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Corticosteroids and Methotrexate as Adjuvants to Costimulation Blockade in Nonhuman Primate Renal Transplantation

Douglas J. Anderson[1], **Denise J. Lo**[1], **Francis Leopardi**[3], **Mingqing Song**[3], **Elizabeth A. Strobert**[2], **Joe B. Jenkins**[2], **Christian P. Larsen**[1], and **Allan D. Kirk**[1,3]

[1]Emory Transplant Center, Emory University, Atlanta, GA

[2]Yerkes National Primate Center, Emory University, Atlanta, GA

[3]Department of Surgery, Duke University, Durham, NC

Abstract

Belatacept, the CD28-B7 costimulation pathway inhibitor, has been approved as a calcineurin inhibitor (CNI) alternative in kidney transplantation. Although costimulation blockade (CoB) allows for CNI avoidance, it is associated with increased rates of early rejection, prompting a search for agents to pair with belatacept. Methotrexate (MTX) is an antimetabolite that has been found to be complimentary with abatacept, a lower affinity CD28-B7-specific analogue of belatacept, in the treatment of rheumatoid arthritis (RA). We examined whether this synergy would extend to prevention of kidney allograft rejection. Rhesus macaques underwent kidney transplantation treated with abatacept maintenance therapy with either a steroid taper, MTX, or both. The combination of abatacept maintenance with steroids prolonged graft survival compared to untreated historical controls and previous reports of abatacept monotherapy. The addition of MTX did not provide additional benefit. These data demonstrate that abatacept with adjuvant therapy may delay the onset of acute rejection, but fail to show synergy between abatacept and MTX beyond that of steroids. These findings indicate that MTX is unlikely to be a suitable adjuvant to CoB in kidney transplantation, but also suggest that with further modification, a CoB regimen used for advanced RA may suffice for RA patients requiring kidney transplantation.

Keywords

methotrexate; costimulation; non-human primate

Correspondence: Allan D. Kirk, MD, PhD, Box 3704, Duke University Medical Center, Durham, NC 27710, Allan.kirk@duke.edu.
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Disclosure

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Supporting Information

Additional supporting information may be found in the online version of this article.

Introduction

Since the introduction of cyclosporine, and later, tacrolimus, calcineurin inhibitors (CNIs) have formed the backbone of maintenance immunosuppression in clinical kidney transplantation. Recently, the development of the B7-CD28 targeted fusion protein, abatacept (CTLA4-Ig), and more importantly the approval of the second-generation molecule, belatacept, has offered an opportunity to replace CNIs and their inherent side effects. Indeed, the BENEFIT (1) and BENEFIT-EXT (2) trials showed improved graft function in belatacept treated groups compared to cyclosporine with similar graft survival. Extended follow-up further suggests those gains in renal function translate to improved graft survival in the long term (3). Despite these promising results, early acute cellular rejection has been seen with increased frequency in patients treated with belatacept (1–4), and this costimulation blockade (CoB) resistant rejection has tended to be more severe than early rejection episodes in CNI treated patients.

Significant effort has been put forth to understand CoB resistant rejection and find additional agents that may ameliorate the associated early rejection without sacrificing long-term salutary effects. Much of this work has focused on targeting other immunomodulatory pathways. Combining CD28-based agents with monoclonal antibodies targeting the CD40-CD154 interaction has met with significant success in animal models (5–10); however, translation to human studies has been hampered by thrombotic events associated with anti-CD154 antibodies (11). Our group recently reported that the combination of belatacept with LFA-1 blockade, a regimen shown to be quite efficacious in models of islet transplantation (12), did not share the same success in non-human primate renal transplantation (13). These targeted biologics have met with only modest success and come with their own set of potential side effects and costs.

Methotrexate (MTX) is a competitive inhibitor of dihydrofolate reductase, halting DNA synthesis and cell division by reducing the availability of donor methyl groups, particularly in the generation of the DNA precursor thymidylate. Methotrexate has a multitude of clinical uses, primarily in autoimmunity such as rheumatoid arthritis (RA) (14–16), asthma (17,18), and psoriasis (19,20), but also in oncology (21–23) as well. It has long been used in hematopoietic transplantation in the prevention of graft-versus-host disease (24–26). Several limited studies in solid organ transplantation also have been completed. Multiple small studies showed MTX to reverse recalcitrant rejection successfully in cardiac (27–31) and lung (32–34) transplantation. Dosing in these studies varied, and was often higher than used for treatment of autoimmunity. One study using methotrexate in addition to cyclosporine and steroid maintenance in renal transplantation studies showed reduction in rejection episodes at 6 months and lower serum creatinine at 12 months (35).

In the treatment of RA, MTX has been one of the most commonly used agents. However, a proportion of patients will continue to have active disease despite appropriate MTX dosing. The addition of CoB using abatacept has been shown to be beneficial to this patient group (36–38). There is reason to believe this combination may also be effective in transplantation. Methotrexate is known to be pro-apoptotic for mitogen activated T cells while leaving resting cells alone, and has been shown to prevent recall responses in T cells exposed to

alloantigen for which they were previously sensitized (39). It is this latter effect that is particularly notable, as memory responses are thought to play a major role in early CoB-resistant rejection. Methotrexate also is known to decrease expression of surface adhesion molecules important for lymphocyte homing and exit into tissues (40). Furthermore, as an anti-proliferative agent, MTX may have synergistic effects with CoB similar to those seen with the anti-purine medications such as mycophenolate or azathioprine. Although belatacept is the approved CoB agent for use in transplantation, abatacept is mechanistically identical, differing only by affinity. Additionally, it is available in a subcutaneous formulation, which could offer an option for patients with poor vascular access or lack of a suitable infusion center. Therefore, we sought to determine whether the addition of MTX to a maintenance regimen of abatacept and methylprednisolone would prolong graft survival in a non-human primate (NHP) model of renal transplantation. We show that abatacept prolongs graft survival in this pre-clinical model, but show no benefit from the addition of MTX beyond that seen with steroids. Our results highlight the potential for prolonged graft survival with CoB monotherapy if early rejection can be avoided, reiterate the efficacy of abatacept in kidney transplantation, and suggest therapeutic options for patients with RA requiring kidney transplantation.

Materials and Methods

Protocols for the care of all experimental animals in this study were approved by the Emory University Institutional Animal Care and Use Committee and designed to comply with the principles laid out in The Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS (41). Veterinary staff were actively involved in the care of all animals, examining them on a regular basis. Rhesus macaques (Macaca mulatta) were obtained from breeding colonies at AlphaGenesis, Inc. (Yemassee, SC, USA) or Yerkes National Primate Research Center (Lawrenceville, GA, USA). Class I and class II MHC typing by 454 pyrosequencing (University of Wisconsin, Madison, WI, USA) was obtained for each individual. Donor-recipient pairs were selected for matched size and maximal MHC disparity. Transplantation was performed in a domino fashion to maximize the utility of the available animals, with each animal serving as a kidney donor prior to receiving a transplant, while avoiding bilateral retroperitoneal dissection within a single operation. Left donor nephrectomy was performed at least 3 weeks prior to transplantation in order to allow sufficient recovery time prior to a second laparotomy. Renal transplantation was performed as previously described (10), with concomitant right nephrectomy leaving each animal entirely dependent on its allograft. Post-transplant monitoring consisted of daily clinical assessment by veterinary staff. Laboratory studies including serum chemistry and complete blood count were performed at least weekly, or more often as dictated by the animal's clinical course.

Experimental Groups

There were three experimental groups (Figure 1): Group 1 received abatacept and methylprednisolone, Group 2 received abatacept and methotrexate, and Group 3 received abatacept, methylprednisolone, and methotrexate. Abatacept was dosed at 20mg/kg on days −1, 3, 7, 14, 21, and 28 relative to the day of transplantation. Abatacept maintenance therapy

was then continued at 10mg/kg weekly until day 56, then biweekly indefinitely. Two groups received a methylprednisolone taper beginning on the day of transplantation with a dose of 15 mg/kg IV. The dose was converted to IM and reduced by half daily until a maintenance dose of 0.5mg/kg daily was reached. Termination of steroid therapy was based on the animals' clinical appearance and ranged from day 30 to day 60. Methotrexate was dosed at 5mg/m2 on days −1, 3, 7, 14, 21, and 28. Body surface area (BSA) for a rhesus macaque was calculated using the DuBois equation, substituting head-to-anus length for height and multiplying by a factor of 1.147 (42).

Flow Cytometric Analysis

Analysis of circulating immune cell phenotypes was performed both prior to transplant and at regular intervals following transplantation. Cell frequencies from flow cytometric analysis were combined with complete blood counts to calculate total numbers of circulating T cells and various T cell subsets. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation (BD Biosciences, Franklin Lakes, NJ) within 6 hours of phlebotomy. PBMCs (1.5×10^6) were then incubated with antibody mixtures at the appropriate titer for 15 minutes and washed twice. For assessment of intracellular markers, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's direction following surface staining. Flow cytometric data was acquired immediately using a BD LSR II multicolor flow cytometer (BD Biosciences). All flow data was analyzed using FlowJo (Tree Star, San Carlos, CA).

Surface markers were stained with the following monoclonal antibodies (mAbs): CD3 PacBlue, CD3 APC-Cy7, CD4 PerCP-Cy5.5, CD8 V500, CD28 PE-Cy7, CD127 PE-Cy7, PD-1 APC, LFA-1 APC, CD20 APC-Cy7 (all BD Biosciences), CD95 PacBlue, CD69 FITC (Invitrogen, Grand Island, NY), and CD25 PE (Miltenyi Biotech, San Diego, CA). Intracellular staining for FoxP3 was performed using FoxP3 Alexa488 (Biolegend, San Diego, CA).

Viral Monitoring

In order to assess treatment effect on protective immunity, weekly analysis of rhesus cytomegalovirus (rhCMV) viral loads was performed by quantitative real-time polymerase chain reaction using DNA isolated from whole blood as previously described (43). Levels of greater than 10,000copies/mL were considered significant and were treated with ganciclovir (6mg/kg IM twice daily) until resolution of the viremia. No routine viral prophylaxis was administered.

Statistics

All statistical analyses were performed in Prism 5 (GraphPad Software, La Jolla, CA, USA). Survival statistics were calculated using the Mantel-Cox method. For all analyses, a two-tailed p-value of < 0.05 was considered statistically significant.

Results

The Tested Regimens are Nondepleting and Preserve Protective Immunity.

Weekly flow cytometry was performed to measure circulating numbers of immune cells. The number of circulating CD3+ cells was not significantly different between any of the treatment groups (Figure 2A). Furthermore, there were no significant changes in the numbers of circulating CD4+ or CD8+ cells (Figures 2B, 2C). As a measure of the effect of these regimens on protective immunity, rhCMV levels were monitored weekly. No animals had significant CMV viremia over the treatment course (Figure S1), and no animals required antiviral treatment.

Maintenance Abatacept with Steroid Induction Prolongs Renal Allograft Survival.

Rejection-free survival was measured for all animals (Table 1, Figure 3). Three animals received a combination of abatacept and methylprednisolone. One animal had rejection at 36 days. The two remaining animals had prolonged graft survival and were sacrificed with functioning grafts at 1 year. Each had a serum creatinine less than 1mg/dL (Figure S2). Historical controls from our lab that were given no immunosuppression had mean graft survival of 6.8 days ($p = 0.01$ for comparison with Group 1) (10).

The Addition of a Short Course of Methotrexate Does Not Provide Additional Benefit.

Two additional groups received a four-week course of MTX, either instead of, or in addition to, the methylprednisolone taper. In the group that received abatacept and MTX, there was one long-term graft survival of greater than 386 days. Two animals rejected their grafts at 9 and 66 days. Four animals were transplanted in the group receiving all three medications. There was one long-term graft survival (>391 days). The remaining animals had graft rejection at 30, 65, and 224 days. Neither group had graft survival significantly different than the group receiving abatacept and methylprednisolone (Figure 3, $p = 0.49$ for Group 1 vs. Group 2, $p = 0.47$ for Group 1 vs. Group 3).

Memory Cell Populations Are Not Affected by Treatment.

The phenotypes of circulating T cells also were measured by flow cytometry. One week following transplant, there was a decline in the relative abundance of circulating CD8+ effector memory T cells (T_{EM}, Figure 4A). This was matched by an increase in the relative abundance of naive CD8+ T cells (T_N, Figure 4B). This effect was reversed completely by post-operative day 14, and CD8+ T_{EM} abundance remained above baseline through post-operative day 70. The kinetics of these changes were essentially similar regardless of treatment regimen, but the amplitude of changes seen in the first week was greatest in the group receiving triple therapy. Over the first 10 weeks, there was a slight downward trend in the percentage of circulating CD8+ central memory T cells (T_{CM}) in all populations (Figure 4C). Interestingly, the groups that received methotrexate had a stabilization in the central memory compartment, while the group that did not get MTX saw continued declines. The relative abundance of these memory subpopulations in CD4+ was unchanged over time and also unaffected by treatment regimen (data not shown).

Regulatory T Cells Are Reduced in All Treatment Groups.

To assess the effect of these treatment regimens on circulating regulatory T cells (T_{REG}), flow cytometric analysis of this population was performed weekly. Lymphocytes were gated for CD3, CD4, and FoxP3. Over the first four weeks, all animals saw decreased levels of circulating T_{REG} cells, both by percentage of circulating CD4+ T cells (Figure 5) and by absolute cell counts (data not shown). Levels of T_{REG} cells remained stable at this reduced level for the duration of the experiment. This effect was seen in all treatment groups and irrespective of the presence of methylprednisolone or MTX.

Discussion

This study was designed to evaluate the effectiveness of a combination of abatacept and MTX in renal transplantation. Several factors provided justification for choosing abatacept. First, abatacept is mechanistically identical to belatacept, albeit with poorer binding affinity (44), and it is already FDA approved for use in combination with MTX in RA. While belatacept would presumably exhibit the same synergy, this has not been proven. Its availability as a subcutaneous injection removes the potential logistic and vascular access barriers inherent to belatacept administration. We sought to determine whether the efficacy of MTX combined with abatacept in the autoimmune disease setting would extend to transplantation.

Transplant pairs were selected to maximize MHC disparity in order to test these regimens in as rigorous a model as possible. It is possible that lesser degrees of MHC mismatch would result in longer graft survival, although it is unknown whether this effect would be independent of immunosuppression regimen. Prior to the availability of Rhesus MHC typing, mixed lymphocyte reactions were typically used to confirm disparity in non-human primate studies. While this was able to confirm the alloreactive response, we feel the direct sequencing of the Rhesus MHC locus allows for better mismatching of the donor-recipient pairs, particularly since the animals come from a small number of colonies and are frequently related.

The clinical dosing of MTX varies significantly with the indication, with some protocols using a simple dose titrated to effect, while others use weight-based or BSA-based calculations. We sought to approximate the dosing typically used in autoimmune disease. Dosing for RA or severe psoriasis starts at 7.5-10mg weekly and can be titrated up to effect. Given an average BSA of adult $1.8m^2$(45), this range is 4.2-5.5mg/m^2. Therefore, we chose a dose of 5mg/m^2. Oncologic use of MTX is often at a much higher dose, and it is possible a higher dosing scheme would yield substantially different results. Similarly, lengthening the treatment course of methotrexate similar to its use in autoimmunoty may have changed the outcomes of those treatment groups. However, we sought to examine the effect of methotrexate as an adjuvant in the early post-transplant period when the risk of rejection is highest and whether avoidance of early rejection would provide a lasting benefit, therefore the course of methotrexate was limited. The dosing of abatacept used here is higher than typically used for treatment of autoimmunity in humans (20mg/kg vs 10mg/kg), but we elected to use the higher dose for consistency with previously published reports using abatacept in the non-human primate model.

Our data show that the use of abatacept with an adjuvant agent was effective at prolonging graft survival when compared to untreated historic controls. Some prolongation of graft survival in these groups was expected based on prior reports of animals receiving abatacept monotherapy having graft survival of 5-58 days depending on the dose used (10,44). Given these previous results, abatacept monotherapy is not a clinically relevant regimen, and a monotherapy group was not performed here. In this study, one animal that received abatacept and steroids rejected its graft at 36 days, similar to those in the aforementioned studies. However, the two remaining animals in that group both had graft survival over 1 year. Additionally, the one animal in the group receiving abatacept and MTX that avoided rejection in this early period also had good long-term survival. This suggests that if early rejection can be prevented prolonged graft survival with CoB monotherapy is possible.

The prolonged graft survival seen in the abatacept/steroid group was a surprising result. Failure of abatacept monotherapy prompted the development of belatacept by Larsen et al. (44), and our results compare favorably to the belatacept cohorts in that study. However, several distinctions can be made. In this study, animals received a slightly higher dose of abatacept, and this was continued indefinitely, whereas the prior study stopped abatacept at day 16 and belatacept at day 70. The steroid taper used here was also significantly higher than the steroid taper used by Larsen et al. It is possible the higher steroid dosing compensated for the lower binding affinity of abatacept compared to belatacept.

The interpretation of the results of our study is limited by a few factors. There are a small number of animals in each cohort. This is further exaggerated by several early rejections, so later time points may only have one or two individuals. The surprising success of the abatacept/steroid group also limits the usefulness of this group as a comparator, as it unlikely any regimen tested would show any significant additional benefit within the design of this study.

Replacing steroids with MTX as the adjuvant to abatacept provided no benefit in terms of graft survival. In fact, this group had the earliest rejection in the study, at 9 days. Addition of MTX to the abatacept/steroid treatment to create a three-agent regimen also provided no graft survival advantage versus abatacept and steroids. Review of the survival curves appears to show increased numbers of rejections seen in the groups receiving MTX. However, given the small sample sizes, it is difficult to determine whether this truly represents a negative effect of MTX. Comparison of the abatacept/steroid group versus all animals receiving MTX, with or without steroids, also failed to show any statistically significant difference ($p = 0.42$, curves not shown).

Interestingly, in the group receiving triple therapy, there was one late rejection at 224 days. It is unclear why this animal had a relatively late rejection, while other animals rejected early or not at all. Analysis of T cell memory subsets during this period revealed that at most time points, compared to the long-term survivor, the late rejector had higher numbers of circulating CD3+ cells and higher percentages of CD4+ and CD8+ T_{EM} (Figure S3). Whether this truly is related to the late rejection experienced by this animal is unclear. Some differences in the relative abundance of the various T cell subsets between treatment groups was seen at later time points, but this did not affect the overall performance of each regimen.

The interpretation of these data is limited severely by a low number of animals reaching the later time points.

Intense immunosuppression can lead to opportunistic infections such as CMV or BK virus, both of which have been associated with increased rates of rejection (46,47). The NHP model is very sensitive to a loss of protective viral immunity, as we have shown (41). Although BK virus or SV40 were not specifically tested, we detected no significant CMV viremia to suggest major impairment of protective immunity in any animal.

The effect of these regimens on T_{REG} populations was expected. There is ample data suggesting that CoB of the CD28 pathway via competitive binding of CD80 and CD86 is deleterious to T_{REG} function (48,49), likely owing to the importance of the complimentary CTLA4-CD80/86 pathway on T_{REG} development and homeostasis (50,51). Progress towards direct inhibition of CD28, which would avoid concomitant blockade of CTLA4, was crippled by the disastrous results of the TGN1412 study (52). However, the development of monovalent antibody fragments which maintain their inhibitory ability but lack the ability to crosslink their targets or act as super antigens has renewed interest in direct CD28 blockade (53,54). Preclinical trials utilizing these new agents are underway (55).

In summary, we have shown that CoB with abatacept can foster long-term graft survival when paired with an adjuvant agent to reduce the risk of early rejection. The choice of agent, either methylprednisolone or MTX, offered no statistical advantage. These data are relevant to the potential use of abatacept in kidney transplantation, and may be of use in designing or considering transplant regimens for patients with RA who are well controlled on a regimen of abatacept and MTX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BSA	body surface area
CNI	calcineurin inhibitor
CoB	costimulation blockade
NHP	non-human primate
rhCMV	rhesus cytomegalovirus

PBMC	peripheral blood mononuclear cell
T_N	naïve T cell
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
T_{REG}	regulatory T cell
MTX	methotrexate

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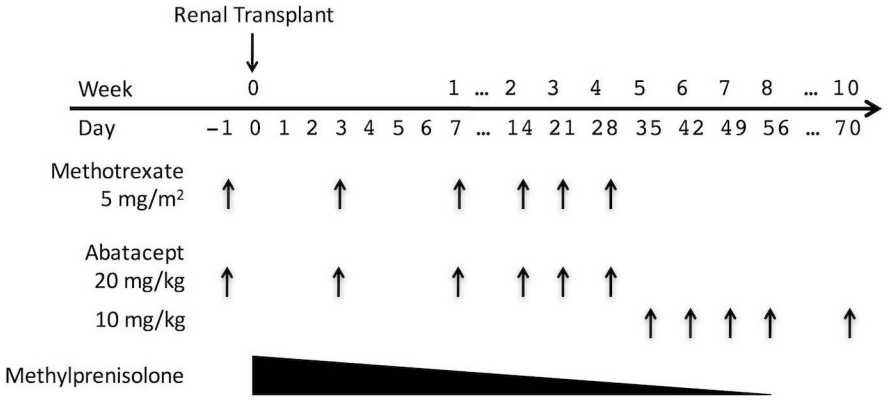


Figure 1:

Experimental Plan. All animals received maintenance abatacept. Groups 1 and 3 received the steroid taper. Groups 2 and 3 received 4 weeks of methotrexate.

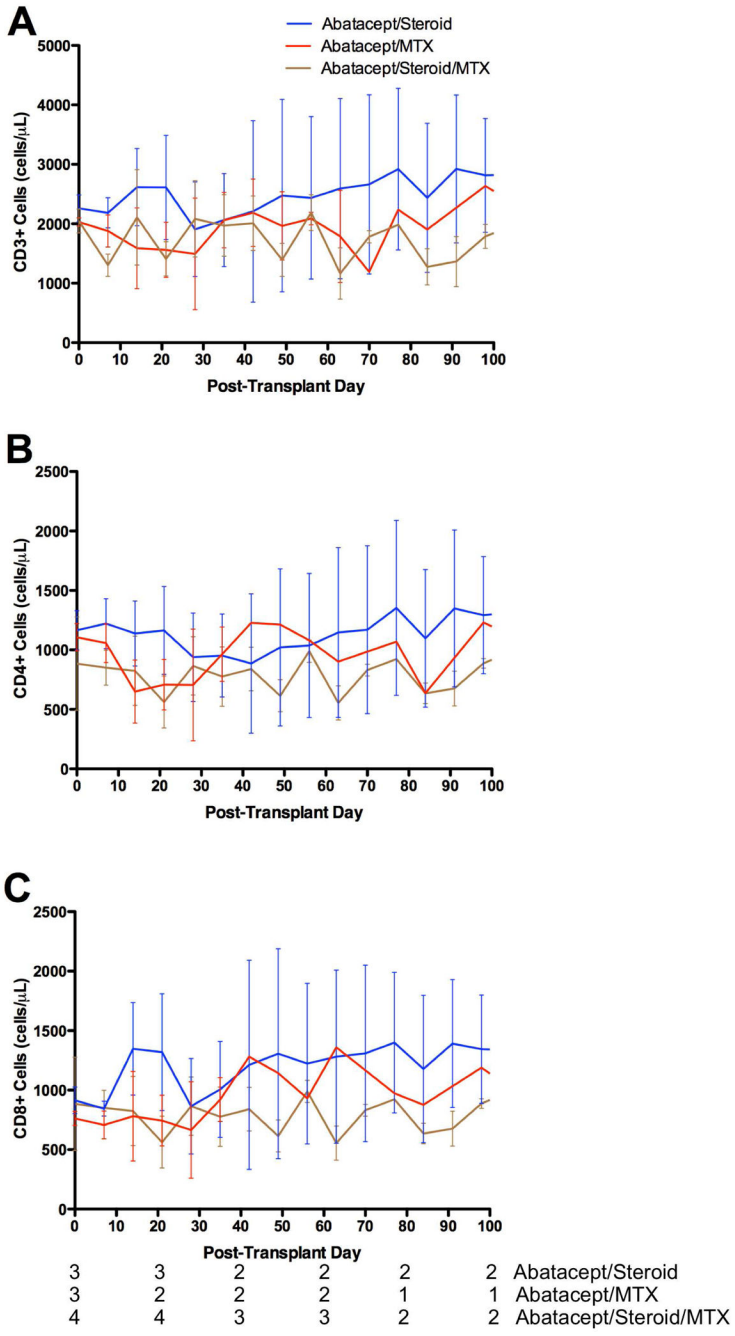


Figure 2:

Absolute counts of (A) CD3+, (B) CD4+, and (C) CD8+ cells were relatively stable over the treatment course and were not affected by treatment regimen.

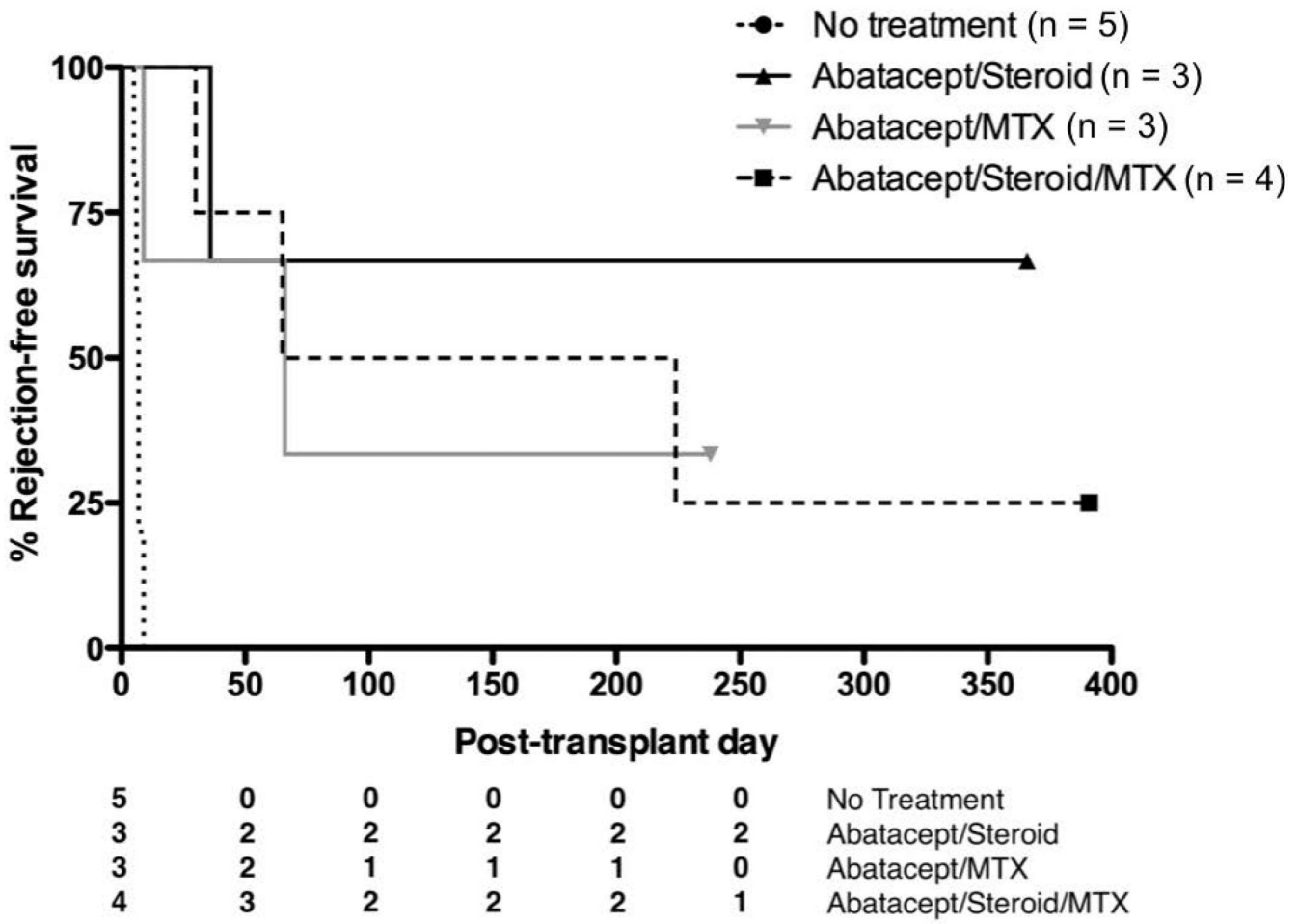


Figure 3:
Survival curves for treatment groups. All regimens prolonged graft survival versus no therapy. There was no statistical difference between any of the treatment groups.

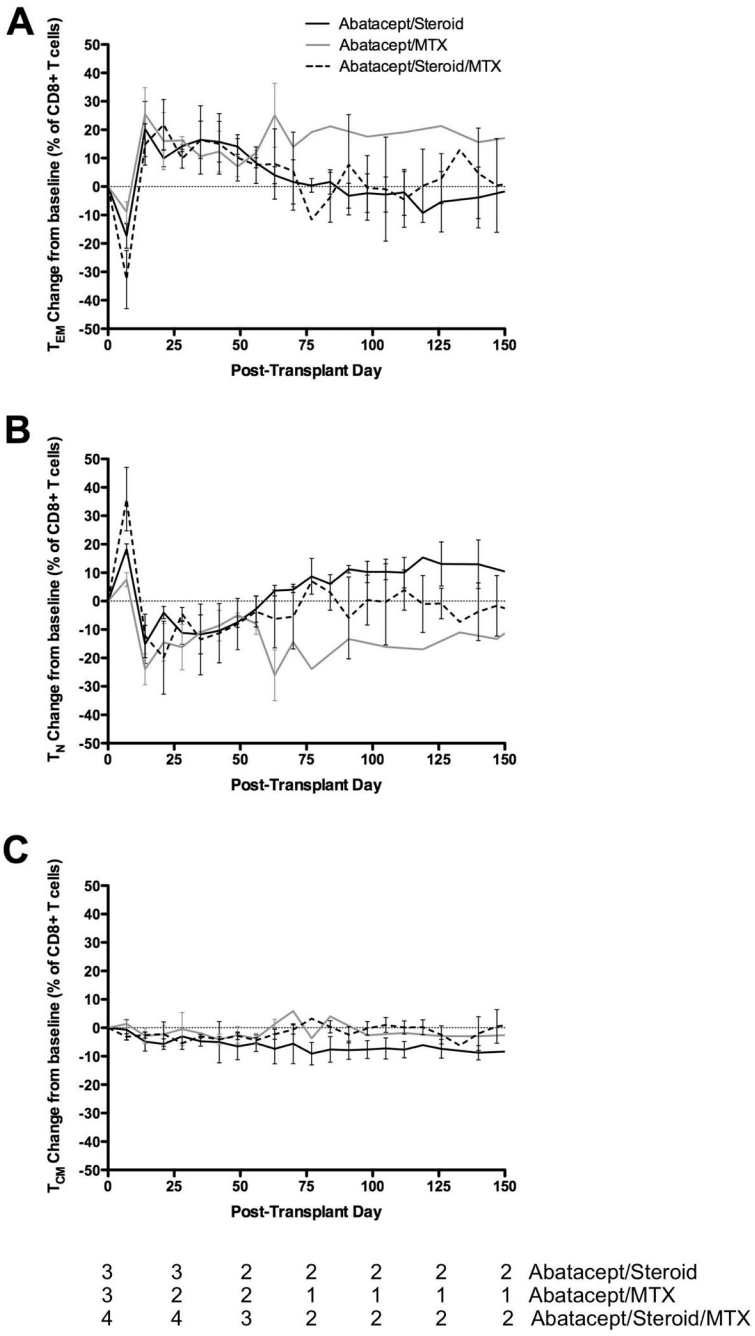
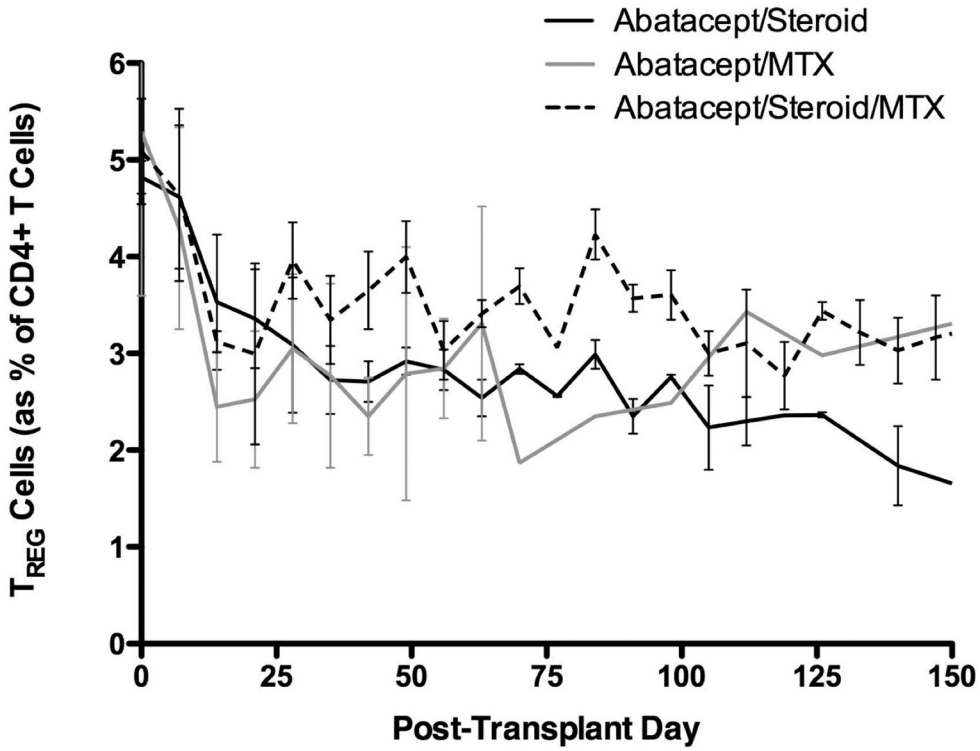


Figure 4:

Memory subpopulations of CD8+ T cells: (A) T_{EM}, (B) T_N, and (C) T_{CM}. The kinetics of changes in memory subpopulations was not affected by treatment regimen



3	3	2	2	2	2	2	Abatacept/Steroid
3	2	2	1	1	1	1	Abatacept/MTX
4	4	3	2	2	2	2	Abatacept/Steroid/MTX

Figure 5:
T_{REG} cells were decreased in all treatment arms.

Table 1:

Rejection-free survival for each treatment group.

Treatment Group	Rejection-Free Survival (days)
No Treatment (Historic Control)	5, 6, 7, 7, 9
Group 1: Abatacept + Steroid	36, >366, >366
Group 2: Abatacept + Methotrexate	9, 66, >238
Group 3: Abatacept + Steroid + Methotrexate	30, 65, 224, >391