SUPPLEMENTAL DATA

Methods

K_i Calculation

6.2 nM of uPA was incubated with various concentrations of Fab (0 -2μM) in assay buffer at room temperature for at least 5 hours to assure steady-state behavior of the system. The activity of uPA was measured for each inhibitor concentration by addition of various concentration of substrate (3.9 μM - 500 μM). The initial reaction velocity was monitored by reading the absorbance at 405 nm.

The apparent Michaelis constants value (Km_{app}) for each concentration of substrate was determined by fitting the data to the equation of Michaelis-Menten for competitive inhibition:
$V_0 = (V_{max}[S])/(Km_{app} + [S])$

Where, $Km_{app} = Km(1+([I]/K_i))$
[S] is the concentration of substrate, [I] is the concentratiom of Fab, and K_m is the Michaelis constant for Spectrozyme® uPA under the assay conditions. The Km_{app} values were then plotted against Fab concentration to obtain the K_i. All data were analyzed using Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA.

Selectivity of U33 IgG for active uPA versus inactive uPA

Proteins were purchased from the following vendors: human single chain uPA/pro-urokinase (American Diagnostica Inc. Cat # 107), human uPAR (R&D Systems Cat # 807-UK); human Serpin E1/PAI-1 Inhibitor (R&D Systems, Cat # 1786-PI-010).

uPA versus pro-uPA: With the following exceptions, the protocol described in Supplemental Figure 1 was utilized: Human pro-uPA is inactive, therefore a MUGB titration was not performed. The protein concentration provided by American Diagnostica was used to prepare a 2.8 nM solution of pro-uPA in PBS.

uPA versus uPA complexed to uPAR: Either 50 μL of 2.8 nM active uPA OR 50 μL of 2.8 nM uPAR in coating buffer was added to each well in a Nunc Maxisorb ELISA plate and incubated O/N at 4°C. Unbound protein was removed by hand washing wells 3x with wash buffer. Wells were incubated in 200 μL of Block Solution per well for 2 h at RT. Block Solution was removed by hand washing wells 3x with wash buffer. 50 μL of 2.8 nM active uPA was added to uPAR coated wells and incubated at RT for 1 h. Unbound protein was removed by hand washing wells 3x with wash buffer. 50 μL of U33 IgG in Block Solution was added for 1 h at RT. The highest U33 IgG concentration was 651 nM, and was decreased in two-fold increments to 5.1 nM. All subsequent steps are identical to the previously described protocol.

uPA versus PAI-1 inhibited uPA: Active uPA was coated to an ELISA plate and wells were blocked as previously described. 50 μL of PAI-1 in Block Solution was added for 1 h at RT. The highest PAI-1 concentration was 133 nM, and was decreased in two-fold increments to 8.3 nM. Unbound protein was removed by hand washing wells 3x with wash buffer. 50 μL of U33 IgG in Block Solution was added for 1 h at RT. The highest U33 IgG concentration was 651

nM, and was decreased in two-fold increments to 5.1 nM. All subsequent steps are identical to the previously described protocol.

Dependence of U33 IgG binding on an accessible uPA active site
Inhibitors used are H-Glu-Gly-Arg-CMK (EMD, Cat 347436) and p-Aminobenzamidine (Fluka, Catalog 06880). Buffers used are 50 mM Tris, 150 mM NaCl, 5 mM CaCl2, 0.05% Tween-20 pH=7.4 (1xTBST+Ca+2) and the wash buffer and block solution described previously.

uPA versus uPA-CMK: Active uPA was coated to an ELISA plate and wells were blocked as previously described. 50 µL of H-Glu-Gly-Arg-CMK in Block Solution was added for 1 h at RT. The highest CMK concentration was 7.5 mM, and was decreased in two-fold increments to 467 µM. Unbound protein was removed by hand washing wells 3x with wash buffer. 50 µL of U33 IgG in Block Solution was added for 1 h at RT. The highest U33 IgG concentration was 651 nM, and was decreased in two-fold increments to 5.1 nM. All subsequent steps are identical to previously described protocol.

p-Aminobenzamidine displacement: Both uPA and p-aminobenzamidine stocks were prepared in 1xTBST+Ca+2. Equal volumes of 100-µM p-aminobenzamidine AND 2 µM uPA OR buffer were combined and incubated at room temperature for 1 hour. 50 µL of the p-aminobenzamidine/uPA complex OR 50 µL of p-aminobenzamidine alone was added per well to a blacked-walled 96 well plate. 50 µL of 12 µM U33 IgG OR 50 µL of 1xTBST+Ca+2 was added to wells containing the p-aminobenzamidine/uPA complex OR p-aminobenzamidine alone. The final concentrations are 25-µM p-aminobenzamidine, 500-nM uPA, and 6-µM U33 IgG. Fluorescence intensity measurements were made on a Tecan plate reader using an excitation wavelength of 325 nM and collecting emission data from 356nm-450nm.

Selectivity of U33 IgG for human uPA compared to highly related serine proteases
Proteases were purchased from the following vendors: human uPA (R&D Systems, Cat# 1310-SE), human tPA (Molecular Innovations Cat# HTPA-TC), human MTSP1 (R&D Systems Cat# 3946-SE-010), human plasmin (Haematologic Technologies, Inc. Cat# HCPM-0140), human thrombin (R&D Systems Cat# 1473-SE-010), human HGFA (R&D Systems Cat# 1514-SE-010), human hepsin (R&D Systems, Cat# 4776-SE-010), human chymotrypsin (Athens Research and Technologies Cat# 16-19-030820) , human trypsin (Athens Research and Technologies Cat# 16-19-032000), mouse uPA (Molecular Innovations Cat# MUPA). The concentration of active protease was determined using the active site titrant MUGB (Knight CG. Active-site titration of peptidases. Methods Enzymol. 1995;248:85-101.). Antibodies used include U33 IgG (produced in-house at CytomX Therapeutics, Inc.) and peroxidase conjugated AffiniPure Anti-Human IgG, Fc fragment specific (Jackson Immuno Research, Cat. 709-035-098). Coating buffer is 1xPBS, wash buffer is 1x PBST, and block solution is 4% (w/v) milk in 1xPBST.

50 µL of 2.8 nM active protease in coating buffer were added to each well in a Nunc Maxisorb ELISA plate and incubated O/N at 4°C. Unbound protease was removed by hand washing wells 3x with wash buffer. Wells were incubated in 200 µL of Block Solution per well for 2 h at RT. Block Solution was removed by hand washing wells 3x with wash buffer. 50 µL of U33 IgG in Block Solution was added for 1 h at RT. The highest U33 IgG concentration was 651 nM, and was decreased in two-fold increments to 5.1 nM. Unbound U33 IgG was removed by hand

washing wells 6x with wash buffer. 50 μL of 1:2000 dilution of secondary antibody in Block Solution was added for 1 h at RT. Unbound secondary antibody was removed by hand washing wells 6x with wash buffer. 100 μL of 1-step Ultra TMB-ELISA was added and then quenched with 100 μL of 1 M HCl. Signal was quantified by measuring A450 on plate reader.