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CD40 Blockade Combines with CTLA4Ig and Sirolimus To Produce Mixed Chimerism in an MHC-defined Rhesus Macaque Transplant Model


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Abstract

In murine models, T-cell costimulation blockade of the CD28:B7 and CD154:CD40 pathways synergistically promotes immune tolerance after transplantation. While CD28 blockade has been successfully translated to the clinic, translation of blockade of the CD154:CD40 pathway has been less successful, in large part due to thromboembolic complications associated with anti-CD154 antibodies. Translation of CD40 blockade has also been slow, in part due to the fact that synergy between CD40 blockade and CD28 blockade had not yet been demonstrated in either primate models or humans. Here we show that a novel, non-depleting CD40 monoclonal antibody, 3A8, can combine with combined CTLA4Ig and sirolimus in a well-established primate bone marrow chimerism-induction model. Prolonged engraftment required the presence of all three agents during maintenance therapy, and resulted in graft acceptance for the duration of immunosuppressive treatment, with rejection resulting upon immunosuppression withdrawal. Flow cytometric analysis revealed that upregulation of CD95 expression on both CD4+ and CD8+ T-cells correlated with rejection, suggesting that CD95 may be a robust biomarker of graft loss. These results are the first to demonstrate prolonged chimerism in primates treated with CD28/mTOR blockade and non-depletional CD40 blockade, and support further investigation of combined costimulation blockade targeting the CD28 and CD40 pathways.

Keywords

Costimulation Blockade; Non-human Primate; Mixed Chimerism; Biomarkers

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Introduction

In murine models, T cell costimulation blockade-based immunosuppression can induce robust immune tolerance to bone marrow, solid organ, and skin allografts. Two costimulation pathways have been predominantly targeted: the CD28:B7 pathway, and the CD154:CD40 pathway. Combined blockade of these two pathways has been demonstrated to be synergistic in promoting immune tolerance in mice, (1–7) and thus, there has been a long-standing interest in translating these results to the clinic. Translation of CD28:B7 blockade has moved the farthest: abatacept, the original formulation of CTLA4Ig, is approved for use in Rheumatoid arthritis, (8–10) and belatacept, a CTLA4Ig analog with higher avidity for CD80 and CD86 compared to abatacept, (11) has now completed Phase III trials as part of a calcineurin-avoiding immunosuppression regimen for renal transplantation. (12)

The trajectory of CD40:CD154 blockade toward the clinic has been decidedly slower, with therapeutic blockade of CD154 currently halted by the occurrence of thrombotic complications during both preclinical and clinical trials, (13) likely due to CD154-mediated platelet aggregation. (14) The lack of expression of CD40 on platelets suggests that these thrombotic complications were CD154-specific and not globally associated with blockade of the CD154:CD40 signaling pathway. Thus, targeting the CD40 component of the CD40:CD154 costimulation axis remains a potentially clinically important strategy for downregulating alloimmunity during transplantation.

Several categories of anti-CD40 reagents are currently available. These include anti-CD40 antibodies that lead to depletion of B cells, (15–17) and those that are non-depleting, (3, 18, 19) and include blocking antibodies (17, 19, 20) that retain partial CD40 agonist activity (20, Badell et al., submitted) and those that are full antagonists. (16, 18) While CD40-directed reagents that deplete B cells have shown efficacy in promoting allograft survival, (15–17, 20) from a mechanistic standpoint, these reagents may be acting outside of their role as inhibitors of costimulation blockade. Finally, while complete CD40 pathway antagonists may avoid untoward immune pathology that may occur with activation of costimulation pathways, partial activation of these pathways may actually be beneficial, if they are better able to trigger activation-induced cell death. (21–23)

As CD40-directed therapies are investigated, it is critical to determine their ability to synergize with CD28 blockade, given the impact of CD28-CD154 dual blockade that has been demonstrated in murine models, and the emerging clinical importance of therapies such as belatacept. While murine experiments have recently demonstrated that, like CD154-blockade, CD40 blockade can synergize with CTLA4Ig to promote the acceptance of both bone marrow and skin allografts (3), to date, studies in non-human primates with non-depleting antibodies targeting human CD40 have failed to demonstrate a similar effect. (18) Here we demonstrate for the first time that a novel, non-depleting CD40 monoclonal antibody, 3A8, can synergize with combined CTLA4Ig and sirolimus to promote donor engraftment in a rhesus macaque mixed-chimerism model.

Methods

Experimental Animals

This study utilized specific pathogen free, juvenile rhesus macaques, obtained either from the Yerkes National Primate Research Center or the NIAID-sponsored Rhesus macaque colony managed by Alphagenesis Inc (Yemassee, SC). All animals were treated in accordance with Emory University and Yerkes National Primate Research Center IACUC.
regulations. For this study, donor and recipient pairs were chosen that were half-siblings and matched for one MHC haplotype, as previously described.(24)

**HSCT protocol**

Hematopoietic stem cells (HSCs) were harvested either by leukopheresis or terminal bone marrow harvest as previously described. (24–26) Details of the total nucleated cell dose, the total CD34+ stem cell dose and the total CD3+ T cell dose that each recipient received are shown in Table 1.

**Immunomodulation**

Recipient animals were prepared for transplant with a single dose of intravenous busulfan (9.5mg/kg, Otsuka Pharmaceuticals, Rockville, MD). They were also given two peri-transplant doses of basiliximab (0.3mg/kg on days -1 and 3 relative to transplant).

Maintenance immunosuppression consisted of the mouse anti-human CD40 monoclonal antibody, 3A8 (American Type Culture Collection, Rockville, MD) produced in serum-free medium, CTLA4Ig (Bristol Myers Squibb, Pennington, NJ) and sirolimus (LC laboratories, Woburn MA) (Figure 1a). In this report the full immunosuppression regimen is referred to as “3CS”. CTLA4Ig was dosed at 20mg/kg on day -6, -1, 0, 3, 5, 9, 16, and every 14 days thereafter, until day 180 post-transplant. 3A8 was dosed as follows: 20mg/kg/dose x 2 doses on day -6 and day -1, 10mg/kg/dose x four doses on days 0, 3, 5, 9, and then maintenance dosing at 5mg/kg/dose twice weekly thereafter, continuing until day 180 post-transplant. Sirolimus was given intramuscularly with serum trough levels targeted at 5–15 ng/dL, until day 180 post-transplant when sirolimus weaning was initiated, with discontinuation of the drug at day 208 post-transplant.

**Chimerism determination**

Chimerism was measured using real-time PCR or DNA microsatellite-based quantification as previously described.(24, 27, 28) Briefly, MHC- or SRY-based chimerism was monitored by real-time SybrGreen PCR (ABI, Foster City, CA, USA). (24, 28) If PCR-based chimerism determination was not possible, divergent donor- and recipient-specific microsatellite markers were used by comparing peak heights of the donor- and recipient-specific amplicons. (24, 27, 28) T cell or B cell chimerism was determined by first sorting CD3+/CD20- cells (T cells) or CD3-/CD20+ cells (B cells) using a FACSAria cell sorter (Becton Dickinson, San Diego, CA). We have previously shown that myeloid chimerism (CD14+/CD45+) mirrors whole blood chimerism. (24) Thus, for this study, whole blood chimerism was used as a surrogate for myeloid chimerism. The day of rejection was defined as the last day that donor chimerism could be detected by PCR or microsatellite analysis.

**Pharmacokinetic analysis of the 3A8 monoclonal antibody**

Serum 3A8 levels were determined by ELISA analysis or the murine IgG2b moiety, using an anti-IgG2B ELISA kit (Bethyl Laboratories, Montgomery TX).

**Evaluation of Rhesus anti-mouse antibodies (RAMA)**

RAMA was quantified by an ELISA assay using 3A8 as the target antigen, adding serial dilutions of recipient serum (starting at 1:10), and detection of binding antibody with an anti-rhesus IgG (1B3)-horse radish peroxidase anti-macaque IgG (obtained from the Non-human Primate Reagent Resource). The presence of RAMA was expressed as an end-point titer, which was defined as the dilution-factor of the recipient serum that generated an ELISA OD that exceeded the OD of the pretreatment sample at the same dilution by 2-fold.
Measurement of anti-donor antibody

Anti-donor antibody was determined by incubating PBMCs from the donor with serum from the recipient which was collected longitudinally. The presence of anti-donor antibody in the recipient serum was detected flow cytometrically on CD3+/CD20− cells by binding of a FITC-labeled goat anti-monkey IgG (KPL Inc, Gaithersburg, Maryland, catalog # 072-11-021) to the donor cells to which anti-donor antibodies were bound.

Measuring anti-donor T-cell alloreactivity using CFSE-MLR

CFSE MLR analysis was performed as previously described. (28) Briefly, recipient peripheral blood lymphocytes (PBL) were enriched for T lymphocytes by depletion of antigen presenting cells by binding to anti-mouse IgG-Dynal Beads (Invitrogen, Carlsbad, CA) after labeling with anti-CD20 and anti-HLA-DR antibodies (BD Biosciences). The enriched T-cell fraction was labeled with 5µM CFSE (Molecular Probes, Eugene, OR) and 2 × 10^5 were incubated for 5 days at 37°C with 2×10^5 donor PBL. Flow cytometric analysis of APC-labeled anti-CD4 and anti-CD8 antibodies (clones SK3 and SK1, respectively) and CFSE was performed. The CD8-APC antibody labels T cells with greater fluorescence intensity than does the CD4-APC and, therefore, both populations could be monitored simultaneously. To quantify the amount of proliferation that occurred, CD8+ cells were gated, and the percent of cells remaining in culture that had divided at least once (resulting in a > 2-fold reduction in CFSE fluorescence compared to the undivided fraction) was determined using FloJo flow cytometry analysis software (Ashland, OR). The fluorescence of highly divided CD4+ responder populations could not be accurately distinguished from the unlabeled stimulator cells, thus, only CD8 proliferation was quantified for this study.

Longitudinal flow cytometric analysis of T cell phenotype

Multicolor flow cytometry panels were used to determine the following cell subpopulations: T cells: CD3+/CD20−; B cells: CD20+/CD3−; CD4+ T cells: CD4+/CD3+/CD8−/CD20−; CD8+ T cells: CD8+/CD3+/CD4−/CD20−; Naïve T cells (Tn): CD28+/CD95− cells in either the CD4 or CD8 T cell subsets. Central memory T cells (Tcm) CD28+/CD95+ cells in either the CD4 or CD8 T cell subsets. Effector/Effector memory T cells: CD28−/CD95+ cells in either the CD4 or CD8 T cell subsets. (29) The sources and clones used for each of these antibodies are as follows: From BD Biosciences (San Jose CA): CD3, Clone SP34-2; CD8, Clone: RPA-T8; CD40, Clone 5C3 (distinct from the 3A8 clone). From eBioscience (San Diego CA): CD4, Clone OKT4; CD20, Clone: 2H7; CD28, Clone: CD28.2; CD95, Clone: DX2. Longitudinal flow cytometric analysis was performed using FloJo flow cytometry analysis software.

Statistical Analysis

Anova and Kaplan-Meier analysis was carried out using the JMP® statistical software package, Version 8 (SAS Institute Inc, Cary, NC, 1989–2009). To determine Anova for multiple parameters, a post-hoc Tukey HST test was used to determine significant differences for pair-wise comparisons.

Results

The 3A8 anti-CD40 antibody synergizes with combined CTLA4Ig and sirolimus for chimerism-induction after nonmyeloablative BMT

As shown in Figure 1b, significant whole blood chimerism was specifically induced in recipients transplanted with hematopoietic stem cells from MHC haploidentical donors when the 3A8 antibody was combined with CTLA4Ig and sirolimus (‘3A8/CTLA4Ig/ sirolimus’, which will henceforth be referred to as “3CS” immunosuppression). The peak
level of whole blood chimerism measured was significant (72%, 81% and 83% in CX16, CX6R, and CW6F, respectively) and donor chimerism persisted for the length of immunosuppression (lasting an average of 218 days post-transplant). For all three recipients, transplant rejection occurred upon immunosuppression withdrawal. For two of the 3CS recipients (CW6F, CX16), rejection was precipitous, with high-level chimerism persisting throughout treatment and rejection occurring rapidly with 3CS washout. For one of the 3CS recipients (CX6R) while significant donor chimerism was maintained until approximately day 135 (mean donor chimerism = 48%), a reduction in chimerism occurred prior to 3CS withdrawal, (mean donor chimerism = 15% between days 142–200 post-transplant) with complete loss of chimerism in the absence of immunosuppression. The presence of all three immunomodulators was necessary for prolonged engraftment: as shown in Figures 1b, and 1c, animals that were transplanted with CTLA4Ig and sirolimus alone, CTLA4Ig and 3A8 alone, sirolimus and 3A8 alone, or 3A8 alone did not exhibit sustained engraftment. These data are the first to demonstrate synergy between antibody-based CD40 blockade and combination CD28 blockade with CTLA4Ig and sirolimus in primates.

Donor-specific down-regulation of alloreactivity accompanied chimerism and 3CS treatment

During treatment with 3CS, and when significant donor chimerism was present, transplant recipients demonstrated down-regulation of donor-directed T cell proliferation, with maintenance of anti-third party proliferative responses, as measured by CFSE MLR analysis. (representative example from CX16 shown in Figure 1d). However, concomitant with transplant rejection, robust anti-donor alloreactivity reappeared, consistent with T cell-mediated rejection of donor hematopoietic stem cells (Figure 1d).

Rejection occurred in the setting of compartmentalized chimerism-induction in these transplant recipients. Thus, as we have previously demonstrated (24, 28) while high-level chimerism could be measured in unfractionated whole blood, significant T cell and B cell chimerism did not develop in the two 3CS-treated recipients in whom sorted chimerism was measured. Thus, as shown in Figure 1e, T and B cell chimerism were 0.48% and 8.3%, respectively, versus 62% whole blood chimerism for CW6F and 0.55% and 0.11%, respectively versus 53% for CX6R. Thus, the T cell alloreactivity that was measured with the CFSE MLR assay derived predominantly from the recipient T cell pool, and represented a rejection response rather than a graft-versus host phenomenon. While T cell alloreactivity was present concomitant with rejection, no alloantibody was detected either during or after immunosuppression withdrawal in 3CS treated recipients (Figure 2). Alloantibody formation was also not detected in recipients treated with CTLA4Ig/sirolimus, 3A8/sirolimus, or 3A8/CTLA4Ig (data not shown). Donor cells were not available to test alloantibody formation in recipient DJ5V (who received 3A8 alone).

CD95 expression on peripheral blood CD4+ and CD8+ T cells correlated with transplant rejection

The primate mixed-chimerism model benefits from the fact that, unlike models of solid organ or islet transplantation, donor alloantigens are persistently present in the peripheral blood. Thus, immune signatures of graft acceptance or rejection may also be discernable in the blood. The data depicted in Figure 3a–c provide evidence that the shift toward CD95-positivity may be a broadly prevalent signature of rejection, occurring across immunosuppression regimens and across increasing degrees of MHC disparity. Thus, for the MHC-haploidentical transplant recipients that received 3CS immunosuppression, during ongoing donor engraftment, the percent of both CD4+ and CD8+ T cells that were CD95+ was stable. However, concomitant with rejection, all three of the 3CS recipients demonstrated a surge in the proportion of T cells that were CD95+.
To determine the extent to which acquisition of CD95 expression correlated with rejection across immunosuppressive platforms, we compared the results that we obtained with the 3CS cohort with a cohort of recipients that we have previously described, who also received MHC haploidentical bone marrow transplants, but who received immunosuppression with an anti-CD154-based immunosuppression platform. As shown in Figure 4, these recipients rejected their allografts earlier than the 3CS cohort (Figure 4a, MST = 137 for the CD154-based transplants versus 218 days for the 3CS cohort, p = 0.018). Concomitant with the earlier rejection was earlier acquisition of CD95 expression on recipient T cells (Figure 4b). These data suggest that expression of CD95 correlates with transplant rejection, and that this signature exists during transplant rejection across multiple immunosuppressive platforms and during transplant across increasing degrees of MHC disparity.

**Pharmacokinetic and Pharmacodynamic studies with 3A8 suggest that the immunogenicity of mouse immunoglobulin may limit its in vivo efficacy**

To evaluate the saturation of CD40 binding by 3A8, we monitored the binding of a cross-blocking anti-CD40 antibody, 5C3. As shown in our companion paper (Badell et al., submitted, Figure 1a,b), 3A8 efficiently blocked 5C3 binding of CD40 in vitro (with 50% blockade observed at 0.01 µg/ml and full blockade at >0.1µg/mL, Badell et al., submitted). The inhibition of 5C3 binding to CD40 by 3A8 is likely due to cross-blocking, rather than CD40 downregulation, given that, in our companion manuscript (Figure 1 c,d in Badell et al., submitted) we show that 3A8 does not inhibit sCD154, indicating that CD40 levels are likely unchanged after 3A8 treatment. Figure 5 demonstrates that, in vivo, in the majority of animals that were tested, the binding of the 5C3 antibody to CD40 was efficiently inhibited in animals treated with 3CS immunosuppression. Thus, as shown in the representative example in Figure 5a, near complete inhibition of 5C3-binding of CD40 occurred during 3CS treatment, indicating saturating levels of 3A8. However, the saturation of CD40 by 3A8 had a short half-life. Thus, as shown in Figure 5a, within one week of discontinuation of 3A8, 5C3 binding to CD40 was restored. This short functional half-life may have increased the risk for breakthrough CD40 expression during 3CS treatment. Indeed, when binding of 5C3 to CD40 was tracked in the three recipients who received 3CS, intermittent but significant binding of 5C3 indicated that periodically, CD40 was not saturated by 3A8. Thus, as shown in Figure 5b, intermittent, low-level breakthrough CD40 staining was observed in all three of the recipients, and in one recipient (CW6F), while efficient inhibition of CD40 saturation by 3A8 was established through day 65 post-transplant, after day 65, intermittent, but significant breakthrough CD40 staining occurred (Figure 5b).

Several observations suggest that rapid clearance of the murine 3A8 antibody may have led to breakthrough CD40 expression: First, we observed that when the 3A8 antibody was used as monotherapy, this mouse immunoglobulin induced a strong rhesus anti-mouse antibody (RAMA) response: As shown in Figure 5c (red triangles), in DJ5V, a transplant recipient treated with 3A8 alone, a high titer RAMA developed (with an end-point titer of >1:63,840). Concomitant with the development of RAMA, this animal developed a progressive serum-sickness syndrome, characterized by significant facial edema, swelling and respiratory distress, necessitating our halting further treatment with 3A8 after day 22 post-transplant. The development of high-titer RAMA (by day 14 post-transplant) correlated with loss of CD40 blockade in this recipient (data not shown). As demonstrated in Figure 5c, 3CS immunosuppression significantly, but not completely, inhibited RAMA formation. Thus, all three animals treated with 3CS (CW6F, blue circles, CX6R, green circles and CX16, purple circles) had evidence for the development of low-titer RAMA at least once during the post-transplant observation period (Figure 5c). While the titers were significantly lower than that observed with 3A8 monotherapy (highest RAMA titer in the 3CS cohort was...
1:160 compared to 1:63840 for 3A8 monotherapy), these data suggest that 3A8 was partially immunogenic even when given in combination with CTLA4Ig and sirolimus. This anti-3A8 response would be expected to reduce circulating concentrations of 3A8, which could lead to decreased in vivo efficacy of this antibody. Serum trough measurements support this hypothesis. As shown for all three animals treated with 3CS, serum trough measurements of 3A8 were variable. Thus, as shown in Figure 5d, these animals showed as much as a 50-fold variability in serum trough values during longitudinal analysis at maintenance dosing (with estimated troughs ranging from 0.02 µg/mL to 1 µg/mL in CW6F, 0.5 µg/mL to 5 µg/mL in CW6R, and 0.16 µg/mL to 1.3 µg/mL in CX16). The variability in antibody levels would be expected to increase the risk of intermittent breakthrough of CD40 blockade. Taken together, these data suggest that the immunogenicity of the mouse 3A8 antibody likely negatively impacted both its in vivo stability and its CD40 blocking efficiency, implying that the data that we have accumulated may actually underestimate the potency of CD40 blockade to enable allograft acceptance.

Discussion

The Phase III BENEFIT trial has demonstrated significantly better renal function in patients treated with a calcineurin-sparing immunosuppressive regimen containing the CTLA4Ig analog, belatacept, highlighting the potential clinical utility of costimulation blockade-based immunosuppression after solid organ transplantation. However, significant numbers of belatacept-resistant rejection episodes were observed, identifying an ongoing need for novel therapeutic approaches that might be capable of synergizing with CD28-directed therapies while continuing to minimize the toxicities associated with calcineurin inhibitors or steroids.

In this study, we have used the well-established, rhesus macaque mixed-chimerism-model (24, 28) to evaluate the ability of a novel, non-depleting anti-CD40 antibody to promote allograft acceptance. Our results demonstrate that a regimen containing the 3A8 anti-CD40 monoclonal antibody can lead to significant donor chimerism after nonmyeloablative hematopoietic stem cell transplant, and are the first to demonstrate synergy between a sirolimus and CTLA4Ig-containing immunosuppression platform and non-depletional CD40 blockade in a primate model. It should be noted that due to the constraints of primate transplantation, multiple dosing regiments of the active components of 3CS were not tested in this study. However, the doses chosen were based on extensive experience with this and similar biologics to achieve biologically active drug levels. While this and the companion study in islet transplantation (Badell et al., submitted) are the first to evaluate the 3A8 antibody for a transplant indication, two other anti-CD40 antibodies have previously been studied in primate transplant models. The IgG2a Chi220 antibody was demonstrated to prolong islet allograft survival both when given as a monotherapy and when given in combination with belatacept. However, this antibody led to significant B cell depletion, complicating the mechanistic interpretation of the impact that its CD40 blockade functionality had on allograft survival. In addition, Haanstra et al investigated the antagonist chimeric ch5D12 anti-CD40 antibody (30, 31) in a rhesus macaque model of renal transplantation. The ch5D12 antibody was able to prolong acceptance of the renal allograft when given as a monotherapy (mean survival time of 99 days compared to 6 days without immunosuppression). However, no synergy was demonstrated when the anti-CD40 antibody was paired with the anti-CD86 antibody chFun-1. These results underscore the functional variability inherent in distinct monoclonal antibody clones, and of variable immunoglobulin isotypes, despite their targeting of the same signaling pathway. It is important to note that the two antibodies that have shown synergistic effects with CD28/B7 blockade (Chi220 and 3A8 (17, 19)) both demonstrate partial agonist behavior, (20, Badell et al., submitted) rather than being purely antagonistic, as has been reported for ch5D12. While 3A8’s partial agonism could also have an untoward effect on transplant
acceptance through the upregulation of CD80/86 on the allograft, and the mechanistic necessity for partial agonism of the CD40/CD154 signaling axis for optimal transplant acceptance has not yet been proven, these observations suggest that the 3A8 clone may have unique structural and functional properties that will make it an attractive target for future clinical development.

The pharmacokinetic and pharmacodynamic studies that we performed show that while initially, and with high antibody doses, high serum levels of the 3A8 antibody could be achieved, this murine-anti-human antibody led to the development of rhesus-anti-mouse-antibodies, which likely significantly decreased its functional half-life. Thus, serum levels of 3A8 were variable, and blockade of CD40 was intermittently absent, and completely reversed within a week of antibody cessation. These results suggest that, despite the significant efficacy observed with the 3A8 antibody, these results likely occurred despite a suboptimal pharmacokinetic profile, and that a primatized antibody containing the 3A8 CD40 binding domain may lead to increased immunosuppressive efficacy. Development of these second-generation 3A8-based reagents, is currently being actively pursued (Reimann and Larsen, unpublished data) and may yield a novel clinically relevant therapeutic for combinatorial therapy with CD28-directed costimulation blockade.

The primate mixed-chimerism model has also allowed us to probe the immune signature that accompanies both ongoing engraftment and transplant rejection. For these studies, the mixed-chimerism model has a potentially important advantage over other primate models of transplantation, in that the site of donor antigen, the peripheral blood, is directly accessible for longitudinal analysis. This has allowed us to determine if an immunophenotype could be discerned that was predictive of transplant rejection. We have observed that with both CD40− and a CD154-based post-transplant immunosuppression, and during both MHC-matched (24) and MHC-mismatched transplantation (Figures 3 and 4), a shift of the T cell phenotype towards high expression of the CD95 memory T cell antigen correlated closely with transplant rejection. The accumulation of CD95+ memory T cells likely occurred due to the prolonged exposure of recipient T cells (in the setting of minimal donor T cell chimerism) to donor hematopoietic antigens. Given that memory T cells rely less on costimulation pathways for activation and effector functions, (32) this shift toward memory-predominance could potentiate costimulation-blockade-resistant rejection. Mechanistically, upregulation of CD95 (Fas) on memory T would be expected to limit their expansion through Fas:FasL-induced apoptosis (33, 34) thus limiting the risk of immunopathology from unrestrained cytotoxicity of the accumulating memory cells.

The observation that recipients treated with a CD154-based immunosuppressive strategy demonstrated an earlier phenotypic shift towards T cell CD95-positivity (Figure 4b), and also demonstrated earlier rejection (Figure 1c) has two clinically important implications. First, it implies that this shift in phenotype may constitute a robust immunologic signature of ensuing rejection, potentially predicting allografts that are at risk of failure. Second, it suggests that CD40 blockade may be well situated to substitute for CD154 blockade in combination therapy with belatacept. Our observations suggest that the CD95+ signature of rejection should be sought in models of solid organ transplant rejection, and in patient trials. They also suggest that clinical translation of CD40-directed therapies may be warranted, to directly assess the ability of combined CD28− and CD40-based costimulation blockade to prevent solid organ and cellular allograft rejection.

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References


CD40 blockade with the 3A8 antibody in combination with CTLA4Ig and Sirolimus induce mixed-chimerism in rhesus macaques.

a. Transplant strategy. MHC haploidentical transplant pairs were chosen, and transplant recipients were prepared for transplant with a single 9.5mg/kg dose of busulfan and two doses of basiliximab (0.3mg/kg/dose). Maintenance immunosuppression with the 3A8 anti-CD40 antibody, CTLA4Ig and sirolimus was given to recipients at the doses and intervals shown.

b. Mixed-chimerism-induction with 3CS immunosuppression. The percentage of donor cells in the whole blood was determined either through qPCR or microsatellite analysis of disparate MHC alleles (described in Methods). Circles: transplant recipients treated with the 3A8 anti-CD40 antibody, CTLA4Ig and sirolimus (“3CS” immunosuppression, n=3, blue circles = CW6F, green circles = CX6R, purple circles = CX16). Squares: Transplant recipients treated with CTLA4Ig and sirolimus, without 3A8 (n=2, yellow squares = DJ2L, orange squares = DJ7G). Triangles: transplant recipient treated with 3A8 alone (n=1, red triangles = DJ5V). Crosses: transplant recipients treated with 3A8 and sirolimus (n =2, blue
cross = CV2R, pink cross = DN9V). Diamonds: transplant recipients treated with 3A8 and CTLA4Ig (n=2, black diamond = DJ7L, red diamond = DN95). The red bar indicates the duration of treatment with immunosuppression.

c. Detailed view of mixed-chimerism-induction in animals receiving CTLA4Ig/sirolimus, 3A8 alone, 3A8/sirolimus, or 3A8/CTLA4Ig. The y-axis scale in this panel is 10-fold lower than the scale in Figure 1b. Squares: Transplant recipients treated with CTLA4Ig and sirolimus, without 3A8 (n=2, yellow squares = DJ2L, orange squares = DJ7G). Triangles: transplant recipient treated with 3A8 alone (n=1, red triangles = DJ5V). Crosses: transplant recipients treated with 3A8 and sirolimus (n =2, blue cross = CV2R, shown in Figure 1b, pink cross = DN9V). Diamonds: transplant recipients treated with 3A8 and CTLA4Ig (n=2, black diamond = DJ7L, red diamond = DN95).

d. Representative CFSE-MLR analysis of alloproliferation. Shown is a representative series of CFSE MLR assays, which were performed for recipient ID# CX16 at three distinct time-points. Assays were performed “Pre-transplant”, before infusion of donor stem cells and before treatment with 3CS; “While chimeric”, during the period of treatment with 3CS, when donor chimerism was present; and “Post-rejection”, after discontinuation of 3CS immunosuppression and after transplant rejection. T cells were enriched from the recipient by bead-based elimination of antigen presenting cells (as described in Methods), and were then labeled with the fluorescent dye, CFSE. These cells were co-incubated either without stimulators (black columns), with irradiated PBMCs derived from the transplant donor (white columns), or irradiated PBMCs derived from a third-party animal (gray columns) for 5 days at 37°C. After 5 days, the cultures were analyzed by flow cytometry to determine the percentage of the transplant recipient’s T cells that had proliferated. The percent of CD4+ and CD8+ T cells that had undergone at least one cell division was determined using FloJo flow cytometry analysis software, and the nonspecific autologous percent proliferation was subtracted to yield the percent specific alloproliferation (y-axis).

e. Compartmentalized chimerism occurred in transplant recipients. Whole blood chimerism, T cell chimerism and B cell chimerism were determined for two of the transplant recipients (CW6F and CX6R). T and B cell chimerism was determined by first sorting CD3+/CD20− T cells or CD3−/CD20+ B cells using a FacsAria flow cytometric cell sorter. Chimerism was then determined either through qPCR or microsatellite analysis of disparate MHC alleles. Grey: whole blood chimerism. Black: T cell chimerism measured within two days of the whole blood chimerism determination. Red: B cell chimerism measured within two days of the whole blood chimerism determination.
Figure 2.
Alloantibody did not develop in 3CS-treated recipients. Two of the 3CS-treated recipients (CX16 and CX6R) were evaluated for alloantibody formation by incubating PBMCs from the donor with serum from the recipient, collected at the indicated time points. The presence of anti-donor antibody in the recipient was tested flow cytometrically on CD3+/CD20− cells by binding of a FITC-labeled goat anti-mouse IgG to the donor cells to which the recipient serum was bound. The positive control was a recipient of a combined tissue allograft from an independent transplant series who developed significant alloantibodies post-transplant.
Figure 3.
Rejection was associated with a shift toward CD95 positivity in both CD4+ and CD8+ T cells.

a. Representative flow cytometric analysis of CD95 expression on both CD4+ and CD8+ T cells. CD3+ T cells were first identified and then were gated for either CD4+/CD8−/CD3+ T cells or CD4−/CD8+/CD3+ T cells. The percent of these cells that expressed CD95 was then determined using FloJo flow cytometry analysis software.

b. The percent of CD4+/CD8−/CD3+ T cells that were either CD95+ (red circles) or CD95− (black circles) was determined longitudinally in all three 3CS-treated recipients (CW6F, CX6R and CX16). Purple arrow: day of transplant rejection.

c. The percent of CD4−/CD8+/CD3+ T cells that were either CD95+ (red circles) or CD95− (black circles) was determined longitudinally in all three 3CS-treated recipients (CW6F, CX6R and CX16). Purple arrow: day of transplant rejection.
Figure 4.
Chimerism induction and phenotypic shifting in animals treated with anti-CD154-based immunosuppression.

- **a.** Transplant recipients treated with an anti-CD154 monoclonal antibody, belatacept and sirolimus develop mixed chimerism that was rejected while the animals were still undergoing immunosuppressive therapy.
- **b.** The percent of CD4+/CD8−/CD3+ (top row) or CD4−/CD8+/CD3+ (bottom row) T cells that were either CD95+ (red circles) or CD95− (black circles) was
determined longitudinally in two of the anti-CD154/belatacept/sirolimus-treated recipients (CW54 and RPh9). Purple arrow: day of transplant rejection.
Figure 5.
Measuring blockade of CD40 on B cells after transplantation with 3CS immunosuppression.

**a.** Flow cytometric analysis of the expression of CD40 on CD20+ B cells in the absence and presence of the 3A8 anti-CD40 antibody. Peripheral blood was drawn longitudinally from transplant recipients receiving 3CS immunosuppression. The saturation of CD40 by 3A8 was determined flow cytometrically by measuring the binding of a fluorescently conjugated cross-blocking anti-CD40 clone (5C3) on CD20+/CD3− B cells.

**b.** Longitudinal flow cytometric analysis of CD40 blockade in the 3CS recipients CW6F, CX6R and CX16. CD20+/CD3− B cells were identified flow cytometrically and the expression of CD40 on these cells was determined by binding of the fluorescent-labeled 5C3 anti-CD40 clone. The degree of masking of CD40 expression by 3A8 is shown as the mean fluorescence intensity in the CD40 fluorescence channel over time.

**c.** Rhesus anti-mouse antibody (RAMA) was determined by ELISA for a Rhesus anti-mouse IgG RAMA was quantified by an ELISA assay using 3A8 as the target antigen, serial dilutions of recipient serum, and detection with a 1B3-horse radish peroxidase anti-macaque IgG (obtained from the Non-human Primate Reagent Resource). The presence of RAMA was expressed as an end-point titer, which was defined as the dilution-factor of the recipient serum that generated an ELISA OD that exceeded the OD of the pretreatment sample at the same dilution by 2-fold. Filled circles: 3CS-treated recipients. Blue circles = CW6F; Green circles = CX6R; Purple circles = CX16. The red triangles show the end-point titer in the animal (DJ5V) treated with the 3A8 anti-CD40 antibody alone.

**d.** Serum trough levels of the 3A8 antibody as measured by ELISA (for the murine IgG2b isotype) for the three 3CS recipients, CW6F, CX6R, and CX16 by manual interpolation from an IgG2b standard curve.
### Table 1

Summary of Transplant Source, Cell Dose and Immunosuppressive Regimens

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Stem Cell Source</th>
<th>Immunosuppression</th>
<th>TNC/kg</th>
<th>CD34+/kg</th>
<th>CD3+/kg</th>
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<tr>
<td>CW6F</td>
<td>CW6H</td>
<td>Bone Marrow</td>
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