**Anti-tumor effect of CTLA-4 antibody is independent of checkpoint blockade**

**Supplementary Materials**

Supplementary Table S1-S4

Supplementary Figure S1-S3

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| **Supplementary Table 1. Data collection and refinement statistics for crystal structures** | | |
|  | **CTLA-4/ipilimumab-Fab** | **CTLA-4/D138-dAb** |
| PDB code | 6RP8 | 6RPJ |
| Crystallization condition | 20% PEG 3000, 0.2M Ca(AC)2, 0.1M Hepes pH 7.0 | 12% PEG 3350, 3% Tascimate pH5.0 |
| **Data collection** | | |
| Space group | P1211 | C121 |
| Cell dimensions |  |  |
| a, b, c (Å)  α, β, γ (°) | 56.91, 129.89, 76.91  90, 96.44, 90 | 309.98, 75.708, 83.047  90, 100.05, 90 |
| Wavelength (Å) | 0.9792 | 0.97915 |
| Resolution (Å) | 48.11-2.60 (2.72-2.60) | 50-3.25 (3.37-3.25) |
| Total NO. of observation | 121541 (15065) | 140556 (27487) |
| Total NO. unique | 34019 (4158） | 30159 (1926) |
| Rmerge | 0.17 (0.87) | 0.15 (0.72) |
| I/σI | 6.4 (1.5) | 8.0 (3.0) |
| Completeness (%) | 99.3 (99.7) | 100 (99.9) |
| Multiplicity | 3.6 (3.6) | 4.7 (4.5) |
| **Refinement** |  |  |
| Resolution (Å) | 48.11-2.60(2.667-2.60) | 47.57-3.25 (3.329-3.25) |
| No. of reflections | 33990 （1911） | 27487 (1926) |
| No. of residues | 1073 | 973 |
| No. of atoms | 8177 | 7424 |
| Rwork/Rfree | 0.211/0.267 | 0.248/0.263 |
| B-factors (Å2) | 45.0 | 98.0 |
| Bond length RMSD (Å) | 0.010 | 0.006 |
| Bond angle RMSD (°) | 1.227 | 0.937 |
| **Ramachandran plot** |  |  |
| In Preferred Region (%) | 96.3 | 94.6 |
| In Allowed Region (%) | 3.7 | 5.33 |
| Outliers (%) | 0 | 0.10 |

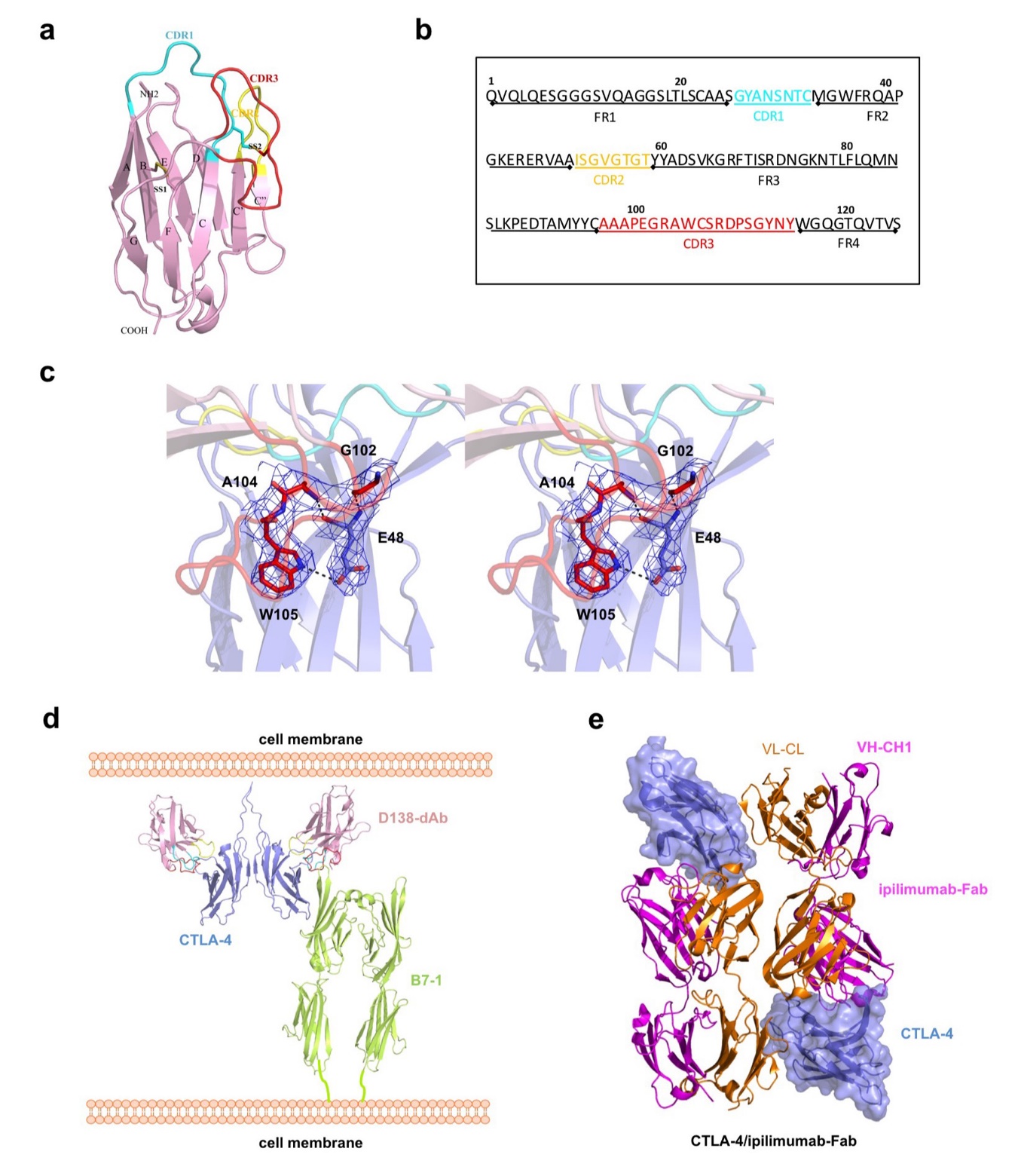
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| **Supplementary Table 2. Hydrogen bonds and salt bridges between CTLA-4 and D138-dAb（distance ≤ 3.7Å）** | | | |
| **CTLA-4 contact residue** | **CTLA-4 residue location** | **D138-dAb contact**  **residue** | **D138-dAb residue**  **location** |
| V46 | C’ strand | N29 | CDR1 |
| T47 | C’ strand | N29 | CDR1 |
| E48 | C’ strand | G102 | CDR3 |
| E48 | C’ strand | A104 | CDR3 |
| E48 | C’ strand | W105 | CDR3 |
| D64 | C’’D loop | R108 | CDR3 |
| D65 | C’’D loop | R47 | C’ strand |
| D65 | C’’D loop | C106 | CDR3 |
| D65 | C’’D loop | R108 | CDR3 |
| G83 | DE loop | T56 | CDR2 |
| D88 | DE loop | G55 | CDR2 |

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| **Supplementary Table 3. Binding affinities of CTLA-4 variants towards D138** | | | |
| **CTLA-4 variants** | **Kd (M)** | **Kon (1/Ms)** | **Koff (1/s)** |
| WT | 5.21E-08# | 1.29E+05 | 6.70E-03 |
| T47A | 7.27E-07 | 2.69E+04 | 1.95E-02 |
| E48A | —\* | — | — |
| V49A | —\* | — | — |
| L63A | 2.91E-08 | 2.74E+05 | 8.00E-03 |
| D64A | 3.15E-05 | 4.17E+02 | 1.32E-02 |
| D65A | —\* | — | — |
| I67A | 1.36E-06 | 6.96E+04 | 9.48E-02 |
| D88A | —\* | — | — |
| Y92A | —\* | — | — |

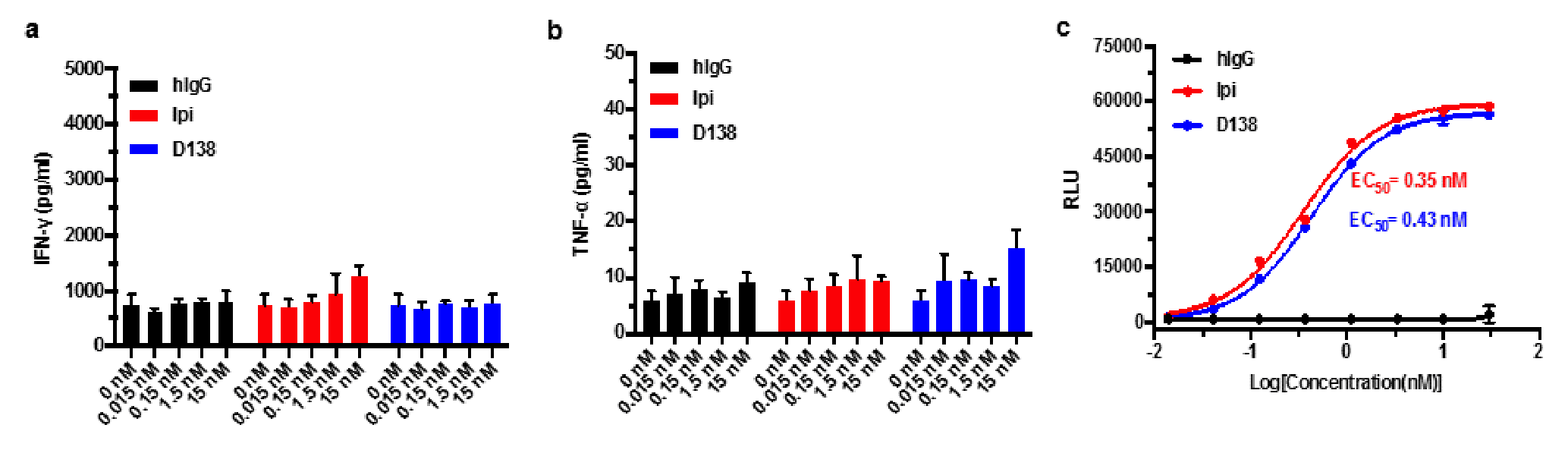
#CTLA-4-His protein has a larger Kd value than that of CTLA-4-mFc (Fig. 1b) due to CTLA-4-mFc is bivalent.

\*No significant binding could be detected when the concentrations of these CTLA-4 variants were increased to 6.6 μM.

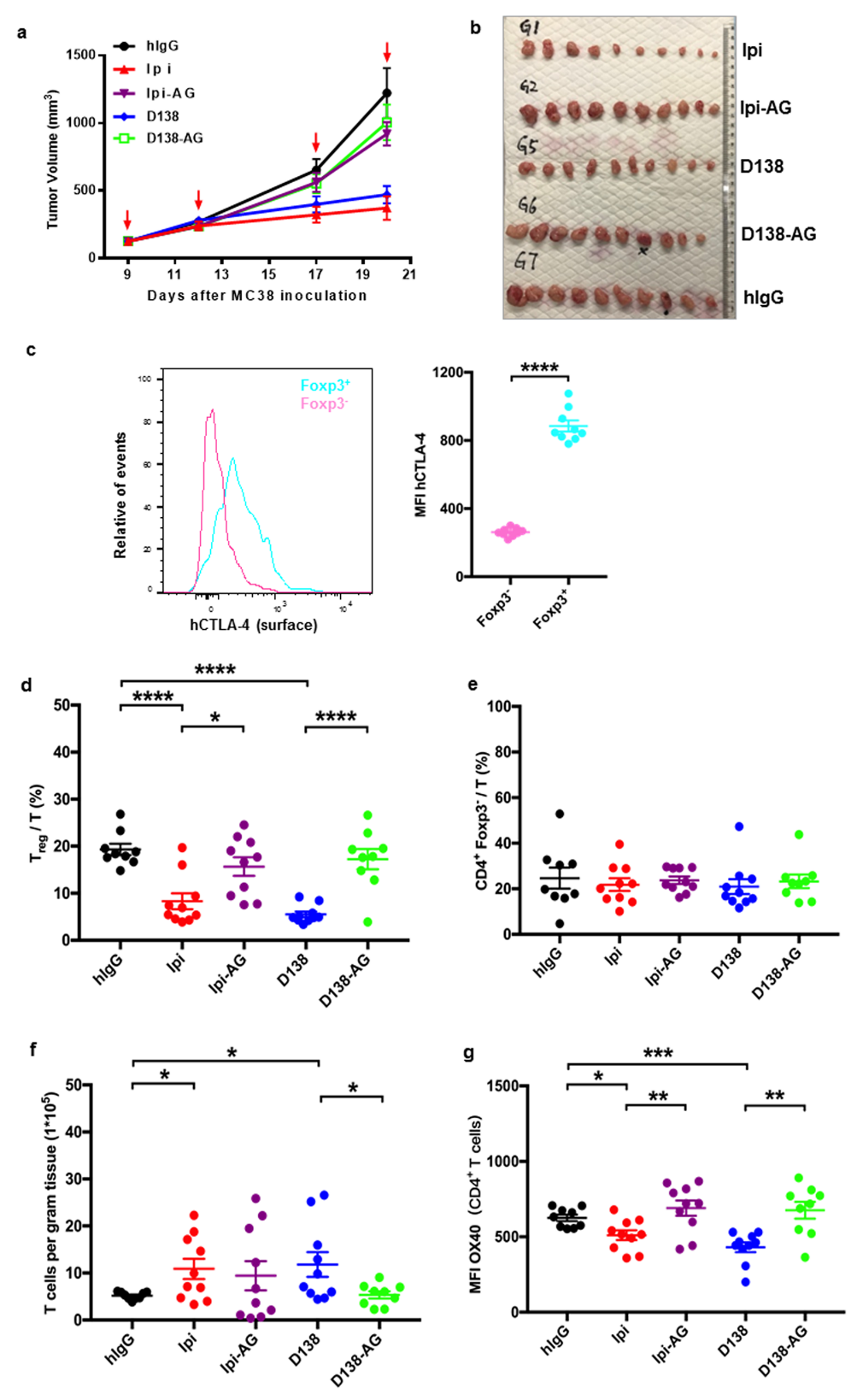
|  |  |  |
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| **Supplementary Table 4. Antibodies used for Flow Cytometry analysis** | | |
| **Reagent** | **Source** | **Cat number** |
| Livedead-BV510 | BD Biosciences | Cat# 564406 |
| Anti-mouse CD45-APC-Cy7 | Biolegend | Cat# 103114 |
| Anti-mouse CD45.2-APC | Biolegend | Cat# 109828 |
| Anti-mouse CD4-APC | eBioscience | Cat# 47-0042-82 |
| Anti-mouse CD8-PerCy5.5 | Biolegend | Cat# 100712 |
| Anti-mouse Foxp3-PE-Cy7 | eBioscience | Cat# 17-5773-82 |
| Anti-mouse ICOS-PE | Biolegend | Cat# 107705 |
| Anti-mouse OX40-FITC | Abcam | Cat# ab33998 |
| Anti-mouse PD1-PE-Cy7 | eBioscience | Cat# 25-9985-80 |
| Anti-human CTLA-4-PE-Cy7 | Biolegend | Cat# 349914 |
| Anti-human CTLA-4 PE | Biolegend | Cat# 349905 |
| Anti-mouse Ki67-PE | BD Biosciences | Cat# 556027 |

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**Supplementary Fig. 1.** **Structures of CTLA-4 antibodies. a.** Structure of D138-dAb with CDR1 loop in cyan, CDR2 in yellow and CDR3 in red**. b.** The sequence of D138-dAb with each CDR region highlighted in corresponding color. **c.** The wall-eye stereo view of the interaction between Trp105 of D138 and Glu48 of CTLA-4 with electron density (blue mesh) contoured at 1σ. **d.** A model of a complex of CTLA-4 dimer with D138 and B7-1 dimer on the cell surface showing D138 binding would not affect the CTLA-4-B7 interaction. **e**. Two copies of CTLA-4 and ipilimumab-Fab complex in the asymmetry unit of the crystal structure.



**Supplementary Fig. 2. INF-γ (a), TNF-α (b) production and ADCC activities (c) by antibodies. a,b.** Freshly prepared PBMCs were plated in serum free RPMI-1640, and incubated in 37°C, 5% CO2 for 2 hours in 96-well plate (2×105 cells). The cells were then treated with different concentrations of ipilimumab or D138 for one hour. Staphylococcus Enterotoxin B (SEB, 200 ng/ml) was subsequently added. The supernatant was collected after five days culture and the levels of INF-γ and TNF-α were evaluated by ELISA kit according to the manufacturer instructions. c. ADCC activities of antibodies were also determined using Fc Effector Reporter Bioassays. CTLA-4 transiently expressed on HEK293T cells were used as targeted cells. The effector cells were co-cultured with target 293-CTLA-4 cells at an effector-to-target ratio of 4:1, and along with different concentrations of anti-human CTLA-4 antibodies or control. After incubation of the 96-well plates at 37°C for 6h, 75 μl Bio-Glo™ luciferase assay reagent was added into each well and the luminescence intensity was measured (n=3).

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**Supplementary Fig. 3. Intra-tumor T cell population analysis. a.** Age-matched *Ctla4h/h* C57BL/6J female mice were inoculated with MC38 (1×106 cells). After tumor size reached about 80~160 mm3, mice were grouped (n=10 each group) and treated (arrowed) via intraperitoneal administration of antibodies. Tumor volume and body weight of tumor were recorded. Data represent mean±S.E.M. **b.** Mice were sacrificed on day 21 (one day after the fourth injection)with tumors extracted. **c.** The shaded histogram (left) or summary graph (right) showing surface CTLA-4 expression on CD4+Foxp3+ or CD4+Foxp3- T cells in tumors from the control hIgG-treated group. **d-g**, Tumors were digested and cells were stained with various mAbs and analyzed with flow cytometry similarly as in Figure 4. **d.** The percentage of Treg cells over total T cells. **e.** The percentage of CD4+Foxp3- cells over total T cells. **f.** Numbers of T cells per gram of tumor tissue. **g.** MFI of OX40 on CD4+ T cells. Statistical significance was determined using unpaired Student’s *t* test. Notably, two mice died one day before the 4th treatment and their tumors (marked with black dot below in **b**) were extracted but were excluded for T cell population analysis.