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IN VITRO TESTING OF AN ANTI-CD40 MONOCLONAL ANTIBODY, CLONE 2C10, IN PRIMATES AND PIGS

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Abstract

Background—The CD40/CD154 and CD28/B7 pathways are important in allo- and xeno-transplantation. Owing to the thrombotic complications of anti-CD154mAb, anti-CD40mAb has emerged as a promising inhibitor of costimulation. Various clones of anti-CD40mAb have been developed against primate species, e.g., clone 2C10 against rhesus monkeys. We have compared the *in vitro* efficacy of 2C10 to prevent a T cell response in primates and pigs.

Methods—The binding of 2C10 to antigen-presenting cells (PBMCs [B cells]) of humans, rhesus and cynomolgus monkeys, baboons, and pigs was measured by flow cytometry, and was also tested indirectly by a blocking assay. The functional capacity of 2C10 was tested by mixed lymphocyte reaction (MLR) with polyclonal stimulation by phytohemagglutinin (PHA) and also with wild-type pig aortic endothelial cells (pAECs) as stimulators.

Results—There was a significant reduction in binding of 2C10 to baboon PBMCs compared to rhesus, cynomolgus, and human PBMCs, and minimal binding to pig PBMCs. The blocking assay confirmed that the binding of 2C10 was significantly lower to baboon PBMCs when compared to the other primate species tested. The functional assay with PHA showed significantly reduced inhibition of PBMC proliferation in humans, cynomolgus monkeys, and baboons compared to rhesus monkeys, which was confirmed on MLR with pAECs.

Conclusions—Since both the binding and functional activity of 2C10 in the baboon is lower than in rhesus monkeys, *in vivo* treatment using 2C10 in the baboon might require a higher dose or more frequent administration in comparison to rhesus monkeys. It may also be beneficial to develop species-specific clones of anti-CD40mAb.

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DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

David Ayares is an employee of Revivacor. No other author has a conflict of interest.

Keywords

Anti-CD40 monoclonal antibody; Costimulation blockade; Nonhuman primate; Pig; Transplantation; Xenotransplantation

INTRODUCTION

The CD28/B7 (CD80/CD86) and CD40/CD154 pathways are important in the immune response to both allo- and xeno-transplantation. Blockade of costimulatory molecules has emerged as one of the most promising forms of immunosuppressive therapy [1-3], and has been associated with prolonged graft survival following both allo- and xeno-transplantation models [4-25]. The development of agents that target costimulatory molecules (e.g., CD28/B7 blockade [belatacept]) has progressed through nonhuman primate models into the clinic [26]. Blockade of the CD28/B7 pathway using human cytotoxic T-lymphocyte-associated protein 4- immunoglobulin (hCTLA4-Ig) (e.g., belatacept), and blockade of the CD40/CD154 (CD40 ligand) pathway have emerged as therapeutic approaches to suppress the T cell immune response [1-3].

Unfortunately, even though anti-CD154mAb is highly effective in inhibiting a T cell response [7, 8, 27, 28], its administration is associated with thrombotic complications [6, 29-32], and it cannot currently be used clinically. As a result, anti-CD40mAb has gained increasing attention, and various clones (e.g., Chi220, 3A8, 5D12, 4D11, and 2C10) have been developed to block the CD40/CD154 pathway [4-6, 9-19, 21-24].

Rhesus and cynomolgus monkeys have often been selected to investigate the effect of immunosuppressive drugs in regards to the prevention of allograft rejection, but the baboon has frequently been used as a recipient for pig organ xenotransplantation [33], whereas the cynomolgus monkey has been used for cell xenotransplantation (e.g., islets) [34, 35]. Studies of pig organ and cell transplantation in nonhuman primates have recently been reviewed [33].

Recombinant mouse-rhesus chimeric forms of 2C10 have been generated using either rhesus IgG1 (2C10R1) or IgG4 (2C10R4) heavy chain and rhesus kappa light chain constant region sequences [19]. The functional activities of 2C10R1 and 2C10R4 have been investigated *in vitro* and *in vivo* using rhesus monkeys. Both agents completely blocked the T cell-dependent antibody response to keyhole limpet hemocyanin (KLH), and prolonged islet allograft survival [19]. 2C10 therefore has considerable potential in clinical transplantation. However, as 2C10 was generated against rhesus cells [19] we felt it important to assess its binding to, and suppressive capacity against, other nonhuman primates, especially the baboon, used in xenotransplantation research.

The aim of the present study was to compare the binding and suppressive capacity of 2C10 to cells from rhesus monkeys and other primates (humans, cynomolgus monkeys, baboons) and pigs.

MATERIALS AND METHODS

Sources of peripheral blood mononuclear cells (humans, monkeys, baboons, pigs)

Buffly coats were obtained from healthy human blood donors (n=7; Institute for Transfusion Medicine, Pittsburgh, PA). Blood was obtained from healthy baboons (n=5; *Papio species*, Oklahoma University Health Sciences Center, Oklahoma City, OK). Blood was collected from healthy rhesus (n=8) and cynomolgus (n=5) monkeys (Alpha Genesis or the NIAID NHP colony, both Yemassee, SC). Blood was also drawn from wild-type (outbred Large White Landrace, n=3) pigs and from α 1,3-galactosyltransferase gene-knockout pigs transgenic for the human complement-regulatory protein CD46 (GTKO/CD46 pigs, n=4), both from Revivicor, Blacksburg, VA [36]. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described [37].

All animal care was in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals.

Preparation of pig aortic endothelial cells (pAECs)

pAECs were collected from wild-type pig aortas, and cultured as previously described [36]. pAECs of passages 3 to 5 were used as stimulators for the mixed lymphocyte reaction (MLR). The sub-confluent pAECs were activated for 72h by co-culture with recombinant pIFN- γ (50ng/mL, R&D Systems, Minneapolis, MN). Activation of the cells was evaluated by staining with swine leukocyte antigen (SLA) class I (mouse anti-pig SLA class I, clone JM1E3, Serotec, Raleigh, NC) and SLA class II (DR) (mouse anti-pig SLA class II, clone 2E9/13, BD Biosciences, San Jose, CA) using flow cytometry [38].

Immunosuppressive agents

Anti-CD40mAb (clone 2C10R4) was kindly provided by Keith Reimann through the NIH NHP Reagent Resource facility. Belatacept (hCTLA4-Ig) was purchased from Bristol-Myers-Squibb (Princeton, NJ). Agents were diluted to the desirable concentration with PBS or AIM-V, a serum-free medium (Life Technologies, Carlsbad, CA) as previously described [19, 39].

Binding of anti-CD40 mAbs

To compare the affinity of different anti-CD40mAbs to PBMCs from various species (i.e., human, rhesus, cynomolgus, baboon, or pig), binding assays were performed. PBMCs (2×10^5) were stained with (i) anti-human CD21 (clone B-ly4, BD) which cross-reacts with all nonhuman primates and pigs, and anti-CD40 of (ii) clone 5C3 (FITC-conjugated, BD), (iii) clone 3A8 (PE-conjugated, NIH), or (iv) clone 2C10R1 (PE-conjugated, NIH) for 30min at 4°C. Appropriate isotype antibodies were used as negative controls. The binding of anti-CD40mAb to CD21⁺B cells in PBMCs was measured by LSR II flow cytometry (BD), and analyzed by FlowJo software (Treestar, Ashland, OR) [38].

CD40 blocking assay

To confirm the specificity of 2C10 for rhesus, other primate, and pig CD40, PBMCs were incubated with a series of concentrations (between 100µg/ml [highest] and 0.001µg/ml [lowest]) using anti-CD40 mAb clone 2C10R4 for 30min at 4°C as previously described [19]. After washing, cells were stained with FITC-conjugated anti-CD40 (clone 5C3) and PE-conjugated anti-human CD22 (clone RFB-4, Invitrogen, Carlsbad, CA) mAbs for 30min at 4°C. An anti-CD22 mAb was also used as a B cell marker, which covered the entire memory B cell phenotype since some memory B cells might not express CD21 [40]. The remaining binding of 5C3 to CD40 was detected by flow cytometry.

Mitogen stimulation of PBMCs

To investigate the potency of 2C10R4 and belatacept by suppression of the proliferation of PBMCs, primate and pig PBMCs were stimulated with phytohemagglutinin (PHA, Roche, Basel, Switzerland) as previously described [38]. Responder PBMCs (1×10^5 /well) from primates and pig were cultured with 10µg/ml PHA with/without 2C10R4 and/or belatacept in AIM V culture media, and incubated at 37°C in 5% CO₂ for 3 days. ³H-thymidine (1 µCi/well, PerkinElmer, Akron, OH) was added for the final 16h of culture, and the mean of triplicate results of cell proliferation was expressed as ³H-thymidine incorporation. The percentage inhibition was calculated using the equation:

$$\% \text{ inhibition} = 100 - ([\text{cpm in drug presence}] / [\text{cpm in drug absence}]) \times 100$$

Mixed lymphocyte reaction

The response of primate cells to the activated pAECs was determined as previously described [37, 38]. In our preliminary study, since it was difficult to define the suppressive capacity of 2C10R4 among species when GTKO/CD46 pAECs were used as stimulators (because the MLR response to GTKO/CD46 pAECs was significantly lower than to wild-type pAECs) (data not shown), wild-type pAECs were used as stimulators. Responder PBMCs (1×10^5 /well) from primates were co-cultured with irradiated (2,800cGy) pAECs as stimulators (at responder-stimulator ratios of 5:1) with/without 2C10R4 and/or belatacept for 6 days. The mean of triplicate results of cell proliferation was expressed as ³H-thymidine incorporation, and the percentage inhibition was calculated.

Statistical analysis

The statistical significance of differences was determined by Student's t or nonparametric tests, as appropriate, using GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Values are presented as mean ± SD. Differences were considered to be significant at $p < 0.05$.

RESULTS

Binding of the anti-CD40mAbs to PBMCs (CD21⁺B cells) from primates and pigs

The binding capacities of the three different clones of anti-CD40 mAb (5C3, 2C10R1, and 3A8) to primates and pig CD21⁺B cells were measured by flow cytometry (Figure 1A). An anti-CD21 mAb (clone B-ly4) was chosen as the B cell marker for the present study because the antibody cross-reacts with all species (primates and pig), and there was CD40 mAb

binding to CD21⁺ cells. In addition, there was also some binding of 2C10R1 to CD21⁻ cells because some CD21⁻ cells (which include monocytes, dendritic cells and some memory B cells) express CD40 on their surface.

Clone 3A8 had a higher binding capacity to all species, including pig, in comparison to the two other clones (5C3 and 2C10R1) (Figure 1B, C, and D). The binding capacities of both 5C3 and 2C10R1 to baboon cells were significantly lower than to cells from other primates, but there was no difference in the binding capacity of 3A8 to rhesus monkey and human cells (Figure 1B, C, and D). There was no significant difference of 2C10R1 binding to the cells from humans, cynomolgus, and rhesus monkeys (Figure 1C). There was no binding of 5C3, minimum binding of 2C10R1, and significant binding of 3A8 to pig cells (Figure 1B, C, and D).

The binding capacity of 2C10 R4 to CD40 on baboon CD22⁺B cells was less than that to rhesus monkey CD22⁺B cells

To confirm the lower binding capacity of 2C10R1 to CD40 antigens on baboon cells, PBMCs from all primates were incubated with several concentrations of 2C10R4 and analyzed for CD40 expression on CD22⁺B cells using the fluorescein-labeled anti-CD40 clone 5C3 (Figure 2A). Pre-incubation with a high concentration of 2C10R4 could completely block the secondary staining of CD40 (binding by 5C3) on the cells from all primates. However, when lower concentrations of 2C10R4 were used (e.g., 0.001 µg/ml), there was a significantly lower blocking capacity of 2C10R4 to baboon cells compared to those from rhesus monkeys (Figure 2B). These results confirmed the specificity of 2C10R4 for rhesus CD40, and suggest that the lower binding capacity of 2C10R4 for baboon cells may be important in preclinical translational studies relating to 2C10R4 if a baboon model is employed.

Since neither 5C3 nor 3A8 can be used for future *in vivo* studies because 5C3 is limited to *in vitro* use and 3A8 has the capacity to be a B cell agonist [19, 23], only 2C10R4 was studied in subsequent *in vitro* functional assays.

The functional suppressive capacity of 2C10R4 and/or belatacept (hCTLA4-Ig)

To investigate the functional capacity of 2C10R4 to suppress the MLR, PBMC proliferation assays were carried out using PHA as a mitogen (Figure 3A) or wild-type pAECs as stimulators (Figure 3B). To block the CD28/B7 pathway, we tested belatacept (Figure 4).

Proliferation of all primate and pig PBMCs was reduced by 2C10R4 in a dose-dependent manner (Figure 3A and 3C). However, when a dose of <20 µg/mL of 2C10R4 was used, there was a significantly weaker suppressive effect on proliferation of cells from humans, cynomolgus monkeys, and baboons compared to those from rhesus monkeys (Figure 3B and D). There was only a minimal suppressive capacity of 2C10R4 on pig PBMCs after PHA stimulation. (Figure 3A and B)

In contrast to 2C10R4, belatacept suppressed proliferation of all primate and pig PBMCs in a dose-dependent manner (Figure 4A and C). Although baboon PBMC proliferation was suppressed significantly more than human PBMC proliferation after PHA stimulation

(Figure 4B), there was no significant difference in the suppressive effect of belatacept among primates after co-culture with pAECs (Figure 4C and D).

We also investigated whether the combination of 2C10R4 and belatacept could significantly increase suppression of PBMC proliferation following stimulation compared to a single drug. Since lower concentrations of belatacept (e.g., 4 μ g/mL) were efficient in suppressing PBMC proliferation following stimulation, it would be difficult to define the effect of 2C10R4 when a high concentration of belatacept (e.g., 20 μ g/mL) was used. Therefore, the combination of a high dose of 2C10R4 (20 μ g/mL) and a low dose of belatacept (4 μ g/mL) was used for this purpose. Although 2C10 was less effective with regard to binding (Figures 1 and 2) and suppressive capacity (Figure 3) in baboons compared to rhesus monkeys, the combination of a high dose of 2C10R4 (20 μ g/mL) and a low dose of belatacept (4 μ g/mL) showed a significant increase in suppression of T cell proliferation compared to that of low dose belatacept alone or 2C10R4 alone (Figure 5).

DISCUSSION

CD40 is a member of the TNF receptor superfamily and is primarily expressed on antigen-presenting cells (e.g., B cells, macrophages, dendritic cells), fibroblasts, and endothelial cells. CD40 has been shown to be involved in a broad variety of immune and inflammatory responses, including B cell and dendritic cell activation, proliferation and differentiation, immunoglobulin isotype class-switching, memory B cell development, and germinal center formation [41].

Targeting the CD40/CD154 pathway using an anti-CD154 mAb resulted in thrombotic complications that occurred in a CD40-independent manner, suggesting that blocking CD40 rather than CD154 could be an alternative method to safely target this pathway [1]. Several anti-CD40mAbs, e.g., Chi220, 3A8, 5D12, 4D12, and 2C10, have shown promise in various transplant models without thromboembolic complication [4-6, 9-19, 21-24]. However, some of these anti-CD40 mAbs (i.e., Chi220, 3A8) have been associated with potentially adverse effects, e.g., as a B cell agonist or associated with substantial peripheral B cell depletion [9, 16, 19, 23]. Therefore, these specific anti-CD40mAbs may not be candidates for clinical translation.

A fully human mAb to CD40, clone 4D11 (IgG4 isotype), has recently been shown to prolong renal, hepatic, and islet allograft survival in cynomolgus monkeys [12, 13, 18, 21, 42]. Phase I clinical trials in renal transplantation using 4D11 are currently in progress.

Human and rhesus CD40 share approximately 95% amino acid identity in the extracellular domains [19]. Human and murine CD40 molecules share 62% amino acid identity [41]. Therefore, a chimeric mouse-rhesus mAb, 2C10, might cross-react with human CD40, and would have potential for clinical application.

In the present study, we demonstrated that there was a similar level of binding of 2C10 (both R1 and R4) to human, cynomolgus, and rhesus monkey cells, but not to baboon cells. Since CD40 proteins from rhesus monkeys were used to generate 2C10, it may have a weaker binding capacity and immunosuppressive effect on baboon cells. However, the lower

expression of CD40 on B cells in baboons might have an effect on the result of 2C10 binding assay by flow cytometry. In fact, the fluorescence intensity for both 2C10R1 and 5C3 was lower in baboons compared to all other primates, although there was no difference in binding capacity of 3A8 between rhesus monkeys and baboons. The lower fluorescence intensity of 2C10 by flow cytometry might not be correlated to the affinity of 2C10 to CD40 among primates. In our personal communication with Reimann, his group found that humans, rhesus monkeys and baboons shared identical amino acid sequence for CD40. Furthermore, their preliminary results using surface plasmon resonance analysis indicated a similar affinity of 2C10 for all 3 species (unpublished).

It is important to mention that the suppressive capacity of 2C10R4 would be higher in baboons if the baboon expresses a lower level of CD40 on their cells in comparison to other primates (with the same affinity of 2C10). However, in contrast to the binding capacity of 2C10 among primates, 2C10R4 showed less functional activity against human, cynomolgus monkey and baboon PBMCs compared to rhesus PBMCs in a proliferation assay. These *in vitro* data suggest that potential low binding capacity of 2C10 in relation to a lower expression level of CD40 on baboon cells and possible low affinity of 2C10 to baboon cells compared to rhesus monkey cells cannot be excluded. Therefore, the lower suppressive capacity of 2C10R4 in baboons proposes that *in vivo* an increased dosage of 2C10R4 or more frequent administration, and/or its combination with another immunosuppressive agent (e.g., blockade of CD28/B7 pathway), might be necessary.

CD28/B7 costimulation blockade (e.g., belatacept) has been used clinically in organ allotransplantation with encouraging results [43]. Our *in vitro* studies indicated that belatacept had a stronger suppressive effect on the primate and pig MLRs than 2C10R4. This observation correlates with a previous *in vitro* study that demonstrated that blockade of the CD28/B7 pathway was more suppressive in regard to the human anti-pig T cell response than blockade of the CD40/CD154 pathway [44]. However, our own studies indicate that this is clearly not the case *in vivo* [32, 45, 46]. A regimen based on blockade of the CD28/B7 pathway did not prevent the development of elicited anti-pig antibodies after pig-to-baboon artery patch transplantation [32] whereas belatacept in combination with 2C10R4 prevented this response [45]. We have also tested the combined 2C10R4-belatacept-based regimen in the pig-to-baboon heart transplantation model with follow-up for up to 18 weeks [46], and demonstrated it prevented an adaptive response as successfully as in the artery patch model [45].

The combination of blockade of the CD40/CD154 and CD28/B7 pathways has also been shown to prevent donor-specific antibody production and islet allograft rejection [9, 15]. Chi220, a chimeric anti-human CD40 mAb, when administered alone, demonstrated modestly prolonged islet and renal allograft survival in rhesus macaques, but was much more effective when combined with belatacept [4, 9].

However, there is increasing evidence that 2C10R4 alone effectively prevents a T cell-dependent anti-pig response in baboons. Mohiuddin et al demonstrated prolonged xenograft survival of pig hearts in baboons receiving high doses of 2C10R4 (50mg/kg) in the absence of belatacept, but in combination with B cell depletion [23, 24], suggesting CD40/CD154

blockade alone at high dosage is sufficient to prevent rejection. Our own group has recently confirmed the good effect of this regimen after pig kidney transplantation in a baboon [47].

The CD40/CD154 pathway plays an important role in T cell-dependent and T cell-independent B cell activation, resulting in increased humoral immunity (i.e., the development of anti-donor antibody) [41]. In particular, soluble CD154 (sCD154) has been shown to contribute to allograft rejection independent of T cells [48]. Platelets are a major source of sCD154, and this molecule can interact with CD40 on endothelial cells, resulting in upregulation of adhesion molecules [41, 49]. Furthermore, binding of sCD154 to CD40 on B cells promotes germinal center formation, B cell activation, and anti-donor IgG antibody production [50]. These results suggest a crucial role for the CD40/CD154 pathway in transplant immunity.

It would be important to investigate how long the 2C10R4 occupies the receptor although we have not investigated it *in vivo*. Mohiuddin's group has recently shown that anti-CD40mAb (2C10R4) was detectable in sera in baboons up to 8-10 weeks following the last injection (50mg/kg) (M. Mohiuddin, personal communication). This observation suggests that 2C10R4 remains in the serum for a long period. Therefore, we believe that the CD40/CD154 pathway could be blocked by 2C10R4 if an adequate concentration of 2C10R4 (50mg/kg) is administered in baboons.

2C10 exhibits several important characteristics for successful clinical translation, such as lack of agonistic properties (no B cell activation), minimal depletion of CD40-expressing target cells (e.g., B cells), and complete inhibition of a T cell-dependent antibody response to KLH [19]. Furthermore, 2C10 administration as induction therapy in rhesus monkeys resulted in significantly prolonged islet allograft survival [19]. Interestingly, 2C10 binds a unique epitope distinct from that bound by either Chi220 or 3A8.

In conclusion, 2C10 cross-reacts with human, cynomolgus monkey, and baboon cells, but only minimally with pig cells. Binding of 2C10 to baboon cells is less than to rhesus monkey cells. The functional suppressive capacity of 2C10 in humans, cynomolgus monkeys, and baboons is weaker than in rhesus monkeys. *In vivo* treatment using 2C10 in the baboon might require a higher dose and/or more frequent administration in comparison to treatment in rhesus monkeys. The administration of 2C10 in combination with belatacept can suppress both T cell-dependent and T cell-independent graft rejection in baboons.

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Abbreviations

AECs aortic endothelial cells

GTKO	α 1,3-galactosyltransferase gene-knockout
hCTLA4-Ig	human cytotoxic T-lymphocyte-associated protein 4- immunoglobulin
mAb	monoclonal antibody
MLR	mixed lymphocyte reaction
p	pig
PBMCs	peripheral blood mononuclear cells
PHA	phytohemagglutinin
sCD154	soluble CD154
SLA	swine leukocyte antigen

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Figure 1. Significantly lower binding capacity of 2C10R1 to baboon CD21⁺B cells in comparison to other primate CD21⁺B cells

The CD40 antigens on CD21⁺B cells in primates (human [n=7], rhesus monkey [n=8], cynomolgus monkey [n=5], and baboon [n=5]) and pigs (n=7; wild-type n=3; GTKO/CD46 n=4) were detected by anti-CD40mAbs (clones 5C3, 2C10R1, and 3A8) using flow cytometry. (A) Representative flow cytometry of binding of anti-CD40mAb (clone 2C10R1) to baboon CD21⁺B cells. All CD21⁺B cells in baboons expressed the CD40 antigen. Binding activity of (B) 5C3, (C) 2C10R1, and (D) 3A8 to primate and pig CD21⁺B cells. There were significantly lower binding capacities of 5C3 and 2C10R1 to baboon cells in comparison to all other primate cells. Binding activity of 3A8 to cynomolgus monkey CD21⁺B cells was significantly higher than to human and baboon CD21⁺B cells. There was no or less binding activity of all three anti-CD40mAbs to pig CD21⁺B cells compared to primate cells. (** p<0.01)

Figure 2. Secondary detection of CD40 antigens on primate CD22⁺B cells after blocking of CD40 antigens by 2C10R4

A blocking assay of CD40 antigens was carried out using 2C10R4. CD40 antigens on primate CD22⁺B cells were then detected by 5C3 by flow cytometry. **(A)** Representative results (of a total of five experiments) of the blocking assay in primates. Secondary staining of CD40 antigens using 5C3 was dose-dependently decreased by prior blocking with 2C10R4 in all primate species. **(B)** When a low concentration (0.001 µg/ml) of 2C10R4 was used in the blocking assay, there was significantly greater residual binding of 5C3 to baboon CD22⁺B cells compared to those of rhesus monkeys. (** p<0.01)

Figure 3. Functional capacity of 2C10R4 to suppress the MLR

The functional capacity of 2C10R4 was tested in MLR with primate and pig PBMC stimulation by PHA (10µg/ml) for 3 days (**A and B**) or by wild-type pAECs as stimulators for 6 days (**C and D**). After stimulation with either PHA or pAECs, the percentage inhibition of responder cell proliferation was increased by blocking of CD40 antigens by 2C10R4 in a dose-dependent manner (**A and C**). At a concentration of 20µg/mL, 2C10R4 suppressed proliferation of rhesus monkey PBMCs after stimulation to a significantly greater extent compared to that of other primate and pig PBMCs (**B and D**) (* $p<0.05$, ** $p<0.01$)

Figure 4. Functional capacity of belatacept (hCTLA4-Ig) to suppress the MLR

The functional capacity of belatacept was tested in MLR with primate and pig PBMC stimulation by PHA (10µg/ml) for 3 days (**A and B**) or by wild-type pAECs as stimulators for 6 days (**C and D**). After stimulation with either PHA or pAECs, the percentage inhibition of responder cell proliferation was increased by blocking the B7 family (CD80/86) antigens by belatacept in a dose-dependent manner (**A and C**). There was significantly higher suppression of baboon PBMC proliferation by belatacept than of human PBMC proliferation (**B**). There was significant suppression of pig PBMC proliferation after PHA stimulation, but this was less than that of human PBMC proliferation (**B**). When PBMCs were stimulated with wild-type pAECs, there was no significant difference in suppressive capacity of belatacept among primates (**D**). (** p<0.01)

Figure 5. The combination of 2C10R4 with belatacept increased suppression of PBMC proliferation after stimulation with pAECs

Different concentrations (0.16 and 20 µg/mL) of 2C10R4 with/without a low concentration of belatacept (4 µg/mL) were tested in MLR using PBMCs from rhesus monkeys (**A**) or baboons (**B**) as responders with wild-type pAECs as stimulators. The percentage inhibition of responder cell proliferation after stimulation with pAECs was reduced by blocking of both B7 (CD80/86) antigens (using a low concentration of belatacept) and CD40 antigens (using high concentration of 2C10R4). (** p<0.01; NS=not significant).