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REVIEW

# Chemical mutagenesis: a new strategy against the global threat of infectious diseases

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**Abstract** The perpetual evolution of drug-resistant microbes, the overwhelming burden of acquired immune suppression due to HIV, the emergence or re-emergence of various pathogens (West Nile virus, pandemic influenza, Creutzfeld-Jacob disease), and increased fears of bioterrorism has drawn a great deal of new attention to infectious diseases. The pathogenesis of infection is characterized by complex interactions of potentially virulent microorganisms with host genetic and acquired factors. Chemical mutagenesis of the mouse genome provides a robust method to unravel this challenging problem. To deepen our understanding of the natural host response to pathogens, our team and others are interrogating the mouse genome to define genes that are crucial to the defense against infectious diseases (pathogen recognition, viral defense, bacterial defense, prion infection). In this review we highlight the current progress of these efforts and propose a toolbox for other groups that are interested in this endeavor.

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## Infectious diseases threat

At the beginning of the 20th century, infectious diseases were the leading cause of death in Eastern Europe and North America (actually worldwide). Better hygiene, vaccination, and the introduction of antibiotics brought a rapid decrease of the infectious disease burden by the 1960s, leading former US Surgeon General William H. Stewart to declare victory in the war against infectious diseases (Garrett 1994). In 2007, infectious diseases continue to be the leading cause of death in most parts of the world (WHO) (http://www.who.int/whosis/whostat/2007/en/index.html). The presence of microorganisms is clearly not sufficient for the development of infection; other genetic and nongenetic factors are required (Casanova and Abel 2004). Several epidemiologic and population studies have shown the contribution of the host genetic background to the risk and progression of infection in humans (reviewed in Hill 2006). These studies demonstrate clearly that the pathogenesis of infectious diseases is multifactorial and represents a complex interaction of microbial virulence determinants and geographical or environmental factors with host resistance/susceptibility genes. In this context, it is more critical than ever to better understand host-pathogen interactions and to develop new ways to modulate the host response. Identification of genes implicated in this relationship is an essential step toward achieving this goal.

## Gene discovery

The mouse is an excellent model organism to study the function of alleles that contribute to complex diseases in vivo and has been extremely useful in dissecting the genetic architecture of the host immune response against various human pathogens (Tuite and Gros 2006). This is true even if mouse and man have ~70 million years of divergent evolution with radically different microbial selection pressures during that time. Some criticisms were raised regarding the usefulness of the mouse model following the observation that some gene mutations, notably those in the IL-12/IFN-γ/STAT1, the TIR/IRAK4/NF-kB, and the TLR3/UNC93B/IFNα pathways affected susceptibility to a broad range of microorganisms in the mouse but only to a very few in the human (Casanova and Abel 2007). The relatively narrow range of human pathogen susceptibility for a given mutation may be due to increasing specialization of human innate immune recognition and response pathways, and/or the development of more robust compensatory immune mechanisms as a result of outbreeding, or other yet to be determined mechanisms. This difference does not negate the seminal insights into host resistance derived through ENU but rather brings out important interspecies variation that actually creates a deeper level of understanding of human immunity.

Characterization of the host immune response to infection enables the dissection of complex networks of interacting cells and biological pathways. For example, elegant studies in the mouse have demonstrated reprogramming of the host transcriptome during infection (Jenner and Young 2005). The Jenner and Young study also showed that several of the modulated pathways are not only involved with the host response against invading microorganisms but also with inflammatory processes, autoimmune diseases, and tissue homeostasis (Inohara and Nunez 2003).

Studies in mice have provided candidate genes for infectious disease association studies in humans and have brought significant advances on mechanisms of pathogenesis and host response to many types of infections (Vidal et al. 2008). Noteworthy examples include two innate immune genes, *Slc11a1* and *Tlr4*, that were identified by positional cloning in the spontaneous mouse mutants *Ity/Bcg/Lsh* and *Lps*, respectively (Poltorak et al. 1998; Qureshi et al. 1999; Vidal et al. 1993). In the laboratory mouse, *Slc11a1* was shown to control the innate response to infection with the intracellular pathogens *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium), *Leishmania donovani*, and several species of mycobacteria. In humans, polymorphic variants within or adjacent to the *SLC11A1* gene were associated or linked with susceptibility to tuberculosis in African, Asian, and Canadian Aboriginal populations as well as pediatric cases from South Africa (Greenwood et al. 2000; Li et al. 2006; Malik et al. 2005). *SLC11A1* is also involved in susceptibility with two other species of mycobacteria, *Mycobacterium leprae* (leprosy) (Alcais et al. 2000) and *Mycobacterium ulcerans*, the etiologic agent of Buruli ulcer (Stienstra et al. 2006). In mice, *Tlr4* was initially shown to be a crucial signal transducer for lipopolysaccharide (LPS, a major constituent of the outer membrane of Gram-negative bacteria) in vivo (Poltorak et al. 1998; Qureshi et al. 1999). In humans, two *TLR4* polymorphisms, Asp299Gly and Thr399Ile, were associated with impaired responsiveness to inhaled LPS in healthy volunteers and with an increased risk of Gram-negative infection, sepsis, severe inflammatory response syndrome, severe malaria, brucellosis, and respiratory syncytial virus disease (reviewed in Miller et al. 2005).

Currently, inbred and recombinant strains of laboratory mice presenting different degrees of resistance to a variety of experimental infections are the major resource available for forward genetic analysis of host resistance. Identification of infectious disease susceptibility genes using this resource is constrained by the finite number of strains and the limited range of natural genetic variation. To capitalize on genetic diversity, novel mouse models of infection have been developed using wild-derived mice (Caron et al. 2002; Turcotte et al. 2006), and a new panel of 1000 strains (the Collaborative cross), derived from an eight-way interstrain cross (Churchill et al. 2004), will soon be available for studies of the host response to infection. In addition, several large-scale initiatives are using the potent chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) to create new mouse mutants for the purpose of analyzing immune regulation and mechanisms of innate immunity (reviewed in Beutler et al. 2006; Hoyne and Goodnow 2006). This approach has led to important discoveries of the genes and pathways involved in the host response to infection. In this article we describe the initiatives and strategies using the germ line mutagen ENU to identify genes for host resistance against infectious challenge.

## ENU mutagenesis

Since the 1970s, the chemical ENU has been recognized as a potent mutagen in multicellular organisms, including plants (Veleminsky et al. 1970), *Drosophila* (Vogel and Natarajan 1979), and mouse (Russell et al. 1979). ENU causes a high rate of random point mutations, most commonly A-to-T transversions and A-to-G transitions (Justice et al. 1999), and a low rate of chromosome breakage compared to other mutagens (Vogel and Natarajan 1979). Using this mutagen, the whole genome of an organism can

be screened for genes that affect phenotypes of interest to an investigator. Theoretically, a mutagenesis screen could define the complete set of genes, recently defined as the "resistome" (Marathe et al. 2004), that are implicated in host resistance to an infectious challenge. Since mutagenesis may result in a complete loss of protein function or alteration of protein activity, genes with a lethal knockout phenotype may be identified through the generation of viable hypomorphic alleles. So far, 80% of the ENU mutations that have been recovered code for missense or nonsense mutations at the protein level (Takahasi et al. 2007) and approximately 70% have resulted in a loss of function phenotype (Cook et al. 2006).

The mutation rate in ENU-injected animals and the actual recovery of homozygous mutations per G3 animal varies depending on the inbred strain background, the mutagenesis protocol, and the breeding scheme used. Although the mutations induced are generally thought to be random (Takahasi et al. 2007), the probability of a mutation in a particular gene will vary depending on its length and the number of exons it contains. It is estimated that the mutagenized G0 mice transmit between 30 (Beutler et al. 2006; Cook et al. 2006) and 100 (Crozat et al. 2006; Nelms and Goodnow 2001) random functional mutations to each G1, resulting in 4-12 homozygous defects in each G3 animal.

## Pool of genes tackled by ENU

One important question concerns the number of genes that are involved in a given resistome. The resistome of the mammalian genome infected with mouse cytomegalovirus (MCMV) has been estimated at approximately 480 genes (Crozat et al. 2006). The same group has predicted the number of ENU targets for the Toll-like receptor (TLR) signaling pathway to 44 genes. This estimation is based on the identification of 11 mutations in ten different genes, including five that were previously known (50%) (Beutler et al. 2005). At that time, 22 genes were known to form the TLR pathway; hence, by extrapolation the total number of genes in the TLR pathway was estimated at 44. Therefore, depending on the nature of the infectious diseases phenotype, 50-500 target genes may be expected for a given resistome, taking into account that these two examples represent a broad phenotype (clinical disease or death following virus inoculation) or the more narrowly defined response of a population of explanted cells to microbial structures. The uncertain size and overlap of individual resistomes also highlights the potential for redundancy of related phenotypic screens in mice, as demonstrated for MCMV and vesicular stomatitis virus (VSV) infections (Beutler et al. 2005). Conversely, the discovery of the molecular basis of several forms of human primary immunodeficiency indicates that mutations in specific genes regulate the response to a very narrow range of pathogens (one gene, one infection) (Casanova and Abel 2007). For example, the IL-12/IL-23–IFNα pathway is essential in the host defense against mycobacterial and *Salmonella* infections but not for other infectious agents (Fieschi and Casanova 2003; MacLennan et al. 2004). This is also the case for herpes simplex virus 1 (HSV-1) encephalitis susceptibility caused by a mutation within the *UNC93B1* (Casrouge et al. 2006) or *TLR3* (Zhang et al. 2007) genes, and for predisposition to certain common infectious diseases by major susceptibility genes (Casanova and Abel 2007).

## Optimization of a breeding scheme for infectious diseases screen

A critical parameter for any ENU screen is to establish an unambiguous phenotype that has minimal background variation to reduce the number of false-positive results. Ideally, the assay used should also be sensitive enough to allow the identification of genes with subtle effects on the phenotype, be nonlethal, and should allow high throughput. Overall, a sensitive and robust phenotype will facilitate efficient screening of a large number of animals and pedigrees that carry a limited number of homozygous functional mutations. Identification of phenodeviants followed by outcrossing to an informative strain is then performed to confirm and map the presence of the mutation.

The choice of a suitable phenotype for an infectious disease screen is one of the substantial challenges to ENU mutagenesis. Frequently, investigators must choose between a lethal endpoint caused by the infectious challenge itself or during the cell/tissue collection process and the use of a nonterminal phenotypic assay. In some cases the rapidity of data collection justifies the use of a survival or death phenotype but does require the propagation of the mutation using viable G3 animals for confirmation and eventual identification of the causative mutation. A clinically relevant phenotype such as severe disease or death also reflects the complexity of the physiologic immune response to a specific pathogen, and consequently will lead to the identification of the most relevant molecular determinants (as noted by Rolf Zinkernagel comment in Zinkernagel 2007). An alternative approach would be to use a sublethal phenotype followed by rescue with antimicrobial therapy, thereby allowing further breeding of the viable G3 animals. Another approach, limited probably to genes of the host essential for a viral infectious cycle, would be to screen for genes that allow the affected animals to survive an otherwise lethal infection, allowing propagation of the mutated line by breeding resistant G3s.

As previously mentioned, each G1 animal will carry approximately 30-100 functional mutations from a pool of

about 300 mutations present in the G0 spermatogonial cells (Bode 1984). Generation of four G2 mice for each G1 should allow recovery of 94% of the mutations in a pedigree. To test the effect of recessive mutations in a three-generation breeding scheme, G2 mice can be either intercrossed or backcrossed to the G1 male. For an intercross, 78% of the expected mutations can be screened by setting up four G2 per G1 pairs and then testing G3 per G2 pairs (Nelms and Goodnow 2001). In this case, the number of testable mutations can be doubled by initially crossing two unrelated G1 animals. Alternatively, an equivalent number of mutations may be evaluated (Nelms and Goodnow 2001) by backcrossing four G2 sisters with their G1 father and then testing four G3 per G2 mother. On the other hand, since generation of G1 males is usually not a limiting step, a greater number of mutations can be identified by backcrossing a single G2 female to its father while increasing the total number of G1 males that are tested and maximizing the number of mutagenized G0. This strategy is advantageous since it simplifies the manipulation of mice and reduces potential handling errors.

### Mapping of causal mutations

Time can be saved in the initial localization of an ENU mutation by directly crossing the G0 and G1 animals to an informative strain. While this is an attractive mapping strategy, an informative breeding scheme may confound subsequent gene identification by introducing background genetic variability that influences the expression of the selected phenotype. In the case of susceptibility to infection, the introduction of a new genetic background early in the pedigree may result in a high degree of variability in the observed phenotype. The introduction of modifier genes may make impossible the identification of true ENU mutants, as we observed in our first MCMV screen (S. Vidal et al., unpublished data). Such an approach has been used for robust phenotypes like organogenesis deviants (Herron et al. 2002).

Fortunately, rapid progress in genome sequencing of several strains of mice and the consequent proliferation of single nucleotide polymorphisms (SNPs) have made possible the use of closely related inbred strains to minimize phenotypic variability while retaining the ability to map the mutations directly in the G3 animals using two to four markers per chromosome. This approach has been used with the two closely related strains C57BL/6J and C57BL/10J (Croker et al. 2007; Crozat et al. 2007) and using 129S1 and the 129X1 inbred strains for which the genetic variability is approximately 7.5–8.4% (based on the Wellcome Trust and the Mouse Phenome SNP databases), as recently done by us. This strategy allows mapping the ENU mutations in a three-generation breeding scheme instead of five, facilitating the establishment of a mutant pedigree for which a new informative cross can then be set up.

A third approach is to screen G3s from a homogeneous background. The mutation can then be fixed in a mutant pedigree by setting random pairs of G3s (if the phenotype is lethal) and subsequently using the resulting progeny to set up an informative cross for mapping the mutation. These G3s will have no genetic variability other than the one introduced by the ENU mutagen and the normal genetic drift, therefore reducing the risk of the introduction of modifier genes affecting the phenotype. This approach was used successfully by several groups, including Beutler's group (Croker et al. 2007; Hoebe et al. 2003a).

## Infectious challenge screens

Table 1 summarizes the known initiatives.

### McGill University group

To deepen our knowledge of the natural host response to pathogens, our team undertook an interrogation of the mouse genome to define genes involved in pathogen recognition, viral [coxsackievirus B3 (CVB3)], and bacterial (*Salmonella*) defense (Table 2). The pathogens were selected based on their effect on human health and the availability of a robust mouse model. Our initial recessive breeding scheme involved breeding four G2 pairs for each G1 pair issued from two independent G0 mutagenized males to produce the phenotyped G3s (Fig. 1A). This was initially selected to maximize the number of phenodeviants identified while maintaining the X chromosome screen. The pedigree was built in a way that the G3 progeny were used for all the phenotypes. 129S1/SvImJ (129S1) mice were selected for mutagenesis (G0) and subsequently bred with C57BL/6J females to generate the first-generation (G1) progeny. Two independent G1s (different G0 parents) were intercrossed to maximize the number of mutations segregating in G2 progeny. For each G1 pedigree, four G2 brother-sister pairs were mated. Using this breeding scheme, we introduced in the G2 population the C57BL/6J *Slc11a1 Salmonella* susceptibility allele ($Slc11a1^{Asp169}$). The G2 mice were then genotyped for *Slc11a1* and selected for the wild-type resistant allele ($Slc11a1^{Gly169}$) for further G3 breeding. Because the endpoints of all the phenotypic screens involving viral and bacterial infections result in death of the G3 animals, G2 fathers of a deviant were outcrossed to wild-type female. $F_1$ female offspring were backcrossed to the G2 male to produce N2 progeny, which were used to map and recover the mutation.

*Screen for defective microbial recognition by the innate immune system* Microbial recognition by the innate

**Table 1** Infectious diseases screen initiatives: Summary of the published and proposed ENU screens involving infectious diseases phenotypes

| | Screen | Model | Web site | G2/mutant |
|---|---|---|---|---|
| GBF | *L. monocytogenes, S. pyogenes, Y. enterolitica* | Proposed | www.gsf.de/ieg/groups/genome/enu.html | |
| McGill | CVB3 | 129S1 survival | www.mcgill.ca/hostres/enu/ | 86 |
| | PAMPs | 129S1 C57BL/6 splenocytes | | 145 |
| | *Salmonella* Typhimurium | 129S1 survival | | 82 |
| McLaughlin | Prions | Proposed | www.montana.edu/wwwmri/enump.html | – |
| Scripps | MCMV | C57BL/6 | www.scripps.edu/imm/beutler/research.html | 83 |
| | PAMPs | C57BL/6 peritoneal macrophage | | 370 |
| | *L. monocytogenes* | Proposed | | |

**Table 2** McGill group summary: screening output from the three phenotype analyzed

| | *Salmonella* | CVB3 | PAMPs | Total |
|---|---|---|---|---|
| Mice tested | 2274 | 1200 | 1207 | 4681 |
| G2 (pairs or female) | 246 | 172 | 145 | 563 |
| G1 | 137 | 139 | 47 | 323 |
| Deviant pedigrees | 11 | 6 | 16 | 33 |
| Confirmed deviant | 3 | 2 | 1 | 6 |
| Mapped deviant | 1 | 0 | 0 | 1 |

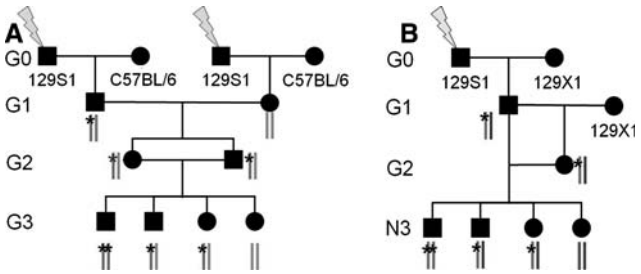


**Fig. 1** Breeding scheme. (**A**) Initial breeding scheme, the G0 129S1 males were mutagenized and outcrossed to C57BL/6 to generate the G1, G2, and the phenotyped G3 animals. Two independent G1 animals were crossed to augment the total number of recovered mutations. (**B**) The actual breeding scheme involves outcrossing mutagenized G0 129S1 males to 129X1 females to generate G1 males that are backcrossed to 129X1 females to generate G2 females. The G2 females are then backcrossed to the G1 male to produce the phenotyped N3 animals

immune system is an essential first step in robust host defense (Akira et al. 2006). To probe the molecular mechanisms that mediate pathogen recognition, an in vitro screen was developed to analyze activation of immune cells from ENU-mutagenized mice following stimulation with a panel of structures, termed pathogen-associated molecular patterns (PAMPs), that are unique and essential to microbial physiology. PAMPs selected for the screen included lipopolysaccharide (LPS), lipoteichoic acid, double-stranded RNA (poly I:C), unmethylated CpG DNA motif oligonucleotide (CpG DNA), and zymosan that represent bacteria, viruses, and fungi. A homogeneous cell suspension was obtained from the spleen of individual G3 mice by mechanical disruption and erythrocyte lysis and stimulated overnight with individual PAMPs. The cell supernatants were assayed for the production of proinflammatory cytokines tumor necrosis factor $\alpha$ and interleukin-6, and polyclonal cell proliferation was determined by radiolabeled 3H-thymidine incorporation. A total of 47 G1 pedigrees yielding 1207 G3 mice have been screened for defects in microbial recognition using this protocol. Several pedigrees produced mice with hyporesponsive phenotypes that were not due to heritable or fully penetrant defects. In one pedigree, a deviant animal demonstrated a reproducible unresponsive phenotype following stimulation with CpG DNA but had normal responses to the other PAMPs. A homozygous mutant line has been established from the G2 progenitor pair, and an outcross-backcross population derived from the relevant G2 male is being generated to localize the mutation.

*Salmonella screen* *Salmonella* causes human diseases of important public health concern (typhoid fever and salmonellosis). After oral infection of the mice, *Salmonella* Typhimurium invade epithelial and M cells of the intestine, migrate to mesenteric lymph nodes (MLN), reaching the blood stream to establish a systemic disease resembling human typhoid fever. After interacting with complement factors, the bacteria reach an intracellular location within the macrophages and the neutrophils. The phase of innate immune response is followed by activation of a complex host response that suppresses the growth of bacteria in tissues and coincides with the formation of macrophage-rich microgranulomas. Clearance of infection requires both CD4 + and CD8+ T lymphocytes (Mastroeni 2006). With the exception of mice of the 129 family (129S1/SvImJ, 129S6/SvEvTac, or 129X1/SvJ), most other inbred mouse strains are susceptible to infection by *Salmonella* Typhimurium. Susceptibility is usually assessed by survival to infection or by measuring the bacterial load in target organs

(liver and spleen). For the ENU recessive screen, the G3 animals were subjected to an infectious challenge of 1000 CFU of *Salmonella* Typhimurium IV. Early susceptibility to infection was used as phenotype to minimize false-positive results: animals moribund or dead before day 8 were considered susceptible. Families in which susceptible animals were identified in at least two litters were then further investigated. Most of the other animals survived the challenge, indicating that the phenotype observed in susceptible animals was most likely not a consequence of background variability.

Eleven deviant pedigrees were identified by screening for *Salmonella* susceptibility a total of 2274 mice issued from 297 G2 pairs. The transmissibility of the causal mutations was demonstrated in three instances by outcrossing the G2 male from each interesting pedigree to a DBA2/J female. The resulting females were then backcrossed to the G2 male and the F2 progeny phenotyped. The choice of DBA/2J mouse strains to generate a fully informative mapping panel was based on the necessity to select a strain that carries wild-type allele for the major innate *Salmonella*-susceptibility genes *Slc11a11* and *Tlr4*. Using this approach the mutant mice gene *Immunity to* Typhimurium *9* (*Ity9*) was mapped to chromosome 6. The mapping was achieved using the Beier group's 768 SNPs panel (Moran et al. 2006) by identifying regions of the genome of eight affected F2s that were homozygous for 129S1 alleles. Uninfected G3s were then genotyped and selected for homozygosity for 129S1 alleles on chromosome 6 and pairs were made, fixing the mutation in a homozygous line. The fact that two independent G1s were used to generate G2 brings the possibility that unaffected animals (G3s or F2s) carry a mutant and a wild-type 129S1 chromosome 6 region for *Ity9*, making the mapping possible only with affected animals. The breeding scheme was then adapted to a backcross strategy to be able to use all animals (affected and unaffected) for finer mapping (Fig. 1B). The region identified on chromosome 6 is large (8.1 MB) and contains 162 genes, a subset of which has been selected for sequencing based on their expression in macrophages or other immunologically relevant cells and function for complete evaluation.

*CVB3 screen* CVB3 is an important human pathogen causing over 30% of the myocarditis cases in North America. Infection by CVB3 is also an important risk factor of acquired heart failure in children and dilated cardiomyopathy in adults (Gauntt and Huber 2003; Roivainen et al. 2000). Experimentally, CVB-infected mice faithfully replicate human disease but display heterogeneous pathology depending on their genetic composition. Mouse strains that are relatively resistant present mild transient myocarditis, while susceptible mice succumb of severe myocarditis. Both viral cardiomyocyte destruction and overstimulated immune infiltrates of the host play a significant role in CVB3 susceptibility (Liu and Mason 2001). In addition, integrity of myocardial structure and of the type I interferon (IFN) response is critical for protection. IFN$\beta$ and IFN-$\alpha/\beta$ receptor (Wessely et al. 2001) and *Ifnb1* knockout mice (Deonarain et al. 2004) have increased viral load and severity of the disease, suggesting a protective role for type I IFNs against CVB3-mediated pathology. Type I IFNs also regulate and enhance the antiviral activities of NK cells (Biron et al. 1999), which play an equally important role in CVB3 clearance and host survival (Godeny and Gauntt 1987). Finally, CD8+ T cells are protective later in the infection, whereas CD4+ T cells seem to contribute to disease in susceptible mice (Ayach et al. 2003).

Our breeding scheme using $129S1^{ENU} \times$ C57BL/6 had the advantage of providing excellent fertility and the opportunity to use mice from the same cross for the three phenotypic screens which simplifies mouse management. However, the use of such a strain combination introduces some genetic variability that may impact the response to viruses. Therefore, we decided to modify our breeding scheme by crossing $129S1^{ENU}$ with 129S1 wild-type to produce G1 male and G2 female, which were backcrossed to G1 to generate homogeneous N3 mice. These mice were screened for susceptibility to CVB3 at 7 weeks of age. The mice were infected by intraperitoneal injection of $10^4$ plaque-forming units of CVB3 (Aly et al. 2007) and monitored for survival over 14 days. To focus on the early phase of host response, we prioritized pedigrees where mice succumbed before day 8 postinfection. The screened 139 G1 pedigrees (about 1200 N3 mice) revealed four deviant pedigrees with heritable susceptibility. Two of these mutant pedigrees, named *Greasy* and *Heartfelt*, were recovered at Mendelian frequencies in an outcross with the closely related mouse strain 129X1. In secondary postinfection phenotyping using histopathologic analysis, *Greasy* was shown to present cardiac steatosis. Accumulation of lipid inclusion in cardiomyocytes was particularly observed at the atria, with spreading into the ventricular wall in most affected animals (ratio 7:33). *Heartfelt* presented a very different phenotype characterized by large areas of cell destruction in the heart (ratio 8:30). Notably, no cell infiltrate was observed in the myocardium. Previous experience with CVB3 and other models of CVB3 susceptibility (e.g., *mdx*) suggest that these ENU-derived phenotypes define two new models of host susceptibility to infection.

## Scripps research institute group

The group directed by Bruce Beutler at the Scripps Research Institute has used a recessive genome-wide

mutagenesis screen to identify genes involved in the host innate immune response (for a comprehensive list of the identified mutants see http://mutagenetix.scripps.edu). One major aim of the project was to identify additional components of the TLR signaling pathway. A TNF-mediated cell-killing assay ex vivo was used to measure the response of peritoneal macrophages to different TLR agonists. In this particular project, C57BL/6J mice were mutagenized and a homogeneous genetic background was maintained. Selected G3 mice were outcrossed to C3H/HeN to confirm the heritability of the deviant phenotype and to map the mutation (Hoebe et al. 2003b). Eleven transmissible mutations affecting ten different genes were identified by screening about 20,000 G3 mice. Eight of the genes were positionally cloned and were identified as important players in the TLR sensing and signaling pathways (for an excellent detailed review, please refer to Beutler et al. 2006). These genes include *Unc93b1*, a gene participating in signaling by "intracellular" TLRs; *Trif*, a gene important in Tlr4 and Tlr3 signaling (Hoebe et al. 2003a); *Cd36*, a new sensor of diacylglycerides (Hoebe et al. 2005); and new mutants of *Cd14*, *Myd88*, *Tlr6*, and *Tlr9* (Jiang et al. 2005, 2006). In a dominant screen for innate immune signaling modifications, *PanR1*, a *Tnf* mutation, was also mapped with a C3H/HeN outcross (Rutschmann et al. 2006).

Mutants identified in the TLR screen of peritoneal macrophages were further tested for susceptibility to bacterial and viral infections. The oblivious (*Cd36*) mutation enhances susceptibility to Gram-positive *Staphylococcus aureus* infections (Hoebe et al. 2005) and the *PanR1* mutation confers susceptibility to *Listeria monocytogenes* (Beutler et al. 2006). The *Unc93b1* mutation causes extreme susceptibility to MCMV infection and enhances susceptibility to *Staphylococcus aureus* (Tabeta et al. 2006). The *Lps2* (*Trif*) (Hoebe et al. 2003b), *CpG1* (*Tlr9*) (Tabeta et al. 2004) and *Pococurante* (*MyD88*) (Beutler et al. 2006) mutations were also shown to increase susceptibility to MCMV infection. These discoveries clearly show the relevance of studying a discrete process such as innate immune signaling to deconstruct the highly complex genetic regulation of host susceptibility to infectious diseases.

Analysis of phenodeviants selected for visible skin defects also allowed this group to identify *Scd1* as a critical gene for defense against two Gram-positive bacteria, *Streptococcus pyogenes* and *Staphylococcus aureus*, which are known to cause a variety of human diseases. This finding is of particular clinical relevance given the emergence of drug-resistant *Staphylococcus aureus*, a major cause of nosocomial infection (Georgel et al. 2005). Finally, the mutant *Rxrα* was also identified using a similar approach and shown to enhance Th1 response to OVA antigens (Du et al. 2005).

For the MCMV screen performed by this group, mutations could not be propagated using the phenotyped G3 animals. To circumvent this problem, more G3s were produced and randomly mated to bring putative mutations to homozygosity. So far, ten mutant pedigrees have been isolated, leading to the identification of a mutation in *Stat1* (Crozat et al. 2006) and the identification of a truncated version of *Unc13d*, the ortholog of MUNC 13-4 implicated in type 3 familial hemophagocytic lymphohistiocytosis (Crozat et al. 2007). Finally, three independent pedigrees with extreme MCMV susceptibility were shown to be defective in Kir6.1, a component of an ATP-sensitive potassium channel. In this particular case, the mutation originated from a partial deletion of *Kcnj8* (encoding Kir6.1) in the mouse stock used in the mutagenesis program (Croker et al. 2007).

## Future of screens

Most large-scale ENU mutagenesis projects around the world are dominant screens that study visible, biochemical, immunologic, and behavioral phenotypes, while very few are focused on the analysis of the immunologic response to microbes using a recessive screen. One limitation of doing a recessive screen is that a large number of animals have to be produced and tested, with approximately one phenodeviant identified for every 1800 G3 phenotyped (Beutler et al. 2006; this article). Even if such an approach appears lengthy, mutagenesis is an efficient method for identification of causative Mendelian genes. In fact, the reported recessive screens have been extremely successful in identifying critical players in the host response to specific pathogens. Bruce Beutler's group has demonstrated that a focused screen (PAMPs signaling) can be successfully used to investigate the genetic architecture of a more complex clinical problem such as infectious disease susceptibility. The advantage of this approach is to decrease phenotyping noise, although it targets a specific biological process rather than approaching a problem from a more general perspective. ENU mutagenesis applied to infectious diseases will identify major gene effects, although it may not be able to identify subtle genetic contributions to the complex response to infectious challenge that may be necessary, but not sufficient, to cause clinical illness. On the other hand, a relevant gene for host defense against a particular infectious disease may have an essential function in mouse development and may not be identified because of embryonic lethality. Chris Goodnow's group has developed a sensitized screen to identify heterozygous loss-of-function variants that confer immunologic tolerance in type 1 diabetes that normally would have lethal effects and remain undetected (Hoyne and Goodnow 2006). As pointed out in

this review, it will also be interesting to develop nonlethal screens with antimicrobial rescue (*Streptococcus pneumonia* infection with blood CFU as phenotype), use of surrogate clinical markers (body temperature, weight), conventional biomarkers (leukocyte count, hemoglobin), and novel biomarkers (e.g., procalcitonin in sepsis) to be able to quickly fix the mutation in a mutant pedigree.

Other groups have initiated or proposed screens for a broad range of infectious diseases. The TUM (Technische Universität München/GBF) initiated a program under the NGFN in which they plan to establish ENU-mutagenized mouse lines selected in immunologic alterations and screen the mice response to *Listeria*, *Streptococcus*, and *Yersinia* (http://www.helmholtz-hzi.de/en/research/research_programme/technological_platforms/infection_challenge_platform/). A screen involving the infectious prion protein has been initiated at the McLaughlin Research Institute by Carlson's group (www.montana.edu/wwwmri/carlson.html) (Clark et al. 2004). The screen has been used to find genes that modify the prion incubation time. They have used a three-generation breeding scheme to bring recessive mutation to homozygozity in G3 mice by initially outcrossing mutagenized C57BL/6J to C3H.SW-H2b/SnJ to produce G1 males that were backcrossed to C3H.SW. The resulting daughters were backcrossed to the G1 male to produce the phenotyped G3s (www.montana.edu/wwwmri/enump.html).

To face the infectious diseases threat, ENU mutagenesis appears to be a valuable tool. The cost effectiveness of this approach is appreciable; although costly to initiate, the main limitation is in the identification of the mutations generated. So far in at least one instance a gene identified by ENU-induced mutation was directly linked to human susceptibility to pathogens by causing a premature nonsense codon of the human ortholog of *Unc93b1* in two Herpes simplex virus-1 encephalitis patients (exons 6 and 8) (Zhang et al. 2007). ENU mutagenesis will provide an invaluable resource of new animal models for the study of host resistance to infection that will result in a deeper understanding of the delicate interplay between the host and the pathogen. Such knowledge is critical to the investigation of novel therapeutic approaches.

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