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ORIGINAL ARTICLE

Acute murine cytomegalovirus disrupts established transplantation tolerance and causes recipient allo-sensitization

Shuangjin Yu[1,2] | Anil Dangi[1] | Melanie Burnette[1] | Michael M. Abecassis[3] | Edward B. Thorp[4] | Xunrong Luo[1,5]

[1]Division of Nephrology, Department of Medicine, Duke University School of Medicine, Durham, North Carolina

[2]Division of Organ Transplantation, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China

[3]College of Medicine, University of Arizona, Tucson, Arizona

[4]Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, Illinois

[5]Duke Transplant Center, Duke University School of Medicine, Durham, North Carolina

Correspondence
Xunrong Luo
Email: xunrong.luo@duke.edu

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We have previously shown that acute cytomegalovirus (CMV) infection disrupts the *induction* of transplantation tolerance. However, what impact acute CMV infection would have on the *maintenance* of established tolerance and on subsequent recipient allo-sensitization is a clinically important unanswered question. Here we used an allogeneic murine islet transplantation tolerance model to examine the impact of acute CMV infection on: (a) disruption of established transplantation tolerance during tolerance maintenance; and (b) the possibility of recipient allo-sensitization by CMV-mediated disruption of stable tolerance. We demonstrated that acute CMV infection abrogated transplantation tolerance during the maintenance stage in 50%-60% recipients. We further demonstrated that acute CMV infection–mediated tolerance disruption led to recipient allo-sensitization by reverting the tolerant state of allo-specific T cells and promoting their differentiation to allo-specific memory cells. Consequently, a second same-donor islet allograft was rejected in an accelerated fashion by these recipients. Our study therefore supports close monitoring for allo-sensitization in previously tolerant transplant recipients in whom tolerance maintenance is disrupted by an episode of acute CMV infection.

KEYWORDS
alloantibody, basic (laboratory) research/science, immunobiology, immunosuppression/immune modulation, infection and infectious agents - viral: Cytomegalovirus (CMV), islet transplantation, rejection: T cell–mediated (TCMR), re-transplantation, tolerance

1 | INTRODUCTION

Organ transplantation is an effective therapy for end-stage organ diseases.[1] Although nonspecific immunosuppressants are the mainstay of anti-rejection therapy, they cause significant comorbidities. Consequently, tolerance-induction strategies have been devised and investigated, with a few showing promise in clinical trials.[2-7] In this context, it is becoming increasingly important to study how inadvertent pathogen infections might affect the outcome of transplantation tolerance.

Abbreviations: DCs, dendritic cells; DSAs, donor-specific antibodies; DST, donor-specific transfusion; ECDI-SP, donor splenocytes treated with ethylenecarbodiimide; eDCs, enrich dendritic cells; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MDSC, myeloid-derived suppressor cell; MLRs, mixed lymphocyte reactions; PAMPs, pathogen-associated molecule patterns; PRRs, pattern-recognition receptors; SD, standard deviation; TCR, T cell receptor; T_{FH}, T follicular helper cells.

Shuangjin Yu and Anil Dangi are co-first authors.

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A frequent clinically encountered opportunistic pathogen in transplantation is cytomegalovirus (or CMV). Using a murine model of CMV infection, we have previously reported that acute MCMV infection abrogates the *induction* of transplantation tolerance by priming alloreactive CD8 T cells through altering myeloid-derived suppressor cell (MDSC) differentiation and promoting their maturation to inflammatory monocytes capable of cross-presentation.[8] Clinically, numerous other bacterial and viral infections have also been known to correlate with allograft rejection[9-13] via additional mechanisms including activation of alloreactive T and B cell responses.[14-17]

Although our proof-of-concept study of acute CMV infection on tolerance *induction* has provided important insights regarding this issue, a far more clinically relevant question is how CMV infection might impact the *maintenance* of established tolerance, since recipients are far more likely to encounter an inadvertent infection during the considerably longer tolerance maintenance period. In addition, if acute rejection is precipitated by an episode of infection during tolerance maintenance, the consequence of tolerance disruption on the future transplantability of the recipients is an immediately important question. Here, possibilities may range from spontaneous reemergence of tolerance after the infection has abated, to recipient allo-sensitization significantly challenging future transplants. Recipient allo-sensitization is a status in which allo-reactive T cells and/or anti-HLA antibodies already exist pretransplant.[18] It is commonly caused by previous transplantations, blood transfusions, or pregnancies. Of interest it is reported that loss of anti-CD154/donor-specific transfusion (DST)–induced transplantation tolerance during its maintenance stage following an infection due to *Listeria monocytogenes* is unexpectedly transient, and is not associated with recipient allo-sensitization.[19] Consequently, a second same-donor transplant is spontaneously accepted in this model provided that the temporary heightened allo-reactivities triggered by the infection have waned.[19] It would therefore be clinically important to determine if this phenomenon occurs with other relevant pathogens in other transplant tolerance settings.

Here we studied the impact of MCMV infection on the maintenance of transplantation tolerance in an allogeneic islet transplant model. We used an established murine model of transplantation tolerance in which DST is induced by recipient peri-transplant infusions of donor splenocytes treated with a chemical cross-linker ethylenecarbodiimide (ECDI-SP).[20,21] We found that during the maintenance stage of stable transplantation tolerance, MCMV infection led to acute allograft rejection in ~50%-60% of tolerized recipients. Concurrently, alloreactive CD4 and CD8 T cell immune responses initially inhibited by ECDI-SP were restored by MCMV infection. It is important to note that these T cells also developed a memory phenotype and rapidly responded to same-donor re-stimulation. Consequently, a second same-donor islet allograft transplanted in these recipients was rejected in an accelerated fashion.

2 | MATERIALS AND METHODS

2.1 | Mice

Eight- to 12-weeks-old male C57BL/6(B6; H2b), BALB/c(H2d) were purchased from Jackson Laboratory. 4C mice[22] were provided by Dr Qizhi Tang (UCSF). Mice were used per protocols approved by the Duke Institutional Animal Care and Use Committee.

2.2 | Diabetes induction and islet transplantation

B6 mice were injected with streptozotocin (Sigma Aldrich) at 170 mg/kg. Diabetes was defined by two consecutive glucose levels >250 mg/dL. Islet transplantation were performed as described.[20] In some recipients, a second BALB/c islet graft was transplanted to the contralateral kidney. Graft function was determined by OneTouch glucometer. Graft rejection was defined as two consecutive glucose levels >250 mg/dL.

2.3 | Tolerance induction by donor ECDI-SP

BALB/c splenocytes were treated with ECDI as described.[23] Briefly, BALB/c splenocytes (SPs) were incubated with ECDI (Calbiochem, 150 mg/mL per 3.2×10^8 cells) on ice for 1 hour with agitation followed by washing. 10^8 BALB/c ECDI-SP were injected intravenously to recipients on day −7 and day +1.

2.4 | MCMV infection

Mice were infected intraperitoneally with the MCMV strain Δm157 (10^8 plaque-forming units) on indicated days. The Δm157 lacks the m157 glycoprotein recognized by the natural killer cell receptor Ly49H, and thus has improved virulence in B6 compared with wild-type MCMV.[24]

2.5 | Adoptive transfer of T cells

T cell receptor (TCR) transgenic 4C ($CD90.1^+$ $V\beta13^+$) CD4 T cells[22] were purified from the spleen of 4C mice, labeled with 10 μM V450 (eBioscience), and injected intravenously into B6 ($CD90.2^+$) mice.

2.6 | Mixed lymphocyte reactions (MLRs)

B6 splenic T cells were purified and labeled with 10 μM V450, plated at 1×10^5 per well, and stimulated with 1×10^5 BALB/c enriched dendritic cells (eDCs) obtained as described.[25]

2.7 | Flow cytometry

Cells were incubated with fluorochrome-conjugated antibodies for 30 minutes on ice. For intracellular staining, cells were permeabilized using Cytofix/Cytoperm buffers (BD) before staining. The following antibodies were used: CD3-BUV 396(UCHT1), CD4-BV650(RM4.5), CD90.1-FITC(OX7), CD90.2-PE-Cy7(53-2.1), CD127-PE(SB/199), all from BD Bioscience; and CD8a-Percp-Cyanine5.5(53-6.7), CD44-APC-eFluor 780(IM7), IFN-γ-APC(XMG 1.2), Dead cells-Aqua live/dead dye, all from eBioscience.

2.8 | Donor-specific antibody (DSA) quantification

Donor-specific antibodies (DSAs) were quantified as described.[20] Briefly, BALB/c splenocytes were incubated with recipient serum for 1 hour on ice and then washed and stained with anti-B220-PE and FITC-conjugated anti-IgG1, anti-IgG2b, or anti-IgG3 mAbs (BD PharMingen). Naive B6 serum was used as a negative control. Serum from a presensitized B6 was used as a positive control. DSA levels were compared between groups after excluding dead and $B220^+$ B cells.

2.9 | Statistical analysis

Graft survival was compared using Kaplan-Meier survival curves with log-rank test. Student's *t* test or analysis of variance (ANOVA) was used to compare means of groups. All statistical analyses were performed in GraphPad Prism 7.0. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Acute MCMV infection disrupts established transplantation tolerance

We first investigated the impact of acute MCMV infection on established transplantation tolerance. As shown in Figure 1A, diabetic C57BL/6 recipients were tolerized with 1×10^8 BALB/c ECDI-SP injections on day −7 and day +1,[20] and grafted with BALB/c islets on day 0. We have previously shown that with donor ECDI-SP injections, stable DST is established by day 60-90 following the first transplantation.[20] However, we were interested in testing the feasibility of shortening our experimental cycle by examining if tolerance can be stably established at an earlier time point. Therefore, we administrated MCMV ($\Delta m157^{24}$) infection on day 14 or 95 posttransplant, and graft outcome was compared to that when MCMV infection was given on day 0 as we published previously.[8] As shown in Figure 1B, acute MCMV infection on either day 14 or 95 led to graft rejection in 50%-60% of the infected recipients over a period of 8-40 days postinfection. The rate and the kinetics of graft rejection were indistinguishable between MCMV infection given either on day 14 or 95, although both were significantly lower or delayed when compared with day 0 infection (~80%, between 8 and 22 days postinfection; Figure 1B). Because of similar rejection rate and kinetics by MCMV infection on either day 14 or 95, for all subsequent experiments, we chose to give MCMV infection on day 14.

3.2 | Second same-donor transplant is rejected in an accelerated fashion

In recipients having rejected their first islet allograft by MCMV infection on day 14, we retransplanted them with a second BALB/c

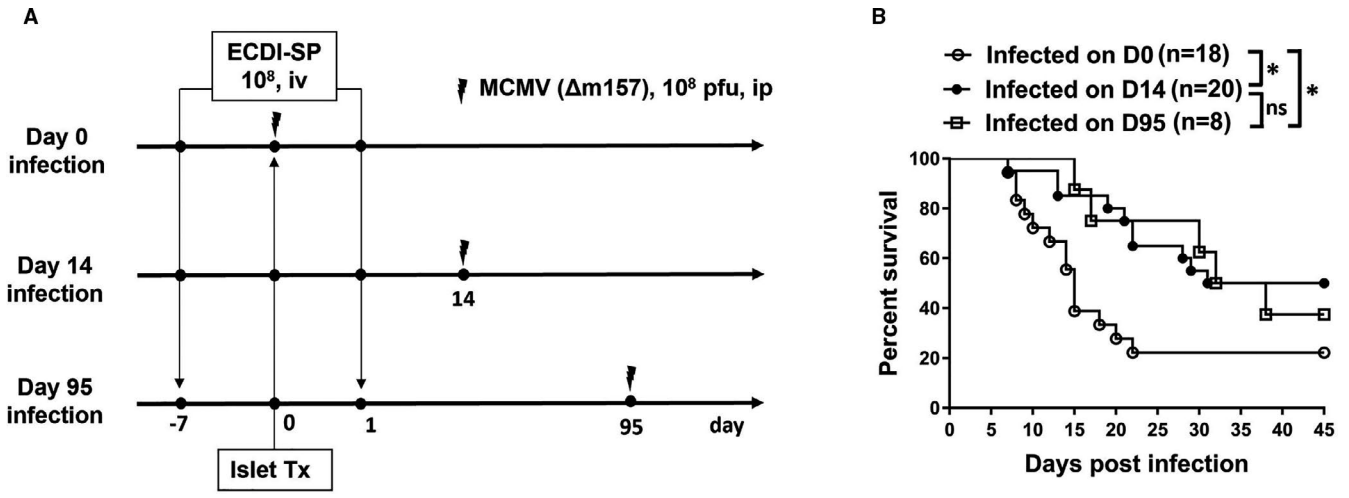

FIGURE 1 Islet allograft survival in donor ECDI-SP–tolerized recipients acutely infected with MCMV on day 0, day 14, and day 95. A, Schematic treatment timeline of different groups. Islet transplantation was performed on day 0 from BALB/c donors to diabetic B6 recipients. Transplantation tolerance was induced by BALB/c ECDI-SP infusions on day −1 and day 7. Recipients were infected with 1×10^8 pfu of the MCMV strain Δm157 intraperitoneally on day 0, day 14, or day 95. B, Islet allograft survival (y axis) plotted as a function of "days post infection" (x axis). ip, intraperitoneal injection; iv, intravenous injection; pfu, plaque forming unit; Tx, transplantation. N = 8-20 in each group. $^*P < .05$; ns = no significance

islet allograft 10 days later (Figure 2A, Tol+MCMV+Rej group). We anticipated possible outcomes to range from spontaneous recovery of ECDI-SP–induced tolerance allowing acceptance of the second islet allograft, to progression to anti-donor memory responses precipitating accelerated rejection of the second graft. As a tolerant control, we transplanted a second BALB/c islet allograft in ECDI-SP–tolerized recipients without MCMV infection (Figure 2B, Tol group). We further transplanted naive B6 recipients with BALB/c islet allografts (without ECDI-SP) as a control for rejection kinetics in unsensitized, untolerized recipients (Figure 2B, Naive group). As we can see, despite being previously tolerized by donor ECDI-SP, all recipients who rejected their first BALB/c islet allograft in the setting of acute MCMV infection rejected their second BALB/c islet allograft in an accelerated fashion (open square), in comparison to the rejection kinetics in unsensitized recipients (filled circle). In contrast, tolerant recipients without acute MCMV infection were able to accept the second BALB/c islet allograft indefinitely without further intervention (filled square). We have reported that following acute MCMV infection in islet transplant recipients, MCMV DNA in the islet allograft or the spleen is cleared by day 7 postinfection, and that MCMV-induced heightened IFN-α level in circulation peaks by day 2 and subsides by day 4.[8] Therefore, we do not think that the accelerated rejection of the second islet allograft was due to an ongoing anti-viral immune response, but rather due to recipient heightened anti-donor immune responses resulting from rejection of the first islet allograft in the setting of acute MCMV infection.

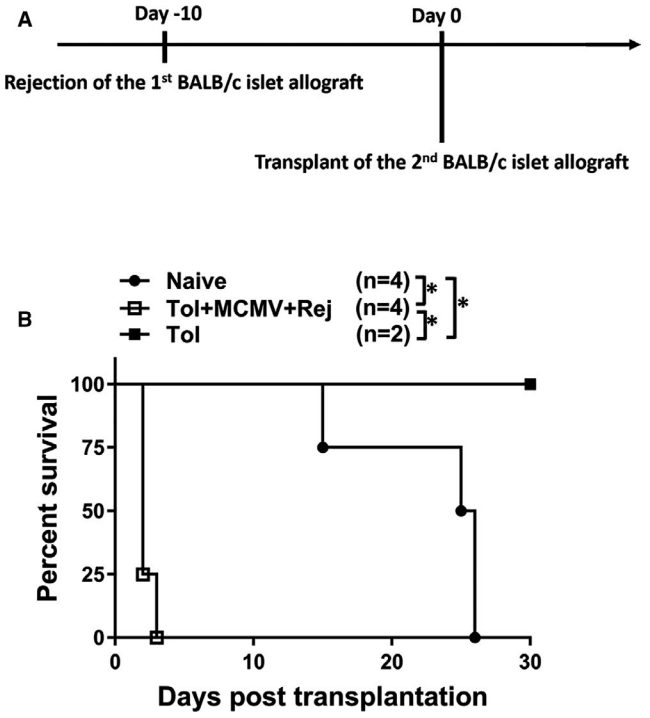

FIGURE 2 Survival of the second BALB/c islet allograft in previously tolerized recipients rejecting their first BALB/c islet allograft in the setting of MCMV infection. A, Schematic timeline of the second islet transplantation. Tolerized recipients were infected with MCMV and subsequently rejected their BALB/c islet allograft (indicated as "Day −10"). Ten days later (indicated as "Day 0"), a second BALB/c islet allograft was transplanted underneath the kidney capsule of the contralateral kidney (the "Tol-MCMV-Rej" group). As a tolerant control, we contemporaneously retransplanted a second BALB/c islet allograft into tolerized recipients without an episode of acute MCMV infection (the "Tol" group). Naive B6 recipients were also transplanted with BALB/c islet allografts without any treatment (the "Naive" group) for comparison of rejection kinetics. B, Islet allograft survival (y axis) plotted as a function of "days post transplantation" of the second BALB/c islet allograft (x axis). N = 2-4 in each group. *$P < .05$

3.3 | Cellular immunity during accelerated graft rejection indicates recipient sensitization

To determine the T cell and humoral immune responses during rejection of the first BALB/c islet allograft in tolerized recipients experiencing acute MCMV infection, we examined graft-infiltrating cells and circulating DSAs at the time of rejection of the first islet allograft. To do so, we sacrificed the recipients as soon as rejection of the first BALB/c islet allograft in the setting of acute MCMV infection was confirmed. The islet allografts and sera were collected and analyzed. This group was labeled as "Tol+MCMV+Rej." Contemporaneously, a tolerized recipient *without* acute MCMV infection in which the transplanted BALB/c islet allograft was stably functioning was also sacrificed for comparison (Tol group). As shown in Figure 3A, at the time of rejection, the number of graft-infiltrating CD4 and CD8 T cells was significantly higher in the Tol+MCMV+Rej group in comparison to those in the control Tol group. However, DSA levels in all IgG sub-classes were essentially undetectable in both groups (Figure 3B). Collectively, these results suggest that acute rejection of the first islet allograft precipitated by acute MCMV infection in tolerant recipients is mediated by T cell immunity, not by humoral immunity.

We next examined the T cell and humoral immune responses during the accelerated rejection of the second BALB/c islet allograft in retransplanted recipients. To do so, we sacrificed recipient mice on day 3 posttransplant of the second BALB/c islet allograft, and collected their second islet grafts as well as sera for analysis. Similarly, tolerized recipients without acute MCMV infection in which the transplanted first BALB/c islet allograft was stably functioning were also transplanted with a second BALB/c islet allograft, and sacrificed on day 3 posttransplant for comparison. An additional "naive" group was also transplanted for comparison in which a BALB/c islet allograft was transplanted to naive B6 recipients and sacrificed on day 3 posttransplant. This additional control would allow us to compare the magnitude of early (day 3) immune responses in naive recipients vs those in previously tolerized recipients who subsequently rejected a donor graft in the setting of MCMV infection. As shown in Figure 3C, on day 3 posttransplant, the number of intragraft CD4 and CD8 T cells was significantly higher in the Tol+MCMV+Rej group than those in the Tol group or the naive group. These data indicate that although graft rejection eventually resulted in both Tol+MCMV+Rej and naive groups, T cell graft infiltration occurred

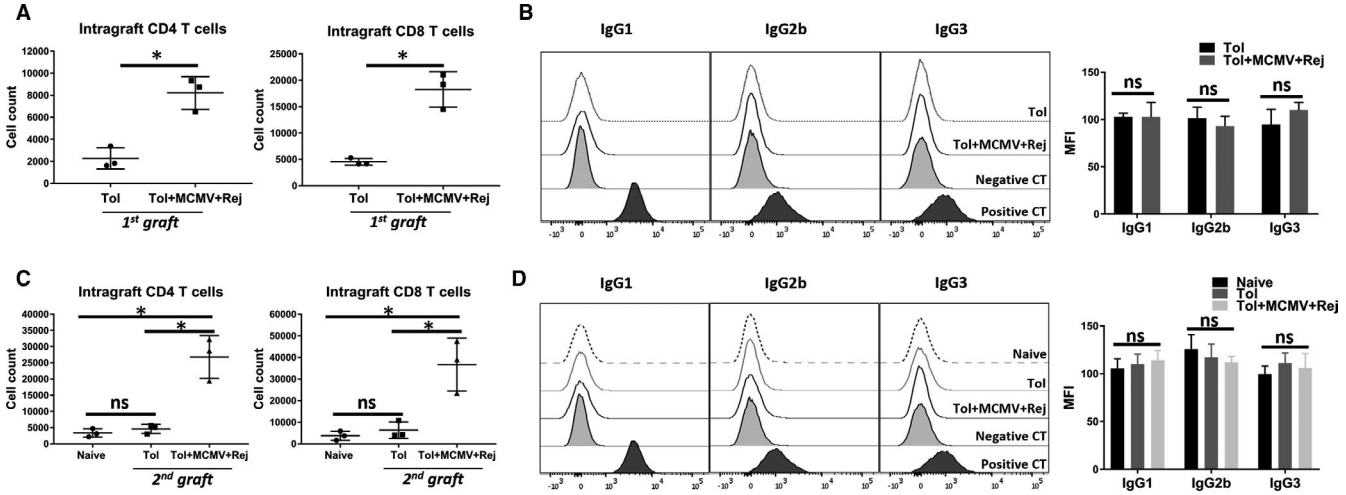

FIGURE 3 Cellular immunity during accelerated graft rejection indicates recipient sensitization. A,B, Tolerant recipients infected with MCMV on day 14 post the first BALB/c islet transplant were sacrificed as soon as graft rejection was confirmed (the "Tol+MCMV+Rej" group). Tolerant recipients without MCMV infection (the "Tol" group) were sacrificed contemporaneously as the control. Islet allografts and sera were collected at the time of sacrifice, and examined for graft-infiltrating T cells (A) and circulating DSAs (B). C,D, Recipients retransplanted with a second BALB/c islet allograft as outlined in Figure 2A were sacrificed on day 3 post the second transplant. The second islet allografts and sera were collected at the time of sacrifice, and examined for graft-infiltrating T cells (C) and circulating DSAs (D). An additional "naive" group was also transplanted for comparison in which a BALB/c islet allograft was transplanted to naive B6 recipients and sacrificed on day 3 posttransplant. Data are presented as mean ± SD in all scatter plots and bar graphs. N = 3 in all groups. MFI, mean fluorescent intensity. $^*P < .05$; ns = no significance

much faster in the Tol+MCMV+Rej group than in the naive group. DSAs in all IgG sub-classes, on the other hand, were again undetectable in all groups (Figure 3D), indicating that the rapid rejection of the second islet allograft in the Tol+MCMV+Rej group was not due to humoral immunity. Collectively, these results suggest that T cell, but not humoral, sensitization has occurred in the setting of acute MCMV infection, leading to accelerated rejection of the same donor graft when the recipient is retransplanted.

3.4 | MCMV infection reverts the inhibitory effects of donor ECDI-SP on allo-specific T cells

Next we determined the impact of MCMV infection on the tolerizing effect of donor ECDI-SP on donor-specific T cell alloimmune responses. As schematically shown in Figure 4A, B6 mice were injected with 1×10^8 BALB/c ECDI-SP on day −7 and +1, and allo-stimulation was provided by intraperitoneal injection of 1×10^6 fresh BALB/c SPs on day 0. MCMV infection was given on day +14. Mice were sacrificed on day 21, and their splenic T cells were purified for BALB/c-stimulated mixed lymphocyte reactions (MLRs). Comparison was made to T cells isolated from similarly treated B6 mice but without MCMV infection. As shown in Figure 4B (upper panels, representative histograms; lower panels, scatter plots showing means and standard deviations), BALB/c-stimulated T cell proliferation was appreciably inhibited by donor ECDI-SP, but was significantly augmented by host MCMV infection on day 14.

To directly interrogate donor-specific T cells, we took advantage of the 4C TCR transgenic CD4 T cells. 4C mice are on the B6 background and the 4C CD4 T cell TCR carries a vβ13 chain that specifically recognizes intact BALB/c MHC-II I-A^d to initiate direct anti-donor alloimmune response.[22] As schematically shown in Figure 4C, we adoptively transferred 5×10^5 V450-labeled CD90.1$^+$ 4C CD4 T cells to B6 mice on day −9, followed by a similar experimental timeline as in Figure 4A. Mice were sacrificed on day 21, and the in vivo 4C cell response to BALB/c stimulation on day 0 was directly analyzed. Comparison was made to 4C CD4 T cells adoptively transferred to similarly treated B6 mice but without MCMV infection. An additional comparison was made to 4C CD4 T cells adoptively transferred to B6 mice only stimulated with BALB/c splenocytes on day 0 without either ECDI-SP infusions or MCMV infection (untreated control). As shown in Figure 4D-F, in comparison to those in the untreated control, donor ECDI-SP treatment significantly reduced the 4C CD4 T cell number in the spleen (Figure 4D), their in vivo proliferation (Figure 4E), and IFN-γ production (Figure 4F) in response to day 0 BALB/c splenocyte stimulation. However, all of these effects by donor ECDI-SP treatment were reversed by host MCMV infection on day 14.

3.5 | Recipient splenic T cells following MCMV infection exhibit memory phenotype and function

We next analyzed the phenotype of splenic T cells in previously tolerized hosts following MCMV infection and graft rejection ("Tol+MCMV+Rej" group) on day 10 post graft rejection. Splenic T cells from ECDI-SP–only treated recipients without MCMV infection ("Tol" group) or from naive B6 mice ("Naïve" group) were

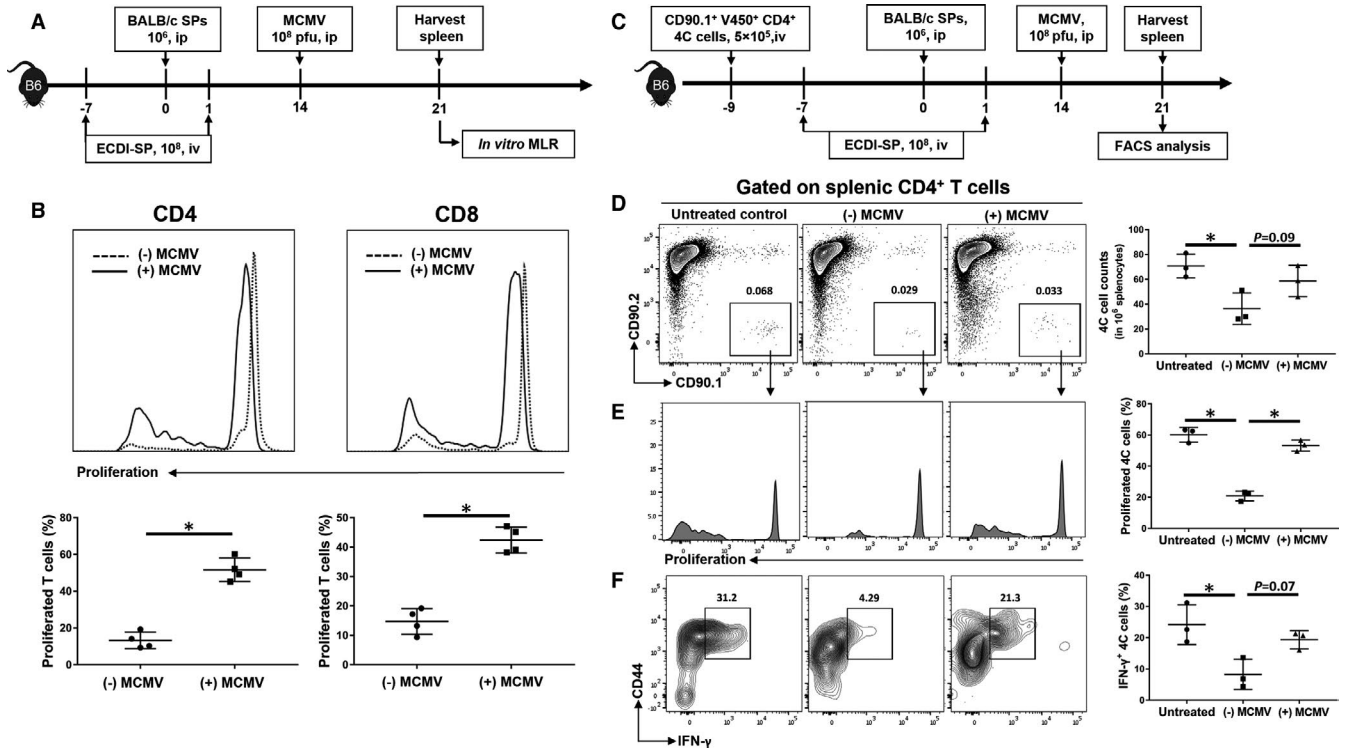

FIGURE 4 MCMV infection reverts the inhibitory effects of donor ECDI-SP on allo-specific T cells. A, Schematic experimental timeline. B6 mice were tolerized by intravenous injection of 10^8 BALB/c ECDI-SP on day −7 and day 1. Allogeneic challenge was provided on day 0 by an intraperitoneal injection of 10^6 fresh BALB/c splenocytes. These mice were then either infected with 10^8 pfu MCMV (Δm157) intraperitoneally on day 14 (the "(+)MCMV" group) or not (the "(−)MCMV" group). All mice were sacrificed on day 21, and splenic T cells were purified for donor-stimulated MLR in vitro. B, Purified T cells were labeled with the proliferation dye V450 and co-cultured with BALB/c enriched dendritic cells (eDCs) at a ratio of 1:1 for 5 d. T cell proliferation was determined by V450 dilution and compared between groups. N = 4 in both groups. C, Schematic experimental timeline. The experimental timeline is the same as in (A) with the exception of additional adoptive transfer of $5x10^5$ V450-labeled 4C CD4 T cells on day −9. D, Number of 4C CD4 T cells in the spleen. E, In vivo proliferation of splenic 4C CD4 T cells. F, In vivo IFN-γ production by splenic 4C CD4 T cells. D-F, N = 3 in all groups. Data are presented as mean ± SD in all scatter plots. *$P < .05$

obtained at the same time and analyzed for comparison. We examined for CD44 as a marker for antigen experience and the development of effector function, and CD127 (IL-7Rα) as a marker for memory T cell differentiation. As shown in Figure 5A, the percentage of antigen-experienced $CD44^+$ T cells among total splenic T cells was significantly higher in transplanted recipients in comparison to that in naive mice, regardless of graft tolerance (in the absence of MCMV infection) or graft rejection (in the presence of MCMV infection). Of interest, among the $CD44^+$ T cells, a significant upregulation of CD127 indicating their differentiation to memory T cells[26] was seen only in recipients with graft rejection precipitated by MCMV infection, not in tolerized recipients in the absence of MCMV infection (Figure 5B). Furthermore, when subjected to a short (3 day) recall MLR stimulated by BALB/c cells in vitro, only T cells from the Tol+MCMV+Rej group exhibited rapid proliferation, but not T cells from the other Tol or the naive groups (Figure 5C).

Collectively, these results indicate that T cells in ECDI-SP–treated recipients following acute MCMV infection and graft rejection not only revert their tolerant characteristics but also acquire memory phenotype and function.

4 | DISCUSSION

In this study, we demonstrate that acute MCMV infection disrupts stably established transplant tolerance induced by donor ECDI-SP via augmenting alloreactive T cell immune responses. Furthermore, this process is allo-sensitizing, demonstrated by the generation of allo-reactive memory T cells and an accelerated kinetics of rejection of the second same-donor allograft. To the best of our knowledge, this is the first report demonstrating allo-sensitization in previously tolerant recipients experiencing acute graft rejection precipitated by a viral infection, and supports close clinical monitoring and intervention for allo-sensitization in this setting.

We have previously reported that acute MCMV infection during the perioperative period (day 0) prevents the *induction* of tolerance by donor ECDI-SP in about 80% of recipients.[8] The current study examines the impact of MCMV infection on the *maintenance* of tolerance induced by donor ECDI-SP. We infected ECDI-SP–treated recipients with MCMV when tolerance was deemed well established (on day 14 or 95), and observed a rejection rate of ~50%-60% in these recipients. Here the rate of graft rejection was significantly lower and the kinetics of graft rejection was significantly delayed

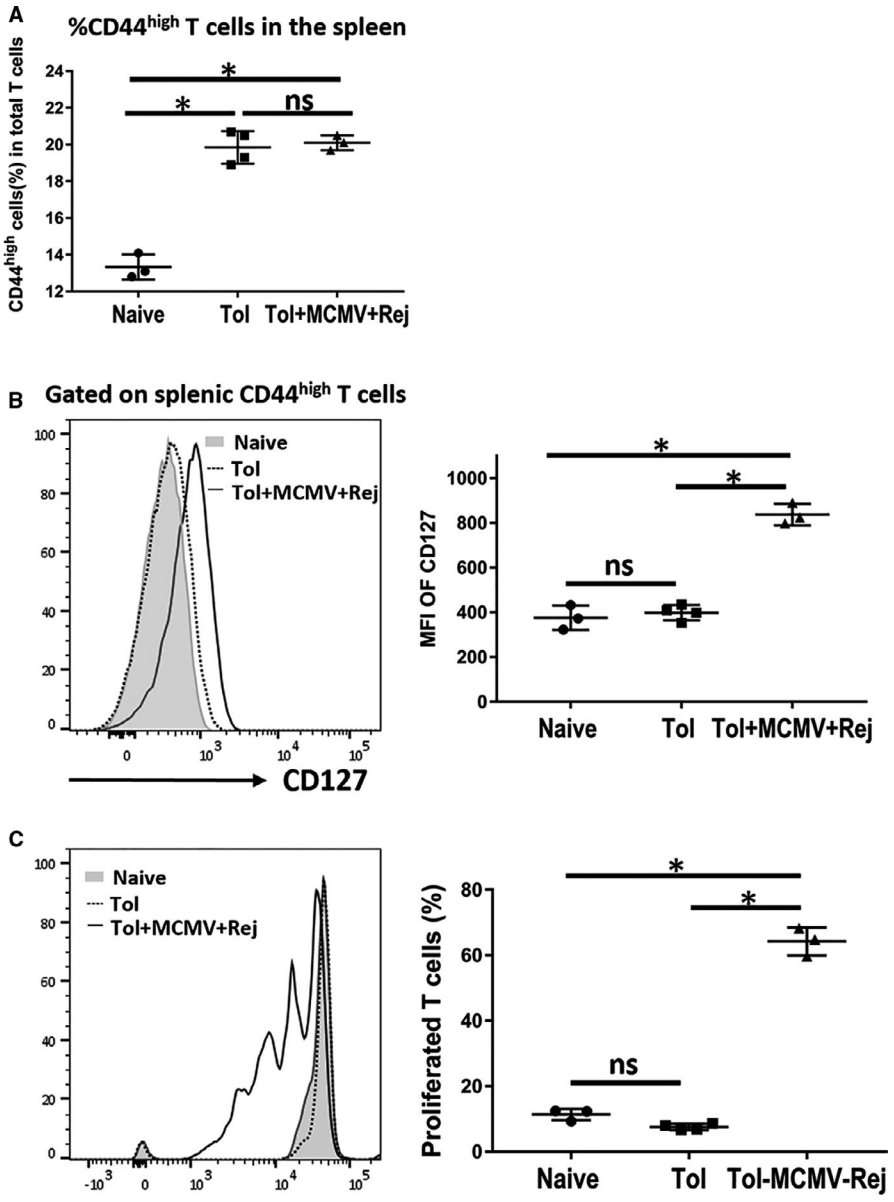

FIGURE 5 Tolerized recipient splenic T cells following MCMV infection exhibit memory phenotype and function. Tolerant recipients infected with MCMV on day 14 post the first BALB/c islet transplant were sacrificed 10 days after graft rejection was confirmed (the "Tol+MCMV+Rej" group). Tolerant recipients without MCMV infection (the "Tol" group) were sacrificed contemporaneously as the control. Naive B6 mice ("Naive" group) were sacrificed and similarly examined for comparison. Spleens from mice of various groups were harvested, and splenic T cells were isolated and analyzed. A, Percentage of $CD44^{high}$ T cells among total splenic T cells. B, Representative histograms (left panel) and scatter plot (right panel) of CD127 expression among $CD44^{high}$ T cells from various groups. C, T cells from various groups were labeled with V450 and subjected to in vitro BALB/c eDC re-stimulation (T:eDC ratio 1:1) for 3 days. T cell proliferation (by V450 dilution) is shown in representative histograms (left panel), and quantified and compared in scatter plot (right panel). A-C, N = 3-4 in each group. Data are presented as mean ± SD in all scatter plots. *$P < .05$; ns = no significance

compared to those in recipients infected on day 0 (Figure 1B). Therefore, tolerance during the maintenance phase appears to be more resistant to disruption by MCMV infection than during the induction phase. Furthermore, once tolerance has been established, it appears to be stable over the period of 14-95 days, as the rejection rate and kinetics are indistinguishable by MCMV infection given on either day 14 or day 95 (Figure 1B).

In our study, all second same-donor islet grafts in these recipients were rejected rapidly (Figure 2B). Contrary to our findings, a previous study shows that cardiac allograft tolerance abrogated by *L. monocytogenes* infection is able to restore spontaneously after the infection has abated, thus allowing acceptance of a second same-donor transplant without further intervention.[19] The different outcomes may be due to several factors. First, mechanisms of tolerance disruption by *L. monocytogenes* vs MCMV infection are different. The adverse effect of *L. monocytogenes* infection on tolerance is mainly mediated by IL-6 and IFN-β, whereas MCMV induces IFN-α to impair graft tolerance.[8,14,17] It is possible that these cytokines have different impacts on alloimmune memory formation. Second, the acute bacterial infection by *L. monocytogenes* is typically thoroughly eradicated by host anti-*L. monocytogenes* immune responses, whereas the acute viral infection by MCMV generally establishes latency and therefore provides persistent antigen exposure to the host.[27,28] This difference may also contribute to the difference in alloimmune memory development. Third, different tolerance induction strategies may also result in differences in the initial quality of tolerance. For instance, in murine allogeneic heart transplantation models, anti-CD154/DST results in longer allograft survival than that by ECDI-SP.[19,29] The magnitude and mechanisms of initial inhibition of allo-reactive T cells may contribute to their subsequent ability to differentiate to allo-reactive memory cells. Finally, the tissue/organ transplanted (islet vs heart) is different. Different tissues/organs contain different resident immune cells and lymphatic vessel phenotypes, which in turn determine the nature and magnitude of

host alloimmune responses to the allograft.[30-34] Consequently, different tissue/organ allografts also display different susceptibility to tolerance induction,[35] and when rejection occurs, result in different tendencies for allo-specific memory formation. Putting our results into the context of the published literature, we conclude that the immunological consequences of pathogen-induced graft rejection in a previously tolerance recipient may be highly variable, depending on the pathogen encountered, the tolerance strategy used, and the tissue/organ transplanted. This perception is consistent with a previous notion that transplant tolerance is not an all-or-none phenomenon, but rather a graded entity determined by a collective mechanisms of T cell tolerance.[36] Depending on the specific tolerance mechanisms disrupted and the magnitude of disruption, recipients may exhibit a range of resulting alloimmunity that influences the quality of residual tolerance and/or their susceptibility to future tolerance.[36]

In this study, we explored the impact of acute primary MCMV infection on established transplantation tolerance and alloimmune memory formation. However, clinically, reactivation from latent CMV infection in transplant recipients is far more common than acute primary infection.[37] Therefore, the implications of our current study in the context of CMV reactivation should be considered. On the one hand, it has been shown that latent MCMV infection impairs the induction of tolerance in a D−/R+ allogeneic murine cardiac transplantation model.[38] Therefore, latent MCMV clearly has its imprint on the host immune system. On the other hand, in a D+/R− allogeneic murine kidney transplantation model, we have recently shown that MCMV reactivation and dissemination from latently infected donor kidneys can in fact be effectively prevented by established transplantation tolerance.[39] Reconciling these information, we speculate that in the presence of established transplantation tolerance, MCMV reactivation and dissemination would be less likely to occur. However, if they do occur, the ensuing immunological consequences would similarly result in recipient sensitization as we show here.

In this study, the roles of T cells and DSAs in interfering with the mechanisms of tolerance maintenance by donor ECDI-SP were investigated. Similar as lymphocytic choriomeningitis virus (LCMV) infection,[14] MCMV infection also resulted in activation of alloreactive CD4 and CD8 T cells (Figure 4). The impact of pathogens on alloreactive T cell response has been thought to be mediated primarily by inflammatory cytokines produced in response to the infection, such as IL-6 during *Staphylococcus aureus* infection, IL-6 and INF-β during *L. monocytogenes* infection, and IFN-α during MCMV infection.[8,14,15] Innate cytokines such as IL-6 are known to augment T cell alloreactivity.[40] In addition, T cell cross-reactivities between pathogen epitopes and structurally similar allo-antigens have also been described as a mechanism for heightened allo-reactivity during infection.[41] It is notable that it has been reported that CMV-specific T cell are capable of responding to allo-antigens in humans.[42] Therefore, cross-reactivity between MCMV-specific T cells and BALB/c alloantigen-specific T cells may be an additional mechanism of tolerance disruption and recipient sensitization in our model. This possibility warrants further investigation. Another important focus for future research is to design strategies for re-establishing transplantation tolerance, particularly if allo-sensitization occurs, such as in our case. As a conventional marker for memory T cells,[26] CD127 has been targeted to effectively control antigen-specific memory T cell–mediated chronic inflammation[43] and to decrease the generation of memory T cells to improve short- and long-term allograft survival.[44,45] In our study, $CD127^+$ T cells are shown to be associated with rapid rejection of the second allograft (Figure 5B). It would be therefore highly valuable to determine the respective contribution of $CD44^+CD127+$ vs $CD44^+CD127-$ cells to the observed accelerated rejection. Such studies are currently underway and will have a direct impact on the utility of employing anti-IL-7R to block memory T cells[44] among other strategies[25] to re-establish transplantation tolerance in such hosts.

In contrast to allo-reactive T cells, DSAs do not appear to play a major role in tolerance disruption or recipient sensitization in the setting of MCMV infection (Figure 3B,D). The absence of DSAs even after MCMV-precipitated rejection of the first islet allograft (Figure 3B) is in sharp contrast to the development of DSAs in naive untolerized B6 recipients following rejection of an islet allograft.[20] This finding suggests that the ability of B cells to differentiate to allo-antibody-producing plasma cells is effectively inhibited by ECDI-SP, and remains inhibited even in setting of MCMV infection. Published studies of the impact of CMV infection on B cells have focused primarily on latent CMV infection.[46-48] In an influenza vaccine study in humans, CMV seropositivity (indicating CMV latency) is shown to be associated with increased B cell–intrinsic inflammation but decreased anti-influenza antibodies.[46,47] Similarly, in a murine study of latent CMV infection, impaired B cell responses and delayed antibody class-switch in response to vesicular stomatitis virus infection are observed.[48] Taking these data into the context of our study, it is therefore not surprising that inhibition of DSA production by ECDI-SP is not reverted by MCMV infection.

However, B cells may still participate in memory T cell development.[49-52] In murine models of infections, absence of B cells results in lower levels of antigen-specific CD4 and CD8 memory T cells.[49,50] More importantly, it is reported that alloreactive T cell memory is impaired in B cell–deficient hosts,[51] as B cells play a critical role in promoting the differentiation, proliferation, and survival of alloreactive T cells, and the generation of an optimal number of functional alloreactive memory T cells.[51] In our model, whether memory T cell generation is also dependent on host B cells; and if so, how MCMV infection alters the function of B cells to promote memory formation, warrants further study.

In summary, our study demonstrates that acute MCMV infection disrupts maintenance of tolerance initially induced by donor ECDI-SP. In this process, allo-reactive $CD127^+$ memory T cells are generated, resulting in recipient allo-sensitization and rapid rejection of subsequent same-donor transplantation. Our work underscores the potential detriment to the recipient in pathogen-precipitated graft rejection, and emphasizes the importance to examine and define such impact under individual circumstances specific to the pathogen encountered, the tolerance strategy used, and the tissue/organ transplanted.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

AUTHOR CONTRIBUTIONS

Contribution: SY, AD, and XL designed the experiments. SY, MB, and AD performed the experiments. SY, AD, and XL analyzed the data. SY and XL wrote the manuscript; and AD, MA, ET, and XL edited and finalized the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ORCID

Michael M. Abecassis https://orcid.org/0000-0001-9333-463X
Edward B. Thorp https://orcid.org/0000-0003-1387-7058
Xunrong Luo https://orcid.org/0000-0001-5581-9409

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