Characterization of Virus-Mediated Inhibition of Mixed Chimerism and Allospecific Tolerance

Matthew A. Williams, Joyce T. Tan, Andrew B. Adams, Megan M. Durham, Nozomu Shirasugi, Jason K. Whitmire, Laurie E. Harrington, Rafi Ahmed, Thomas C. Pearson and Christian P. Larsen

J Immunol 2001; 167:4987-4995; doi: 10.4049/jimmunol.167.9.4987
http://www.jimmunol.org/content/167/9/4987

References

This article cites 41 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/167/9/4987.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Characterization of Virus-Mediated Inhibition of Mixed Chimerism and Allospecific Tolerance

Matthew A. Williams, Joyce T. Tan, Andrew B. Adams, Megan M. Durham, Nozomu Shirasugi, Jason K. Whitmire, Laurie E. Harrington, Rafi Ahmed, Thomas C. Pearson, and Christian P. Larsen

Simultaneous blockade of the CD28 and CD40 T cell costimulatory pathways has been shown to effectively promote skin allograft survival in mice. Furthermore, blockade of one or both of these pathways has played a central role in the development of strategies to induce mixed hematopoietic chimerism and allospecific tolerance. It has recently been observed that the beneficial effects of CD40 blockade and donor splenocytes in prolonging skin graft survival can be abrogated by some viral infections, including lymphocytic choriomeningitis virus (LCMV). In this study, we show that LCMV infection prevents prolonged allograft survival following CD28/CD40 combined blockade. We further show that LCMV prevents the induction of allospecific tolerance and mixed hematopoietic chimerism, while delay of infection for 3–4 wk posttransplant has no effect on tolerance induction. Because of reports of anti-I-2β activity following LCMV infection, we assayed the ability of LCMV-specific T cells to respond to alloantigen at a single cell level. Although we confirm that LCMV infection induces the generation of alloreactive cells, we also demonstrate that LCMV-specific T cells do not divide in response to alloantigen. The allosresponse suppressed by costimulation blockade is restored by LCMV infection and correlates with increased dendritic cell maturation. We hypothesize that the costimulation blockade-resistant rejection mediated by LCMV could be partly attributable to the up-regulation of alternative costimulatory pathways subsequent to LCMV-induced dendritic cell maturation. *The Journal of Immunology, 2001, 167: 4987–4995.*

In recent years, it has become clear that at least two types of signals are required for optimal T cell activation. Signal one, delivered through the TCR, provides specificity to the immune response. The second signal is mediated by numerous costimulatory molecules, most notably the CD28 and CD40 pathways (1–4), and allows for optimal generation of immune responses. Manipulation of costimulatory pathways through Ab or fusion protein blockade has been shown to be a promising therapy for the amelioration of experimental autoimmune diseases in mice (5–8) and the delay of allogeneic transplant rejection in both mice (9–12) and nonhuman primates (13, 14). More recently, costimulatory blockade has played a crucial role in strategies to generate mixed hematopoietic chimeras (15, 16), promote peripheral deletion of reactive T cells (15, 17), and establish a permanent state of central tolerance to donor Ag. Indefinite donor graft survival and donor-specific hyporesponsiveness induced by radiation (15) or administration of hematopoietic stem cell selective toxins like busulfan (18), as well as in strategies involving administration of high levels of donor bone marrow without recipient conditioning (16, 19).

As these strategies move closer to the clinic, one cause for concern is the effect that tolerance induction protocols might have on protective immunity to foreign pathogens. Disruption of the CD40/CD40L pathway has been shown to inhibit effective Ab responses in several settings (10, 20–23). Furthermore, blockade of the CD40 and/or CD28 pathways as a method to achieve allogeneic bone marrow engraftment leads to the selective deletion of donor-reactive T cells (15, 16). Administration of reagents blocking these costimulatory interactions could therefore potentially impair both antiviral humoral and cellular responses. Alternatively, the presence of acute viral infections and their associated immune responses might interfere with the induction of allogeneic tolerance due to up-regulation of alternative costimulatory pathways, enhancement of inflammatory cytokines and stimuli, increased availability of T cell growth factors, or activation of viral Ag-specific T cells that also have specificity for donor MHC. In support of the latter possibility, several mouse pathogens stimulate allogeneic effector T cells following a primary infection (24–26).

Welsh et al. (6) recently showed that acute infection with lymphocytic choriomeningitis virus (LCMV) inhibits long-term skin graft survival and donor-specific hypersensitivity induced by administration of donor splenocytes and anti-CD40L (27). Interestingly, this effect was observed following LCMV and pichinde virus (PV) infections, but not following infection with other viruses (e.g., vaccinia virus (VV) and mouse CMV (MCMV)). Previous reports established that primary infection of C57BL/6 (B6)

---

*Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; PV, pichinde virus; VV, vaccinia virus; MCMV, mouse CMV; GVD, graft-vs-host disease; MMTV, mouse mammary tumor virus; MST, median survival time; NP, nucleoprotein.*
mice with LCMV induces a substantial number of H-2d-reactive T cells at the peak of the antiviral response (28). The ability of virus-infected mice to generate an alloresponse was subsequently mapped to the MHC locus, but independent of class II (24). Further studies have characterized T cell clones having dual specificity for H-2d alloantigens and syngeneic infected targets, as well as clones specific for alloantigen but not syngeneic infected targets (24). One possible explanation for the ability of LCMV to induce early graft rejection is the generation of activated T cells specific for both viral Ags and donor MHC. Conversely, at least two mouse pathogens that have been shown to induce allogeneic responses during primary infection, VV and MCMV, do not induce early graft rejection, suggesting that cross-reactivity may be either not required or not sufficient to prevent allograft survival. The role of TCR cross-reactivity in the virus-induced rejection of allogeneic tissue therefore remains unclear. In this study we extend previous results to show that LCMV infection disrupts the beneficial effects of combined blockade of the CD40 and CD28 costimulatory pathways. Furthermore, we extend these findings to a mixed chimerism tolerance induction model. We have recently reported that administration of donor bone marrow, costimulation blockade, and the hematopoietic stem cell selective toxin busulfan around the time of transplant leads to indefinite skin graft survival, deletion of donor-reactive T cells, and the induction of high levels (>50%) of mixed hematopoietic chimerism (18). We report in this work that acute infection with LCMV causes prompt graft rejection, failure to generate mixed chimerism, and an inability to delete donor-reactive CD4 T cells. Rejection can be mediated by either CD4+ or CD8+ T cells, and a delay of infection following transplantation has no effect on tolerance induction. We present a detailed quantitative assessment of allogeneic and antiviral responses using a graft-vs-host disease (GVHD) model and MHC tetramer analysis, as well as ELISPOT and intracellular IFN-γ staining. Although LCMV infection allows for the generation of costimulation blockade-resistant alloresponses, cross-reactivity of LCMV-specific CD8 T cells to H-2d alloantigen is minimal at a single cell level. Although mice receiving LCMV infection without a skin graft do generate high numbers of alloreactive cells at the peak of the response, their presence is largely associated with acute infection, as they are reduced 50- to 100-fold by day 30 postinfection, while normal LCMV-specific T cells are reduced 10- to 12-fold over the same time period. To explore alternative reasons for the virus-induced abrogation of tolerance induction, we assess dendritic cell activation in the spleen following transplantation and concurrent viral infection. We show that LCMV drives dendritic cell maturation, regardless of the presence of costimulation blockade. We propose that one possible explanation for the LCMV-induced rejection of skin allografts is that infection leads to dendritic cell maturation and an increased capacity for stimulating CD28/CD40-independent T cell responses.

Materials and Methods

Mice and virus infections

Adult male 6- to 8-wk old BALB/c, B6, and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were infected with 2 × 10⁵ PFU LCMV Armstrong injected i.p. Virus stocks were grown and quantitated as previously described (29).

Skin grafting

Full thickness skin grafts (~1 cm²) were transplanted on the dorsal thorax of recipient mice and secured with a Band-Aid (Johnson & Johnson, Arlington, TX) for 7 days. Graft survival was then followed by daily visual inspection. Rejection was defined as the complete loss of viable epidermal graft tissue. Statistical analyses were performed using a Mann-Whitney U test.

Bone marrow preparation and treatment protocols

Skin graft 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. CD4- and CD8-depleted experimental groups received 100 μg of rat anti-mouse CD4 (GK1.5) or rat anti-mouse CD8 (TIB105) i.p. on days −3, −2, −1, and weekly until harvest. Mice treated with busulfan (Busulfex; Ortho Medical, Minnetonka, MN) received 600 μg on postoperative day 5. Bone marrow was flushed from tibiae, femurs, and humeri, and red blood cells were lysed using a Tris-buffered ammonium chloride solution. Cells were resuspended in saline and injected i.v. at 2 × 10⁶ cells/dose on postoperative days 0 and 6.

CFSE labeling and adoptive transfers

Labeling of naive or immune B6 T cells and adoptive transfer into irradiated BALB/c recipients were performed as previously described (30). Harvested splenocytes were analyzed by flow cytometry.

Intracellular IFN-γ assay

Intracellular IFN-γ expression in response to restimulation with LCMV peptides was analyzed essentially as described (31). In the case of irradiated recipients of CFSE-labeled cells, harvested splenocytes were incubated for 5 h with LCMV-infected or uninfected MC57 fibroblasts in the presence of brefeldin A (GolgiPlug; BD PharMingen, San Diego, CA). In the GVHD assay, peptide-specific IFN-γ production was assessed by restimulating with uninfected IC21 macrophage cells pulsed with the appropriate LCMV peptide at 0.1 μg/ml. After surface staining, cells were permeabilized and stained for IFN-γ expression using the Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer’s instructions.

IFN-γ ELISPOT assays

Allospecific T cell responses were measured by IFN-γ ELISPOT assay. Three-fold dilutions of recipient splenocytes (H-2b or H-2d) were stimulated overnight with 5 × 10⁵ irradiated donor splenocytes (H-2d) per well in ester-cellulose-bottom plates (Millipore, Bedford, MA) that had been previously coated with IFN-γ capture Ab. To measure LCMV-specific responses, splenocytes were restimulated overnight with infected L929 (H-2b) or MC57 (H-2d) cells. Plates were coated and developed as previously described (30).

Cell preparations and flow cytometry

MHC class I tetramers were prepared and refolded with β2-microglobulin and the appropriate peptide as described previously (31). Analyses of splenocytes of irradiated recipients of CFSE-labeled T cells were conducted using fluorochrome-conjugated Abs (rat IgG2a PE, rat IgG2b PE, anti-CD4 PE, anti-CD8 PE; BD PharMingen) and APC-labeled tetramers. For intracellular staining, cells were labeled with anti-CD8 FITC, anti-I-A b FITC, anti-CD40 FITC, anti-CD54 FITC, rat IgG1 FITC, anti-CD11c FITC, anti-CD11b FITC, and APC (BD PharMingen). Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (rat IgG2a APC, anti-CD4 APC, mouse IgG2a FITC, anti-H-2K b FITC, mouse IgG1 FITC, anti-Vβ8 FITC, rat IgG2b FITC, anti-Vβ11 FITC; BD PharMingen), followed by red blood cell lysis and washing with a whole-blood lysis kit (R&D Systems, Minneapolis, MN). Splenic dendritic cells were enriched on an Optiprep column (Nycomed, Oslo, Norway) as previously described (32) and analyzed using fluorochrome-conjugated Abs (hamster IgG PE, anti-CD11c PE, ham IgM FITC, anti-CD40 FITC, anti-CD54 FITC, rat IgG2a FITC, anti-CD80 FITC, anti-CD86 FITC, mouse IgG4a FITC, anti-H-2K b FITC, anti-I-A b FITC; BD PharMingen). Flow cytometry was performed using a FACSCaliber, with CFSE fluorescence data being collected on the FL1 (FITC) channel. Data were analyzed using CellQuest software (BD Biosciences, Braintree, MA).

Cell lines

The fibrosarcoma cell line MC57 (H-2b) and the liver-derived cell line L929 (H-2b) were grown and passaged in RPMI 1640 supplemented with 10% FBS, antibiotics, and 2-ME.
**Results**

Acute LCMV infection disrupts prolongation of allograft survival induced by blockade of the CD28/CD40 T cell costimulatory pathways

Recent evidence has indicated that some viral infections (e.g., LCMV and PV) inhibit the prolongation of skin allograft survival mediated by blockade of the CD40 pathway and administration of donor splenocytes (27). We sought to assess whether acute LCMV infection could alter skin graft survival time when the CD28 and CD40 T cell costimulatory pathways were blocked simultaneously. We previously reported that C3H/HeJ mice receiving a BALB/c skin allograft enjoy substantial prolongation of graft survival when treated with CTLA4-Ig and anti-CD40L for a short time course at the time of engraftment, with median survival times (MSTs) often exceeding 100 days (9). In this experiment, C3H/HeJ mice receiving BALB/c skin allografts and costimulation blockade survived >80 days. In contrast, mice receiving the same procedure and treatment, along with a concomitant infection of 2 × 10^5 LCMV Armstrong on or near the day of transplant, rejected their grafts promptly (MST = 20 days; Fig. 1).

To determine the relative contributions of each T cell subset to the costimulation blockade-resistant rejection of skin allografts following acute LCMV infection, we depleted CD4^+^ and CD8^-^ T cells in vivo with GK1.5 and TIB105 Abs, respectively. As seen in Fig. 1, depletion of CD4^+^ T cells did not alter the ability of C3H/HeJ mice to reject BALB/c skin grafts following infection with LCMV (MST = 18 days). Depletion of CD8^-^ T cells resulted in a slight delay of skin graft rejection (MST = 26 days). Depletion of both subsets simultaneously resulted in long-term allograft survival (MST > 60 days), indicating both that the depletions were effective and that the virus was not directly harmful to the allograft.

These results parallel and extend the observations previously made, indicating that LCMV induces accelerated graft rejection in the face of combined blockade of the CD28 and CD40 T cell costimulatory pathways. This suggests that the mechanism of graft failure in the previous report is not likely due to the CD40-independent up-regulation of B7 molecules. Furthermore, they suggest that either CD4^+^ or CD8^-^ T cells are sufficient to mediate LCMV-induced skin graft rejection in this setting.

Acute LCMV infection prevents the establishment of partial hematopoietic chimerism, deletion of alloreactive T cells, and the induction of donor-specific tolerance

We next sought to determine whether LCMV infection had the same effect in a more robust tolerance induction model. Specifically, we sought to determine whether acute LCMV infection could disrupt the costimulation blockade-mediated establishment of mixed hematopoietic chimerism and donor-specific tolerance. Recent work in our lab has demonstrated that administration of donor bone marrow following treatment with the selective stem cell toxin busulfan, together with blockade of the CD40/CD28 costimulatory pathways, result in high levels of chimerism, deletion of donor-reactive T cells, and indefinite donor-specific tolerance (18).

As seen in Fig. 2A, 5/5 B6 mice receiving BALB/c skin and bone marrow, as well as busulfan and costimulatory blockade treatment, had >200-day skin graft survival in 100% of mice tested. Conversely, 5/5 mice receiving the same treatment concurrently with an acute LCMV infection rejected their grafts promptly (MST = 14 days). These results are representative of three separate experiments. As in the previous model, predepletion of CD8^-^ T cells demonstrated that CD4^+^ T cells could mediate graft rejection, although in a somewhat delayed fashion. Following depletion, no CD8^-^ T cells could be detected in the peripheral blood, while simultaneous depletion of both subsets during infection resulted in indefinite graft survival, indicating that the depletions were effective (data not shown).

Following the aforementioned procedure, uninfected mice proceeded to develop substantial levels of hematopoietic chimerism (Fig. 2B). By day 125, >60% of peripheral blood leukocytes were H-2K^d^+^ in all the mice (n = 5). Chimerism was seen in all lineages tested, including CD4^+^, CD8^-, B220^+, CD11b^-, and GR-1^-^ cells (data not shown). Conversely, mice receiving the same treatment along with LCMV at the time of engraftment never developed detectable long-term chimerism. Predepletion of CD8^-^ T cells (Fig. 2B) did not alter the ability of the infection to abort chimerism.

Donor-specific tolerance following bone marrow engraftment and treatment with costimulation blockade is due at least in part to deletion of alloreactive T cells (15, 16). To determine whether LCMV-induced skin graft rejection was associated with impaired peripheral deletion of donor-reactive T cells, we compared the use of Vβ11 and Vβ5.1/2 by CD4^-^ T cells from B6 recipients in the uninfected group (accepted both bone marrow and skin grafts) and from the infected groups (rejected bone marrow and skin grafts). BALB/c mice delete Vβ11 and Vβ5-bearing T cells in the thymus due to their high affinity for endogenous retroviral superantigens (mouse mammary tumor virus (MMTV)) presented by I-E MHC class II molecules. B6 mice do not express I-E and thus use Vβ11 on ~5–7% of CD4^-^ T cells and Vβ5.1/2 on ~3–5% of CD4^-^ T cells. In this experiment, uninfected mice treated with costimulation blockade, bone marrow, and busulfan following skin engraftment showed decreased percentages of Vβ11^-^CD4^-^ and Vβ5^-^CD4^-^ T cells in the peripheral blood by day 28 posttransplant. At day 60 posttransplant, expression of these cell populations was nearly undetectable in the peripheral blood, comprising similar percentages of the total CD4^-^ population as those found in BALB/c mice. In contrast, mice receiving 2 × 10^6 PFU LCMV Armstrong at the time of engraftment failed to delete Vβ5^-^CD4^-^ and Vβ11^-^CD4^-^ T cells at any time posttransplant (Fig. 2C and D). Failure to delete these cell populations occurred regardless of the presence of CD8^-^ T cells. This correlates with earlier
observations noting an LCMV-induced inhibition of peripheral deletion of alloreactive T cells following disruption of the CD40/CD40L pathway (33).

These results indicate a role for LCMV in overcoming the tolerizing effects of combined costimulation blockade and bone marrow administration. We show rapid rejection of skin and hematopoietic allografts following acute infection, preventing the induction of donor-specific tolerance. We have also performed heterotopic heart allografts using the same treatment, and again LCMV inhibits the generation of donor-specific tolerance (data not shown). This effect cannot be attributed to either CD8− or CD4+ T cells alone, as either subpopulation appears capable of inducing rapid CD40/CD28-independent graft rejection following acute infection. As predicted by graft survival, donor-reactive T cells are not deleted in infected mice, whereas uninfected mice receiving the tolerizing regimen delete donor MMTV superantigen-reactive T cell subpopulations within 60 days.

**LCMV infection does not abrogate established tolerance**

Based on previous studies (27) and our own conclusions concerning the peripheral deletion of alloreactive T cells, we considered it unlikely that a delayed infection with LCMV could induce rejection of skin or bone marrow grafts in tolerant chimeric mice. To test this hypothesis, mice were infected with LCMV 4–5 wk following transplantation and tolerance induction. 5/5 mice were significantly decreased from uninfected mice (p < 0.01). B. Uninfected mice proceeded to develop >60% H-2Kd+ cells in the peripheral blood in all animals by day 120 posttransplant. Infected mice, with or without depletion of CD8 T cells, failed to develop mixed chimerism. CD4+ T cell subsets expressing Vβ5 (C) and Vβ11 (D) are deleted in uninfected mice by postoperative day 60. These subsets are normally deleted in BALB/c but not B6 mice due to the expression of MMTV superantigens in conjunction with I-E by BALB/c cells. Infected mice, with or without depletion of CD8 T cells, fail to delete these T cell subsets. All error bars represent the SEM.

**FIGURE 2.** Acute LCMV infection prevents tolerance, mixed chimerism, and deletion of donor-reactive T cells following disruption of the CD40 CD40L pathway (33).

A. B6 mice received a BALB/c skin graft along with BALB/c bone marrow on postoperative days 0 and 6. All groups also received anti-CD40L and CTLA4-Ig on days 0, 2, 4, and 6. Mice were further treated with 600 µg of the hematopoietic stem cell selective toxin busulfan on day 5 posttransplant. Skin grafts in uninfected mice proceeded to indefinite skin graft survival (MST > 300 days, n = 5). Mice infected with LCMV at the time of transplant rejected their grafts promptly (MST = 15 days, n = 5), while infected mice depleted of CD8 T cells rejected their grafts in a slightly delayed manner (MST = 26 days, n = 5). Both groups were significantly decreased from uninfected mice (p < 0.01). B. Uninfected mice proceeded to develop >60% H-2Kd+ cells in the peripheral blood in all animals by day 120 posttransplant. Infected mice, with or without depletion of CD8 T cells, failed to develop mixed chimerism. CD4+ T cell subsets expressing Vβ5 (C) and Vβ11 (D) are deleted in uninfected mice by postoperative day 60. These subsets are normally deleted in BALB/c but not B6 mice due to the expression of MMTV superantigens in conjunction with I-E by BALB/c cells. Infected mice, with or without depletion of CD8 T cells, fail to delete these T cell subsets. All error bars represent the SEM.
B6 mice received BALB/c skin grafts and bone marrow, along with costimulatory blockade and busulfan treatment. Control mice received the same treatment regimen following receipt of syngeneic bone marrow and skin grafts. On day 28 posttransplant, mice were infected with LCMV. Eight days later splenocytes were harvested, restimulated for 5 h with LCMV peptides in the presence of brefeldin A, and stained for intracellular IFN-γ. Eight days later, mice were infected with LCMV on postoperative day 28 also maintained well-healed skin grafts indefinitely in all the mice tested (n = 5). Both uninfected mice and those infected on postoperative day 28 proceeded to develop >60% mixed hematopoietic chimerism by day 120 in all animals tested. Error bars represent the SEM.

**FIGURE 3.** Delayed LCMV infection does not impair tolerance induction or the development of mixed chimerism. All mice were treated as in Fig. 2. A. Uninfected controls enjoyed long-term graft survival (>250 days, n = 4). Mice infected with LCMV on postoperative day 28 also maintained well-healed skin grafts indefinitely in all the mice tested (n = 5). B. Both uninfected mice and those infected on postoperative day 28 proceeded to develop >60% mixed hematopoietic chimerism by day 120 in all animals tested. Error bars represent the SEM.

**FIGURE 4.** The antiviral T cell response following delayed infection is moderately decreased but epitope hierarchy remains unchanged. B6 mice received the tolerance induction protocol as before. One group received allogeneic (BALB/c) bone marrow and skin grafts, while another group received syngeneic (B6) bone marrow and skin grafts. Mice were infected on postoperative day (POD) 28. Eight days later, splenocytes were harvested and assessed for their antiviral T cell responses to the indicated epitopes by intracellular IFN-γ staining. The y-axis represents the total number of epitope-specific T cells per spleen. All epitopes in the animals receiving allogeneic transplants were decreased roughly 2-fold, with no striking diminution in the response to any particular epitope. Error bars represent the SEM (n = 3 for all groups).

**LCMV-specific T cells fail to divide in response to alloantigen**

To directly address the question of whether LCMV-specific CD8 T cells were also alloreactive, we used a previously described GVHD model (34). T cells from LCMV-immune B6 mice (>30 days postinfection) were labeled with the fluorescent dye CFSE (Molecular Probes, Eugene, OR) and injected i.v. into irradiated (1800 rad) allogeneic BALB/c hosts. In this model, allogeneic T cells responding to Ag lose fluorescence with each successive division, allowing for quantitation and analysis of highly divided alloreactive cells by flow cytometry. By using LCMV-immune mice as donors, we could assess whether LCMV-reactive T cells also divided in response to alloantigen by direct staining with the D^b/np396–404, D^b/gp33–41, and K^b/gp34–43 class I MHC tetramers. Splenocytes were harvested 72 h posttransfer, stained with anti-CD8 Abs and tetramers, and analyzed by flow cytometry.

CD8^+ T cells from both naive and immune mice divided significantly in response to alloantigen, with large numbers of cells from both groups reaching at least eight divisions. In contrast, CD8^+ T cells from either group injected into irradiated syngeneic recipients did not divide more than three times (data not shown). Therefore, we gated undivided and maximally divided (four to eight divisions) CD8^+ T cells and analyzed tetramer binding in each population (Fig. 5). LCMV-specific CD8 T cells were readily detectable within the undivided population in the recipients of LCMV-immune T cells for each tetramer tested. However, we failed to detect discernible staining above background for any of the tetramers in the maximally divided population (Fig. 5). The results are summarized in Table I. As a control to verify that our failure to detect tetramer binding was not simply a result of TCR down-modulation in highly divided cells, we stained for expression of TCRβ. We did not observe decreased expression of the TCR in the maximally divided cells by this assay (data not shown). Furthermore, a previous study has established that proliferating LCMV-specific CD8 T cells in lymphopenic hosts do not show decreased binding to MHC tetramers (35).

Although this experiment excludes the three principal epitopes as candidates for alloreactivity, we sought to determine whether cross-reactivity could be detected in donor cells from immune mice following restimulation with whole LCMV and specific
FIGURE 5. Tetramer-positive LCMV-immune CD8 T cells do not divide in response to alloantigen. Recipient BALB/c mice were irradiated with 1800 rad. Naive and LCMV-immune donor splenocytes were enriched for T cells using nylon wool. Cells were labeled with the fluorescent dye CFSE (Molecular Probes) and injected into irradiated recipients. Splenocytes were harvested on day 3 posttransfer and stained for expression CD8 and tetramers. In the first column, splenocytes are gated for CD8 expression and the histogram displays CFSE fluorescence. Peaks to the right of the histogram represent highly fluorescent, undivided cells, while successive peaks to the left measure loss of fluorescence with each cell division. We gated on undivided (middle column) and highly divided (four to eight divisions, right column) CD8 T cells and assessed their ability to bind class I tetramers folded with two immunodominant LCMV peptides. Representative samples from three mice per group are shown. No tetramer binding was detected in highly divided cells.

LCMV peptides in vitro. To achieve this, mouse splenocytes were harvested from BALB/c recipients on day 3 as above, restimulated for 5 h with infected or uninfected MC57 cells and brefeldin A, permeabilized and stained for intracellular IFN-γ expression, and analyzed by flow cytometry. As seen in Fig. 6, no IFN-γ expression was observed above background except in the undivided CD8 T cells stimulated with infected MC57 cells. We further analyzed responses to four LCMV epitopes in the same manner. In these experiments, rather than stimulating with infected cells, harvested splenocytes were restimulated with MC57 cells pulsed with LCMV peptides (NP396–404, gp33–41, gp276–286, NP205–214). None of these peptides induced IFN-γ production above background in the divided, alloreactive CD8 T cells. In contrast, LCMV-specific CD8 T cells could be readily detected in the undivided population following restimulation with these peptides (data not shown). One caveat to these experiments is the high background IFN-γ production in the highly divided cells, presumably due to the continued cycling and low-level stimulation of these cells during brefeldin A incubation.

We are unable to detect any allospecific proliferation by LCMV-reactive CD8+ T cells in this model. Although cross-reactivity to alloantigen has been demonstrated to exist at some level (24), it seems unlikely that this is the sole mechanism of LCMV-mediated graft failure.

LCMV facilitates the CD28/CD40-independent generation of alloreactive IFN-γ-producing cells

To better characterize the generation of allogeneic and antiviral T cell responses following LCMV infection, splenocytes were monitored for their ability to produce IFN-γ after restimulation in vitro by an ELISPOT assay. In this experiment, C3H/HeJ mice receiving BALB/c skin grafts generated ~3–4 × 10^5 allospecific T cells in the spleen by day 8 posttransplant, and these cell numbers dropped slightly at day 15. Treatment with costimulation blockade completely abolished the allogeneic response at both time points. Mice receiving skin grafts and costimulation blockade concurrent with an acute LCMV infection generated small numbers (~9 ×

Table I. LCMV-specific T cells fail to divide in response to alloantigen

<table>
<thead>
<tr>
<th>Tetraper</th>
<th>LCMV–Immune Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–1 div.</td>
</tr>
<tr>
<td>D^b/NP396–404</td>
<td>1.83 ± 0.046%</td>
</tr>
<tr>
<td>D^b/gp33–41</td>
<td>1.59 ± 0.088%</td>
</tr>
<tr>
<td>K^b/gp34–43</td>
<td>1.73 ± 0.250%</td>
</tr>
</tbody>
</table>

CFSE-labeled T cells (2 × 10^7) from LCMV-immune (>30 days postinfection) B6 mice were injected i.v. into irradiated (1800 rad) BALB/c mice. Recipient splenocytes were harvested 72 h later and analyzed by three-color flow cytometry for expression of CD8, CFSE fluorescence, and MHC tetramer binding. Numbers indicate the percent of CD8+ T cells in the indicated divided populations that bound MHC tetramer. The indicated error represents the SEM (n = 3).
10^6) of allospecific cells in the spleen by day 8. By day 15, these mice had overcome the immunosuppressive effects of costimulation blockade and had generated an alloresponse comparable to untreated controls (~2.5 x 10^7). As reported previously, acute LCMV infection in the absence of a skin graft resulted in the generation of some allospecific IFN-γ-producing cells by day 8 (~3 x 10^3). By day 15, this effect had diminished markedly to ~4 x 10^4 IFN-γ+ cells per spleen (Fig. 7).

To measure the LCMV-specific response, splenocytes from each group were incubated with infected L929 cells overnight on an ELISPOT plate. As expected, LCMV infection alone induced a potent antiviral response, generating ~1.2 x 10^7 IFN-γ-producing T cells in the spleen, whereas mice that received concurrent combination blockade and a BALB/c skin graft, while still generating a large response, had an ~3-fold drop in the number of LCMV-specific cells in the spleen (~4 x 10^6). By day 15, spleens from LCMV-infected mice showed a 3- to 4-fold decrease in the number of LCMV-specific cells. In mice receiving combination blockade, the drop was somewhat greater (Fig. 7).

To assess whether LCMV-infected mice generated memory to alloantigen, we infected B6 mice and quantitated the number of allospecific cells in the spleen at the peak of the infection (day 8) and following the development of immune memory (>30 days postinfection) by IFN-γ ELISPOT. LCMV-infected mice generated allospecific T cells (7.29 x 10^5 ± 1.78 x 10^5, n = 3) at the peak of the infection, but by day 30 postinfection, the number of these cells in the spleen dropped 50- to 100-fold (1 x 10^5 ± 9.9 x 10^3, n = 3). In contrast, the number of T cells specific for several known immunodominant and subdominant LCMV epitopes (NP396–404, gp33–41, gp276–286, NP205–214) dropped 10- to 12-fold in the spleen over the same period (1.52 x 10^7 ± 1.13 x 10^7 to 1.40 x 10^6 ± 1.38 x 10^5, n = 3 for both groups). This level of LCMV memory is similar to previous reports (31).

We conclude that LCMV infection stimulates the activation of at least a subset of allogenic T cells by CD40/CD28-independent mechanisms, thereby overcoming the immunosuppressive effects of costimulation blockade and leading to early graft rejection. Based on our CFSE and ELISPOT results, we propose that the frequency of virus-specific T cells also bearing TCR specificity to alloantigen is low.

**LCMV infection induces the CD28/CD40-independent maturation of splenic dendritic cells**

We next sought to explore other potential mechanisms whereby LCMV infection could abrogate transplant tolerance and stimulate the activation of alloreactive T cells. Our previous experiments studying deletion of Vβ subsets made it clear that in the presence of LCMV infection, CTLA4-Ig and anti-CD40L are unable to initiate the deletion of alloreactive T cells. A possible explanation was that LCMV infection was able to influence the induction and/or up-regulation of T cell costimulatory pathways by APCs. Furthermore, LCMV might induce the expression of molecules or survival factors that prevented deletion of alloreactive T cells. To test the merit of this hypothesis, we analyzed the effects of LCMV infection on costimulatory molecule and MHC expression by CD11c+ dendritic cells in the spleen.

Mice received BALB/c skin grafts and bone marrow, costimulatory blockade therapy, and busulfan. One group was infected with LCMV Armstrong on day 0, while the other remained uninfected. Splenocytes were harvested on day 6 and separated based on cell density using an Optiprep column (Nycomed) as previously described (32). The low-density fraction, which is enriched for dendritic cells, was harvested and stained for CD11c expression, along with MHC class I and II, ICAM-1, CD40, CD80, and CD86. Following analysis by flow cytometry, expression of these molecules among CD11c+ cells was analyzed. As seen in Fig. 8, LCMV infection resulted in the increased expression of all of these molecules, regardless of the presence of costimulatory blockade. We conclude that LCMV infection induces a higher activation state among dendritic cells. We suggest that one explanation for the deleterious effects of LCMV infection on tolerance induction could be the increased ability of APCs to stimulate and activate alloreactive T cells.

**Discussion**

In this study, we show that LCMV infection causes rapid allograft rejection following combined therapy with CTLA4-Ig and anti-

---

**FIGURE 7.** LCMV stimulates the CD28/CD40-independent generation of alloreactive IFN-γ-producing T cells. C3H/HeJ mice either received a BALB/c skin graft (SG) or a skin graft with costimulation blockade (CB). A third group received a skin graft and costimulation blockade concurrent with an LCMV infection, while a fourth group received an LCMV infection without further manipulation. Mouse spleens were harvested on the indicated days, and the frequency of IFN-γ-producing cells specific for LCMV or alloantigen was determined using an ELISPOT assay. For this assay, splenocytes were restimulated in vitro with either irradiated donor splenocytes or infected L929 cells. Error bars represent the SEM (n = 3 for all groups).

**FIGURE 8.** LCMV infection drives the CD28/CD40-independent maturation of dendritic cells. B6 mice received either BALB/c bone marrow and costimulation blockade, or the same regimen concurrent with an LCMV infection. Splenocytes were harvested on day 6 posttransplant. CD11c+ dendritic cells were enriched using Optiprep columns (Nycomed), stained with the indicated Abs, and analyzed by flow cytometry. Histograms represent expression of the indicated molecules among cells gated for CD11c expression. Filled histograms represent mice treated with bone marrow and costimulation blockade, solid lines represent mice receiving a concurrent LCMV infection, and dotted lines are isotype controls. These histograms are representative of two separate experiments.
CD40L. This effect can be extended to a robust tolerance induction model, as LCMV infection prevents both indefinite skin allograft survival as well as mixed hemopoietic chimerism following administration of donor bone marrow, busulfan, CTLA-Ig, and anti-CD40L. Although this effect is somewhat delayed in the absence of CD8 T cells, it nonetheless occurs without detectable CD8 expression in the blood, and depletion of CD4 T cells has little to no effect on graft survival. LCMV-induced allograft rejection correlates with a failure to delete donor-reactive CD4 T cells, as measured by tracking superantigen-reactive Vβ T cell subsets. We show that infection must occur at or around the time of transplant, as a delay of 3–4 wk in the onset of infection has no effect on graft survival or the induction of mixed chimerism. These studies confirm prior reports of the LCMV-mediated abrogation of skin graft survival following administration of donor splenocytes and anti-CD40L (27), and extend upon them by showing that LCMV-induced graft rejection is not mediated by CD40-independent up-regulation of B7.1 or B7.2. One concern with the use of tolerance induction strategies is the potential to induce tolerance to concurrent viral infections. It is of considerable interest that the immune responses to LCMV are not rendered tolerant following the use of costimulation blockade-based tolerance induction regimens, a finding consistent with previous observations that LCMV T cell responses are largely independent of CD28 and CD40 (23, 36, 37).

It has been proposed that one possible explanation for the deleterious effects of LCMV infection on graft survival could be the presence of cross-reactivity to alloantigen at the level of TCR/MHC recognition during an antiviral response (27). In this scenario, antiviral responses would include some cells also bearing specificity for alloantigen. In support of this hypothesis, it has been shown that LCMV induces H-2d-specific CD8 T cells at the peak of the T cell response (28). Although we make similar observations, we find little evidence for substantial cross-reactivity of LCMV-specific CD8 T cells generated in vivo to H-2d alloantigen. A delayed primary infection (4 wk posttransplant) elicits an antiviral response with unchanged epitope hierarchy, although the numbers of activated CD8 T cells are somewhat globally diminished. Furthermore, using a sensitive single cell assay using intracellular cytokine staining and MHC tetramers, we are unable to detect the division of LCMV-immune CD8 T cells in response to alloantigen. Nevertheless, as has been previously reported, LCMV primary infection does generate alloreactive cells. These cells drop greatly in number by day 15 postinfection and are barely detectable in LCMV-immune mice (>30 days postinfection). These experiments suggest that the frequency of LCMV-specific CD8 T cells that are cross-reactive to alloantigen is low. We cannot rule out the possibility of high levels of cross-reactive CD4 T cells using this assay.

The primary mechanism by which alloreactive T cells are activated during LCMV infection remains uncertain. Studies in recent years have shown that the great majority of activated CD8 T cells generated during an antiviral response are Ag-specific (31, 38, 39). Given the high frequency of alloreactive CD8 T cells in naive mice, substantial cross-reactivity at the level of TCR/MHC interaction would not be surprising. However, we are unable to detect significant levels of allospecific activation of CFSE-labeled LCMV-specific CD8 T cells following injection into irradiated BALB/c donors. Furthermore, LCMV-induced alloreactive cells do not behave as other virus-specific populations, as they have an exaggerated death phase following the peak of the response. Both CD4 and CD8 T cell subsets in isolation are capable of preventing tolerance induction and mixed chimerism. Interestingly, disruption of costimulatory pathways during LCMV infection has little effect on CD8+ T cell responses but almost entirely prevents the generation of CD4+ antiviral T cells (40). Nevertheless, CD4+ T cells are sufficient to mediate the LCMV-induced prevention of tolerance induction to alloantigen. Although cross-reactivity to alloantigens likely exists at some level, our data suggest that this is a relatively infrequent event during LCMV infection. It is of interest to note that LCMV responses are diminished in mice receiving the tolerance induction regimen. This observation could be due to non-specific immunosuppressive effects of allogeneic bone marrow and costimulation blockade treatment. Alternatively, the influx of H-2d+ donor APCs in the immune compartments could dilute the available Ag for stimulating an H-2b-restricted response. Further studies are warranted to assess the long-term effects of tolerance induction on immune responses to other pathogens.

Regardless of the extent to which alloreactive cells are generated during primary LCMV infection through TCR cross-reactivity, other mechanisms clearly play an indispensable role in the LCMV-mediated circumvention of the CD28/CD40 pathways. For example, MCMV and VV both generate allogeneic responses during primary infection (26), yet infection with these viruses has been shown not to impair graft survival (27). The primary CD8 anti-LCMV response itself has been shown to be largely independent of the CD28 and CD40 pathways (23, 36, 37). Interestingly, a recent study demonstrates that LCMV-specific responses, but not those directed toward VV, can be driven by parenchymal cells (41). This suggests that LCMV, but not VV, can lower the threshold required for full activation of effector cells. One possibility is that LCMV triggers specific innate immune mechanisms that allow for the circumvention of these pathways in generating T cell responses. Also, anti-LCMV responses may provide cytokines and growth factors that aid the generation of CD28/CD40 independent alloresponses. Another possibility is that LCMV infection induces the expression of CD40/CD28-independent costimulatory pathways. In support of this latter possibility, we show in this study that LCMV infection mediates the CD28/CD40-independent up-regulation of MHC and costimulatory molecules on dendritic cells. We hypothesize that infection with LCMV facilitates the activation of alloreactive cells in the face of costimulatory blockade through the up-regulation of alternative costimulatory molecules on the surface of APCs. In this model, the need for costimulation and activation of dendritic cells by the CD28 or CD40 pathways would be abrogated by infection with LCMV. Further studies are required to elucidate the precise mechanisms by which some viral infections (e.g., LCMV, PV) but not others (e.g., MCMV, VV) mediate early graft rejection in the face of costimulation blockade.

Infections have long been a problem during attempts to immunosuppress transplant recipients. It is likely that any type of immunosuppression used in the future will carry the risk of predisposing the recipient to opportunistic pathogens and viral infections. As protocols involving the use of costimulatory molecule-blocking reagents move closer to clinical application, it will be of vital importance that we understand the mechanisms whereby viral infections can interfere with attempts to prevent graft rejection. Of particular interest is the ability of LCMV infection, but not all viral infections, to overcome attempts at tolerance induction through the use of costimulatory blockade. Our data suggest that the prevention of tolerance induction following LCMV infection is not solely due to cross-reactivity to alloantigen. Other mechanisms likely play a role, as evidenced by our observation that LCMV infection elevates the activation and maturation level of host dendritic cells. Future studies should elucidate the precise pathways by which viral infections mediate the costimulation blockade-resistant activation of alloreactive T cells.
Acknowledgments

We thank Diane Hollenbaugh, Robert Peach, and Alejandro Aruffo (Bristol-Myers Squibb) for providing CTLA4-Ig.

References