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Primary and Secondary Immunocompetence in Mixed Allogeneic Chimeras[1]

Matthew A. Williams,* Andrew B. Adams,* Melody B. Walsh,* Nozomu Shirasugi,* Thandi M. Onami,† Thomas C. Pearson,* Rafi Ahmed,[2†] and Christian P. Larsen[2*]

Targeted disruption of T cell costimulatory pathways, particularly CD28 and CD40, has allowed for the development of minimally myeloablative strategies for the induction of mixed allogeneic chimerism and donor-specific tolerance across full MHC barriers. In this study we analyze in depth the ability of mixed allogeneic chimeras in two strain combinations to mount effective host-restricted and donor-restricted antiviral CD4 and CD8 responses, as well as the impact of development of mixed chimerism on the maintenance of pre-existing memory populations. While antiviral CD8 responses in mixed chimeras following acute viral infection with lymphocytic choriomeningitis virus Armstrong or vaccinia virus are largely host-restricted, donor-restricted CD8 responses as well as host- and donor-restricted CD4 responses are also readily detected, and virus is promptly cleared. We further demonstrate that selection of donor-restricted T cells in mixed chimeras is principally mediated by bone marrow-derived cells in the thymus. Conversely, we find that mixed chimeras exhibit a deficit in their ability to deal with a chronic lymphocytic choriomeningitis virus clone 13 infection. Encouragingly, pre-existing memory populations are largely unaffected by the development of high level mixed chimerism and maintain the ability to control viral rechallenge. Our results suggest that while pre-existing T cell memory and primary immunocompetence to acute infection are preserved in mixed allogeneic chimeras, MHC class I and/or class II tissue matching may be required to fully preserve immunocompetence in dealing with chronic viral infections. ***The Journal of Immunology,*** **2003, 170: 2382–2389.**

A state of mixed hemopoietic chimerism has long been associated with life-long transplantation tolerance (1–3). Attempts to induce mixed chimerism and donor-specific tolerance in animal models have become feasible with the advent of therapies aimed at disrupting the CD28 and CD40 T cell costimulatory interactions. These strategies rely on recipient conditioning with gamma irradiation to create space for stem cell engraftment (4). Other studies have shown that administration of high doses of donor bone marrow without recipient conditioning can lead to mixed hemopoietic chimerism and donor-specific tolerance (5, 6). Recent experiments in our laboratory have identified an alternative method for establishing indefinite allospecific tolerance and high level mixed chimerism involving recipient conditioning with the stem cell-selective toxin busulfan, administration of donor bone marrow, and blockade of the CD28 and CD40 costimulatory pathways (7).

Although the induction of high level mixed chimerism is emerging as a promising strategy for the establishment of donor-specific tolerance, questions remain regarding the long term immune status of recipients. Presumably, such mice would require the generation of T cell responses restricted to donor and recipient MHC, as each cell type is present in the periphery. Furthermore, little is known about the maintenance of pre-existing memory populations in mixed allogeneic chimeras. Previous studies have identified that recipient-restricted antiviral CD8 responses are readily detectable in mixed chimeras (8, 9), although little has been done to study antiviral CD4 responses in these animals. However, despite the development of T cell chimerism, early studies failed to detect donor-restricted cytolytic activity following viral infection (8). Several factors could play a role in determining the ability of donor- or host-restricted cells to respond to antiviral infections, among which are potential defects in the ability of the recipient thymus to positively select donor-restricted T cells as well as the replacement of recipient professional APCs with APCs of donor origin.

As chimerism studies enter clinical trials and move beyond complete MHC matching, we believe further investigation of this area is critical. While these early studies provide an important foundation, new tools (e.g., MHC tetramers and intracellular cytokine staining) provide opportunities for more detailed single-cell analysis of host- and donor-restricted responses in mixed hemopoietic chimeras. Furthermore, our current methods to establish chimerism are significantly different from the lethal irradiation models in these early studies (3). The goal in establishing mixed chimerism is to promote transplant tolerance while preserving robust immunocompetence. In this light, the ability of MHC-mismatched chimeras to successfully combat a range of infections requires further study.

In this report we analyze in detail the preservation of secondary and primary immunocompetence in high level (40–60%) mixed hemopoietic chimeras. We find that while primary antiviral CD8 responses in mixed chimeras are largely dominated by recipient-restricted epitopes, donor-restricted responses can also be readily

*Emory Transplant Center and Department of Surgery, and †Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, 30322

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[2] Address correspondence and reprint requests to Dr. Christian P. Larsen, Emory Transplant Center and Department of Surgery, Emory University School of Medicine, Suite 5105 WMB, 1639 Pierce Drive, Atlanta, GA 30322. E-mail address: clarsen@emory.org; or Dr. Rafi Ahmed, Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, G211 Rollins Research Building, 1510 Clifton Road, Atlanta, GA 30322. E-mail address: ra@microbio.emory.edu

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detected, albeit at much lower levels. Moreover, mixed chimeras are capable of generating host- and donor-restricted CD4 responses. Mixed chimeras in two-strain combinations successfully controlled acute lymphocytic choriomeningitis virus (LCMV)[3] and vaccinia virus (VV) infection. However, they were impaired in their ability to control a potent chronic infection with LCMV clone 13, indicating that in some cases the donor-restricted response is not sufficient to promote viral clearance. In analyzing secondary immunocompetence, we found that despite the development of high levels of chimerism, pre-existing CD8 and CD4 memory populations are preserved at levels comparable to those in wild-type controls. Furthermore, LCMV-immune mixed chimeras effectively generate recall responses, simultaneous donor-restricted naive responses, and rapidly control virus following rechallenge.

Classic experiments have demonstrated the dominant role played by thymic epithelium in positively selecting T cell repertoires (10–12), although bone marrow-derived cells may also contribute to this process to a lesser extent in some circumstances (13, 14). While the restriction of the immune response in the mixed chimeras analyzed here is largely determined by the haplotype of the selecting thymus, we also observed that donor-restricted responses can be selected by the recipient thymus and define a role for bone marrow-derived cells in this process. We conclude that the generation of host- and donor-restricted CD8 and CD4 responses following viral infection is critical in preserving the immunocompetence of mixed allogeneic chimeras. However, we also found that fully MHC-mismatched mixed chimeras may display some immunodeficiency in the face of persistent infection.

Materials and Methods

Mice and virus infections

Adult male 6- to 8-wk-old wild-type or thymectomized BALB/c, C57BL/6 (B6), or $CB6F_1$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were infected with 2×10^5 PFU of LCMV Armstrong injected i.p. Mice receiving a secondary challenge or a primary chronic infection were injected i.v. with 2×10^6 PFU of LCMV clone 13, a strain and dose combination that establishes a chronic infection in naive mice. Infectious LCMV in serum and spleen was quantitated by plaque assay on Vero cell monolayers as described previously (15). For VV experiments, mice were injected with 5×10^6 PFU of wild-type VV i.p. Virus stocks were grown and quantitated as previously described (15). Effector responses were analyzed in the spleens of infected mice on day 8 postinfection. Infections of euthymic mixed chimeras were performed 90–150 days post-transplant. Infections of thymectomized mixed chimeras were performed 6–8 wk post-transplant.

Bone marrow preparation and treatment protocols

Bone marrow recipients were treated with 500 μg each of hamster anti-murine CD40L Ab (MR1) and human CTLA4-Ig (provided by D. Hollenbaugh, Bristol Myers Squibb, Princeton, NJ) administered i.p. on the day of transplantation (day 0) and on postoperative days 2, 4, and 6. Mice treated with busulfan (Busulfex; Orphan Medical, Minnetonka, MN) received 600 μg on postoperative day 5. Bone marrow was flushed from tibiae, femurs, and humeri, and RBC were lysed using a Tris-buffered ammonium chloride solution. Where indicated, bone marrow was depleted of $CD3^+$ cells using biotin-labeled CD3 Ab (BD PharMingen, San Diego, CA) and strepavidin-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA), as previously described (7). In our hands, depletion of >95% of T cells in the bone marrow is routinely achieved using this method. Cells were resuspended in saline and injected i.v. at 2×10^7 cells/dose on post-transplant days 0 and 6.

Cell preparations and flow cytometry

Spleens were teased apart, and RBC were lysed with Tris-buffered ammonium chloride. Cell surface staining was performed with fluorochrome-conjugated Abs (anti-H-$2K^d$-PE, anti-CD4-allophycocyanin, anti-CD8- allophycocyanin, antiCD44-PE, anti-CD62L-PE; BD PharMingen). MHC class I tetramers were prepared and refolded with β_2-microglobulin and the appropriate peptide and used to stain splenocytes as described previously (16). Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (rat IgG2a-allophycocyanin, anti-CD4-allophycocyanin, fat IgG2b-PE, anti-CD8-PE, anti-H-$2K^d$-FITC, anti-H-$2K^b$-FITC, mouse IgG1-FITC; BD PharMingen), followed by RBC lysis and washing with a whole blood lysis kit (R&D Systems, Minneapolis, MN). Flow cytometry was performed on a FACSCalibur (BD Biosciences, Braintree, MA), and data were analyzed using CellQuest software (BD Biosciences).

Cell lines and in vitro infections

A20 cells are a B cell lymphoma line that expresses both MHC class I and II of the H-2^d haplotype, and IC21 cells are an i.p. macrophage line that expresses MHC class I and II of the H-2^b haplotype. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (complete RPMI). Cells were infected with wild-type VV at a multiplicity of infection of ~1.0. Infected cells were harvested along with uninfected controls ~10–12 h postinfection for use as stimulators in the IFN-γ assay.

Intracellular IFN-γ assays

Intracellular IFN-γ expression was induced in response to ex vivo restimulation with LCMV peptides as previously described (16). For VV-infected animals, 1×10^6 splenocytes were incubated with 5×10^5 cells from the uninfected or virus-infected cell lines in a 96-well, flat-bottomed plate. All stimulations were performed for 4–5 h at 37°C in the presence of 10 ng/ml human IL-2 (BD PharMingen) and brefeldin A (GolgiPlug; BD PharMingen). Cells were stained with anti-IFN-γ-FITC and anti-CD4-APC or anti-CD8-allophycocyanin using the Cytofix/Cytoperm kit (BD PharMingen). Use of VV-infected cell lines to stimulate ex vivo IFN-γ production has been previously described (17).

Results

Analysis of host- and donor-restricted primary CD8 responses in mixed allogeneic chimeras

Since positive selection in the thymus is thought to primarily take place on parenchyma-derived epithelial cells, a primary concern regarding the ability of mixed chimeras to combat infection is that they may be impaired in their ability to positively select donor-restricted T cells. While donor-specific tolerance is facilitated by negative selection in the thymus, an impairment in the positive selection of T cells capable of recognizing foreign Ag in the context of donor MHC might result in an inability to control pathogens harbored in donor-derived cells.

To assess the ability of mixed chimeras to generate recipient- and donor-restricted CD8 responses, we infected BALB/c→B6 chimeras (BALB/c donors, B6 recipients) at >90 days post-transplant with 2×10^5 PFU of LCMV Armstrong. Acute infection with this strain is resolved within 1 wk in wild-type mice, and we can observe H-2^b (B6)-restricted and H-2^d (BALB/c)-restricted responses by tracking the generation of IFN-γ-producing or tetramer binding T cells specific for D^b-restricted responses to nucleoprotein (NP)396–404 and gp33–41, and L^d-restricted responses to NP118–126. B6 mice generated a high number of IFN-γ-producing CD8 T cells specific for the immunodominant D^b-restricted epitope NP396–404 by day 8 postinfection, while BALB/c mice generated a potent response directed toward the L^d-restricted NP118–126 epitope. Potent responses to both epitopes were detected in B6 × BALB/c F_1 mice ($CB6F_1$), although they were largely dominated by the NP118–126 epitope. In contrast, antiviral responses in BALB/c→B6 chimeras were directed largely to the recipient-restricted NP396–404 epitope, while responses to the NP118–126 epitope in these mice were decreased (Fig. 1*A*). BALB/c→B6 chimeras generated ~10-fold fewer donor-restricted NP118–126-specific T cells at the peak of infection compared with BALB/c and $CB6F_1$ mice (Fig. 1*B*). Nevertheless, despite the decreased numbers of donor-restricted T cells in these mice, virus was readily cleared by day 8 postinfection in the spleen

[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; VV, vaccinia virus.

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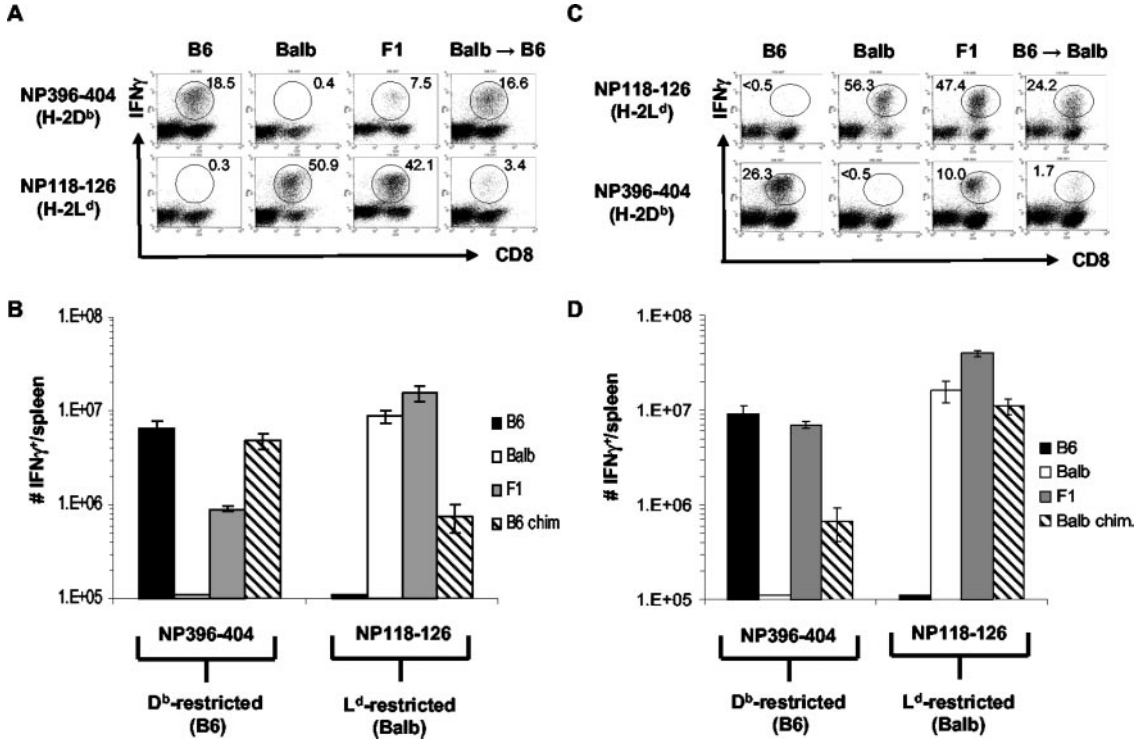


FIGURE 1. Host-restricted and donor-restricted CD8 responses in mixed chimeras following acute LCMV infection. B6, BALB/c, B6 × BALB/c F_1, BALB/c→B6 chimeric, or B6→BALB/c chimeric mice were infected with 2×10^5 PFU LCMV Armstrong. Eight days later splenocytes were restimulated for 5 h ex vivo in the presence of brefeldin A with an H-2D^b (B6)-restricted peptide, NP396–404, or an H-2L^d (BALB/c)-restricted peptide, NP118–126, and stained for intracellular IFN-γ expression. *A*, Representative flow plots display CD8$^+$ (*x*-axis), IFN-γ$^+$ (*y*-axis) splenocytes following restimulation with the indicated peptides in B6, BALB/c, F_1, and BALB/c→B6 chimeric mice. *B*, The total number of CD8$^+$IFN-γ$^+$ cells in the spleen is displayed on the *y*-axis following restimulation with the peptide indicated on the *x*-axis. Error bars represent the SEM ($n = 4$ for each group). Results are representative of three separate experiments. *C*, Representative flow plots display CD8$^+$ (*x*-axis), IFN-γ$^+$ (*y*-axis) splenocytes following restimulation with the indicated peptides in B6, BALB/c, F_1, and B6→BALB/c chimeric mice. *D*, The total number of CD8$^+$IFN-γ$^+$ cells in the spleen is displayed on the *y*-axis following restimulation with the peptide indicated on the *x*-axis. Error bars represent the SEM ($n = 4$ for each group). Results are representative of two separate experiments.

and serum (data not shown). The results shown are representative of four separate experiments. To verify that donor-restricted CD8 responses in mixed chimeras were not observed simply due to contaminating mature donor-derived T cells in the original bone marrow inoculum, gated CD8$^+$ or gated CD8$^+$IFN-γ$^+$ T cells were costained for expression of donor MHC with anti-H-2K^d Abs throughout our experiments. The levels of chimerism in the IFN-γ$^+$ population roughly correlated with the overall levels of chimerism in the CD8 population. In a representative experiment, 51.3 ± 3.6% of CD8$^+$ T cells were donor-derived, while 48.8 ± 3.3% of NP396–404 (recipient-restricted)-stimulated and 56.7 ± 4.0% of NP118–126 (donor-restricted)-stimulated IFN-γ$^+$CD8$^+$ T cells were donor-derived ($n = 4$). None of the B6 control animals stained positively for H-2K^d (data not shown). These results indicate that both host- and donor-derived cells are emerging that are capable of recognizing both host- and donor-restricted foreign epitopes, and that donor-restricted responses are not likely to arise solely from contaminating T cells in the bone marrow inoculum.

To ascertain whether the same observation applied to other strain combinations, we infected B6→BALB/c chimeras (B6 donors, BALB/c recipients) >90 days post-transplant with 2×10^5 PFU of LCMV Armstrong. On day 8 postinfection, splenocytes were harvested, restimulated with LCMV peptides in the presence of brefeldin A, and stained for intracellular IFN-γ production. As expected, infection of BALB/c mice induced a potent response to the L^d-restricted NP118–126 epitope, while B6 mice generated a strong response to the D^b-restricted NP396–404 epitope. As in the previous experiment, antiviral CD8 responses in CB6F_1 mice were largely dominated by the NP118–126 epitope; however, easily detectable responses were generated to the NP396–404 epitope. In contrast, B6→BALB/c chimeras, while generating potent host-restricted responses to the NP118–126 epitope, were reduced in their ability to generate responses to the donor-restricted epitope NP396–404 (Fig. 1*C*). When the total numbers of Ag-specific cells per spleen were calculated, we again observed an ~10-fold decrease in the number of donor-restricted T cells specific for the NP396–404 epitope compared with B6 and CB6F_1 mice, while the number of T cells specific for the host-restricted NP118–126 epitope was comparable to that in BALB/c mice (Fig. 1*D*). Once again, viral clearance was not impaired (data not shown). We concluded that while mixed allogeneic chimeras had a reduced ability to generate donor-restricted CD8 responses, they were nevertheless fully protected from infection with LCMV.

BALB/c→B6 or B6→BALB/c mixed allogeneic chimeras also generated host and donor-restricted CD8 responses to VV infection. Chimeras were infected with 5×10^6 PFU of VV, and their spleens were harvested 8 days later. Splenocytes were restimulated with VV-infected A20 cells (for H-2^d-restricted responses) or VV-infected IC21 cells (for H-2^b-restricted responses). Both strain combinations allowed for the generation of donor- and recipient-restricted CD8 responses (Fig. 2, *A* and *B*, representative of three separate experiments) and control of the infection (data not shown). These results indicate that even in the absence of an MHC-matched thymus, mixed allogeneic chimeras are capable of

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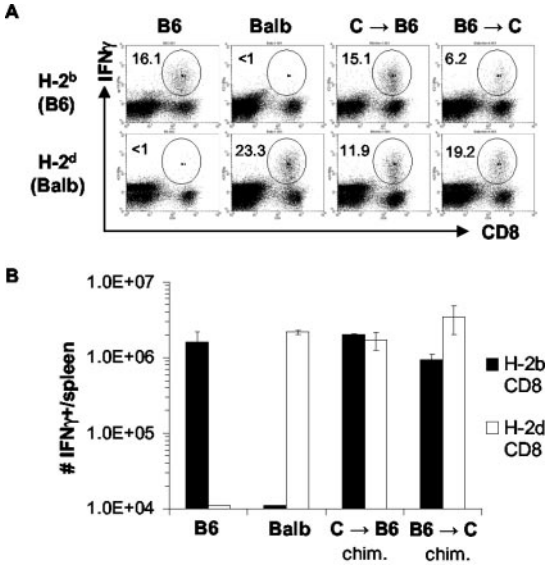


FIGURE 2. Host-restricted and donor-restricted CD8 responses in mixed chimeras following acute VV infection. B6, BALB/c, BALB/c→B6 chimeric, or B6→BALB/c chimeric mice were infected with 5×10^6 PFU wild-type VV. Eight days later splenocytes were restimulated ex vivo with infected or uninfected A20 (H-2^{d+}) or IC21 (H-2^{b+}) cells in brefeldin A cells and stained for IFN-γ expression. *A*, Representative flow plots display $CD8^+$ (*x*-axis), IFN-γ^+ (*y*-axis) splenocytes following restimulation with the indicated peptides in B6, BALB/c, BALB/c→β6, and B6→BALB/c chimeric mice. *B*, The total number of $CD8^+$IFN-γ^+ cells in the spleen are displayed on the *y*-axis following restimulation with infected cells. MHC restriction of the response is indicated in the legend. Error bars represent the SEM ($n = 4$ for chimeras, $n = 3$ for wild-type animals). Results are representative of two separate experiments.

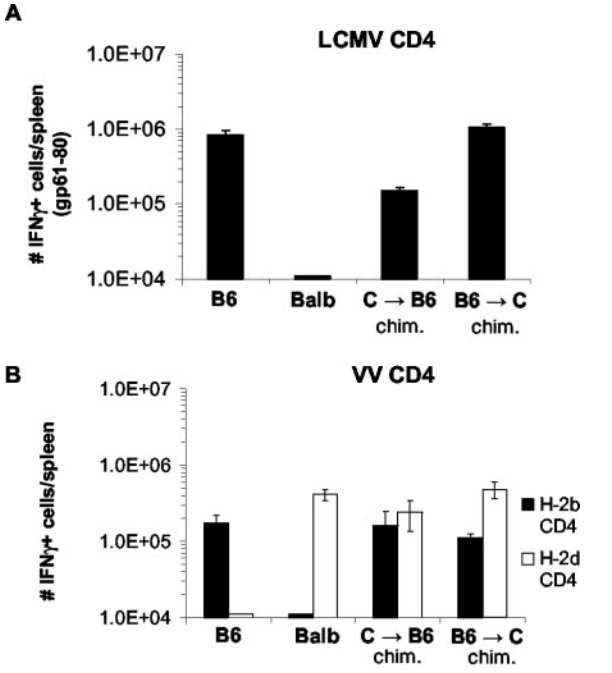


FIGURE 3. Host- and donor-restricted CD4 responses in mixed chimeras following acute viral infection. B6, BALB/c, BALB/c→B6 chimeric, or B6→BALB/c chimeric mice were infected with 2×10^5 PFU of LCMV Armstrong or 5×10^6 PFU of wild-type VV. Eight days later splenocytes were restimulated ex vivo with either the IA^b-restricted peptide gp61–80 (for LCMV infected mice; *A*) or infected or uninfected A20 (H-2^{d+}) or IC21 (H-2^{b+}) in brefeldin A cells (*B*) and stained for IFN-γ expression. The total number of $CD4^+$IFN-γ^+ cells in the spleen is displayed on the *y*-axis following restimulation with the peptide (for LCMV-infected mice) or infected cells (for VV-infected mice, MHC restriction of response indicated in legend). Error bars represent the SEM ($n = 4$ for each group). Differences between host-restricted responses to gp61–80 following LCMV infection in B6 mice vs BALB/c→B6 mixed chimeras are significant ($p < 0.01$), but no significant differences in the host-restricted CD4 responses made by B6 and BALB/c mice vs the mixed chimeras following VV infection were observed. Results are representative of two separate experiments.

generating T cell repertoires that can functionally respond to viral pathogens in the context of both recipient and donor MHC. While the ability of mixed allogeneic chimeras to generate host-restricted CD8 responses has been previously documented, donor-restricted CD8 responses were not detectable in chromium release cytolytic assays (8). In contrast, we found, using either MHC tetramers or intracellular cytokine staining, that mixed chimeras can also generate readily detectable donor-restricted CD8 T cell responses to at least two viruses in two strain combinations.

Analysis of host- and donor-restricted primary CD4 responses in mixed allogeneic chimeras

We further sought to analyze anti-viral CD4 responses in mixed chimeras. Mice were infected with LCMV or VV and assessed for their ability to respond to class II-restricted peptides. Following LCMV infection, B6 or $CB6F_1$ mice generated 5–10 $\times 10^5$ IFN-γ-producing CD4 cells directed to the I-A^b-restricted gp61–80 epitope. However, responses to this epitope in BALB/c→B6 chimeras were partially impaired, resulting in an ~5-fold decrease in the number of virus-specific CD4 cells compared with normal B6 mice or $CB6F_1$ mice (Fig. 3*A*). Conversely, when B6→BALB/c chimeras were assessed for their ability to generate donor-restricted antiviral CD4 effector cells to the I-A^b-restricted gp61–80 epitope, we found that they developed a strong response, comparable in number to that generated in wild-type B6 and $CB6F_1$ mice (Fig. 3*A*).

We next infected both BALB/c→B6 and B6→BALB/c mixed chimeras with 5×10^6 PFU of VV, then restimulated splenocytes 8 days later with VV-infected A20 or IC21 cells. Both strain combinations were able to generate readily detectable IFN-γ-producing CD4 cells restricted to either host or donor MHC (Fig. 3*B*). In this scenario, the host-restricted responses were unimpaired in mixed chimeras compared with B6 controls, indicating that the impairment of host-restricted responses to the gp61–80 epitope following LCMV infection is not a universal phenomenon. These results indicate that even in the absence of a MHC-matched thymus, mixed allogeneic chimeras are capable of selecting a CD4 T cell repertoire able to respond to foreign Ag in the context of both donor and recipient MHC.

Mixed chimeras have impaired ability to control chronic viral infection

Injection with LCMV Armstrong results in an acute infection that is CD8 dependent, but largely CD4 independent (18). Therefore, it is not surprising to note that mice generating potent host-restricted responses also successfully controlled virus. However, we considered the possibility that mixed chimeras may have difficulty in dealing with more robust chronic infections. To test this hypothesis, BALB/c→B6 mixed chimeras were infected i.v. with 2×10^6 PFU of LCMV clone 13. This variant induces a long term chronic infection that is slowly cleared from the serum over the course of 2–3 mo (15). Eventual control of the infection is reliant on the presence of CD4 T cells (19). Mice deficient in either CD4 or CD8

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responses fail to control the infection and develop a life-long carrier state. Following infection, mice were bled at various time points and tested for presence of viral titers in the serum. By day 60 postinfection, five of five B6 control mice had either controlled the virus or had very low serum viral titers. In contrast, five of five mixed chimeras failed to clear virus from the serum (Fig. 4). These results are representative of two separate experiments. Mixed chimeras failed to clear the infection throughout the course of the experiment (>90 days), while all untreated B6 animals eventually cleared the virus from the serum (data not shown). Our data indicate that mixed chimerism in fully MHC-mismatched recipients may result in a reduced capacity for generating protective immune responses to chronic infection.

Donor bone marrow-derived cells mediate selection of donor-restricted T cells in mixed allogeneic chimeras

We considered at least two possibilities to explain the ability of mixed chimeras to generate donor-restricted responses. First, donor-restricted responses in mixed allogeneic chimeras could be due to positive selection mediated by bone marrow-derived cells. Alternatively, we considered the possibility that due to degeneracy in TCR-peptide-MHC interactions, some donor-restricted T cells could be selected by the recipient haplotype. To explore this question we performed thymectomies in 4- to 6-wk-old B6 and BALB/c mice, induced allogeneic mixed chimerism with T cell-depleted bone marrow and CD28/CD40 blockade, and measured their ability to mount donor-restricted and host-restricted antiviral responses following LCMV infection. Presumably, the T cell repertoire in the thymectomized animals at the time of infection would be entirely selected by host MHC. Therefore, the existence of donor-restricted responses in this setting would indicate that some donor-restricted T cells can be selected by thymic epithelial cells bearing host MHC. Conversely, if thymectomized mixed chimeras had deficient donor-restricted responses compared with euthymic mixed chimeras, this would implicate donor bone marrow-derived cells in the positive selection of donor-restricted T cell repertoires.

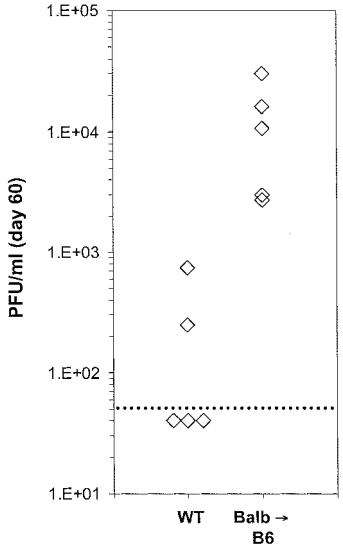


FIGURE 4. Mixed chimeras fail to control a chronic LCMV clone 13 infection. Wild-type B6 or BALB/c→B6 mixed chimeras were infected with 2×10^6 PFU of LCMV clone 13 i.v. Mice were bled 60 days postinfection, and the serum was tested for the presence of LCMV titers on Vero cell monolayers. The *y*-axis displays PFU per milliliter for the indicated groups on the *x*-axis. Results are representative of two separate experiments.

Eight days following infection with LCMV Armstrong, splenocytes were restimulated ex vivo with the H-2D^b-restricted peptide NP396–404 or the H-2L^d-restricted peptide NP118–126 in the presence of brefeldin A and stained for CD8 and intracellular IFN-γ expression to gauge the magnitude of the antiviral CD8 response. Potent host-restricted CD8 responses to the H-2^b-restricted epitope NP396–404 were seen in wild-type B6 mice, euthymic BALB/c→B6 chimeras, and thymectomized BALB/c→B6 chimeras. In the opposite strain combination, potent host-restricted CD8 responses to the H-2^d-restricted epitope NP118–126 were seen in wild-type BALB/c, euthymic B6→BALB/c chimeras and thymectomized B6→BALB/c chimeras (Fig. 5*A*). As expected, euthymic mixed chimeras in both strain combinations also generated lower, but readily detectable, donor-restricted antiviral CD8 responses. In contrast, thymectomized mixed chimeras generated markedly reduced numbers of CD8 T cells specific for donor-restricted epitopes that were not significantly different from those seen in nonchimeric wild-type negative controls (Fig. 5*A*). As seen earlier, costaining with donor MHC confirmed that both host and donor-derived CD8 T cells responded to both host and donor-restricted epitopes in the euthymic mixed chimeras, demonstrating that these responses are probably due to newly emerging T cells, not to contaminating cells in the bone marrow inoculum (data not shown). The lack of donor-restricted responses in thymectomized animals further confirms that donor-derived T cell contamination of bone marrow inoculum is not likely to play a significant role in this system.

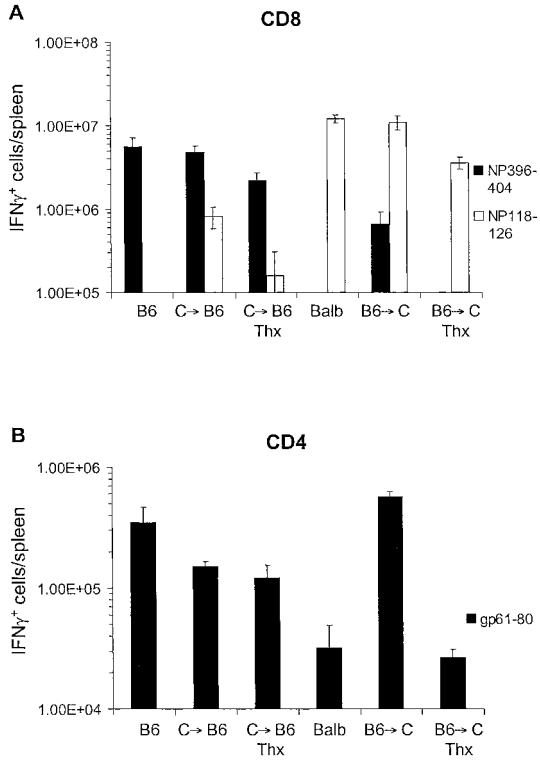


FIGURE 5. Donor-restricted T cells in mixed chimeras are positively selected in the thymus by bone marrow-derived cells. Wild-type B6 and BALB/c, euthymic B6→BALB/c and BALB/c→B6 mixed chimeras, and thymectomized B6→BALB/c or BALB/c→B6 mixed chimeras were infected with 2×10^5 PFU of LCMV Armstrong. Splenocytes were restimulated with the indicated LCMV peptide 8 days postinfection and stained with IFN-γ Ab. *A*, The number of CD8$^+$IFN-γ$^+$ cells in the spleen is indicated on the *y*-axis for the peptides in the legend. *B*, The number of CD4$^+$IFN-γ$^+$ cells in the spleen is indicated on the *y*-axis for the IA^b-restricted gp61–80 peptide. Error bars represent the SE ($n = 4$ for all groups). Results are representative of two separate experiments.

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To assess host- and donor-restricted CD4 responses, splenocytes were stimulated with the I-A^b-restricted epitope gp61–80 and stained for intracellular IFN-γ expression. As shown previously in this report, wild-type B6 animals generated readily detectable CD4 responses to this epitope. Host-restricted CD4 responses to this epitope in euthymic and thymectomized BALB/c→B6 mixed chimeras were both reduced, but detectable. Wild-type BALB/c mice, as expected, failed to generate a CD4 response to this epitope, while euthymic B6→BALB/c mixed chimeras generated a donor-restricted CD4 response similar in magnitude to that in wild-type B6 animals. However, thymectomized B6→BALB/c chimeras failed to generate detectable donor-restricted CD4 responses to the gp61–80 epitope (Fig. 5*B*).

We concluded from these results that thymectomized mixed chimeras were unable to generate significant donor-restricted CD4 and CD8 responses and that donor bone marrow-derived cells probably played an important role in the positive selection of donor-restricted T cell repertoires in mixed allogeneic chimeras. We propose that these donor-restricted populations, albeit small in number, could have a crucial role in clearing infection from donor-derived cells and preserving immunocompetence in MHC-mismatched chimeras.

CD8 T cell memory populations are stable following the development of hemopoietic macrochimerism

One unresolved issue in the application of strategies involving the generation of high levels of chimerism is the fate of pre-existing memory lymphocyte populations in the periphery. Therefore, we sought to evaluate the impact of a mixed chimerism-based tolerance induction regimen on the numbers and function of LCMV-specific memory cells in tolerant mice over a long time course. B6 mice with established memory to LCMV were rendered tolerant to BALB/c donors by treatment with busulfan, CTLA4-Ig, and anti-CD40 ligand and infusion of donor bone marrow. Over the course of several experiments, 37 of 45 mice developed high levels of hemopoietic chimerism, with overall levels exceeding 50% of peripheral blood leukocytes in nearly all cases. Chimerism was detected in all lineages tested, including $CD4^+$, $CD8^+$, $GR1^+$, $CD11b^+$, and $B220^+$ cells in the peripheral blood and spleen (data not shown). It has previously been reported that infection with LCMV at the time of transplantation abrogates tolerance induction (20, 21). In contrast, we find here that prior acute LCMV infection and established T and B cell memory do not preclude the induction of chimerism and tolerance.

Next we tracked the fate and function of virus-specific CD8 T cell memory populations following the induction of high level hemopoietic chimerism using MHC tetramers and intracellular IFN-γ staining. We compared memory populations in three groups of mice: LCMV-immune B6 mice; immune mice receiving allogeneic (BALB/c) bone marrow, costimulation blockade, and busulfan; and immune mice receiving syngeneic (B6) bone marrow, costimulation blockade, and busulfan as treatment controls. Mice received the standard tolerance induction protocol 30 days postinfection, and spleens were harvested at 60, 90, and 150 days postinfection. Splenocytes were assessed for their ability to make IFN-γ in response to ex vivo peptide restimulation (Fig. 6) or for their ability to bind MHC tetramers (data not shown). Results are shown for four LCMV epitopes: NP396–404, gp33–41, gp276–286, and NP205–214. As expected, CD8 T cell responses rose rapidly following infection and peaked on day 8. The number of LCMV-specific CD8 T cells then declined, forming a stable population by day 30. Following induction of chimerism, the number of LCMV-specific memory CD8 cells in the spleen remained similar among the three treatment groups, demonstrating that the maintenance of CD8 memory populations is relatively unaffected by the influx of donor leukocytes (Fig. 6). The results for the NP396–404 and gp33–41 epitopes were further confirmed by MHC tetramer staining (data not shown). We observed modest, but reproducible, declines in memory populations specific to the subdominant epitope NP205–214 in chimeric mice. The factors contributing to the loss of a portion of this memory population are unclear. Nevertheless, the overall levels of CD8 memory cells are similar among the three groups.

LCMV-immune chimeric mice maintain protective immunity to secondary challenge

To confirm that these mice maintained functional immunity, we rechallenged them with 2×10^6 PFU of LCMV clone 13 at 150 days postinfection, a viral strain and dose that normally result in a long term chronic infection. Five days following rechallenge we

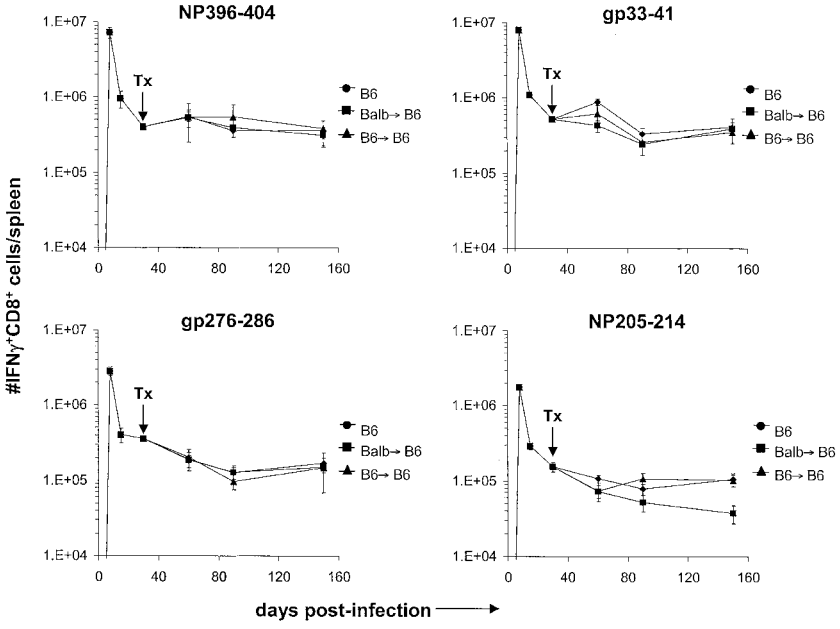


FIGURE 6. CD8 memory T cells are unaffected by the establishment of high levels of hemopoietic macrochimerism. B6 mice were infected with 2×10^5 PFU of LCMV Armstrong and 30 days later received either no treatment (immune) or busulfan, CTLA4-Ig, anti-CD40L, and syngeneic bone marrow (B6→B6) or busulfan, CTLA4-Ig, anti-CD40L, and BALB/c bone marrow (BALB/c→B6). Splenocytes were harvested 30, 60, and 120 days later (60, 90, and 120 days postinfection) and restimulated with the indicated LCMV peptides. The *y*-axis represents the number of IFN-γ^+CD8^+ cells in the spleen specific for each epitope. Error bars represent the SEM (n = 3 for each group at each time point). The data are representative of two separate experiments.

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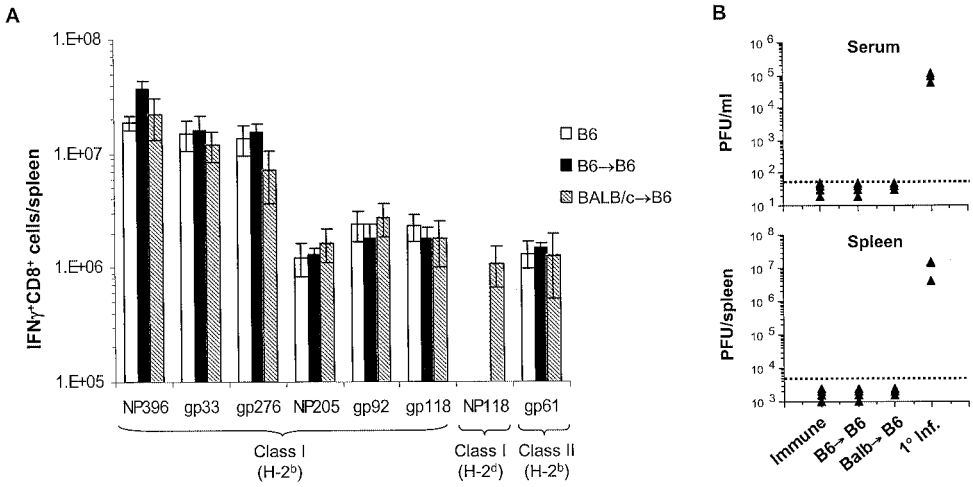


FIGURE 7. LCMV-immune mixed chimeras mount protective recall responses upon viral challenge. Treatment groups are the same as in Fig. 2. Mice were challenged 150 days postinfection (120 days post-transplant) with 2×10^6 PFU of LCMV clone 13. *A*, Five days following challenge, splenocytes were harvested and restimulated with the indicated peptide. The *y*-axis represents the number of IFN-γ^+CD8$^+$ cells in the spleen. Recipients are H-2^{b+}, and donors are H-2^{d+}. Error bars represent the SEM ($n = 4$ for immune and syngeneic; $n = 3$ for allogeneic). *B*, Viral titers were assessed in the spleen and serum by their ability to lyse Vero cell monolayers. Day 5 titers following a primary infection with LCMV clone 13 are provided for the sake of comparison. Dashed lines represent the limit of detection.

harvested spleens and serum to assess secondary antiviral responses and viral clearance. We quantitated LCMV epitope-specific responses by measuring the frequency of virus-specific IFN-γ-secreting cells following ex vivo peptide restimulation (Fig. 7*A*) or by using MHC tetramers (data not shown). Secondary responses to all six of the H-2^b class I-restricted epitopes tested (NP396–404, gp33–41, gp276–286, NP205–214, gp92–99, gp118–128) were profound and comparable in size among the three treatment groups, usually generating at least a 10- to 20-fold increase over the number of pre-existing Ag-specific memory cells per spleen. Similarly, the H-2^b class II-restricted peptide gp61–80 also generated a significant secondary response in all three groups (Fig. 7*A*). These data confirm that the induction of mixed chimerism does not impair the ability of either CD4 or CD8 memory T cells to respond to viral rechallenge.

Interestingly, we also detected primary CD8 T cell responses to a donor (H-2^d)-restricted class I epitope (NP118–126; Fig. 7*A*). This response consisted of $\sim 10^6$ Ag-specific cells by day 5 post-challenge and was only detected in allogeneic mixed chimeras. In this setting a primary H-2^d-restricted response may represent a crucial line of defense for controlling infection of H-2^{d+} leukocytes.

The most important test of functional memory is viral clearance. Viral titers were measured in the spleen and serum 5 days after rechallenge. As expected based on the potent T cell responses elicited, virus was undetectable in either the spleen or serum of all mice from the three treatment groups, whereas virus could be detected at high levels in both the spleen and serum of naive mice receiving a primary challenge with LCMV clone 13 (Fig. 7*B*). Taken together, these studies demonstrate that the induction of allospecific tolerance and hemopoietic macrochimerism do not impact functional immunity to secondary rechallenge.

Discussion

A major concern in the development of strategies to induce mixed allogeneic chimerism and transplantation tolerance is their potential to significantly impair protective primary and secondary immunity. Here we analyze in detail both the maintenance of pre-existing memory populations following the development of chimerism as well as the ability of mixed allogeneic chimeras to mount effective antiviral responses to three distinct pathogens. Our finding that host-restricted CD8 responses to LCMV and VV in mixed chimeras are largely unimpaired is in accordance with previous studies (8). However, using sensitive modern tools we report here that donor-restricted antiviral responses are detectable by either MHC tetramer staining or intracellular IFN-γ expression following peptide restimulation in two separate strain combinations. Furthermore, both host-restricted and donor-restricted CD4 responses are readily detectable by intracellular IFN-γ production. Interestingly, high level mixed chimeras infected with LCMV Armstrong generated significantly decreased host-restricted CD4 responses, although this was not observed following VV infection. Therefore, we conclude that host-restricted CD4 responses are in general likely to be unimpaired in mixed chimeras.

Whereas control of LCMV Armstrong is CD4 independent, control of a chronically infecting strain, LCMV clone 13, is CD4 dependent (19, 22). Following clone 13 infection, virus is typically cleared from the serum over the course of 2–3 mo in wild-type animals, but can be detected in peripheral organs such as the kidney throughout the life of the animal (15). Mixed chimeras exhibited a deficit in their ability to bring the infection under control, with serum titers remaining high after 60 days. It is unclear whether this deficiency is due to problems with the donor-restricted or host-restricted response, and whether CD4 or CD8 responses are principally to blame. Additional experiments using haplotype tissue matching (F_1 donor) or MHC class I and/or class II tissue matching will shed light on potential areas of immunodeficiency in MHC-mismatched mixed chimeras.

T cell repertoire restriction is largely determined by the haplotype of the selecting thymus (10–12). However, in recent years, some studies have demonstrated a potential role for bone marrow-derived cells in the positive selection of classically restricted T cell repertoires in some scenarios (14, 23). Selection of T cells restricted to nonclassical MHC molecules has also recently been shown to be mediated by bone marrow-derived cells (24). Our experiments demonstrate that bone marrow-derived cells can mediate positive selection in the thymus of donor-restricted T cells in

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the setting of mixed allogeneic chimerism. The selection of donor-restricted T cells may be critical for the long term immunogenic health of mixed hemopoietic chimeras. Interestingly, however, bone marrow cell-mediated positive selection was far less efficient than that mediated by thymic epithelial cells. A better understanding of the role of this phenomenon in preserving immunocompetence in mixed chimeras will aid in selecting appropriate tissue matches as mixed chimerism strategies are applied clinically.

CD8 memory populations are clearly maintained at normal levels in chimeric mice, despite the development of high levels of donor chimerism. While more limited, our data also suggest that CD4 memory populations are preserved. These observations are consistent with previous studies that have shown memory T cell homeostasis to be relatively independent of naive T cell maintenance (25, 26). Furthermore, other reports have shown that CD4 and CD8 memory T cells, unlike naive T cells, do not require engagement of MHC for survival in the periphery (27, 28). Significantly, the development of chimerism does not impair the ability of LCMV-immune mice to clear LCMV clone 13 upon rechallenge. While primary infection with this viral strain results in long term persistent infection, all the immune mice were protected from rechallenge regardless of the development of high level hemopoietic chimerism. One concern with the development of chimerism might be that a recipient-restricted recall response would prove ineffective at clearing virus harbored in donor-derived cells. However, following rechallenge of immune chimeric mice, a substantial donor-restricted primary T cell response developed to the L^d/NP118–126 epitope by day 5, and virus was cleared from the host. These results are in accordance with a recent study showing that primary T cell responses can occur simultaneously with a potent recall response (29).

While hemopoietic chimerism has the potential to treat or cure life-threatening conditions (30) and to facilitate tolerance induction to transplants, it also has the potential to adversely affect the ability of a recipient to generate effective immune responses, particularly against intracellular pathogens. It is critical that we understand the degree to which chimerism-based tolerance induction regimens create immunologic blind spots and impair the ability of a recipient to effectively control intracellular pathogens, particularly those that cause persistent or latent infections.

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