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Defining dose–response relationships in the therapeutic blockade of B7RP-1-dependent immune responses

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ABSTRACT

The ICOS (Inducible T cell Co-Stimulator)/B7RP-1 (B7-related protein 1) interaction is critical for the proper activation of a T lymphocyte. In this manuscript we describe a systematic *in vivo* approach to determine the level of blockade required to impair the generation of a T cell-dependent antibody response. We have developed an overall strategy for correlating drug exposure, target saturation, and efficacy in a biological response that can be generalized for most protein therapeutics. Using this strategy, we determined that low levels of B7RP-1 blockade are still sufficient to inhibit the immune response. These data suggest that contact between the T cell and the antigen-presenting cell during antigen presentation is much more sensitive to inhibition than previously believed and that ICOS/B7RP-1 blockade may be efficacious in the treatment of autoimmune diseases.

1. Introduction

An important consideration in designing clinical studies is determining the dose range for the Phase I trial. Ideally, one would begin the regimen with the lowest dose to ensure a large safety margin that would also be known to elicit the relevant on-mechanism biological effect. This is very difficult to establish. Although modeling human doses using *in vivo* animal data is a frequently-used approach, quality differences between the actual therapeutic and its experimental surrogate complicate direct comparisons. In addition, animal disease models that accurately reflect human disease are rare. Many clinical trials depend on the correlation of pharmacokinetic analyses with efficacy in disease outcome. Although this approach has proven to be successful in many cases, the lack of assays that reflect on-mechanism effects of the therapeutic make these estimations approximate. A multi-step approach to improve the accuracy of determining dosing in humans can be designed based on the use of similar mechanism-based model systems in preclinical animal studies and in patients. The first step is to use *in vitro* functional and binding assays to compare the therapeutic with its species surrogate. A mechanism-based *in vivo* assay can then be designed to test efficacy of a species surrogate in a preclinical species; subsequent functional and pharmacokinetic results can then be used to design Phase I experiments using the same *in vivo* assay. One critical limitation is that using pharmacokinetic studies to measure exposure does not provide information on the relative saturation of the target at a given dose. Knowledge of this important parameter would enable the investigator to relate partial target saturation with partial efficacy in the *in vivo* functional assay and thus provide a direct functional relationship between exposure and the pharmacodynamic readout.

To study these correlations further, we have used as a model system, antibody blockade of the ICOS/B7RP-1 pathway in the immune system. Proper activation of the immune response requires the presentation of foreign antigen to the T cell by an antigen-presenting cell. We and others have characterized a costimulatory receptor–ligand pair that is critical for T cell activation (Yoshinaga et al., 1999; Swallow et al., 1999; Ling et al., 2000). The receptor ICOS (inducible T cell co-stimulator) is expressed only on activated T cells, whereas the ligand, B7RP-1 (B7-related protein also referred to as ICOSL, B7h, and GL50), is expressed constitutively on antigen-presenting cells. Data from knock-out mice (Dong et al., 2001; Mak et al., 2003; McAdam et al., 2001; Nurieva et al., 2003a; Tafuri et al., 2001; Yoshinaga et al., 1999) as well as therapeutic intervention of this

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pathway (Akbari et al., 2002; Coyle et al., 2000; Gonzalo et al., 2001; Greenwald et al., 2002; Iwai et al., 2002, 2003; Nurieva et al., 2003b; Ozkaynak et al., 2001; Rottman et al., 2001) suggest that the ICOS/B7RP-1 interaction is required for T cell-dependent B cell responses and therefore it has been hypothesized that blockade of this pathway may prove efficacious in treating autoimmune diseases that are driven by the activation of autoreactive lymphocytes (Sporici and Perrin, 2001). A subset of patients with combined variable immunodeficiency has been shown to be null for ICOS (Grimbacher et al., 2003a,b), confirming that blockade of the ICOS/B7RP-1 pathway may be efficacious in inhibiting the immune response in humans.

In this manuscript, we characterize the role of ICOS/B7RP-1 in the immune response using pharmacological intervention with an anti-B7RP-1 monoclonal antibody. We have developed an approach for relating target saturation to both pharmacokinetic and pharmacodynamic endpoints in a therapeutic system. We have determined that only low levels of B7RP-1 occupancy by the antibody are required for significant inhibition of the immune response. These data suggest that the ICOS/B7RP-1 interaction is very susceptible to inhibition, further indicating that this pathway may be a good target for pharmacological intervention in autoimmune disease. In addition, these data suggest that dose levels of an anti-B7RP-1 reagent needed to obtain a pharmacologic effect in humans may be much lower than previously believed.

2. Materials and methods

2.1. Generation of 1B7v2

The hybridoma that produced the original monoclonal antibody generated against B7RP-1, referred to as 1B7, expressed two light chains and thus the resulting antibody preparation contained multiple species. Because of this, the genes encoding the VH and VK regions for the species of 1B7 that bound to B7RP-1 were cloned into expression vectors containing the murine IgG1 heavy chain and kappa light chain constant region genes, respectively. Stable CHO cell lines were generated with these expression constructs and the resulting chimeric mouse-rat mAb, referred to as 1B7v2, was produced and compared in functional studies with other anti-B7RP-1 mAbs.

2.2. Plate assays

Tissue culture plates were coated with anti-CD3 (2 μg/ml) and anti-mu Fc (10 μg/ml) overnight at 4 °C, washed with PBS, recoated with a B7RP-1 Fc fusion protein (5 μg/ml) for 4 h at 37 °C, and rewashed with PBS. 1×10^5 T cells were then added per well, along with the indicated amounts of mAb and incubated for 48 h. ^{3}H-thymidine was then added to each culture, incubated for 16 h, and the cells harvested and analyzed for ^{3}H-thymidine incorporation.

2.3. Pharmacokinetic analysis

A standard colorimetric solid-phase sandwich ELISA was developed to measure serum anti-B7RP-1 levels. BALB/c mice were given single intraperitoneal administrations of 1.03 μg/mouse (0.05 mg/kg; Group 1), 10.3 μg/mouse (0.5 mg/kg; Group 2) and 291 μg/mouse (15 mg/kg; Group 3), and followed for 7 days by collecting blood samples at the appropriate time points. The data were analyzed using non-compartmental methods. The assay has a lower and upper quantitation limits of 10 and 625 ng/ml, respectively.

2.4. Flow cytometry

Cells were stained following a standard protocol as previously described. In brief, 2 ml of Red Blood Cell Lysis Buffer (Stemcell Technologies Inc, Cat.# 07850) were added to 200 μl of blood and incubated on ice for 10 min. The cells were then pelleted by centrifugation at 5 min at approximately 350 g and washed twice with PBS. Cell concentration was then adjusted to 5×10^6/ml with staining buffer (0.2% BSA and 0.01% Na Azide in PBS). Approximately 5×10^5 to 1×10^6 cells were added to each well of a 96-well plate along with 2 μg/well of FcR blocking antibody for mouse (2.4G2; Becton-Dickenson cat # 01241D) to block non-specific binding. Cells were incubated on ice for 15 min, and the appropriate staining mAb, either 1 μg/well of the biotinylated mAb 1B7v.2 or 18A5-C5, was added. The plate was then incubated on ice for 20–30 min, and the cells were washed twice with staining buffer. The cell pellet was resuspended in 100 μl staining buffer and Strepavidin-PE (1:1000 dilution; Becton-Dickenson cat# 554061) and 1 μg FITC-conjugated CD19 (mouse; Becton-Dickenson cat # 553088). Cells were incubated on ice for 20–30 min, washed twice in staining buffer, resuspended in staining buffer, transferred to FACS tubes, and analyzed using a FACS Calibur flow cytometer. B lymphocytes were identified by CD19 expression.

2.5. Occupancy assays

We administered single doses of PBS, 10 mg/kg of isotype-matched control, or 0.1, 1, and 10 mg/kg of 1B7v2 i.p. into female BALB/c mice. Blood samples were collected 24 h and 7 days after dosing. In addition, lymph nodes and spleens were collected at necropsy on day 7, red blood cell-lysed peripheral blood (PBMCs), lymph node and spleen mononuclear cells were stained with PE-labeled 1B7v2 and FITC-labeled 18A5 and analyzed by flow cytometry for target saturation.

To calculate occupancy of the cell surface B7RP1 with dosed unlabeled 1B7v2, the mean fluorescent intensity (MFI) of cells stained with labeled antibody was divided by the MFI for the same antibody in the no dose antibody control (data not shown). These values represent the fractional fluorescence relative to the zero dose. The ratio of the relative 1B7v2 fluorescence to relative 18A5-C5 fluorescence yields the fraction of free (un-occupied) receptors; one minus the fraction of free receptors equals the occupied receptors. A correction is subsequently made for background binding by the isotype control; In essence, we note that the isotype control contributes about 14% of the overall fluorescence by examining a region of the flow cytometric plots where there is no specific binding of the 1B7v2 or 18A5-C5 antibodies. The ratio of isotype control antibody MFI to 1B7v2 antibody MFI lets us calculate this value.

2.6. Mice

Female BALB/c mice 7–8 weeks old were purchased from Charles River Laboratories. Mice were housed under pathogen-free conditions at the Laboratory Animal Research Facility at Amgen. Animal experiments were conducted as approved by the Amgen Institutional Animal Care and Use Committee.

2.7. Antigen challenge

Female BALB/c mice were immunized i.p. at day 0 with 1 mg/kg KLH (Keyhole Limpet Hemocyanin, Pierce #77600) plus 1 mg of alum (Aluminum Hydroxide, JT. Baker Inc. #0518-01) and subsequently challenged on day 21 with 5 mg/kg KLH only. Mice were dosed i.p. starting at day −1 and every subsequent 5 days with either 1B7v2 (anti-B7RP1) or isotype-matched control antibody. A positive control (CTLA4-Fc protein) was dosed i.p. starting at day −1 and every subsequent 3 days of the study; this dosing regimen was previously determined to ensure adequate target coverage (data not shown).

2.8. KLH-specific ELISA

Serum samples from days 7, 14, and 21 were serially diluted in 1× PBS at 1:50/200/800/3200/12800. Serum samples from days 28, 35, and 42 were serially diluted 5–6 times in 1× PBS. Unknown KLH standards were prepared from pooled day 42 sera of isotype-treated control mice. KLH-

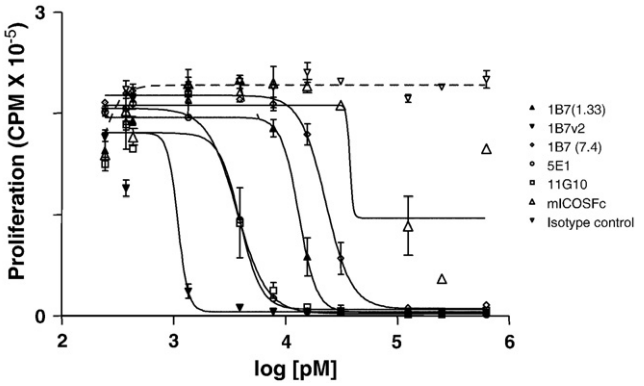


Fig. 1. Functional characteristics of monoclonal antibodies against murine B7RP-1. Rat anti-mouse monoclonal antibodies were generated, produced, and tested in a plate costimulation assay (see Materials and methods for details). Proliferation of T cells in response to costimulation with suboptimal levels of α-CD3 and a fusion protein of murine B7RP-1 fused to an Fc fragment are shown on the *Y* axis; concentration of added blocking reagent are shown on the *X* axis. Two different preparations of the rat anti-mouse mAb 1B7 are shown along with the 5E1, 11G10 mAbs generated from the same fusion. 1B7v2 consists of the variable regions of the 1B7 mAb cloned onto mouse constant regions. A murine ICOS-Fc fusion protein is included as a blocking reagent control. Error bars represent standard error of the mean of triplicate points. This experiment was conducted three times with similar EC_{50} results as those shown.

specific standards were prepared per manufacturer's protocol (Mouse IgG1 Isotype standard, R&D Systems #MAB002; Mouse IgG2a Isotype standard, R&D Systems #MAB003; Mouse IgM isotype standard, BD Pharmingen #557275). ELISA plates were coated overnight at 4 °C with KLH at 4 μg/ml in 1X PBS (Nunc C-bottom 96-well Immunoplates, VWR #62409-002 or 384-well Maxi-sorp plate, Corning Costar #3700). Plates were blocked for 1 h at RT (10% BSA Block solution, KPL #50-61-00). Samples and standards were incubated for 1 h at room temperature. Ig-horseradish peroxidase conjugated antibody was incubated at room temperature for 1 h (Goat anti-mouse IgG1, Southern Biotech #1070-05; Goat anti-mouse IgG2a, Southern Biotech #1080-05; Goat anti-mouse IgM, Southern Biotech #1020-05). Tetramethylbenzidine developing solution was incubated approximately 5–20 min at room temperature in the dark depending on the Ig analyzed (TMB Peroxidase (solution A), KPL #50-76-01, Peroxidase Substrate (solution B), KPL #50-65-00). Reaction was stopped with 2 M sulfuric acid. Plates were read on a spectrophotometer at 450 nm.

2.9. Cytokine analysis

Mouse sera were collected at day 21, before KLH challenge and at day 22, 24 h post-KLH challenge. A premixed mouse cytokine 22-plex kit (Linco Research# MCYTO-70K-PMX22) was used to measure cytokine levels before and after KLH challenge. This kit was used according to the manufacturer's specifications.

2.10. Statistical analysis

Mann–Whitney *U* or *t*-tests were used to analyze statistical significance between treatment groups. These tests were performed using either STATISTICA (Statsoft, Inc) or SigmaStat (Systat Software, Inc) analysis software. Determining the fraction of target occupancy takes into account the slight increase in B7RP-1 expression that occurs when this pathway is blockaded *in vivo*.

3. Results

3.1. A monoclonal antibody against murine B7RP-1

We have generated a monoclonal antibody to murine B7RP-1, 1B7v2, that inhibits its interaction with ICOS but does not delete the antigen-presenting cell *in vivo* or downregulate B7RP-1 from the cell surface (data not shown). In addition, plate costimulation assays show that 1B7v2 is very effective in inhibiting ICOS/B7RP-1-dependent T cell activation, with an EC50 in the sub-nanomolar range (Fig. 1). Thus, 1B7v2 can be considered to be a blocking monoclonal antibody. In addition, these results show efficacy of 1B7v2 at least 5-fold greater than that observed with other anti-B7RP-1 mAbs that have been generated. Importantly, 1B7v2 is almost two orders of magnitude more efficacious at inhibiting the B7RP-1/ICOS interaction than the ICOS-Fc fusion protein (Fig. 1 and data not shown). BIAcore binding data indicate that 1B7v2 has the highest affinity for B7RP-1 of all the antibodies tested and successfully blocks the binding of ICOS (S. Steavenson and Q. Chen, data not shown), further confirming that 1B7v2 is the best candidate mAb for *in vivo* studies.

3.2. 1B7v2 displays nonlinear PK parameters

To determine the exposure of 1B7v2, we conducted pharmacokinetic studies of 1B7v2 in normal BALB/c mice. Fig. 2 shows serum concentration-time profiles of 1B7v2 in mice up to 7-days following a single intraperitoneal (i.p.) administration of 1B7v2 at Group 1, 0.05 mg/kg, Group 2, 0.5 mg/kg, or Group 3, 15 mg/kg. Following i.p. administration, serum levels of 1B7v2 increased rapidly, reaching a peak at approximately 8 to 12 h post-dose, and then declining in a monophasic fashion for all groups. Analyses of these profiles suggest nonlinear pharmacokinetics, with clearance decreasing as the dose increases. Increases in peak serum levels of 1B7v2 were greater (1402-fold increase) than the increases in dose (283-fold increase), with levels of 160, 4992, and 224,459 ng/ml following doses of 0.05 mg/kg, 0.5 mg/kg and 15 mg/kg respectively. Serum levels of 1B7v2 were quantifiable up to 24 h after dosing with 0.05 mg/kg and up to 7 days following a single dose of 0.5 mg/kg.. At the highest dose (15 mg/kg), serum levels of 1B7v2 were maintained above approximately 10,000 ng/ml from 2 to 192 h (8 days) post-dose. Based on these data, we simulated multiple-dose regimens designed to maintain specific trough-level concentrations of mAb in the blood for use in our antigen-challenge studies (see below).

3.3. An occupancy assay to determine target saturation by an antibody

To correlate exposure of the therapeutic to the level of occupancy of the target, we have developed a flow cytometric assay to measure the extent of *in vivo* target saturation by an administered protein therapeutic. This assay requires two protein reagents that bind the same target: the first is the potential therapeutic, whereas the second is directed against a second, non-competing epitope on the target (Fig. 3). In this case, the therapeutic is a monoclonal antibody directed against B7RP-1, 1B7v2, and the second is a mAb that recognizes another, non-

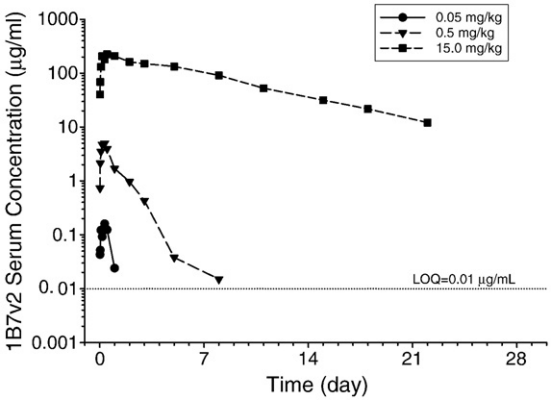
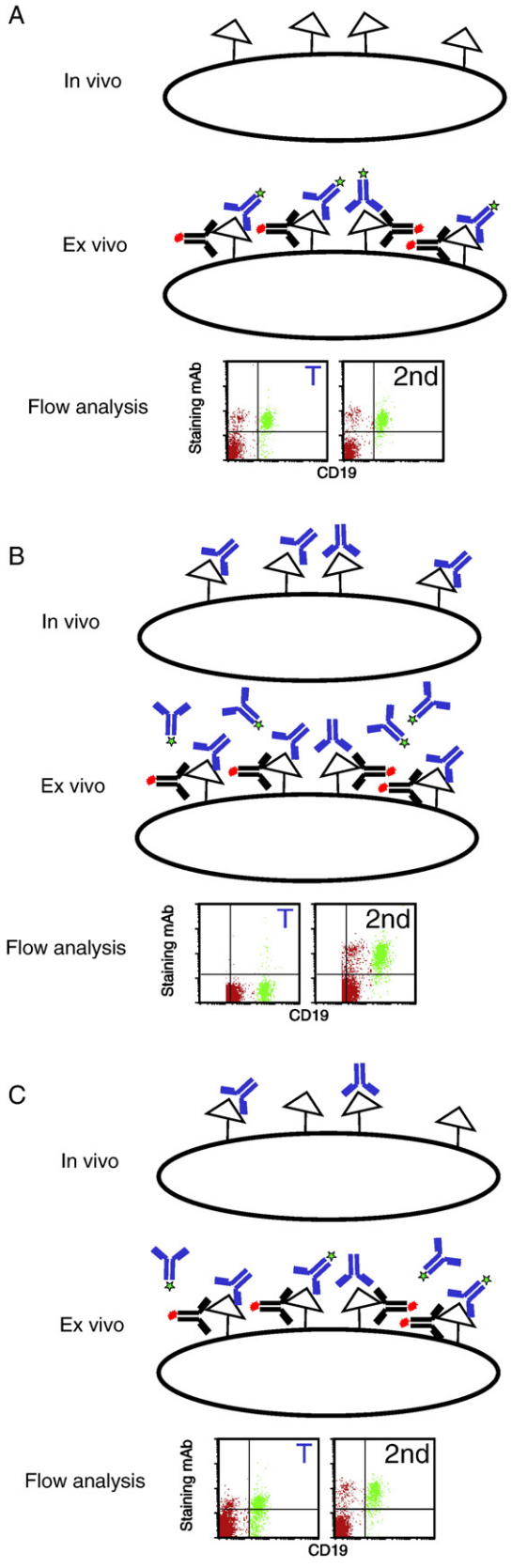


Fig. 2. Pharmacokinetic analysis of the 1B7v2 mAb. Single-dose injections of 1B7v2 were administered to BALB/c mice and serum levels of mAb were detected by ELISA at the indicated time points. Each time point consists of the average of data from two mice.

competing epitope on the same target (18A5). Cells are harvested from the blood of dosed subjects and the target B cells identified using an antibody against the B cell-specific antigen CD19. The B cells are then analyzed for staining with fluorescently-labeled 1B7v2 and 18A5. For a subject that has not received a dose of the therapeutic antibody, the B cells stain equally well with both mAbs, as all epitopes on B7RP-1 will be available for binding (Fig. 3A). Alternatively, cells from a subject given a dose of the therapeutic which saturates 100% of its target will not stain with fluorescently-labeled 1B7v2 *in vitro*, as all of the available sites will be occupied by the originally dosed therapeutic 1B7v2 (Fig. 3B); however, the mAb against the second epitope, 18A5, will still bind at high levels, since it is unaffected by the presence of the therapeutic mAb. Finally, cells recovered from a subject given a dose of the therapeutic mAb that does not cover the target *in vivo* completely will stain partially *ex vivo* with the fluorescently-labeled therapeutic mAb; as for the other examples, binding of the mAb against the second epitope will be unaffected and thus no differences should be seen in *ex vivo* staining with this mAb (Fig. 3C). By measuring the differences in staining of the therapeutic mAb, we can estimate the degree that the dosed mAb has saturated its target in the patient.



In vivo occupancy was measured in mice using this approach. We used the two previously discussed mAbs, 1B7v2, the surrogate therapeutic against B7RP1 and 18A5, a rat anti-mouse B7RP-1 mAb, which binds a second epitope on the target and thus does not compete for binding with 1B7v2 (Fig. 4A). B cells were analyzed, as B7RP-1 is expressed at high levels in this cell type. As expected, $CD19^+$ B cells from mice dosed with either PBS or the isotype-matched control mAb stained at high levels with both 1B7v2 and 18A5. B cells from mice dosed with either 1 or 10 mg/kg of 1B7v2, however, showed partial or no staining with the PE-labeled 1B7v2 respectively, whereas the 18A5 mAb still bound at high levels to B cells. These data suggest that the administered 1B7v2 has completely saturated the available B7RP-1 molecules at the highest dose; the staining of 18A5 suggests that despite the binding of the dosed 1B7v2, B cells are still present at normal levels (data not shown) and are expressing cell surface B7RP-1. Thus, saturation binding by 1B7v2 does not lead to depletion of B cells or the internalization of B7RP-1. To the contrary, expression of B7RP1 is increased on animals dosed with saturating concentrations of 1B7v2 (10 mg/kg) indicated by elevated 18A5 mAb staining levels. A recent publication shows that tonic interactions between B7RP1 on the B cell and ICOS on the T cell lead to shedding of B7RP1 (Watanabe et al., 2008). It is likely that the presence of saturating concentrations of 1B7v2 prevents B7RP1–ICOS interactions and block shedding. This would lead to the appearance of increased levels of B7RP1 on the surface of the B cells. Mice dosed with 1 mg/kg of 1B7v2 showed partial staining of the PE-labeled 1B7v2, suggesting that even at this low dose we can detect partial occupancy 24 h after 1B7v2 administration. To determine the kinetics of the target occupancy, we analyzed B cells obtained from the blood of mice at 3, 4, 5 and 7 days after intraperitoneal administration of a single 1 mg/kg dose (Fig. 4B). Consistent with the PK results, B cells obtained from mice at these time points show a gradual decrease in receptor occupancy. This can be quantified by comparing Median Fluorescence Intensity, thus allowing for direct comparisons with pharmacokinetic and functional efficacy parameters (see below).

Although $B7RP\text{-}1^+$ cells exist in the peripheral blood, antigen presentation occurs in the lymph node and the spleen, and thus blood measurements of B7RP-1 coverage may not reflect saturation in the target organ. To address this concern, B cells were harvested from the spleen and lymph nodes of the mice dosed 7 days earlier, analyzed for

Fig. 3. An assay for determining target occupancy. A.) Theoretical analysis of an undosed test subject. Both fluorescently-labeled T and 2nd bind to target *ex vivo*, leading to positive staining (*Y* axis in the flow cytometric plots). B.) Theoretical analysis of a test subject dosed to 100% coverage. The fluorescently-labeled T does not bind to the target *ex vivo*, as its binding sites are completely occupied by the dosed therapeutic. The fluorescently-labeled 2nd still binds efficiently, as it binds to a different site than the dosed therapeutic and thus does not compete for binding to the target. C.) Theoretical analysis of a test subject dosed to partial coverage. The fluorescently-labeled T binds at lower levels to the target *ex vivo*, as it can only bind to sites that are not already bound by the dosed therapeutic. As discussed above, the fluorescently-labeled 2nd still binds efficiently.

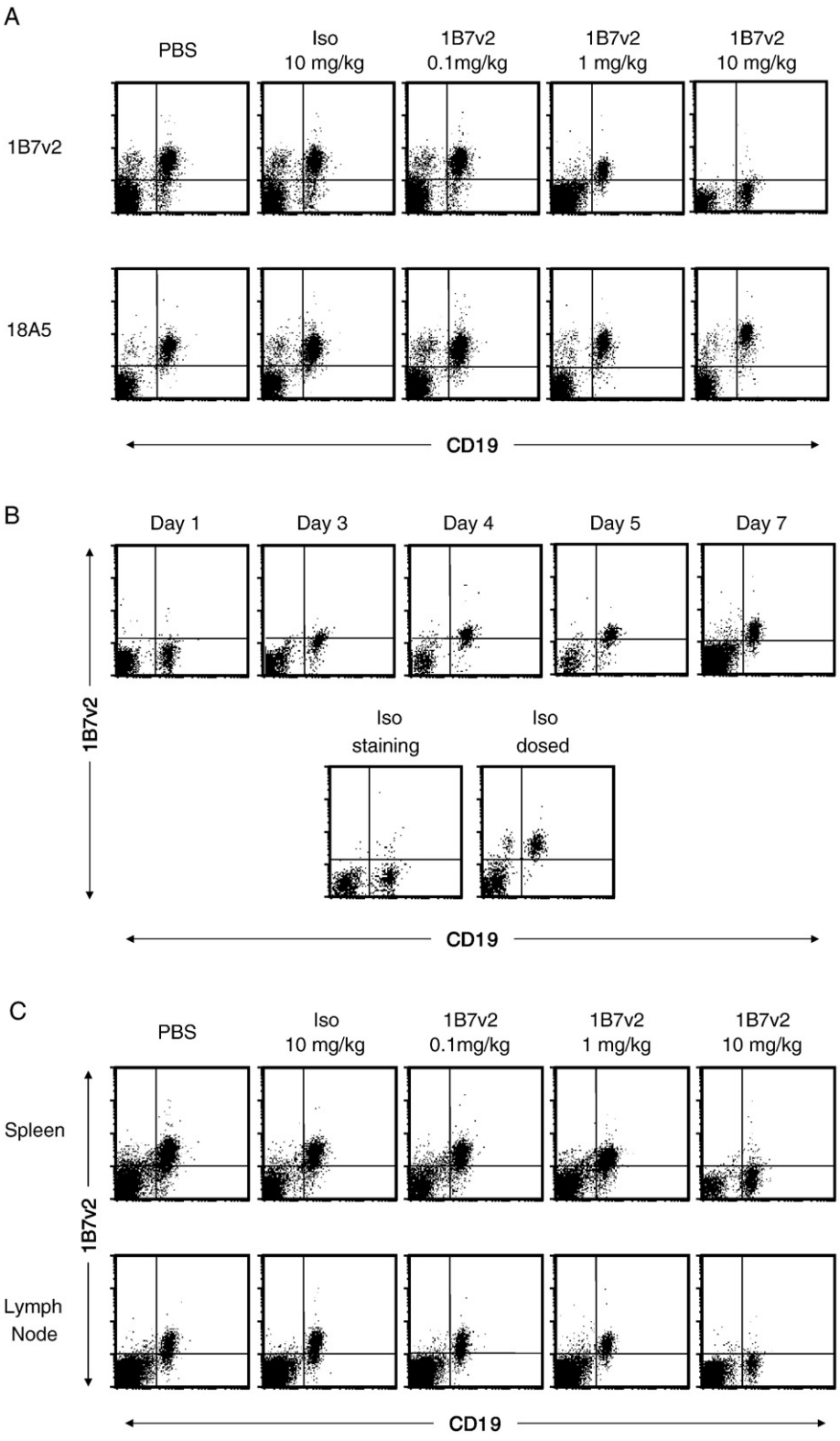


Fig. 4. *In vivo* validation of the occupancy assay. A.) BALB/c mice were dosed with isotype-matched control, PBS, or different doses of 1B7v2 and peripheral blood harvested 7 days later and analyzed with either PE-conjugated 1B7v2 (top row) or FITC-conjugated 18A5 (bottom row) and with α-CD19 to visualize B cells. Representative data are shown. B.) BALB/c mice were given a single dose of 1 mg/kg of 1B7v2 and peripheral blood harvested on the indicated days and analyzed with either the 1B7v2 mAb (top row) or the 18A5 control (data not shown). Control undosed mice or mice injected with 1 mg/kg of isotype-matched control were also analyzed with either 1B7v2 (for iso-dosed) or with fluorescently-labeled nonbinding isotype-matched control (for iso-staining) along with 18A5 (data not shown). Staining with 18A5 in all experiments was similar to that observed in (A). C.) Blood and peripheral lymph tissue were harvested from the BALB/c mice from (A) and analyzed as above for 1B7v2 or 18A5 staining (data not shown).

receptor occupancy, and compared to the results obtained from the blood. As can be seen in Fig. 4C, results from all of these tissues are identical: we can still detect full coverage at the 10 mg/kg dose and substantial loss of coverage at the 0.1 and 1 mg/kg doses. This indicates that peripheral blood values can indeed be used to estimate mAb coverage in the target tissues.

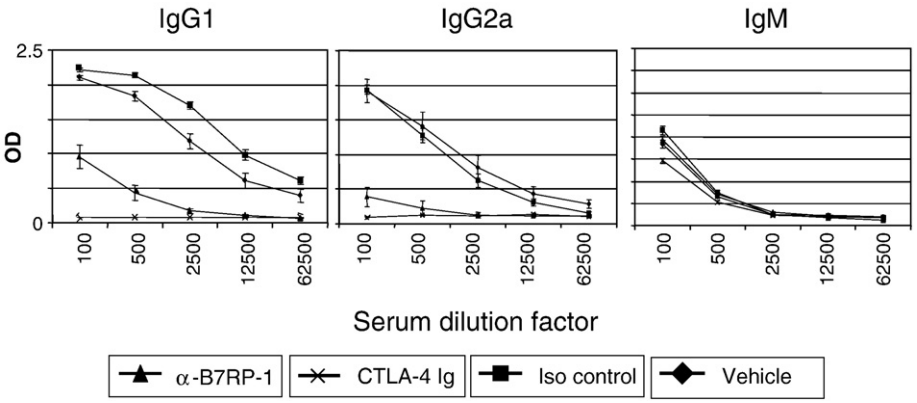


Fig. 5. Complete blockade of the ICOS/B7RP-1 interaction leads to inhibition of antigen-specific immunoglobulin production. BALB/c mice were primed and challenged with KLH and dosed with 200 μg/mouse (10 mg/kg) 1B7v2, 100 μg/mouse (5 mg/kg) CTLA-4-Ig, 200 μg/mouse (10 mg/kg) isotype-matched control, or PBS vehicle alone as described in Materials and methods. Serial dilutions from the serum harvested from each test mouse on day 35 were analyzed for antigen-specific IgG1, IgG2A, or IgM using ELISA; each point represents the average of fifteen mice in each treatment group. Error bars represent the standard error of a sample of means (S.E.M.).

3.4. Partial blockade of the ICOS/B7RP-1 interaction is effective in inhibiting a T cell-dependent immune response

To study the relationship of the target saturation to the functional efficacy of *in vivo* blockade, we conducted studies using the 1B7v2 mAb in Keyhole Limpet Hemocyanin (KLH)-antigen-challenge studies in mice. As can be seen in Fig. 5, we can detect high titers of anti-KLH IgM, IgG1, and IgG2A 2 weeks after challenge when mice are dosed with either PBS or the isotype-matched control mAb. Mice dosed with 10 mg/kg of 1B7v2 or 5 mg/kg of CTLA-4-Ig, however, show significant inhibition of KLH-specific IgG1 and IgG2A titers, indicating that blockade of these pathways significantly inhibits T cell-dependent antibody production. Consistent with this hypothesis, blockade with either 1B7v2 or CTLA-4-Ig does not inhibit the induction of antigen-specific IgM, which is T cell independent (Fig. 5).

ICOS/B7RP-1 is believed to be important in the induction of T cell responses, we attempted to determine if we could detect repression of the secretion of T cell cytokines with blockade with 1B7v2 (Fig. 6). We collected serum from the test mice 24 h before and after antigen challenge and assayed them for cytokines using Luminex. In the control mice, we could detect elevated levels of the T helper 2-specific cytokine IL-5 after challenge but not before, consistent with the hypothesis that the KLH challenge has led to the activation of effector T cells and thus secretion of high levels of IL-5. In mice treated either with 1B7v2 or CTLA-4-Ig, we can detect statistically significant decreases in serum IL-5. These data are consistent with the hypothesis that blockade of the ICOS/B7RP-1 axis leads to the inhibition of T cell activation and thus the repression of T cell-dependent antibody responses.

To determine if lower doses of 1B7v2 can also inhibit T cell-dependent antibody production, we conducted additional antigen-challenge experiments using the same dosing schedule but with lower doses (Fig. 7). These lower doses were selected on the basis of pharmacokinetic modeling developed from the data presented in Fig. 2 and were chosen to give steadily decreasing levels of serum 1B7v2 5 days after the last dose. Pharmacokinetic and Target Occupancy analyses confirm that the doses chosen conveyed decreasing serum levels of 1B7v2 as well as target saturation; as can be seen in Fig. 7. We observe a correlation between decreasing serum concentrations of 1B7v2 and target occupancy, confirming that these two assays are thus reflective of each other. Although the correlation is not direct in that a precise percentage of receptor occupancy does not lead to the same precise percentage reduction in a pharmacodynamic effect, a clear relationship between the loss of exposure and receptor occupancy and the loss of pharmacodynamic effect can be seen. No statistically significant differences can be detected in the KLH-specific IgM levels, consistent with our data above. We can detect almost complete blockade of KLH-specific IgG1 and IgG2A at doses as low as 1 mg/kg (Fig. 7). In addition, we can detect significant decreases of serum IL-5 levels 24 h after KLH challenge (Fig. 6B), confirming that we are indeed inhibiting T cell responses at this dose. Further decreases in dosing leads to further decreased efficacy in inhibiting the formation of KLH-

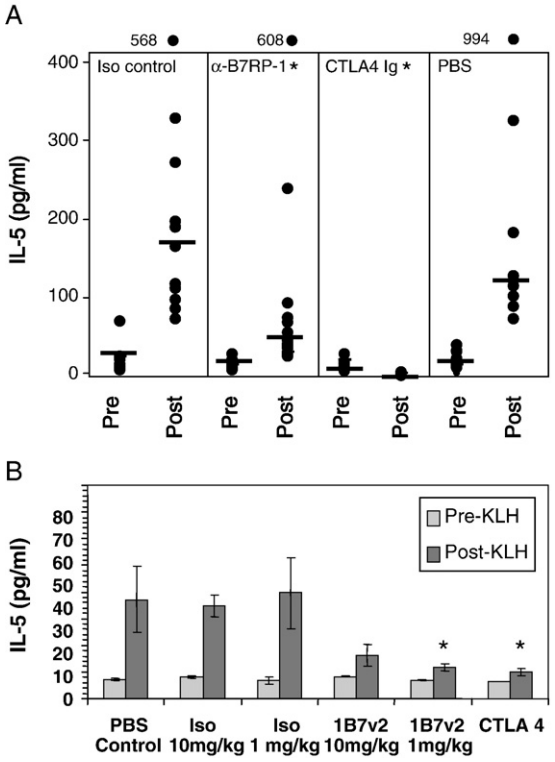


Fig. 6. Blockade of the ICOS/B7RP-1 interaction leads to inhibition of serum IL-5 levels. A.) Sera from the mice described in Fig. 6 were harvested and analyzed for IL-5 levels using Luminex. Sera were harvested from the same mouse on day 21 just before challenge (labeled 'pre') or 24 h later (labeled 'post'); each symbol represents the data from each individual mouse ($n = 15$ per treatment group). The circles above the graph indicate off-scale outliers and are nonetheless included in the subsequent analysis. Horizontal bars indicate the mean; asterisks indicate statistically significant ($P<0.05$) differences between the treatment groups and the isotype or vehicle controls. B.) BALB/c mice were primed and challenged with antigen and dosed with 100 μg/mouse (5 mg/kg) CTLA-4-Ig or with two different doses of either 1B7v2 or isotype-matched control as described in the Materials and methods section. Sera were harvested as described above and analyzed for IL-5 levels using Luminex. Although a panel of cytokines was analyzed, only IL-5 gave detectable levels. Asterisks indicate statistically significant ($P<0.05$) differences between the treatment groups and the isotype or vehicle controls.

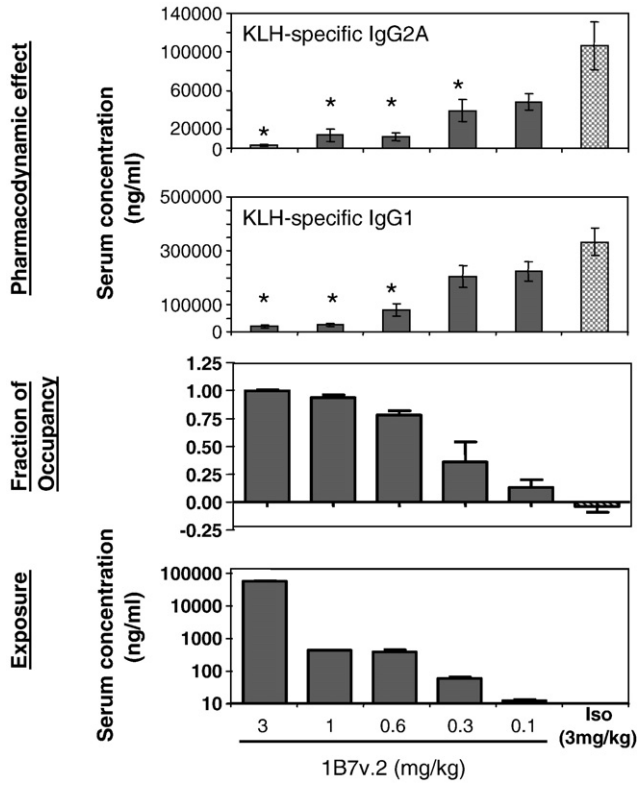


Fig. 7. Decreasing target occupancy correlates with decreasing pharmacodynamic effect and pharmacokinetic exposure. BALB/c mice ($n = 15$) were primed and challenged with KLH as described and dosed either with 3, 1, 0.6, 0.3, or 0.1 mg/kg 1B7v2 or 3 mg/kg isotype-matched control. On day 35 sera were harvested and analyzed for antigen-specific IgG2A (top row), and IgG1 (2nd row) using ELISA. Data presented are an average of fifteen mice per group; error bars represent the standard error of a sample of means (S.E.M.). Asterisks indicate statistical significance ($P<0.002$). Fraction of target occupancy was determined on day 35 (3rd row) from 3/15 treated mice. Bars in each group indicate staining with either fluorescently-labeled 1B7v2 (blue) or with isotype-matched control (maroon). Serum 1B7v2 levels were also determined from treated mice on day 35 (4th row). Data presented are an average of fifteen mice per group; error bars represent the standard error of a sample of means (S.E.M.).

specific antibodies; indeed, we can observe a correlation between serum concentrations of 1B7v2, target occupancy, and levels of antigen-specific KLH antibodies. These data indicate that all three assays are consistent with each other and further suggest that this approach can be used to establishing dosing regimens.

Surprisingly, we can still detect reduction of levels of KLH-specific antibodies using treatment regimens with doses as low as 0.1 mg/kg (Fig. 7). At these doses, serum levels of 1B7v2 are very low and we can detect only partial target occupancy, even only 24 h after dosing (Fig. 4A). We therefore conclude that ICOS/B7RP-1 is so sensitive to blockade that even low levels of target saturation are sufficient to inhibit the proper development of the immune response. These data in turn suggest that the ICOS/B7RP-1 axis is essential for a full T cell-dependent antibody response, and that even low levels of B7RP-1 blockade may prove to be efficacious in treating autoimmune disease.

4. Discussion

4.1. Establishing a PK–occupancy–PD relationship to help determine dosing regimens

One of the more difficult tasks in drug discovery is to establish a relationship between the exposure of the therapeutic to the patient, the degree to which the endogenous target is saturated by the therapeutic, and how these two parameters affect the biology and ultimately the disease condition of the patient. Here we have designed a general approach in which this can be done for protein therapeutics. In principle, we can more easily estimate doses of therapeutic that are likely to have a biological effect in humans, which in turn can be used to establish dosing regimens in patients with autoimmune disease. In this case, the therapeutic is a monoclonal antibody that blocks the interaction of the cell surface molecule B7RP-1 with its receptor ICOS, thus inhibiting the cell–cell interaction necessary for the activation of the immune response. We compare the extent of exposure using pharmacokinetic studies and the efficacy of the therapeutic by using an assay based on the mechanism of action of the target. We have also developed a target occupancy assay that provides a critical functional link between exposure and efficacy. This assay allows us quantitatively to measure the extent to which the therapeutic saturates its target on white blood cells, which are easily obtainable from a patient. We have also demonstrated that the occupancy results obtained from the blood reflect those obtained from cells harvested from the spleen and lymph node. Since these two organs are the primary site for antigen presentation, these data indicate that results from the blood can be used to estimate the level of target occupancy at the relevant site, even if the cells from the site cannot be readily accessed.

This integrated strategy can be used to translate experimental results from preclinical animal models to assist in estimating the dosing range for humans for the Phase I trials. A complete characterization of the physical properties of both the proposed therapeutic and its surrogate for the animal model species is first conducted; this includes binding affinities for the target and efficacy in multiple *in vitro* assays. The surrogate therapeutic is then tested in an *in vivo* preclinical model that can ultimately be translated into humans. In this case, the KLH antigen-challenge system is ideal since it is a standard *in vivo* model whose parameters are well-known and can be easily monitored. The critical mediating cell types are the targets of the therapeutic being tested and thus the efficacy readouts are known to be on-mechanism. Once these experiments are completed, the relationship between concentration of the therapeutic in the blood, the level of target saturation achieved, and the ultimate effect of these levels on the biological mechanism of the pathway *in vivo* can be determined. From these studies we can determine the minimal levels of target saturation at the dosing trough necessary for the optimal pharmacodynamic effect. Similar *in vitro* and *in vivo* experiments using the proposed therapeutic in non-human primates can also be conducted, either independently or as a part of the IND-enabling pharmacokinetic and toxicology studies. In humans, pharmacokinetic and target occupancy data can be obtained during the single-dose Phase I studies. By using the target occupancy results obtained from the preclinical studies, we can then determine the dosing necessary to obtain the same target occupancy in humans at the dosing trough. A KLH antigen-challenge experiment can be designed into the multi-dose portion of the Phase I trial; the dosing regimen can be established starting at the dose determined in the single-dose Phase I trial necessary for obtaining the required trough target occupancy level from the preclinical experiments. Although the surrogate therapeutic used in the animal model experiments may differ from the proposed therapeutic in its physical or pharmacological characteristics, the basic biology behind costimulation – formation of the synapse, induction of signaling – is similar between mouse and human; thus partial blockade of the ICOS/B7RP-1 pathway will likely lead to similar efficacy in these two systems. Nonetheless, this strategy allows for a thorough and systematic approach for establishing a range of dosing in humans based on the mechanism of action of the therapeutic, and thus can help devise more rational experiments for the determination of the proof of biological activity.

4.2. Sub-saturating levels of anti-B7RP-1 antibody are sufficient to inhibit the immune response

Antigen presentation requires cell–cell contact that is dependent upon the interaction of different receptor–ligand pairs expressed on

the T cell and the antigen-presenting cell. During the initial steps of antigen presentation, molecules important in T cell antigen recognition as well as their ligands on the antigen-presenting cell are directed towards the contact point between the two cells, where they are subsequently organized into the immunological synapse. In this process a significant amount of these molecules are collected in a relatively small space, and are kept there both by their interactions with their ligands on the opposing cell surface as well as by their own cytoskeletal machinery. Thus, these interactions greatly favor the continued integrity of the synapse. Since many of the receptor–ligand interactions are of relatively low affinity, synapse formation constrains these molecules in juxtaposition, thus forcing the continued interaction of the receptor–ligand pairs long enough for a meaningful signal to be sent to the corresponding cells.

Given the strong thermodynamic as well as cytoskeletal forces working to maintain the synapse, interdiction of the immune response by blocking any one receptor–ligand interaction would appear to be difficult. Since this requires the inhibition of binding of the receptor to its ligand, blocking antibodies need to overcome synapse formation as well as the inherent affinity of the receptor for its ligand. Previous studies of the ICOS/B7RP-1 interaction have been hampered by this problem. Although both null mice and pharmacological intervention have demonstrated the absolute importance of this pathway in T cell activation, its relative importance to the immune response had not been clearly delineated. Much of the analyses were based in disease model systems, which are often hampered by the uncertain linkage between the disease endpoint and the underlying basic immunological mechanism mediating the effect (Akbari et al., 2002; Gonzalo et al., 2001; Iwai et al., 2002, 2003; Nurieva et al., 2003b; Ozkaynak et al., 2001; Rottman et al., 2001; Tesciuba et al., 2001). The most complete basic mechanism *in vivo* model utilized ICOS-Fc to block the ICOS/B7RP-1 interaction (Coyle et al., 2000); in addition to uncertainties surrounding the exposure of the drug *in vivo*, the use of soluble ICOS as a blocking reagent does not provide an affinity advantage over endogenous ICOS. Thus, it is unclear that the modest phenotype observed was the result of suboptimal target coverage in the experiment or the lesser importance of this pathway in the immune response.

We have determined that even low levels of exposure and target saturation by an anti-B7RP-1 blocking antibody are sufficient to inhibit T cell-dependent immune responses. These data thus suggest that a full signal through the ICOS/B7RP-1 pathway is critical for optimal T cell activation – even low-level blockade of this pathway will be reflected in the subsequent immune response. These observations are consistent with the hypothesis that the ICOS/B7RP-1 interaction is of fundamental importance in the activation of the T cell as opposed to being an instrument for the fine-tuning of the response. These conclusions thus suggest that blocking the ICOS/B7RP-1 interaction may be useful in the treatment of autoimmune disease; its importance in the maintenance of the T cell-dependent antibody response suggests that blockade of this pathway would be effective in treating B cell-mediated diseases. Blockade of the CD28/B7 pathway has been proposed as a potential target for therapeutic intervention; indeed, preliminary clinical reports involving the use of CTLA-4-Ig in rheumatoid arthritis have been encouraging. However, CD28/B7 blockade has the potential of generating many more safety issues. For example, CD28/B7 blockade affects the activation of all T cells, whereas ICOS/B7RP-1 affects primarily T cell-dependent B cell responses. Second, the CD28/B7 pathway is believed to be important in tumor surveillance; thus, blockade at this point may also lead to the inhibition of adequate protection to tumor formation. Blockade of CD28/B7 during antigen activation leads to anergy of the responding T cell (Appleman and Boussiotis, 1992; Bachman et al., 1998); chronic treatment with CTLA-4-Ig may therefore lead to the generation of holes in the T cell repertoire (Tamada and Chen, 2000). Because of the importance of ICOS/B7RP-1 in the generation of a B cell response, blockade at this point may prove efficacious, whereas its more limited scope of action implies that interdiction of this pathway would lead to less side effects than the blockade of the CD28/B7 axis.

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