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A Novel Monoclonal Antibody to CD40 Prolongs Islet Allograft Survival

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Abstract

The importance of CD40/CD154 costimulatory pathway blockade in immunosuppression strategies is well documented. Efforts are currently focused on monoclonal antibodies specific for CD40 because of thromboembolic complications associated with monoclonal antibodies directed towards CD154. Here we present the rational development and characterization of a novel antagonistic monoclonal antibody to CD40. Rhesus macaques were treated with the recombinant anti-CD40 mAb, 2C10, or vehicle before immunization with keyhole limpet hemocyanin (KLH). Treatment with 2C10 successfully inhibited T cell-dependent antibody responses to KLH without significant peripheral B cell depletion. Subsequently, MHC-mismatched macaques underwent intraportal allogeneic islet transplantation and received basiliximab and sirolimus with or without 2C10. Islet graft survival was significantly prolonged in recipients receiving 2C10 (graft survival time 304, 296, 265, 163 days) compared to recipients receiving basiliximab and sirolimus alone (graft survival time 8, 8, 10 days). The survival advantage conferred by treatment with 2C10 provides further evidence for the importance of blockade of the CD40/CD154 pathway in preventing alloimmune responses. 2C10 is a particularly attractive candidate for translation given its favorable clinical profile.

Keywords

Co-stimulation Blockade; Islet Transplantation; Type 1 Diabetes Mellitus; Immunosuppressive therapy

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Disclosures

The authors of the manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation: RW, CPL and KAR have a patent application pending for technologies described in this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article: Supplemental Figure 1: Activation of rhesus B cells in vitro as measured by changes in expression of CD23 or CD80.

Introduction

Current immunosuppression strategies to prevent allograft rejection have substantial drawbacks. Chronic toxicities associated with calcineurin inhibitor (CNI) based regimens contribute to increased morbidity and mortality resulting from both cardiovascular disease(1–3) and allograft failure(4). Belatacept, a high affinity derivative of CTLA4-Ig, was recently approved by the Food and Drug Administration and European Medicines Agency for the prevention of rejection in renal transplantation. Belatacept preserved excellent patient and graft survival over the first three years while achieving 27–33% better renal function compared with CNI(5, 6). Belatacept also provided improved cardiovascular and metabolic risk profiles compared with CNI(7). However, patients treated with belatacept experienced higher rates and grades of acute rejection and a higher incidence of post-transplant lymphoproliferative disorders(5). Furthermore, belatacept is approved for use with concomitant steroid maintenance therapy. Thus, while belatacept represents an important advance for the field of transplantation, there are significant opportunities to develop novel immunosuppressive therapies that further improve quality of life and reduce morbidity after transplantation.

Therapeutic manipulation of CD40/CD154 pathway has been an appealing but elusive target since its discovery. Monoclonal antibodies (mAb) directed against CD154 demonstrate potent effects in preventing rejection and inducing long-term graft acceptance in nonhuman primates (NHP), particularly when coupled with CD28 pathway blockade (8–13). Unfortunately, clinical development of anti-CD154 mAb was halted due to thromboembolic complications observed in human studies, which are now linked to the expression of CD154 on platelets(14–16). While advances in mAb engineering may permit the development of monovalent, non-cross-linking CD154-specific antibody constructs that avoid thromboembolism, an alternative approach is the development of therapeutic mAb specific for CD40.

CD40 is constitutively expressed on B cells, macrophages and dendritic cells and is critical for B cell activation, immunoglobulin class switching and dendritic cell activation. A monoclonal antibody directed at CD40 ideally inhibits B cell activation without agonism or substantial peripheral B cell depletion. Several anti-CD40 mAbs have shown promise in various transplant models, but their progression to human translation is limited because of potentially adverse effects. Chi220, a chimeric IgG1 CD40-specific mAb, produced prolonged graft survival in both islet and renal models of transplantation in NHP (17, 18); however, treatment with Chi220 resulted in significant peripheral B cell depletion(17). Our group recently showed that the mouse anti-human CD40 mAb 3A8 significantly prolongs islet allograft survival in NHP(19); this mAb can, however, act as a partial agonist of B cells. Although the clinical importance of partial agonism and peripheral B cell depletion is unclear, anti-CD40 mAbs that neither agonize the B cell response nor cause substantial depletion may be more appealing candidates for clinical translation.

A fully human mAb to CD40, 4D11, has recently been shown to prolong both renal and islet allograft survival in NHP(20, 21). Phase I clinical trials in renal transplantation with this agent are currently in progress. Taken in total, the success of these anti-CD40 mAbs confirms the importance of targeting this pathway to prolong allograft survival and underscores the need to continue preclinical investigation of agents that block CD40. Here we present the development and characterization of 2C10, a novel mAb to CD40. This chimeric mouse-rhesus mAb lacks agonistic properties, binds to an epitope of CD40 unique from several other anti-CD40 mAbs, prevents antigen-specific antibody formation, and results in significantly prolonged islet allograft survival in NHP. These results provide additional support for efforts to develop clinically relevant CD40/CD154 pathway blockade.

Materials and Methods

Generation of anti-rhesus CD40 antibodies

A fusion protein consisting of the terminal 113 amino acids of rhesus CD40 protein fused to maltose binding protein (MBP) was expressed in bacteria and used to immunize A/J mice. Hybridomas were generated by fusion of splenocytes with SP2/0 myeloma cells and selected by screening for reactivity to rhesus CD40-glutathione synthase transferase fusion protein by enzyme-linked immunosorbent assay (ELISA) and by demonstrating binding to rhesus B-lymphoblastoid cell lines and to human and rhesus B cells by flow cytometry.

Generation and production of rhesus chimeric anti-CD40 antibodies

The immunoglobulin variable region genes were cloned from the hybridomas secreting anti-CD40 antibody clone 2C10 and from anti-human CD40 clone 3A8(22) (obtained from the American Type Culture Collection, ATCC, Vienna, VA) using 5' rapid amplification of cDNA ends-polymerase chain reaction. The immunoglobulin heavy and light chain variable regions were subcloned into expression vectors containing rhesus IgG1 or rhesus IgG4 heavy chain and rhesus kappa light chain constant region sequences.

Recombinant heavy and light chains were subcloned into expression vectors and packaged in retroviral vectors used to transduce Chinese hamster ovary cells using the GPEx expression technology (Catalent Pharma Solutions, Middleton, WI). A pool of transduced cells was grown in serum-free medium and secreted antibody was purified by protein A affinity chromatography. The purified chimeric rhesus IgG1 (2C10R1, 3A8R1) and IgG4 (2C10R4) antibodies were diafiltered into phosphate buffer; endotoxin levels were confirmed to be less than 1 endotoxin unit/mg.

CD40 and CD154 blocking assays

To confirm the specificity of 2C10 for rhesus and human CD40 in vitro, rhesus or human peripheral blood mononuclear cells (PBMC) were incubated with escalating concentrations of 2C10 or an isotype control then incubated with a fluorophore-labeled antibody to CD40 (clone 5C3, BD Bioscience, San Jose, CA) known to bind to CD40 of both species and analyzed by flow cytometry. To test the ability of 2C10 to block binding of CD154 in vitro, rhesus or human PBMC were incubated with escalating concentrations of 2C10 or an isotype control and incubated with soluble histidine-tagged recombinant human CD154 (R&D Systems, Minneapolis, MN). PBMC were analyzed by flow cytometry for anti-histidine secondary (R&D Systems, Minneapolis, MN) to detect cell-bound CD154.

Epitope comparison of anti-CD40 mAb

To determine if various mAb to CD40 (2C10, 3A8 and Chi220) bind similar epitopes, an in vitro competitive blockade assay was performed. 2C10 was conjugated to allophycocyanin (APC) using the Lightning Link antibody labeling kit (Novus Biologics, Littleton, CO). Human PBMCs were incubated with escalating concentrations of 2C10, 3A8 or Chi220, and then stained with the APC-conjugated 2C10 to assess the ability of each antibody to cross-block 2C10.

Functional assays for B cell blockade or activation

To test the ability of anti-CD40 mAb to functionally block activation via the CD154-CD40 pathway, rhesus PBMC were co-cultured with CD154⁺ Jurkat D1.1 cells (ATCC) at a 1:1 ratio in the presence of anti-CD40 or anti-CD154 mAbs. To test the ability of anti-CD40 antibodies to activate B cells via CD40 engagement, PBMC were cultured with varying concentrations of antibody in the absence of CD154 stimulation. Cells were stained with

CD20-FITC (L27, BD Bioscience), CD23-ECD (9P25, Beckman Coulter, Brea, CA), CD80-PE (L307.4, BD Bioscience) and CD86-APC (FUN-1, BD Bioscience), and analyzed by flow cytometry. Activation of B cells was determined by measuring changes in expression of CD23, CD80 and CD86 on CD20⁺ B cells.

T cell-dependent antibody response

Nine rhesus macaques (*Macaca mulatta*) were immunized once on day zero with 4-hydroxy-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (KLH, 10 mg IM) antigen (Biosearch Technologies, Novato, CA). Prior to immunization and at one week, cohorts of 3 animals received an intravenous dose (50 mg/kg) of 2C10R1, 2C10R4, 3A8R1 or saline. All animals were observed for 70 days, and flow cytometry was performed weekly.

T cell-dependent antibody responses to KLH-NP were tested by ELISA. Plates were coated with KLH (0.01 mg/ml, Sigma, St. Louis, MO) and blocked with Super Block (Thermo Scientific, Woodstock, GA). Pre- and post-treatment plasma samples were serially diluted, plated for 1 hr, and washed with phosphate-buffered saline/0.05% Tween. Anti-KLH antibodies were detected by incubating for 1 hr with monoclonal anti-rhesus IgG-horseradish peroxidase (clone 1B3, NHP Reagent Resource, Boston, MA). Plates were then incubated with Peroxidase Substrate Solution (KPL). Stop solution (KPL) was then added and optical density read on an ELISA plate reader at 450 nm. A sample was considered positive at a given dilution if the optical density reading of the post-treatment plasma exceeded the optical density of the pre-treatment plasma at the same dilution by 2-fold.

Donor pancreatectomy and islet isolation

Rhesus macaques weighing 10–20 kg underwent donor pancreatectomy one day prior to transplantation via a midline laparotomy. The pancreas was isolated and placed on ice after the animals were terminally exsanguinated. Islet isolation was performed using Collagenase/Neutral protease (950 Wunsch units and 63 units, respectively; Serva, Heidelberg, Germany). The digested pancreas was purified on a four layer, discontinuous Euroficoll gradient (Mediatech, Manassas, VA) and Cobe 2991 blood cell processor (CaridianBCT, Lakewood, CO). Samples of the final islet preparation were counted and expressed as islet equivalents (IEQ). Isolated islets were cultured overnight, counted and suspended in Transplant Media (Mediatech).

Diabetes induction and islet transplantation

Rhesus macaques weighing 3–5 kg were rendered diabetic using streptozotocin (1250 mg/m² IV; Zanosar, Teva Parenteral Medicines, Irvine, CA) four weeks prior to transplantation. Diabetes was confirmed by intravenous glucose tolerance test (IVGTT) with a 500 mg/kg bolus of dextrose and measurement of primate C-peptide. Glucose levels were monitored and C-peptide was measured at baseline and 10, 30, 60 and 90 after injection of dextrose. Diabetes was confirmed by measurement of elevated blood glucose levels in the absence of detectable serum C-peptide. Diabetic recipients underwent MHC-mismatched islet allotransplantation. A mean of 15,745 ($\pm$ 4,063) IEQ were infused via a small midline laparotomy and cannulation of a mesenteric vein.

Diabetes management and post-transplant monitoring

Blood glucose levels were measured twice daily by earstick; NPH (Novolin; Novo Nordisk, Princeton, NJ) and glargine (Lantus; Sanofi-Aventis, Bridgewater, NJ) insulin were administered to maintain fasting blood glucose (FBG) less than 300 mg/dL pre-transplant and following graft rejection. IVGTT was performed periodically post-transplant to monitor

graft function. Transplant recipients underwent weekly flow cytometric analysis to monitor T cell (CD3 V450, CD4 PerCP-Cy5.5, CD8 PerCp; BD Bioscience) and B cell (CD20 PE, BD Bioscience) populations. After islet engraftment rejection was defined as FBG greater than 130 mg/dL on two consecutive days. Primary endpoint was rejection-free islet graft survival. All animals used in these experiments were treated in compliance with the Emory University IACUC and the Guide for the Care and Use of Laboratory Animals(23).

Immunosuppression regimens

Transplant recipients received either 2C10R4, basiliximab (Simulect, Novartis, Basel, Switzerland) and sirolimus or basiliximab and sirolimus alone. 2C10R4 (50 mg/kg) was administered intravenously on post-operative day (POD) 0 and 7. Basiliximab (0.3 mg/kg) was administered intravenously on POD 0 and 3. Sirolimus was administered intramuscularly daily to achieve trough levels of 5–15 ng/mL through POD 120. All three animals receiving basiliximab and sirolimus alone are historic controls(24). Two of these historic controls (RQz6 and RIb7) underwent diabetes induction by pancreatectomy and received oral sirolimus.

Results

Rational generation of an anti-CD40 mAb

To generate a mouse mAb specific for CD40, mice were immunized with a fusion protein containing the extracellular domain of rhesus CD40. The extracellular domain of rhesus CD40 shares approximately 95% amino acid identity with human CD40; we therefore hypothesized that most of the rhesus mAbs generated would cross-react with human CD40. Seven mouse mAb clones specific for rhesus CD40 were produced; all seven cross-reacted with human CD40 as evidenced by binding to human B cells (data not shown). However, clone 2C10 was found to lack agonistic activity (data not shown) and to cause the most significant suppression of CD154-mediated B cell activation as measured by changes in expression of CD23 (Figure 1), CD80, and CD86 (data not shown). The inhibition of B cell activation by 2C10 was markedly more potent than the other six clones and was similar to the inhibition resulting from blockade with an anti-CD154 mAb.

2C10 blocks CD40 and prevents binding of CD154

To assess the ability of 2C10 to bind to both rhesus and human CD40, B cells from both species were incubated with escalating concentrations of 2C10 and analyzed for CD40 expression using the fluorophore-labeled anti-CD40 clone 5C3. 2C10 blocked binding of 5C3 to CD40 in a dose-dependent manner on both rhesus (Figure 2a) and human (data not shown) B cells compared to an isotype control. These results further confirm the specificity of 2C10 for rhesus CD40 and suggest similar binding affinity of 2C10 for both rhesus and human CD40, which is important for potential clinical translation of 2C10.

To confirm the ability of 2C10 to block binding of its cognate ligand, CD154, rhesus and human B cells were incubated with escalating concentrations of 2C10 and then incubated with histidine-tagged soluble CD154 and analyzed for histidine expression. 2C10 blocked the binding of CD154 in a dose-dependent manner on both rhesus (Figure 2b) and human (data not shown) B cells, suggesting that 2C10 can effectively block the interaction of T cell-bound CD154 with CD40 on B cells and antigen-presenting cells.

Unique properties of 2C10 distinguish it other anti-CD40 mAb

In an effort to compare CD40 epitope binding, we tested the ability of 2C10 to block binding of Chi220 and 3A8. Rhesus PBMC were incubated with escalating concentrations of 2C10, Chi220 and 3A8 followed by incubation with APC-conjugated 2C10. Binding of APC-

conjugated 2C10 decreased with increasing concentrations of 2C10 but not Chi220 or 3A8 (Figure 3), suggesting that 2C10 binds a unique epitope distinct from either Chi220 or 3A8.

We next sought to determine if the unique epitope of 2C10 resulted in distinct functional properties. When rhesus or human B cells were incubated with CD154⁺ Jurkat cells, 2C10 prevented the activation of B cells, evidenced by inhibition of CD23, CD80 and CD86 expression, as effectively as a mAb specific for CD154 (Supplemental Figure 1a). This in vitro inhibition of B cell activation was not seen with 3A8 and Chi220 when tested on either rhesus (Supplemental Figure 1a) or human (data not shown) B cells. In contrast, incubation of rhesus B cells with 3A8 and Chi220 resulted in upregulation of activation markers, whereas incubation with 2C10 or an anti-CD154 antibody had no effect on B cell activation (Supplemental Figure 1b). These findings suggest that 2C10 blocks B cell activation and acts primarily as a CD40 antagonist; this antagonism contrasts with 3A8 and Chi220, which we have previously shown to act as partial agonists with weak stimulatory potential(17, 19).

2C10 is minimally depleting and blocks a T cell-dependent antibody response

Having established that 2C10 binds to a unique epitope on CD40, inhibits B cell activation similarly to an anti-CD154 mAb, and lacks agonistic properties, we sought to further characterize the effects of 2C10 in vivo. Recombinant mouse-rhesus chimeric forms of 2C10 were generated using either rhesus IgG1 (2C10R1) or IgG4 (2C10R4) heavy chain and rhesus kappa light chain constant region sequences. Recombinant antibodies constructed with a rhesus IgG1 constant region have the potential for potent cytolytic effector function in vivo(25), whereas the in vivo effector function of rhesus IgG4 is substantially modulated (unpublished data). A chimeric rhesus IgG1 form of 3A8 (3A8R1) was also generated for use as a control.

To test the ability of these anti-CD40 mAb to block a T cell dependent antibody response, rhesus macaques were immunized with KLH and treated with 2C10R1, 2C10R4, 3A8R1 or vehicle (n=3 per group). Treatment with either recombinant 2C10 isotype resulted in modest change in peripheral B cell counts (Figure 4) compared to the previously reported significant and prolonged depletion of peripheral B cells occurring in animals receiving either 3A8R1(26) or Chi220(17).

Following KHL immunization, control animals developed high-titer KLH-specific IgG (Figure 5) and IgM (data not shown) responses. Animals that received 3A8R1 also developed anti-KLH responses but titers were approximately 10-fold lower than controls despite significant depletion of B cells. In contrast, the generation of both IgG and IgM anti-KLH antibodies was nearly completely blocked through day 56 in all animals that received either 2C10R1 or 2C10R4.

2C10 prolongs islet graft survival

We have thus far shown that 2C10 possesses several important characteristics for successful translation; specifically 2C10 lacks agonistic properties, causes modest depletion of CD40-expressing target cells such as B cells, and completely blocks a T cell-dependent antibody response to KLH. These characteristics justified proceeding with testing 2C10R4 in a NHP alloislet transplant model. 2C10R4 was administered as induction therapy with basiliximab induction and sirolimus maintenance therapy. Treatment with this regimen resulted in significantly prolonged islet graft survival (Figure 6a) compared to controls receiving only basiliximab induction and sirolimus maintenance therapy (Figure 6b). Median rejection-free graft survival time for animals receiving 2C10R4 is 280 days compared to 8 days for control animals (p=0.010, Table 1).

Pharmacokinetic data predict that plasma 2C10R4 levels would be less than 1 µg/ml by POD 100; since sirolimus was discontinued at POD120, the recipient with the longest survival (304 days) received no immunosuppression for approximately 24 weeks prior to rejection. No animals treated with 2C10R4 developed clinically relevant infectious complications or weight loss.

These results reflect animals that received the IgG4 isotype of 2C10. Two additional animals that received the IgG1 isotype of 2C10 (2C10R1) in combination with basiliximab and sirolimus achieved similarly prolonged graft survival of 220 and 162 days (data not shown).

Discussion

The importance of the CD40-CD154 pathway in transplant immunology has been well established. To achieve clinical translation of an immunosuppression regimen based on blockade of this pathway, we developed 2C10, a novel antagonistic mAb to CD40. We have shown here that 2C10 blocks the binding of CD154 to CD40 and inhibits CD154-mediated B cell activation. Primatized forms of this antibody caused nearly complete inhibition of the T cell-dependent antibody response to KLH. When combined with background immunosuppression, 2C10 significantly prolonged islet allograft survival in NHP. These results confirm the therapeutic potential of this anti-CD40 mAb.

Human and rhesus CD40 share approximately 95% amino acid identity in the extracellular domains; therefore, when generating the 2C10 antibody mice were immunized with rhesus CD40 protein with the expectation that any resulting antibody would retain cross-reactivity with human CD40. Indeed, all seven clones characterized did cross-react with human CD40. Although the specific CD40 epitope recognized by 2C10 has not yet been identified, binding of a cross-blocking antibody was inhibited on human and rhesus B cells by 2C10 at similar concentrations suggesting that the affinity of 2C10 for human CD40 is retained. Moreover, 2C10 was able to block CD154-mediated activation of human and rhesus B cells in vitro at similar antibody concentrations indicating similar potency in both species. The cross-reactivity of 2C10 against human and rhesus CD40 coupled with the demonstration of similar binding and functional properties in the two species provide important advantages for preclinical translational studies.

Previously studied mAbs to CD40 have demonstrated markedly different agonizing or antagonizing effects on activation signaling via CD40(17, 19). These varying biological activities likely result from differences in the epitope recognized by each mAb. Our data indicate that 2C10 binds to a CD40 epitope that is unique from both 3A8 and Chi220. Furthermore, 2C10 showed marked CD40 antagonism whereas 3A8 and Chi220 have agonistic activity in vitro. Intuitively, antagonistic anti-CD40 antibodies would seem to be preferred as immunosuppressive therapies. However, several previously tested anti-CD40s that caused partial agonism(17, 19) were also capable of significantly prolonging graft survival.

The potential for a therapeutic antibody to deplete target cells depends upon both epitope binding and the effector function of the antibody. The anti-CD40 mAbs 3A8 and Chi220 were both shown to cause potent B cell depletion in rhesus macaques when engineered with primate IgG1 heavy chains. However, 2C10 caused a relative lack of B cell depletion as both IgG1 and IgG4 recombinant antibodies while retaining strong immunosuppressive properties.

We postulate that the unique epitope specificity of 2C10 accounts for this lack of B cell depletion. When the identical rhesus IgG1 Fc was engineered into 3A8, another CD40-specific mAb that binds to a different epitope, substantial depletion was observed. In

addition, the identical IgG1 Fc has been engineered into three other mAb specific for CD4, CD8 α , and CD8 β ; all three of these antibodies cause complete target cell depletion, further implicating the uniqueness of the 2C10 epitope as opposed to a failure of Fc effector function.

For preclinical NHP testing, 2C10 was engineered as a mouse-rhesus chimeric antibody to minimize immunogenicity and better model the effect of humanized antibodies administered to humans. We chose to use a rhesus IgG4 constant region to minimize Fc receptor and complement binding(27) thereby limiting effector function. Of the native rhesus IgG isotypes, IgG4 has reduced cytotoxicity. In addition, the heavy chain constant region of native rhesus IgG4 has a stabilizing proline at hinge position 228 which may stabilize Fab arm exchange that can occur with human IgG4 antibodies (28, 29). The humanized form of 2C10 will retain an immunologically silent Fc with a stabilized Fab hinge.

To test the ability of recombinant 2C10 to block costimulation, macaques were administered this antibody concurrently with the T cell-dependent antigen KLH. Treatment with 2C10 resulted in nearly complete inhibition of the T cell-dependent antibody response to KLH despite preservation of peripheral B cells. This effect was similar for both the IgG1 and IgG4 chimeric antibodies. In contrast, administration of 3A8 as an IgG1 mouse-rhesus chimeric was only capable of blunting the anti-KLH antibody response despite substantial depletion of B cells(26).

We have also shown that a short course of treatment with 2C10 can significantly prolong allograft survival in a nonhuman primate model. When used as induction therapy with basiliximab and sirolimus, MHC-mismatched islets survive almost 300 days without significant weight loss or infectious complications. In this protocol, all immunosuppressive drugs were withdrawn at 120 days post-transplantation. In several 2C10-treated animals the allograft survived over 160 days after all immunosuppression was halted. Given these excellent results with use as induction therapy, we plan to assess the effects on graft survival by administering 2C10 as maintenance therapy.

Blockade of the CD40/CD154 pathway may prove useful in conjunction with other costimulation blockade agents. Belatacept, a high affinity version of CTLA4-Ig designed to block the CD28/B7 costimulatory pathways, has shown efficacy in NHP models of renal and islet transplantation and in phase II and III clinical trials in renal transplantation(5, 6, 17, 30–32). The BENEFIT trial revealed superior renal function in patients treated with belatacept; however, these patients had a higher incidence and more severe grade of biopsy-proven acute rejection(5, 6). In light of this increased rate of acute rejection and the synergy between CD40 and B7 blockade(11), we plan to test the efficacy of combined 2C10 and belatacept therapy in NHP kidney transplantation.

Antibodies targeting CD40 hold significant promise for translation to immunosuppressive regimens for use in human allotransplantation. The antibody 2C10 exhibits unique properties that are likely the result of unique epitope binding. These results provide support for clinical targeting of the CD40/CD154 pathway and for clinical translation of 2C10.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	allophycocyanin
CNI	calcineurin inhibitor
ELISA	enzyme-linked immunosorbent assay
FBG	fasting blood glucose
IEQ	islet equivalent(s)
IVGTT	intravenous glucose tolerance test
KLH	keyhole limpet hemocyanin
MBP	maltase binding protein
NHP	nonhuman primate(s)
PBMC	peripheral blood mononuclear cell
POD	post-operative day

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Figure 1. B cell activation of seven novel anti-CD40 mAb

Of seven novel anti-rhesus CD40 mAb clones, 2C10 caused the most significant inhibition of CD154-mediated B cell activation when compared to activated B cells without anti-CD40 mAb and with a positive control, as shown by CD23 expression (mean fluorescent intensity). The inhibition seen with 2C10 is similar to that seen with an anti-CD154 mAb (right top panel).

Figure 2. 2C10 cross-blocks CD40 and inhibits CD154 binding

Rhesus PBMCs were incubated with escalating concentrations of 2C10 or an isotype control. (A) B cells were analyzed for CD40 expression using the fluorophore-labeled anti-CD40 clone 5C3. 2C10 blocked binding of 5C3 to CD40 in a dose-dependent manner. (B) B cells were analyzed for CD154 binding by incubating with histidine-tagged soluble CD154 and analyzing for histidine expression. 2C10 blocked the binding of CD154 in a dose-dependent manner. Results of (A) and (B) are representative of multiple repetitions of both experiments.

Figure 3. 2C10 binds to a unique CD40 epitope

To test the ability of 2C10 to cross-block two previously described anti-CD40 mAb, rhesus PBMC were incubated with increasing concentrations of 2C10, Chi220 and 3A8 followed by incubation with APC-conjugated 2C10. Binding of APC-conjugated 2C10 decreased with increasing concentration of 2C10 but not with Chi220 or 3A8.

Figure 4. Treatment with 2C10 results in minimal B cell depletion compared to chimeric 3A8 and Chi220

Following treatment with 2C10 or vehicle control and immunization with KLH, peripheral B cell counts were monitored for 56 days. Treatment with 2C10 resulted in negligible peripheral B cell depletion when compared to the significant and prolonged B cell depletion seen with chimeric IgG1 forms of 3A8 (3A8R1) and Chi220 (data previously published). Error bars represent standard error of the mean.

Figure 5. 2C10 prevents a T cell-dependent antibody response

Following treatment with 2C10 or vehicle control and immunization with KLH, treatment with the chimeric 3A8R1 resulted in ten-fold decrease in T cell-dependent antibody response compared to treatment with saline control. However, treatment with 2C10 completely prevented a T cell-dependent antibody response. Error bars represent standard error of the mean.

Figure 6. 2C10 significantly prolongs islet allograft survival

(A) The immunosuppression regimen containing 2C10 induction therapy resulted in significantly prolonged islet allograft survival compared to (B) control recipients not receiving 2C10. Serum levels of 2C10 from a representative recipient are shown in the solid line on the right y-axis. FBG: fasting blood glucose.

Table 1

Recipient treatment cohorts, transplant characteristic and survival.

Recipient	Therapy	IEQ/kg	Graft Survival (days)	Comment
DP4A	2C10R4/Basiliximab/Sirolimus	21,973	296	Rejection
RAol3	2C10R4/Basiliximab/Sirolimus	14,388	304	Rejection
RZql3	2C10R4/Basiliximab/Sirolimus	15,881	265	Rejection
RRql3	2C10R4/Basiliximab/Sirolimus	20,596	163	Rejection
RQz6	Basiliximab/Sirolimus	12,980	8	Rejection
RIb7	Basiliximab/Sirolimus	10,903	8	Rejection
RMc11	Basiliximab/Sirolimus	13,796	10	Rejection