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Abstract

The advent of costimulation blockade provides the prospect for targeted therapy with improved graft survival in transplant patients. Perhaps the most effective costimulation blockade in experimental models is the use of reagents to block the CD40/CD154 pathway. Unfortunately, successful clinical translation of anti-CD154 therapy has not been achieved. In an attempt to develop an agent that is as effective as previous CD154 blocking antibodies but lacks the risk of thromboembolism, we evaluated the efficacy and safety of a novel anti-human CD154 domain antibody (dAb, BMS-986004). The anti-CD154 dAb effectively blocked CD40-CD154 interactions but lacked Fc binding activity and resultant platelet activation. In a non-human primate kidney transplant model, anti-CD154 dAb was safe and efficacious, significantly prolonging allograft survival without evidence of thromboembolism (MST 103 days). The combination of anti-CD154 dAb and conventional immunosuppression synergized to effectively control allograft rejection (MST 397 days). Furthermore, anti-CD154 dAb treatment increased the frequency of CD4+CD25+Foxp3+ regulatory T cells. This study demonstrates that the use of a novel anti-CD154 dAb that lacks Fc binding activity is safe without evidence of thromboembolism and is equally as potent as previous anti-CD154 agents at prolonging renal allograft survival in a non-human primate preclinical model.
Abbreviations

biot-IZhCD154; CD62P; CHO; Ci; dAb; F(ab)2; Fc; FCγR; FcRn; FCS; H&E; hu85c8-mod; ITP; Iz; Kd; MMF; MST; PAC-1; PT; PTT; Rad; rhCMV; SEM; SPR; TEMRA; Treg; Vκ

Introduction

Although current immunosuppressive regimens provide excellent short-term outcomes, long-term graft survival remains less than ideal. Immune and non-immune mediated chronic graft injury almost universally results in progressive loss of allograft function. A key element of chronic graft injury can be attributed to the non-immune side effects associated with current immunosuppressive therapy. In recent years many of the pathways that are critical for T cell activation and function have been elucidated including those involved in T cell costimulation(1,2). In an effort to more specifically inhibit T cell mediated rejection and avoid the side effects associated with current immunosuppressive agents, novel biologic agents directed against these key pathways have been developed and tested.

Perhaps the single most effective therapy tested to prevent transplant rejection in animal models has been antibody blockade of the CD40/CD154 pathway. Antibodies directed against CD154 or CD40 have been shown to effectively prolong heart, kidney and islet allografts in both mouse and non-human primate models(1,3–10), usually with increased efficacy compared to CD28 blockade, and is a critical component of many tolerance induction regimens(11–13). Unfortunately clinical translation of anti-CD154 therapy was complicated by safety concerns as initial trials in both transplantation and autoimmunity demonstrated an increased incidence of thromboembolic complications(14–16). Further studies have suggested potential mechanisms for the increased risk of thromboembolism including an important role for the CD40/CD154 pathway in platelet activation(17,18). Moreover there is evidence to suggest that soluble CD154/anti-CD154 mAb immune complexes are responsible for the pro-thrombotic effects of anti-CD154 therapy via an FcγRIIa-dependent process(17,19). It is now clear that higher ordered (i.e. clustered) anti-CD154 immune complexes are able to bind and trigger FcγRIIa signaling which results in platelet activation ultimately leading to thrombotic complications(20). Accordingly, the development of an anti-CD154 reagent which lacks Fc binding activity could eliminate unintended platelet activation and resultant thrombosis while still providing potent immunosuppressive activity.

Recently a novel blocking domain antibody construct (dAb) that targets CD154 (anti-CD154 dAb) was generated utilizing a mutated IgG1 construct that is devoid of effector functions, including Fc binding and complement fixation(21). In murine studies a surrogate anti-CD154 dAb prolonged graft survival and augmented frequencies of Foxp3+ regulatory T cells (Tregs) with or without concomitant CTLA-4-Ig administration(22). Importantly, extensive evaluation confirmed the lack of thromboembolic properties of the Fc-silent anti-CD154 dAb, providing the impetus for further pre-clinical testing(23,24). Given these promising data an anti-human CD154 dAb (BMS-986004) that lacked Fc binding activity was generated. We evaluated the efficacy and safety of the newly generated anti-human
CD154 dAb both in vitro and in an in vivo pre-clinical model of kidney transplantation. In this report, we demonstrate that anti-CD154 dAb is a safe and effective therapy to prevent transplant rejection. There were no observed thromboembolic events and anti-CD154 dAb was as potent as hu5C8, one of the anti-CD154 clones that had been previously tested in clinical trials (14). Additionally, when we combined anti-CD154 dAb with conventional immunosuppression it was highly effective at preventing allograft rejection. These data suggest that an anti-CD154 therapy devoid of Fc function may allow for a clinically applicable anti-CD154-based immunosuppressive regimen for use in transplant recipients.

Methods

Anti-CD154 antibodies

Anti-CD154 dAb (BMS-986004) is a dimeric anti-human CD154 dAb fused to the Fc portion of a modified IgG1 lacking effector function (2,25). The genes encoding selected dAbs were cloned as previously described and V region genes for kappa L chains (Vκ) dAbs were selected for binding by phage display (26). Vκ dAbs were purified on a column of immobilized protein L (23). For these studies, an additional form of hu5c8 (hu5c8-mod) was generated using the identical modified IgG1 that was used for the anti-CD154 dAb.

Kinetic Binding Analysis of anti-CD154 dAb

Surface Plasmon Resonance (SPR) was used to characterize the kinetics and binding affinity of anti-CD154 dAb. All experiments used a biotinylated version of CD154 fused to an N-terminal isoleucine zipper motif (biot-IZ-hCD154), which facilitates the specific assembly of the CD154 molecule into the native trimeric form.

Platelet Activation Assays

Platelet activation was detected by flow cytometry using antibodies against platelet activation markers P-selectin (anti-CD62P PE) and activated GPIIb/IIIa (anti-PAC-1 FITC, BD Biosciences, Franklin Lakes, NJ, USA).

CD154-dependent Immune Activation Assays

Human or monkey B cells were incubated with soluble hCD154 trimer along with the indicated concentration of dAb or mAb. The plates were incubated at 37°C for 72 hours, at which time thymidine (3H; 0.5μci/well) was added for 6 hours and proliferation was assessed based on thymidine incorporation. Chinese hamster ovary (CHO) cells were transfected with human CD154 to generate a stable cell line expressing high levels of CD154 on the cell surface. Irradiated CHO-CD154 cells were incubated with human or monkey B cells (1:100 ratio of CHO-CD154:B cells).

Rhesus Macaque Renal Transplantation

The care and treatment of all experimental animals in this study was conducted with the approval of the Emory University institutional animal care and use committee and in adherence with the principles laid out in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, U.S.)
Department of Health and Human Services). Rhesus macaques (Macaca mulatta) were obtained from breeding colonies at AlphaGenesis, Inc. (Yemassee, SC) or Yerkes National Primate Research Center (Lawrenceville, GA). All animals underwent class I and class II MHC typing by 454 pyrosequencing (University of Wisconsin, Madison, WI). Post-transplant monitoring consisted of daily clinical assessment by veterinary staff and laboratory studies, including serum chemistry and complete blood count assessments performed at least weekly. Treatment groups, pairing, and necropsy information are in the supplemental methods.

Cellular Staining and Flow Cytometry

Weekly peripheral blood samples were drawn for immunophenotyping including flow cytometric analysis of T cell subsets and other markers consistent with immune activation. Peripheral blood cells and cells from tissue taken at the time of necropsy (splenocytes, graft infiltrating cells, and cells from the draining lymph nodes) were stained for CD3, CD4, CD8, CCR7, and CD45RA (Biolegend, San Diego, CA). Intracellular staining with anti-Foxp3 was performed using a Foxp3 intranuclear staining kit (eBiosciences, San Diego, CA), according to manufacturer’s instructions. Samples were analyzed using an LSR II FACS machine (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Statistics

Survival data were plotted on Kaplan–Meier curves. Non-parametric Mann-Whitney tests were performed. T cell differences between groups over time were analyzed using a two-way analysis of variance (ANOVA) with an alpha of 0.05. Analyses were done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Results

In-vitro characterization of anti-human CD154 dAb

After concerns that the pro-thrombotic effects of anti-CD154 therapy were mediated through an FcγRIIa dependent process, we sought to engineer a reagent that targeted human CD154 but lacked Fc binding activity. The Fc-domain used in the construction of anti-CD154 dAb is a mutated IgG1 engineered to retain the ability to bind FcRn but to disrupt the binding to FcγRs. To confirm that the engineered molecule had the desired Fc receptor binding profile, the binding affinity of anti-CD154 dAb was compared to hu5c8, an anti-CD154 antibody that was previously tested in clinical trials, and a mutated version of hu5c8 (hu5c8-mod) that contained the same modified, non-Fc binding IgG1 tail as the anti-CD154 dAb. All three constructs were evaluated for binding to human FcγRs (CD64 (FcγRI), CD32a (FcγRIIa), CD32b/c (FcγRIIb/c), CD16a (FcγRIIIa), and CD16b (FcγRIIIb)) using SPR.

FcγR binding properties for hu5C8 exhibited very strong binding to the high affinity FcγRI hCD64 (dissociation constant (Kd) < 0.5 nM), and weaker binding to the low affinity FcγRs (Table 1). The anti-CD154 dAb and hu5C8-mod with the mutant Fc were found to have at least 10-fold lower affinity for hCD64 and undetectable binding to the low affinity FcγRs, confirming that the Fc-domain modifications had the intended effect of disrupting FcγR
binding. Both anti-CD154 dAb and hu5C8-mod had fairly weak affinities for FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIb while the hu5C8 bound more strongly to the activating FcγRs, FcγRIIa and FcγRIIIa. In addition to evaluating the binding affinity to the various Fc receptors we also performed SPR analysis to determine equilibrium and kinetic binding properties of the antibodies to hCD154 (Table 2). All three constructs exhibited similar binding properties to human CD154 confirming that the addition of the modified Fc region did not impact their ability to bind CD154.

Next in order to assess whether the inactivation of Fc activity on the CD154 dAb influenced the likelihood of thrombosis we tested each of these compounds for their ability to activate platelets. Blood was obtained from human donors and incubated with hu5C8, hu5C8-mod, antigen binding fragments (F(ab)2) from hu5C8, or the newly constructed anti-CD154 dAb. Only platelets treated with hu5C8 had significantly elevated expression of PAC-1 or CD62P, suggesting the elimination of Fc binding activity may prevent anti-CD154 induced platelet activation (Figure 1). This held true for human donors with the H/R FcγRIIA-131 polymorphism (28).

After confirming that anti-CD154 dAb exhibited similar affinity to CD154 as hu5c8 but did not readily bind FcγRs nor cause platelet activation in vitro, we next assessed whether anti-CD154 dAb could effectively inhibit CD154-dependent immune cell activation and proliferation. We compared anti-CD154 dAb and hu5c8 for their ability to inhibit primary human and non-human primate CD154-dependent B cell proliferation. The primary human and non-human primate B cell proliferation assays were conducted two ways: recombinant CD154 trimer was used to drive B cell proliferation; or CHO cells expressing CD154 on the membrane (CHO-CD154) were utilized to induce B cell proliferation. The utility of CHO-CD154 cells was particularly important to ensure that signals from membrane-bound CD154 were inhibited equally well when compared to the soluble CD154 trimer. Anti-CD154 dAb demonstrated similar potency to hu5c8 in these assays whether a recombinant CD154 trimer or CHO-CD154 cells were used to induce B-cell proliferation (Table 3). Given these data suggesting that anti-CD154 dAb is effective but unlikely to possess thromboembolic potential as it did not bind Fc receptors well nor activate platelets in vitro, we sought to test this compound in a pre-clinical kidney transplant model.

**Dose escalation of anti-CD154 dAb and assessment of thromboembolism activity**

In an effort to ascertain the effective dose of anti-CD154 dAb we performed a dose escalation study. Rhesus macaques underwent life-sustaining renal transplant and were treated with either 2 (n=2), 10 (n=1), or 20 mg/kg (n=5) IV doses of anti-CD154 dAb for 10 weeks (Figure 2A). Animals underwent protocol sacrifice at POD 77, or sooner if they met clinical endpoints of the study. Similar to prior reports with other anti-CD154 antibodies, only the 20 mg/kg dose of anti-CD154 dAb was effective at prolonging allograft survival (Figure 3A) (4,29). Animals treated with the lower doses experienced early acute cellular rejection (Figure 3A). In contrast, all animals treated with 20 mg/kg survived over 60 days with excellent renal function (Figure 3B). Serial serum samples were collected and the levels of anti-CD154 dAb were measured (Figure 3C).
Given the prior association of anti-CD154 therapy and the development of thromboembolic complications, the safety profile of CD154 dAb was assessed. Clinical and laboratory markers for hypercoagulability or thromboembolism were measured for the duration of therapy. During the daily clinical assessments, animals were evaluated for any observable evidence of thromboembolic events including, but not limited to, unilateral limb swelling, respiratory changes, changes in appetite or activity, and skin changes. Analysis of serum coagulation factors, including prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, D-dimer, and anti-thrombin III levels were measured for the first 30 days after transplant in the animals that received the higher dose (Figure 4A–D). None of the animals that received the higher dose of anti-CD154 dAb demonstrated aberrations in their coagulation factor profile that would suggest increased susceptibility to thromboembolism formation. PT, PTT, fibrinogen, and antithrombin III levels were within normal ranges while on therapy. D-dimer levels did rise above normal ranges on the date of surgery (Figure 4C), but after day 7 levels began to fall and normalize back to baseline values (0.85μg/mL). This is not a surprising finding following major surgery as D-dimer is a nonspecific marker of inflammation and ongoing activation of hemostasis(30,31), and levels were comparable to control animals. The timing and peak levels of D-dimer we encountered are also in line with published data in post-surgical human patients(32). Mean platelet counts were maintained above 100,000/μL in all animals that were treated with 20mg/kg dose. The two animals treated with the lowest dose, 2mg/kg, did exhibit thrombocytopenia at the time of sacrifice with ongoing graft rejection (Figure 4E), which can be seen in the context of severe rejection(33).

As a part of the dose escalation study all animals underwent sacrifice and necropsy at the time of rejection or 11 weeks after transplant, whichever came first. All necropsies were performed by a certified veterinary pathologist employed at the Yerkes National Primate Center. During the necropsy there was extensive tissue sampling for gross and histologic evaluation for evidence of thromboembolism. All of the major vasculature was closely examined for any evidence of thromboembolism. There was no evidence of thromboembolic complications (data not shown).

**Anti-CD154 dAb significantly prolongs allograft survival without compromising protective immunity**

We next evaluated the impact of anti-CD154 dAb therapy on survival either alone or in combination with a clinically applicable immunosuppression regimen. Despite an impressive immunosuppressive effect (MST 103 days) (Figure 5A), monotherapy anti-CD154 dAb was insufficient to prevent rejection. We next evaluated anti-CD154 dAb in a clinically relevant immunosuppression regimen that included anti-IL-2R antibody induction as well as daily MMF and steroids (Figure 2B). Animals received MMF and daily steroid dosing for 20 weeks, after which the animals were maintained on monthly doses of anti-CD154 dAb. Ultimately all therapy was discontinued at day 300 and anti-CD154 dAb levels became immeasurable. The combination of anti-CD154 dAb and conventional immunosuppression (n=5) was significantly more potent than anti-CD154 dAb alone (n=5). (MST 397 days vs. 103 days, Figure 5A–B).
To assess the possibility of over immunosuppression we evaluated a surrogate marker, serum rhesus cytomegalovirus (rhCMV) viral load, in all recipients (Figure 5C). Only one out of five animals receiving monotherapy anti-CD154 dAb developed significant viremia post-transplant. Viremia was quickly controlled with anti-viral therapy suggesting minimal effect on protective immunity. No animals in the anti-CD154 dAb + conventional immunosuppression group developed significant rhCMV viral loads. In addition, no significant infectious episodes, including urinary tract infection, pneumonia, bacteremia, fungemia, etc were observed.

Protocol biopsies of the renal allograft were performed at 5, 10 and 20 weeks post-transplant in both treatment groups. Biopsies from days 35, 70, and 140 were examined. There was minimal lymphocytic infiltrate in those animals with preserved renal function. When the combination group was compared to biopsies taken from anti-CD154 monotherapy treated animals, Banff scores were less severe with lower t and i scores, and there was no evidence of acute cellular rejection unlike animals that received anti-CD154 dAb alone. This suggests that the addition of conventional immunosuppression to anti-CD154 therapy decreases early infiltration and reduces the risk of rejection (Figure 6A–D). Biopsies taken at later time points in the anti-CD154 dAb + conventional treatment group show preserved renal architecture and minimal infiltrate (data not shown).

**Anti-CD154 dAb reduces levels of memory T cells and augments the number of Tregs**

In an effort to investigate the changes in immune phenotype with anti-CD154 dAb therapy we performed longitudinal flow cytometric analysis of peripheral blood lymphocytes from the date of transplant until time of sacrifice. There was no significant difference in the percentage of naïve CD4+ or CD8+ T cells over time (defined as CCR7+CD45RA+) in either animals treated with anti-CD154 dAb alone or in combination with conventional immunosuppression (Figure 7A). Interestingly when analyzing the frequencies of CCR7-CD45RA+ CD4+ effector memory T cells (TEMRA), we noted significantly higher frequencies in the monotherapy group as compared to anti-CD154+ conventional (Figure 7B). Thus, the addition of the conventional immunosuppressants to anti-CD154 dAb therapy seemed to reduce the frequencies of CD4+ TEMRA T cells, corresponding to a decrease in rejection and associated with an increase in survival. A caveat to these data is that there are smaller, but still significant, differences at baseline between the CD4+ TEMRA T cells in animals treated with anti-CD154 dAb alone versus those that received anti-CD154 dAb + conventional therapy.

Anti-CD154 therapy has been associated with an increase in regulatory T cell numbers after transplant(22,34,35). In order to assess whether anti-CD154 dAb treatment augmented Tregs we assessed the frequency of CD25+Foxp3+ CD4+ cells (Tregs) in animals post-transplant. We compared the anti-CD154 dAb-based therapy to another historic control group of experimental animals that received belatacept (n=9) instead of anti-CD154 dAb (n=5). Those animals treated with anti-CD154 dAb had significantly higher frequencies of Tregs post-transplantation (Figure 8). This finding was present as early as the first week post-transplantation and persisted throughout the study, suggesting a possible mechanism for the effects of anti-CD154 dAb therapy.
Discussion

The clinical application of novel immunosuppression strategies which target T cell costimulatory signals has proven to be an effective method to improve long-term outcomes in renal transplant recipients (36–38). In addition to the CD28-CD80/86 pathway, the CD40-CD154 pathway is one of the most potent and well-studied costimulatory pathways. Despite initial promise, the clinical development of reagents to block this pathway was halted due to unexpected thromboembolic complications. Recent work has suggested that one important contribution to the development of thromboembolism associated with anti-CD154 antibodies is due to Fc-receptor dependent mechanism; other novel anti-CD154 monoclonal antibodies lacking Fc domains have shown efficacy in autoimmune disease without thromboembolic complications (20,39–42). Thus an anti-CD154 antibody that is devoid of Fc function may provide potent immunosuppression in transplantation without thromboembolic complications (24). We tested this hypothesis in a pre-clinical model of kidney transplantation. We evaluated a novel domain antibody without Fc receptor activity that targets CD154 in a non-human primate kidney transplant model. Although previous reports suggested that the effects of anti-CD154 therapy were potentially due to antibody-directed lysis of activated T cells expressing CD154, ample data now exists to suggest intact Fc function is not required to provide immunomodulatory capacity (19,24). An aglycosylated anti-CD154 has been effectively used in mice to prolong allograft survival and promote tolerance (24). In this study, we show that the anti-CD154 dAb, which lacks Fc activity, effectively prolonged allograft survival without evidence of thromboembolic potential. It was particularly effective when combined with conventional immunosuppression in a clinically applicable regimen.

The potential for thromboembolism has prompted reasonable concern for the use of therapies targeting the CD40-CD154 pathway. However, reservations surrounding this critical pathway have spurred investigation into the underlying mechanisms, which in turn informed the development of safe and efficacious next-generation therapies targeting the CD40-CD154 pathway. Here we describe an Fc silent domain antibody specific for CD154 that effectively prevents rejection but is also devoid of platelet activation and thromboembolism. These data in part supported the decision to initiate a clinical trial to evaluate the efficacy of the anti-CD154 dAb to treat patients with immune thrombocytopenic purpura (ITP, clinicaltrials.gov NCT02273960). In the phase I clinical study, there have been no untoward effects with clinically relevant doses. This preliminary experience in patients combined with the pre-clinical data outlined in our study of non-human primate renal transplantation supports continued interest in the CD40-CD154 blockade as a method to prevent rejection in clinical solid organ transplantation. Furthermore, CD154 blockade may be synergistic with existing costimulation blockade reagents that are currently being used clinically in transplant patients. The adoption of belatacept in renal transplantation has proven beneficial in prolonging graft survival and function compared to standard immunosuppression regimens; however, use of belatacept has been associated with higher rates of acute rejection in the early postoperative period (36,38,43–45). We and others have previously described the added benefits of combined costimulatory blockade in both mouse and non-human primate transplant models (1,46). The use of anti-CD154 dAb in
combination with belatacept and/or other costimulation blockade reagents may provide a strategy to reduce the rates of acute rejection seen with belatacept alone and promote effective, more targeted yet less toxic immunosuppressive strategies.

Another strategy that we and others have employed to block this pathway is the use of antibodies specific for CD40 instead of CD154. Previous studies have reported on the use of CD40 blockade in prolonging graft survival in both kidney and islet transplantation using murine and non-human primate models(3,5–10). Even though anti-CD40 therapies have proven effective, blockade of the pathway ligand (CD154) may offer benefits over therapies specific for CD40 receptor. There are now numerous reports describing the relationship between targeting CD154 and increased frequency of Tregs(22,34,35,47), and it is known that increased frequency of Tregs may provide immune-protection, and perhaps even tolerance, in transplantation. Consistent with these prior reports we found that treatment with anti-CD154 dAb increased the frequency of CD25+Foxp3+CD4+ regulatory T cells when compared to other costimulatory molecule blocking agents. This may be one important mechanism that distinguishes anti-CD154 therapy. We plan to perform CD3, CD4, and Foxp3 staining for graft- infiltrating T cells and Treg-specific demethylated region analysis in future studies to strengthen these observations.

In a more clinically relevant set of studies, we combined anti-CD154 dAb therapy with conventional agents used in transplant induction and maintenance immunosuppression. Anti-CD154 combined with conventional immunosuppression significantly improved long-term rejection free survival. Furthermore, animals treated with anti-CD154 dAb alone had a consistently higher frequency of terminally differentiated effector memory (TEMRA) cells present in the peripheral blood compared to animals that received anti-CD154 combined with conventional immunosuppressive therapies, suggesting that one mechanism supporting prolonged survival in the combined treated animals may be a synergistic attenuation of costimulation independent TEMRA cells. The addition of conventional immunosuppressive therapy to anti-CD154 dAb may influence T cell differentiation and as a result reduce TEMRA frequency in the peripheral blood, a hypothesis that requires further testing. Animals treated with anti-CD154 dAb and conventional therapy maintained low levels of CD4+ TEMRA cells for a prolonged period. It is unclear whether the mechanism for this observation is related to an alteration of T cell maturation whereby T cells are restrained to a naïve phenotype or if the addition of conventional therapy actually accelerates terminal differentiation to such a degree that they are more rapidly cleared from the peripheral blood.

In conclusion, we describe important preclinical findings that demonstrate that anti-CD154 dAb safely and effectively targets the CD154/CD40 pathway in nonhuman primates resulting in prolonged survival with preserved graft function particularly when it is used in combination with conventional immunosuppression. Importantly, there was no evidence of thromboembolism or other untoward complications. Anti-CD154 therapy remains one most potent and targeted therapies to prevent allograft rejection. The use of anti-CD154 dAb in patients in the context of a clinical trial to prevent ITP suggests that this therapy may be closer to clinical use than was previously thought. The potential clinical translation of anti-CD154 dAb in combination with existing approved therapies that target costimulation

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pathways may allow for effective control of the allo-immune response and improved long-term outcomes by promoting preservation of renal function.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


Figure 1. Anti-CD154 dAb does not activate human platelets in vitro
Platelets from human volunteers were incubated with hu5c8, hu5c8-mod with a mutated IgG construct, the F(ab)2 fragment of hu5c8, and the novel anti-CD154 dAb agent with the mutated IgG construct. Only hu5c8 resulted in platelet activation by PAC-1 (■) or CD62P (□) expression.
Figure 2. Treatment schedules for anti-CD154 dAb and conventional immunosuppression
(A) To determine the safety and efficacy of optimal dosing of anti-CD154 dAb, animals were treated with either 2, 10, or 20 mg/kg and underwent protocol sacrifice on day 77 after surgery while still on therapy. (B) To determine the potential survival benefit of anti-CD154 dAb, animals were treated with 30 mg/kg regimen of anti-CD154 dAb in combination with a conventional immunosuppression regimen of basiliximab, mycophenolate mofetil, and steroids.
Figure 3. 20mg/kg anti-CD154 dAb is a safe and effective dose
(A) Animals treated with 20mg/kg IV anti-CD154 dAb (n=5) (——) had significantly prolonged graft survival than those treated at lower doses (10mg/kg (n=1) (---), 2mg/kg (n=2) (····)). (B) Animals treated with 20mg/kg maintained good renal function as measured by serum creatinine. Animals treated with lower doses had accelerated rises in their laboratory values consistent with their shortened rejection-free survival times. (C) After the first infusion on the date of transplant, the level of anti-CD154 dAb peaked on post-operative day 1 and subsequently decreased until day 7 when they received their next infusion. The 20mg/kg dose maintained trough levels >20ug/ml.
Figure 4. Coagulation factors and platelet counts were maintained in the normal range in animals treated with the optimal dosing of anti-CD154 dAb.

(A–D) Serum levels of coagulation factors were measured from animals on the optimal dosing regimen of anti-CD154 dAb (n=5) for the first 30 days after transplant. Coagulation factors (PTT, PT/Fibrinogen/D-Dimer/Antithrombin III) were maintained in the normal range. Animals who did not receive anti-CD154 dAb as part of their treatment regimen were also tested as controls. (E) Platelet counts were maintained above 100 (103/μL) with the 20mg/kg dosing regimen (20mg/kg —, 10mg/kg ——, 2mg/kg ———) and comparable to control animals who did not receive anti-CD154 dAb (••••).
Figure 5. The addition of conventional immunosuppression to anti-CD154 dAb improves graft survival versus anti-CD154 dAb alone

(A) The combination of anti-CD154 dAb with basiliximab, mycophenolate mofetil, and steroids (n=5) (---) significantly improved rejection-free allograft survival than anti-CD154 dAb monotherapy (n=5) (-----) (MST 397 vs MST 103). (B) Animals treated with anti-CD154 and conventional immunosuppression had excellent renal function as assessed by serum creatinine. (C) CMV viral load was used as a surrogate for overall level of immunosuppression. Only one animal in the monotherapy group developed detectable CMV viremia which was quickly controlled with anti-viral therapy. No animal in the combined group developed detectable levels of virus.
Figure 6. Protocol biopsies revealed less infiltrate in the anti-CD154 dAb + conventional therapy group at both earlier and later time points.

(A) Early protocol H&E stain biopsy of allograft at day 35 receiving only anti-CD154 dAb.
(B) Late protocol H&E biopsy of allograft at day 70 receiving only anti-CD154 dAb. (C) Early protocol H&E biopsy of allograft at day 70 receiving anti-CD154 dAb + conventional therapy. (D) Late protocol H&E biopsy of allograft at day 140 receiving anti-CD154 dAb + conventional therapy. Monotherapy animals demonstrated greater severity in Banff scoring manifested by increased t and i scores compared to anti- CD154 dAb + conventional therapy animals. Monotherapy animals also had evidence of acute cellular rejection on earlier biopsies while those that received combination therapy did not.
Figure 7. A lower frequency of terminally differentiated CCR7-CD45RA+ CD4+ T cells was observed when conventional immunosuppression was added to anti-CD154 dAb

(A) No difference in the percentage of phenotypically naïve CD4+ or CD8+ T cells was observed between animals receiving anti-CD154 dAb alone (n=5) (●) or anti-CD154 dAb + conventional therapy (n=5) (●●). (B) Animals receiving conventional immunosuppression in addition to anti-CD154 dAb demonstrated a significantly lower frequency of terminally differentiated CCR7-CD45RA+ CD4+ T cells during their time on therapy. Graphs depict mean ± standard error of the mean (SEM).
Figure 8. Treatment with anti-CD154 dAb increases the frequency of CD25+Foxp3+ regulatory T cells

For the duration of therapy with anti-CD154 dAb (n=5) (●●●●), the frequency of CD25+Foxp3+ phenotypically regulatory T cells was increased compared to standard costimulation blockade therapy (n=9) (■■■■). Differences between treatment groups were significant for time points over the entire duration of therapy. Graphs depict mean ± SEM.
**Table 1**

FcγR Binding Affinity as Determined Using Surface Plasmon Resonance

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<th></th>
<th>FcγRI CD64 (K&lt;sub&gt;d&lt;/sub&gt; nM)</th>
<th>FcγRIIa CD32a (K&lt;sub&gt;d&lt;/sub&gt; nM)</th>
<th>FcγRIIb/c CD32b/c (K&lt;sub&gt;d&lt;/sub&gt; nM)</th>
<th>FcγRIIIa CD16a (K&lt;sub&gt;d&lt;/sub&gt; nM)</th>
<th>FcγRIIIb CD16b (K&lt;sub&gt;d&lt;/sub&gt; nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CD154 dAb</td>
<td>0.6</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>hu5c8</td>
<td>&lt;0.5</td>
<td>~10&lt;sup&gt;-7&lt;/sup&gt; M&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&gt;3000</td>
<td>240</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>Hu5c8-mod</td>
<td>0.9</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
</tr>
</tbody>
</table>

<sup>*</sup>CD32a binding to hu5c8 was biphasic. K<sub>d</sub> was estimated as ~10<sup>-7</sup> M based on steady state fit to dominant binding event. This K<sub>d</sub> is consistent with the K<sub>d</sub> reported in the literature for FcγRIIa binding to IgG1(27).
Table 2

hCD154 Kinetic and Affinity Values as Determined Using Surface Plasmon Resonance

<table>
<thead>
<tr>
<th></th>
<th>$K_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CD154 dAb</td>
<td>2.3 E+06</td>
<td>2.6 E-04</td>
<td>0.11</td>
</tr>
<tr>
<td>hu5c8</td>
<td>5.4 E+05</td>
<td>2.3 E-04</td>
<td>0.42</td>
</tr>
<tr>
<td>hu5c8-mod</td>
<td>5.8 E+05</td>
<td>1.3 E-04</td>
<td>0.22</td>
</tr>
</tbody>
</table>
## Table 3

In Vitro Potencies of Anti-CD154 dAb and hu5c8 in Human and Non-Human Primary B Cell Assays

<table>
<thead>
<tr>
<th></th>
<th>Human B Cell Proliferation IC₅₀ (nM)</th>
<th>Non-human Primate B Cell Proliferation IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD40L trimer</td>
<td>CHO-CD40L</td>
</tr>
<tr>
<td>anti-CD154 dAb</td>
<td>3.6 ± 1.3</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>3.8 ± 1.3</td>
<td>7.3 ± 4.6</td>
</tr>
<tr>
<td>hu5c8</td>
<td>5.4 ± 1.4</td>
<td>1.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>7.8 ± 1.8</td>
<td>5.7 ± 3.3</td>
</tr>
</tbody>
</table>