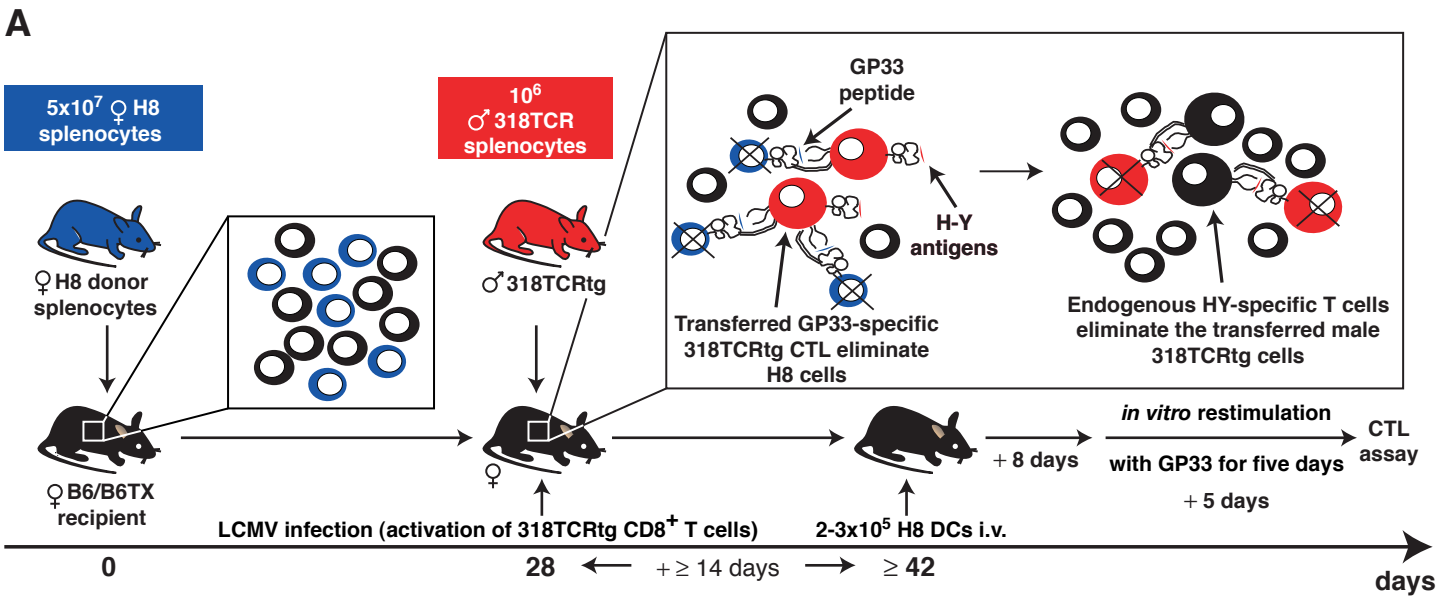
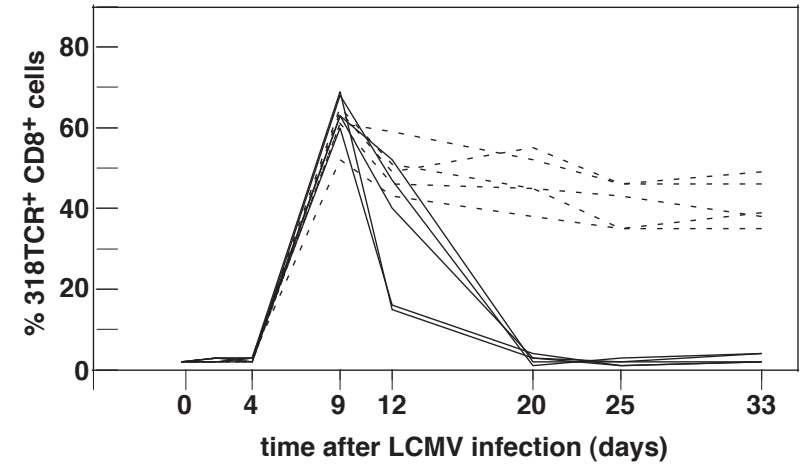
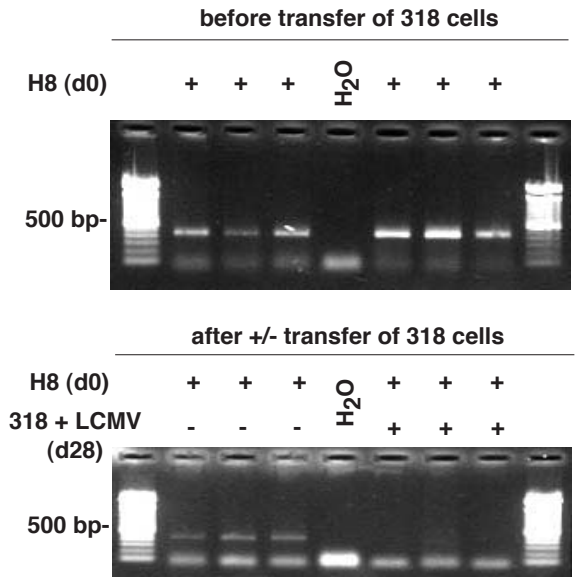
Supplementary Figure 1. Alternative experimental protocol for removing H8 donor cells from B6 recipients.







Supplementary Figure 1. Alternative experimental protocol for removing H8 donor cells from B6 recipients.

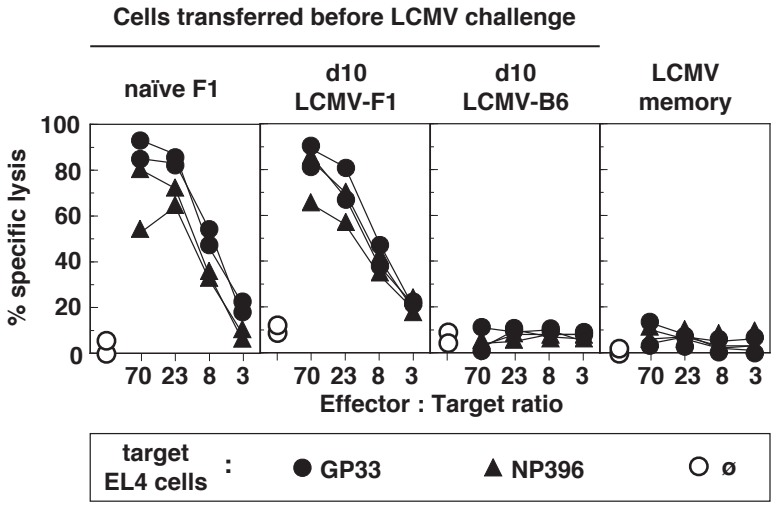
(A) Description of the experimental procedure used in Supplementary Figure 2 and Supplementary Figure 3 and its rationale. Female euthymic (B6) or adult thymectomized (B6TX) mice were transfused with sex-matched H8 splenocytes (blue). This resulted in a long-lasting lymphohematopoietic chimerism schematically represented by mixed endogenous (black) and donor cells (blue). Twenty-eight days later, the recipients were additionally transfused with 10^6 male 318 TCR transgenic splenocytes (red) and were simultaneously infected with LCMV. Immediate activation by LCMV infection induced expansion (compare B) of 318TCRtg $CD8^+$ T cells, prevented their deletion (1, 2) and resulted in clearance of the viral infection and in elimination of GP33 bearing H8 donor cells (compare C). Notably, only the transferred GP33-specific $CD8^+$ T cells could be induced by this procedure whereas endogenous GP33-specific T cells were already rendered unresponsive or had been deleted (see discussion section) due to H8 cell chimerism. Over the course of 21 days after 318TCRtg splenocyte transfer and infection, male 318TCRtg donor cells were themselves efficiently rejected due to the male (H-Y) minor histoincompatibility target (compare B). At different time points after 318TCRtg splenocyte transfer and LCMV infection, the recipients were immunized i.v. with $2\text{-}3x10^5$ bone marrow derived H8 DCs, that prime naïve GP33-specific CTL precursors (3). Eight days later, the recipient's splenocytes were collected and tested in a secondary CTL assay against GP33

after *in vitro* restimulation with GP33 for five days. It is important to note that LCMV infection simultaneously with 318TCRtg splenocytes transfer induced endogenous LCMV-specific CTLs of various epitope specificities other than GP33 (e.g. NP396). Hence, these mice would have been protected against LCMV, excluding the evaluation of GP33-specifc $CD8^+$ T cell reactivity by a second LCMV challenge (compare "LCMV-memory" group in Supplementary Figure 1). This is unlike in the first protocol that was based on removal of chimeric H8 donor cells by d10-LCMV-F1 (B6xBALB/b) cells. In this first protocol, all transferred T cells were rejected irrespective of their specificities and hence none of them could contribute to the clearance of the subsequent LCMV infection.

(B) Female B6 mice received either transgenic male (solid line) or female (dashed line) 318 splenocytes i.v. and were simultaneously infected with LCMV. 318TCR transgenic ($V\alpha2^+V\beta8^+$) $CD8^+$ T cells in blood were enumerated by flow cytometry. After LCMV induced activation and vigorous expansion, male 318TCRtg splenocytes were rapidly rejected by female hosts whereas female 318 cells persisted after the contraction phase of the CTL response. Each line represents an individual mouse. One representative of 3 experiments is shown.

(C) Peripheral blood DNA was isolated prior to and after removing H8 donor cells from recipient mice (male 318TCRtg splenocyte transfer and LCMV infection on day 28) and served as template for H8-specific PCR analysis. Each lane represents an individual mouse.

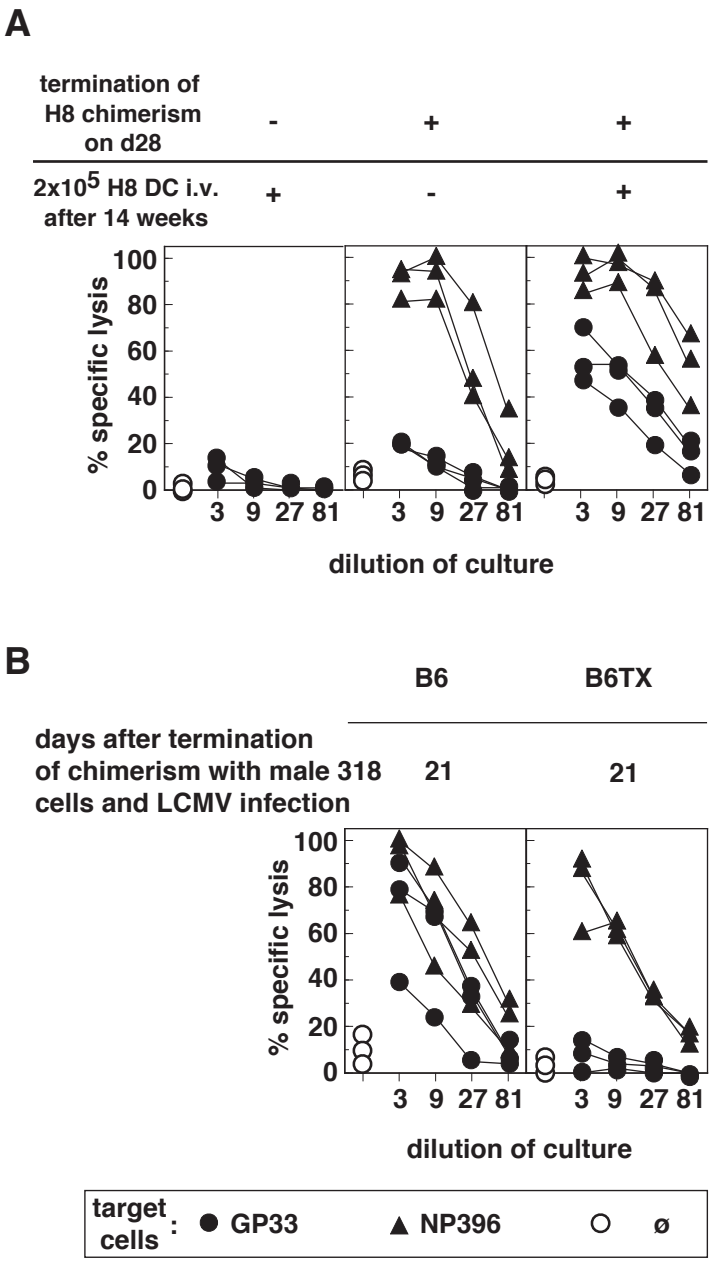
Supplementary Figure 2. Absence of LCMV immunity in recipients of d10-LCMV-F1 cells



Supplementary Figure 2. Absence of LCMV immunity in recipients of d10-LCMV-F1 cells.

Minor disparate splenocytes from naïve (B6 x BALB/b)F1 (naïve F1), day 10 LCMV-immune (B6 x BALB/b)F1 (d10-LCMV-F1), or syngeneic day 10 LCMV-immune B6 donors were adoptively transferred to sex-matched B6 recipients. Alternatively, B6 mice were immunized with LCMV (LCMV memory). Twenty days later, all mice were challenged with LCMV. Eight days later GP33- (●) and NP396-specific (▲) CTL activity was tested in a primary *ex vivo* CTL assay. Unpulsed targets (○) were incubated with effector cells at the highest effector to target ratio. LCMV immune mice (by transfer of syngeneic d10-LCMV-B6 splenocytes or by previous LCMV immunization) showed no detectable responses eight days after LCMV challenge due to premature elimination of the challenge virus inoculum whereas GP33- and NP396-specific CTL activity followed normal kinetics with a peak around day 8-9 when naïve or LCMV-immune splenocytes from minor disparate mice had been adoptively transferred.

Supplementary Figure 3. Thymus- and time-dependent re-emergence of GP33-specific CD8+ T cell precursors after removing H8 donor cells by the alternative protocol.



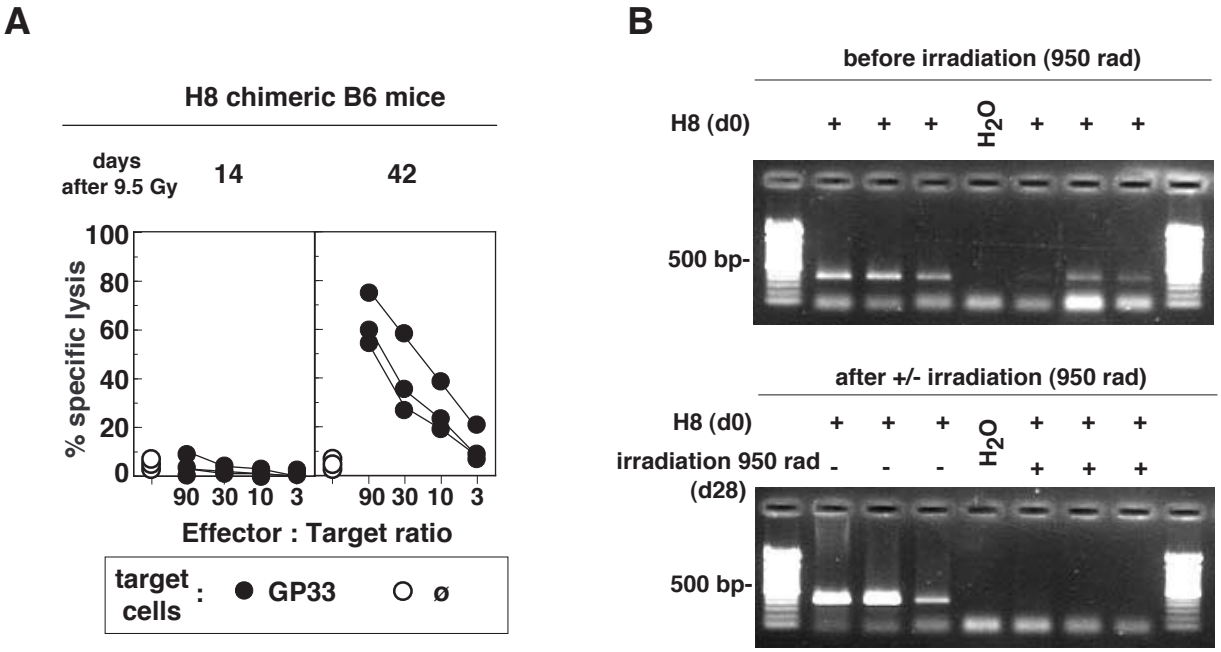
Supplementary Figure 3. Thymus- and time-dependent re-emergence of GP33-specific CD8$^+$ T cell precursors after removing H8 donor cells by the alternative protocol.

(A) H8 female splenocytes were adoptively transferred to female B6 recipients. Chimerism was either allowed to persist or was terminated 28 days later by an additional transfer of male 318TCRtg splenocytes and simultaneous infection with LCMV. Fourteen weeks later, three 318TCRtg splenocyte recipient mice were immunized i.v. with H8 DCs whereas another three 318TCRtg splenocyte recipients were left untreated. Additional eight days later, splenocytes from all mice were collected and were restimulated for five days *in vitro* with either GP33 (●, left, center and right panel) or NP396 (▲, center and right panel) before testing in a secondary CTL assay. (○: unlabeled target control for highest effector to target ratio).

(B) H8 splenocytes were adoptively transferred into euthymic (B6) and adult thymectomized (B6TX) female recipients. H8 donor cells were removed by additionally transferring male 318TCRtg splenocytes and simultaneous infection with LCMV. Twenty-one days later, all mice were immunized with H8 DCs i.v. Eight days after DC immunization, the mice were sacrificed and splenocytes were tested in a secondary CTL assay after *in vitro* restimulation for five days with GP33 (●) or NP396 (▲) (○ unpulsed EL-4 control targets at highest effector to target ratio). Note that the NP396-specific CTL response represents an internal positive control for the restimulation procedure and that unlike the re-

emerging GP33-specific response (induced by H8 DC immunization) the NP396-specific CTLs were endogenous cells that had been induced by the earlier LCMV infection. Each line represents an individual mouse.

Supplementary Figure 4. Re-emergence of GP33-specific CTL precursors after deletion by lethal irradiation and bone marrow reconstitution parallels the kinetics observed after termination of chimerism.



Supplementary Figure 4. Re-emergence of GP33-specific CTL precursors after deletion by lethal irradiation and bone marrow reconstitution parallels the kinetics observed after termination of chimerism.

The experiments presented in Figure 4 were strongly suggestive for *de novo* generation of antigen-specific T cell precursors by thymic T cell maturation. This prompted us to compare the above kinetics of T cell repertoire reconstitution with a typical situation of physical T cell depletion and re-population.

A: H8 splenocytes were adoptively transferred to sex-matched B6 mice. Four weeks later, the recipients were lethally irradiated (9.5 Gy) and reconstituted the following day with T cell-depleted syngeneic sex-matched bone marrow cells (to rule out any possible effect of co-transferred naïve donor T cells). Fourteen or 42 days later, mice were infected with LCMV to assess re-appearance of GP33-specific CTLs. Eight days after infection, CTL activity was measured in a primary ex vivo CTL assay on GP33-pulsed (●) unpulsed (○) target cells. LCMV challenge 14 days after bone marrow reconstitution elicited only background levels of GP33-specific CTL activity (left panel) whereas reactivity was readily observed when the mice were allowed to rest for 42 days between bone marrow reconstitution and LCMV challenge (right panel). Hence, the reappearance of CTL responsiveness after lethal irradiation and bone marrow reconstitution followed the kinetics of GP33-specific responsiveness in H8 chimeric mice upon removal of the grafted H8 donor cells (compare to Figure 4C). This provided additional support for our conclusion that H8 splenocyte engraftment induced and

maintained GP33-specific CTL unresponsiveness by clonal deletion of specific $CD8^+$ T cells.

B: Irradiation with 9.5 Gy not only caused elimination of mature endogenous lymphocytes and their precursors but also eliminated the H8 chimerism. This was shown by H8-specific PCR analysis of DNA isolated from peripheral blood before and after removing H8 donor cells by lethal irradiation. Each lane represents an individual mouse.

Supplementary Methods

Mice

TCR transgenic mice specific for the LCMV GP33 epitope (TCR318) (4) were obtained from the Institut für Labortierkunde (University of Zurich, Switzerland). TCR318 mice had a pure B6 background and were kept under specific pathogen free conditions.

In vitro restimulation of CTLs

For restimulation, responder splenocytes ($5x10^6$) were incubated with $5x10^6$ irradiated (20 Gy) B6 stimulator splenocytes. 5-day cultures were set up in M24 tissue culture wells in the presence or absence of 60U/ml recombinant mouse IL-2. The relevant peptides were used at 10^{-8} M final concentration in the standard cultures. Serial threefold dilutions of washed effector cultures were prepared and tested in Cr^{51} release assays as described in the main paper.

Bone marrow reconstitution

For bone marrow reconstitution, B6 mice were lethally γ-irradiated with 9.5 Gy the day before reconstitution. Bone marrow was prepared from tibiae and femora of sex-matched B6 mice. The obtained cell populations were T cell depleted by MACS technology using Thy 1.2 (30-H12) specific antibodies coupled to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and $4x10^7$ cells were injected i.v. in 500 μl BSS.

Flow cytometry

For detection of transferred $CD8^+$ TCR318 T cells we used purified monoclonal antibodies (BD PharMingen) specific for CD8α (53-6.7), Vα2 (B20.1), or Vβ8.1/8.2. They were conjugated either to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC).

Supplementary References

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