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Minireview

Signaling advances from immunogenetics to immunogenomics
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Abstract

Recent studies describe new genome-wide mutagenesis strategies, coupled with phenotypic screening, and demonstrate the power of such approaches to provide new insights into the genetics of the immune response.

A spate of recent articles in *Nature, Journal of Biology*, and *Immunity* [1-4] herald the ripened fruit of mutagenic and genomic approaches to dissecting the molecular mechanisms of immunity. Genetics has long been of central importance in elucidating the nature of immune responses, its roots extending at least as deeply as the establishment of systems for understanding histocompatibility and immune regulation. The 'pre-genomic' era produced insights into autoimmune-disease susceptibility using the non-obese diabetic (NOD) strain of mice [5]. Somatic-cell mutants unresponsive to interferons were generated and characterized, thereby leading understanding of Jak-STAT signaling pathways [6-8]. The *TLR4* gene was identified as the basis for lipopolysaccharide (LPS) unresponsiveness in C3H/HeJ mice [9], and a mutated NF-κB-inducing kinase gene was found to be the cause of the alymphoid (*aly*) mutant mouse strain [10]. Moving into the post-genomic era, these exciting precedents have inspired systematic attacks on the generation and analysis of mutant mouse strains to achieve a greater saturation of the gene pool and screening of these strains for immune system phenotypes. Papers exemplifying each of several mutational approaches establish that global mutagenic strategies are already paying dividends in new understanding and promise even greater yields in the future.

At least four systematic approaches are now in use, employing oncoretroviruses or chemical mutagenesis, in cell lines or mice. Because of the greater ease and lower costs of screening and selection strategies with mammalian cell lines rather than whole animals, cellular approaches have provided some of the earliest targets and success stories of genome-wide approaches [6,7,11,12]. Evidence that Carma1/CARD11, a member of the MAGUK (signaling-associated scaffold) family of proteins, is essential for induction of NF-κB transcription factors by the T-cell receptor (TCR) and CD28, is one recent finding that exemplifies the success of such strategies for studying immune regulation [13]. In this study, a powerful screening strategy was implemented in a widely used human T-cell line, Jurkat. A synthetic NF-κB-dependent promoter was used to govern the expression of green fluorescent protein (GFP) and chemically mutagenized cells were identified that were deficient in the induction of GFP in response to activation of the TCR but normal in terms of the response to stimulation by tumor necrosis factor α (TNFα). From a panel of such cells with a selective block to signaling, one variant was shown to be deficient in Carma1, a defect reversed by restoration of Carma1 expression. This mutation caused a loss of NF-κB induction as a result of defective relays between the cell surface and the transcription factor, and also diminished TCR-induced activation of the JNK but not the ERK subtype of mitogen activated protein (MAP) kinase [13].

Retroviral mutagenesis methods

Although an antecedent to results from concerted mouse-based mutagenesis work, the ability to use cell lines in genome-wide approaches is impacted by issues of ploidy, genetic variation, and barriers to mapping studies using somatic cells. Instead of an approach such as that described above, which is oriented to recessive mutations, more recent

work has used a screening strategy based on dominant mutations in Jurkat cells [2]. For this study, a novel retrovector was generated that permits tetracycline regulation of the expression of inserted cDNAs (Figure 1). The vector is replication-deficient because the promoters of the retroviral long terminal repeats have been mutated, and it carries a GFP cDNA to allow selection of transduced cells. After insertion of mutated cDNA libraries into this retrovector downstream of a tetracycline-inducible promoter and transduction of the Jurkat T-cell line, individual cells were screened by fluorescence-activated cell sorting to identify those exhibiting decreased expression of a marker, CD69, known to be downstream from TCR ligation. Importantly, this study validated the ability to identify scores of genes for which mutations (generated while creating the cDNA library) attenuated TCR signaling to the CD69 gene [2]. Furthermore, the incorporation of an on-off system controlling expression of the cDNA made a critical screening step possible by simple reversion testing - loss of the mutant phenotype when the expression of the cDNA insert was turned off. The completion of genome sequences and availability of tools sufficiently robust to handle the numbers of cDNAs screened (2,828 in the study in question [2], of which 1,323 were validated by reversion) link this approach to the post-genomic era.

Such a cell-based, dominant selection system offers a powerful tool for the identification of molecules that impact a signaling pathway, albeit at the cost of several drawbacks. Discovery will be limited to those molecules for which dominant negatives have an effect on the pathway, and there will be inherent pitfalls in the use of any cell line: for example, lipid-kinase signaling in most Jurkat T cells will be abnormal because of pre-existing loss-of-function mutations in the *PTEN* lipid phosphatase/tumor suppressor genes. Nonetheless, this or analogous retrovector systems can be used for the transduction of primary lymphocytes to perform further validation. Intriguingly, variations on this approach offer potentially great power in structure-function analyses with selected molecules. Single cDNAs could be subjected to high-density mutagenesis and, through the on-off capabilities of the approach, microarray analyses would allow measurement of the changes in global gene-expression profiles that result from graded interference with a given pathway.

The applicability of a finding to immune regulation ultimately depends on its validation in the immune response of animals. In principle, such retroviral technologies could also be applied to the transduction of hematopoietic cells (for example, lymphocytes or dendritic cells) derived from mouse lines in which loss-of-function mutations have been engineered by other approaches. This extension of the approach could allow high-throughput structure-function screening in primary cells independent of the need to achieve dominant-negative effects. In parallel, however, enhanced techniques for random mutagenesis, phenotypic screening, and gene identification have been important goals. To facilitate the process of cloning mutated genes after mutagenesis, another class of mutagen takes advantage of the fact that retrovirus insertion in the genome can be engineered so as to achieve loss-of-function mutations at high efficiencies ([14]; reviewed in [15]). In particular, vectors based on oncoretroviruses are modified to encode a selectable trait whose expression depends on insertion into an active transcription unit. After bulk infection of pluripotent embryonic stem (ES) cells with high-titre retrovirions, individual clones can be micro-injected and transmitted to the germ line, so that progeny can be mated to homozygosity. As an example of the early success and potential of this strategy of retroviral mutagenesis, a novel link was identified between the mouse *FUS* gene, chromosomal stability, and B-lymphoid proliferation [16]. This technology promises to provide further successes, which will be facilitated by the creation of banks of mutated ES cells for which a sequence tag identifying the mutated gene is publicly accessible. Two examples of ES-cell banks currently available are from a for-profit company, Lexicon Genetics [17], and a program funded by the National Heart Lung and Blood Institute and linked to BayGenomics [18].

Ethylnitrosourea mutagenesis screens
Progress in genome sequencing and annotation, and saturation with sufficient genetic markers for gene mapping, have dramatically enhanced our capacity to identify a gene mutated by chemical mutagens that create point mutations. These capabilities, coupled to recognition of the powerful advantages of ethylnitrosourea (ENU) as a mutagen [19], set the stage for several of the recent successes noted at the beginning of this article. ENU is a highly efficient mutagen, able to create multiple mutations in the germ line of each male breeder, which can be coupled with breeding strategies to perform recessive screens (Figure 2) [20-22]. In the case of mice with mutations in the *Lps2* gene, this approach has revealed an essential component of signaling by some, but not all, Toll-like receptors (TLRs) operating as part of a pathway independent of the MyD88 relay protein [1,23]. Mutagenesis and screening for defects in *ex vivo* production of TNFα by macrophages were initiated on an extensively used inbred genetic background, C57BL/6. Notably, this work involved approximately 2,650 G1 mice, heterozygous for any mutation, and around 4,600 G3 mice, bred for the generation of homozygotes, underscoring some of the cost and logistical issues in such screens. The authors of these studies [1,23] focused on a homozygote whose macrophage response to endotoxin (lipopolysaccharide) and double-stranded RNA was abrogated while activators of other TLRs elicited normal TNFα responses. Gene mapping and high-throughput sequencing identified a mutation in the gene encoding a previously known adapter protein, Trif/Ticam-1, that leads to a small carboxy-terminal substitution. Although this gene product had already been implicated in TLR signaling, the *Lps2* mutant mouse lines establish several novel features of the both the protein (Trif/Ticam-1) and the
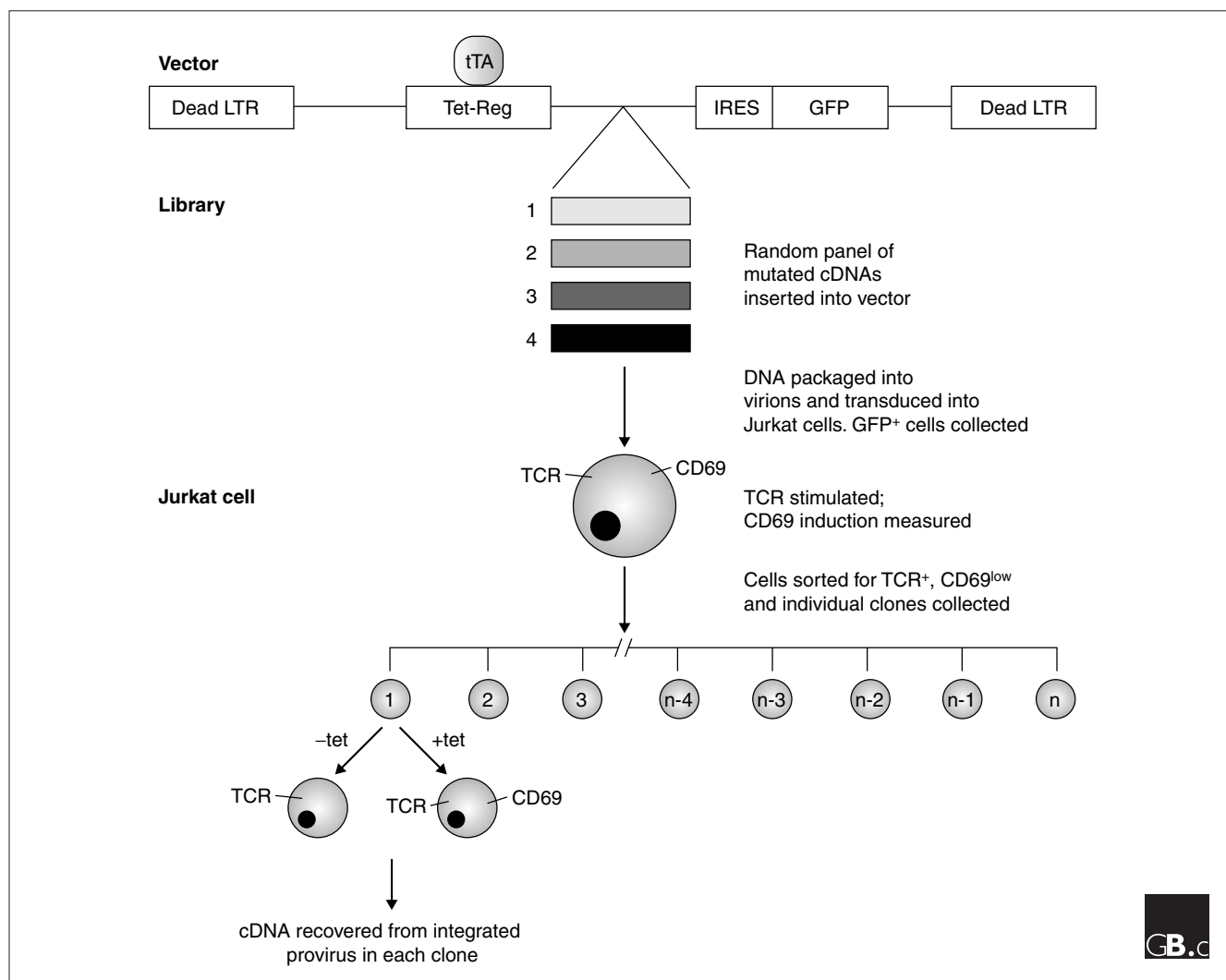
Vector

| Dead LTR | Tet-Reg | | IRES | GFP | Dead LTR |

tTA

Library

1
2 — Random panel of mutated cDNAs inserted into vector
3
4

DNA packaged into virions and transduced into Jurkat cells. GFP+ cells collected

Jurkat cell

TCR — CD69

TCR stimulated; CD69 induction measured

Cells sorted for TCR+, CD69low and individual clones collected

1   2   3   n-4   n-3   n-2   n-1   n

−tet   +tet

TCR

TCR — CD69

cDNA recovered from integrated provirus in each clone

GB.c

Figure 1
A dominant strategy for the identification of signaling molecules using a tetracycline-regulated retrovector. A replication-defective retrovector was generated in which the normal transcriptional promoters of the long terminal repeats were crippled ('dead' LTR), and a promoter (Tet-Reg) repressible by the tetracycline trans-activator protein (tTA) in the presence of a tetracycline antibiotic, such as doxycycline, was inserted upstream from cloning sites, an internal ribosomal entry sequence (IRES), and GFP cDNA. After cloning a library of mutated cDNAs into the vector, the DNA was packaged into virions, and a large population of modified Jurkat T cells (carrying the tTA) was transduced with the virions; GFP expression was used to select clones expressing a unique cDNA. Cells stimulated through their antigen receptor (TCR) were scored for retention of receptor expression and loss of induction of a molecule downstream from TCR signaling, CD69. Individual clones could be re-scored for reversibility of the phenotype by comparison of results in the presence (+tet) or absence (-tet) of doxycycline. Individual, sorted clones could then be used for isolation of the cDNA cloned into the retrovector. Adapted from [2].

MyD88-independent signaling pathway. In particular, these studies make clear the essential role of the Trif/Ticam-1 protein in signaling by several members of the TLR gene family, including some whose signaling also uses MyD88. The results also indicate that other TLRs use an Lps2-independent, MyD88-dependent means of signaling.

Another triumph of functional screening at a genome-wide level has emerged from a separate program, resulting from similar ENU mutagenesis and recessive screening in the same C57BL/6 background [3,4]. This large-scale program focussed on the identification of mutations leading to immune dysregulation [22]. Founder mice were identified by a quantitative screen that detected differences in the profile of surface immunoglobulin M (sIgM) on circulating B lymphocytes that also carried IgD. In addition to a two-fold increase in sIgM, closer examination also revealed a modest increase in another molecule linked to signaling, CD21. Gene mapping localized the point mutation to the Carma1/CARD11 gene. Rather than a loss of expression, as
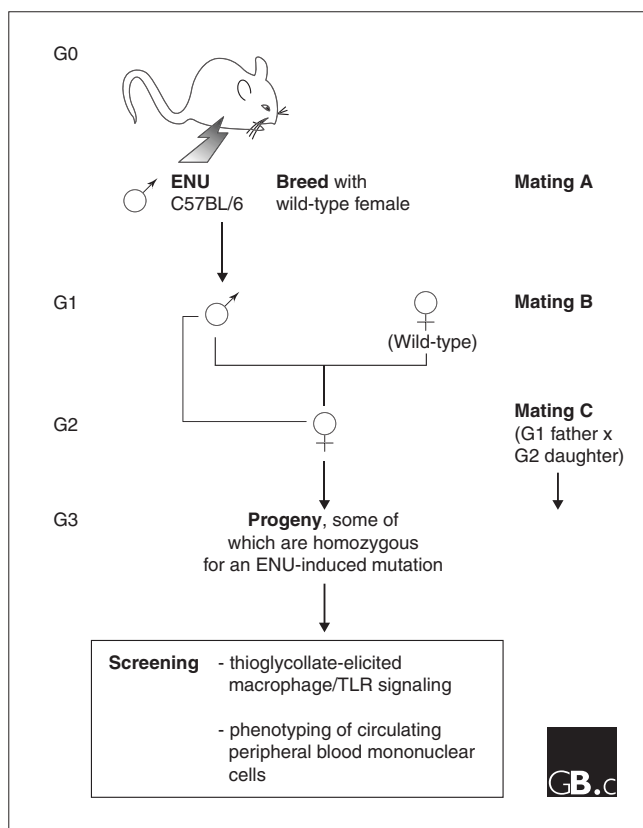
Figure 2
Recessive screens for defects in mouse immunity. One of two breeding strategies that can be used for large-scale screening programs is illustrated. Ethylnitrosourea (ENU) is used to generate a high burden of mutations in the germ line of male mice in the G0 generation (approximately 100 per male). These mutations are transmitted by breeding with wild-type females (Mating A), creating a panel of progeny in the G1 generation that are heterozygous for some of the mutated alleles. Males of this G1 generation can again be bred to wild-type females (Mating B), leading to a large family of individuals in the G2 generation, many of which are heterozygous for mutated alleles. Female G2 mice, when bred with their G1 father (Mating C), bear progeny of whom some will be homozygous for an ENU-induced mutation. Alternatively, G1 males and unrelated G1 females can be crossed, and the G2 progeny of these mice can participate in brother-sister matings that can generate homozygotes. Adapted from [22].

found in the prior work in Jurkat cells (see above, [13]) and concomitant papers using gene targeting [24,25], this ENU-induced point mutation led to normal levels of a full-length protein with altered function. Comparison of the consequences of this mutation to the outcome of gene-targeting experiments which eliminated Carma1 expression [24] is illuminating in terms of what each strategy can offer. The ENU mutant, which affects a coiled-coil region of the protein, led to a phenotype far more prominent in B lymphocytes than their T-cell counterparts, while the lack of Carma1 in the loss-of-expression studies led to a profound impairment of TCR/CD28-induced production of interleukin-2 (IL-2) and proliferation, along with B-cell signaling defects

[13,22,23]. Conversely, the point-mutant Carma1 led to a partially penetrant hyper-IgE syndrome with other atopic manifestations in the mice, whereas this abnormality was not noted in the conventional knockouts. Thus, the random generation of a point mutant uncovered new features of the protein's function *in vivo* and some selectivity in the impact of these functional traits on particular lymphoid-cell types.

So, what are the global lessons from these advances, and what future refinements incorporating genome-wide approaches may be possible? The first lesson is that these approaches establish the ability of large, well-funded, and well-organized operations to generate new insights into immune regulation using functional-genomic screens. In addition, some of the findings will differ from those obtained using conventional knockouts. Subsequent to these initial 'test-of-principle' papers, the pace of discovery using this type of approach will accelerate. The second lesson is that although there undoubtedly is some selection bias, a strikingly high frequency of 'old friends' turn up in these screens. Thus far, most of the listed gene products have been previously identified and implicated in the relevant signaling pathways by conventional approaches. This suggests that intensive gene discovery by pre-genomic techniques has been quite successful. Nonetheless, because a substantial fraction of predicted genes do not fall into known functional groups it is to be expected that the gene products uncovered will extend more into groups not previously linked to the functions being screened. Finally, the collected findings indicate that it is already timely to be generating systems-based strategies to exploit these advances. Approaches of this type will gain power as they are combined and linked to other forms of genomics [26-28], proteomics and systems analysis. Thus, for example, the tetracycline-regulated dominant retrovector system can be combined with transductions of hematopoietic cells from mutant mice and to microarray analyses of gene-expression profiles. Such approaches may allow the identification of selective roles of protein modules and quantitation of the effects on function of a wide range of sequence variants generated *in vitro*. Similarly, when coupled to screens of protein-protein interactions in cell lines, genome-scale approaches will rapidly increase our insights into structure-function relationships in immunity and disease.

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