Rapid analysis of T-cell selection *in vivo* using T cell–receptor retrogenic mice

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Although T-cell receptor (TCR) transgenic as well as knockout and knockin mice have had a large impact on our understanding of T-cell development, signal transduction and function, the need to cross these mice delays experiments considerably. Here we provide a methodology for the rapid expression of TCRs in mice using 2A peptide–linked multicistronic retroviral vectors to transduce stem cells of any background before adoptive transfer into RAG-1$^{-/-}$ mice. For simplicity, we refer to these as retrogenic mice. We demonstrate that these retrogenic mice are comparable to transgenic mice expressing three commonly used TCRs (OT-I, OT-II and AND). We also show that retrogenic mice expressing male antigen–specific TCRs (HY, MataHari and Marilyn) facilitated the analysis of positive and negative selection in female and male mice, respectively. We examined various tolerance mechanisms in epitope-coupled TCR retrogenic mice. This powerful resource could expedite the identification of proteins involved in T-cell development and function.

The TCR is a complex structure that mediates many diverse functions in the thymus and the periphery, such as positive and negative selection, peripheral tolerance and anergy, T helper–cell differentiation, proliferation, cytotoxicity and cytokine production. Understanding the molecular mechanism of these crucial events is an important goal for immunologists. The TCR is composed of at least eight polypeptide subunits, heterodimers of TCRαβ, CD3εδ, CD3εγ and CD3ζζ homodimer, which are necessary for efficient TCR surface expression and signal transduction[1,2]. Although TCR ligation with broadly reactive stimulators, such as CD3 antibodies and bacterial superantigens, and the use of *in vitro* experiments have provided valuable insight, these complex events are best analyzed *in vivo* after cognate or self antigen stimulation.

The development of TCR transgenic mice has provided a source of clonotype-specific T cells that can be analyzed after stimulation with their cognate antigen. They have also facilitated the analysis of both central and peripheral tolerance *in vivo*[3]. With the advent of knockout and knockin technologies, the role of many molecules in these events can now be analyzed. But crossing transgenic and knockout mice is expensive and delays experiments by approximately six months. As a consequence, only one or two TCRs are typically examined because of these constraints. Several studies have also yielded conflicting results when different TCR Tg mice were examined. For instance, P14 T cells were positively selected in CD3ε$^{-/-}$ mice expressing a nonsignaling CD3ε transgene, whereas HY T cells were not[4]. Likewise, loss of CD5 expression was found to have a variable effect on thymocyte selection depending on the TCR used[5]. These observations emphasize the importance of using multiple TCRs to assess the efficiency of T-cell selection and function in any new mutant mouse models.

There is clearly a need to develop new methodology that would facilitate the rapid generation of TCR transgenic mice and analysis of key events *in vivo*, such as central and peripheral tolerance. Others have attempted to generate such mice by adoptive transfer of hematopoietic precursors that have been transduced using separate retroviral vectors for TCRα and TCRβ, which necessitated the use of recombination-activating gene (RAG)-deficient bone marrow, or single vectors containing an internal ribosome entry site (IRES), which led to nonstoichiometric production of the TCRβ chain[6,7].

We recently developed a method for the expression of multiple proteins *in vivo* using retrovirally mediated stem cell gene transfer and multicistronic, 2A peptide–linked retroviral vectors[8,9]. Several viruses use 2A peptides, or 2A–like sequences, to mediate protein 'cleavage'[8–10]. These include members of the *Picornaviridae* family, such as the porcine teschovirus-1 (PTV-1), and other viruses, such as the insect *Thosea asigna* virus (TaV)[10]. Through a ribosomal 'skip' mechanism, the extremely rare 2A consensus motif appears to impair normal peptide bond formation between the glycine in the 2A peptide and the proline in the 2B peptide, whose gene is located immediately downstream of that encoding the 2A peptide, without affecting the translation of the 2B peptide[11] (**Fig. 1a**). Use of 2A peptides to link the sequences encoding several proteins in the same open reading frame results in near-complete separation and stoichiometric production of the encoded proteins.

In this study, we used this system to encode both TCRα and TCRβ in a single vector and expressed these in mice using retrovirally mediated stem cell gene transfer. To avoid confusion with conventional transgenic (Tg) mice, we refer to these as 'retrogenic' (Rg) mice ('retro' from retrovirus and 'genic' from Tg). Here we describe seven TCR retroviral vectors, including some that also contain a selecting ligand. We demonstrate that Tg and Rg mice are

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comparable for immunological studies, and that this system can be used to rapidly monitor T-cell selection and function *in vivo*.

RESULTS

Comparison of TCR Rg and Tg mice

We first assessed whether T-cell development and function in TCR Rg mice was comparable to that in their Tg counterparts. We focused on two frequently used TCRs, OT-I[12] and OT-II[13] (**Supplementary Table 1** online). The cDNAs encoding the TCRα (lacking the stop codon) and TCRβ chains were linked via a 2A peptide into a single open reading frame by recombinant PCR and cloned into a mouse stem cell virus (MSCV)-based retroviral vector containing an IRES-GFP cassette to facilitate the identification of marked cells[8,9] (**Fig. 1a**). Given the stoichiometric production of 2A peptide–linked proteins[9], this system is ideally suited for the expression of heterodimeric TCRs. The 2A peptide remains attached to the TCRα carboxy terminus and can be used to verify the complete separation of the two chains (**Supplementary Fig. 1** online). Inclusion of a Gly-Ser-Gly (GSG) spacer between TCRα and the 2A peptide ensures complete 'cleavage'. We have not seen any deleterious effects of the 2A peptide on the function of any proteins examined thus far[8,9,14].

We used the retroviral producer cell lines expressing OT-I and OT-II TCRs to transduce C57BL/6 donor bone marrow before reconstitution into lethally irradiated RAG-1$^{-/-}$ recipient mice. T cells in recipient mice can be analyzed 5–8 weeks after transfer. Approximately 50% of thymocytes and splenocytes were GFP^{+} by 5 weeks after transfer (**Supplementary Fig. 1**). In vector control Rg mice (Vec. Rg), the distribution of CD4^{+} and CD8^{+} T cell populations in the thymus and spleen was comparable to that in the thymus and

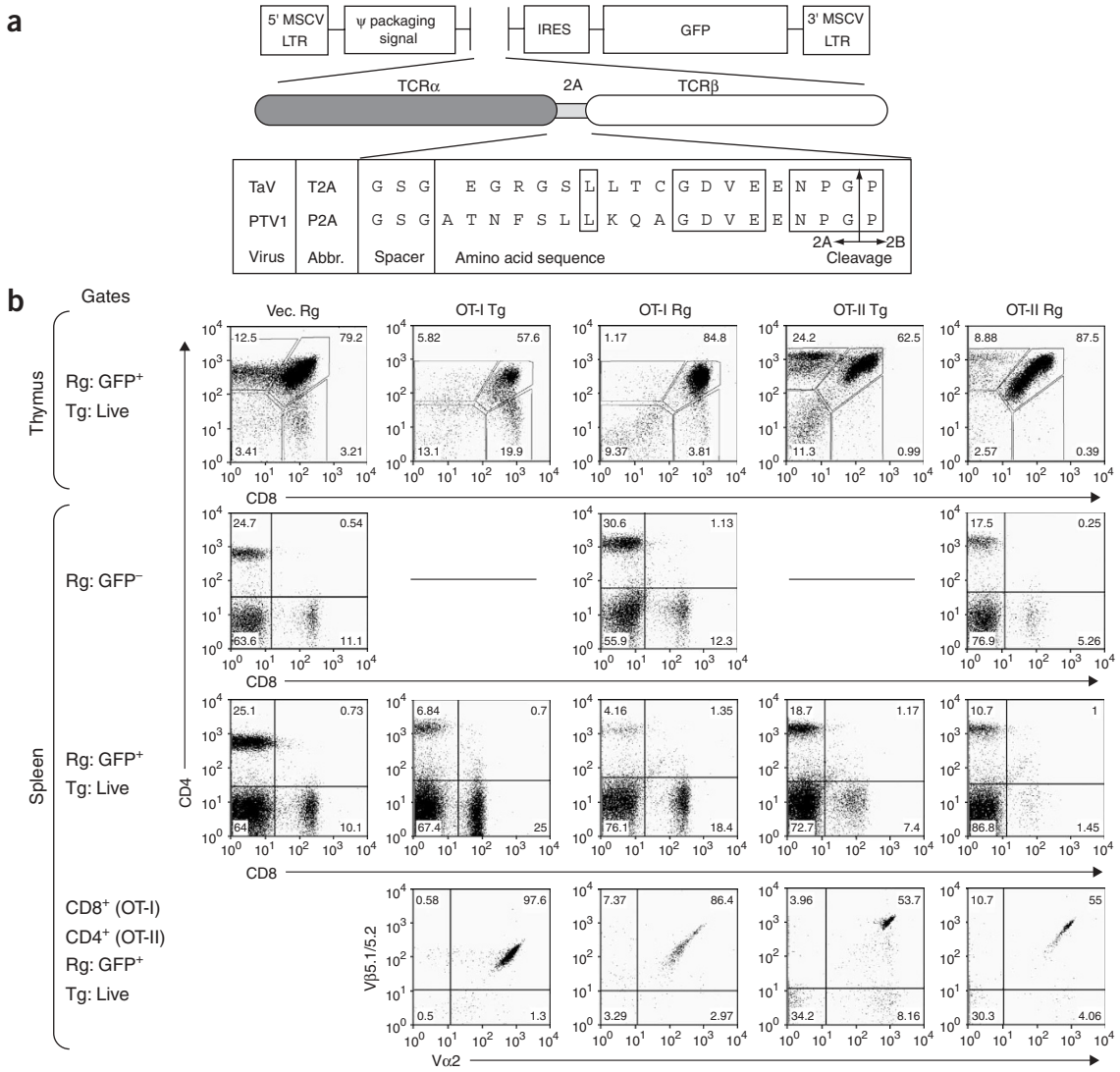


Figure 1 | Comparison of TCR Rg and Tg mice. (**a**) Schematic of the TCRα–2A–TCRβ constructs, including the amino acid sequence of the 2A regions of *Thosea asigna* virus (T2A) and porcine teschovirus-1 (P2A), spacer and insertion point in the MSCV–IRES–GFP (pMIG) vector. Conserved residues are boxed, with the 'cleavage' point between the 2A and 2B peptides indicated by the arrow. (**b**) Rg mice were generated from C57BL/6 bone marrow by retrovirally mediated stem cell gene transfer. Mice were analyzed 5–8 weeks after transfer and compared with either conventional OT-I or OT-II Tg equivalents. Thymocytes and splenocytes were analyzed by flow cytometry using antibodies against the markers indicated and gated as shown on the left. Dot plots for the Rg mice are representative of data from 5–15 mice per group.

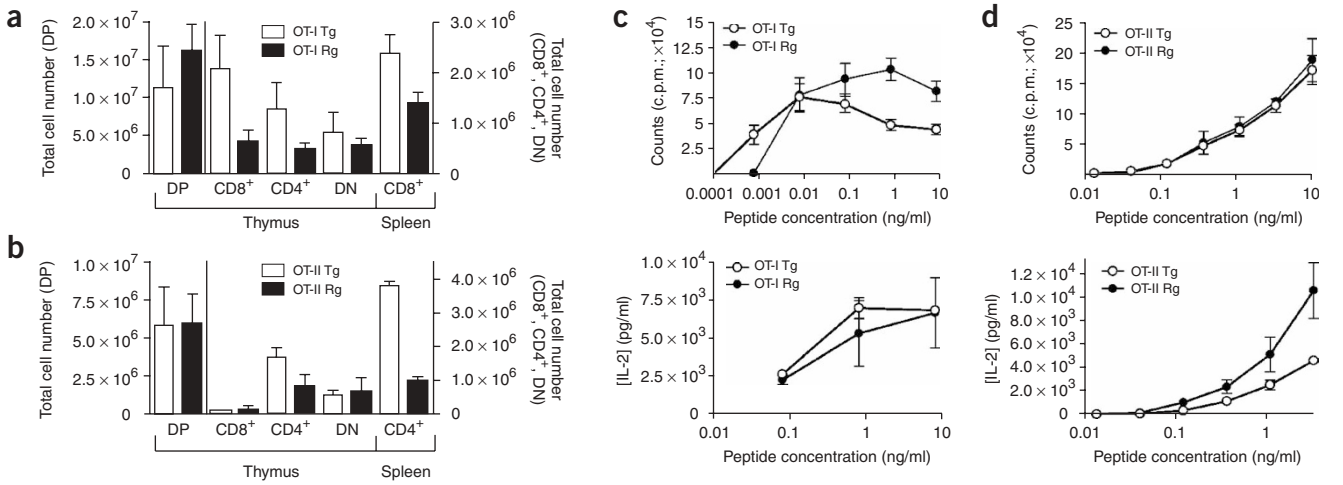


Figure 2 | Comparison of TCR Rg and Tg mice. (**a**,**b**) Thymocytes and splenocytes from **Figure 1** (OT-I, **a**; OT-II, **b**) were counted and the number of cells at each developmental stage determined by flow-cytometric analysis. Data represent the mean ± s.e.m. of 2–3 mice (Tg) or 5–10 mice (Rg). Tg mice were 5–12 weeks old when analyzed. Rg mice were analyzed 5–6 weeks after transfer. (**c**,**d**) Splenic GFP$^+$ (Rg only), CD8$^+$ (OT-I only; **c**) and CD4$^+$ (OT-II only; **d**) T cells were purified by FACS or MACS. OT-I or OT-II T cells (1×10^5 per well) were stimulated with varying peptide concentrations plus irradiated C57BL/6 splenocytes (5×10^5) in serum-free AIMV medium. After 24 h (OT-I) or 48 h (OT-II), 50 μl of the supernatant was removed to assay for IL-2 secretion, and the cells were pulsed with [^{3}H]thymidine for an additional 8 h before collecting them to determine [^{3}H]thymidine incorporation. Data are the mean ± s.e.m. of 2–6 separate experiments.

spleen of C57BL/6 mice (**Fig. 1b** and data not shown). Whereas we observed some differences between Tg and Rg mice in the thymus (which may result from differences in the promoters driving TCR expression), the T-cell phenotype and distribution in the spleen were very similar (**Fig. 1b**). The percentage of CD4$^+$ and CD8$^+$ T cells found in each Rg mouse was consistent throughout all the experiments, demonstrating the reliability of this approach (**Supplementary Fig. 1**). Furthermore, T-cell development and CD8 or CD4 coreceptor skewing occurs in RAG-1$^{-/-}$ donor bone marrow demonstrating that T cells were derived from the thymus (**Supplementary Fig. 1**). Expression of the OT-I or OT-II TCR skewed T-cell development toward either the CD8 or the CD4 lineage, respectively, in both Tg and Rg mice (**Fig. 1b**). The percentage of CD8$^+$ (for OT-I) or CD4$^+$ (for OT-II) T cells that were Vα2$^+$/Vβ5$^+$ was also very similar between Tg and Rg (GFP$^+$) mice (**Fig. 1b**). There were generally fewer T cells in Rg mice than in Tg mice (**Fig. 2a**,**b**). This was in part due to early analysis (5–8 weeks), and more cells could be obtained >10 weeks after transfer. Notably, TCR surface expression level was similar (**Supplementary Fig. 1**).

To assess their functionality, we purified T cells from Tg and Rg mice by fluorescence-activated cell sorting (FACS). The ability of these T cells to proliferate and produce IL-2 in response to their cognate peptide was remarkably similar (**Fig. 2c**,**d**). We have also generated Rg mice using the AND[15] and TEa[16] TCRs (**Supplementary Figs. 2** and **3** online). We observed strong CD4 skewing, allelic exclusion and functional responsiveness with both of these TCRs, suggesting the general applicability of this approach for many different TCRs. Furthermore, when we expressed TEa, AND or OT-I TCRs in RAG-1$^{-/-}$ bone marrow, only clonotype T-cells developed (**Supplementary Figs. 1–3**). Taken together, these data show that T cells derived from Rg and Tg mice are comparable. Notably, these Rg mice could be used within 5 weeks of their production, facilitating the simultaneous analysis of multiple TCR and donor genotype combinations.

Analysis of selection using male antigen-specific TCR

One of the most widely used tools to study negative selection *in vivo* has been the HY TCR Tg mouse model[17]. This TCR is specific for a male minor histocompatibility antigen, allowing negative selection to be studied in male Tg mice, whereas HY clonotype positive T cells develop normally in female mice. We exploited this dichotomy by generating three multicistronic retroviral vectors that encoded HY, MataHari and Marilyn male-specific TCRs[17–19] (**Supplementary Table 1**). Using these vectors with male or female donors, we generated Rg mice and compared tolerance induction (**Figs. 3** and **4**).

In female HY Rg mice, using either C57BL/6 or RAG-1$^{-/-}$ donor bone marrow, a distinct population of CD8$^+$ T3.70$^+$ (HY clonotype) T cells develop and proliferate in response to male but not female antigen-presenting cells (APCs) (**Figs. 3a**, **4d** and **Supplementary Fig. 4** online). In contrast, there was a reduction in the percentage of CD8$^+$ T3.70$^+$ thymocytes in male HY Rg mice (**Fig. 3a**). Notably, there was a twofold increase in the number of T3.70$^+$ HY T cells in the periphery of male mice (**Fig. 4a**). These cells, however, expressed reduced levels of TCR and CD8, and did not respond to male APCs (**Figs. 3a** and **4d**,**g**). The lack of complete deletion and emergence of clonotype-positive T cells in the periphery of male HY Rg mice is consistent with previous descriptions of their Tg equivalents[17]. The surface expression of TCR during thymic development was also comparable between HY Rg and Tg mice, but deletion of double positive (DP) thymocytes and loss of CD8 expression in the HY Tg mice appears to be more pronounced than in the HY Rg mice (**Supplementary Fig. 4**).

In contrast to HY Rg mice, we observed profound deletion in MataHari and Marilyn Rg mice. In female recipients, CD8$^+$ MataHari T cells and CD4$^+$ Marilyn T cells developed normally and exhibited robust coreceptor skewing and allelic exclusion (**Fig. 3b**,**c** and **Supplementary Fig. 4**). Furthermore, both T-cell populations proliferated and produced IL-2 in response to male, but not female, APCs (**Fig. 4e**,**f**,**h**,**j**). In contrast, there was a

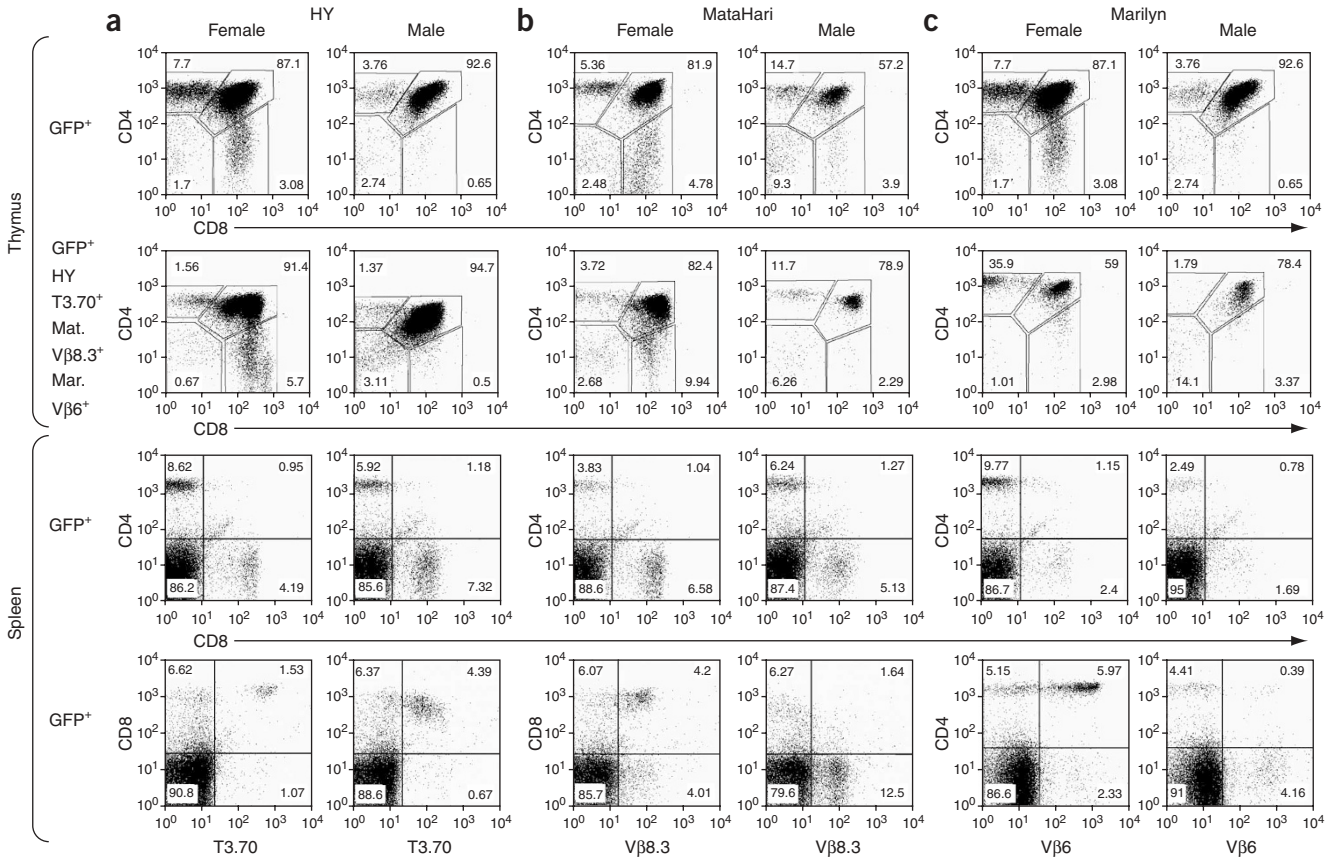


Figure 3 | Analysis of tolerance induction using male HY antigen–specific Rg mice. (**a**–**c**) Retrovirally mediated stem cell gene transfer was used to generate HY (**a**), MataHari (**b**) and Marilyn (**c**) Rg mice using male or female donor C57BL/6 bone marrow and corresponding male or female RAG-1$^{-/-}$ recipient mice as indicated. Thymocytes and splenocytes were analyzed by flow cytometry 6–8 weeks after transfer (data representative of 10–15 mice per group). The second panel is gated as shown in **Supplementary Fig. 4** to ensure cells expressing low levels of TCR are included.

substantial reduction in the relevant single positive (SP) thymocyte population in male MataHari and Marilyn TCR Rg mice, which was more pronounced in the GFP$^+$ Vβ8.3$^+$ and Vβ6$^+$ populations, respectively (**Fig. 3b**,**c** and **Supplementary Fig. 4**). In addition, very few splenic T cells (MataHari–CD8$^+$Vβ8.3$^+$; Marilyn–CD4$^+$Vβ6$^+$) were generated in male Rg mice (**Figs. 3b**,**c** and **4b**,**c**). The few TCR–Vβ8.3$^+$ (MataHari) and TCR–Vβ6$^+$ (Marilyn) T cells that do migrate to the periphery lack coreceptor expression (**Fig. 3b**,**c**) and are unresponsive to male APCs, reflecting additional mechanisms of tolerance induction (**Fig. 4e**,**f**,**h**,**i**).

Analysis of selection using H-2Eα–coupled TEa TCR Rg mice

The use of TCRs specific for endogenous antigens in the study of T-cell selection has its limitations as it does not allow for the manipulation of the selecting ligand. Thus, we asked if this system could be adapted to include a selecting ligand in the retroviral construct. We chose to use the H-2Eα 52–68-specific TCR, TEa[16]. As the *H2-Ea* gene is defective in C57BL/6 and C57BL/10 mice, TEa T cells develop normally in these strains. To verify that TEa T cells are deleted in the presence of the H-2Eα protein, we generated Rg mice using either C57BL/10 or B10.A(5R) bone marrow, the latter having an intact *H2-Ea* gene. Whereas TEa T cells developed normally in C57BL/10 Rg mice, they were almost completely deleted on a B10.A(5R) background (**Supplementary Fig. 3**).

We then asked whether effective tolerance induction could be exerted by inserting the deleting ligand in the retroviral construct. We linked the coding sequence for H-2Eα to the TEa TCRβ sequence via T2A in a single open reading frame (**Fig. 5a**). As a negative control, we generated a second version of this construct that contained an L60T mutation in the H-2Eα protein that would produce, after processing, a null peptide for the TEa TCR (A. Rudensky, personal communication). We generated TEa, TEa + Eα and TEa + EαL60T Rg mice using C57BL/6 donor bone marrow and RAG-1$^{-/-}$ mice as recipients. We observed surface expression of H-2Eα on B cells from TEa + Eα but not TEa Rg mice (**Supplementary Fig. 3**). Consistent with this cognate ligand expression, we observed substantial deletion of TEa Vα2$^+$Vβ6$^+$ 'clonotypic' T cells in TEa + Eα mice but not in the 'null' TEa + EαL60T control (**Fig. 5b** and **Supplementary Fig. 3**). The few T cells that did remain had reduced Vα2/Vβ6 surface expression and were unresponsive to antigenic stimulation (**Fig. 5c**).

Analysis of selection using peptide-coupled OT-I Rg mice

Several elegant studies have used fetal thymic organ culture (FTOC) to assess the ability of different OVA 257–264 peptide analogs to mediate either positive or negative selection of OT-I Tg T cells *in vitro*[12,20]. Despite the strengths of this approach, it requires removal of the thymus, and whereas negative selection can be analyzed on

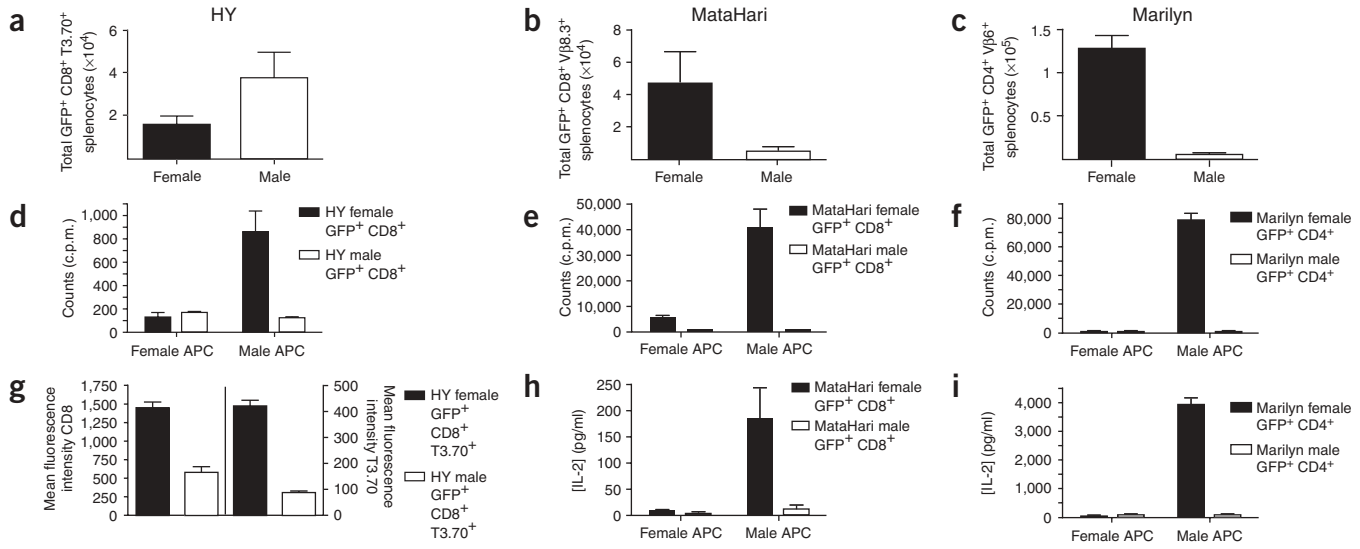


Figure 4 | Analysis of tolerance induction using male HY antigen–specific Rg mice. (**a**–**c**) The total number of splenic HY (**a**, $CD8^+T3.70^+$), MataHari (**b**, $CD8^+V\beta8.3^+$) and Marilyn (**c**, $CD4^+$ $V\beta6^+$) 'clonotypic' T cells in female and male Rg mice is shown (mean ± s.e.m. of 10–15 mice from 2–3 experiments). (**d**–**f**) GFP^+CD8^+ (MataHari and HY) and GFP^+CD4^+ (Marilyn) splenocytes were sorted by FACS and stimulated (at 1×10^5 cells per well) with 5×10^5 irradiated male or female C57BL/6 splenocytes in AIMV media. After 24 h (**d**,**e**) or 48 h (**f**) cells pulsed with $[^3H]$thymidine for an additional 24 h to determine the extent of proliferation. Purified female C57BL/6 $CD4^+$ or $CD8^+$ T cells cultured with male APCs were used as a control for background proliferation mediated by natural occurring male reactive T cells (data not shown). Data are mean ± s.e.m. of 2–4 separate experiments (2–3 mice per group). (**g**) Female and male $GFP^+CD8^+T3.70^+$ from HY Rg splenocytes were analyzed for mean fluorescence of both CD8 and T3.70 to determine their surface expression levels. Data are mean ± s.e.m. from 5 mice. (**h**–**i**) Supernatant samples taken from proliferation assays after 24 h (**e**) or 48 h (**f**) were assayed for IL-2. Data are mean ± s.e.m. of 2–4 separate experiments (2–3 mice per group).

any background, positive selection requires the use of mice on either a $TAP^{-/-}$ or $\beta2M^{-/-}$ background[12,21].

To allow control of the selecting ligand, we used the OT-I TCR system and attached peptide epitopes to the C terminus of the OT-I TCRαβ chain via P2A (**Fig. 6a**). Major histocompatibility complex class I epitopes are cleaved at the C terminus by the 26S proteasome but trimmed at the amino terminus by aminopeptidases in the cytosol and the endoplasmic reticulum[22]. Thus, the OVA 257–264 epitope was preceded by a six amino acid N-terminal extension of the natural OVA sequence plus Flag tag to facilitate natural processing. In addition to the wild-type SIINFEKL agonist peptide (WT), we included a null mutation (K4), intermediate agonist (G4), weak agonist-antagonist (E1), potent antagonist (R4) and natural 'self' selecting peptide ligand from β-catenin, which is an antagonist[23–25] (**Fig. 6a**). Irradiated splenocytes from the OT-I + WT mice effectively stimulated OT-I T cells demonstrating that these peptide epitopes were efficiently processed and presented (**Supplementary Fig. 5** online). We then performed retrovirally mediated stem cell gene transfer using C57BL/6 bone marrow and $RAG\text{-}1^{-/-}$ recipients to express all seven OT-I constructs.

OT-I + K4 Rg mice possessing the 'null' peptide were identical to the OT-I Rg mice, as expected (**Fig. 6b**,**c** and **Supplementary Fig. 5**). In contrast, T cells in OT-I + WT Rg mice were completely deleted, as we observed essentially no $CD8^+$ thymocytes, and there was a clear lack of $V\alpha2^+V\beta5.2^+$ 'clonotypic' T cells in the spleen (**Fig. 6b**,**c** and **Supplementary Fig. 5**). Mice expressing the other four analog peptides gave a graded outcome commensurate with the affinity of these peptides for the OT-I TCR and previously published observations in FTOC experiments[12,24]. Thus, expression of the G4 partial agonist resulted in substantial but incomplete

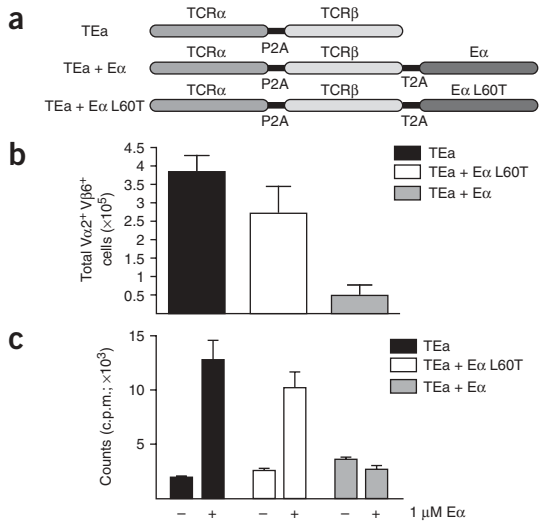
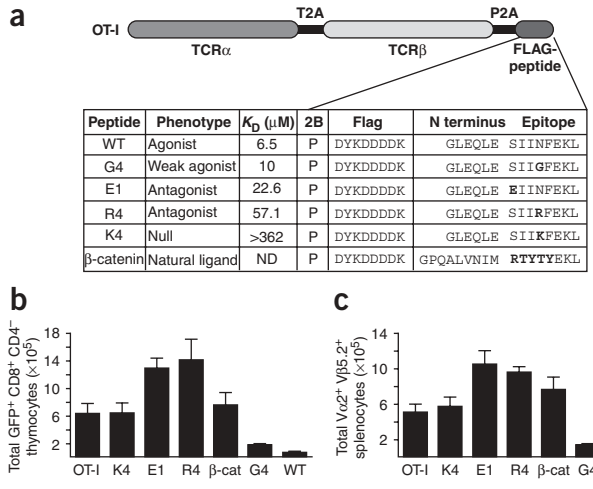


Figure 5 | Analysis of CD4+ T-cell selection using H-2Eα–coupled TEa TCR Rg mice. (**a**) Schematic of the TEa constructs used. TEa, TEa + Eα and TEa + EαL60T Rg mice were generated by retrovirally mediated stem cell gene transfer using donor C57BL/6 bone marrow and $RAG\text{-}1^{-/-}$ mice as recipients. Thymocytes and splenocytes were analyzed by flow cytometry 6–8 weeks after transfer. (**b**) The total number of splenic $V\alpha2^+V\beta6^+$ 'clonotypic' T cells is shown (mean ± s.e.m. of 5–10 mice from 2 experiments). (**c**) Splenocytes were subjected to negative depletion on an AutoMACS to enrich for CD4+ T cells (2×10^5 cells per well) and stimulated with 1 μM H-2Eα 52–68 peptide plus 5×10^5 irradiated C57BL/6 splenocytes in AIMV media. After 48 h the cells were pulsed with [3H]thymidine for an additional 24 h to determine the extent of proliferation. Data are the mean ± s.e.m. of 2–4 separate experiments (2–3 mice per group).



Peptide	Phenotype	K_D (μM)	2B	Flag	N terminus Epitope
WT	Agonist	6.5	P	DYKDDDDK	GLEQLE SIINFEKL
G4	Weak agonist	10	P	DYKDDDDK	GLEQLE SII**G**FEKL
E1	Antagonist	22.6	P	DYKDDDDK	GLEQLE **E**IINFEKL
R4	Antagonist	57.1	P	DYKDDDDK	GLEQLE SII**R**FEKL
K4	Null	>362	P	DYKDDDDK	GLEQLE SII**K**FEKL
β-catenin	Natural ligand	ND	P	DYKDDDDK	GPQALVNIM **RTYTY**EKL

Figure 6 | Analysis of CD8+ T-cell selection using peptide-coupled OT-I Rg mice. (**a**) Schematic of the OT-I constructs generated. The amino acid sequence detailed in the table immediately follows the C-terminal proline of the P2A sequence. The OVA 257–264 epitope was preceded by a six amino acid N-terminal extension of the natural OVA sequence to facilitate natural processing. We had included a Flag tag to allow for the monitoring of peptide production, but subsequent analysis showed that processing was too rapid to allow for detection. Residues in bold indicate differences from the wild type OVA peptide. Phenotype and K_D values are as previously published[23–25]. (**b**–**c**) Retrovirally mediated stem cell gene transfer was used to generate OT-I ± peptide retrogenic mice. The total number of thymic GFP+CD8+CD4− cells (**b**) and splenic Vα2+Vβ5.2+ 'clonotypic' T cells (**c**) is shown (mean ± s.e.m. of 5–10 mice from 1–2 experiments). β-cat, β-catenin.

T-cell deletion, and those T cells that did emerge in the periphery were very poorly responsive to antigenic stimulation *in vitro* (**Fig. 6b,c** and **Supplementary Fig. 5**). Notably, expression of all three antagonist peptides, R4, E1 and β–catenin, led to an increase in the number of $CD8^+$ thymocytes (except for β-catenin), increased CD5 expression on $CD4^+CD8^+$ and $CD8^+$ thymocytes and increased number of $V\alpha2^+V\beta5.2^+$ splenic T cells, all indicative of enhanced positive selection (**Fig. 6b,c** and **Supplementary Fig. 5**). Previous studies have suggested that thymic cortical epithelial cells are the primary mediators of positive selection[26,27]. Given that only the hematopoetic cells in these mice express the retroviral construct, it is presently unclear what acts as the presenting and selecting cell type. Recent studies have suggested that thymocytes can mediate positive selection[28,29]. Alternatively, it is possible that peptides are picked up by other cells in the thymus.

Mature peripheral T cells from OT-I + E1/R4/β-catenin Rg mice proliferate less than OT-I or K4 Rg T cells in response to OVA 257–264 stimulation *in vitro* (**Supplementary Fig. 5**). This partial unresponsiveness to peptide was also seen in E1 FTOC experiments[30] suggesting that enhanced positive selection may lead to a decreased threshold of signaling in peripheral mature T cells. It is also possible, however, that splenic T cells are 'influenced' by the peripheral expression of these antagonist 'self' ligands, which in turn leads to less activation by peptide.

DISCUSSION

We have described a strategy for the rapid analysis of T-cell tolerance and peripheral function using Rg mice. This approach has five key advantages over the more traditional approach of crossing the desired knockout and knockin mutant model with TCR Tg mice. First, our approach is substantially faster, with Rg mice ready to analyze after ~6 weeks. In contrast, it can take more than 6 months to generate the required Tg-mutant mice. Second, multiple TCR and/or TCR-epitope combinations can be examined simultaneously, ensuring a more comprehensive analysis of T-cell development and function. Indeed, in this study we examined seven TCR and eight epitope-tagged constructs, an endeavor that would have been logistically challenging and costly using the traditional transgenic approach. Third, as these mice are individually generated, each mouse is in essence a founder, limiting the potential bias introduced by a founder effect. Fourth, our system allows for the development and inclusion of additional TCR vector systems pertinent to the study in question. Fifth, this approach facilitates the analysis of positive and negative selection *in vivo* by using male antigen–specific or epitope-coupled TCR vectors.

There are four considerations when using this system. First, Rg mice cannot be propagated by breeding. Second, the 2A tag remains attached to the N-terminal cistron. However, we have seen no effect of this tag on T-cell development and function. Third, the number of T cells in Rg mice is generally less than in Tg mice. The amount obtained, however, is sufficient to perform most standard immunological experiments. Fourth, T cells develop in an adult rather than neonatal environment. Consequently, peripheral T cells have a 'memory-like' phenotype reminiscent of cells undergoing homeostatic expansion. Although our results suggest considerable consistency with previously published data, this concern could be circumvented by using these constructs for retroviral or lentiviral transgenesis followed by RAG-$1^{-/-}$ recomplementation to allow for normal thymic ontogeny in neonates.

Although the constructs we have described can effectively be used to analyze many T-cell processes, we envisage additional modifications that could be made to enhance these analyses and even broaden their usefulness to other cell systems. Given that our peptide-based approach proved successful for the analysis of major histocompatibility complex class I–restricted T-cell tolerance, could a simplified system be developed for $CD4^+$ T cells? Although TEa Rg mice were used effectively to study negative selection, T-cell deletion was incomplete, most likely due to low H-2Eα expression.

In summary, our system will allow investigators to rapidly assess the contribution of target molecules in T-cell signal transduction as well as central and peripheral tolerance using multiple TCR systems. Furthermore, we see no reason why this system could not be modified to assess the selection and function of B cells using multicistronic immunoglobulin (Ig) vectors. We also believe that this approach could facilitate a 'high'-throughput functional analysis of genes involved in T-cell selection and function.

METHODS

Generation of TCR multicistronic vectors. We cloned the genes encoding TCRα and TCRβ chains by PCR from splenocytes of the appropriate Tg mice, except for the gene encoding OT-I TCR, which we generated using plasmid templates (provided by F. Carbone, Monash University, Australia). We generated the genes encoding 2A peptide–linked constructs by recombinant PCR and cloned them into pMIG, an MSCV-based retroviral vector containing an IRES–GFP cassette, as described previously[9]. We included a GSG or SG spacer between TCRα and the 2A peptide to ensure

complete 'cleavage', the GSG is now included in all new constructs (**Supplementary Table 1**).

Mice. OT-I, OT-II, AND and TEa (provided by A. Rudensky, HHMI, University of Washington School of Medicine, Seattle, USA) Tg mice were maintained at St. Jude Children's Research Hospital. Spleens from HY and HY × RAG–2$^{-/-}$ were provided by J. Ihle, E. Parganas and S. Gingras (HHMI, St. Jude Children's Research Hospital). Spleens from MataHari and Marilyn were provided by P. Matzinger (US National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, USA). RAG-1$^{-/-}$, C57BL/6J, C57BL/10 and B10.A(5R) mice were obtained from Jackson Laboratories. All animal experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited, *Helicobacter*-free, specific pathogen–free facility following national, state and institutional guidelines. Animal protocols were approved by St. Jude Institutional Animal Care and Use Committee.

FACS analysis and cell sorting. We stained splenocytes and thymocytes with antibodies for magnetic cell sorting (MACS; AutoMACS; Miltenyi Biotec) or FACS (MoFlo; DakoCytomation). For negative MACS depletion, we used biotinylated anti-Mac1, anti-Ter119, anti-Gr1, anti-B220, anti-NK1.1 and either anti-CD4 (for CD8+ TCR) or anti-CD8α (for CD4+ TCR) (all BD-PharMingen). For FACS and flow cytometry, we used anti-CD4.APC (RM4-4), anti-CD4.cychrome, anti-CD8α.PE (53–6.7), anti-Vα2.PE, anti-Vβ6.biotin, anti-Vβ5.1/5.2.biotin, anti-Vβ3.biotin, anti-Vβ8.1/8.2.PE, anti-Vβ8.3.PE, anti-Vα11.1.PE, streptavidin-APC (BD-PharMingen) and anti-HY.PE (T3.70) (eBioscience). We performed flow cytometric analysis as detailed previously[31], using a FACSCalibur (Becton Dickinson).

Additional methods. Descriptions of retrovirally mediated stem cell gene transfer, western blotting and proliferation assays are available in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Weiss, A. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein–tyrosine kinases. *Cell* **73**, 209–212 (1993).
2. Call, M.E. & Wucherpfennig, K.W. The T-cell receptor: critical role of the membrane environment in receptor assembly and function. *Annu. Rev. Immunol.* **23**, 101–125 (2005).
3. Mondino, A., Khoruts, A. & Jenkins, M.K. The anatomy of T-cell activation and tolerance. *Proc. Natl. Acad. Sci. USA* **93**, 2245–2252 (1996).
4. Sommers, C.L. *et al.* Function of CD3 epsilon–mediated signals in T-cell development. *J. Exp. Med.* **192**, 913–919 (2000).
5. Azzam, H.S. *et al.* Fine tuning of TCR signaling by CD5. *J. Immunol.* **166**, 5464–5472 (2001).
6. Yang, L., Qin, X.F., Baltimore, D. & Van, P.L. Generation of functional antigen-specific T cells in defined genetic backgrounds by retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells. *Proc. Natl. Acad. Sci. USA* **99**, 6204–6209 (2002).
7. Yang, L. & Baltimore, D. Long–term *in vivo* provision of antigen-specific T-cell immunity by programming hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **102**, 4518–4523 (2005).
8. Arnold, P.Y., Burton, A.R. & Vignali, D.A. Diabetes incidence is unaltered in glutamate decarboxylase 65–specific TCR retrogenic nonobese diabetic mice: generation by retroviral–mediated stem cell gene transfer. *J. Immunol.* **173**, 3103–3111 (2004).
9. Szymczak, A.L. *et al.* Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide–based retroviral vector. *Nat. Biotechnol.* **22**, 589–594 (2004).
10. Donnelly, M.L. *et al.* The 'cleavage' activities of foot-and-mouth disease virus 2A site–directed mutants and naturally occurring '2A-like' sequences. *J. Gen. Virol.* **82**, 1027–1041 (2001).
11. Donnelly, M.L. *et al.* Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J. Gen. Virol.* **82**, 1013–1025 (2001).
12. Hogquist, K.A. *et al.* T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17–27 (1994).
13. Barnden, M.J., Allison, J., Heath, W.R. & Carbone, F.R. Defective TCR expression in transgenic mice constructed using cDNA–based alpha– and beta–chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76**, 34–40 (1998).
14. Szymczak, A.L. *et al.* The CD3epsilon proline-rich sequence, and its interaction with Nck, is not required for T cell development and function. *J. Immunol.* **175**, 270–275 (2005).
15. Kaye, J. *et al.* Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* **341**, 746–749 (1989).
16. Grubin, C.E., Kovats, S., deRoos, P. & Rudensky, A.Y. Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II–bound self-peptides. *Immunity* **7**, 197–208 (1997).
17. Kisielow, P., Bluthmann, H., Staerz, U.D., Steinmetz, M. & von Boehmer, H. Tolerance in T cell–receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature* **333**, 742–746 (1988).
18. Lantz, O., Grandjean, I., Matzinger, P. & Di Santoz, J.P. Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation. *Nat. Immunol.* **1**, 54–58 (2000).
19. Valujskikh, A., Lantz, O., Celli, S., Matzinger, P. & Heeger, P.S. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* **3**, 844–851 (2002).
20. Hogquist, K.A., Jameson, S.C. & Bevan, M.J. Strong agonist ligands for the T cell receptor do not mediate positive selection of functional CD8+ T cells. *Immunity* **3**, 79–86 (1995).
21. Ashton–Rickardt, P.G. *et al.* Evidence for a differential avidity model of T cell selection in the thymus. *Cell* **76**, 651–663 (1994).
22. Saveanu, L., Fruci, D. & van Endert, P. Beyond the proteasome: trimming, degradation and generation of MHC class I ligands by auxiliary proteases. *Mol. Immunol.* **39**, 203–215 (2002).
23. Santori, F.R. *et al.* Rare, structurally homologous self–peptides promote thymocyte positive selection. *Immunity* **17**, 131–142 (2002).
24. Alam, S.M. *et al.* T cell–receptor affinity and thymocyte positive selection. *Nature* **381**, 616–620 (1996).
25. Gascoigne, N.R., Zal, T. & Alam, S.M. T cell–receptor binding kinetics in T-cell development and activation. *Exp. Rev. Mol. Med.* **3**, 1–17 (2001).
26. Starr, T.K., Jameson, S.C. & Hogquist, K.A. Positive and negative selection of T cells. *Annu. Rev. Immunol.* **21**, 139–176 (2003).
27. Laufer, T.M., DeKoning, J., Markowitz, J.S., Lo, D. & Glimcher, L.H. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* **383**, 81–85 (1996).
28. Li, W. *et al.* An alternate pathway for CD4 T cell development: thymocyte–expressed MHC class II selects a distinct T cell population. *Immunity* **23**, 375–386 (2005).
29. Choi, E.Y. *et al.* Thymocyte–thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity* **23**, 387–396 (2005).
30. Jameson, S.C., Hogquist, K.A. & Bevan, M.J. Specificity and flexibility in thymic selection. *Nature* **369**, 750–752 (1994).
31. Vignali, D.A. & Vignali, K.M. Profound enhancement of T cell activation mediated by the interaction between the TCR and the D3 domain of CD4. *J. Immunol.* **162**, 1431–1439 (1999).

Erratum: Rapid analysis of T-cell selection *in vivo* using T cell–receptor retrogenic mice

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In the version of this article initially published, the name of one of the receptors mentioned in the abstract was incorrectly stated as OY-II instead of OT-II. The correct sentence is: "We demonstrate that these retrogenic mice are comparable to transgenic mice expressing three commonly used TCRs (OT-I, OT-II and AND)."

This error has been corrected in the HTML and PDF versions of the article.