Supplemental Info File

Supplemental Materials and Methods

Cell culture and reagents

Cell lines were cultured using standard conditions (5% CO_2; 37^{o}C). The 4T1 mammary tumor line was purchased from Karmanos Cancer Institute, and was propagated in high glucose DMEM (11965-118, ThermoFisher) containing 10% (v/v) fetal bovine serum (ThermoFisher) and 1% (v/v) penicillin/streptomycin antibiotic (15140122, ThermoFisher). All 4T1 sublines (4T1-high, 4T1-low, 4T1-ffLUC-eGFP) were cultured as above.

Cell line generation

Lentivirus preparation

Lentivirus was produced following 293T transfection at ~50% confluency with Fugene 6 (Promega) at a ratio of 3:1 Fugene/DNA in Opti-MEM. Constructs used to generate lentivirus included donor plasmids pFUGW-FerH-ffLuc2-eGFP (Addgene plasmid #71393; a kind gift from Glenn Merlino (Day et al., 2009)) or pFUGW-FerH-eGFP (generation described below), packaging construct psPAX2 and envelope construct pMD2.G (Addgene plasmids #12260, #12259; kind gifts from Didier Trono). Media was changed 24 h post-transfection and collected at 72 h and 96 h post-transfection. Following centrifugation and filtration, 1 volume of Lenti-X (Clontech) concentrator was added to 3 volumes media and the viral supernatant stored at 4^{o}C overnight. Virus was collected following centrifugation (1,500 x g, 45 min, 4^{o}C), resuspended in sterile PBS at 200x concentration and stored at -80^{o}C.

Generation of GFP-expressing 4T1-low and 4T1-high sublines

For experiments requiring GFP expression alone (no ffLUC), the pFUGW-FerH-ffLuc2-eGFP vector underwent double restriction digestion with EcoRI (2624 bp) and BamHI (5764 bp) to remove a 3140 bp fragment containing FerH-ffLUC. Gibson cloning was performed with the remaining 6601 bp vector backbone to insert a 1270 bp fragment containing the FerH promoter. The resulting 7787 bp lentiviral vector construct (pFUGW-FerH-eGFP) is available upon request. Sequencing was performed to verify the final construct.

4T1 mammary tumor cells were transduced with pFUGW-FerH-eGFP lentivirus. Following transduction, cells were passaged for 2+ weeks prior to flow sorting the top 1.5% of GFP-expressing 4T1 cells. Flow sorted cells were subsequently grown in culture for >1 month prior to undergoing a second round of flow sorting, to ensure stable integration of lentivirus. On

the 2nd round of sorting, the bottom 6% of GFP+ cells were collected; this subline was subsequently referred to as '4T1-low' or '4T1-FerH-eGFP$^{low'}$. The top 5% of GFP+ cells were also collected; this subline was subsequently referred to as '4T1-high', or '4T1-FerH-eGFP$^{high'}$. GFP positivity was determined by including a 4T1 parental cell sample (GFP-negative). Only events higher than the GFP-negative parental lines were collected at either the bottom, or top, spread of GFP intensity. Following sorting, the 4T1-low and 4T1-high sublines were expanded to a T225 and when 70-80% confluent were frozen down (7-9 days post-sort) to be used in experiments.

Generation of ffLUC/GFP-expressing lentivirally transduced line: 4T1-ffLUC-eGFP

4T1 mammary tumor cells were transduced with pFUGW-FerH-ffLUC-eGFP lentivirus. Following transduction, cells were passaged for 2+ weeks prior to flow sorting the top 1-2% of GFP-expressing cells. Luciferase expression was confirmed using the IVIS Spectrum (Perkin Elmer). Flow sorted cells were subsequently grown in culture for >1 month prior to undergoing a second round of flow sorting (top 5% GFP+), to ensure stable integration of lentivirus. These cells (referred to as '4T1-ffLUC-eGFP') were used in the following experiments.

Determining GFP intensity of 4T1 sublines

Parental (GFP$^{-negative}$; 4T1 parental) and GFP-expressing cell lines (4T1-high, 4T1-low, 4T1-ffLUC-eGFP) were grown in culture and concurrently harvested at ~70-90% confluency. Cells (~2.0x10^6) were resuspended at 1.0x10^6/mL in FACS buffer (1xPBS, pH 7.4 + 2% (v/v) FBS + 0.1% (v/v) sodium azide), and kept on ice. All cell lines were run concurrently on a BD LSR FortessaX50 flow cytometer. Laser intensity to detect GFP was set using the 4T1 parental (negative control) and 4T1-ffLUC-eGFP (GFP-high expressing) lines. A size gate (FSC-A vs. SSC-A) was set to gate around the cells (and gate out debris), followed by a second size gate (SSC-W vs. SSC-A) to eliminate doublets. A FSC gate was also set (FSC-W vs. FSC-H) as an additional measure to select for single cells only. All cell lines were then run (100,000 events/sample) and events recorded.

Animal studies

IACUC and mouse strains. All mouse work was performed in accordance with institutional, IACUC and AAALAS guidelines, under the Fred Hutchinson Cancer Research Center (FHCRC) approved protocols 50928, 50865 and 51075. Mouse strains used for these studies included BALB/c (BALB/cAnNCrl; strain code 028, Charles River), NOD-SCID mice (NOD.CB17-

Prkdcscid/J; bred in house - CCEH, FHCRC; or NOD.CB17-*Prkdcscid*/NCrCrl; strain code 394, Charles River).

The C.FVB-Tg(*Gnrhr-luc/EGFP)L8Mrln*/LmwJ (aka 'Glowing Head'; GH) transgenic strain, was kindly obtained from Dr. Glenn Merlino (NIH). GH are a transgenic strain where eGFP/ffLUC is driven by the rat growth hormone promoter. Expression is limited to the pituitary gland (and testes for males) throughout development and in the adult, to prevent interference with whole body bioluminescence imaging, whilst reported to concurrently tolerize the mouse to GFP/ffLUC antigens (Day et al., 2014). The GH strain was backcrossed from a founder FVB/N Glowing Head strain (FVB/N-Tg(*Gnrhr-luc/EGFP)L8Mrln*/J) by the Merlino group prior to arriving at FHCRC.

The B6.129(Cg)-*Cx3cr1tm1Litt Ccr2tm2.1Ifc*/JernJ (also known as $CCR2^{RFP}Cx3cr1^{GFP}$ (Jung et al., 2000; Saederup et al., 2010)) transgenic mouse strain was obtained from Dr. Eric Holland (Fred Hutch). This C57BL/6 strain was backcrossed (in house) a minimum of 10 times onto the BALB/c background, before use in experiments. We refer to the BALB/c backcrossed strain as '*Cx3cr1-GFP;CCR2-RFP*' or '*Cx3cr1-GFP'* throughout the manuscript. Importantly, *Cx3cr1-GFP;CCR2-RFP* express eGFP in monocytes, NK cells, brain microglia and dendritic cells (Jung *et al.*, 2000; Saederup *et al.*, 2010). To generate progeny for experiments, BALB/c female mice (Charles River) were paired with *Cx3cr1-GFP;CCR2-RFP* heterozygote (HET) male mice. As such, only female *Cx3cr1-GFP* HET (never homozygotes) were used for experiments, which ensured a functional copy of the *Cx3cr1* and *CCR2* genes remained intact.

GH mouse genotyping. GH breeders were initially screened for luciferase expression (pituitary gland and testes); breeders had detectable luciferase (~10^4 p/sec/cm^2/sr) in the pituitary and/or testes as assessed via whole body imaging (IVIS Spectrum). For the experimental cohorts generated, mice were genotyped for presence of the GFP transgene using PCR. gDNA was extracted from tail snips using the DNeasy Blood and Tissue Kit (Qiagen, 10416) according to the manufacturer's instructions. The following primer sets were used to assess for the presence of GFP and an endogenous glucagon sequence.

<u>eGFP:</u>
1. Forward primer - eGFP-214F: 5'-GCAGTGCTTCAGCCGCTAC-3'
2. Reverse primer - eGFP-309R: 5'-AAGAAGATGGTGCGCTCCTG-3'

<u>Glucagon:</u>
1. Forward primer: GLCOE4-F 5'-AACATTGCCAAACGTCATGATG-3'
2. Reverse primer: GLCOE4-R 5'-GCCTTCCTCGGCCTTTCA-3'

For each mouse sample, PCRs were set up in duplicate and both GFP and glucagon (endogenous control) assessed using a quantitative PCR assay. A 20 µL reaction was set up which included: gDNA (50 ng), 1x QuantiTect SYBR Green master mix (Qiagen), 0.5 µM forward primer, 0.5 µM reverse primer, and nuclease-free water up to 20 µL total. Positive controls included known GH homozygous and heterozygous gDNA samples. Negative controls - no template/water, or wildtype gDNA - were also included. Real-time cycler conditions included an initial activation step (95°C for 15 min); followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec. ΔCt, ΔΔCt, and gene copy number were calculated for each sample: mice homozygous for GFP have a transgene copy number of ~2.0 whereas heterozygotes are ~1.0.

Cx3cr1-GFP mouse genotyping. gDNA was extracted from tail snips using DirectPCR Lysis Reagent (Viagen Biotech), according to the manufacturer's instructions. The following primer and probe sets were used to assess for the presence of GFP and an internal control gene (CD86):

Primer/Probe	Sequence (5'to 3')
qGFP-2 Fwd	TGTGATCGCGCTTCTCGTT
qGFP-2 Rev	CTGCTGCCCGACAACCA
qGFP Probe	TACCTGAGCACCCAGTCCGCCCT
qCD86 Fwd	CTTGATAGTGTGAATGCCAAGTACCT
qCD86 Rev	TGATCTGAACATTGTGAAGTCGTAGA
qCD86 Probe	CCGCACGAGCTTTGACAGGAACAACT

Probes were FAM-MGB-NFQ labelled. For each mouse sample, PCRs were set up in duplicate and both GFP and CD86 (internal control) were assessed using a Taqman quantitative PCR assay. A 20 µL reaction was set up which included: 1x TaqMan master mix (4369016, Thermo), 0.25 uM GFP or CD86 probe, 0.9 uM forward primer (GFP or CD86), 0.9 uM reverse primer (GFP or CD86), 1.0 uL of gDNA sample (extracted with DirectPCR Lysis) and nuclease-free water up to 20 µL total. Positive controls included known *Cx3cr1-GFP* heterozygous gDNA samples. Negative controls - no template/water, or wildtype gDNA - were also included. Real-time cycler conditions included a UNG incubation step (50°C for 2 min); followed by a polymerase activation step (95°C for 10 min) followed by 40 cycles of 95°C for 15 sec then 60°C for 60 sec. ΔCt, ΔΔCt, and gene copy number were calculated for each sample: mice homozygous for GFP have a transgene copy number of ~2.0 whereas heterozygotes are ~1.0.

4T1-high vs. 4T1-low study. The 4T1-high (4T1-FerH-GFP^{high}) and 4T1-low (4T1-FerH-GFP^{low}) lines were generated as described above, following infection with GFP-expressing lentivirus and flow sorting to select for high vs. low GFP expressers. 4T1-high or 4T1-low (750,000 cells/mouse) were orthotopically injected into the 4th mammary fat pad of 6-8 week old immune-competent female (BALB/c WT) or immune-compromised female NOD-SCID (*n*=8 mice/group) in a 50 µL solution of 1:1 LrECM (growth-factor reduced Cultrex; Trevigen): 1xPBS. Tumor volume was measured starting at 4 days post-orthotopic injection and continued every 2-3 days until study endpoint (day 21). The primary tumor and lungs were collected and flash frozen, and spleen processed to permit cryopreservation for ImmunoSpot assessment at a later date.

Glowing Head study. 4T1 parental (unlabelled) or 4T1-ffLUC-eGFP tumor cells (750,000 cells/mouse) were orthotopically injected into the 4th mammary fat pad of ~7 week old female NOD-SCID (immune-compromised), BALB/c WT (immune-competent) or GH ('tolerized') mice in a 50 µL solution of 1:1 LrECM (growth-factor reduced Cultrex; Trevigen): PBS. Tumor volumes were recorded three times per week, starting from day 4 post-injection. Mice were euthanized 3 weeks following orthotopic injection, with the primary tumor intact. PBS perfusion and organ collection (liver, lung, brain, tibia/femurs) is described below in '*Organ bioluminescent imaging ex vivo'*. Lymph nodes and spleen were also taken for tetramer analyses, described below in '*tetramer and antibody staining'*.

Central tolerization study: Cx3cr1-GFP. The 4T1-high (aka 4T1-FerH-GFP^{high}) subline was generated as described above. To enable assessment of metastasis at a later timepoint, 4T1-high (7,500 cells/mouse) were orthotopically injected into the 4th mammary fat pad of 6-8 week old female immune-compromised (NOD-SCID), immune-competent (BALB/c WT: Charles river, or *Cx3cr1-GFP* WT littermates) or GFP-tolerized (*Cx3cr1-GFP*; HET) (*n*=9-12 mice/group) in a 50 µL solution of 1:1 LrECM (growth-factor reduced Cultrex; Trevigen): 1xPBS. A smaller cohort of BALB/c WT (*n*=4/group) were injected with 4T1 parental (7,500/mouse) as an additional positive control. Mice in cohort 1 (*n*=7 Cx3cr1-GFP WT; *n*=6 Cx3cr1-GFP HET) were harvested at day 14 or 15 to assess the number of GFP-specific CD8$^+$ T cells present (via a class I tetramer assay) at peak immune response, to confirm if Cx3cr1-GFP HET mice were centrally tolerized to GFP.

In cohort 2, tumor volumes were measured starting at day 4 post-orthotopic injection, every 2-3 days until study completion. Mice were harvested at day 30, at maximum tumor

volume. The primary tumor and lungs were flash frozen. Spleens were processed and cryopreserved for ImmunoSpot assessment at a later date.

Mammary tumor measurements. Mammary tumors were measured *in vivo* and *ex vivo* using digital calipers to measure the long (L) and short (w) axes of the tumor. Volume was calculated using the equation $V= 0.5Lw^2$. Tumor measurements were typically recorded starting from day 4 post-tumor cell injection and were taken every 2-3 days (minimum 3 times/week).

Organ bioluminescent imaging ex vivo. Prior to organ harvest, mice were injected with 100 µL D-Luciferin (10 mg/mL, BioVision, Inc.) via IP. Mice were harvested ~20-25 min following D-Luciferin delivery. Following euthanasia, mice were perfused with 3 mL PBS via the inferior vena cava with simultaneous ligation of the portal vein (to directly target the liver). This was followed by ~15 mL PBS intracardiac perfusion via the left ventricle to target the remaining organs. The liver, lung, brain, bones (femurs and tibias) were collected. Connective tissue was dissected away, and these organs imaged on the IVIS Spectrum in a 12 well plate (using 0.1 cm focus, FOV C, auto exposure). Bioluminescent signal was calculated as total flux (photons/sec) using Living Image software (Perkin Elmer).

Detection of specific anti-eGFP and anti-ffLUC CD8$^+$ T cells by class I tetramers

Tetramer generation. MHC-class I tetramers were generated by the Immune Monitoring Lab (led by Jianhong Cao) at FHCRC. H-2K(d) APC-labelled tetramers were generated using peptides for the following major antigenic sequences: eGFP (HYLSTQSAL; sequence position 200-208 (Gambotto et al., 2000)), ffLUC (GFQSMYTFV; sequence position 160-168 (Limberis et al., 2009)). A tetramer generated to the Influenza A virus nucleoprotein (FLU) (TYQRTRALV; sequence position 147-155 (Rotzschke et al., 1990)) was included as a negative control.

Tetramer and antibody staining. A staining protocol based largely off the previously published protocol by Moon *et al*. was employed (Moon et al., 2009). Spleens and lymph nodes (including the inguinal, axillary, brachial, cervical, and mesenteric lymph nodes) were processed into single cell suspension using mechanical digestion. The exception to this is the *Cx3cr1-GFP* tetramer study, where only spleens were collected. Following pelleting, cells were treated with 100 µg/mL DNase I (D4527-10KU, Sigma) + 5 mM $MgCl_2$ in HBSS at room temperature for 15 min. Cells were washed using 5 mM $MgCl_2$ in HBSS (300xg 5 min 4^{o}C) and resuspended in a volume of Fc block (10 µg/mL CD16/32 (#101320, BioLegend) + 2% FBS + 0.1% sodium azide

in PBS, pH 7.4) equal to the size of the pellet. Cells were incubated in the appropriate amount of tetramer (GFP, ffLUC or FLU @ 4 ng/µL final) for 30 min at 4°C. Each splenic/lymph node sample from an entire mouse was stained with 1 tetramer only, in order to quantify the entire $CD8^+$ T cell population present in the LNs and spleen for that antigenic epitope. Following washing, cells were resuspended in sorter buffer and anti-APC magnetic beads (130-090-855, Miltenyi Biotec) added for 30 min at 4°C. Following washing, samples underwent positive selection ('Posselds separation') on an autoMacs Pro Separator (Miltenyi Biotec). Bound and unbound fractions were collected. All samples were washed in PBS, and then resuspended in Zombie NIR Viability Dye (423105, Biolegend) for 15 min at room temperature, in the dark. Following washing with EHAA media, the bound fraction was resuspended to 95 µL exactly in Fc block. The unbound fraction was resuspended in 2 mL. A small portion from each sample (5 µL) was removed and mixed with 200 µL AccuCheck counting beads (200,000 beads/mL; PCB-100, ThermoFisher). To the remaining bound and unbound samples, the following antibody cocktail was added: B220-PacBlue (1:100, 103227, BioLegend), CD11b-PacBlue (1:100, 101224, BioLegend), CD11c-PacBlue (1:100, 117322, BioLegend), F4/80-PacBlue (1:40, 123124, BioLegend), CD8-PE (1:333, 100708, BioLegend), CD3-FITC (1:200, 100306, BioLegend), CD4-BV605 (1:166, 100547, BioLegend), CD44-AlexaFluor 700 (1:80, 103026, BioLegend). For the *Cx3cr1-GFP* tetramer experiment, the antibody/fluorescence panel required modification due to the presence of endogenous GFP^+ and RFP^+ immune populations. In this case, the antibody panel consisted of: CD45-FITC (1:300, 103108, BioLegend), B220-PacBlue (1:100, 103227, BioLegend), CD11b-PacBlue (1:100, 101224, BioLegend), CD11c-PacBlue (1:100, 117322, BioLegend), F4/80-PacBlue (1:40, 123124, BioLegend), CD4-PacBlue (1:166, 100531, BioLegend), CD3-BUV395 (1:200, 563565, BD Biosciences), CD8-PE (1:333, 100708, BioLegend), CD44-AlexaFluor 700 (1:80, 103026, BioLegend). Cells were incubated in this antibody cocktail for 30 min at 4°C. Following washing and resuspension in sorter buffer, samples were immediately run on a BD LSR FortessaX50 flow cytometer (BD Biosciences). Events for the entire bound fraction were collected for each sample; up to $2x10^6$ events were collected for the unbound. A minimum of 10,000 events were run for each counting bead sample.

Flow cytometry analysis. Compensation and gating to detect $tetramer^+$ cells was set using FloJo v10 software. For the GH tetramer studies, both bound and unbound sample analyses were gated initially off of time vs. FSC-A, to exclude electronic noise. Following this selection, a non-stringent lymphocyte gate was set using SSC-A vs. FSC-A. Cells were then gated off SSC-A vs.

SSC-W to select single cells only. Viable cells were gated off of FSC-A vs. Zombie-NIR, with Zombie-NIR$^-$ cells selected only. CD3$^+$ cells were gated away from a dump gate (B220, CD11b, CD11c, F4/80), in order to eliminate cell populations known to non-specifically bind to class I tetramers. Only dump$^-$ CD3$^+$ events were included. Next, CD4 vs. CD8 were gated in order to eliminate CD4$^+$ cells which can also non-specifically bind to class I tetramers. CD8$^+$ T cells were selected only. Epitope-specific CD8$^+$ T cells were finally gated off CD44 vs. APC. Gates to collect tetramer$^+$ epitope-specific CD8$^+$ T cells were set based off gating of unbound CD44 vs. APC (internal negative control, containing minimal positive events). A tetramer$^+$ population was enriched in the bound fraction of each sample only.

For the *Cx3cr1-GFP* tetramer study, a modified gating strategy was applied in order to account for endogenous GFP$^+$ and RFP$^+$ immune populations in these samples. A size gate was applied to loosely select lymphocytes, and subsequently single cells. Viable cells (Zombie NIR$^-$) were then selected, followed by selecting both endogenous GFP$^+$ expressing immune cells and CD45-FITC$^+$ populations together. A BV421$^-$ dump gate (B220, CD11b, CD11c, F4/80, CD4) was then applied to exclude all cell populations known to non-specifically bind to class I tetramers. CD3$^+$ BV421$^-$ T cells were selected only. CD8$^+$ T cells were then selected. Finally, tetramer-APC$^+$ cells can be discerned. The final gate was set based off unbound CD44 vs. APC (internal negative control, containing minimal positive events).

To determine total cell number, a gate was set around the number of beads collected (on SSC-A vs. FSC-A). The number of cell events equated to the number of total events – number of bead events.

Calculating total cell number. As outlined in Moon *et al.* total cell number was determined by the following calculation: Total cell number = (cell events/bead events) (bead concentration) (bead volume/cell volume) (volume of cell sample) (Moon *et al.*, 2009).

Calculating the specific number of epitope-specific CD8$^+$ T cells. As based off the calculation outlined in Moon *et al.* the total number of GFP-, ffLUC- or FLU- specific CD8 T cells was determined by the following calculation: Epitope-specific T cell number = (total cell number) (proportion time) (proportion lymphocyte) (proportion single cells) (proportion viable cells) (proportion CD3$^+$) (proportion CD8$^+$) (proportion cells in tetramer-positive gate) (Moon *et al.*, 2009).

Additional controls. As a negative control, a tetramer generated using the same H-2K(d) backbone and APC-conjugated fluorophore was used – in this case, the tetramer was generated against a FLU peptide, which BALB/c mice should not have been previously exposed to. Addition of the H-2K(d) FLU-APC conjugated tetramer detected between 48 – 122 epitope-specific $CD8^+$ T cells in inoculated (WT or GH) mice. This was similar to the 44 epitope-specific $CD8^+$ T cells collected in an uninoculated BALB/c mouse.

As additional negative controls, mice that had not previously been exposed to GFP or ffLUC antigens (both WT and GH backgrounds) were used.

Detection of functional $CD8^+$ GFP-specific T cells by ImmunoSpot

Cryopreservation of mouse splenocytes. Following mouse harvest, spleens were placed into 5 mL of 1xCTL-Wash media + 1% (v/v) penicillin/streptomycin (#CTLW-010, CTL) on ice in a 6 cm dish until ready to process. To ensure high viability of samples, the time spleens were left on ice between harvesting and processing was kept to a minimum. Spleens were processed according to manufacturer instructions (CTL), in order to cryopreserve using the CTL-Cryo™ ABC media kit (CTLC-ABC-100, CTL). Splenocyte samples were frozen back at $2.0x10^7$/vial and stored in the liquid N_2 until all mouse samples were collected for an entire experiment.

ImmunoSpot assay. Mouse IFNγ single color FluoroSpot assays (mT1000Fp and mT01; CTL) were purchased pre-coated from CTL, and carried out according to the manufacturer's instruction. Specifically, following sample cryo-revival and washing in 1x CTL anti-aggregate wash medium (CTL-AA-005, CTL), viable splenocyte samples (400,000 cells/well) were plated in CTL-Test medium (#CTLT-005, CTL) in duplicate with the following: 1) concanavalin A (1 ug/mL, C0412-5MG, Sigma); 2) $eGFP_{(200-208)}$ peptide (HYLSTQSAL, 1 ug/mL, Elim Biopharm) or; 3) $FLU_{(147-155)}$ peptide (TYQRTRALV, 1 ug/mL, Elim Biopharm). Concanavalin A (positive control) is an antigen-independent mitogen, stimulating T cell activation. eGFP (HYLSTQSAL) is the major antigenic epitope to eGFP, presented by H-2K(d). FLU (TYQRTRALV) is a peptide to influenza nucleoprotein presented by H-2K(d), used as a negative control peptide. Following culture at 37°C with concanavalin A, eGFP or FLU peptide for 24 h, IFNγ detection and development was carried out according to the manufacturer's instruction. After the plate was dry, IFNγ-CTLRed fluorescent spots were visualized and counted using an ImmunoSpot S6 Analyzer (CTL) with ImmunoSpot software. Fluorescent signal was captured using the -690 filter, and SmartCount™ applied to determine the number of red fluorescent IFNγ spots per well.

The average number of spots were determined per well and plotted in Prism. Samples were excluded if the concanavalin A control did not respond appropriately.

Tissue immunofluorescence

Sectioning of primary tumor or lung tissue

Following perfusion with 1xPBS, primary tumors were dissected, and half of the tumor was flash frozen in OCT (with the middle of the tumor, where it had been dissected in half, orientated to be immediately exposed to the cryostat blade) using liquid nitrogen following dissection from the mouse. Lung tissue was also excised from the mouse, and the entire lung flash frozen in OCT using liquid nitrogen.

Cryosections (12 µm) of tumor or lung were cut using a Leica Cryostat CM3050 S (Leica Microsystems) and placed onto Superfrost Plus glass slides. For the lung, 12 um sections were collected at intervals of 100 um between sections, with 12 sections (6 slides, 2 sections/slide) cut for each sample. Typically, slides 2 and 5 of the lung (4 sections/mouse) were stained for metastasis quantification. After the sections had dried, slides were placed back-to-back, wrapped in Kim wipes, wrapped in foil, and stored unfixed at -80^{o}C until required.

Immunofluorescence - primary tumor and lung tissue

Wrapped slides were placed at 37^{o}C for 10 min to allow any condensation to evaporate. Sections were placed into a Coplin jar then immediately fixed with 10% neutral buffered formalin (HT501128-4L, Sigma) for 20 min at room temperature (RT). Following washing with copious 1xPBS with agitation (3 x 5 min), sections were permeabilized using 0.5% (v/v) Triton X-100/PBS for 20 min at RT. Slides were washed again, and then blocked using 10% (v/v) donkey serum + Li-COR Odyssey blocking buffer (927-40000, Li-COR) for 1 h at RT. The following primary antibodies were then added to the slides (overnight, 4^{o}C), diluted in Odyssey blocking buffer at the following concentrations: CD45 (1:50, 550539, BD Biosciences), CK18 (1:200, ab53118, Abcam or 1:100, GP11, Progen), GFP (1:1000, ab13970, Abcam), or phospho-histone 3 (pH3, 1:100, 06-570, Millipore). The following day, slides were washed (3 x 5 min) and the appropriate highly cross-adsorbed secondary antibodies were added at 1:400, diluted in Odyssey blocking buffer: either donkey anti-chicken CF488A (20166, Biotium), donkey anti-guinea pig CF568 (20377, Biotium), donkey anti-guinea pig CF633 (20171, Biotium), donkey anti-rabbit Alexa Fluor 647 (A-31573, ThermoFisher), or donkey anti-rat Alexa Fluor 647 (712-605-153, Jackson ImmunoResearch). Following incubation in secondary antibody for 45 min at RT, slides were washed for a final time in PBS (3 x 5 min) and nuclei stained with Hoechst

(1:10,000 in PBS) for 5 min RT. Following another PBS wash, they were mounted using Fluoromount-G (0100-01, ThermoFisher), sealed using nail polish and stored at 4^{o}C prior to imaging.

Tumor imaging

An entire cross section of each primary tumor was obtained (2.5x/0.12 M27 objective lens, 6x6 tile scan, 1024 x 1024 frame, speed 8) using -405, -488, -555 and -639 laser lines on a Zeiss LSM700 confocal microscope. Typically, Hoechst (-405), GFP (-488), CK18 (-555) and CD45 (-639) signal was captured following excitation with the listed laser. For the *Cx3cr1-GFP* study, both CK18 and CD45 signal were captured with the -639 laser in order to avoid capture of endogenous RFP signal. Identical imaging acquisition settings were applied to all samples within the same study.

GFP per cytokeratin 18 (CK18) area

In (Fiji Is Just) Image J, tumor tile scans (2.5x objective, 6x6) were opened ('File' → 'Import' → 'Bioformats') and background around the tumor was removed on the -488 (GFP) and -555 (or -639; CK18) channels by selecting the tumor using the freehand selection tool and 'Edit' →'Clear Outside'. On the CK18 channel, CK18 signal was thresholded to select positive signal ('Edit' → 'Selection' → 'Create selection'). The GFP window was then brought up, GFP signal thresholded, and CK18 signal superimposed onto the GFP window using ''Restore selection'. % area was then measured and recorded. This provided the % of GFP per CK18 area.

Lung imaging and metastasis quantification

<u>4T1-high vs. 4T1-low study</u> – An entire cross section of lung (*n*=4 sections/mouse) was obtained (10x/0.3 M27 objective lens, 16x16 tile scan, 256 x 256 frame, speed 8) using -405, -488 and -639 laser lines on a Zeiss LSM700 confocal microscope. Hoechst (-405), GFP (-488), and pH3 (-639) signal was captured following excitation with the listed laser. Detection of pH3 signal and lowly expressed GFP by the 4T1-low line meant imaging required use of a higher 10x objective lens. Identical imaging settings were used for all samples within the same study.

The number of GFP^{+} lesions per section were counted, and the average number of GFP^{+} lesions per section plotted for each mouse. Alternatively, the number of pH3^{+} lesions per section were counted, and the average number of pH3^{+} lesions per section plotted for each mouse.

Cx3cr1-GFP study – An entire cross-section of the lung (*n*=4 sections/mouse) was imaged (2.5x/0.12 M27 objective lens, 6x6 tile scan, 1024 x 1024 frame, speed 8) using the -405 (Hoechst) and -488 (GFP) lasers. Note that these imaging conditions only resolved metastatic lesions from approximately >100 um diameter; anything smaller was not readily detected using these imaging settings. To confirm GFP^+ lesions were proliferative metastases, a single snap using a 10x/0.3 M27 objective lens to capture a 2048x2048 frame was taken (-405, -488, -639), to permit the resolution of pH3 signal on -639 using a higher objective lens. Identical imaging settings were used for all samples within the same study.

The number of GFP^+ lesions per section were counted, and the average number of lesions per section plotted for each mouse. Importantly, only GFP^{high} were counted – which excluded much lower intensity, endogenous GFP signal from *Cx3cr1-GFP*$^+$ immune cells from the analysis. Further, differentiation of GFP^{high} metastases from GFP^{low} *Cx3cr1-GFP* endogenous signal was confirmed via the co-expression of GFP^{high} metastatic lesions with pH3 and a lack of CD45 expression.

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 9 software (Version 9.1.0). All data are reported as mean ± standard error of the mean (SEM), unless otherwise noted. A $p<0.05$ was considered significant. Please refer to the figure legend for individual *n* and *P* values, and statistical test performed. For mouse experiments, all data points from each biological sample in the study are shown. Typically, at a minimum two experimental repeats, but frequently three or more, were performed for mouse studies. This was achieved by enrolling mice over staggered experimental cohorts.

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Supplemental Figure Legend

Figure S1. GFP elicits a metastasis suppressive immune response in wild-type and peripherally tolerized hosts that is eliminated in centrally tolerized transgenics. (A) 4T1-high vs. 4T1-low experimental schematic and GFP expression levels. **(B)** Primary tumor volumes for 4T1-high or 4T1-low GFP-expressing sublines in immune-compromised (NOD-SCID) or immune-competent (BALB/c WT) mice. Mean±SEM (*n*=8/group). One-way ANOVA with Tukey's multiple comparisons test. **(C)** Tumor immunofluorescence assessing GFP per cytokeratin 18 (CK18) area (%). Mean±SEM; One-Way ANOVA, Tukey's multiple comparisons test. **(D)** ImmunoSpot assay. Cryopreserved splenocytes from inoculated 4T1-high or 4T1-low BALB/c WT mice at day 21 post-injection, following incubation with H-2K(d) eGFP peptide (HYLSTQSAL) or FLU peptide (TYQRTRALV) as a negative control. The number of IFNγ spots indicates the number of *functional* eGFP-specific $CD8^+$ T cells per well. Mean±SEM; One-Way ANOVA with Tukey's multiple comparisons test, or unpaired t-test (two-tailed) for 4T1-low GFP vs. 4T1-low FLU. **(E-F)** Lung metastasis quantification. Immunofluorescence for GFP^+ tumor cells **(E)** or proliferation marker phospho-histone 3 (pH3) **(F)** was performed in lung sections taken from day 21 mice. 10x objective, 16x16 tile scans. One-Way ANOVA with Tukey's multiple comparisons test. **(G)** Glowing Head experimental schematic and 4T1-ffLUC-eGFP GFP-expression level. **(H)** Primary tumor volumes for 4T1-ffLUC-eGFP or 4T1 parental lines injected into immune-compromised (NOD-SCID), immune-competent (BALB/c WT) or Glowing Head (GH) mice. Mean±SEM (*n*=6-11/group); One-way ANOVA with Tukey's multiple comparisons testing. **(I-J)** Class I tetramer assay to detect eGFP- **(I)** or ffLUC- **(J)** specific $CD8^+$ T cells per mouse in lymph nodes/spleen. FLU tetramer in inoculated mice - negative control. The FLU group graphed on I and J contain the same mice. Uninoc = uninoculated. Mean±SEM; Kruskal-Wallis with Dunn's multiple comparisons test. **(K)** Bioluminescent signal in organs *ex vivo* at day 21 post injection of 4T1-ffLUC-eGFP. Mean±SEM. Kruskal-Wallis with Dunn's multiple comparisons test. **(L)** Central tolerization experimental schematic. **(M)** Primary tumor volumes for GFP-expressing 4T1-high or 4T1 parental line injected into immune-compromised (NOD-SCID), immune-competent (BALB/c WT) or GFP-tolerized (Cx3cr1-GFP) mice. Mean±SEM (*n*=4-12/group). 2way ANOVA with Tukey's multiple comparisons test; ***$p<0.001$, ****$p<0.0001$. **(N)** GFP/CK18 area (%) in primary tumors. Mean±SEM; One-Way ANOVA, Tukey's multiple comparisons test. **(O)** Class I tetramer assay to detect eGFP-specific $CD8^+$ T

cells per spleen at day 14-15 post inoculation. Left - representative gating of $CD45^+$ $CD3^+$ $CD8^+$ $tetramer^+$ cells in uninoculated immune-competent vs. inoculated WT littermates ('immune-competent') vs. inoculated *Cx3cr1-GFP* ('GFP-tolerized') mice. Right – Number of GFP-specific $tetramer^+$ cells graphed. FLU tetramer in inoculated mice - negative control. Mean±SEM; Unpaired t-test, two-tailed. **(P)** ImmunoSpot assay. Cryopreserved splenocytes from mice (immune-competent WT or *Cx3cr1-GFP*) either uninoculated or inoculated with 4T1-high GFP-expressing tumor cells were obtained at day 30 post-injection. Splenocytes were incubated with H-2K(d) eGFP or FLU (negative control) peptide. Left - representative wells following incubation with eGFP peptide; IFNγ spots are in red. Right – IFNγ spots/well graphed. Mean±SEM; One-Way ANOVA with Tukey's multiple comparisons test. **(Q)** Lung metastasis. Left - immunofluorescence of representative 2.5x objective tile scan of lung. Inset: immune marker CD45 (gray) to confirm GFP^{high} metastatic lesion. Scale bars: tile scan 500 um; inset 100 um. Right – total average lesions/section/mouse graphed. Mean±SEM; unpaired t-test, two-tailed. Below - metastatic incidence tabulated. Fisher's exact test, two-tailed. **(R)** Graphical summary. Only immune-competent mice centrally tolerized to GFP (*Cx3cr1-GFP*) entirely eliminate the GFP-specific $CD8^+$ T cell response, permitting GFP^+ metastatic progression.

Figure S1 - GFP elicits a metastasis-suppressive immune response in wild-type and peripherally tolerized hosts, that is eliminated in centrally tolerized transgenics