Reporter Alleles that Inform on Differences in Cre Recombinase Expression

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J Immunol published online 28 April 2010
http://www.jimmunol.org/content/early/2010/04/28/jimmunol.1000089

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/04/28/jimmunol.1000089.DC1

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Throughout embryogenesis, cells adopt different fates as a consequence of signaling in response to secreted, matrix-associated, or cell-bound factors. For a class of factors that includes morphogens, individual cell fate depends both on factor identity and how much of it the cell encounters (1). It follows, therefore, that cells of different but related lineages should be distinguishable from one another on the basis of how much signaling they experienced from individual morphogen receptors during their development and the transcriptional output of genes that were induced by this signaling. Analytical procedures that inform on the gene expression history of cells are therefore of great interest for understanding how differences in gene expression result in divergent developmental fates during the development and differentiation of lymphocytes and other cells. The Journal of Immunology, 2010, 184: 000–000.

Controversy in determining the significance of signal strength in the control of lineage choice derives in part from difficulties associated with manipulating signaling in vivo in a fashion that avoids the possibility of artifact [e.g., due to complicating effects of premature loss or gain of signaling, prolonged or constitutive signaling when extinguishing it is a critical part of regulation (10), or simply overexpression outside of the normal range experienced by cells]. Cells actively undergoing commitment are also typically short-lived intermediates within minority populations, and such cells often lack markers that unambiguously define them as those that are experiencing the critical signaling processes. It can sometimes be difficult, therefore, to redirect commitment experimentally in a fashion that can be reliably interpreted or to identify and study cells undergoing commitment events.

An alternative approach to studying lineage commitment depends on marking cells that undergo specific experiences during commitment (16). If the mark placed on the cells is stable once imparted, then the fate of the cells can be traced and correlated to the commitment experiences they had. In its most sophisticated form, this type of approach can inform on highly specific experiences that occur in cells in narrowly defined periods of their differentiation. Fate mapping of this sort has been widely used and has shed light on diverse commitment processes (17).

We have designed a new fate-mapping scheme that is intended to provide an enhanced perspective on differences in gene expression that occur during commitment events in vivo. This scheme involves use of the Cre recombinase and novel reporter alleles that can adopt different states as a function of recombinase concentration in cells. In this study, we describe the design and properties of the reporter alleles and establish the foundation for their use in mice. We demonstrate that the alleles perform as expected in vivo by showing they can discriminate populations of T cells that differ in their prior expression of a gene (Tnfrsf4) that is variably induced as a function of TCR signaling. The study makes clear the properties of the new reporter alleles and their potential benefits for the analysis of cell-fate decisions within and beyond the immune system.

Materials and Methods

Generation of gene targeting vectors

The Stop element in the reporter alleles was generated by inserting a PGK-puro gene (18) just after the 5’ loxP site of an existing Stop element (19) that had been modified such that the 3' splice acceptor (SA) sequence was replaced with an SV40 polyadenylation sequence. An adenovirus SA sequence was isolated from pSA-βgeo (20) and inserted immediately upstream of this. DNA fragments containing the tdTomato (21) and human...
CD2 (hCD2) open reading frames (ORFs) were generated by PCR and inserted downstream of the Stop element. An internal ribosome entry site (IRES)-enhanced GFP (eGFP) element (derived from pIGCN21) (22) was flanked by recombinase recognition sites (FRT, 5172, and 3373) by conventional ligation with fragments taken from plasmids containing these sites. The modified IRES-eGFP was then cloned downstream of either SA-Stop-tdTomato or SA-Stop-hCD2. SV40 polyadenylation sequences were added downstream of the eGFP or hCD2 ORFs, and, finally, the fully assembled reporter inserts were cloned into a polylinker inserted into the XbaI site of a modified form of pRosa26-1.

Embryonic stem cell culture, gene targeting, and generation of mice

E14 mouse embryonic stem (ES) cells (23) were cultured and transfected using standard conditions. The cells were cultured in Glasgow MEM supplemented with penicillin, streptomycin, 2-ME, sodium pyruvate, nonessential amino acids (all from Invitrogen, Carlsbad, CA), 15% FCS (Cambrex, East Rutherford, NJ), and 0.3% LIF-containing supplement. Cells were cultured on gelatinized tissue culture-treated plates without feeder cells. The targeting vector (25 μg DNA linearized with AscI) was electroporated into 2 × 10^7 E14 ES cells in PBS (Invitrogen) using cuvettes with a 0.4-cm electrode gap at 250 V and 950 μF. Puromycin (2 μg/ml) selection was imposed after 2 d, and single ES cell colonies were picked after an additional 7–9 d in selection. The colonies were expanded in 96-well plates, after which one-half of the culture was frozen down, whereas the other half was used for isolation of DNA. The DNA was screened by Southern blot using the mini-Southern procedure (24) and a radiolabeled Xhol fragment of the ROSA26 locus from the pRosa26 plasmid (25). Mice were generated from targeted ES cells by microinjection of the ES cells into C57BL/6 (C57BL/6xDBA/2 F1) eight-cell mouse embryos using a laser-assisted technique (26). Germine transmission was accomplished by breeding chimeric males to C57BL/6 females. Mice were used between 6 and 10 wk of age for experiments. All mice were maintained and bred under specific pathogen-free conditions under the approval of the University of California, San Francisco, Institutional Animal Care and Use Committee (San Francisco, CA).

Transient transfections and use of cell-permeant Cre

For transient transfections, targeted E14 cells were trypsinized, washed, and resuspended at 2.5 × 10^6/ml. A total of 40 μg circular DNA was mixed with 0.8 ml cells, after which the suspension was transferred to a cuvette (0.4-cm electrode gap) and electroporated at 250 V and 950 μF. To purify cells exhibiting a partially recombined phenotype, targeted ES cell clones were transfected with pMCre-Cre (27), incubated for 5 d, and then flow-sorted using a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA). After further expansion, the sorted tdTomato-eGFP cells were transfected with a Cre-estrogen receptor (ER) plasmid (28) and a human CD2 expression vector as a tracer for transfected cells. Twenty-four hours later, the cells were incubated with varying concentrations of 4-hydroxy-tamoxifen, the cells were incubated with varying concentrations of 4-hydroxy-tamoxifen for 24 h. After an additional 24 h of culture in the absence of 4-hydroxy-tamoxifen, the cells were analyzed for human CD2 and reporter CD2 expression vector as a tracer for transfected cells. Twenty-four hours later, the cells were transfected with a Cre-estrogen receptor (ER) plasmid (28) and a human

Flow cytometry and immunofluorescence

Conjugated Abs were purchased from BD Biosciences and eBioscience (San Diego, CA). Single-cell suspensions were prepared from mouse spleens using 0.45-μm cell strainers (BD Falcon, BD Biosciences) and PBS containing BSA (0.3% w/v). T cell stimulations were performed with purified anti-CD3e (clone 145-2C11) and plate-bound goat anti-armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) with or without purified anti-mouse CD28 (clone 37.51, BD Biosciences). Cells were washed by centrifugation and then incubated on ice for 30 min with saturating concentrations of Ab specific for cell surface molecules (B220, CD4, CD8, CD25, and CD44). DAPI (0.3 μM) was used for live-dead cell discrimination. Cells were analyzed using either BD LSRII or FACSCalibur flow cytometers (BD Biosciences). All data were acquired using BD FACS Diva software (BD Biosciences) and further analyzed with FlowJo software (TreeStar, Ashland, OR).

Spleens or lymph nodes were embedded in 4% low-melting agarose/PBS, and 150–250-μm sections were cut in cold PBS with a Vibratome (Vibratome, Bannockburn, IL) at high amplitude and slow speed with a blade angle of 25–28°, mounted onto slides, and imaged. Fixed tissues were prepared by incubating spleen quarters or entire lymph nodes in 2–4% (w/v) paraformaldehyde/PBS at room temperature for 1–3 h. Tissues were washed briefly in PBS, floated on cold 30% (w/v) sucrose, frozen in OCT (Sakura Finetek, Torrance, CA) and stored at −80°C until sectioning on a Leica CM3050 S cryostat (Leica Microsystems, Deerfield, IL). eGFP was detected by fixed samples using a rabbit anti-GFP antiserum (Novus Biologicals, Littleton, CO), followed by a biotinylated donkey anti-rabbit F(ab')2 Ab (Jackson ImmunoResearch) and a tyramide signal amplification-based FITC detection system (PerkinElmer, Waltham MA). Confocal imaging was performed using a modified Axiovert 200M microscope (Zeiss, Oberkochen, Germany) equipped with a spinning-disk confocal scanner (Yokogawa, Sugar Land, TX) with a 40×/1.3 NA oil immersion objective and an iXon EMCCD camera from Andor (South Windsor, CT). Data collection and processing were performed using MetaMorph software with the “Scan Slide” drop-in application (Molecular Devices, Downingtown, PA).

Results

loxp sites are 34 bp in length and are composed of two inverted repeats flanking a central spacer sequence (30) (Fig. 1A). Mutations introduced into the spacer or repeat sequences do not necessarily prevent recombination, but they can affect both the efficiency and specificity of recombination. Some mutant loxp sites recombine
efficiently with the native loxP sequence, whereas others will only recombine with loxP sites that are similarly mutated (31, 32).

We selected two mutant loxP sites as the basis of a novel reporter allele design. A sensitive in vitro recombination assay previously established that these two sites do not recombine detectably with native loxP sites (32). The sites also show reduced homotypic recombination efficiencies relative to loxP: 10% and 30% of native efficiency for 3373 and 5172, respectively (Fig. 1). We designed two novel reporter alleles (one incorporating 3373 sites and the other 5172 sites) based on a successful design used previously by many laboratories. A conventional component of the design was the use of the Git(Rosa)26Sor locus (25) (referred to in this study as Rosa26) as a site into which the reporter elements would be inserted. This locus was attractive because it is transcriptionally active in most cell types in the mouse, and it shows useful expression levels in hematopoietic lineages (25, 33). A second conventional component of the design was the placement of a loxP-flanked disruption (commonly referred to as a Stop element) (19, 34) within an intron of the Rosa26 locus immediately downstream of a strong SA sequence. The purpose of this element was to terminate transcription such that any reporter ORFs appended to it would not be expressed (unless the element was first removed by Cre recombination). The two reporter ORFs we chose were those that encoded the tdTomato variant of dsRed (21) and eGFP (35). A human CD2 cDNA was also used in place of the tdTomato gene in alternative versions of the reporter alleles. We placed an IRES between the two reporter ORFs to allow for their cotranslation (Fig. 1B). tdTomato and eGFP were favored as reporter proteins because they can be readily detected and discriminated by flow cytometry and fluorescence microscopy.

The most important aspect of the reporter design was the placement of mutant Cre recombination sites (3373 or 5172) (32) around the IRES-eGFP element. This was done so that the IRES-eGFP could be selectively excised from the reporter allele with an efficiency that was reduced relative to excision of the upstream loxP-flanked Stop element. The most favored recombination event in cells expressing Cre, therefore, would be loss of the Stop element resulting in gain of both eGFP and tdTomato expression (Fig. 1C). Loss of eGFP would then occur in some but not all of the tdTomato+ cells, and the fraction of them undergoing this event would be a function of the amount of Cre recombinase they expressed (29, 36). Reporter phenotype should therefore reflect the amount of Cre expressed by a population of cells with tdTomato+eGFP− cells expressing lower levels of Cre than tdTomato+eGFP+ cells. Because 3373 sites are recombined less efficiently than 5172 sites (32), loss of eGFP should occur with lower efficiency in populations carrying the 3373 reporter allele than in cells carrying the 5172 allele.

The Rosa26 locus was mutated in mouse ES cells (Fig. 1D) by gene targeting using targeting vectors that carried the indicated reporter configurations (Fig. 1B). Initial versions of the reporter alleles incorporating a dsRed ORF upstream of the IRES-eGFP failed to generate detectable red fluorescence in targeted ES cells following transient Cre expression, even though we could readily detect eGFP in the transfected populations. To correct this problem, we replaced the dsRed ORF with ORFs encoding either the tdTomato variant of dsRed (21) or human CD2. Targeted ES cells carrying these alternative versions of the alleles showed Cre-dependent expression of both eGFP and tdTomato or human CD2 (Fig. 1E). These preliminary experiments established that the reporter alleles were functional in ES cells, so additional experiments were performed to analyze their properties in more detail.

We performed a series of experiments to explore the relationship between Cre recombinase expression levels and reporter recombination status. These involved various strategies to titrate Cre activity in the cells, of which the most robust proved to be treatment with varying doses of Tat-Cre, which is a form of the recombinase that crosses the membrane because it contains a short N-terminal extension from the HIV Tat protein (29).

Both reporter alleles (3373 and 5172) were recombined in treated ES cells in a Tat-Cre dose-dependent fashion (Fig. 2A). A key observation was that at low doses of Cre, cells that had undergone full recombination of the reporter alleles were less common than at higher doses of Cre. That is, the ratio of single-positive cells (SP; tdTomato+eGFP− cells carrying fully recombined alleles) to double-positive cells (DP; tdTomato+eGFP+ carrying partially recombined alleles) was lower at the low doses of Cre than at the high doses. Incubation of Tat-Cre with cells carrying a variant of the reporter allele lacking the mutant loxP sites rendered the cells positive for both tdTomato and eGFP (Fig. 2B), showing that the tdTomato+eGFP+ phenotype depended on Cre recombination. A second important observation was that the frequency of cells carrying fully recombined forms of the 5172 reporter allele was higher than that of cells carrying fully recombined forms of the 3373 allele at all concentrations of Cre (Fig. 2C). Both observations were consistent with the anticipated behavior of the alleles based on the fact that both of the mutant loxP sites are recombined less efficiently by Cre than native loxP and that 3373 shows one-third the efficiency of 5172 (32).

To focus specifically on the efficiency of recombination that was dependent on 3373 or 5172 sites, we sorted tdTomato+eGFP− cells from clones of targeted ES cells that had been transiently transfected with a Cre expression vector. These sorted cells were ∼95% pure and retained the partially recombined reporter phenotype during prolonged culture (Fig. 3A). We treated these cells transiently with varying doses of Tat-Cre and then cultured them before analysis by flow cytometry. Loss of eGFP occurred in the...
cells in a Cre recombinase dose-dependent fashion (Fig. 3A), and it was more prevalent in cells carrying the 5172 allele than in those carrying the 3373 allele (Fig. 3B). Similar results were obtained when Cre was delivered to the cells by other means, including transient transfection of a vector expressing a Cre-ER fusion protein followed by induction of Cre activity by treatment of the cells with varying doses of 4-hydroxy-tamoxifen (28) (Supplemental Fig. 1). Thus, as above, these data showed that the reporter alleles behaved in the expected fashion and that the ratio of cells carrying fully recombined alleles to those carrying partially recombined alleles (SP/DP) informs on how much Cre recombinase the cells expressed.

In vitro recombination data predicted that the reporter alleles would adopt one of only two possible recombined states because loxP sites do not recombine detectably with either 3373 or 5172 sites. It was nonetheless of interest to determine whether such unfavorable recombination might be prevalent in the context of the reporter constructs stably integrated into the genome of ES cells. The 3373 and 5172 sites were inserted into the constructs in inverted orientation relative to the upstream loxP sites, so heterotypic recombination would result in inversion of the DNA between the involved sites. Of six possible inversion events (four without prior excision of the Stop element and two with it), two had the potential to allow for reporter expression (eGFP without tdTomato), whereas four would result in an absence of reporter expression. We detected no cells with a tdTomato eGFP+ phenotype following Cre recombination in cells carrying either of the two reporters (Figs. 2A, 3A, Supplemental Fig. 1A). Moreover, when Tat-Cre was added to the flow-sorted tdTomato+eGFP− cells, we noted no accumulation of cells with a tdTomato+eGFP+ phenotype (Fig. 3C) such as would be expected if unfavorable recombination events occurred at a significant frequency. Thus, heterotypic recombination is not a prevalent event, and the reporter alleles behave as predicted.

We generated lines of mice with the ES cells carrying the tdTomato forms of the reporter alleles (26). Lymphocytes from these mice responded to treatment with Tat-Cre by expressing tdTomato and eGFP in a similar fashion to what we had observed in the ES cells. Specifically, the ratio of fully to partially recombined alleles (SP/DP) increased as a function of Cre concentration, and the 5172 allele was more readily recombined than the 3373 allele (Fig. 4A).

To determine whether the reporter alleles would respond to concentrations of Cre that are useful experimentally, we crossed the reporter mice to Ox40-cre mice. These mice express Cre from the native Tnfrsf4 locus and show persistent high expression of the recombinase in CD4+ regulatory T cells, transient high expression in precursors of CD4+ memory T cells, and transient weak expression in a subpopulation of precursors of CD8+ memory T cells and the thymic precursors of a small subpopulation of naive T cells (37). Ox40-cre induced recombination of the reporter alleles in a fashion that reflected the relative levels of Cre expression in the different types of T cells. Specifically, populations that expressed high amounts of Cre (CD4+ regulatory and memory T cells) showed the highest ratios of fully to partially recombined reporter alleles (Fig. 4B, 4C). By contrast, fully recombined alleles were less prevalent in naive and CD8+ T cells, which expressed lower levels of the recombinase (Fig. 4B, 4C).

Cre expression levels should be elevated in mice that are homozygous for Ox40-cre relative to heterozygous mice. This predicts that reporter-positive cells should be more numerous and recombination ratios higher in T cells from the former than in those from the latter. The Ox40-cre allele expresses no OX40, and this would be expected to compromise the survival of some T cells (such as CD4+ memory T cells) (38). Despite this, however, we found increases in reporter-positive T cells in the homozygous mice (Supplemental Fig. 2A), and, associated with this, there were increases in the SP/DP recombination ratios (Supplemental Fig. 2B).

T cells were purified from Ox40-cre mice and stimulated in vitro with an anti-CD3 Ab. Induction of OX40/Ox40-cre in this context occurs as a function of anti-CD3 concentration (37) and is potentiated by costimulatory signaling delivered by inclusion of an anti-CD28 Ab. Consistent with this, we found a higher frequency of cells carrying fully recombined reporter alleles in cultures treated with high amounts of anti-CD3 compared with those treated with low amounts or in cultures that received costimulation compared with those that did not (Fig. 4D). Cumulatively, therefore, we conclude that the reporter alleles behave as expected and can be used to inform on differences in the levels of Cre expressed by populations of cells in vivo.

As a final test of the utility of the alleles, we determined whether they could be detected by fluorescence confocal microscopy in sections of lymphoid tissue from Ox40-cre reporter-positive mice. Whereas the tdTomato and eGFP moieties could be readily detected in fresh tissue sections (Supplemental Fig. 3), eGFP fluorescence was typically low and became undetectable by confocal microscopy upon routine fixation. This problem could be overcome by use of fluorescently labeled anti-GFP antisera, and, using this approach, we could easily distinguish lymphocytes carrying fully recombined reporter alleles from those with partially recombined alleles in frozen sections (Fig. 4E). These data show that the alleles can be used for determining the localization of cells that differ in their reporter expression and thus gene expression history.

Discussion

Reporter alleles that inform on Cre activity in cells are typically designed to transition between inactive and active states (34, 39, 40). The active state arises when Cre excises a loxP-flanked element.
from the allele, rendering the cell positive for persistent expression of a reporter protein from a downstream ORF. Populations of cells that express the reporter have previously expressed Cre, whereas those that do not are enriched for cells that never expressed Cre or expressed insufficient levels of it for recombination to occur efficiently. Reporters that have been used in this sort of context include β-galactosidase, eGFP and variants of eGFP, placental alkaline phosphatase, and wheat-germ agglutinin (33, 34, 39–42).

The reporters we have described in this paper include the core components of the conventional design and thus they can be used for all of the same purposes. They also include an additional component that can be used to discriminate between populations of cells that differ in how much Cre they previously expressed. Thus, instead of two possible configurations (i.e., inactive versus active), the reporters are capable of adopting three states (i.e., inactive and two distinct active states) depending on the amount of Cre expressed in cells. If Cre expression is governed in a physiologically significant fashion (e.g., by elements taken from a developmentally regulated gene), then the reporter alleles can be used to discriminate populations that have different gene expression histories. This type of discrimination can be exploited in the study of lineage commitment decisions because it allows for cell fate to be correlated with prior gene expression levels.

To analyze the performance of the reporter alleles, we employed various strategies to deliver graded amounts of Cre recombinase activity to the targeted ES cells. These included transient transfection of expression vectors encoding Cre or transient transfection (or retroviral transduction) of vectors encoding Cre-ER (28) followed by induction of Cre activity with 4-hydroxy-tamoxifen. Relative to these, the cell-permeant Tat-Cre protein (29) provided the highest efficiency Cre recombination and the greatest experimental dynamic range. Using Tat-Cre, we could show that the penetrance of complete recombination at the reporter alleles increased as a function of Cre recombinase activity, and, consistent with our

![Figure 4](http://www.jimmunol.org/)
expectations from in vitro recombination assays (32), we found that recombination of 3373 sites occurred less efficiently than that of 5172 sites. Complete recombination of the 3373 reporter allele therefore depended on higher levels of Cre activity in cells than the 5172 allele.

The characteristics of the reporter alleles just summarized make clear their value for informing on prior Cre expression in cells, and thus, as argued above, their potential applicability to the study of lineage commitment decisions in which fate determination is a function of quantitative differences in signaling and downstream gene expression. The decision to generate two types of alleles, one involving 3373 sites and the other 5172 sites, was made with two related considerations in mind. The first was that for any given Cre expression level from a cre allele, the population of cells carrying a reporter in its partially recombined configuration would be larger with the 3373 allele than with the 5172 allele. If expression of the cre allele was induced differentially as part of a differentiation process, then correlations between reporter state and differentiated phenotype would likely be higher with one allele than with the other. This is simply because the partially recombined versus fully recombined states would more closely align with differentiated phenotypes in one case than in the other. The second related consideration was that different levels of Cre will be expressed in cells depending on what cre allele has been used. In general, the 3373 reporter allele is likely to be more useful than the 5172 allele when Cre expression is high because the penetrance of complete recombination in affected populations of cells would approach 100% with the 5172 allele (and thus the allele would not discriminate different populations of Cre-expressing cells). When Cre expression is lower, the potential utility of the 5172 allele would increase.

Cre recombination approached 100% penetrance in regulatory T cells in Ox40-cre mice because these cells express the Ox40 gene in a constitutive fashion. Recombination penetrance was lower in other types of cells that express Ox40 transiently and/or at sub-maximal levels. These characteristics allowed us to examine the behavior of the two reporter alleles in mice as a function of differing Cre expression levels. Our analysis included an examination of reporter allele recombination in cells that had been stimulated in vitro with anti-CD3ε (to induce Ox40 expression). In all cases, the recombination status of the alleles correlated well with the known prior/ongoing expression of the Ox40 gene in the populations under analysis. Furthermore, the two alleles (3373 and 5172) consistently differed in their sensitivity to Cre levels as expected, and consequently, they discriminated different subpopulations of cells (i.e., the relative frequencies of cells with fully versus partially recombined alleles were different in the two types of mice for all lineages examined). The results of these experiments therefore substantiate the rationale behind making the two types of alleles, while also providing a clear demonstration of their utility for lineage marking.

In addition to providing information about absolute levels of Cre expression, the new reporter alleles are also expected to inform on differences in the duration of Cre expression in cells. That is, the probability that they will adopt the fully recombined state will increase the longer the cells stay positive for expression of Cre, even if they only express low levels of the recombinase. This aspect will limit the utility of the alleles in discriminating between effects caused by transient high versus prolonged low gene expression, and this must be borne in mind when considering their usefulness for addressing specific biological problems. It is also important to consider that even though the reporter alleles inform on prior Cre activity in single cells, they were nonetheless designed for the analysis of populations of cells rather than single cells. For a given subsaturating level of Cre, a population of cells may be highly enriched for a particular recombination outcome (i.e., full or partial recombination of a reporter allele), but it is expected that some cells within it will harbor alternative outcomes. The extent to which this is the case will differ for the two alleles as a function of Cre expression levels as discussed above. Despite these potential limitations, however, it seems likely that the alleles will provide useful information in many settings and that mice carrying them will be beneficial when studying the activity of multiple cre alleles expressed in a broad range of cell types and developing tissues.

Acknowledgments

We thank members of the Killeen laboratory for helpful discussions and Ron Basco and Quinn Walker for expert technical assistance. We also thank Max Krummel and members of his laboratory for access to a confocal microscope and assistance with data acquisition and analysis.

Disclosures

The authors have no financial conflicts of interest.

References

7. Krummel and members of his laboratory for access to a confocal microscope and assistance with data acquisition and analysis.


**Supplementary Figure Legends**

**Fig. S1.** Recombination of the reporter alleles in response to expression and induction of Cre-ER. 
(A) ES cell clones carrying the 5172 or 3373 alleles were transiently cotransfected with an expression vector encoding Cre-ER (1) and human CD2. Cre recombination was induced by incubation with varying concentrations of 4-hydroxy-tamoxifen for 24 hours after transfection. The cells were then incubated for 72 hours before analysis by flow cytometry. The plots show expression of tdTomato and eGFP on cells that were gated for human CD2 expression as a transfection marker. Numbers on the plots refer to the percentages of cells in the marked fluorescence gates. 
(B) Graph showing the ratio of cells (carrying either the 3373 or 5172 reporter alleles) exhibiting the fully recombined phenotype (tdTomato⁺eGFP⁻) versus the partially recombined phenotype (tdTomato⁺eGFP⁺) as a function of 4-hydroxy-tamoxifen concentration (after treatment and analysis as in A). Statistical significance, p=0.0002, calculated by Student’s t-test.

**Fig. S2.** A two-fold increase in the number of Cre recombinase alleles increases the frequency of cells exhibiting the fully recombined reporter phenotype. 
(A) Reporter activity in peripheral blood CD4⁺CD25⁻CD44lo lymphocytes from either heterozygous Ox40-cre/+ (left) or homozygous Ox40-cre/cre (right) mice as assessed by flow cytometry. Numbers above the gates indicate the frequency of cells with fully or partially recombined alleles. 
(B) The graph shows the ratios of fully to partially recombined alleles from 4 mice of each genotype. The data are representative of two independent experiments. p=0.009, calculated by Student’s t-test.

**Fig. S3.** eGFP fluorescence in fresh tissue sections. The images show tdTomato (left), eGFP (middle) and merged fluorescence (right) from a section of popliteal lymph node. A
tdTomato/eGFP double positive lymphocyte is marked in the overlay with an arrow. Scale bar = 8μM.

References
