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Immunosuppressive effect of ASP2408, a novel CD86-selective variant of CTLA4-Ig, in rats and cynomolgus monkeys

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ABSTRACT

The CTLA4-Ig fusion proteins abatacept and belatacept inhibit CD28-mediated T cell activation by binding CD80 (B7-1) and CD86 (B7-2) costimulatory ligands and are clinically proven immunosuppressants used for rheumatoid arthritis and renal transplantation, respectively. Abatacept and belatacept preferentially bind CD80, yet CD86 has been implicated as the dominant ligand for CD28-mediated costimulation of T cells. We investigated the immunosuppressive effects of ASP2408, a novel CTLA4-Ig with CD86 selectivity and high potency created by directed evolution methods. Here we evaluated the effect of ASP2408 *in vitro* using cynomolgus monkey and rat T cell proliferation assays and *in vivo* using cynomolgus monkey tetanus toxoid (TTx) immunization and a rat rheumatoid arthritis model. ASP2408 was 290-fold and 21-fold more potent in suppressing *in vitro* monkey T cell proliferation than abatacept and belatacept, respectively. ASP2408 inhibited anti-TTx immunological reactions in cynomolgus monkey at a 10-fold lower dose level than belatacept, through complete CD86 and partial CD80 receptor occupancies, and also suppressed inflammation in the rat collagen-induced arthritis model. Overall, improved immunosuppressive potency of ASP2408 relative to abatacept and belatacept correlated well with improved CD86 binding affinity. These results may support the advantage of preferential enhancement of CD86 binding affinity to inhibit T cell-mediated immune response and improved dosing convenience in humans relative to abatacept or belatacept.

1. Introduction

T cells play a pivotal role in initiating and modulating cellular and humoral immune responses in rheumatoid arthritis (RA) ([1] Cush JJ and Lipskey PE; [2] Tran CN et al.). Inhibition of costimulatory signals required for T cell activation is now a clinically validated immunosuppressive strategy for RA therapy. Abatacept is a fusion of the extracellular domain (ECD) of human cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, also known as CD152) with the Fc domain of modified human IgG1 (CTLA4-Ig). CTLA-4, an inhibitory receptor on the T cell with high homology to the costimulatory receptor CD28, serves as a negative regulator of CD28-mediated activation of CD4+ and CD8+ T cells ([3] Chambers et al.). Abatacept acts by disrupting engagement of CD28 with B7 molecules CD80 (also known as B7-1) and CD86 (also known as B7-2) on antigen-presenting cells (APC) ([4] Herrero-Beaumont G et al.). Belatacept, a second generation CTLA4-Ig, is a modified version of abatacept with two amino acid changes in the ECD of CTLA-4 (A29Y and L104E) that were reported to confer improved CD80 and CD86 affinity. Belatacept was shown to have improved efficacy in non-human transplant models relative to abatacept ([5] Larsen et al.) and is now an approved medicine for the prophylactic prevention of renal transplant rejection ([6] Wekerle et al.; [7] Chopra et al.).

A number of biological and structural studies have shown that CD86 plays a more significant role than CD80 in engaging CD28 on the T cell ([8] Stamper et al.; [9] Pentcheva-Hoang et al.; [10] Bhatia et al.; [11] Jansson et al.; [12] Latek et al.; [13] Xu et al.), suggesting that increased

Abbreviations: APC, antigen presenting cells; BrdU, 5-bromo-2′-deoxyuridine; CI, confidence interval; ConA, Concanavalin A; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; ECD, extra-cellular domain; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; EPT, end-point titer; FBS, fetal bovine serum; MFI, median fluorescence intensity; HLA-DR, human leukocyte antigen - antigen D related; NHP, non-human primate; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; RA, rheumatoid arthritis; SC, subcutaneous; Treg, T regulatory cell; TTx, tetanus toxoid.

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affinity for CD86 may provide increased immunosuppressive efficacy of CTLA4-Ig therapeutics. Indeed, this hypothesis was the rationale for development of belatacept ([12] Latek et al.), although belatacept retains preferential binding to CD80 relative to CD86. Furthermore, because CD80-mediated signaling is related to several protective immune functions including control of infection and malignancy ([14] Lumsden et al.; [15] Suvas et al.; [16] Ando et al.; [17] Rozanski et al.), which are known complications for abatacept and belatacept therapies ([7] Chopra et al., [18] Keating et al., [19] Wojciechowski and Vincenti), a drug candidate with less inhibition of CD80 signaling may mitigate such complications.

ASP2408 is a next-generation CTLA4-Ig therapeutic created by directed evolution (gene shuffling) methods. ASP2408 is an IgG2 Fc fusion protein that contains six amino acid changes in the CTLA-4 ECD relative to wild-type that confer dramatically improved binding affinity and selectivity to human CD86 ([20] Oshima et al.). Here we show that ASP2408 has improved immunosuppressive potency relative to abatacept and belatacept *in vitro* and *in vivo* using cynomolgus monkey immunization and rat arthritis models.

2. Material & methods

2.1. Creation of ASP2408, reagent and control proteins

ASP2408, abatacept IgG2 (wild-type human CTLA-4 ECD fused with human IgG2 Fc), belatacept IgG2 (human CTLA-4 ECD with A29Y L104E mutations fused with human IgG2 Fc), belatacept (human CTLA-4 ECD with A29Y L104E mutations fused with modified human IgG1 Fc), rhesus macaque CD80-Ig (rhesus CD80 ECD fused with human IgG1 Fc), rhesus macaque CD86-Ig (rhesus CD86 ECD fused with human IgG1 Fc) and human CD80-mIg (human CD80 ECD fused with murine Fc) were produced at Perseid Therapeutics (Redwood City, CA, USA) ([20] Oshima et al.). Rat CD80-Ig (R&D systems, Minneapolis, MN, USA, catalog number 1214-B7), rat CD86-Ig (R&D systems, catalog number 1340-B2) and abatacept (Bristol-Myers Squibb, Princeton, NJ, USA) were purchased. Endotoxin level of ASP2408 and belatacept were ensured to be within the limits of exposure (<5 EU/kg/h) for *in vivo* experiments.

2.2. Biacore analysis for monkey and rat CD80 and CD86

Biacore kinetics and equilibrium-binding studies of CTLA4-Ig variants and data analysis were performed as described previously ([20] Oshima et al.). CD80-Ig and CD86-Ig were immobilized on CM-5 biosensor chips (GE Healthcare, Little Chalfont, UK, catalog number BR-1000-14) via capture with mouse anti-human IgG1 antibody (US Biological, catalog # I1904-75K, clone 2B2.9) and CTLA4-Ig variants were flowed over these surfaces. Data were analyzed by BIAevaluation software (v4.1, available from GE Healthcare) to determine the association rate constant (k_a) and the dissociation rate constant (k_d) and to calculate the equilibrium dissociation constant, K_D.

2.3. Animals

Tetanus toxoid (TTx)-induced immune response experiments in cynomolgus monkeys (*Macaca fascicularis*) were conducted at Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). Collagen-induced arthritis experiments in rats were performed at PharmaLegacy Laboratories (Shanghai, China). Both of these studies and all experimental procedures conducted on animals at Astellas Pharma Inc. were approved by the institutional Animal Care and Use Committee of each facility and were performed in accordance with the ethics criteria contained in the bylaws of the committee. Furthermore Astellas Pharma Inc., Tsukuba Research Center and Shin Nippon Biomedical Laboratories, Ltd. are accredited Accreditation Status by the AAALAC International.

2.4. Monkey mixed lymphocyte reaction (MLR)

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of normal cynomolgus monkeys using Ficoll-Paque gradient technique as per manufacture's recommended conditions. PBMC were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA, catalog number P0781), and 10% fetal bovine serum (FBS). Responder PBMC (1.5×105/well) were cultured in triplicate in 96-U-well culture plates for 4 days at 37 °C with an equal number of irradiated (20Gy) allogeneic PBMC to provide co-stimulatory signaling via endogenous APC, and with different concentrations of CTLA4-Ig variants. The cells were pulsed with 1 μCi/well ^{3}H-thymidine (GE Healthcare Inc.) six hours before the termination of culture, and proliferation was assessed by ^{3}H-thymidine incorporation using a scintillation counter. Plates were harvested and the counts per minute analyzed for IC_{50} using a Sigmoid-Emax model non-linear regression analysis. The percent inhibition of proliferation was calculated by defining the no test compound treated group as 0% and the no stimulator cell control group as 100%. Geometric mean values of IC_{50} for each test compound were calculated from four independent experiments along with a two-sided 95% confidence interval (CI).

2.5. ConA induced rat T-cell proliferation

Splenocytes of Lewis rats (Charles River Labs, Kanagawa, Japan) isolated by standard technique were prepared in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, catalog number P0781) and 10% FBS, and 1.5×10^5 of splenocytes were stimulated with 2 μg/mL of Concanavalin A (ConA) (Vector Laboratories, Inc., Burlingame, CA, USA) in 96-well culture plates. Test compounds were serially diluted in the culture medium and added to the wells. For controls, splenocytes were cultured in the presence or absence of ConA without test compounds. After incubating at 37 °C in a CO_2 incubator for 2 days, splenocyte proliferation was assayed using a cell proliferation enzyme-linked immunosorbent

Table 1
Summary of binding profile of CTLA4-Ig variants on monkey ligands.

	CD86_monkey						CD80_monkey					
CTLA4-Ig variants	k_a (1/Ms)	k_d (1/s)	K_D (M)	Fold vs abatacept	Fold vs belatacept	Selectivity	k_a (1/Ms)	k_d (1/s)	K_D (M)	Fold vs abatacept	Fold vs belatacept	Selectivity
Abatacept-IgG2[a]	1.60E + 06	2.96E − 03	1.85E − 09	1	0.089	0.28	9.65E + 05	4.96E − 04	5.15E − 10	1	0.12	3.6
Belatacept-IgG2[b]	3.05E + 06	5.02E − 04	1.65E − 10	11	1	0.39	1.63E + 06	1.04E − 04	6.39E − 11	8.1	1	2.6
ASP2408[b]	3.54E + 06	1.04E − 04	2.94E − 11	63	5.6	9.5	1.29E + 06	3.61E − 04	2.80E − 10	1.8	0.23	0.11

k_a represents the association rate constant, k_d represents the dissociation rate constant and K_D represents the equilibrium dissociation constant.
Values are expressed as the mean of 2 or more independent measurements. Selectivity is expressed as the ratio of K_Ds for each ligand.
[a] Analyzed with 5 min dissociation phase.
[b] Analyzed with 20 min dissociation phase.