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Allograft dendritic cell p40 homodimers activate donor-reactive memory CD8+ T cells

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Recipient endogenous memory T cells with donor reactivity pose an important barrier to successful transplantation and costimulatory blockade–induced graft tolerance. Longer ischemic storage times prior to organ transplantation increase early posttransplant inflammation and negatively impact early graft function and long-term graft outcome. Little is known about the mechanisms enhancing endogenous memory T cell activation to mediate tissue injury within the increased inflammatory environment of allografts subjected to prolonged cold ischemic storage (CIS). Endogenous memory CD4+ and CD8+ T cell activation is markedly increased within complete MHC-mismatched cardiac allografts subjected to prolonged versus minimal CIS, and the memory CD8+ T cells directly mediate CTLA-4Ig–resistant allograft rejection. Memory CD8+ T cell activation within allografts subjected to prolonged CIS requires memory CD4+ T cell stimulation of graft DCs to produce p40 homodimers, but not IL-12 p40/p35 heterodimers. Targeting p40 abrogates memory CD8+ T cell proliferation within the allografts and their ability to mediate CTLA-4Ig–resistant allograft rejection. These findings indicate a critical role for memory CD4+ T cell–graft DC interactions to increase the intensity of endogenous memory CD8+ T cell activation needed to mediate rejection of higher-risk allografts subjected to increased CIS.

Introduction

The generation of T cell memory provides effective protection to recurrent infectious agents. When compared with antigen-reactive naive T cells, memory T cells have many distinct features enhancing their function to rapidly respond to and eliminate viral and bacterial infections, including rapid trafficking into peripheral tissue inflammatory sites and activation that occurs independently of the many costimulatory signals required for naive T cell activation (1–3). Moreover, memory CD8+ T cell responses within peripheral tissue inflammatory sites often occurs without the CD4+ T cell generated helper signals required for optimal naive antigen–reactive CD8+ T cell responses (4–6). Memory T cells with reactivity to allogeneic MHC molecules are an important barrier to the function and survival of organs transplanted to treat end-stage organ disease (7–9). Seminal studies by Heeger and colleagues indicated that the presence of high numbers of memory T cells with donor reactivity in the peripheral blood of kidney transplant patients prior to the transplant resulted in higher incidence of delayed graft function, acute graft rejection, and poorer long-term graft outcomes (10, 11). Memory CD4+ and CD8+ T cell repertoires generated during exposure to viral and bacterial infections often contain high frequencies of T cells reactive to allogeneic class II or class I MHC molecules, providing donor-reactive memory T cells that can undermine successful transplantation (12–15). Mice also contain readily detectable populations of CD4+ and CD8+ T cells with memory phenotypes, and a proportion of this memory T cell repertoire in naive mice infiltrate cardiac allografts within hours of graft reperfusion and respond to donor class I and class II MHC alloantigens (16–18). Endogenous memory CD8+ T cells infiltrating allografts subjected to minimal periods of cold ischemic storage (CIS) are activated to produce IFN-γ and other mediators that increase graft inflammation. This endogenous memory CD8+ T cell response occurs independently of CD4+ T cells and CD28/CD80/86- and CD40/CD154-mediated costimulation, but it is insufficient to directly mediate graft rejection.

The initial inflammation induced in transplanted organs arises from their removal from a source of oxygen following graft harvest and the subsequent reexposure to oxygen during graft revascularization that generates ROS and danger-associated molecules, potent activators of innate immunity (19–21). Imposition of longer ischemic storage times on clinical transplants generates marked increases in graft inflammation during reperfusion and negatively impacts early graft function and long-term survival (22–26). We recently reported that increasing the time of CIS imposed on cardiac allografts quickly provokes a highly inflammatory environment after revascularization that increases endogenous memory CD4$^+$ and CD8$^+$ T cell numbers within the allograft and increases in endogenous memory CD8$^+$ T cell expression of IFN-γ, perforin, and granzyme B (27). Whereas peritransplant CTLA-4Ig treatment extended the survival of allografts subjected to minimal (e.g., 30 minutes) CIS by more than 7 weeks, this treatment extended the survival of allografts subjected to prolonged (e.g., 6–8 hours) CIS by only a few days; this rejection was mediated by the endogenous memory CD8$^+$ T cells. These results were reminiscent of the costimulatory blockade–resistant endogenous memory CD8$^+$ T cell responses that mediate acute rejection and decrease graft survival in nonhuman primate (NHP) and human allograft recipients (28–31). How such endogenous memory CD8$^+$ T cells are activated within allografts to mediate this early severe graft injury and failure has not been determined. The goal of the current study was to identify mechanisms promoting endogenous memory CD8$^+$ T cell activation within allografts subjected to increased CIS. Overall, the results indicate an intricate series of activation events within higher-risk allografts during the first 1–2 days after transplant initiated by endogenous memory CD4$^+$ T cell interaction with graft DCs to generate a unique cytokine required for activation of donor-reactive memory CD8$^+$ T cells to mediate acute allograft injury and rejection.

Results

Donor-specific alloreactivity of early graft–infiltrating endogenous memory CD8$^+$ T cells. To begin to further assess polyclonal donor-reactive memory CD8$^+$ T cell infiltration into highly ischemic cardiac allografts, responses of adoptively transferred donor-sensitized memory CD8$^+$ T cells were studied. Briefly, memory CD8$^+$ T cells were enriched from the spleens and lymph nodes of CD45.2$^+$ C57BL/6 recipients of A/J skin transplants and adoptively transferred to congenic CD45.1$^+$ recipients that were then transplanted with A/J cardiac allografts subjected to minimal or prolonged (0.5 vs.8 hours) CIS. Similar to the endogenous memory CD8$^+$ T cells from recipients without such adoptively transferred memory CD8$^+$ T cells (27), transferred donor antigen–primed memory CD45.2$^+$CD8$^+$ T cells were detectable in A/J heart allografts subjected to either minimal or prolonged CIS within 12–16 hours after graft reperfusion, but transferred memory CD8$^+$ T cell numbers were increased in allografts subjected to longer CIS (Figure 1A). The adoptively transferred CD8$^+$ T cells from A/J skin allograft–primed mice also infiltrated third-party (DBA/1) allografts subjected to prolonged CIS but at the decreased levels seen in A/J cardiac allografts subjected to minimal CIS.

The donor reactivity of endogenous memory CD8$^+$ T cells infiltrating A/J cardiac allografts subjected to 8 hours of CIS was directly investigated by isolating the CD8$^+$ T cells from the allografts on day 3 after transplant, labeling the T cells with CFSE, and testing their ability to proliferate in response to various splenocyte stimulator cells in vitro. The purified graft-infiltrating memory CD8$^+$ T cells exhibited little reactivity to syngeneic stimulator cells but robustly proliferated to graft donor A/J stimulator cells (Figure 1B). Consistent with their infiltration into DBA/1 cardiac allografts, the A/J graft–infiltrating memory CD8$^+$ T cells also demonstrated a lower but significant response to third-party DBA/1 stimulator cells. Mixing A/J and DBA stimulators did not yield a synergistic proliferative response when compared with the response to the A/J stimulators alone, suggesting that the third-party alloreactive memory CD8$^+$ T cells are contained within the A/J donor–reactive population. Overall, these results establish the donor reactivity of endogenous memory CD8$^+$ T cells infiltrating heart allografts and complement studies investigating the infiltration of donor-reactive transgenic CD8$^+$ T cells into allografts (32).

The substantial percentage of allograft-infiltrating CD8$^+$ T cells that did not respond to donor cells prompted a more comprehensive analysis of the endogenous CD4$^+$ and CD8$^+$ T cell populations infiltrating complete MHC-mismatched heart allografts subjected to minimal vs. prolonged CIS 48 hours after reperfusion of the allografts. The greatest percentage of CD4$^+$ T cells infiltrating the allografts subjected to either minimal or prolonged CIS were effector memory (CD62L^{low}CD44^{high}) cells with a lower percentage of central memory (CD62L^{high}CD44^{high}) cells (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.96940DS1). Naive (CD62L^{low}CD44^{high} and CD62L^{low}CD44^{high}) CD4$^+$ T cells were also prominent in the allografts. In contrast, naive cells constituted
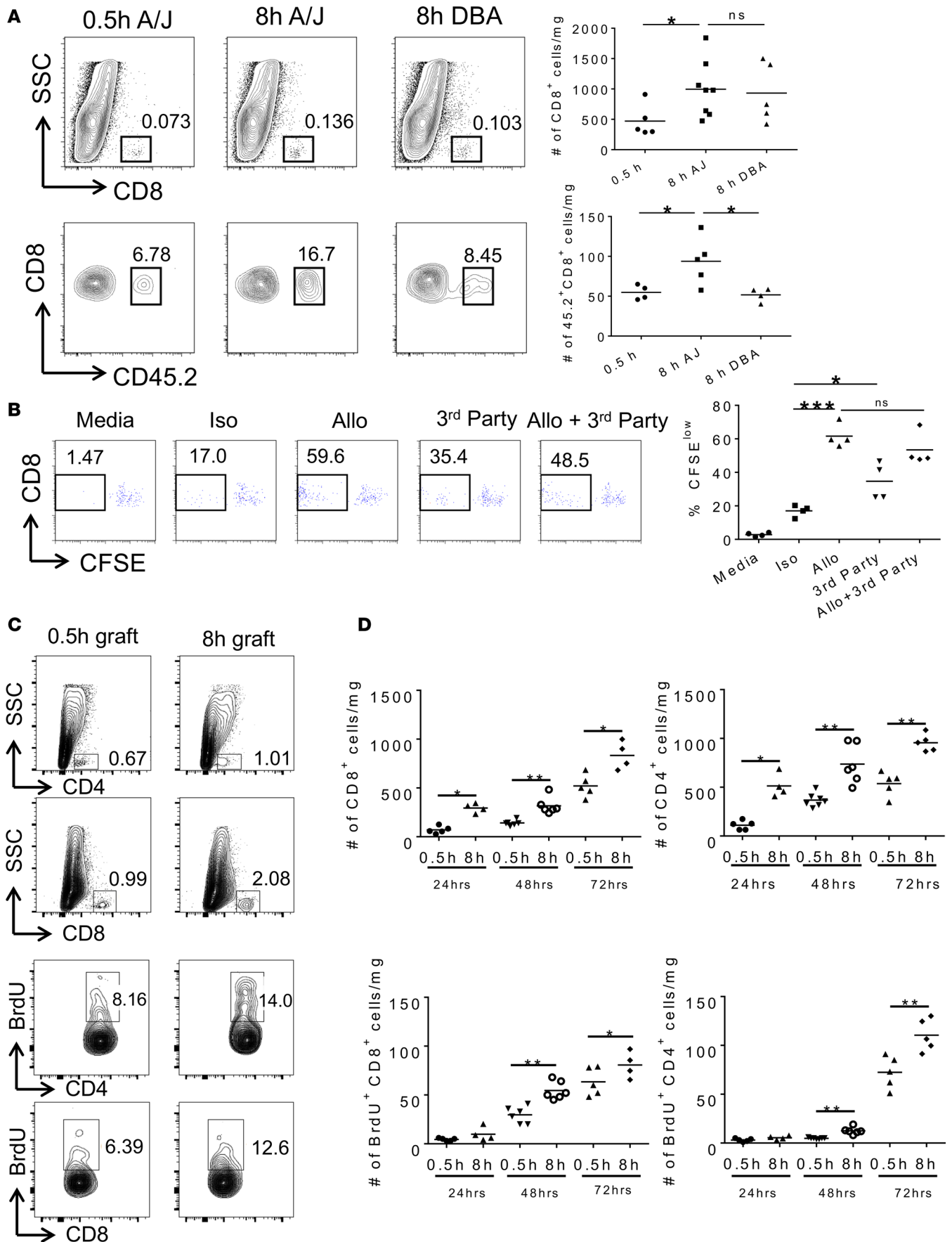
Figure 1. Heart allografts subjected to greater ischemic storage have increased accumulation of endogenous memory CD8+ T cells with donor reactivity at early times after transplant. (**A**) Memory CD8+ T (CD8+CD44high) cells were purified from the spleens and lymph nodes of CD45.2 C57BL/6 (H-2b) mice 6 weeks after A/J (H-2a) skin transplants, and 2 × 10^6 aliquots of cells were transferred i.v. to groups of CD45.1 C57BL/6 (n = 5–8/group) mice. Three days later, the CD45.1 C57BL/6 mice received A/J cardiac allografts subjected to either 0.5 or 8 hours of CIS or DBA/1 (H-2q) heart allografts subjected to 8 hours of CIS. Cardiac allograft recipients were sacrificed 12–16 hours after transplant, the allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample to assess and quantitate the infiltration of memory CD8+ memory T cells and the transferred CD45.2 memory CD8+CD44high T cells into the allografts. *P < 0.05, as determined by the Mann-Whitney nonparametric test. (**B**) A/J hearts subjected to 8 hours of CIS were transplanted to a group of four C57BL/6 mice, and on day 3 after transplant, the graft-infiltrating memory CD8+CD44high T cells were purified, labeled with CFSE, and cultured in media or in a 1:4 mixture with spleen cells from C57BL/6 (Iso), A/J (Allo), and/or DBA/1 (3rd party) mice. After 96 hours, the cultured cells were collected and washed, and the dilution of CFSE by the CD8+ T cells was analyzed by flow cytometry. *P < 0.05, ***P < 0.001, as determined by 1-way ANOVA with Bonferroni's multiple comparison post-test. (**C**) A/J hearts subjected to 0.5 or 8 hours of CIS were transplanted to groups of C57BL/6 mice (n = 4–6/group). BrdU was injected i.p. on days 0 and 1. The allografts were harvested after 24, 48, or 72 hours and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample. (**D**) The total number of gated infiltrating memory CD4+ and CD8+ T cells and their incorporation of BrdU was quantitated. *P < 0.05, ** P < 0.01, as determined by the Mann-Whitney nonparametric test.

the largest percentage of CD8+ T cells in allografts subjected to minimal vs. prolonged CIS, but infiltrating effector memory (CD62LlowCD44high) CD8+ T cells were prominent with a smaller percentage of central memory (CD62LhighCD44high) CD8+ T cells (Supplemental Figure 1B).

Consistent with previous studies (27), macrophages (F4/80+) and neutrophils (Ly6G+) also infiltrated allografts subjected to minimal and prolonged CIS, with greater numbers in the allografts subjected to prolonged CIS (Supplemental Figure 2, A and B). Smaller but equivalent numbers of B lymphocytes (CD19+B220+) and barely detectable numbers of NK (CD3ε–NK1.1+CD49b+) cells were observed in allografts subjected to minimal and prolonged CIS (Supplemental Figure 2C and data not shown).

Increased proliferation of endogenous memory CD8+ T cells within allografts subjected to prolonged CIS. The greater numbers of endogenous memory CD8+ T cells rapidly accumulating in allografts subjected to prolonged vs. minimal CIS led us to hypothesize that reperfusion of allografts subjected to prolonged CIS generated an inflammatory environment promoting the proliferation of early graft–infiltrating memory T cells. To test this, recipients of A/J cardiac allografts subjected to 0.5 or 8 hours of CIS were pulsed with BrdU, and its incorporation into DNA of graft-infiltrating memory CD8+ and CD4+ T cells was assessed on day 2 after transplant. As previously reported (27), increasing CIS time prior to transplant markedly increased endogenous CD4+ and CD8+ T cell numbers in the allografts (Figure 1C). Compared with T cells infiltrating allografts subjected to minimal CIS, CD8+ and CD4+ T cells within allografts subjected to prolonged CIS exhibited a roughly 2-fold increase in proliferation at this time point. Interestingly, even in allografts subjected to prolonged CIS prior to transplant, a relative minority (< 30%) of total graft-infiltrating T cells was proliferating on day 2 after transplant. Endogenous CD4+ and CD8+ T cell proliferation was not observed 24 hours after reperfusion of the highly ischemic allografts, but it was clearly evident and increased with time after transplant thereafter (Figure 1D).

The populations of allograft-infiltrating endogenous CD4+ and CD8+ T cells proliferating in allografts subjected to minimal and prolonged CIS were examined (Supplemental Figure 3, A and B). Effector memory CD4+ and CD8+ T cell proliferation was stronger in allografts that had been subjected to prolonged vs. minimal CIS. In contrast to the absence of central memory CD4+ and CD8+ T cell proliferation within allografts subjected minimal CIS, central memory CD4+ and CD8+ T cells proliferated within allografts that had been subjected to 8 hours of CIS.

Anti-CD154 mAb prolongs survival of cardiac allografts subjected to prolonged CIS and inhibits endogenous memory T cell proliferation within allografts. Endogenous memory CD8+ T cells infiltrating allografts subjected to prolonged CIS are activated to directly mediate graft rejection that is resistant to CTLA-4Ig (27). Consistent with this resistance, peritransplant CTLA-4Ig treatment had a minimal effect on the early proliferation of endogenous memory CD4+ and CD8+ T cells within A/J allografts subjected to 8 hours of CIS (Figure 2A). In contrast to CTLA-4Ig, peritransplant treatment with a different costimulatory blockade agent, anti-CD154 mAb, markedly attenuated total numbers and numbers of proliferating/BrdU+ endogenous memory CD4+ and CD8+ T cells within allografts subjected to 8 hours of CIS when assessed 48 hours after transplant (Figure 2B). Similar to our previous results (27), peritransplant treatment with CTLA-4Ig extended the survival of heart allografts subjected to minimal CIS (Figure 2C) but had little impact on the survival of allografts subjected to 8 hours of CIS (median time of survival [MTS], 7 vs. 11 days; control Ig vs. CTLA-4Ig treatment) (Figure 2C). In contrast, peritransplant
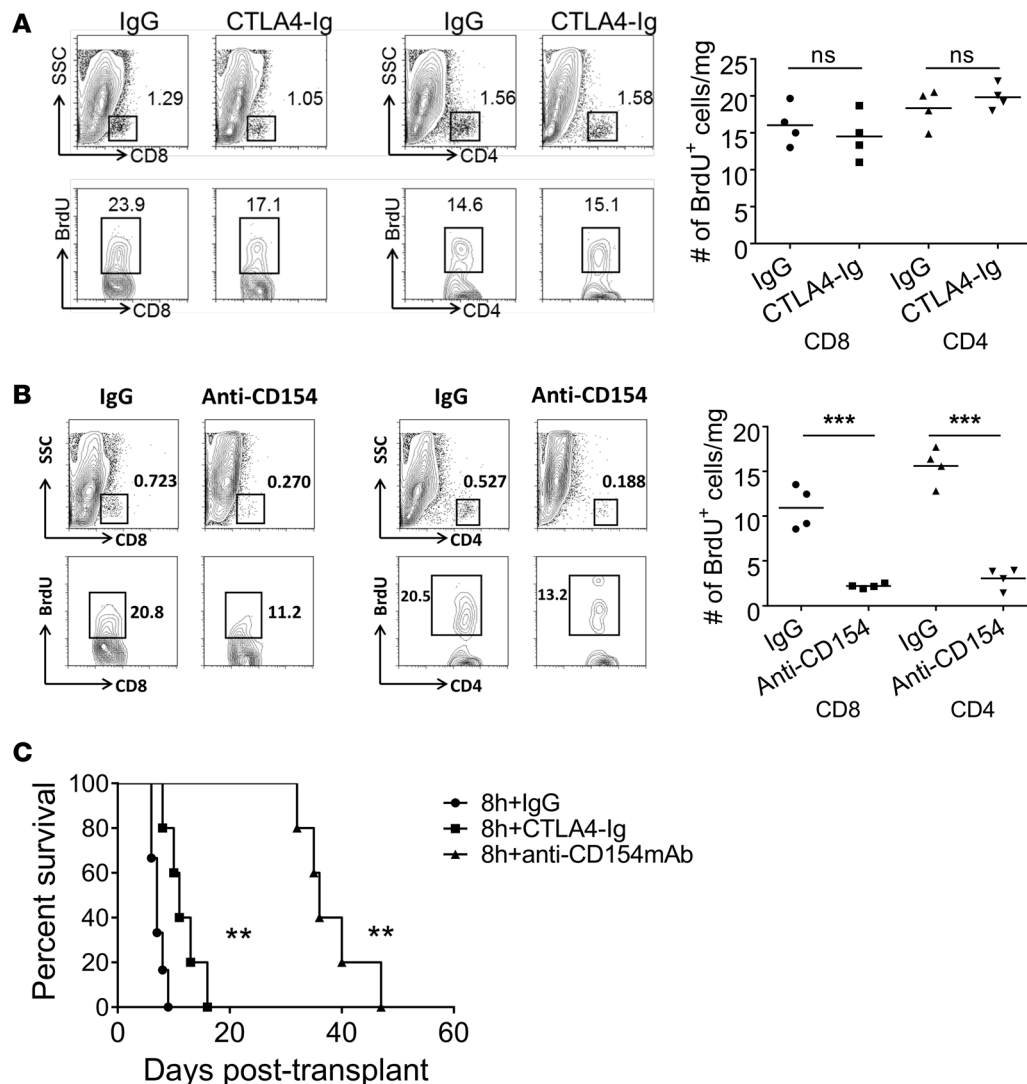
Figure 2. Peritransplant treatment with anti-CD154 mAb, but not CTLA-4Ig, inhibits early memory T cell proliferation in allografts subjected to 8 hours of CIS. Groups of C57BL/6 (n = 4/group) mice received A/J cardiac allografts subjected to 8 hours of CIS. On days 0 and 1 after transplant, all recipients were injected with 100 μg BrdU i.p. and either 250 μg control rat IgG or (**A**) 250 μg CTLA-4Ig or (**B**) 250 μg anti-CD154 mAb. After 48 hours, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample to assess and quantitate the BrdU incorporation of infiltrating memory CD4+ and CD8+ T cells.***P < 0.001, as determined by the Mann-Whitney nonparametric test. (**C**) Groups of C57BL/6 recipient mice (n = 5–6/group) received A/J cardiac allografts subjected to 8 hours of CIS and, on days 0 and 1 after transplant, were treated with 250 μg control rat IgG, 250 μg CTLA-4Ig, or 250 μg anti-CD154 mAb i.p. Allograft survival was monitored by daily abdominal palpation, and rejection was confirmed by laparotomy. **P < 0.01 versus 8 hours of CIS + IgG or + CTLA-4Ig, as determined using the Log-rank/Mantel-Cox test.

treatment with anti-CD154 mAb resulted in a substantial prolongation in survival of the highly ischemic cardiac allografts (MTS, 36 days).

Endogenous memory CD8+ T cell proliferation within highly ischemic cardiac allografts is dependent on CD4+ T cells. The anti-CD154 mAb–mediated inhibition of endogenous memory CD8+ T cell proliferation within highly ischemic cardiac allografts suggested a role for CD4+ T cells in this response. Groups of C57BL/6 recipients were treated with control Ig or with depleting anti-CD4 mAb on 3 consecutive days prior to transplant, with A/J heart allografts subjected to 0.5 or 8 hours of CIS, and endogenous memory CD8+ T cell proliferation within the allografts was examined 48 hours after transplant. In recipients depleted of CD4+ T cells, the increases in endogenous memory CD8+ T cell proliferation in highly ischemic allografts were reduced to the proliferation observed in allografts subjected to minimal CIS (Figure 3A) and were accompanied by decreases in total memory CD8+ T cell numbers in the allografts (not shown).
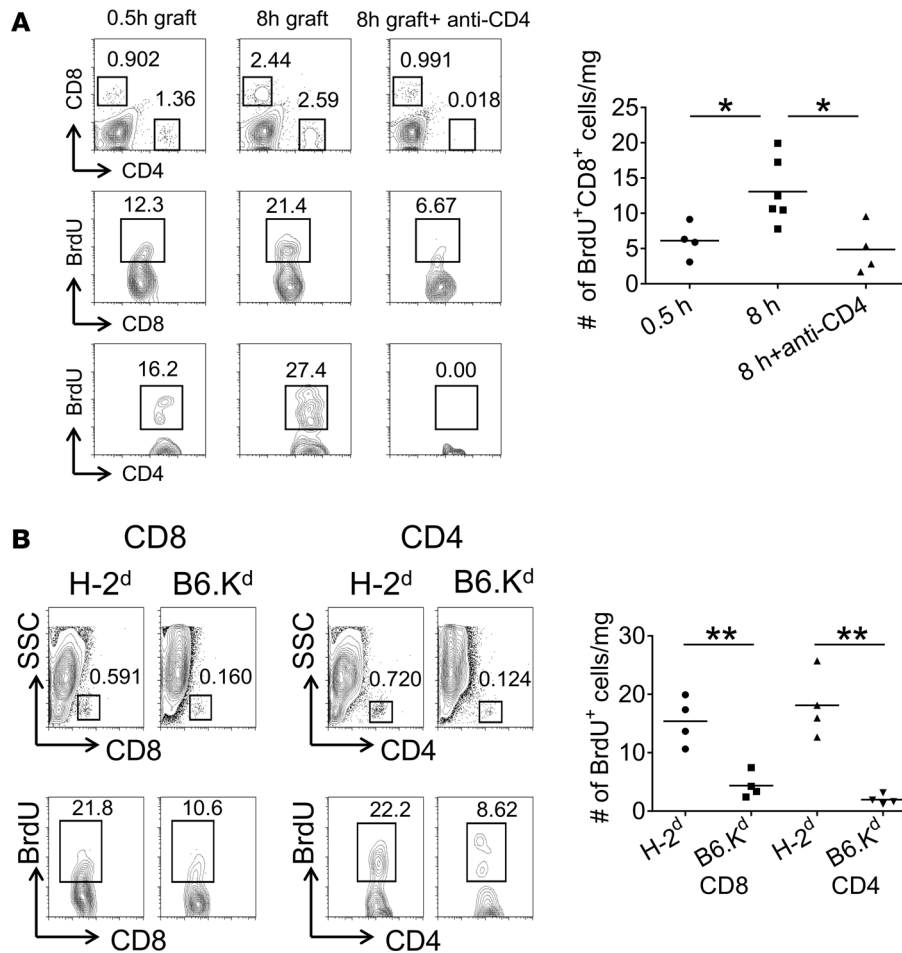
Figure 3. Endogenous memory CD8+ T cell proliferation in allografts subjected to prolonged CIS requires recipient CD4+ T cells. (**A**) Groups of C57BL/6 (*n* = 4–6/group) mice were treated with 200 μg control rat IgG or anti-CD4 mAb on days –3, –2, and –1 prior to transplant and then received A/J cardiac allografts subjected to 0.5 or 8 hours of CIS on day 0. On days 0 and 1 after transplant, all mice were injected with 100 μg BrdU i.p. The next day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample to assess and quantitate the BrdU incorporation of infiltrating memory CD8+ T cells. *P < 0.05, as determined by the Mann-Whitney nonparametric test. (**B**) Groups of C57BL/6 mice (*n* = 4/group) received complete MHC-mismatched BALB/c (H-2d) or single class I MHC disparate Kd (B6.Kd) cardiac allografts subjected to 8 hours of CIS. On days 0 and 1 after transplant, all graft recipients were injected with 100 μg BrdU i.p. The next day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample to assess and quantitate the BrdU incorporation of infiltrating memory CD4+ and CD8+ T cells. **P < 0.01, as determined by the Mann-Whitney nonparametric test.

The role of memory CD4+ T cells in promoting the increased endogenous memory CD8+ T cell proliferation within allografts subjected to prolonged CIS was further tested by comparing C57BL/6 recipient endogenous memory CD4+ and CD8+ T cell responses within cardiac allografts from complete MHC-mismatched BALB/c donors (H-2d) vs. single class I MHC–mismatched B6.Kd donors (transgenic Kd on H-2b background) that had been subjected to 8 hours of CIS. Total numbers and proliferation of endogenous memory CD4+ and CD8+ T cells were significantly reduced in single class I MHC–disparate B6.Kd allografts when compared with complete MHC-mismatched allografts (Figure 3B). These results are consistent with the need for graft class II MHC alloantigen expression to activate the recipient memory CD4+ T cells required for infiltrating memory CD8+ T cell activation to the donor class I MHC alloantigen.

The requirement for allograft class II MHC expression was directly tested by comparing endogenous memory CD4+ and CD8+ T cell proliferation within WT C57BL/6 vs. B6.class II MHC–deficient cardiac allografts subjected to prolonged CIS before transplantation to A/J recipients. When compared with WT allografts, there was a marked decrease of endogenous memory CD4+ and CD8+ T cell numbers and proliferation in the highly ischemic class II MHC−/− allografts that were similar to the numbers and proliferation observed in WT allografts subjected to minimal CIS and in A/J isografts subjected
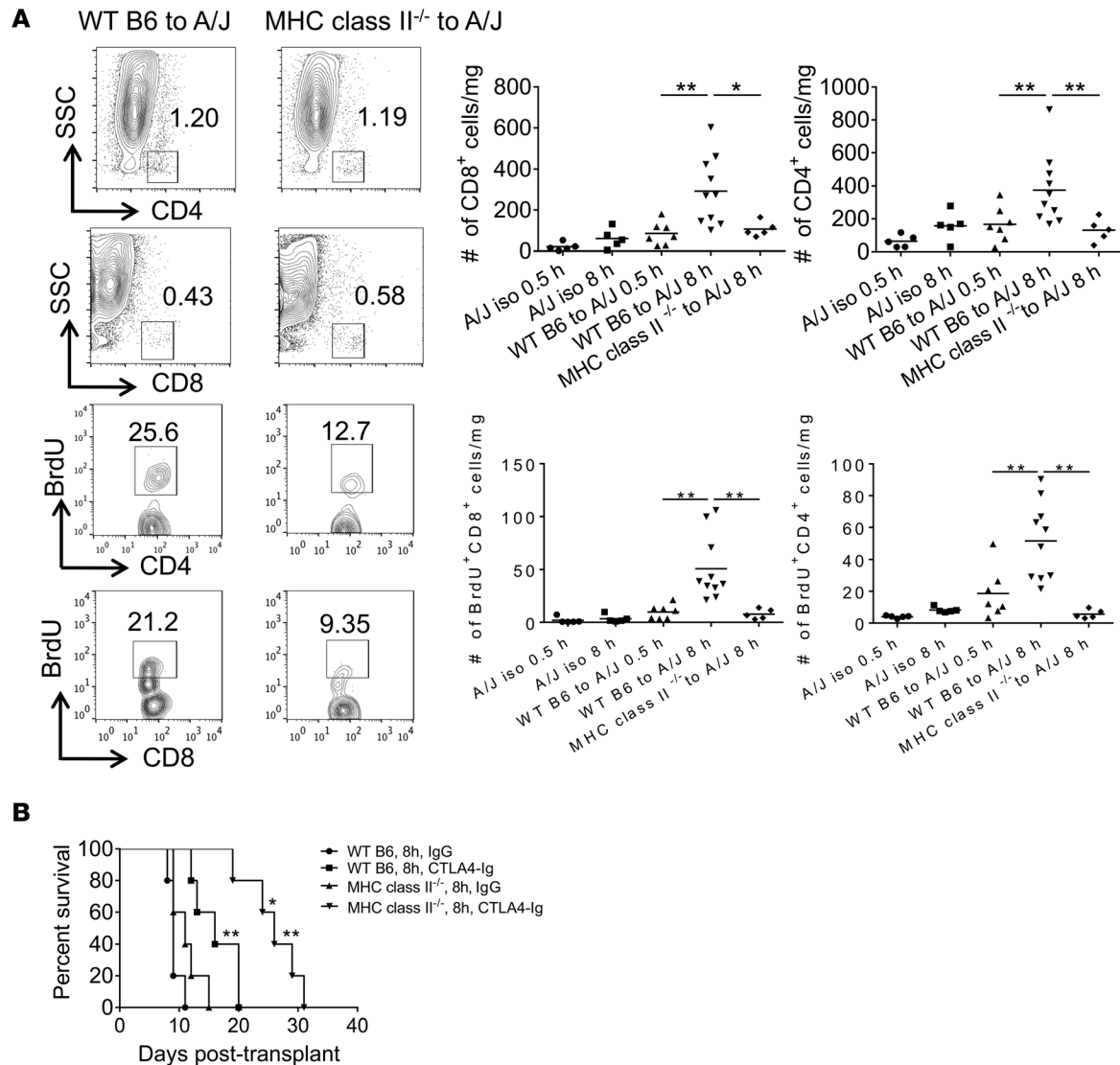
Figure 4. Endogenous memory CD8⁺ T cell proliferation in allografts subjected to prolonged CIS requires recipient graft class II MHC expression. (A) Cardiac allografts from WT C57BL/6 or B6.class II⁻/⁻ mice or from A/J hearts were subjected to 0.5 or 8 hours of CIS and transplanted to groups of A/J mice (*n* = 5–10). On days 0 and 1 after transplant, all graft recipients were injected with 100 μg BrdU i.p. The next day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry, with examples of gating as shown for each allograft sample to assess and quantitate the BrdU incorporation of infiltrating memory CD4⁺ and CD8⁺ T cells. *P < 0.05, **P < 0.01, as determined by the Mann-Whitney nonparametric test. (B) The survival of MHC class II–deficient allografts or WT C57BL/6 allografts subjected to 8 hours of CIS in A/J recipients treated with 250 μg control rat IgG or CTLA-4Ig i.p. on days 0 and 1. Allograft survival was monitored by daily abdominal palpation, and rejection was confirmed by laparotomy. **P < 0.01 versus survival of WT C57BL/6 and MHC class II⁻/⁻ allografts in IgG-treated A/J recipients; *P < 0.05 vs. survival of WT C57BL/6 allografts in CTLA-4Ig–treated recipients.

to 0.5 or 8 hours of CIS (Figure 4A). Consistent with the memory CD8⁺ T cell responses in the WT vs. class II MHC–deficient allografts, mRNA expression of the effector CD8⁺ T cell mediators IFN-γ, FasL, perforin, and granzyme B was markedly increased in WT allografts subjected to 8 vs. 0.5 hours of CIS but was significantly decreased in class II MHC⁻/⁻ allografts subjected to prolonged CIS (Supplemental Figure 4). In contrast, no significant difference was observed in the mRNA expression of the neutrophil chemoattractant CXCL1 in any of the iso- and allograft groups. Whereas CTLA-4Ig treatment only extended survival of WT C57BL/6 allografts subjected to prolonged CIS in A/J recipients from day 9 (MTS) to day 16 MTS, CTLA-4Ig treatment significantly extended survival of highly ischemic B6.class II MHC⁻/⁻ allografts from day 11 MTS to day 26 MTS (Figure 4B).

Endogenous memory CD4⁺ and CD8⁺ T cell proliferation within highly ischemic heart allografts requires graft expression of CD40 and graft DCs. The inhibition of endogenous memory CD4⁺ and CD8⁺ T cell activation within highly ischemic allografts by anti-CD154 mAb suggested a requirement for CD40 engagement for this response.
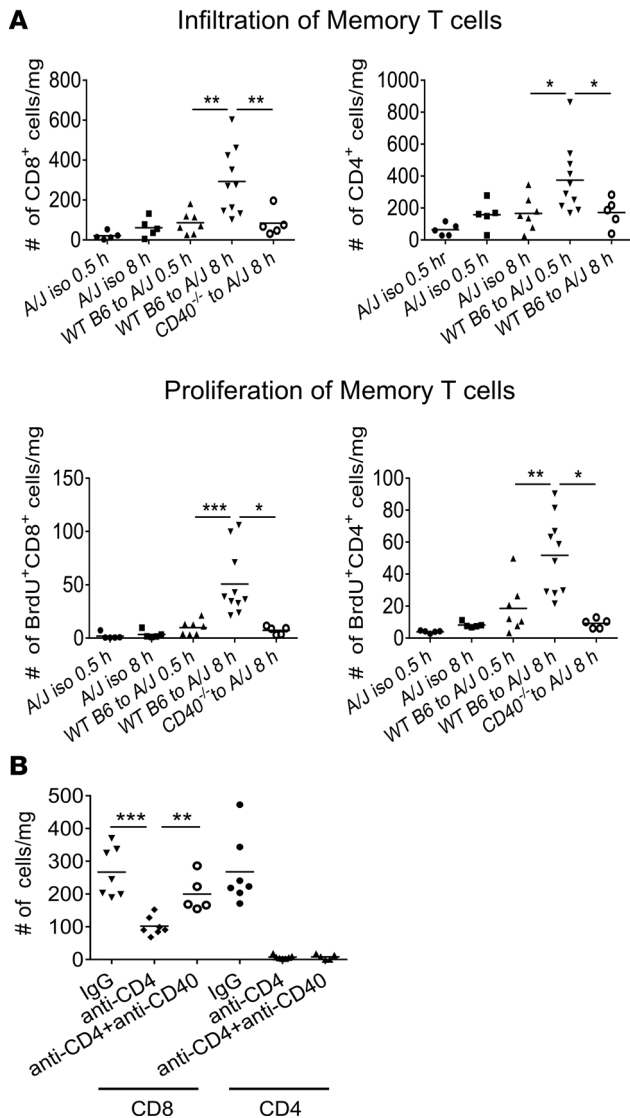
A

Infiltration of Memory T cells

Proliferation of Memory T cells

B

Figure 5. Endogenous memory CD8+ T cell proliferation in allografts subjected to prolonged CIS requires graft CD40 expression. (**A**) Groups of A/J mice (*n* = 5–10) received WT C57BL/6 or B6.CD40−/− cardiac allografts subjected to 0.5 or 8 hours of CIS and were injected with 100 μg BrdU i.p. on days 0 and 1 after transplant. The next day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry to quantitate the infiltration and proliferation of memory CD8+ and CD4+ T cells. *P < 0.05, **P < 0.01, ***P < 0.001, as determined by the Mann-Whitney nonparametric test. (**B**) Groups of C57BL/6 (*n* = 5–7/group) mice were treated with 200 μg control rat IgG or anti-CD4 mAb on days −3, −2, and −1 prior to transplant and then received A/J cardiac allografts subjected to 8 hours of CIS. On days 0 and 1, recipients were injected where indicated with 100 μg control rat IgG or agonist anti-CD40 mAb i.v. The next day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry to quantitate the infiltrating memory CD4+ and CD8+ T cells. **P < 0.01; ***P < 0.001, as determined by the Mann-Whitney nonparametric test.

When compared with memory T cell responses within WT C57BL/6 allografts subjected to 8 hours of CIS, marked decreases in total numbers and proliferating endogenous memory CD4+ and CD8+ T cells were observed in B6.CD40−/− allografts 48 hours after transplant to A/J recipients (Figure 5A). The importance of CD40-mediated signaling for increased endogenous memory CD8+ T cell accumulation within allografts subjected to prolonged CIS was further indicated by the ability of an agonist anti-CD40 mAb to restore endogenous memory CD8+ T cell numbers in highly ischemic A/J allografts in C57BL/6 recipients depleted of CD4+ T cells (Figure 5B).

Endogenous memory T cell proliferation was compared in control and DC-depleted C57BL/6 cardiac allografts subjected to 8 hours of CIS and transplanted to A/J recipients. Diphtheria toxin (DT) receptor–CD11c transgenic (B6.DTR-CD11c) mice were treated with vehicle or with DT on 2 consecutive days prior to harvest of hearts and transplant to A/J recipients, and endogenous memory CD4+ and CD8+ T cell proliferation within the allografts was determined 48 hours later. The absence of graft DCs markedly decreased both endogenous memory CD4+ and CD8+ T cell numbers and proliferation within the heart allografts (Figure 6A). Graft DC depletion also resulted in decreased IFN-γ and granzyme B mRNA expression in allografts, as well as in decreased expression of proinflammatory cytokine genes including IL-1β, CXCL2, and IL-12 p40, but not IL-6 (Figure 6B). On day 8 after transplant, the impact of allograft DC depletion on the de novo priming of donor-reactive naive T cells was also determined by enumerating donor-reactive T cells producing IFN-γ and IL-17 in the spleens from each allograft recipient group. While IFN-γ–producing cell numbers were significantly decreased in recipients of the DC-depleted allografts, Il-17–producing T cell numbers were similar to recipients of WT allografts (Figure 6C).

p40 homodimers mediate proliferation of endogenous memory CD8+ T cells within highly ischemic allografts. Candidate factors driving the CD4+ T cell–dependent proliferation of endogenous memory CD8+ T cells within the highly ischemic cardiac allografts were next investigated. First, mRNA expression of different cytokines within cardiac allografts subjected to either 30 minutes or 8 hours of CIS was compared on day 2 after transplant. Of a large panel of test cytokines, IL-1β, IL-2, IL-12 p40, IL-21, and CCL5 mRNA had the largest increases in cardiac allografts subjected to prolonged vs. minimal CIS (Figure 7A). Depletion of recipient CD4+ T cells prior to the transplant reduced mRNA expression levels of these cytokines in the highly ischemic cardiac allografts to the levels observed in isografts and in allografts subjected to minimal CIS. In contrast, treating CD4+ T cell–depleted allograft recipients with an agonist anti-CD40 mAb restored the expression of IL-12 p40 mRNA in the highly ischemic allografts but not IL-1β, IL-2, IL-21, or CCL5 mRNA. Similar to the observed mRNA expression levels, increases in proinflammatory cytokine protein were observed in allografts subjected to increased CIS, and this increase was decreased to background by depletion of recipients CD4+ T cells (Supplemental Figure 5).
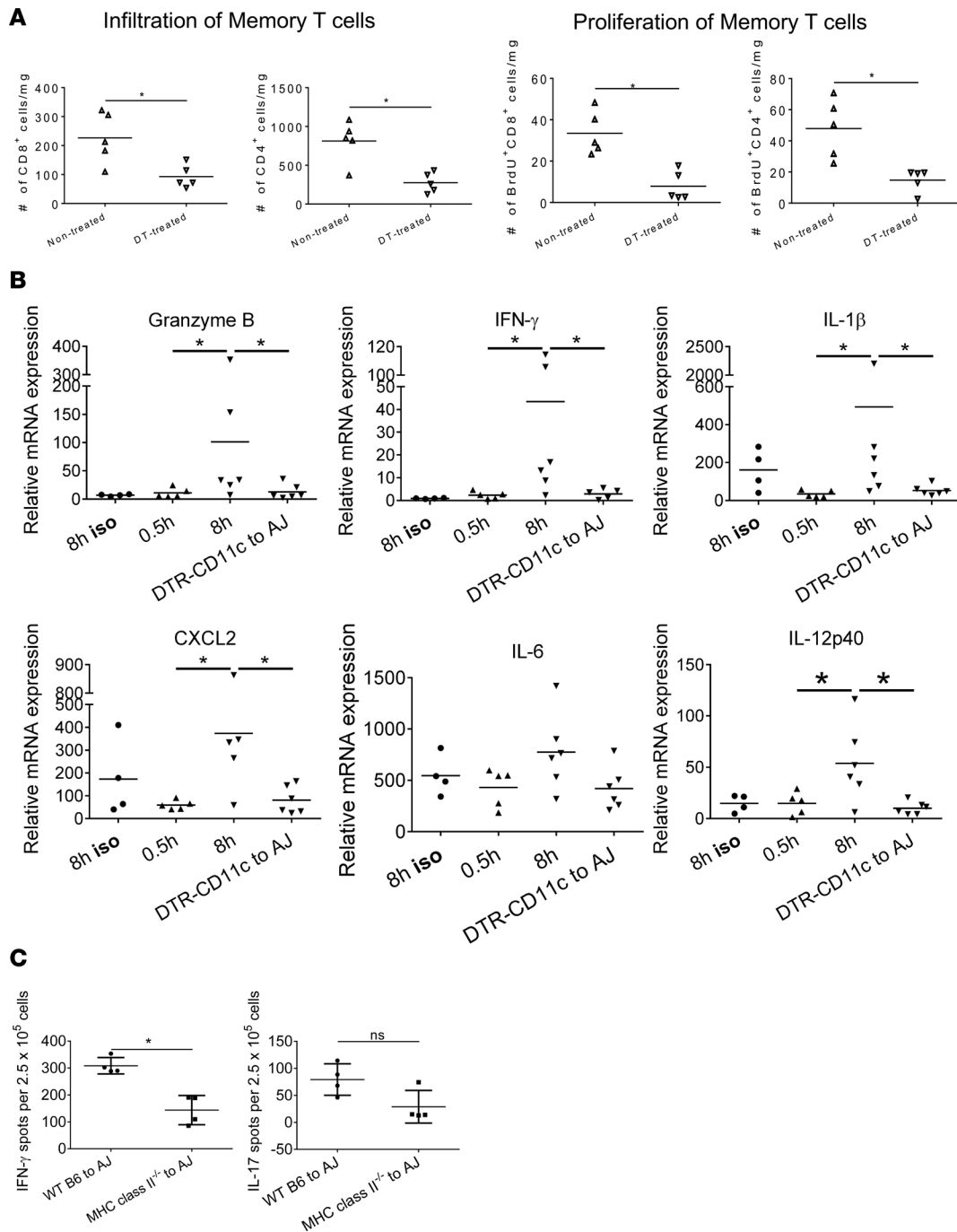
Figure 6. Endogenous memory CD8⁺ T cell proliferation in allografts subjected to prolonged CIS requires graft DCs. (A) Groups of A/J mice (*n* = 5/group) received cardiac allografts subjected to 8 hours of CIS from B6.DTR-CD11c transgenic mice that had been treated with or without diphtheria toxin (DT; 4 ng/g body weight) on days –2 and –1 prior to transplantation. On days 0 and 1 after transplant, all recipients were injected with 100 μg BrdU i.p. The following day, allografts were harvested and digested, and aliquots of single cell suspensions were stained with antibody and analyzed by flow cytometry to quantitate the infiltration and proliferation of memory CD4⁺ and CD8⁺ T cells. *P < 0.05, as determined by the Mann-Whitney nonparametric test. **(B)** Groups of A/J mice (*n* = 4–6/group) received cardiac isografts or allografts subjected to either 0.5 or 8 hours of CIS from WT C57BL/6 mice or B6.DTR-CD11c transgenic mice treated with DT prior to harvest and transplant. On day 2 after transplant, RNA was isolated from harvested grafts and expression of the indicated cytokines tested by qPCR. Results shown indicate relative expression vs. expression in naive hearts of C57BL/6 mice. *P < 0.05, as determined by the Mann-Whitney nonparametric test. **(C)** Groups of A/J mice (*n* = 5/group) received cardiac allografts subjected to 8 hours of CIS from B6.DTR-CD11c transgenic mice treated with or without DT on days –2 and –1 prior to transplantation. On day 8 after transplant, recipient spleen cells were tested to determine numbers of donor-reactive T cells producing IFN-γ or IL-17 by ELISPOT assay. *P < 0.05, as determined by the Mann-Whitney nonparametric test.

To begin to test a potential role for IL-12 in endogenous memory CD8+ T cell proliferation within cardiac allografts subjected to prolonged CIS, infiltrating memory CD8+ T cell expression of the IL-12 receptor β1 and β2 subunits (IL-12R β1 and IL-12R β2, respectively) was examined. CD8+ T cells within allografts subjected to either minimal or prolonged CIS expressed both IL-12R β1 and IL-12R β2 (Figure 7B), with greater memory CD8+ T cell numbers expressing both IL-12 receptor subunits in allografts subjected to prolonged CIS, and these numbers decreased in the absence of recipient CD4+ T cells. The expression of both IL-12 receptor subunits was restricted to those infiltrating memory CD8+ T cells with intermediate incorporation of BrdU and was absent on memory CD8+ T cells with the highest incorporation of BrdU. Expression of IL-12R β1 was expressed on a minority of memory CD4+ T cells infiltrating allografts subjected to either minimal or prolonged CIS, whereas prolonged CIS induced marked increases in allograft-infiltrating memory CD4+ T cells expressing IL-12R β2 (data not shown).

The roles of IL-12 p35/p40 heterodimers and p40 on the proliferation and accumulation of endogenous memory CD4+ and CD8+ T cells within allografts was then investigated. Endogenous memory CD8+ T cell, but not memory CD4+ T cell, proliferation and accumulation was significantly reduced in B6.p40$^{-/-}$ cardiac allografts that had been subjected to 8 hours of CIS before transplantation to A/J recipients when compared with WT C57BL/6 allografts (Figure 8A). Similarly, recipient C57BL/6 treatment with p40 neutralizing mAb resulted in significant reductions of endogenous memory CD8+ T cell proliferation and numbers in A/J allografts subjected to 8 hours of CIS and more modest reductions of endogenous memory CD4+ T cell proliferation and accumulation (Figure 8A). However, no differences in endogenous memory CD8+ and CD4+ T cell proliferation and accumulation were observed in B6.p35$^{-/-}$ and WT C57BL/6 heart allografts that had been subjected to 8 hours of CIS before transplantation to A/J recipients (Figure 8B).

To further investigate the indicated role of p40 but not p35 in endogenous memory CD8+ T cell proliferation within allografts subjected to prolonged CIS, mRNA levels of IL-12 p35, IL-23 p19, and IL-12 p40 in cardiac iso- and allografts were compared 2 days after transplantation. Although p35 mRNA was expressed at 6- to 8-fold higher levels than p19 mRNA, expression of IL-23 p19 and IL-12 p35 mRNA did not change in allografts subjected to either minimal or prolonged CIS or in isografts subjected to prolonged CIS time before transplant (Figure 8C). Only expression of p40 mRNA increased in allografts subjected to the prolonged vs. minimal CIS time before transplantation.

When assessed 48 hours after transplant, p40 protein was increased in allografts subjected to prolonged vs. minimal CIS, as well as in the recipient serum, and these increases were dependent on recipient CD4+ T cells (Figure 9A). In contrast, IL-12 p70 heterodimer protein levels were equivalent in heart allografts subjected to either minimal or prolonged CIS, and the absence of recipient CD4+ T cells did not alter p70 levels in the allografts subjected to prolonged CIS.

Macrophages and DC often produce p40 homodimers during inflammatory stimulation and antagonize the delivery of IL-12 p35/p40 heterodimer signaling to T cells during antigen priming (33, 34). A role for p40 homodimers in the proliferation of the endogenous memory CD8+ T cells within the highly ischemic cardiac allografts was directly addressed by testing the ability of p40 homodimers to induce endogenous memory CD8+ T cell proliferation within cardiac allografts subjected to minimal or prolonged CIS. Injection of p40 homodimers into mice on the day of and 1 day after transplant provoked a marked increase in memory CD8+ T cell proliferation in allografts subjected to 0.5 hours of CIS when assessed on day 2 after transplant but had no effect on memory CD8+ T cell proliferation within allografts subjected to prolonged CIS (Figure 9B). Injection of the p40 homodimers had little to no effect on proliferation and numbers of endogenous memory CD4+ T cells in allografts subjected to either minimal or prolonged CIS (not shown).

Finally, the impact of p40 neutralization on the CTLA-4Ig–resistant rejection of allografts that had been subjected to 8 hours of CIS prior to transplantation was tested. As previously reported (27), CTLA-4Ig treatment of recipients of allografts subjected to minimal CIS prior to transplant extended graft survival from 8–10 days in control-treated recipients to 50–60 days, and administration of anti-p40 mAb had no impact on the CTLA-4Ig enhanced allograft survival (Figure 9C). In contrast, peritransplant CTLA-4Ig plus anti-p40mAb significantly extended survival of allografts subjected to prolonged CIS from days 20–22 in recipients treated with CTLA-4Ig alone to between days 30–50 after transplant.
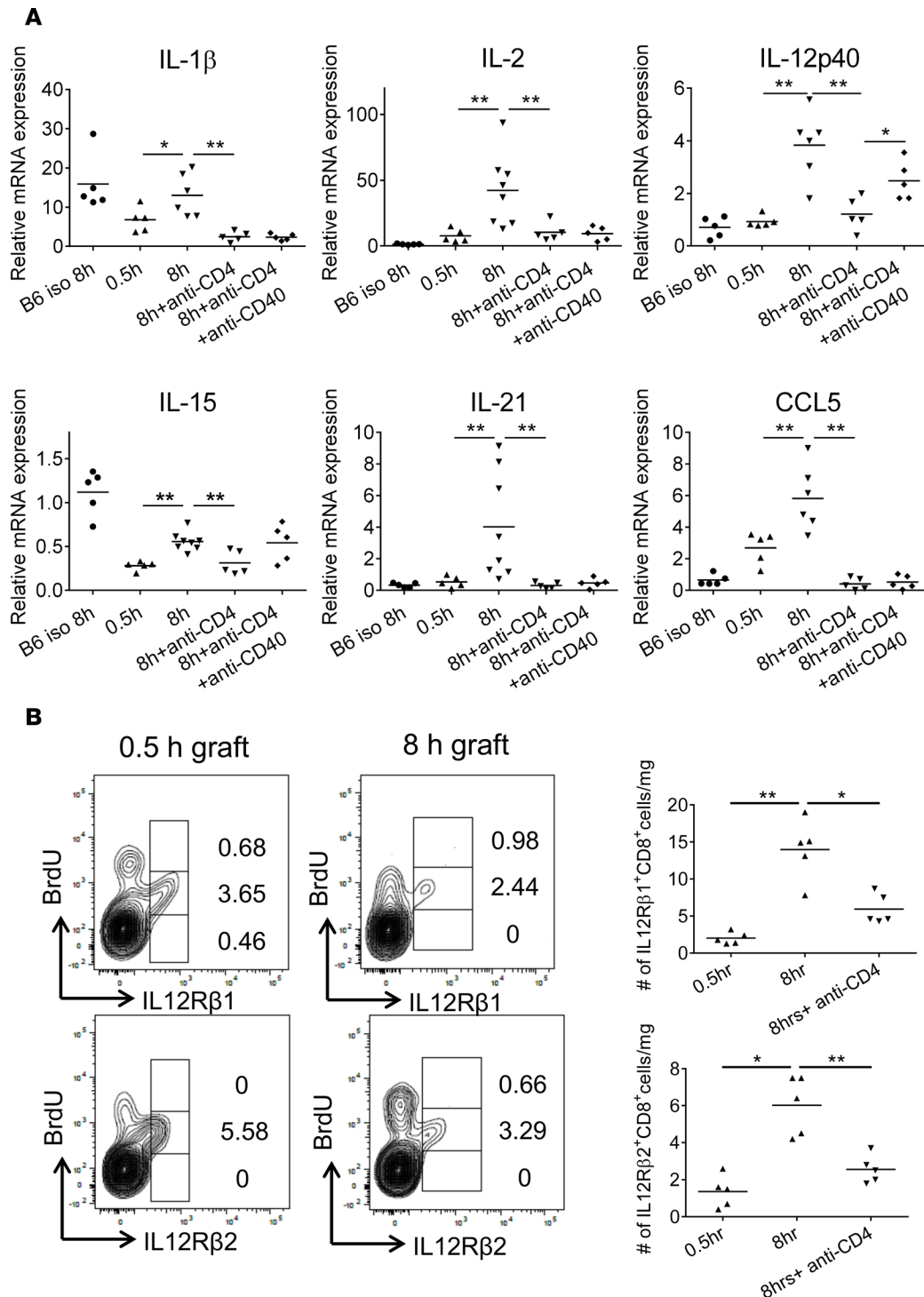
Figure 7. Elevated expression of IL12p40 mRNA in allografts subjected to 8 hours of CIS. (**A**) Groups of C57BL/6 (*n* = 4–6/group) mice received C57BL/6 isografts subjected to 8 hours of CIS or A/J cardiac allografts subjected to either 0.5 or 8 hours of CIS. The indicated recipients of 8-hour CIS allografts were treated with 200 μg control rat IgG or anti-CD4 mAb on days –3, –2, and –1 prior to transplant. They then received A/J cardiac allografts subjected to 8 hours of CIS and then either 100 μg control rat IgG or agonist anti-CD40 mAb i.v. on days 0 and 1 after transplant. Grafts were harvested on day 2 after transplant, total RNA was isolated, and expression of the indicated cytokine mRNAs was tested by qPCR. Results shown indicate relative expression vs. expression in naive hearts of C57BL/6 mice. *P < 0.05, as determined by the Mann-Whitney nonparametric test. (**B**) For the indicated groups of C57BL/6 (*n* = 5/group) allograft recipients in **A**, 100 μg BrdU was given i.p. on days 0 and 1 after transplant. The next day, all allografts were harvested and digested, and aliquots of single cells were stained with antibody and analyzed by flow cytometry to assess infiltrating memory CD8+ T cell proliferation and their expression of IL-12R β1 and IL-12R β2. *P < 0.05, **P < 0.01, as determined by the Mann-Whitney nonparametric test.
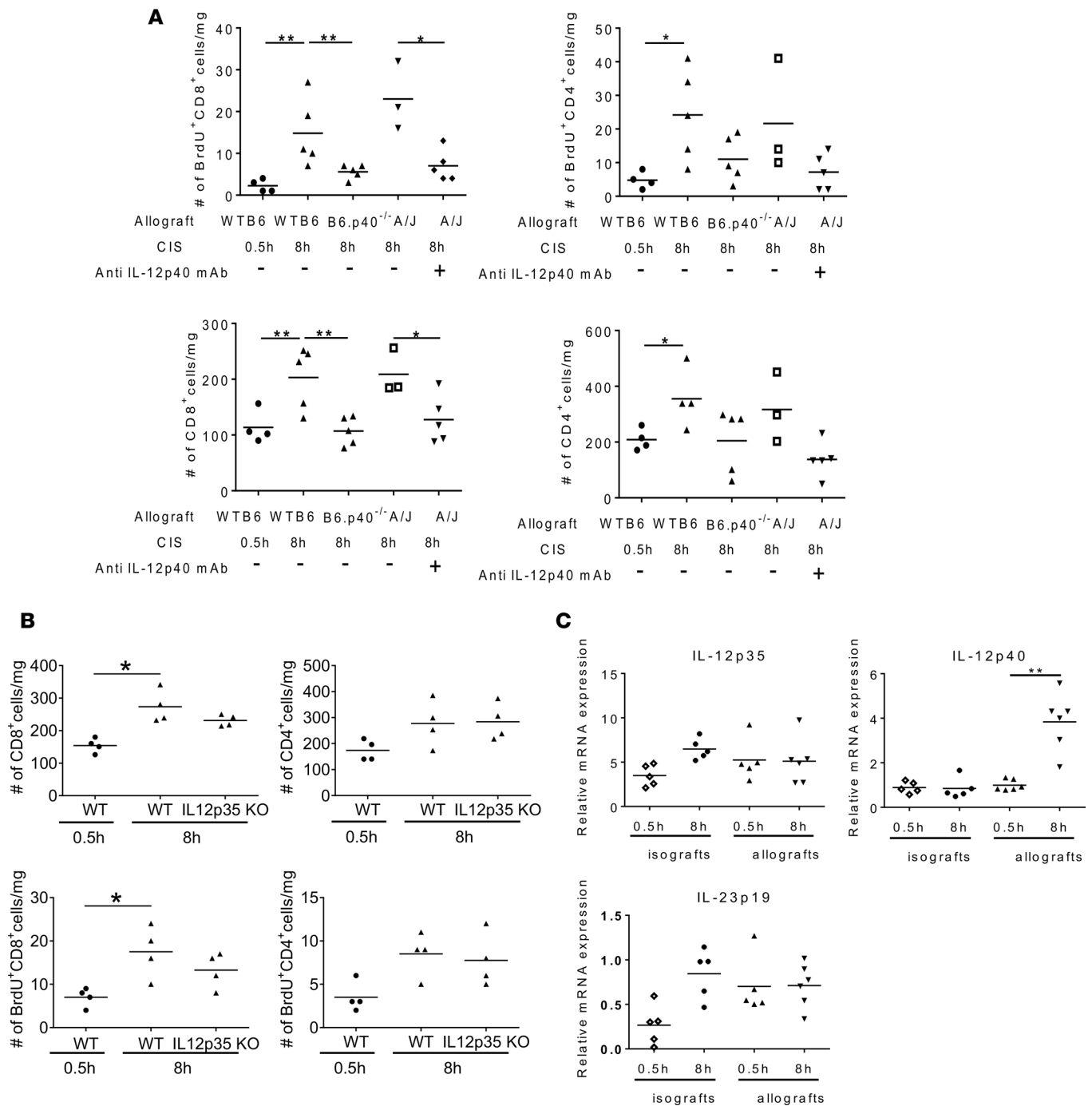
Figure 8. Endogenous memory CD8+ T cell proliferation within allografts subjected to prolonged CIS requires graft p40 but not p35. (**A**) Groups of A/J mice (*n* = 4–8/group) received cardiac allografts subjected to 0.5 or 8 hours of CIS from either WT C57BL/6 (WTB6) or B6.IL12p40^{−/−} donors. The indicated recipients of WT allografts were treated with 200 μg anti–IL-12 p40 mAb i.p. on days 0 and 1. All recipients were injected with 100 μg BrdU i.p. on days 0 and 1 after transplant. The following day, allografts were harvested and digested, and cell aliquots were stained with antibody and analyzed by flow cytometry to assess total numbers and proliferation of infiltrating memory CD4+ and CD8+ T cells. *P < 0.05; **P < 0.01, as determined by the Mann-Whitney nonparametric test. (**B**) Groups of A/J mice (*n* = 4/group) received cardiac allografts subjected to 0.5 or 8 hours of CIS from WT C57BL/6 or B6.IL12p35^{−/−} donors. All recipients were injected with 100 μg BrdU i.p. on days 0 and 1. On day 2 after transplant, allografts were harvested and digested, and cell aliquots were stained with antibody and analyzed by flow cytometry to assess total numbers and proliferation of infiltrating memory CD4+ and CD8+ T cells. *P < 0.05 as determined by the Mann-Whitney nonparametric test. (**C**) Groups of C57BL/6 mice (*n* = 5–6/group) received C57BL/6 cardiac isografts or A/J cardiac allografts subjected to 0.5 or 8 hours of CIS. Grafts were harvested on day 2 after transplant, total RNA was isolated, and expression of the indicated cytokine mRNA was tested by qPCR. Results shown indicate relative expression vs. expression in naive hearts of A/J mice. **P < 0.01, as determined by the Mann-Whitney nonparametric test.
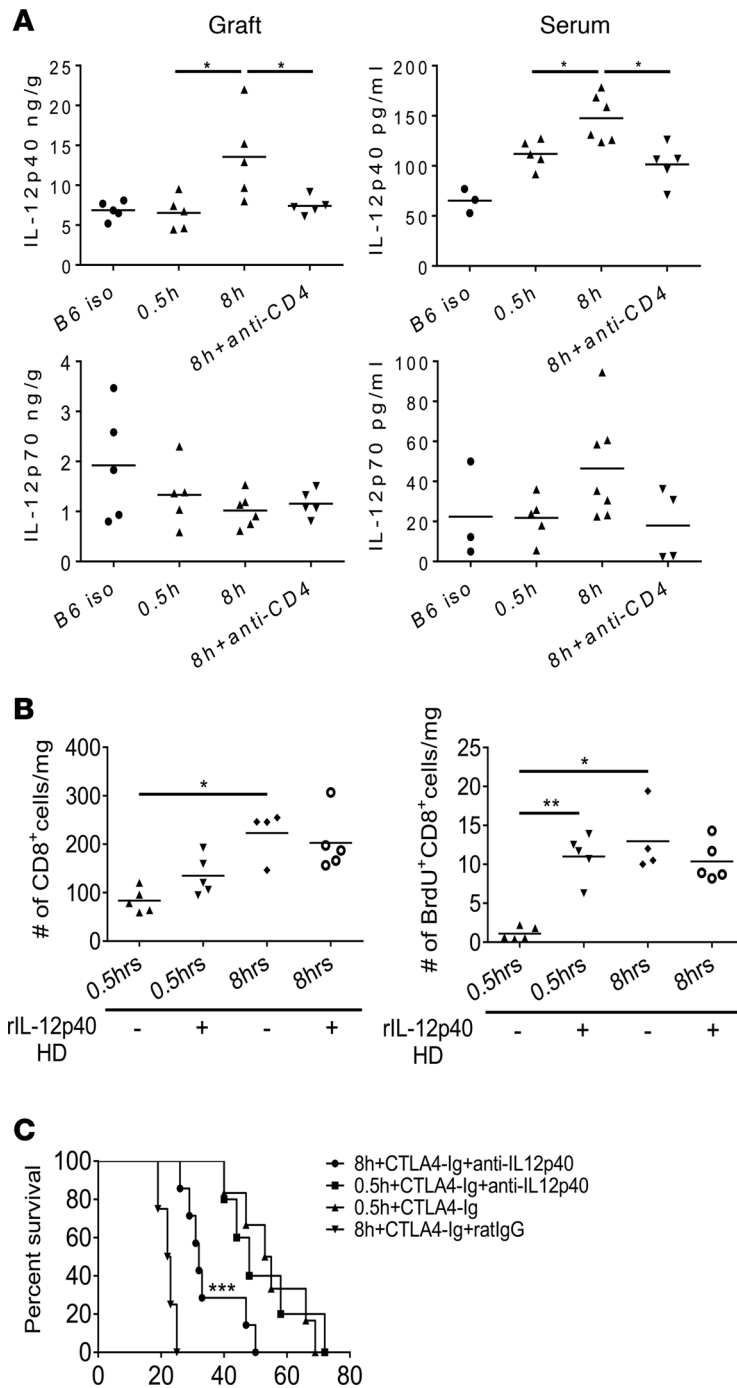
Figure 9. Endogenous memory CD8+ T cell proliferation within cardiac allografts subjected to prolonged CIS and CTLA-4Ig–resistant allograft rejection requires p40 homodimers. (A) Groups of C57BL/6 mice (*n* = 5/group) received A/J cardiac allografts or C57BL/6 isografts subjected to 0.5 or 8 hours of CIS. The indicated recipients of 8 hours of CIS allografts were treated with 200 μg control rat IgG or anti-CD4 mAb on days –3, –2, and –1 prior to transplant. On day 2 after transplant, serum and graft lysates were prepared and quantities of p40 homodimers and IL-12 p70 heterodimers were tested by ELISA. *P < 0.05, as determined by the Mann-Whitney nonparametric test. **(B)** Groups of C57BL/6 mice (*n* = 5/group) received A/J cardiac allografts subjected to 0.5 or 8 hours of CIS and the indicated recipients were injected with 200 μg recombinant p40 homodimers i.p. on days 0 and 1. All recipients were injected with 100 μg BrdU i.p. on days 0 and 1 after transplant; the next day, allografts were harvested and digested, and cell aliquots were stained with antibody and analyzed by flow cytometry to assess total numbers and numbers of proliferating memory CD8+ T cells infiltrating the grafts. *P < 0.05; **P < 0.01, as determined by the Mann-Whitney nonparametric test. **(C)** Groups of C57BL/6 mice (*n* = 4–7/group) received A/J cardiac allografts subjected to 0.5 or 8 hours of CIS, and on days 0 and 1, were treated with 200 μg control rat IgG or 250 μg CTLA-4Ig i.p. with or without 200 μg recombinant p40 homodimers i.p. Allograft survival was monitored by daily abdominal palpation and rejection confirmed by laparotomy. ***P < 0.001 vs. all other groups, as determined using the Log-rank/Mantel-Cox test.

Discussion

This study investigated the early posttransplant function of polyclonal donor-reactive memory CD4+ and CD8+ T cells that comprise the endogenous memory T cell repertoire in unprimed heart allograft recipients. Our previous studies had indicated the CD4+ T cell–independent activation of endogenous memory CD8+ T cells to produce IFN-γ within allografts transplanted as quickly as possible from donor harvest to revascularization in the recipient, but this activation was insufficient to mediate acute rejection (16, 18). In contrast, prolonging CIS time prior to transplant increased endogenous memory CD4+ and CD8+ T cell numbers within the allografts and activation of the memory CD8+, but not CD4+, T cells to directly reject the allograft (27). Such direct memory CD8+ T cell activation within tissue sites is often dependent on inflammatory signals that compensate for the absence of CD4+ T cell–generated helper signals (35–37). A key finding of the current study is that the increased activation of endogenous memory CD8+ T

cells to mediate acute injury and rejection of allografts subjected to prolonged CIS requires endogenous memory CD4^{+} T cell activation, suggesting that a critical difference within allografts subjected to minimal vs. prolonged CIS is that the increased inflammation in the latter grafts generates an environment promoting memory CD4^{+} T cell interaction with graft DC to produce helper signals required for optimal donor-reactive memory CD8^{+} T cell activation.

While memory T cells cross-reactive to allogeneic MHC are able to disrupt strategies administered to recipients to induce allograft tolerance (30, 38, 39), it had remained unclear if such endogenous memory CD8^{+} T cells have the ability to directly mediate graft injury independently of CD4^{+} T cell help. Although a requirement for CD4^{+} T cell help to elicit donor-reactive memory CD8^{+} T cell responses to complete MHC-mismatched allografts has not been observed, previous studies investigating memory CD4^{+} and CD8^{+} T cell activation to allografts have primarily utilized donor-sensitized recipients or transfer of donor-primed memory T cells to naive recipients (39–43). In addition to CD4^{+} T cell–independent activation, these donor alloantigen–primed memory CD8^{+} T cell responses are typically resistant to both CD28- and CD154-mediated costimulatory blockade, and we have also observed that memory T cells primed to donor skin allografts are resistant to CD40-CD154 costimulatory blockade when transferred to recipients of donor heart allografts subjected to prolonged CIS (H. Tsuda, unpublished results). Clearly, these donor antigen priming strategies bias the reactive memory CD8^{+} T cell repertoire to those cells with strong reactivity to the sensitizing antigen, and this is likely to confer their CD4^{+} T cell–independent activation and resistance to many different costimulatory blockade strategies. Alternatively, the endogenous memory T cell repertoire is likely to be composed of cells with a range of avidities for allogeneic MHC, and the current study demonstrates that peritransplant treatment with anti-CD154 mAb inhibits endogenous memory CD8^{+} T cell activation within allografts subjected to prolonged CIS and enhances graft survival.

A second key finding of the current study is the identification of unique memory CD4^{+} T cell–induced helper factors, IL-12 p40 homodimers, required for the proliferation and accumulation of endogenous memory CD8^{+} T cells within the highly ischemic allografts. This increase in infiltrating memory CD8^{+} T cell numbers within allografts is not a trivial matter, as previous studies titrating donor antigen–primed memory T cells have demonstrated that a critical threshold is needed to mediate allograft rejection and resistance to costimulatory blockade (39). IL-12 p40/p35 (i.e., p70) heterodimers direct naive antigen–specific CD4^{+} T cell differentiation to IFN-γ–producing cells during initiation of T cell responses to intracellular bacterial infections and potentiate elicitation of primary and memory CD8^{+} T cell responses (44–49). Although IL-12 p70 heterodimers are required for rejection of single minor antigen OVA-expressing heart allografts, their absence does not decrease donor-reactive effector CD4^{+} and CD8^{+} T cell priming or rejection of complete MHC-mismatched cardiac allografts (50–52). The absence of allograft p40, but not p35, or antibody neutralization of p40 abrogates endogenous memory CD8^{+} T cell activation within allografts subjected to increased CIS, implicating p40 homodimers, and not IL-12 p70 heterodimers, as critical helper factors driving endogenous memory CD8^{+} T cell proliferation within the allografts. The upregulation of p40, but not p35, mRNA and the absence of this upregulation in allografts depleted of DC in cardiac allografts subjected to prolonged, but not minimal, CIS suggests that the increased inflammatory environment promotes memory CD4^{+} T cell interaction with graft DCs to induce p40, but not p35, transcription and translation. Indeed, p40 homodimers were initially viewed as antagonists for the IL-12R β1 and –β2 heterodimer, resulting in modulation of the potency of the IL-12 p70 heterodimer to promote antigen-specific CD4^{+} T cell development to IFN-γ–producing cells (33, 34). Recent studies suggest that p40 homodimer binding to dimers or oligomers of IL-12R β1 on macrophages and DCs activates NF-κB to induce chemokine production (53, 54).

A final key finding is the requirement for graft cells producing p40 and graft DCs for endogenous memory CD8^{+} T cell activation within the highly ischemic allografts. DCs in heart and kidney allografts subjected to minimal CIS are rapidly replaced by recipient DCs — up to 90% within 24 hours (55, 56) — and we have observed similar replacement in the cardiac allografts subjected to prolonged CIS prior to transplant (H. Tsuda, data not shown). The results of the current study suggest that the recipient DC infiltrating p40-deficient allografts cannot supply the IL-12 p40 homodimers required for memory CD8^{+} T cell activation. Recipient DCs have been shown to be required for de novo priming of naive donor-reactive T cells through acquisition of graft DC–derived exosomes expressing graft class I and class II MHC alloantigens (57, 58), but recipient DC acquisition of donor exosomes is unlikely to be the main pathway of alloantigen presentation activating infiltrating memory CD4^{+} and CD8^{+} T cells within the highly ischemic allografts at early times after transplant.

Collectively, the results of this study document a unique donor-reactive memory T cell response that is a consequence of increased ischemia-reperfusion injury within the organ allograft and provide potentially novel insights into the early mobilization and expansion of donor-reactive endogenous memory CD8^{+} T cells within cardiac allografts subjected to prolonged CIS. This response requires the coordinated and inter-dependent activation of donor-reactive endogenous memory CD4^{+} and CD8^{+} T cells within the ischemic allografts that generates functional memory CD8^{+} T cells that directly mediate acute tissue injury and graft rejection. The initiation and progression of this endogenous memory CD8^{+} T cell response is resistant to CTLA-4Ig but is sensitive to agents that block the CD4^{+} T cell–graft DC interactions generating the p40 homodimers. It is worth noting that these activation events are analogous to those occurring in secondary lymphoid organs during de novo activation of reactive CD8^{+} T cells by antigen-presenting DCs, where CD4^{+} T cell licensing of antigen-presenting DC is required for optimal naive antigen–reactive CD8^{+} T cell development to functional effector cells. Considering the high incidence of acute rejection episodes observed in kidney transplant patients treated with CTLA-4Ig in place of cyclosporine A (59, 60), the results suggest other targets to inhibit or attenuate this rejection. Given the detrimental impact of ischemia-reperfusion injury and memory T cells on allograft function and survival, strategies to minimize the postreperfusion inflammatory milieu of higher-risk grafts by inhibiting early memory T cell proliferation and activation to express the effector functions mediating graft tissue injury should prove beneficial to transplant outcomes.

Methods

Mice. C57BL/6 (H-2[b]), congenic B6.PL-Thy1a/CyJ mice (H-2[b]), B6.MHC class II^{−/−}, B6.CD40^{−/−}, B6.p40^{−/−}, B6.p35^{−/−}, and B6.diphtheria-toxin receptor–CD11c (DTR-CD11c) transgenic mice and A/J (H-2[a]), BALB/c (H-2[d]), and DBA/1 (H-2[q]) mice were purchased from the Jackson Laboratory. The generation of C57BL/6 mice transgenically expressing K[d] (i.e., B6.K[d]) has been previously detailed (61), and colonies of B6.K[d] mice were established and maintained at our facility. Male mice, 8–12 weeks of age, were used in the studies.

Heterotopic cardiac transplantation and recovery. Murine heterotopic intraabdominal cardiac transplantation and recovery were adapted from the method of Corry and colleagues as previously reported by our laboratory (18, 62). Briefly, the donor aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava in the peritoneal cavity. Following harvest from the donor, hearts were stored for either 0.5 or 8 hours in cold UW solution (BUW-0001, Bridge to Life) on ice prior to transplant. Graft survival was monitored daily by recipient abdominal palpation, and rejection was confirmed visually by laparotomy. At the time of cardiac graft recovery, 10 ml of Ringer's solution (Ringer's Irrigation, USP, Baxter Healthcare Corporation) was flushed into the recipient circulatory system. Grafts and recipient spleens were removed and were snap-frozen in liquid nitrogen or placed in media for digestion and analysis of graft-infiltrating cells.

In vivo antibody treatments. A list of all antibodies used in this study and their sources is provided in Supplemental Table 1. CD4^{+} T cell depletion in graft recipients was performed using a 1:1 cocktail of anti-CD4 mAb, (YTS191 and GK1.5, Bio X Cell), 0.2 mg given i.p. on days −3, −2, and −1 prior to transplant. CD4^{+} T cell depletion was ≥ 98% in peripheral blood and spleen in treated sentinel animals. CTLA-4Ig (Bio X Cell) was given at a daily dose of 0.25 mg i.p. on days 0 and +1 after transplant. Anti-CD154 mAb (MR-1, Bio X Cell) was administered at 0.25 mg i.p. daily on days 0 and +1. Agonist anti-CD40 mAb (FGK4.5, Bio X Cell) was administered at 0.1 mg i.v. daily on days 0 and +1. Anti-p40 mAb (C17.8, catalog 505305, BioLegend) was given at 0.2 mg i.p. daily on days 0 and +1. In all cases, equal amounts of normal rat IgG (catalog I4131, MilliporeSigma) were administered to control groups. Recombinant p40 homodimers (BioLegend) were given at 0.2 µg i.v. on day +1 after transplant.

BrdU labeling and flow cytometry analysis of graft-infiltrating CD4^{+} and CD8^{+} T cells. To assess memory CD4^{+} and CD8^{+} T cell proliferation within allografts, recipient mice were injected i.p. with 100 µg BrdU (BD Biosciences) on days 0 and +1 after transplant. Flow cytometric detection of graft-infiltrating cells was performed using a modification of the method of Afanasyev and colleagues (18, 63, 64). On the indicated day after transplant, harvested grafts were weighed, cut into pieces, and incubated for 1 hour at 37°C in RPMI with Type II collagenase (MilliporeSigma). The tissue was then pressed through a 40-µm filter, the collected cells were washed and counted, and the aliquots were stained with PECy7-anti-CD45 (30-F11, eBiosciences, catalog 20-0451-81), PE-anti-CD4 (RM4-5, BD Biosciences, catalog

553049), and APC-anti-CD8 mAb (53-6.7, BD Biosciences, catalog 553045). Intracellular staining to detect BrdU incorporation was performed using the FITC BrdU Kit according to the manufacturer's protocol (BD Biosciences). Total numbers of each leukocyte population were determined by the following equation: (the total number of leukocytes counted) × (% of the leukocyte population counted in the CD45+ cells)/100.

Memory T cell priming, purification, and adoptive transfer. To generate donor-reactive memory T cell populations, WT CD45.2+ C57BL/6 recipients received full thickness A/J skin allografts. Recipient spleen suspensions were prepared 6 weeks later, and total CD8+ T cells were enriched by negative selection (Stemcell Technologies). Purified CD8+ T cell populations (roughly 50% were of an activated/memory phenotype, CD44high) were transferred i.v. (2×10^6/mouse) to congenic CD45.1+ B6 recipients. After 72 hours, recipient mice remained untransplanted or received cardiac allografts subjected to minimal or prolonged CIS.

In vitro alloreactivity assay. Graft-infiltrating CD8+ T cells were purified from allografts on day 3 after transplant by surface staining with PE-anti-CD8 mAb, followed by positive selection with anti-PE mAb microbeads (Miltenyi Biotec). Purified responder CD8+ T cells were labeled with 3 μM CFSE (Invitrogen) in PBS for 10 minutes at room temperature. An equal volume of FCS (Atlanta Biologicals) was added to stop the staining. Stimulator spleen cells from C57BL/6, A/J, or DBA (third party) were depleted of T cells using mouse pan T Dynabeads (Invitrogen) and treated with 25 μg/ mitomycin C (MilliporeSigma). A 1:4 mixture of responder and stimulator cells was cultured for 4 days in 200 μl complete RPMI (Mediatech Inc.) in a U-bottomed 96-well plate, and CFSE dilution on the graft-purified responder CD8+ T cells was detected by flow cytometry analysis.

RNA extraction and quantitative analysis of chemokines. RNA was isolated from harvested iso- and allografts using Fibrous Tissue kits (QIAGEN) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as previously described (65). Duplicate runs of isolated RNA from 3–5 individual grafts per group were tested, and the data for each group are expressed as mean test cytokine expression level ± SEM.

IL-12 p70 and –p40 ELISA. Harvested grafts were immediately snap-frozen, and lysates were prepared in 1.5% Triton-X in PBS with protease inhibitors as previously detailed (18). Quantification of IL-12 heterodimer and p40 homodimer protein was performed on heart iso- and allograft lysates, and serum was collected at 48 hours after transplant using Mouse IL-12 p70 and Mouse p40 Quantikine ELISA Kits, respectively, from R&D Systems.

Statistics. Data analysis was performed using GraphPad Prism Pro (GraphPad Software Inc.). Cardiac allograft survival was plotted using Kaplan-Meier cumulative survival curves, and differences in survival between groups were determined using the Log-rank (Mantel-Cox) test. Differences between experimental and control or naive groups for cellular infiltration and proliferation were evaluated by the Mann-Whitney nonparametric test to determine significance. Statistical comparison between groups for the in vitro alloreactivity assay was determined by 1-way ANOVA with Bonferroni's multiple comparison post test. $P < 0.05$ was considered a significant difference between groups. Error bars indicate ± SEM.

Study approval. All procedures involving animals were approved by the IACUC at the Cleveland Clinic.

Author contributions

HT, CAS, TT, KA, and BM performed experiments. AV provided advice on experimental strategies, and RLF designed the research and wrote the paper.

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