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# Genetic dissection of innate immunity to infection: the mouse cytomegalovirus model

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Resistance to infection is largely inherited rather than acquired, and is encoded by a definable set of host genes designated the 'resistome'. Logically speaking, piecemeal disruption of the resistome gives us the best chance to define it, and the most spectacular advances in understanding innate immunity have grown from spontaneous or induced germline mutations of the resistome. Mutations induced by random germline mutagenesis have now become so numerous that we are nearly in a position to define the size of the resistome, and both random and targeted mutations give us a fairly nice sketch of its components and how they interact. Our own N-ethyl-N-nitrosourea mutagenesis effort, which recently showed that components of Toll-like receptor signaling are essential constituents of the arsenal against MCMV infections, validated the forward genetic approach as a powerful tool to define the resistome.

## Addresses

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## Abbreviations

<b>APC</b>	antigen-presenting cell
<b>CTL</b>	cytotoxic T lymphocyte
<b>DC</b>	dendritic cell
<b>ENU</b>	N-ethyl-N-nitrosourea
<b>IFN</b>	interferon
<b>IL</b>	interleukin
<b>LT-<math>\beta</math></b>	lymphotoxin- $\beta$
<b>MCMV</b>	mouse cytomegalovirus
<b>MyD88</b>	myeloid differentiation antigen 88
<b>TIR</b>	Toll/IL-1 receptor
<b>TLR</b>	Toll-like receptor
<b>TNF</b>	tumor necrosis factor
<b>TRIF</b>	TIR-domain-containing adapter inducing IFN- $\beta$

## Introduction

The innate immune system recognizes a restricted collection of molecules that are indispensable for microbial

life and are shared by essentially all microbes. The molecular basis of innate immune sensing and response, which not only protects the host but paradoxically also produces many of the symptoms of infectious disease, is now partly understood, and in many instances involves the Toll-like receptors (TLRs) and their signaling pathways. But there are specialized pathways that do not depend upon TLRs [1\*,2]. Beyond its ability to sense infection, the innate immune system is endowed with effector functions, whereby it creates conditions that are anathema to most microbes. Utilizing a restricted set of genes, far fewer in number than the number of microbes with which it must cope, the innate immune system defends the host very effectively.

A central goal of the science of innate immunity is to identify all of the sensing and effector mechanisms that comprise the innate immune response. It would be desirable to know what resistance entails from the outset. Hence, we may ask, 'How many genes are involved in innate immunity? How do their products interact with one another? And, which products can be categorized as having sensory versus effector function?'

These questions can best be addressed using a model system in which the host must respond to the execution of a genetic program resident within the pathogen. Viruses, which have lower genomic complexity than bacteria, offer the best opportunity to achieve this. The complement of host genes required for effective containment of viral infection during the early hours following inoculation is currently sought. The tool used to identify these genes and enumerate them is random germline mutagenesis. The mutagen of choice, in the mouse, is N-ethyl-N-nitrosourea (ENU).

## Pathogenesis of mouse cytomegalovirus infection: the agent

Mouse cytomegalovirus (MCMV) is a member of the  $\beta$ -herpes virus family, and as such is a relatively large double stranded (ds)DNA virus (230 kb in length). There are approximately 170 genes in the MCMV genome, and simultaneous bidirectional transcription of many of them assures that dsRNA will be produced in abundance during the course of an infection. This, in turn, is certain to alert the host via specialized proteins (including TLR3) that detect dsRNA. The virus is cytosine/guanine rich (58.7%) but shows no evidence of CpG suppression along most of its length [3]. Despite the fact that the unmethylated CpG DNA sensor TLR9

contributes to viral recognition, a CpG-rich genome has been maintained.

MCMV has broad tissue tropism, and can infect epithelial cells of the host, such as salivary gland tissue, as well as macrophages and lymphoid cells. It is transmitted among mice in the wild state by biting. Approximately 90% of wild mice are infected with MCMV [4], suggesting that the virus might be, or might once have been, a strong selective agent. Yet, infection is generally contained in the wild. Accordingly, it can be concluded that a substantial and ultimately definable fraction of the mouse genome has evolved to cope with this pathogen and, indeed, both 'generic' and 'specific' innate immune mechanisms are known to combat it.

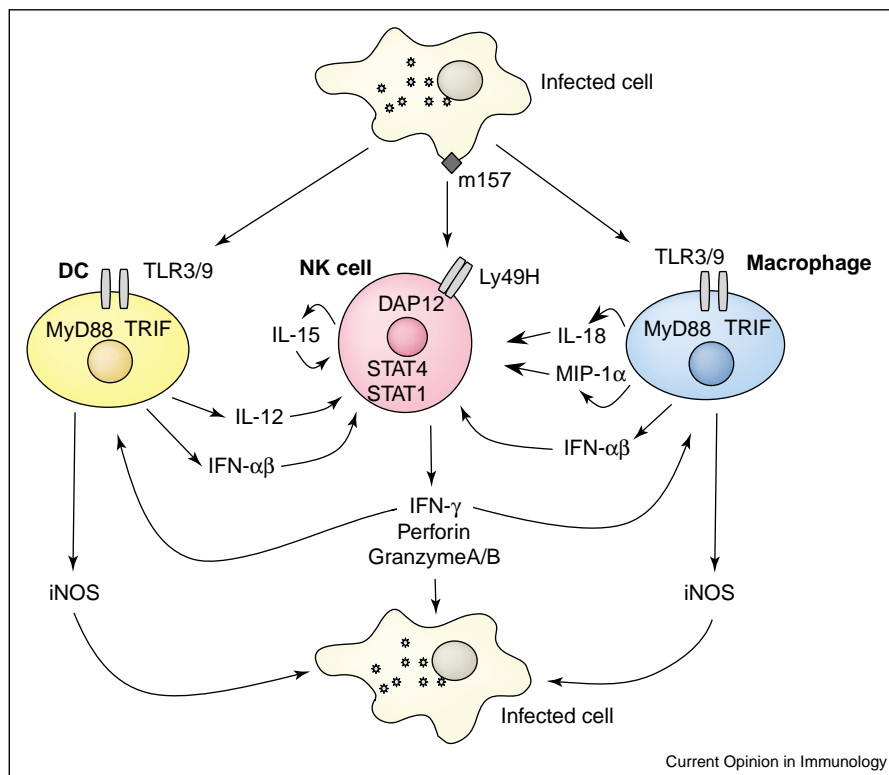
### Pathogenesis of mouse cytomegalovirus infection: the host

The immune control of MCMV infection requires elements from both innate and adaptive immune systems. Innate defenses (illustrated in Figure 1) provide early protection, whereas adaptive defenses provide delayed protection. Neither alone is sufficient to permit survival of the host.

### The cellular defenses

A selective lack of T cells and B cells, as occurs in mice with severe combined immunodeficiency disorder (SCID), is associated with a transient period of viral containment followed by relapse and persistent infection 14 days post-inoculation [5], ultimately leading to the death of the host. Depletion of NK cells, however, renders a normal host highly susceptible to MCMV early on, so that infection leads to death within a few days following inoculation. Similarly, in humans with quantitative NK-cell deficiency [6], overwhelming CMV infection is a frequent cause of death. These observations reveal an essential and non-redundant protective role for NK cells in the early immune response to MCMV infection and CMV infection alike, indicating that the murine virus is, at least in some ways, an excellent model for the human virus. They also suggest that T cells and B cells are dispensable for host defense during the early days that follow infection. As described below, however, they do not exclude a crucial role for other cellular elements and, in fact, it appears that myeloid and/or plasmacytoid dendritic cells make an equally important contribution to defense against MCMV.

Figure 1



Cellular responses and cross-talk during the early innate immune response against MCMV. Infected cells express the virally encoded protein m157, whose binding to the receptor Ly49H triggers the activation of NK cells and the subsequent release of IFN- $\gamma$ , perforin and granzyme A/B. NK cell activation, however, also requires cytokines (IL-12, IL-18, MIP-1 $\alpha$ , IFN  $\alpha/\beta$ ) produced by DCs or macrophages. TLR3 and TLR9 are essential sensors of the infection in these APCs. Activated DCs and macrophages also exert a protective effect through nitric oxide production as a result of inducible NO synthase (iNOS) gene upregulation.

### Genes and their interactions

Classical genetic methods have led to the identification of molecules essential both for the recognition of MCMV, and for the activation and function of NK cells and cytotoxic T lymphocytes (CTLs). Some mechanisms of MCMV resistance are strain dependent, and their elucidation by positional cloning has yielded important insights into NK activation mechanisms. Although C57BL/6 mice are relatively resistant to MCMV, BALB/c mice (and most other *Mus musculus* strains) are highly susceptible. The difference in susceptibility can be traced to a single locus, termed *cmv1*, which was recently found to encode the NK-activating receptor Ly49H, a C-type lectin that is expressed by both NK cells and CTLs [7–9].

Direct recognition of infected tissues by NK cells occurs in the MCMV-resistant C57BL/6 strain, which express the plasma membrane protein Ly49H. This protein specifically recognizes the MCMV-encoded protein m157, a molecule with distant homology to class I MHC antigens [10,11]. Because Ly49H is not expressed in BALB/c mice, animals of this strain are highly susceptible to MCMV infection. Restoring Ly49H expression by transgenesis partially rescues the susceptibility phenotype of BALB/c mice [12\*]. MCMV susceptibility is also observed in mice carrying a loss-of-function allele at the Ly49H-associated adaptor protein KARAP–DAP12, confirming the requirement of the Ly49H–KARAP–DAP12 pathway in MCMV detection [13].

MCMV infection is simultaneously perceived by TLRs expressed in the endosomal compartment within antigen presenting cells (APCs), such as macrophages or dendritic cells (DCs) [14\*,15\*]. Plasmacytoid DCs, as key sources of type I IFN, might be especially important in this ‘indirect’ arm of the innate immune sensing process. Mice homozygous for mutations in TLR3 or TLR9 proteins, which recognize dsRNA and CpG patterns, respectively, are hypersusceptible to MCMV, CpG more so than dsRNA. Both Toll/IL-1 receptor (TIR)-domain-containing adapter inducing IFN- $\beta$  (TRIF; the TLR3 adaptor protein) and myeloid differentiation antigen 88 (MyD88; an adaptor for TLR9 and most other TIR domain receptors) are important to a commensurate extent in the activation of this pathway [14\*,16\*\*]. Both pathways lead to the production of type I IFN.

IFN- $\alpha$  and IFN- $\beta$  interact with the IFN- $\alpha/\beta$  receptor (IFNAR) expressed on NK cells and CTLs. On a C57BL/6 or 129 background, IFNAR deficiency confers up to 800-fold higher susceptibility to MCMV [17] despite the fact that IFN- $\alpha/\beta$  limits IL-12 activation of NK cells [18]. IFN- $\alpha/\beta$  also enhances IL-15 production in a signal transducer and activator of transcription (STAT-1)-dependent manner, and IL-15, among other cytokines,

contributes to the maintenance and/or accumulation of NK cells [19].

Interestingly, although both the TLR3–TRIF signaling axis and the TLR9–MyD88 signaling axis result in type I IFN production, neither pathway alone suffices to provide full protection. Hence, other cytokines that are yet to be identified might also be important, and some of these might be unique products of each pathway.

The migration to and activation of NK cells in response to an infection is chemokine and cytokine dependent. In liver, the macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) and IL-18 are both required to recruit NK cells to inflammatory foci [20,21].

Data from other models of viral infection imply the importance of an IL-12–STAT-4–IFN- $\gamma$  activation loop within APCs in the NK response as well, and, by implication, this loop might apply to MCMV infection. In spleen and liver, lymphochoriomeningitis virus (LCMV)-infected APCs produce high levels of IL-12. The IL-12 receptor (IL-12R), when engaged by ligand, activates STAT-4, leading to IFN- $\gamma$  production by NK cells and CTLs. This, in turn, activates APCs through a feedback loop. Hence, STAT-4-deficient mice are hypersusceptible to LCMV infection [22]. The crosstalk between APCs and NK cells or CTLs involving IL-12–IL-12R and IFN- $\gamma$ –IFN- $\gamma$ R pathways is essential in order to mount a strong immune response to MCMV infection [17,20].

IL-15 deficiency affects the development of specific lymphoid lineages by limiting NK cell survival and accumulation [19,23] suggesting its requirement for the response to MCMV. IL-15-deficient mice lack most of their NK and NKT cells, which explains their susceptibility to MCMV infection [19,24]. A role for lymphotoxin- $\beta$  (LT- $\beta$ ), its receptor (LT- $\beta$ R), and the type I TNF receptor in the response to MCMV has also been reported [25]. Although the mechanism of protection by type I TNFR signaling is obscure, the disordered lymphoid architecture observed in mice lacking LT- $\beta$ R, LT- $\beta$  or LT- $\alpha$  might play a contributory role in the case of defects involving any of these proteins.

The cytotoxicity of NK cells is mostly due to their capacity to lyse infected cells by secreting granzymes A and B, and perforin molecules. Perforin is important for clearance of MCMV in salivary glands only [26]; however, the formation and trafficking of secreting vesicles is essential for NK cell cytotoxic activity in *beige* mice, where a mutation in the lysosomal trafficking (*lyst*) regulator gene confers susceptibility to MCMV [27]. *Beige* mice have a light coat color, demonstrating a link between melanosome and lysosome trafficking. In humans, the Chediack-Higashi syndrome (CHS) is equivalent to the murine *beige* syndrome.

### The genomic footprint of resistance to murine cytomegalovirus

Only a handful of genes have been named in the preceding paragraphs. In all, how many genes are actually required to defend the host against MCMV? Many approaches might conceivably be used to address the question, but few are equal to the task. One might, for example, monitor the changes in gene expression that occur in diverse host tissues in the wake of infection; however, the number of genes that show altered expression might greatly exceed the number that is essential for effective containment of the pathogen. Moreover, the expression of some genes that are of key importance to the innate immune response (e.g. TLR3 and TLR9) might very well be static.

A mutagenic approach to the issue of susceptibility is surely more robust, and can be pursued in an *in vitro* system, or using germline mutagens. *In vitro* systems are relatively inexpensive, and have the virtue of permitting deep saturation of the genome, but miss the cell–cell interactions that are known to occur during infection. Such interactions are known to be of key importance to the immune response and, in some respects, are the very essence of host resistance. For example, the production of type I IFNs by plasmacytoid DCs and the effect of type I IFNs on NK cells [28<sup>\*\*</sup>,29<sup>\*</sup>] could not be appreciated if only one cell type was used in an *in vitro* mutagenesis experiment. Germline mutagenesis is far more costly, but offers hope of finding all genes that contribute to innate immune defense, provided that the mutations in question are compatible with survival to the age of assay. As viable allelic variants of virtually any gene can be produced, this condition is probably a realistic one. Less certain is the ability of a single germline mutagen to hit each and every gene. ENU is presently the mutagen of choice; it might or might not be capable of changing every gene.

Because MCMV is transmitted in nature by biting, the laboratory model of *in vivo* infection (intraperitoneal injection of the virus) does not represent a marked departure from what actually occurs. Although parenteral models of *Salmonella typhimurium* infection might be criticized as they circumvent the normal route by which the pathogen is acquired, MCMV gains access to the blood in the laboratory in a more or less ‘natural’ manner. Hence, a screen for MCMV susceptibility, based on the injection of virus into germline mutant mice, is likely to disclose most of the genes that guard against infection in the wild state.

Genomic saturation is ultimately required to identify all genes that are required for a given phenotype (in the present example, enhanced susceptibility to MCMV infection). Short of achieving saturation, a reasoned estimate of genomic saturation must be made to calculate the number of target genes that exist.

### Progressive destruction of the mouse genome to find the murine cytomegalovirus resistome

Direct sequencing estimates suggest that ENU mutagenesis (performed by administering three injections of ENU at a dose of 100 mg/kg body weight to G0 male mice at weekly intervals) causes the introduction of approximately one mutation per million base pairs of haploid genomic DNA [30]. As the mouse genome is 2600 Mb in length, and about 1.3% of the mouse genome has coding function [31], this rate of mutation corresponds to an alteration of coding sequence in about 45 genes in each G1 mouse born to a mutagenized father. As approximately 76% of random nucleotide changes within a coding region constitute missense or nonsense mutations, it might be expected that approximately 34 proteins are structurally altered in each F1 animal. When F1 mice are crossed and backcrossed to yield a G3 generation, an average of 1/8 of all typeable loci are rendered homozygous; hence the average G3 mouse is homozygous for approximately four missense, nonsense, or splicing errors induced by ENU.

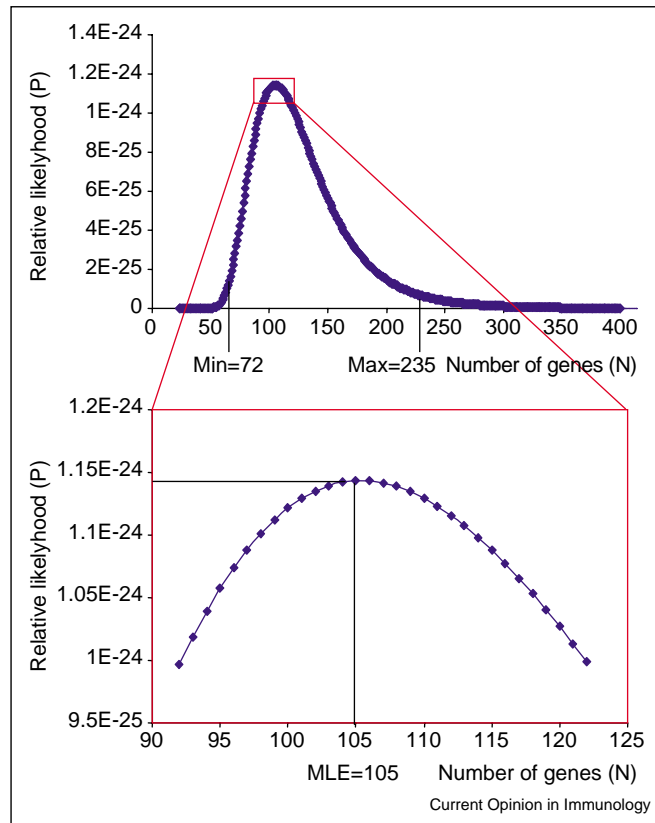
It must then be asked what percentage of random coding changes yield a discernable phenotype (in this case, the conditional lethality observed when a mouse is inoculated with MCMV). The answer is unknown, but very general estimates can be made from mutagenesis studies in which unconditional lethality is detected as a phenotype through the use of balancer chromosomes [32<sup>\*</sup>] or hemizygous deletion [33] covering defined genomic intervals. A rough estimate of saturation can then be made using a model in which it is assumed that all genes have an equal probability of being destroyed by the mutagen (an assumption that is certainly incorrect, but one that serves as a starting point for analysis). Furthermore, it is assumed that conditional lethality (i.e. early death following inoculation with MCMV) is as easily created by ENU as unconditional lethality on a per-gene basis.

The problem of determining saturation can be likened to the problem of determining the number of slots in a roulette wheel. Assuming that the number of slots (N, or number of genes conferring a given phenotype) is unknown, if the ball is rolled 50 times (the number of mutations, Y) and single hits (non allelic mutations, Z) are observed 40 times, it is possible to calculate the relative likelihood (P) for different values of N, according to the formula:

$$P = N! / (N - Z)! N^Y.$$

If there is a maximum likelihood estimate (MLE) of 105 slots in the wheel, one could say, with 95% confidence, that there are no fewer than 72 and no more than 235 slots (Figure 2)

Figure 2



Graphical representation of the formula  $P=N!/(N-Z)!N^Y$ , with Z (number of genes mutated) = 40, Y (number of mutations recovered) = 50, for values of N (number of genes involved in the phenotype which is considered) varying between 0 and 400. The graph was generated with an MS Excel sheet to calculate the relative likelihood (P) for each value of N. A 95% confidence interval defines a minimal (Min) and maximal (Max) value for N. The insert at the bottom of the figure represents the top values of P and shows that the maximal likelihood estimate (MLE) is obtained for N=105.

Kile *et al.* [32<sup>•</sup>], observed a single allelic lethal mutation in a panel of 24 lethals recovered from a region containing 731 genes. Imposing the limitation that  $N_{\text{Max}}=731$ , there is an MLE of 268 genes (95% confidence limits between 104 and 711) with lethal alleles residing within the target region. Hence 36% of all genes are expected to have lethal alleles. And, as 23 genes were converted to a lethal phenotype among the 268 that are estimated to exist, 8.5% saturation was achieved.

As to the conditionally lethal phenotype of MCMV susceptibility and the number of target genes wherein variant alleles can cause it, a total of 1513 micropedigrees have so far been examined for mutations that cause MCMV susceptibility (P Georgel, K Crozat and B Beutler, unpublished). The recovery of genes was not 100% efficient, as is the case of the balancer approach used by Kile *et al.* [32<sup>•</sup>], but approximately 49.3% efficient, as two daughters were produced from each F1 male, and three progeny were tested from each daughter. The strain employed (C57BL/6) was the same as that used by Kile *et al.* [32<sup>•</sup>], and the number, schedule and dose of

ENU injections was the same. Assuming that the ENU was equipotent when used in each laboratory, we would imagine that we have achieved 8.6% phenotypic saturation. A total of 25 transmissible mutations have been detected by screening (P Georgel, K Crozat and B Beutler, unpublished). If we assume non-allelism among these mutations, it follows that 290 target genes exist in all. A direct estimate of the number of MCMV resistance genes, independent of the saturation estimate made by Kile *et al.* [32<sup>•</sup>], awaits determination of allelism among those mutations that cause MCMV susceptibility.

It must be acknowledged that the 'roulette' model is not strictly applicable to the problem at hand, because not all genes are equally mutable. Gene size is certainly an influential factor; the adenine–thymine content of the gene is also important, as ENU chiefly targets adenine–thymine pairs [34]. The susceptibility of the coding region (in terms of the actual percentage of changes that yield a coding error) is important as well. Intangible factors related to chromatin structure might also be influential. Within these parameters, corrections could be made for

each except the influence of chromatin structure, based on information presently available about all of the annotated genes within the genome. Hence, a roulette wheel with 'variable slot size' could be envisioned and the estimate of saturation could be adjusted accordingly.

### The nature of the genes that offer protection

Approximately 25 000 genes are annotated in the mouse genome at present. A fraction of them have conditionally lethal alleles, in the sense that, when they are altered, they create susceptibility to infection by MCMV. There are two types of these mutations. On the one hand, there are effector mutations that permit unbounded growth of the pathogen, yet make the host ineffectual in coping with it. On the other hand, some mutations affect the host ability to recognize infection, and also permit the virus to reach a high titer, but do not ultimately suppress the response. From published reports, it is known that a collection of additional genes (Table 1) participate in the elimination of MCMV as well. These genes can similarly be classified as serving sensory or effector functions.

On the sensory side, the TLRs and Ly49H have key roles, as already discussed. On the effector side, perforin, IFN- $\gamma$ , and other molecules that permit killing of the infected target cells are of key importance. Much remains to be learned about both sensing and effector arms,

however, and, to cite one example of this, the genes required for vesicle formation and trafficking, and endosome maturation, are very little understood. Many (although not all) of the Hermansky-Pudlak mutations create susceptibility to MCMV, for example, as do mutations of the *Lyst* locus. These mutations jointly affect pigmentation and susceptibility. Some mutations, however, seem to affect only the endosomes rather than melanosomes. Ultimately, a divergent pathway must be postulated, and the details of organelle formation and function have not yet been elucidated.

Other genes, encoding GTP-binding proteins, such as Mx and its relatives [35], also offer resistance to viral infection (albeit not necessarily to MCMV infection) and are IFN responsive, but there is presently little understanding as to how they might function. Clearly there is room for improvement in our understanding, and, if approximately 290 genes do comprise the MCMV resistome, it must be said that only about 8% of them have been found.

### The overlap of resistomes and the degeneracy of the innate immune system: how many genes offer resistance to all infectious agents?

So far, we have considered resistance to MCMV. But the innate immune system has inherent degeneracy. Some of the receptors utilized by the innate immune system (for example, receptors of the TLR class) are known to initiate responses to such diverse pathogens such as bacteria and viruses. In mice there are twelve TLRs. Three of these have functions that are still unknown. But there are only five adaptors serving TLR responses, and the destruction of two of these adaptors is sufficient to silence signaling by all of the known TLRs. Only two protein kinases are linked to the TIR domain adaptors, and these kinases represent a further level of degeneracy. If one steps beyond the level of the kinases, it must be considered that the host cell is ignorant of the stimulus that initially triggered the sensing reaction. Hence, mutations of MyD88 compromise responses to fungal, bacterial and viral infections alike. The size of the 'universal resistome' is obviously much smaller than the sum of all individual resistome sizes.

Mutations that cause immunocompromise to *Listeria* infection often have very similar effects on MCMV sensing. For example, mutations of TNF, the p55 TNF receptor, MyD88, and IFN- $\gamma$  cause host susceptibility to these very disparate pathogens. The size of the universal resistome might ultimately be estimated through forward genetic analysis of resistance to several unrelated pathogens.

### Conclusions

We provide here a first approximation of the MCMV resistome size, on the basis of the number of mutations

**Table 1**

**Genes required for innate immune response against mouse cytomegalovirus infections.**

	Gene name	Evidence	Refs
<b>Signals</b>	<i>IL-12p40</i>	KO	[36]
	<i>IL-18</i>	KO	[20]
	<i>IFN-<math>\gamma</math></i>	KO	[17]
	<i>MIP-1<math>\alpha</math></i>	KO	[21]
<b>Sensors</b>	<i>Ly49h</i>	QTL	[7,9]
	<i>TLR3</i>	KO	[14*]
	<i>TLR9</i>	ENU	[14*]
	<i>IL-2/IL-5R<math>\beta</math></i>	Antibody depletion	[19]
	<i>IFN-<math>\gamma</math>R</i>	KO	[17]
<b>Transmitters</b>	<i>IFN-<math>\alpha\beta</math>R</i>	KO	[17]
	<i>DAP12</i>	KO	[13]
	<i>MyD88</i>	KO	[14*]
	<i>TRIF</i>	ENU	[16**]
	<i>Lyst</i>	QTL	[27]
	<i>Sandy</i>	ENU	(a)
	<i>IRF-1</i>	KO	[37]
	<i>STAT-4</i>	KO	[38]
<b>Effectors</b>	<i>STAT-1</i>	KO	[38]
	<i>Perforin</i>	KO	[26]
	<i>Granzyme A</i>	KO	[26]
	<i>Granzyme B</i>	KO	[26]
	<i>iNOS</i>	KO	[39]
	<i>LT-<math>\alpha</math></i>	KO	[25]

Abbreviations: iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor-1; KO, knock-out; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ .

(a) P Georgel, K Crozat, B Beutler, unpublished.

recovered after a germline mutagenesis screen encompassing more than 9000 G3 mice. The discrepancy between our estimate of resistome size and the number of genes that have been experimentally proven to be required for antiviral resistance emphasizes the need for unbiased, phenotype-driven methods to uncover those molecules that serve non-redundant functions in antiviral immunity. ENU mutagenesis is one such method; however, inbred strains of mice constitute an enormous source of genetic diversity and we might also expect that thorough phenotypic analysis of the innate immune responses in these animals will also lead to the identification of relevant loci. Although neither method may disclose all elements of the resistome, each will provide leads upon which hypothesis-driven methods can successfully operate.

### Acknowledgements

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