# Bivalent engagement of endothelial surface antigens is critical to prolonged surface targeting and protein delivery *in vivo*

Kiseleva R.Yu.[1], Glassman P.G.[1], LeForte K.M.[1], Walsh L.R.[1], Villa C.H.[1], Shuvaev V.V.[1], Myerson J.W. [1], Aprelev P.A. [1], Marcos-Contreras O.A.[1], Muzykantov V.R.[1], Greineder C.F.[2*]

[1]Department of Pharmacology, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States of America

[2]Departments of Emergency Medicine and Pharmacology, University of Michigan, Ann Arbor, MI, United States of America

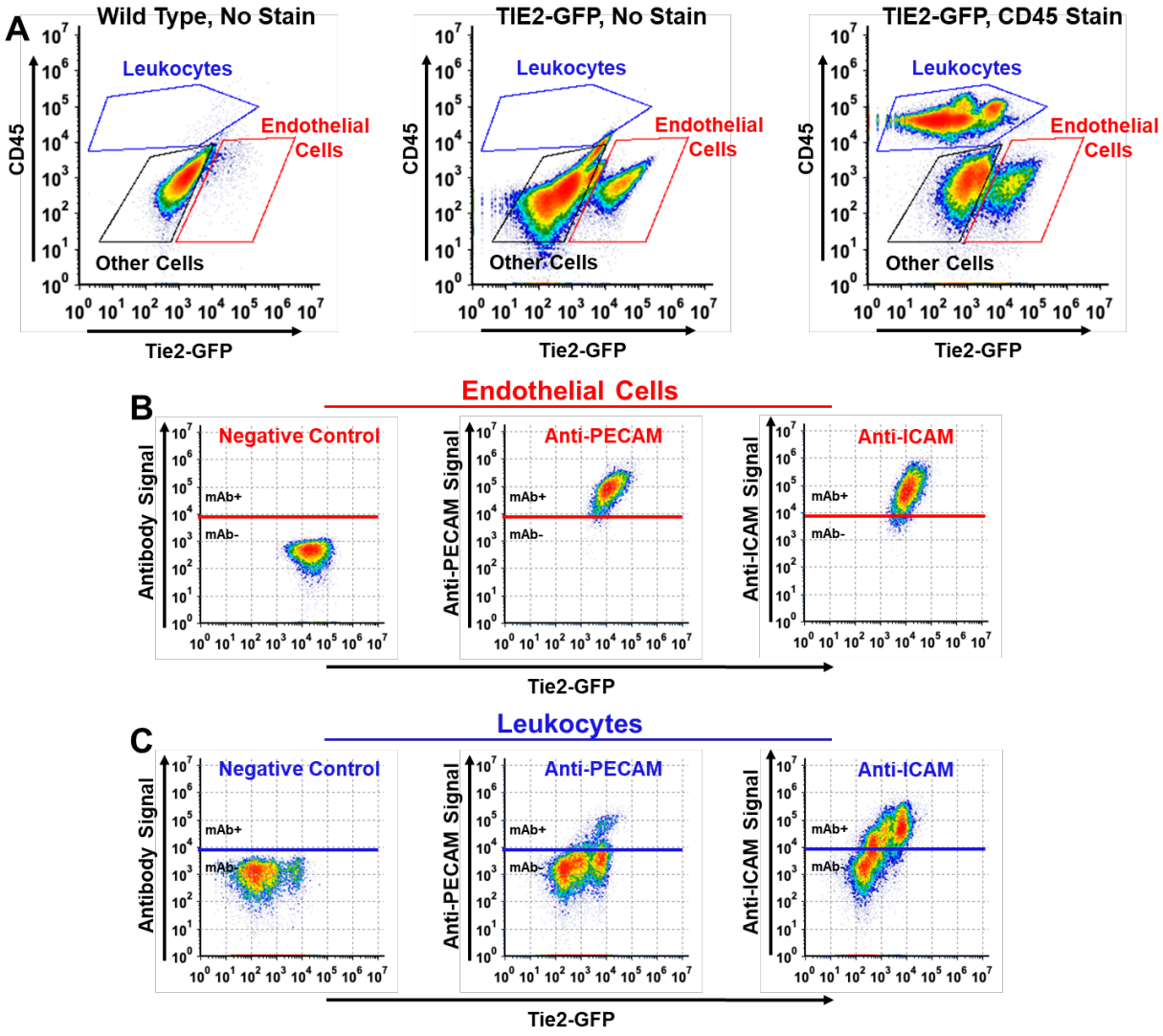



**Figure S1. Gating strategy for flow cytometry analysis of αICAM and αPECAM antibody distributions among cell types in mouse lungs.** A) Density plots showing cells localized to double-negative gate for unstained wild-type mouse lungs, to endothelial cell and double-negative gates for unstained TIE2-GFP mouse lungs, and to leukocyte, endothelial cell, and double-negative gates for TIE2-GFP mouse lungs stained with CD45 antibody. B) Density plots showing that all endothelial cells (as defined by gating in (A)) fall below negative-positive antibody fluorescence threshold in TIE2-GFP mice that don't receive antibody. C) Density plots showing that all leukocytes (as defined by gating in (A)) fall below negative-positive antibody fluorescence threshold in TIE2-GFP mice that don't receive antibody.

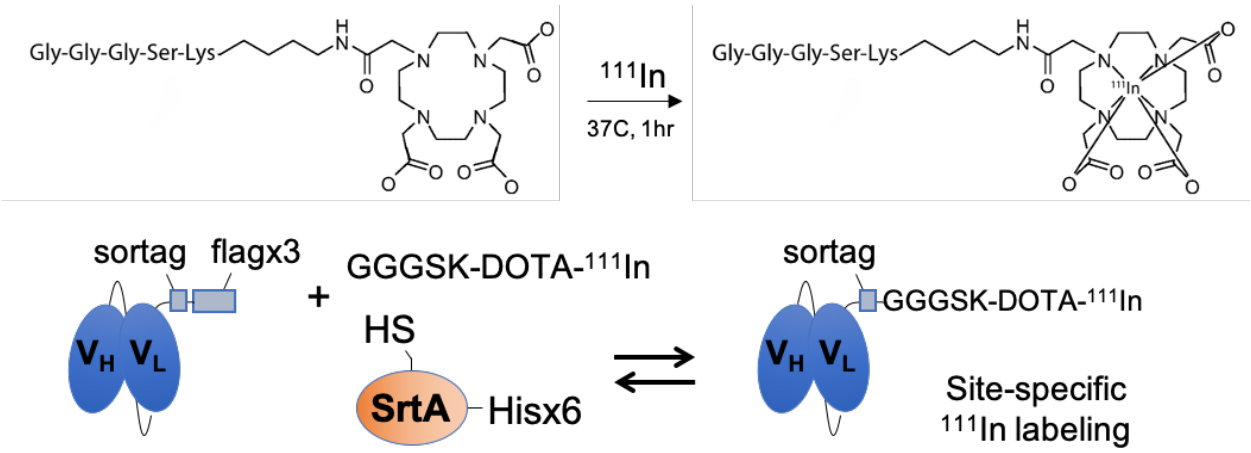



**Figure S2. Two-step, site-specific radiolabeling technique using sortase A transpeptidation.** Small peptide incorporating the radiometal chelating group, DOTA (1,4,7,10-tetrazacyclododecane-N,N',N'',N'''-tetraacetic acid) was labeled with $^{111}$In and then attached by SrtA to the C-terminal end of the scFv.

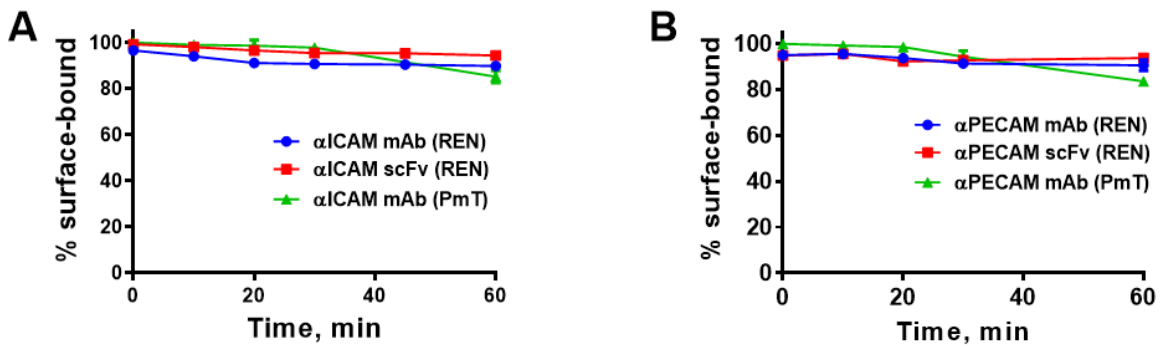
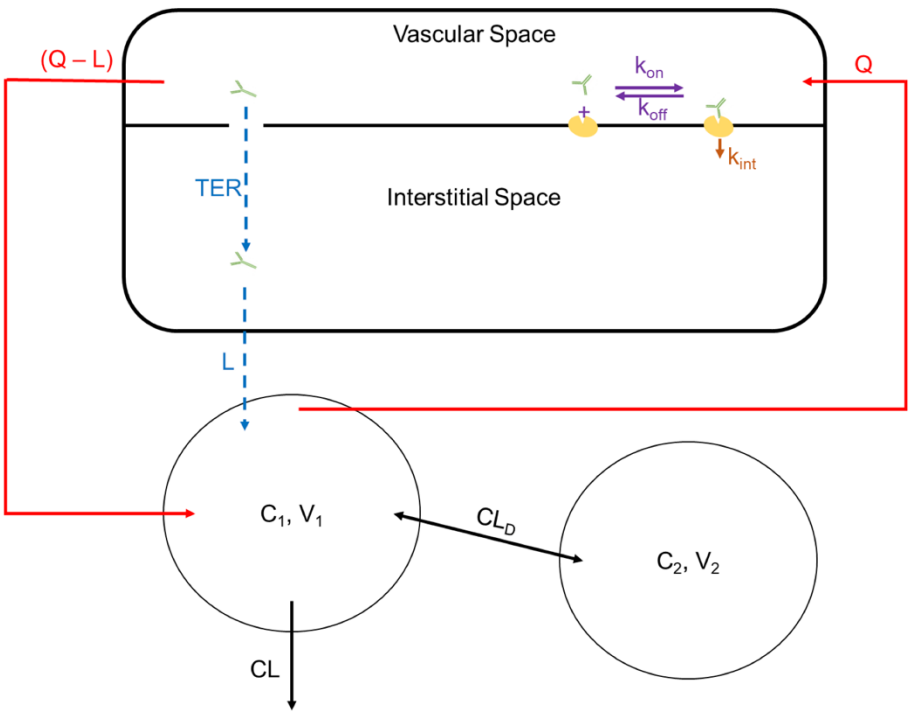



**Figure S3. Radiointernalization assay of αICAM and αPECAM affinity ligands.** The percent of surface-associated affinity ligand was determined by glycine elution after various periods of incubation at 37C. Internalization rates of monovalent and bivalent affinity ligands were compared on REN cells expressing the relevant target antigen and further compared to PmT mouse lung endothelial cells. In all cases, cells demonstrated similar, low rates of endocytosis of affinity ligands, with < 20% of internalized at 1 hour. Data presented as mean ± SD with n = 4.

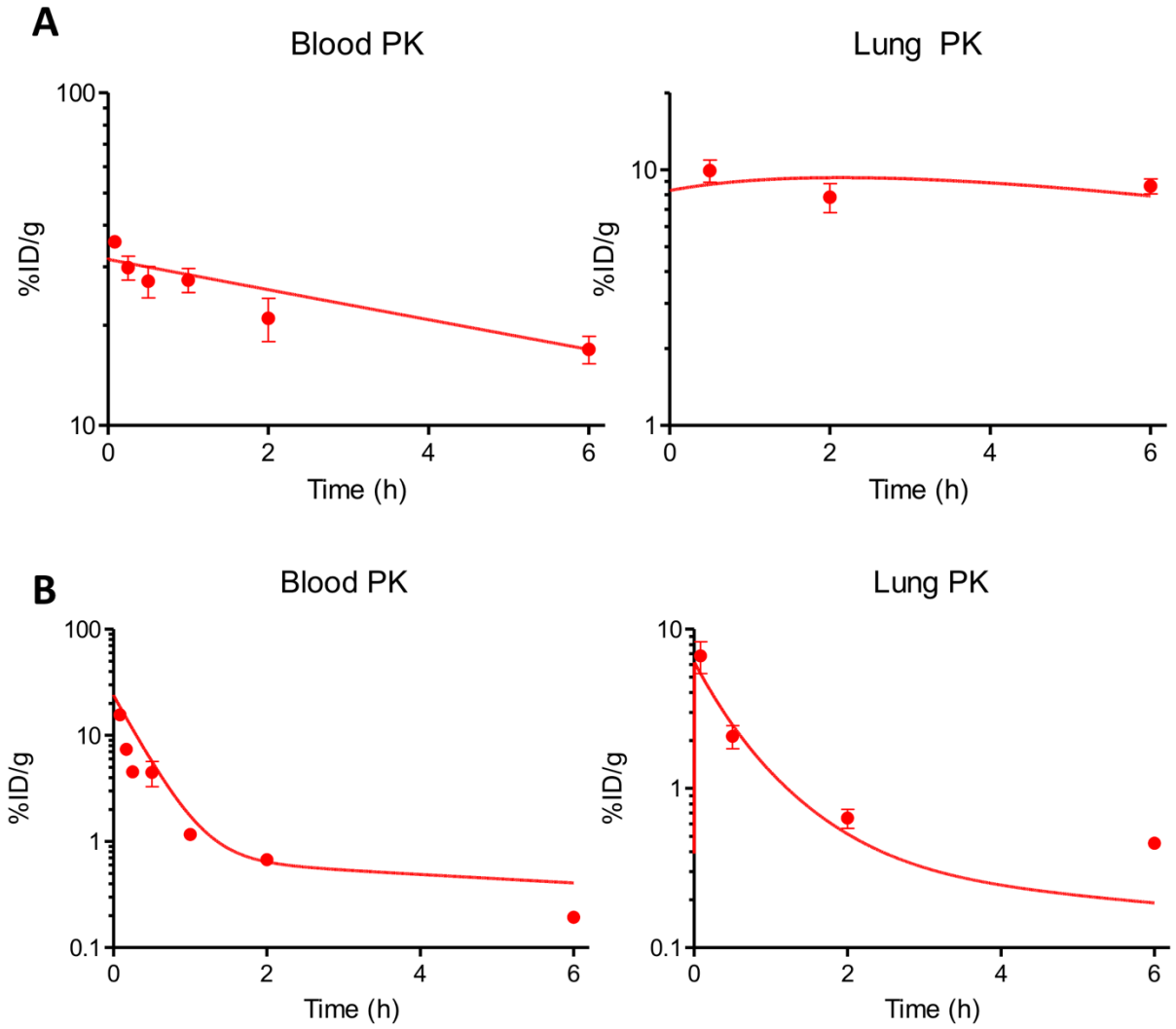
A




B

| Tissue | Blood Flow (mL/min) | Total Volume (mL) | Vascular Volume (mL) | Interstitial Volume (mL) |
|---|---|---|---|---|
| Lung | 11.3 | 0.204 | 0.0536 | 0.0384 |

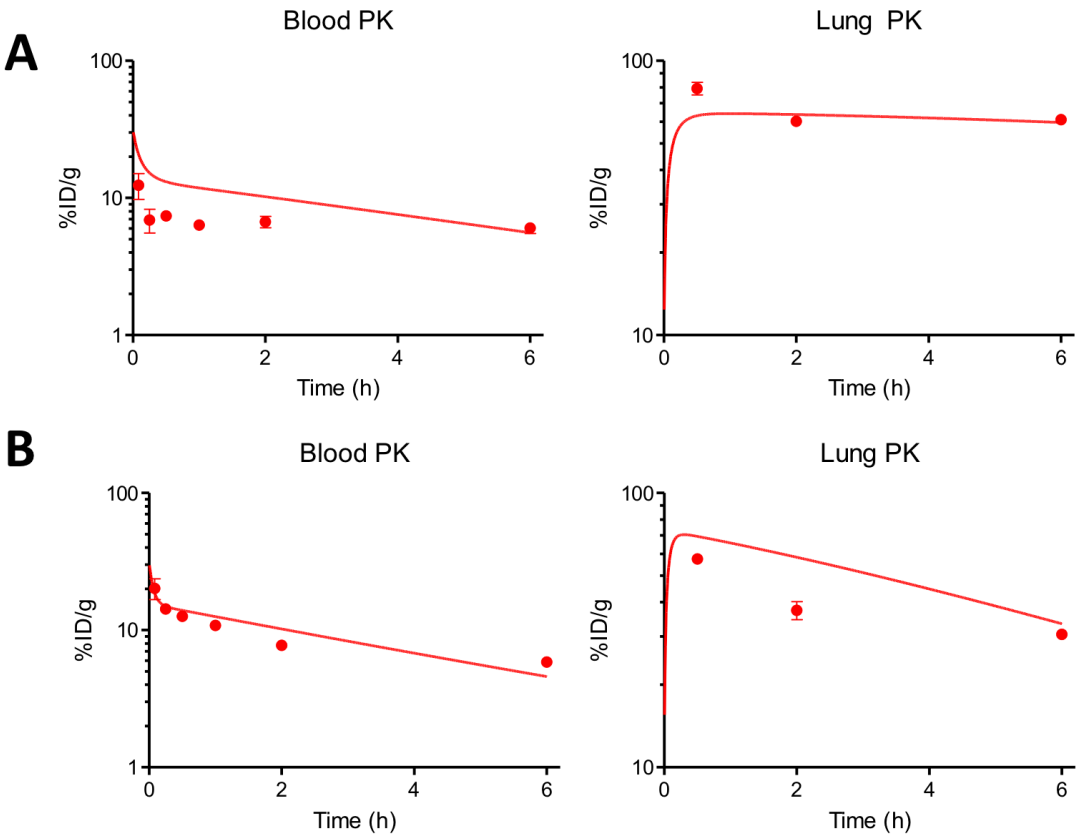
**Figure S4: Structure of Semi-physiologically based pharmacokinetic (PBPK) model.** (A) The proposed model structure links a 2-compartment mammillary model to a physiologically-based lung model. The lung model is described using physiologically-relevant blood flow rates and volumes, as summarized in (B). Within the lung space, protein is able to bind to target via estimated binding rate constants, pass into the interstitial space via bulk fluid flow, or return to the central compartment via lung blood flow rate.




**C**

| Parameter | IgG | scFv |
|---|---|---|
| $V_1$ (mL) | 3.13 (2.95%) | 4.16 (3.62%) |
| $V_2$ (mL) | N.E. | 32.9 (66.7%) |
| CL (mL/h) | 0.326 (8.46%) | 6.54 (23.1%) |
| $CL_D$ (mL/h) | N.E. | 5.88 (24.4%) |
| TER | 0.00950 (32.1%) | 0.0352 (19.9%) |

**Figure S5: Model-fitted blood and lung concentration vs. time curves** for untargeted IgG (A) and scFv (B) as well as Final Parameter Estimates for Untargeted IgG and scFv (C). Symbols represent observed data and solid lines represent model fitted profiles.




C

| Parameter | YN1 (anti-ICAM) | 390 (anti-PECAM) |
|---|---|---|
| $k_{deg}$ ($hr^{-1}$) | 0.0344 (81.0%) | 0.210 (9.49%) |
| $R_{tot, lung}$ (nM) | 81.5 (0.639%) | 185 (0.974%) |
| $R_{tot, central}$ (nM) | 5.31 (0.926%) | 9.17 (2.70%) |

**Figure S6: Blood and lung observed and model fitted concentration vs. time curves** following a 5 µg dose of α ICAM (A) and α PECAM (B) mAbs. Final Parameter Estimates for Targeted mAbs (C). Symbols represent observed data and solid lines represent model fitted profiles.

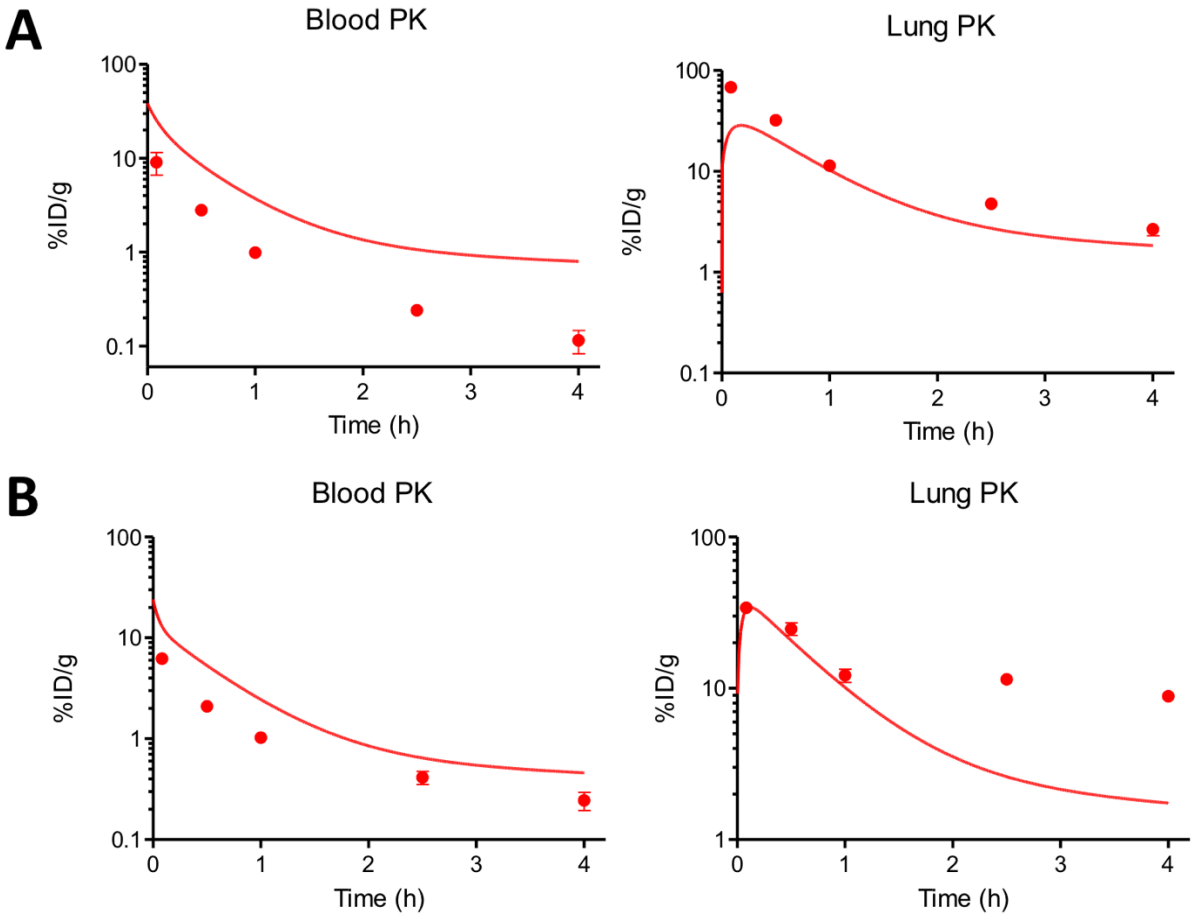



**Figure S7: Blood and lung observed and model predicted concentration vs. time curves** for α ICAM (A) and α PECAM (B) scFv. Symbols represent observed data and solid lines represent model fitted profiles.

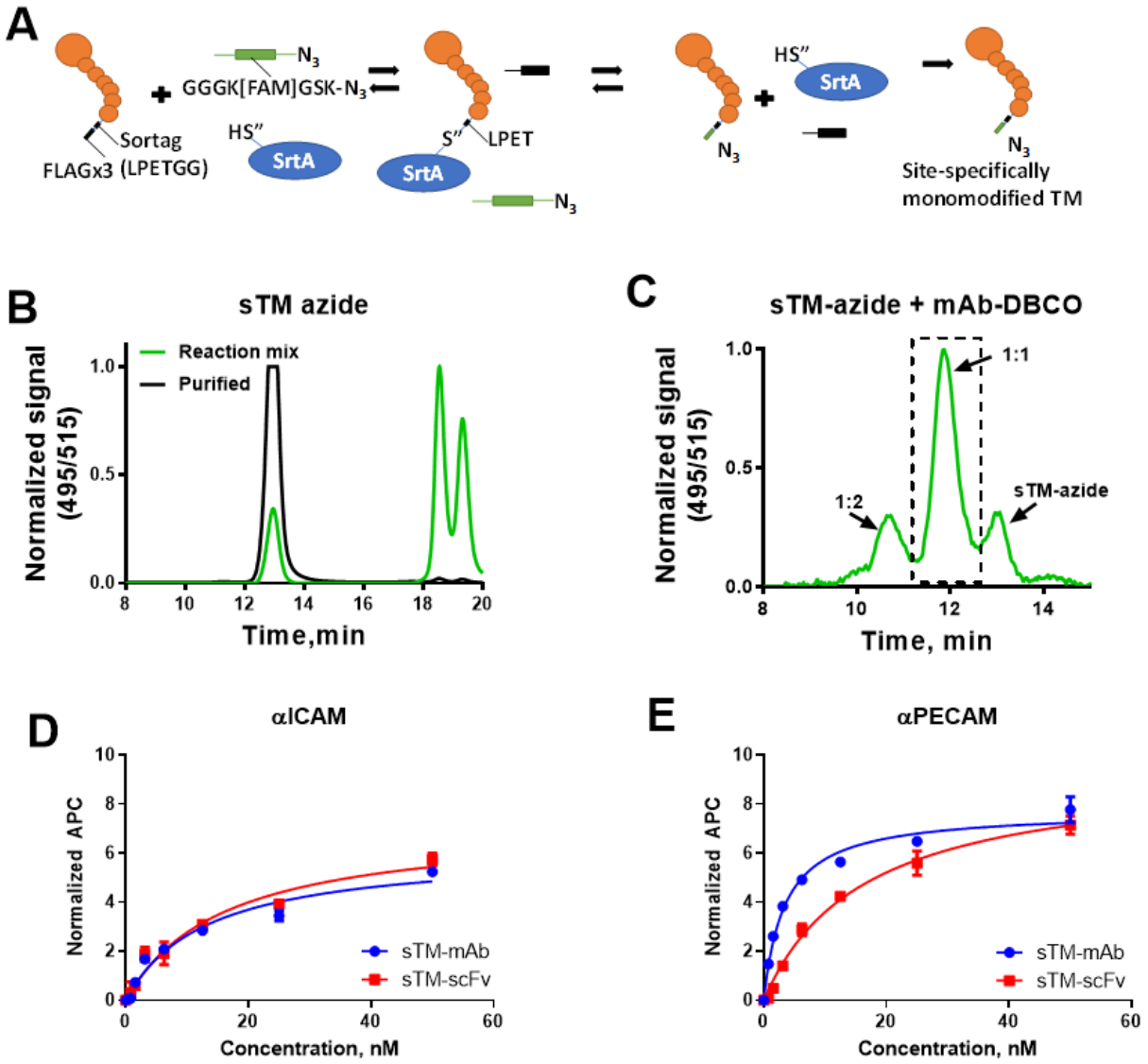
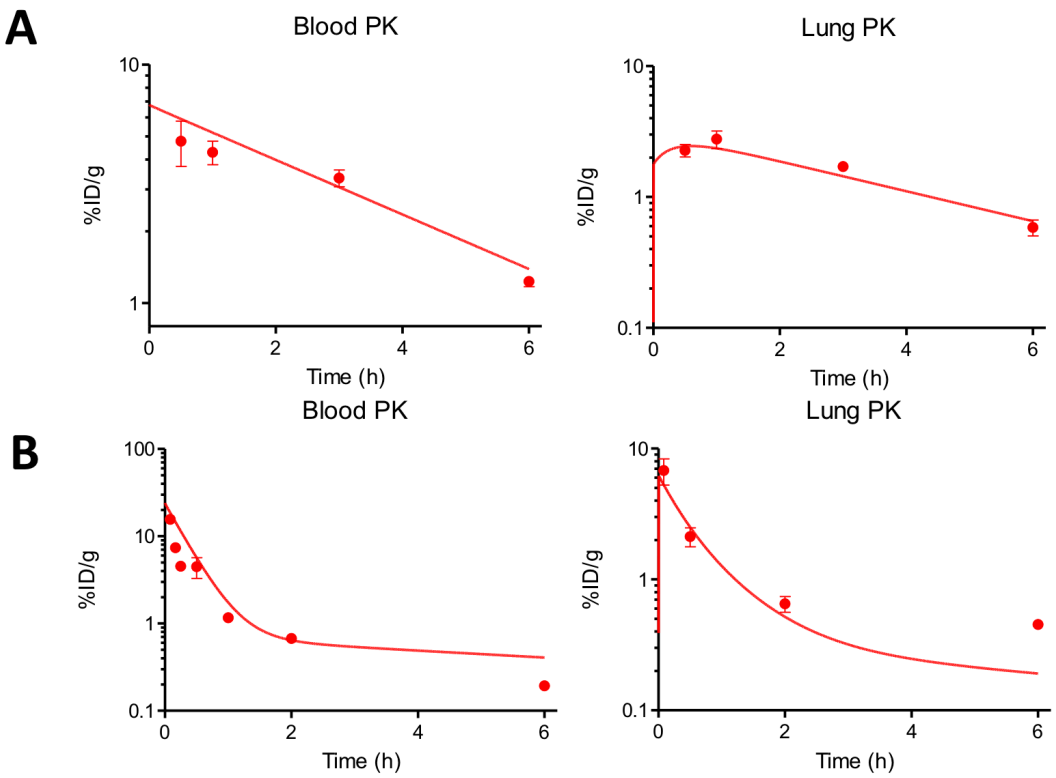



**Figure S8. Site specific modification of sTM and conjugation to DBCO-modified mAb.** A) Schematic showing sortase A modification of sTM-LPETGG – the enzyme removes the C-terminal FLAG tag and attaches a fluorescent, azidolysine containing peptide. B) SEC HPLC trace showing unpurified sTM reaction mixture (with excess peptide) and purified sTM-azide. C) HPLC trace of reactions of sTM-azide and mAb-DBCO at 1:3 molar ratio. A small excess of antibody produced the optimal amount of 1:1 conjugate, which was subsequently purified by HPLC (black dotted lines). D and E) *In vitro* generation of APC by thrombin stimulated by ICAM- (D) and PECAM- (E) targeted TM conjugates.




C

| Parameter (Units) | IgG | scFv |
|---|---|---|
| CL (mL/h) | 3.90 (3.65%) | 6.54 (23.1%) |
| $CL_D$ (mL/h) | N.E. | 5.88 (24.4%) |
| $V_1$ (mL) | 14.8 (4.42%) | 4.16 (3.62%) |
| $V_2$ (mL) | N.E. | 32.9 (66.7%) |
| TER (mL/h) | 0.0794 (48.0%) | 0.0352 (19.9%) |

**Figure S9: Model fitted concentration vs. time profiles for untargeted sTM conjugates. Upper panel: IgG conjugates, Lower panel: scFv conjugates. Estimated parameters for untargeted sTM conjugates. Symbols represent observed data and solid lines represent model fitted profiles.**

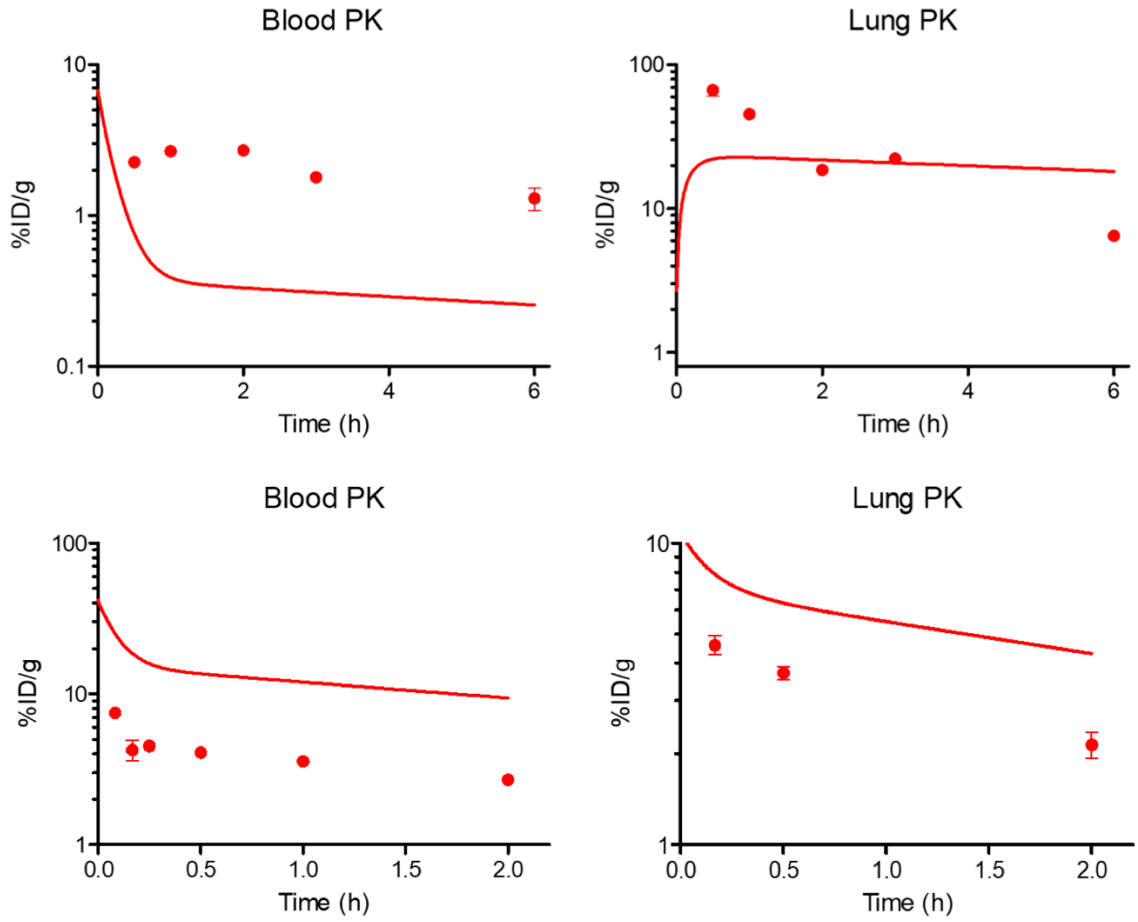



**Figure S10: Observed and model-predicted blood and lung concentration vs. time profiles for YN1-sTM. Upper panel: mAb conjugates, Lower panel: scFv conjugates. Symbols represent observed data and solid lines represent model fitted profiles.**

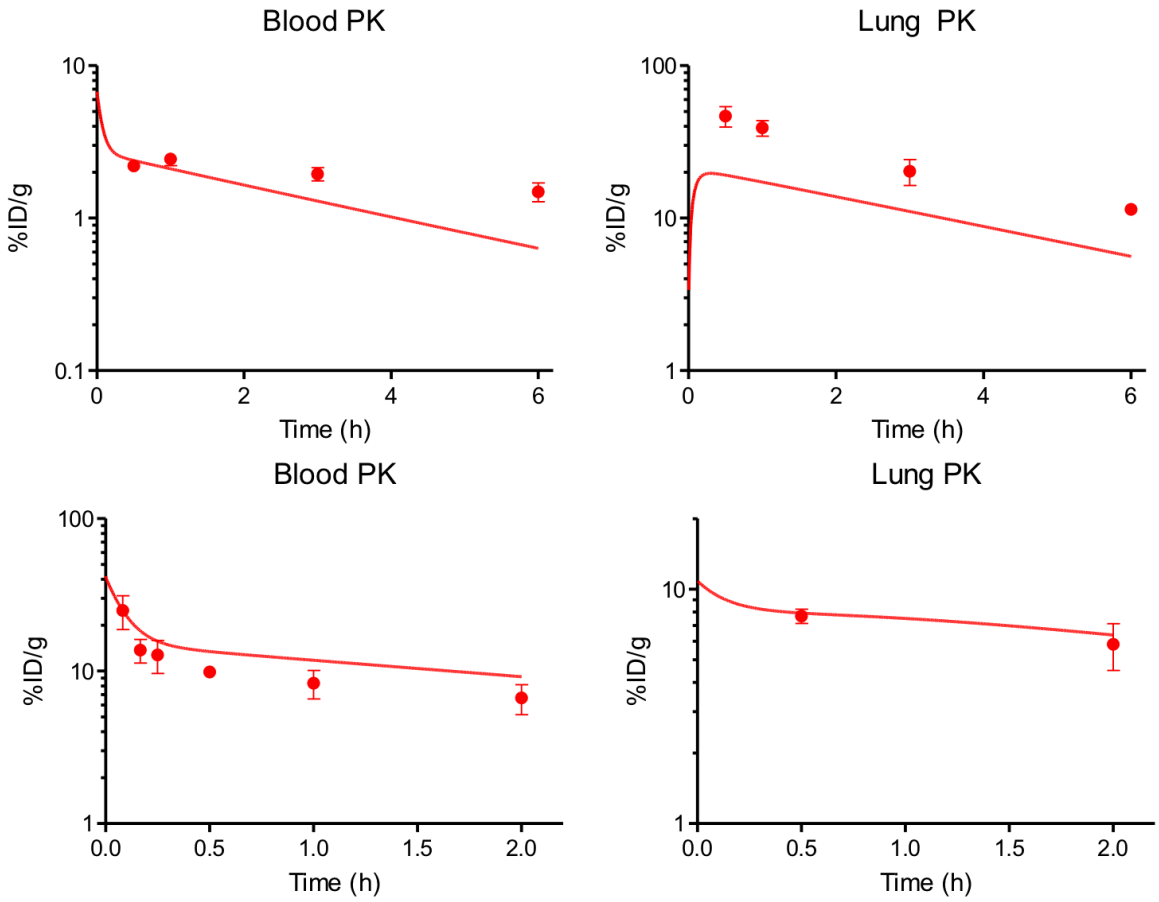



**Figure S11: Observed and model-predicted blood and lung concentration vs. time curves for 390-sTM. Upper panel: mAb conjugates, Lower panel: scFv conjugates. Symbols represent observed data and solid lines represent model fitted profiles.**

**Table S1. Characteristics of affinity ligands.** Binding parameters (calculated Kd, nM), Blood and Lung concentrations (%ID/g), calculated values of circulation half-life (Hrs) and area under the lung concentration vs. time curve for monovalent vs bivalent affinity ligands to ICAM and PECAM.

| | | Monovalent | | | Bivalent | | |
|---|---|---|---|---|---|---|---|
| | | PECAM-1 | ICAM-1 | Untargeted | PECAM-1 | ICAM-1 | Untargeted |
| Affinity (RIA), nM | | 33.2±1.9 | 12±1.2 | - | 4.2±0.5 | 0.12±0.01 | - |
| Blood concentration, % ID/g | 30 min | 2.5±0.2 | 3.5±0.8 | 1.9±0.4 | 10.6±0.13 | 7.4±0.4 | 28.9±3.0 |
| | 60 min | 1±0.13 | 1±0.1 | 1.1±0.1 | 10.7±0.15 | 6.3±0.4 | 27.4±2.3 |
| | 120 min | 0.4±0.1 | 0.24±0.03 | 0.7±0.07 | 8.9±0.15 | 6.7±0.6 | 25.7±1.9 |
| T ½ in blood, hrs | | 1.50 ± 0.24 | 0.984 ± 0.153 | 1.97 ± 0.21 | 4.3 ± 0.3 | 21.0 ± 4.6 | 11.4 ± 2.7 |
| Lung concentration, % ID/g | 30 min | 47.7±5.8 | 80.4±10.6 | 1.5±0.5 | 58.9±3.6 | 79.3±4.2 | 9.9±0.9 |
| | 60 min | 13.4±1.7 | 11.4±1.71 | - | - | - | - |
| | 120 min | 11.5±0.8 | 4.8±0.9 | 0.6±0.1 | 37.3±2.8 | 60.1±2.1 | 7.8±1.0 |
| | 180 min | 8.9±0.8 | 4.0±0.7 | 0.4±0.04 | - | - | - |
| | 360 min | - | - | - | 30.5±0.3 | 60.9±2.1 | 8.6±0.6 |
| $AUC_{inf}$ lung | | 114±37 | 53.3±7.9 | 8.9±0.3 | 1023±507 | 1698±352 | 263±39 |

**Table S2. Characteristics of TM conjugates.** Binding parameters (calculated Kd, nM), Blood and Lung concentrations (%ID/g), calculated values of circulation half-life (Hrs) and area under the lung concentration vs. time curve for monovalent vs bivalent TM conjugates targeted to ICAM or PECAM.

| | | Monovalent | | | Bivalent | | | sTM azide | ss |
|---|---|---|---|---|---|---|---|---|---|
| | | PECAM-1 | ICAM-1 | Untargeted | PECAM-1 | ICAM-1 | Untargeted | | |
| Affinity (RIA), nM | | 48±8.1 | 16.7±4.1 | - | 4.1±1.1 | 12.4±2.9 | - | | |
| Blood concentration, % ID/g | 30 min | 9.9±1.2 | 4.1±0.4 | 8.2±0.8 | 2.2±0.1 | 2.1±0.3 | 4.8±2.0 | 10.4±0.5 | 2.0±0.6 |
| | 60 min | 8.3±3.0 | 3.6±0.2 | 7.1±0.5 | 2.4±0.5 | 2.7±1.9 | 4.2±0.9 | 8.3±1.5 | 2.5±0.4 |
| | 120 min | 6.7±2.6 | 2.7±0.1 | 5.8±0.6 | 1.9±0.4 | 2.7±0.5 | 3.2±0.5 | 8.5±0.6 | 1.8±0.6 |
| T ½ in blood | | | | | | | | | |
| Lung concentration, % ID/g | 30 min | 7.6±0.9 | 3.7±0.3 | 4.6±0.4 | 46.7±14.2 | 66.6±11.1 | 2.7±0.5 | 4.7±1.2 | 93.6±8.9 |
| | 60 min | - | - | - | 39.0±9.1 | 45.4±2.3 | 2.8±0.9 | | 68±0.9 |
| | 120 min | 5.8±2.2 | 2.1±0.4 | 2.8±0.3 | 20.3±7.9* | 18.7±3.5 | 1.7±0.3 | 3.4±0.1 | 45.2±8.4 |
| $AUC_{inf}$ lung | | 34.7±19.9 | 12.4±4.2 | 14.5±1.9 | 188±90 | 141±3.2 | 11±1.5 | 46.6±10.8 | 253±18 |

**Table S3. Fold difference of $AUC_{inf}$ calculated for both affinity ligands and their corresponding conjugates.**

| | | Bivalent/Monovalent | Mono/IgG | Bivalent/IgG |
|---|---|---|---|---|
| Affinity ligand | ICAM | 32 | 6 | 6.5 |
| | PECAM | 8.9 | 12 | 4 |
| Conjugate | ICAM | 12 (21) | 0.9 | 12 |
| | PECAM | 5.4 | 2.4 | 17 |

**Table S4: Model-estimated binding parameters for scFv-sTM conjugates**

*Due to repeated convergence to binding parameters approaching 0.00 for both $k_{off}$ and for $k_{on}$ ($<1x10^{-6}$), binding affinity was assumed to be negligible and values were fixed.

| Parameter | YN1 (anti-ICAM) | 390 (anti-PECAM) |
|---|---|---|
| $K_D$ (nM) | N.E.* | 364 (41.4%) |
| $k_{off}$ ($h^{-1}$) | 0.00 (FIX) | 0.910 (85.7%) |
| $k_{on}$ ($nM^{-1}h^{-1}$) | 0.00 (FIX) | 0.00250 (CALC) |