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The Role of the IL-2 Pathway in Costimulation Blockade-Resistant Rejection of Allografts

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Blockade of the CD40 and CD28 costimulatory pathways significantly prolongs allograft survival; however, certain strains of mice (i.e., C57BL/6) are relatively resistant to the effects of combined CD40/CD28 blockade. We have previously shown that the costimulation blockade-resistant phenotype can be attributed to a subset of CD8+ T cells and is independent of CD4+ T cell-mediated help. Here we explore the role of the IL-2 pathway in this process using mAbs against the high affinity IL-2R, CD25, and IL-2 in prolonging skin allograft survival in mice receiving combined CD40/CD28 blockade. We have also investigated the effects of treatment on effector function by assessment of cytotoxicity and the generation of IFN-γ-producing cells in response to alloantigenic stimulators as well as proliferation in an in vivo graft-vs-host disease model. We find that additional blockade of either CD25 or IL-2 significantly extends allograft survival beyond that in mice receiving costimulation blockade alone. This correlates with diminished frequencies of IFN-γ-producing alloreactive T cells and reduced CTL activity. Anti-CD25 therapy also synergizes with CD40/CD28 blockade in suppressing proliferative responses. Interestingly, depletion of CD4+ T cells, but not CD8+ T cells, prevents prolongation in allograft survival, suggesting an IL-2-independent role for regulation in extended survival.


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Until recently the IL-2 pathway was considered a necessary component of adaptive immune responses to transplanted tissue. Blockade of either the cytokine or the IL-2R prolongs heterotopic heart allograft survival in rats and mice (17–20). Recently, growing doubt has mounted regarding the necessity of its role, given the evidence that absence of IL-2 expression in knockout models and that specific intragraft inhibition of IL-2 mRNA fails to delay allograft rejection (21–24). Further, the finding that cytokines IL-2, -4, -7, -9, -13, and -15 use a common signaling domain (γc) supports the idea that these cytokines are partially interchangeable in immune responses, with the loss of a single type having only a minor impact on overall immune function (25). Despite this evidence, Abs directed against the α-chain of IL-2R (CD25) have shown clinical efficacy when used in combination with standard immunosuppressive therapies (26–28). For this reason we hypothesized that the use of Abs directed against CD25 may prove efficacious in combination with costimulation blockade.

In the current study we demonstrate that treatment with blocking Abs to CD25 or with IL-2-neutralizing Abs synergizes with costimulation blockade in prolonging skin allograft survival in
C57BL/6 mice. Enhanced graft survival was associated with a significant decrease in the frequency of T cells producing IFN-γ and a decreased cytotoxic response to allogeneic stimulator cells. We further demonstrate that the observed prolongation of allograft survival is dependent on the presence of CD4+ cells, but not CD8+ cells. Finally, we show that in vivo proliferation is significantly attenuated when using costimulation blockade or anti-CD25, and that this attenuation is further enhanced when using these treatments together.

Materials and Methods

Mice

Adult male BALB/c (H-2b) and C57BL/6 (H-2b) mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals received humane care in accordance with Emory University institutional animal care and use committee protocols and the Principles of Laboratory Animal Care (29).

Treatment protocols

Skin graft recipients were treated with 500 μg each of hamster anti-mouse CD40L Ab (MR1) and human CTLA4-Ig administered i.p. on the day of transplantation (day 0) and on postoperative days 2, 4, and 6. Additional experimental groups were treated with 250 μg anti-CD25 mAb (PC61) or 250 μg rat IgG2b administered i.p. on days 0, 2, 4, and 6 or 100 μg rat anti-mouse CD8 (53-6-72) or rat anti-mouse CD4 (GK1.5) administered i.p. on days −2, −1, and 0 and weekly thereafter until the time of rejection. Depletion of CD4 and CD8 cells was confirmed as >99% by flow cytometry of peripheral blood leukocytes. Refer to Table I for a summary of the treatment protocols as they appear in Figs. 1 and 2.

Flow cytometry

Analysis of splenocytes and peripheral blood was conducted using fluorochrome-conjugated Abs (rat IgG2a-APC, rat IgG2b-APC, anti-CD4-APC, and anti-CD8-APC, PharMingen, San Diego, CA). Flow cytometry was performed using a FACSCalibur, and data were analyzed using CellQuest software (both BD Immunocytometry Systems, San Jose, CA).

Skin grafting

Full-thickness skin grafts (~1 cm2) were transplanted on the dorsal thorax of recipient mice and secured with a plastic adhesive bandage for 7 days. Graft survival was then followed by daily visual inspection. Rejection was defined as the complete loss of viable epidermal graft tissue. Statistical analyses were performed using a Mann-Whitney U test.

IFN-γ ELISPOT assays

Allospecific T cell responses were measured by IFN-γ ELISPOT assay using splenocytes obtained from skin-grafted C57BL6 mice. Rat anti-mouse IFN-γ (clone R4-6A2; BD PharMingen), the capture Ab, was incubated at 4 μg/ml in PBS (100 μl/well) at 4°C overnight in ester-cellulose-bottom plates (Millipore, Bedford, MA). Following PBS wash, 1×105, 3×105, 1×106, 3×106, 1×107, 3×107, 1×108, and 3×109 dilutions of splenocytes were added to the plate in duplicate. Stimulators were irradiated (2000 rad) donor (BALB/c) splenocytes added at a 1:1 stimulator to effector ratio. Effector cells were incubated for 14–16 h at 37°C with or without stimulators. After the culture period cells were removed by washing the plate in PBS-Tween (0.05%). Biotinylated anti-mouse IFN-γ (clone XMG1.2; BD PharMingen) was added at 4 μg/ml (100 μl/well), and the plates were incubated for 2–3 h at 4°C. Unbound Ab was then removed, and HRP-avidin D (Sigma, St. Louis, MO) was added. Spots were developed with the substrate 3-amino-9-ethyl-carbazole (Sigma) with 0.015% H2O2. Each spot represents an IFN-γ-secreting cell, and the frequency of these cells was determined by dividing the number of spots counted in each well by the total number of cells plated at that dilution. Naive splenocytes produce IFN-γ at a frequency of ≤3/106 cells with or without stimulation. Statistical analysis was performed using two-tailed Student’s t test.

Cytotoxicity assay

BALB/c CL.7 cells were used as targets. Target cells were suspended in saline and 5% FCS (−1×107/ml) with 750 μCi 51Cr (sp. act., 470 mCi/mg; NEN Life Science Products, Boston, MA) for 90 min at 37°C. Target cells were washed three times and plated in 96-well round-bottom plates at 1×103 targets/well in R10 medium (RPMI 1640 supplemented with penicillin, streptomycin, 2-ME, and 10% FCS (HyClone, Logan UT)). Effector cells were prepared by harvesting splenocytes and lysing RBC. Effectors were plated at the appropriate ratios in quadruplicate. Four wells were set aside for total lysis by addition of 2% Triton-X to the targets, and four wells were set aside for spontaneous lysis by the addition of R10 without effector cells. After 5 h, the supernatant was harvested and analyzed by gamma counting. The percent specific lysis was determined using the following formula: 100 × ((cpmunknown − cpmspontaneous)/(cpmtotal − cpmspontaneous)).

CFSE staining

Nylon wool-passed C57BL6 splenocytes were incubated in 10 μM CFSE (Molecular Probes, Eugene, OR). After 10 min the staining was halted by the addition of cold RPMI. Irradiated (18 Gy) BALB/c mice then received 3×105 CFSE-labeled cells i.v. by penile vein injection.Recipient mice were treated on the day of transfer (day 0) and on day 2 with CTLA4-Ig and anti-CD40L, anti-CD25 alone, rat IgG alone, or CTLA4-Ig and anti-CD40L with the addition of anti-CD25 or rat IgG.

Determination of precursor frequencies

Precursor frequency was determined as previously described (30). In short, peaks were labeled with the number of times the cells had divided (n). A T cell that divides n times generates 2n daughter cells. Therefore, to obtain the number of precursors for each individual peak, the number of peaks in a peak, n, is divided by 2. For allogeneic transfers, the original number of precursors resulting in each of peaks 4–8 was determined. These resulting values were added together for peaks 4–8 and divided by the total number of initial precursors, which yielded a precursor frequency for divisions 4–8. These divisions were chosen based on the observation that syngeneic

Table I. Treatment protocols for Figs. 1 and 2

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transferred cells do not proceed detectably beyond three divisions. Statistical analysis was performed using Student’s two-tailed t test.

Results

The IL-2 pathway plays a role in costimulation blockade-resistant skin allograft rejection

The observation that CD8$^+$ T cells mediate costimulation blockade-resistant rejection led us to postulate that these cells use CD28/CD40-independent costimulatory pathways. IL-2 has long been considered a central factor in the function of T cell responses, but experiments in IL-2 knockout mice have failed to demonstrate a primary role for the cytokine in allograft survival (21, 22). Further, Dai et al. (31) showed that IL-2$^{-/-}$ mice are refractory to costimulation blockade in the setting of vascularized heart allografts. However, since the observations in IL-2$^{-/-}$ animals may be due to abnormal lymphocyte development in the absence of IL-2, we

![Figure 1](http://www.jimmunol.org/) Blockade of the IL-2 pathway inhibits costimulation blockade-resistant rejection. A, C57BL/6 (H-2$b^*$) recipients of BALB/c skin grafts (H-2$d^*$) were treated with anti-CD25 (○), 250 μg rat IgG (●), 500 μg each of CTLA4-Ig and anti-CD40L (costimulation blockade; ▲), costimulation blockade and 250 μg rat IgG (■), or costimulation blockade and 250 μg anti-CD25 (▲) on days 0, 2, 4, and 6 following engraftment (n = 5 for each group). Grafts were monitored by visual inspection until rejection occurred. Animals receiving only costimulation blockade or costimulation blockade with the rat IgG isotype control reject skin grafts relatively quickly (MST, 34 and 32 days, respectively). Animals receiving additional anti-CD25 have significantly prolonged allograft survival (MST, 76 days). B, C57BL/6 recipients of BALB/c skin grafts were treated with costimulation blockade (●), costimulation blockade and 250 μg anti-CD25 (▲), or costimulation blockade and 250 μg anti-IL-2 (○) on days 0, 2, 4, and 6 following engraftment (n = 6 for each group). Animals treated with costimulation blockade reject with a MST of 29 days. Additional anti-IL-2 significantly prolongs skin graft survival (MST, 61 days), as does additional anti-CD25 (MST, 76 days).

![Figure 2](http://www.jimmunol.org/) Depletion of CD4$^+$ cells, but not CD8$^+$ cells, inhibits the prolongation in allograft survival achieved with additional anti-CD25 therapy. A, C57BL/6 recipients of BALB/c skin grafts were treated with costimulation blockade (▲); costimulation blockade and 250 μg anti-CD25 (□) on days 0, 2, 4, and 6 postengraftment; 250 μg anti-CD25 on days 0, 2, 4, and 6 and 100 μg anti-CD8 depleting Ab on days −2, −1, and 0 and weekly thereafter (●); or costimulation blockade, 250 μg anti-CD25 on days 0, 2, 4, and 6, and 100 μg anti-CD8 depleting Ab on days −2, −1, and 0 and weekly thereafter (○). Depleted animals receiving anti-CD25 reject skin grafts with an MST of 11 days, whereas depleted animals receiving costimulation blockade with anti-CD25 and nondepleted animals receiving costimulation blockade and anti-CD25 show marked prolongation in skin graft survival (MST, 63 and 76 days, respectively). B, C57BL/6 recipients of BALB/c skin grafts were treated with costimulation blockade and 250 μg anti-CD25 (□) on days 0, 2, 4, and 6 postengraftment; 250 μg anti-CD25 on days 0, 2, 4, and 6 and 100 μg anti-CD4 depleting Ab on days −2, −1, and 0 and weekly thereafter (■); or costimulation blockade, 250 μg anti-CD25 on days 0, 2, 4, and 6, and 100 μg anti-CD4 depleting Ab on days −2, −1, and 0 and weekly thereafter (○). CD4 depletion with anti-CD25 treatment provided a modest delay in rejection (MST, 21 days); however, CD4 depletion eliminated the extended graft survival in animals treated with costimulation blockade and anti-CD25 (MST, 35 vs 76 days).
considered the possibility that interruption of the IL-2 pathway in normal animals might inhibit the generation of CD8+ T cell-dependent costimulation blockade-resistant rejection responses. We therefore used either anti-IL-2 Ab (S4B6) or anti-CD25 Ab (PC61) to target the IL-2 pathway during concomitant treatment with CTLA4-Ig and anti-CD40L. Animals treated with anti-CD25 rejected skin grafts (mean survival time (MST), 13 days) with the same kinetics as those receiving isotype control (rat IgG; MST, 15 days; Fig. 1A). As in our previous experiments, B6 recipients treated with costimulation blockade alone had a modest prolongation of BALB/c skin allograft survival (MST, 34 days; Fig. 1A). In contrast, allograft survival in recipients treated with costimulation blockade in addition to anti-CD25 Ab was dramatically prolonged (MST, 76 days) compared with that in recipients treated with costimulation blockade plus isotype control (MST, 32 days; p < 0.008; Fig. 1). To distinguish between the mechanisms of inhibition of the IL-2 pathway or targeted depletion of CD25+ cells, we treated animals with costimulation blockade and Ab directed against IL-2 cytokine. This treatment resulted in a similar prolongation in allograft survival (MST, 61 days; p < 0.004 vs CB) as treatment with CB and anti-CD25.

Extended allograft survival mediated by anti-CD25 is dependent on CD4+ T cells

We next sought to determine whether the effects were duplicated in the absence of specific T cell populations. The treatment protocols were repeated in animals depleted of either CD4+ or CD8+ cells by mAb (GK1.5 or 53-6.72, respectively). Depletions were confirmed as >99% by flow cytometry of peripheral blood leukocytes. As expected, animals depleted of CD8+ cells treated with anti-CD25 rejected their allografts promptly (MST, 11 days; Fig. 2A). Also as expected, animals depleted of CD8+ cells and treated with both costimulation blockade and anti-CD25 showed significant prolongation of allograft survival (MST, 59 days; Fig. 2A). This is consistent with previous findings demonstrating prolonged allograft survival in animals depleted of CD8+ cells and treated with anti-CD40L and CTLA4-Ig alone (8).

Animals depleted of CD4+ cells showed relatively rapid rejection when treated with only anti-CD25, and surprisingly, no enhancement of allograft survival over costimulation blockade alone when treated with anti-CD25 and costimulation blockade together (Fig. 2B). These results clearly demonstrate that a simple model in which costimulation blockade impacts CD4+ T cells and anti-CD25 treatment impacts CD8+ is not sufficient to explain the mechanism of these effects.

To explore the effects of anti-CD25 mAbs on the generation of breakthrough T cell responses in mice receiving CTLA4-Ig and anti-CD40L, IFN-γ ELISPOT assays were performed 20 and 35 days post-transplant. We compared the precursor frequency of T cells producing IFN-γ in response to BALB/c stimulator cells between animals receiving various treatment regimens (Figs. 3 and 4). Animals receiving costimulation blockade alone demonstrate a breakthrough IFN-γ response on day 20, which persists on day 35. Conversely, animals receiving additional anti-CD25 showed no breakthrough IFN-γ response at these time points (p < 0.05 vs CB alone on day 20; p < 0.01 on day 35). Interestingly, animals depleted of CD4+ T cells and receiving both costimulation blockade and anti-CD25 showed no response on day 20, but demonstrated a

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Development of effector functions by alloreactive cells is inhibited by additional anti-CD25 treatment. C57BL/6 recipients of BALB/c skin grafts received the indicated treatments (n = 3 for each experiment). Animals were sacrificed, and splenocytes were harvested on days 20 and 35 for use in an IFN-γ ELISPOT assay or a CTL 51Cr release assay. *A*, Recipient splenocytes were incubated with irradiated BALB/c splenocytes for 13 h to stimulate IFN-γ production from primed alloreactive cells. Secreted IFN-γ was captured by plate-bound Abs and detected using a second biotinylated anti-IFN-γ. Each spot developed represents an IFN-γ-secreting cell. Represented is the average ± SEM of the number of spots per million recipient cells plated of the three animals in each group. Costimulation blockade-treated animals demonstrate a breakthrough response on day 20, while additional anti-CD25 therapy results in naive levels of IFN-γ production. *B*, Recipient splenocytes were incubated with 51Cr-labeled BALB clone 7 cells for 5 h. Supernatant was drawn off and analyzed for 51Cr release. Presented is the percent specific lysis, which is determined by the following: [(sample 51Cr release − spontaneous release)/(total release − spontaneous release)] × 100%). Values are the average of the three animals ± SEM. Animals were treated with costimulation blockade (■) or costimulation blockade with 250 μg anti-CD25 (▲) or were left untreated (●). Lysis was observed for costimulation blockade-treated and untreated animals, while additional anti-CD25 treatment resulted in lysis indistinguishable from the naive controls (▲). aIL-2R, Anti-IL-2R; aCD4, anti-CD4.
Synergistically with anti-CD25 to inhibit both CD4 effector function is dependent on the presence of CD4+ T cells. Next, we studied the effects of anti-CD25 Ab on the expansion of T cells in vivo in a graft-vs-host disease model using the fluorescent dye CFSE (32). Cells labeled with CFSE lose half their fluorescence each time they divide, and thus cell divisions can be tracked in vivo using flow cytometry (33, 34). Nylon wool-passed, CFSE-labeled C57BL/6 T cells were adoptively transferred into lethally irradiated BALB/c recipients that were treated with anti-CD25 Ab, rat Ig control Ab, CTLA4-Ig and anti-CD40L, or CTLA4-Ig, anti-CD40L, and anti-CD25 Ab. Recipient splenocytes were harvested 3 days after adoptive transfer and analyzed by flow cytometry for CD4 or CD8 expression and CFSE fluorescence.

For both CD4+ and CD8+ populations, only populations with fluorescence corresponding to greater than four divisions were included in the analysis, as syngeneic T cells transfers undergo up to three divisions, but not detectably more, in the same time period. The precursor frequency of cells undergoing more than four divisions was calculated as described in Materials and Methods.

As shown in the histograms in Fig. 5, costimulation blockade and anti-CD25-treated animals show reduced numbers of cells in the highly divided population of CD4+ and CD8+ cells compared with isotype controls. Combination of these therapies further reduced the frequency of highly divided cells. This is reflected in the precursor frequency of cells that undergo greater than four divisions (Fig. 6). Control treated animals had a precursor frequency of 6.7 ± 0.52% of transferred CD4 cells and 4.1 ± 0.10% of transferred CD8 cells. Both costimulation blockade and CD25 blockade alone significantly reduce the precursor frequency of divided cells (CD4, 1.95 ± 0.092% with CB and 3.85 ± 0.69% with anti-CD25; CD8, 2.34 ± 0.17% with CB and 2.67 ± 0.64% with anti-CD25; p < 0.05 for both CD4 and CD8). A combination of the two treatments together further decreases proliferation (CD4, 0.96 ± 0.11% (p < 0.0001); CD8, 1.42 ± 0.087% (p < 0.005 vs CB alone)).

Discussion

Diversity in alloimmune responses between different strains of inbred mice will probably be reflected in outbred human populations. It is clear from our work and that of others that costimulation blockade alone fails to completely inhibit the alloimmune response in many settings. One approach to overcome these limitations and potentially improve clinical utility is through the simultaneous use of other clinically efficacious agents selected to complement costimulation blockade. In this study we have shown blockade of IL-2R α-chain, a clinically approved therapeutic modality, to be an effective supplement to costimulation blockade regimens that simultaneously target CD28-B7 and CD40L-CD40 interactions.

For a direct ex vivo CTL assay was performed by measuring the release of 51Cr from a BALB/c fibroblast cell line in the presence of splenocytes derived from engrafted animals receiving the indicated treatments (Fig. 3B). By day 20 costimulation blockade-treated animals demonstrated effective killing of allogeneic targets. Animals receiving additional anti-CD25 were indistinguishable from naive animals in their ability to lyse targets (p < 0.01 vs CB alone). Along with the results of ELISPOT assays, these findings demonstrate that addition of anti-CD25 to costimulation blockade abrogates the costimulation blockade-resistant phenotype by inhibiting the generation of allospecific T cells (i.e., cytotoxicity and IFN-γ secretion). Like prolonged allograft survival, inhibition of effector function is dependent on the presence of CD4+ T cells.

Costimulation blockade using anti-CD40L and CTLA4-Ig acts synergistically with anti-CD25 to inhibit both CD4+ and CD8+ T cell proliferation in a graft-vs-host disease model

FIGURE 4. CD4 depletion in CB- and anti-CD25-treated animals leads to a breakthrough IFN-γ (IFNg) response similar to that seen in CB-treated animals. A, Costimulation blockade-treated animals continue to display IFN-γ production far above that of naive animals, whereas additional anti-CD25 treatment on days 0, 2, 4, and 6 prevents this response. B, Analysis on a smaller scale (omitting untreated animals) reveals that CD4 depletion also gives rise to a breakthrough IFN-γ response in costimulation blockade- and anti-CD25-treated animals, which is concurrent with the MST of their skin grafts, as shown in Fig. 2. aIL-2R, Anti-IL-2R; aCD4, anti-CD4.

A

Day 35 IFNg ELISpot

Table

B

Day 35 IFNg ELISpot

Table
In contrast to our initial hypothesis, the beneficial effect of anti-CD25 therapy is not mediated solely by an inhibitory or depleting effect on activated CD8$^+$ T cells, but also perhaps through a synergistic action with costimulation blockade to inhibit both CD4$^+$ and CD8$^+$ T cell responses. Two observations argue against an underlying depleting mechanism for the effect of anti-CD25 therapy. First, neutralization of IL-2 cytokine has a similar effect in prolonging allograft survival. Second, costimulation blockade or anti-CD25 (H9251) have substantially reduced cell proliferation, whereas a combination of costimulation blockade and anti-CD25 treatment nearly eliminates the population of highly divided cells in both the CD4$^+$ and CD8$^+$ T cell populations. Animals receiving costimulation blockade or anti-CD25 (oCD25) have substantially reduced levels of proliferation, whereas a combination of costimulation blockade and anti-CD25 treatment nearly eliminates the population of highly divided cells in both T cell subsets.

Multiple previous experiments have shown the IL-2 pathway to be dispensable in allograft rejection, an observation largely attributed to the redundancy inherent in the IL-2R system (21, 23). The $\gamma$c component of the IL-2R, which mediates intracellular signaling following cytokine binding through the Janus kinase-STAT signaling cascade, is shared by the cytokines IL-4, -7, -9, -13, and -15 (25). IL-15 also shares the IL-2R $\beta$-chain, a target that is now of considerable interest. Others have shown prolonged allograft survival through blockade of the $\gamma$c and through selective blockade of IL-15 (35–38). Our findings confirm that anti-CD25 therapy alone has little effect on skin allograft survival, but when used in concert with costimulation blockade at the time of engraftment, it achieves significant prolongation in survival. The absence of indefinite survival in our model may be due to activation signals delivered by any of the cytokines sharing the $\gamma$c or other as yet to be defined costimulatory pathways. The recent findings of Ferrari-Lacraz et al. (38) demonstrate that IL-15 blockade inhibits costimulation blockade-resistant rejection in allografts. Thus, it appears that both IL-2 and IL-15 are important in the alloimmune response. Indeed, the recent work of Li et al. (39) demonstrates a staggered role for the two cytokines, in which IL-15 is critical to early rounds of T cell proliferation, and IL-2 is critical to late proliferation and accompanying apoptosis. Therefore, combined or staggered treatments focused on both the IL-2 and IL-15 pathways may prove beneficial.

Our findings are somewhat surprising in light of the studies by Lakkis et al. (31) where blockade or absence of IL-2 eliminated an extension in heart allograft survival mediated by CTLA4-Ig. In this model the antagonistic effects were attributed to the role of IL-2 in preparing CD8$^+$ cells for activation-induced cell death (AICD), which was later shown to be mediated by up-regulation of Fas ligand and down-regulation of $\gamma$c (40). On the surface, it would appear that if this model held in our experiments, we would expect anti-CD25 therapy to exacerbate costimulation blockade-resistant rejection, but this was clearly not the case. There are several differences in the experimental design of the earlier experiments and our current results, including the strain combinations used, tissue types transplanted, and timing of costimulation blockade, any of which might account for the differential effects of anti-IL-2R Abs in our studies. Further, Lakkis et al. (31) made use of knockout animals, in which IL-2 signaling is never possible, whereas blockade of these signals with Ab in our experiments may be incomplete. Such differences in design preclude direct comparisons between these studies; however, the results of these experiments would be consistent if IL-2 blockade in our model results in passive T cell death through IL-2 starvation, vs the active IL-2R-dependent AICD required for tolerance in IL-2-deficient animals, where alternative pathways may substitute for IL-2 to prevent starvation.

**FIGURE 5.** Representative samples from a flow cytometric assay for T cell proliferation in an in vivo graft-vs-host disease model. Irradiated BALB/c mice received 3 $\times$ 10$^7$ CFSE-labeled C57BL/6 nylon wool-passed T cells i.v. and the indicated treatments. Cells were harvested at 70 h postinfusion. With each division, the fluorescent intensity diminishes by half, and therefore each peak beyond the highest intensity represents a division. Highly divided cells have the lowest fluorescent intensity. Animals receiving rat IgG only show the largest proportion of highly divided cells in both the CD4$^+$ and CD8$^+$ T cell populations. Animals receiving costimulation blockade or anti-CD25 (oCD25) have substantially reduced levels of proliferation, whereas a combination of costimulation blockade and anti-CD25 treatment nearly eliminates the population of highly divided cells in both T cell subsets.

**FIGURE 6.** Costimulation blockade and anti-CD25 have a synergistic effect on inhibition of proliferation in a graft-vs-host disease (GVHD) model. Shown is the precursor frequency of highly proliferating cells in the in vivo GVHD model (mean ± SEM; n = 6 for each treatment group). Animals were treated with 250 $\mu$g rat IgG ( ), 250 $\mu$g anti-CD25 ( ), costimulation blockade ( ), or costimulation blockade and 250 $\mu$g anti-CD25 ( ) on days 0 and 2 after infusion. Cells were harvested at 70 h and were analyzed by flow cytometry for diminution of CFSE signal and CD4$^+$ or CD8$^+$ cells. Data represent the precursor frequency of cells undergoing more than four divisions.
Like many cytokines, it is apparent that IL-2 plays multiple roles, including involvement in both expansion and death of activated T cells. Our data indicate that concurrent blockade of costimulatory signals and the IL-2 pathway effectively prevents the generation of alloreactive effector cells. This highlights IL-2’s role in the activation and expansion of costimulation blockade-resistant CD8+ T cells. In contrast, the absence of the IL-2 pathway has a very different effect. In this setting, where IL-2’s role in proliferation may be diminished, IL-2 signals appear to play an important role in the elimination of alloreactive T cells by enhancing AICD (31). Taken together these findings indicate that combinations of costimulation blockade and anti-CD25 mAbs may be used to inhibit undesirable immune responses. However, it appears that concomitant therapy must be given at the outset of the response. Extrapolation of these approaches to treatment of ongoing responses, such as autoimmunity, may exacerbate, rather than ameliorate, disease. Further study of this issue is clearly warranted.

Our data showing diminished survival when CD4+ T cells are depleted from recipients treated with CB and anti-CD25 are consistent with a growing body of data implicating an important role for regulatory CD4+ T cells in allografts. CD25+ CD4+ regulatory cells have received considerable attention and are particularly relevant in light of our use of anti-CD25 mAbs. However, the role of IL-2 signaling in these regulatory cells has not been elucidated (41, 42). Our laboratory is currently pursuing the role of regulatory CD4+ T cells in this and other models of costimulation blockade-based therapies. Further study of this incompletely understood phenomenon may allow targeted utilization of regulatory effects to ameliorate allograft rejection.

Our data clearly demonstrate that blockade of the IL-2 pathway inhibits costimulation blockade-resistant rejection. Clinically available anti-CD25 mAbs may therefore serve as a useful adjunct to emerging costimulation blockade strategies. Future study of the effect of the IL-2 pathway may also elucidate the role of regulation in certain rejection responses.

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