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Pilot Study of Delayed ICOS/ICOS-L Blockade With αCD40 to Modulate Pathogenic Alloimmunity in a Primate Cardiac Allograft Model

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Background. Inducible costimulator (ICOS) is rapidly upregulated with T-cell stimulation and may represent an escape pathway for T-cell costimulation in the setting of CD40/CD154 costimulation blockade. Induction treatment exhibited no efficacy in a primate renal allograft model, but rodent transplant models suggest that the addition of delayed ICOS/ICOS-L blockade may prolong allograft survival and prevent chronic rejection. Here, we ask whether ICOS-Ig treatment, timed to anticipate ICOS upregulation, prolongs NHP cardiac allograft survival or attenuates pathogenic alloimmunity. **Methods.** Cynomolgus monkey heterotopic cardiac allograft recipients were treated with αCD40 (2C10R4, d0-90) either alone or with the addition of delayed ICOS-Ig (d63-110). **Results.** Median allograft survival was similar between ICOS-Ig + αCD40 (120 days, 120-125 days) and αCD40 (124 days, 89-178 days) treated animals, and delayed ICOS-Ig treatment did not prevent allograft rejection in animals with complete CD40 receptor coverage. Although $CD4^+$ T_{EM} cells were decreased in peripheral blood (115 ± 24) and mLNs (49 ± 1.9%) during ICOS-Ig treatment compared with monotherapy (214 ± 27%, $P = 0.01$; 72 ± 9.9%, $P = 0.01$, respectively), acute and chronic rejection scores and kinetics of alloAb elaboration were similar between groups. **Conclusions.** Delayed ICOS-Ig treatment with the reagent tested is probably ineffective in modulating pathogenic primate alloimmunity in this model.

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Costimulation pathway blockade with belatacept is an established alternative therapeutic strategy in clinical transplantation, allowing safe reduction in exposure to nephrotoxic calcineurin inhibitors. Blockade of the CD40/CD154 pathway in preclinical models via αCD40 treatment has demonstrated prolonged allograft survival and delayed production of donor specific antibodies in nonhuman primate (NHP) renal,[1] islet cell,[2,3] and cardiac[4] models. However, αCD40 (or αCD154[4-7]) monotherapy has been associated with chronic rejection, and a minority of allografts even reject during treatment, identifying existence of rejection mechanisms that are resistant to CD40/CD154-directed costimulation pathway blockade.

Based on prior work by us and others in preclinical models and in man, we hypothesized that immune injury associated with CD40/CD154-directed costimulation blockade is mediated by one of several known alternative costimulation pathways. One pathway of particular interest is the inducible

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T.Z., X.C., A.M.A., and R.N.P. participated in research design. K.A.R. designed and developed the drugs used in this study. N.A.O. wrote the article, and A.M.A. and R.N.P. critically edited the article. N.A.O., T.Z., G.B., X.C., A.H., W.S., S.D., N.K., W.H., C.L., and A.C. performed all experiments and data analysis.

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costimulator (ICOS)/ICOS-ligand (ICOS-L) pathway. ICOS is rapidly induced after T-cell receptor cross-linking. ICOS is expressed on activated $CD4^+$ and $CD8^+$ T cells and effector $CD4^+$ T follicular helper (Tfh) cells within the germinal center,[8] enhances T-cell responses to foreign antigens, and ICOS expression is increased in acutely[9-11] and chronically[5,10,12] rejected allografts. One benefit of ICOS blockade over other costimulation targets may be the pathway's relative specificity for upregulation on activated, pathogenic T cells.

In support of this paradigm, ICOS-Ig monotherapy moderately prolonged allograft survival in mice,[13,14] and the combination blockade of the CD40/CD154 pathway with ICOS/ICOS-L pathway demonstrated significantly prolonged survival in rodent transplant models.[10,12,15,16] Murine transplant and autoimmune models have further demonstrated benefit with delayed treatment of ICOS-Ig on both allograft survival, disease pathology, and suppression of $CD4^+$ and $CD8^+$ effector memory cell populations.[14,17-20] In contrast, the first NHP preclinical trial of ICOS-Ig, administered for one month (until day 28),[21] in combination with ongoing CD28/B7 blockade, did not prolong kidney allograft survival. Based on our observation that ICOS expression generally becomes detectable in cynomolgus monkey cardiac allografts about 2 months after transplant, here we evaluate whether delayed ICOS-Ig treatment, timed to anticipate ICOS upregulation in the graft, prolongs allograft survival or attenuates pathogenic alloimmunity in that model.

METHODS

Animal Model

Captive-bred cynomolgus monkeys (*Macaca fascicularis*) of Chinese and Indonesian origin were used. Males weighing 5.0 to 9.5 kg received ABO blood type-compatible hearts from donors that were selected based on stimulation index >5 in mixed lymphocyte reaction to confirm major histocompatibility complex (MHC) class II mismatch. MHC class I mismatch was confirmed retrospectively by detection of antidonor class I (T cell) alloantibodies, or in exceptional cases where antibody was not detected, by genomic DNA Illumina sequencing.[4] All protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and were conducted in compliance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Surgical Procedures and Allograft Monitoring

Heterotopic intra-abdominal cardiac transplantation was performed as described previously.[22] Cardiac biopsies were obtained by protocol on postoperative days (d) 14, 42, 90, and 150; biopsies were occasionally omitted or delayed due to recipient anemia or weight loss. Mesenteric lymph nodes (mLNs) were usually sampled at the time of biopsy.

Core temperature and graft heart rate, systolic blood pressure, and diastolic blood pressure were assessed at least once daily using intra-abdominal telemetry (D70-PCTP or L11, Data Sciences International, St. Paul, MN) until time of graft explant for failure. Allograft failure was defined by one or more of the following criteria for 2 consecutive days: decline in heart rate greater than 20% below baseline or less than 120 beats per minute, pulse pressure less than 30 mm Hg, or nonpalpable graft contraction.

Immunosuppression

All animals (n = 9) received αCD40 treatment using 2C10R4, a mouse-rhesus IgG4κ anti-CD40 antibody,[2] intravenously (IV) at 30 mg/kg on d0, d3, d7, and d14; 10 mg/kg on d21, d28, d35, and d42; and 20 mg/kg on d56 and d84 (total 200 mg/kg). Six animals received αCD40 alone, as previously reported,[4] and 3 animals additionally received ICOS-Ig, a rhesus recombinant Ig-fusion protein, in combination with αCD40 beginning on d63 (Figure 1). Both 2C10R4 and ICOS-Ig were obtained from NIH Nonhuman Primate Reagent Resource (Boston, MA).

The ICOS-Ig protein, comprising the 141 extracellular amino acids of rhesus ICOS (Genbank accession NM_001266989.1) and the hinge, CH2 and CH3 domains of rhesus IgG1, was expressed using the GPExÒ technology (Catalent Pharma Solutions, Madison, WI). Briefly, DNA encoding the Ig fusion protein was inserted into an expression vector and Chinese hamster ovary (CHO) cells were transduced with this vector using replication incompetent retrovirus. A pool of transduced CHO cells was grown in serum free medium and ICOS-Ig purified using conventional protein A affinity chromatography. The final product was formulated in PBS, pH 7.0.

ICOS-Ig dosing was progressively adjusted in the 3 animals receiving combination treatment based on interval graft fates and in response to pharmacokinetic data showing low trough levels (Figure 2). The first animal, DV8T, received 5 mg/kg weekly from d63 to d90 (25 mg/kg total). The second animal, DM4XX, received 10 mg/kg on d63, 70; 5 mg/kg on d77, d84, and d90; and 10 mg/kg on d98 and d105 (55 mg/kg). The third animal, DW4P, received 5 mg/kg twice weekly from d63 until d109 (70 mg/kg).

Routine monitoring for CMV was not performed, and antiviral medications were not used.

Drug Trough Levels and CD40 Receptor Coverage

Serum trough αCD40 levels were checked retrospectively by enzyme-linked immunosorbent assay and peripheral blood CD40 receptor saturation was measured by flow cytometry, as described previously.[4]

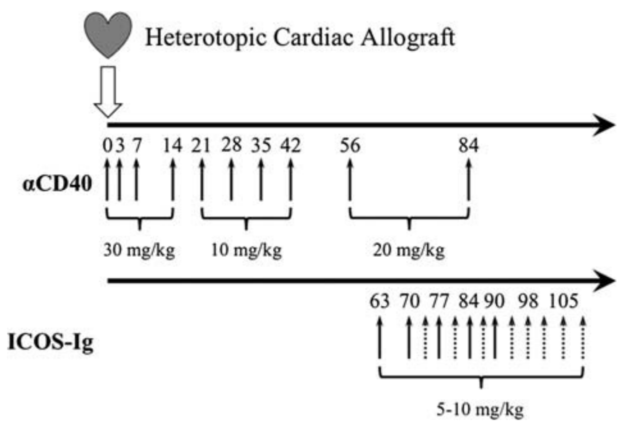

FIGURE 1. αCD40 and ICOS-Ig dosing schematic. Heterotopic cardiac allograft transplantation in NHP was performed on d0. All animals received αCD40 from d0 to d84 (total of 200 mg/kg). ICOS-Ig (n = 3) was given in a delayed fashion from d63 to d109, and dosing was progressively escalated in each animal. Solid black arrows indicate doses that each animal within the treatment group received. Dotted black arrows indicate additional ICOS-IG doses given during the dose escalation.

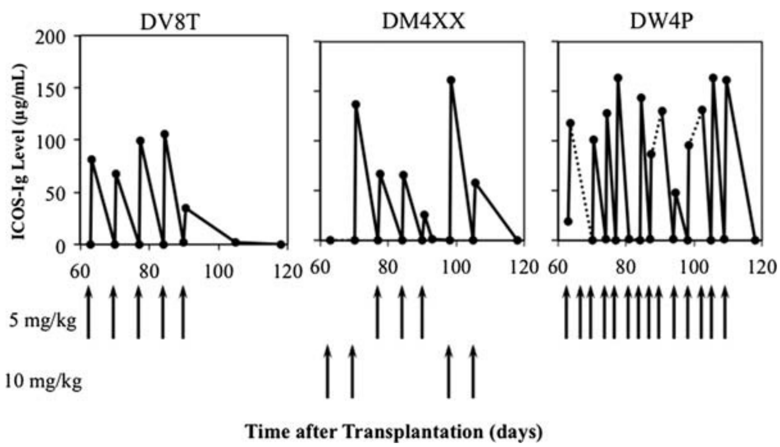

FIGURE 2. Serum ICOS-Ig peak and trough levels. ICOS-Ig treatment was started on d63 in each animal. Solid arrows represent ICOS-Ig doses corresponding to either 5 mg/kg or 10 mg/kg. ICOS-Ig treatment was progressively escalated in each animal from a total of 25 mg/kg (DV8T) to 55 mg/kg (DM4XX) to 70 mg/kg (DW4P). Trough levels were drawn before ICOS-Ig administration intravenously. Peak serum levels were sampled approximately 30 minutes after ICOS-Ig administration. Dotted lines represent time points when either a peak or trough level was not drawn between data points. Serum trough levels (DV8T: 0.9 ± 0.4, DM4XX: 0.3 ± 0.03, DW4P: 0.9 ± 0.1; one way ANOVA rank $P = 0.03$) and serum peak levels (DV8T: 78 ± 11, DM4XX: 86 ± 19, DW4P: 123 ± 10; $P = 0.08$) increased with dose escalation. ANOVA, analysis of variance.

Serum peak and trough ICOS-Ig levels were also analyzed retrospectively by enzyme-linked immunosorbent assay. Maxisorp 96- well plates were coated with ICOS-L (ICOSLG Sino Biological Inc, Beijing, China) at 1 µg/mL in PBS and were incubated at 4°C overnight. Plates were washed with PBS-Tween 20 0.05% (Sigma-Aldrich, St. Louis, MO), blocked with PBS containing 3% bovine serum albumin (Sigma-Aldrich), and serum samples were incubated followed by goat anti-human IgG secondary antibody and HRP (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Due to its inducible nature, reliable flow cytometry methods to detect ICOS receptor coverage have not been developed.

Histological Grading of Acute and Chronic Rejection

Biopsy and explant cardiac tissue specimens were fixed with 10% formalin, processed for paraffin embedding, and stained with hematoxylin and eosin, as described previously.[23] Acute cellular myocardial infiltration was graded using the 2005 International Society for Heart and Lung Transplantation (ISHLT) revised criteria for cardiac allograft rejection.[24] Cardiac allograft vasculopathy (CAV) severity with hematoxylin and eosin staining was scored by 3 independent evaluators (N.A.O. or L.B., T.Z., and R.N.P.), who were blinded with respect to treatment group.[23]

Lymphocyte Detection and Memory T- and B-Cell Analysis

Routine blood collection was performed per protocol to monitor cell blood counts, B and T lymphocyte subsets, blood chemistry, and antidonor alloantibody (alloAb) production, as previously described.[4]

Peripheral blood was stained for cell surface markers CD3 (clone SP34-2), CD4 PerCP (clone L200), CD8 APC (clone SK1), CD28 (clone 28.2), CD62L (clone SK11), CD45RA (clone 5H9), and CD95 (clone DX2). Samples were collected using FACSCalibur cytometer (BD Biosciences), and data analysis was conducted using FlowJo 10.1r5 (Treestar, Ashland, OR) software.

Lymph nodes were blocked with pure human IgG (Jackson ImmunoResearch), stained with e780 fixable viability dye (eBioscience), and then stained with the T-cell surface markers described above and CD20 (clone L27), CD27 (clone MT271), CD38 (clone OKT10), IgD (goatαhuman; Southern Biotech, Birmingham, AL), and IgM (W6/32, NIH Nonhuman Primate Reagent Resource). $CD3^+$ T memory cell phenotypes were defined as naive (T_N) $CD45RA^+CD95^-CD28^+CD62L^+$, central memory ($T_{CM}$) $CD45RA^-CD95^+CD28^+CD62L^+$, or effector memory ($T_{EM}$) $CD45RA^{+/-}CD95^{+/-}CD28^-CD62L^-$.[25] $CD20^+$ B memory cell phenotypes were defined as naive $IgD^+CD27^-CD38^+IgM^{intermediate}$, isotype-switched B memory cell $CD27^+IgD^-$, and nonswitched B memory $CD27^+IgD^+$.[26,27]

alloAb Detection

alloAb was measured retrospectively by flow cytometry using archived frozen donor splenocytes and recipient sera. AlloAb elaboration was defined as consistently detected IgM-positive or IgG-positive donor $CD3^+CD20^-$ T cells greater than 10% relative to donor serum before transplant, as described previously.[23]

Statistical Analysis

Survival analysis was performed by the Kaplan-Meier method and compared using the log-rank test. Continuous variables were expressed as the mean ± standard error of the mean (SEM) or median and interquartile ranges, and these were compared using either the 2-tailed Mann-Whitney *U* test to compare 2 groups or 1-way analysis of variance on ranks for comparing 3 or more groups. Nominal variables were compared using a contingency table and the Fisher exact test. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with GraphPad InStat (version 3.01; GraphPad Software, San Diego, CA).

RESULTS

ICOS-Ig Dose Escalation

For the αCD40 + ICOS-Ig–treated animals, serum ICOS-Ig levels were analyzed retrospectively to determine serum drug levels before and 30 minutes after ICOS-Ig dosing. Although peak levels (mean ± SEM 78 ± 11 µg/mL) achieved

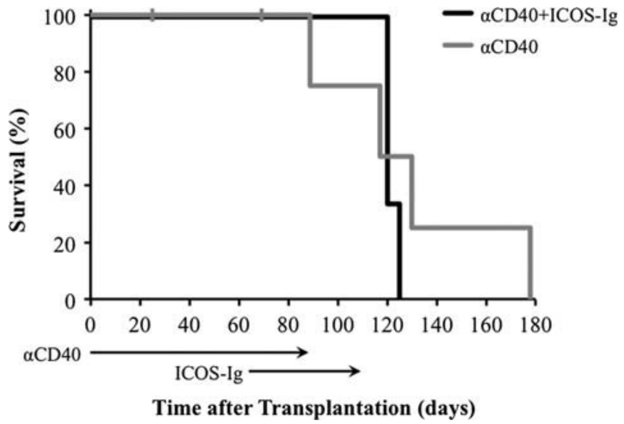

FIGURE 3. Kaplan-Meir allograft survival curve. Graft rejection was defined by one or more of the following criteria for 2 consecutive days: decline in heart rate >20% below baseline or <120 beats per minute, pulse pressure <30 mm Hg, or nonpalpable graft contraction. MST is similar between the 2 treatment groups. (+) represent allografts removed without evidence of rejection, and are censored from survival analyses. αCD40 (d0-d84) and ICOS-Ig (d63-d109) treatment time periods are denoted with arrows. αCD40 monotherapy control group was previously reported.[4]

our expected target (>50 μg/mL), ICOS-Ig serum trough levels in the first animal, DV8T (0.9 ± 0.4 μg/mL) were lower than anticipated. Given this observation, ICOS-Ig dosing was escalated for subsequent animals (Figure 2). Although peak levels increased proportionate to the administered dose after each infusion, trough levels remained lower than expected (DW4P: 0.9 ± 0.1 μg/mL; P = 0.03). These results suggest that the half-life of this molecule was shorter than expected.

Allograft Survival

Median survival time (MST) of the combination αCD40 + ICOS-Ig–treated animals (n = 3, MST 120 days; range, 120-125) was similar to αCD40 monotherapy (n = 6, 124, 89-178; P = 0.6, Figure 3). Specifically, ICOS-Ig treatment did not have a measureable effect on allograft survival. Two animals in the 2C10R4 treatment group (FB9N and DV36) had functional, beating allografts removed for health reasons (Table 1), as reported previously.[4]

Before αCD40 re-dosing, CD40 receptor coverage was regularly assessed. CD40 receptor coverage was nearly 100% for at least 35 days after the final αCD40 treatment, and similar between αCD40 alone (98.8 ± 0.8% on d118) and αCD40 + ICOS-Ig (99.0 ± 1.0% on d118, P = 0.8; Figure 4). One of 4 evaluable animals in the αCD40-treatment group (FA53) and 2 of 3 animals in the combination group (DM4XX and DW4P) exhibited graft rejection despite 100% receptor coverage (Table 1). These data demonstrate existence of graft injury mechanisms escaping control by CD40-directed treatment, alone or with ICOS-Ig, despite persistent, prevalent CD40 receptor coverage.

TABLE 1.

Individual animal survival and receptor coverage at time of allograft explant

Treatment groups	Animal ID	Allograft survival (d)	Receptor coverage (%)[a]
αCD40	FB9N	>25	100
	DV36	>69	100
	FC9J	89	88
	FA53	117	100
	DV2R	130	48
	FB6E	178	0
αCD40 + ICOS-Ig	DW4P	120	100
	DM4XX	120	99
	DV8T	125	17

Animals with adequate CD40 receptor coverage at the time of explant (FA53, DW4P, DM4XX) may represent costimulation blockade resistant rejection. Shaded boxes represent animals with functional, beating allografts removed for other health reasons.

[a] CD40 receptor coverage at the time of allograft explant.

Acute and Chronic Rejection Acute Cellular Infiltration

Protocol biopsies and explanted cardiac allografts were evaluated for evidence of acute and chronic rejection, and time points were grouped by αCD40 alone (d0-60 for both groups), during treatment with ICOS-Ig (d61-110), or after ICOS-Ig treatment (d111-180). Acute cellular infiltration graded by ISHLT score[24] was similar between αCD40 monotherapy and αCD40 + ICOS-Ig–treated animals at each time point (Figure 5A). Chronic allograft vasculopathy (CAV) severity was also similar at each time point between the 2 groups (Figure 5B). These data demonstrate participation of both acute (cellular) and chronic (as CAV) rejection mechanisms that escape control by CD40-directed treatment and are not modulated by ICOS-Ig as administered.

alloAb Production

Because others have shown that αICOS inhibits alloAb isotype switching,[16] we evaluated IgM and IgG alloAb production after transplantation. IgM alloAb elaboration from d0 to d60 was significantly higher in the αCD40 + ICOS-Ig (7 of 22 time points with IgM alloAb >10%; P = 0.0004) than the αCD40 monotherapy treatment group (0 of 38) because one animal (DV8T) within the αCD40 + ICOS-Ig group developed IgM antidonor alloAb on d7 (d7-118 mean ± SEM: 18 ± 1.4%; Figure 6).

During ICOS treatment (d61-110) and after ICOS treatment (d111-180), IgM and IgG alloAb detection was similar between the 2 groups and elaboration occurred just prior or at the time of allograft rejection. Together, these data demonstrate participation of humoral (as anti-donor alloantibody) rejection mechanisms that escape control by CD40-directed

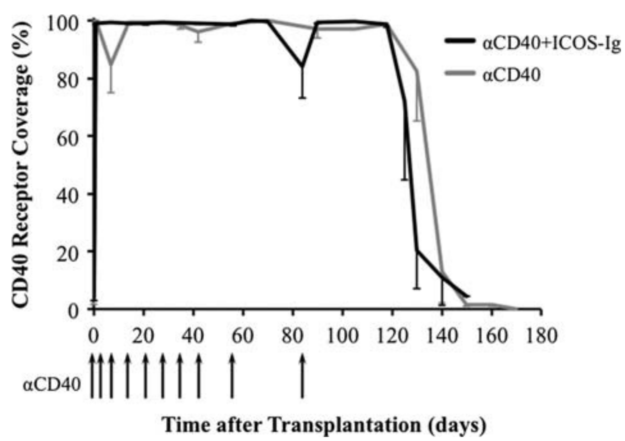

FIGURE 4. Mean peripheral blood CD40 receptor coverage (%) detected by flow cytometry. SEM is demonstrated in error bars. Solid arrows represent αCD40 dosing. Receptor coverage was nearly 100% through d118, after which point coverage becomes somewhat variable across animals.

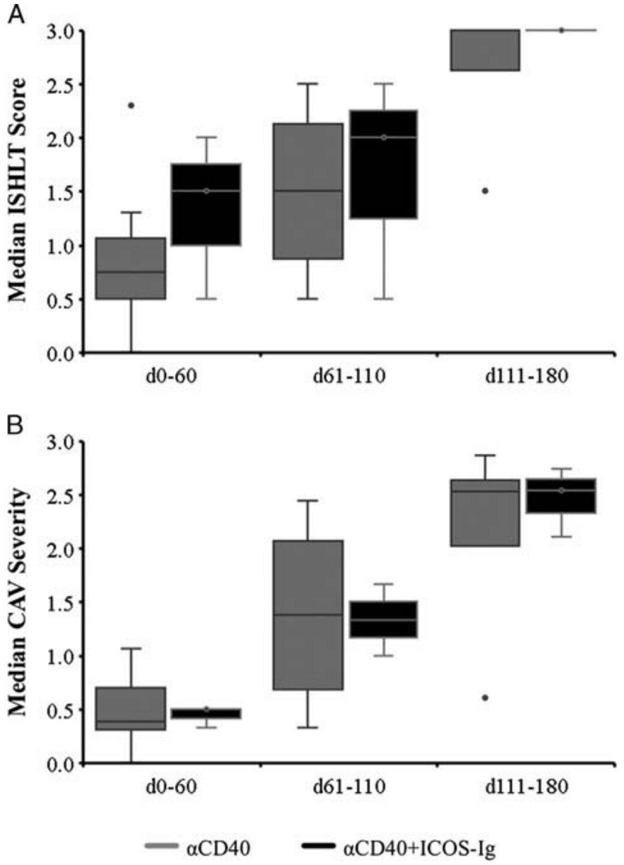

FIGURE 5. Acute and chronic rejection histology grading of protocol biopsies and explanted allografts shown as box and whisker plots. From d0 to d60, both experimental groups received only αCD40. From d61 to d100, the combination group (black) received αCD40 + ICOS-Ig. A, Median ISHLT acute cellular infiltration score was similar between the 2 groups. B, Median CAV, a measure of chronic rejection, severity scores were also similar between both groups. Outlier data points are shown as dots.

treatment and suggest that the addition of delayed ICOS-Ig treatment, as administered, was unable to prevent alloAb isotype switching.

mLN Memory B-Cell Prevalence

Given the failure of ICOS-Ig treatment to prevent alloAb isotype switch, we evaluated the prevalence of memory B cells in mLN after ICOS-Ig treatment. The proportion of naive B cells detected by flow cytometry within lymph node was similar between the 2 groups (Figure 7 and **SDC Figure S1,** http://links.lww.com/TXD/A57). The proportion of isotype-switch memory B cells significantly increased in the αCD40 + ICOS-Ig–treated animals in mLN from d90 and d120 (42 ± 9.1%) compared with αCD40-treated animals (5.8 ± 1.1%; P = 0.04), and, reciprocally, nonisotype switched memory B cells trended down in the combination group (2.0 ± 0.6% vs. 8.0 ± 2.3%; P = 0.07). Together with the alloAb data, delayed ICOS-Ig treatment did not prevent development of isotype switched memory B cells within the regional lymph node and subsequent humoral rejection.

Memory T Cell Kinetics in Blood and Lymph Nodes

Several murine models have demonstrated a decrease in T_{EM} cells with disruption of the ICOS/ICOS-L pathway,[14,28] which prompted us to study the kinetics of T_N, T_{CM}, and T_{EM} cells in peripheral blood. From d70 to d130, time points during and after ICOS-Ig treatment, $CD4^+$ T_{EM} cells were significantly lower in the αCD40 + ICOS-Ig animals (115 ± 24 cells/μL blood) compared with αCD40–treated animals (214 ± 27; P = 0.01), and a similar trend was observed in the $CD8^+$ T_{EM} cells (527 ± 60 vs. 736 ± 120; Figure 8 and **SDC Figure S2,** http://links.lww.com/TXD/A58). $CD8^+$ T_{CM} cells were significantly higher in the αCD40 + ICOS-Ig group (102 ± 8) than in αCD40 monotherapy (65 ± 5; P = 0.0004); however, this cell population was also elevated from d0 to d65 in the combination group (83 ± 7) than in the αCD40 monotherapy group (50 ± 5; P = 0.0001).

Similarly, $CD4^+$ T_{EM} cell comprised a significantly lower proportion of $CD4^+$ cells in mLN in association with ongoing ICOS-Ig treatment (49 ± 1.9% at d90 and d120) compared to αCD40 monotherapy (72 ± 9.9%; P = 0.01, Figure 9 and **SDC Figure S3,** http://links.lww.com/TXD/A59). This relative decrease in the mLN $CD4^+$ T_{EM} cell population was accompanied by a relative increase in $CD4^+$ T_N proportion (45 ± 1.4 vs. 21 ± 6.3%; P = 0.002), whereas $CD4^+$ T_{CM} proportions remained stable and similar between the 2 groups. These results suggest that ICOS-Ig treatment significantly skews the $CD4^+$ T_{EM} cell trafficking in blood and secondary lymphatic compartments, as expected based on rodent studies.

DISCUSSION

ICOS was discovered as a CD28-homologous structure expressed on activated T cells. ICOS is absent on naive T cells but is rapidly upregulated on both $CD4^+$ and $CD8^+$ T cells after T cell receptor cross-linking. CD28 ligation augments ICOS expression, but is not necessarily required.[29,30] Its ligand, ICOS-L (B7RP-1, B7h, GL50),[9,31] is constitutively expressed on B cells and immature dendritic cells (DC). ICOS-L expression is induced on monocytes, macrophages, and other nonhematopoietic cells like fibroblasts and endothelial cells after inflammatory stimuli.[12,32,33]

The ICOS/ICOS-L pathway appears to play a nonredundant role in T-cell costimulation, proliferation, and expansion.[34,35] ICOS enhances T-cell response to an antigen by increasing cytokines such as IL-5, TNF-α, IFN-γ,[12,29,31,36] and Th2 cytokines IL-4[29,37] and IL-10.[9,12] ICOS also appears to play a role in chemokine receptors CCR1, CCR2, CCR5, CXCR3, CXCR4, and CXCR5 expression.[12,38,39] Together, these cytokines and chemokines are induced upon T-cell activation and play a role in allograft rejection. These considerations justify efforts to explore blockade of the ICOS/ICOS-L pathway to prevent alloimmune injury in transplantation.

Early ICOS blockade (αICOS or αICOS-L) in murine cardiac[13-15] or hepatic[40] transplant models found that early treatment, initiating on d0, was associated with a modest, though significant, increase in allograft survival compared to untreated controls; however, survival was similar between untreated controls and ICOS-Ig–treated NHP.[21] In autoimmune mouse models of EAE, asthma, and diabetes, early αICOS treatment during the antigen priming phase was found to increase the severity of disease, cell infiltration, and damaging cytokine expression.[17,18,41] When αICOS treatment was delayed and given after T-cell activation, clinical disease was abrogated at the clinical and cellular level.[17,18] Delayed blockade of the ICOS/ICOS-L pathway in mouse cardiac models significantly prolonged allograft survival and decreased cellular infiltration,

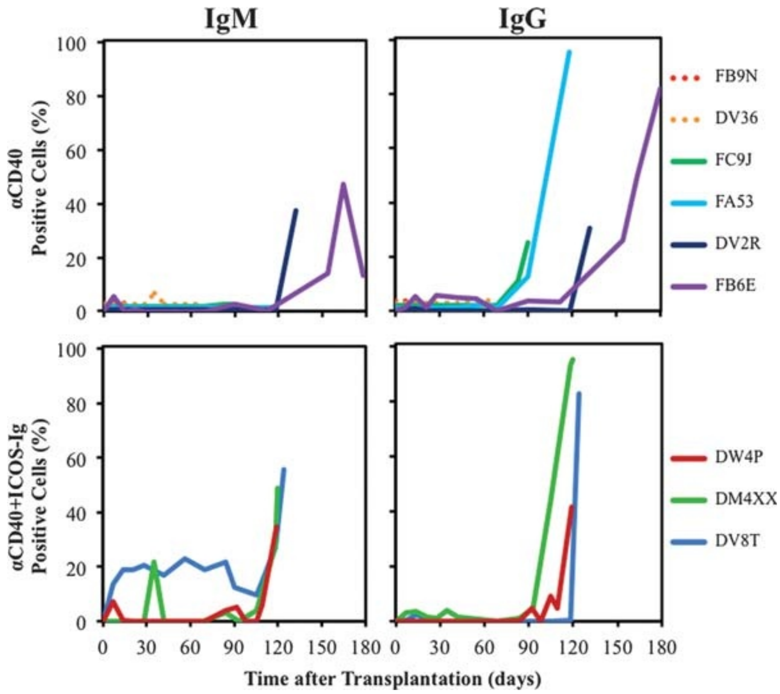

FIGURE 6. Anti-donor alloantibody production after transplantation. The last data point for each animal represents the time of allograft explant. IgG alloAb elaboration was attenuated in both groups until the time of allograft rejection.

vasculopathy severity, and IgG alloAb production.[14,19,20] Given these encouraging results, we chose to evaluate delayed ICOS-Ig treatment in a MHC mismatched cardiac allograft NHP model.

Efficacy appears to be most consistent when the ICOS/ICOS-L pathway is targeted in conjunction with CD40/154 pathway blockade[10,12,15] whereas heterogeneous results,[10,13,20,21,42] including deleterious effects with shorter graft survival,[14,43] have been reported when ICOS/ICOS-L targeting is combined with αCD28/B7 blockade. These considerations informed our current experimental design, and we chose to combine delayed ICOS-Ig with αCD40, rather than calcineurin inhibition or αCD154, based on several considerations. In our NHP model, αCD40 consistently prolongs allograft survival as a monotherapy during treatment, with reproducible detection of ICOS at around 60 days, followed by progressive chronic rejection leading to graft loss.[4] In contrast, either 5C8H1 or cyclosporine A are associated with failure of some grafts during treatment[4,5,23] and inconsistent kinetics of first ICOS detection by PCR and IHC (AA, unpublished observations). However, our resulting pilot data suggest that delayed ICOS-Ig, as applied here, does not significantly attenuate cardiac allograft rejection mechanisms associated with αCD40 costimulation blockade.

T cells expressing ICOS are phenotypically resting or T_{EM} cells,[9,28,31,39] and these $ICOS^+$ T memory cells can undergo rapid expansion independent of CD28/B7 or CD40/CD154 ligation.[44] As predicted from ICOS-deficient human and mice,[28,39] in this study ICOS-Ig treatment demonstrated a decrease of $CD4^+$ T_{EM} cells in peripheral blood and mLNs during treatment. In contrast to rodent studies,[14,15] the decrease of T_{EM} cells in our study did not affect allograft survival or acute or chronic rejection after transplantation, which may be due to intact cytotoxic $CD8^+$ T cell responses of the

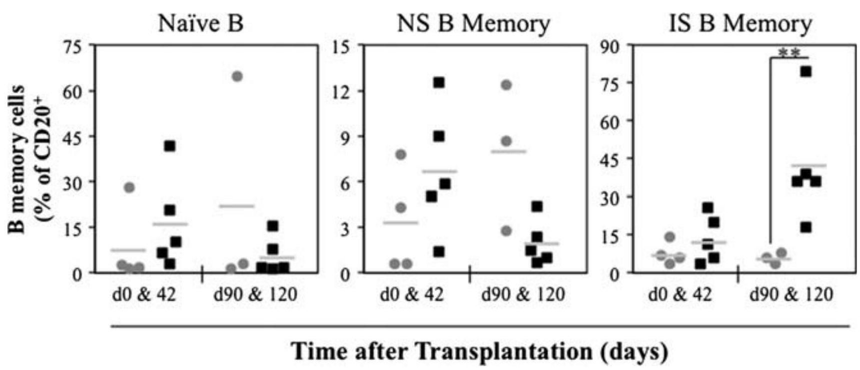

FIGURE 7. mLN B memory cell phenotype by flow cytometry with αCD40 alone (grey) or αCD40 + ICOS-Ig (black). Lymph nodes from protocol biopsies on d90 and d120 represent samples after delayed ICOS-Ig treatment. Naive B cells were defined as $CD20^+IgD^+CD27^-CD38^+IgM^{intermediate}$, nonswitched (NS) B memory $CD20^+CD27^+IgD^+$, and isotype-switched (IS) B memory cell $CD20^+CD27^+IgD^-$. After ICOS-Ig treatment, IS B memory proportion significantly increased (42 ± 9.1%), NS B memory trended downward (2.0 ± 0.6%), and naive B cells were similar (5.7 ± 2.4%) compared with αCD40 monotherapy (5.8 ± 1.1%, 8.0 ± 2.3%, 23 ± 17%, respectively). Light grey lines represent the mean. ** P = 0.04.

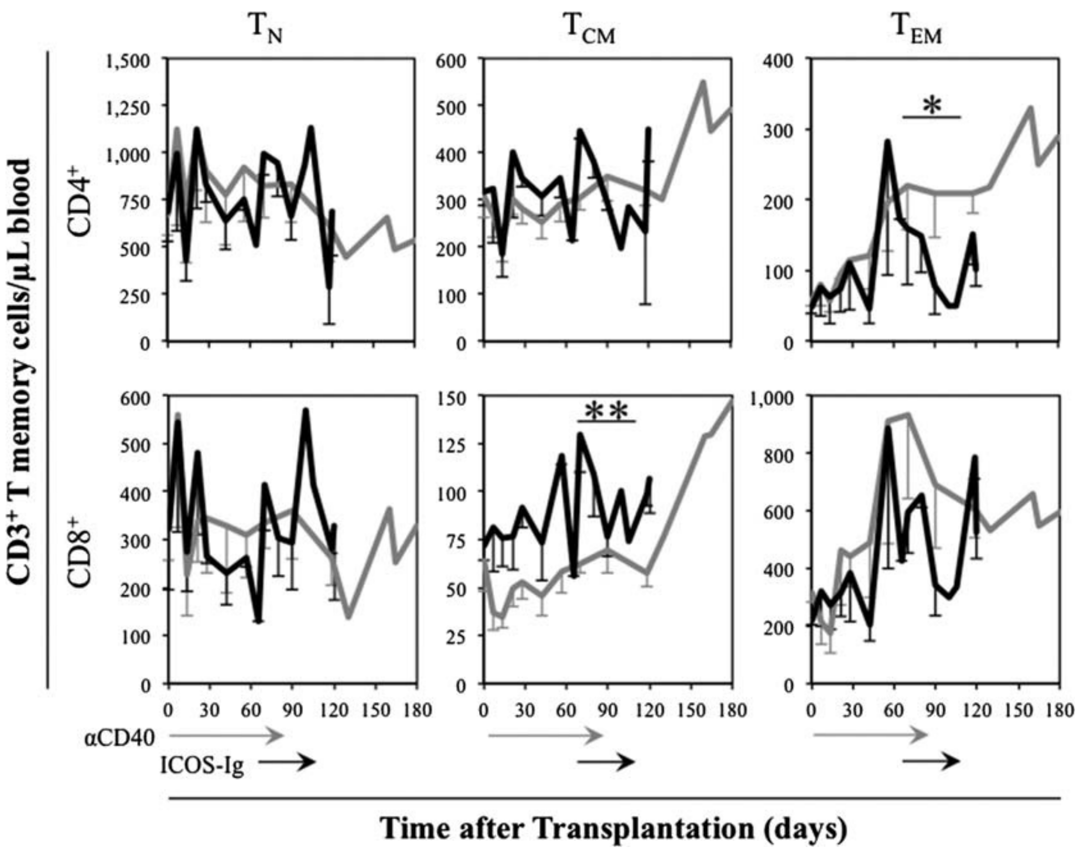

FIGURE 8. Peripheral blood T memory cell kinetics after transplantation. $CD3^+CD4^+$ and $CD8^+$ T cells were identified using flow cytometry. T memory cell phenotypes were characterized as naive (N: $CD45RA^+CD62L^+CD28^+$ or $CD95^-CD62L^+CD28^+$), T_{CM} ($CD45RA^-CD62L^+CD28^+$ or $CD95^+CD62L^+CD28^+$), or T_{EM} cell ($CD45RA^{+/-}CD62L^-CD28^-$ or $CD95^{+/-}CD62L^-CD28^-$). During ICOS-Ig treatment (black, n = 3), $CD4^+$ T_{EM} cells significantly decreased compared to αCD40 monotherapy (grey, n = 6). No significant difference was seen in the $CD8^+$ T_{EM} phenotype. SEM is demonstrated in error bars. **P* = 0.01, ***P* = 0.0004.

remaining T_{EM} cell,[10] $CD8^+ICOS^+$ T_{EM} cells that divide within the allograft after crossing the endothelial barrier,[45] or the loss of inhibitory effector functions of regulatory T cells, which is dependent on ICOS/ICOS-L signaling.[18,28,41] Our results do not provide compelling evidence regarding any of these nonexclusive hypotheses.

ICOS expression within lymph nodes is predominately on Tfh cells within the germinal center where T cells induce the terminal differentiation patterns of B cells into plasma or memory cells.[8,9,46,47] Although we did see a similar effect of ICOS-Ig on T_{EM} cell phenotype in mLNs as has been described in mice, we did not observe a difference in IgM alloAb development or IgG alloAb class switch between αCD40 and combination ICOS-Ig + αCD40–treated NHP. Failure to modulate class switching was unexpected, as others have demonstrated limited alloAb isotype switching with disruption of ICOS/ICOS-L.[16,36,37] Furthermore, ICOS-deficient mice and human have reduced Tfh ($CD4^+CXCR5^+$) cells[39] suggesting B memory cells may be affected by ICOS; however, we found an increased proportion of isotype switch B memory cells after ICOS-Ig treatment within regional lymph nodes. The continued persistence of B memory cells and ability to produce alloAb likely contributed to the ineffectiveness of ICOS-Ig in our study, although we cannot exclude the possibility that either the dose or pharmacologic characteristics of our molecule were inadequate to efficiently block the ICOS/ICOS-L pathway in vivo.[48]

The lack of synergistic effects with delayed ICOS-Ig in this NHP study compared with previous rodent studies may be due to several other factors. The reagent target itself, ICOS-Ig, combines with its ligand on APC, preventing ligation of ICOS on T cells, whereas αICOS binds the ICOS receptor on T cells, potentially leading directly to effects on the ICOS-expressing T-cell population. Although an ICOS-Ig for human use is not currently available,[49] 2 different αICOS monoclonal antibodies, MEDI-570 (ClinicalTrials.gov Identifier: NCT01127321 and NCT02520791) and AMG 557 (ClinicalTrials.gov Identifier: MCT02334306),[50] are in phase 1 and phase 2, respectively, clinical trials for SLE, Sjögren's syndrome, and refractory T-cell lymphoma. Alternatively, the ICOS-Ig dose may not have been sufficiently intensive to saturate the pervasive constitutive expression of ICOS-L on APC and nonhematopoietic cells. This interpretation is supported by the dose escalation that was required[15] to demonstrate modest survival improvement reported in a similar model,[42] and our inability to achieve increased ICOS-Ig trough levels despite dose escalation. Finally, the most sensitive method to detect ICOS expression remains unclear, and, therefore, the optimal timing of delayed ICOS-Ig treatment may not have been achieved in this study.

In summary, this pilot study of delayed ICOS-Ig treatment in combination with αCD40 failed to prolong primate cardiac allograft survival, or to significantly modulate acute rejection, chronic rejection, or alloAb elaboration, despite an

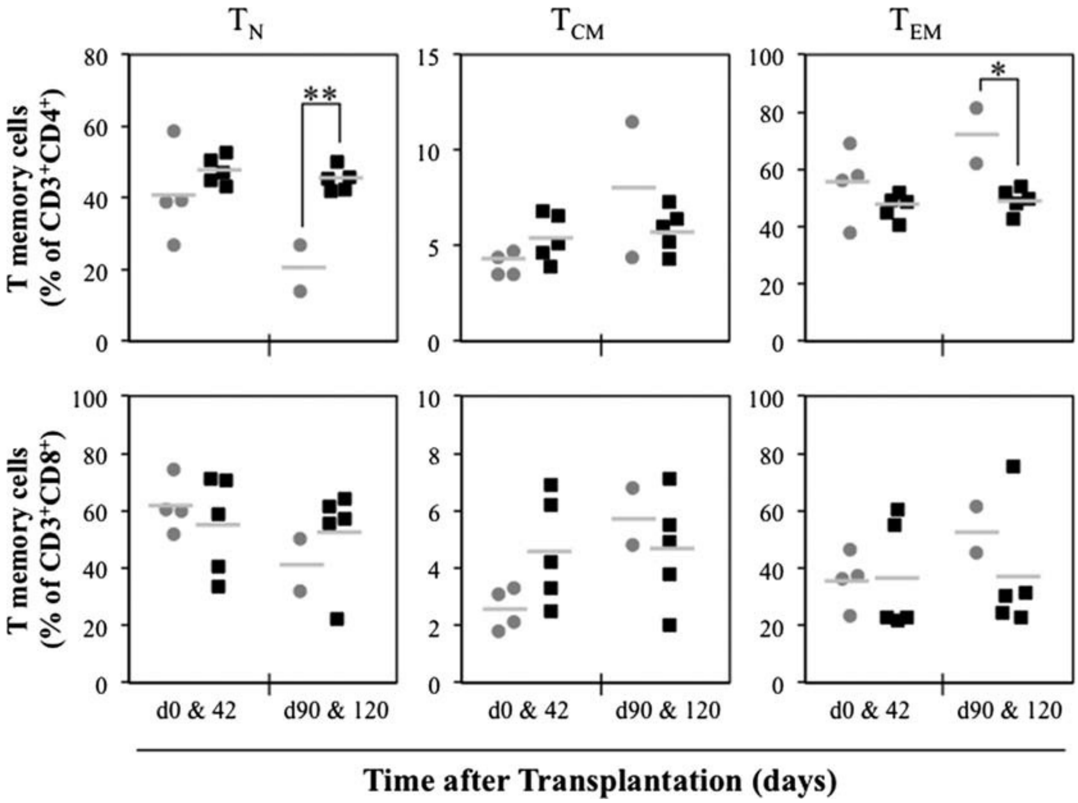

FIGURE 9. mLN T memory cell phenotype by flow cytometry with αCD40 alone (grey) or αCD40 + ICOS-Ig (black). Lymph nodes from protocol biopsies on d90 and d120 represent samples after delayed ICOS-Ig treatment. The proportion of $CD3^+CD4^+$ (top row) and $CD3^+CD8^+$ (bottom row) T_{EM} cell phenotypes were defined as N ($CD45RA^+CD62L^+CD28^+$ or $CD95^-CD62L^+CD28^+$), T_{CM} ($CD45RA^-CD62L^+CD28^+$ or $CD95^+CD62L^+CD28^+$), or T_{EM} cell ($CD45RA^{+/-}CD62L^-CD28^-$ or $CD95^{+/-}CD62L^-CD28^-$). After ICOS-Ig treatment, LN $CD4^+$ T_{EM} cells (49.1 ± 1.9%) significantly decreased compared to αCD40 monotherapy (grey, 71.7 ± 9.9%). Light grey lines represent the mean. ** $P = 0.002$, *$P = 0.01$.

observed decrease in T_{EM} cell in blood and lymph nodes during treatment. Together with the pilot study results from Lo et al. showing similar allograft results with early ICOS-Ig treatment with CTLA-4Ig, we provisionally conclude that ICOS-Ig, as tested, is ineffective in preventing costimulation blockade resistant rejection. Although a reagent with improved pharmacodynamics or demonstrated efficacy in another model would arguably justify reconsideration in transplantation, our future study regarding mechanisms to better protect grafts in recipients treated with costimulation blockade will focus on alternative pathways.

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