Supporting Information for

IgG-engineered protective antigen for cytosolic delivery of proteins into cancer cells

Zeyu Lu,† Nicholas L. Truex,† Mariane B. Melo, Yiran Cheng, Na Li, Darrell J. Irvine, and Bradley L. Pentelute*

1Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

2The Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 500 Main Street, Cambridge, Massachusetts 02142, USA.

3Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

4Department of Materials Science and Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

5Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

6Howard Hughes Medical Institute, 4000 Jones Bridge Rd, Chevy Chase, Maryland 20815, USA.

7Center for Environmental Health Sciences, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

8Broad Institute of MIT and Harvard, 415 Main Street, Cambridge, Massachusetts 02142, USA.

†These authors contributed equally to this work.

*To whom correspondence should be addressed: blp@mit.edu
I. **SUPPLEMENTAL TABLES**

**Table S1.** Amino acid sequences of mPAC and mPAC [F427A] mutants. S5

**Table S2.** Amino acid sequences of LC and HC fragments for Tmab. S6

**Table S3.** Amino acid sequences of LC and HC fragments for Cmab. S7

**Table S4.** Amino acid sequences of mPAC-uPA and mPAC-MMP mutants. S8

II. **SUPPLEMENTAL FIGURES**

**Fig. S1.** SDS-PAGE analysis of mPAC and mPAC [F427A]. S9

**Fig. S2.** LC/MS analysis of mPAC and mPAC [F427A]. S10

**Fig. S3.** LC/MS analysis of Tmab-LPSTGGK and Cmab-LPSTGGK. S11

**Fig. S4.** LC/MS analysis of the purified linker peptides 1, 2, and 3. S12

**Fig. S5.** Mass spectrometry analysis of G3-mPAC with peptides 1, 2, and 3. S13

**Fig. S6.** SDS-PAGE analysis of Cmab-mPAC. S14

**Fig. S7.** SDS-PAGE analysis of pore formation by Tmab-mPAC. S15

**Fig. S8.** SDS-PAGE analysis of serum stability for Tmab-mPAC when prepared with linker peptides 1 and 2. S16

**Fig. S9.** Translocation activity against HER2-positive cells (BT474) of Tmab-mPAC, when prepared with linker peptides 1, 2, and 3. S17

**Fig. S10.** Translocation activity against HER2-positive cells (BT474) for Tmab-mPAC vs. Tmab-(mPAC)2. S18

**Fig. S11.** SDS-PAGE analysis of Tmab-LF\textsubscript{N}-DTA conjugate. S19

**Fig. S12.** WT PA translocation activity against BT549 and Jurkat cells. S20

**Fig. S13.** Tmab-mPAC activity against HER2-positive EGFR-negative (BT474) and EGFR-positive HER2-negative (A431) cells. S21

**Fig. S14.** Tmab-mPAC activity against HER2-positive EGFR-negative (BT474) and EGFR-positive HER2-negative (A431) cells. S21
Fig. S15. Translocation activity against HER2-negative EGFR-positive cells (A549 and HCT-116) for Tmab-mPAC and Cmab-mPAC.

Fig. S16. SDS-PAGE analysis of mPAC-uPA and mPAC-MMP.

Fig. S17. LC/MS analysis of mPAC-uPA and mPAC-MMP.

Fig. S18. LC/MS analysis of G3-mPAC-uPA and G3-mPAC-MMP.

Fig. S19. SDS-PAGE analysis of Cmab-mPAC-uPA.

Fig. S20. SDS-PAGE analysis of Cmab-mPAC-MMP.

Fig. S21. Characterization of Cmab-mPAC-uPA and Cmab-mPAC-MMP.

Fig. S22. Whole-body animal PET images showing biodistribution of mPAC-uPA, Cmab, and Cmab-mPAC-uPA.

Fig. S23. Tissue distribution comparison of Cmab and Cmab-mPAC-uPA.

Fig. S24. Tissue distribution of mPAC-uPA at 24 h post injection.

Fig. S25. Histological analysis of kidney and liver tissue from mice.

Fig. S26. Extended in vivo analysis of administering Cmab-mPAC variants long term.

Fig. S27. Extended treatments with Cmab-mPAC do not promote inflammation.

Fig. S28. Extended treatments with Cmab-mPAC do not promote signs of liver toxicity, based on aspartate transaminase (AST) and alanine transaminase (ALT) activity.

Fig. S29. Extended treatments with Cmab-mPAC do not promote tissue damage, based on histological analysis of liver, lung, heart, kidney, and spleen.
### III. MATERIALS AND METHODS

| Materials                                                                 | S37 |
|--------------------------------------------------------------------------|-----|
| Synthesis and purification of peptides                                   | S37 |
| Plasmid construction of PA and IgG mutants                              | S38 |
| Expression and purification of PA mutants, EF, and LFN-RRSP             | S38 |
| Expression and purification of Trastuzumab and Cetuximab                | S39 |
| Conjugation of mPAC to IgG antibodies                                   | S39 |
| Evaluation of serum stability                                            | S39 |
| Furin, uPA, and MMP-9 cleavage analysis of Cmab-mPAC variants            | S40 |
| Cell culture                                                             | S40 |
| Cell viability analysis                                                  | S40 |
| cAMP assay                                                               | S41 |
| Western blot analysis                                                    | S41 |
| Animal work                                                              | S42 |
| Endotoxin Testing and Removal                                            | S42 |
| Pharmacokinetics and Biodistribution analysis                            | S43 |
| Histological analysis                                                    | S44 |
| Inflammation analysis                                                    | S45 |
| Alanine Aminotransferase (ALT) Activity Assay                           | S46 |
| Aspartate Aminotransferase (AST) Activity Assay                         | S47 |

### IV. REFERENCES

| S48 |
## I. Supplemental Tables

### Table S1. Amino acid sequences of mPAC and mPAC [F427A] mutants.

| Name         | Mutations         | Amino acid sequence¹ ² |
|--------------|-------------------|------------------------|
| mPAC         | N682A, D683A, K563C | MEVKQENRRLLNESSSSQGLLGYFSDNFLFQAPMVVTSTTGDLSPSSELENIPSENQYFQSAIW  |
|              |                   | SGFIKVKSDEYTFATSADNHVTMVDDQEVINKASNSNKRLKEGRLYQIKIYQRENPTKGL      |
|              |                   | DFKLYWTDQNKKEVSSDDNQLPELQKQLSNRAKKRSTSAQTPVDRDNGIPDSLVEGEYTV     |
|              |                   | DVKKRTFLSPWNIHEKKGKTKSSPKWSTASDPYSDFEKVTGRIDKNSPEARHPJAVAAY       |
|              |                   | PIVHVDMMIISSKNEQDSQTNDSETRTIKNTSTSRTHSTSEVHNGAEVHASSFIDGGSVAGFSN  |
|              |                   | SNSTTVADHSLSLAGERTWAETMGLNTADTNARLANIRYVNTGAPIYYNLPTTSLLVGKRNQTL |
|              |                   | ATIKAKENQLSEQILAPNLYYPSKLANPALNAQDDGSSSTPIITMNVQFLELTKQRLEDTVQV  |
|              |                   | GNIATYNFENGRVVRVDTSNWSYELPQIEQTTARIIFNGKDLNLVERIAAVPSDPLETTKPDMT |
|              |                   | LKEALKIAFGFNEPNQGKDIETDFNDQQTSEQNLCN pinELNATINIVTLDKIKLNAKM    |
|              |                   | NILIRDKRFHYDRNNAVGADSEVSKEAHREVINSSTENGLLNLKDIRHLSGYIYEIDTEGLKE   |
|              |                   | VINDRYDMLNISLSRQDGKTFIDFKYAAKLPLYSNPYNVKVNYAVTKENTINIPSENGDSTNG  |
|              |                   | IKKILFSSKGYEIG                                                  |
| mPAC [F427A] | N682A, D683A, K563C | MEVKQENRRLLNESSSSQGLLGYFSDNFLFQAPMVVTSTTGDLSPSSELENIPSENQYFQSAIW  |
|              |                   | SGFIKVKSDEYTFATSADNHVTMVDDQEVINKASNSNKRLKEGRLYQIKIYQRENPTKGL      |
|              |                   | DFKLYWTDQNKKEVSSDDNQLPELQKQLSNRAKKRSTSAQTPVDRDNGIPDSLVEGEYTV     |
|              |                   | DVKKRTFLSPWNIHEKKGKTKSSPKWSTASDPYSDFEKVTGRIDKNSPEARHPJAVAAY       |
|              |                   | PIVHVDMMIISSKNEQDSQTNDSETRTIKNTSTSRTHSTSEVHNGAEVHASSFIDGGSVAGFSN  |
|              |                   | SNSTTVADHSLSLAGERTWAETMGLNTADTNARLANIRYVNTGAPIYYNLPTTSLLVGKRNQTL |
|              |                   | ATIKAKENQLSEQILAPNLYYPSKLANPALNAQDDGSSSTPIITMNVQFLELTKQRLEDTVQV  |
|              |                   | GNIATYNFENGRVVRVDTSNWSYELPQIEQTTARIIFNGKDLNLVERIAAVPSDPLETTKPDMT |
|              |                   | LKEALKIAFGFNEPNQGKDIETDFNDQQTSEQNLCN pinELNATINIVTLDKIKLNAKM    |
|              |                   | NILIRDKRFHYDRNNAVGADSEVSKEAHREVINSSTENGLLNLKDIRHLSGYIYEIDTEGLKE   |
|              |                   | VINDRYDMLNISLSRQDGKTFIDFKYAAKLPLYSNPYNVKVNYAVTKENTINIPSENGDSTNG  |
|              |                   | IKKILFSSKGYEIG                                                  |

¹Mutated residues are indicated by bold text with one line underneath, as shown here.

²The Furin cleavage site is indicated by bold text with two lines underneath, as shown here.
Table S2. Amino acid sequences of LC and HC fragments for Tmab.

| Name         | Fragment | Amino acid sequence                                                                 |
|--------------|----------|-------------------------------------------------------------------------------------|
| Tmab         | LC       | ADIQMTQSPSSLSASVGDRVITCRASQDVNTAVAWYQQPKGAPKLLILYASASFLYSVGVPSTSFSGSRSFGTXTLSSQPEFATYCCQHYTTPFGQGTKVEIKRTVAAPSVFIPPSDEQLKSATSVVCLLNNFYPREAVQYKVDNALQSGNSQVEQDSKADSTLKLADYKHKVYACEVTIQGLSSPVTKSFGEC |
|              | HC       | EVQLVESGGGLVPGGLSRLSCLASGQNIKTDTIHWVRQAPKGSLEWVARQITPNYTRYADSVKGRFTISADTSKNTAYLMNSLAEDTAVYCASRGGDFGAMDYWGQTLTVSASSKTGPVFPLAPSSKSTSGTAALGCLKDYFEPEPVTVSWNSGALTSGHVTFFPVLQSSGLYLSSVTVYPSLGLGTQYICIVHKPSNKVDKKVEPKSDCDKHTCPPCPAPELLGGPSVFLFPPKDTLLMSRTPEVTCVVDVEDPEVKFNYWDGVEVHNAKTTPREEQNYSNTYRVSIVSLTTLQHDWNLGKEYCKCVSNAKLPAIEKTISAKGQPREPVYTLPPRSLTNQVSLTCVKGYPSDIAYELESNGQVENNYKTTPVLDGFSFLYKLTVDKRSWQGNSCGSVMEALHNHYTQKSLSLSPGK |
| Tmab-LPSTGGK | LC       | ADIQMTQSPSSLSASVGDRVITCRASQDVNTAVAWYQQPKGAPKLLILYASASFLYSVGVPSTSFSGSRSFGTXTLSSQPEFATYCCQHYTTPFGQGTKVEIKRTVAAPSVFIPPSDEQLKSATSVVCLLNNFYPREAVQYKVDNALQSGNSQVEQDSKADSTLKLADYKHKVYACEVTIQGLSSPVTKSFGEC |
|              | HC-LPSTGGK | EVQLVESGGGLVPGGLSRLSCLASGQNIKTDTIHWVRQAPKGSLEWVARQITPNYTRYADSVKGRFTISADTSKNTAYLMNSLAEDTAVYCASRGGDFGAMDYWGQTLTVSASSKTGPVFPLAPSSKSTSGTAALGCLKDYFEPEPVTVSWNSGALTSGHVTFFPVLQSSGLYLSSVTVYPSLGLGTQYICIVHKPSNKVDKKVEPKSDCDKHTCPPCPAPELLGGPSVFLFPPKDTLLMSRTPEVTCVVDVEDPEVKFNYWDGVEVHNAKTTPREEQNYSNTYRVSIVSLTTLQHDWNLGKEYCKCVSNAKLPAIEKTISAKGQPREPVYTLPPRSLTNQVSLTCVKGYPSDIAYELESNGQVENNYKTTPVLDGFSFLYKLTVDKRSWQGNSCGSVMEALHNHYTQKSLSLSPGK |

1Mutated residues are indicated by bold text with one line underneath, as shown here.
Table S3. Amino acid sequences of LC and HC fragments for Cmab.

| Name               | Fragment | Amino acid sequence<sup>1</sup> |
|--------------------|----------|----------------------------------|
| Cmab               | LC       | DILLTQPVLISVSPGERVSFSGRASQQTIGTNIHWYQRTNGSRFRLLIKYASEGISGIPSFSGSGGSFGTDFTLSINESEDIADYYCQMNNNWPFTTFAAPTSAVGFPVFIPPSDEQLSGTASVVCLLNFFYPEAKVQWKVDNALQSGSQQESVTEQDSKDTSTSSSTLTLTSDKARYEHKVKYACEVTHQGLSSPVTKSFNRGEC |
| Cmab               | HC       | QVQLKQSGPLVQPSQLSLITCTSVGSLTNYGVHWQPSGRGKLEYWGLVIGWGGTDYNTPTSFRLSINKDNSKSVQFFKMNSLSQNNTSIYCARALTYDYEAYWGGTFLVTSAASTKGPSVFPLAPSKSTSGTAALGCLVDYFPEPVTQWSWNSGALTSGVHTFAPLVQSSGLYLSSTVTPSSSLGTVQYCNHPSNKTVKKEPSCDKTHCPCPAPELLLGSPVFLPPPKDPDTLMISRTPEVTCCVVDYFSHDEPVKFNWYVGGVEVHNAKTKFPEEQYNSTRYVSVLTLHQDVLWNGKEYKCKVSNKALPAPIKTISKAGGPQREPQQYTVLPSREDLTKQVSLTCLVKGYPDSIAVEWESNGQPENNYKTTPVLDSDLGFLYSLKLTVDSRQWQNVFSCSVMHEALHNHYTQKSLSLPSGK |
| Cmab-LPSTGGK      | LC       | DILLTQPVLISVSPGERVSFSGRASQQTIGTNIHWYQRTNGSRFRLLIKYASEGISGIPSFSGSGGSFGTDFTLSINESEDIADYYCQMNNNWPFTTFAAPTSAVGFPVFIPPSDEQLSGTASVVCLLNFFYPEAKVQWKVDNALQSGSQQESVTEQDSKDTSTSSSTLTLTSDKARYEHKVKYACEVTHQGLSSPVTKSFNRGEC |
| Cmab-LPSTGGK      | HC       | QVQLKQSGPLVQPSQLSLITCTSVGSLTNYGVHWQPSGRGKLEYWGLVIGWGGTDYNTPTSFRLSINKDNSKSVQFFKMNSLSQNNTSIYCARALTYDYEAYWGGTFLVTSAASTKGPSVFPLAPSKSTSGTAALGCLVDYFPEPVTQWSWNSGALTSGVHTFAPLVQSSGLYLSSTVTPSSSLGTVQYCNHPSNKTVKKEPSCDKTHCPCPAPELLLGSPVFLPPPKDPDTLMISRTPEVTCCVVDYFSHDEPVKFNWYVGGVEVHNAKTKFPEEQYNSTRYVSVLTLHQDVLWNGKEYKCKVSNKALPAPIKTISKAGGPQREPQQYTVLPSREDLTKQVSLTCLVKGYPDSIAVEWESNGQPENNYKTTPVLDSDLGFLYSLKLTVDSRQWQNVFSCSVMHEALHNHYTQKSLSLPSGK |

<sup>1</sup>Mutated residues are indicated by bold text with one line underneath, as shown here.
Table S4. Amino acid sequences of mPAC-uPA and mPAC-MMP mutants.

| Name   | Mutations | Amino acid sequence |
|--------|-----------|---------------------|
| mPAC-uPA | N682A, D683A, K563C | MEVKQENRLLNESESSSQQGLLGYFSDLNQAPMVVTSSSGSLESNPGSSRSASTGVTVDKLYWTDQNKKEVISSLNQLPGKKSQDMPSLPWNSIHIEKKGLYKSSPEKWTASDPSYDFEKVTRIDKNSHEAPRLVAAYPIVHVDMENIIILSKNEDQSTQNTDSETRTKNTSTSTRTHTSEVHNAGNVEHHASDFDIGGSPAGFSNNSSTVAIDHSLAGERTWAETMGNLNTADTKLANIRYTVNTGDATIPIYVLPTSLVGLKNOQTUATLAQKNSQILAPNNYPSKLAPLIALNAQDDDFSTPITMNYNFLEETKQQLRLDTDQVYGNIAIYTFENGVRVDGTSGNWEVLQPIQETTARIIFNGKDLNLVERAAVNPDSPLEETKPDMLKEALKIAFGNEPNGNLQYQKIDTEFDNFDQTSQNTQCNQALENATNYTVLDDKLNAMCNILIRDKFHYDRLNIAVGADESVKEAHREVSSTEGLLLIDKIRKLSGYIEEIDTEGLKEVINDRYDLMNSSLRQDGKTFIDKKYAAKLPLYISNPYKVNYAVTKENTIINPSENGDTSTNGIKKILFSKGGYIEG |
| mPAC-MMP | N682A, D683A, K563C | MEVKQERNLLESESSSQQGLLGYFSDLNQAPMVVTSSSGSLESNQGPGSSRSASTGVTVDKLYWTDQNKKEVISSLNQLPGKKSQDMPSLPWNSIHIEKKGLYKSSPEKWTASDPSYDFEKVTRIDKNSHEAPRLVAAYPIVHVDMENIIILSKNEDQSTQNTDSETRTKNTSTSTRTHTSEVHNAGNVEHHASDFDIGGSPAGFSNNSSTVAIDHSLAGERTWAETMGNLNTADTKLANIRYTVNTGDATIPIYVLPTSLVGLKNOQTUATLAQKNSQILAPNNYPSKLAPLIALNAQDDDFSTPITMNYNFLEETKQQLRLDTDQVYGNIAIYTFENGVRVDGTSGNWEVLQPIQETTARIIFNGKDLNLVERAAVNPDSPLEETKPDMLKEALKIAFGNEPNGNLQYQKIDTEFDNFDQTSQNTQCNQALENATNYTVLDDKLNAMCNILIRDKFHYDRLNIAVGADESVKEAHREVSSTEGLLLIDKIRKLSGYIEEIDTEGLKEVINDRYDLMNSSLRQDGKTFIDKKYAAKLPLYISNPYKVNYAVTKENTIINPSENGDTSTNGIKKILFSKGGYIEG |

1Mutated residues are indicated by bold text with one line underneath, as shown here.
2The uPA and MMP cleavage sites are indicated by bold text with two lines underneath, as shown here.
II. SUPPLEMENTAL FIGURES

![SDS-PAGE analysis of mPAC and mPAC [F427A]](image)

**Figure S1. SDS-PAGE analysis of mPAC and mPAC [F427A].** Coomassie-visualized SDS-PAGE gel analysis of fractions from preparative anion exchange chromatography for (top) mPAC and (bottom) mPAC [F427A]. Lane 2 shows the supernatant collected after resuspending the pellet in sucrose buffer (Suc). Lane 3 shows the supernatant collected after extracting protein from the pellet with Mg buffer (Mg).
Figure S2. LC/MS analysis of mPAC and mPAC [F427A]. Each protein (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH$_3$CN in H$_2$O with 0.1% FA and a flow rate of 0.8 mL/min. The proteins were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. The TIC peak was integrated and the mass was deconvoluted using maximum entropy algorithm.
Figure S3. LC/MS analysis of Tmab-LPSTGGK and Cmab-LPSTGGK. Prior to LC/MS analysis, the IgG light (LC) and heavy (HC) chains were reduced by incubating with 20 mM TCEP in PBS at 37°C for 20 minutes. Each antibody (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH₃CN in H₂O with 0.1% FA and a flow rate of 0.8 mL/min. The antibodies were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. The TIC peak was integrated and the mass was deconvoluted using maximum entropy algorithm. For the HC, the difference between the observed and calculated mass is attributed to post-translational glycosylation.
Figure S4. LC/MS analysis of the purified linker peptides 1, 2, and 3. Each peptide (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH₃CN in H₂O with 0.1% FA and a flow rate of 0.8 mL/min. The peptides were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. D-amino acids are indicated by lowercase letters. Maleimide group is indicated by Me.
Figure S5. **Mass spectrometry analysis of G3-mPAC variants with peptides 1, 2, and 3.** Each protein (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH3CN in H2O with 0.1% FA and a flow rate of 0.8 mL/min. The proteins were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. The TIC peak was integrated and the mass was deconvoluted using maximum entropy algorithm.
Figure S6. SDS-PAGE analysis of Cmab-mPAC. Coomassie-visualized SDS-PAGE gel analysis of Cmab-mPAC prepared with linker peptide 1. Fractions from preparative (top) size exclusion chromatography and (bottom) anion exchange chromatography for Cmab-mPAC.
Figure S7. SDS-PAGE analysis of pore formation by Tmab-mPAC. Coomassie-visualized SDS-PAGE gel analysis of mixtures of Tmab-(mPAC$_{63}$)$_2$ and Tmab-(mPAC$_{63}$)$_1$ after 30 min incubations at pH 7, 6, and 5. Lane 1 shows the initial mixture of Tmab-(mPAC$_{83}$)$_2$ and Tmab-(mPAC$_{83}$)$_1$. Lane 2 shows the mixture used for this pore-formation study of Tmab-(mPAC$_{63}$)$_2$ and Tmab-(mPAC$_{63}$)$_1$ that was prepared by adding trypsin to the mixture of Tmab-(mPAC$_{83}$)$_2$ and Tmab-(mPAC$_{83}$)$_1$, followed by purification with anion exchange chromatography.
Figure S8. SDS-PAGE analysis of serum stability for Tmab-mPAC when prepared with linker peptides 1 and 2. SDS-PAGE gel analysis with Western blot visualization of Tmab-mPAC variants prepared with linker peptides 1 and 2. Both variants were analyzed on the same gel, but were separated for visual clarity. Each lane shows the remaining amounts of Tmab-mPAC after incubation in MEM medium with 10% fetal bovine serum at 37°C for 0, 1, 24, 72, and 168 h. An anti-PA antibody was used to visualize the mPAC protein, which also exhibited non-specific binding to protein from the medium (indicated).
Figure S9. Translocation activity against HER2-positive cells (BT474) of Tmab-mPAC, when prepared with linker peptides 1, 2, and 3. Relative cell viability after 72 h incubations with 10 nM LF_N-DTA, and with ten-fold serial dilutions of an Tmab-mPAC conjugate. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean of three replicate wells ± the standard deviation (± s.d.).
Figure S10. Translocation activity against HER2-positive cells (BT474) for Tmab-mPAC vs. Tmab-(mPAC)$_2$. Relative cell viability after 72 h incubation with 10 nM LF$_N$-DTA, and with ten-fold serial dilutions of a Tmab-mPAC$_n$. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells.
Figure S11. SDS-PAGE analysis of Tmab-LF<sub>N</sub>-DTA conjugate. Coomassie-visualized SDS-PAGE gel analysis of Tmab-LF<sub>N</sub>-DTA fractions from preparative size exclusion chromatography. Tmab-LF<sub>N</sub>-DTA was prepared using sortase-mediated ligation with SrtA* from Tmab-HC-LPSTGGK and G<sub>5</sub>-LF<sub>N</sub>-DTA.
Figure S12. WT PA translocation activity against BT549 and Jurkat cells. Relative cell viability after 72 h incubation with 10 nM LF_N-DTA, and with ten-fold serial dilutions of WT PA. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells.
Figure S13. Tmab-mPAC activity against HER2-negative EGFR-positive (BT474) and EGFR-positive HER2-negative (A431) cells. Relative cell viability after 72 h incubation with the indicated constructs. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells. The curves and EC$_{50}$ values reflect a variable slope sigmoidal model fitted to the data.

Figure S14. Cmab-mPAC activity against HER2-negative EGFR-positive (BT474) and EGFR-positive HER2-negative (A431) cells. Relative cell viability after 72 h incubation with the indicated constructs. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells. The curves and EC$_{50}$ values reflect a variable slope sigmoidal model fitted to the data.
Figure S15. Translocation activity against HER2-negative EGFR-positive cells (A549 and HCT-116) for Tmab-mPAC and Cmab-mPAC. Relative cell viability after 72 h incubation with 10 nM LF_N-DTA, and with ten-fold serial dilutions of either Cmab-mPAC or Tmab-mPAC. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells.
Figure S16. SDS-PAGE analysis of mPAC-uPA and mPAC-MMP. Coomassie-visualized SDS-PAGE gel analysis of fractions from preparative anion exchange chromatography for (top) mPAC-uPA and (bottom) mPAC-MMP. Lane 2 shows the supernatant collected after resuspending the pellet in sucrose buffer (Suc). Lane 3 shows the supernatant collected after extracting protein from the pellet with Mg buffer (Mg).
**Figure S17.** LC/MS analysis of mPAC-uPA and mPAC-MMP. Each protein (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH₃CN in H₂O with 0.1% FA and a flow rate of 0.8 mL/min. The proteins were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. The TIC peak was integrated and the mass was deconvoluted using maximum entropy algorithm.
Figure S18. LC/MS analysis of $G_3$-mPAC-uPA and $G_3$-mPAC-MMP. These proteins were prepared with linker peptide 1. Each protein (200 ng) was loaded onto an Agilent Zorbax 5 μm 300SB-C3 column (2.1 × 150 mm) and was eluted with a gradient of 5–65% CH$_3$CN in H$_2$O with 0.1% FA and a flow rate of 0.8 mL/min. The proteins were detected on an Agilent 6520 ESI-Q-TOF mass spectrometer. The TIC peak was integrated and the mass was deconvoluted using maximum entropy algorithm.
Figure S19. SDS-PAGE analysis of Cmab-mPAC-uPA. Coomassie-visualized SDS-PAGE gel analysis of Cmab-mPAC-uPA fractions that eluted after preparative (top) size exclusion chromatography and (bottom) anion exchange chromatography.
**Figure S20. SDS-PAGE analysis of Cmab-mPAC-MMP.** Coomassie-visualized SDS-PAGE gel analysis of Cmab-mPAC-MMP fractions that eluted after preparative (top) size exclusion chromatography and (bottom) anion exchange chromatography.
Figure S21. Characterization of Cmab-mPAC-uPA and Cmab-mPAC-MMP. (a) SDS-PAGE gel analysis with Western blot visualization of protease-treated Cmab-mPAC, Cmab-mPAC-uPA, and Cmab-mPAC-MMP (indicated as Cmab-uPA and Cmab-MMP). (b) H2030 cells (EGFR positive, uPA/MMP negative) treated with 10 nM LF$_N$-DTA, and with ten-fold serial dilutions of Cmab-mPAC, Cmab-mPAC-uPA, or Cmab-mPAC-MMP. (c) Normal human microvascular endothelial cell line (HMEC-1) cells treated with 10 nM LF$_N$-DTA, and with ten-fold serial dilutions of WT PA, Cmab-mPAC, Cmab-mPAC-uPA, or Cmab-mPAC-MMP. Cell viability was determined by the relative luminescence from a CellTiter-Glo assay, which was normalized to untreated cells. Data represent the mean ± s.d. of three replicate wells.
Figure S22. Whole-body animal PET images showing biodistribution of mPAC-uPA, Cmab, and Cmab-mPAC-uPA. Images were acquired with a PET preclinical imaging system. Animals with mPAC-uPA were only monitored for 24 h due to the rapid clearance.
Figure S23. Tissue distribution comparison between Cmab and Cmab-mPAC-uPA. Radioactivity analysis of animal tissue harvested from individual organs.
Figure S24. Tissue distribution of mPAC-uPA at 24 h post injection. Radioactivity analysis of animal tissue harvested from individual organs.
Figure S25. Histological analysis of kidney and liver tissue from mice. Representative histological images of kidney and liver sections visualized with hematoxylin and eosin (H&E) from female nude mice (n = 3) i.v. administered with 1 mg/kg LF₅-DTA alone or in combination with 3 mg/kg Cmab-mPA-uPA or 3 mg/kg Cmab-mPA-MMP. Animals were monitored over 48 h for outward signs of toxicity, then tissue samples from kidney and liver were subjected to histological analysis.
Figure S26. Extended in vivo analysis of administering Cmab-mPAC variants long term. (A) Protein constructs were i.v. (tail vein) administered six times over three weeks to tumor-free animals (C57BL/6 mice, n = 5). On days 9, 17, and 21, sera samples from peripheral blood were collected and analyzed for inflammatory cytokine levels (Fig. S27); aspartate aminotransferase (AST) activity (Fig. S28); and alanine transaminase (ALT) activity (Fig. S28). On day 21, animals were harvested and processed to analyze toxicity across five major organs: liver, lung, heart, kidney, and spleen. Throughout the entire experiment, animals were monitored for (B) survival and (C) body weight. Body weight measurements were normalized to day 0, and represent the mean ± s.d. of the surviving animals. The asterisk (*) indicates that this group was harvested on day 7, because the surviving animals showed signs of morbidity.
Figure S27. Extended treatments with Cmab-mPAC do not promote inflammation. Mouse sera were analyzed using a luminex panel for measuring relative levels of 13 inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF. Data represent the mean ± s.d. of the surviving animals.
Figure S28. Extended treatments with Cmab-mPAC do not promote signs of liver toxicity. Mouse sera were analyzed for signs of liver toxicity based on levels of (A) AST activity and (B) ALT activity. Data represent the mean ± s.d. of the surviving animals.
Figure S29. Extended treatments with Cmab-mPAC variants do not promote tissue damage. Representative histological images (visualized with H&E) from the surviving groups (indicated), showing mouse liver, lung, heart, kidney, and spleen. Organs were harvested and fixed (formalin) on day 21, followed by embedding in paraffin, sectioning, and staining. The asterisk (*) indicates that this group (mPAC + LFN-DTA) was harvested on day 7.
III. MATERIALS AND METHODS

Materials. Fmoc-protected L- and D-amino acids used for peptide synthesis were purchased from Chem-Impex. All antibodies used for western blots were purchased from Cell Signaling, unless stated otherwise. All media used in cell culture were purchased from Thermo Fisher. Mouse inflammation was analyzed using a LEGENDplex™ Mouse Inflammation Panel (13-plex) from BioLegend (cat. # 740150). Liver function enzymes were measured using Aspartate Aminotransferase Activity Assay (cat. # MAK055-1KT) and Alanine Aminotransferase Activity Assay (cat. # MAK052-1KT) kits that were purchased from Millipore Sigma.

Synthesis and purification of peptides. Linker peptides 1, 2, and 3 were synthesized by solid-phase peptide synthesis on ChemMatrix resin with a Rink amide linker. The first residue, Fmoc-Lys(alloc)-OH, was manually coupled onto the resin, and successive rounds of couplings were completed as previously described.\textsuperscript{1,2} When the synthesis was complete, the alloc group was removed using Pd(PPh\textsubscript{3})\textsubscript{4} and phenyltrihydrosilane (PHSiH\textsubscript{3}).\textsuperscript{3} A maleimide group was coupled on the side chain of L- or D-lysine by incubating with 1.5 equivalents of N-\gamma-maleimidobutyryl-oxysuccinimide ester (GMBS, Thermo Fisher) dissolved in DMF for 3 h. Similarly, a bromoacetic acid was coupled on the side chain of D-Lys by incubating with 0.4 M bromoacetic acid, 0.38 M HATU, and 0.38 M DIEA in DMF for 20 minutes.

The peptides were cleaved with a solution of TFA/H\textsubscript{2}O (97.5:2.5) and purified by semi-preparative RP-HPLC with Agilent Zorbax 300SB C18 column (9.4 x 250 mm, 5 \textmu m) at a flow rate of 4 mL/min using the gradient of 1-31% acetonitrile over 80 min. Pure HPLC fractions were pooled, lyophilized, and analyzed by LC/MS.
**Plasmid construction of PA and IgG mutants.** The plasmids of mPA (N682A, D683A), mPAC (N682A, D683A, K563C), and mPACA (N682A, D683A, K563C, F427A) were prepared from WT PA using QuikChange multi site-directed mutagenesis kit (Agilent). The furin cleavage site RKKR (164–167) in mPAC was replaced by the uPA substrate sequence PGSGRSA or gelatinase substrate sequence GPLGMLSQ to give mPAC-uPA or mPAC-MMP using QuickChange single site-directed mutagenesis kit (Agilent). Using the same kit, the sortase-specific conjugation tag LPSTGG was inserted at the C-terminus of the heavy chain of Tmab and Cmab.

**Expression and purification of PA mutants, EF, and LF<sub>N</sub>-RRSP.** All non-IgG proteins were expressed in E. coli BL21 (DE3) cells from Thermo Fisher. The PA variants were expressed at New England Regional Center of Excellence/Biodefense and Emerging Infectious Diseases (NERCE) and were purified according to previously described methods. After PA expression, the cell pellets were prepared by suspension and incubation in a sucrose buffer (20 mM Tris pH 8.5, 1 mM EDTA, 20% sucrose), followed by suspension and incubation in a 5 mM MgSO<sub>4</sub> buffer. After pelleting the cell lysate, the supernatant was purified by anion exchange chromatography. The fractions were analyzed by SDS-PAGE at room temperature and 165 V for 36 min on an Invitrogen Bolt™ 4-12% Bis-Tris Plus Gel with Bolt™ MES SDS Running Buffer (1x). The gel was visualized by SimplyBlue™ SafeStain (Coomassie) and the clean fractions were pooled and concentrated with Amicon® Ultra-15 Centrifugal Filter Units.

EF and LF<sub>N</sub>-RRSP were expressed in Champion PET-SUMO vector with a His tag in E. coli BL21(DE3) and were induced at OD 0.7–0.9 with 0.4 mM IPTG at 20°C for 16 h. The proteins were purified by a HisTrap FF Ni-NTA column. The SUMO was cleaved by SUMO protease and removed by size-exclusion chromatography.
**Expression and purification of Trastuzumab and Cetuximab.** Tmab and Cmab in gWiz vector were transiently transfected using PEI and expressed in FreeStyle 293-F cells (Thermo Fisher) according to the manufacturer’s protocol. The IgGs were subsequently purified from the medium by Protein A affinity chromatography (Genscript) and stored in PBS at -80°C.

**Conjugation of mPAC to IgG antibodies.** mPAC (400 μM) was incubated with linker peptide 1, 2, or 3 (2 mM) in 20 mM Tris and 150 mM NaCl (pH 8.5). After 1 h, the excess peptide was removed by three rounds of buffer exchange with a 30 kDa Amicon ultra-15 centrifugal filter (Millipore). The resulting G3-mPAC (100 μM) was incubated with IgG-LPSTGGK (40 μM) in the presence of 5 μM of triple mutant sortase (SrtA*) in sortase buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5) for 1 h at room temperature. The reaction mixture was then loaded onto HiLoad 16/600 Superdex 200 pg size exclusion column (GE). The fractions containing IgG-mPAC₁ and IgG-(mPAC)₂ were pooled and purified again by 5-mL HiTrap Q anion exchange column (GE) to give the purified IgG-mPAC₁.

**Evaluation of serum stability.** IgG-mPAC variants were incubated in MEM cell culture medium supplemented with 10% fetal bovine serum (FBS) at 37°C. At 0, 1, 24, 72, and 168 h, aliquots were collected and frozen in liquid nitrogen for storage at -80°C. Upon analysis, the aliquots were thawed on ice, followed by running an SDS-PAGE gel at 165 V for 36 min on an Invitrogen Bolt™ 4-12% Bis-Tris Plus Gel with Bolt™ MES SDS Running Buffer (1x). The gels were visualized by Western blot using an anti-PA antibody (Santa Cruz Biotechnology).
**Furin, uPA, and MMP-9 cleavage analysis of Cmab-mPAC variants.** The Cmab-mPAC, Cmab-mPAC-uPA, and Cmab-mPAC-MMP conjugates were subjected to proteolytic cleavage conditions, as previously described. Cleavage was evaluated by SDS-PAGE gel analysis with Western blot visualization after incubating each conjugate (10 µg) at 37°C and a volume of 40 µL under the following conditions: (1) Furin protease, which was analyzed with 1 µl of furin (>2000 unit/ml, Sigma) in 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1.0 mM CaCl$_2$, and 1.0 mM MgCl$_2$. (2) uPA protease, which was analyzed with 1 µg of uPA (Millipore) in 20 mM Tris·HCl (pH 7.5) and 150 mM NaCl. (3) MMP-9 protease, which was analyzed with 0.2 µg of MMP-9 (Millipore) in 50 mM HEPES (pH 7.5), 10 mM CaCl$_2$, 200 mM NaCl, 0.05% (v/v) Brij-35, and 50 µM ZnSO$_4$.

**Cell culture.** Cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in serum-containing medium at 37°C with 5% CO$_2$. All cells were maintained and passaged according to the manufacturer’s instructions, except MDA-MB-231 cells were grown in DMEM with 10% FBS.

**Cell viability analysis.** Cells were seeded in a 96-well plate at a density of 5×10$^3$ cells per well, then allowed to adhere overnight. Protein treatments were prepared in 10% FBS cell culture medium with 10-fold serial dilutions of protein, either with or without LF$_N$-DTA. The treatments were incubated with the cells for 72 h at 37 °C and 5% CO$_2$. Cell viability was measured by CellTiter-Glo luminescent assay (Promega) by following the manufacturer’s protocol. Relative viability was inferred from the luminescence values, which were normalized to the values from untreated cells.
**cAMP assay.** MDA-MB-231 cells were seeded in a 96-well plate at a density of $1.5 \times 10^3$ cells per well, then allowed to adhere overnight. The protein treatments were prepared in 10% FBS cell culture medium with 100 nM IgG-mPAC and with either 20 nM EF or LF_N-DTA. The treatments were incubated with cells for 2 h at 37 °C and 5% CO$_2$, followed by cell lysis with a solution of 0.1 M HCl and 0.5% Triton X-100. The intracellular cAMP levels were measured with a colorimetric competitive ELISA kit (Enzo Life Science) according to the manufacturer’s instructions.

**Western blot analysis.** MDA-MB-231 cells were plated at $2 \times 10^5$ cells/well in a 12-well plate. After 24 h, the cells were treated with 50 nM LF$_N$-RRSP (LR) in the presence or absence of an IgG-mPAC variant for another 24 h. Subsequently, the medium was removed and the cells were lysed by RIPA buffer (10 mM pH 8.0 Tris-Cl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF) supplemented with Roche protein inhibitor for 20 minutes on ice. The lysate was separated by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad) using a Bio-Rad Trans-Blot turbo transfer system. The membrane was blocked by incubating with LI-COR blocking buffer on an orbital shaker for 1 h at room temperature (RT), followed by incubation with the primary antibodies in TBST buffer and 0.1% Tween overnight at 4°C. The following day, the membrane was washed by TBST (3x), followed by incubation with a fluorescent secondary antibody in TBST on an orbital shaker for 1 h at RT. Blot images were collected on a ChemiDoc MP imaging system (Bio-Rad). Subsequent proteins were detected by stripping the membrane with stripping buffer (Thermo Fisher) and repeating the above staining procedure.
Animal work. All animal work was conducted in accordance with a protocol approved by the MIT Committee on Animal Care (CAC). Female nude (immune compromised) and C57BL/6 mice were used. Animals were anesthetized prior to any procedures by isofluorane inhalation and were euthanized by exposure to CO2, followed by cervical dislocation. While being treated, the mice were monitored daily for clinical signs indicating a moribund condition, which included one or more of the following indicators: (1) impaired ambulation preventing access to food or water, (2) excessive weight loss and emaciation, (3) lack of physical or mental alertness, (4) difficult or labored breathing, (5) inability to remain upright, and (6) skin ulcerations. Animals were euthanized by CO2 asphyxiation if their appearance indicated an unlikely survival until the next scheduled injection/observation.

Endotoxin Testing and Removal. All materials for injection were prepared with < 5 EU/kg (i.e., ~0.4 EU for a 20 g mouse). Endotoxin was measured with single-use cartridges (0.05 EU/mL, PTS2005) for an Endosafe® nexgen-PTS™ (Charles river) reader. Several procedures to remove endotoxin were followed if endotoxin level were > 5 EU/kg (after adjusting for injection amount).

(1) Pierce™ High Capacity Endotoxin Removal Resin: This resin reduced endotoxin levels for: Cmab, mPAC, mPAC-uPA, mPAC-MMP, WT PA, and LFN-DTA. Prior to use, an aliquot of resin was centrifuged (14000 rpm × 5 min) and resuspended in an equivalent volume of endotoxin-free phosphate-buffered saline (PBS). The resuspended resin was then added to each sample at a 1:4 or 1:8 v/v ratio (resin volume/protein volume). After mixing gently for 1 hr at room temperature, the resin was removed by filtering the samples through a 0.2 μm filter. This procedure typically
provided high protein recovery (~90%) and was repeated (1–3x) until sufficient endotoxin levels were reached (< 5 EU/kg).

(2) HiTrap Q (GE) anion-exchange purification: After sortase-mediated ligation reactions, chromatography was used to reduce endotoxin levels for Cmab-mPAC, Cmab-mPAC-uPA and Cmab-mPAC-MMP. Prior to using this method, a Q column (5 mL) was flushed with a solution of 0.5 M NaOH for 1 h at 1 mL/min and rinsed with a solution of endotoxin-free water. In addition, 0.5 M NaOH was incubated overnight with: glass media bottles (for buffers), the protein purification system, and the HiLoad 16/600 Superdex 200 pg size-exclusion column. Endotoxin-free water was used the following day to rinse all materials and prepare fresh buffers. After completing the sortase reaction, endotoxin-free IgG-mPAC was obtained after purifying the reaction mixture by size-exclusion chromatography and ion-exchange chromatography (above).

**Pharmacokinetics and Biodistribution analysis.** Pharmacokinetics and biodistribution were analyzed with $^{89}$Zr-labelled (Washington University School of Medicine in St. Louis) mPAC-uPA, Cmab, and Cmab-mPA-uPA. Pharmacokinetics was determined by collecting peripheral blood samples and measuring radioactivity over time. Groups of female nude mice were i.v. injected through the tail vain with mPAC-uPA ($n = 1$), Cmab antibody ($n = 4$), or Cmab-mPA-uPA ($n = 4$) at 1 mg/kg. Blood samples (5–15µL) were collected by tail snipping, then radioactivity was measured using a PerkinElmer Wizard 2 gamma counter. PET images were also acquired for evaluating biodistribution, in which whole-body animal PET scans were collected on a G8 PET/CT preclinical imaging system (PerkinElmer). After 166 h, the mice were sacrificed by CO$_2$ followed by cervical dislocation. The organs and tissues were collected and measured by the gamma counter.
**Histological analysis.** Initial safety analysis was performed by dosing tumor-free animals with one treatment of Cmab-mPAC-uPA or Cmab-mPAC-MMP, followed by histological staining and imaging. Groups of female nude mice ($n = 3$, 8 to 12 weeks old) were i.v. administered with PBS or 1 mg/kg LF$_N$-DTA, followed by i.v. injection with 3 mg/kg Cmab-mPAC-uPA/Cmab-mPAC-MMP or 15 mg/kg Cmab-mPAC-uPA/Cmab-mPAC-MMP. The mice were closely monitored over 48 h for any signs of toxicity, then were euthanized. The tissues were fixed in 4% formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) for microscopic analysis.

An extended safety study was also performed with repeated dosing, while monitoring survival, body weight, inflammation, liver function, and histological toxicity (Fig. S26). The study design is modeled after previous work by Leppla and coworkers on the rigorous analysis of bacterial toxin safety in vivo.$^8$ Protein constructs were i.v. administered six times over three weeks to tumor-free animals (C57BL/6 mice, $n = 5$). During this time, the mice were checked twice daily for any signs of outward toxicity and were periodically monitored for changes in body weight. Sera samples from peripheral blood were collected on days 9, 17, and 21, which were stored at -80°C for subsequent analysis of inflammatory cytokine levels (Fig. S27); aspartate aminotransferase (AST) activity (Fig. S28); and alanine transaminase (ALT) activity (Fig. S28). On day 21, animals were harvested and processed to analyze toxicity across five major organs: liver, lung, heart, kidney, and spleen.
**Inflammation analysis.** Mouse inflammation was analyzed using a LEGENDplex assay kit (BioLegend), which provides multiplex fluorescent beads that enable measurement of cytokine levels by flow cytometry. The beads in this particular kit allowed simultaneous quantification of 13 cytokines and chemokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF.

On the day of the experiment, sera samples were thawed slowly on ice and were diluted two-fold with the assay buffer provided (50 µL total volume). The standard provided was reconstituted in the assay buffer, then used to prepare five additional diluted solutions (four-fold serial dilutions). In a 96-well V-bottom plate, standard solutions (25 µL) and a solution matrix (25 µL) were added in duplicate to columns 1 and 2 of the plate. Sample solutions (25 µL) and the assay buffer solution (25 µL) were added to columns 3–10. Fluorescent beads (25 µL) were added to all of the wells, then incubated for 2 h at room temperature. After washing (2x) with the washing buffer provided, the detection antibody (75 µL) solution was added to all of the wells and incubated for 1 h at room temperature. Without washing, the streptavidin detection solution (25 µL) was added to each of the wells and incubated for 0.5 h at room temperature. After washing (1x) with the washing buffer, the beads were analyzed immediately with an LSR Fortessa (BD) flow cytometer (4 laser, 18 color analyzer) equipped with an autosampler. Analyzer setup and data collection were performed according to the instructions provided. Data processing, analysis, and cytokine quantification was performed using the accompanying LEGENDplex (v8.0) software.
**Alanine Aminotransferase (ALT) Activity Assay.** ALT activity was analyzed using a measurement kit (Millipore Sigma), which enabled detection of serum ALT enzyme levels as an indicator for liver damage. Activity was determined based on fluorometric detection of the assay analyte ($\lambda_{ex} = 535/\lambda_{em} = 587$) and was plotted as nmol/min/mL (milliunit/mL). In addition, this experiment was performed on the same day as the inflammation analysis to avoid loss of enzyme activity from multiple freeze/thaw cycles.

For this experiment, an aliquot of each serum sample (5 µL) was added directly to wells of a 96-well clear bottom white plate (columns 3–10) and were diluted to 20 µL with the assay buffer provided. A standard curve was prepared using the pyruvate standard solution provided (100 nmol/µL), which was diluted to 0.1 nmol/µL by a performing a 100-fold dilution and a subsequent 10-fold dilution. This solution was added in duplicate to the 96-well plate (columns 1 and 2), generating 0, 0.2, 0.4, 0.6, 0.8, and 1.0 nmole/well standards at 20 µL total volume.

Prior to analysis, 100 µL of the assay master reaction mix was added to all of the wells (columns 1–10). The plate was immediately transferred to a fluorescent plate reader, in which the incubation chamber was pre-equilibrated to 37 °C. Time-course fluorescent measurements were recorded every 2.5 min until the fluorescence readings exceeded the range of detection.

Activity was determined by calculating the change in fluorescence over time ($\Delta$FLU = FLU$_{final}$ - FLU$_{initial}$) and subtracting the background fluorescence. Using the standard curve generated (right), the $\Delta$FLU values were converted to the amount of pyruvate generated (nmol). ALT activity was than calculated using the equation below:

$$\text{ALT activity} = \frac{\text{pyruvate generated (nmol)}}{\text{(reaction time) $\times$ (serum volume)}}$$
**Aspartate Aminotransferase (AST) Activity Assay.** AST activity was analyzed using a measurement kit (Millipore Sigma), which enabled detection of serum AST enzyme levels as an indicator for liver damage. Activity was determined based on absorbance detection of the assay analyte (A = 450) and was plotted as nmol/min/mL (milliunit/mL). In addition, this experiment was performed on the same day as the inflammation analysis to avoid loss of enzyme activity from multiple freeze/thaw cycles.

For this experiment, an aliquot of each serum sample (10 µL) was added directly to wells of a clear 96-well plate (columns 3–10) and were diluted to 50 µL with the assay buffer provided. A standard curve was prepared using the glutamate standard solution provided (100 nmol/µL), which was diluted to 1 nmol/µL by a performing a 100-fold dilution. This solution was added in duplicate to the 96-well plate (columns 1 and 2), generating 0, 2, 4, 6, 8, and 10 nmole/well standards at 50 µL total volume.

Prior to analysis, 100 µL of the assay master reaction mix was added to all of the wells (columns 1–10). The plate was immediately transferred to a plate reader, in which the incubation chamber was pre-equilibrated to 37 °C. Time-course absorbance (λ = 450 nm) measurements were recorded every 2.5 min for 1 h.

Activity was determined by calculating the change in absorbance over time ($\Delta A_{450} = (A_{450})_{\text{final}} - (A_{450})_{\text{initial}}$) and subtracting the background absorbance. Using the standard curve generated (right), the $\Delta A_{450}$ values were converted to the amount of glutamate generated (nmol). AST activity was than calculated using the following equation:

$$\text{AST activity} = \frac{\text{glutamate generated (nmol)}}{(\text{reaction time}) \times (\text{serum volume})}$$
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