Finally, the authors applied the strategy to the identification of drug-resistant mutants from the *Saccharomyces cerevisiae* deletion collection. A total of 128 strains were smart-pooled into 14 pools of 64 with a sevenfold redundancy, and assayed against two drugs. Both previously known resistant strains were unambiguously identified despite the ninefold increase in efficiency.

Although the smart-pooling design used by the authors is intuitive and highly efficient, it can be optimized further. It can be described as the *n*-dimensional hypercube extension of the grid design illustrated in **Figure 1**, restricted to side length 2 and without the diagonal pools. Two potential weaknesses are that the pool sets of some pairs of probes are too similar and others are complementary. Consequently, decoding is ambiguous in the presence of noise or of multiple positives. Another limitation is that choosing the pools' size entirely determines the design: for example, pools of 8 probes necessarily have a fourfold redundancy. This is problematic in the two-hybrid experiment for instance, in which small pools are required for sensitivity but the high error rates would call for more redundancy.

Alternative designs[3–5], which have been described and await experimental validation, may overcome these limitations. For example, a recently proposed design[3] can be precisely adapted to the characteristics of any experiment (for example, pool size and redundancy are set independently). Of course, these more mathematical constructions are less intuitive, and decoding must be done *in silico*, but they scale up extremely well.

Naturally, the biological characteristics drive the choice of the smart-pooling design. The expected fraction of true positive probes and the error rates should be carefully estimated beforehand, and one must also determine the maximal pool size that can be used without excessive degradation of the assay's sensitivity: in many applications, this essentially determines the achievable efficiency. Based on this information, an optimized design—powerful enough yet not wasteful—can be selected and the method applied on a large scale.

Smart-pooling can reduce the number of experiments and yet considerably increase sensitivity and specificity, because the redundancy allows the investigator to identify and correct both false positive and false negative observations. This strategy's feasibility and versatility in real-world applications is now demonstrated, using three different high-throughput technologies. It opens new perspectives for systems biology experiments seeking to detect low-frequency events in the presence of noise: substantial improvements in the quality and breadth of such datasets are now within close reach.

1. Jin, F. *et al. Nat. Methods* **3**, 183–189 (2006).
2. Uetz, P. *et al. Nature* **403**, 623–627 (2000).
3. Thierry-Mieg, N. *BMC Bioinformatics* **7**, 28 (2006).
4. Balding, D., Bruno, W., Knill, E. & Torney, D. In *Genetic mapping and DNA sequencing*. (Speed, T. & Waterman, M.S., eds.) 133–154 (Springer, New York,1996).
5. Ngo, H. & Du, D.Z. *DIMACS Ser. Discrete Math. Theoret. Comput. Sci.* **55**, 171–182 (2000).

Retroviral TCR gene transduction: 2A for two

Rémy Bosselut

A recently developed multigene viral expression system is put to work to generate mice carrying a single T-cell receptor (TCR) specificity. Complementing the transgenic-mice technique, this method offers new practical options to researchers studying T-cell development.

Receptors and ligands that mediate T cell–antigen recognition are remarkable because of their staggering diversity. Thus, a defining breakthrough in T-cell immunology was the generation of transgenic mice whose T cells all carry the same antigen receptor[1], thereby fixing the 'receptor side' of the interaction. An elegant trick reported in this issue of *Nature Methods* should facilitate the generation of monospecific T-cell populations by retroviral transduction of hematopoietic stem cells[2].

Most T cells use a TCR comprising two antigen-specific chains (α and β) to recognize antigenic peptides bound to classical major histocompatibility complex (MHC) molecules[3]. Neither the α nor the β chain is germline encoded; rather, each is produced from an open reading frame generated by random rearrangement of TCRα and TCRβ loci during T-cell development in the thymus[4] (a process called V(D)J recombination). This process results in extensive TCR diversity, so that each developing T cell (thymocyte) carries a distinct TCR specificity.

Because of the extreme allelic diversity of MHC genes, most TCR$\alpha\beta$ specificities generated in any given individual are of inappropriate avidity for self peptide–self MHC complexes[5] (**Fig. 1**). Low-avidity receptors are useless and fail to rescue thymocytes from death by neglect, whereas receptors with high avidity for self are potentially harmful and trigger active thymocyte deletion (negative selection). In the end, only the small subset of thymocytes carrying intermediate-avidity TCRs survive and differentiate into mature T cells (a process called positive selection), which normally react against foreign peptide–self MHC complexes.

Key to the study of T-cell development and responses has been the development of mice in which all (or most) T cells express the same TCR$\alpha\beta$ specificity, mediating either positive or negative selection and responding to a defined antigen[1]. Note that expression of such recombinant TCR chains prevents or substantially impairs endogenous TCR gene rearrangement[4]. Such mice have so far been generated using conventional transgenic procedures, by introducing into the mouse genome recombinant TCRα and TCRβ minigenes controlled by their own regulatory elements. More recently[6], retroviral

Rémy Bosselut is at the Laboratory of Immune Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.
e-mail: remy@helix.nih.gov
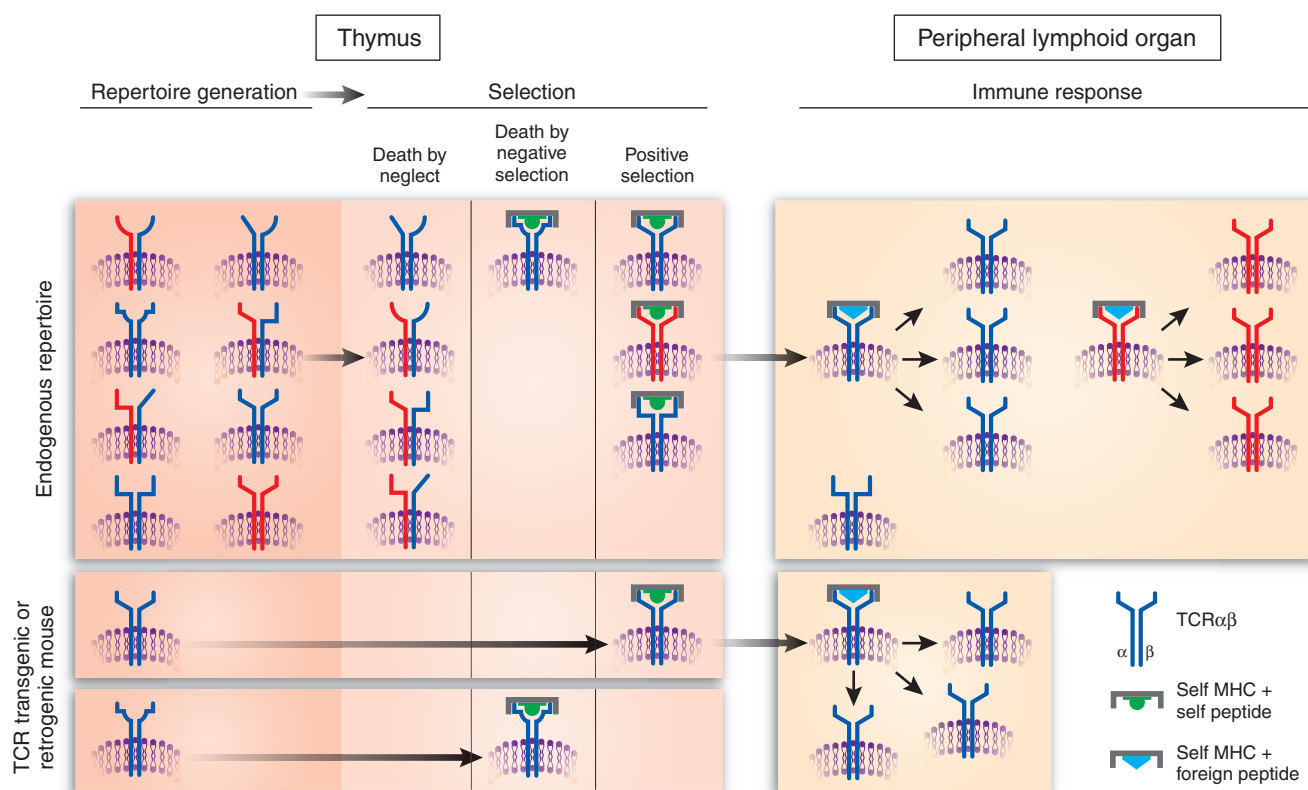
Figure 1 | Generating mice with the same T cell receptors. The fate of thymic T-cell precursors (normally expressing diverse TCR specificities; top) is dictated by the avidity of their TCR for self–MHC peptide complexes: death by neglect, active deletion (death by negative selection) or survival and differentiation (positive selection), followed by colonization of peripheral lymphoid organs and immune responses to antigenic peptides. Analyses of T-cell differentiation are facilitated in mice in which precursors all carry the same TCR specificity, mediating either positive (middle) or negative (bottom) selection. A new procedure bypasses the need for transgenesis to achieve this goal, using instead retroviruses to express both TCRα and β from a single composite open reading frame.

vectors have been used to transduce TCR genes into bone marrow stem cells whose development into T cells is subsequently analyzed after transfer into recipient mice (irradiated beforehand to eliminate their own hematopoietic cells—in effect, a bone marrow transplant). In such vectors, however, one of the two chains must be translated from an internal ribosome entry site (IRES), resulting in somewhat reduced TCR expression[6].

An elegant improvement by Holst *et al.*[2] bypasses this obstacle by retrovirally expressing both polypeptides from a single composite open reading frame. The strategy involves insertion of short protein motifs, referred to as 2A or 2A-like, between TCRα- and TCRβ-encoded sequences (these motifs are present in some viral genomes, such as that of the foot and mouth disease virus). As the polypeptide is synthesized, the local structure of the nascent chain apparently prevents peptide-bond formation at the last amino acid of the motif, without arresting translation[7,8]. The lack of peptide bond releases the upstream polypeptide chain (TCRα-2A), but ribosomes remain on the mRNA and translate sequences downstream of the motif into a separate polypeptide (TCRβ). As a result, near stoichiometric amounts of both chains are produced from the single open reading frame[9].

The composite cDNA is carried by a conventional retroviral vector, upstream of an IRES–fluorescent protein cassette to identify transduced cells. Surface expression of the receptor remains T cell–specific because it requires association of TCRαβ with endogenous T lineage–specific CD3 chains[3]. Using several previously characterized TCRs, Holst *et al.* demonstrate that their approach reconstitutes T-cell development (both positive and negative selection) in a manner similar to that in the corresponding TCR transgenic strain.

The obvious benefit of this 'retrogenic' approach is to streamline *in vivo* analyses of TCR-induced responses, bypassing the labor-intensive generation of new TCR transgenic lines, or saving the time and resources needed to breed existing ones onto new genetic backgrounds. Another potential advantage of retrogenic mice is the ability to control the frequency of transduced precursors in the transplanted bone marrow; this should avoid the massive imbalance between receptor and ligand observed in TCR transgenic mice, in which, for instance, thymic selection efficiency is reduced because of limiting MHC-peptide ligands[10]. Finally, because multiple 2A-like motifs can be introduced in a single cDNA, retrogenic vectors can simultaneously carry additional genes. Building on that possibility, Holst *et al.* transduced selecting MHC peptides together with TCRαβ, thereby providing both receptor and ligand specificities[2]. Although this approach improved positive selection, such experiments must be interpreted with caution, as positive

selection is normally driven by the thymus epithelium[5], whereas retrogenic vectors express MHC-peptide in donor-derived bone marrow cells.

Are the days of TCR transgenic mice numbered? Not yet. Retrogenic mice have their limitations as well. Although the 19 2A-motif residues appended carboxy-terminally to TCRα should not affect TCR assembly or signaling[3], it is necessary to irradiate the recipient mouse before transplantation and this is not without deleterious effects on lymphoid organs. Perhaps more significant, however, are workload considerations, and the fact that bone marrow transduction and transplantation introduce their own experimental variability. As a result, the convenience and reproducibility of TCR transgenic strains will probably remain paramount for large-scale or long-term projects. But retrogenic TCRs should find multiple niches, for instance in pilot studies, to conduct TCR mutagenesis studies *in vivo* or to introduce TCR specificities on complex genetic backgrounds. In addition, retrogenic vectors should facilitate investigations inaccessible to TCR transgenic mice, for instance immunotherapy attempts by transferring into mature T cells TCRs of defined specificity (including those that would fail intrathymic selection, for example, high-avidity receptors against infectious or tumor antigens)[11].

1. von Boehmer, H. *Annu. Rev. Immunol.* **8**, 531–556 (1990).
2. Holst, J., Vignali, K., Burton, A. & Vignali, D. *Nat. Methods* **3**, 191–197 (2006).
3. Davis, M.M. & Chien, Y.H. In *Fundamental Immunology* (ed., Paul, W.E.) 227–258 (Lippincott Wiliams and Wilkins, Philadelphia, 2003).
4. Livak, F. & Petrie, H.T. *Semin. Immunol.* **14**, 297–309 (2002).
5. Starr, T.K., Jameson, S.C. & Hogquist, K.A. *Annu. Rev. Immunol.* **21**, 139–176 (2003).
6. Yang, L. & Baltimore, D. *Proc. Natl. Acad. Sci. USA* **102**, 4518–4523 (2005).
7. Ryan, M.D. & Drew, J. *EMBO J.* **13**, 928–933 (1994).
8. Donnelly, M.L. *et al. J. Gen. Virol.* **82**, 1013–1025 (2001).
9. Szymczak, A.L. *et al. Nat. Biotechnol.* **22**, 589–594 (2004).
10. Huesmann, M., Scott, B., Kisielow, P. & von Boehmer, H. *Cell* **66**, 533–540 (1991).
11. Moss, P. A. *Nat. Immunol.* **2**, 900–901 (2001).