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Non-Depleting Anti-CD40-Based Therapy Prolongs Allograft Survival in Nonhuman Primates

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Abstract
Costimulation blockade of the CD40/CD154 pathway has been effective at preventing allograft rejection in numerous transplantation models. This strategy has largely depended on mAbs directed against CD154, limiting the potential for translation due to its association with thromboembolic events. Though targeting CD40 as an alternative to CD154 has been successful at preventing allograft rejection in preclinical models, there have been no reports on the effects of CD40-specific agents in human transplant recipients. This delay in clinical translation may in part be explained by the presence of cellular depletion with many CD40-specific mAbs. As such, the optimal biologic properties of CD40-directed immunotherapy remain to be determined. In this report, we have characterized 3A8, a human CD40-specific mAb and evaluated its efficacy in a rhesus macaque model of islet cell transplantation. Despite partially agonistic properties and the inability to block CD40 binding of soluble CD154 (sCD154) in vitro, 3A8-based therapy markedly prolonged islet allograft survival without depleting B cells. Our results indicate that the allograft-protective effects of CD40-directed costimulation blockade do not require sCD154 blockade, complete antagonism or cellular depletion, and serve to support and guide the continued development of CD40-specific agents for clinical translation.

Keywords
Islet transplantation; immunosuppressive therapy; nonhuman primate; monoclonal antibodies

Introduction
Since elucidation of the costimulatory interactions critical for optimal T cell activation (1), therapeutic targeting of T cell costimulation has been of great interest to the transplant community (2, 3). While manipulation of the CD28/CD80/86 pathway by abatacept (CTLA4Ig) was FDA approved in 2005 and phase III studies of belatacept (a high affinity variant of CTLA4Ig) have been completed, clinical development of blockers of the CD40/CD154 pathway has been slow and fraught with challenges. Though targeting this pathway...
has shown profound experimental effects on alloimmunity in preclinical models, attempts at clinical translation were nearly abandoned due to an association with thromboembolic events in preclinical studies and clinical trials (4, 5). The recognition that anti-CD154 mAbs may cause thromboembolism by binding CD154 on platelets has left open the possibility that therapeutic targeting of CD40 may achieve the immunosuppressive effects of inhibiting this pathway without disrupting hemostatic mechanisms (6–8).

CD40 is a member of the TNF receptor family and is constitutively expressed on B cells, dendritic cells (DCs) and macrophages. Both cell-surface-bound and soluble CD154 (sCD154) ligation of CD40 enhances antigen presentation and is required for T cell dependent humoral immunity (9). Specifically, CD40 signaling promotes cytokine production and the induction of costimulatory molecules on the surface of DCs, allowing them to mature and effectively activate T cells. Engagement of CD40 on B cells is essential for antibody production, isotype switching, affinity maturation, and the generation of plasma cells and B cell memory. Overall, CD40-CD154 interactions augment antigen presenting cell (APC) differentiation, proliferation and survival, processes critical to the generation of an acquired immune response (10).

The mechanisms underlying the effects of anti-CD154 therapy have not been entirely defined. While the potent immunosuppression observed with agents targeting CD154 is generally thought to result from blockade of CD40/CD154-mediated costimulation, induction of CD4+ T cell apoptosis (11) and T cell depletion (12) have been proposed as alternative mechanisms of action. Hence the ability of CD40-specific therapy to reproduce the immunosuppressive effects of CD154-directed blockade has been questioned. Nonetheless, successful targeting of CD40 as an alternative to CD154 has been achieved in preclinical animal models, including renal allograft protection in nonhuman primates (NHPs) (13–17). Moreover, our group reported extended graft survival using a B cell depleting chimeric anti-CD40 (Chi220) in a rhesus macaque model of allogeneic islet cell transplantation (18). Despite these preclinical observations, a CD40-specific agent has yet to be tested in humans. This delay in translation can in part be explained by the presence of cellular depletion in instances of anti-CD40-mediated graft survival, and variability amongst mAbs used in regards to degree of CD40 antagonism, isotype, and immunogenicity. Thus, the ideal biologic properties of a CD40-specific antibody for clinical translation remain to be determined.

In this study we report on 3A8, aCD40-specific mouse IgG2b mAb. Here we have characterized its effects on CD40, specifically showing that 3A8 has the ability to inhibit T cell alloreactivity despite allowing binding of recombinant sCD154 and the induction of at least partial CD40 signaling in vitro. We have also evaluated 3A8 for its efficacy in preventing allograft rejection in a NHP model of pancreatic islet cell transplantation. We observed that 3A8 prolonged islet allograft survival without evidence of cellular depletion, indicating that non-depleting CD40-specific blockade is capable of protecting transplanted grafts via targeting of the CD40/CD154 pathway.

**Materials and Methods**

**Polychromatic flow cytometry (PFC)**

Surface staining was performed on peripheral whole blood or lymphocytes isolated from rhesus macaques. After red blood cell lysis, cells were incubated with surface marker antibodies specific for CD3 [SP34-2], CD20 [2H7], CD40 [5C3] (BD Biosciences, San Jose, CA), and mouse IgG2b (Southern Biotech, Birmingham, AL), washed twice, processed on the LSR II (BD Biosciences), and analyzed with FlowJo software (Tree Star, Ashland, OR).
Binding and activation assays

**CD40 Binding**—Macaque lymphocytes were incubated with indicated doses of 3A8 or IgG2b isotype control (Bio × Cell, West Lebanon, NH), washed and then stained with surface marker-specific antibodies, including a fluorochrome-conjugated antibody competitive with 3A8 for CD40 [5C3] (BD Biosciences) at 5 μl of commercial antibody in 200 μl of cells.

**sCD154 Blockade**—Human peripheral blood lymphocytes were first incubated with indicated doses of 3A8, IgG2b isotype control or Chi220 (Bristol-Myers Squibb, Princeton, NJ), washed and then incubated with histidine-tagged recombinant sCD154 at 1 μg/ml (R&D Systems, Minneapolis, MN). Cells were then stained with surface marker-specific antibodies, including an anti-poly-histidine antibody (R&D Systems) for the detection of cell-bound sCD154.

**B cell Activation**—Rhesus lymphocytes were incubated for 24 hours with 3A8 or IgG2b isotype control at 10 μg/ml, washed and then stained with surface marker-specific antibodies, including fluorochrome-conjugated antibodies specific for CD80 [L307.4] and CD86 [FUN-1] (BD Biosciences).

PFC analysis was used for all assays as described.

Mixed lymphocyte cultures (MLCs)

Lymphocytes isolated from rhesus macaques were designated “responders” or “stimulators”. Responder cells were depleted of APCs by magnetic cell sorting using MACS LD columns and HLADR and CD20 beads according to package inserts (Miltenyi Biotec, Auburn, CA). Responders were then labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE), while non-CFSE labeled stimulators were γ-irradiated to ensure unidirectional proliferation. Responder-stimulator pairs combined at a 1:2 ratio (2 × 10^5 responders: 4 × 10^5 stimulators) were cultured in 96-well plates in 10% FBS/RPMI. After 5 days cells were harvested and surface stained for proliferation analysis by PFC.

NHPs

Captive bred rhesus macaques (Macaca mulatta) used as donors (10–20 kg) and recipients (3–5 kg) were obtained from Yerkes National Primate Research Center (Atlanta, GA). All recipients were specific pathogen-free and seropositive for rhesus cytomegalovirus (CMV). Donor-recipient pairs had at least one MHC class I and/or one MHC class II allele mismatch (Table 1). All pairs were alloreactive by MLCs. All experiments were conducted in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals and approved by Emory University’s Institutional Animal Care and Use Committee.

Donor pancreatectomy and islet isolation

Donor pancreatectomies were performed one day prior to transplantation. Via a midline laparotomy incision, the pancreatic tail and spleen were mobilized, the short gastric vessels divided, and the pancreatic body dissected free. Heparin (200 units/kg) was administered, the infrarenal aorta cannulated and the animal exsanguinated. Cold saline slush was immediately packed around the pancreas. Using sharp dissection, the common bile and pancreatic ducts were ligated, and the remainder of the pancreas mobilized and removed en bloc.
Pancreatic islet isolation was achieved through minor modifications of the automated method for human islet isolation (19) using Liberase HI (0.71 mg/mL; Roche Applied Science, Indianapolis, IN). The pancreas was enzymatically and mechanically disrupted, and the digest purified on a four layer, discontinuous Euroficol gradient (densities 1.108, 1.097, 1.069 and 1.037; Mediatech, Herndon, VA) and Cobe 2991 blood cell processor (Caridian BCT, Lakewood, CO). Samples of the final islet preparation were stained with dithizone, counted and expressed as islet equivalents (IEQs)(20).

**Diabetes induction and islet transplantation**

Diabetes was induced by streptozocin (STZ, 1250 mg/m² IV; Zanosar, Teva Parenteral Medicines, Irvine, CA) 4 weeks prior to transplant. Two historical control animals (RQz6, Rlb7) underwent duodenal-sparing total pancreatectomies for diabetes induction 2–4 weeks prior to transplant, as previously described(21). Post-diabetes care consisted of blood glucose control and supportive measures.

After overnight culture, islets were counted and re-suspended in transplant media. Recipient abdomens were opened via a midline mini-laparotomy incision, a mesenteric colic vein cannulated with a 20-gauge catheter and the islet suspension infused into the portal vein and liver.

**Glucose management**

Fasting and non-fasting blood glucose levels were measured daily via ear-stick. Insulin NPH (Novolin; Novo Nordisk, Princeton, NJ) and glargine (Lantus; Sanofi-Aventis, Bridgewater, NJ) were administered three times daily with the goal of maintaining fasting blood glucose (FBG)<300 mg/dL in pre-transplant diabetic monkeys and in those that had rejected their grafts. Intravenous glucose tolerance tests (IVGTTs) were performed pre-transplant to confirm diabetes and periodically post-transplant to monitor graft function. After transplant and islet engraftment, rejection was defined as FBG >130 mg/dL on two consecutive days.

**Experimental groups and immunosuppression**

Islet recipients received 3A8 plus basiliximab and sirolimus, 3A8 alone, or basiliximab and sirolimus alone. 3A8 was given intravenously on postoperative day (POD) 0 and 3 at 20 mg/kg, 7, 10 and 14 at 10 mg/kg, and 17, 21, 24, 28, 31 and 35 at 5 mg/kg. Basiliximab was administered on POD 0 and 3 (0.3 mg/kg IV), and sirolimus dosed IM once daily to achieve trough levels of 5–15 ng/mL until initiation of withdrawal on POD 120 and complete discontinuation on POD 134. Anti-viral prophylaxis consisting of oral valganciclovir (60 mg twice daily) was administered to all recipients while on immunosuppressive therapy. The basiliximab-and sirolimus-treated group consisted of one contemporaneous (RMc11) and two historical (RQz6 and Rlb7) controls that received oral sirolimus for target trough levels of 8–12 ng/mL (21). The hybridoma producing 3A8 was obtained from the American Type Culture Collection (Manassas, VA). Antibody was produced in vitro in serum-free medium and purified by protein A chromatography. Endotoxin level was < 1 EU/mg. Basiliximab (Simulect; Novartis, East Hanover, NJ), and valganciclovir (Valcyte; Roche, Nutley, NJ) were purchased from the Emory University Hospital pharmacy, and sirolimus (Rapamune) from LC Laboratories (Woburn, MA).

**Donor-specific antibody(DSA)detection**

Isolated lymphocytes (5 × 10⁵ cells) from donor whole blood were blocked with goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), incubated with serial recipient sera, and washed twice before incubating with FITC-labeled goat anti-monkey IgG (KPL,
Statistical Analysis

Islet allograft survival curves were created using the product limit method of Kaplan and Meier, and the logrank (Mantel-Haenszel) test was used to compare survival between groups. A P value < 0.05 was considered statistically significant.

Results

3A8 binds to rhesus CD40, but does not block sCD154

3A8 was originally generated in mice immunized with cells expressing recombinant human CD40 (22). To evaluate the ability of 3A8 to bind rhesus CD40, we used a binding assay where lymphocytes isolated from rhesus monkeys were incubated with increasing doses of 3A8 and then a competitive fluorochrome-conjugated anti-CD40 antibody to measure CD40 saturation. In contrast to mouse IgG2b isotype control, 3A8 saturated CD40 and prevented its detection by the competitive antibody on CD20<sup>+</sup> B cells (Figure 1A). While CD40 MFI decreased in the presence of 3A8, CD40 levels did not change with increasing doses of IgG2b (Figure 1B). As such, this dose-dependent saturation of CD40 establishes the specificity of 3A8 for rhesus CD40.

3A8 has been shown to inhibit cell contact-dependent human B cell proliferation induced by a murine thymoma cell line (22). However, the direct effect of 3A8 on sCD154 binding has not been examined. We therefore sought to characterize the relationship between 3A8 and sCD154 using PFC. Lymphocytes incubated with recombinant sCD154 in the presence of 3A8, IgG2b or Chi220, a chimeric CD40-specific mAb previously shown by our group to prevent sCD154 binding (18), were assessed for bound sCD154. Surprisingly, 3A8 did not block CD40 engagement of sCD154 (Figure 1C). CD40-bound sCD154 persisted despite increasing doses of 3A8, whereas Chi220 prevented sCD154 binding in a dose-dependent manner (Figure 1D).

3A8 inhibits T cell alloreactivity

Given the established ability of 3A8 to block the effects of cell contact-dependent, CD40-surface CD154 interactions (22), and the unexpected inability of 3A8 to inhibit CD40 engagement of recombinant sCD154, we investigated the functional effect of 3A8 on CD40-sCD154 interactions using MLCs. CFSE-labeled responder lymphocytes were cultured in the presence of increasing doses of 3A8 or IgG2b isotype control, and the degree of T cell proliferation in response to alloantigen was determined. 3A8 suppressed T cell allo-proliferation, as the percentage of proliferating cells declined in the presence of 3A8 (Figure 2A). In contrast, IgG2b isotype control, 3A8 abrogated CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferative responses in a dose-dependent manner (Figures 2B and 2C). These results suggest that despite permitting sCD154 binding, 3A8 is most likely inhibiting T cell alloreactivity by blocking functional CD40-surface CD154 interactions.

3A8 induces costimulatory molecule upregulation

In order to ascertain whether 3A8 elicits any signaling activity through CD40 or is a functionally inert blocker, we used PFC to analyze surface expression of the activation markers CD80 and CD86 on CD20<sup>+</sup> lymphocytes incubated with 3A8 or IgG2b isotype control. In 3A8-treated lymphocytes, we observed an increase in the percentage of CD80<sup>hi</sup> and CD86<sup>hi</sup> CD20<sup>+</sup> B cells, an effect not observed in untreated or IgG2b-treated samples (Figure 3A). This 3A8-induced upregulation of costimulatory molecules was a more than 1.5-fold increase relative to an untreated control (Figure 3B). Additionally, the MFI of
CD80 and CD86 on B cells approximately doubled in response to 3A8 (Figure 3C), supporting the observed rise in CD80^hi and CD86^hi cells. These findings suggest that 3A8 may act as a partial agonist by inducing stimulatory signals on CD40-bearing cells.

**3A8 promotes islet allograft survival**

Having defined 3A8 as a partially agonistic CD40-specific mAb with immunosuppressive potential via inhibition of T cell allo-proliferation in vitro, we set out to test the ability of 3A8 to extend allograft survival in a NHP model of islet cell transplantation. We combined 3A8 with basiliximab (anti-IL-2Rα mAb) and sirolimus, base therapy that does not confer a survival advantage to transplanted islets (21). By using this regimen, the experimental effect of 3A8 could be directly interpreted from any prolongation of graft function.

STZ-induced diabetic rhesus macaques were transplanted allogeneic islets (>10,000 IEQ/kg) from MHC-mismatched donors and immunosuppressed with 3A8, basiliximab and sirolimus maintenance therapy (Table 1). All animals experienced immediate return to euglycemia following transplant and prolonged islet allograft survival (Figures 4A and 4B). In contrast, controls treated with 3A8 alone, or basiliximab and sirolimus rejected shortly after transplant despite initial islet engraftment and resolution of hyperglycemia (Figures 4A, 4C and 4D). Daily FBG levels and periodic glucose tolerance tests with c-peptide measurements confirmed glycemic control and graft function in surviving islet allograft recipients (Figure 4B and data not shown).

Notably, all recipients remained insulin independent while on immunosuppressive therapy, and did not proceed to reject their grafts until after the discontinuation of sirolimus on POD 134. 3A8 facilitated islet allograft survival to 312 days in one animal, almost six months beyond the cessation of immunosuppression (Figure 4A). Histologic analysis of recipient livers after the loss of insulin independence showed dense focal lymphocytic infiltrates and destruction of transplanted islets, findings consistent with graft rejection (data not shown).

Given concerns over the potential for anti-CD40 therapy to disrupt the maintenance of protective immunity to latent viral infections, we monitored peripheral blood levels of rhesus CMV. With the use of anti-viral prophylaxis in this regimen, no recipients developed latent virus reactivation (data not shown). Furthermore, monkeys exhibiting extended islet allograft function experienced excellent weight retention and growth, both sensitive markers of primate health (data not shown). Pathologic analysis of all 3A8-treated islet recipients at necropsy was grossly and microscopically negative for evidence of thromboembolism.

**3A8 is non-depleting**

Past success targeting CD40 to protect transplanted allografts has been associated with B cell depletion (13, 16–18), leaving open the possibility that depletion is an important mechanism underlying the immunosuppressive effects observed with CD40-directed therapy. Examination of the various leukocyte lineages in 3A8-treated monkeys by PFC revealed no alteration in circulating lymphocyte counts. The absolute numbers of CD3^+ and CD20^+ cells in the peripheral blood did not decrease during or after 3A8 administration (Figure 5A). This was in stark contrast to the profound depletion seen in previous studies with the depleting anti-CD40 mAb Chi220 (Figure 5B) (13, 18).

**3A8 achieves CD40 saturation in vivo**

CD40 saturation was assessed in 3A8-treated animals by serial pharmacodynamic monitoring of peripheral blood using PFC. CD20^+ B cell CD40 saturation was achieved in 3A8-treated recipients as determined by CD40 MFI using the described competitive binding assay; unbound CD40 was significantly greater pre-treatment than while on 3A8 (Figure
3A8 was detected on the surface of CD40-bearing cells by staining lymphocytes from animals receiving 3A8 with an anti-mouse IgG2b antibody (Figure 6B), indicating that 3A8 coated targeted cells. This effect was drug-dependent and resolved upon discontinuation of 3A8 (Figure 6C). Although 3A8 bound CD40, we cannot exclude the possibility that the decrease in unbound CD40 could in part be due to lower CD40 expression levels.

**3A8-based therapy attenuates DSA formation**

Given the crucial role CD40 plays in the generation of antibody responses, we used PFC to test recipient monkey sera for the presence of donor-specific IgG antibodies. In relation to pre-transplant levels, no recipient with prolonged graft survival formed detectable antibodies during therapy and only one had developed alloantibodies at the time of immunosuppression withdrawal (Figure 7). Three monkeys had DSAs present at the time of rejection. Interestingly, two of the five long-term survivors had not generated alloantibodies despite histologic rejection.

**Discussion**

While several anti-CD40 mAbs have been shown to promote allograft survival in preclinical models, the optimal biologic properties of CD40-specific agents have not been defined. Here we show that 3A8, a CD40-specific mAb extends islet allograft survival in rhesus monkeys without evidence of cellular depletion. Treatment with 3A8 was well-tolerated and prevented alloantibody production in most recipients while receiving immunosuppression. Mechanistic studies demonstrated that 3A8 was efficacious despite allowing CD40 ligation of recombinant sCD154 and partial agonistic activity in vitro.

Several CD40-specific mAbs have been evaluated in preclinical transplant models. Functional antibody properties that can be rationally modified include 1) blockade of ligand binding, 2) a range of agonistic effects, 3) cellular depletion, and 4) serum half-life. Determinants of these properties might include the target epitope, the parent isotype, or engineered mutations that modify complement and Fc receptor binding. Additionally, humanization might affect immunogenicity. Thus far, complete antagonists and partial agonists, engineered as IgG1(Chi220) or IgG4 (ch5D12) chimeric isotypes, or fully human IgG4(4D11) have all prolonged allograft survival in primates despite different characteristics (13–18). Furthermore, our group has shown that IgG1 and IgG2b isoforms of the rat anti-mouse CD40 antibody 7E1 have variable effects on B cell proliferation in vitro, and opposite effects in vivo, as only the IgG2b isotype promoted allogeneic bone marrow chimerialsm and skin graft survival in mice (23). Taken together with our findings, it is clear that the mechanisms underlying the immunosuppressive effects of targeting the CD40 pathway are not yet fully understood. Hence, candidate CD40-specific therapeutics will continue to require careful characterization of effective anti-CD40-mediated mechanisms.

It is well established that CD154 is the ligand for CD40 and that it plays a critical role in generation of the alloimmune response (2, 24), at least in large measure by stimulating APCs expressing CD40. CD154 exists and is biologically active in both cell-surface and soluble forms (25). sCD154 has been shown to activate APCs in vitro (9) and induce allograft rejection independent of cell-bound CD154 in vivo (26). 3A8 was originally described for its inhibition of cell contact-dependent, CD40-mediated human B cell proliferation induced by a murine cell line putatively expressing surface-bound CD154 (22). This effect occurred in the absence of human T cell supernatant, making it unlikely that inhibition of sCD154 binding was an important mechanism of action in that assay system. Our results showing that 3A8 does not block CD40 binding of a recombinant sCD154 are compatible with these prior observations, albeit unanticipated, considering the in vivo efficacy of 3A8. These findings do not minimize the relevance of sCD154 in immunity nor preclude an advantage...
of blocking its function, but rather highlight a unique aspect of targeting CD40 for immunosuppressive purposes. Namely, a CD40-specific agent need not block sCD154 engagement of CD40 to prevent allograft rejection.

Although the thromboembolic complications associated with antibodies directed against CD154 are generally thought to be CD40-independent (7, 8), the role of CD40-dependent mechanisms in thrombosis remains unclear. While there is evidence CD154 can regulate arterial thrombus stability via pathways unrelated to CD40 (6), sCD154 ligation of CD40 on platelets and endothelial cells has been proposed to activate platelets (27) and incite endothelial cell-mediated inflammation (28) two processes critical to hemostasis. Therefore, designing an anti-CD40 mAb with the sCD154-permissive characteristic of 3A8 might provide a theoretical advantage over agents that block sCD154 by avoiding potential disruption of any as yet undiscovered CD40-sCD154-driven mechanisms pivotal to thrombus homeostasis.

Agonistic antibodies targeting CD40 have traditionally been used to boost immune responses against infectious agents or malignancies, but more recent data has described instances of reduced immunity resulting from CD40 agonism (29). Moreover, our group has reported prolongation of allograft survival in primate kidney and islet models with Chi220, a partially agonistic anti-CD40 mAb (13, 18). Although the effects of partial stimulatory signaling via CD40 in the setting of alloantigen exposure are unknown, they may limit proliferation of CD40-bearing cells or predispose APCs to activation-induced cell death. In fact, activation of CD40 on some cancer cell lines has led to growth inhibition and apoptosis (30, 31), and the differential activation of two distinct but reciprocal CD40 signaling pathways has induced counteractive immune responses in a mouse model of parasitic infection (32). Though CD80 and CD86 are indicators of APC activation, their expression may be markers of an early or incompletely activated state requiring additional stimulation to avoid anergy or apoptosis. Existing data (22) suggests that 3A8 acts at least in part as a functional blocker of CD40-surface CD154 interactions, but it is possible that mechanisms altering APC maturation and/or survival contribute to its effect on the alloimmune response.

Previous examples of allograft protection in NHPs by CD40-specific mAbs have included cellular depletion (13, 16–18). This concurrent finding has made it difficult to discern the relative mechanistic contribution, if any, of depletion on anti-CD40-mediated graft survival. Our group did show that B cell depletion was not solely responsible for the immunosuppressive effects of Chi220 (18); however, analysis was limited by the inability to dissect out the role of other CD40-bearing cell populations. The present study shows that the depletion of cells expressing CD40 is not necessary to achieve allograft protection when using mAbs directed against CD40.

Conceptually, depleting antibodies are clinically less desirable in that they have potential to excessively compromise protective immunity, induce homeostatic proliferative mechanisms (33), and limit dosing regimens. The lack of a depletion requirement makes the administration of CD40-specific agents for longer treatment periods more feasible and clinically attractive, opening up the possibility for use as an alternative or companion to belatacept as either induction or maintenance immunotherapy.

It has been repeatedly shown that CD40/CD154 pathway inhibition can have potent effects on experimental alloimmunity. Here we provide additional evidence that therapeutics directed against CD40 as an alternative to CD154 can be highly efficacious. Our data not only support the existing wealth of science that promotes continued pursuit of manipulating CD40/CD154 interactions for use in human transplantation, but also provide new
mechanistic insight regarding the biologic properties of CD40-specific therapy needed to guide clinical development.

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List of Non-standard Abbreviations

- **APC**: antigen presenting cell
- **CFSE**: carboxyfluorescein diacetate succinimidyl ester
- **CMV**: cytomegalovirus
- **DC**: dendritic cell
- **DSA**: donor-specific antigen
- **FBG**: fasting blood glucose
- **IEQ**: intravenous glucose tolerance test
- **IFN-γ**: interferon-γ
- **IVGTT**: intravenous glucose tolerance test
- **MFI**: mean fluorescence intensity
- **MLC**: mixed lymphocyte culture
- **NHP**: nonhuman primate
- **PFC**: polychromatic flow cytometry
- **POD**: postoperative day
- **sCD154**: soluble CD154
- **STZ**: streptozocin

References


Figure 1. 3A8 binds to rhesus CD40, but does not block sCD154
PFC was used to determine the specificity of 3A8 for rhesus CD40. (A) A competitive CD40-specific antibody was used to detect saturation of CD40 by 3A8 on CD20+ lymphocytes incubated with 3A8 (10 μg/ml). (B) CD40 saturation was measured using a competitive CD40-specific antibody as above at the indicated doses of 3A8 and IgG2b isotype control. (C) To test the ability of 3A8 to block sCD154 binding to CD40, lymphocytes were incubated with 3A8, IgG2b isotype control or Chi220 (chimeric anti-CD40 mAb) at 10 μg/ml and then recombinant sCD154 at 1 μg/ml. Cells were then secondarily stained for sCD154. (D) Ability of various concentrations of 3A8, IgG2b and Chi220 to interfere with sCD154 binding. Data is representative of 3 independent experiments.
Figure 2. 3A8 inhibits alloreactive T cell proliferation
CFSE-labeled responder T cells were allo-stimulated in MLCs for 5 days in the presence of 3A8 or IgG2b isotype control and analyzed by PFC. (A) The percentage of proliferating cells (top left PFC plot corner) is depicted in the presence of 3A8 at the indicated doses. CD3^+CD8^+ PFC plots shown are representative. Similar findings were observed with CD4^+ T cells. % Maximum (B)CD4^+ and (C) CD8^+ T cell proliferation (n = 5) at increasing doses of 3A8 and IgG2b isotype control. % Maximum is the percentage of proliferating cells at the indicated dose of 3A8 or IgG2b divided by the percentage of cells proliferating in the absence of antibody. Data represent mean ± SEM from 4 independent experiments.
Figure 3. 3A8 induces upregulation of CD80 and CD86 on B cells in vitro

PFC was used to analyze the surface expression of CD80 and CD86 on CD20⁺ lymphocytes incubated for 24 hours with 3A8 or IgG2b isotype control at 10 μg/ml. (A) Percentage of CD80⁺hi and CD86⁺hi CD20⁺ cells in untreated, IgG2b- and 3A8-treated lymphocytes. (B) Fold change in the % of CD80⁺hi and CD86⁺hi B cells (n = 3). (C) Fold change in MFI of CD80 and CD86 on untreated and treated B cells (n = 3). Data represent mean ± SEM from 3 independent experiments.
Figure 4. 3A8 prolongs islet allograft survival
STZ-induced diabetic rhesus monkeys were transplanted allogeneic islets under cover of 3A8 (5–20 mg/kg) plus basiliximab (0.3 mg/kg) and sirolimus (target trough levels 5–15 ng/ml). Rejection was defined as FBG > 130 mg/dL on two consecutive days after initial islet engraftment. (A) Kaplan-Meier survival curves of 3A8 plus basiliximab/sirolimus-, 3A8-, and basiliximab/sirolimus-treated groups (n = 5, 2 and 3, respectively). 3A8 was discontinued on POD 35 (white arrow) and sirolimus on POD 134 (black arrow). Statistical analysis using the logrank test for graft survival between groups showed superiority of 3A8 plus basiliximab/sirolimus as compared to 3A8-and basiliximab/sirolimus-treated controls (P = 0.0082 and 0.0046, respectively). (B–D) Representative FBG graphs of islet recipients. Survival times (days) are listed for each corresponding graph.
Figure 5. 3A8 is non-depleting in vivo
Peripheral leukocyte counts were serially monitored using PFC in 3A8-treated monkeys. (A) Absolute numbers of CD3+ and CD20+ cells during and after 3A8 administration (n = 5). (B) Depletion of CD20+ lymphocytes observed in a previous study with the depleting CD40-specific mAb Chi220 (n = 5) (18). The black bars depict 3A8 and Chi220 dosing. Data represent mean ± SEM.
Figure 6. 3A8 achieves CD40 saturation in vivo
CD40 saturation was assessed in 3A8-treated animals by serial pharmacodynamic monitoring of peripheral blood using PFC. (A) Representative PFC plots of unbound CD40 on recipient CD20⁺ lymphocytes pre-transplant (PreTx, red) and while on 3A8 (blue) on POD 7 are shown, as determined by staining cells with a competitive anti-CD40 mAb (as described in Figure 1). (B) CD20⁺ lymphocytes were stained with anti-mouse IgG2b to demonstrate the presence of 3A8 on targeted cells after treatment. Data shown was obtained on POD 7 (4 days after last 3A8 dose). (C) Representative longitudinal graph of CD40 saturation by 3A8 on CD20⁺ lymphocytes in a 3A8-treated recipient, as determined by the competitive binding assay described above (3A8 dosing depicted by the black bars). The possibility that less unbound CD40 represents a combination of 3A8 saturation and lower CD40 expression levels cannot be excluded.
Figure 7. 3A8-based therapy attenuates DSA formation
Donor lymphocytes were incubated with corresponding islet recipient sera at the indicated time points and then analyzed by PFC to test for donor-specific IgG antibody formation. Each line represents one individual recipient (n = 5) and the order is maintained amongst the designated time points. Detectable antibody levels are shown pre-transplant (yellow), during therapy (green), and at immnosuppression withdrawal (blue) and rejection (red).
### Table 1

Recipient Groups and Islet Allograft Survival

<table>
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<tr>
<th>Therapy</th>
<th>Recipient ID</th>
<th>IEQ/kg</th>
<th>Graft Survival (days)</th>
<th>MHC Mismatch (n)</th>
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\(^\text{A} \) - Historical control (21)

ND – Non-detected amongst typed alleles