome permit MCMV to evade innate immunity, proliferate, and ultimately kill the host [4]. MCMV is capable of infecting many cell types [5], and persists in mice in a latent state in the salivary gland epithelium [6], from which it may be reactivated to initiate further infectious cycles. It is transmitted in the wild by bites, and is endemic in mouse populations [7,8]. Like human cytomegalovirus, MCMV can cause death of individuals with different forms of immune compromise.

We set out to identify mutations that permit death following intraperitoneal inoculation with 10^5 PFU of MCMV. Approximately 22,600 mice of the third generation (G3) of C57BL/6J animals carrying mutations induced by *N*-ethyl-*N*-nitrosourea were tested in this manner. Mice with immunological or visible phenotypes detected in other screens (such as defects of TLR signaling, adaptive immunity, pigmentation, and/or development) were cross-tested as well so as to be as inclusive as possible. Our conclusions support a model of collaboration between NK cells, inflammatory monocytes, and dendritic cells in the establishment of resistance to MCMV (Figure 1). However, other proteins extrinsic to the innate immune system also contribute to survival during an infection. Here we present a concise portrait of how resistance is exercised, drawing on our own mutational evidence¹ and the studies of others.

NK cells

When an infection first occurs, NK cells play a critical, non-redundant role in sensing and containing it [9,10]. This was first deduced from depletion studies, in which antibodies were used to transiently eliminate most NK cells *in vivo* prior to inoculation [3,11,12]. When interstrain differences in susceptibility were noticed between BALB/c and C57BL/6 strains [13], positional cloning ascribed the preponderance of the difference to a single locus [14], and ultimately to a single gene encoding the NK-activating receptor Ly49H, one of two NK-activating receptors in C57BL/6J mice and absent in BALB/c mice [15,16]. Ly49H engages a virally encoded protein called m157 [17,18], stimulating NK cell proliferation [19], without which the antiviral response is inadequate.

Recent studies have identified at least four other NK-activating Ly49 receptors that mediate recognition and confer resistance to MCMV. Ly49P [20,21], Ly49L, Ly49P1, and Ly49D2 [22], expressed respectively in mouse strains MA/My, BALB/c (BALB.K and BALB.F), NOD/LtJ, and PWK/Pas, all engage a complex composed of the viral glycoprotein m04 and an H-2 molecule. An NK cell subset expressing the inhibitory receptor Ly49G2 was shown to be the first to expand and kill infected cells after MCMV infection [23,24]. Ly49G2 thus

¹Mutations without journal references are described at <http://mutagenetix.utsouthwestern.edu>.

appears to mark activated NK cells, although its role in NK cell activation remains unknown. NK cells are also activated through the activating receptor NKG2D, which engages MULT-1, RAE-1, and H60, surface proteins that are upregulated in cells infected by MCMV.

Classically, activated NK cells respond to MCMV by expanding, producing IFN γ , and mediating cytotoxicity through release of granules containing perforin and granzymes. NK cells also regulate the adaptive immune response through modulation of the plasmacytoid dendritic cell (pDC) and conventional dendritic cell (cDC) compartments, as discussed in the following section.

Finally, our studies detected a mutation in *Gimap5* (*sphinx*) that impaired resistance to MCMV through an impact on NK cell development [25]. *Gimap5* is necessary for NK cell development, as found in *Gimap5*-null mice [26], and for NK cells to leave the bone marrow and gain access to the periphery [25]. Similarly a lack of NK cells occasioned by common gamma chain or IL-15 deficiency produces a lethal outcome [27] (C. Eidenschenk and B. Beutler, unpublished data).

The DC compartment

The NK cell is not the sole site of MCMV perception. Two nucleic acid sensing Toll-like receptors, TLR3 and TLR9 (but not other TLRs), are crucial for survival. TLR3 detects dsRNA, produced by bidirectional transcription from the virion [28], while TLR9 detects viral DNA. Even though the MCMV genome exhibits some level of CpG suppression [29], it is evidently capable of being “seen” by TLR9, and host survival depends on such detection. cDCs and pDCs detect the virus via TLR9 [30]. However, pDC depletion using antibodies does not interfere with host survival following MCMV infection [30].

A key antiviral cytokine promoting survival is type I IFN, produced in large quantities by pDCs, but as inferred from the depletion studies just mentioned, in adequate quantities by other cells. Mutations in IRAK4 (*otiose*), MyD88 (*pococurante*), TRIF (*Lps2*), and Unc93b1 (*3d*) all impair survival through an impact on type I IFN production. Unc93b1 transports both TLR3 and TLR9 to the endosomal sites from which they signal. IRAK4 and MyD88 cooperate through direct interaction to allow signaling from TLR9, whereas TRIF allows signaling from TLR3, leading to nuclear translocation of NF- κ B, IRF-7 and possibly other IRFs, as well as AP-1 activation that contribute to expression of type I IFN. Downstream, mutations in IRF1 (*endeka*), IRF7 (*inept*), and IRF8 (*gemini*) have all been found to compromise survival. IRF8 is necessary for the development of most cDCs, whereas IRF1 and IRF7 are proximally involved in the IFN response.

Despite the importance of type I IFN in defense against viruses, in the context of MCMV infection activated NK cells moderate type I IFN production by pDCs [31] and favor cDC function, which promotes the activation of virus-specific adaptive immune (CD8 T cell) responses [32]. Conversely, cDCs co-activate NK cells through trans-presentation of the NK trophic factor IL-15, promoting NK cell expansion [33,34]. A mutation in *Flt3* (*warmflash*), the function of which is necessary for the development of cDCs to full competence for NK cell co-activation, impairs host survival after MCMV infection [35]. The details of this

process have not been fully elucidated, but the mutant cDCs produce less IFN α/β , IL-12, and IL-15R α than wild type controls in response to TLR activation. Although crosstalk between NK cells and cDCs is an important factor in innate immune control of MCMV, the effect of this interaction may not be so simple. Indeed, another report provided evidence that NK cells limit the extent to which DCs are infected and mediate their elimination, thereby reducing the duration and effectiveness of virus-specific CD4⁺ and CD8⁺ T cell responses [36].

The type 1 interferon response depends upon signaling by both chains of the type I IFN receptor complex, IFNAR1 and IFNAR2, and mutations in either (*macro-1* and *macro-2*) causes severe immune compromise during infection. STAT1, acting together with STAT2, translocates to the nucleus after phosphorylation by JAK1 and TYK2 kinases, and drives the transcription of many genes that mediate the interferon response. The *domino* and *poison* alleles of *Stat1* both impair NK cell function and cause a rapidly fatal outcome during MCMV infection [37].

NF- κ B activation is most probably essential for survival of the host as well, because *Panr2*, a mutation affecting *Ikbkg* and thus limiting the nuclear translocation of NF- κ B, is strongly compromising during MCMV infection [38]. While the number of NK cells is normal in these mice, NKT numbers are much diminished. It is not clear whether multiple cellular compartments are adversely affected by the *Panr2* mutation, but diminished TLR signaling in cDCs would likely be sufficient to account for susceptibility.

Inflammatory monocytes

The *elektra* mutation was detected in the standard screen for host survival during MCMV infection, and found to affect T cell survival during antigen-driven and homeostatic activation [39]. However, since mice deficient in both T and B cells survive MCMV infection perfectly well for several weeks, another cause of death had to be sought. NK cell numbers and function, and cDC function appear perfectly normal in *elektra* homozygotes. But inflammatory monocytes, like T cells, are fragile and undergo programmed death when activated by a variety of infections. On this basis, it has been proposed that they are important for containing MCMV infection. *Elektra* is a missense mutation of *Schlafen2*, a member of the poorly understood Schlafen (Slfn) family represented in humans and mice, providing unprecedented evidence of the importance of Slfns in viral resistance. The function of Slfn2, its subcellular location, and its intermolecular contacts remain obscure.

Effector proteins

The lysosome related organelle (LRO) system is critically important for surviving MCMV infection. Mutations affecting the *Lyst* gene, encoding lysosomal trafficking regulator, the adaptor protein 3 complex (*bullet gray*), and at least some of the Hermansky-Pudlak syndrome proteins including dysbindin and *Hps6* (*salt and pepper* and *Toffee*, respectively), all cause a lethal outcome. These mutations also cause hypopigmentation, reflecting the shared function of the LRO system in NK granule exocytosis and melanin export to the hair shaft. Some proteins related to NK granule export are not involved in pigmentation,

however. The *jinx* mutation was identified in *Unc13d*, a protein dedicated to exocytosis of toxic granules from NK cells and CD8 T cells of the immune system. While it is necessary for survival during MCMV infection, *Unc13d* also is needed for the clearance of other viruses such as LCMV. *Jinx* mutants develop a severe disease similar to human hemophagocytic lymphohistiocytosis (HLH) when inoculated with LCMV [40], and in humans, mutations in the orthologous gene are associated with childhood-onset HLH as well [41]. Perforin is also needed to survive infection by MCMV [42], and perforin mutations are associated with an HLH-like disease [43]. Rab27a is also needed to survive infection by MCMV as indicated by the compromising effect of the *concrete* mutation [44], and Rab27a mutations in humans are associated with the HLH-related Griscelli syndrome [45].

Homeostatic functions during infection

The changes that occur during infection by MCMV entail cytokine production with peak levels measured in the plasma between 36 and 48 hours after inoculation. We discovered through study of the *mayday* mutation that previously unknown accommodations must be made in the cardiovascular system to allow the host to survive during this period. The *mayday* mutation was detected in mice that died suddenly, apparently from cardiac arrest during this time interval. The phenotype was ascribed to a rearrangement of the *Kcnj8* locus, encoding one of two subunits that form an ATP-sensitive potassium channel expressed in the heart and coronary artery smooth muscle cells [46]. Absent this channel, the host cannot cope with its own innate immune response and sudden cardiac death occurs. Not only viral infection but administration of minute quantities of LPS can cause sudden death. To date, no single cytokine has been identified as the critical mediator of a lethal outcome. However, suppression of TLR signaling (combined *Myd88* and *Ticam1* mutation) is strongly protective against LPS-induced death. Neither mutation by itself is sufficient to suppress death induced by LPS in a *mayday* homozygote [46].

Cell autonomous immunity

In vitro screening for diminished macrophage resistance to infection by a GFP-tagged MCMV strain disclosed certain mutations that dramatically enhance viral proliferation in a setting where NK cells play no protective role. *Ifnar1* and *Ifnar2* mutations, already mentioned, were detected in this manner, and *Stat1* mutations also scored in this screen. More interesting was the mutation *atchoum*, which affects *Eif2ak4*, the eukaryotic translation initiation factor 2 alpha kinase 4, also known as GCN2 [47]. GCN2 is related to PERK, PKR, and HRI, all of which phosphorylate serine 51 of eIF2 α , leading to repression of translation initiation. While PKR is well known to respond to viral infection and downregulate translation, GCN2 has not generally been implicated in viral resistance but responds to attenuation of cytoplasmic amino acids. Occurring in the context of MCMV infection, this may limit proliferation of the virus, and thus benefit the host. We found that homozygous *atchoum* mice show modest but significant susceptibility to systemic infection by MCMV, indicating that even before the immune system is engaged, the host tries to repress infection, perhaps buying time to mobilize professional virus killers, including the cDC $\rightarrow$ NK axis.

Mutations that don't matter during MCMV infection

It is perhaps more remarkable that certain mutations that ablate whole classes of cells do not have a strong effect: for example, mutations eliminating T and B lymphocytes are without immediate effect when MCMV inoculation is performed. Some other mutations that might have been thought deleterious seem not to matter during MCMV infection. For example, the *feeble* mutation, which prevents pDC responses to nucleic acids by ablating expression of the solute channel Slc15a4 [48] has no effect on MCMV susceptibility, notwithstanding the pronounced deficiency of type I IFN in plasma observed in these mice in the course of infection. The *Joker* mutation in *Itgb2*, encoding integrin beta 2 or CD18, also has no effect on survival, despite the hypothesized importance of CD18 on formation of the synapse between NK cells and their infected targets [49].

Summary and perspective

Our current understanding of resistance to MCMV incorporates the functions of dozens of genes. Yet because mutational saturation of the genome has not been achieved in our screen, we believe that the classical genetic approach has not revealed all that it can about MCMV resistance. We do not know precisely how many genes with deleterious mutations were tested in our screen of 22,600 G3 mice. In the future, a far more precise estimate may be made, based on “up front” sequencing of G1 mutant mice fed into the G3 production pipeline. At present, we can only say that many discoveries likely lie in waiting.

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Figure 1. Genes required for innate resistance to MCMV identified by classical genetic studies Their protein products are depicted in the cellular compartments in which they function to mediate resistance. In red are the mutations that caused susceptibility. Sensing of MCMV infection occurs primarily in cDCs. Mutations affecting sensing were identified in genes encoding two nucleic acid-sensing TLRs (TLR3 and TLR9); in *Unc93b1*, required for transporting TLR3 and TLR9 to endosomes; in the signaling molecules Myd88, TRIF (Ticam1), IRAK4, and NEMO (Ikbkg); and in the transcription factors IRF1 and IRF8. Signaling from Flt3 ligand, which engages the receptor tyrosine kinase Flt3, is required for cDCs to gain competence to activate NK cells to full cytolytic potential. Within NK cells, responses to MCMV require IFN receptors *Ifnar1* and *Ifnar2*, the receptor for IL-12, and STAT1. NK cell degranulation is mediated by *Unc13d*, which is involved in vesicle priming, and numerous proteins also required for melanosome biogenesis and transport including *Lyst*, *Rab27a*, *dysbindin*, *Hps5*, and *Ap3b1*. *Slfn2* is required for resistance to MCMV, and likely acts in inflammatory monocytes in the context of this infection. *Gimap5* and *IRF8* are necessary for the development of NK cells and inflammatory monocytes, respectively. *GCN2*, encoded by *Eif2ak4*, likely contributes to resistance by limiting viral proliferation in infected cells. Finally, the ATP-sensitive potassium channel subunit *Kir6.1* encoded by *Kcnj8* is necessary for cardiac homeostasis during the innate immune response to MCMV.