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Supplementary Text

Site-directed mutagenesis of CTLA-4

CTLA-4 mutants based on wild type human CTLA-4 ECD (K36-D153, UniProt accession code: P16410) with a C terminal His tag were constructed using site-directed mutagenesis. For each single mutation, two pairs of primers were employed to produce PCR fragments that harbor the mutation site and share overlapping sequences. After template DNA was digested by DpnI (NEB, R0176L), two PCR fragments were cloned using pEASY®-Uni Seamless Cloning and Assembly Kit (Transgen, CU101-03).

Wildtype and mutant CTLA-4 were expressed in HEK293 cells (Gibco, R79007). After transfection, the cells were grown at 37°C for 6-7 days in FreeStyle F17 expression medium (Gibco, A1383502). Cell viability and density were measured every other day. When the viability was less than 70%, cell culture medium was harvested. Wild type and mutant CTLA-4 proteins were purified using HisTrap excel (GE Healthcare, 29048586) and then dialyzed against PBS, pH7.4 (Sangon, E607016).

Crystallization of CTLA-4/4003-1 (VH) and determination of its structure

HCAb 4003-1 was selected for its excellent binding affinities to both human and cynomolgus monkey CTLA-4 proteins (Table s1) as measured by surface plasmon resonance (SPR). To investigate the molecular interaction of anti-CTLA-4 HCAb 4003-1 to human CTLA-4 protein, we crystallized the complex of human CTLA-4 and 4003-1 (VH) and determined its structure at 2.38 Å (PDB code: 7DV4). The statistics for the data collection and refinement are shown in Table s11. There are four human CTLA-4 and four 4003-1 (VH) molecules in one asymmetric unit (Figure s5) but we focused only on CTLA-4 and 4003-1 (VH) within one heterodimer (Figure s5). The interactions within 1.90-3.90 Å between CTLA-4 and 4003-1 (VH) were explored using open-source PyMOL (The PyMOL Molecular Graphics System, Version 2.4 Schrödinger, LLC). The interacting interface between 4003-1 (VH) and human CTLA-4 consisted of 15 residues from 4003-1 (VH) and 14 from hCTLA-4, respectively (Figure 1b and Table s12). All three CDRs of 4003-1 (VH) took part in the interaction with human CTLA-4 and CDR3 as the dominant interaction region.

Superimposition of CTLA-4/4003-1 (VH) and CTLA-4/ipilimumab

To investigate the unique binding domain of CTLA-4/4003-1 (VH) to CTLA-4/ipilimumab, the structures of CTLA-4/4003-1 (VH) and CTLA-4/ipilimumab (PDB code: STRU) were superimposed and compared using PyMOL by overlaying 4003-1 (VH) onto the heavy chain of ipilimumab (RMSD: 0.55 Å). Residues Y34 (CDR1), Y53 and S54 (CDR2), and R98, H102, S103 and F107 (CDR3) were involved in more interacting pairs compared to other residues. On CTLA-4, residues E33, R35, E48, T53, T61, L63, E97, M99, Y100 and Y104 made a major contribution to the interaction with 4003-1 (VH), while C50, A51, Y54 and M55 did not.
To validate the binding epitopes on CTLA-4 disclosed in crystal structure, we mutated CTLA-4 at particular amino acid positions predicted to be involved in binding and measured the affinities of antibody to mutated versions of CTLA-4 protein using Bio-Layer Interferometry (BLI). As shown in Table s12, mutagenesis of CTLA-4 at R35, E97, M99 and Y104 to alanine completely abolished the binding of CTLA-4 to 4003-1 (HCAb), demonstrating essential roles played by these residues in antibody-antigen interaction. Especially, mutation of R35K or E97D resulted in a dramatic reduction on its binding to 4003-1, suggesting that side chains of R35 and E97 in CTLA-4 proteins are essential to the binding of 4003-1 (HCAb). CTLA-4 residues E33, E48, L63 and Y100 are also important, since mutagenesis of these four residues to alanine led to a 42 to 80-fold decrease in binding activity.

Superimposition of CTLA-4/4003-1 (VH) and CTLA-4/CD80 (CD86)

In order to study whether 4003-1 (HCAb) would block binding of CTLA-4 to CD80 and CD86, the structure of the CTLA-4/4003-1 (VH) complex was superposed onto CTLA-4/CD80 and CTLA-4/CD86 (PDB code: 1I8L and 1I85), respectively, using PyMOL. The VH of 4003-1 partially overlapped with CD80 or CD86 (Figure s2c and s6b,c) which explains why it blocks binding. As revealed by analysis of the interactions using PyMOL, five CTLA-4 residues (E33, R35, M99, Y100 and Y104) are involved in the interaction with the VH of 4003-1 (Figure 2b). To validate whether these residues on CTLA-4 are virtually involved in binding with CD80 and CD86, the binding of several CTLA-4 mutants to CD80 and CD86 were measured using the BLI assay (Table s13). CTLA-4 mutants M99A and Y104A did not associate with either CD80 or CD86, E97A and E97D did not bind CD80, and Y100A did not interact with CD86 (Figure 2b). This suggests these residues are the most important ones interacting with CD80 or CD86, and these are also the key residues recognized by HCAb 4003-1 (VH). However, when binding with CD80, CTLA-4 mutants E33A, E33D, R35A, R35K and Y100A showed only a decrease in affinity of 4.7- to 11-fold compared to wild type CTLA-4. As for binding affinities to CD86, the CTLA-4 mutation E33A showed a reduction about 4.8 fold; R35A about 3.3 fold; while E33D, R35K and T53S kept almost the same binding affinities as wild type CTLA-4.
HCAB 4003-2 comparison with BMS-986218 and Agen1181

The binding, blocking, ADCC killing activity and Tumor growth inhibition of HCAb 4003-2 was also compared to that of BMS986218 and Agen1181 using the same methods as described for Ipilimumab (Figures 2-4). HCAb 4003-2 showed a higher binding, blocking and ADCC activity when compared with BMS986218 and Agen at the same concentration (Figure s1, s2).

Supplementary reagents

An Ipilimumab analogue was generated using the available ipilimumab patented sequence. The resulting antibody was compared to the commercially available ipilimumab (Yervoy) by gel electrophoresis, HPLC, B7-1 and B7-2 blocking by Elisa and Binding to Jurkat cerlls and an ADCC reporter assay.

*Stimune: Fresh PBS (antigen) in oil emulsion was prepared before injection by mixing 4 parts of the water phase containing antigen with 5 parts of Stimune by adding the water phase slowly to the oil phase (Stimune) during vigorous mixing, 20 micrograms of antigen in 100μl volume was injected subcutaneously.

*Ribi: Content of standard vial was dissolved in 2 ml of PBS. Antigen amount to be injected per mouse was in 200 ml. 100 ml was injected s.c between two different sites and 100 ml was given i.p.

Supplementary methods

Antigen specific ELISA

To check whether the mice responded, ~100 μl of blood was collected from the mouse facial veins for ELISA. ELISA was carried out in 96 well plate (Nunc™ MaxiSorp®). Coating was done with 5 μg/ml antigen in PBS overnight in the fridge followed by blocking with 300 μl PBS with 1% BSA, 1% non-fat milk and 0.1% Tween-20 per well for 30 minutes at room temperature and washing 3 times with PBS with 0.1% Tween-20. 3 μl of serum was diluted to 600μl in PBS with 1% BSA, 1% non-fat milk and 0.1% Tween-20. This is a 200-fold dilution, next dilution is 1000 fold, then 3000, 7500, 15000, 30000. 50 μl of each serum dilution is added to the wells and incubated for at least 2 hours at room temperature. Plate was washed 5 times with PBS with 0.1% Tween-20. Washing volume was 300 μl each time. 50 μl of rat anti-mouse IgG1 antibody peroxidase labeled (BD Pharmigen Cat 559626) diluted 1:1000 in PBS with 1% BSA, 1% non-fat milk and 0.1% Tween-20 was incubated for 2 hours at room temperature, washed 5 times with 300 μl of PBS with 0.1% Tween-20 followed by addition of the 50 μl of POD substrate (Roche, BM Blue POD substrate soluble, # 11484281001). After incubation of 3-5minutes, reaction was stopped with 50 μl of 1N H2SO4. The plates were analyzed on Varioscan ELISA reader at 450nm.
Mice were briefly anesthetized by Isoflurane inhalation and injected with 10 μl of Evans blue (stock 500mg/10ml PBS) in foot pads. 10-15 min later, they were sacrificed (by cervical dislocation) and lymph nodes (stained blue) dissected. A single cell suspension was made, and antigen specific lymphocytes were isolated.

Antigen (huCTLA4) was biotinylated using EZ-LINK NHS-PEG12-Biotin (Thermo Scientific#21312) according to the manufacturer’s instruction. Biotinylated antigen was concentrated and free label removed by using Amicon centrifugal filter (10kDa cut off) and used for binding of antigen specific cells. Cells were washed by PBS/0.5%BSA/2mM EDTA (washing includes adding the buffer, re-suspending the cells, centrifugation at 1000rpm for 5min and discarding the supernatant).

RNA extraction from antigen specific B cells

Lymphocytes bound to biotinylated antigen on Streptavidin beads were treated with TRI Reagent (Sigma-Aldrich, T9424) by repeated pipetting. 1 ml of the reagent is sufficient to lyse 5-10 × 10⁶ animal cells. Total RNA was made according to manufacturer’s instruction. In short, 0.2 ml of chloroform per ml of TRI Reagent was used, shacked vigorously for 15 seconds allowed to stand for 2–15 minutes at room temperature and the resulting mixture centrifuged at 12,000 × g for 15 minutes at 4 °C. Colorless upper aqueous phase (containing RNA) was transferred to a new tube and precipitated with 0.6V of 2-propanol. After centrifuging at 12,000 × g for 10 minutes at 4 °C, RNA precipitate was washed the 75% ethanol, centrifuged at 7,500 × g for 5 minutes at 4 °C and dissolved in RNAse free dH₂O. RNA concentration was measured on Nanodrop.

cDNA generation from RNA

92 ng RNA was used for making the cDNA in a 20 µl reaction volume for first strand cDNA synthesis using SuperScript TM II RT (Cat. No. 18064-022 Invitrogen) and oligo dT, using the following protocol: mix 1 µL Oligo(dT) 12-18 (500 µg/mL), 1 µL 92 ng total RNA, 1 µL dNTP Mix (10 mM each) and 9 µL sterile, distilled water. Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add 4 µL 5X First-Strand Buffer, 2 µL 0.1 M DTT, 1 µL RNaseOUT™ (Cat. No. 10777-019) (40 units/µL). Incubate at 42°C for 2 min. 1 µL (200 units) of SuperScript™ II RT was added and incubated at 42°C for 50 min. Finally, the reaction was inactivated by heating at 70°C for 15 min.

PCR reactions were performed using the Phusion high Fidelity DNA polymerase (Finnzymes, F530L, now BioEnland) using the following cycling instructions:

- Initial denaturation 98°C 30sec 1 cycle
- Denaturation: 98°C 10sec
- Annealing 65°C 30sec
- Extension 72°C 30sec 30 cycles
- Final extension: 72°C 10min 1 cycle
6 reactions were set in 50 µl volume (per reaction: template cDNA 2 µl; 5X HF Phusion buffer 10 µl, 1mM dNTPs 1µl, primers at 0.5MicroM final conc, DMSO 1.5 µl-final 0.3%, and 0.5 µl Phusion

Primers used

Forward primers (from the leader sequences with PVUII site):

lib-3-23/S3-S: 5’-GTGTCCAGTGAGGTGCAGCTG
lib-3-11-S: 5’-GTGTCCAGTGCAGGTGCAGCTG

reverse primer (from the hinge of mouse IgG1):

a rev IgG1 hinge BSTEII: AATCCCTGGCAGTAAGAGACGGTGACC

The PCR product was cleaned up by using Nucleospin Extract II (Macherey-NagelGmbH) according to protocol provided by the manufacturer. Double PVUII /BSTEII digest at 37 °C for 1 hour was performed using BSTEII HF with cut SMart buffer (NEB #R3162). Digested VH fragments were gel purified using Nucleospin gel/ PCR clean up kit and ligated to PvuII / BstEII cut phosphatase treated pCAG hygro mG1 vector (Harbour Ab). Ligation was performed as following:

2100 ng vector (PvuII /Bstell, digested CIP treated vector)
140 ng PVUII/BStEII digested amplified VHs
10X ligation buffer (4 µl)
T4DNA Ligase Promega (4µl )
H2O up to 55 µl

The Hind III digest was performed directly on the ligation after linearization of the vector.

The digest was performed in 100µl (10 units of enzyme, 10 µl of 10X buffer and H2O up to 100, for 1h at 37°C). The digest sample was run on 0.6 % agarose gel and linear fragment was cut out and cleaned up on a spin column (Nucleospin) and concentration measured on NanoDrop.

Transfection and positive clone selection by ELISA

840ng of linearized vector was used to transflect 1x 24well with 90-100% confluent HEK cells plated in advance using Lipofectamin2000. Invitrogen protocol was followed making sure to have the HEK cells without antibiotics from the day before transfection. The transfection was left overnight in the incubator. Next day the transfected cells were re-suspended in 750ml of fresh medium (with antibiotics but not the Hygromycin yet) and plated on 72x96well plates, 100µl per well. Next day 100µl of medium containing 240 µg/ml Hygromicin was added to each well to end up with a concentration of 120µg/ml Hygromicin.

After incubating for 10 days, all supernatants (wells) were screened with an ELISA assay. ELISA was performed as before on the serum, but in a 384-well-plate. Plates were coated with 15 µl
(5µg/ml of antigen), 50 µl of supernatants were tested, 20 µl of goat anti mouse IgG-PO 1:2500 (Abcam) was used and 25 µl of POD substrate was used.

**Affinity screen on ForteBio Octet system**

Biotinylated antigen (10 µg/ml) was loaded to streptavidin tips after the baseline in PBS, probes were washed in PBS (5min), dipped into the wells with supernatants or plain medium as a negative control for association step, followed by dissociation step in PBS. All volumes were kept at 200 µl.

**Crystallization, data collection and structure determination**

Human CTLA-4 and 4003-1(VH) proteins were prepared by Viva biotech. The two proteins were mixed at a molar ratio of 1:1 and incubated at 4oC overnight to allow complex formation. Human CTLA-4/4003-1(VH) complex (8.9 mg/mL) was crystallized at 18°C using the sitting-drop vapor diffusion method in a reservoir solution of 100 mM sodium citrate, 10% (w/v) PEG 6000, pH 5.0 (sodium citrate: Alfa Aesar, A12274; PEG6000: Alfa Aesar, A17541). One diffraction dataset, which was collected at the Shanghai Synchrotron Radiation Facility (SSRF), was processed using XDS package (Kabsch, 2010). One Fab fragment (PDB code: 5AZE) and huCTLA-4 apo homodimer (PDB code: 3OSK) were used as searching models for molecular replacement in Phaser in CCP4 suite. Refmac5 was employed for refinement.

**Determination of binding affinity between 4003-1 (HCAb) and CTLA-4**

To validate the binding epitope on CTLA-4 revealed by crystal structure, interaction between HCAb 4003-1 and wild type and mutant huCTLA-4 were determined using BLI (Octet Red96e, ForteBio). HCAb 4003-1 was loaded using AHC biosensors (ForteBio, 18-5060) to 0.8 -1.0 nm. CTLA-4 proteins at 100 nM (WT, R35A, E97A, E97D, M99A, Y104A, Y104F) or at 180, 90 and 45 nM (WT, E33A, E33D, R35K, E48A, E48D, T53A, T61A, L63A, Y100A) were used as analytes. Double reference was applied when doing the 3-dose measurement.

**Measurement of interaction between CTLA-4 and its receptors B7-1 and B7-2**

Affinities between CTLA-4 and two receptors (B7-1 and B7-2) were measured using Octet Red96e to check whether 4003-1, B7-1 and B7-2 bind to adjacent sites on CTLA-4. His-tagged wildtype and mutant CTLA-4 proteins were loaded using Anti-Penta-HIS (HIS1K) biosensors (ForteBio, 18-5120) to 0.2 -0.3 nm. B7-1 or B7-2 of different concentrations (50, 25 and 12.5 nM) were used as analytes. Double reference was employed to minimize the signal fluctuation.

**In vivo efficacy study in CT26 model**

CT26 cells (from Gempharmatech) were cultured in RPMI-1640 Medium (Gibco) with 10% FBS (Gibco), 100U/mL penicillin and 100µg/mL Streptomycin (AMRESCO) and cultured at 37°C with 5% CO2 in air. 5×10^5 CT26 cells were collected, resuspended in DPBS and transplanted in BALB/c-huCTLA-4 KI animals. Animals were randomized with 6 mice per group and treated with human IgG1 0.5 mg/kg, ipilimumab 0.5 mg/kg and HCAb 4003-2 0.1 mg/kg when the average tumor size
reached 81 mm\(^3\) with dose administration on Days 0, 6 and 12 post treatment. The various doses applied in the study has been normalized to an equal molar concentration of antibodies.

**Suppl. Reference**

**Kabsch W.** Acta Crystallogr D Biol Crystallogr. 2010;66(Pt 2):125-32
**Supplementary Figures**

**Figure S1**

Comparison of HCAb 4003-2 to BMS-986218. HCAb 4003-2 shows better binding to CTLA4 (panel A) and blocking of CD80 and CD86 binding to CTLA4 (panel B) and shows comparable ADCC activity on primary Treg cells (panel C) and Tcell activation in a PBMC assay using two different donors (panel D).
Figure S2

Figure S2 Comparison of HCAb 4003-2 to BMS-986218. HCAb 4003-2 shows better binding to CTLA4 (panel A) and blocking of CD80 and CD86 binding to CTLA4 (panel B) and shows comparable ADCC activity on primary Treg cells (panel C) and T-cell activation in a PBMC assay using two different donors (panel D).

Figure S3

Figure S3 Tumor growth inhibition of HCAb 4003-2 when compared to BMS-986218 or Agen 1181. The left panel show that HCAb 4003-2 and BMS-986218 have a comparable tumor growth inhibition using the same MC38 model as used for Ipilimumab. The right panel shows that HCAb 4003-2 and Agen 1181 also have a comparable tumor growth inhibition.
Figure s4. Potent in vivo efficacy of 4003-2 in MC38 bearing huCTLA-4 KI C57BL/6 mice. (a) Tumor volume change over time with different antibodies (ipilimumab at 1 mg/kg and 0.1 mg, HCAb 4003-1 and 4003-2 at 0.54 mg/kg and 0.054 mg/kg) dosed twice per week. Each group is randomized with 6 mice. Tumor volume is presented as means ± SEM.*P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA test. Treatment time is shown by arrows below the horizontal axis. (b) Tumor volume change over time with different antibodies (HCAb 4003-2 at 0.1 mg/kg and 0.03 mg/kg) dosed every 10 days. Each group is randomized with 6 mice. Tumor volume is presented as means ± SEM.*P < 0.05, **P < 0.01 by one-way ANOVA test. Treatment time is shown by arrows.
Figure s5. Crystal structure of human CTLA-4/4003-1(VH) complex in one asymmetric unit (ASU). Four CTLA-4 molecules are shown in magenta, hot pink, blue and purple blue, and four 4003-1 (VH) are shown in green, forest green, yellow and yellow orange. The above structure can be regarded as two heterotetramers (chains ABGH and CDEF), each consisting of two CTLA-4 and two 4003-1 (VH), or four heterodimers (chains AB, GH, CD and EF), each of which is composed of one CTLA-4 and one 4003-1 (VH). However, the contact between two heterotetramers probably resulted from crystal packing. Meanwhile, the interaction between two heterodimers within one heterotetramer is also thought to be artificial because the orientation of two CTLA-4 molecules in the heterotetramer is totally different from that in apo CTLA-4 homodimer (PDB code: 3OSK). Thus, we only focused on CTLA-4/4003-1 (VH) heterodimer.
Figure s6. Structure overlay.

(a) Superposition of CTLA-4/4003-1(VH) and CTLA-4/ipilimumab-Fab. Ipilimumab’s heavy and light chains are colored in dark and light blue, respectively. CTLA-4 bound with ipilimumab is shown in cyan ribbon. (b) Superposition of CTLA-4/4003-1(VH) and CTLA-4/CD80. CD80 is shown in blue ribbon and bound CTLA-4 in cyan cartoon, 4003-1(VH) in green and CTLA-4 bound with it in purple (color scheme is the same in panels b and c unless stated otherwise). Only one CTLA-4/4003-1(VH) is superposed in structure for simplicity. (c) Superposition of CTLA-4/4003-1(VH) and CTLA-4/CD86. CD86 is presented in grey ribbon.
Figure S7. Structure of CTLA-4/4003-1 (VH) complex within one heterodimer.

All three CDRs of 4003-1 (VH) took part in the interaction with human CTLA-4 and CDR3 as the dominant interaction region.

Figure S8. Pharmacokinetics of 4003-1 and ipilimumab at 3mg/kg following single intravenous (IV) administration to female C57BL/6 mice (n=6).
Figure s9 Gel electrophoresis comparing Yervoy and the ipilimumab analogue. 2 μg/well, 150 V, 50 min., R: Reduced, NR: Non-Reduced, SurePAGE 4-12% (Genscript, M00654), Coomassie Blue R-250(Genscript, P0017F), Marker: PageRuler Unstained Protein (Thermo, 26614)

| Sample Name | Lot No. | Concentration (mg/mL) | Mw(KD) | %Aggregates | % Monomer | %Fragments |
|-------------|---------|-----------------------|--------|-------------|-----------|------------|
| ipilimumab  | NA      | 4.93                  | ~150   | 0.85        | 98.75     | 0.41       |
| YERVOY      | NA      | 5                     | ~150   | 0.17        | 99.36     | 0.47       |

Figure s10. HPLC of Yervoy and the ipilimumab analogue. HPLC System: Agilent 1260. Column: Zenix-C SEC-300 (3 μm, 7.8 x 300 mm, Part No. 233300-7830, S/N 7F51911, Lot No. CM094). Mobile phase: 1 X PBS, pH 7.0 (Diluted 10 times by purified water from 10 X PBS, Beyotime, ST476; Adjust pH 7.0 by 85% phosphoric acid). Flow rate: 1 mL/min; Column temperature: 25 °C; Autosampler temperature: 8 °C; Gel Filtration Standard: Bio-Rad, #151-1901. Injection volume: 20 μL for standards and 20 μg for samples
Figure s11. Blocking of B7-1 or B7-2 binding to CTLA4 by Yervoy and the ipilimumab analogue using Elisa.

Figure S12 Binding of Yervoy and the ipilimumab analogue to Jurkat cells by FACS and ADCC reporter assay.
### Supplementary Tables

**Table s1. Binding kinetics of interaction between HCAb 4003-1 and ipilimumab to human and cynomolgus monkey CTLA-4 proteins**

| Antibody     | Antigen     | $ka$ (1/Ms) | $kd$ (1/s) | $K_D$ (M) |
|--------------|-------------|-------------|------------|-----------|
| HCAb 4003-1 | Human CTLA-4| $5.35 \times 10^6$ | $2.29 \times 10^{-4}$ | $4.28 \times 10^{11}$ |
| ipilimumab   |             | $1.23 \times 10^6$ | $8.98 \times 10^{-5}$ | $7.32 \times 10^{11}$ |
| HCAb 4003-1 | Cyno CTLA-4 | $5.21 \times 10^6$ | $3.08 \times 10^{-4}$ | $5.91 \times 10^{11}$ |
| ipilimumab   |             | $3.73 \times 10^6$ | $1.29 \times 10^{-3}$ | $3.47 \times 10^{10}$ |

**Table s2. Maximal IL-2 release of HCAb 4003-1, 4003-2 and ipilimumab at 2 µg/ml**

| Donors | 4003-1 | 4003-2 | ipilimumab |
|--------|--------|--------|------------|
| 1#     | 99 pg/ml | 858 pg/ml | 151 pg/ml |
| 2#     | 181 pg/ml | 1883 pg/ml | 345 pg/ml |
| Sample Type | Treatment                              | T Cells | CD4+ T Cells | Treg  |
|-------------|----------------------------------------|---------|--------------|-------|
| **Tumor**   | Group 1: hlgG (10 mg/kg)                | 5.76    | 2.11         | 16.94 |
|             | Group 2: Ipilimumab analogue (10 mg/kg) | 6.29    | 2.22         | 12.05 |
|             | Group 3: hlgG1 HCAb (5.4 mg/kg)        | 5.79    | 1.85         | 15.50 |
|             | Group 4: 4003-2 (5.4 mg/kg)            | 6.01    | 2.20         | 4.88  |
|             | Group 5: 4003-2 (1.5 mg/kg)            | 5.95    | 1.80         | 2.74  |
| **Spleen**  | Group 1: hlgG (10 mg/kg)                | 28.28   | 15.32        | 13.93 |
|             | Group 2: Ipilimumab analogue (10 mg/kg) | 30.55   | 17.52        | 15.87 |
|             | Group 3: hlgG1 HCAb (5.4 mg/kg)        | 29.30   | 14.91        | 14.24 |
|             | Group 4: 4003-2 (5.4 mg/kg)            | 27.16   | 15.46        | 14.62 |
|             | Group 5: 4003-2 (1.5 mg/kg)            | 30.60   | 16.02        | 13.68 |
| **Blood**   | Group 1: hlgG (10 mg/kg)                | 30.35   | 18.45        | 0.60  |
|             | Group 2: Ipilimumab analogue (10 mg/kg) | 33.39   | 19.77        | 1.15  |
|             | Group 3: hlgG1 HCAb (5.4 mg/kg)        | 36.06   | 20.39        | 0.49  |
|             | Group 4: 4003-2 (5.4 mg/kg)            | 29.03   | 17.43        | 0.72  |
|             | Group 5: 4003-2 (1.5 mg/kg)            | 33.35   | 18.79        | 0.39  |
Table S4. Treg percentage in tumor, spleen and blood

| Group                | Antibody          | Dosing Date | Sample Collection Date | Tumor (%) | Spleen (%) | Blood (%) |
|----------------------|-------------------|-------------|------------------------|-----------|------------|----------|
| G-hlgG-D0S2          | hlgG1 10 mg/kg    | D0          | D2                     | 63        | 7          | 3        |
| G-4003-5.4-D0S2      | 4003-2 5.4 mg/kg  | D0          | D2                     | 30        | 8          | 3        |
| G-4003-1.0-D0S2      | 4003-2 1.0 mg/kg  | D0          | D2                     | 23        | 8          | 3        |
| G-hlgG-D0D3D7S8      | hlgG1 10 mg/kg    | D0, D3, D7  | D8                     | 59        | 8          | 3        |
| G-4003-5.4-D0D3D7S8  | 4003-2 5.4 mg/kg  | D0, D3, D7  | D8                     | 23        | 12         | 4        |
| G-4003-1.0-D0D3D7S8  | 4003-2 1.0 mg/kg  | D0, D3, D7  | D8                     | 21        | 11         | 4        |
| G-4003-5.4-D0S8      | 4003-2 5.4 mg/kg  | D0          | D8                     | 21        | 11         | 4        |
| G-4003-5.4-D0D3S8    | 4003-2 5.4 mg/kg  | D0, D3      | D8                     | 27        | 12         | 4        |
### Table S5. Pharmacokinetic Parameters of 10 mg/kg Human IgG1, 10 mg/kg Ipilimumab and 5.4 mg/kg HCAb 4003-2 with an Intravenous Dosing in Female C57BL/6 Mice

| PK Parameters | Iso hlgG1 | HCAb 4003-2 | Ipilimumab |
|---------------|-----------|-------------|------------|
| AUC<sub>all</sub> (µg/mL*hr) | 50,902±1,384 | 1,465±45.4 | 13,584±157 |
| C₀ (µg/mL) | 286 | 103 | 161 |
| Vₜ (mL/kg) | 68.3 | 149 | 88.8 |
| Cl (mL/hr/kg) | 0.15 | 3.09 | 0.37 |
| T<sub>1/2</sub> (hr) | 315 | 41.0 | 169 |

### Table S6. Pharmacokinetic Parameters of HCAb 4003-1 and Ipilimumab After an Intravenous Dose of 3 mg/kg in Female C57BL/6 Mice

| PK parameters | Unit | 4003-1 | Ipilimumab |
|---------------|------|--------|------------|
| CL | mL/day/kg | 13.9 | 7.12 |
| Vss | mL/kg | 194 | 127 |
| V1 | mL/kg | 64.0 | 47.2 |
| Alpha t<sub>1/2</sub> | day | 0.113 | 0.271 |
| Beta t<sub>1/2</sub> | day | 9.92 | 12.8 |
| AUC | day*µg/mL | 216 | 421 |
| MRT | day | 14.0 | 17.8 |
Table s7: [3H] 4003-2 and [3H] H2L2\textsubscript{TAAl} concentration in tumor/tissue and ratio of 4003.2 to H2L2\textsubscript{TAAl} at 1h or 24 h post 10 mg/200 \(\mu\)Ci/kg antibody dosing in tumor bearing mice (n=6)

| Time point | Tissue type | Concentration (ng Eq/g) | Tumor/tissue: plasma portion |
|-----------|-------------|-------------------------|-------------------------------|
|           | H2L2\textsubscript{TAAl} | HCAb 4003.2 | H2L2\textsubscript{TAAl} | HCAb 4003.2 | Ratio of 4003.2 to H2L2\textsubscript{TAAl} |
| 1 hour after [3H] antibody dosing | Tumor | 11727±1802 | 11985±2375 | 4.50% | 8.61% | 1.91 |
| | Brain | 2059±383 | 1333±221 | 0.79% | 0.96% | 1.22 |
| | Heart | 29327±2210 | 13665±2705 | 11.27% | 9.82% | 0.87 |
| | Pancreas | 3831±1024 | 4496±1110 | 1.47% | 3.23% | 2.20 |
| | Lung | 36567±6218 | 31364±5939 | 14.05% | 22.53% | 1.60 |
| | Kidney | 32907±3413 | 35989±7386 | 12.64% | 25.86% | 2.05 |
| | Liver | 14526±2763 | 27468±4588 | 5.58% | 19.74% | 3.54 |
| | Plasma | 260325±24285 | 139182±27666 | 100% | 100% | 1.00 |
| 24 hour after [3H] antibody dosing | Tumor | 20641±2476 | 20203±3818 | 14.01% | 53.94% | 3.85 |
| | Brain | 1269±169 | 1114±210 | 0.86% | 2.97% | 3.45 |
| | Heart | 14767±1377 | 4300±1523 | 10.02% | 11.48% | 1.15 |
| | Pancreas | 7696±1447 | 3895±723 | 5.22% | 10.40% | 1.99 |
| | Lung | 29020±12195 | 12459±1574 | 19.69% | 33.27% | 1.69 |
| | Kidney | 18254±2057 | 11719±3132 | 12.39% | 31.29% | 2.53 |
| | Liver | 7439±519 | 11498±1663 | 5.05% | 30.70% | 6.08 |
| | Plasma | 147352±14159 | 37452±7277 | 100% | 100% | 1.00 |

Table s8. Ka, Kd, and K\textsubscript{D} Analysis of HCAb 4003-1 and 4003-2 to Human Fc Receptor Proteins

| Antibodies | Receptor | ka (1/Ms) | kd (1/s) | K\textsubscript{D} (M) | Antibodies | Receptor | ka (1/Ms) | kd (1/s) | K\textsubscript{D} (M) |
|------------|----------|-----------|---------|-----------------|------------|----------|-----------|---------|-----------------|
| HCAb 4003-1 | CD16a | 1.158E+6 | 0.076 | 6.568E-8 | CD16a | 7.836E+6 | 7.212E-3 | 9.203E-10 |
| | CD16b | 2.867E+6 | 0.221 | 7.695E-8 | CD16b | 2.159E+7 | 5.313E-2 | 2.460E-9 |
| | CD32a | N/D | N/D | 2.622E-7 | CD32a | N/D | N/D | 1.645E-8 |
| | CD32b | N/D | N/D | 1.023E-6 | CD32b | N/D | N/D | 2.600E-8 |
| | FcRn (pH6) | N/D | N/D | 7.705E-9 | FcRn (pH6) | N/D | N/D | 5.298E-9 |

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Table s9. Ka, Kd, and K_D Analysis of ipilimumab and 4003-2 to Human CD16a, 158V/F

| Sample ID | K_D (M) | Ka (1/Ms) | Kd (1/s) |
|-----------|---------|-----------|----------|
| CD16a,158V |         |           |          |
| ipilimumab | 6.45E-08 | 7.16E+05  | 4.62E-02 |
| 4003-2     | 4.23E-09 | 4.77E+05  | 2.02E-03 |
| CD16a,158F |         |           |          |
| ipilimumab | 5.94E-08 | 7.58E+05  | 4.50E-02 |
| 4003-2     | 8.30E-09 | 8.13E+05  | 6.75E-03 |

Table s10: Ka, Kd, and K_D analysis of HCAb 4003-1 and 4003-2 to mouse Fc receptor proteins

| Antibodies | Receptor | Ka(1/Ms) | Kd (1/s) | K_D (M) | Antibodies | Receptor | Ka (1/Ms) | Kd (1/s) | K_D (M) |
|------------|----------|----------|----------|---------|------------|----------|------------|----------|---------|
| HCAb 4003-1 | mouse CD16 | No binding |          |         | HCAb 4003-2 | mouse CD16 | No binding |          |         |
|            | mouse CD16-2 | N/D      | N/D      | 2.60E-07 |            | mouse CD16-2 | 2.44E+06  | 1.35E-03 | 5.52E-10 |
|            | mouse CD32b  | No binding |          |         |            | mouse CD32b  | N/D      | N/D      | 5.54E-06 |
|            | mouse CD64  | N/D      | N/D      | 3.03E-07 |            | mouse CD64  | 3.12E+06  | 1.68E-02 | 5.37E-09 |
|            | mouse FcRn  | 3.22E+06 | 4.97E-03 | 1.54E-09 |            | mouse FcRn  | 2.86E+06  | 5.05E-03 | 1.76E-09 |
Table s11. X-ray diffraction data collection and refinement statistics

| Data collection                      |          |
|--------------------------------------|----------|
| Wavelength (Å)                       | 0.979183 |
| Resolution (Å)                       | 44.73 – 2.38 (2.46 – 2.38) |
| Completeness (%)                     | 99.8 (100.0) |
| Redundancy                           | 6.4 (5.7) |
| R_{merge} (%)                        | 3.4 (78.3) |
| I/σ(I)                               | 23.5 (1.9) |
| CC_half                              | 0.999 (0.693) |
| Space group                          | P2_12_1 |
| a, b, c (Å)                          | 75.59, 110.34, 134.18 |
| α, β, γ (°)                          | 90.00, 90.00, 90.00 |

| Refinement statistics                |          |
| Resolution (Å)                       | 42.33 - 2.38 |
| Unique reflections                   | 43345    |
| Residues/ASU                         | 935      |
| Water molecules/ASU                  | 45       |
| R_{work} (%)                         | 21.7     |
| R_{free} (%)                         | 26.3     |
| RMSD bond length (Å)                 | 0.004    |
| RMSD bond angle (°)                  | 1.331    |
| Average B-factors (Å²)               | 74.325   |
| Protein                              | 81.23    |
| Water molecules                      | 69.93    |

| Ramachandran Plot                    |          |
| In most favoured regions (%)         | 96.5     |
| In allowed regions (%)               | 3.39     |
| In disallowed regions (%)            | 0.11     |
Table s12. Binding kinetics of interactions between CTLA-4 and 4003-1 (HCAb) obtained from Octet assay

| CTLA-4 | CTLA-4 Conc. (nM) | Highest response (nm) | K_d (M) | Ka (1/Ms) | kd (1/s) |
|--------|-------------------|-----------------------|---------|-----------|---------|
| WT*    | 100               | 0.1156                | <1.0E-12| 1.83E+05  | <1.0E-07|
| WT$^+$ | 180,90,45         | 0.1093                | 8.91E-11| 1.58E+05  | 1.41E-05|
| E33A   | 180,90,45         | 0.0945                | 4.42E-09| 1.33E+05  | 5.89E-04|
| E33D   | 180,90,45         | 0.0740                | 3.79E-09| 7.99E+04  | 3.03E-04|
| R35A   | 100               | NA                    | NA      | NA        | NA      |
| R35K   | 180,90,45         | 0.0774                | 2.01E-08| 1.52E+05  | 3.06E-03|
| E48A   | 180,90,45         | 0.0526                | 3.78E-09| 4.83E+04  | 1.83E-04|
| E48D   | 180,90,45         | 0.0474                | 2.07E-09| 6.78E+04  | 1.40E-04|
| T53A   | 180,90,45         | 0.0517                | 1.34E-09| 6.40E+04  | 8.54E-05|
| T61A   | 180,90,45         | 0.0844                | 1.54E-09| 1.17E+05  | 1.79E-04|
| L63A   | 180,90,45         | 0.0780                | 7.13E-09| 1.44E+05  | 1.03E-03|
| E97A   | 100               | NA                    | NA      | NA        | NA      |
| E97D   | 100               | NA                    | NA      | NA        | NA      |
| M99A   | 100               | NA                    | NA      | NA        | NA      |
| Y100A  | 180,90,45         | 0.1426                | 5.88E-09| 2.28E+05  | 1.34E-03|
| Y104A  | 100               | NA                    | NA      | NA        | NA      |
| Y104F  | 100               | NA                    | NA      | NA        | NA      |

Note: WT*: purchased from Novoprotein (Cat.: CP33); WT$: protein produced by Harbour BioMed.
Table s13. Binding kinetics of interactions between CTLA-4 and its receptors (CD80 and CD86) obtained from Octet assay.

| CTLA-4 | Receptor | Highest Response (nm) | $K_D$ (M) | $Ka$ (1/Ms) | $kd$ (1/s) |
|--------|----------|-----------------------|------------|-------------|------------|
| WT*    | CD80     | 0.6596                | 6.47E-10   | 6.94E+05    | 4.49E-04   |
| WT$^5$ | CD80     | 0.6056                | 2.29E-09   | 5.92E+05    | 1.36E-03   |
| E33A   | CD80     | 0.0625                | 1.93E-08   | 2.32E+06    | 4.48E-02   |
| E33D   | CD80     | 0.2682                | 1.67E-08   | 9.80E+05    | 1.64E-02   |
| R35A   | CD80     | 0.1295                | 1.07E-08   | 2.30E+06    | 2.46E-02   |
| R35K   | CD80     | 0.2101                | 2.54E-08   | 1.30E+06    | 3.29E-02   |
| E97A   | CD80     | <0.01                 | NA         | NA          | NA         |
| E97D   | CD80     | <0.01                 | NA         | NA          | NA         |
| M99A   | CD80     | <0.01                 | NA         | NA          | NA         |
| Y100A  | CD80     | 0.2580                | 1.93E-08   | 8.54E+05    | 1.65E-02   |
| Y104A  | CD80     | <0.01                 | NA         | NA          | NA         |
| Y104F  | CD80     | 0.0406                | 1.02E-08   | 4.27E+06    | 4.38E-02   |
| WT*    | CD86     | 0.4817                | 4.92E-09   | 9.22E+05    | 4.53E-03   |
| WT$^5$ | CD86     | 0.3264                | 9.26E-09   | 1.42E+06    | 1.31E-02   |
| E33A   | CD86     | 0.1204                | 4.43E-08   | 2.34E+05    | 1.04E-02   |
| E33D   | CD86     | 0.1937                | 7.06E-09   | 2.17E+06    | 1.54E-02   |
| R35A   | CD86     | 0.0682                | 3.08E-08   | 1.77E+05    | 5.46E-03   |
| R35K   | CD86     | 0.0946                | 2.42E-08   | 2.27E+05    | 5.48E-03   |
| T53A   | CD86     | 0.0905                | 4.88E-09   | 2.68E+06    | 1.31E-02   |
| T53S   | CD86     | 0.3081                | 6.44E-09   | 1.48E+06    | 9.54E-03   |
| M99A   | CD86     | <0.01                 | NA         | NA          | NA         |
| Y100A  | CD86     | <0.01                 | NA         | NA          | NA         |
| Y104A  | CD86     | <0.01                 | NA         | NA          | NA         |
| Y104F  | CD86     | 0.0378                | 1.06E-08   | 2.62E+05    | 2.78E-03   |

Note 1: WT*: purchased from Novoprotein (Cat.: CP33); WT$^5$: protein produced by Harbour BioMed.

Note 2: Experimental curves with very low response (<0.01 nm) were not fitted.
Table s14. The residues involved in interaction between 4003-1 (VH) and huCTLA-4 within four 4003-1 (VH)-huCTLA-4 heterodimers. Only residues which took part in binding in at least two out of four heterodimers are listed in this table.

| Antibody    | Interacting via... | from CDR1 | from CDR2 | from CDR3          |
|-------------|--------------------|-----------|-----------|--------------------|
| 4003-1 (VH) | Mainchain (MC)     | --        | G55       | --                 |
|             | Sidechain (SC)     | N33, Y34  | Y53, S57, T59 | R98, V100, H102, F107 |
|             | Both MC and SC     | K32       | S54       | S103, S105, S106  |
| Antigen     | Interacting via... |           |           | Residues          |
| huCTLA-4    | MC                 | C50, Y54, M55 |               |                    |
|             | SC                 | E33, R35, E48, T53, T61, E97, Y100, Y104 |               |                    |
|             | Both MC and SC     | A51, L63, M99 |               |                    |