Supporting Information

Biosynthesis of Multifunctional Transformable Peptides for Downregulation of PD-L1

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Experimental

Materials

E. coli TransB (DE3) chemically competent cells were obtained from TransGen Biotech Co., Ltd. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and ampicillin were purchased from Beijing solarbio science and technology Co., Ltd. NaH_2PO_4 and modified BCA Protein Assay Kit were purchased from Sangon Biotech (Shanghai) Co., Ltd. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo, Kumamoto, Japan. Dialysis membrane biotech cellulose ester tubing (MWCO 10-12 KDa, flat width 31 mm, length 5 m) was purchased from Spectrum® Laboratories, Inc. M3 was purchased from QYAOBIO(ChinaPeptides Co.，Ltd.）, whose purity is greater than 96%. A375 and Jurkat T cells were obtained from Procell Life Science & Technology Co., Ltd (Wuhan, China). Cathepsin B (CtsB) were purchased from Sigma Aldrich Chemical Company. Ni-NTA, Gibco Fetal Bovine Serum (FBS), and Western blot related products were purchased from Thermo Fisher Scientific Inc. All antibodies were supplied from Cell Signaling Technology (Beverly, MA). All reagents and organic solvents purchased from commercial suppliers were used without further purification unless otherwise noted.

Instruments

UV-Vis absorption spectra were recorded on a Thermo Evolution 201 spectrophotometer. The particle size was obtained on a Nano ZS90 (Malvern, UK) system. TEM images were conducted on a Hitachi S-7700 transmission electron microscope. Fluorescence spectra were measured on a Hitachi F-4500 fluorometer with a xenon lamp excitation source (Thermo Scientific Company, USA). Cell counting was performed on an automated cell counter (Countess, Invitrogen). The images of the western blot were taken on a Bio-Rad Molecular Imager ChemiDoc XRS system. CD spectra were collected on JASCO J-1700 CD spectrometer. Confocal laser scanning microscopy images were measured with a confocal laser scanning microscope (FV1000-IX81, Olympus, Japan).

Plasmid construction

The expression plasmid pET21a-His-T22-GFP-GO203-KLVFF-RGD was constructed by inserting total synthetic DNA sequence with 6×His tag (protein sequence shown below) into the pET-21a, using the NdeI [CATATG] / XhoI [CTCGAG] multiple cloning sites. The plasmid of control group was constructed in a similar manner.

Sequence of M1

HMHHHHHHRRWCYRKCYKGYCYRKCRGGSSRSSMSKGEELFTGVVPILVELDGDVNG
HKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFAYGVQCFSRYPDHMKRHDFF
KSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
YNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST
QSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGGGSGGGSGGGSGGGSGGGSGFL

GRRRRRRRRRCQCRRKN KLVFFAEGFFYGFLGGGSSRSSCRGDK

Sequence of control group M2

HMHHHHHHRRWCYRKCYKGYCYRKCRGGSSRSSMSKGEELFTGVVPILVELDGDVNG
HKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFAYGVQCFSRYPDHMKRHDFF
KSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
YNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLST
QSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGGGSGGGSGGGSGGGSGGGSGGG
GRRRRRRRRRCQCRRKNKLVFFAEGFFYGGGGGGSSRSSCRGDK

Sequence of M3

LGRRRRRRRRRCQCRRKN KLVFFAEGFFYGF

Expression and purification of His-T22-GFP-GO203-KLVFF-RGD Plasmid

pET21a-His-T22-GFP-GO203-KLVFF-RGD was transformed into *E. coli Trans*B (DE3) cells, which were cultured at 37°C in LB medium with 100 μg/mL ampicillin. When OD_{600} reached 0.6 - 0.8, protein expression was induced at 16°C overnight by 0.75 mM IPTG. Bacterial cells were harvested by centrifugation at 8000 rpm for 7 min at 4°C. Then lysis buffer (50mM NaH_2PO_4, 500 mM NaCl, 10 mM Imidazole, pH 8.0) was used to re-suspend the cell pellets. The cell suspension was lysed in an ice-water bath for 20 min by sonication. The cell lysate then was centrifuged at 9000 rpm at 4°C for 45 min. The supernatant was filtered through a 0.45 μm membrane, which was loaded in a Ni-NTA column and washed with wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 80 mM Imidazole, pH 8.0) for 10 column volume. The protein was then eluted with elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 500 mM Imidazole, pH 8.0). Finally, the proteins were dialyzed at ice-water in dialysis buffer overnight. The purificatory protein solution was concentrated by ultrafiltration centrifugation (5 mL, GE Healthcare, MWCO =10 kDa), which was checked by SDS-PAGE and western blot, respectively. The protein concentration was determined by BCA assay. All proteins were frozen in liquid nitrogen and stored at −80°C until use.

Morphology transformation property

The M1 (0.5 mg/mL) was dissolved in acetate buffer solution (AB, 0.01 M, pH 5.0) with CtsB (1.0 μg/mL) and gently stirred at 37°C for 10 h. To the cuvette was added 1 mL liquid of each group then the diameter was measured by DLS. The structure of nanofibers was analysed by CD spectra.

Transmission electron microscopy (TEM) characterizations

8 μL of solution in each group was added on copper meshes. After 10 min, unnecessary liquid was removed and the copper meshes were evaporated in the hood overnight. The morphology transformation of M1 and M2 assemblies were analyzed by TEM (HT 7700).

Cytotoxicity assay

A375 cells were cultured in 96-well plates at a density of 6×10^3 cells/ well and cultured overnight. The supernatant was removed and the cells were incubated with M1 at a series of concentrations for 48 h. Subsequently, MTT (0.5 mg/mL in medium, 100 μL/well) was added to each well followed by incubation at 37°C for 4 h. Then, 100 μL DMSO was added to each well after the supernatant was removed. After shaking the plate for 2 min, absorbance values of formazan at 570 nm were read with a microplate reader.

Confocal laser scanning microscopy (CLSM)

A375 cells were cultured in confocal microscope dish at a density of 8×10^4 cells per dish in DMEM with 10% FBS, 1% penicillin-streptomycin at 37°C in a humified atmosphere containing 5% CO_2 overnight. The adherent cells were washed with FBS-free DMEM one time. The culture medium was replaced with DMEM containing (0.2 mg/mL) M1 (λ_{ex} = 488 nm) and further cultured for 24 hours. The supernatant was removed and the dishes were washed with PBS three times. Then, the dishes were added DMEM containing 200 nM Lyso Tracker DND 99 (λ_{ex} = 633 nm) and 10 μg/mL Hoechst 33342 (λ_{ex} = 405 nm) followed by incubation at 37°C for 20 min. The supernatant was removed and the dishes were washed with PBS three times. Subsequently, the fluorescence in cells was observed and analyzed by CLSM.

Western blot analysis

A375 cells were cultured in dishes and incubated overnight. The culture medium was replaced by fresh culture medium that contained 1 mg/mL M1 for 48 h. Subsequently, the cells were harvested and washed with precooling of PBS. After centrifugation at 4°C, the cells were re-suspended in lysis buffer (Beyotime Biotechnology). Then, the cytolysis was centrifugated at 13000 rpm for 30 min. After the total protein concentration of the supernatant were estimated by BCA kit, the lysate proteins (40 μg) were subjected to SDS-PAGE and then transferred to a PVDF membrane. Next, the membrane was blocked with a blocking buffer at room temperature for 1 h. Then the membrane was incubated with primary antibodies overnight at 4°C, and incubated with secondary antibody for 2 h at room temperature. Finally, Bio-Rad Molecular Imager ChemiDoc XRS system was used to image.

Bio-TEM observation

The treated cells were collected and fixed in 2.5% glutaraldehyde at 4℃ for 24 h, then washed with PB saline (PBS, 0.01 M) three times and further fixed with 1% osmium containing PBS for 2 h. Then washed with PBS three times, the tumor tissue was dehydrated with a graded series of alcohol (30, 50, 70, 90, 95, 100, 100%) for 10 min and acetone twice for 10 min. Subsequently, acetone/ epoxy resin (1:3) were used for infiltration 3 h, and tumor tissue were immersed in pure resin overnight. Then, the tumor tissue was transferred to embedding mould for solidification at 35, 45 and 60°C for 24 h, respectively. The tumor tissue was sectioned with 70 nm and attached to copper grids with Formvar film (300 mesh). At last, the sections were performed by staining with 2% uranyl

acetate for 30 min and 3% lead citrate for 30 min, respectively. HT7700 TEM (Hitachi, Japan) was used for observation. The characterization results were supported by Beijing Zhongkebaice Technology Service Co., Ltd (www.zkbaice.cn).

Intercellular interaction in vitro

A375 cells were cultured in 96-well plates at a density of 6×10^3 cells/ well and cultured overnight. The supernatant was removed and the cells were incubated with M1 (1 mg/mL) for 24 h. Subsequently, Jurkat T cells at a density of 5×10^4 or 6×10^4 cells/ well were incubated with the A375 cells for 24 h. Then, 10 μL CCK8 solution was added to each well followed by incubation at 37°C for 1 h. After shaking the plate for 2 min, absorbance values of formazan at 450 nm were read with a microplate reader.

Supporting Figures

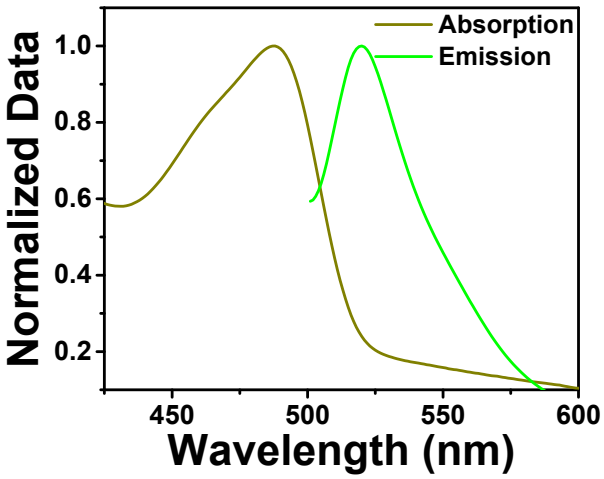


Figure S1. Absorption and emission spectra of the purified M1.

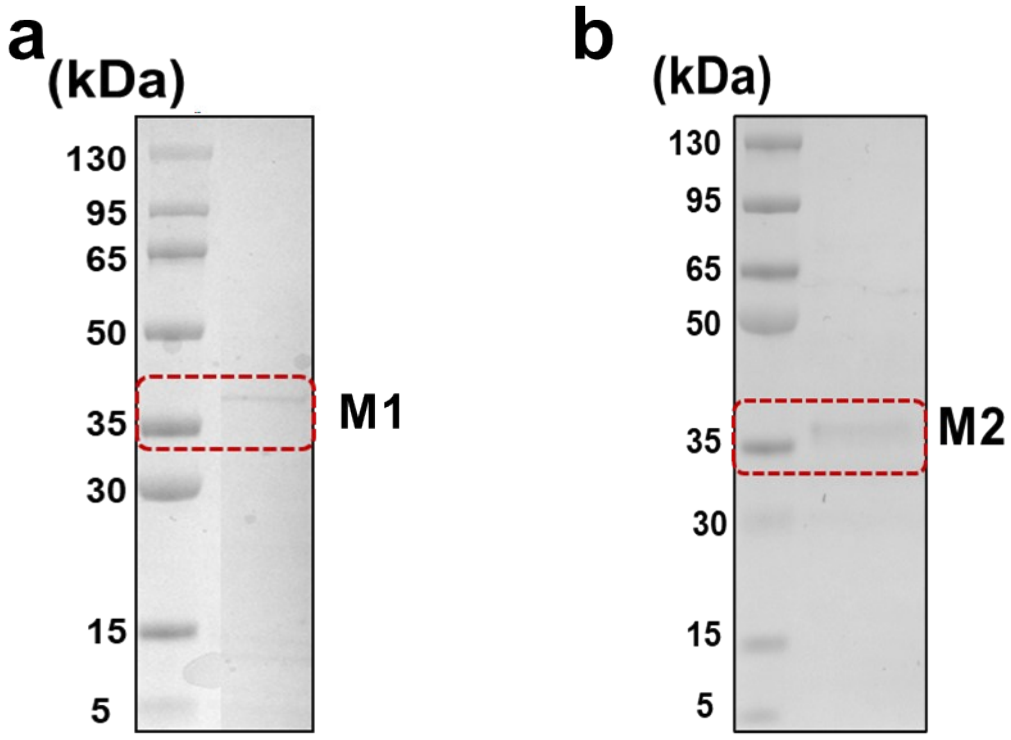


Figure S2. SDS-PAGE-based assessment of the molecular weight of M1 and M2.

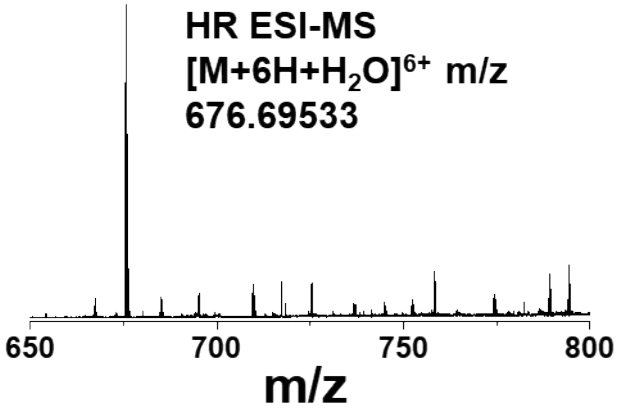


Figure S3. High-resolution LC-MS of M1 incubated with CtsB in an acetate buffer solution (AB, 0.01 M, pH 5.0) for 10 h.

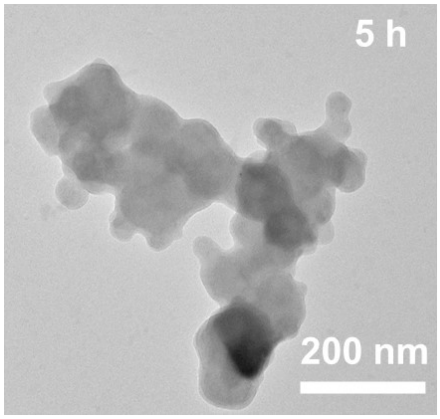


Figure S4. TEM images of M1 in the presence of CtsB (1.0 μg mL-1, AB, 0.01 M, pH 5.0, 37℃) for 5 h. Scale bars, 200 nm.

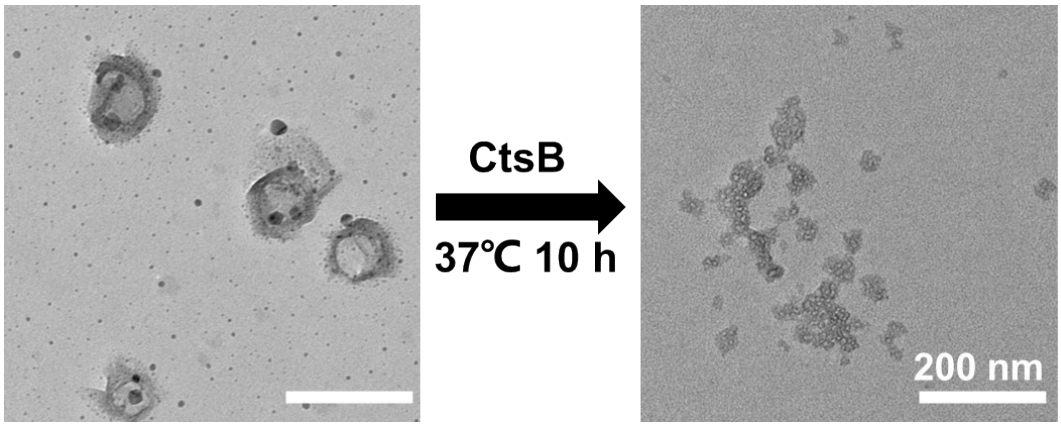


Figure S5. TEM images of M2 in the presence of CtsB (1.0 μg mL-1, AB, 0.01 M, pH 5.0, 37℃) for 10 h. Scale bars, 200 nm.

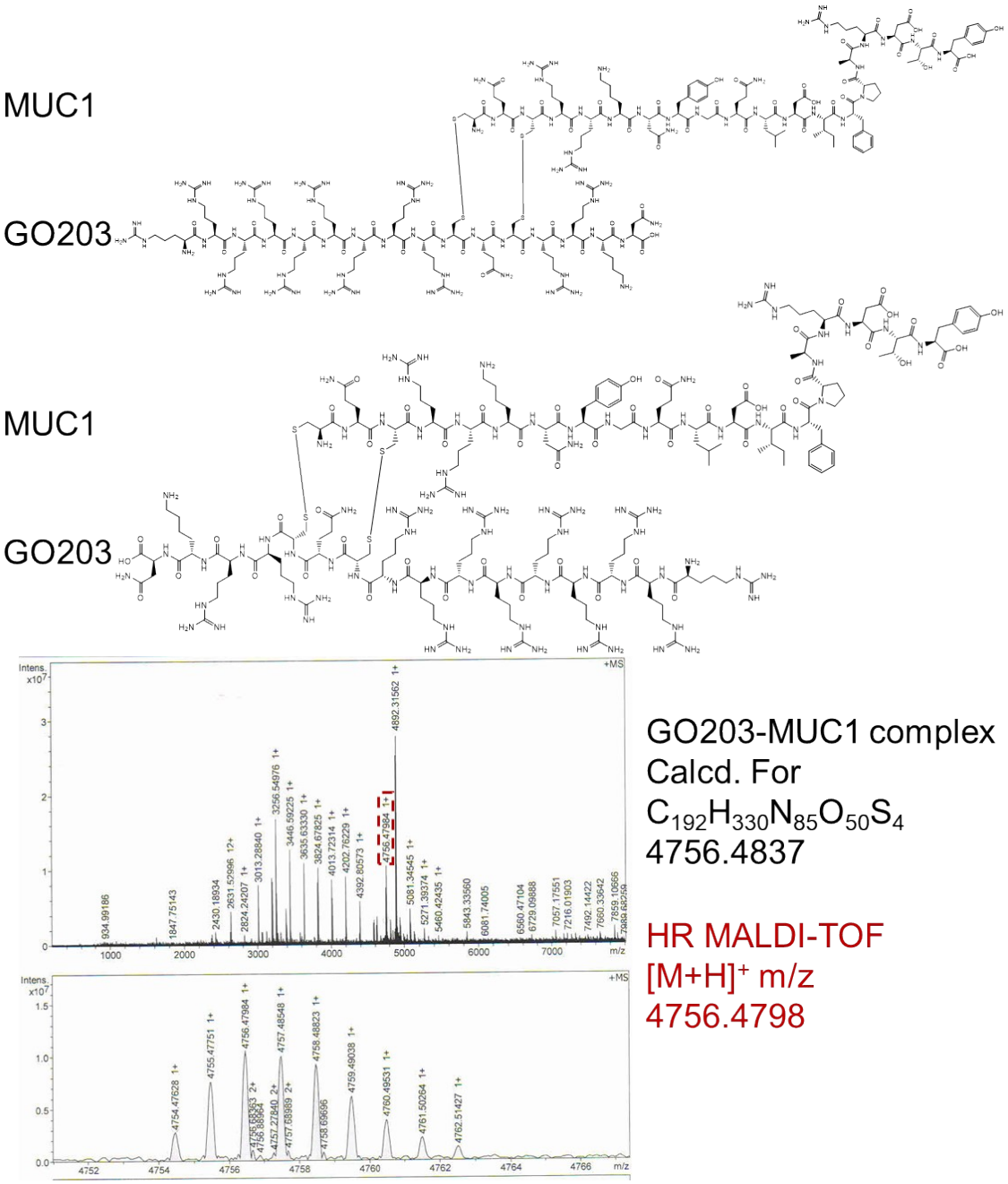


Figure S6. Mass spectrometry analysis of GO203-MUC1 fragment complex.

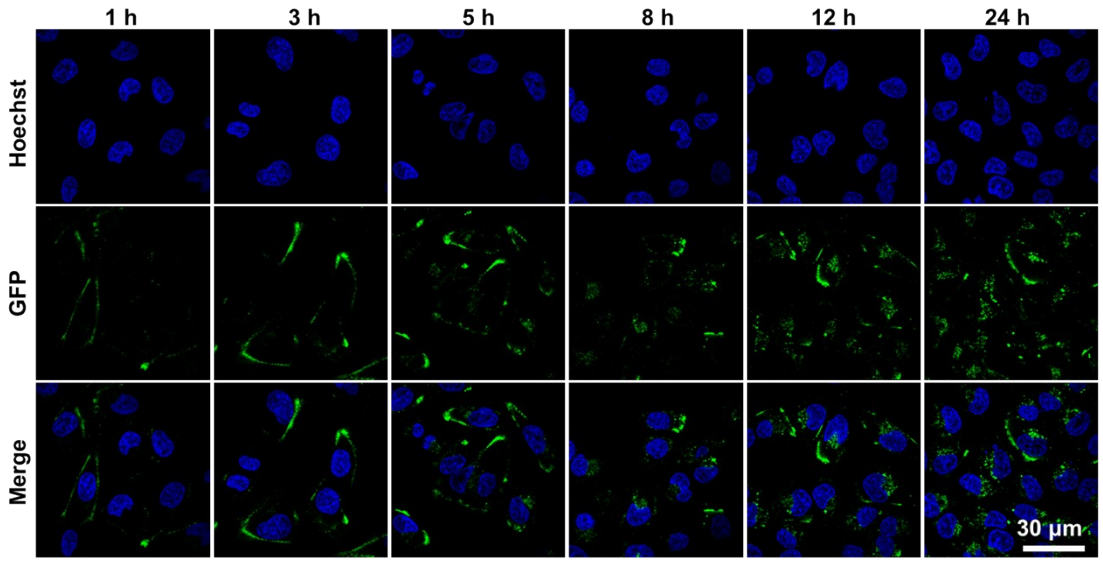


Figure S7. Confocal laser scanning microscope (CLSM) images of A375 cells treated with M1 with different incubation time.

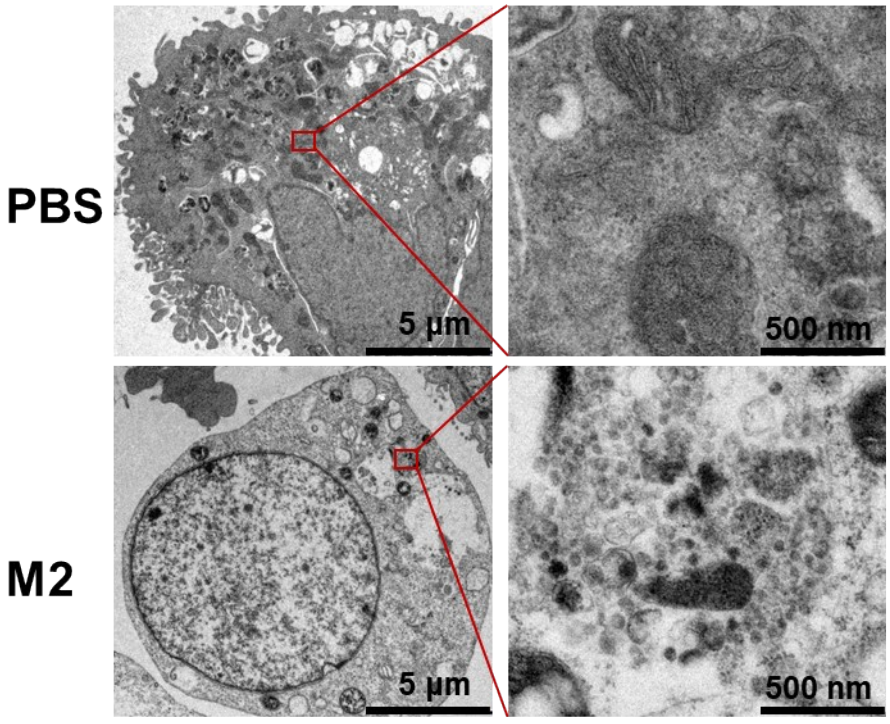


Figure S8. Bio-TEM images of A375 cells after incubated with PBS and M2.

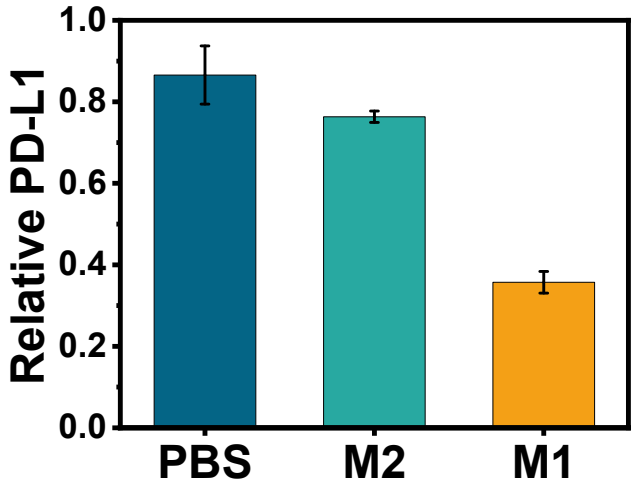
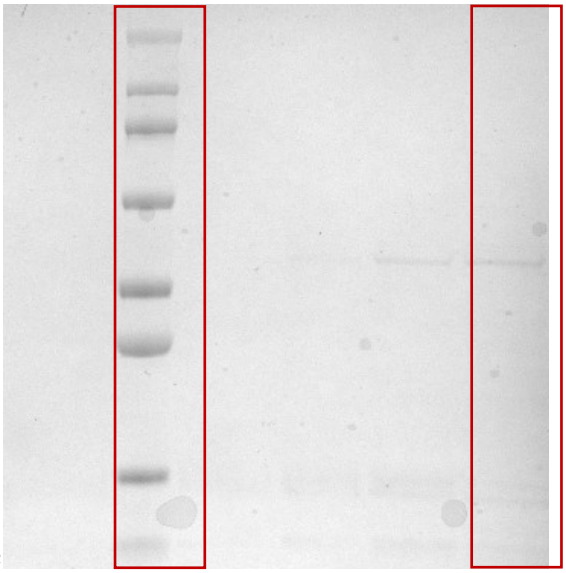


Figure S9. Quantification of PD-L1 expression levels from Figure 4a.

Uncropped versions of gels and blots

Fig 2a

Coomassie



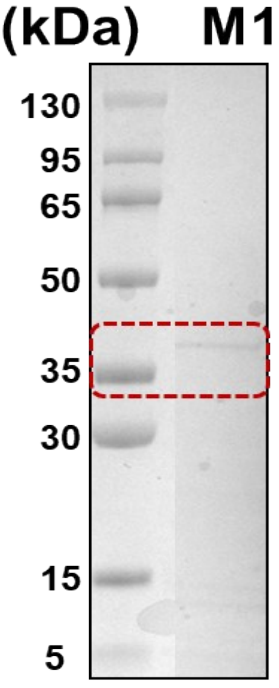
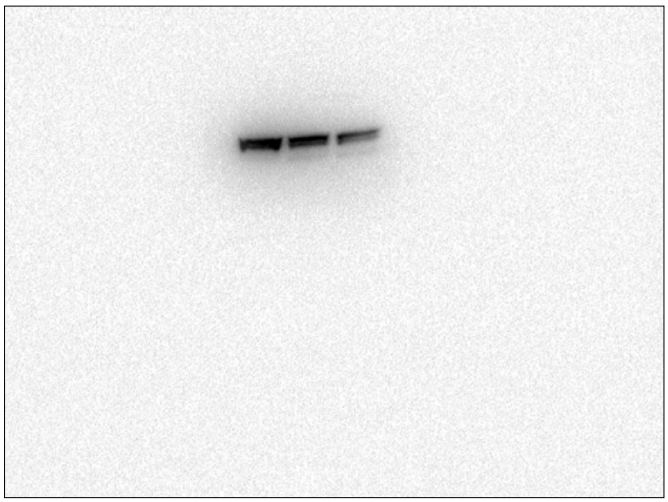
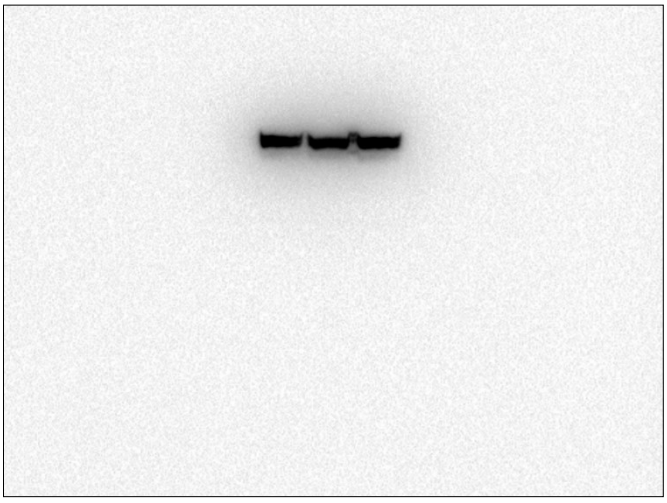


Fig 4b



IB:**PD-L1**



IB:**β-actin**