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# Transplant Acceptance Following Anti-CD4 Versus Anti-CD40L Therapy: Evidence for Differential Maintenance of Graft-Reactive T Cells

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**Inductive therapy with anti-CD4 or anti-CD40L monoclonal antibodies (mAb) leads to long-term allograft acceptance but the immune parameters responsible for graft maintenance are not well understood. This study employed an adoptive transfer system in which cells from mice bearing long-term cardiac allografts following inductive anti-CD4 or anti-CD40L therapy were transferred into severe combined immunodeficiency (SCID) allograft recipients. SCID recipients of cells from anti-CD4-treated mice (anti-CD4 cells) did not reject allografts while those receiving cells from anti-CD40L-treated mice (anti-CD40L cells) did reject allografts. Carboxyfluorescein succinimidyl ester (CFSE) labeling of transferred cells revealed that this difference was not associated with differential proliferative capacities of these cells in SCID recipients. Like cells from naïve mice, anti-CD40L cells mounted a Th1 response following transfer while anti-CD4 cells mounted a dominant Th2 response. Early (day 10) T-cell priming was detectable in both groups of primary allograft recipients but persisted to day 30 only in recipients treated with anti-CD4 mAb. Thus, anti-CD40L therapy appears to result in graft-reactive T cells with a naïve phenotype while anti-CD4 therapy allows progression to an altered state of differentiation. Additional data herein support the notion that anti-CD40L mAb targets activated, but not memory, cells for removal or functional silencing.**

**Key words: Basic immunology, cardiac allograft tolerance, CD4 + T cells, costimulation blockade**

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## Introduction

While various clinical and experimental immunosuppressive therapies promote the acceptance of transplanted organs, it is intuitive that graft acceptance is likely achieved by distinct immunologic mechanisms depending upon the therapy. The nature of the immune mechanisms responsible for graft acceptance likely reflects various immune deviations induced by different therapies that may vary in strength and resistance to reversal. A better understanding of immune parameters responsible for maintaining various forms of graft acceptance would facilitate the development of strategies aimed at achieving vigorous and nonreversible transplant acceptance.

For example, we have previously reported that inductive therapy with either anti-CD4 (1,2) or anti-CD40L (2,3) monoclonal antibodies (mAb) markedly prolongs cardiac allograft survival. However, at 60 days posttransplant, functioning allografts in anti-CD4-treated recipients develop signs of chronic rejection (CR) including transplant-associated vasculopathy (TAV) and interstitial collagen deposition (4), while allografts in anti-CD40L-treated recipients do not (3). These differences correlate with the intragraft expression of TGFβ and induction of connective tissue growth factor (CTGF) in recipients treated with anti-CD4 (that develop CR), but not in recipients treated with anti-CD40L (that do not develop CR) (5). This study explored additional immunologic differences that result from these inductive mAb therapies and sheds further light on the mechanisms of action underlying the resulting states of graft acceptance. To this end, an adoptive transfer system revealed differential cytokine production by graft-reactive T cells as well as distinct sensitivities to reversal of allograft acceptance. In this system, cells obtained from anti-CD4-treated primary recipients (anti-CD4 cells) were skewed toward a Th2 phenotype and failed to mediate rejection following adoptive transfer into secondary severe combined immunodeficiency (SCID) allograft recipients. In contrast, cells from anti-CD40L-treated recipients (anti-CD40L cells) exhibited a Th1 phenotype and mediated rejection following transfer. Thus, the robust and nonreversible graft acceptance mediated by anti-CD4 cells correlated with altered T-cell function whereas anti-CD40L cells maintained the functional capacities of naïve T cells.

## Materials and Methods

### *Mice*

Female wild-type (WT) and SCID C57BL/6 (H-$2^b$) and BALB/c (H-$2^d$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and used between 6 and 12 weeks of age. Mice were housed under pathogen-free

conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. Animal use was approved by the University of Michigan's Committee on the Use and Care of Animals.

### *Culture medium*

Dulbecco's modified Eagle's medium (DMEM) was supplemented with 2% fetal calf serum (FCS), 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.6 mM L-glutamine, 10 mM HEPES buffer (all from Invitrogen, Grand Island, NY), 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μM folic acid and 50 μM 2-mercaptoethanol (all from Sigma Chemicals, St. Louis, MO).

### *Vascularized cardiac transplantation*

C57BL/6 mice were transplanted with intact BALB/c cardiac allografts (6). Transplant function was monitored by abdominal palpation. H & E stained sections of graft tissues were assessed to determine the degree of the cellular infiltrate, vascular involvement and myocyte death as determined by loss of nuclei and cross-striation.

### *Inductive anti-CD4 and anti-CD40L mAb therapies*

Anti-CD4 (GK1.5, American Type Culture Collection, Manassas, VA) and anti-CD40L (MR1, kindly provided by Dr. Randy Noelle, Dartmouth) mAb were purified and resuspended in phosphate-buffered saline (PBS) by Ligocyte Pharmaceuticals (Bozeman, MT). To transiently deplete CD4+ cells, allograft recipients were injected i.p. with 1 mg of anti-CD4 mAb on days –1, 0 and 7 relative to transplantation (1,2,4). Following depletion, CD4+ cells begin to repopulate the periphery between 3 and 4 weeks posttransplant (4,7). For inductive anti-CD40L therapy, allograft recipients were injected i.p. with 1 mg of anti-CD40L on days 0, 1 and 2 relative to transplantation (2,3). Both of these inductive protocols promote allograft survival for >60 days.

### *Adoptive transfer of splenocytes into SCID allograft recipients*

C57BL/6 SCID mice were transplanted with BALB/c cardiac allografts. Two days posttransplant, SCID recipients were injected i.v. with $5 \times 10^7$ splenocytes obtained from the following groups of C57BL/6 mice: (1) naïve, non-transplanted mice as a positive control for rejection, (2) mice bearing functioning allografts on day 60 posttransplant that received inductive anti-CD4 mAb, (3) mice bearing functioning allografts on day 60 posttransplant that received inductive anti-CD40L mAb, (4) unmodified mice that were rejecting cardiac allografts on day 8 posttransplant as a source of activated donor-reactive effector cells, (5) mice that were sensitized with BALB/c skin grafts 70 days previously as a source of donor-reactive memory cells. Where indicated, SCID allograft recipients were given inductive anti-CD40L mAb or control hamster IgG (hIgG).

### *Splenocyte proliferation following transfer into SCID allograft recipients*

Splenocytes from naïve mice, anti-CD4-treated allograft recipients or anti-CD40L-treated allograft recipients were labeled with carboxyfluorescein succinimidyl ester (CFSE) dye using the Vybrant CFDA SE Cell Tracer Kit (Invitrogen, Carlsbad, CA) as per manufacturer's directions. $5 \times 10^7$ CFSE-labeled splenocytes were i.v. injected into SCID allograft recipients. On day 7 and 14 posttransfer, cells were recovered from the spleens and mesenteric lymph nodes (LN) of SCID recipients and CFSE dilution was assessed by flow cytometry to measure *in vivo* proliferation of transferred cells.

### In vivo *neutralization of IL-4*

SCID allograft recipients were injected i.p. with 1-mg anti-IL-4 mAb (11B11; purified by Ligocyte Pharmaceuticals) or control rat IgG (Sigma) three times per week until the termination of the experiment. To verify the presence of circulating anti-IL-4 mAb in these mice, sera were tested for IL-4 neutralizing capacity in a competitive IL-4 ELISA as described (2). In this assay, 1:2000 dilutions of sera neutralized 5 ng/ml IL-4, thereby verifying the effectiveness of this IL-4 neutralizing regimen.

### In vitro *T-cell subset depletion*

CD4+ or CD8+ cells were depleted using Dynal Beads (Invitrogen). Single-cell suspensions of splenocytes were incubated with anti-CD4 or anti-CD8-coated beads for 30 min. Bead-bound cells were removed magnetically. Unbound cells were confirmed to be subset depleted by flow cytometry.

### *ELISPOT assays for* in vivo *primed and precursor graft-reactive cells*

ELISPOT assays were employed to quantify in vivo primed and quiescent donor-alloantigen-reactive IFNγ (Th1) and IL-4 (Th2) producing cells (8). Briefly, primed cells produce detectable cytokine when stimulated with donor Ag in short-term overnight ELISPOT cultures while quiescent cells require stimulation with donor Ag for 72 h in MLC prior to detection in overnight ELISPOT cultures.

Capture and detection mAb for IFNγ and IL-4 were purchased from Pharmingen (San Diego, CA). Irradiated (1000 rads) donor splenocytes ($4 \times 10^5$) were added to each well followed by $1 \times 10^6$ recipient splenocytes for primed responses or $5 \times 10^5$ recipient splenocytes for precursor responses. After an 18-h incubation, plates were developed and spots were quantified with an Immunospot Series 1 ELISPOT analyzer (Cellular Technology Ltd., Cleveland, OH).

### *Real-time PCR for intragraft IFNγ and IL-4 expression*

Allografts were homogenized in 1-mL TRIzol® (Invitrogen) and RNA was isolated as per manufacturer's protocol. Five μg of total RNA were reverse transcribed using 10× PCR buffer (Roche, Indianapolis, IN). Ten mM dNTPs, Oligo (dT), M-MLV-RT (all from Invitrogen) and RNAsin (Promega, Madison, WI). Products were then cleaned with 1:1 phenol/chloroform/isoamyl (25:24:1) and reprecipitated with 2 M $NH_4OAC$ in pure EtOH overnight at $-80^\circ C$.

Real-time PCR was performed on cDNA using a Rotor-Gene 3000™ (Corbett Life Science, San Francisco, CA). Primer binding to DNA was detected by SYBR Green I™ dye (BioRad). Relative expression of the gene of interest was expressed as the concentration of the gene product compared to GAPDH product as calculated by accompanying Rotor-Gene software. Significance was determined with an unpaired *t*-test with Welch's correction.

Primer sequences:

GAPDH sense: 5′ CTGGTGCTGAGTATGTCGTG,
anti-sense: 5′ CAGTCTTCTGAGTGGCAGTG.
IFNγ sense: 5′ GGCCATCAGCAACAACATAAGC,
anti-sense: 5′ CCCCGAATCAGCAGGGACTC.
IL-4 sense: 5′ GCCAAACGTCCTCACAGCAA,
anti-sense: 5′ GCATGGTGGCTCAGTACTACGA.

### *Statistical analyses*

Data were analyzed with Statview 5.0.1 software using analysis of variance (ANOVA) with a post *ad hoc* Fischer's PLSD test. The p-values $\leq 0.05$ were considered statistically different.

## Results

While inductive treatment with either anti-CD4 or anti-CD40L mAb results in prolonged allograft survival, treatment with anti-CD4 results in CR while treatment with

anti-CD40L does not (3–5). The development of CR is associated with the intragraft expression of TGFβ and CTGF in recipients treated with anti-CD4 and the absence of CR correlates with the lack of expression of these cytokines in recipients treated with anti-CD40L (5). This study explores additional immune parameters that are associated with prolonged allograft survival under these conditions.

### *Donor-reactive precursor cells are detectable in mice bearing long-term allografts*

In Figure 1, we employed ELISPOT (8) to quantify *in vivo* primed (left panel) and precursor (right panel) donor-reactive Th1 (IFNγ) and Th2 (IL-4) using splenocytes obtained from mice bearing functioning allografts on day 60 following inductive therapy with either anti-CD4 (anti-CD4 cells) or anti-CD40L (anti-CD40L cells) mAb. Splenocytes obtained from unmodified allograft recipients on day 8 posttransplant were used as a positive control for T-cell priming while naïve splenocytes served as a negative control for priming. Anti-CD4 and anti-CD40L cells resembled naïve cells in that primed cells were not detectable at this late time point posttransplantation. Precursor Th1 and Th2 were detectable at levels similar to those seen in naïve mice, suggesting that donor-reactive cells were maintained in a quiescent state in mice bearing long-term allografts.

### *Anti-CD40L cells, but not anti-CD4 cells, adoptively transfer acute rejection responses in SCID allograft recipients*

We have reported that anti-CD4 cells mediate chronic rather than acute rejection when adoptively transferred into secondary SCID allograft recipients (4). Hence, we used this adoptive transfer system to further explore immunologic differences in mice bearing long-term allografts following inductive anti-CD4 versus anti-CD40L treatment (Figure 2). As expected, transfer of naïve cells resulted in acute rejection in SCID allograft recipients while transfer of anti-CD4 cells did not. Somewhat unexpectedly, transfer of anti-CD40L cells resulted in rejection, albeit slightly delayed relative to transfer of naïve cells. It should be noted that this 'delayed acute rejection' mediated by anti-CD40L cells histologically resembled the more brisk acute rejection mediated by naïve cells (data not shown). Hence, anti-CD40L cells behaved like naïve cells in this setting. The difference in the ability of these cell populations to mediate rejection (anti-CD40L cells) or not (anti-CD4 cells) was highly reproducible ($n = 21$ and 28, respectively) and significant ($p < 0.0001$).

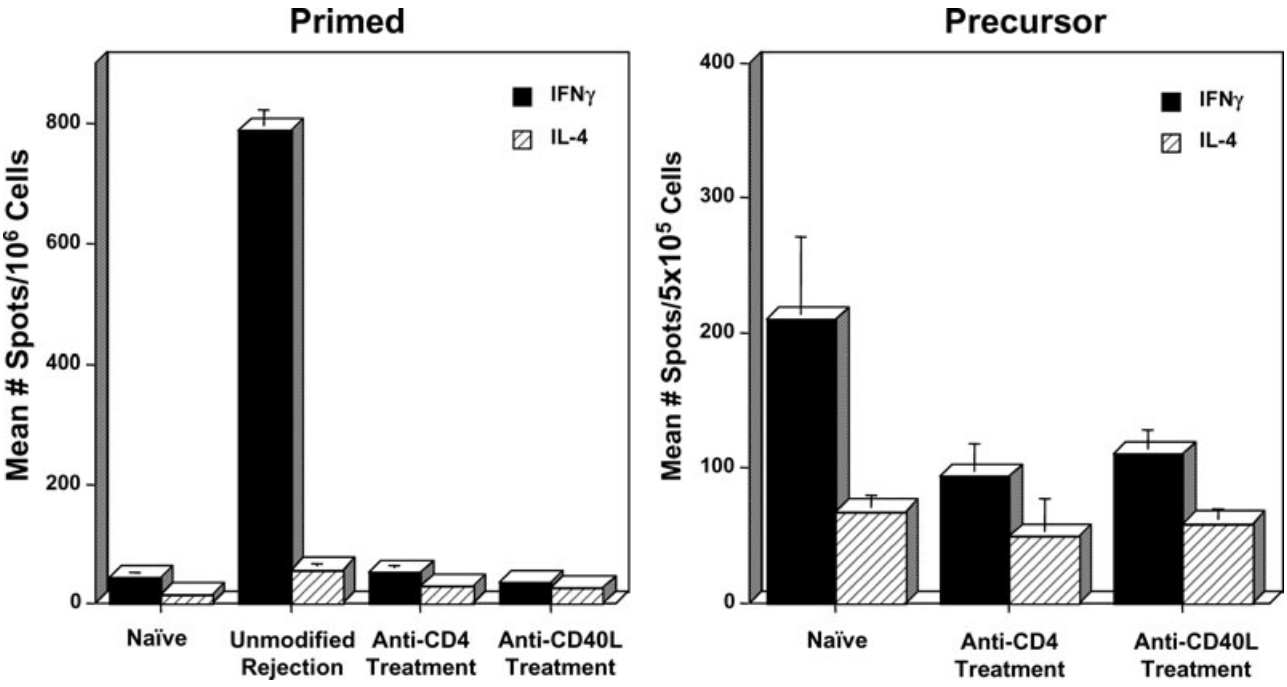


**Figure 1: Donor-reactive T-cell responses in mice bearing long-term allografts following inductive therapy with anti-CD4 or anti-CD40L mAb.** C57BL/6 mice were transplanted with BALB/c cardiac allografts and given inductive anti-CD4 or anti-CD40L therapy. Sixty days posttransplant, splenocytes from three mice per group were pooled for each experiment and assessed for primed and precursor donor-reactive Th1 and Th2 by ELISPOT. The averages of 5–11 experiments (three mice per experiment) are depicted for primed responses and the averages of 10–16 experiments (three mice per experiment) are represented for precursor responses. Error bars represent the SEM. Naïve splenocytes from nontransplanted mice served as negative controls for T-cell priming while splenocytes obtained from unmodified allograft recipients that were actively rejecting their grafts on day 8 posttransplant served as positive controls for T-cell priming.

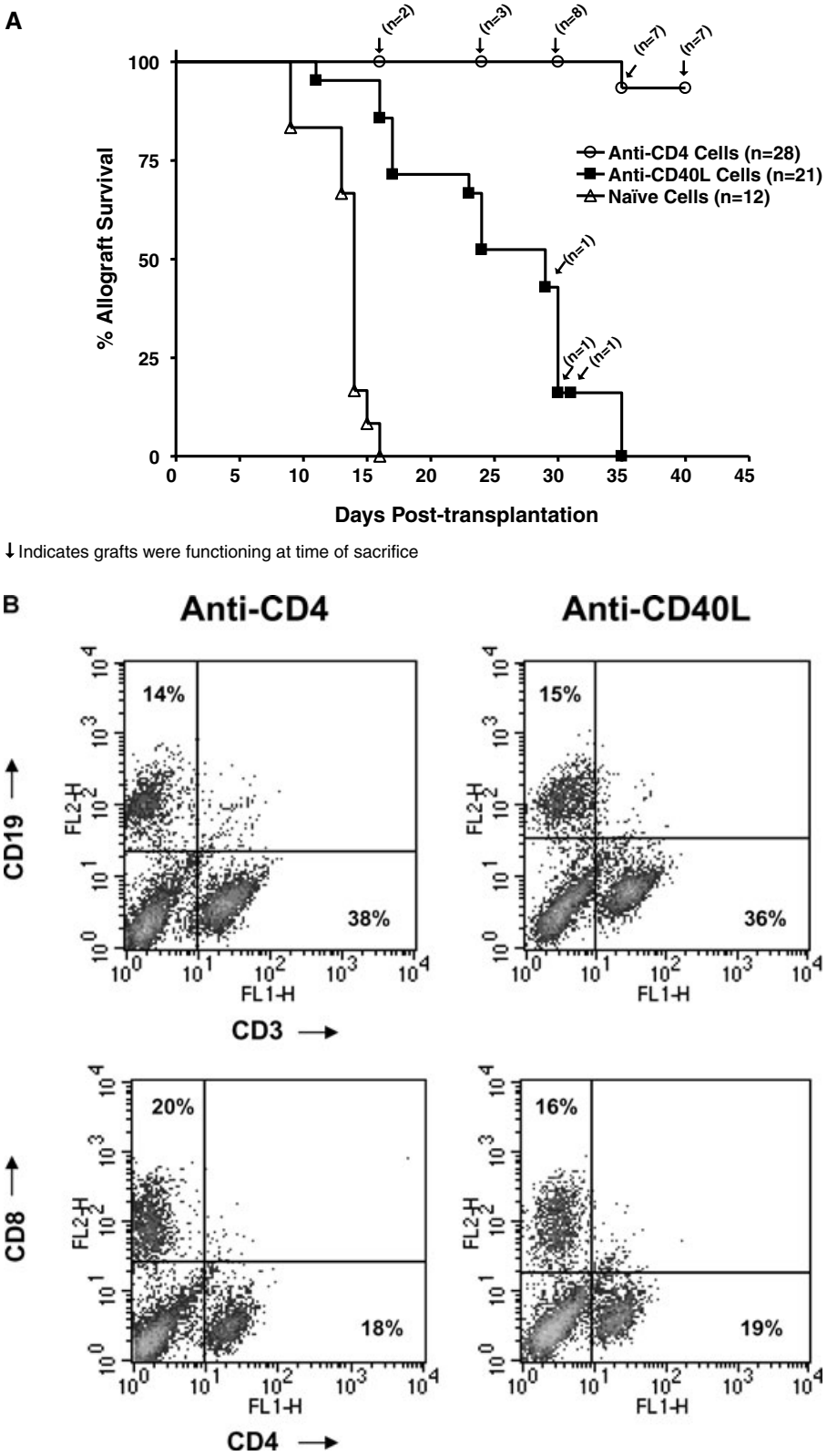


**Figure 2: Cells from long-term anti-CD40L, but not anti-CD4 mAb-treated primary allograft recipients transfer rejection to secondary SCID allograft recipients.** C57BL/6 SCID mice were transplanted with BALB/c cardiac allografts. Two days posttransplant, SCID mice were infused i.v. with $5 \times 10^7$ naïve cells obtained from nontransplanted mice or cells from mice bearing functioning allografts 60 days following inductive therapy with anti-CD4 mAb (anti-CD4 cells) or anti-CD40L mAb (anti-CD40L cells). Panel A depicts allograft survival. The number of individual SCID recipients for each group is presented in parentheses, $p < 0.0001$ for recipients of anti-CD4 versus anti-CD40L cells. Panel B depicts representative flow cytometry data for splenocytes from SCID allograft recipients receiving anti-CD4 cells (left) or anti-CD40L cells (right) and verifies the persistence of T and B cells following adoptive transfer. These analyses were performed on each SCID recipient depicted in Figure 2A at the time of rejection for recipients of anti-CD40L cells or at the termination of the experiment for recipients of anti-CD4 cells. Panel C depicts *in vivo* proliferation of CFSE labeled naïve, anti-CD4 or anti-CD40L cells on day 7 posttransfer into SCID allograft recipients. Flow cytometry data are representative of four separate experiments evaluating proliferation as assessed by CFSE dilution within the spleens of SCID recipients. Similar data were obtained for CFSE-labeled cells recovered from the mesenteric LN and for cells obtained on day 14 postadoptive transfer.

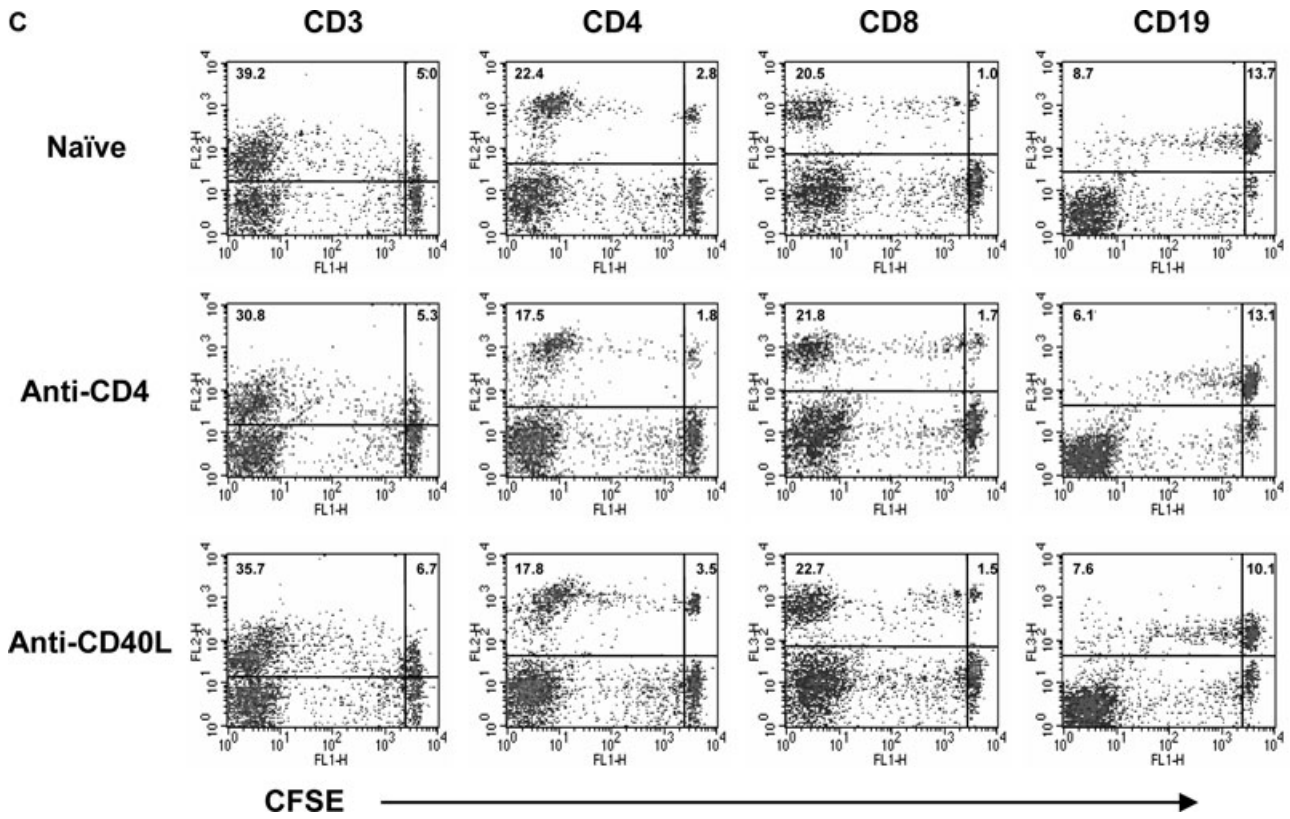


**Figure 2: (Continued)**

The differential ability of anti-CD4 versus anti-CD40L cells to mediate rejection was not due to differences in T- and B-cell reconstitution of the SCID recipients by transferred cells. As shown in Figure 2B, the percentages of CD19 + B cells and CD3+ T cells (top panel) were virtually identical in SCID recipients of these cell populations. In the T-cell compartment, the percentages of CD4+ and CD8+ cells (bottom panel) were also similar between the groups. Similar flow cytometry data were generated for each individual SCID recipient depicted in Figure 2A.

Further, the differences in the ability to mediate rejection were not associated with differential proliferative capacities of these cells following transfer into SCID recipients. Cell populations were labeled with CFSE prior to transfer and the expansion of CD3+, CD19+, CD4+ and CD8+ cells was assessed by flow cytometry in the spleens (Figure 2C) and mesenteric LN (data not shown). Virtually identical proliferative responses were observed for all cell populations on day 7 (Figure 2C) and day 14 (data not shown) posttransfer.

### *In vivo primed Th1 and Th2 responses in SCID allograft recipients following adoptive transfer*

We next asked whether differences in effector functions correlated with allograft rejection versus acceptance under these conditions. Splenocytes were recovered from the SCID allograft recipients and used as responder cells in ELISPOT to quantify *in vivo* primed donor-reactive Th1 and Th2. Figure 3A illustrates that a primed Th1 dominant response correlated with graft rejection following transfer of naïve cells into SCID recipients. This Th1 > Th2 pattern is characteristic of unmodified rejection in normal mice (Figure 1, left panel and (Reference 9)). Similarly, this Th1 > Th2 profile of cytokine production was observed when anti-CD40L cells were used for transfer and rejection ensued. Hence, anti-CD40L cells resembled naïve cells in this assay. Of interest, the cytokine pattern was reversed with Th2 > Th1 in SCID recipients of anti-CD4 cells that did not reject their grafts.

To determine whether IL-4 production was required for allograft acceptance when anti-CD4 cells were used for transfer, SCID allograft recipients were treated with 1-mg anti-IL-4 mAb three times per week (2) for the duration of the experiment (30 days). Out of seven SCID recipients of anti-CD4 cells that were treated with anti-IL-4 mAb, none rejected their allograft indicating that Th2 production of IL-4 was not required for graft acceptance. High levels of neutralizing anti-IL-4 mAb were detectable in the sera of these mice (see Methods). Further, ELISPOT revealed that the primed Th2 response was significantly reduced in SCID recipients of anti-CD4 cells that were treated with anti-IL-4 mAb (Figure 3A).

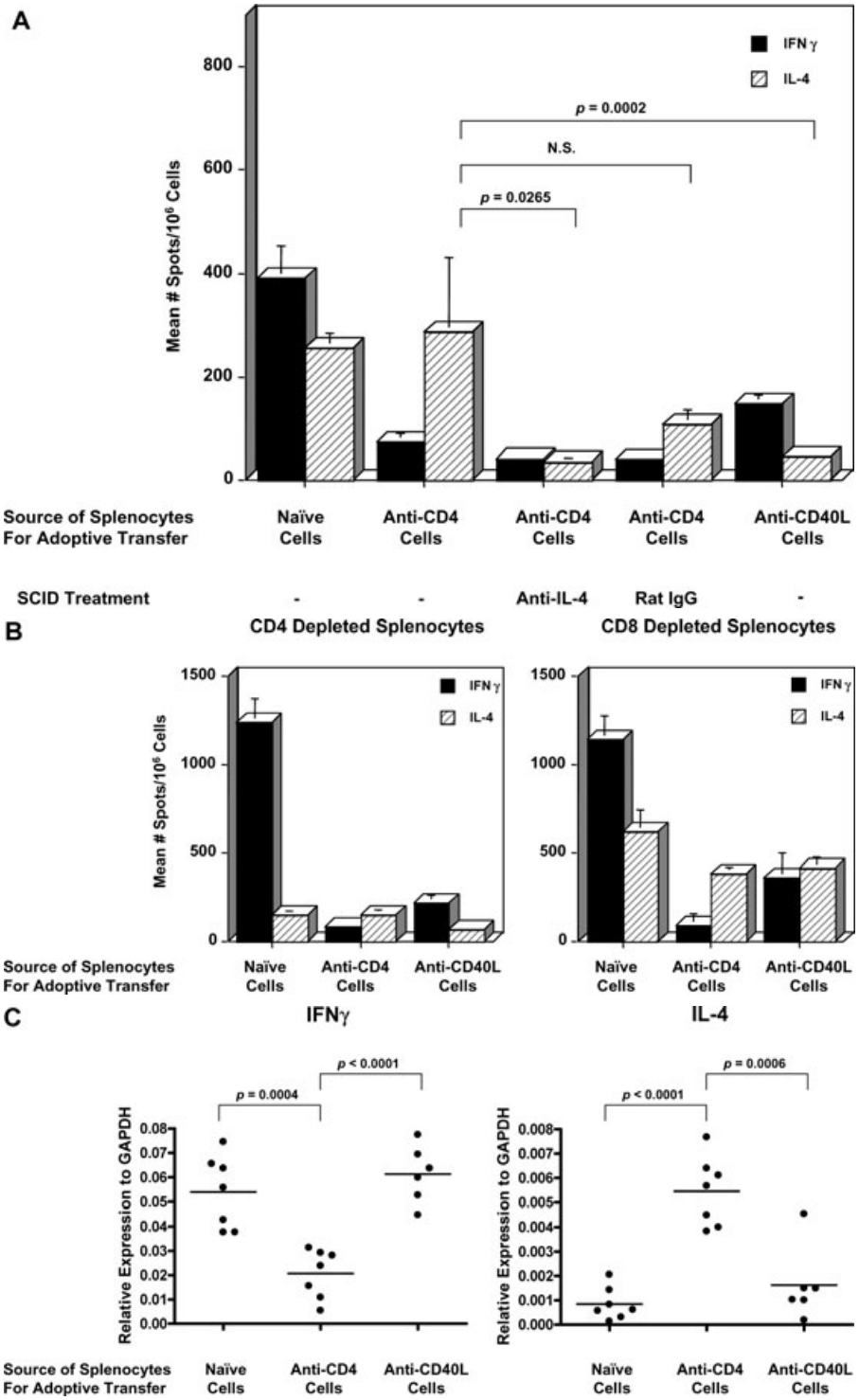


**Figure 3: Differential cytokine production by primed donor-reactive cells in SCID allograft recipients following transfer of anti-CD4 or anti-CD40L cells.** Panel A: Splenocytes were harvested from SCID allograft recipients depicted in Figure 2 and assessed for primed donor-reactive Th1 and Th2 responses by ELISPOT. Data represent the average of responses + SEM for 6–21 individual SCID recipients. Where indicated, 7 SCID recipients of anti-CD4 cells were treated with 1-mg anti-IL-4 mAb three times per week for 30 days. All 7 allografts in anti-IL-4 mAb-treated recipients continued to function normally until the termination of the experiment. Panel B: T-cell subsets were depleted from splenocyte populations with immunomagnetic beads before culture in ELISPOT assays to determine the contributions CD4+ and CD8+ T cells to cytokine profiles. Bars represent the average responses + SEM of 5–8 separate experiments. Panel C: Intragraft IFNγ and IL-4 gene expression was assessed by real-time PCR. Each data point represents the cytokine gene expression level of an individual allograft. Horizontal bars depict the mean expression level for each group.

The contributions of T-cell subsets to cytokine profiles were determined by depleting CD4+ and CD8+ cells prior to addition to the ELISPOT assays (Figure 3B). When CD4+ cells were depleted, the profiles of cytokine production were maintained for both anti-CD4 and anti-CD40L cells. When CD8+ cells were depleted prior to ELISPOT, Th2 responses were induced in all cell populations, in keeping with previous reports that depleting CD8+ cells results in Th2 responses (i.e. Reference 10). These data indicate that CD8+ cells influence Th1/Th2 balance in this system by suppressing CD4+ Th2 function.

We next asked whether the cytokine profiles assessed by ELISPOT analyses of splenocytes were reflective of

cytokine profiles within the transplant. However, ELISPOT analyses were precluded due to insufficient numbers of graft-infiltrating cells. Hence, we performed real-time PCR to quantify intragraft IFNγ and IL-4 expression levels in the allografts of SCID recipients of naïve, anti-CD4 or anti-CD40L cells (Figure 3C). IFNγ expression levels were significantly higher in the grafts of SCID recipients of anti-CD40L or naïve cells relative to recipients of anti-CD4 cells. Similarly, IL-4 expression levels were significantly higher in the grafts of recipients of anti-CD4 cells when compared to recipients of anti-CD40L or naïve cells. Thus, the ELISPOT data obtained from splenocytes reflected the patterns of cytokine gene expression within the allografts.

### *Donor-reactive T-cell priming following inductive anti-CD4 and anti-CD40L in primary allograft recipients*

The finding that anti-CD4 and anti-CD40L cells transferred distinct cytokine profiles to secondary SCID allograft recipients suggested that these cell populations experienced distinct events in the primary allograft recipients. The anti-CD4 mAb GK1.5 depletes peripheral CD4+ cells (11). When mice are treated with our inductive anti-CD4 regimen, CD4+ cells are transiently depleted and begin to repopulate the periphery between 3 and 4 weeks (4,7). Hence, we assessed donor-reactive T-cell priming in anti-CD4-treated primary allograft recipients on day 30 posttransplant and compared these responses to their anti-CD40L counterparts (Figure 4B). While responses in anti-CD40L-treated recipients day 30 posttransplant were similar to those seen with naïve cells from nontransplanted mice, primed donor-reactive Th1 and Th2 were readily detectable in allograft recipients on day 30 following inductive anti-CD4 therapy. These data, in conjunction with the day 60 posttransplant data in Figure 1, indicate that anti-CD4 cells undergo a transient priming phase as they repopulate the periphery.

We also asked whether T-cell priming was evident early posttransplantation in primary allograft recipients (Figure 4A). Low, albeit detectable Th1 and Th2 priming was observed in both groups at day 10 posttransplant. Thus, T-cell priming was short-lived in primary allograft recipients treated with anti-CD40L mAb. This *in vivo* finding is in keeping with the *in vitro* observation of Blair et al. (12) who reported that cross-linking CD40L results in cytokine production followed by apoptosis of activated T cells.

### *Assessing the effects of anti-CD40L mAb on donor-reactive T cells*

It has been reported that memory, but not naïve T cells are resistant to the suppressive effects of anti-CD40L mAb (13). Therefore, if anti-CD40L cells are maintained in a naïve state, then rejection following transfer of these cells into secondary SCID recipients should be ablated by anti-CD40L mAb treatment of these SCID mice. To this end, SCID allograft recipients were infused with naïve or anti-CD40L cells and treated with anti-CD40L or control hIgG. As shown in Figure 5A, treatment of SCID allograft recipients of either naïve or anti-CD40L cells with anti-CD40L mAb, but not hIgG, prevented graft rejection. Prolonged graft survival by anti-CD40L treatment correlated with reduced T-cell priming relative to that observed in SCID recipients of naïve or anti-CD40L cells that were treated with hIgG (Figure 5B). These observations further support the notion that anti-CD40L cells are maintained in a naïve phenotype. As a control for memory cell resistance to anti-CD40L therapy, SCID cardiac allograft recipients were infused with cells obtained from C57BL/6 mice that had rejected BALB/c skin grafts 70 days previously. As expected (13), anti-CD40L was ineffective at preventing allograft rejection (Figure 5A) or inhibiting primed Th1 responses (Figure 5B) mediated by memory cells. However, an interesting observation was made when SCID recipients of activated effector cells were treated with anti-CD40L. Effector cells were obtained from unmodified cardiac allograft recipients that were in the process of rejecting their allografts on day 8 posttransplant. Unlike in recipients of memory cells, anti-CD40L mAb prevented rejection in recipients of effector cells and suppressed primed donor-reactive T-cell responses (Figure 5A and B). Thus, activated effector cells were targeted by anti-CD40L mAb, while memory cells were not. An alternate explanation for this observation is that effector cells, but not memory cells, undergo activation-induced cell death upon adoptive transfer into SCID allograft recipients as opposed to being controlled by anti-CD40L mAb. While we cannot definitively rule out this possibility, it should be noted that perturbation of CD40L on activated T cells has been reported to induce apoptosis (12) in keeping with the idea that anti-CD40L mAb contributes to the elimination of effector cells.

## Discussion

Multiple effector mechanisms of allograft rejection exist (reviewed in References 14–17). For example, unmodified cardiac allograft rejection is characterized by a prominent CD8+ T-cell response (1,10). These CD8+ cells produce IFNγ and little IL-4 (9,10,18), which suggested that Th1 were critical to the rejection process and that inducing Th2 may promote graft acceptance (reviewed in Reference 14). However, depleting CD8+ cells induces Th2 that recruit granulocytes resulting in a nonclassical form of rejection (10,19,20). Similar observations have been made in liver transplant patients undergoing acute rejection (21). Further, complete elimination of IFNγ results in IL-4 production by CD8+ cells and a granulocytic infiltrate that is not controlled by depleting CD4+ cells or anti-CD40L therapy (2). Thus, while IFNγ may contribute to rejection, this cytokine also appears to be required for allograft acceptance under certain conditions. These observations point to a delicate balance between allograft rejection and acceptance.

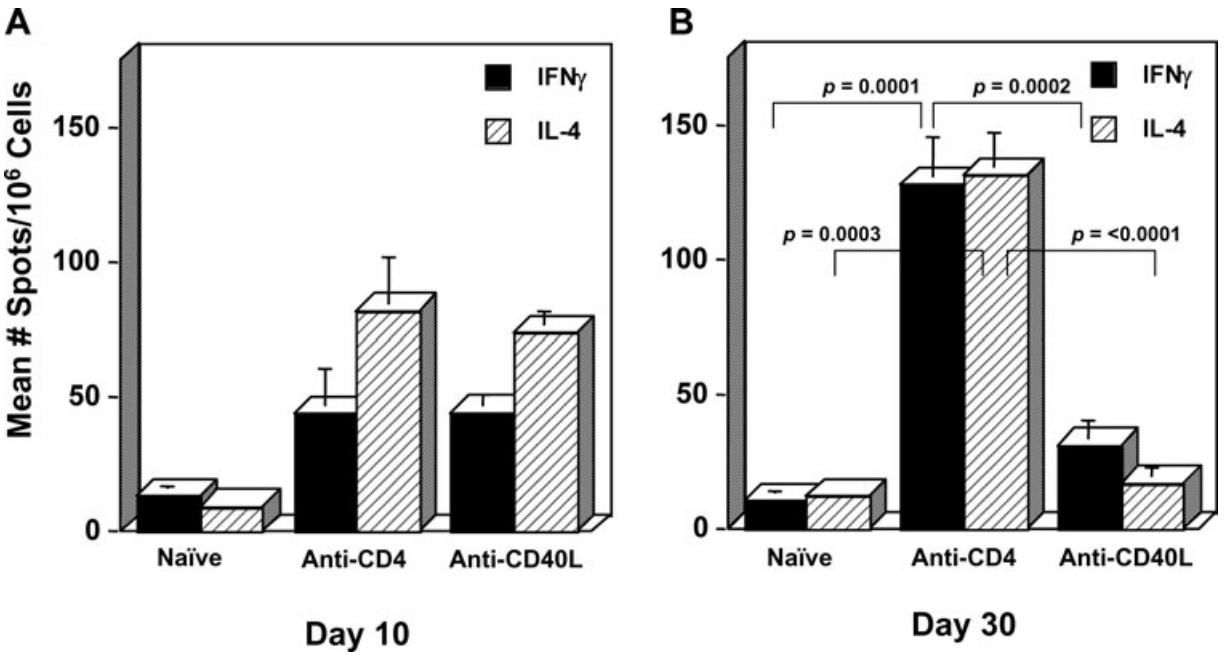


**Figure 4: Donor-reactive T-cell priming following inductive anti-CD4 and anti-CD40L mAb therapy.** Primary allograft recipients were given inductive anti-CD4 or anti-CD40L mAb therapy. On day 10 (Panel A) or day 30 (Panel B) posttransplant, splenocytes were harvested and assessed for primed donor-reactive Th1 and Th2 responses by ELISPOT. Data are presented as the mean + SEM For day 10 posttransplant, data represent responses for 6 anti-CD4 mAb and 9 anti-CD40L mAb-treated allograft recipients. For day 30 posttransplant, data represent responses for 10 anti-CD4 mAb and 7 anti-CD40L mAb-treated allograft recipients. Naïve splenocytes obtained from nontransplanted mice (n = 4) served as negative controls for T-cell priming.

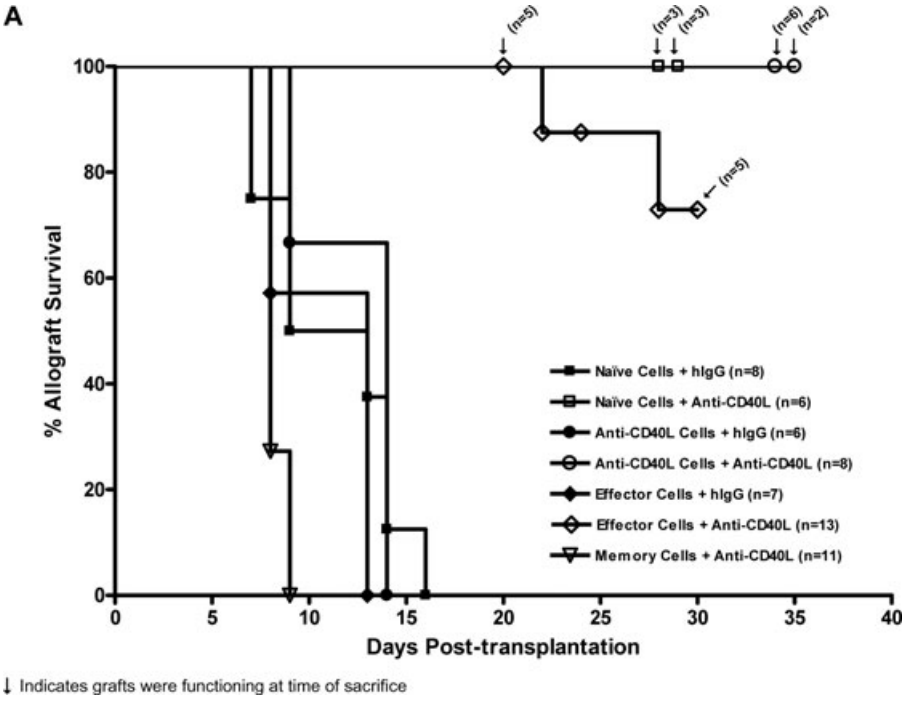


**Figure 5: Rejection transferred by anti-CD40L cells is inhibited by anti-CD40L mAb therapy.** SCID allograft recipients were infused with (1) naïve cells, (2) anti-CD40L cells, (3) effector cells obtained from unmodified allograft recipients that were actively rejecting their grafts on day 8 posttransplant or (4) memory cells obtained from mice sensitized with skin allografts 70 days previously. SCID recipients were given inductive anti-CD40L therapy or control hIgG. Panel A depicts allograft survival. The number of individual transplants in each group is given in parentheses. Panel B depicts primed donor-reactive Th1 and Th2 responses (ELISPOT, mean + SEM) mounted by splenocytes obtained from these SCID recipients. 'Too few cells' indicates that adequate cell numbers were not recovered for the ELISPOT assay.

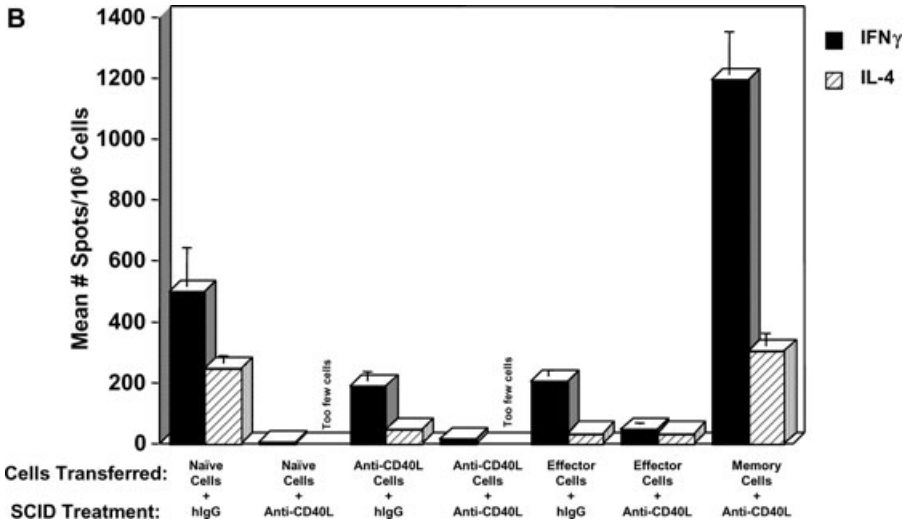


**Figure 5: (Continued)**

Since there are multiple effector mechanisms of rejection (reviewed in References 14–17), it comes as no surprise that sustained allograft 'tolerance' has been difficult to achieve (22,23). While various therapies promote allograft 'acceptance' (reviewed in References 24–27), the mechanisms by which allograft acceptance is maintained likely varies depending on the acceptance-inducing regimen. Similarly, the vigor of allograft maintenance mechanisms and their susceptibility to reversal may also vary depending upon how allograft acceptance is induced. This study explored elements of this hypothesis by comparing mechanisms of allograft maintenance resulting from two inductive mAb therapies that primarily target CD4+ T cells: the anti-CD4 mAb GK1.5 and the anti-CD40L mAb MR1.

The anti-CD4 mAb GK1.5 (rat IgG2b) transiently depletes peripheral CD4+ T cells *in vivo* and is a cytolytic mAb that fixes complement (C′) (11). When allograft recipients are given our inductive anti-CD4 regimen, CD4+ cells begin to repopulate the periphery between 3 and 4 weeks following initial depletion (1,4,7). Transient depletion of CD4+ cells does not appear to require the classical complement pathway, in that treatment of C1q-deficient mice (28) with anti-CD4 mAb is as effective at depleting CD4+ cells as it is in WT mice (Csencsits et al. Am J Transplant, in press). Similarly, Ghobrial et al. (11) reported that GK1.5 was effective at depleting CD4+ T cells in C5-deficient mice.

Regardless of how CD4+ cells are initially depleted by anti-CD4 mAb, their return to the periphery in allograft bearing mice is associated with donor-reactive T-cell priming (Figure 4). Hence, the immune status of anti-CD4 cells is modified relative to that of naïve cells. It is of interest that both Th1 and Th2 responses were induced since Th2 are not readily detectable during unmodified acute rejection (Figure 1) (9). Similarly, Bass et al. (29) observed that when CD4+ cells repopulate the periphery following total lymphoid irradiation that their function is skewed toward Th2 and that Th1 function is reduced. Further, donor-reactive Th2 dominated the response when anti-CD4 cells were adoptively transferred into allograft-bearing SCID recipients (Figure 3A), and this Th2 response was associated with prolonged graft survival (Figure 2A). Since the protective effects of Th2 are questionable (reviewed in References 14–17), we neutralized IL-4 in SCID recipients to determine whether IL-4 was required for prolonged graft survival (Figure 3A). Neutralizing IL-4 inhibited Th2 emergence, verifying the biologic activity of this regimen. However, neutralizing IL-4 did not induce rejection, suggesting that IL-4 was not essential for allograft acceptance by anti-CD4 cells. A likely candidate cytokine for the suppressive activity of anti-CD4 cells is TGFβ. Anti-CD4 cells transfer CR to SCID allograft recipients (4) and TGFβ has been implicated in the progression of CR (reviewed in Reference 30). TGFβ has numerous immunosuppressive activities that are beneficial in inflammation and transplantation (reviewed in References 31 and 32). Further, TGFβ induces expression of Foxp3 and can convert Foxp3- effector cells into Foxp3+ Treg (reviewed in References 32 and 33). Indeed, we observed that intragraft Foxp3 expression was greater in SCID recipients of anti-CD4 relative to recipients of anti-CD40L cells at the termination of each experiment (data not shown), which further suggests a role for TGFβ. Finally, it should be noted that TGFβ is expressed within long-term allografts in recipients that receive anti-CD4, but not anti-CD40L mAb therapy (5). Hence, future studies will be aimed at assessing the role of TGFβ in promoting allograft survival in this setting.

The anti-CD40L mAb MR1 (hamster IgG3) was originally described for its ability to inhibit B-cell activation by CD4+ cells (34). CD40L is expressed on activated, but not resting CD4+ T cells (reviewed in References 35–38). CD40 has a wide tissue distribution and the consequences of stimulating CD40 vary depending on the cell (reviewed

in References 36, 38–40). Perhaps the most immunologically important CD40-CD40L interactions occur between CD40 expressing B cells or other antigen-presenting cells (APC) and CD40L expressing CD4+ T cells (35,41). CD40 stimulation on B cells promotes proliferation, induces Ig isotype switching and enhances APC function by upregulating the costimulatory molecules CD80/86. CD40 stimulation of macrophages and dendritic cells augments their APC function through upregulating CD80/86 and stimulating IL-12 production. Hence, CD40 stimulation confers APC functions that are critical in driving T-cell responses.

The anti-CD40L mAb MR1 is believed to interfere with CD40-CD40L interactions in what is often referred to as 'costimulatory blockade'. This infers a physical disruption of CD40-CD40L interactions. However, reports suggest that cross-linking CD40L may have direct effects on T cells. For example, we have reported that CD40−/− BALB/c mice reject CD40−/− C57BL/6 cardiac allografts (42). Interestingly, anti-CD40L therapy prolongs allograft survival in this setting, which is completely devoid of CD40. Blotta et al. (43) reported that cross-linking CD40L on T cells results in increased production of IL-4 and IL-10, suggesting a role for CD40L in immune deviation. Similar observations were reported by van Essen et al. (44), who employed soluble CD40 and CD40−/− mice to demonstrate that CD40L stimulation was required for isotype switching and germinal center formation. Further, Blair et al. (12) reported that cross-linking CD40L results in the release of IL-10, IFN$\gamma$ and TNF$\alpha$, and subsequent apoptosis of the T cells. Hence, CD40L functions at levels beyond simple perturbation of CD40.

Our observation that anti-CD40L mAb ablates rejection mediated by activated effector cells (Figure 5) supports this notion. One would assume that the initial CD40-CD40L interactions required for effector cell generation would have occurred and that subsequent costimulatory blockade would be without consequence. However, the kinetics, level and duration of CD40L expression by effector cells may make these cells susceptible to removal and/or silencing by anti-CD40L mAb. Upon activation, naïve T cells rapidly upregulate CD40L at both the mRNA and surface protein levels (45–48). CD40L expression is enhanced and prolonged on Th1, but not Th2 (49). Indeed, the effector cells used in this study were generated from unmodified allograft recipients and exhibit a polarized Th1 phenotype (Figure 1) (9) that may facilitate removal by anti-CD40L mAb *in vivo*. This notion is supported by reports that C′ is required for optimal effectiveness of anti-CD40L mAb *in vivo* (50,51).

Treg play an important role in allograft acceptance and maintenance (reviewed in References 25, 52 and 53) and it is likely that adaptive Treg are generated by both anti-CD4 and anti-CD40L therapies. Indeed, quiescent precursor donor-reactive cells are present in long-term allograft bearing mice that received either of these inductive regimens (Figure 1) (3,7), supporting a role for Treg suppression of effector cell emergence. However, Treg are heterogeneous with respect to phenotype, function and trafficking behavior (reviewed in References 54–58). Hence, an in-depth assessment of Treg and their persistence in anti-CD4 versus anti-CD40L cells is warranted.

In summary, this study documents differences in the functional capacities of donor-reactive cells obtained from mice bearing accepted allografts following inductive therapy with anti-CD4 versus anti-CD40L mAb. These distinct populations of donor-reactive cells exhibit differential capacities to lose or reverse their allograft accepting behavior and subsequently mediate acute rejection. Thus, an assessment of the vigor and persistence of allograft maintenance factors would have utility in formulating inductive therapies that yield enduring allograft acceptance.

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