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A CRISPR-Cas9 delivery system for in vivo screening of genes in the immune system

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Therapies that target the function of immune cells have significant clinical efficacy in diseases such as cancer and autoimmunity. Although functional genomics has accelerated therapeutic target discovery in cancer, its use in primary immune cells is limited because vector delivery is inefficient and can perturb cell states. Here we describe CHIME: CHimeric IMmune Editing, a CRISPR-Cas9 bone marrow delivery system to rapidly evaluate gene function in innate and adaptive immune cells in vivo without ex vivo manipulation of these mature lineages. This approach enables efficient deletion of genes of interest in major immune lineages without altering their development or function. We use this approach to perform an in vivo pooled genetic screen and identify Pten2 as a negative regulator of CD8+ T cell-mediated responses to LCMV Clone 13 viral infection. These findings indicate that this genetic platform can enable rapid target discovery through pooled screening in immune cells in vivo.

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Understanding the mechanisms that regulate innate and adaptive immunity has accelerated the development of immunotherapies for autoimmune and allergic diseases, transplant rejection and cancer. The dramatic clinical success of immune checkpoint blockade in a broad range of cancers illustrates how fundamental knowledge of immunoregulation can translate to therapy. However, limitations in the tools available for perturbing genes of interest in immune populations has hindered the discovery and validation of new therapeutic targets for immune-mediated diseases.

The use of functional genomics and genetic perturbation strategies has provided an effective tool for the rapid discovery of new therapeutic targets in cancer. In particular, shRNA-based screening enabled the classification of tumor suppressors and essential genes in cancer. However, shRNA approaches are limited by the issues of incomplete knockdown and a high degree of off-target effects. Targeted nucleases, such as TALENs and zinc finger nucleases, have enabled the complete knockout of genes with improved specificity but require custom design of proteins for each target gene. CRISPR-Cas9 genome editing methods to knockout genes in mammalian cells have the advantages of targeted nuclease editing with improved modularity.

Genetic perturbation approaches in immune cells have the potential to accelerate the discovery and validation of new therapeutic targets. One current approach is to stimulate T cells to transduce with a shRNA/sgRNA-expressing lentiviral vector followed by in vitro analysis or in vivo transfer of edited T cells. Although this method is rapid, in vitro stimulation of T cells perturbs their long-term differentiation, does not allow for the study of genes expressed during T cell priming, and is only applicable to immune cell populations that are easily transferred intravenously for analysis in disease models. To circumvent some of these issues, we have previously used a system of lentiviral transduction of bone marrow precursors and subsequent creation of bone marrow chimeras for shRNA-based perturbation of naive T cells without disrupting their differentiation or homeostasis. CRISPR-Cas9 transduction of bone marrow precursors has enabled editing of genes involved in oncogenesis to model hematologic malignancies and in the development of hematopoietic precursors. However, these approaches have not been used for studying the immune response in different disease models or discovery of regulators of T cell responses during cancer and viral infection.

Here we describe CHIME, a bone marrow chimera-based Cas9-sgRNA delivery system that enables rapid in vivo deletion of immunologic genes of interest without altering the differentiation of mature immune cells. We demonstrate the versatility of this system to delete genes of interest in all major immune cell lineages. As a proof of concept, we perform a curated in vivo screen in the LCMV Clone 13 infection model and show that deletion of Ptpn2 enhances CD8+ T cell responses to LCMV Clone 13, thereby revealing a negative regulatory role for Ptpn2 in CD8+ T cell-mediated responses to LCMV Clone 13. Our results illustrate the ability of this genetic platform to enable rapid discovery of therapeutic targets in immune cells using pooled loss-of-function screening.

Results
CHIME enables efficient deletion of immunologic genes. To create gene deletions in hematopoietic lineages, we developed a single guide RNA (sgRNA) chimera delivery system using bone marrow from Cas9-expressing mice. We isolated Cas9-expressing Lineage− Sca-1+ c-Kit+ (LSK) cells from donor mice (Supplementary Fig. 1a), transduced the LSK cells with a lentiviral sgRNA expression vector containing a Vex (violet-excited GFP) fluorescent reporter, and transferred the LSK cells to irradiated recipients to create bone marrow chimERIC mice. Following 8 weeks of immune reconstitution we isolated immune cells that express Cas9 and the sgRNA (marked by Vex). To determine whether candidate genes from major immune lineages could be deleted in vivo we designed sgRNAs to canonical genes expressed by B cells (Ms4a1), macrophages (Fcgr1), and dendritic cells (Ly75), and created chimeric mice using either these sgRNAs or control sgRNAs. We found that CD20 was significantly reduced on B cells in the presence of two Ms4a1 (Cd20) sgRNAs but not a control sgRNA, demonstrating that in vivo deletion of genes in B cells was possible (Fig. 1b, c and Supplementary Fig. 1b). We next confirmed that Fcgr1 (Cd64) could be deleted in red-pulp splenic macrophages by showing that Cd64 expression was significantly reduced for two Fcgr1 targeting sgRNAs but not a control sgRNA (Fig. 1b, c and Supplementary Fig. 1c). Lastly, we created bone marrow chimeras with two Ly75 (Dec205) targeting sgRNAs or a control sgRNA and showed a significant reduction in DEC205 expression on splenic cross-presenting dendritic cells with Ly75 targeting sgRNAs but not control sgRNAs (Fig. 1b, c and Supplementary Fig. 1d). Thus, this chimeric system can be used to efficiently delete genes of interest in both innate and adaptive immune populations in vivo.

CHIME does not alter immune reconstitution or responses. To determine if the presence of Cas9 protein, the lentiviral sgRNA vector, or the process of transducing hematopoietic stem cells affected the reconstitution of immune cells, we generated chimeric mice using either mock-transduced WT LSK cells or Cas9-expressing LSKs that were transduced with a lentiviral sgRNA vector containing a non-targeting sgRNA. The stem cells were transduced at an MOI such that approximately half of the immune cells expressed the fluorescent reporter Vex, indicating the presence of the sgRNA vector (Supplementary Fig. 1e). We analyzed chimeras after immune reconstitution and found that the percentages of B cells, CD4+ and CD8+ T cells, CD11b+ myeloid cells, and dendritic cells in the spleen were similar in WT and Cas9 + non-targeting sgRNA chimeras (Fig. 1d). To determine if the chimeric system altered the response of the immune system to a pathogen, we challenged chimeric mice (WT and Cas9 + non-targeting sgRNA as above) with LCMV Clone 13 virus and examined immune responses. WT and Cas9 + sgRNA chimeric mice had similar weight loss kinetics (Fig. 1e), indicating that the sgRNA delivery system did not alter the susceptibility of the mice to LCMV Clone 13. Serial viral titers in the blood and viral titers in the kidney at day 30 were comparable between WT and Cas9 + sgRNA chimeras, indicating a similar response to the viral infection (Fig. 1f and Supplementary Fig. 2a). These chimeras also had similar CD8+ T cell responses as assessed by expression of T cell functional markers, co-inhibitory receptors, and P3 antigens in mice (Supplementary Fig. 2b, 2c). These findings demonstrate that our Cas9-sgRNA delivery system does not affect immune reconstitution or immune responses to LCMV Clone 13 viral infection.

Candidate genes are efficiently deleted in naive T cells. To determine if CHIME could be used for studying genes in naive unperturbed T cells, we first examined whether this system altered T cell development. To examine this we analyzed thymic subsets in WT and Cas9 + non-targeting sgRNA chimeras and found no differences in the frequencies of double-negative (DN),
double-positive (DP), or CD4/CD8 single-positive (SP) populations in WT and Cas9 + non-targeting sgRNA chimeras (Fig. 2a). We also examined the naive status of CD8+ T cells from these chimeric mice and found no differences in CD44, CD62L, and CD69 percentages (Fig. 2b–d). To determine if CHIME enabled efficient deletion of genes in naive CD4+ and CD8+ T cells, we created chimeras carrying two non-targeting control sgRNAs or three Pdcd1 targeting sgRNAs. We stimulated T cells from these chimeric mice with αCD3/CD28 to induce PD-1 expression and found a significant reduction of PD-1 expression in the presence of targeting sgRNAs but not control sgRNAs. On average 80% deletion was achieved in both CD4+ and CD8+ T cells (Fig. 2e, f). Analyses of naive CD4+ and CD8+ T cells from these mice prior to stimulation using the TIDE assay confirmed that these T cells had ~80% aberrant sequences, indicating efficient CRISPR-mediated indel formation (Fig. 2g and Supplementary Fig. 2d). We next analyzed off-target effects in this system by performing the TIDE assay on the top three predicted off-target sites for each of the three Pdcd1 sgRNAs, and found minimal off-target editing above background in both CD4+ and CD8+ T cells (Fig. 2h, i). Thus, this system can be used to knockout genes in naive T cells without altering their development or state.

**T cell-intrinsic functions can be examined in disease models.** To assess whether this Cas9-sgRNA delivery system can be used to

![Diagram](https://via.placeholder.com/150)
identify intrinsic regulators of T cell function in CD8<sup>+</sup> T cells, we used two models: LCMV Clone 13 viral infection as a model of T cell exhaustion, and MC38-OVA tumors as a model of tumor immunity. T cells responding to LCMV Clone 13 viral infection encounter multiple inhibitory mechanisms<sup>26,27</sup>, many of which are conserved in the tumor microenvironment (TME)<sup>17,28</sup>. The MC38-OVA tumor model was used to directly assess antigen-specific T cell suppressive mechanisms in the TME. To analyze antigen-specific T cells in vivo, we used Cas9-expressing donor mice with the transgenic T cell receptors P14 (specific to the LCMV CD8

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**Fig. 2** Deletion of genes in naive T cells with minimal off-target editing using CHIME. **a** Quantification of thymic subsets (CD4<sup>+</sup> CD8<sup>−</sup>, CD4<sup>−</sup> CD8<sup>+</sup>, CD4<sup>−</sup> CD8<sup>−</sup>, CD4<sup>+</sup> CD8<sup>+</sup>) from WT or Cas9 + control sgRNA chimeric mice. **b** Representative flow cytometry plots of CD44, CD62L, and CD69 from splenic CD8<sup>+</sup> T cells. **c** Quantification of naive status of CD8<sup>+</sup> T cells in (b, c). **d** Flow cytometry plots of PD-1 expression in CD4<sup>+</sup> T cells (top panel) and CD8<sup>+</sup> T cells (bottom panel) from representative non-targeting control sgRNA or Pdcd1 sgRNA chimeras following αCD3/CD28 stimulation. **e** Quantification of PD-1 expression for two control and three Pdcd1 sgRNAs from (e). **f** TIDE assay on naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells for three Pdcd1 targeting sgRNAs. **h** TIDE assay on naïve (h) CD4<sup>+</sup> and (i) CD8<sup>+</sup> T cells designed to detect the top three predicted off-target sites (1st, 2nd, 3rd) for three Pdcd1 targeting sgRNAs. Dashed line represents the aberrant sequence (%) when comparing two non-targeting control sgRNAs (background aberrant sequence). All experiments had at least three biological replicate animals per group and are representative of two independent experiments. Bar graphs represent mean and error bars represent standard deviation. Statistical significance was assessed among the replicate bone marrow chimeras by one-way ANOVA (**p < .05, ***p < .01, ****p < .001, *****p < .0001). See also Supplementary Fig. 2. Source data are provided as a source data file.
epitope GP33-41) or OT-1 (specific to the ovalbumin CD8 epitope OVA257–264). We transferred equal numbers (1:1 ratio) of congenically-marked antigen-specific gene-deleted naive CD8+ T cells and control cells to an unmanipulated host that was subsequently challenged with viral infection or tumor (Fig. 3a and Supplementary Fig. 2d) to compare the phenotype and function of the gene-deleted and control T cells in the same microenvironment.

We first evaluated the effect of deleting Batf, an essential transcription factor for effector T cell differentiation19,29 during LCMV Clone 13 viral infection. Analysis of the Batf sgRNA containing cells pre-transfer (input) by the TIDE assay indicated that the indel percentage was on average 90% (Supplementary Fig. 2e). We infected recipient mice with LCMV Clone 13 and analyzed the ratio of control sgRNA-containing P14 T cells to control sgRNA or to Batf sgRNA-containing P14 T cells (Fig. 3b, c and Supplementary Fig. 2f) in the spleen on day 8 post infection. The ratios of P14 T cells with control sgRNA vs. control sgRNA remained unchanged compared with the input ratios (Fig. 3b). In contrast, P14 TCR transgenic T cells containing the Batf sgRNA were significantly depleted for three different Batf sgRNAs, which recapitulates both germline knockout and shRNA knockdown phenotypes of BATF in CD8+ T cells during LCMV viral infection.
infection\textsuperscript{19,29}. Batf sgRNA-containing T cells were similarly depleted in the liver of recipient mice, indicating a robust effect in multiple organs (Fig. 3c). Thus, the chimera system can be used to evaluate the cell-intrinsic role of genes that regulate T cell differentiation and function in the LCMV Clone 13 infection model.

To determine if we could also examine the cell-intrinsic function of genetically perturbed CD8\textsuperscript{+} T cells in the TME, we transferred naive OT-I transgenic CD8\textsuperscript{+} T cells into recipient mice and implanted MC38-OVA tumor cells. We again used sgRNAs targeting Batf, as an example of a gene deletion that reduced T cell expansion. The ratios of control sgRNA to control sgRNA-containing OT-1 T cells in the tumor did not change significantly at day 7 post-tumor implantation, compared with input (Fig. 3d, e and Supplementary Fig. 2g). In contrast, OT-1 T cells with the three Batf sgRNAs had a significantly reduced expansion compared with control cells (Fig. 3d, e). These results indicate that the chimera system can be used to perturb genes and evaluate cell-intrinsic effects in multiple disease models.

Performing an in vivo CRISPR screen in T cells with CHIME. We sought to determine if we could evaluate the function of multiple genes in a pooled fashion\textsuperscript{30}. In order to determine the number of genetic perturbations that could feasibly be evaluated in a single animal, we performed bandwidth experiments to determine the number of integrated sgRNAs (barcodes) that could be successfully delivered to WT LSK cells and recovered from major immune populations (Supplementary Fig. 1b and Supplementary Fig. 3a–b). We transduced LSK cells with a pool of vectors expressing 20,000 distinct barcodes and determined the number of recovered barcodes in bone marrow, thymic, and splenic immune populations by sequencing (Supplementary Fig. 3c–d). The number of recovered barcodes varied considerably between cell types (least in thymic DP subset, and greatest in B cells), which is likely a consequence of the different differentiation paths from LSK progenitors (Supplementary Fig. 3d). In the spleen we recovered approximately 500 sgRNAs per mouse in all of the lineages analyzed (Supplementary Fig. 3d). Thus the number of recovered barcodes from many of the evaluated cell populations was sufficiently high to enable pooled in vivo screens using this system. Assuming recovery of 500 sgRNAs per mouse, we performed a Monte Carlo simulation to determine the number of pooled chimeras necessary to recover a 5000 sgRNA library. We found that theoretically pooling 30 chimeras enabled recovery of greater than 96% of a 5000 sgRNA library (Supplementary Fig. 3e).

We next designed a proof-of-concept pooled screen in effector CD8\textsuperscript{+} T cells to identify genes that regulate CD8\textsuperscript{+} T cell responses to LCMV Clone 13 infection. We generated 10 bone marrow chimeras from P14-transgenic Cas9-expressing CD45.1\textsuperscript{+} LSK cells transduced with a library of 110 sgRNAs targeting 21 genes relevant to T cell biology and 50 non-targeting control sgRNAs (Fig. 4a). The 21 genes were chosen to target three broad classes: TCR/cytokine signaling, coinhibitory/costimulatory receptors, and metabolism-associated. These classes were of particular interest given the important established roles of these pathways in T cell activation and immunosuppression during T cell responses. We confirmed that the recipient mice had a normal response to LCMV Clone 13 viral infection by assessing weight loss during the infection as well as kidney viral titer day 8 post infection (Supplementary Fig. 3f and 3g). We next analyzed CD44 expression, a T cell activation marker, on the CD45.1\textsuperscript{+} transferred population and confirmed that all transferred T cells containing the sgRNA library were CD44\textsuperscript{hi} (Supplementary Fig. 3h, 3i), indicating all transferred cells were responding to LCMV Clone 13.

We recovered the majority of the 110 sgRNA pool from each LCMV-infected mouse (Supplementary Fig. 3j). Comparison of the 30 replicate mice revealed that sgRNA abundance (fold change relative to input) in both the spleen and lung were highly correlated (Pearson correlation coefficient 0.96), indicating high replicate animal reproducibility of library representation (Fig. 4b and Supplementary Fig. 3k). Further, we identified both enriched and depleted sgRNAs with approximately a normal distribution (Fig. 4c). The enrichment and depletion of sgRNAs was similar in spleen and lung, suggesting that the effect on CD8\textsuperscript{+} T cell differentiation was not tissue-dependent (Fig. 4d). We visualized the distribution of FDR values and identified marked depletion of sgRNAs targeting Zap70, Cd28, and Dlat (Fig. 4e, i, and Supplementary Table 1). We found that Zap70 was the most significantly depleted gene in the screen, consistent with its essential role in TCR signaling. Cd28 is a positive regulator of CD8\textsuperscript{+} T cell responses, and primary CD8\textsuperscript{+} T cell responses to LCMV are attenuated in CD28 depleted mice\textsuperscript{31}, supporting our screen results.

sgRNAs targeting Kdr, Pdcd1, Adora2a, and Ptpn2 were markedly enriched in CD8\textsuperscript{+} T cells (Fig. 4g, h, and Supplementary Table 1). Enrichment of Pdcd1 is consistent with its negative regulatory role in CD8\textsuperscript{+} T cell responses to LCMV Clone 13\textsuperscript{32}. Adora2a and Kdr have both been implicated as negative regulators of CD8\textsuperscript{+} T cells in the TME\textsuperscript{33,34}, but have not been studied in the context of LCMV viral infection. The phosphatase Ptpn2 was also an enriched hit, consistent with its established negative regulatory role in CD8\textsuperscript{+} T cells in autoimmunity and tolerance\textsuperscript{35,36}.

Pdcd1 and Ptpn2 negatively regulate anti-viral CD8\textsuperscript{+} T cells. We focused on Pdcd1 given its established negative regulatory role in the LCMV model\textsuperscript{32} and on Ptpn2 for validation studies. The role of Ptpn2 in regulating CD8\textsuperscript{+} T cell responses to chronic viral infection has not been examined, although it is known to attenuate T cell responses to maintain tolerance and prevent autoimmunity\textsuperscript{35,36}.

To evaluate the role of Pdcd1 in regulating anti-viral CD8\textsuperscript{+} T cell responses, we performed a 1:1 competitive assay with control sgRNA or Pdcd1 sgRNA-containing P14 CD8\textsuperscript{+} T cells during LCMV Clone 13 viral infection. Using three sgRNAs, we found that Pdcd1 sgRNA-containing P14 CD8\textsuperscript{+} T cells significantly outcompeted control sgRNA-containing cells in the spleen of infected recipient animals (Fig. 5a, b). Analysis of Pdcd1 sgRNA-containing P14 CD8\textsuperscript{+} T cells following LCMV Clone 13 infection revealed significantly reduced PD-1 expression levels, as expected (Fig. 5c). To evaluate Ptpn2, we first confirmed efficient deletion (~80%) of Ptpn2 using the TIDE assay (Fig. 5d) and by western blot (Fig. 5e). Using two sgRNAs, we found that Ptpn2 sgRNA-containing P14 CD8\textsuperscript{+} T cells significantly outcompeted control sgRNA-containing cells in the spleen of infected recipient animals (Fig. 5f, g). These data indicate that both Pdcd1 and Ptpn2 have cell-intrinsic negative regulatory roles on CD8\textsuperscript{+} T cells in the LCMV Clone 13 infection model. In addition, this validates our screening approach for recovering (Pdcd1) and discovering (Ptpn2) regulators of T cell function in vivo.

Discussion
The discovery of new regulators of immune cell function using functional genomics has been limited by the difficulty of genetically perturbing immune cells without extensive ex vivo manipulation\textsuperscript{19}. Here we describe CHIME, a chimera-based delivery system that enables rapid deletion of candidate genes in both innate (macrophages and dendritic cells) and adaptive (B cells, CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells) immune populations without perturbing
cell states. In vivo analysis of gene function in immune populations through ES cell-targeted generation of knockout mice\textsuperscript{37,38} is a lengthy process, while activation or cytokine stimulation of T cells to enable transduction\textsuperscript{15} results in altered effector T cell differentiation\textsuperscript{19}. Our system improves on available approaches by allowing deletion of genes in immune cell lineages in 8 weeks while maintaining normal immune development and function. Thus, we can rapidly analyze genes in immune cells in both physiological and disease contexts for the discovery of therapeutic targets. This system also enables high throughput screening using pooled sgRNA libraries\textsuperscript{15,18,39} for the rapid identification and prioritization of new therapeutic targets. Our proof-of-concept screen with a 110 sgRNA library in conjunction with our band-width experiments suggest that this system enables screening of thousands of genes. In addition, our system expands the classes of immune cell lineages that can be screened, allowing efficient editing of macrophages and dendritic cells in vivo - two cell populations that are difficult to study in vivo without the use of knockout mice. Macrophages in particular have garnered attention as a source of cancer immunotherapy targets\textsuperscript{40}, and our system enables discovery of targets that alter macrophage function. Moreover, our system expands the scope of phenotypes that can be evaluated by maintaining normal differentiation and homeostasis of the immune system. For example, our system makes it possible to target genes important for the earliest stages of T cell activation and differentiation, which has implications for developing new vaccine strategies. Our results suggest that CHIME is a versatile genetic platform can enable rapid discovery of therapeutic targets in immune cells in a spectrum of disease models.

Methods

Mouse breeding and production. Seven- to ten-week-old female or male mice were used for all experiments and 7- to 14-week-old female or male mice were used as donors for bone marrow chimera experiments. WT C57BL/6 mice were purchased from The Jackson Laboratory. LoxP-STOP-LoxP Cas9 mice (B6.129(B6N)-Gt(ROSA)26Sortm1(CAG-cas9*,-EGFP)Feh/J)\textsuperscript{14} were a generous gift from Dr. Feng Zhang, Massachusetts Institute of Technology. These mice were bred to Zp3-Cre mice (C57BL/6-Tg(Zp3-cre)1Gwh/$\beta$J) to delete the loxp-STOP-LoxP in the female germline. The resulting Cas9-expressing strain was then bred to OT-1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J) or P14 (Taconic B6.Cg-Tcratm1Mom Tg (TcrlCMV)5327Sd4 backcrossed 10 generations to Jackson C57BL/6J) TCR transgenic mice on the CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ) congenic background. All strains used were backcrossed at least 10 generations to Jackson C57BL/6J.
Fig. 5 Pdcd1 and Ptpn2 are negative regulators of CD8+ T cell responses to LCMV. a Representative output flow cytometry plots of P14 T cells containing control sgRNA vs. control or Pdcd1 sgRNAs in the spleen day 8 post LCMV Clone 13 viral infection. b Quantification of (a) with normalization of the output flow cytometry plots to the input ratios at day 0 and log2 transformation of the data. c Quantification of PD-1 expression on P14 T cells containing control sgRNA or Pdcd1 sgRNAs in the spleen day 8 post LCMV Clone 13 viral infection. d TIDE assay on naive CD8+ T cells containing control sgRNA or Ptpn2 sgRNAs. e Cropped western blot of splenic CD8+ T cells from control sgRNA or Ptpn2 sgRNA-containing chimeras. f Representative output flow cytometry plots of P14 T cells containing control sgRNA vs. control or Ptpn2 sgRNAs in the spleen day 8 post LCMV Clone 13 viral infection. g Quantification of (f) with normalization of the output flow cytometry plots to the input ratios at day 0 and log2 transformation of the data. All experiments (except western blot) had five biological replicate animals per group and are representative of two independent experiments. Western blot had two pooled mice per group and is representative of three independent experiments. Bar graphs represent mean and error bars represent standard deviation. Statistical significance was assessed among the replicate recipients of transferred T cells by one-way ANOVA (b, c, g) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Source data are provided as a source data file.

Sample size was chosen to ensure the possibility of statistical analysis and to also minimize the use of animals. Data exclusion was not used. Age and sex-matched animals were used for each experiment. Animals were also co-housed when possible. All attempts to reproduce our findings were successful. The LCMV Clone 13 infection experiment (Fig. 1e, f) was blinded during data collection. All experimental mice were housed in specific pathogen-free conditions and used in accordance with the ethical regulations and animal care guidelines preapproved by the Harvard Medical School Standing Committee on Animals and the National Institutes of Health.

Guide RNA design and cloning. The sgRNA oligonucleotides (sequences in Supplementary Table 2), were designed using the Broad CRISPR algorithm41. Off-target sites were identified using the Benchling CRISPR design tool which incorporates off-target rules from the MIT CRISPR algorithm42. sgRNAs were cloned into our sgRNA vector using a BsmBI restriction digest. This sgRNA vector incorporates off-target rules from the MIT CRISPR algorithm42. sgRNAs were validated based on expression of a selectable marker protein. MC38-OVA cells were selected for 2 days with 2 µg/mL puromycin prior to use. MC38-OVA cells were validated based on expression of a selectable marker and exome sequencing. BHK-21 cells (gift from E. John Wherry, University of Pennsylvania) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 5% tryptose phosphate broth. Vero cells (gift from Cigall Kadoch, Dana Farber Cancer Institute) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 µg/ml gentamicin.

Cell lines. MC38-OVA cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 µg/ml gentamicin. MC38-OVA cells (gift from Dario Vignali, University of Pittsburgh) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 5% tryptose phosphate broth. Vero cells (gift from E. John Wherry, University of Pennsylvania) were cultured in EMEM supplemented with 10% FBS, and 1% penicillin/streptomycin. 293x cells (gift from Cigall Kadoch, Dana Farber Cancer Institute) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 µg/ml gentamicin.

Bone marrow isolation and chimera setup. Femurs, tibias, hips, and spines were isolated from CD45.1+ donor mice, crushed, and ACK-lysed. LSK (lineage- Sca-1+ Kit+) were enriched with a CD317 MACS isolation kit and then sorted to purity. The LSK were spin transduced with lentiviral constructs on a Retroviren-coated plate. LSK cells were then transfected intravenously into irradiated CD45.2+ recipients.

TIDE assay. The TIDE (Tracking of Indels by DEcomposition) assay25 was performed on isolated naive T cells. To do this, DNA was extracted from cells (Quagen DNeasy kit) and a 500 base pair product spanning the sgRNA cut site amplified by PCR. This PCR product was purified (Quagen PCR Purification kit) and analyzed by Sanger sequencing. All TIDE primers are listed in Supplementary Table 2.
Adaptive T cell transfer. Spleens were isolated from chimeric mice (>8 week post reconstitution) and naïve CD8+ T cells were purified using a naïve CD8+ MACs kit (95% purity). Cells were stained with lineage-specific antibodies (TERR1-199, B220, and Gr-1) and 7-Aminomycin D (7-AAD) and then sorted (Lineage-, 7-AAD-, Vex+ cells). For LCMV studies, cells were transferred (500:500 mix) to recipient mice on day -1, and mice were infected with LCMV Clone 13 (as below) on day 0. For tumor studies, cells were transferred (1000:1000 mix) to recipient mice on day -1, and mice were injected with MC38-OVA (as below) on day 0.

LCMV production and plaque assay. LCMV Clone 13 virus was produced by infecting BHK-21 cells with an LCMV Clone 13 virus stock at an MOI of 0.03 and harvesting viral supernatants 48 h after. Viral titers were determined by plating diluted viral stocks or serum/tissue samples on Vero cells with an agarose overlay. Four days later the Vero cells were stained with neutral red dye, and 14 h later plaques quantified.

LCMV infection and analysis. Mice were infected with 4 × 10^6 PFU LCMV Clone 13 i.v., monitored for weight loss, and bled or sacrificed at day 8, 15, or 30 post infection for flow cytometry analyses. For viral titer studies, mice were bled at days 8, 15, and 30 post infection. Liver lymphocytes were isolated by dissociation of the liver followed by a 40%/60% Percoll gradient. Lung lymphocytes were isolated by dissociation of the lung on a gentleMACS Dissociator followed by a 37°C incubation in collagenase for 30 min. Lymphocytes were enriched on a 40%/60% Percoll gradient. To deplete CD4+ T cells, mice were injected i.p. with 200 µg antiCD4 (BioXCell Cat# BE00003-1) on days -1 and -1 (relative to LCMV Clone 13 injection on day 0).

Tumor injection. Mice were anesthetized with 2.5% 2,2,2-Trimethoxyethanol (Avertin) and injected in the flank subcutaneously with 2 × 10^6 MC38-OVA tumor cells. Tumors were measured every 2–3 days once palpable using a caliper. Tumor volume was determined by the volume formula for an ellipsoid: 1/2 × D × d^2 where D is the longer diameter, and d is the shorter diameter. Mice were sacrificed when tumors reached 2 cm or upon ulceration.

Tumor infiltrating lymphocyte isolation. Tumors were excised and dissociated using a gentleMACS Dissociator. Tumors were then incubated in collagenase for 20 min at 37°C. Lymphocytes were enriched using a 40%/70% Percoll gradient.

Flow cytometry and cell sorting. Flow cytometry analyses were performed on a BD LSR II or BD FACSymphony A5 and cell sorting was performed on a BD Aria using a gentleMACS Dissociator. To deplete CD4+ T cells, mice were injected i.p. with 200 µg antiCD4 (BioXCell Cat# BE00003-1) on days -1 and -1 (relative to LCMV Clone 13 injection on day 0).

Bandwidth and in vivo screening experiments. For bandwidth experiments a 20,000 sgRNA library (Brie library from the Genetic Perturbation Platform at the Broad Institute) was used to transduce WT OT1 mice to create bone marrow chimeras as described above. Following immune reconstitution, immune subsets were sorted based on lineage markers and Vex. DNA was isolated using the Qiagen DNeasy kit, the sgRNA region PCR amplified, and sequenced on an Illumina HiSeq to determine sgRNA abundance. Recovery of sgRNAs was assessed using a 25 read cutoff. For calculations of the number of chimeras needed for recovery of a 5000 sgRNA library we performed a Monte Carlo simulation where we sampled 5000 sgRNAs with replacement from a pool of 50,000 sgRNAs. We determined the number of samples of 5000 sgRNAs it would take to recover ~95% of the 5000 sgRNA library with a probability of ~95%. This single sample was performed 10,000 times and was increased by 1 h at room temperature. The membrane was washed and visualized using the Li-Cor Cle Imaging System (Li-Cor). Full uncropped blot is available in the source data file.

Western blotting. Spleen and lymph nodes (cervical, axillary, inguinal) were isolated from control or experimental animals. Lysates were generated using a mixture of Pierce RIPA buffer and protease/phosphatase inhibitor at a final concentration of 1 mg/mL. Protein concentration was measured with a BCA protein assay kit. Subsequently, 30 µg of protein was run on a NuPage 4–12% bis-tris protein gel and then transferred to a nitrocellulose membrane. The membrane was incubated overnight in Odyssey blocking buffer followed by staining with anti-TC-PTP (C term) mouse monoclonal IgG antibody (Medimabs Cat MM-00195) and anti-β actin rabbit polyclonal IgG antibody (Acbam Cat# ab8227) at a 1:1000 dilution for 1 h at room temperature. The membrane was washed with TBS-T and then incubated with secondary antibodies IRDye 680RD Goat anti-Mouse IgG (LI-COR Biosciences Cat# 925-68070) and IRDye 800CW Donkey anti-Rabbit IgG (H + L) (LI-COR Biosciences Cat# 926-80022), at a 1:10000 dilution for 1 h at room temperature. The membrane was washed and visualized using the Li-Cor Cle Imaging System (Li-Cor). Full uncropped blot is available in the source data file.
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Author contributions

M.W.L., W.N.H. and A.H.S. conceived the project and wrote the manuscript with assistance from T.H.N., M.A.C., and J.E.G. M.W.L., T.H.N., M.A.C., W.N.H. and A.H.S. designed experiments; M.W.L., T.H.N. and M.A.C. performed and analyzed all experiments with assistance from K.B.Y., J.D.T., F.D.B., and J.E.G. J.G.D. helped with bandwidth/screen library construction, experimental design, sequencing, and computational analysis of results. S.A.W. helped with analysis and visualization of screen data. D.J.C. helped with computational analyses of bandwidth experiments.

Additional information

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Competing interests: A.H.S. has patents on the PD-1 pathway licensed by Roche Genentech and Novartis, and consults for Novartis. W.N.H. has a patent application on in vivo Cell exclusion-specific enhancers. A.H.S. and W.N.H. have a patent application on PTNP2 as a therapeutic target held/submitted by Dana-Farber Cancer Institute. J.G.D. and W.N.H. consult for Tango Therapeutics. The remaining authors declare no competing interests.

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