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ORIGINAL ARTICLE

Safety and pharmacodynamics of anti-CD2 monoclonal antibody treatment in cynomolgus macaques – an experimental study

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SUMMARY

Anti-CD2 treatment provides targeted immunomodulatory properties that have demonstrated clinical usefulness to condition the immune system and to treat transplant rejection. The treatment is species-specific due to structural CD2 antigen differences between nonhuman primates and humans. Herein, we report the safety profile and efficacy of two modifications of the same anti-CD2 monoclonal antibody in cynomolgus macaques. Twelve subjects received one i.v. anti-CD2 (of rat or rhesus type) dose each, range 1–4 mg/kg, and were followed for 1–7 days. Treatment effects were evaluated with flow cytometry on peripheral blood and histopathological evaluation of secondary lymphoid organs. *In vitro* inhibitory activity on primary MHC disparate mixed lymphocyte reactions (MLRs) was determined. Upon anti-CD2 treatment, $CD4^+$, $CD8^+$ memory subsets were substantially depleted. Naïve T cells and Tregs were relatively spared and exhibited lower CD2 expression than memory T cells. Early immune reconstitution was noted for naïve cells, while memory counts had not recovered after one week. Both antibodies displayed a concentration-dependent MLR inhibition. Lymph node examination revealed no significant lymphocyte depletion. None of the animals experienced any significant study drug-related adverse events. This study outlines the safety and pharmacodynamic profile of primate-specific anti-CD2 treatment, relevant for translation of anti-CD2-based animal models into clinical trials.

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Key words
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Introduction

Treatment with anti-CD2 antibody is an attractive approach to condition the immune system or treat rejection, as this antibody possesses T-cell depleting, co-stimulatory blocking, and donor-specific regulatory T cell enhancing properties [1–7]. In humans, the anti-CD2 monoclonal antibody BTI-322 (rat IgG2b) has

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been shown to reverse first-time kidney allograft acute rejections (AR), steroid and antithymocyte globulin (ATG) resistant rejections [8], and significantly decrease AR rates compared to standard-of-care when used as induction therapy [9]. Both BTI-322 and siplizumab (humanized BTI-322, IgG1k) have favorable safety profiles [5,10]. The most significant benefit of siplizumab has been as a key component in a phase I/II tolerance induction clinical trial where the majority of HLA-mismatched kidney allograft recipients could successfully be weaned off all chronic maintenance immunosuppression for at least 5 years [11,12]. To bring anti-CD2 treatment to the clinic as a standardized approach, it is important to characterize interventions on this signaling pathway. Cynomolgus macaques are widely used in translational studies due to the biological similarities to humans. To enable experimental use of anti-CD2 treatment in cynomolgus macaque-based animal models, homologous antibodies must be used in lieu of siplizumab and BTI-322, due to the latter's specificity for a binding site present only on the human and chimpanzee CD2 antigen [13]. Herein, we report the safety profile and *in vitro* and *in vivo* efficacy of two anti-CD2 mAbs, one rodent and one CDR-grafted rhesus version, respectively, in cynomolgus macaques.

Materials and methods

Test substances

The test substances were manufactured to target the CD2 antigen and included two modifications of the same anti-CD2 mAb: a rat anti-primate CD2 IgG2b (Immerge BioTherapeutics) (hereafter termed RT-CD2), and the further modified rhesus recombinant anti-primate CD2 IgG1 (hereafter termed RH-CD2). The latter was developed by grafting the CDRs from the RT-CD2 antibody into rhesus frameworks and using rhesus IgG1 and kappa constant regions (NIH Nonhuman Primate Reagent Resource, Boston, MA, USA). RT-CD2 was expressed from stably-transduced Chinese hamster ovary cells. Whereas BTI-322 and its human analogue siplizumab have a narrow species specificity, restricted to only the human and chimpanzee CD2 antigens, the two anti-CD2 antibodies studied herein have a broader primate reactivity. The anti-CD2 mAbs were administered as i.v. infusions over 60 min, preceded by prednisolone 0.6 mg/kg and diphenhydramine 1 mg/kg i.v. (Table 1). Antibody dosing was based on the body weight taken on the day of dosing.

Table 1. Animal characteristics and treatment regimens

Group	ID	Gender	Weight (kg)	Age (year)	Other treatments	Anti-CD2	Dose (mg/kg)	Days followed post-anti-CD2 (day)
A	A1	M	7.3	7	Naïve	Rhesus	1*	7
	A2	M	10.2	8	Naïve	Rhesus	2*	1§
	A3	M	7.1	7	Naïve	Rhesus	2*	7
	A4	M	7.4	9	Naïve	Rhesus	2*	7
B	B1	M	8.5	7	TBI†, rituximab‡	Rat	1*	1
	B2	M	8.3	8	TBI†, rituximab‡	Rat	1*	1
C	C1	M	10.3	7	TBI†, rituximab‡	Rhesus	1*	1
	C2	M	10.1	8	TBI†, rituximab‡	Rhesus	2*	1
	C3	M	7.1	9	TBI†, rituximab‡	Rhesus	2*	1
	C4	M	8.1	6	TBI†, rituximab‡	Rhesus	2*	1
	C5	M	9.0	8.5	TBI†, rituximab‡	Rhesus	2*	1
	C6	M	12.5	7	TBI†, rituximab‡	Rhesus	4*	1

ID, animal identification code; M, male; TBI, total body irradiation.

*All animals received premedication with 0.6 mg/kg methylprednisolone and 1 mg/kg diphenhydramine before anti-CD2 dosing on day 0.

†TBI 1.5 Gy at day −3.

‡Rituximab 20 mg/kg at day −3.

§Severe adverse reaction to ketamine on day 1, unrelated to test substance. Animals B1–B2, C1–C6 could only be followed for one day as they were also included in a separate study as part of a tolerance induction regimen in which they received additional T- and NK cell acting agents following the anti-CD2 dose.

Animals and experimental design

Three *naïve* Indonesian-origin cynomolgus macaques (*Macaca fascicularis*), obtained from AlphaGenesis, Inc (Yemassee, SC, USA), and one Mauritius-origin cynomolgus macaque (Bioculture Ltd, Senneville, Mauritius) were used to evaluate the effect of RH-CD2 monotherapy during seven consecutive study days (group A, Table 1). Eight additional Mauritius cynomolgus monkeys (selected from Bioculture Ltd), also part of a longer term liver tolerance study, were evaluated at day 1 postdosing with either RT-CD2 or RH-CD2 (groups B and C). Groups B and C received low-dose total body irradiation and rituximab (targeting B-cells only) three days prior to anti-CD2 dosing as part of the liver tolerance inducing regimen (Table 1). During the 24 h that Groups B and C were monitored for the effects of administered anti-CD2, no other T cell depleting agents had been infused. All macaques were housed and treated at the Institute of Comparative Medicine (ICM, Columbia University Medical Center, New York, NY, USA). The ICM facility has USDA assurance and is AAALAC-accredited. All experimental procedures were performed in accordance with NIH guidelines for the care and use of primates, and approved by the Columbia University IACUC.

All animals underwent baseline testing before administration of the test substance, consisting of complete blood cell count, serum chemistry, and flow cytometry. Inguinal lymph nodes were excised predose for routine histopathological examination to evaluate the potential treatment effect. Starting on day 0, the animals received i.v. infusions containing the test substance. Flow cytometric analyses were performed on peripheral blood to determine changes in lymphocyte subsets. When applicable, postdose lymph node biopsies were collected and microscopically assessed by a pathologist for any gross lymphocyte depletion. Safety assessments included careful clinical observation and hematologic and clinical chemistry evaluation before and after dosing.

Flow cytometric immune phenotyping

Fresh peripheral blood samples were stained using a direct cell surface technique to determine the leukocyte subpopulations. The following antibodies were included in serial polychromatic flow cytometric (PFC) measurements: anti-CD2 BV786 (RPA-2-10), MHC-I PE (DX17), MHC-I PeCy7 (G46-2.6), CD3 PerCP Cy5.5 (SP34-2), CD4 APC (L200), CD4 AmCyan (L200), CD8 Pacific Blue (RPA-T8), CD8 APC (RPA-T8), CD56 PE (NCAM16.2), CD95 PE (DX2) (BD Biosciences, San Jose, CA, USA); CD11b AmCyan (M1/70.15.11.5), CD20 APC-Cy7 (LT-20), CD45RA APC-Cy7 (T6D11) (Miltenyi Biotec, Auburn, CA, USA); CCR7 PeCy7 (G043H7), CD28 Pacific Blue (CD28.2) (BioLegend, San Diego, CA, USA). The first step to assess naïve and memory T cells was based on CCR7 and CD45RA gating; naïve being defined as $CCR7^{+}CD45RA^{+}$, and all other cells as memory T cells. The populations were further gated on CD95 and CD28 to define naïve ($CD95^{low/-}CD28^{+}$), central memory ($CD95^{+}CD28^{+}$), and effector memory ($CD95^{+}CD28^{-}$) cells, respectively. Cells were permeabilized using the BioLegend FoxP3 Fixation/Permeabilization Buffer Set according to the manufacturer's protocol. Prior to permeabilization, cells were stained with CD3 PerCP Cy5.5 (SP34-2) (BD Biosciences), CD4 AmCyan (L200) (BD Biosciences), CD8 PeCy7 (BW135/80) (Miltenyi), CD127 (HL-7R-M21) (BD Biosciences), CD25 Pacific Blue (BC96) (BioLegend), and CD45RA APC-Cy7 (T6D11) (Miltenyi Biotec). Permeabilized cell staining included FOXP3 (PCH101) (Invitrogen, Carlsbad, CA, USA). Regulatory T cells (Tregs) were defined as $CD3^{+}CD4^{+}CD25^{high}FoxP3^{+}$. Isotype controls were included throughout, as well as fluorescence minus one (FMO) controls for CD2, and FOXP3. Data were acquired on a BD FACSCanto II flow cytometer (BD Bioscience) and BD Fortessa (BD Bioscience) flow cytometer and analyzed with FlowJo v10.1 software (TreeStar Inc., Ashland, Ashland, OR, USA) and FCS Express (De Novo Software, Glendale, CA, USA). Anti-CD2 BV786 (RPA-2-10, BD Biosciences) was used for the detection of CD2 expression. Anti-CD2 BV786 and RH-CD2 (secondary detecting antibody, clone HP6017, FITC anti-human IgG Fc; BioLegend) showed partial competitive binding to the CD2 epitope (Fig. 1h).

Lymph node immunopathology

One portion of each lymph node biopsy specimen was fixed in 10% formalin, and 70% ethanol, paraffin-processed, sectioned, and stained with Gill's hematoxylin and eosin (H&E) or CD3 (LN10; Leica, Newcastle, UK). Another portion of the biopsy was placed in RPMI-1640 medium (Gibco; Life Technologies, Grand Island, NY, USA), and lymphocytes were collected by grinding and passing through a 70 μm cell strainer (Corning, NY, USA). Isolated lymphocytes were immunophenotyped by flow cytometry using the same antibody panels as for PBMCs. After euthanasia of each dosed subject, necropsies were performed and major lymphoid tissues were collected for standard histopathological evaluation, performed by a clinical pathologist.

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Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) responses were evaluated on freshly isolated PBMCs from four cynomolgus macaques (including animals A2–A4), creating four MHC-mismatched responder-stimulator pairs, in the presence of increasing doses (0–20 μg/ml) of RT-CD2 and RH-CD2, respectively. Stimulator cells (1×10^5 cells/well) were irradiated (35 Gy) and plated in triplicate wells together with responder cells (1×10^5 cells/well) in 200 μl MLR media of a 96-well U-bottom plate (Costar, New York, NY, USA). MLR media contained RPMI-1640 (Gibco; Life Technologies), supplemented with 10% human serum (Gemini, Bioproducts, Sacramento, CA, USA), and 4% Nutrient mixture (MEM nonessential amino acids, Life Technologies), L-glutamine (Life Technologies), sodium pyruvate (Corning Cellgro, Manassas, VA, USA), and penicillin/streptomycin (Life Technologies). Responders stimulated with anti-CD2CD3CD28 beads (Miltenyi Biotec) (0.5×10^5 beads/well) and responders with MLR media alone served as positive and negative controls, respectively. After a four-day-long incubation at 37 °C and 5% CO_2, plates were pulsed with 1 μCi of ^{3}H-thymidine per well (Perkin, Elmer, Waltham, MA, USA), and harvested 24 h later (Tomtec, Hamden, CT, USA). Thymidine incorporation was measured on a ß-counter (1450 MicroBeta Scintillation-Luminescence Counter; PerkinElmer). Stimulation indexes (SI) were calculated as: (mean counts per minute (CPM) of experimental wells)/(mean CPM of negative control).

Mechanistic FcγR-binding and CDC assays

To determine Fcγ receptor (FcγR) affinity, 6x serial dilutions of RT-CD2 and RH-CD2 were incubated with reporter cells stably transfected with human FcγRI, FcγRIIA-H, or FcγRIIIA-V and a luciferase reporter gene (Promega, Madison, WI, USA). Luciferase reporter gene expression was triggered if reporter cells bound to the Fc-fragment of a target-bound IgG antibody with their respective FcγR. The reporter cells were from a human Jurkat $CD2^+$ acute T cell leukemia cell line. Reporter cell binding was flow cytometrically confirmed, for RH-CD2 using a BV421 anti-human IgG Fc (clone HP6017; BioLegend), and for RT-CD2 using a BV421 goat anti-rat IgG (clone Poly4054; BioLegend). Since the antibodies have affinity for the reporter cells they can act as a target and reporter in the assay. A human anti-CD2 IgG1 monoclonal antibody was used as a positive control (ITB-Med AB, Stockholm, Sweden), while anti-CD2 Fab fragments (enzymatically produced from the positive control) were used as a negative control. After 23 h of incubation at 37 °C and 5% CO_2, Bio-Glo Luciferase assay reagent was added and luminescence measured after 10–15 min of incubation at room temperature and shielded from light using a Synergy HTX Multi-mode plate reader (BioTek). Luminescence values were normalized to the luminescence signal obtained with the highest concentration of the positive control. Each run contained triplicate serial dilutions of each antibody.

For assessment of complement-dependent cytolysis (CDC), Ficoll density-gradient centrifugation isolated PBMCs were incubated with 10× serial dilutions of the respective antibody agent in 10% Ultra low-IgG FBS (Gibco) in PBS at room temperature. Alemtuzumab (Sanofi, Cambridge, MA, USA) served as a positive control while a human anti-CD2 IgG1 mAb (ITB-Med AB) known to not activate complement was used as a negative control. Subsequently, an equal volume of reconstituted rabbit complement (InnoTrain) was added followed by incubation at room temperature for 60 min. PBMCs were washed in PBS and stained with 7-AAD (Life Technologies), anti-CD56 BV421 (BD, Clone NCAM16.2), anti-CD89 PE (Miltenyi, Clone REA234), anti-CD3 APC, and anti-CD19 APC-H7 (BD; Clone SJ25C1). Samples were incubated on ice and shielded from light until sample acquisition using a FACSVerse flow cytometer (BD Biosciences). FLOWJO software (FlowJo LLC; Ashland) and GRAPHPAD PRISM 8 (GraphPad Software, San Diego, CA, USA) were used for sample processing and data analysis.

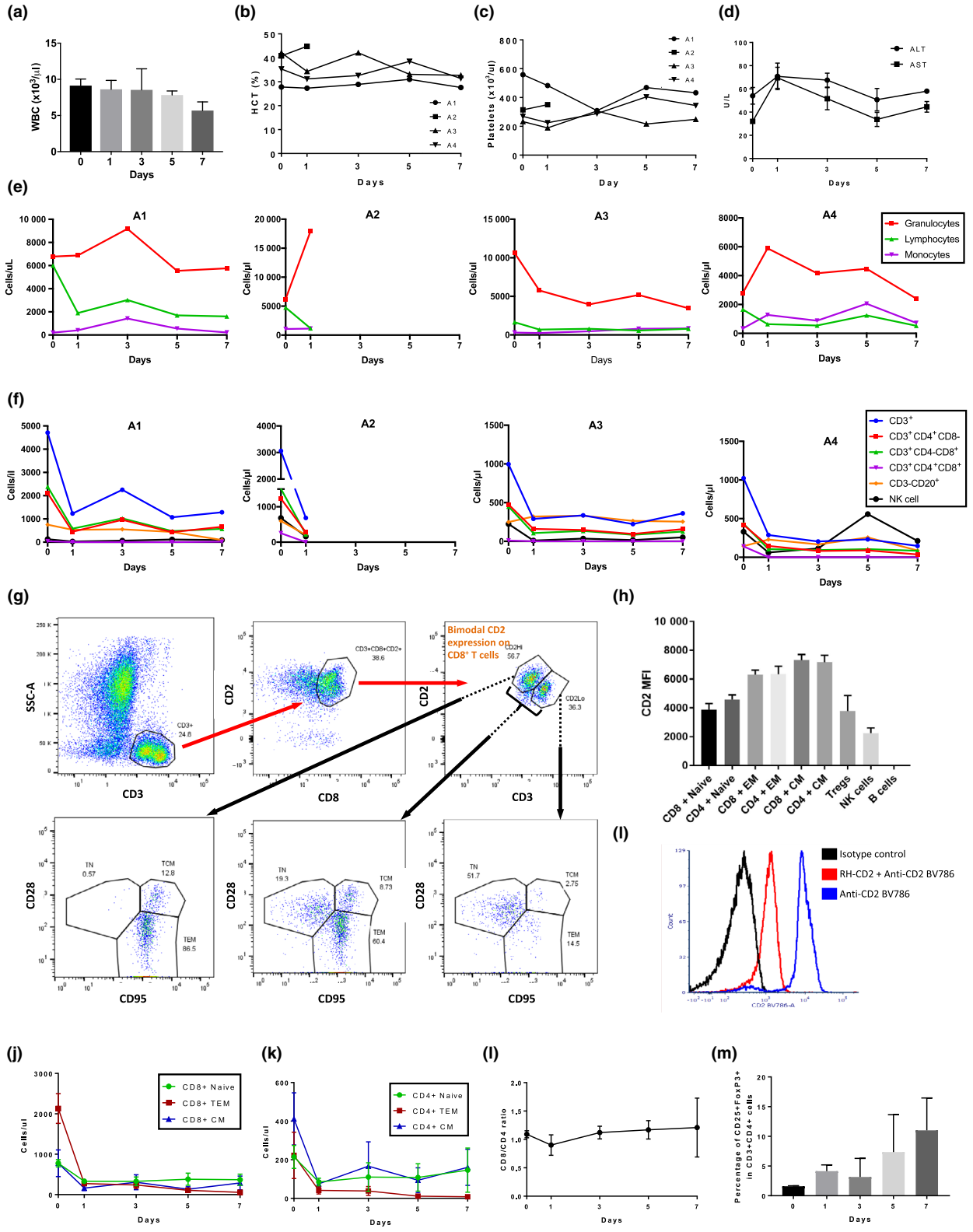
Statistical analysis

To establish statistically significant changes before and after treatment, Student's *t*-test or one-way ANOVA were used, and *P*-values <0.05 were considered significant, where *n.s.* denoted not significant. Data are expressed as mean ± standard error of the mean (SEM) unless stated otherwise.

Results

Clinical data

The study subjects were treated according to Table 1. None of the animals (groups A–C, Table 1) experienced any adverse events (AEs) related to the study drugs. Animal A2, however, had a severe apneustic event following ketamine injection on day 1, during sedation performed for peripheral blood collection. Prior to RH-CD2 infusion, the animal had shown signs of delayed



recovery after ketamine injections. The animal was euthanized on day 1. No significant weight loss was noted in animals A1, A3, and A4, which were followed for 7 days.

Chemistry and hematology

Blood chemistry panels and complete blood counts were followed serially. The total WBC did not significantly

Figure 1 Anti-CD2 treatment pharmacodynamics and CD2 expression on untreated freshly isolated lymphocytes. (a) WBC counts in all treated animals, groups A–C (*n.s.*). (b,c) Showing treatment impact on hematocrit (Hct%) or platelet levels (*n.s.*). (d) ALT and AST levels pre- and post-anti-CD2 infusion, group A. (e,f) Post-treatment levels of leukocyte subsets. (g) Showing bimodal CD2 and CD3 T cell expression in naïve cynomolgus monkeys, with memory cells having a higher $CD2^{hi}$ and $CD3^{lo}$ expression. Shown is a representative gate defining the bimodal distribution, and the three subsets of T cells (T_N, T_{EM}, T_{CM}) gated for CD2 and CD3. Predominantly, TN are $CD3^{hi}CD2^{lo}$ and memory cells $CD3^{lo}CD2^{hi}$. CCR7/CD45RA gating step not shown. (h) Average median fluorescence intensities (MFI) of cell surface CD2 on $CD4^+$-, $CD8^+$-, NK-, B-, and regulatory T cells from naïve animals ($n = 4$). (i) Showing partial competitive binding of RH-CD2 and CD2-BV786 antibodies. (j,k) RH-CD2 effectively depletes memory cells, while relatively sparing naïve lymphocytes in group A. (l) Illustrating the CD8/CD4 ratio before and after RH-CD2 in group A. (m) Showing a 695% $CD25^{hi}FoxP3^+$ cell enrichment from day 0 to day 7 after RH-CD2 infusion ($P = 0.052$) in group A. Data are presented as average (±SEM). CD, cluster of differentiation; CM, central memory; EM, effector memory; NK cell, natural killer cell; WBC, white blood cell count.

change post-treatment (*n.s.*, Fig. 1a). No clinically significant changes in hemoglobin or hematocrit were observed (Fig. 1b). Animal A1 had a mild transient decrease in platelet count only on day 3 postantibody infusion, which had normalized by day 5 (Fig. 1c). Groups B and C were sampled 24 h postinfusion, and showed stable WBC, which was in line with group A day 1 measurements. There was a transient low-grade increase in serum transaminases in several animals, which normalized within a few days (Fig. 1d). All other chemistry parameters were maintained within normal limits. The RH-CD2 infusion did not alter the number of circulating monocytes (Fig. 1e). The granulocyte count was stable in animals A1 and A4, while it was about halved in animal A3, and more than doubled in animal A2 (Fig. 1e).

Cell surface CD2 expression in untreated animals and circulating lymphocyte composition before and after anti-CD2 treatment

Polychromatic flow cytometric was used to serially evaluate the effect of anti-CD2 mAb on circulatory lymphocyte subsets post-treatment. All animals developed lymphopenia, characterized by decreased T cell numbers ($CD3^+$, $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^+$), fewer NK cells, with no change in B cell counts ($CD3^-CD20^+$) (Fig. 1f). Freshly isolated PBMCs from untreated cynomolgus macaques showed bimodal CD2 expression patterns among $CD3^+CD8^+$ and $CD3^+CD4^+$ cells, with naïve cells exhibiting lower expression than memory cells (Fig. 1g). There was no significant CD2 MFI difference between naïve cells, Tregs, and NK cells (*n.s.*), whereas naïve $CD3^+CD8^+$ and $CD3^+CD4^+$ cells had lower CD2 expression than their T_{CM} counterparts ($P = 0.001$ and $P = 0.017$, respectively), and T_{EM} and T_{CM} cells within both the CD4 and CD8 compartments had significantly higher expression than Tregs (Fig. 1h). Due to partial competitive binding between the treatment (RH-CD2) and detecting (CD2 BV786) antibodies, exact CD2 expression postinfusion could not be determined in group A (Fig. 1i).

Post-RH-CD2 treatment, we observed a substantial depletion within circulatory $CD4^+$ and $CD8^+$ memory subsets. Naïve cells were relatively spared, while T_{EM} counts were impacted the most (Fig. 1j,k). After one week, at the time of euthanasia, the most pronounced depletion was seen within the $CD8^+$ T_{EM} and $CD4^+$ T_{EM} compartments, with less effect on the naïve cells. There was no major shift in the overall $CD3^+CD8^+$ to $CD3^+CD4^+$ ratio (Fig. 1l). Anti-CD2 treatment relatively spared Tregs, enriching $CD25^{hi}FoxP3^+$ cells among $CD3^+CD4^+$ cells. Between day 0 (pretreatment) and 7 days after RH-CD2 infusion, the average relative Treg frequency had increased 695% ($P = 0.052$, Fig. 1m). Groups B and C were part of a separate tolerance study. Their peripheral lymphocytic depletion could therefore only be tracked 24 h post-anti-CD2 treatment, which supported a similar early (24 h) depletion profile as in Group A, without any dose-dependent effects observed (data not shown).

Primary mixed lymphocyte reaction effects of rat and rhesus anti-CD2 antibodies

To assess the immunosuppressive effects of RH- and RT-CD2 antibodies, varying concentrations of each mAb were added to a primary MLR using MHC disparate PBMCs from untreated macaques. Both antibodies displayed concentration-dependent inhibition of the primary MLR. The addition of beads elicited a strong proliferative response as expected, while culture with either autologous cells or MLR media did not induce proliferation (Fig. 2).

Histopathological and flow cytometric tissue examination

Light microscopic analyses and PFC performed on iliac and mesenteric lymph node samples obtained before

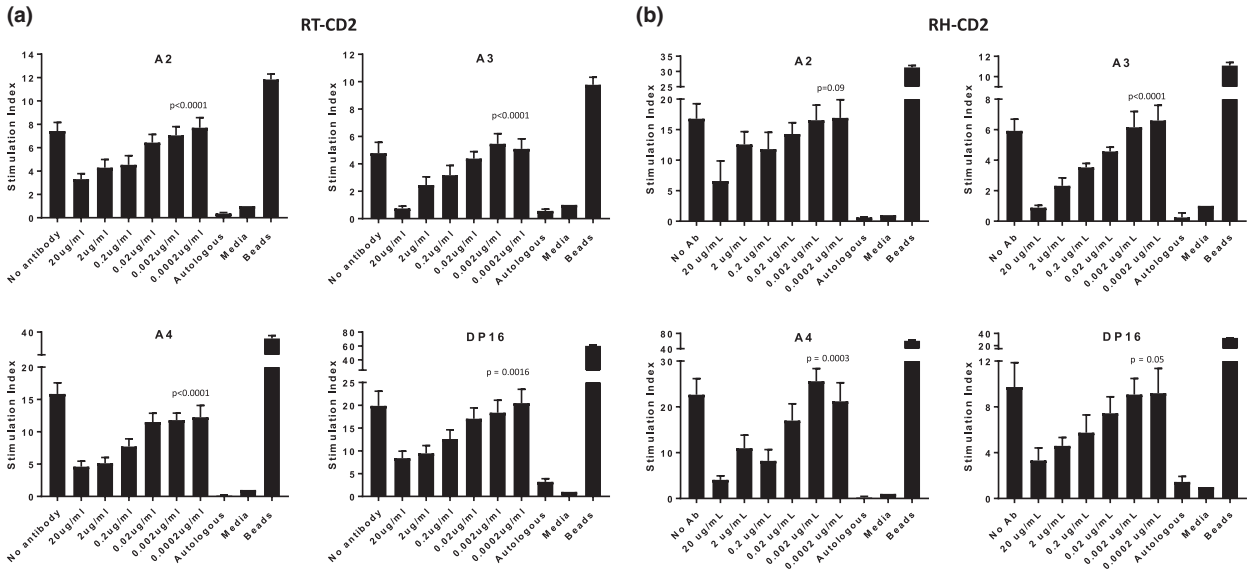


Figure 2 Immunosuppressive effects of rat and rhesus anti-CD2 mAb on a primary mixed lymphocyte reaction (MLR). The allogeneic MLRs were inhibited by varying concentrations of RT- and RH-CD2, respectively, at the onset of the host effector cell cultures. After four days, MLRs were pulsed for 24 h with 1 μCi ^{3}H-thymidine per well. The cellular radioactivity was measured in triplicates samples. Statistical analysis was performed of antibody and no antibody treatments using one-way ANOVA. (a) RT-CD2 (b) RH-CD2.

and after the treatment with RH-CD2 (group A). No significant cellular reduction was evident, although the presence of a potential minor depletion could not be ruled out. The normal thymus appearance of 6–9 years old cynomolgus monkeys (age span included in this study) is not fully known, but no major cellular depletion could be appreciated at the time of sacrifice (Fig. 3). A similar result was noted within the bone marrow, which showed a high cellularity on the last study day.

Mechanistic FcγR-binding and CDC assays

RH-CD2 induced a clear dose-dependent signaling through human FcγRI, FcγRIIA, and FcγRIIIA, respectively, although at a lower level than the human anti-CD2 IgG1 reference antibody that was used as a positive control. In contrast, RT-CD2 induced minimal FcγR-mediated signaling (Fig. 5a–c). Both RT-CD2 and RH-CD2 induced significant dose-dependent CDC of lymphocytes ($P < 0.05$; Fig. 5d).

Discussion

Blocking the interaction between the CD2 molecule on T cells and LFA-3 (CD58) on antigen-presenting cells has dual effects, leading to inhibition of T cell proliferation and co-stimulation blockade [1,2,14,15]. Depending on the structure of anti-CD2 mAbs, they can mediate T- and NK-cell depletion [5]. The humanized anti-CD2 monoclonal antibody Siplizumab induces lytic antibody-dependent cell-mediated cytotoxicity (ADCC) [3,16] via Fc receptor binding. More recently, using quantitative functional analyses and high-throughput T-cell receptor sequencing, we have shown that pre-existing donor-specific Tregs are expanded early in human recipients of combined kidney transplant and bone marrow cell infusion, who received a nonmyeloablative preparative regimen in which siplizumab was a key component [6]. *In vivo*, the enrichment of $CD3^{+}CD4^{+}CD25^{high}CD127^{low}$ $Foxp3^{+}$ Tregs in blood constituted almost 75% of the $CD3^{+}CD4^{+}$ PBMCs at the peak [4]. These results were further supported by *in vitro* cellular assays, where the ability of siplizumab monotherapy to enrich donor-specific Tregs while effectively depleting memory T cells was demonstrated [7]. This differential response is likely an effect of the lower CD2 expression on human Tregs and naïve cells and higher CD2 cell surface expression on memory lymphocyte subsets [7]. The current work built on these previous findings about the anti-CD2 effects in humans to determine if similar effects can be generated through intravenous administration of homologous anti-CD2 mAbs in nonhuman primates.

None of the cynomolgus monkeys dosed with either the RH- or RT-CD2 showed any study drug-related AEs. However, all animals received premedication with methylprednisolone and diphenhydramine, potentially masking any first-dose effects. The only detectable change in serum chemistries postdosing was a transient

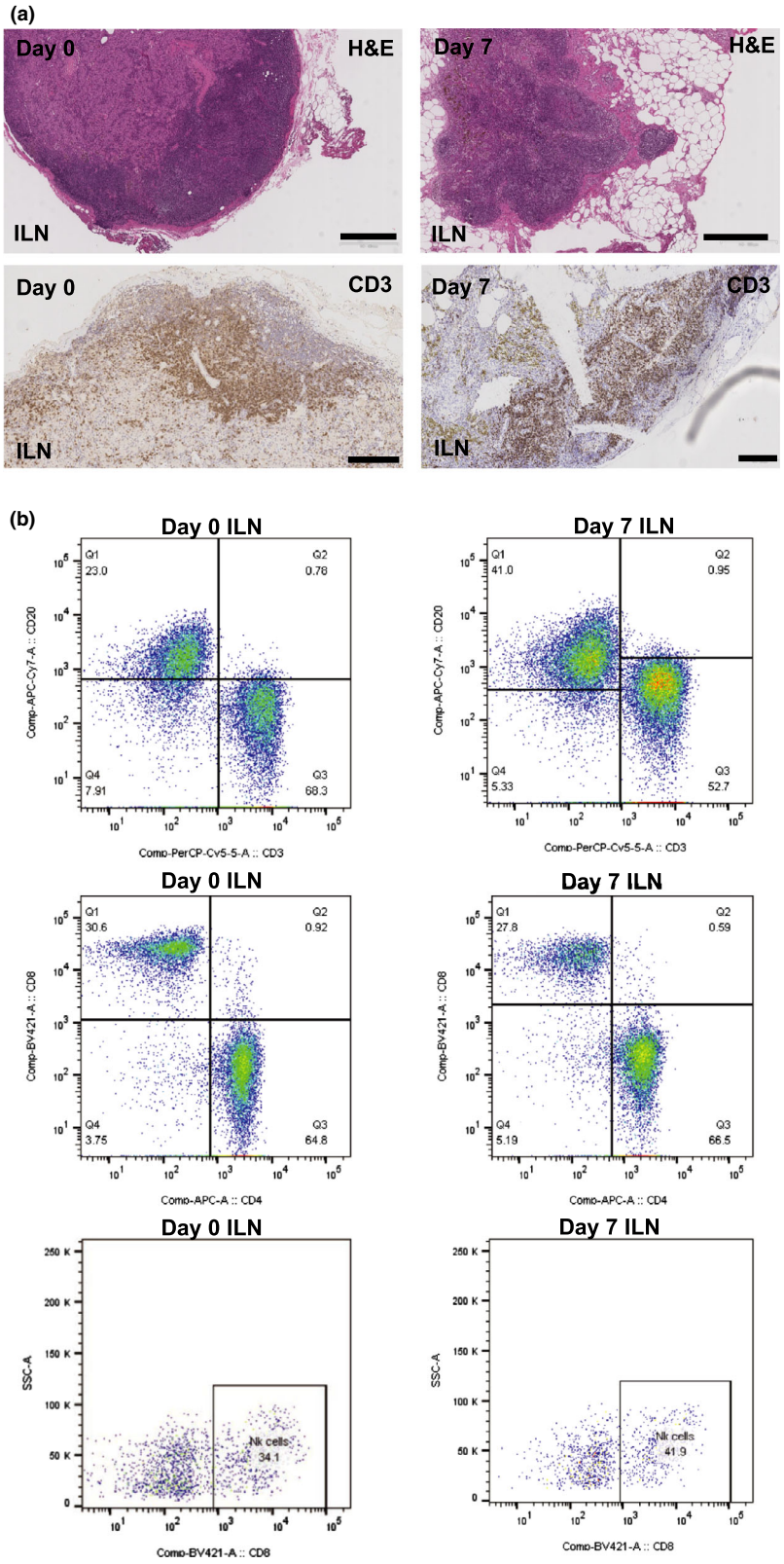


Figure 3 Histomorphological analysis before (n = 2) and after (n = 4) anti-CD2 treatment (Group A). (a) Light microscopic analysis showing representative iliac lymph node H&E and CD3 staining, suggesting no significant lymphocyte reduction seven days post-treatment. Bar width, 500 μm. (b) Polychromatic flow cytometric analysis of lymph nodes showing no major treatment impact on $CD3^+$, $CD4^+$, $CD8^+$, and NK-cells subpopulations. BM, bone marrow; ILN, iliac lymph node.

low-grade increase in serum transaminases, which normalized spontaneously without clinically impacting the animals (Fig. 1d).

Both anti-CD2 mAbs (RT- and RH-derived) effectively and significantly depleted circulating $CD8^+$ and $CD4^+$ effector memory T cells within 24 h postdosing,

while relatively sparing naïve cells and Tregs (Fig. 1j,k, m). The depletion profile for memory T cells lasted during at least the studied seven days and is likely reflected by the high CD2 expression on cynomolgus monkey PBMCs (Fig. 1h), which has similarities to the findings on human PBMCs [7]. The postdosing CD2 expression analysis was incomplete due to partial competing binding between the treatment (RH-CD2) and detection (CD2 BV786) antibodies (Fig. 1i). The low-dose TBI given in Groups B and C is not expected to significantly influence lymphocyte number at the time of anti-CD2 administration. Therefore, the early (24 h) anti-CD2 depletion profile in Group B–C further supports the effects seen in Group A. However, no dose-response conclusions can be drawn.

Results obtained from MLR assays that compared the effect of titrated doses of RT- and RH-CD2 on the proliferation of stimulated PBMCs confirmed that the *in vitro* addition of the anti-CD2 mAbs resulted in a concentration-dependent inhibition (Fig. 2a,b), suggesting the same effect could occur *in vivo*. This in vitro inhibition was not as strong as for siplizumab treatment of human PBMCs in equal dose ranges [7].

Histopathological data that compared lymph node samples obtained before and after RH-CD2 treatment (group A) showed no significant changes in cell number, cell type or distribution in lymph nodes (Fig. 3). Similarly, thymus and bone marrow samples obtained at the time of sacrifice suggested no significant changes in the cell populations compared to the standard cell distribution in cynomolgus macaques in these tissues [17].

The mode of depletion for RT-CD2 is mainly mediated via CDC while RH-CD2 mediated both CDC and FcγR signaling (indicating ADCC/ADCP). However, consideration must be taken toward possible differences in the affinity of the Fc-region of the respective antibodies toward human Fc-receptors used in the assays (Fig. 4).

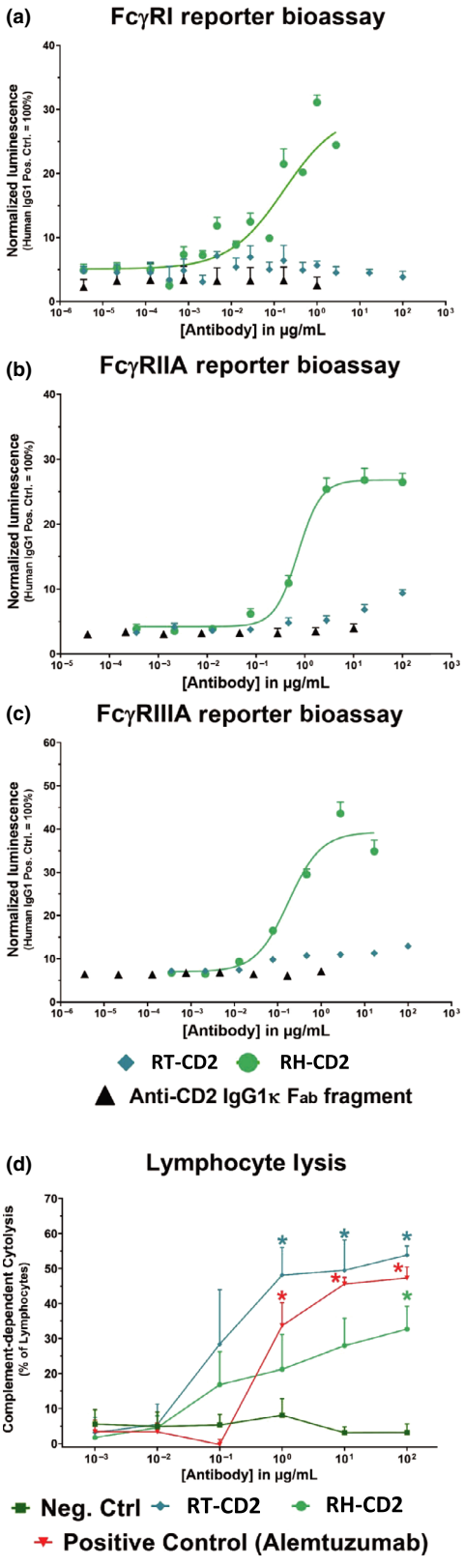


Figure 4 FcγR Reporter Bioassay and flow cytometry-based assessment of CDC. Effector cells stably transfected with (a) FcγRI (n = 3), (b) FcγRIIA (n = 3) or (c) FcγRIIIA (n = 2) and a luciferase reporter gene were incubated with serial dilutions of antibody. Upon binding of a target-bound IgG antibody (the reporter cells expressed CD2) luciferase expression was induced. Luminescence values were normalized to the highest concentration of the positive control. RH-CD2 displayed clear dose-dependent FcγR-mediated signaling but RT-CD2 did not. (d) Both antibodies induced dose-dependent CDC of lymphocytes relative to untreated controls (n = 4).

Taken together, there is a resemblance of CD2 expression patterns on naive cynomolgus monkeys, rhesus macaques, and human PBMCs. The safety

and pharmacodynamic profiles are similar with both antibodies, and with no obvious dose-response effects.

Authorship

EB, FS and CB: participated in research design; writing of the paper; performance of the research; contributed new reagents or analytic tools; participated in data analysis. PA-G, MD, NL and AG: participated in research design; writing of the paper; performance of the research; data analysis. RF, AI and DHS: participated in the writing of the paper; data analysis. DB and MS: participated in research design; writing of the paper; data analysis. HS and DE-A: participated in the performance of the research. KAR: participated in the writing of the paper; contributed new reagents or analytic tools; participated in data analysis.

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Conflict of interest

EB, DHS, and DB are shareholders in ITB-MED AB. MS is a scientific advisor to ITB-MED AB. FS, CB are employees of ITB-MED AB. All other authors have no conflict of interest to disclose.

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