Supporting Online Material
Materials and methods
Figs. S1, S2 & S3

Materials and Methods

BAC clone modification and purification

A 197 kb BAC clone (RP23-184A18) containing the *gzmB* gene was purchased from BACPAC resources (http://bacpac.chori.org/). The BAC was modified by introducing CreER^{T2}-SV40 polyadenylation signal into the start ATG of the *gzmB* gene by homologous recombination (*1*). BAC DNA was purified from 200ml bacterial cultures by alkaline lysis (Qiagen buffers), and circular DNA was separated by CsCl ultracentrifugation. Briefly, 4.04g of CsCl were added to 4ml resuspended DNA and CsCl dissolved at 40^{o}C. 25 µl 10mg/ml EtBr and 75µl water were added. Samples were spun in a bench tube centrifuge at 3000 rpm for 15 min to remove remaining proteins. The DNA CsCl solution was spun at 70,000g for 6 hours. EtBr was removed by n-butanol extraction and the DNA precipitated. Successful recombination was confirmed by PCR, restriction digestion analysis and Southern blot analysis. A linear 155kb fragment was cut using the Sbf I enzyme (New England Biolabs) and separated by pulse field gel electrophoresis (1% Seaplaque GTG agarose (Cambrex) in 1x TAE, 4.5 V/cm, switch time 1-25, 120^{o} angle, 24 hours) using a Bio-Rad Chef Mapper. DNA was electroeluted from the gel fragment (*2*) and spot dialyzed on Millipore VSWPO2500 filters into polyamine buffer (10mM Tris-Cl, pH 7.5, 0.1mM EDTA, 100mM NaCl, 30µM spermine, and 70µM spermidine).

Mice and viral infections

GzmBCreER^{T2} mice were identified by quantitative real-time PCR (Taqman real-time universal PCR mix (Applied Biosystems) using primers/probe specific for CreER^{T2} and normalised using primers/probes specific for an endogenous gene, *fap*. Using this method we were able to screen for homozygosity. ROSA26EYFP mice were a kind gift from Prof. Doug Winton (CRUK, Cambridge). The ROSA26EYFP gene was screened for using the protocol described elsewhere (*3*). Primary HKx/31 (H3N2, 10^4 pfu) and secondary A/PR8/34 (PR8, H1N1, 2 x 10^3 pfu) influenza infections were administered via the intranasal route (*4*). Mice received 1mg tamoxifen daily in 10% EtoH/90% sunflower seed oil (Sigma Aldrich) via i.p. injection. BrdU was administered by i.p. injection (2mg every 12 hours).

Tissue preparation and flow cytometry

Lungs, mediastinal lymph nodes and spleens were homogenised in RPMI/10%FBS/2 mM EDTA and erythrocytes lysed (PUREGENE RBC lysis buffer). Antigen specific cells were identified using PE and APC conjugated H-2D^{b}/NP ASNENMETM pentamers (Proimmune). Cells were counted using Caltag counting beads and dead cells gated out using 7-AAD (unfixed samples) or Invitrogen's live/dead acqua stain (fixed samples). Cell surface staining was performed in FACS buffer (PBS, 1% FBS, 2mM EDTA) at 4^{o}C using the following antibodies: CD8 APC/APC-alexa Fluor 750, CD44 PE, CD3ε pacific blue, CD19 PE/PE-Cy5, CD62L PE/APC-alexa fluor 750, CD127 PE, B220 APC, CD49b PE, CD11c PE, CD11b APC, Gr-1 PE, CD25 PE. All antibodies were from

ebioscience unless otherwise stated. BrdU incorporation was assessed according to manufacturers instructions (BD pharmingen). To stain for intracellular granzyme B (anti-human granzyme B APC, Caltag) cells were fixed (10 min, RT) and permeabilized (BD fix/perm). Data was collected using BD FACSCalibur and Dako Cyan flow cytometers and analysed using Flowjo software (Treestar inc.). Statistical comparisons used the unpaired two-tailed Student's t-test.

Cell culture experiments

Naïve $CD8^+$ T cells ($CD8^+$, $CD44^{low}$, $CD62L^{high}$) were FACS purified using a Moflo cell sorter. Cells were stimulated using anti-CD3ε coated tissue culture plates (BD Pharmingen) and the following cytokines added: IL-2 (0.5-20ng/ml), IFN-β (100 U/ml), IFN-γ (2ng/ml ebioscience), IL-7 (2 ng/ml from day 2), IL-12 (2 ng/ml), IL-6 (2 ng/ml). All cytokines were from R&D Biosystems unless otherwise stated. The following blocking antibodies were used at 5μg/ml: anti-CD25, anti-IL-2, anti-IL-12, anti-IFN-γ and anti-IFN-γRI (ebioscience). Trimeric soluble CD70 (sCD70) was used at a saturating dose, as assessed by flow cytometry (*5*). The tamoxifen metabolite 4-hydroxytamoxifen was used to stimulate recombination in vitro at 300nM.

Primers

Homology box A for	GGCGCGCCTAAGAGGAAGTGGGGAAGCAG
Homology box A rev	CTTCCCCGGAAGGCCGCCTAG
Homology box B for	TCTGGTCGACTCTAGACTCTAAGATCCTCCTGCTACTGCTG
Homology box B rev	ATTTGCGGCCGCTCCTTCACAGTGAGCAGCAG
CreERT2 overlap for	CGGCCTTCCGGGGAAGATGTCCAATTTACTGACCG
CreERT2 overlap rev	AGAGTCGACCAGACATGATAAG
Integration confirmation A for	CTCTGGACACTAAGTAAGAATAG
Integration confirmation A rev	CAAACGGACAGAAGCATTTTC
Integration confirmation B for	ATCTTTCTAGGCAGGTATGG
Integration confirmation B rev	GTCCAAACTCATCAATGTATC
CreERT2 genotype for	GCCACCAGCCAGCTATCAA
CreERT2 genotype rev	CGTAAATCAATCGATGAGTTGCTT
CreERT2 genotype probe	FAM-TCGCGCCCTGGAAGGGATTTTT-TAMRA
Fap genotype for	CTGGAAAGGCTCCAGCAAATC
Fap genotype rev	CTTCTCCTTCCCCACAGAGCTT
Fap genotype probe	FAM- CCGCCAGACCAGTGGAACACAATCA-TAMRA
ROSAEYFP for	AAAGTCGCTCTGAGTTGTTAT
ROSAEYFP rev 1	GCGAAGAGTTTGTCCTCAACC
ROSAEYFP rev 2	GGAGCGGGAGAAATGGATATG

Supplementary references

1. T. Sparwasser, S. Gong, J. Y. Li, G. Eberl, *Genesis* **38**, 39 (2004).
2. S. J. Strong, Y. Ohta, G. W. Litman, C. T. Amemiya, *Nucleic Acids Res* **25**, 3959 (1997).
3. S. Srinivas *et al.*, *BMC Dev Biol* **1**, 4 (2001).
4. P. M. Manders *et al.*, *Proc Natl Acad Sci U S A* **102**, 7418 (2005).
5. J. M. Carr *et al.*, *Proc Natl Acad Sci U S A* **103**, 19454 (2006).

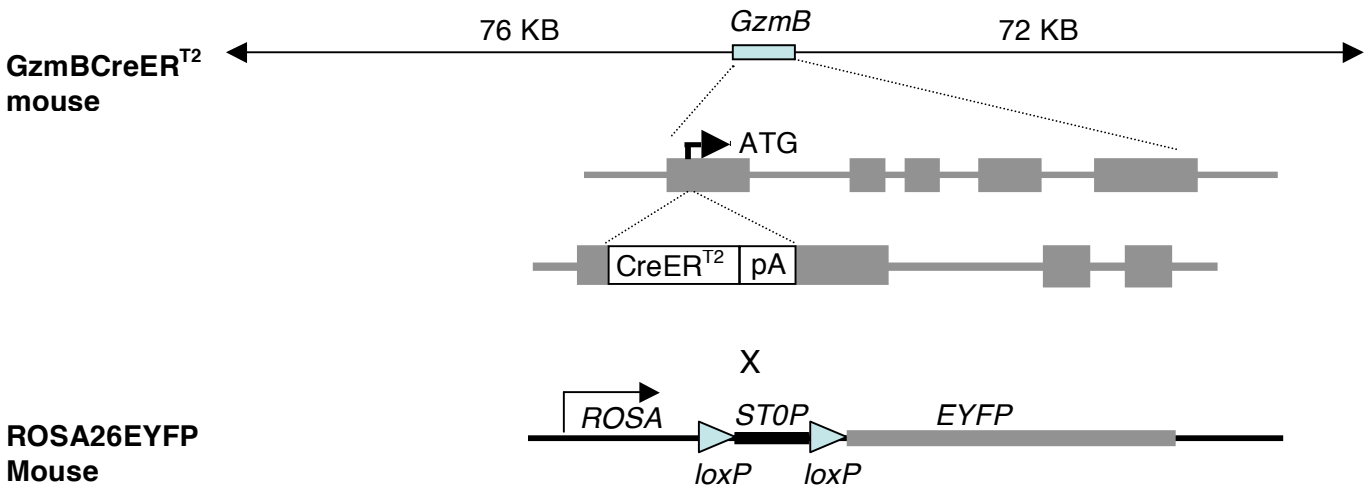

Fig. S1. A transgenic mouse model for indelible marking of $CD8^+$ T cells that express granzyme B during a defined period**.** We generated a BAC transgenic mouse using a 155kb fragment of the murine chromosome 14 which we modified by introducing the tamoxifen dependent recombinase $CreER^{T2}$ into the start codon of the granzyme B gene. The $GzmBCreER^{T2}$ mouse was crossed to the ROSA26EYFP reporter line so that *gzmB* transcription by a cell in the presence of tamoxifen may lead to the $CreER^{T2}$-mediated excision of the loxP-flanked stop codon and permit irreversible expression of EYFP.

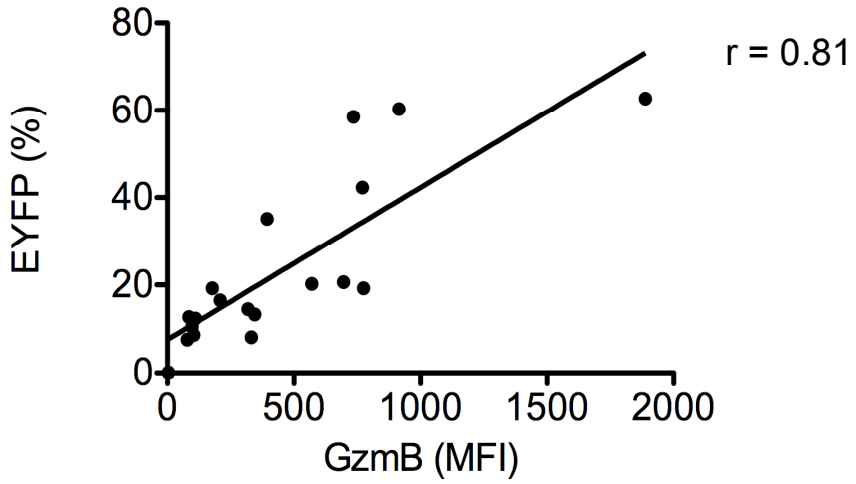

Fig. S2. EYFP frequency correlates with the level of granzyme B expression. Naive $CD8^+$ T cells from $gzmBER^{T2}$/ROSAEYFP mice were stimulated in vitro with immobilized anti-CD3ε either in the presence of IL-2/CD25 block and soluble CD70 or with IL-2 added (0.5, 2 or 20 ng/ml) in the presence or absence of various inflammatory cytokines (IL-12, IL-6, IFN-γ or IFN-β) to induce a range of granzyme B expression levels. Cells were analysed for granzyme B mean fluorescence intensity (MFI) and EYFP frequency after 4 days of culture.

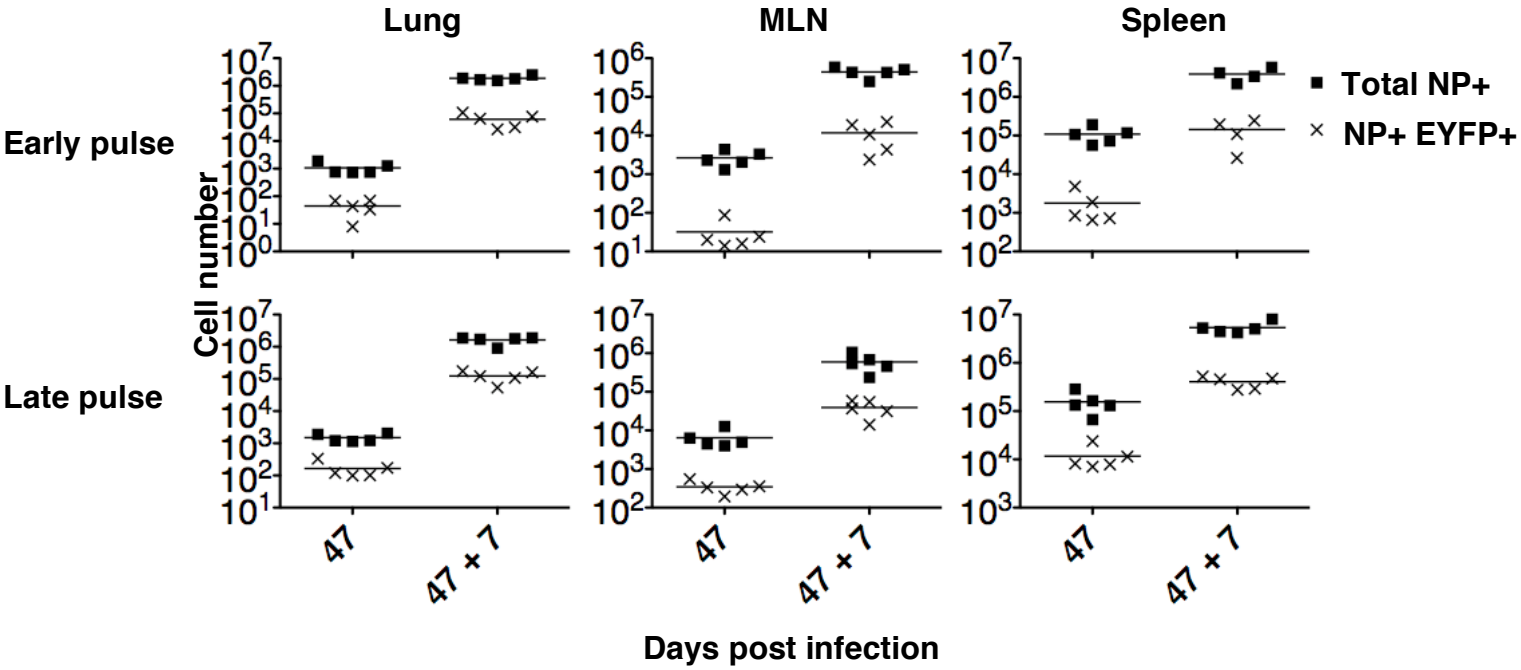

Fig. S3. Expression of gzmB by CD8+ T cells early during primary infection does not impair their capacity for secondary clonal expansion. gzmBCreER^{T2}/ROSA26EYFP mice were infected intranasally with the HKx/31 strain of influenza and treated with tamoxifen either early in the response (days 1-3 p.i.) or late in the response (days 7-9 p.i.). Mice were rechallenged 47 days later with the PR8 strain of influenza. The numbers of total (squares) and EYFP^{+} (crosses) D^{b}/NP-specific CD8^{+} T cells in the lungs, MLNs, and spleens of each mouse before and 7 days after secondary challenge are shown.