2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses

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Mounting evidence in models of both autoimmunity and chronic viral infection suggests that the outcome of T cell activation is critically impacted by the constellation of co-stimulatory and co-inhibitory receptors expressed on the cell surface. Here, we identified a critical role for the co-inhibitory SLAM family member 2B4 (CD244) in attenuating primary antigen-specific CD8+ T cell responses in the presence of immune modulation with selective CD28 blockade. Our results reveal a specific up-regulation of 2B4 on antigen-specific CD8+ T cells in animals in which CD28 signaling was blocked. However, 2B4 up-regulation was not observed in animals treated with CTLA-4 Ig (abatacept) or CD28 blockade in the presence of anti–CTLA-4 mAb. 2B4 up-regulation after CD28 blockade was functionally significant, as the inhibitory impact of CD28 blockade was diminished when antigen-specific CD8+ T cells were deficient in 2B4. In contrast, 2B4 deficiency had no effect on CD8+ T cell responses during unmodified rejection or in the presence of CTLA-4 Ig. We conclude that blockade of CD28 signals in the presence of preserved CTLA-4 signals results in the unique up-regulation of 2B4 on primary CD8+ effectors, and that this 2B4 expression plays a critical functional role in controlling antigen-specific CD8+ T cell responses.

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Abbreviations used: CTLA-4, cytotoxic T lymphocyte antigen 4; dAb, domain antibody; DLN, draining LN; ICOS, inducible co-stimulator; MST, median survival time; PD-1, programmed death 1; PD-L1, programmed death ligand 1.

T cell–specific co-stimulation blockade is an attractive alternative to traditional immunosuppression to mitigate unwanted immune responses during transplantation and autoimmunity. Owing to the limited tissue distribution of its targets, T cell co-stimulation blockade offers a potential advantage over calcineurin inhibitors (CNI) in that it is associated with lower nephrotoxicity, hyperlipidemia, and development of type 2 diabetes (Vincenti et al., 2005, 2010a,c, 2012; Durrbach et al., 2010; Larsen et al., 2010). For example, under current CNI-based immunosuppressive regimens, the half-life of a transplanted kidney is just over 10 yr (Lamb et al., 2011; Lodhi et al., 2011), and chronic dysfunction associated with the use of CNIs has been causally linked to graft loss. Freedom from these off-target toxicities offers a potential quantity and quality of life benefit for transplant recipients. However, the T cell co-stimulation blocker belatacept, recently FDA approved for use in renal transplantation, is also associated with a higher incidence and severity of acute rejection as compared with standard CNI-based immunosuppression (Vincenti et al., 2010b). Thus, addressing the increased incidence of acute rejection is an important goal in optimizing the use of T cell co-stimulation blockade to improve outcomes in transplantation.

Accumulating evidence over the last decade in models of both autoimmunity and chronic viral infection suggests that the outcome of T cell activation during priming and recall is critically impacted by the constellation of co-stimulatory and co-inhibitory receptors expressed on the surface of those cells (Blackburn et al., 2009; Crawford and Wherry, 2009). However, how the balance of signals from co-stimulatory and co-inhibitory molecules affects primary

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and secondary responses in transplantation is not well understood, and new knowledge in this area is needed to facilitate therapeutic manipulation of the anti-donor T cell response. One such co-inhibitory molecule recently identified as being expressed on exhausted cells after chronic viral infection is 2B4 (CD244, SLAMf4), a 38-kD type I transmembrane protein and member of the CD2 subset of the immunoglobulin superfamily molecules (Lee et al., 2004; Vaidya et al., 2005). 2B4 is expressed on NK cells, monocytes, basophils, and eosinophils, and is inducibly expressed on a subset of $CD8^+$ T cells in both mice and humans (Rey et al., 2006; Wherry et al., 2007; Blackburn et al., 2009; Bengsch et al., 2010; Raziorrouh et al., 2010; Waggoner et al., 2010; Wang et al., 2010). In NK cells, 2B4 has been reported to have both activating and inhibitory functions (Laouar et al., 2007); however recent evidence in both murine and human models indicates that its role in T cells is co-inhibitory. 2B4 expression is reduced in patients with systemic lupus erythematosus (SLE; Kim et al., 2010), and 2B4 deficiency in mice resulted in spontaneous development of a SLE-like disease in autoimmune-prone genetic backgrounds (Brown et al., 2011). However, the regulation of expression of this co-inhibitor is not well understood, particularly with regard to how the balance of initial co-stimulatory and co-inhibitory signals during T cell activation impacts 2B4 expression to further fine-tune the response.

Understanding how this initial balance of co-stimulatory and co-inhibitory signals impacts T cell responsiveness is particularly clinically relevant because both T cell co-stimulation blockers that are currently approved for use in transplantation (belatacept) and autoimmunity (abatacept) are CTLA-4-Ig fusion proteins that bind both CD80 and CD86, thus inhibiting both co-stimulatory signaling through CD28 as well as co-inhibitory signaling through CTLA-4 (Salomon and Bluestone, 2001). In this study, we used a novel domain antibody that selectively blocks CD28 while leaving CTLA-4 signals intact to dissect how the balance of CD28 co-stimulatory and CTLA-4 co-inhibitory pathways engaged during T cell activation impacts the subsequent expression of additional co-stimulatory and co-inhibitory molecules on donor-reactive T cell responses after transplantation. Therapeutic use of anti-CD28 antibody was previously attempted in the TGN1412 study, in which an agonistic anti-CD28 F_c-intact monoclonal antibody resulted in massive T cell activation and "cytokine storm" in a Phase I clinical trial (Suntharalingam et al., 2006). This study highlighted the complexities of developing CD28-specific blocking reagents (Waibler et al., 2008a,b). However, recent advances in the development of novel domain antibodies, in which the F_c portion is completely absent, have allowed the development of novel blocking, nonactivating reagents to safely and specifically block CD28 co-stimulatory signals, while leaving CTLA-4 co-inhibitory signals intact. Recent work has shown that sc28AT, a monovalent CD28-specific fusion antibody modestly prolonged cardiac and renal allograft survival in nonhuman primates (NHP) as monotherapy and was more effective in combination with CNIs (Poirier et al., 2010). Work in a murine model demonstrated that the graft-prolonging effects of sc28AT were CTLA-4 dependent (Zhang et al., 2011). However, a mechanistic understanding of the effects of selective CD28 blockade in transplantation and autoimmunity is lacking. Here, we used a TCR transgenic model of minor antigen disparity to specifically identify and characterize donor-reactive T cell responses after transplantation to interrogate the ability of a novel domain antibody (dAb) that selectively blocks CD28 to alter T cell programming by impacting the subsequent expression of other co-stimulatory and co-inhibitory molecules on antigen-specific T cell responses. Our results indicate that the enhanced efficacy of selective CD28 blockade in prolonging graft survival as compared with CTLA-4 Ig is dependent on the specific up-regulation of the 2B4 co-inhibitor on antigen-specific $CD8^+$ T cell responses after transplantation.

RESULTS

Selective CD28 blockade results in superior graft survival as compared with CTLA-4 Ig, where both CD28 co-stimulatory and CTLA-4 co-inhibitory signals are blocked

To test the hypothesis that selective CD28 blockade with preserved CTLA-4 co-inhibitory signals may result in enhanced prolongation of graft survival after transplantation, we made use of a novel recombinant domain antibody specific for CD28, which contains only the antigen-binding Vκ variable domain and lacks an F_c domain. To test the efficacy of this anti-CD28 dAb in inhibiting donor-reactive T cell responses in a fully MHC disparate model of endogenous polyclonal alloreactivity, B6 recipients of BALB/c skin grafts were treated with CTLA-4 Ig or CD28 dAb in the presence of anti-CD154 mAb (Fig. 1 A). As previously reported (Ford et al., 2007), the median survival time (MST) of animals treated with CTLA-4 Ig and anti-CD154 was 32 d, significantly longer than that of untreated animals (MST 14 d). In contrast, 100% of the animals treated with the CD28 dAb in the presence of anti-CD154 exhibited graft survival of >50 d. To compare the effects of selective CD28 blockade in the absence of the additional immune modulation provided by the anti-CD154, we assessed skin graft rejection in a published model of minor antigen disparity (Fig. 1 B; Ehst et al., 2003). B6 recipients were grafted with skin from OVA-expressing transgenic donors, which results in rejection in control Vκ dAb-treated animals with an MST of 19 d (Fig. 1 C; Ford et al., 2007). Rejection in CTLA-4 Ig-treated recipients was prolonged to 34 d. In contrast, treatment of graft recipients with the anti-CD28 dAb resulted in better long-term graft survival (MST > 100 d; Fig. 1 C). To confirm the specificity of this reagent for CD28, B6 splenocytes were incubated with increasing doses of an unlabeled control Vκ dAb or an unlabeled anti-CD28 dAb, and then stained with a FITC-conjugated anti-CD28 mAb (clone E18) that competes with anti-CD28 dAb for binding to CD28. Results demonstrated a titratable reduction in fluorescence with increasing concentrations

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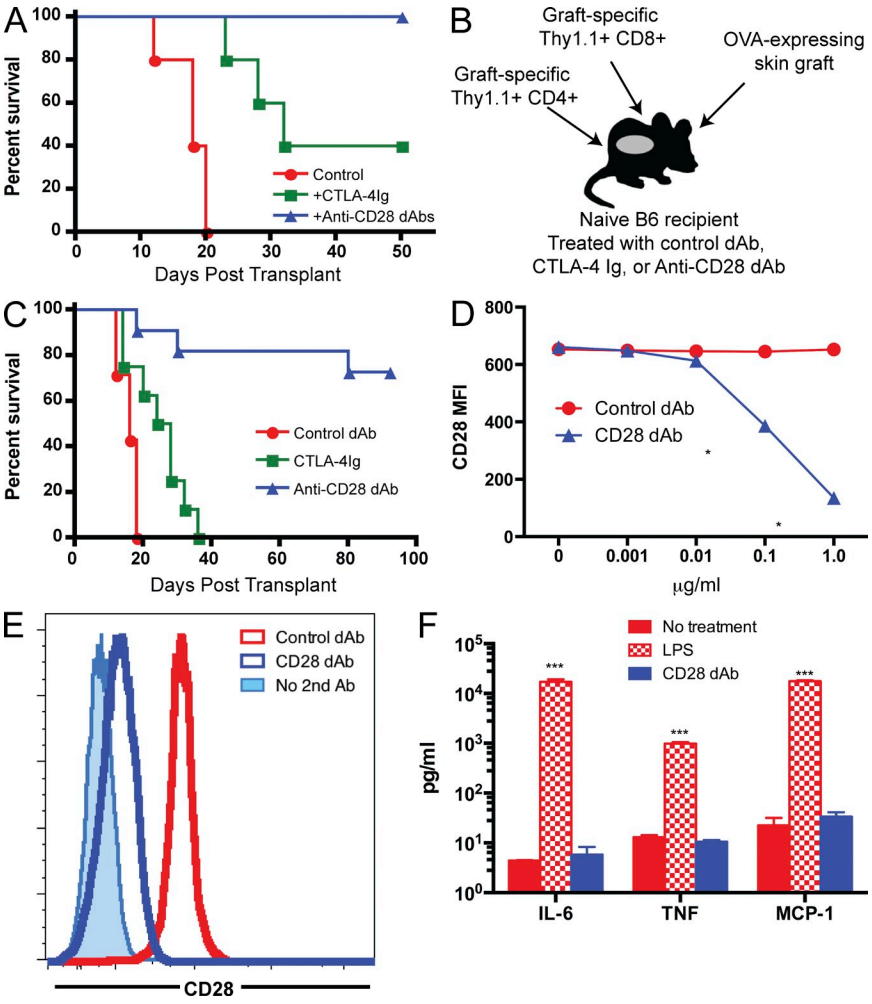

Figure 1. Selective CD28 blockade results in superior graft survival as compared with CTLA-4 Ig, where both CD28 co-stimulatory and CTLA-4 co-inhibitory signals are blocked. (A) B6 recipients of BALB/c skin grafts were treated with CTLA-4 Ig or anti-CD28 dAb in the presence of anti-CD154 mAb on days 0, 2, 4, and 6, and then three times per week continuously thereafter until day 50, as described in the Materials and methods. Anti-CD154 alone–treated animals (red line, control) served as negative controls. MST of control-treated animals was 14 d and MST of animals treated with CTLA-4 Ig and anti-CD154 was 32 d. MST of animals treated with anti-CD28 dAbs and anti-CD154 > 50 d ($P = 0.0013$ as compared with CTLA-4 Ig/anti-CD154; $n = 5$/group). (B) Experimental design of TCR transgenic model of minor antigen disparity wherein 10^6 Thy1.1+ OT-I and 10^6 Thy1.1+ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of either control dAb, CTLA-4 Ig, or anti-CD28 dAb, which were dosed on days 0, 2, 4, and 6 and then three times per week continuously thereafter as described in Materials and methods. (C) Graft survival data from the experimental design depicted in B. Control dAb and CTLA-4 Ig-treated animals rejected their grafts with MSTs of 19 and 34 d, respectively, and anti-CD28 dAb-treated animals exhibited an MST of >100 d ($P < 0.0001$ compared with CTLA-4 Ig; $n = 8$–10 animals/group for all groups). (D) Splenocytes were incubated with increasing doses of the CD28 dAb and a control dAb, and then secondarily stained with a fluorophore-conjugated anti-CD28 mAb competitive with the anti-CD28 dAb for CD28 to assess blockade in vitro. Data shown are representative of three independent experiments. (E) Similarly, mice were injected with 100 μg of anti-CD28 dAb or control dAb and their splenocytes were harvested 3 h later to assess in vivo CD28 blockade. Splenocytes from each mouse were stained with the same anti-CD28 mAb competitive for CD28. A sample without the secondary anti-CD28 antibody was included as an additional control (shaded blue). (F) To evaluate for cytokine release after anti-CD28 dAb administration, mice were injected with anti-CD28 dAb (100 μg) or LPS (20 μg) as a positive control. Serum cytokine levels were measured at baseline before injection (no treatment) and 6 h after injection ($n = 3$ per group, error bars SEM). *, $P < 0.05$; ***, $P < 0.001$.

of anti-CD28 dAb (Fig. 1 D). In addition, we assessed the degree of CD28 blockade in vivo in animals treated with the dose used in the aforementioned transplant experiments (Fig. 1 E). Animals were treated with 100 μg of control or anti-CD28 dAb and splenocytes were harvested and stained with the same competitive anti-CD28 mAb (clone E18). Results indicated that, at the dose used, the anti-CD28 dAb successfully blocked CD28. Lastly, we confirmed that this anti-CD28 dAb is devoid of the cross-linking–induced cytokine storm that was associated with TGN1412, the super-agonist anti-CD28 mAb that resulted in major morbidity in a pilot clinical trial (Suntharalingam et al., 2006; Waibler et al., 2008a,b). Animals were injected with anti-CD28 dAb (or LPS as a positive control) and serum cytokine levels were measured 6 h after injection, the time point at which cytokine storm began in the human recipients of the TGN1412 anti-CD28 mAb that induced cross-linking. As shown in Fig. 1 F, whereas the LPS-injected positive controls contained serum IL-6, TNF, and MCP-1 levels in the range of 10^3–10^4 pg/ml, serum cytokine levels detected in recipients of anti-CD28 dAb were similar to those observed in uninjected negative control animals.

Donor-reactive CD4+ and CD8+ T cell accumulation and differentiation are more profoundly attenuated by selective CD28 blockade than by CTLA-4 Ig

To more precisely quantify the effects of selective CD28 blockade on donor-reactive T cell responses, we used a TCR tg system in which OVA-specific CD4+ and CD8+ congenically labeled Thy1.1+ T cells are adoptively transferred into naive B6 animals which are then challenged with OVA-expressing skin grafts (Fig. 1 B). Mice were then treated with

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a control Vκ dummy dAb, anti-CD28 dAb, or CTLA-4 Ig as described in Materials and methods and sacrificed at 10 d after transplant, which we have previously shown is the peak of the donor-reactive T cell response in this system (Ford et al., 2007). Draining lymph nodes were assessed for the expansion, differentiation, and effector functions of donor-reactive $CD4^+$ and $CD8^+$ T cells. Results indicated that although frequencies and absolute numbers of donor-reactive $CD4^+$ T cell responses were effectively reduced in CTLA-4-Ig–treated animals as expected (Ford et al., 2008), there was a significant further reduction in anti-donor $CD4^+$ T cell responses in the presence of selective CD28 blockade (Fig. 2, A–C). Further examination of the phenotype of these cells revealed that selective CD28 blockade resulted in significantly impaired differentiation of naive $CD44^{lo}$ $CD62L^{hi}$ cells into $CD44^{hi}$ $CD62L^{lo}$ effectors as compared with CTLA-4 Ig (Fig. 2, D and E). These results suggest that the provision of CTLA-4 signals in the absence of CD28 signals further impedes differentiation of naive $CD4^+$ T cells into effector cells. We next assessed the effect of selective CD28 blockade on the effector function of antigen-specific $CD4^+$ T cells, and found that CD28 blockade in the presence of preserved CTLA-4 signals did not result in a further reduction in IL-2 production (as measured by intracellular cytokine staining) beyond that observed with CTLA-4 Ig (Fig. 2 F).

Analysis of donor-reactive $CD8^+$ T cell responses revealed similar results. However, consistent with previously published studies (Trambley et al., 1999; Coley et al., 2009), we observed that CTLA-4 Ig less adequately suppressed donor-reactive $CD8^+$ T cells when compared with the effect on alloreactive $CD4^+$ T cells, suggesting that $CD8^+$ T cells may be responsible for co-stimulation blockade-resistant breakthrough rejection. In contrast, selective CD28 blockade resulted in a much more profound diminution of the donor-reactive $CD8^+$ T cell response (Fig. 3, A–C). As observed with donor-reactive $CD4^+$ responses, selective CD28 blockade resulted in significantly impaired differentiation of naive $CD44^{lo}$ $CD62L^{hi}$ cells into $CD44^{hi}$ $CD62L^{lo}$

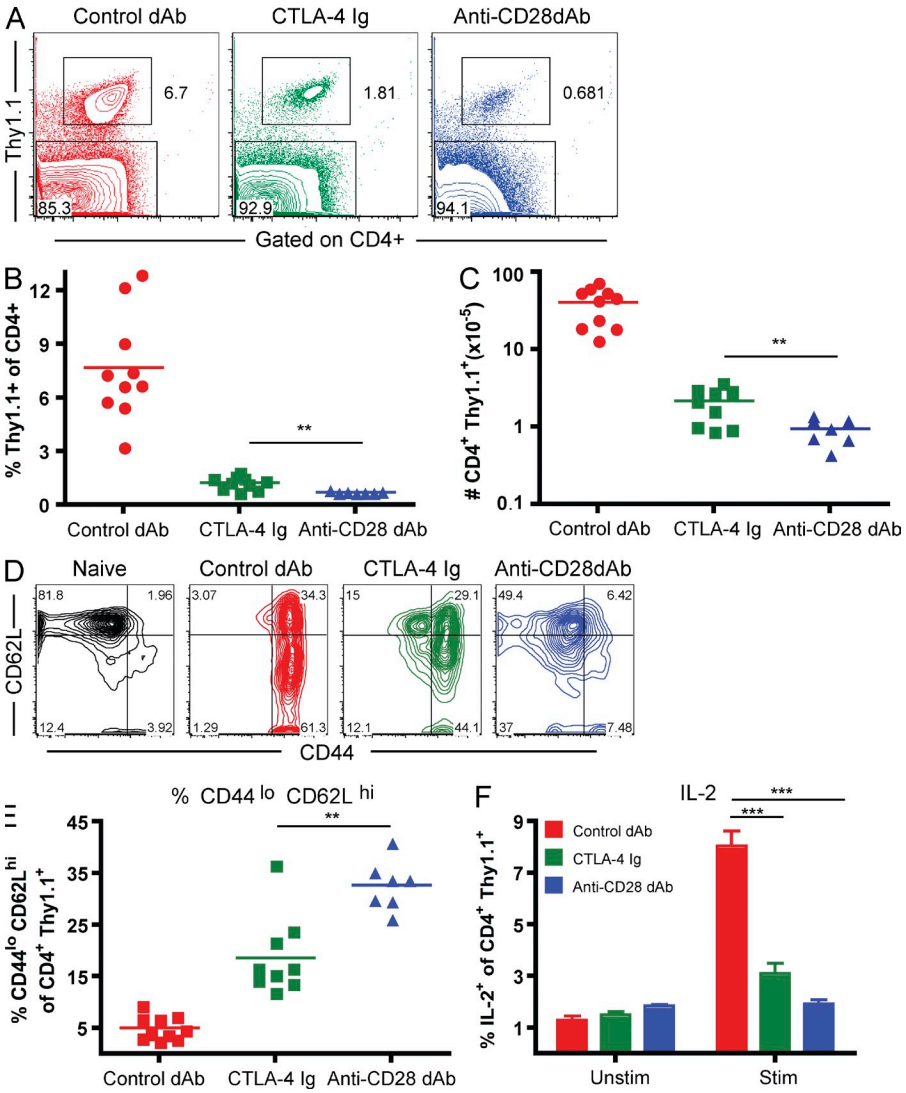

Figure 2. Donor-reactive $CD4^+$ T cell accumulation and differentiation are more profoundly attenuated by selective CD28 blockade versus CTLA-4 Ig. 10^6 $Thy1.1^+$ OT-I and 10^6 $Thy1.1^+$ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control Vκ dAb, CTLA-4 Ig, or anti-CD28 dAb, and then dosed on days 0, 2, 4, and 6 and three times per week continuously thereafter, as described in the Materials and methods. (A) Assessment of frequencies of donor-reactive $CD4^+$ $Thy1.1^+$ T cells on day 10 after transplant in draining lymph nodes. Data shown are representative and gated on $CD4^+$ T cells. (B and C) Summary data of 3 independent experiments with a total of 8–10 mice per group. Frequencies (B) and absolute numbers (C) of $CD4^+$ $Thy1.1^+$ T cells in anti-CD28 dAb-treated animals as compared with CTLA-4 Ig-treated animals (B, $P = 0.0031$; C, $P = 0.00185$). (D) Analysis of CD44 and CD62L expression on $CD4^+$ $Thy1.1^+$ T cells in DLN on day 10 after transplant. Data shown are representative. (E) Summary data of 3 independent experiments with a total of 8–10 mice per group ($P = 0.0004$). (F) IL-2 production by $CD4^+$ $Thy1.1^+$ T cells isolated from DLN on day 10 after transplant after ex vivo restimulation with OVA 323–339 ($P < 0.0001$). **, $P < 0.01$; ***, $P < 0.001$.

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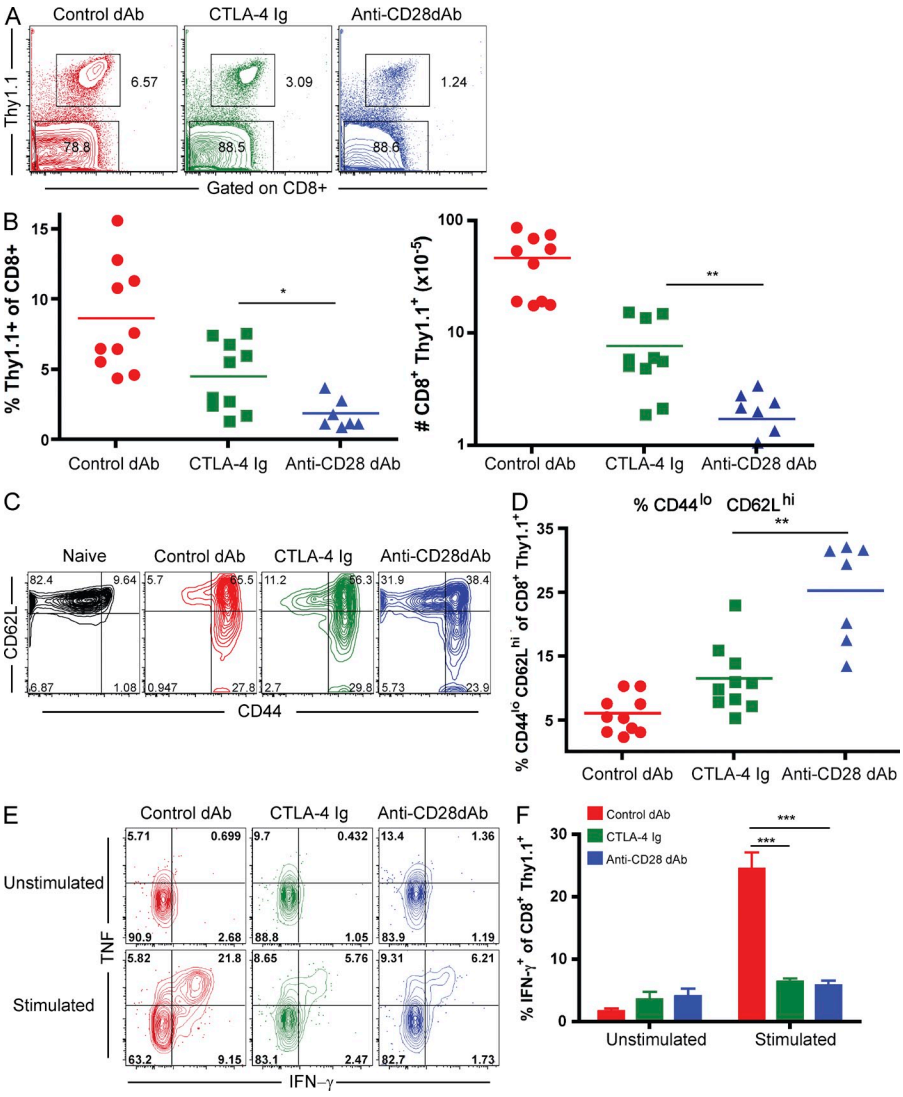

Figure 3. Donor-reactive CD8+ T cell accumulation and differentiation are more profoundly attenuated by selective CD28 blockade versus CTLA-4 Ig. 10^6 Thy1.1+ OT-I and 10^6 Thy1.1+ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control Vκ dAb, CTLA-4 Ig, or anti-CD28 dAb, and then dosed on days 0, 2, 4, and 6 and three times per week continuously thereafter, as described in the Materials and methods. (A) Assessment of frequencies of donor-reactive CD8+ Thy1.1+ T cells on day 10 after transplant in draining lymph nodes. Data shown are representative and gated on CD8+ T cells. (B) Summary data of three independent experiments with a total of 8–10 mice per group. Frequencies (P = 0.0185) and absolute numbers (P = 0.0021) are shown. (C) Analysis of CD44 and CD62L expression on CD8+ Thy1.1+ T cells in DLN on day 10 after transplant. Data shown are representative. (D) Summary data of 3 independent experiments with a total of 8–10 mice per group. (P = 0.0004). (E) IFN-γ and TNF production by CD8+ Thy1.1+ T cells isolated from DLN on day 10 after transplant after ex vivo restimulation with SIINFEKL. (F) IFN-γ production in both CTLA-4 Ig– and anti-CD28 dAb-treated animals is shown relative to control Vκ dAb-treated animals (P < 0.0001). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

effectors as compared with CTLA-4 Ig (Fig. 3, C and D). These results suggest that, as observed in CD4+ T cell responses, the provision of CTLA-4 signals in the absence of CD28 signals further impede differentiation of CD8+ T cells into effector cells. To further investigate the impact of selective CD28 blockade on effector status, we examined the ability of donor-reactive CD8+ T cells to produce IFN-γ and TNF after ex vivo restimulation with cognate antigen. Results demonstrated a similar reduction in the frequency of cytokine-producing effector cells in animals treated with CTLA-4 Ig as compared with anti-CD28 dAb (Fig. 3, E and F). Overall, these results suggest that selective blockade of CD28 co-stimulatory signals in the presence of intact CTLA-4 co-inhibitory signals more profoundly inhibits the expansion and differentiation (but not effector function) of the donor-reactive CD8+ T cell responses known to be a major mediator of co-stimulation blockade-resistant rejection (Trambley et al., 1999).

CD28 co-stimulatory blockade in the presence of CTLA-4 signals results in diminished ICOS expression and enhanced 2B4 expression

To determine why donor-reactive CD8+ T cell responses were more profoundly inhibited after treatment with anti-CD28 dAb as compared with CTLA-4 Ig, we examined the phenotype of donor-reactive CD8+ T cells under both treatment conditions. We observed two important differences between these groups. First, we observed that although CTLA-4 Ig treatment resulted in only a modest reduction in the up-regulation of the inducible co-stimulatory molecule ICOS relative to untreated animals at day 10 after transplant, treatment with anti-CD28 dAb resulted in a significantly greater reduction in up-regulation on both CD4+ and CD8+ donor-reactive T cells (Fig. 4, A and B). We confirmed that this reduction in ICOS detection in anti-CD28 dAb-treated recipients was not simply the result of antibody cross-reactivity (unpublished data). These data suggest that the subsequent transmission of inducible co-stimulatory signals

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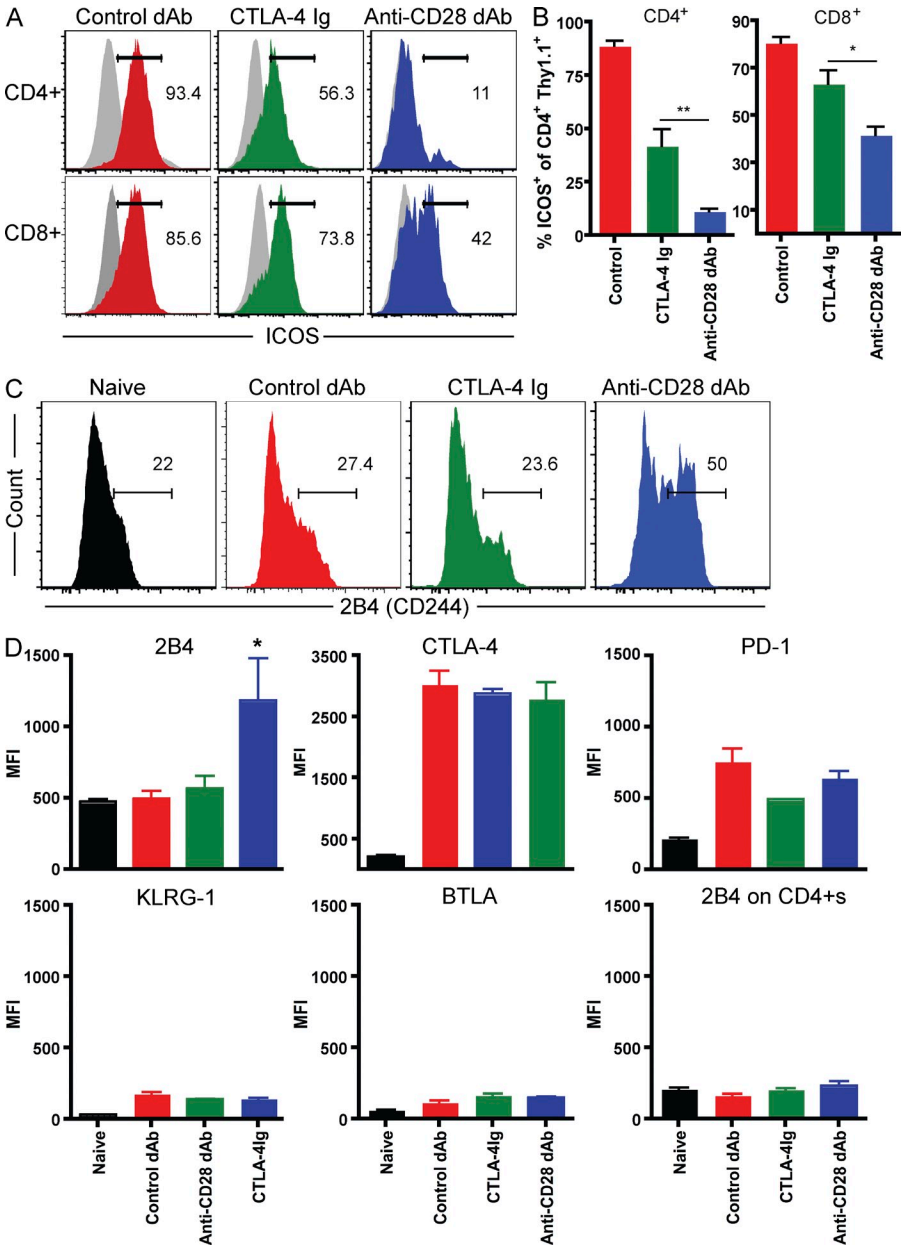

Figure 4. CD28 co-stimulatory blockade in the presence of CTLA-4 signals results in reduced up-regulation of ICOS and increased expression of the co-inhibitor 2B4. 10^6 Thy1.1+ OT-I and 10^6 Thy1.1+ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control dAb, CTLA-4 Ig, or anti-CD28 dAb, and then dosed on days 0, 2, 4, and 6 and three times per week continuously thereafter, as described in the Materials and methods. Graft-draining LNs were harvested on day 10 after transplant and analyzed by flow cytometry. (A) ICOS expression on naive donor-reactive T cells (gray) or donor-reactive CD4+ and CD8+ T cells isolated from control Vκ dAb dAb-treated animals (red), CTLA-4 Ig-treated animals (green), and anti-CD28 dAb-treated animals (blue). Data shown are representative and gated on CD4+ Thy1.1+ cells (top) and CD8+ Thy1.1+ cells (bottom). (B) Summary data from 3 independent experiments with 8–10 animals per group. CD4+ T cells, P = 0.0079; CD8+ T cells, P = 0.0317. (C) Up-regulation of 2B4 on donor-reactive CD8+ T cells isolated from untreated animals (red), CTLA-4 Ig-treated animals (green), or anti-CD28 dAb-treated animals (blue). Naive CD8+ T cells are shown in black. Data shown are representative and gated on CD8+ Thy1.1+ cells. (D) Summary data from 3 independent experiments with 8–10 animals per group. 2B4 expression on CD8+ T cells isolated from animals treated with anti-CD28 dAb as compared with control Vκ dAb or CTLA-4 Ig ($P < 0.05$). *, $P < 0.05$; **, $P < 0.01$.

through ICOS are more greatly diminished under conditions in which CD28 co-stimulatory signals are blocked but CTLA-4 co-inhibitory signals are maintained, as compared with when both co-stimulatory and co-inhibitory signals are blocked.

Second, CD8+ T cells from mice treated with anti-CD28dAb exhibited a significant and selective increase in the expression of the co-inhibitory receptor 2B4 (Fig. 4, C and D). 2B4 up-regulation occurred specifically on antigen-specific CD8+ T cells in animals treated with selective CD28 blockade and was not observed on antigen-specific CD4+ T cells in these recipients (Fig. 4 D). Importantly, expression of CTLA-4 was not altered in any of the treatment groups (Fig. 4 D), suggesting that co-inhibitory signals through this receptor could still be transmitted if CD80/CD86 ligands were available. Thus, based on the altered profile of positive and negative regulatory receptors on donor-reactive CD8+ T cells in which CD28 is selectively blocked, as compared with cells in which both CD28 and CTLA-4–mediated signals were attenuated, we conclude that selective CD28 blockade alters CD8+ T cell programming by skewing the balance of co-stimulatory and co-inhibitory molecule expression.

2B4 is also up-regulated on endogenous, polyclonal alloreactive CD8+ T cells after treatment with anti-CD28 dAb

To confirm the aforementioned findings in a more physiologically relevant fully allogeneic system, we used an MHC-mismatched skin graft model where BALB/c skin grafts were

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placed onto B6 recipients that were treated with anti-CD28 dAb or left untreated. Animals were sacrificed at day 7 after transplant, and as shown in Fig. 5, treatment with anti-CD28 dAb markedly inhibited the alloreactive CD8$^+$ T cell response as assessed by IFN-γ secretion after ex vivo restimulation with irradiated BALB/c stimulators (Fig. 5 A). Importantly, we observed a significant increase in the expression of 2B4 on CD8$^+$ T cells isolated from BALB/c graft recipients that were treated with anti-CD28 dAb as compared with untreated controls (Fig. 5, B and C). Similarly, we observed a significant reduction in the frequency of ICOS$^+$ CD8$^+$ T cells in these recipients relative to untreated controls (Fig. 5, D and E). These results confirm our findings in the transgenic model

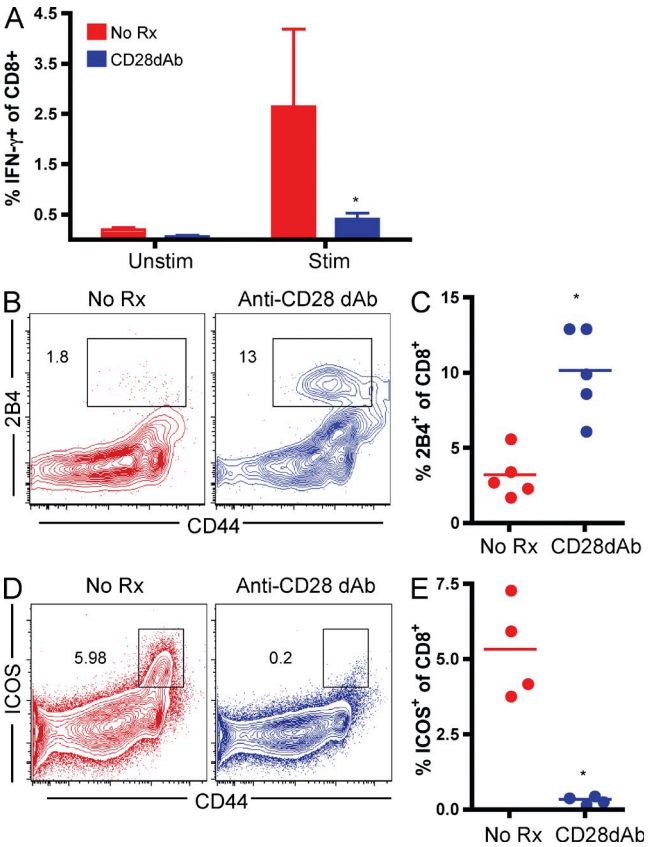

Figure 5. 2B4 is up-regulated on endogenous, polyclonal alloreactive CD8$^+$ T cells after treatment with anti-CD28 dAb. BALB/c skin grafts were placed onto B6 recipients that were treated with 100 µg anti-CD28 dAb on days 0, 2, 4, and 6 or left untreated. Animals were sacrificed at day 7 after transplant. (A) Splenocytes were restimulated for 4 h ex vivo with irradiated BALB/c stimulator cells, and intracellular IFN-γ was assessed. Results indicated significantly fewer CD8$^+$ H-2K^d$^-$ IFN-γ–secreting alloreactive T cells in CD28 dAb-treated recipients. (B) 2B4 expression on CD8$^+$ T cells in grafted recipients was assessed. Data shown are representative and gated on CD8$^+$ H-2K^d$^-$ cells. (C) Percent 2B4$^+$ of CD8$^+$ T cells. (D) ICOS expression on CD8$^+$ T cells in grafted recipients was assessed. Data shown are representative and gated on CD8$^+$ H-2K^d$^-$ cells. (E) Percent ICOS$^+$ of CD8$^+$ T cells. All graphs are summary data from $n = 5$ animals per group (*, $P < 0.05$).

and show that in a more physiological, fully allogeneic setting, selective CD28 blockade functions to up-regulate 2B4 on alloreactive CD8$^+$ T cells.

Increased efficacy and 2B4 up-regulation after selective CD28 blockade is mediated by CTLA-4– but not PD-L1–mediated signals

To directly test the hypothesis that preserved CTLA-4 co-inhibitory signals are required for the observed increased efficacy of the anti-CD28 dAb, including the observed up-regulation of 2B4, we conducted experiments in which anti-CD28 dAb was given in the presence of anti–CTLA-4 blocking antibodies. As shown in Fig. 6, blockade of CTLA-4 signals significantly impaired the efficacy of anti-CD28 dAb, both in terms of its ability to inhibit donor-reactive CD4$^+$ (Fig. 6, A and C) and CD8$^+$ (Fig. 6, B and D) T cell responses, and its ability to inhibit cytokine production in those cells (Fig. 6, E and F). Blocking CTLA-4 co-inhibitory signals also restored ICOS expression on both CD4$^+$ and CD8$^+$ graft-specific T cells (Fig. 6 G) treated with anti-CD28 dAb, and importantly, reduced the expression of 2B4 on donor-reactive CD8$^+$ T cells back down to baseline levels (Fig. 6, H and I).

The interaction of CD80 with the alternate ligand PD-L1 has also been shown to be co-inhibitory for T cells (Butte et al., 2007). By targeting CD28 instead of CD80/CD86, CD80 is theoretically free to bind PD-L1, thus we sought to determine whether this interaction might also play a role in the observed superiority of the anti-CD28 Ab. To directly test the role of the PD-L1 pathway in the efficacy of selective CD28 blockade, animals were treated with anti-CD28 dAb in the presence or absence of anti–PD-L1 antibody to block PD-L1–CD80 interactions after transplantation. Results showed that donor-reactive CD4$^+$ (Fig. 7, A and C) and CD8$^+$ T cell accumulation (Fig. 7, B and D) and effector function (Fig. 7 E) were similarly reduced by anti-CD28dAb whether or not PD-L1–B7-1 interactions were blocked, thus suggesting that this interaction is not critical for the observed efficacy of selective CD28 blockade. Likewise, the reduction in ICOS expression observed on both CD4$^+$ and CD8$^+$ donor-reactive T cells was not restored after PD-L1 blockade (Fig. 7 F). Thus, along with the data presented in Fig. 6 demonstrating that CTLA-4 interactions are critical for the observed efficacy of the anti-CD28 dAb, these data definitively demonstrate that the increased efficacy of anti-CD28 dAb relative to CTLA-4 Ig is dependent on the preservation of CTLA-4 but not PD-L1 co-inhibitory signals.

2B4$^{-/-}$ CD8$^+$ T cells are relatively resistant to the effects of selective CD28 blockade

To determine the role of 2B4 up-regulation in mediating increased allograft survival after selective CD28 blockade, we generated donor-reactive CD8$^+$ T cells that were deficient in 2B4. 2B4$^{-/-}$ mice were bred to OT-I Thy1.1$^+$ animals, and 2B4$^{-/-}$ OT-I T cells (along with WT Thy1.1$^+$ OT-II) were adoptively transferred into B6 recipients that were then

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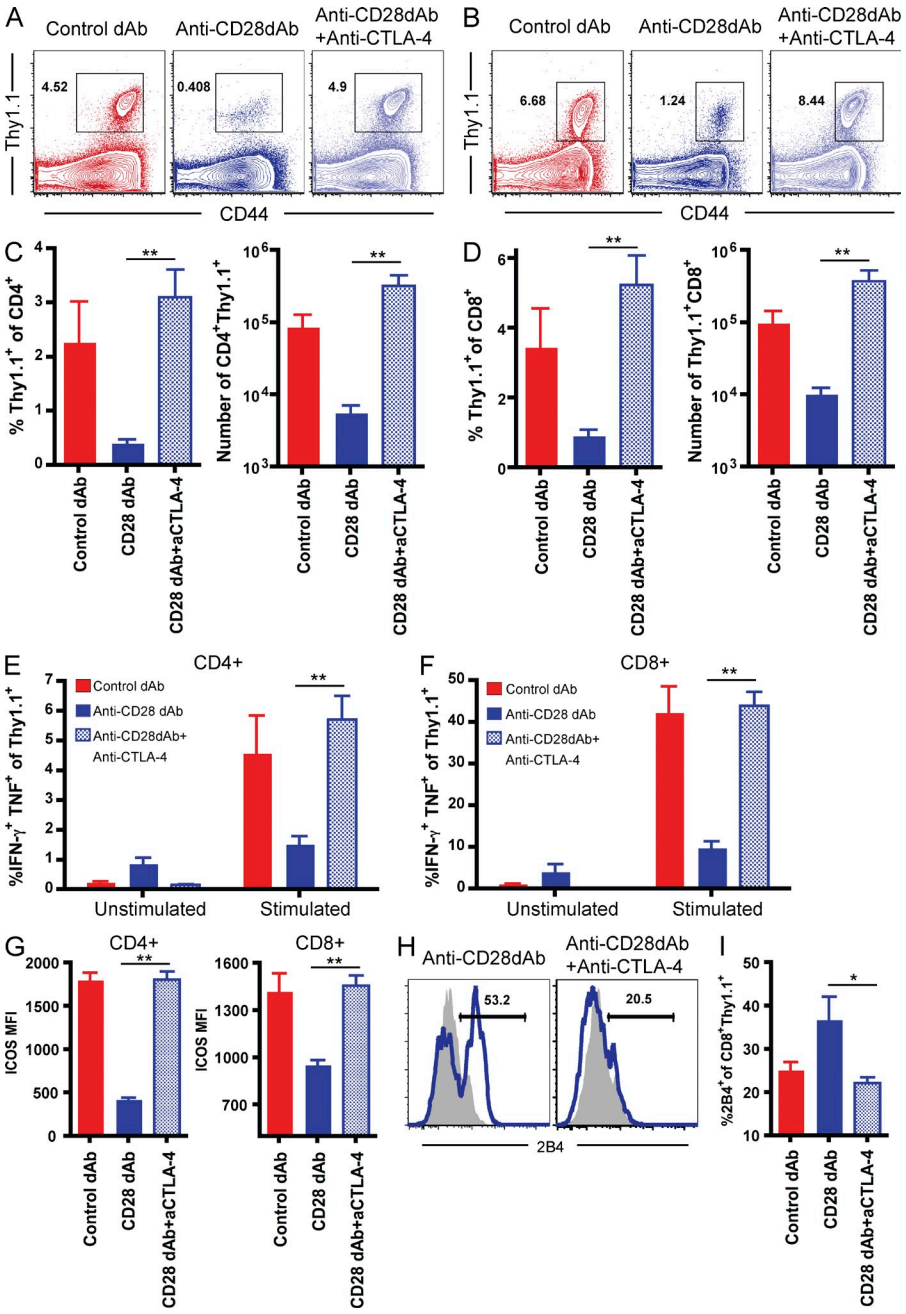

Figure 6. Increased efficacy and 2B4 up-regulation after selective CD28 blockade is dependent on CTLA-4–mediated signals. 10^6 Thy1.1+ OT-I and 10^6 Thy1.1+ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control Vκ dAb, anti-CD28 dAb, or anti-CD28 dAb + anti–CTLA-4 mAb dosed on days 0, 2, 4, and 6 as described in the Materials and methods. (A and B) Assessment of frequencies of donor-reactive CD4+ (A) and CD8+ (B) Thy1.1+ T cells on day 10 after transplant in draining lymph nodes. Data shown are representative and gated on CD4+ (A) or CD8+ (B) T cells. (C and D) Frequencies and absolute numbers of both CD4+ (C) and CD8+ (D) Thy1.1+ T cells in anti-CD28 dAb + anti–CTLA-4–treated animals as compared with animals treated with anti-CD28 dAb alone (P = 0.0079 for CD28 dAb versus CD28 dAb + anti–CTLA-4 groups). (E and F) IL-2 production by CD4+ Thy1.1+ T cells isolated from DLN on day 10 after transplant after ex vivo restimulation with OVA 323–339 (P = 0.0079 for CD28 dAb versus CD28 dAb + anti–CTLA-4 groups). (F) IFN-γ and TNF production by CD8+ Thy1.1+ T cells isolated from DLN on day 10 after transplant after ex vivo restimulation with SIINFEKL (P = 0.0079 for CD28 dAb vs. CD28 dAb + anti–CTLA-4 groups). (G) ICOS expression is shown on both CD4+ and CD8+ Thy1.1+ T cells isolated on day 10 after transplant from anti-CD28 dAb + anti–CTLA-4 treated animals relative to animals treated with anti-CD28 dAb alone (P = 0.0079). (H and I) 2B4 expression is shown on CD8+ Thy1.1+ T cells isolated on day 10 after transplant from anti-CD28 dAb + anti–CTLA-4–treated animals relative to animals treated with anti-CD28 dAb alone (P = 0.0159). All graphs are summary data of 2 independent experiments with a total of 8–10 mice per group. *, P < 0.05; **, P < 0.01.

challenged with OVA-expressing skin grafts. $2B4^{-/-}$ OT-I T cells analyzed before adoptive transfer were phenotypically similar to WT OT-I T cells in terms of their expression of the activation markers CD44, CD62L, ICOS, and PD-1 (unpublished data). Animals were left untreated or treated with anti-CD28 dAb, and mice receiving WT OT-I T cells in the presence or absence of the same reagents served as controls. Mice were sacrificed at 10 d after transplant, and donor-reactive Thy1.1+ CD8+ T cells in draining LNs were analyzed. Results indicated that accumulation of donor-reactive CD8+ Thy1.1+ T cells in untreated animals was similar in those that received WT or $2B4^{-/-}$ T cells (Fig. 8, A and B), indicating that 2B4 deficiency in CD8+ T cells has no effect on the donor-reactive primary CD8+ T cell response during unmodified rejection. These results are consistent with the fact that 2B4 is expressed at very low levels on primary antigen-specific CD8+ T cells in the absence of immune modulation (Fig. 4, C and D). In contrast, in the presence of selective CD28 blockade, we observed a significant increase in the accumulation of donor-reactive $2B4^{-/-}$ antigen-specific CD8+ T cells as compared with WT CD8+ T cells, demonstrating that the efficacy of anti-CD28 dAb in diminishing the expansion of the donor-reactive T cell response was impaired when CD8+ T cells lacked 2B4. Similar results were observed after

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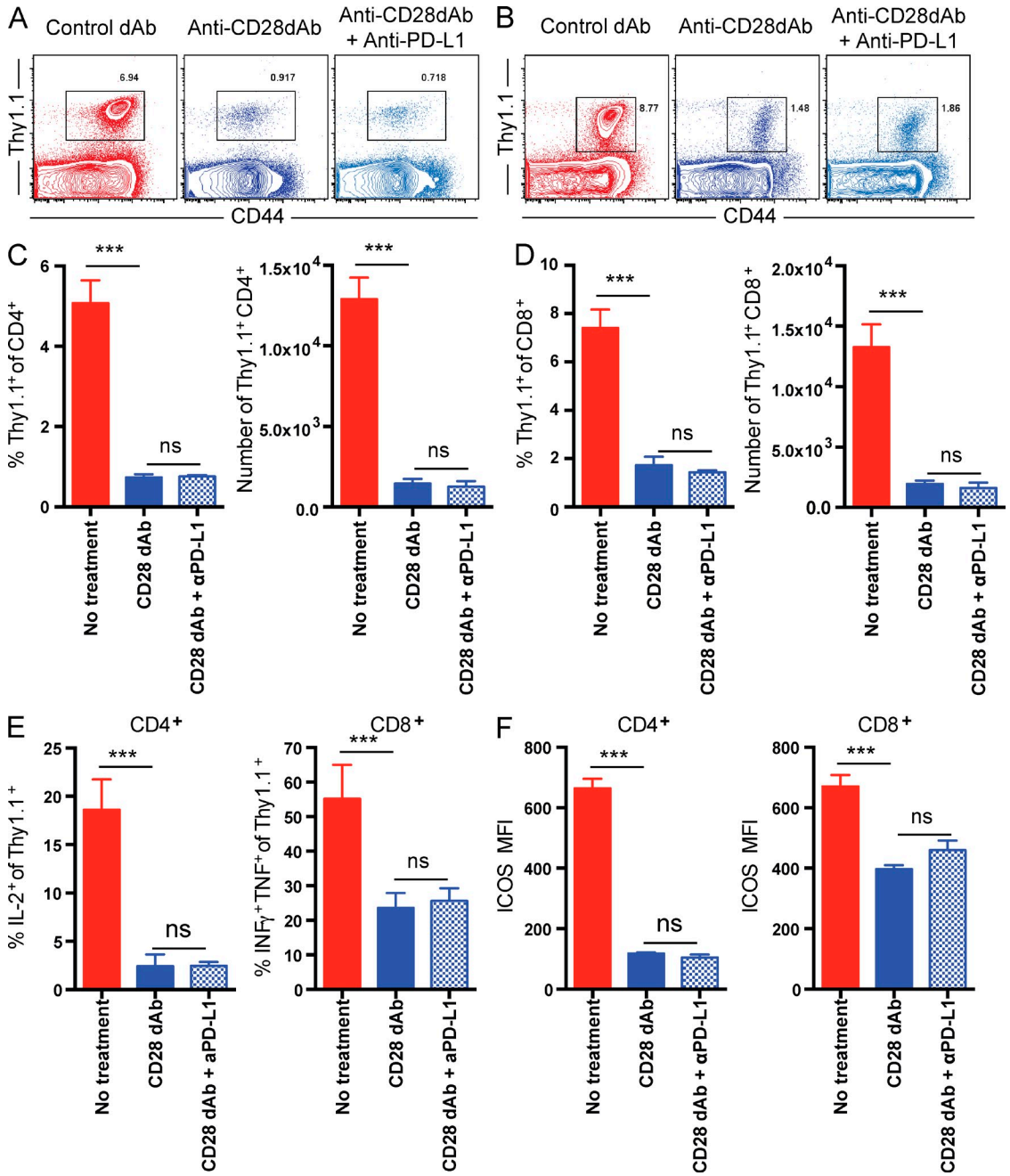

Figure 7. Increased efficacy and ICOS down-regulation after selective CD28 blockade is independent of PD-L1–mediated signals. 10^6 Thy1.1+ OT-I and 10^6 Thy1.1+ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control Vκ dAb, anti-CD28 dAb, or anti-CD28 dAb + anti-PD-L1 mAb dosed on days 0, 2, 4, and 6 as described in Materials and methods. (A and B) Assessment of frequencies of donor-reactive CD4+ (A) and CD8+ (B) Thy1.1+ T cells on day 10 after transplant in draining lymph nodes. Data shown representative and are gated on CD4+ (A) or CD8+ (B) T cells. (C and D) Frequencies and absolute numbers of either CD4+ (C) or CD8+ (D) Thy1.1+ T cells in anti-CD28 dAb + anti-PD-L1 treated animals as compared with animals treated with anti-CD28 dAb alone are shown. (E) Cytokine production by CD4+ (IL-2) or CD8+ (IFN-γ+ TNF+) Thy1.1+ T cells isolated from DLN on day 10 after transplant after ex vivo restimulation with cognate antigen is shown. (F) ICOS expression on both CD4+ and CD8+ Thy1.1+ T cells isolated on day 10 after transplant is shown. All graphs are summary data of a total of 4–5 mice per group from two independent experiments. ***, $P < 0.0001$; ns, not significant.

assessment of antigen-specific CD8+ T cell effector function. We observed no difference in the frequency of either IFN-γ producers or TNF producers in splenocytes isolated from untreated recipients containing WT vs. 2B4$^{-/-}$ OT-I T cells after ex vivo restimulation with cognate antigen (Fig. 8, C and E). However, in anti-CD28 dAb-treated recipients, the frequencies

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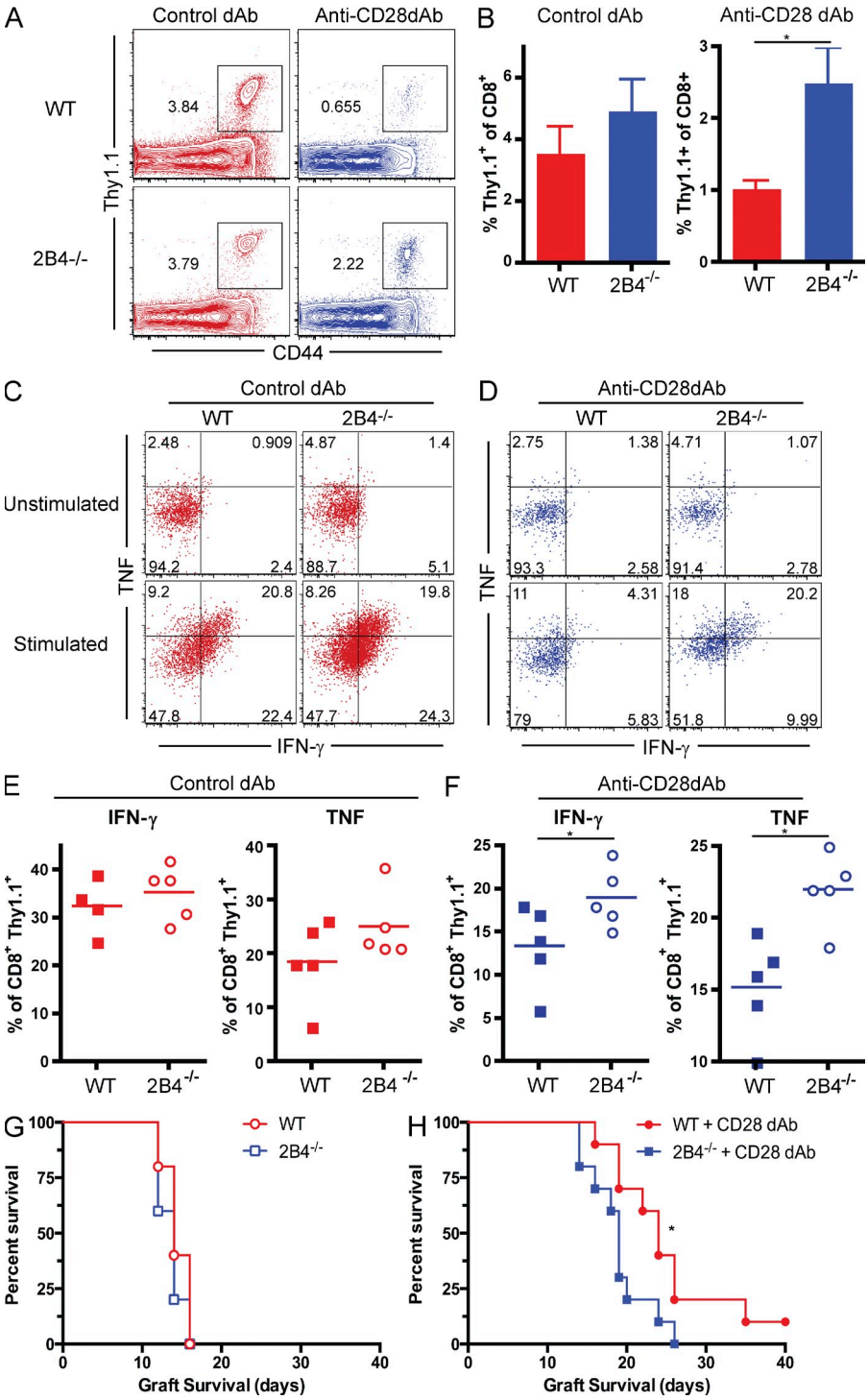

Figure 8. 2B4$^{-/-}$ CD8$^+$ T cells are resistant to the effects of selective CD28 blockade. 10^6 Thy1.1$^+$ OT-I or 10^6 Thy1.1$^+$ 2B4$^{-/-}$ OT-I and 10^6 Thy1.1$^+$ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control Vκ dAb or anti-CD28 dAb, and then dosed on days 0, 2, 4, and 6 and three times per week continuously thereafter as described in the Materials and methods. Graft-draining LNs were harvested on day 10 after transplant and analyzed by flow cytometry. (A) Frequencies of donor-reactive CD8$^+$ Thy1.1$^+$ T cells WT and 2B4$^{-/-}$ donor-reactive T cells in the presence or absence of anti-CD28 dAb. Data shown are gated on CD8$^+$ T cells. (B) Summary data showing expansion of 2B4$^{-/-}$ donor-reactive CD8$^+$ T cells after treatment with anti-CD28 dAb as compared with expansion of WT OT-I T cells ($P = 0.03$). (C) IFN-γ and TNF production of donor-reactive CD8$^+$ Thy1.1$^+$ T cells in untreated animals that received WT or 2B4$^{-/-}$ T cells. Data shown are gated on CD8$^+$ Thy1.1$^+$ T cells. (D) IFN-γ and TNF production of donor-reactive CD8$^+$ Thy1.1$^+$ T cells in anti-CD28 dAb–treated animals that received WT or 2B4$^{-/-}$ T cells. Data shown are gated on CD8$^+$ Thy1.1$^+$ T cells. (E) Frequencies of IFN-γ$^+$ and TNF$^+$ donor-reactive CD8$^+$ T cells in untreated recipients of WT versus 2B4$^{-/-}$ OT-I T cells. (F) Frequencies of IFN-γ$^+$ ($P = 0.0343$) and TNF$^+$ ($P = 0.0159$) donor-reactive CD8$^+$ T cells in anti-CD28 dAb–treated recipients of WT versus 2B4$^{-/-}$ OT-I T cells. Flow plots are representative and graphs are summary data from two independent experiments with a total of 10 animals per group. (G and H) Recipients of WT or 2B4$^{-/-}$ OT-I were left untreated (G) or treated with anti-CD28 dAb (H) and monitored for skin graft survival ($P = 0.0299$). *, $P < 0.05$.

of both IFN-γ and TNF producers were significantly augmented in antigen-specific 2B4$^{-/-}$ CD8$^+$ populations as compared with WT antigen-specific CD8$^+$ T cells (Fig. 8, D and F). Finally, we assessed skin graft survival in recipients of WT versus 2B4$^{-/-}$ donor-reactive CD8$^+$ T cells and observed that although skin graft rejection was indistinguishable between untreated recipients of WT versus 2B4$^{-/-}$ T cells (Fig. 8 G), graft survival was significantly longer in anti-CD28 dAb-treated recipients of WT as compared with 2B4$^{-/-}$ T cells (Fig. 8 H; $P = 0.0299$). These data indicate that in the absence of immunomodulation, WT and 2B4$^{-/-}$ T cells are similarly capable of mediating graft rejection, but that anti-CD28dAb treatment is less efficacious at preventing graft rejection when 2B4 is lacking on donor-reactive CD8$^+$ T cells. Collectively, these results suggest that 2B4$^{-/-}$ donor-reactive T cells are less susceptible to the inhibitory effects of anti-CD28

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dAb relative to WT T cells, thus implicating engagement of the 2B4 co-inhibitory pathway as a mechanism underlying the efficacy of selective CD28 blockade in attenuating donor-reactive CD8$^+$ T cell responses.

Decreased ICOS up-regulation after selective CD28 blockade is dependent on engagement of the 2B4 pathway

As shown in Fig. 4 (A and B), up-regulation of the inducible co-stimulator ICOS on donor-reactive CD8$^+$ T cells was significantly reduced in animals treated with anti-CD28 dAb as compared with control dAb or CTLA-4 Ig. Thus, we hypothesized that the decreased up-regulation of ICOS on antigen-specific CD8$^+$ T cells isolated from anti-CD28 dAb-treated mice might be dependent on increased 2B4 co-inhibitory signals in these cells. To test this hypothesis, we adoptively transferred either WT or 2B4$^{-/-}$ Thy1.1$^+$ OT-I cells along with WT Thy1.1$^+$ OT-II into naive B6 recipients, which were then challenged with an OVA-expressing graft and left untreated or treated with anti-CD28 dAb. At day 10 after graft challenge, mice were sacrificed and the expression of ICOS on CD8$^+$ Thy1.1$^+$ donor-reactive T cells was assessed. We observed that CD8$^+$ 2B4$^{-/-}$ antigen-specific T cells failed to exhibit decreased ICOS up-regulation after treatment with anti-CD28 dAb as compared with WT OT-I T cells treated with anti-CD28 dAb (Fig. 9, A and B). This effect was specific to CD8$^+$ T cells, as we observed no difference in the degree of ICOS up-regulation on donor-reactive CD4$^+$ T cells between these groups (Fig. 9 C). Thus, we conclude that decreased ICOS co-stimulatory expression on CD8$^+$ T cells is a result of enhanced T cell–intrinsic 2B4 co-inhibitory signaling after selective CD28 blockade.

DISCUSSION

Our results have identified a novel and critical role for the 2B4 co-inhibitor in controlling donor-reactive CD8$^+$ T cell responses in the presence of immune modulation with selective CD28 blockade. Consistent with our findings that 2B4 was not expressed on donor-reactive CD8$^+$ T cells in the absence of immune modulation, we observed no differences in the expansion or effector function of WT versus 2B4$^{-/-}$ T cells during unmodified rejection. These results suggest that the 2B4 co-inhibitory pathway does not play a major role in programming primary T cell responses in transplantation in the absence of immunosuppression. In contrast, we observed a specific up-regulation of 2B4 on donor-reactive CD8$^+$ T cells in animals treated with selective CD28 blockade, and demonstrated significantly reduced efficacy of selective CD28 blockade in controlling donor-reactive T cell responses when the T cells were deficient in 2B4. However, the reliance on 2B4 in controlling donor-reactive T cell responses was not a property of all types of immunomodulation, as 2B4 was not up-regulated in the presence of CTLA-4 Ig, nor was the efficacy of CTLA-4 Ig in inhibiting donor-reactive T cell responses diminished in recipients of 2B4$^{-/-}$ T cells (unpublished data). Because 2B4 was not up-regulated in the presence of CTLA-4 Ig (where CTLA-4 signals are blocked; Fig. 4, C and D), we tested the hypothesis that CTLA-4 co-inhibitory signals are required to induce expression of 2B4 using CTLA-4–blocking antibodies and found that 2B4 failed to be up-regulated after CD28 dAb administration in the absence of CTLA-4 mediated signals (Fig. 6, H and I). Thus, we conclude that the net sum of blockade of CD28 signals in the presence of preserved CTLA-4 signals results in the unique up-regulation of 2B4 on primary effectors during transplantation, and that this up-regulation plays a critical functional role in controlling donor-reactive CD8$^+$ T cell responses.

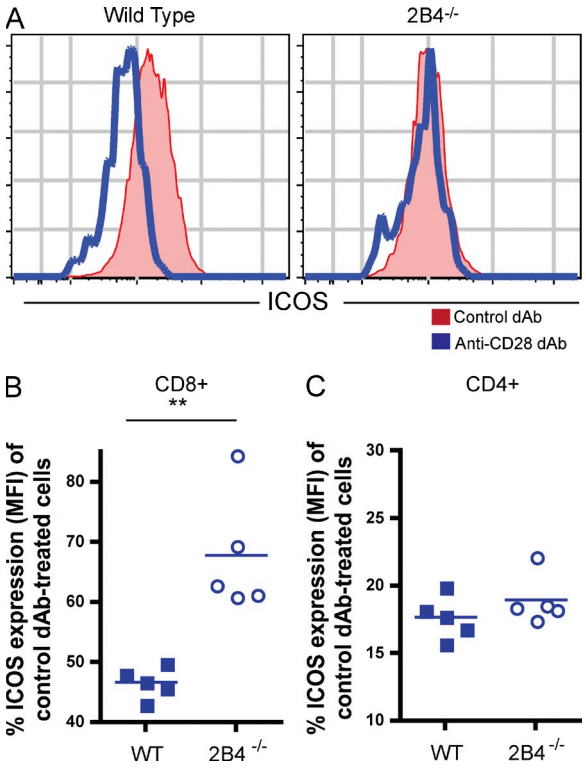

Figure 9. Reduced up-regulation of ICOS after selective CD28 blockade is dependent on engagement of the 2B4 pathway. 10^6 Thy1.1$^+$ OT-I or 10^6 Thy1.1$^+$ 2B4$^{-/-}$ OT-I and 10^6 Thy1.1$^+$ OT-II T cells were adoptively transferred into naive B6 recipients, which were then challenged with an OVA-expressing skin graft in the presence of control dAb or anti-CD28 dAb, and then dosed on days 0, 2, 4, and 6 and three times per week continuously thereafter as described in the Materials and methods. Graft-draining LNs were harvested on day 10 after transplant and analyzed by flow cytometry. (A) ICOS expression on WT (left) and 2B4$^{-/-}$ (right) donor-reactive CD8$^+$ Thy1.1$^+$ T cells in the presence and absence of anti-CD28 dAb. (B) Summary data from n = 5/group (representative of 2 independent experiments with a total of 10 mice per group) show the percent reduction in ICOS expression after anti-CD28 dAb treatment as compared with control dAb treatment in WT versus 2B4$^{-/-}$ CD8$^+$ T cells ($P = 0.0018$). Data shown are gated on CD8$^+$ Thy1.1$^+$ T cells. (C) Percent reduction in ICOS expression after anti-CD28 dAb treatment on CD4$^+$ donor-reactive T cells in mice that received WT or 2B4$^{-/-}$ OT-I T cells. Data shown are gated on CD4$^+$ Thy1.1$^+$ T cells. Summary data shown are from n = 5/group (representative of two independent experiments with a total of 10 mice per group). **, $P < 0.01$.

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As noted above, we demonstrated that the increased expression of 2B4 on anti-CD28dAb–treated mice as compared with CTLA-4 Ig-treated mice is dependent on CTLA-4–mediated signaling. These findings are consistent with recently published work from Zhang et al. (2011) demonstrating that the efficacy of selective CD28 blockade was dependent on an intact CTLA-4 pathway. However, because CD80–PD-L1 interactions have also been shown to inhibit T cell responses (Butte et al., 2007), we tested the alternative hypothesis that the increased expression of 2B4 on $CD8^+$ T cells in anti-CD28 dAb-treated mice is dependent on an intact PD-L1–CD80 pathway, and found that PD-L1 blockade failed to rescue donor-reactive $CD4^+$ or $CD8^+$ T cell responses in this system (Fig. 7). These data demonstrate that sparing of CD80–PD-L1 interactions is not the mechanism of superiority of CD28 dAb relative to CTLA-4 Ig, and also suggest that there is no role for PD-1 mediated co-inhibition of donor-reactive T cell responses at this early time point after transplantation. These findings are consistent with the observation that PD-1 is not up-regulated at early time points on donor-reactive $CD8^+$ T cells isolated from CD28 dAb-treated graft recipients (Fig. 4 D). In contrast, our previously published work has demonstrated a critical role for the PD-1–PD-L1 pathway in the suppression of donor-reactive $CD8^+$ T cell responses at late time points after transplantation (Koehn et al., 2008). Specifically, at >100 d after transplant, donor-reactive $CD8^+$ T cell populations in recipients of surviving allografts express high levels of PD-1, and administration of either anti–PD-1 or anti–PD-L1 at this time point rapidly precipitates graft rejection. Along with the results of the studies presented here, these data suggest temporally segregated roles for CD28-family co-inhibitory receptors in transplantation, with CTLA-4–mediated signals functioning early and PD-1–PD-L1-mediated signals functioning late to control donor-reactive $CD8^+$ T cell responses and inhibit graft rejection.

The role of 2B4 as a co-inhibitor is controversial. Its intracellular domain contains an immunotyrosine switch motif (ITSM), thus potentially allowing both co-stimulatory and co-inhibitory properties. In NK cells, 2B4 has been reported to have both activating and inhibitory functions (Laouar et al., 2007); however consistent with our results its activity on $CD8^+$ T cells has thus far been reported to be co-inhibitory (Kim et al., 2010; Brown et al., 2011; West et al., 2011). Factors that influence the co-stimulatory versus co-inhibitory function of 2B4 signaling include the density of surface 2B4 expression (where increased expression equates to increased co-inhibitory function), degree of ligation by its ligand CD48 (where increased CD48 ligation results in increased co-inhibitory function), and the level of intracellular association with the adaptor molecule SLAM-associated protein (SAP; where decreased association with SAP is associated with increased co-stimulatory function; Laouar et al., 2007). Additional manipulation of these parameters might be exploited to further enhance the co-inhibitory properties of 2B4 in transplantation and autoimmunity.

We also identified a reduction in the up-regulation of the inducible co-stimulator ICOS in the presence of CTLA-4 Ig but to a greater degree in the presence of selective CD28 blockade. Our results using $2B4^{-/-}$ antigen-specific T cells demonstrate that the up-regulation of 2B4 is functionally responsible for this further reduction in the level of ICOS up-regulation. Recent studies have identified an AP-1 responsive site within the ICOS promoter and have demonstrated that expression of the AP-1 related molecule Fos-related antigen-2 (Fra2) is highly correlated with ICOS expression in T cells after TCR/CD28 stimulation (Watanabe et al., 2012), raising the possibility that 2B4-mediated signals might alter Fra2 binding to diminish ICOS up-regulation. In addition, a recent report demonstrated the ability of CTLA-4 Ig and ICOS antagonism to synergize in prolonging graft survival in a murine transplant model (Schenk et al., 2009). Our data showing that selective CD28 blockade results in 2B4 up-regulation, which drives reduced ICOS up-regulation and leads to prolonged graft survival, are consistent with these results. Determining whether reduced ICOS up-regulation is functionally responsible for the improved efficacy of CD28 dAb in inhibiting donor-reactive T cell responses and prolonging graft survival or rather is an indicator of reduced T cell activation is an area of ongoing investigation.

In sum, our study highlights how the dynamic interplay of co-stimulatory and co-inhibitory signals received during T cell priming orchestrate and further fine tune antigen-specific $CD8^+$ T cell responses that ultimately result in either tolerance or immunity. Furthermore, our data suggest that selective CD28 blockade through the use of domain antibodies may hold promise as a clinically translatable strategy for the mitigation of unwanted immune responses in transplantation and autoimmunity.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (Hogquist et al., 1994) and OT-II (Barnden et al., 1998) transgenic mice, purchased from Taconic Farms, were bred to $Thy1.1^+$ background at Emory University. mOVA mice (C57BL/6 background, H-2^b; Ehst et al., 2003) were a gift from M. Jenkins (University of Minnesota, Minneapolis, MN). 2B4 $(CD244)^{-/-}$ animals on a B6 background were a gift from C. Terhorst (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA), and were bred onto OT-I x Thy1.1 background at Emory University (Atlanta, GA). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

Characterization of CD28 dAb. The anti-CD28 dAb (BMS-1m74-14982) precursor was identified by phage display from a library of human heavy and light chain dAbs. The identified Vκ chain then underwent random mutagenesis followed by site-directed mutagenesis in the complementarity-determining regions and was selected for increased affinity for murine CD28 using a surface plasmon resonance assay. To determine affinity and kinetics, a streptavidin chip (GE Healthcare) surface was preconditioned with 1 M NaCl and 50 mM NaOH. Mouse and human CD28-biotinylated monomers (1 μg/ml) were immobilized on a flow cell with 15-s contact time at 10 μl/min to give ~180 RUs. The mouse anti-CD28 dAb was injected at different concentrations (800–1.56 nM) for 3 min at 30 μl/min

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Table 1. CD28 dAb affinity and kinetic parameters

	Receptor	k_a (1/Ms)	k_d (1/s)	K_d (nM)	R_{max}
CD28 dAb	Mouse CD28	$8.4 \pm 6.4 \times 10^4$	$2.2 \pm 1.6 \times 10^{-5}$	2.6 ± 2.8	5.9 ± 0.3
CD28 dAb	Human CD28	N.D.	N.D.	N.D.	0

N.D., none detected; R_{max} = Biacore maximum response signal.

followed by 15 min of dissociation. Surfaces were regenerated with two 45-s pulses of 10 mM Glycine. Kinetic analysis was performed using the Biacore T100 evaluation software using a global analysis 1:1 Langmuir binding model. Calculation of the average kinetic parameters and standard errors was determined using three replicates (Table 1). The anti-CD28 dAb is formatted with a 40-kD branched polyethylene glycol, and had an EC_{50} (2.2 ± 0.6 nM) comparable to that of human CTLA4Ig (abatacept, 4.25 ± 2 nM) in murine mixed lymphocyte reactions.

CD28 competitive binding assay. B6 splenocytes were incubated ex vivo with increasing concentrations of unconjugated anti-CD28 dAb at 37 degrees for 1 h and then were washed and stained with a FITC-conjugated anti-CD28 mAb (clone E18; BD) that competes with anti-CD28dAb for binding to CD28 and analyzed by flow cytometry. For assessment of CD28 blockade in vivo, animals were treated with 100 µg of control Vκ dAb or anti-CD28 dAb and sacrificed 3 h later. Splenocytes were harvested and stained with fluoresceinated anti-CD28 mAb clone E18 (BD) and analyzed by flow cytometry.

Measurement of serum cytokines. For assessment of serum cytokines, animals were injected with 100 µg of anti-CD28 dAb or 20 µg LPS as a positive control and were sacrificed 6 h later. Peripheral blood was harvested and serum was isolated. Cytokines were measured using the Mouse Inflammatory Cytokine Cytometric Bead Array kit from BD according to manufacturer's instructions.

Donor-reactive T cell adoptive transfers. For adoptive transfers of donor-reactive T cells, spleen, and mesenteric LNs isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were processed and stained with monoclonal antibodies for CD4 and CD8 (both from Invitrogen), Thy1.1, and Vα2 (BD) for flow cytometry analysis. Cells were resuspended in PBS and 1.0×10^6 of each Thy1.1$^+$ OT-I and OT-II were injected i.v. 24–48 h before skin transplantation.

Skin transplantation and in vivo co-stimulatory molecule blockade. Full-thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (Trambley et al., 1999). Where indicated, mice were injected with 100 µg control Vκ dAb, 100 µg anti-CD28 dAb or 250 µg CTLA-4 Ig (all Bristol-Myers Squibb) on days 0, 2, 4, 6, and three times per week continuously thereafter until the mice were sacrificed or until day 50 (for skin graft survival experiments). In some experiments, mice were also treated with 500 µg hamster monoclonal anti–mouse CD154 (MR-1, BioXCell) on days 0, 2, 4, and 6 and then weekly thereafter until day 50 after transplantation. For CTLA-4 and PD-L1 studies, grafted recipients were treated with a short course of 100 µg of anti-CD28dAb on days 0, 2, 4, and 6. Where indicated, mice also received either 500 µg of anti–CTLA-4 (clone 9H10) or anti-PD-L1 (clone 10F.9G2; both from BioXCell).

Allostimulation assay. At day 7 after transplant, B6 recipients of BALB/c skin grafts recipients were euthanized and splenocytes were isolated. To assess for donor-reactive T cells, 10^6 recipient splenocytes were incubated with 2×10^6 BALB/c splenocytes per well in flat-bottom 96-well plates in the presence of 10 µg/ml Brefeldin A for 4 h at 37°C. Subsequently, cells were stained with anti-CD4, anti-CD8, and anti-K^d (to exclude stimulator cells), and then fixed, permeabilized, and stained with anti–IFN-γ (BD) according to the manufacturer's instructions (BD). All cells were acquired on an LSR-II flow cytometer (BD), and flow data were analyzed using FlowJo software (Tree Star).

Flow cytometry and intracellular cytokine staining. Spleens or graft-draining axillary and brachial LNs were stained for CD4 and CD8 (both from Invitrogen) and Thy1.1 (BD). For phenotypic analysis, cells were also surface-stained with anti-ICOS, anti-2B4, anti-PD-1, anti-KLRG-1, and anti-BTLA (all BD). CTLA-4 expression was measured intracellularly using an intracellular staining kit (BD) after ex vivo restimulation. Absolute numbers were calculated using TruCount bead analysis according to the manufacturer's instructions. Samples were analyzed on an LSRII flow cytometer (BD). Data were analyzed using FlowJo software (Tree Star). For intracellular cytokine staining, splenocytes were stimulated with 10 nM OVA257-264 (SIINFEKL) or 10 µM OVA323-339 (ISQAVHAAHAEINEAGR; GenScript, Inc.) where indicated, in the presence of 10 µg/ml Brefeldin A for 4 h. An intracellular staining kit was used according to manufacturer's instructions to detect TNF and IFN-γ (all from BD).

Statistical analysis. Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For analysis of T cell responses, nonparametric Mann-Whitney *U* tests were performed. Results were considered significant if $P < 0.05$. All analyses were done using GraphPad Prism software (GraphPad Software Inc). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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