November 2002

Cutting Edge: Persistent Viral Infection Prevents Tolerance Induction and Escapes Immune Control Following CD28/CD40 Blockade-based Regimen

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THE JOURNAL OF IMMUNOLOGY

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J Immunol 2002;169;5387-5391

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CUTTING EDGE

Cutting Edge: Persistent Viral Infection Prevents Tolerance Induction and Escapes Immune Control Following CD28/CD40 Blockade-Based Regimen[1]

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A continuing concern with CD28 and/or CD40 blockade-based strategies to induce tolerance and mixed chimerism is their potential to disrupt protective immunity to preexisting infections. In this report, we find that preexisting persistent infection with lymphocytic choriomeningitis virus (LCMV) clone 13 prevents the induction of tolerance, mixed chimerism, and donor-reactive T cell deletion. Mice continue to be refractory to tolerance induction even after viremia has been resolved and virus is present only at very low levels in peripheral tissues. Conversely, we find that the full tolerance regimen, or costimulation blockade alone, specifically inhibits already ongoing antiviral immune responses, leading to an inability to control viremia. These findings suggest that ongoing T cell responses continue to depend on costimulatory interactions in the setting of a chronic infection and provide insight into potential risks following costimulation blockade posed by chronic or latent viral infections such as hepatitis C, EBV, and CMV. *The Journal of Immunology*, 2002, 169: 5387–5391.

In recent years, costimulation blockade therapies have played a central role in strategies to induce mixed allogeneic chimerism and transplantation tolerance. Successful strategies have included the use of preconditioning regimens consisting of sublethal gamma-irradiation (1), as well as administration of large numbers of donor bone marrow cells without preconditioning (2, 3). We have recently developed a model in which mice are preconditioned with a minimally myelosuppressive dose of the stem cell-selective toxin busulfan, allowing for the engraftment of donor bone marrow under the cover of costimulation blockade. Skin grafts given at the same time as bone marrow survive indefinitely (4).

A continuing concern in clinical transplantation is the effect tolerance induction regimens might have on immunity to pathogens. One possible consequence is that in attempting to induce tolerance to the allograft, antiviral immune responses could be compromised, leading to undesirable consequences for the transplant recipient. Conversely, ongoing viral infections could prove to be a barrier to the successful induction of allograft tolerance. In support of this latter scenario, it has recently been reported that acute infection with lymphocytic choriomeningitis virus (LCMV)[4] Armstrong at the time of transplantation prevents tolerance induction and induces rapid graft rejection (5, 6).

As preexisting chronic or latent infections are prevalent in transplant recipients (e.g., EBV, CMV, polyoma, hepatitis C), we sought to apply a model of chronic viral infection. LCMV clone 13, in contrast to LCMV Armstrong, causes a long-term persistent infection in mice that is cleared from serum over the course of 2–3 mo (7). In this study, we analyze the impact of an ongoing chronic infection on the induction of tolerance. We also assess the ability of recipient mice to maintain control of the infection following costimulation blockade-based treatment. We find that the presence of a chronic LCMV infection prevents the establishment of tolerance and deletion of donor-reactive T cells as long as 4 mo postinfection, long after viremia has been controlled. Moreover, analysis of the antiviral T cell response reveals that administration of the tolerance regimen during persistent infection, and specifically short-term costimulation blockade, results in sustained impairment of responses to a panel of CD8 epitopes and a failure to control viremia. To our knowledge, these data provide the first evidence that persistent infections may prove to be a barrier to the clinical application of costimulation blockade-based tolerance induction regimens, and perhaps even more importantly that in the absence of antiviral therapy, such regimens also have the potential to hamper protective antiviral immunity. Furthermore, we find that costimulation via the CD28 and CD40 pathways continues to be essential for T cell responses to chronic infection well after initial Ag exposure, expansion, and differentiation.

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Received for publication August 9, 2002. Accepted for publication September 20, 2002.

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[1] This work was supported by grants from the National Institutes of Health and from the Carlos and Marguerite Mason Trust.

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[4] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; CD40L, CD40 ligand; MST, median survival time.

0022-1767/02/$02.00

Materials and Methods

Mice and virus infections

Adult male 6- to 8-wk-old BALB/c and C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 LCMV carrier mice were bred at Emory University (Atlanta, GA) as previously described (8). Mice were infected with 2×10^5 PFU of LCMV Armstrong injected i.p. to induce acute infection, or with 2×10^6 PFU of LCMV clone 13 (i.v.) to induce chronic infection. Virus stocks were grown and quantitated as previously described (7). Infectious LCMV in serum and tissues was measured by plaque assay on Vero cell monolayers as described (7).

Skin grafting

Full thickness skin grafts (~1 cm^2) were transplanted on the dorsal thorax of recipient mice and secured with a band-aid for 7 days. Graft survival was then followed by daily visual inspection. Rejection was defined as the complete loss of viable epidermal graft tissue.

Bone marrow preparation and treatment protocols

The mixed chimerism tolerance regimen, involving treatment with donor bone marrow, busulfan (Busulfex; Orphan Medical, Minnetonka, MN), CTLA4-Ig, and anti-CD40 ligand (CD40L) (MR1), has been described elsewhere (4). The dose of MR1 (2 mg total over four treatments) has been shown to generate therapeutically effective levels for >50 days (9).

Cell preparations and flow cytometry

Intracellular IFN-γ expression was induced in response to ex vivo restimulation with LCMV peptides as described (8). Cells were stained with anti-IFN-γ and anti-CD8 (BD PharMingen, San Diego, CA) using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD PharMingen). Peripheral blood was analyzed by staining with the indicated fluorochrome-conjugated Abs (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 and data were analyzed using CellQuest software (BD Biosciences, Braintree, MA).

Results

Ongoing chronic infection, but not prior acute infection, prevents tolerance induction, mixed chimerism, and deletion of donor-reactive T cells

We have previously described a method for establishing indefinite allospecific tolerance and 50–70% mixed hemopoietic chimerism following treatment with the stem cell-selective toxin busulfan, administration of donor bone marrow, and blockade of the CD28 and CD40 costimulatory pathways (4). However, we have recently reported that tolerance induction could be inhibited by a concurrent acute LCMV infection (6). In the present experiments, we sought to determine the effects of preexisting long-term chronic viral infections on tolerance induction, the development of hemopoietic chimerism, and the deletion of donor-reactive T cells. Mice infected with LCMV clone 13 typically develop high viral titers in the serum that are gradually controlled by the host T cell response over the course of 2–3 mo. Virus is generally controlled and cleared from the serum by day 90 postinfection and persists at very low levels in peripheral organs indefinitely (7). We assessed the efficacy of the tolerance regimen during the course of a chronic

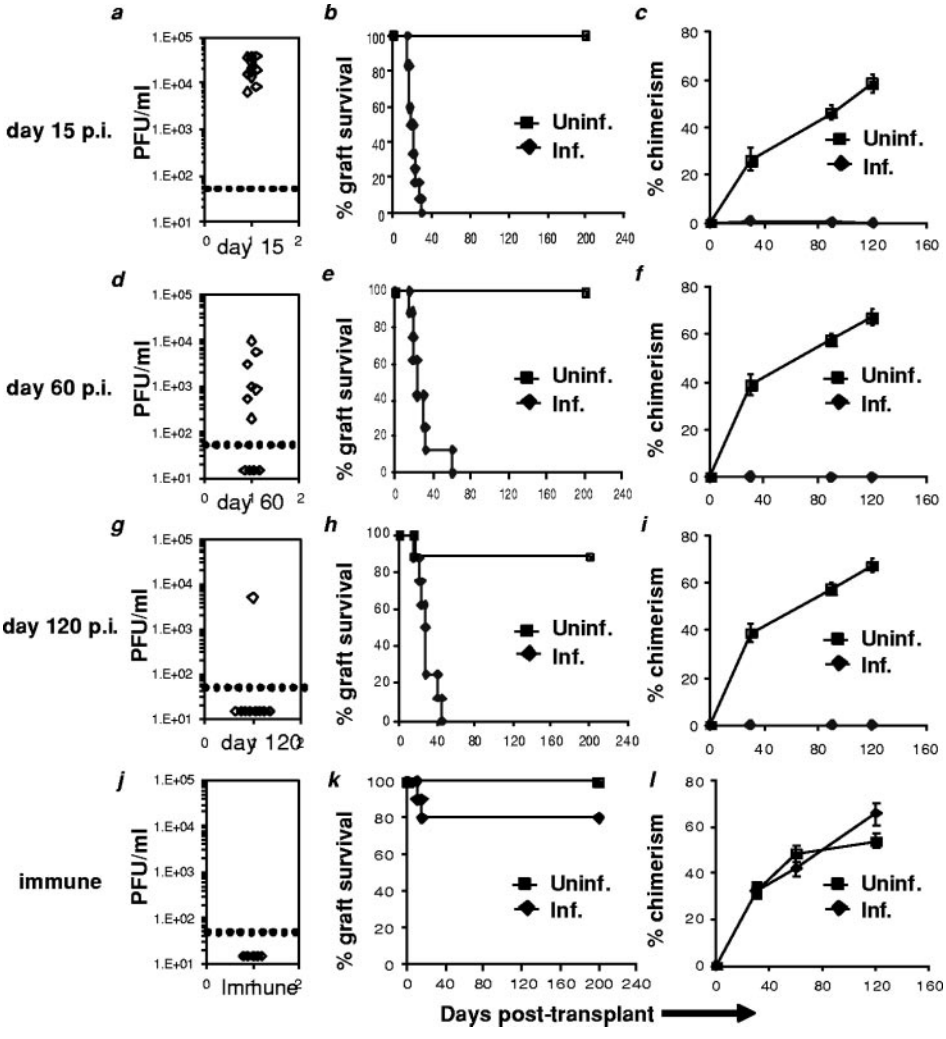

FIGURE 1. Chronic infection with LCMV clone 13 prevents tolerance induction and chimerism over a long time course. B6 mice were infected with 2×10^6 PFU of LCMV clone 13. At 15 (*a–c*), 60 (*d–f*), or 120 (*g–i*) days postinfection, mice were treated with the tolerance induction regimen and given a BALB/c skin graft. *j–l*, LCMV immune mice also received the tolerance regimen 30 days after an acute infection with LCMV Armstrong. *a*, *d*, *g*, Mice were bled on days 15, 60, or 120 postinfection, respectively, and tested for the presence of LCMV in the serum. The dotted line represents the limit of detection. Mice transplanted at these time points were assessed for skin graft survival (*b*, *e*, and *h*) and donor cell chimerism (*c*, *f*, and *i*) over a long time course (at day 15, $n = 15$ for infected, $n = 10$ for uninfected; at days 60 and 120, $n = 9$ for infected, $n = 8$ for uninfected). Similar assays were performed for LCMV-immune mice. *j*, Serum titers were measured 30 days after acute infection with LCMV Armstrong ($n = 5$). Mice received the tolerance regimen and a BALB/c skin allograft >30 days postinfection and were assessed for skin graft survival (*k*) and chimerism (*l*) ($n = 10$ for infected, $n = 8$ for uninfected). All error bars represent the SEM ($p < 0.001$ for all infected vs uninfected groups). Skin graft survival and chimerism data are pooled from two separate experiments.

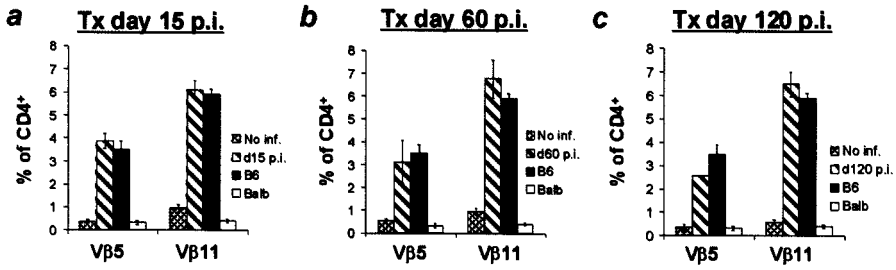

FIGURE 2. Chronic LCMV infection prevents deletion of donor-reactive T cells. B6 mice were infected with 2×10^6 PFU of LCMV clone 13, and then received the tolerance regimen and a BALB/c skin graft 15 (*a*), 60 (*b*), or 120 (*c*) days postinfection. Mice were bled 120 days posttransplant and peripheral leukocytes stained for the presence of $V\beta5^+CD4^+$ and $V\beta11^+CD4^+$ cells. The *y*-axis is the percentage of $CD4^+$ cells that are also $V\beta5^+$ or $V\beta11^+$ for infected ($n = 5$ for day 15, $n = 4$ for day 60, $n = 4$ for day 120), uninfected tolerant ($n = 4$ for all time points), B6 ($n = 3$), and BALB/c ($n = 3$) mice. Error bars represent the SEM ($p < 0.001$ between all infected and uninfected tolerant groups).

infection by administering the tolerance induction protocol to mice 15, 60, and 120 days postinfection. As expected, viral titers in mice infected with LCMV clone 13 gradually decrease and disappear from the serum by day 120 postinfection (Fig. 1, *a*, *d*, and *g*). However, low levels of virus persisted in the kidneys of infected mice as late as 250 days postinfection (data not shown). Conversely, mice infected with LCMV Armstrong, an acutely infecting strain, rapidly clear the virus from the serum (Fig. 1*j*) and peripheral tissues (data not shown).

B6 mice receiving a BALB/c skin graft along with donor bone marrow, costimulation blockade, and busulfan 15 days postinfection rejected their grafts promptly (Fig. 1*b*, median survival time (MST) = 18 days). In contrast, 10 of 10 uninfected control mice displayed indefinite graft survival (MST >200 days). Infected mice also failed to develop detectable levels of stable mixed chimerism, while uninfected controls generally developed ≥50% chimerism in the peripheral blood by day 60 (Fig. 1*c*). Mice also rejected skin grafts promptly when receiving the tolerance regimen 60 (MST = 23 days) or 120 (MST = 27 days) days postinfection (Fig. 1, *e* and *h*). Failure to accept skin grafts was further reflected in a failure to generate mixed chimerism following transplantation of infected mice, while uninfected controls established high levels of stable mixed chimerism (Fig. 1, *f* and *i*). In comparison, 8 of 10 mice with a prior acute LCMV Armstrong infection (>30 days postinfection) accepted donor skin grafts (>200 days) indefinitely and developed stable mixed chimerism (Fig. 1, *k* and *l*).

In aggregate, while 25 of 26 uninfected B6 mice were rendered tolerant and chimeric, none of the 33 infected animals accepted skin allografts or developed chimerism. It is notable that even mice with no detectable virus in the serum rapidly rejected skin allografts following administration of donor bone marrow, busulfan, and costimulation blockade.

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) presented by I-E MHC class II molecules, whereas 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. Treatment with costimulation blockade and the subsequent development of chimerism in B6 recipients results in the deletion of the donor-reactive Vβ5 and Vβ11 subsets (2, 3). Mice receiving the tolerance regimen 15, 60,

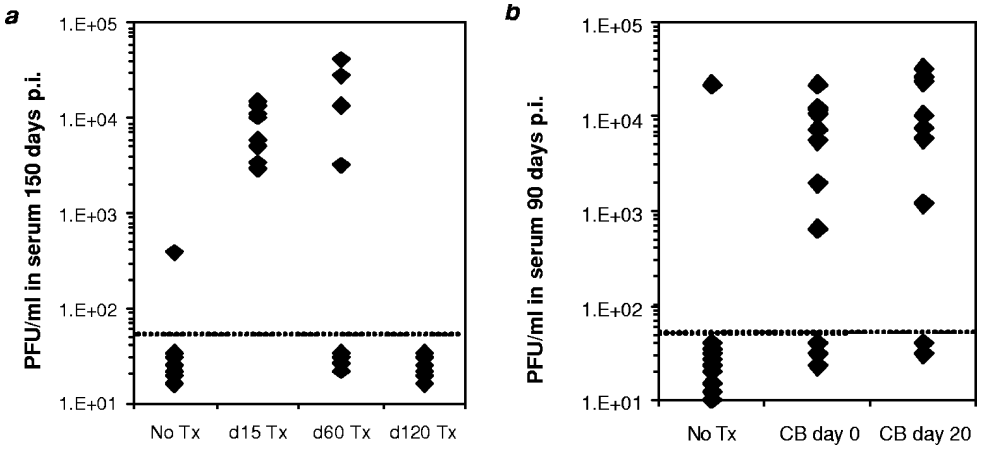

FIGURE 3. Costimulation blockade-based therapies prevent control of ongoing chronic infection. *a*, B6 mice were infected with LCMV clone 13 and received either no further treatment (No Tx; $n = 7$) or the tolerance regimen on days 15 (d15 Tx; $n = 8$, $p < 0.01$ vs No Tx), 60 (d60 Tx; $n = 8$, $p < 0.05$ vs No Tx), or 120 (d120 Tx; $n = 6$) postinfection. Mice were bled at day 150 postinfection and serum was tested for the presence of LCMV. The *y*-axis displays plaque forming units per milliliter. The dotted line indicates the limit of detection for this assay. *b*, B6 mice were treated with LCMV and received either no further treatment (No Tx; $n = 10$) or administration of CTLA4-Ig and anti-CD40L starting on day 0 (CB day 0; $n = 10$, $p < 0.05$ vs No Tx) or day 20 (CB day 20; $n = 10$, $p < 0.05$ vs No Tx) postinfection. Mice were bled on day 90 postinfection and serum was tested for the presence of LCMV. The *y*-axis displays plaque forming units per milliliter in the serum. The dotted line indicates the limit of detection for this assay. Results are pooled from two separate experiments.

or 120 days postinfection were bled 120 days posttransplant and tested for the presence of Vβ5- and Vβ11-bearing CD4$^+$ T cells. Chronic infection with LCMV prevented the deletion of donor-reactive T cells at all time points (Fig. 2). In contrast, tolerant control animals deleted these subsets. We concluded that inhibition of tolerance induction by chronic LCMV infection was associated with a failure to delete donor-reactive T cells from the periphery.

Costimulation blockade impairs ongoing antiviral immunity

The observation that chronic LCMV infection could induce alloresponses sufficient for graft rejection suggested that administration of costimulation blockade-based immunosuppressive therapy was unlikely to disrupt antiviral responses and prevent effective viral clearance. To test this, we measured viral titers in the serum of animals that had received either no treatment or the full tolerance regimen (skin allograft, donor bone marrow, busulfan, CTLA4-Ig, and anti-CD40L) either 15, 60, or 120 days postinfection. Eight of eight mice receiving the regimen at day 15 and four of eight mice receiving the regimen at day 60 failed to clear virus from the serum by day 150 postinfection (Fig. 3*a*) and throughout the course of the experiment (>200 days, data not shown). Significantly, mice treated 120 days postinfection that had already resolved viremia but maintained low levels of virus in the kidney did not display reemergence of the virus in the peripheral blood at day 150 postinfection (Fig. 3*a*) or at later time points (data not shown). As a treatment control, mice given bone marrow and busulfan without costimulation blockade on day 15 postinfection successfully controlled viremia with normal kinetics (data not shown). We further found that treatment with costimulation blockade alone (CTLA4-Ig and anti-CD40L), beginning at day 0 or 20 postinfection, also prevented normal viral clearance from the serum by day 90 postinfection (Fig. 3*b*).

To determine whether the treatment regimen could specifically blunt ongoing antiviral CD8 T cell responses to chronic LCMV, we treated mice with the full tolerance regimen or costimulation blockade alone 20 days postinfection, then assessed by IFN-γ staining the frequency of LCMV-specific T cells in the spleen 15 days later. We observed ~3- to 4-fold decreased numbers of CD8 T cells specific for the gp33–41, gp276–286, and gp118–128 epitopes in the spleens of mice receiving the tolerance regimen or costimulation blockade treatment alone, as compared with untreated controls (Fig. 4). Responses to the nuclear protein 396–404 epitope were virtually nonexistent in all groups, as previously documented (10).

We concluded from these experiments that treatment with costimulation blockade-based tolerance regimens during the course of a chronic viral infection can significantly impair antiviral CD8 T cell immune responses and control of viremia even well after initial priming, expansion, and differentiation. Importantly, however, this treatment does not lead to reemergence of the virus in the serum when initiated after viremia has been controlled, and the virus is confined to peripheral tissues.

Discussion

In this report, we demonstrate that chronic LCMV infection, but not prior acute infection, prevents the induction of chimerism and donor-specific tolerance and predisposes toward the CD28/CD40-independent rejection of skin allografts. Remarkably, even mice receiving transplants as long as 120 days postinfection were resistant to the effects of the tolerance regimen, despite the fact that viremia had been resolved and virus persisted only at very low levels in peripheral tissues such as the kidney. Graft rejection correlated with a failure to delete donor-reactive T cells. Given that concurrent infection with acute LCMV has been shown to prevent

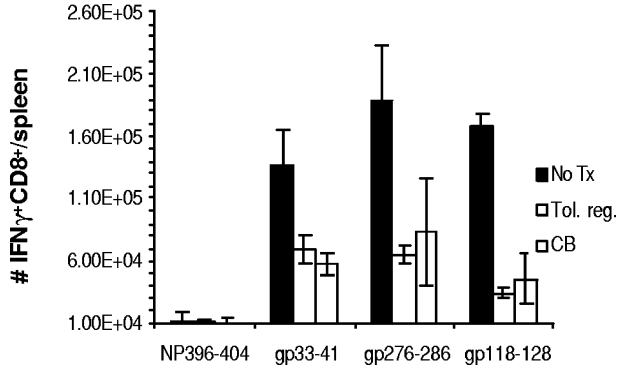

FIGURE 4. Costimulation blockade blunts ongoing antiviral CD8 T cell responses. B6 mice were infected with LCMV clone 13. At 20 days postinfection, they either received no further treatment (No Tx; $n = 3$), the tolerance regimen (Tol. reg.; $n = 5$), or costimulation blockade alone (CB; $n = 4$). Fifteen days later, splenocytes were harvested, restimulated with the indicated peptides in the presence of brefeldin A, and stained for intracellular expression of IFN-γ. Splenocytes were restimulated with the gp33–41, nuclear protein 396–404, gp276–286, or gp118–128 peptides, as indicated on the *x*-axis. The total number of CD8$^+$IFN-γ^+ cells per spleen of each treatment group is indicated on the *y*-axis, as calculated based on the percentage of CD8$^+$IFN-γ^+ and the total number of splenocytes harvested. Error bars represent the SEM ($p < 0.001$ for uninfected group vs either infected group for gp33–41, gp276–286, and gp118–128).

the induction of tolerance in a variety of costimulation blockade-based models (5, 6, 11), it seems likely that chronic LCMV infection will also disrupt other CD28/CD40 blockade-based tolerance induction regimens.

Although CD28 and CD40 have been found to be largely dispensable for clearance of an acute LCMV infection (12–14), T cell costimulatory pathways are much more important for dealing with more potent persistent infections (12). Our report indicates that Ag-specific CD8 T cells continue to require CD28/CD40 costimulation in the setting of chronic infection even well after priming, activation, and expansion (as late as 60 days into the response), as demonstrated by our observation that ongoing CD8 responses are down-modulated following costimulatory blockade. Interestingly, blockade of costimulatory pathways has been shown to be effective in preventing the onset of autoimmunity in mouse models (15, 16). Our data provide a rationale for such treatment in ameliorating ongoing disease (17).

Latent or persistent viral infections are very common in adult human transplant recipients, and loss of immune control of these viruses following transplantation results in significant morbidity and mortality. It is crucial to understand the effects of tolerance regimens on other chronic persistent or latent infections, such as hepatitis C, polyoma, EBV, and CMV. Further studies should further dissect the pathways whereby chronic LCMV promotes the generation of CD28/CD40-independent alloresponses and eventual graft rejection.

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