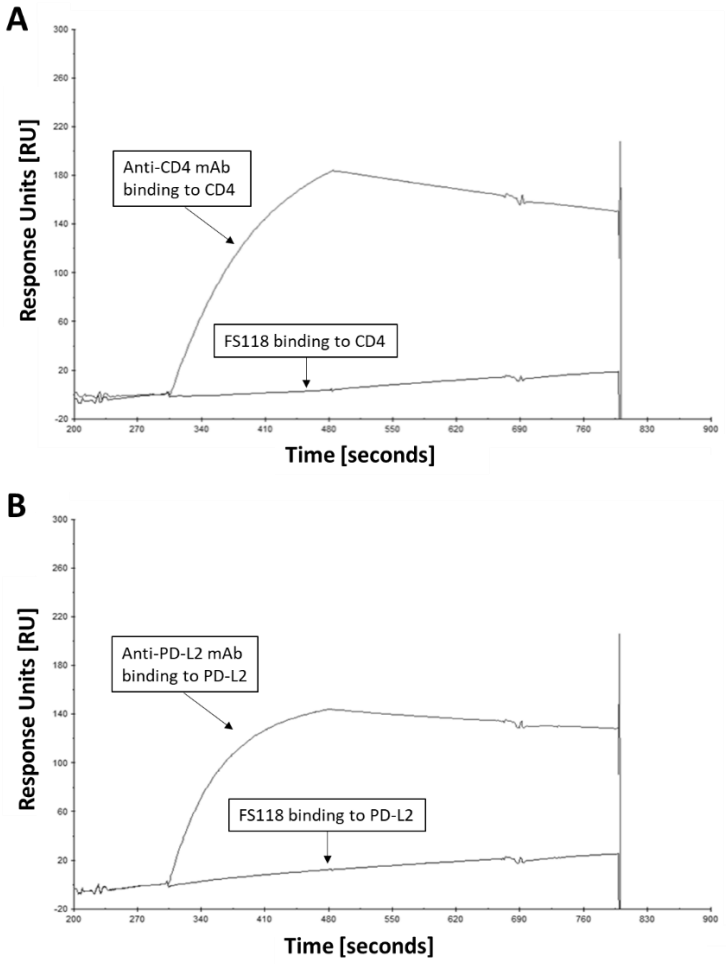
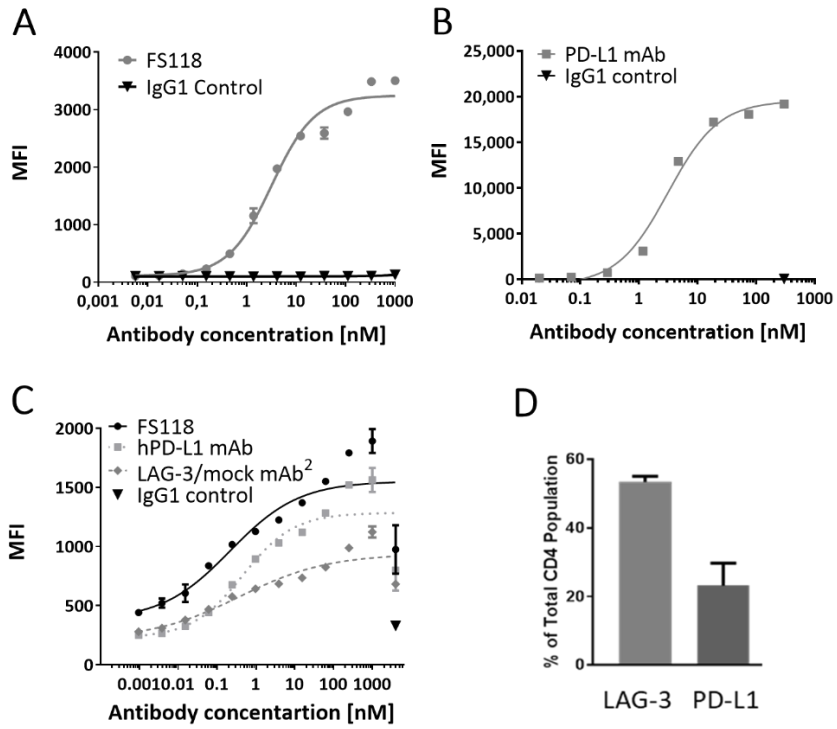
SUPPLEMENTARY DATA



Supplementary Figure S1. No binding of FS118 to closely related proteins, CD4 and PD-L2, as detected by Surface Plasmon Resonance (SPR).

(A-B) FS118 was assessed by SPR to examine binding to (A) recombinant human CD4 or (B) programmed death-ligand 2 (PD-L2), two closely related proteins to human LAG-3 and human PD-L1, respectively. FS118 and the positive control antibodies (A) anti-human CD4 mAb (BioLegend) and (B) anti-human PD-L2 mAb (BioLegend) were captured directly to the SPR chip, followed by the injection of the analytes (A) recombinant human CD4 (R&D Systems, 514-CD-050/CF) or (B) his-tagged recombinant human PD-L2 (R&D Systems). FS118 did not bind to human CD4 or human PD-L2.



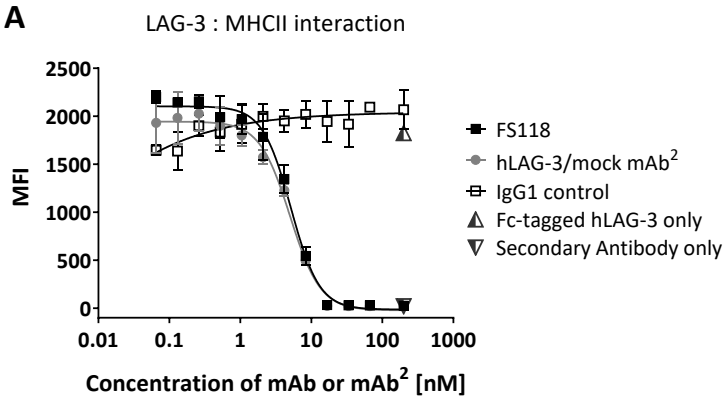
Supplementary Figure S2. FS118 binds to cells expressing LAG-3 and PD-L1.

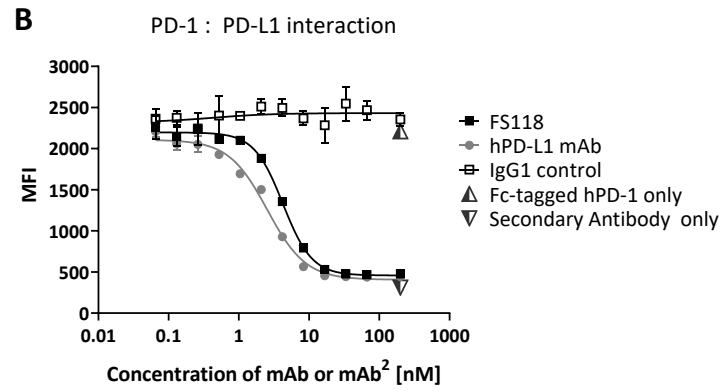
(A-B) Flow cytometry binding analysis to LAG-3 and PD-L1 expressed on HEK cells (Fig. S1A and S1B, respectively) to generate concentration-response plots.

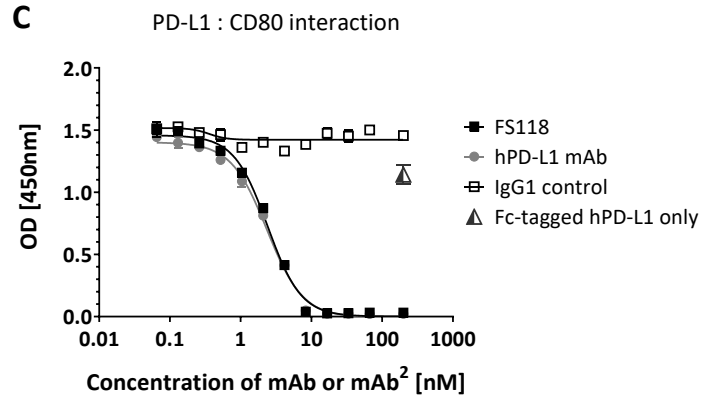
(C) Flow cytometry binding analysis to the native LAG-3 and PD-L1 molecule on activated $CD4^+$ T cells to generate concentration-response plots to derive EC_{50} values.

(D) It was confirmed by flow cytometry that 52% to 55% of activated $CD4^+$ T cells expressed LAG-3 and 17% to 30% expressed PD-L1.

Assays were performed in duplicate and data are presented as mean +/- SEM. hPD-L1 mAb is the anti-human PD-L1 mAb used to generate the human LAG-3/PD-L1 mAb^2 (FS118).







Supplementary Figure S3. FS118 blocks the binding of human LAG-3 to MHC class II, of human PD-L1 to human PD-1, and of human PD-L1 to human CD80.

(A) Flow-based binding assay to assess the ability of FS118 to block the interaction between LAG-3 and MHCII. FS118 blocked this interaction in a concentration-dependent manner. hLAG-3/mock mAb^2 was used as a positive control to bind to MHCII expressed on the cell surface of Raji cells.
(B) Flow-based binding assay to assess the ability FS118 to block the interaction between PD-1 and PD-L1. FS118 blocked this interaction in a concentration-dependent manner. hPD-L1 mAb was used as a positive control to bind to human PD-L1 over-expressed on HEK293 cells (HEK-hPD-L1).

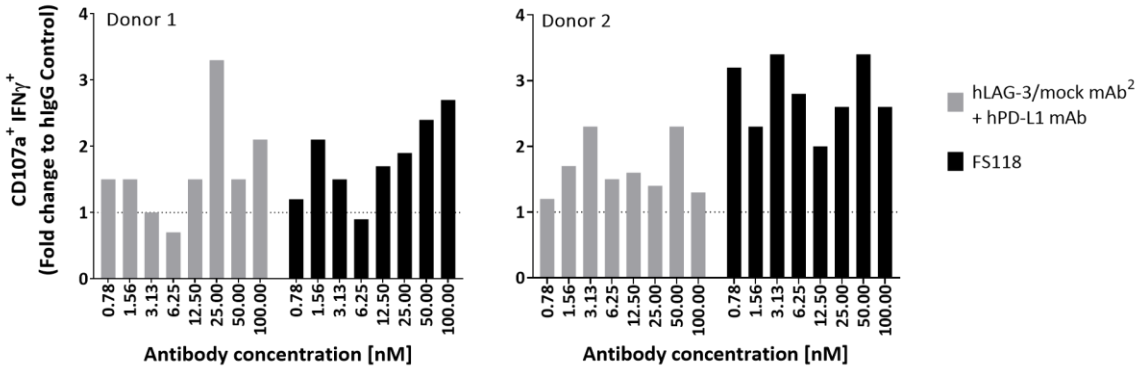
(C) ELISA-based blocking assay to assess the ability of FS118 to block the interaction between PD-L1 and CD80. Both FS118 and hPD-L1 mAb blocked this interaction in a concentration-dependent manner.

Assays were performed in duplicate, and data are presented as mean +/- SD with a non-linear regression curve fit (log[inhibitor] vs response) calculated using GraphPad Prism.

Supplementary Table S1. EC_{50} Values from DO11.10 T Cell Activation Assay

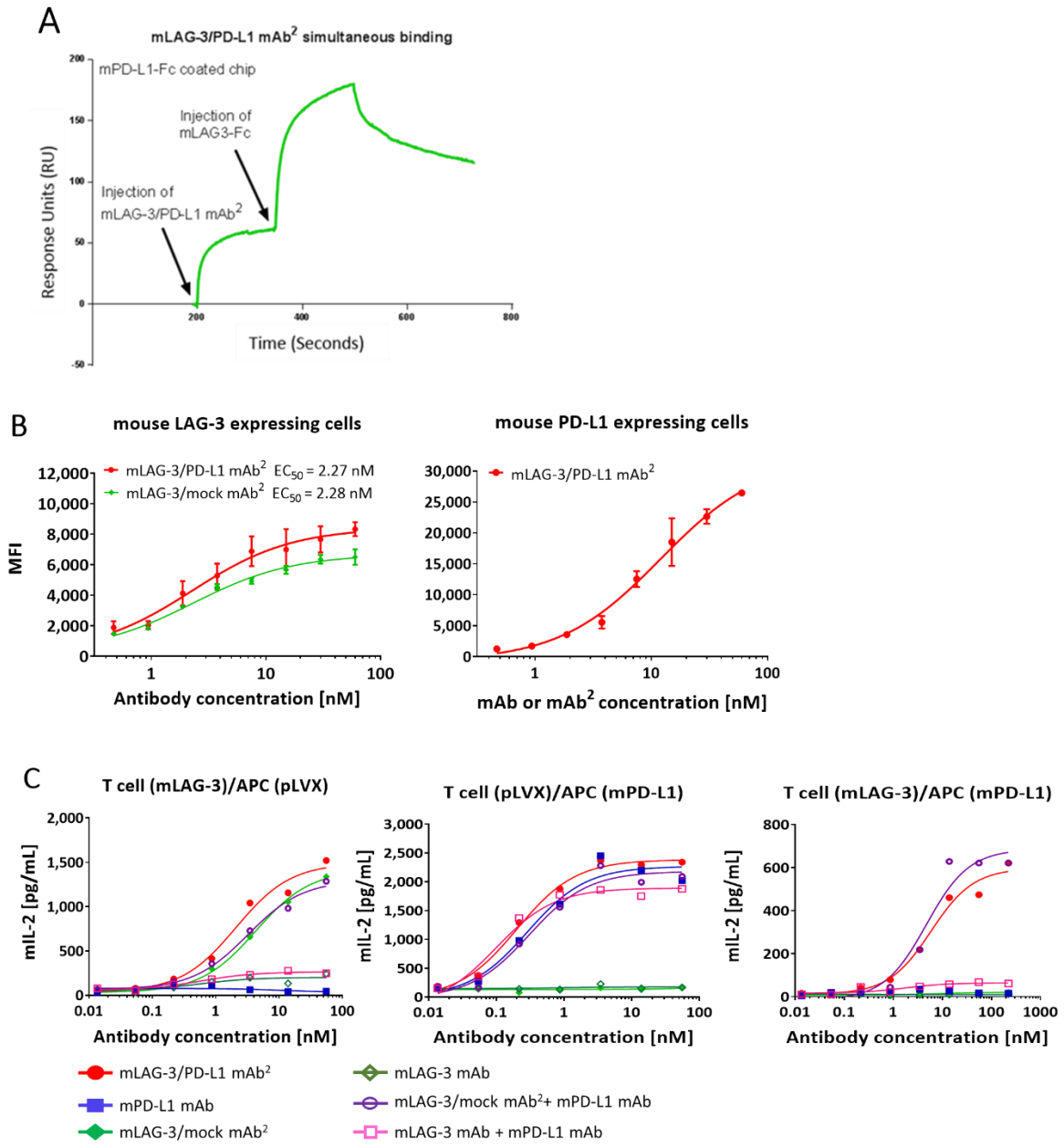
Antibody	**EC_{50} (nM)**		
	Expression of Human LAG-3 + PD-L1	**Expression of Human LAG-3 only**	**Expression of Human PD-L1 only**
FS118	0.75	1.25	0.47
hLAG-3/mock + hPD-L1 mAb	2.07	1.10	0.52
hLAG-3/mock	N.F.	1.23	N.F.
IgG	N.F.	N.F.	N.F.
hPD-L1 mAb	N.F.	N.F.	0.35

Abbreviations: EC_{50} = concentration that achieves a half-maximal response; mAb = monoclonal antibody; N.F. = no fit.



Supplementary Figure S4. FS118 stimulates antigen-driven CD8$^+$ T cell responses

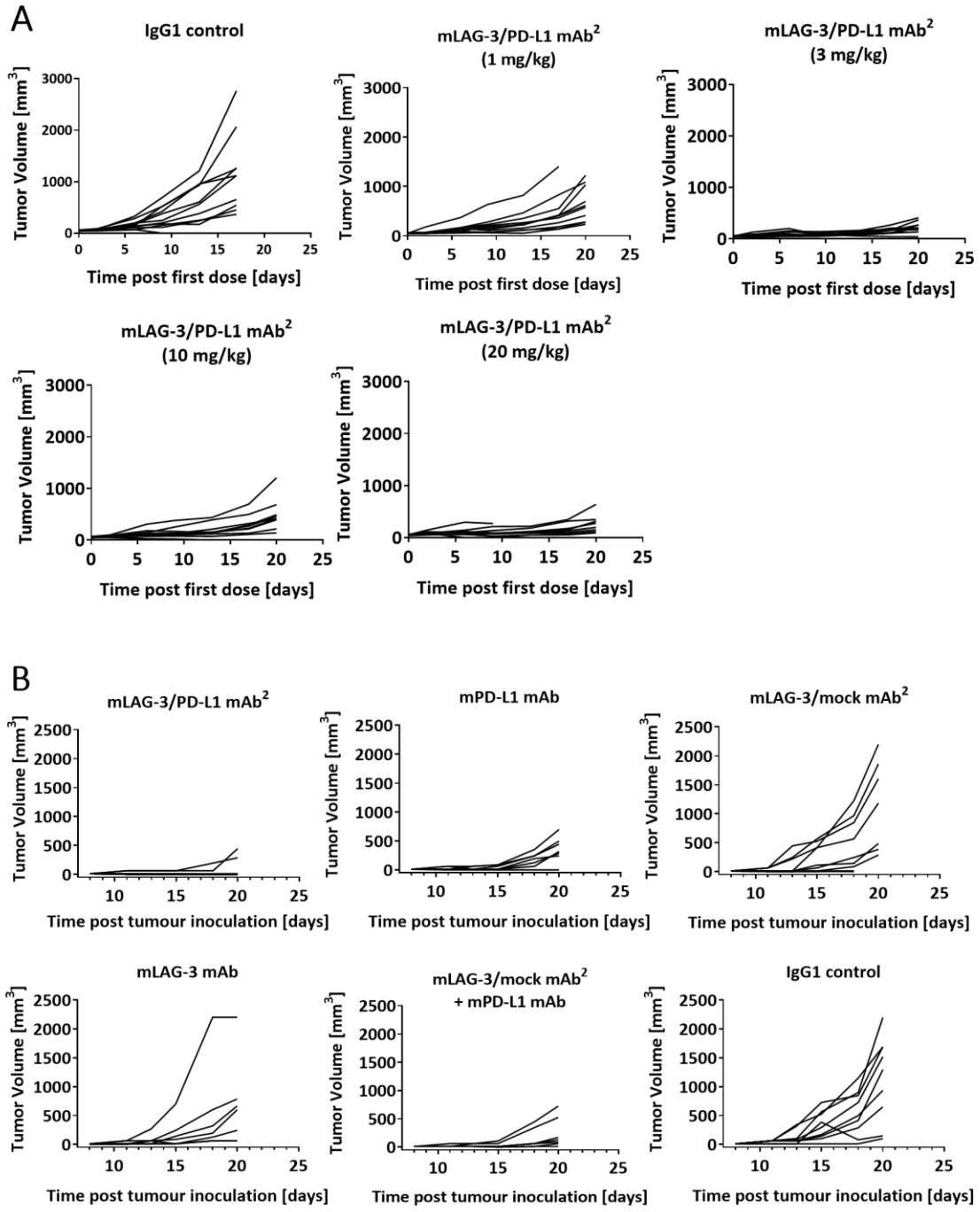
PBMC were cultured with CEFT peptide pool for 7 days in the presence of test antibodies (8-point 2-fold dilution from 100nM) prior to re-stimulation with peptides in the presence of protein export inhibitors (monensin and brefeldin A) for 24h and stained for CD8$^+$ activation markers (CD107a and IFNγ). The level of expression of intracellular CD107a and IFNγ was normalised to the level of expression in the IgG1 isotype control treated samples. The expression level in IgG1 isotype control treated samples is indicated by the dashed line on each bar graph.



Supplementary Figure S5. Biophysical and functional characterisation of the mLAG-3/PD-L mAb^2.

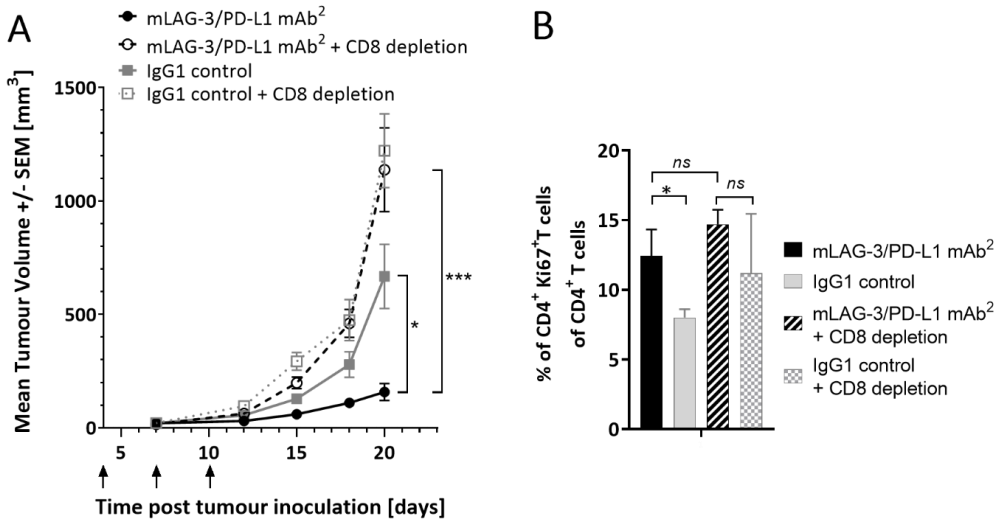
(A) The ability of mLAG-3/PD-L1 mAb^2 to bind simultaneously to murine LAG-3 and murine PD-L1 was tested by SPR. Murine PD-L1-Fc was immobilized on flow cell of a CM5 chip. The mLAG-3/PD-L1 mAb^2 was flowed at a rate of 20 µL/min for 150 sec. Subsequently, 50 nM of murine recombinant LAG-3-Fc protein was flowed across at a flow rate of 20 µL/min for 150 sec. After each binding step, a dissociation was performed for 3 min.
(B) The *in vitro* cell binding affinity of mLAG-3/PD-L1 mAb^2 was assessed using stable HEK293 cell lines engineered to express high-levels of mouse LAG-3 and mouse PD-L1. Flow cytometry binding analysis to mLAG-3 (left) and mPD-L1 (right) expressing cells were used to generate dose-response plots. Assay were performed in duplicate and data are presented as mean +/- SD.
(C) DO11.10 OVA-specific T cells overexpressing mouse LAG-3 (mLAG-3) or empty vector (pLVX) were mixed with the antigen presenting cell LK35.2 overexpressing mouse PD-L1 (APC (mPD-L1)) or untransduced in the presence of the $OVA_{324-339}$ peptide and with various concentration of antibodies

for 24 h. Culture supernatant was collected and assessed for secreted levels of mouse IL-2 by ELISA. IL-2 production was determined for LAG-3 mediated inhibition (left), PD-L1 mediated inhibition (middle), or inhibition mediated by both LAG-3 and PD-L1 (right).
Assays were performed in duplicate and data are presented as mean +/- SEM.



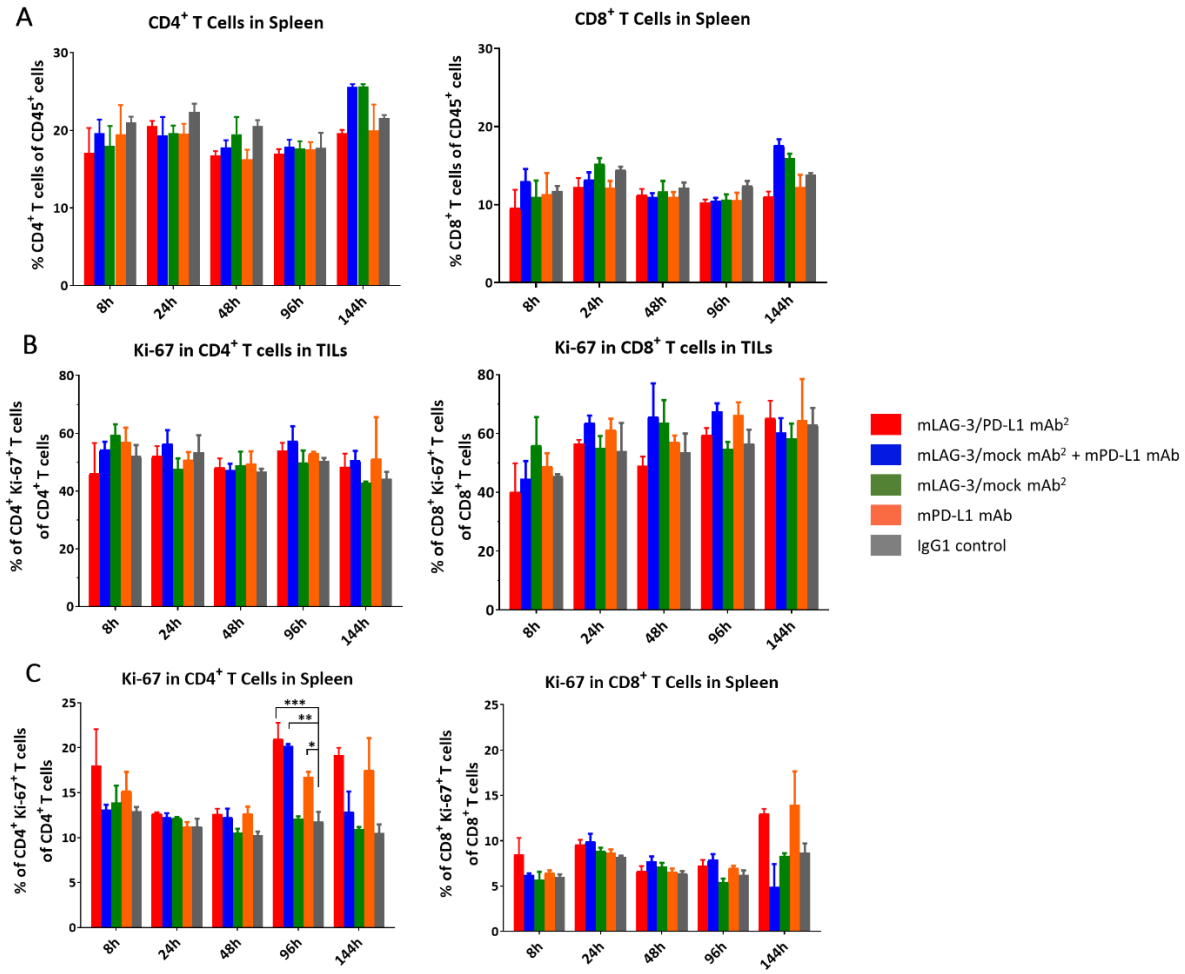
Supplementary Figure S6. mLAG-3/PD-L1 mAb² profoundly impacts early tumour establishment in MC38 tumours.

Female C57/BL6 mice were subcutaneously injected with MC38 tumour cells until a palpable tumour formed and randomised to treatment cohorts. Eight days after tumour cell implantation, the mice were administrated intraperitoneally with either (A) IgG1 isotype control (10 mg/kg) or anti-mLAG-3/PD-L1 mAb² at 1, 3, 10 or 20 mg/kg at day 0, day 3, and day 6 post-randomisation, or (B) a total of 20 mg/kg (10mg/kg antibody X + 10 mg/kg antibody Y or IgG control) at day 8, day 11, and day 14 post-inoculation. Shown are the individual tumour volume growth curves over time.



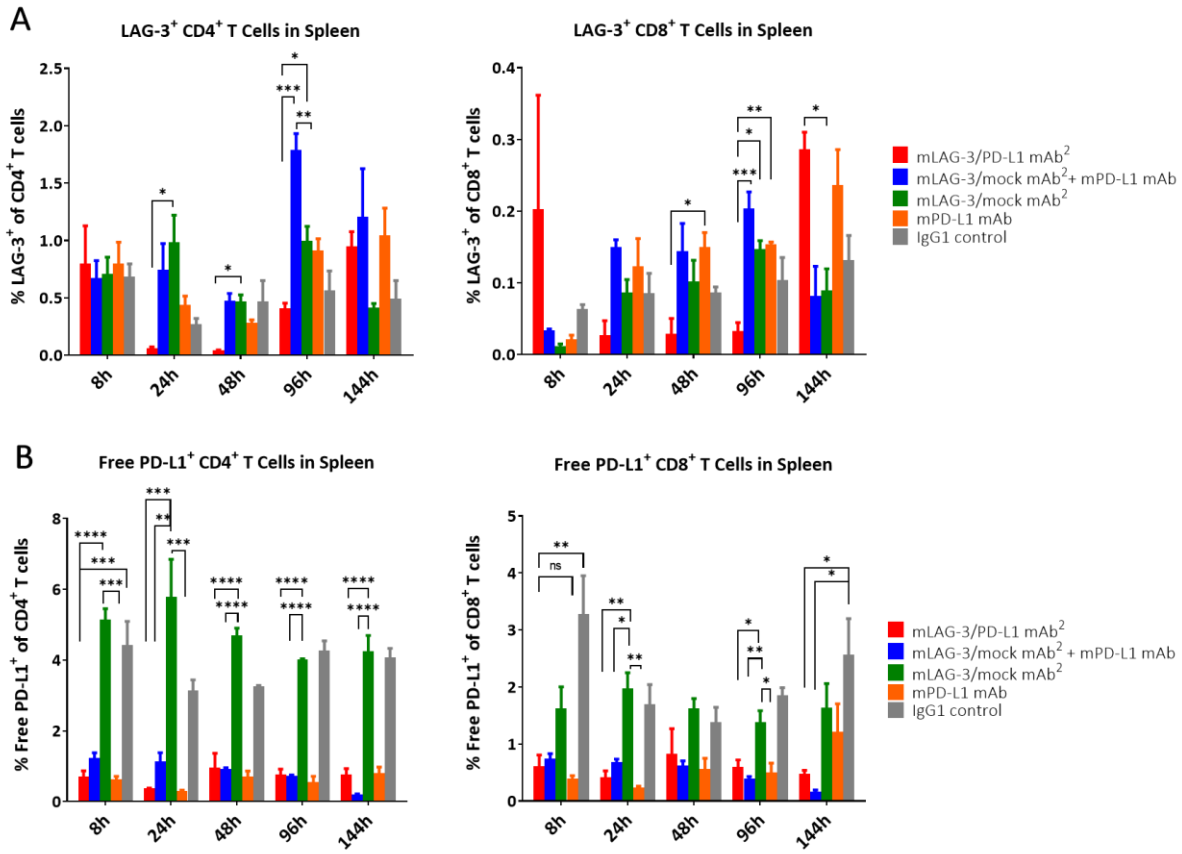
Supplementary Figure S7. Depletion of CD8⁺ T cells in MC38 tumour-bearing mice eliminates anti-tumour activity of mLAG-3/PD-L1 mAb² but has no effect on pharmacodynamic modulation by mLAG-3/PD-L1 mAb² on peripheral CD4⁺ T cells.

(A) Female C57/BL6 mice were subcutaneously injected with MC38 tumour and randomised to treatment cohorts. At day 2, day 3, day 6, day 9 and day 11 after tumour cell implantation, the mice received intraperitoneally an anti-CD8 monoclonal antibody (10 mg/kg, clone 2.43) to deplete CD8⁺ T cells. IgG1 isotype control (10 mg/kg) or anti-mLAG-3/PD-L1 mAb² (10 mg/kg) was administered intraperitoneally at day 4 post implantation to a dosing regimen of Q3Dx3 (arrows).
Data are presented as mean tumour volume +/- SEM of 24 mice per group. A mixed model analysis was used to determine statistical significance *** denotes $p<0.001$ * denotes $p<0.05$, ns = non-significant.
(B) Ki-67 proliferation marker expression by CD4⁺ T cells in the spleen of MC38-tumour bearing mice 96 hours following a single intraperitoneal injection of anti-mLAG-3/PD-L1 mAb² or IgG1 control, as measured by flow cytometry. CD8⁺ T cells were previous depleted by 3 injections of 10 mg/kg anti-CD8 mAb (clone 2.43).
Assay was performed as n=4 and data are presented as mean +/- SD. Statistical significance (p-value) was determined using two-tailed unpaired, non-parametric Mann-Whitney Test. * denotes $p<0.05$, ns = non-significant.



Supplementary Figure S8. CD4⁺ and CD8⁺ T cell proportion and proliferation in spleen and TILs after treatment of test antibodies.

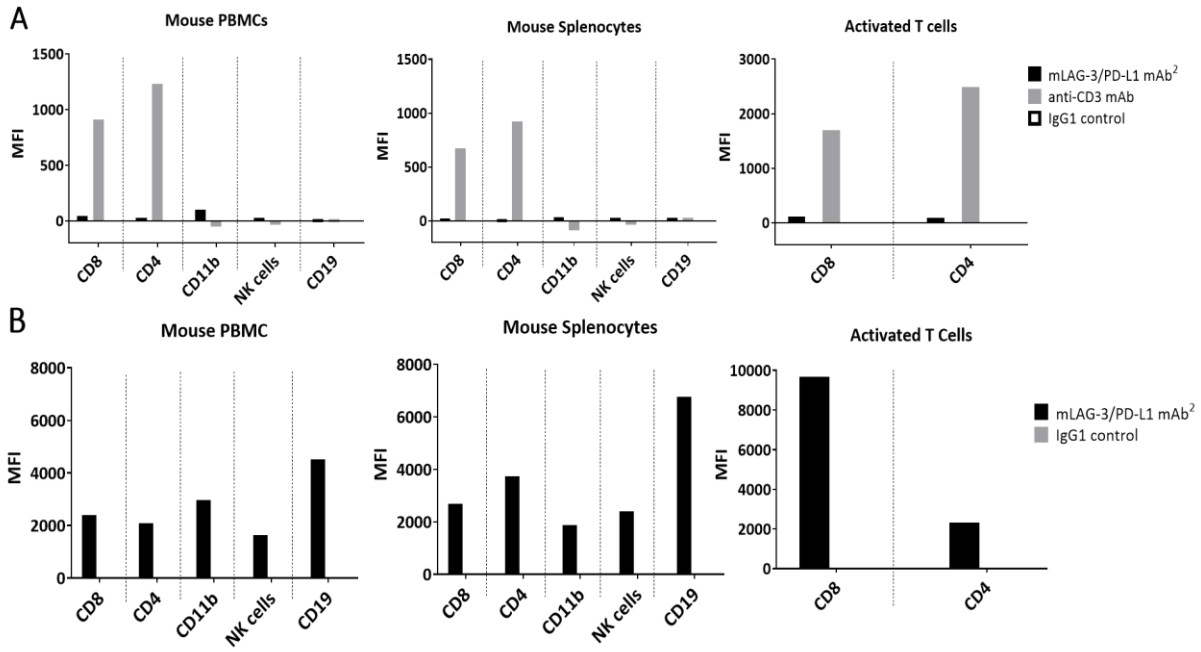
Proportion of (A) CD4⁺ and CD8⁺ T cells in splenic population, (B) Ki-67 proliferation marker expression in CD4⁺ and CD8⁺ T cells in TILs and (C) Ki-67 proliferation marker expression in CD4⁺ and CD8⁺ T cells in spleen from MC38.OVA tumour bearing mice treated following single dosing of test antibodies as measured by flow cytometry. When subcutaneous tumours reached around 65 mm^3 mice were administrated with one intraperitoneal injection of mLAG-3/PD-L1 mAb^2 or controls antibodies. At designated timepoints following dosing, 3 mice were sacrificed and spleen and tumours were processed for flow cytometry. Assays were performed in triplicate and data are presented as mean +/- SEM. Statistical significance (p-value) was determined using One-Way ANOVA with Tukey's correction for multiple comparisons. Significant p-values are labelled with one or more '*', denoting *p < .05, **p < .01, and ***p < .001.



Supplementary Figure S9. Loss of LAG-3 expression and decrease of free PD-L1 after treatment with mLAG-3/PD-L1 mAb^2 antibody.

(A) Total mLAG-3 expression and (B) free mPD-L1 expression on murine CD4$^+$ splenocyte populations (left) or CD8$^+$ splenocyte populations (right) following single dosing of test antibodies in MC38.OVA tumour model as measured by flow cytometry. When subcutaneous tumours reached 65 mm^3, mice were administrated with one intraperitoneal injection of anti-mLAG-3/PD-L1 mAb^2 or controls antibodies. At designated timepoints following dosing 3 mice were sacrificed and spleens were processed for flow cytometry.

Assays were performed in triplicates and data are presented as mean +/- SEM. Statistical significance (p-value) was determined using One-Way ANOVA with Tukey's correction for multiple comparisons. Significant p-values are labelled with one or more '*', denoting *p < .05, **p < .01, ***p < .001 and ****p < .0001.



Supplementary Figure S10. mLAG-3/PD-L1 mAb^2 is not highly internalised in mouse PBMCs, mouse splenocytes, and *ex-vivo* activated T cells.

(A) Mouse PBMCs, mouse splenocytes, and activated mouse pan T cells were used to assess internalisation of mLAG-3/PD L1 mAb^2 in different cellular subsets. The internalisation of mLAG-3/PD-L1 mAb^2 was compared to an anti-mouse CD3 mAb, which was expected to show internalisation by CD3 positive T cells.
(B) A separate batch of mLAG-3/PD-L1 mAb^2 and IgG control antibodies were directly labelled with Lightning-Link® Rapid DyLight® 488 (Innova Biosciences) to determine surface binding.
The samples were acquired on a Cytoflex flow cytometer (Beckman Coulter). The mean fluorescence intensity (MFI) of each sample were quantified and the MFI of the IgG1 control group was subtracted from the MFI of the mLAG-3/PD-L1 mAb^2 or anti-CD3 mAb.

Supplementary Methods

Blocking assays

For the assay to assess the blocking of the interaction between LAG-3 and MHCII by FS118, FS118 was titrated and pre-incubated with a final concentration of 2 µg/mL biotinylated recombinant human LAG-3 Fc-tagged protein (BPS Bioscience, 71147) for 1 hour. This antibody mix was then incubated with Raji cells expressing MHC class II for 1 hour, followed by the addition of streptavidin-Alexa647 solution (Invitrogen, S-32357) at 2 µg/mL for 30 minutes. The binding of biotinylated LAG-3 to MHCII on the cell surface was analysed by flow cytometry.

To assess FS118 blocking PD-L1 binding to PD-1, FS118 was titrated and incubated with HEK293 cells overexpressing human PD-L1 (HEK-hPD-L1) for 1 hour followed by the addition of 1 µg/mL (final concentration) of biotinylated recombinant human PD-1-Fc-tagged protein (BPS Bioscience, 71109) for another hour. To detect binding of the antibodies to cell surface PD-L1, streptavidin-Alexa647 solution (Invitrogen, S-32357) at 2 µg/mL was added for 30 minutes. The binding of biotinylated PD-1 to cell surface PD-L1 was analysed by flow cytometry.

The CD80 blocking assay was generated using an ELISA-based method. FS118 was titrated and pre-incubated with 1 ug/mL of biotinylated recombinant PD-L1 Fc-tagged protein (BPS Bioscience, 71105). The pre-incubated mAb^2 and PD-L1 mixture was then added to ELISA plates coated with recombinant human CD80-Fc protein (R&D Systems, 140-B1). The binding of biotinylated PD-L1 to the coated CD80 was analysed using a BioTEK plate reader by measuring the absorbance at 450nm.

***In vitro* binding assay with HEK293 cells overexpressing targets LAG-3 and PD-L1**

The binding on HEK cells was assessed by incubating the HEK293-hPD-L1 or HEK293-hLAG-3 cells with serial dilution of FS118 or control antibodies. The cells were washed, and bound antibody was detected using a fluorescently conjugated anti-human Fc secondary antibody (anti-human Fc-AlexaFluor488 antibody, Jackson Immuno Research). For the binding on T cells, $CD4^+$ T cells isolated from human peripheral blood mononuclear cells (PBMCs) (supplied by NHS Blood and Transplant Service (NHSBT, UK) as anonymized samples from consenting donors) were activated and expanded using Dynabeads Human T-Activator CD3/CD28 kit (Invitrogen) for 8 days. The binding assay was then performed as described for modified HEK293 cells. The samples were acquired on a BD FACSCanto II (Becton-Dickinson). Flow Jo software (TreeStar) was used to quantify the mean fluorescence intensity (MFI) of each sample. Prism software (GraphPad) was used to plot the measured MFI as a function of antibodies concentration.

$CD8^+$ T cell antigen recall assay

PBMC were separated over a Ficoll density gradient from 3 single donor buffy coats and stimulated with a pool of CD8 (MHC class I-restricted) peptides. Cells were exposed to either a pool of pathogen-derived peptides (CEFT peptide pool, JPT), or to a ”non-stimulatory” pool of self-derived peptides (HLA

peptide pool, JPT). Cytokines (IL-7/IL-15) were added to expand the number of responding $CD8^+$ T cells. FS118 and comparator antibodies serially diluted from 100 nM were tested on CEFT stimulated PBMC for 7 days. All cultures were then washed free of peptides and compounds and rested for 4 days. Cultures were harvested and re-stimulated with the peptide pool in the absence of test compounds but in the presence of monensin and brefeldin A for 24h. $CD8^+$ T cells were then assessed for levels of surface CD107a (as a correlate for degranulation and direct killing) and for intracellular IFNγ (as a readout for indirect killing) by flow cytometry with CD8a, IFNγ, CD107a antibodies (all from BioLegend). Single cultures were performed for each concentration point for each of the three donors. Data was acquired using a BD FACSCanto™ II system (BD Biosciences) flow cytometer.

Internalisation assay in mouse PBMCs, splenocytes, and activated T cells

The pan T cells were isolated from mouse spleen using a mouse pan T cell isolation kit (Miltenyi Biotec) and activated for 4 days using Dynabeads mouse T-Activator CD3/CD28 (ThermoFisher) to upregulate LAG-3 and PD-L1 expression. To determine the extent of internalisation of mLAG-3/PD-L1 mAb^2 or control antibodies (anti-CD3 mAb and IgG1 control mAb), the molecules were directly labelled with a pH-sensitive fluorescent dye (pHrodo Green, Life Technologies), which increases in fluorescence in acidic environments and can therefore be used to measure uptake into intracellular vesicles. The labelled test articles were incubated with cells at 37°C to determine whether any internalisation was detected over a 3-hour assay window in the different mouse immune cell subsets.