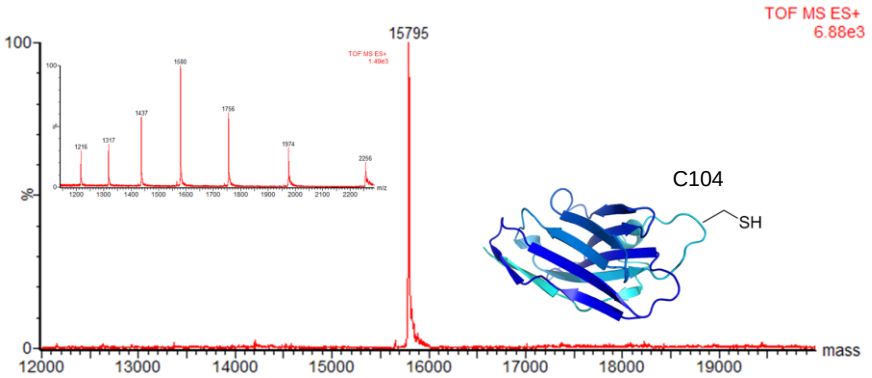
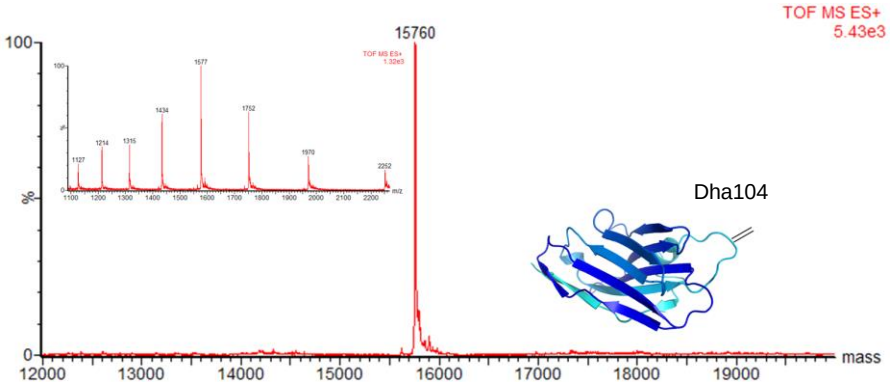
# Supplementary Information

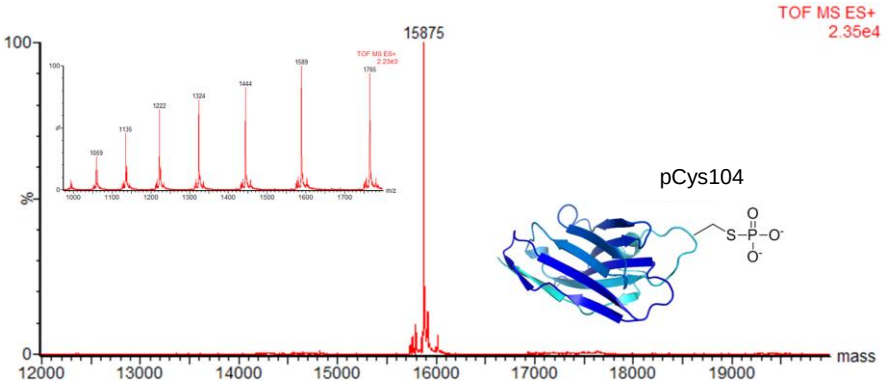
## Supplementary Figures




**Supplementary Figure 1. cAb-Lys3-Cys104.** Calculated mass 15797, observed mass 15795.




**Supplementary Figure 2. cAb-Lys3-Dha104.** Calculated mass 15763, observed mass 15760.



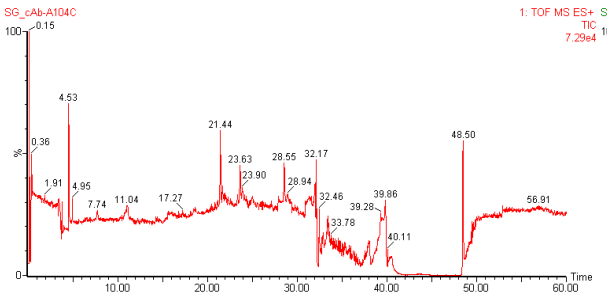

**Supplementary Figure 3. cAb-Lys3-pCys104.** Calculated mass 15874, observed mass 15875.

a

**Associated Datafile: SG_cAb-A104C (50 - 1990 amu)**
**AspN:-/D**

| Frag# | Res# | Sequence | Theor (Bo) | [M+H] | [M+2H] | [M+3H] |
|---|---|---|---|---|---|---|
| D4 | 90-98 | (E)DTAIYYBAA(D) | 1046.44 | 1047.45 | 524.23 | 349.82 |
| D2 | 62-72 | (A)DSVKGRFTISQ(D) | 1236.65 | 1237.65 | 619.33 | 413.22 |
| D3 | 73-89 | (Q)DNAKNTVYLLMNSLEPE(D) | 1949.94 | 1950.95 | 975.98 | 650.99 |
| ■D5 | 99-120 | (A)DSTIYBSYYEBGHGLSTGGYGY(D) | 2508.98 | 2509.99 | 1255.50 | 837.34 |
| D6 | 121-146 | (Y)DSWGQGTQVTVSRENLYFQGHHHHHH(-) | 3093.41 | 3094.42 | 1547.71 | 1032.14 |
| D1 | 1-61 | (-)DVQLVESGGGSVQAGGSLRLSBAASGYTIGPYBMGWFRQAPGKEREGVAAINMGGGITYYA(D) | 6331.12 | 6332.13 | 3166.57 | 2111.38 |

b

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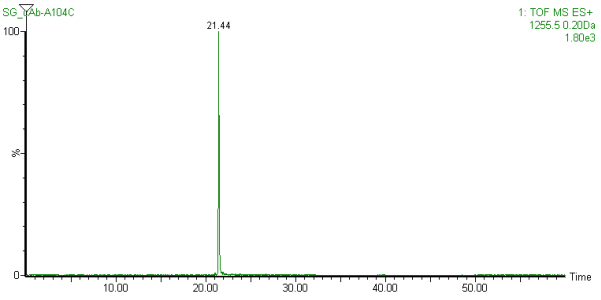


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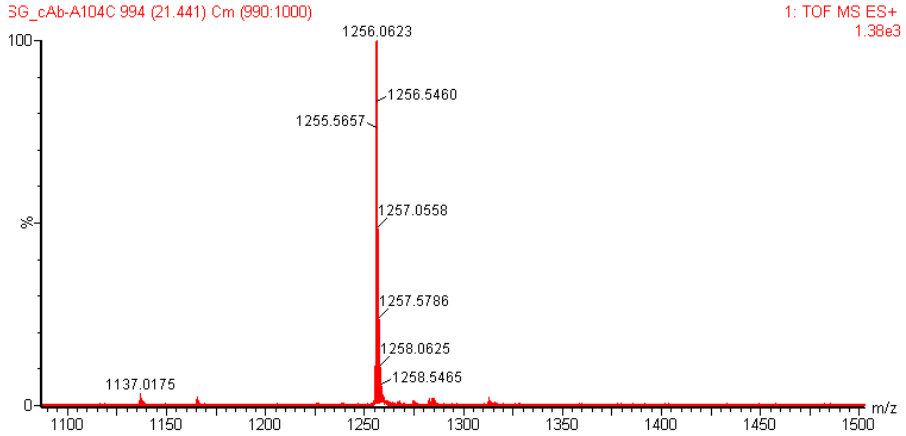


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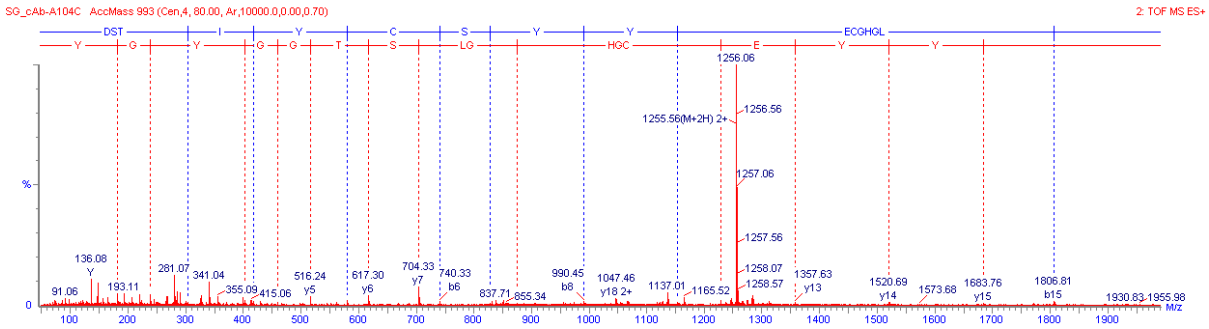



**Supplementary Figure 4. Enzymatic digest of cAb-Lys3-Cys104 and sequencing.** a) Expected sequences and theoretical masses of the peptides resulting from digestion of cAb-Lys3-Cys104 with Asp-N. Masses identified in the mass spectrum integrated over the whole LC-MS chromatogram are highlighted in black (ΔDa = 0.1). B corresponds to carboxymethylamide cysteine. b) LC-MS chromatogram. c) Extracted mass chromatogram

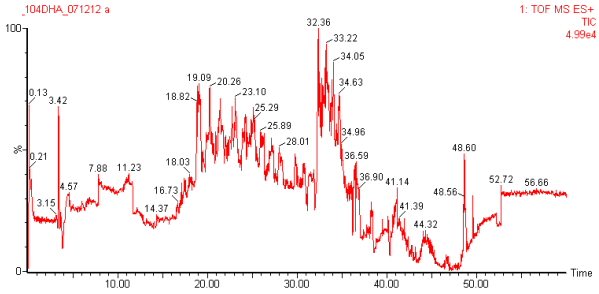
for the theoretical mass $[D5+2H]^{2+}$: M = 1255.50. d) MS of peak at 21.44 minutes. e) MS/MS of peptide D5, Cys is observed at position 104.

a

Associated Datafile: SG_104DHA_071212 a (50 - 1990 amu)
AspN:-/D

| Frag# | Res# | Sequence | Theor (Bo) | [M+H] | [M+2H] | [M+3H] |
|---|---|---|---|---|---|---|
| D4 | 90-98 | (E)DTAIYYBAA(D) | 1046.44 | 1047.45 | 524.23 | 349.82 |
| D2 | 62-72 | (A)DSVKGRFTISQ(D) | 1236.65 | 1237.65 | 619.33 | 413.22 |
| D3 | 73-89 | (Q)DNAKNTVYLLMNSLEPE(D) | 1949.94 | 1950.95 | 975.98 | 650.99 |
| ■D5 | 99-120 | (A)DSTIYZSYYEBGHGLSTGGYGY(D) | 2417.97 | 2418.98 | 1210.00 | 807.00 |
| D6 | 121-146 | (Y)DSWGQGTQVTVSRENLYFQGHHHHHH(-) | 3093.41 | 3094.42 | 1547.71 | 1032.14 |
| D1 | 1-61 | (-)DVQLVESGGGSVQAGGSLRLSBAASGYTIGPYBMGWFRQAPGKEREGVAAINMGGGITYYA(D) | 6331.12 | 6332.13 | 3166.57 | 2111.38 |

b

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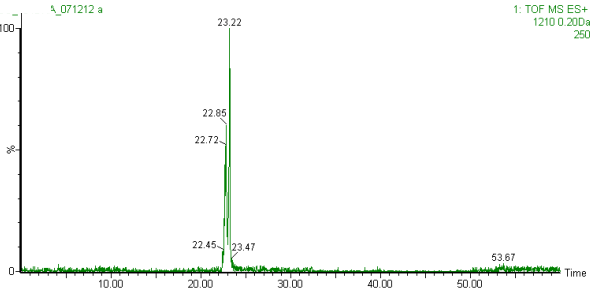


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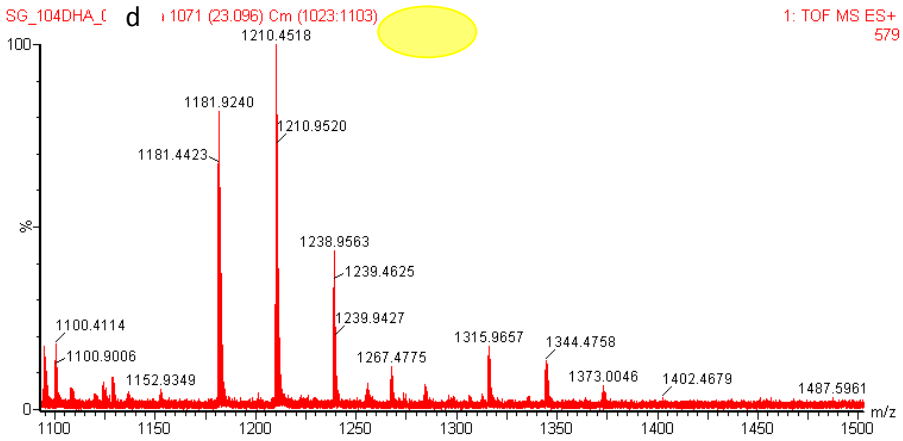


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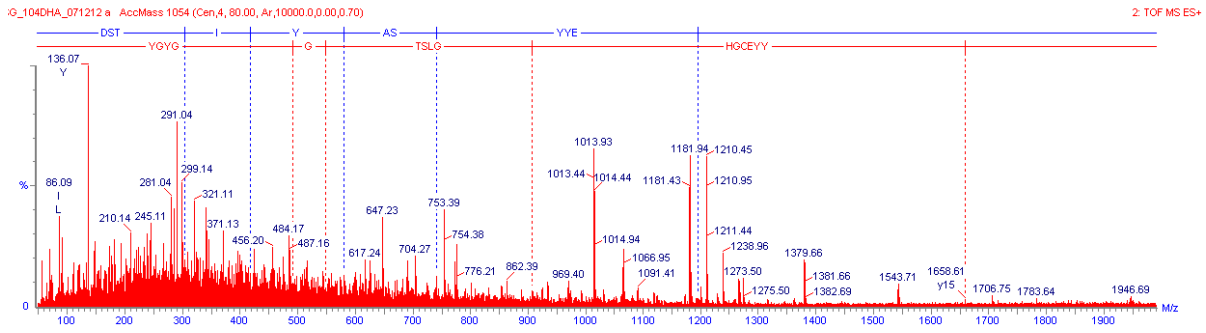


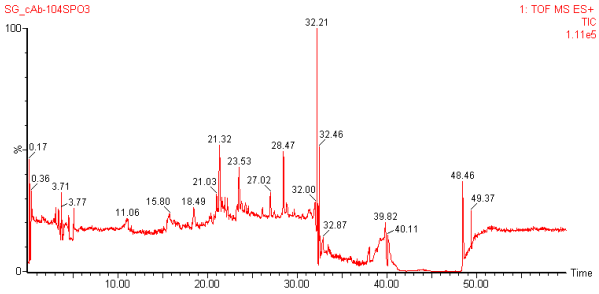

**Supplementary Figure 5. Enzymatic digest of cAb-Lys3-Dha104 and sequencing.** a) Expected sequences and theoretical masses of the peptides resulting from digestion of cAb-Lys3-Dha104 with Asp-N. Masses identified in the mass spectrum integrated over the whole LC-MS chromatogram are highlighted in black (ΔDa = 0.1). B corresponds to carboxymethylamide cysteine, Z is Dha. b) LC-MS chromatogram. c) Extracted mass

chromatogram for the theoretical mass $[D5+2H]^{2+}$: M = 1210.00. d) MS of peak at 22.72 – 23.22 minutes. e) MS/MS of peptide D5, Dha (denoted as A) is observed at position 104.

a **Associated Datafile: SG_cAb-104SPO3 1 (50 - 1990 amu)**
**AspN:-/D**

| Frag# | Res# | Sequence | Theor(Bo) | [M+H] | [M+2H] | [M+3H] |
|---|---|---|---|---|---|---|
| D4 | 90-98 | (E)DTAIYYBAA(D) | 1046.44 | 1047.45 | 524.23 | 349.82 |
| D2 | 62-72 | (A)DSVKGRFTISQ(D) | 1236.65 | 1237.65 | 619.33 | 413.22 |
| D3 | 73-89 | (Q)DNAKNTVYLLMNSLEPE(D) | 1949.94 | 1950.95 | 975.98 | 650.99 |
| ■D5 | 99-120 | (A)DSTIYXSYYEBGHGLSTGGYGY(D) | 2530.92 | 2531.93 | 1266.47 | 844.65 |
| D6 | 121-146 | (Y)DSWGQGTQVTVSRENLYFQGHHHHHH(-) | 3093.41 | 3094.42 | 1547.71 | 1032.14 |
| D1 | 1-61 | (-)DVQLVESGGGSVQAGGSLRLSBAASGYTIGPYBMGWFRQAPGKEREGVAAINMGGGITYYA(D) | 6331.12 | 6332.13 | 3166.57 | 2111.38 |

b

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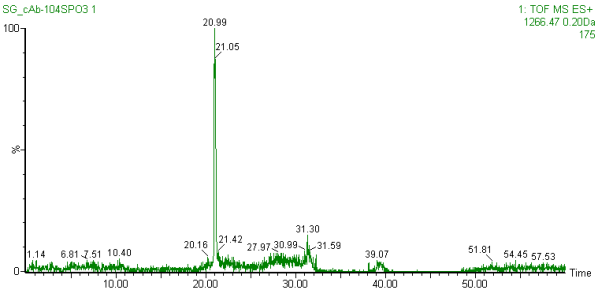


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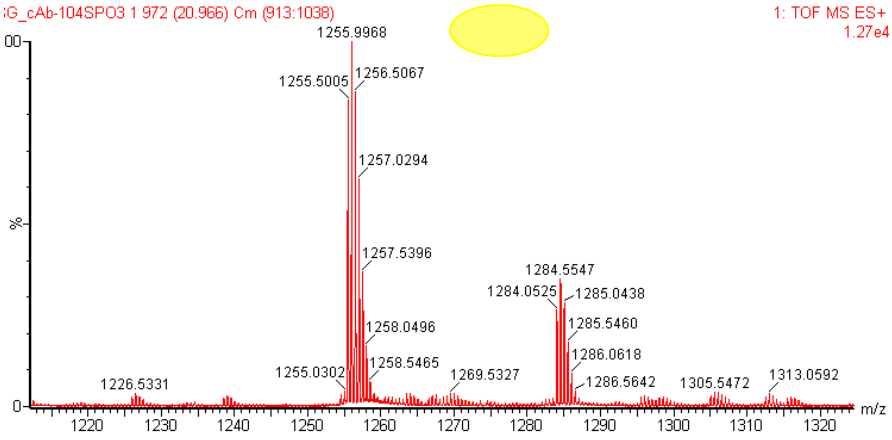


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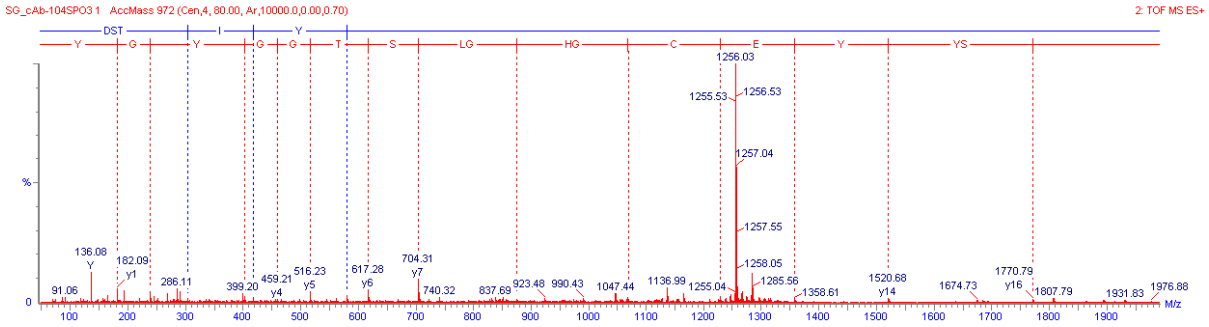

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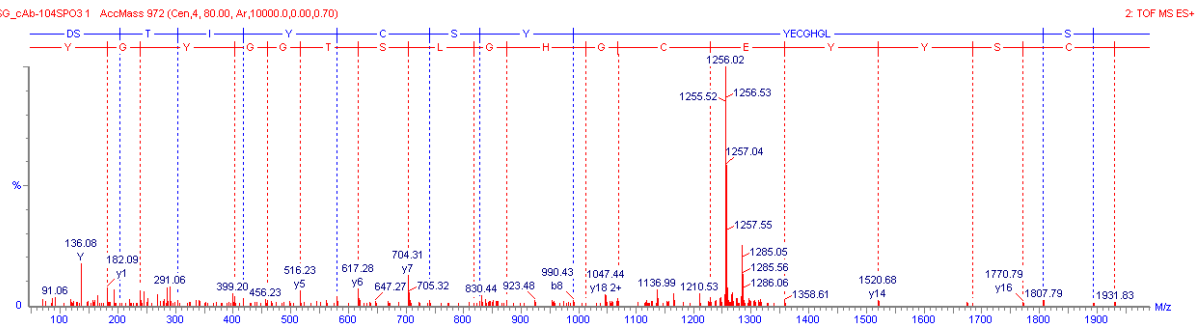
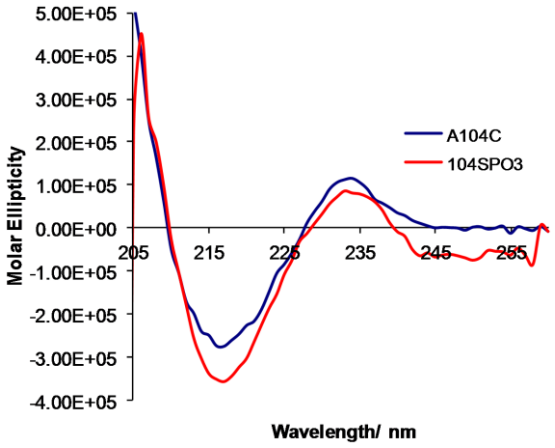
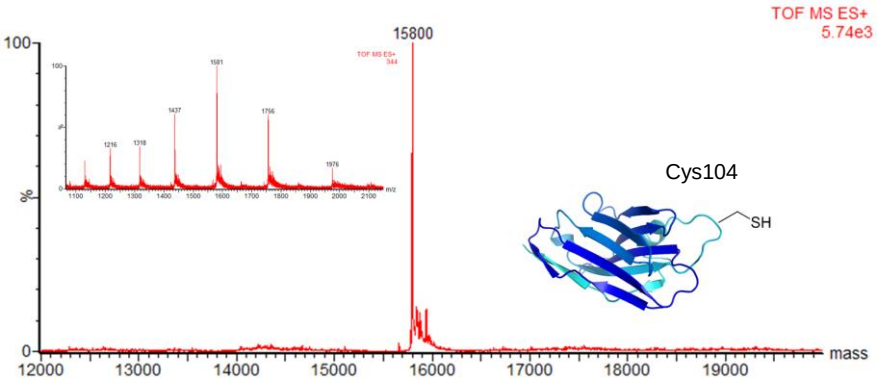


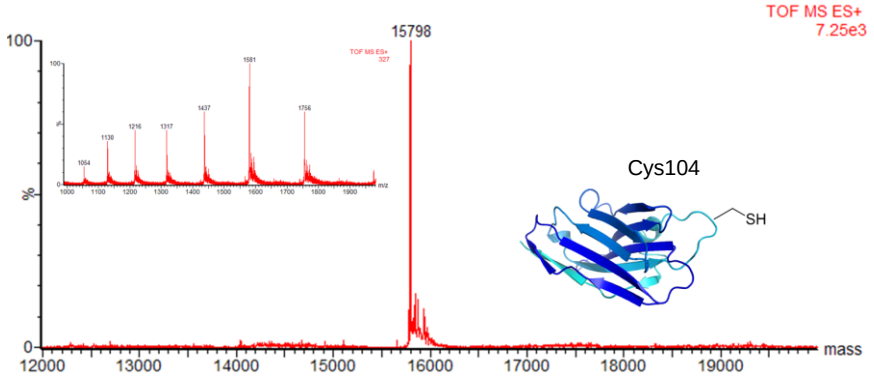

**Supplementary Figure 6. Enzymatic digest of cAb-Lys3-pCys104 and sequencing.** a) Expected sequences and theoretical masses of the peptides resulting from digestion of cAb-Lys3-pCys104 with Asp-N. Masses identified in the mass spectrum integrated over the whole LC-MS chromatogram are highlighted in black (ΔDa = 0.1). B corresponds to carboxymethylamide cysteine, X is phosphocysteine. b) LC-MS chromatogram. c) Extracted mass chromatogram for the theoretical mass $[D5+2H]^{2+}$: M = 1266.47. d) MS of peak at 20.99 minutes. e) MS/MS of peptide D5, phosphocysteine is not observed at position 104, even though the mass was detected. f) MS/MS of peptide D5, where Cys is observed at position 104. No Dha is detected over the chromatogram, indicating the phosphate group is labile during data acquisition.[2]



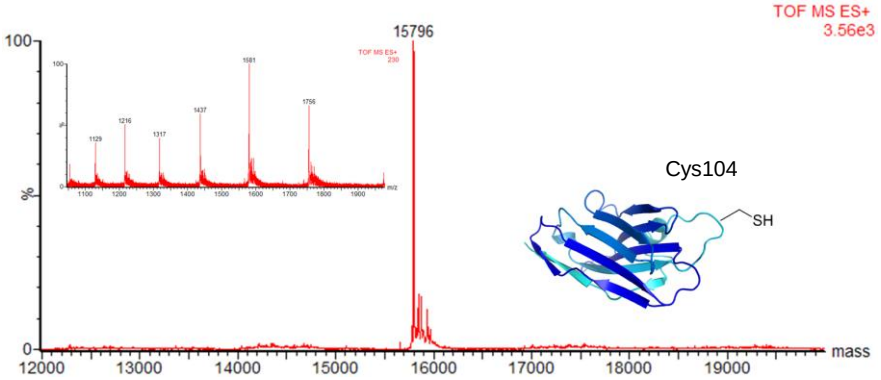

**Supplementary Figure 7. CD spectrum of cAb-Lys3.** Predominant β-sheet character is retained following chemical modification.



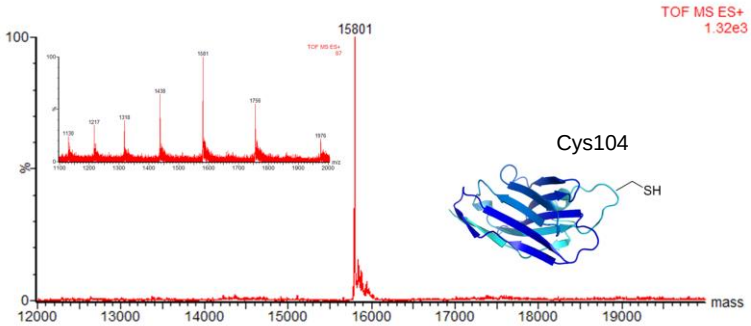

**Supplementary Figure 8. Regeneration of cAb-Lys3-Cys104 by reaction with huPLAP.** Calculated mass 15797, observed mass 15800.



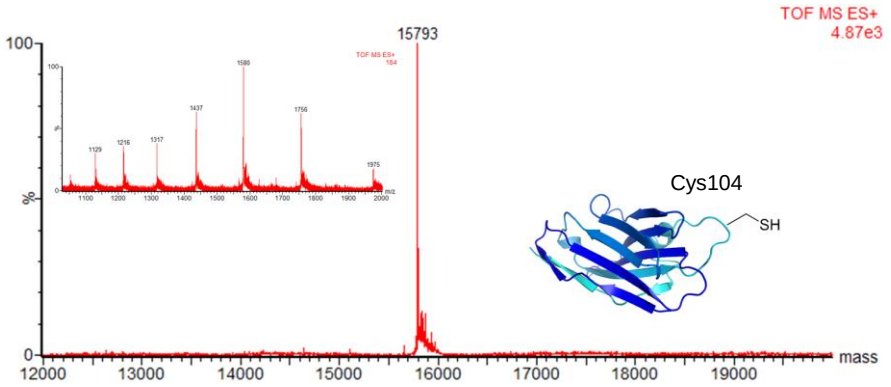

**Supplementary Figure 9. cAb-Lys3-Cys104 can be regenerated by reaction with human acp-1.** Calculated mass 15797, observed mass 15798.



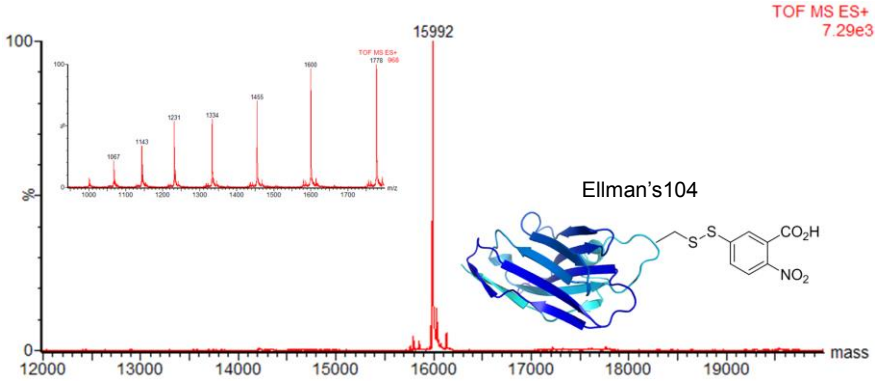

**Supplementary Figure 10. Regeneration of cAb-Lys3-Cys104 by reaction with alp from bovine intestinal mucosa.** Calculated mass 15797, observed mass 15796.



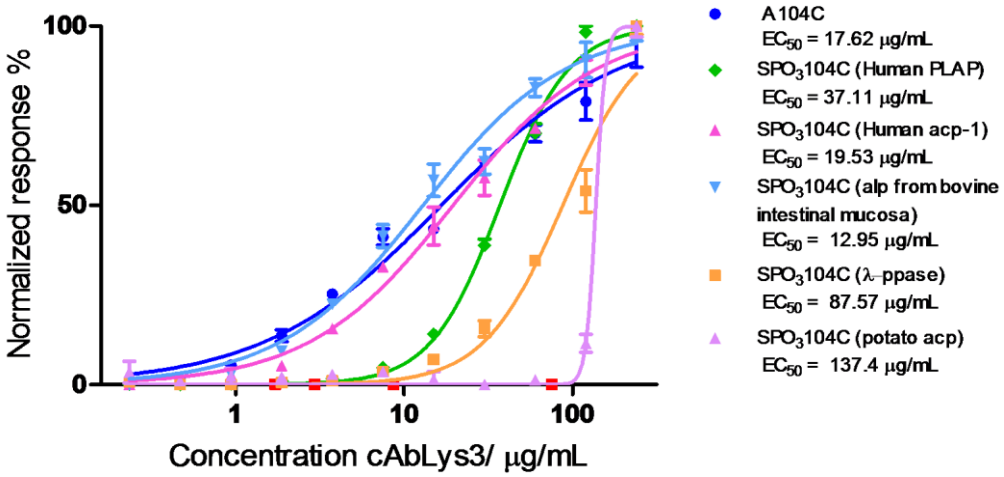

**Supplementary Figure 11. Regeneration of cAb-Lys3-Cys104 reaction with λ-ppase.** Calculated mass 15797, observed mass 15801.



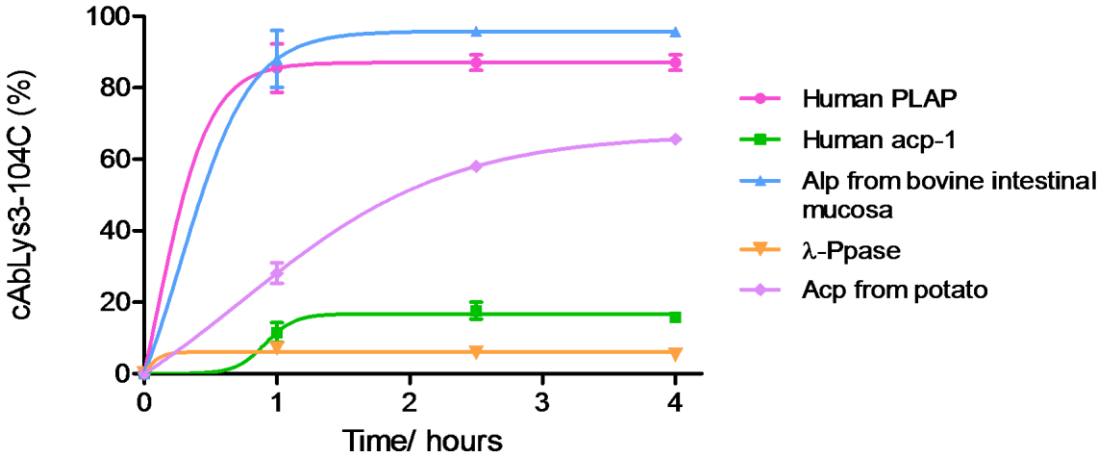

**Supplementary Figure 12. Regeneration of cAb-Lys3-Cys104 by reaction with acp from potato.** Calculated mass 15797, observed mass 15793.



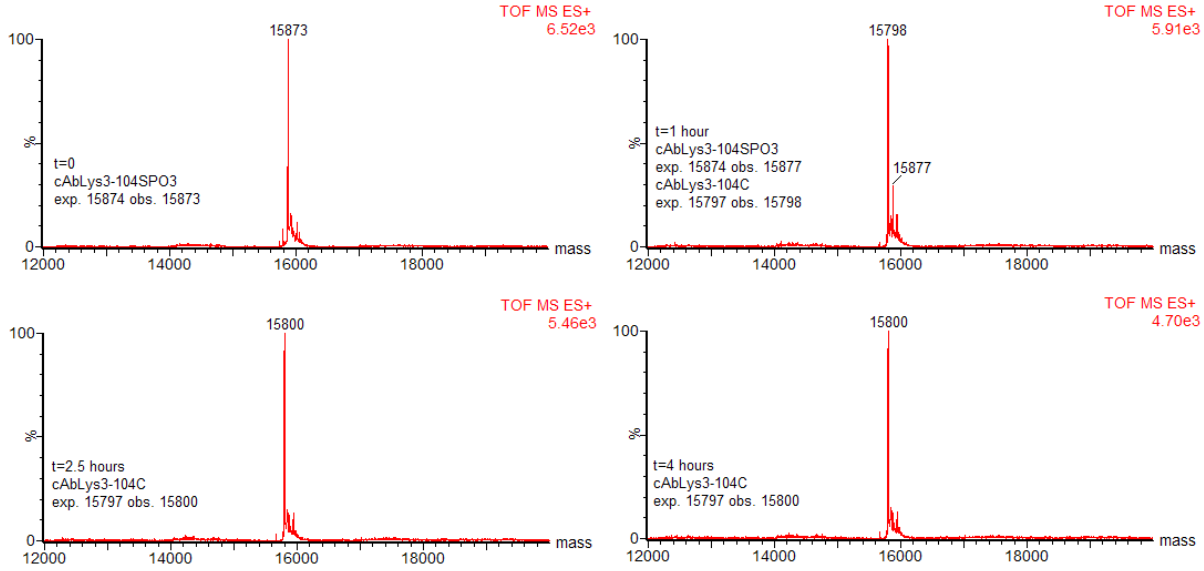

**Supplementary Figure 13. Regenerated cAb-Lys3-Cys104 is able to react with Ellman's reagent.** This indicates that the antibody's reactivity is regenerated. Calculated mass 15994, observed mass 15992.



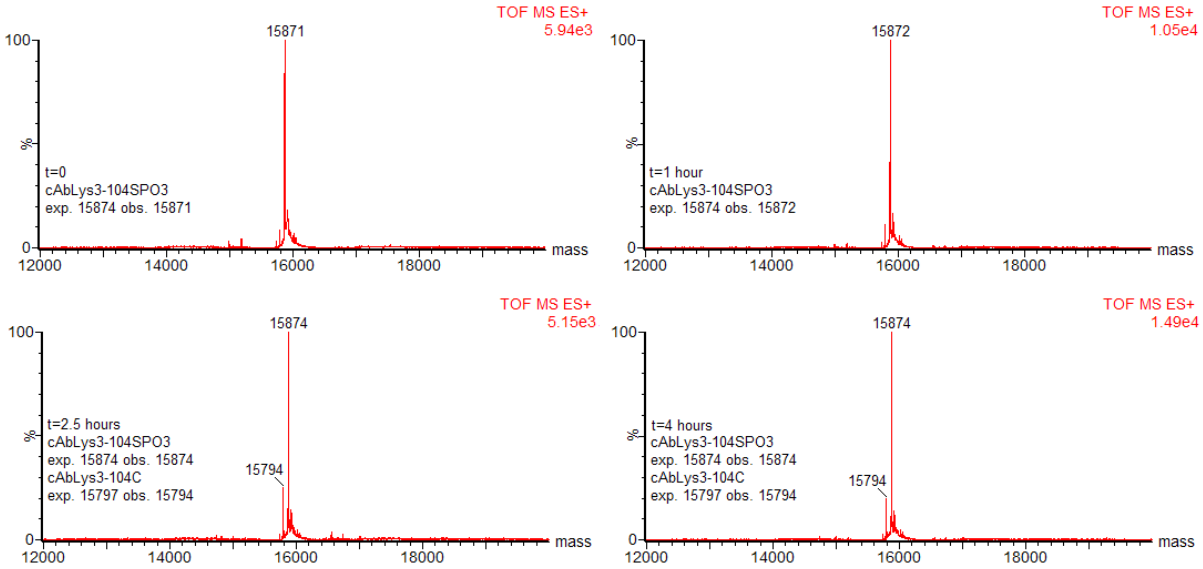

**Supplementary Figure 14. ELISA of regenerated cAb-Lys3-Cys104.** cAb-Lys3 containing regenerated Cys at position 104 is able to bind to lysozyme at similar levels to the original mutant.



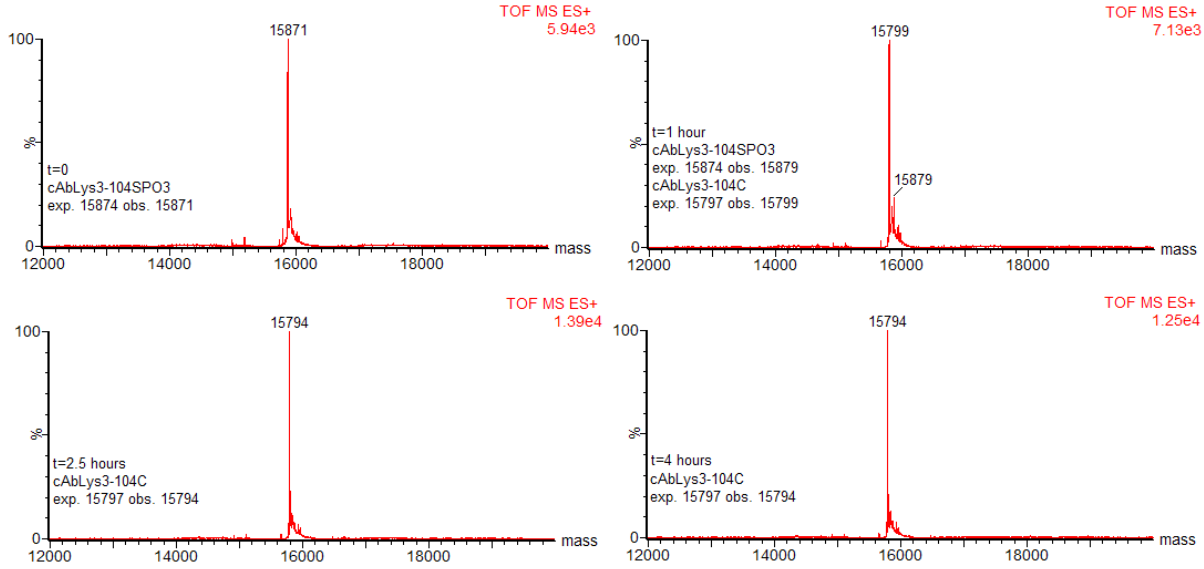

**Supplementary Figure 15. Monitoring the formation of regenerated cAb-Lys3-Cys104 by mass spec with different phosphatases.** Efficient thiophosphate cleavage is observed at physiological conditions with huPLAP and bIMP. For both λ-ppase and human acp-1, limited thiophosphate removal is observed.



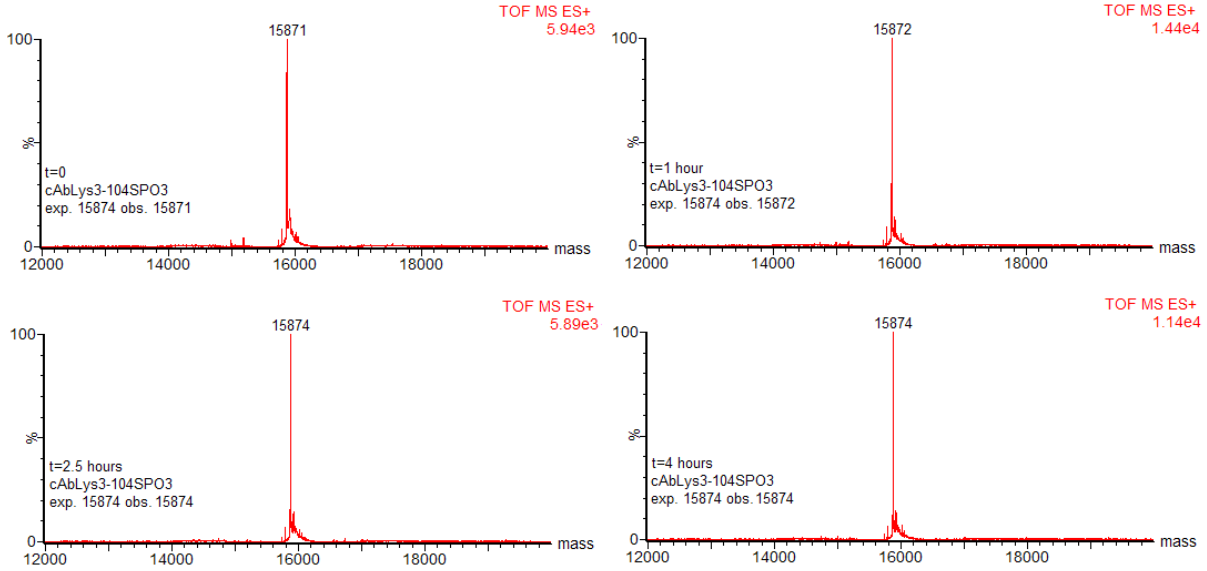

**Supplementary Figure 16. MS data for cAb-Lys3-pCys104 treated with huPLAP in 50 mM Tris-HCl (pH 7.4) over 4 hours.**



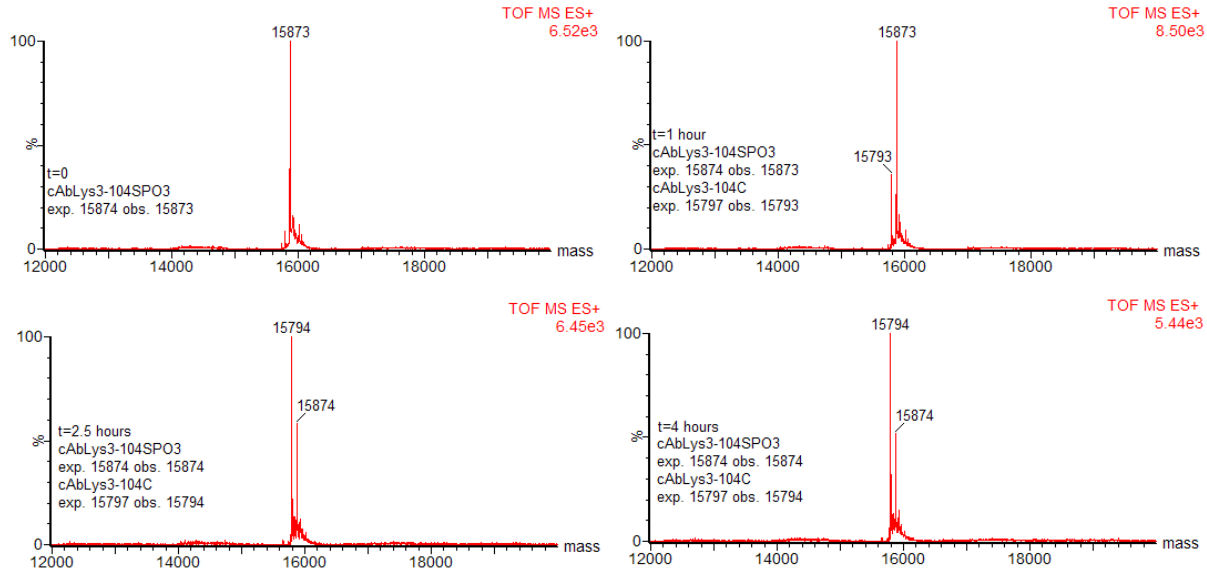

**Supplementary Figure 17. MS data for cAb-Lys3-pCys104 treated with human acp-1 in 50 mM Tris-HCl (pH 7.4) over 4 hours.**



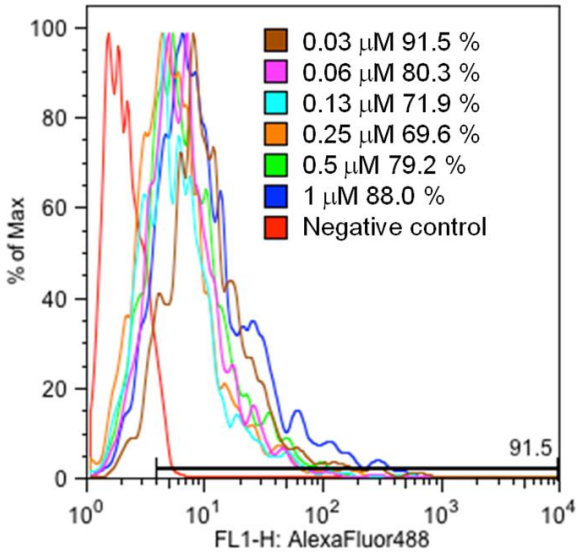

**Supplementary Figure 18. MS data for cAb-Lys3-pCys104 treated with bIMP in 50 mM Tris-HCl (pH 7.4) over 4 hours.**



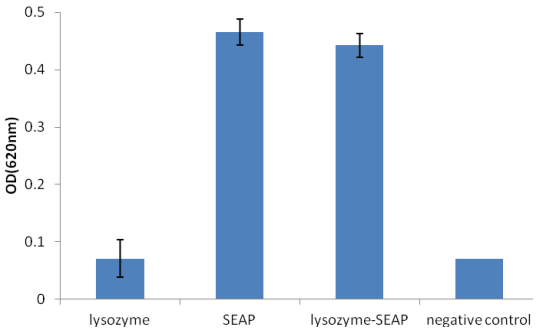

**Supplementary Figure 19. MS data for cAb-Lys3-pCys104 treated with λ-ppase in 50 mM Tris-HCl (pH 7.4) over 4 hours.**



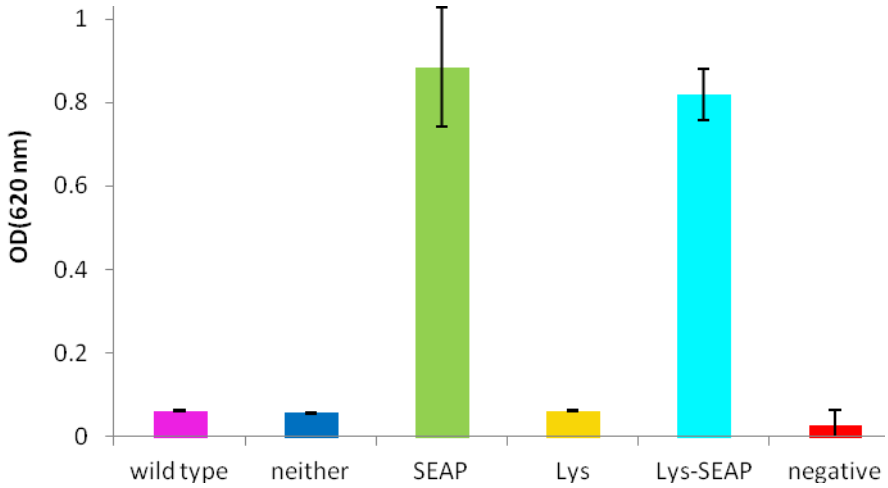

**Supplementary Figure 20. MS data for cAb-Lys3-pCys104 treated with acp from potato in 50 mM Tris-HCl (pH 7.4) over 4 hours.**



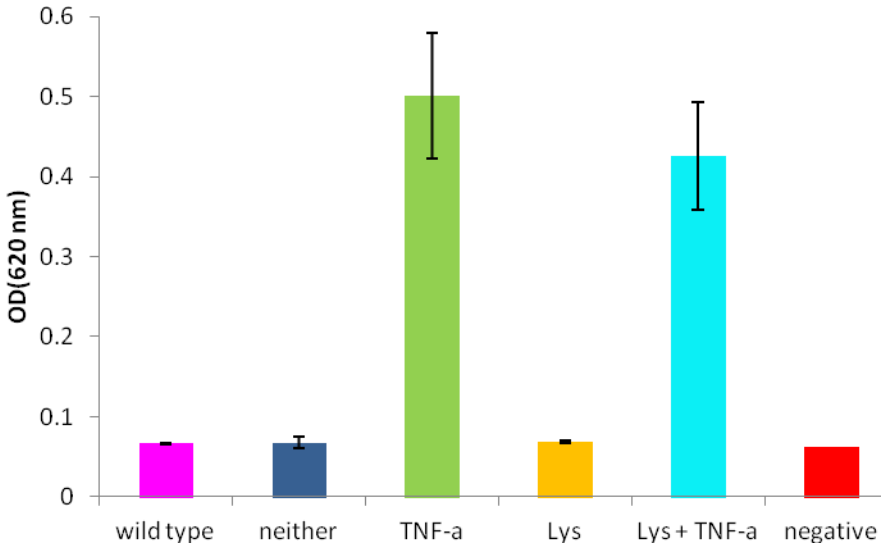

**Supplementary Figure 21. Determination of cell surface lysozyme expression by flow cytometry.** WT-cAb-Lys3 was added in varying concentrations to HEK293T cells transfected with lysozyme followed by PentaHis™ Alexa Fluor® 488. Similar percentages of labelled cells are observed with the varying antibody concentrations, indicating sufficient removal of excess non binding antibody after washing steps.



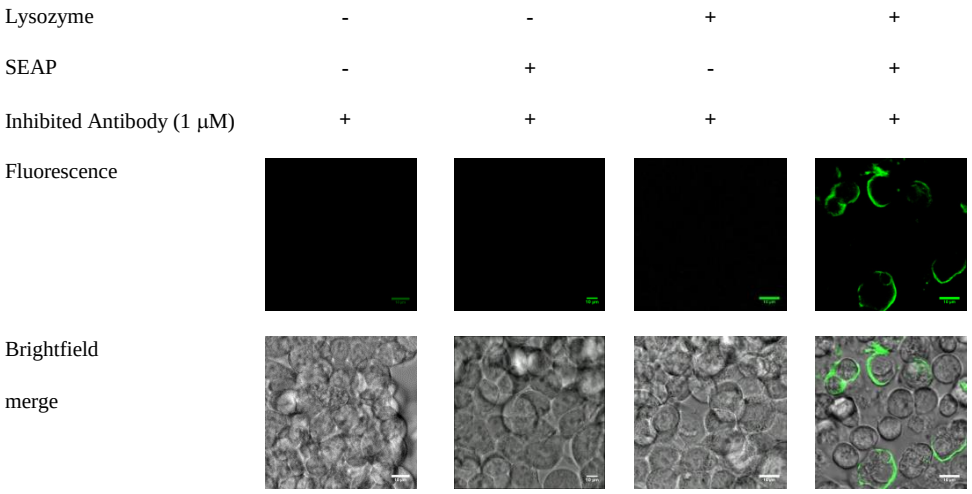

**Supplementary Figure 22. Testing SEAP expression with CMV-SEAP.** HEK293T cells were transfected with lysozyme, SEAP and co-transfected with lysozyme and SEAP. Cell supernatant was incubated with QUANTI-Blue™, and OD (620 nm) measured. An increase in OD is observed with the SEAP sample, indicating that Xfect transfection reagent is successful for the plasmid. A similar increase in OD is observed for the co-transfected sample, essential for testing AND gate conditions.



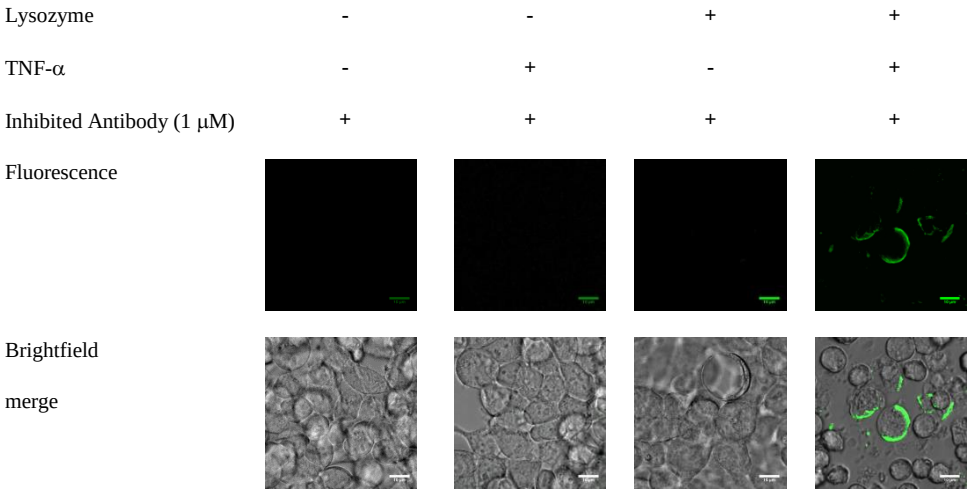

**Supplementary Figure 23. Testing SEAP expression in HEK293T cell AND gate (Fig. 2 a - c).** Following incubation of transfected cell supernatant with QUANTI-Blue™, an increase in OD is observed only for samples where cells were transfected with SEAP.



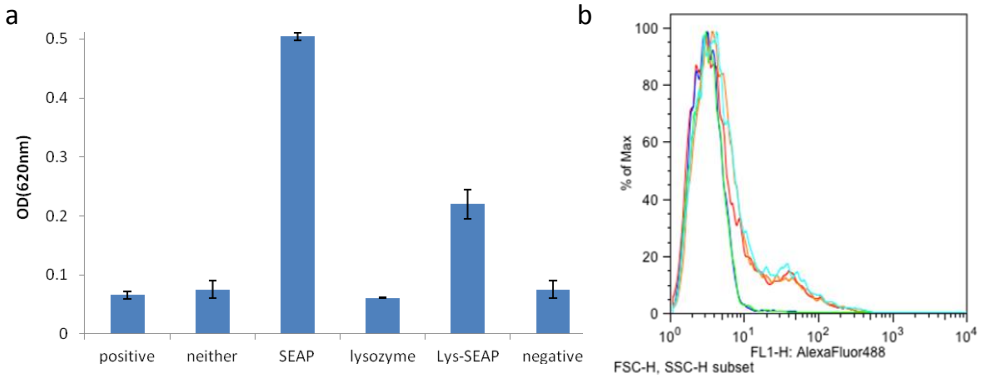

**Supplementary Figure 24. Testing SEAP expression in HEK-Blue Null1 (NF-κB-*seap*+) cell AND gate (Fig. 2 a, d, e).** Following incubation of transfected cell supernatant with QUANTI-Blue™, an increase in OD is observed only for samples where TNF-α has been added to induce SEAP expression.



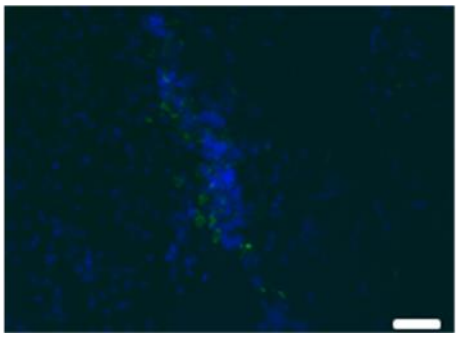

**Supplementary Figure 25. Displaying cell surface labelling of the HEK293T cell AND gate.** Cells transfected with the varying combinations of inputs are stained with PentaHis™ Alexa Fluor® 488 after the addition of cAb-Lys3-pCys104 (inhibited antibody). Scale bar reads 10 μm.



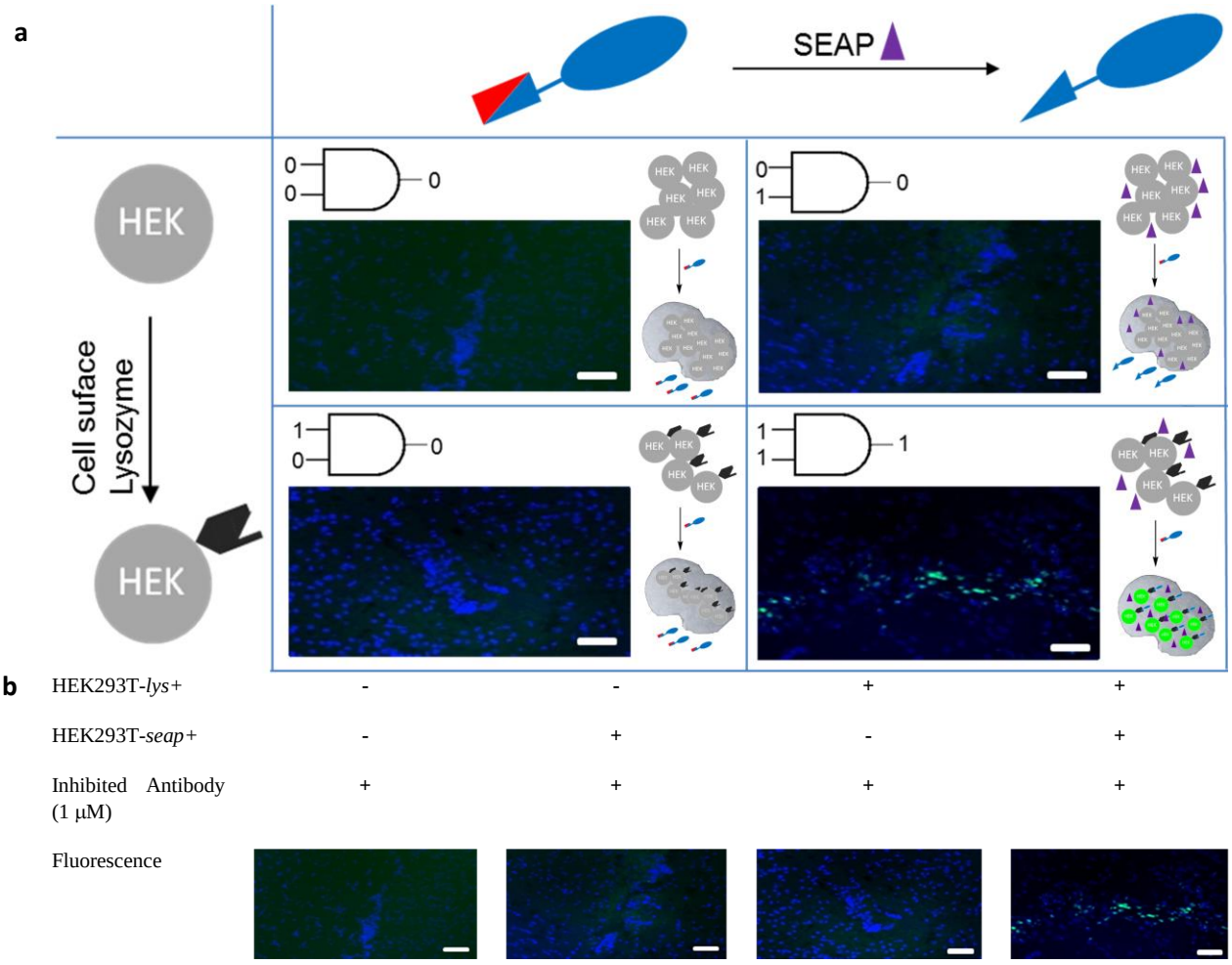

**Supplementary Figure 26. Displaying cell surface labelling of the HEK-Blue Null1 (NF-κB-*seap*+) cell AND gate.** Cells with the varying combinations of inputs are stained with PentaHis™ Alexa Fluor® 488 after the addition of cAb-Lys3-pCys104 (inhibited antibody). Scale bar reads 10 μm.




**Supplementary Figure 27. Testing SEAP and lysozyme expression in HEK293T cells before injection (Supplementary Figure 29).** a) Following incubation of transfected cell supernatant with QUANTI-Blue™, an increase in OD is observed only for samples where cells were transfected with SEAP. b) WT-cAb-Lys3 and PentaHis™ Alexa Fluor® 488 was added to an aliquot of each sample to confirm successful lysozyme expression pre-injection. Red – positive control, dark blue – neither, green – SEAP, turquoise – lysozyme, orange – lysozyme-SEAP.



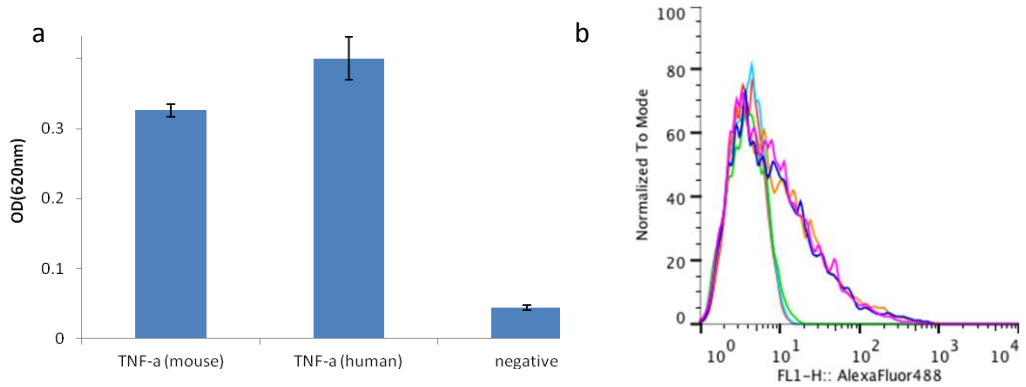
**Supplementary Figure 28. Testing presence of HEK293T-*lys*+ cells in tissue section.** WT-cAb-Lys3 was added to a tissue section injected with HEK293T-*lys*+. Subsequent addition of PentaHis™ Alexa Fluor® 488 allowed visualisation of fluorescent cells by microscopy. Scale bar reads 50 μm.



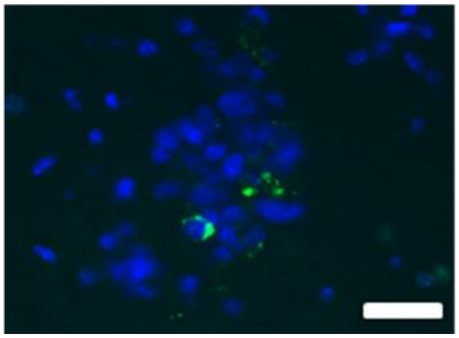

b

| | | | | |
|---|---|---|---|---|
| HEK293T-*lys*+ | - | - | + | + |
| HEK293T-*seap*+ | - | + | - | + |
| Inhibited Antibody (1 μM) | + | + | + | + |
| Fluorescence | | | | |

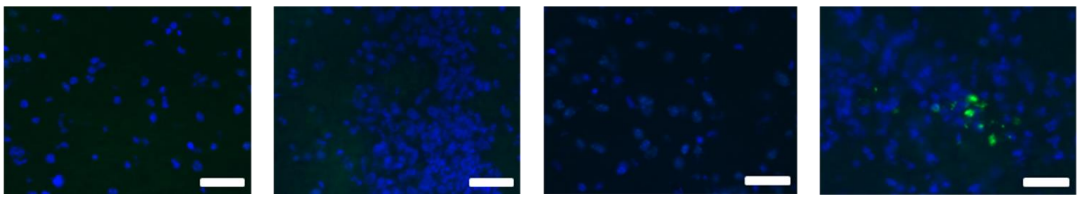
**Supplementary Figure 29. a) Mammalian 'truth table' for the AND antibody, b) table format.** The AND antibody logic gate allowed 'dual-input'-only imaging of mammalian brain tissue. Mice brains were generated containing HEK293T cells expressing varying combinations of input states (no input (0+0), antigen input only (1+0), phosphatase input only (SEAP, shown by a purple triangle in cartoons) (0+1), and dual input (1+1). After overnight incubation, mice were sacrificed and the brains sliced into 10 μm thick sections. Only dual input (1+1) gave rise to output (fluorescent cells observed, bottom right) from the AND antibody. Nuclei staining by DAPI in blue. Scalebar reads 50 μm.



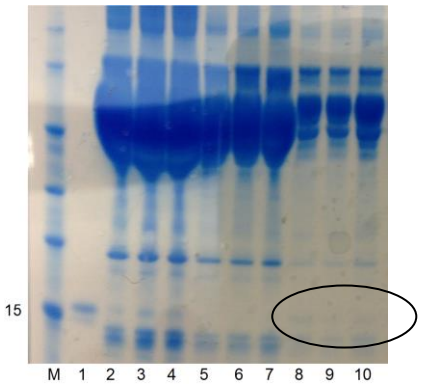

**Supplementary Figure 30. Testing SEAP and lysozyme expression in HEK-Blue Null1 cells before injection (Fig. 3).** a) Following incubation of cell supernatant with QUANTI-Blue™, an increase in OD is observed only for samples where cells have had either mouse or human TNF-α added to them, thereby inducing the expression of SEAP. b) WT-cAb-Lys3 and PentaHis™ Alexa Fluor® 488 was added to an aliquot of each cell sample to confirm successful lysozyme expression pre-injection. Red – negative control, dark blue, pink and orange – cells transfected with lysozyme, green and turquoise – non-transfected cells.



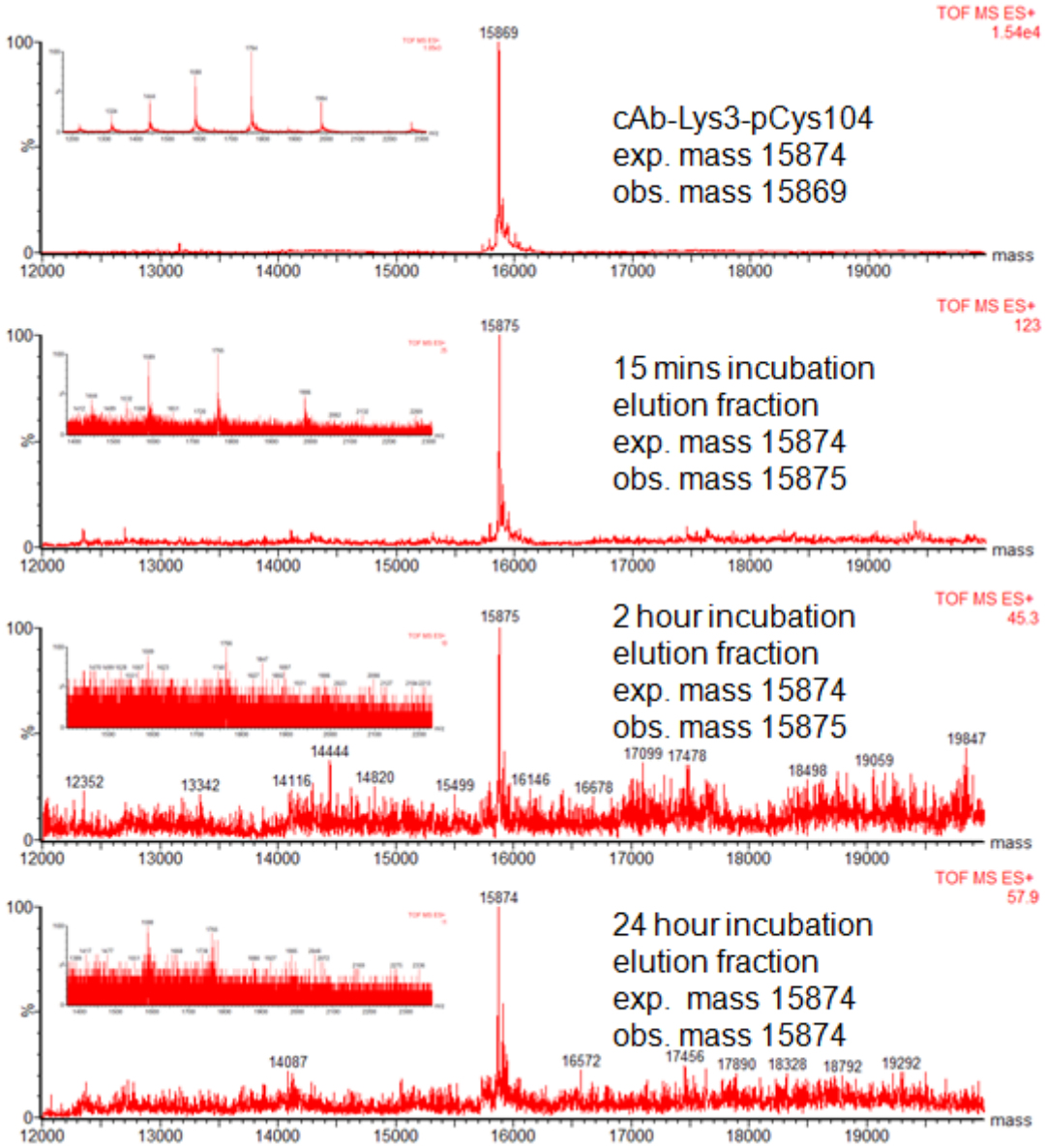
**Supplementary Figure 31. Testing presence of HEK-Blue Null1-*lys*+ cells in tissue section.** WT-cAb-Lys3 was added to a tissue section injected with HEK-Blue Null1-*lys*+. Subsequent addition of PentaHis™ Alexa Fluor® 488 allowed visualisation of fluorescent cells by microscopy. Scale bar reads 50 μm.

| | | | | |
|---|---|---|---|---|
| HEK Blue Null 1-*lys+* | - | - | + | + |
| LPS, inducing endogenous TNF-α | - | + | - | + |
| Inhibited Antibody (1 μM) | + | + | + | + |
| Fluorescence |  | | | |

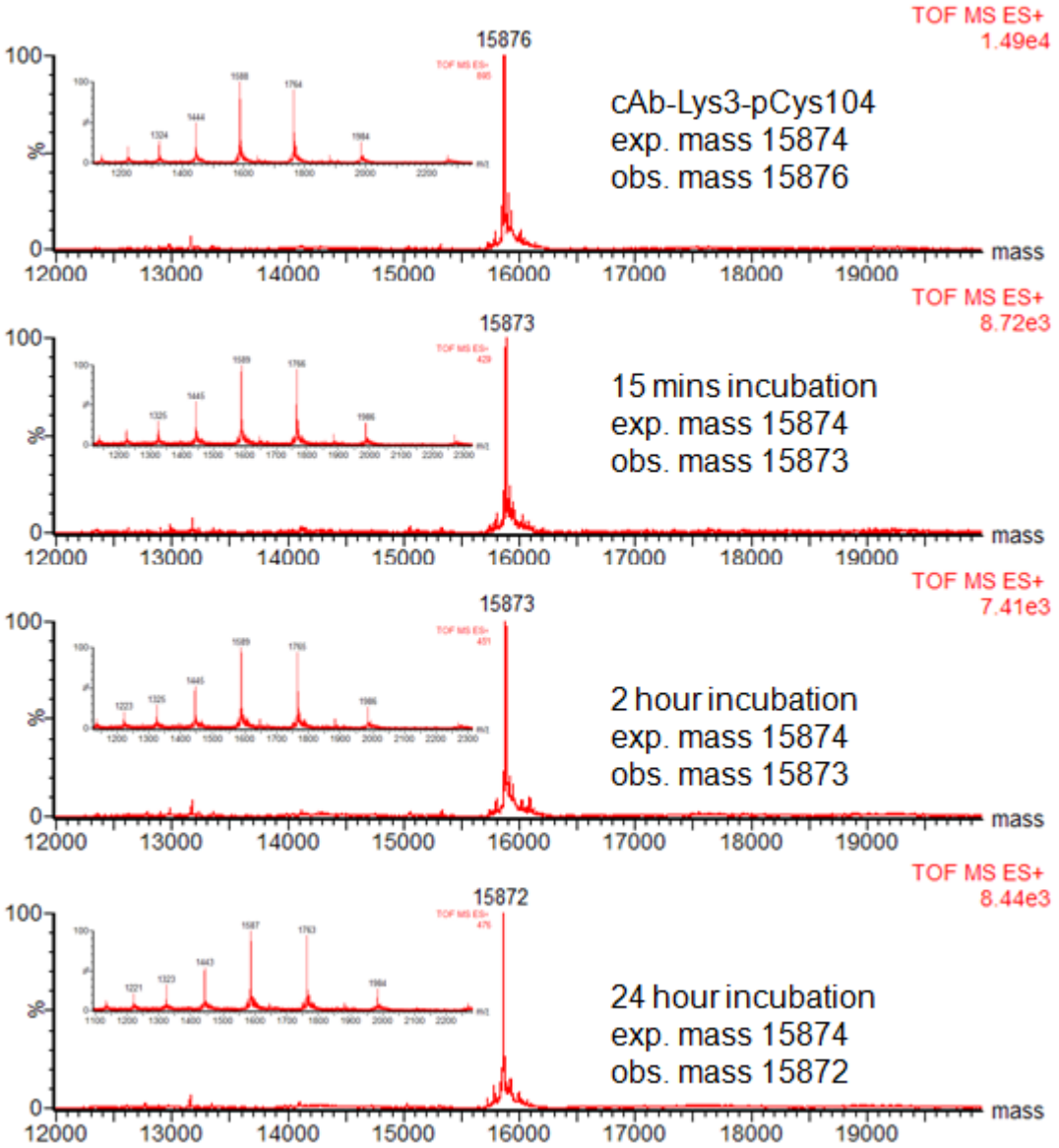
**Supplementary Figure 32. Table format of results shown in Figure 3.** Scale bar reads 50 μm.



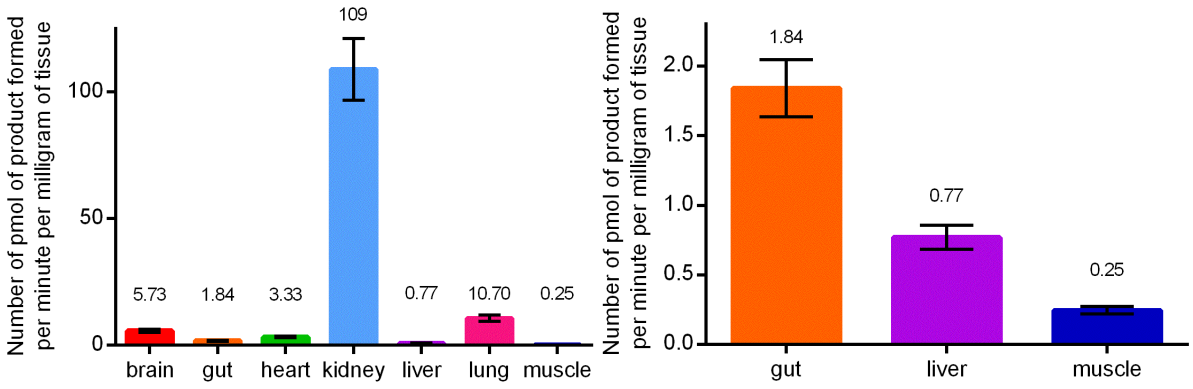

**Supplementary Figure 33. SDS-PAGE of cAb-Lys3-pCys104 and plasma.** 1 – cAb-Lys3-pCys104, 2 – 4 – first wash with binding buffer for samples taken at 15 minutes, 2 hours and 24 hours, 5 – 7 – second wash with binding buffer for samples taken at 15 minutes, 2 hours and 24 hours, 8 – 10 – elution fractions for samples taken at 15 minutes, 2 hours and 24 hours.



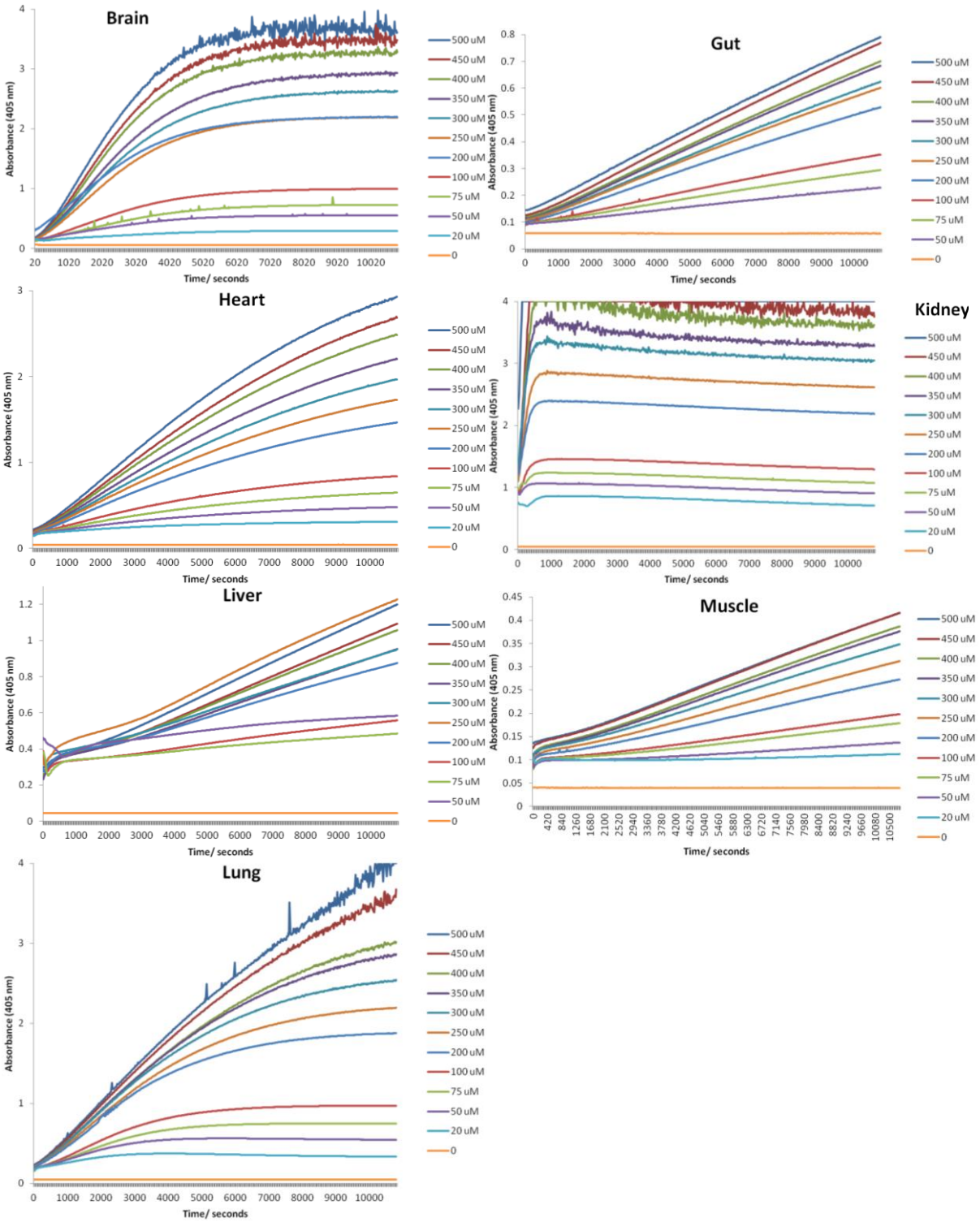

**Supplementary Figure 34.** MS for cAb-Lys3-pCys104 incubated with plasma (5 μM antibody) at various time points. Plasma (3 μL, 10 % of total volume) was added to cAb-Lys3-pCys104 (27 μL, 1.28 mg/ml), and the solution incubated at 37 °C. Samples were taken at 15 minutes, 2 hours and 24 hours, and analysed by LC-MS. No evidence of dephosphorylation or degradation of the antibody was detected at all time points.



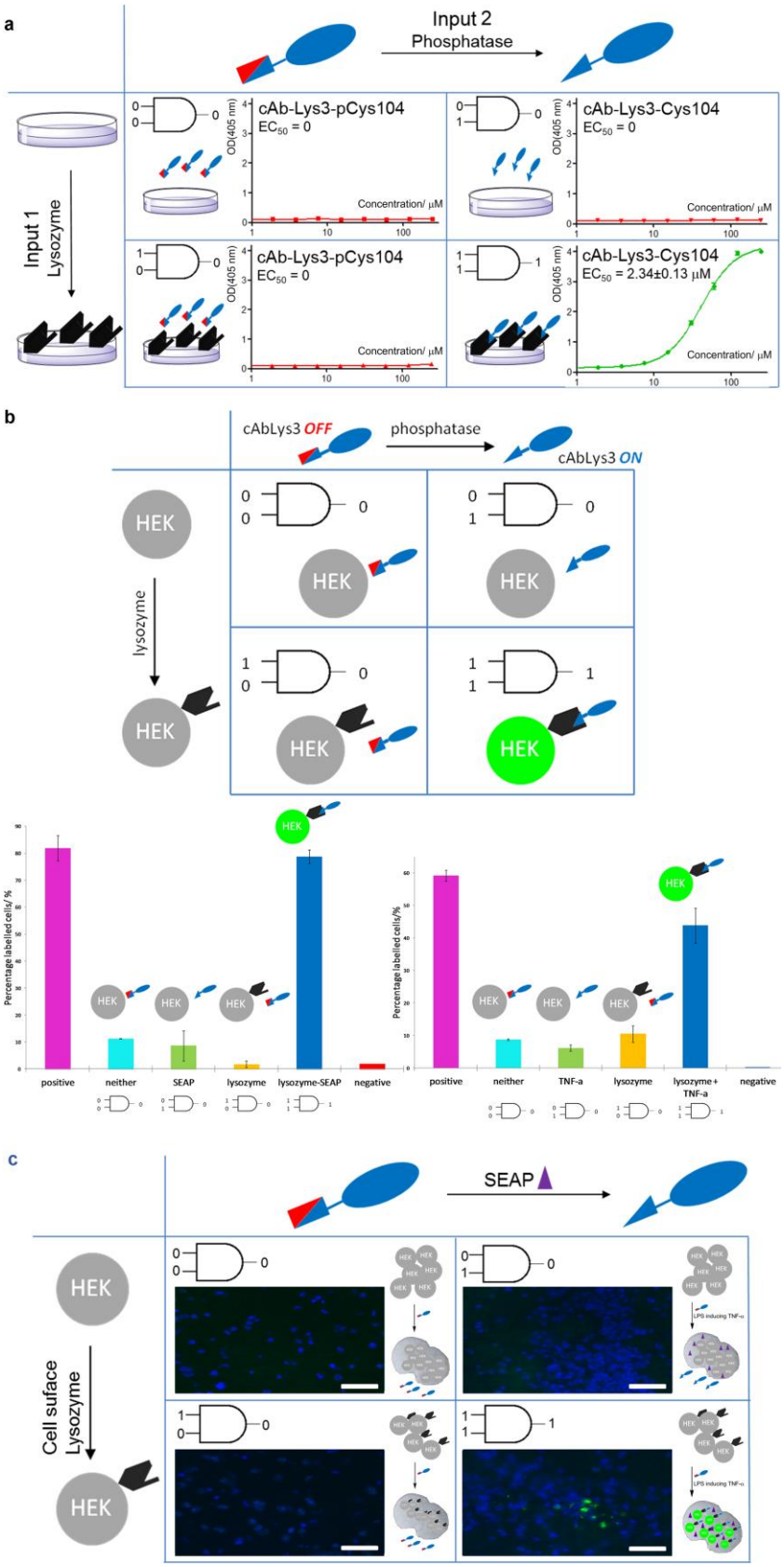

**Supplementary Figure 35.** MS for cAb-Lys3-pCys104 incubated with plasma (10 %) at various time points.




**Supplementary Figure 36.** a) relative phosphatase activities in different organ tissues, expressed as number of pmol of product formed per minute per milligram of tissue; b) expansion of gut, liver and muscle activities.




**Supplementary Figure 37. Raw Phosphatase Activity Data.** Measurement of UV/Vis absorbance at 405 nm for incubation of various tissue organ homogenate samples with chromophoric substrate *para*-nitrophenyl phosphate at 37 °C for 3 hours.




**Supplementary Figure 38. Boolean Truth Table Analysis of the Function of the Gated AND-Ab.** Boolean truth tables compiled considering antigen and enzyme as controllable

inputs showing binding only in response to antigen and enzyme for (a) *in vitro* ELISA analysis (cf Figure 1 main text) (b) on cell surfaces (cf Figure 2 main text) and (c) in mammalian tissue (cf Figure 3 main text). These analyses in a variety of challenging environments confirmed that only upon input of a second state modulator (antigen lysozyme + enzyme phosphatase, 1+1) to this multi-state protein was functional output stimulated; the synthetic antibody cAbLys3-pCys104 may therefore be considered to be an 'AND antibody' (AND-Ab).

## Supplementary Tables

DSTIY**X**SYYECGHGLSTGGYGY

| Residue at position 104 (X) | Calculated monoisotopic mass of neutral peptide |
|---|---|
| Cys | 2508.98 |
| Dha | 2417.97 |
| Phosphocysteine | 2530.92 |

**Supplementary Table 1. Calculated monoisotopic masses of neutral peptides with specified modification at position 104.**

## Supplementary Methods

**Protein Modification and Mass Spectrometry** Milli-Q purified water was used for protein and cellular manipulations. Protein concentrations were determined by Nanodrop. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass LCT (ESI-TOF-MS) coupled to an Agilent 1100 Series HPLC using a Phenomenex Jupiter 5 μm C4 column (250 × 4.6 mm). Water (solvent A) and acetonitrile (solvent B), each containing 1% formic acid by volume, were used as the mobile phase at a flow rate of 1.0 mL/min. The gradient was programmed as follows: 95% A for 5 min to desalt and then a linear gradient to 100% B over 15 min followed by 100% B for an additional 5 min. A linear gradient over 10 minutes back to 95% A was used to re-equilibrate the column. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l/hr. Under these conditions, all protein material typically co-elutes in a single peak between 13 and 18 minutes. For reaction analysis, the mass spectra for all protein material in this peak were combined using MassLynx software (v. 4.1 from Waters). Mass spectra were then calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin obtained at a cone voltage of 25V. The calibrated, combined ion series was deconvoluted using a maximum entropy algorithm that is preinstalled on the MassLynx software.

**ELISA (Enzyme Linked Immunosorbent Assay)** Each well of a 96-well plate (Greiner Bio-one half-area high-binding plates) was coated with 1 μg of hen egg white lysozyme (Sigma), and incubated at 4 °C overnight. All subsequent steps were performed at room temperature. The plate was washed four times with PBS (pH 7.4) containing 0.05% Tween20 (Sigma), blocked for 90 minutes with 3% BSA in PBS and then washed once with PBS (pH 7.4) containing 0.05% Tween20. Antibody (0.48 mg/mL in 1% BSA, 0.02% Tween20 in PBS) dilutions were made across the plate for final concentrations of 240 μg/mL to 234 ng/mL. The plate was incubated with gentle rocking for 2 hours. Unbound antibody was removed by washing four times with PBS (pH 7.4) containing 0.05% Tween20. The secondary antibody (Anti-polyHistidine-alkaline phosphatase conjugate, Sigma) was prepared at a 1:1000 dilution in PBS (pH 7.4) and 50 μL was added to each well. The plate was incubated with gentle rocking for 1 hour. Each well was then washed with 50 μL of diethanolamine buffer

(Phosphatase substrate kit, Thermo Scientific). PNPP phosphatase substrate (Phosphatase substrate kit, Thermo Scientific) was prepared by dissolving one tablet in 6 mL of diethanolamine buffer. 50 μL of the PNPP substrate solution was added to each well and incubated for 15 min. After this time, absorbance was measured at 405 nm. Absorbance was plotted against concentration. Sigmoidal regression analysis and normalisation was carried out using GraphPad Prism 5.01.

**CD (Circular Dichroism)** CD measurements were made using a Chirascan spectropolarimeter fitted with a Peltier temperature controller. Protein solutions were each made up in 10 mM PBS at pH 8. CD spectra were measured in a 1 mm quartz cuvette at room temperature using a scan rate of 50 nm/min, 1 nm interval, 1 nm bandwidth and a response time of 0.5 s. After baseline correction, ellipticities in deg were converted to molar ellipticities (deg $cm^2$ dmol-$res^{-1}$) by normalizing for the concentration of peptide bonds and path length.

**Biological Manipulations** All biological manipulations were undertaken under sterile conditions in a HERAsafe KSP12 laminar flow hood (Thermo Scientific).

**Microscopy** HEK293T cells were visualised on a Leica Microsystems SP5 Inverted Confocal Microscope. All experiments were performed with the pinhole set at 1 Airy diameter. All images were captured at 512x512 pixels at 400 Hz or 1400 Hz capture rates. Z stacks of cell samples were obtained with the 63x oil objective with further magnification being achieved with the optical zoom. PentaHis™ Alexa Fluor® 488 was excited at 488 nm (Ar laser) and emission collected between 500-640 nm. Images were processed using Image J. Gains were kept constant throughout measurements, at a point where saturation was not reached in the most fluorescent sample.

Immunostained sections were examined with a Leica CM5000B fluorescent microscope and images were captured with a Hamamatsu Orca-ER B/W CCD digital camera.

**Flow Cytometry** Flow cytometry was performed on a BD FACSCalibur flow cytometer with excitation at 488 nm (Ar-laser) and fluorescence detected in the FL-1 channel. 10,000 events were collected for each experiment, with live cells being detected with an FL-1 intensity of $10^0$-$10^3$. Data was collected in CellQuest Pro and processed in FlowJo.

**Protein digestion analysis by LC-MS/MS** Peptides were analysed using a nanoLC-MS/MS system with nanoLockspray (nanoAcquity Synapt-HDMS; Waters Corporation, Milford, MA, USA). Peptide digests were injected from a sample manager and trapped on a 5 μm symmetry C18 column (180μm x 20 mm) and washed for 1 min at 15 μl/min with mobile phase A (0.1% formic acid). Peptides were then separated and eluted for MS analysis using a 90 min reverse phase gradient at 400 nl/min (0.1–60% ACN over 70 min) on a BEH 130 C18 1.7μm particle size 75μm x 150mm nanoAcquity UPLC column. The column temperature was set at 300°C. The reference for the nanolockspray was set to the 13C peak of reserpine at a concentration of 3 mg/L flowing at 20 μL/min. The reference was constantly infused and sampled at 10 sec intervals. The eluted peptide spectra were ionised using nano-electrospray and measuring using a Synapt-HDMS (Quadrupole-Time of Flight mass spectrometer). The sample was analyzed in positive, V mode over a mass range between 140–4300 m/z with a scan time of 1s. The on-line eluted peptides were analyzed using a MSe method collecting MS data with a low collision energy (6 eV) and MS/MS data using collision enery ramping between 15 and 35 volts. Spectra were processed using ProteinLynx and Biolynx (Waters Corporation, Milford, MA, USA).

## cAb-Lys3 expression, purification and characterisation

cAb-Lys3-Cys104 was expressed, purified and characterised as described elsewhere.[1]

cAb-Lys3-Cys104 sequence:

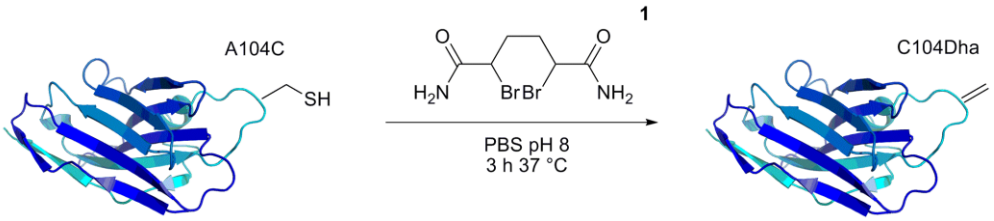
DVQLVESGGGSVQAGGSLRLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYYAD
SVKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTIYCSYYECGHGLSTGGYGYDSWG
QGTQVTVSRENLYFQGHHHHHH

Average Mass = 15797

PDB = 1mel (for WT-cAb-Lys3)

## Chemical modification of cAb-Lys3

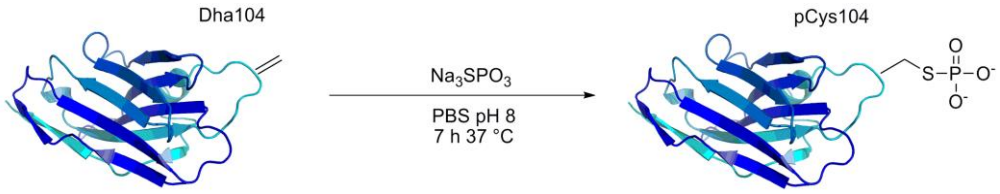
***cAb-Lys3-Dha104***[1]




cAb-Lys3-Cys104 was first reduced with DTT to remove any contaminant disulfide at A104C. DTT (1.2 mg, 7.8 μmol) was added to 0.5 mL of cAb-Lys3-Cys104 (c = 1 mg/mL in 10 mM PBS, pH 8.0) and shaken (600 rpm) at room temperature for 5 minutes. After this time, the protein solution was passed through a PD minitrap (GE Healthcare), previously equilibrated with 10 mM PBS (pH 8.0), eluting with 1 mL of the same buffer. The reduced protein was used immediately. The protein was analyzed by LC-MS, revealing a mass corresponding to the cAb-Lys3-Cys104 mutant (calc. mass 15797; obs. mass 15795). ESI-MS is shown in Supplementary Fig. 1.

Conversion of 104Cys to 104Dha: 2,5-dibromohexanediamide **1** (1.2 mg, 3.3 μmol) was added to a 1.5 mL plastic tube. A 990 μL aliquot of the reduced cAb-Lys3-Cys104 prepared above (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was added to the same tube. The reaction was shaken (600 rpm) at 37 °C for 3 hours. The reaction was then cooled to room temperature and insoluble material was removed by centrifugation (1 minute, 16K g). LC-MS analysis of the supernatant revealed full conversion to a protein with a mass corresponding to the formation of dehydroalanine (calc. mass 15763; obs. mass 15760). ESI-MS are shown in Supplementary Fig. 2. Chemical tests to corroborate the introduction of Dha at position 104 are described elsewhere.[1]

***cAb-Lys3-pCys104***



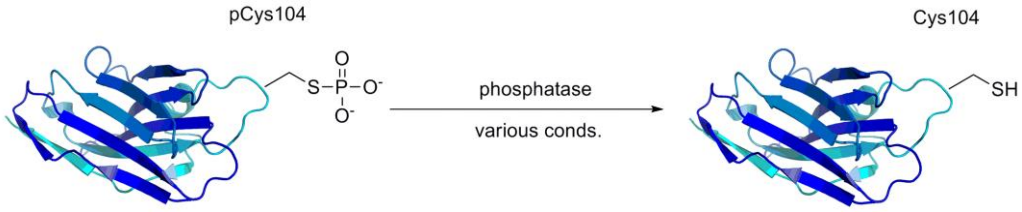
cAb-Lys3-Dha104 (980 µL, c = 0.5 mg/mL in PBS pH 8) was concentrated down to 500 µL (Vivaspin, 5K MWCO, Sartorius Stedim) and then split into 5 aliquots of 100 µL each. A solution of $Na_3SPO_3$ (59 mg, 328 µmol) was made in 18.6 µL MQ $H_2O$ and 20 µL 5 M HCl. 4 x 2.5 µL of the $Na_3SPO_3$ solution (84.9 µmol, ~ 2700 eq.) was added to each aliquot over 30 minutes whilst shaking (600 rpm) at 37 °C. The reactions were continued for 7 hours in total, after which analysis by LC-MS revealed full conversion to cAb-Lys3-pCys104 (calc. mass 15874; obs. mass 15875) as shown in Supplementary Fig. 3. The reaction solution was buffer exchanged and desalted using a PD10 minitrap.

## LC-MS/MS analysis of Asp-N digests of cAb-Lys3-104

cAb-Lys3 (100 µL, 0.2 mg/mL in ammonium bicarbonate pH 8.5) was heated to 95 °C for 15 minutes, reduced with DTT (5 mM) at 60 °C for 15 minutes, then alkylated with iodoacetamide (15 mM) for 15 minutes at room temperature. Asp-N (0.1 µg, 2.5 µL, Sigma) was added to the protein solution, and then shaken at 37 °C overnight.

The sequence of the peptide containing the modification site (104) after digestion with Asp-N are shown in Supplementary Table 1 and Supplementary Figs 4-6.

## Phosphatase activity on cAb-Lys3-pCys104




***Human Placental Alkaline Phosphatase (huPLAP, Sigma)***

A solution of huPLAP (1 mg, 1.3 U) in 50 µL 50 mM Tris-HCl pH 7.4 was prepared. 17 µL of the enzyme solution (0.44 U) was added to cAb-Lys3-pCys104 (150 µL, 4.6 nmol, c = 0.45 mg/mL in 50 mM Tris-HCl pH 7.4), and the reaction shaken at 37 °C (600 rpm). After 2 hours, LC-MS analysis revealed full conversion to regenerate cAb-Lys3-Cys104 (calc. mass 15797; obs. mass 15800, Supplementary Fig. 8).

***Human acid phosphatase-1 (Human acp-1, Source BioScience)***

To a solution of cAb-Lys3-pCys104 (140 μL, 4.4 nmol, c = 0.48 mg/mL in 20 mM MES pH 6.0) was added human acp-1 (1 μL, 80 U). After 2.5 hours at 37 °C (600 rpm), LC-MS analysis revealed full conversion to regenerated cAb-Lys3-Cys104 (calc. mass 15797; obs. mass 15798, Supplementary Fig. 9).

***Alkaline phosphatase (alp) - bovine intestinal mucosa phosphatase (bIMP, Sigma)***

To a solution of cAb-Lys3-pCys104 (140 μL, 4.4 nmol, c = 0.48 mg/mL in 10 mM PBS pH 9.8) was added bIMP (1.5 μL, 209 U). After 20 minutes at 37 °C (600 rpm), LC-MS analysis revealed full conversion to regenerated cAb-Lys3-Cys104 (calc. mass 15797; obs. mass 15796, Supplementary Fig. 10).

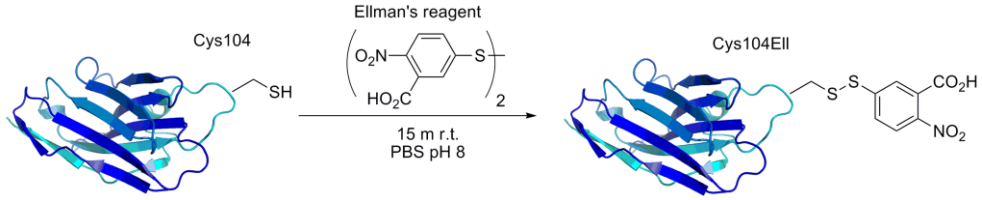
***λ-Protein Phosphatase (λ-ppase, New England Biolabs)***

cAb-Lys3-pCys104 (150 μL, 4.6 nmol, c = 0.48 mg/mL in 50 mM Tris-HCl pH 7.4) was concentrated down to 40 μL (Vivaspin, 5KDa MWCO, Sartorius Stedim), and then desalted into 50 mM Tris-HCl pH 7.4 using a Micro Bio-Spin 6 column (Bio-Rad Laboratories). To the protein solution was added λ-ppase (1 μL, 400 U, Sigma), 10x λ-ppase buffer (5 μL) and 2 mM $MnCl_2$ (5 μL, 20 mM). After 10 minutes at 30 °C (600 rpm), the reaction was analysed by LC-MS, and revealed full conversion to regenerated cAb-Lys3-Cys104 (calc. mass 15797; obs. mass 15801, Supplementary Fig. 11).

***Acid phosphatase (acp) from potato (Sigma)***

A solution of acp from potato (1 mg, 3 U) in 50 μL 10 mM MES pH 4.8 was prepared. 5 μL of the enzyme solution (0.3 U) was added to cAb-Lys3-pCys104 (150 μL, 4.6 nmol, c = 0.45 mg/mL in 10 mM MES pH 4.8), and the reaction shaken at 37 °C (600 rpm). After 2 hours, LC-MS analysis revealed full conversion to regenerated cAb-Lys3-Cys104 (calc. mass 15797; obs. mass 15793, Supplementary Fig. 12).

**Testing of cAb-Lys3-Cys104 with Ellman's Reagent**




A stock solution of Ellman's reagent was prepared by dissolving 0.6 mg in 108 µL 10 mM PBS, pH 8.0. A 75 µL aliquot of regenerated cAb-Lys3-Cys104 (c = 0.5 mg/mL in 10 mM PBS, pH 8.0) was diluted with 75 µL 10 mM PBS, pH 8.0. A 10 µL aliquot of the Ellman's reagent stock solution was added to the diluted protein. The solution was shaken at room temperature for 15 minutes. After this time, the reaction was analysed directly by LC-MS, and revealed full conversion to the Ellman's adduct, confirming consumption of the thiol (calc. mass 15994; obs. mass 15992). ESI-MS are shown in Supplementary Fig. 13.

**Comparative phosphatase activity.**

Each of the phosphatases' ability to cleave the thiophosphate group from cAb-Lys3 were compared directly by conducting reactions under identical conditions with a low amount of enzyme equivalents. Physiological pH was chosen. Accordingly, enzyme solutions were prepared (2.5 – 13.9 U in 50 µL 50 mM Tris-HCl pH 7.4) and the volume corresponding to 0.17 U added to cAb-Lys3-pCys104 (64 µL, 1.7 nmol, c = 0.42 mg/mL in 50 mM Tris-HCl pH 7.4). Reactions were shaken at 37 °C (600 rpm), and monitored over 4 hours. Conversion to product was extrapolated by measuring the relative intensities of peaks obtained in the MS. See Supplementary Figs 15-20.

**Cell Culture.** Lysozyme (pIMMs2011LysD52S) under the CMV (Cytomegalovirus) promoter was obtained from H. Finney (UCB). CMV-SEAP in pBluescript was obtained from Addgene (plasmid 24595). Human Embryonic Kidney (HEK293T) cells were cultured in Dulbecco's modified Eagle Medium (DMEM, Life Technologies) supplemented with 10 % (v/v) FBS (Sigma), 50 U/mL penicillin, 50 µg/mL streptomycin (Sigma) and 100 µg/mL Normocin™ (Invivogen) in a T25 flask (Greiner Bio-One). HEK-Blue™ Null1 cells (HEK-Blue Null1 (NF-κB-*seap*+), Invivogen) were cultured in DMEM supplemented with 10 %

(v/v) FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL Normocin™ and 100 μg/mL Zeocin™ (Invivogen) in a T25 flask. All cells were maintained at 37 °C under 5 % $CO_2$ with passaging every 3 – 4 days. Xfect™ transfection reagent was purchased from Clontech, PentaHis™ Alexa Fluor® 488 from Qiagen, recombinant human TNF-α from Invivogen and recombinant mouse TNF-α from eBioscience.

Xfect-mediated transfection of HEK293T cells with lysozyme and SEAP was carried out according to the manufacturers' protocol. Accordingly, one day prior to transfection, cells were plated in 500 μL complete growth medium (in each well of a 12-well plate) to reach 50 – 70 % confluency. After 24 hours, plasmid (2.5 μg lysozyme or SEAP, in the case of co-transfections, 1.2 μg of each plasmid was used) was diluted with Xfect reaction buffer to a final volume of 50 μL and vortexed. Xfect Polymer was added to the diluted plasmid DNA (0.3 μL for every 1 μg plasmid) and vortexed. The polymer-plasmid solutions were incubated at room temperature for 10 minutes to allow nanoparticle complexes to form. 50 μL of the nanoparticle complex solution was added dropwise to the cell culture medium (per well). Plates were rocked gently back and forth, and then incubated at 37 °C for 4 hours. After this time, cell culture medium was replaced with 1 mL of fresh complete growth medium, and returned to 37 °C for approximately a further 40 hours. For the induction of SEAP expression in HEK-Blue Null1-*seap*+ cells, TNF-α was added to relevant wells at 100 ng/mL at approximately 24 hours into expression. Antibody (WT-cAb-Lys3 or cAb-Lys3-pCys104) was added to relevant wells at 1 μM at approximately 28 hours into expression. For SEAP detection, 18 μL test medium (containing 10 % (v/v) heat-inactivated FBS) was added to wells of a 96-well plate, with 2 μL cell supernatant. 180 μL QUANTI-Blue™ (chromophoric phosphatase substrate) was added, and the plate incubated at 37 °C for 1 – 3 hours, after which SEAP activity could be observed by the naked eye (pink – purple), and quantitatively determined using a spectrophotometer at 620 nm. After expression (~ 44 hours), cells were washed three times with 500 μL of washing buffer (PBS + 2 % FBS) and incubated at room temperature with PentaHis™ Alexa Fluor® 488 (0.5 μg/mL) for 30 minutes. Cells were washed three more times with washing buffer, and then analysed by flow cytometry and microscopy.

***Ex vivo* Experiments** Mouse serum, TWEEN® 20, a PAP pen for immunostaining and formalin solution were purchased from Sigma. Vectashield HardSet mounting medium with DAPI was obtained from Vector Laboratories.

HEK293T cells were transfected in a 12-well plate with varying combinations of inputs as described above (using pIMMs2011LysD52S (*lys*) and SEAP in pBluescript (*seap*)). 48 hours after transfection, media was removed. A sample was taken for confirmation of SEAP expression (as page S22, **Supplementary Figure 27a.**). Cells were washed three times with DMEM (no additives) and lifted with 500 μL DMEM and counted. An aliquot of $10^4$ cells was taken to test for lysozyme expression (as page S22 and S23, **Supplementary Figure 27b.**), and a sample containing $10^6$ cells was spun down (5 mins, 1.2 krpm). Supernatant was removed, and the cell pellet resuspended in 2 μL DMEM.

**Stereotaxic Surgery** Eight-week-old male C57BL/6 mice (Harlan, UK) were allowed to acclimatize for 7 days prior to injection. All surgical procedures were performed under an operating microscope (Wild M650, Leica, Milton Keynes, UK) with UK Home Office and local ethical approval. Animals were anaesthetised with isoflurane (Rhodia Organique Fine Ltd, Bristol, UK) for induction and maintenance at 2.5–3 % in oxygen. Stereotaxic surgery was performed as described previously[3] using a Hamilton syringe with a 30FG needle for the injection of the cells. Briefly, anaesthetised mice were held in a stereotaxic frame, a burr hole was drilled in the skull and a 2 μL volume of the cell suspension was unilaterally microinjected into the left striatum through a glass microcapillary needle over a period of 5 minutes. The stereotaxic co-ordinates were: bregma +0.5 mm, lateral 1.8 mm, and the cells were injected from a rising depth of 2.5-1.5 mm. During the surgical procedures, body temperature was maintained on a heated blanket throughout the period of anaesthesia, and the animals were allowed to recover in a heated chamber. Mice were killed after 24 hours by deep anaesthetic with sodium pentobarbitone and transcardial perfusion with cold saline (4 °C). Brains were frontally cut into 10 μm thick cryostat sections through the injection site onto RNAse free Super Frost Plus slides (Thermo Scientific, Germany).

Tissue sections on slides were removed from -20 °C and 50 μL blocking buffer (50 mM Tris-HCl pH 7.4, 2% mouse serum, 0.01% TWEEN® 20) was added to immerse each tissue slice in a well constructed with a PAP pen for immunostaining. Blocking was allowed to occur for 10 minutes at room temperature after which it was replaced with antibody solution (1 μM in

blocking buffer). Slide-mounted tissue was incubated at 37 °C 5% $CO_2$ in a humidity chamber for 2 hours. As a positive control, WT-cAb-Lys3 was added to a single tissue section injected with HEK293T-*lys*+ cells (**Supplementary Figure 28**). Slides were washed to remove excess antibody by dipping in a beaker of 50 mM Tris-HCl pH 7.4 ten times and then each tissue slice fixed with 50 μL 4% formaldehyde for 5 minutes at room temperature, and then washed three times. Blocking was repeated for 10 minutes at room temperature. PentaHis™ Alexa Fluor® 488 in blocking buffer (0.5 μg/mL) was added to each slice in the humidity chamber and staining was allowed to occur for 1 hour at room temperature. Again, slides were dipped ten times into 50 mM Tris-HCl pH 7.4 to remove excess antibody. Excess liquid was removed from the slides with a medical wipe, each slice was covered with a coverslip containing one drop of mounting medium (with DAPI) and allowed to harden overnight at 4 °C. Immunostained sections were tested for fluorescence by microscopy (**Supplementary Figure 29**).

For *ex vivo* experiments involving the injection of HEK-Blue Null1 cells, cells were either transfected with lysozyme (using pIMMs2011LysD52S (*lys*) as described above) or cultured in the absence of any transfection reagent. 48 hours after transfection, media was removed. A sample was taken for confirmation of SEAP expression inducible by mouse TNF-α (as described above, **Supplementary Figure 30a.**). Cells were washed three times with DMEM (no additives) and lifted with 500 μL DMEM and counted. An aliquot of $10^4$ cells was taken to test for lysozyme expression (as described above, **Supplementary Figure 30b.**), and a sample containing $10^6$ cells was spun down (5 mins, 1.2 krpm). Supernatant was removed, and the cell pellet resuspended in 2 μL DMEM.

Stereotaxic surgery was performed as described on page S26, but in this instance 0.5 μl (10 μg/μl) LPS (*E. coli* 026:B6, Sigma-Aldrich) or saline vehicle was injected stereotaxically via finely-drawn glass microcapillaries as previously described[4] 12 hours prior the microinjection of the cells. The microinjection of LPS induces TNF-α expression in the brain,[5] which is an inducer of SEAP. Thus the presence of LPS induced inflammation provides the AND for the logic gate. The mice were culled 24 hours after the injection of cells and the brains were removed, frozen and 10 μm-thick cryosections from the injection site were mounted on poly-L-lysine coated glass slides. The sections were processed with antibody as described on pages S26 and S27. As a positive control, WT-cAb-Lys3 was added to a single tissue section

injected with HEK-Blue Null1-*lys*+ cells (**Supplementary Figure 31**) as described on page S27.

**Tests with plasma.** Blood was withdrawn from adult C57BL/6 mice by cardiac puncture under terminal anaesthesia with isoflurane and collected in heparin-containing paediatric blood tubes. The blood was centrifuged at 13 krpm for 5 minutes to generate plasma. cAb-Lys3-pCys104 (6.3 μL, stock 1.28 mg/mL) was added to plasma (93.7 μL, 5 μM final antibody concentration) and the solution incubated at 37 °C. Samples were taken at 15 minutes, 2 hours and 24 hours. Samples were diluted with binding buffer (50 mM $KH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8), and then added to HIS-Select® Cobalt Affinity Gel in a 1.5 mL plastic tube. The gel-plasma solutions were incubated at 4 °C for 1 hour, and then washed with 2 x 200 μL binding buffer by centrifugation (10 mins, 14 kprm), and eluted with 3 x 200 μL elution buffer (50 mM $KH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8) by centrifugation. Samples from washes and elutions were analysed by SDS-PAGE and LC-MS. Trace amounts of cAb-Lys3-pCys104 were found in eluted samples, but no dephosphorylated cAb-Lys3-Cys104. See Supplementary Fig. 33.

**Phosphatase activity in organ tissue.** Adult C57BL/6 mice were intracardially perfused with saline under terminal anaesthesia. Organs were quickly harvested and stored at 4ºC. Between 35 and 160 mg of each organ tissue was homogenised with 300 μL diethanolamine, then centrifuged (2 x 20 minutes, 14 krpm). For each organ tissue homogenate, the resulting supernatant was divided equally over 12 wells of a 96-well plate. *para*-Nitrophenylphosphate was added to each tissue homogenate set at concentrations of 500, 450, 400, 350, 300, 250, 100, 75, 50, 20 and 0 μM in adjacent wells. Phosphatase activity of tissue was tested by the detection of the formation of *para*-nitrophenol by taking absorbance measurements at 405 nm every 20 seconds for 3 hours at 37 °C. The number of moles of product formed per minute per milligram of wet tissue was calculated by comparison to standard curves obtained by incubating *para*-nitrophenol (hydrolysed product) under the same conditions as tissue samples. For the kidney, a Bradford assay gave 13.6mg/ml protein. This equates to 2209.2 pmoles/min/mg phosphatase activity. The activity of a range of commercial phosphatases on cAb-Lys3-pCys104 dephosphorylation were assayed (see Supplementary Fig. 15) and the calculated phosphatase activities were 58.0 pmoles/min/mg for acid phosphatase from potato,

306.9 pmoles/min/mg for human PLAP, 1168.8 pmoles/min/mg for human acp-1, 7083.0 pmoles/min/mg for λ–ppase, 2529.8 nmoles/min/mg for bIMP. The tissue activities lie within this range and suggest that endogenous phosphatase activity would be sufficient if the AND requirements are met for alternatively targeted phosphorylated antibodies. See Supplementary Figs 36, 37.

## Supplementary References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