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Danya Liu, Emory University
Scott M. Krummey, Emory University
Idelberto Badell, Emory University
Maylene Wagener, Emory University
Lumelle A. Schneeweis, Bristol-Myers Squibb Company
Dawn K. Stetsko, Bristol-Myers Squibb Company
Suzanne J. Suchard, Bristol-Myers Squibb Company
Steven G. Nadler, Bristol-Myers Squibb Company
Mandy Ford, Emory University

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

Danya Liu,1 Scott M. Krummey,1 I. Raul Badell,1 Maylene Wagener,1 Lumelle A. Schneeweis,2 Dawn K. Stetsko,2 Suzanne J. Suchard,2 Steven G. Nadler,2 and Mandy L. Ford1

1Emory Transplant Center and Department of Surgery, Emory University, Atlanta, GA 30322
2Bristol-Myers Squibb Company, Princeton, NJ 08543

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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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 Fc 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 allograft rejection, B6 recipients of BALB/c skin grafts were treated with CTLA-4 Ig or CD28 dAb in the presence of anti–CD154 mAb (Fig. 1A). 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. 1B; Ehst et al., 2003). B6 recipients were grafted with skin from OVA-expressing transgenic donors, which 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.
mAb that induced cross-linking. As shown in Fig. 1F, 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. 1B). Mice were then treated with

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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 10^5 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 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.
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 cells 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 CD44lo CD62Lhi cells into CD44hi CD62Llo 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 CD44lo CD62Lhi cells into CD44hi CD62Llo T cells

Figure 2. Donor-reactive CD4+ T cell accumulation and differentiation are more profoundly attenuated by selective CD28 blockade versus CTLA-4 Ig. 106 Thy1.1+ OT-I and 106 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 DNL 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. (F) IL-2 production by CD4+ Thy1.1+ T cells isolated from DNL on day 10 after transplant after ex vivo restimulation with OVA 323–339 (P < 0.0001). **, P < 0.01; ***, P < 0.001.
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). 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).
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-CD28 dAb 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
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 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**<sup>–/–</sup> 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<sup>–/–</sup> mice were bred to OT-I Thy1.1<sup>+</sup> animals, and 2B4<sup>–/–</sup> OT-I T cells (along with WT Thy1.1<sup>+</sup> OT-II) were adoptively transferred into B6 recipients that were then
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...
untreated recipients containing WT vs. 2B4−/− OT-IT cells after ex vivo restimulation with cognate antigen (Fig. 8, C and E). However, in anti-CD28 dAb-treated recipients, the frequencies 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-IT 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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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−/− donor-reactive CD8+ T cells are similarly capable of mediating graft rejection, but that anti-CD28 dAb 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...
Ig (where CTLA-4 signals are blocked; Fig. 4, C and D), we Because 2B4 was not up-regulated in the presence of CTLA-4 diminished in recipients of 2B4 cacy of CTLA-4 Ig in inhibiting donor-reactive T cell responses property of all types of immunomodulation, as 2B4 was not up-regulated in controlling donor-reactive CD8+ 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 knockout 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.

**DISCUSSION**

Our results have identified a novel and critical role for the 2B4 co-inhibitory pathway 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 immunosuppression, we observed no differences in the expansion or effector function of WT versus 2B4 knockout 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 knockout 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 knockout 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, 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 knockout (right) donor-reactive CD8+ Thy1.1+ T cells in the presence 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 knockout CD8+ T cells (P = 0.0018). Data shown are gated on CD8+ Thy1.1+ T cells. (C) Percent re-duction in ICOS expression after anti-CD28 dAb treatment on CD4 donor-reactive T cells in mice that received WT or 2B4 knockout 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.
As noted above, we demonstrated that the increased expression of 2B4 on anti-CD28 dAb–treated mice is dependent on 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 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-2b) and BALB/c (H-2d) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (H-2d) (Hoggquist 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-2b; 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.
2 × 10⁶ BALB/c splenocytes per well in flat-bottom 96-well plates in the presence of donor-reactive T cells, 10⁶ recipient splenocytes were incubated with skin grafts. Recipients were euthanized and splenocytes were isolated. To assess Allostimulation assay.

10F.9G2; both from BioXCell). 100 µg of anti-CD28 dAb on days 0, 2, 4, and 6. Where indicated, mice also and PD-L1 studies, grafted recipients were treated with a short course of 6 and then weekly thereafter until day 50 after transplantation. For CTLA-4 monoclonal anti–mouse CD154 (MR-1, BioXCell) on days 0, 2, 4, and 50 µg of 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 (Trambley et al., 1999). Where indicated, mice were injected with 100 µg 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 BD) and analyzed by flow cytometry. Cells were acquired on an LSR-II flow cytometer (BD), and flow data were analyzed using FlowJo software (Tree Star).}

Table 1. CD28 dAb affinity and kinetic parameters

| Receptor   | kₐ (1/μM) | kₐ (1/s) | kₐ (nM) | Rₘax
|------------|-----------|----------|---------|------
| CD28 dAb   |           |          |         |      |
| Mouse CD28 | 8.4 ± 6.4 × 10⁶ | 2.2 ± 1.6 × 10⁻⁵ | 2.6 ± 2.8 × 5.9 ± 0.3 | 0.9
| Human CD28 | N.D.      | N.D.     | N.D.    | 0    |

N.D., none detected; Rₘax = Biaco maximum response signal.

Table 1

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 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.

Skin transplantation and in vivo co-stimulatory molecule blockade. Full-thickness tail and ear skins were transplanted onto the dorsal thorax of C57BL/6 splenocytes were incubated ex vivo with a 40-kD branched polyethylene glycol, and had an EC₅₀ (2.2 ± 0.6 nM) comparable to that of human CTLA4Ig (abatacept, 4.25 ± 2 nM) in murine mixed lymphocyte reactions.

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Flow cytometry and intracellular cytokine staining. Spleens or graft-draining axillary and brachial LNs were stained for CD4 and CD8 (both from Intraventrix) and Thy1.1 (BD). For phenotypic analysis, cells were also surface-stained with anti-IFN-γ, 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 TrueCount bead analysis according to the manufacturer’s instructions. Samples were analyzed on an LSR II 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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