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Expansion of Effector Memory TCR Vβ4+CD8+ T Cells Is Associated with Latent Infection-Mediated Resistance to Transplantation Tolerance

Dale Stapler,* Eun D. Lee,* Saranya A. Selvaraj,* Andrew G. Evans,† Leslie S. Kean,* Samuel H. Speck,† Christian P. Larsen,* and Shivaprakash Gangappa2*

Therapies that control largely T cell-dependent allograft rejection in humans also possess the undesirable effect of impairing T cell function, leaving transplant recipients susceptible to opportunistic viruses. Prime among these opportunists are the ubiquitous herpesviruses. To date, studies are lacking that address the effect of viruses that establish a true latent state on allograft tolerance or the effect of tolerance protocols on the immune control of latent viruses. By using a mixed chimerism-based tolerance-induction protocol, we found that mice undergoing latent infection with γHV68, a murine γ-herpesvirus closely related to human γ-herpesviruses such as EBV and Kaposi’s sarcoma-associated herpesvirus, significantly resist tolerance to allografts. Limiting the degree of virus reactivation or innate immune response did not reconstitute chimerism in latently infected mice. However, γHV68-infected mice showed increased frequency of CD8+ T cell alloreactivity and, interestingly, expansion of virus-induced, alloreactive, “effector/effector memory” TCR Vβ4+CD8+ T cells driven by the γHV68-M1 gene was associated with resistance to tolerance induction in studies using γHV68-M1 mutant virus. These results define the viral gene and immune cell types involved in latent infection-mediated resistance to allograft tolerance and underscore the influence of latent herpesviruses on allograft survival. The Journal of Immunology, 2008, 180: 3190–3200.

Current strategies for inducing durable allograft survival involve long-term use of immunosuppressive drugs following transplantation. Although improvements in immunosuppressive drug regimens have decreased the risk of rejection in bone marrow (BM)3 and solid organ transplant recipients, all such drugs carry with them an increased risk of herpesvirus reactivation (1, 2). Earlier studies have shown that EBV establishes latent infection in >90% of the human adult population and that latent infections present in transplant recipients or originating from donor tissues can threaten the survival of both the allograft and the host (3–5). Latency, defined as the presence of reactivatable viral genome in the absence of detectable infectious virus, is the hallmark of herpesviruses infections (6). Members of the Herpesviridae family establish latency at different sites in the host and carry the potential to reactivate under immunodeficiency (6–9).

In recent years, major advances have been made in animal models and pilot clinical trials to induce donor-specific immunological tolerance using donor BM infusion combined with blockade of critical costimulatory signals required for T cell activation (10–14). Mixed chimerism-based tolerance-induction protocols established in rodent models by several investigators involve donor-specific transfusion (15), rapamycin (16), or busulfan (14) combined with costimulation blockade with CTLA4-Ig and anti-CD40L Ab. These protocols are effective in inducing donor-specific tolerance to allografts across MHC barriers, and the mechanisms of allograft tolerance by these regimens include central deletion (14) and peripheral regulation (16). Although these different regimens show efficacy for donor-specific tolerance induction in naive mice with different MHC haplotypes, their effects on mice with virally induced alloreactive memory T cells (heterologous immunity) are less profound (17–19). These studies suggest that a critical threshold of memory T cells is needed to promote graft rejection, suggesting a potential mechanism to explain why transplant tolerance induction has been more difficult in humans than in mice. However, the studies described above have tested the effects of nonpersistent infections on transplantation tolerance and, to date, no studies have addressed the effects of latent infection with homologs of human viral pathogens on tolerance induction.

Many γ-herpesviruses, including the human pathogens EBV and Kaposi’s sarcoma-associated herpesvirus (KSHV) and the murine γHV68 (γ-herpesvirus 68), establish life-long latent infections in hemopoietic cells (3, 20, 21). Previous studies have revealed the structural homology between these human and murine γ-herpesviruses and have addressed the utility of using γHV68 infection in mice as a model to study γ-herpesvirus latency (22, 23). The relevance of γ-herpesvirus-associated diseases in immunocompromised hosts (24, 25), including the transplant population (26), combined with the availability of viral mutants that fail to reactivate efficiently (27), strongly points toward the utility of this
mouse model of latent infection. Through use of this small animal model combined with costimulation blockade-based tolerance protocols, we found that latently infected mice resist induction of tolerance to allografts and that the resistance is not mediated by tolerance protocol-induced virus reactivation or the host NK cell-mediated lysis. Furthermore, B6 mice carrying latent infection showed an increase in alloreactive CD8⁺ T cells, and, interestingly, failure to expand a CD8⁺ T cell subset (TCR Vβ⁺ CD8⁺ T cells) as a result of infection with a HV68 mutant that lacks the M1 gene did not show resistance to allo graft tolerance. Moreover, tolerance resistance-associated Vβ⁺ CD8⁺ T cells demonstrated effector/effect memory phenotype (CD44high, CD127high, CD62Llow/lo) (28) and increased alloreactive responses compared with Vβ⁻CD8⁺ T cell populations.

To determine the inhibitory effects of latent infection with a homolog of human viral pathogen on induction of tolerance to allografts, and 2) identify the viral gene and consequent immune cell types associated with latent infection-mediated resistance to tolerance induction. These results highlight the need to define mechanisms by which other latent infections influence allo graft survival and to identify strategies to overcome latent infection-mediated barriers and achieve tolerance to allografts in the face of latent infection.

Materials and Methods

Mice and viral infection

C57BL/6 (B6; CD45.2 or congeneric CD45.1), BALB/c mice were purchased from The Jackson Laboratory. To induce latent infection, mice were inoculated i.p. with 10⁶ PFU of wild-type HV68 (29), HV68-open reading frame (ORF) 72 mutant (27), HV68-M1 mutant (M1-LacZ and M1-D51) (30), and HV68-M1 marker rescue (HV68-M1-MR) (30) in 0.5 ml of DMEM. Virus stocks were grown and quantitated as previously described (21, 32). Animals received humane care and treatment in accordance with institutional guidelines.

Chimerism, skin grafting, and tolerance-induction protocols

Donor BM harvest, full-thickness skin grafts, and the mixed chimerism tolerance protocols involving CTLA4-Ig (Bristol-Myers Squibb), anti-CD40L (BioXCell), and busulfan were administered as previously described (14). For NK cell depletion, as described earlier (31), anti-NK1.1 Ab (PK136) was administered on days 1, 0, 2, 4, and 6 relative to tolerance-induction protocol and plate d in a serial 2-fold dilution (starting at 4 × 10⁵ cells/well) onto mouse embryonic fibroblast monolayers in 96-well plates. Twenty-four wells were plated per dilution, and 12 dilutions were plated per sample. Wells were scored microscopically 21 days later for viral cytopathic effect. Preformed virus in tissues was detected by plating parallel cell samples that had been subjected to mechanical disruption and scored microscopically 21 days later for viral cytopathic effect. Mechanical disruption does not inactivate virus but kills >99% of cells, and thus samples treated in this way detect preformed virus rather than virus reactivating from latency (21, 32).

Results

Latent infection interferes with costimulation blockade-based chimerism and tolerance induction

In recent years, major advances have been made in animal models and pilot clinical trials to induce donor-specific immunological tolerance using mixed chimerism-based regimens (10–14, 33). To determine the effects of latent infection on mixed chimerism-based allograft tolerance, as shown in the schematic (Fig. 1), we used a minimally myeloablative costimulation blockade-based chimerism-induction protocol described by Adams et al. to induce donor-specific tolerance in a mouse model (14, 19). Briefly, recipient mice (B6) received busulfan and allogeneic BALB/c donor BM under short-term CD28/B7 and CD40L/CD40 costimulation blockade. As shown in Fig. 2 for the naive uninfected control group, chimerism was tested by assessment of donor hemopoietic chimerism and tolerance induction protocols described by Adams et al. to induce donor-specific tolerance in a mouse model (14, 19). Briefly, recipient mice (B6) received busulfan and allogeneic BALB/c donor BM under short-term CD28/B7 and CD40L/CD40 costimulation blockade. As shown in Fig. 2 for the naive uninfected control group, chimerism was tested by assessment of donor hemopoietic chimerism and tolerance induction protocols described by Adams et al. to induce donor-specific tolerance in a mouse model (14, 19).

Cell preparation and flow cytometry for chimerism, T cell phenotypic characterization, and alloreactivity assays

Splenocytes and/or peripheral blood leukocytes were prepared from experimental and naive mice and 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). Intracellular IFN-γ and TNF-α expression was induced in response to 5 h of ex vivo restimulation with allogeneic stimulators, syngeneic stimulators, HV68 peptides (Kb, ORF61/p79 and Dα, ORF6/p56; 1 μg/ml), or PMA/ionomycin (PMA, 50 ng/ml; ionomycin, 500 ng/ml). In brief, responders were re suspended in cell culture media containing brefeldin (GolgiPlug, BD Pharmingen) and recombinant hIL-2 (10 ng/ml). To enumerate the allogeneic response, responders were plated in a 96-well plate at a 1:1 ratio (10⁶ cells/well) with stimulators (splenocyte preparation from naive allogeneic (BALB/c or C3H) or syngeneic (B6) mice). Responders alone, viral peptides, or stimulators with syngeneic splenocytes were used as specificity controls. All stimulations were performed for 5 h at 37°C. Intracellular staining for IFN-γ and TNF-α was performed as per the manufacturer’s instructions (Cytofix/Cytoperm kit, BD Pharmingen). Flow cytometry was performed on either FACS Calibur or BD LSR II, and data were analyzed using CellQuest (BD Biosciences) or FlowJo (TreeStar) software.

Statistical analyses

All data were analyzed by using GraphPad Prism software. Frequencies were obtained from the cell number at which 63% of the wells scored positive for reactivating virus based on Poisson distribution. Percentage chimerism, percentage skin graft survival, percentage Vβ⁺ CD8⁺ T cells, and absolute numbers of cytokine-producing cells were statistically analyzed by using the Mann-Whitney U test.

FIGURE 1. Allograft tolerance-induction protocol. Groups of naive or γHV68-infected mice received allogeneic (BALB/c) BM and tolerance regimen (costimulation blockade (CTLA4-Ig, anti-CD40L mAbs) and busulfan) on days as indicated. Chimerism was analyzed on days 8, 15, 30, and 60 relative to the tolerance-induction protocol, and tolerance to allogeneic skin graft was tested by placing BALB/c skin graft at day 70 relative to tolerance-induction protocol.
γHV68 infection is cleared by day 14 and latency is established (23, 32). The day 120 time point was included to test whether the effects of latent infection on tolerance induction varies depending on the time point considered to be “latent” infection. We have used a virus inoculum (10^5 PFU) that has been used to study γHV68 latency by the i.p. route of infection by other investigators (21, 29). As shown in Fig. 2, unlike the naive uninfected control group that resulted in high-level mixed chimeras, most latently infected mice in both days 40 and 120 p.i. groups resisted chimerism (top panels) and tolerance to subsequently placed skin grafts at day 70 after chimerism induction (bottom panels). To determine whether the latent infection-mediated resistance was specific to the allogeneic BM, a group of γHV68-infected B6 mice were tested for chimerism by substituting donor BALB/c allogeneic BM infusion with congenic CD45.1-B6 BM in the above-stated tolerance regimen. Chimerism analysis by flow cytometry using H-2d, CD45.1, and B220 staining showed that while latently infected B6 mice resisted chimerism of allogeneic BALB/c BM, they readily accepted congenic cells (data not shown). These data suggest that latent infection interferes with costimulation blockade-based tolerance to allografts and that the resistance is specific to allogeneic grafts.

Latent infection-mediated resistance to chimerism is not mediated by recipient NK cells or altered virus reactivation

As shown above, latent infection of mice with γHV68, a structural homolog of EBV, inhibits chimerism and tolerance to allogeneic skin grafts. The inhibition of chimerism seems to occur at the level of engraftment because our assay failed to detect any chimerism at day 15, 30, or 60 after chimerism induction in all the mice that resisted chimerism (Fig. 2). Previous work from other groups has shown that NK cells respond early after infection and that certain viral infections can strongly stimulate NK cells and can lyse allogeneic cells (36, 37). Also, NK cells have been shown to act as a barrier to tolerance induction and that their numbers increase upon infection in mice with γHV68 (31, 38). Therefore, we asked whether the recipient NK cells eliminate allogeneic donor BM immediately following BM infusion and treatment with the tolerance regimen. Naive or γHV68-infected B6 mice received allogeneic BM and the tolerance regimen as described above, along with anti-NK1.1 Ab or isotype control (31). As shown in Fig. 3, depletion of NK1.1 cells (left panel) at the time of BM engraftment failed to increase chimerism incidence in the latently infected group of mice (right panel), suggesting that γHV68-mediated resistance is not mediated by recipient NK1.1-positive cells.

Previously published results from Adams et al. suggested that sequential acute infection of mice with three different viruses (lymphocytic choriomeningitis virus, vaccinia virus, vesicular stomatitis virus) prevents chimerism and tolerance (19). Note that the effect we observed with latent infection is due to infection with a single virus that has the unique feature of establishing latency at different sites (20, 21, 39) and is amenable by ex vivo reactivation assay to measure the frequency of ex vivo virus reactivation (21, 27, 32, 40). To determine whether the costimulation blockade-based mixed chimerism tolerance-induction protocol triggered viral reactivation and resulted in resistance to tolerance induction, we used a limiting-dilution assay that has been used previously by other investigators (27, 29, 40, 41) to determine the frequency of ex vivo virus reactivation in cells obtained from γHV68-infected mice receiving components of the tolerance-induction protocol.
Interestingly, as shown in Fig. 4A, latently infected mice receiving allogeneic BM, CTLA4-Ig and anti-CD40L mAb, and busulfan showed more than a 5-fold increase in the frequency of ex vivo reactivation compared with latently infected mice that did not receive the tolerance-induction regimen and with latently infected mice receiving costimulation blockade alone. Also of interest, latently infected mice receiving costimulation blockade alone did not show a significant difference in ex vivo reactivation compared with latently infected mice. Furthermore, the γHV68-infected mice receiving the complete tolerance regimen (busulfan, costimulation blockade, and allogeneic BM) showed an increase in the preformed virus compared with latently infected mice or with mice receiving costimulation blockade (CTLA4-Ig and anti-CD40L mAb) alone (Fig. 4A). These findings suggest that the tolerance regimen used led to increased virus reactivation and preformed virus in peritoneal cells harvested from γHV68-infected B6 mice. The splenocytes harvested at this time point for ex vivo virus reactivation, as reported earlier (32, 40), failed to show the levels of reactivation (data not shown) required to determine the frequency of reactivating cells based on Poisson distribution (Fig. 4A, dotted horizontal line at 63.5%).

Because we observed increased ex vivo virus reactivation and increased levels of preformed infectious virus in busulfan and allogeneic BM recipient mice (Fig. 4A), we next examined whether infection of mice with a reactivation-defective γHV68 (γHV68-ORF72 mutant) (27) reconstitutes chimerism in latently infected mice receiving allogeneic BM and the tolerance regimen. The γHV68-ORF72 mutant has been shown to replicate normally in vitro and during acute infection in mice but was significantly compromised in its capacity to reactivate from latency (27). Naive or

FIGURE 3. Latent infection-associated resistance to allograft tolerance is not mediated by NK1.1 cells. Groups of naive (n = 10) or γHV68-infected B6 mice (n = 20) received allogeneic BM and the tolerance-induction regimen. Additionally, the γHV68-infected group also received either isotype control Ab (n = 10) or anti-NK1.1 Ab (n = 10) as described in Materials and Methods. Percentage chimerism (day 60 relative to tolerance-induction protocol; right panel) and NK1.1-cell depletion (days 8 and 16; left panel) was assayed in the peripheral blood. The decrease in percentage chimerism at day 60 in mice infected with γHV68 that received either isotype control Ab or anti-NK1.1 Ab along with the tolerance-induction regimen compared with naive uninfected mice was statistically significant as shown (*). NS (**), Not significant. Data from one of two independent experiments performed are shown.

FIGURE 4. A. Minimally myelosuppressive costimulation blockade-based tolerance-induction regimen leads to increase in frequency of virus-reactivating cells. Groups of mice (n = 5 mice/group) infected with γHV68 (day 40 p.i.) received allogeneic BM and/or the tolerance regimen (CTLA4-Ig + anti-CD40L antibody) and were assayed for quantitation of the frequency of peritoneal cells reactivating from latency at day 50 p.i. (day 10 relative to tolerance-induction protocol). The increase in frequency of virus-reactivating peritoneal cells as a result of administration of allogeneic BM and the tolerance regimen compared with latently infected alone or latently infected mice receiving costimulation blockade (CoB) was statistically significant as follows: latently infected mice receiving allogeneic BM and the tolerance regimen vs latently infected mice receiving costimulation blockade, p = 0.003; latently infected mice receiving allogeneic BM and the tolerance regimen vs latently infected mice, p = 0.0004. Data represent results from two independent experiments, with each experiment containing cells pooled from five mice. B, Infection with reactivation-defective γHV68 does not overcome latent infection-mediated barrier to allograft tolerance. Groups of naive or γHV68-infected B6 mice (day 40 p.i., wild-type γHV68 or reactivation-defective γHV68-ORF72 mutant) received allogeneic BM and the tolerance regimen. Percentage chimerism was analyzed in peripheral blood at day 60 relative to the tolerance-induction protocol. One of two independent experiments is shown.
Virus-primed CD8\(^+\) T cells demonstrate increased alloreactivity

The experimental results discussed above suggest that \(\gammaHV68\)-mediated resistance to allograft tolerance is not mediated by increased virus reactivation as a result of the tolerance regimen used or by the host NK cells. Previous work addressing the effects of viral infections on mixed chimerism-based tolerance induction in mice has suggested that sequential acute infection with different viruses inhibits chimerism due to virus-induced alloreactive memory T cells (19). A number of investigations in humans also have documented the influence of EBV-, CMV-, and HSV-specific T cells on the alloreactive T cell repertoire (42–45). Therefore, we tested whether \(\gammaHV68\)-infected mice show increased alloreactive T cells during the latent phase of infection. Groups of naive or \(\gammaHV68\)-infected B6 mice splenocytes were harvested at day 40 p.i. (the latent infection time point when resistance to tolerance induction was observed, see Fig. 2A) and tested for IFN-\(\gamma\) and TNF-\(\alpha\) production in response to allogeneic (BALB/c or C3H) splenocyte stimulation or \(\gammaHV68\) peptides (K\(^b\), ORF61/p79 and D\(^b\), ORF6/p56) as previously described (19, 46). Interestingly, \(\gammaHV68\)-infected mice showed a significant increase in the total numbers of CD8\(^+\) T cells producing IFN-\(\gamma\) and TNF-\(\alpha\) in response to allogeneic stimulation, indicating augmented CD8\(^+\) T cell alloreactivity in infected mice compared with noninfected mice (Fig. 5, B and C). Although not statistically significant when compared with syngeneic stimulation, \(\gammaHV68\)-infected mice showed an increase in the percentage and total number of CD8\(^+\) T cells producing IFN-\(\gamma\) and TNF-\(\alpha\) in response to allogeneic stimulation (Fig. 5). As expected, CD8\(^+\) T cells from both groups of mice demonstrated IFN-\(\gamma\) and TNF-\(\alpha\) production in response to PMA/ionomycin stimulation, whereas only the infected group of mice showed cytokine response to viral peptide stimulation.

Expansion of TCR V\(\beta^4\) CD8\(^+\) T cells is associated with \(\gammaHV68\)-mediated resistance to chimerism and tolerance induction

Based on our finding that CD8\(^+\) T cells demonstrate increased alloreactivity in mice latently infected with a \(\gamma\)-herpesvirus, we sought to determine the CD8\(^+\) T cell subsets responsible for latent infection-mediated resistance to tolerance induction. Previous reports have indicated that \(\gammaHV68\) infection in mice leads to a significant increase in the percentage of CD8\(^+\) T cells expressing a TCR V\(\beta^4\) chain (>50% in peripheral blood, >30% in spleen at...
day 21 p.i (47)) and that the expansion requires the latent virus (20, 47–49). Because the experimental approach of adoptive transfer of virus-induced CD8⁺ T cells into naive hosts to test resistance to allograft tolerance could have the potential to transfer low/undetectable levels of latent virus, we considered preventing expansion of a predominant subset of CD8⁺ T cells (TCR Vβ4⁺ CD8⁺ T cells) by using a γHV68 mutant (γHV68-M1 mutant (30)) that fails to expand Vβ4⁺ CD8⁺ T cells population (A. G. Evans and

**FIGURE 6.** Absence of Vβ4⁺ CD8⁺ T cell expansion in γHV68-M1 mutant-infected mice. Splenocytes from groups of naive or γHV68-infected (wild-type γHV68, γHV68-M1 mutant, γHV68-M1-MR, or γHV68-ORF72 mutant) B6 mice were analyzed for Vβ4⁺ CD8⁺ T cell expansion at day 60 relative to tolerance-induction protocol. Representative flow cytometry plots from each group (A) and percentage Vβ4⁺ CD8⁺ T cells with mouse numbers for each group (B) are shown. The decrease in percentage of Vβ4⁺ CD8⁺ T cells with γHV68-M1 mutant compared with wild-type γHV68, γHV68-M1-MR, and γHV68-ORF72 mutant was statistically significant as shown above (+). Data represent results from one of two independent experiments performed.

**FIGURE 7.** γHV68-M1 mutant-infected mice achieve costimulation blockade-mediated tolerance to allografts. Groups of naive or γHV68-infected (wild-type γHV68, γHV68-M1 mutant, or γHV68-M1-MR) B6 mice receiving allogeneic BM and the tolerance-induction regimen were analyzed for chimerism at 60 day relative to the tolerance-induction protocol and followed for skin graft survival after placing skin grafts on day 70 relative to tolerance-induction regimen. Representative flow cytometry plots for chimerism from each group (A), percentage chimerism with each dot representing an individual mouse under each group (B), and percentage skin graft survival for each group of mice (C) are shown. The increase in percentage chimerism (B) and skin graft survival (C) with γHV68-M1 mutant-infected mice compared with wild-type γHV68 group and γHV68-M1-MR-infected group were statistically significant as shown above (+). Data from one of two independent experiments are shown.
Furthermore, γHV68-M1 mutants (M1.LacZ, M1-Δ511, M1.Stop) are shown to establish latent infection comparable to wild-type γHV68, and they demonstrated an approximately 5-fold increase in efficiency of reactivation from latency (30). The data shown in Fig. 6, using a γHV68 mutant in which M1 ORF has been disrupted (M1-511, 511 bp deletion at the 5' end of the M1 ORF), confirm that M1 mutant viruses are defective in priming V\textsuperscript{β4}'CD8\textsuperscript{+} T cells. Interestingly, we found that infecting mice with γHV68-M1 mutant reverted the tolerance resistance phenotype in most of the infected mice to a graft-accepting phenotype (Fig. 6). In contrast, mice infected with wild-type γHV68, γHV68-M1-MR, or γHV68-ORF72 mutant led to efficient expansion of V\textsuperscript{β4}'CD8\textsuperscript{+} T cells (Fig. 6) and inhibition of chimerism (Figs. 4B and 7, A and B). Therefore, these results highlight the critical role of CD8\textsuperscript{+} T cells and a strong association of the virus-induced V\textsuperscript{β4}'CD8\textsuperscript{+} T cell expansion to latent infection-mediated resistance to allograft tolerance (47, 49, 50). The peripheral blood samples from γHV68-M1 mutant or wild-type γHV68-infected mice also were analyzed to determine whether the mutation in the γHV68-M1 gene also led to defective expansion of other candidate V\textsuperscript{β} family within the CD8\textsuperscript{+} or CD4\textsuperscript{+} T cell populations. We found that analysis of T cells bearing other TCR V\textsuperscript{β}-chains such as V\textsuperscript{β}6 CD8\textsuperscript{+} T cells and V\textsuperscript{β}4 CD4\textsuperscript{+} T cells in γHV68-M1 mutant or wild-type γHV68-infected mice did not show any significant change in these subsets at days 15, 30, and 60 after chimerism induction (data not shown).
experiments using infection of B6 mice with a γHV68 mutant (γHV68-M1 mutant) demonstrate that the γHV68-M1 gene confers resistance to allograft tolerance, implying that latent infection-mediated resistance to tolerance induction is associated with virus-induced Vβ4⁺CD8⁺ T cells. To determine the phenotypic characteristics of Vβ4⁺CD8⁺ T cells at peak of infection (day 21) and latent infection (days 40 and 157 p.i.), peripheral blood and splenocytes from wild-type γHV68-infected mice were assayed for CD8⁺ T cell subsets based on the markers described to characterize naïve (T naïve, CD44loCD127highCD62Lhigh), effector (T eff, CD44highCD127lowCD62Llow), and subsets of memory CD8⁺ T cell populations (central memory or T CM, CD44highCD127highCD62Lhigh and effector memory or T EM, CD44highCD127highCD62Llow/lo) (28). Before our experiments, a similar phenotypic analysis had been performed by A. G. Evans et al. (A. Evans and S. Speck, unpublished observations), and, as shown in Fig. 8A, Vβ4⁺CD8⁺ T cells attain an “effector” phenotype at the peak of infection, maintain T eff phenotype at day 40 p.i., and transition to T EM phenotype by day 157 p.i. in peripheral blood (Fig. 8A) and spleen (data not shown). To understand the mechanisms by which expansion of Vβ4⁺CD8⁺ T cells lead to virus-induced resistance to tolerance induction, we determined the alloreactive potential of virus-induced Vβ4⁺CD8⁺ T cells for their ability to produce IFN-γ in response to allogeneic stimulation (BALB/c or C3H) at the time point when we observed γHV68-mediated resistance to tolerance induction (day 40 p.i.). As shown in Fig. 8B, we found that compared with all other Vβ family of CD8⁺ T cells, splenocytes harvested from day 41 p.i. had increased numbers of Vβ4⁺CD8⁺ T cells that produced IFN-γ as well as TNF-α in response to allogeneic stimulation (BALB/c or C3H splenocytes). Furthermore, compared with Vβ4⁺CD8⁺ T cells, Vβ4⁺CD8⁺ T cells produced significantly higher levels of IFN-γ in response to viral peptide (p56/p79) stimulation. Taken together, these results demonstrate that the cell types associated with latent infection-mediated resistance to tolerance induction (i.e., the virus-induced Vβ4⁺CD8⁺ T cells) demonstrate an effector/effecter memory phenotype and produce IFN-γ in response to allogeneic stimulation. On the whole, this is the first demonstration that latent infection with γHV68, a murine γ-herpesvirus closely related to EBV and KSHV, interferes with tolerance induction and that the resistance requires the presence of γHV68-M1 gene and is associated with an increase in alloreactivity of virus-induced effector/effecter memory Vβ4⁺CD8⁺ T cells.

Discussion

In this report, we examined the effects of latent infection on transplantation tolerance using a mixed chimera-based tolerance-induction protocol. In this model, infection of mice with a candidate herpesvirus bearing significant structural homology to EBV and KSHV interfered with tolerance to allogeneic BM and skin grafts. The virus-induced resistance was not due to increased reactivation because the use of a reactivation-defective virus failed to improve chimeraism incidence or levels in γHV68-infected mice. Our results also show that a γHV68 mutant that has been previously shown to reactivate with higher reactivation efficiency (γHV68-M1 mutant) (30) did not show resistance to allograft tolerance. These findings suggest that increased efficiency of virus reactivation and/or persistence of persistent lytic virus are not associated with latent infection-mediated resistance to tolerance induction. Furthermore, we found that reconstitution of chimeraism as a result of infection with γHV68-M1 mutant was due to the inability of infected mice to expand a subset of CD8⁺ T cells (TCR Vβ4⁺CD8⁺ T cells) that demonstrated effector (day 40 p.i.) and effecter memory (day 157 p.i.) phenotype and increased alloreactivity.

Recent studies by a number of investigators have led to the recognition that the immune history of a transplant recipient may be a major determinant of the success or failure of tolerance-induction strategies (17, 51–54). Studies in the murine model have addressed the effects of acute infection (nonpersistent) using infection with single or multiple pathogens (17, 19). So far, no studies have addressed the effects of true latent infection on transplant tolerance. In this study, we used a clinically relevant herpesvirus homolog and convincingly demonstrate the ability of latent infection at two time points postinfection (days 40 and 120 p.i.) to resist a mixed chimeraism-based allograft tolerance regimen. However, a smaller number, ~20% of mice infected with the virus became chimeric and accepted allogeneic skin grafts. Given the previous findings from Hardy and colleagues on the CDR3 size analysis of the Vβ4⁺CD8⁺ T cells (55), it is tempting to speculate that latently infected chimeric mice may have distinct clonotypes of Vβ4⁺CD8⁺ T cells that are or are not involved in resistance to tolerance. Note that the effect we observed with latent infection is due to infection with a single virus that has the unique feature of establishing latency at different sites (20, 21, 32) and is amenable by limiting dilution ex vivo reactivation assay to measure the frequency of ex vivo virus reactivation (21, 29). Using this assay, we found that latently infected mice receiving allogeneic BM and the tolerance regimen showed a significantly higher frequency of reactivating cells compared with infected mice receiving costimulation blockade, suggesting that the conditioning protocol involving the use of busulfan or the infusion of allogeneic BM triggers an increase in frequency of reactivating cells in the infected mice. It will be interesting to test other, previously proven deletional and nonchimerism regimens (14–16) for the effects of latent infection on resistance to prolonged graft survival, expansion of tolerance resistant-associated Vβ4⁺CD8⁺ T cells, and frequency of cells capable of virus reactivation.

In the mechanistic studies reported here, we first considered virus reactivation, a unique feature of herpesviruses, as the possible mechanism by which latent virus could cause resistance to tolerance induction. Previous studies in humans and preclinical animal models have demonstrated increased herpesvirus reactivation in transplant recipients as a result of immunosuppressive drug regimen (1, 2, 4, 56, 57). Experimental evidence also indicates a positive correlation between immunosuppression and herpesvirus reactivation in transplant recipients. We found that limiting the efficiency of virus reactivation as a result of our tolerance-induction protocol played a role in inducing the tolerance resistance phenotype seen in our studies, we tested a previously described γHV68 mutant that reactivates less efficiently (27, 60) for its ability to induce resistance to allograft tolerance. Our anticipation was that a severe defect in virus reactivation would allow mice receiving allogeneic BM to achieve chimeraism and tolerance to allografts. We found that limiting the efficacy of virus reactivation failed to induce chimeraism in allogeneic BM recipient mice. Moreover, our finding that a mutant virus previously shown to reactivate approximately 5-fold (30) more efficiently in B6 mice did not induce resistance to tolerance further support that virus reactivation does not have a role in resistance to tolerance induction.

Because virus reactivation did not account for the tolerance resistance phenotype observed with γHV68-infected mice, we questioned the possible contribution of the role of the innate immunity in γHV68-mediated interference with tolerance induction. Previous work from Kean et al. showed that NK cells represent a potent barrier to engraftment of allogeneic BM (31). Also, studies have shown that NK cells respond early after infection and that certain viral infections can strongly stimulate NK cells and can lyse allogeneic cells (36, 37). With respect to γHV68 infection, Usherwood
et al. found that even though NK cells contributed little to control of infection, their numbers increase upon infection in mice with γHV68 (38). Therefore, in the current study, we tested whether depletion of NK1.1 cells reconstitutes chimerism in latently infected mice. Even though NK1.1 cell depletion was effective up to 2 wk, latently infected mice failed to achieve chimerism, indicating that NK1.1 cells are not responsible for preventing tolerance in this model. It is possible that other antiviral innate immune mediators such as IFN-α/β, previously shown to be involved in regulation of γHV68 latency (61), may have a role latent infection-mediated resistance to allograft tolerance.

Alloreactive T cells play an important role in mediating allograft rejection (62), and investigations in murine models have documented the influence of virus-specific T cells on the alloreactive T cell repertoire. For example, studies by Brehm and colleagues established the cross-reactive responses between lymphocytic choriomeningitis virus-specific T cells and allogeneic Ags from different haplotypes (18). Another study by Adams et al. used sequential infection of mice with multiple pathogens and demonstrated that virally induced alloreactive memory T cells (heterologous immunity) inhibit donor-specific tolerance (19). Also, in humans, beginning with the observations by Burrows et al. showing the dual specificity of CTL clones for the EBV epitope (EBNA3A, FLRGRAYGL) presented on the HLA-B8 with alloantigen (HLA-B4402) (42), there have been other studies in herpesvirus seropositive individuals demonstrating the dual specificity of virus-specific T cells with alloantigen: 1) HLA-DR7-restricted human CMV-gB-specific CD4+ T cells to alloantigen HLA-DR4 (43), and 2) HLA-A201-restricted HSV-2-specific CD8+ T cells to alloantigen HLA-B44 (44). However, the molecular and structural basis for the dual specificity (cross-reactivity) has been less clear. A recent study by Archbold et al. used avidity measurements, peptide substitution studies, and x-ray crystallography and showed structural mimicry as the mechanism of the cross-reactive T cell response between the FLRGRAYGL complexed to HLA-B08 and the alloantigen HLA-B35 (45). The vast majority of these studies have been performed in vitro with T cell clones from herpesvirus-seropositive individuals, and the in vivo significance of the observed dual specificity of herpesvirus-specific T cells has not yet been explored. To determine whether increased allosreactivity due to γHV68 infection played a role in latent infection-mediated inhibition of tolerance, we established whether latently infected mice manifested increased allosreactivity in vitro in response to allogeneic stimuli and found that CD8+ T cells produced increased levels of IFN-γ and TNF-α when stimulated with allogeneic splenocytes. To identify the CD8+ T cell subset involved in conferring resistance, based on unpublished observations by A. G. Evans and S. H. Speck, we considered preventing expansion of a predominant subset of CD8+ T cells (TCR Vβ4+CD8+ T cells) by using a γHV68 mutant (γHV68-M1 mutant (30)) that fails to expand the population of Vβ4+CD8+ T cells. This approach was chosen because the conventional approach of adoptive transfer of virus-induced CD8+ T cells to test resistance to allograft tolerance could have the potential to transfer low/undetectable levels of latent virus in addition to issues with transfer efficacy and cell numbers. In the experiments presented here, using the γHV68-M1 mutant, we found that prevention of the expansion of Vβ4+CD8+ T cells by infection of B6 mice with the γHV68-M1 mutant successfully reconstituted tolerance to allografts.

During the past several years, major advances have helped unravel the intricacies of T cell memory. Memory T cells are phenotypically and functionally distinct from their naive counterparts, and the most detailed studies to date have characterized CD8 memory T cells, which are often segregated into two subsets, central memory (Tcm) and effector memory (Tem) (28). Because the γHV68-induced resistance to tolerance induction was dependent on the CD8+ T cells carrying the TCR Vβ4+ chain, we analyzed the phenotype and allosreactive potential of the tolerance resistance-associated cell types and found that Vβ4+CD8+ T cells maintained an effector/effector memory phenotype and demonstrated increased levels of IFN-γ in response to allogeneic stimulation at the time point when resistance to allograft tolerance was observed. Although we do not know the precise requirements of IFN-γ and other CD8+ T cell effector molecules (perforin, granzyme B) or the contribution of Tem vs Tcm cells for the Vβ4+ CD8+ T cell-associated phenomenon, note that while absent at birth, memory T cells compromise 40–50% of the T cell pool in socially housed nonhuman primates by 3 years of age and in adult humans (63–65). This is consistent with studies in patients who after undergoing aggressive T cell depletion with pretransplant alemtuzumab therapy went on to have clinical rejection episodes and did so with a peripheral expansion of Tem cells only (66). Furthermore, both γHV68 and EBV elicit a large T cell response resulting in an expanded Tem pool (50, 67). Although EBV does not have a γHV68-M1 homolog, the fact that 1) the two γ-herpesviruses have a large complex homologous genome that encodes a number of proteins in latently infected B cells (20, 23, 68), 2) both elicit a large T cell response that results in an effector memory pool (50, 67), 3) the CD8+ T cell response appears to be preferentially focused on only a few epitopes (46, 47, 49, 69–71), 4) both are associated with tumor development (72–74), and that 5) EBV does not infect inbred strains of mice strongly suggest the utility of γHV68 infection of mice as a model to study the effects of latent infection on transplantation tolerance.

In the studies presented herein, we used γHV68, a homolog of human viral pathogen, to study the influence of latent infection on allograft tolerance induced by a mixed chimerism tolerance regimen. Our recent experiments also indicate that latent infection can interfere with allograft tolerance induction in a mixed chimerism-independent tolerance regimen (S. Selvara and S. Gangappa, unpublished observations). Previous works from other investigators suggest that infection of mice with murine CMV, a homolog of human CMV, establishes latent infection in myeloid cells (75–77), and, interestingly, immune response to two epitopes (m139, IE3) retained an effector memory phenotype (78). Because the viral persistence (79), latent gene expression (80, 81), reactivation sites (79), reactivating cell types (77, 82), and antiviral immune responses (78, 83) for mCMV and γHV68 infection are distinct (20, 23, 29, 32, 39, 40, 84, 85), the influence of these widely prevalent latent pathogens on allograft function/survival and mechanisms are expected to be diverse and distinct and will be an interesting area of study. Considering the observations that CD8+ T cell response in the two phases (primary and persistent) of EBV infection in humans is qualitatively different (67, 86), and the studies showing CTL response to primary EBV infection appear to be focused on only a few epitopes (EBV-encoded nuclear Ags 3, 4, and 6) (69, 70), together with limited TCR-Vβ gene usage and a high degree of clonality in both CD4+ and CD8+ T cell responses to CMV-pp65 matrix protein (87) in chronically infected individuals, the identification of the allo-cross-reactive latent pathogen-specific memory T cells, their requirement for latent virus control, and their suppression/elimination by treatment or vaccination against allosreactivity are critical to minimize allograft loss and improve host survival. The information acquired from such studies not only will lead to a knowledge-based reduction of the risks related to latent infections in transplant patients harboring a multitude of latent pathogens, but it also could help in designing clinically useful tolerance-induction regimens by plummeting the
magnitude and/or function of virus-induced cross-reactive T cell subsets responsible for direct or indirect interference with tolerance induction.

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Disclosures

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