

Microchimerism maintains deletion of the donor cell–specific CD8⁺ T cell repertoire

Weldy V. Bonilla, ... , Hans Hengartner, Rolf M. Zinkernagel

J Clin Invest. 2006;116(1):156-162. <https://doi.org/10.1172/JCI26565>.

Research Article

Immunology

Rare cases of stable allograft acceptance after discontinuation of immunosuppression are often accompanied by macrochimerism (> 1% donor cells in blood) or microchimerism (< 1% donor cells in blood). Here, we have investigated whether persistence of donor cells is the cause or the consequence of long-lasting CTL unresponsiveness. We found that engraftment of splenocytes bearing a single foreign MHC class I–restricted epitope resulted in lifelong donor cell microchimerism and specific CTL unresponsiveness. This status was reversed in a strictly time- and thymus-dependent fashion when the engrafted cells were experimentally removed. The results presented herein show that microchimerism actively maintains CTL unresponsiveness toward a minor histocompatibility antigen by deleting the specific repertoire and thus excluding dominant, T cell extrinsic mechanisms of CTL unresponsiveness independent of systemically persisting donor cell antigen.

Find the latest version:

<https://jci.me/26565/pdf>





Microchimerism maintains deletion of the donor cell–specific CD8⁺ T cell repertoire

Weldy V. Bonilla, Markus B. Geuking, Peter Aichele, Burkhard Ludewig, Hans Hengartner, and Rolf M. Zinkernagel

Institute of Experimental Immunology, Department of Pathology, University Hospital Zurich, Zurich, Switzerland.

Rare cases of stable allograft acceptance after discontinuation of immunosuppression are often accompanied by macrochimerism (> 1% donor cells in blood) or microchimerism (< 1% donor cells in blood). Here, we have investigated whether persistence of donor cells is the cause or the consequence of long-lasting CTL unresponsiveness. We found that engraftment of splenocytes bearing a single foreign MHC class I–restricted epitope resulted in lifelong donor cell microchimerism and specific CTL unresponsiveness. This status was reversed in a strictly time- and thymus-dependent fashion when the engrafted cells were experimentally removed. The results presented herein show that microchimerism actively maintains CTL unresponsiveness toward a minor histocompatibility antigen by deleting the specific repertoire and thus excluding dominant, T cell extrinsic mechanisms of CTL unresponsiveness independent of systemically persisting donor cell antigen.

Introduction

Abundant literature has correlated donor cell chimerism with graft acceptance and T cell unresponsiveness in various clinical and experimental settings (1–12). This includes chimerism combined with skin acceptance in freemartin cattle and mice (1, 4, 6), tolerance to liver grafts in mice (5), and a steadily increasing number of cases where allograft acceptance after liver or kidney transplantation in humans remained stable when immunosuppression was discontinued (2, 3, 7, 10, 12). Whether chimerism maintains T cell unresponsiveness or whether it is merely a consequence thereof, however, remains controversial (2, 8, 9, 12–15). Among the supporters of the former hypothesis, it remains an open question whether microchimerism (< 1% donor cells in blood) is sufficient to maintain CTL unresponsiveness or whether macrochimerism (> 1% donor cells in blood) is required.

To date, graft acceptance and T cell unresponsiveness have been explained by a variety of mechanisms (16, 17). They can be grouped into dominant, T cell extrinsic mechanisms, including regulatory T cells and antigen presentation by immature DCs, and into recessive, T cell intrinsic mechanisms, such as ignorance, anergy, and deletion (17, 18). The latter (clonal deletion of antigen-specific immune cells) has long been discussed (19, 20) as an explanation for unresponsiveness to antigens expressed in fetal (21, 22) and neonatal life (23). Initially brought up by Owen in his groundbreaking work on freemartin cattle (20), this concept was later fostered by studies on repetitive antigen application and also by observations of a persistent viral infection (24, 25). Yet, CTL unresponsiveness against identical viral epitopes was transient when induced by treatment with nonpersisting peptide (26), suggesting without directly demonstrating a critical role for persisting antigen in maintenance of unresponsiveness.

Insight into the role of donor cell chimerism in transplantation tolerance is important for future refinements of clinical

organ transplantation protocols (13, 27). In the present study, we therefore set out to analyze the causal relationship between donor cell persistence and specific CTL unresponsiveness as well as the underlying mechanisms. For this purpose, we made use of a well-established and molecularly defined model of lymphohematopoietic cell engraftment across a strong minor histocompatibility (miH) antigen mismatch in adult mice (28, 29). We used donor splenocytes of transgenic H8 mice constitutively expressing the H-2D^b-restricted GP33 epitope (amino acids 33 to 41) of the lymphocytic choriomeningitis virus (LCMV) glycoprotein. The engraftment of H8 splenocytes in C57BL/6 (B6) mice has previously been shown to elicit CTL unresponsiveness as measured by *in vitro* and functional *in vivo* readouts for antigen-specific T cells. It was also noted at the time that the administration of the corresponding MHC class I binding peptide resulted in only transient CTL unresponsiveness whereas the same functional status was long-lived when induced by the engraftment of H8 donor cells. This was correlated with the relatively short *in vivo* half-life of peptides as compared with H8 donor cells persisting in the host for at least 60 days, lending support to the hypothesis that persisting H8 cells may help to maintain CTL unresponsiveness (4, 29). It was, however, not possible at the time to discriminate between antigen persistence as an epiphenomenon of CTL unresponsiveness and antigen persistence as an active principle for the maintenance of CTL unresponsiveness (4, 12, 29). Nor had the mechanisms been studied that led to CTL unresponsiveness in this experimental setting. Here we addressed these 2 key questions and found that specific CTL unresponsiveness was irreversible when foreign antigen persisted systemically at macro- or even microchimeric levels. Reconstitution of the endogenous donor cell–specific T cell repertoire required the engrafted cells to be experimentally removed. This reconstitution of CTL responsiveness after a phase of unresponsiveness was strictly thymus- and time-dependent, suggesting clonal deletion of the donor cell–specific T cell repertoire as the responsible mechanism of CTL unresponsiveness.

Results

Long-lasting donor cell chimerism. Systemic persistence of donor cells was compared after adoptive transfer of miH-disparate, H8-trans-

Nonstandard abbreviations used: B6, C57BL/6; B6TX, thymectomized C57BL/6; d10-LCMV-F1, splenocytes of B6 × BALB/c F1 mice infected with LCMV 10 days before; LCMV, lymphocytic choriomeningitis virus; miH antigen, minor histocompatibility antigen; NP, nucleoprotein.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 116:156–162 (2006). doi:10.1172/JCI26565.

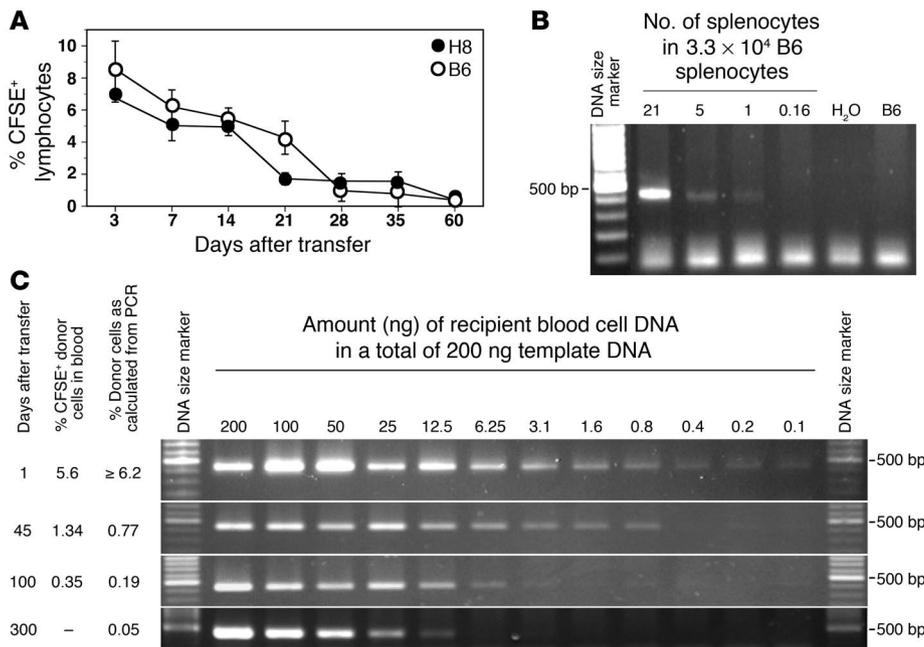


Figure 1 Long-lasting chimerism after adoptive transfer of H8 splenocytes and its detection. **(A)** Sex-matched, CFSE-labeled splenocytes from H8 transgenic (filled circles) or control B6 mice (open circles) were transferred to B6 recipient mice. CFSE-positive cells in the recipient's blood were quantified at the indicated time points by flow cytometry. Symbols represent the mean \pm SD of 4–16 mice per group and time point. **(B)** B6 splenocytes (3.3×10^4) were mixed with titrated numbers of H8 splenocytes as indicated. DNA was isolated from the different cell mixtures and used as template for H8-specific PCR analysis. **(C)** Blood samples were collected at the indicated time points to measure CFSE-positive cells by flow cytometry and to extract DNA. Two-fold serial dilutions of isolated DNA (in wild-type B6 DNA) were performed and used as templates for H8-specific PCR analysis. One representative experiment of 3 is shown.

genic, or syngeneic B6 splenocytes to sex-matched B6 recipients. Both transferred cell populations were fluorescently labeled with CFSE. Flow cytometric analysis over the first 60 days after transfer revealed no apparent differences in donor cell levels between the 2 experimental groups (Figure 1A). At later time points, detection by flow cytometry became unreliable, apparently due to the limited half-life of the fluorescent dye. Homogenous fluorescence of the entire transferred cell population ruled out cell division as an important mechanism (not shown). We therefore established a PCR protocol to detect H8-transgenic donor cells in blood of recipient mice. To assess the sensitivity of this assay, titrated numbers of H8 splenocytes were mixed with fixed numbers of B6 splenocytes for extraction of DNA and PCR analysis. Approximately 1 H8 cell in 3.3×10^4 B6 splenocytes (0.003%) was reliably detected by this method (Figure 1B), matching the sensitivity of similar protocols applied in clinical and experimental transplantation medicine (11, 30). By this method, H8 donor cells remained detectable for more than 700 days after grafting (Figure 1C and data not shown). To distinguish between macro- and microchimerism, DNA isolated from recipient blood was serially diluted in wild-type B6 DNA before H8-specific PCR analysis. The number of 2-fold template dilution steps necessary to reach the detection limit of the PCR test was compared with the percentage of CFSE-positive cells as measured by flow cytometry (Figure 1C). Both methods supported the notion that around 45–100 days after transfer of 5×10^7 miH-disparate H8-transgenic splenocytes, the arbitrary border of macro-

to microchimerism (1% of peripheral blood cells) was crossed. Moreover, preliminary data suggest that donor cells persisted not only in blood but also in the parenchyma of various organs, including lung, liver, spleen, thymus, and lymph nodes (data obtained on day 168 after transfer not shown).

Chimerism-induced antigen-specific CTL unresponsiveness at multiple levels. To assess GP33-specific CTL responsiveness in chimeric recipient mice, these mice were challenged with LCMV 28, 45, or 300 days after engraftment of H8 splenocytes. The GP33-specific response was measured directly ex vivo 8 days later (Figure 2A). The CTL response against a second but donor cell-independent target, i.e., the LCMV nucleoprotein-derived (NP-derived) epitope NP396, served as an internal positive control. LCMV-infected control mice without cell engraftment mounted a vigorous CTL response against both epitopes. The NP396-specific response in H8 chimeric mice was of equivalent strength, but GP33-specific lytic activity remained undetectable at all time points tested, even when peptide restimulation was carried out in the presence of exogenous IL-2 (ruling out CTL anergy; data not shown). Notably, 45 and 300 days after engraftment of H8 splenocytes,

chimerism was still detectable by PCR (Figure 2B, same mice). To distinguish between different possible phenotypes of CTL dysfunction (18, 31), we also assessed binding of MHC class I tetramer complexes (Figure 2C) by LCMV immune splenocytes of chimeric mice and production of IFN- γ in response to in vitro peptide restimulation (Figure 2D). Twenty-eight days after engraftment of H8 donor cells, neither of these alternative readouts detected GP33-specific CD8⁺ T cells. Following the pattern of primary ex vivo cytotoxicity (Figure 2A), NP396-specific CTL responses of H8 chimeric mice were unimpaired or even slightly higher than those of control mice, probably due to the lack of competition by GP33-specific CTLs.

Experimental removal of donor cells. To study the fundamental question of whether chimerism is the cause or a consequence of CD8⁺ T cell unresponsiveness, we developed 2 independent protocols to experimentally remove the engrafted H8 cells from B6 recipients (Figure 3 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI26565DS1). The first protocol (illustrated in Figure 3A) was based on adoptive transfer on day 28 (Figure 3B) or day 700 of splenocytes from B6 \times BALB/b F1 mice infected with LCMV 10 days before (d10-LCMV-F1). The GP33-specific CTLs in the transferred d10-LCMV-F1 cells cleared the grafted H8 cells to below detection levels of flow cytometry (Figure 3B) and PCR (Figure 3C) within days 7 and 29, respectively (earlier time points not tested). Due to the multiple BALB/b miH differences, d10-LCMV-F1 cells were themselves efficiently

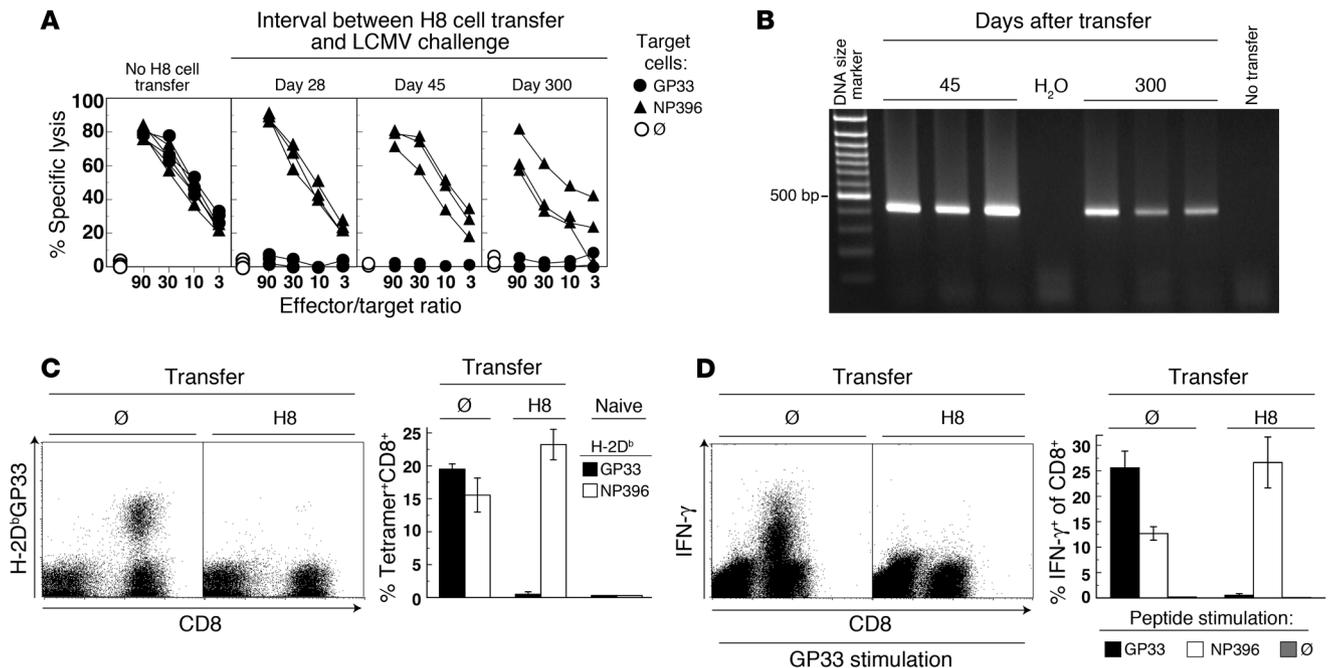


Figure 2 H8 chimerism-induced antigen-specific CTL unresponsiveness. (A–D) Sex-matched H8 splenocytes were adoptively transferred to B6 mice. At the indicated time points (A) or 28 days after transfer (C and D), recipient and control mice without cell transfer were infected with LCMV. (A) At 8 to 9 days after infection with LCMV, splenocytes were tested for GP33-specific (filled circles) and NP396-specific (filled triangles) CTL activity in a standard primary ex vivo CTL assay. Uncoated EL-4 target cells (open circles) are shown at the highest effector to target ratio. Each line represents an individual mouse. One representative experiment of 3 (day 45) or 2 (day 300) is shown. (B) DNA was isolated from blood samples taken from chimeric recipients on days 45 and 300 as shown in Figure 2A. The presence of H8 chimerism was confirmed by H8-specific PCR analysis. (C) Frequencies of GP33- and NP396-specific CD8⁺ T cells in spleen 8 days after LCMV infection were enumerated using MHC class I H-2D^bGP33 (black bars) and H-2D^bNP396 (white bars) tetramers. (D) Splenocytes from the same mice as in C were tested for IFN-γ production upon restimulation with GP33 or NP396 or without restimulation. Representative FACS plots are shown, and bars indicate the mean ± SD of 3 mice (C and D).

cleared from the recipient (Figure 3D). This ensured that no transferred GP33-specific reactivity or other LCMV-specific immunity remained in the recipient that could have interfered with the subsequent assessment of endogenous GP33-specific CD8⁺ T cell responsiveness (Supplemental Figure 2). Transfer of infectivity together with d10-LCMV-F1 effector cells was also excluded since the recipient mice were primed against neither LCMV NP396 nor GP33 epitopes (Supplemental Figure 2).

A second, alternative protocol was also used to remove H8 donor cells from chimeric mice to confirm and extend the conclusions reached with the first protocol. It was based on adoptive transfer of male splenocytes from GP33-specific TCR transgenic mice to female recipients. Further details with a schematic description and validation are provided in Supplemental Figure 1.

Removal of H8 donor cells leads to reemergence of GP33-specific CTL precursors. With these tools in hand, we went on to analyze whether H8 donor cells at macro- or microchimeric levels maintained the recipient's GP33-specific CTL unresponsiveness. As described above, we established H8 chimerism by adoptive transfer of 5×10^7 CFSE-labeled H8 splenocytes to sex-matched B6 recipients. These donor cells were then experimentally eliminated from the recipients by adoptive transfer of d10-LCMV-F1 cells on day 28 (macrochimeric mice) or day 700 (microchimeric mice). Controls were left chimeric. The disappearance of grafted H8 cells was monitored for each individual mouse by H8-specific PCR analysis and, in addition, by

flow cytometry for early time points (Figure 3, B and C, and data not shown). Mice were challenged with LCMV 5 to 6 weeks after d10-LCMV-F1 cell transfer. GP33- and NP396-specific cytotoxic responses were monitored in a primary ex vivo CTL assay 8 days after challenge (Figure 4A). As expected, all mice exhibited high NP396-specific CTL activity, and control mice transfused only with H8 splenocytes and not with d10-LCMV-F1 cells mounted no detectable GP33-specific response (Figure 4A; compare also Figure 2A). Unresponsiveness was found even when the same splenocytes from LCMV-challenged H8 chimeric mice were restimulated in vitro for 5 days with GP33 in the presence or absence of exogenous IL-2 (not shown). MHC class I tetramer binding and intracellular IFN-γ staining after peptide restimulation supported this notion (Figure 4B). Importantly, when H8 donor cell chimerism had been eliminated before LCMV challenge, GP33-specific CTL reactivity was restored (Figure 4A). The physical presence and functionality of GP33-specific CTLs were verified by MHC class I tetramer binding and IFN-γ production upon in vitro restimulation with peptide (Figure 4B). Analogous results were obtained using the alternative protocol (Supplemental Figure 1) to remove the grafted H8 cells (Supplemental Figure 3). Together, these results demonstrate that H8 donor cells at macro- but also at microchimeric levels maintain GP33-specific T cell unresponsiveness.

Reemergence of GP33-specific CTL precursors is strictly thymus and time dependent. The CD8⁺ T cell unresponsiveness found here was

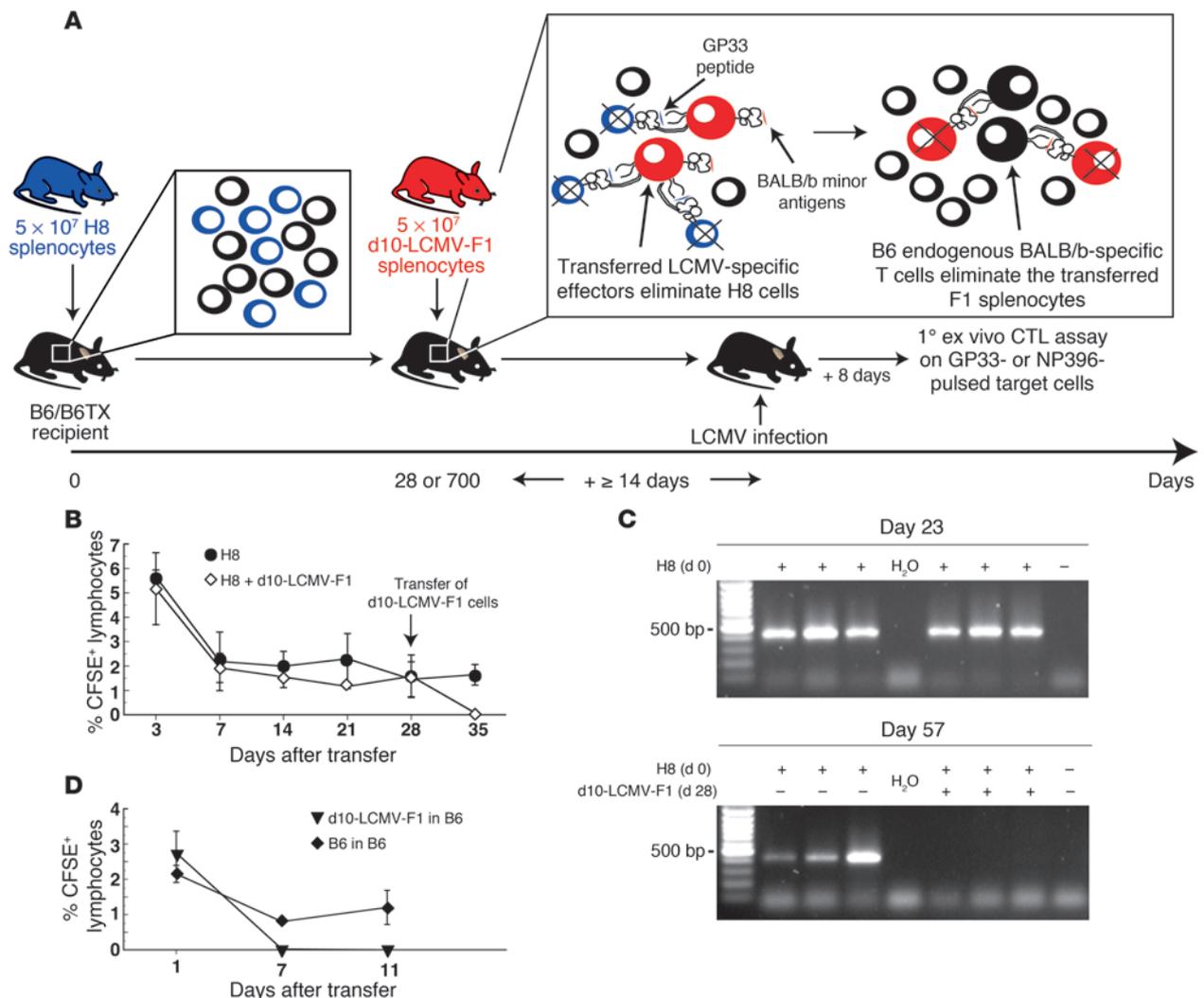


Figure 3

Protocol used for termination of chimerism by adoptive transfer of LCMV-specific CTLs. **(A)** Euthymic (B6) or thymectomized (B6TX) mice (black) were transfused with sex-matched H8 splenocytes (blue). This resulted in permanent mixed chimerism, represented by endogenous (black) and donor (blue) cells. Either 28 or 700 days later, recipients were additionally transfused with splenocytes from B6 × BALB/b F1 mice that had been infected with LCMV 10 days previously (red, d10-LCMV-F1). GP33-specific d10-LCMV-F1 CTLs eliminated H8 donor cells in an H-2D^b-restricted manner. BALB/b miH epitopes expressed by the transferred cells elicited a BALB/b-specific response, rejecting the transferred d10-LCMV-F1 cells within 7 days. At different time points after transfer of d10-LCMV-F1 cells, recipients were challenged with LCMV, and endogenous GP33-specific and NP396-specific CTL responses were measured 8 days later. **(B)** B6 recipients of CFSE-labeled H8 splenocytes were transfused i.v. with d10-LCMV-F1 (B6 × BALB/b) splenocytes (open diamonds) or were left untreated (filled circles). The percentage CFSE-positive lymphocytes in blood was determined by flow cytometry. Symbols represent the mean ± SD of 3 mice. **(C)** DNA from blood of H8 donor cell recipients was isolated before (day 23) and after (day 57) transfer of d10-LCMV-F1 cells on day 28 and from control H8 donor cell recipients without d10-LCMV-F1 transfer for H8-specific PCR analysis. **(D)** CFSE-labeled d10-LCMV-F1 (B6 × BALB/b) (filled triangles) or control B6 splenocytes (filled diamonds) were adoptively transferred to sex-matched B6 recipients, and the percentage of CFSE-positive lymphocytes in blood was determined by flow cytometry. Symbols represent the mean ± SD of 4 mice. One representative experiment of 3 (**B** and **C**) or 2 (**D**) is shown.

distinct from anergy (tetramer-positive CD8⁺ T cells without lytic capacity) (31), and restoration of reactivity after removal of donor cells ruled out any dominant-negative mechanism independent of persisting antigen. We therefore speculated that (a) GP33-specific unresponsiveness in H8 chimeric mice was initially established by peripheral clonal deletion, and (b) therefore, the reappearance of CTL reactivity after removal of the engrafted H8 cells should depend on thymic selection of new CD8⁺ precursor T cells. If this were the case, GP33-specific unresponsiveness should also have

been induced after transfer of sex-matched H8 splenocytes into thymectomized B6 (B6TX) mice whereas some dominant-negative mechanisms of T cell tolerance have been reported to depend on a functional thymus (32). In contrast, the reappearance of GP33-specific CD8⁺ T cell responsiveness upon removal of the engrafted cells should only have been observed in thymus-competent mice. We thus established H8 donor cell chimerism in euthymic (B6) and in B6TX mice and subsequently terminated chimerism by adoptive transfer of d10-LCMV-F1 cells. When euthymic mice were

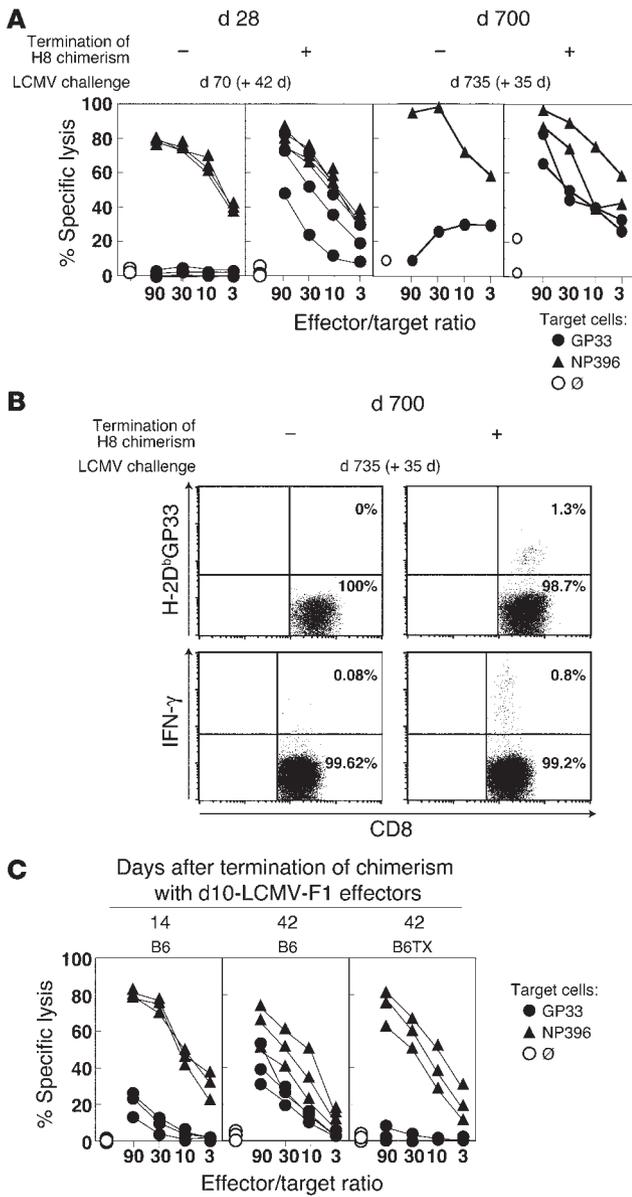


Figure 4

Thymus- and time-dependent reemergence of antigen-specific CTL precursors after removal of H8 donor cells. **(A)** Sex-matched H8 splenocytes were transferred to B6 recipients. Chimerism was either allowed to persist or was terminated at the indicated time point by transfer of d10-LCMV-F1 splenocytes. Forty-two or 35 days after termination of H8 chimerism, i.e., on day 70 or 735 after initial H8 cell transfer, respectively, mice were challenged with LCMV. Specific CTL activity was measured 8 days after infection in a primary ex vivo CTL assay on GP33-coated (filled circles), NP396-coated (filled triangles), and uncoated (open circles) EL-4 target cells. Effector and target cells were incubated for 5 hours (day 70) or 20 hours (day 735). The longer incubation time of 20 hours was necessary to detect the lower response in these old mice. Each line represents an individual mouse. **(B)** MHC class I H-2D^bGP33 tetramer binding and GP33-specific IFN-γ production on gated CD8⁺ cells from the same mice as in **A** after LCMV challenge on day 735. **(C)** Four weeks after transfer of H8 splenocytes into euthymic or thymectomized recipients, chimerism was eliminated in all animals by an additional transfer of d10-LCMV-F1 splenocytes. At 14 or 42 days after this second transfer, mice were infected with LCMV. GP33-specific (filled circles) and NP396-specific (filled triangles) CTL activities were measured 8 days later in a standard primary ex vivo CTL assay on peptide-coated or noncoated (open circles) EL-4 target cells. One representative experiment of 3 is shown.

additional support for thymic education of GP33-specific cells as the mechanism underlying reemergence of CTL responsiveness.

Discussion

To our knowledge, the present report is the first to formally show that donor cell chimerism represents the active principle for maintenance of T cell unresponsiveness and not only a consequence thereof. Moreover, we found that even microchimeric levels of donor cells can suffice in this function.

These insights are of pivotal importance because they indicate that not only macrochimerism but also microchimerism should be considered an important parameter and probably a worthy goal for improved clinical transplantation protocols. A number of regimens are clinically effective in inducing short-term T cell unresponsiveness, among them total body irradiation, antibody-mediated T cell depletion, blockade of costimulation, and anti-mitotic chemotherapy in various combinations (27). Long-term graft acceptance represents, however, an even more delicate task that seems to require somewhat different strategies. It is therefore important to understand the mechanisms for maintenance of T cell unresponsiveness and not only the mechanisms operating in the establishment of T cell unresponsiveness.

In the present model, loss of CTL reactivity was T cell intrinsic (as defined in the Introduction). It seems likely to result from clonal deletion (33, 34) and not from anergy (35-37) or from T cell extrinsic mechanisms (reviewed in refs. 16, 38). Considering that the induction of CTL unresponsiveness was unaffected in thymus-incompetent recipients, central tolerance seemed unlikely to play a key role in these early events. In contrast, peripheral clonal deletion of the specific CD8⁺ T cell repertoire appeared to be sufficient for inducing CTL unresponsiveness. The mechanisms thereof were, however, not the subject of our study. We were primarily interested in understanding the mechanisms operating to maintain CTL unresponsiveness. Following the induction of T cell unresponsiveness, donor cells persisting at initially macrochimeric and later at microchimeric levels prevented the reemergence of GP33-reactive naive thymic emigrants for more than 700 days. Accordingly, thymecto-

challenged with LCMV 14 days later, only marginal GP33-specific CTL responses were elicited while NP396-specific responses were within normal ranges (Figure 4C). GP33-specific responses became considerably stronger when euthymic chimeric recipients of d10-LCMV-F1 cells were allowed to rest for 42 days or longer before LCMV challenge (Figure 4C and data not shown). Importantly, reappearance of GP33-specific CTL responsiveness was not observed in thymectomized recipients (Figure 4C). Analogous findings were made when chimerism was terminated by the alternative protocol (Supplemental Figure 3). Thus, induction of CTL unresponsiveness was thymus independent and was maintained by persisting chimerism. In contrast, restoration of GP33-specific CTL responsiveness was strictly thymus and time dependent and occurred only after elimination of chimerism, suggesting clonal deletion as the underlying mechanism. In addition, the time window needed for restoration of CTL responsiveness after termination of chimerism resembled the kinetics observed after lethal irradiation (Supplemental Figure 4). This correlative evidence provides



mized recipients failed to recover GP33-specific CTL reactivity even when the engrafted H8 cells were experimentally removed. It was therefore impossible to dissect whether H8 donor cells maintained specific CTL unresponsiveness of recipient mice by acting primarily at the stage of thymic negative selection or predominantly via peripheral clonal deletion of new thymic emigrants. A contribution of both mechanisms to maintenance of CTL unresponsiveness appears, however, very likely.

As for the initial induction of T cell unresponsiveness (see above), anergy seems also excluded as a mechanism for the maintenance of this status that could not be reversed by *in vitro* restimulation in the presence of exogenous IL-2 (36, 37). Moreover, specific CD8⁺ T cells were not even physically detectable by MHC class I tetramer binding. Reappearance of CTL responsiveness *in vivo* upon removal of the H8 donor cell population rules out antigen-independent, T cell extrinsic mechanisms, among them suppressor or regulatory CD8⁺ or CD4⁺ T cells, that may, however, contribute to transplantation tolerance in experimental and clinical settings with higher antigenic complexity (16, 17, 38–41). Similarly, mechanisms based on T cell regulation cannot explain the thymus dependence of reemerging GP33-specific CTL responsiveness after elimination of H8 donor cells. In contrast, thymus dependence, the kinetics of reappearing GP33-specific reactivity after removal of the H8 donor cell population (resembling repopulation after lethal irradiation), and, notably, also the naive status of the reemerging GP33-reactive repertoire suggest that microchimerism maintained CTL unresponsiveness via clonal deletion of donor cell-specific CD8⁺ T cells. Whether GP33-specific precursor CTLs were physically deleted or whether unresponsiveness resulted from an irreversible functional inactivation of these cells, e.g., as a result of “veto cell” activity (39) by H8 cells, can, however, not be determined if these functionally deleted cells persisted at a frequency below the detection level of MHC class I tetramers.

While providing a mechanistic proof of principle, low levels of microchimerism as in our study may be less efficient in protecting against graft rejection in clinical organ transplantation. These settings commonly involve many more CTL and T helper cell determinants or even disparate major histocompatibility antigens. It will therefore be important to carry out analogous studies (i.e., the removal of chimerism) in antigenically more complex systems, possibly involving immunosuppressive therapy to induce CTL unresponsiveness. Under such conditions, long-term graft acceptance after withdrawal of immunosuppression may depend on a variety of additional mechanisms for maintenance of graft-specific tolerance (e.g., regulatory T cells and anergy of certain T cell specificities). Accordingly, multiple parameters may likely be needed to predict the safe withdrawal of immunosuppression in clinical settings, a clinically important issue that has not been addressed in our study. In summary, lymphohematopoietic chimerism appears to represent 1 key mechanism for maintenance of specific CTL unresponsiveness. Therefore, persisting donor cells even at the microchimeric level should be considered an important goal on the way to optimized allograft acceptance even under reduced or discontinued immunosuppression.

Methods

Mice and adult thymectomy. B6, BALB/b, and H8 mice were obtained from the Institut für Labortierkunde (University of Zurich, Zurich, Switzerland). H8 mice had a pure B6 background. All mice were kept under specific pathogen-free conditions throughout the experiments. For adult thymectomy

of B6 mice, animals were anesthetized and the thymus made accessible by supraclavicular incision of skin and sternum followed by blunt dissection. A sharp suction pipette was then inserted, and each individual thymus lobe was removed by suction under visual control. Mice were used for experiments no earlier than 14 days after thymectomy. All animal experiments were ethically approved and authorized by the Kantonales Veterinäramt Zürich (Zurich, Switzerland) in accordance with the Swiss laws on animal protection.

Viruses and infections. LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, Germany), and LCMV strain Armstrong was originally provided by M.B.A. Oldstone (The Scripps Research Institute, La Jolla, California, USA) (42). The LCMV strains WE and Armstrong were propagated on L929 and BHK-21 cells, respectively, at a low multiplicity of infection. For the individual experiments presented, mice were infected *i.v.* with 200 PFU of one or the other LCMV strain.

Adoptive transfer protocols and fluorescent cell labeling. For adoptive transfer of spleen cells, single-cell suspensions were prepared from spleens of donor mice, washed twice, and counted, and 5×10^7 cells were injected *i.v.* in a volume of 500 μ l balanced salt solution into recipient mice. For fluorescent labeling, splenocytes were resuspended at about 5×10^7 /ml in RPMI medium without additives. Erythrocytes were lysed by adding an equal volume of 1.66% ammoniumchloride/H₂O solution and incubating for 3 minutes at room temperature. To stop the lysis process, RPMI medium was added. After 2 washes with RPMI medium, splenocytes were resuspended at 5×10^6 cells/ml PBS containing 2.5 μ M CFSE (Invitrogen Corp.) and incubated for 10 minutes at 37°C. After labeling, FCS was added to a 1% final concentration. Cells were washed twice and injected *i.v.* in 500 μ l balanced salt solution.

Isolation of genomic DNA and detection of H8 chimerism by PCR. Samples of whole blood (250 μ l) were collected in heparinized collection tubes (Fischer Scientific International). Erythrocytes were lysed by a 30- to 60-minute incubation step in 10 ml blood lysis solution (140 mM NH₄Cl, 17 mM TrisCl, pH 7.2) at room temperature. Cells were digested overnight at 56°C in 750 μ l proteinase K solution (50 mM TrisHCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% w/v SDS, 400 μ g/ml proteinase K). Genomic DNA was precipitated with ethanol after proteins had been removed with saturated NaCl solution and was resuspended in 30–40 μ l Tris-EDTA buffer (10 mM TrisCl, 1 mM EDTA). For PCR reactions, 200 ng of genomic DNA was used as template. A 434-bp product was amplified using primer 5'-GATCAGAACTCGGAGACG-3' specific for the promoter region of the H8 transgene and primer 5'-CTGACCAGTGCTAATATCCC-3' specific for LCMV-WE-GP nucleotides 124–143.

CTL assay and peptides. LCMV-specific primary *ex vivo* CTL responses were assayed as described previously (43). Briefly, single-cell suspensions were prepared from the spleens of mice at day 8 (unless stated differently) after infection with 200 PFU LCMV and were used directly in a standard ⁵¹Cr-release assay. Target cells, unloaded or peptide pulsed (10^{-6} M) EL-4 cells (dimethylbenzanthrene-induced thymoma cells of B6 origin), were incubated with the effector cells for 5 hours (unless stated differently). LCMV-derived GP33 (KAVYNFATM) and NP396 (FQPQNGQFI) peptides were purchased from Neosystem Laboratoire.

Flow cytometry. For determination of antigen-specific or cytokine-producing CTL, we used purified monoclonal antibodies (BD Biosciences – Pharmingen) specific for CD8 α (53–6.7) or IFN- γ (XMG1.2). They were conjugated to either FITC, PE, allophycocyanin, or biotin. Streptavidin-Tricolor was purchased from CALTAG Laboratories. Tetrameric complexes containing biotinylated H-2D^b, β_2 -microglobulin, and the relevant peptide were generated and used for staining as previously described (44).

For intracellular staining of IFN- γ , 10^6 splenocytes were incubated for 6 hours at 37°C with 1 μ g/ml of the specific peptide or with medium alone in the presence of 10 U/ml IL-2 and 1 μ g/ml Brefeldin A (Sigma-Aldrich).



Staining of cell-surface antigen and intracellular antigens was performed as described (45). Data were acquired on a FACScalibur flow cytometer using Cell Quest Pro software version 4.0.2 (BD).

Statistics. Data were analyzed using GraphPad Prism 3.0cx (GraphPad Software Inc.). All error bars represent mean ± SD.

Acknowledgments

We thank Kathrin Tschannen for the preparation of MHC class I tetramers and Maries van den Broek, Daniel Pinschewer, and Kathy McCoy for helpful discussions and for carefully reading the manuscript. This study was supported by the Swiss National Science Foundation and by the Kanton of Zurich.

Received for publication August 11, 2005, and accepted in revised form October 25, 2005.

Address correspondence to: Weldy V. Bonilla, Prionics AG, Wagistrasse 27a, 8952 Schlieren, Switzerland. Phone: 41-442002128; Fax: 41-442002010; E-mail: welbon@gmx.ch.

Weldy V. Bonilla's present address is: Prionics AG, Schlieren, Switzerland.

Peter Aichele's present address is: Universität Freiburg, Freiburg, Germany.

Burkhard Ludwig's present address is: Kantonsspital St. Gallen, St. Gallen, Switzerland.

Weldy V. Bonilla and Markus B. Geuking contributed equally to this work.

1. Anderson, D., Billingham, R.E., Lampkin, G.H., and Medawar, P.B. 1951. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity*. **5**:379-397.
2. Buhler, L.H., et al. 2002. Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation*. **74**:1405-1409.
3. Cosimi, A.B., and Sachs, D.H. 2004. Mixed chimerism and transplantation tolerance. *Transplantation*. **77**:943-946.
4. Ehl, S., et al. 1998. Antigen persistence and time of T-cell tolerization determine the efficacy of tolerization protocols for prevention of skin graft rejection. *Nat. Med.* **4**:1015-1019.
5. Lu, L., et al. 1995. Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **182**:379-387.
6. Lubaroff, D.M., and Silvers, W.K. 1973. Importance of chimerism in maintaining tolerance of skin allografts in mice. *J. Immunol.* **111**:65-71.
7. Millan, M.T., et al. 2002. Mixed chimerism and immunosuppressive drug withdrawal after HLA-mismatched kidney and hematopoietic progenitor transplantation. *Transplantation*. **73**:1386-1391.
8. Monaco, A.P. 2002. Tolerance and chimerism: separate and unequal concepts. *Transplant. Proc.* **34**:1991-1997.
9. Starzl, T.E. 2004. Chimerism and tolerance in transplantation. *Proc. Natl. Acad. Sci. U. S. A.* **101**(Suppl. 2):14607-14614.
10. Starzl, T.E., et al. 1992. Cell migration, chimerism, and graft acceptance. *Lancet*. **340**:1579-1582.
11. Starzl, T.E., et al. 1993. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology*. **17**:1127-1152.
12. Wood, K., and Sachs, D.H. 1996. Chimerism and transplantation tolerance: cause and effect. *Immunol. Today*. **17**:584-587; discussion 588.
13. Claas, F. 2004. Chimerism as a tool to induce clinical transplantation tolerance. *Curr. Opin. Immunol.* **16**:578-583.
14. Elster, E.A., et al. 2004. The road to tolerance: renal transplant tolerance induction in nonhuman primate studies and clinical trials. *Transpl. Immunol.* **13**:87-99.
15. Monaco, A.P. 2004. Prospects and strategies for clinical tolerance. *Transplant. Proc.* **36**:227-231.
16. Wood, K.J., and Sakaguchi, S. 2003. Regulatory T cells in transplantation tolerance. *Nat. Rev. Immunol.* **3**:199-210.
17. Walker, L.S., and Abbas, A.K. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* **2**:11-19.
18. Redmond, W.L., and Sherman, L.A. 2005. Peripheral tolerance of CD8 T lymphocytes. *Immunity*. **22**:275-284.
19. Burnet, F.M. 1959. *The clonal selection theory of acquired immunity*. Cambridge University Press. New York, New York, USA. 209 pp.
20. Owen, R.D. 1945. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. **102**:400-401.
21. Billingham, R.E., Brent, L., and Medawar, P.B. 1953. Actively acquired tolerance of foreign cells. *Nature*. **172**:603-606.
22. Weigle, W.O. 1973. Immunological unresponsiveness. *Adv. Immunol.* **16**:61-122.
23. Billingham, R.E., and Brent, L. 1956. Acquired tolerance of foreign cells in newborn animals. *Proc. R. Soc. Lond. B. Biol. Sci.* **146**:78-90.
24. Moskophidis, D., Lechner, F., Pircher, H., and Zinkernagel, R.M. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature*. **362**:758-761.
25. Critchfield, J.M., et al. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science*. **263**:1139-1143.
26. Aichele, P., Brduscha-Riem, K., Zinkernagel, R.M., Hengartner, H., and Pircher, H. 1995. T cell priming versus T cell tolerance induced by synthetic peptides. *J. Exp. Med.* **182**:261-266.
27. Fehr, T., and Sykes, M. 2004. Tolerance induction in clinical transplantation. *Transpl. Immunol.* **13**:117-130.
28. Ehl, S., et al. 1998. Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8+ T cells to cause immunopathology. *J. Exp. Med.* **187**:763-774.
29. Ehl, S., et al. 2000. Donor cell persistence and activation-induced unresponsiveness of peripheral CD8+ T cells. *Eur. J. Immunol.* **30**:883-891.
30. Yano, Y., et al. 2001. Microchimeric cells from the peripheral blood associated with cardiac grafts are bone marrow derived, long-lived and maintain acquired tolerance to minor histocompatibility antigen H-Y. *Transplantation*. **71**:1456-1462.
31. Welsh, R.M. 2001. Assessing CD8 T cell number and dysfunction in the presence of antigen. *J. Exp. Med.* **193**:F19-F22.
32. Picca, C.C., and Caton, A.J. 2005. The role of self-peptides in the development of CD4+ CD25+ regulatory T cells. *Curr. Opin. Immunol.* **17**:131-136.
33. Webb, S.R., and Sprent, J. 1990. Induction of neonatal tolerance to Mlsa antigens by CD8+ T cells. *Science*. **248**:1643-1646.
34. Sterzl, J., and Silverstein, A.M. 1967. Developmental aspects of immunity. *Adv. Immunol.* **6**:337-459.
35. Rammensee, H.G., Kroschewski, R., and Frangoulis, B. 1989. Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. *Nature*. **339**:541-544.
36. Ramsdell, F., and Fowlkes, B.J. 1992. Maintenance of in vivo tolerance by persistence of antigen. *Science*. **257**:1130-1134.
37. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science*. **248**:1349-1356.
38. Waldmann, H., et al. 2004. Regulatory T cells and organ transplantation. *Semin. Immunol.* **16**:119-126.
39. Fink, P.J., Shimonkevitz, R.P., and Bevan, M.J. 1988. Veto cells. *Annu. Rev. Immunol.* **6**:115-137.
40. Walsh, P.T., Taylor, D.K., and Turka, L.A. 2004. Tregs and transplantation tolerance. *J. Clin. Invest.* **114**:1398-1403. doi:10.1172/JCI200423238.
41. Joffre, O., Gorsse, N., Romagnoli, P., Hudrisier, D., and van Meerwijk, J.P. 2004. Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes. *Blood*. **103**:4216-4221.
42. Dutko, F.J., and Oldstone, M.B. 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J. Gen. Virol.* **64**:1689-1698.
43. Zinkernagel, R.M., Leist, T., Hengartner, H., and Althage, A. 1985. Susceptibility to lymphocytic choriomeningitis virus isolates correlates directly with early and high cytotoxic T cell activity, as well as with footpad swelling reaction, and all three are regulated by H-2D. *J. Exp. Med.* **162**:2125-2141.
44. Altman, J.D., et al. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. **274**:94-96.
45. Maloy, K.J., et al. 2000. CD4(+) T cell subsets during virus infection. Protective capacity depends on effector cytokine secretion and on migratory capability. *J. Exp. Med.* **191**:2159-2170.