Complete Methods

Isolation and identification of mouse hybridoma antibody sequences

TGF-β mAb 1D11 [22] hybridoma clone, 1D11.16.8 (ATCC® HB¬9849™), was purchased from ATCC (Manassas, VA). The 1D11 mAb is reported to neutralize TGF-β1, TGF-β2, and TGF-β3 isoforms (22, 40). Secreted mouse antibody was isolated and purified by protein G affinity chromatography. RNA was extracted from cell pellets using a Qiagen RNeasy kit (Qiagen, Valencia, CA). RNA was reverse-transcribed and cDNA antibody sequences amplified using SMARTer™ RACE cDNA kit (Clonetech, Mountain View, CA) using sequence-specific reverse primers that recognize a sequence in the IgG constant domain (AbbVie, North Chicago, IL). Purified PCR products were gel isolated and purified using a Qiagen Gel Extraction kit (Qiagen, Valencia, CA). DNA was cloned into TOPO TA cloning vectors (ThermoFisher Scientific, Waltham, MA), and used to transform TOP 10 chemically competent bacteria (Thermo Fisher Scientific, Waltham, MA). DNA from isolated colonies was sequenced using Applied Biosystems model 3730S DNA Analyzer. Putative 1D11 mAb heavy and light chain variable domain DNA sequences were used to generate a mouse-human Fc chimeric mAb produced by co-transfection of expression vectors encoding the heavy and light chain IgGs in HEK293.6E cells. To verify comparability to the hybridoma-derived mAb 1D11, the chimeric mAb was assessed for TGF-β binding and neutralization potency.

An antibody that binds to FnEDA was generated at AbbVie. Recombinant antibody was made using vectors encoding the heavy and light chain IgGs of the FnEDA mAb and were coexpressed in HEK293.6E cells (Biotechnology Research Council of Canada, Montreal) followed by Protein A purification of the cell supernatants. An ELISA was used to verify the binding affinity of the synthesized FnEDA mAb to FnEDA (see below).

Generation of TGF-β + FnEDA DVD-Ig

DVD-Igs were engineered from the two recombinant versions of the "parental" monoclonal antibodies TGF-β mAb 1D11 and FnEDA mAb. Briefly, multiple orientations of the mAb heavy and light chain variable domains were fused in tandem by a variety of linker sequences based on *in silico* design to enable generation of cDNAs by gene synthesis. cDNAs were fused to human IgG1 constant domains containing the L234A, L235A hinge region mutation to complete expression vector synthesis. Multiple DVD-Ig molecules were generated by co-transfection of DVD-Ig heavy and light chain plasmids in HEK293.6E cells, followed by Protein A purification of the cell supernatants DVD-Igs were subsequently characterized by expression level, affinity to FnEDA, and neutralization potency to mouse TGF- β.

Affinity measurement of FnEDA mAb and DVD-Ig

Binding of mAb and DVD-Ig to FnEDA was measured using ELISA. Briefly, 0.5 μg/ml recombinant human FnEDA was allowed to bind overnight to easy wash Costar EIA/RIA 96-well plates (high bind) (Thermo Fisher Scientific, Waltham, MA), followed by washing of un-adhered protein using PBS + 0.05% Tween 20. Wells were blocked with Superblock (Thermo Fisher Scientific, Waltham, MA) for 2 hr at room temperature (RT), followed by washing. Antibody or DVD-Igs were added to blocked wells and incubated for 1 hr at RT. Plates were washed again, and goat-anti-human (H+L) HRP detection antibody (Thermo Fisher Scientific, Waltham, MA) was added and incubated for 1 hr at room temperature. Following washing, TMB substrate (Thermo Fisher Scientific, Waltham, MA) was added and incubated for approximately 5-10 min at room temperature, followed by addition of 2N H_2SO_4, followed by spectrophotometric analysis at OD 450nM using a Softmax pro spectrophotometer.

Affinity measurement of TGF-β mAb and DVD-Ig

Affinity measurements of mAb and DVD-Ig binding to TGF-β was carried out using surface plasmon resonance (Biacore) using goat anti-human IgG (ThermoFisher Scientific, Waltham, MA) covalently immobilized on a CM5 biosensor chip. mAb and DVD-Ig (at a concentration of 1 μg/ml) was

injected over the goat anti-human Fc-coated chip surface at a flow rate of 50 μl/min for 60-90 sec to achieve a capture level of 80-100 RU. The net difference in the baseline signal and the signal after the completion of the antibody injection was taken to represent the amount of bound mAb or DVD-Ig. Each kinetic experiment consisted of TGF-β association and dissociation phases. TGF-β (R and D Systems, Minneapolis, MN) was injected at different concentrations at a flow rate of 50 μl for 5 min over captured mAb and DVD-Ig or the reference surface alone to determine association rates. Dissociation of TGF-β was monitored for 15 min at flow rate of 50 μl/min. Association (ka) and dissociation (kd) rate constants as well as overall affinity (KD) were calculated by the instrument evaluation software based on the values extracted from the data using global fit analysis allowing identical values for each parameter in the data set.

TGF-β potency assay

An HT-2 cell proliferation assay was used to measure the potency of TGF-β mAb or DVD-Ig (41). Briefly, HT-2 (Clone A5E, ATCC, Manassas, VA) cells proliferate in the presence of mouse IL-4 (R and D Systems, Minneapolis, MN) and then undergo apoptosis when exposed to TGF-β. TGF-β-induced apoptosis is blocked in the presence of a neutralizing TGF-β antibody. The number of cells following IL-4 and TGF-β stimulation was determined using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). HT-2 cells were rinsed in PBS, re-suspended at $0.15x10^6$ cells/ml in assay media (phenol red-free RPMI 1640 supplemented with 2% FBS, penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively)), and 50 μM beta-mercaptoethanol. Titrations of mAb or DVD-Ig were pre-incubated with mouse TGF-β (12 pg/ml final concentration in assay media + 0.1% BSA). Following a 60 min preincubation, 50 μl of the mAb or DVD-Ig + TGF-β complex was added to the cells, followed immediately by 50 μl of assay media containing 6.0 ng/ml murine IL-4. Cells were incubated for 20-48 hr at 37° C, then 150 μl of CellTiter 96® Aqueous One solution was added. Plates were quantified on a luminescence plate reader.

Animal model of renal interstitial fibrosis

Male CD1 mice (25-30 g, Charles River Laboratories, Wilmington, MA) were used in this study. All animals were kept in a temperature-regulated environment under a controlled 12-h light-dark cycle. Food and water were provided ad libitum. All procedures were performed in an Association for Assessment and Accreditation of Laboratory Animals Care (AAALAC) approved facility and approved by AbbVie's Institutional Animals Care and Use Committee.

The unilateral ureter obstruction (UUO) model was used to induce renal fibrosis. Mice were placed under isoflurane (Baxter Healthcare Corporation, Deerfield, IL) anesthesia, the left ureter was visualized via a flank incision, and ligated using two 5-0 silk sutures. The ureter was incised between sutures. Sham animals underwent similar surgery but without ureter ligation.

Biodistribution and efficacy studies

Studies were conducted to assess either the biodistribution or efficacy of the FnEDA mAb, TGF-β mAb, and the TGF-β + FnEDA DVD-Ig in UUO or sham animals. The half-lives were 3.8 days for the TGF-β mAb, 3.4 days for the DVD-Ig, and 5.2 days for the FnEDA mAb. The parental mAbs were used as the benchmarks/controls for the DVD-Ig in terms of both efficacy and distribution. These were used as the controls since the focal questions of this study were to determine if the TGF-β + FnEDA DVD-Ig at least maintained the efficacy of the TGF-β mAb but with a more renal restricted distribution, and did the DVD-Ig distribution resemble the distribution of the FnEDA mAb. The analytical techniques for these studies are described following the description of the studies.

Tissue biodistribution of the mAbs, and the DVD-Ig molecule was examined for kidneys (ipsilateral and contralateral), liver, spleen, colon, and urine extracted from UUO or sham mice on day 6 post-surgery by electro-chemiluminescent detection, or by immunohistochemistry (kidney only)

techniques (Figures 2 and 4). Additionally, *in vivo* SPECT imaging was used to detect the whole-body distribution of radiolabelled FnEDA mAb, TGF-β mAb, and the TGF-β + FnEDA DVD-Ig (Figures 3 and 6).

Three efficacy studies were performed. One study was undertaken to determine effects of the TGF-β + FnEDA DVD-Ig, TGF-β mAb, and vehicle on markers of renal fibrosis in UUO and sham mice (Figure 5). mRNA expression of fibrosis related genes (Col1a1 FnEDA, and Timp1) and collagen deposition was assessed in this study. The TGF-β + FnEDA DVD-Ig (n = 9 or 10 per dose group), TGF-β mAb (n = 10 per dose group), and vehicle (n = 10 for UUO, and n = 5 for sham) were administered the day before UUO or sham surgery and once daily thereafter up to day 5 post-surgery at doses of 1 and 10 mg/kg in 10 mg/ml (i.p.). A second efficacy study determined if the FnEDA mAb (1 and 10 mg/kg, i.p.) alone could alter the expression of the same markers in UUO mice. The FnEDA mAb and vehicle were administered the day before UUO or sham surgery and everyday thereafter up to day 5 post-surgery at 10 mg/ml (n = 10 per group, except for sham n = 5). The final efficacy study was conducted to determine the effects of the TGF-β mAb on renal levels of FnEDA protein and collagen deposition (Figure 7). The FnEDA mAb (5 mg/kg, i.p.) was administered once on the day of UUO surgery, and TGF-β mAb or vehicle was administered the day before UUO surgery and everyday thereafter up to day 5 post-surgery at doses 1, 3 and 10 mg/kg in 10 mg/ml (i.p.) (n = 8 per group). All animals in efficacy studies were euthanized on day 6 post-surgery and tissues were extracted for immunohistochemical and mRNA expression analysis.

mRNA analysis

For mRNA analysis, fresh renal cortex samples were collected at the end of UUO studies using a 2 mm diameter biopsy punch (Integra, Plainsboro, NJ), and were placed in RNALater solution (Life Technologies, Carlsbad, CA) at 4°C prior to RNA extraction. Total RNA was isolated from homogenized cortex punches using the Qiagen RNeasy®-96 Universal Tissue Kit (Qiagen, Valencia, CA) following the

manufacturer's protocol. The quantity (UV absorbance at 260 nm) and quality (260/280 ratios) of the purified RNA samples were measured simultaneously using a Nanodrop-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A subset of the samples were loaded into an Agilent RNA 6000 Nano LabChip and assessed with the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA) to test RNA integrity number (RIN). RNA was stored at -80°C until thawed the day of reverse transcription.

Reverse transcription of RNA to cDNA was done using Bio-Rad iScript™ cDNA Synthesis kits (Bio-Rad, Hercules, CA). The final RNA concentration of tissue samples was 0.0167 mg/ml. Reactions were prepared, scaled, and run according to the thermal cycling conditions outlined by the manufacturer. The first batch of cDNA was kept at 4°C overnight, while the second was used in PCR immediately.

All genes of interest were targeted with TaqMan® Assays-on-Demand™ Gene Expression primers with FAM dye-labeled MGB probes from Applied Biosystems (Foster City, CA): Hprt (quality control, Assay ID Mm0145399_m1), Col1a1 (Mm00801666_g1), Eda+ isoform of Fn (Fn-Eda+; Mm00692655_m1), and Timp1 (Mm00441818_m1). TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA) was used in PCR reactions. Each 10 µl reaction consisted of 4.5 µl cDNA (75 ng total), 5 µl 2x TaqMan® Universal Master Mix with UNG, and 0.5 µl primer-probe, in accordance with manufacturer's specifications. Each sample was run in duplicate. To verify efficiencies, standard curves were generated for each target gene using commercially available control mouse universal cDNA (BioCat GmbH, Heidelberg, Germany). Real-time PCR was performed using the Bio-Rad CFX384 Touch system (Bio-Rad, Hercules, CA). Thermal cycling conditions for the reactions were: a) 50°C for 2 min (UNG incubation), b) 95°C for 10 min (enzyme activation), and c) 40 cycles of 95°C for 15 sec (denature), and 60°C for 1 min (anneal/extend). Raw data was analyzed in CFX Manager™ software (Bio-Rad, Hercules, CA). Cq values were exported into a Microsoft Excel worksheet configured to

calculate target gene expression relative to the sham control group using the 2-ΔΔCt method (71) Individual data points with fold change values ± 2 SD from group mean were designated outliers and removed from analysis.

Protein biodistribution and analysis

To profile the biodistribution of the FnEDA mAb (5 mg/kg, i.p.), TGF-β mAb (10 mg/kg i.p.), and the TGF-β + FnEDA DVD-Ig (10 mg/kg, i.p.), the molecules were injected immediately after UUO or sham surgery and levels were measured 6 days later. For analysis of tissue antibody levels, tissue samples were processed to recover intact antibodies. Tissue samples were collecting into 2 ml homogenization tubes (Lysing Matrix S tubes, MP Biomedicals, Santa Ana, CA), flash-frozen in liquid nitrogen, and stored at < -70 °C. Four volumes (weight/volume, assuming tissue densities of 1.00 on average) of cold RIPA lysis buffer (50 mM Na-Citrate (pH 3), 1% Triton X-100 , 0.5% Na-deoxycholate, 150mM NaCl, 5% Glycerol; Boston Bioproducts, Ashland, MA) containing 1X protease inhibitors (Sigma-Aldrich St. Louis, MO) were added. Samples were homogenized in the FastPrep-24 (MP Biomedicals, Santa Ana, CA) for 2 cycles of 60 sec at 6 m/s, cooling on ice for 3-5 min between cycles. Supernatants were clarified by centrifugation twice at 10,000 RPM for 10 min in a table top centrifuge, and neutralized by adding 10% the final volume of 2M Tris base solution.

Tissue homogenates and biofluid samples (serum and urine) were analyzed for antibody levels using Mesoscale Discovery (MSD, Rockville, MD) chemiluminescent detection. The human FnEDA mAb and TGF-β + FnEDA DVD-Ig were measured with a generic anti-human IgG assay. MSD high-bind 96-well plates were coated overnight with a goat anti-human-IgG-Fc antibody (4 µg/ml in carbonate buffer, Thermo Fisher Scientific, Waltham, MA). Plates were then washed 5 times (1X PBS plus 0.05% Tween-20). Samples were diluted at least 1:100 in assay buffer (1% MSD Blocker A in Tris-buffer saline with 0.02% Tween-20) containing 1% CD-1 mouse serum, and transferred in duplicate to the MSD

plate. Standards and quality control samples prepared from stocks of mAb and DVD-Ig molecules were also diluted in assay buffer containing 1% CD-1 mouse serum. After 1 hr with shaking at room temperature, the plates were washed as before, and bound human IgG's detected with a second anti-human detection antibody (Sulfo-Tag goat anti-human antibody, diluted to 1 µg/ml in assay buffer, MSD, Rockville, MD). After an additional 1 hr incubation at room temperature, plates were washed again, 150 µl of 2X MSD read buffer added to all wells, and the plates read on the MSD Sector Imager.

For the measurement of murine TGF-β mAb, MSD high-bind 96-well plates were coated overnight with recombinant human TGF-β (PeproTech, Rocky Hill, NJ) in assay buffer. After washing, a 1 hr sample incubation, and another wash step, bound murine TGF-β mAb were detected with an anti-mouse IgG detection antibody (Sulfo-Tag goat anti-mouse IgG antibody, diluted to 2 µg/ml in Assay Buffer, MSD, Rockville, MD). After an additional 1 hr incubation at room temperature, plates were washed again, 150 µl of 2X MSD read buffer added to all wells, and the samples read on the MSD Sector Imager.

MSD standard curve fitting and data evaluation was performed using XLfit4 software (Version 4.2.1 Build 16). A calibration curve was plotted from MSD luminescence units versus theoretical standard concentrations. A four-parameter logistic model was used for curve fitting. The regression equation for the calibration curve was then used to back calculate the measured concentrations. Tissue samples were normalized as µg of mAb (or DVD-Ig) per gram of tissue, while biofluid samples were normalized as µg of mAb per ml of biofluids.

Histology and immunohistochemistry

Kidney tissues extracted from UUO mice in the efficacy studies were initially fixed by transcardiac perfusion with normal saline followed by 10% neutral buffered formalin. Kidneys were subsequently excised, bisected down the longitudinal axis, allowed to postfix in formalin for 4 hr, then

dehydrated, cleared, embedded in paraffin, sectioned at 5 μm, and mounted on adhesive coated slides. Picrosirius red (PSR) staining was used to examine the extent of tubulointerstitial (TI) fibrosis. Briefly, sections were deparaffinized in xylenes, hydrated through a graded series of ethanol solutions to water, then treated for 2 min in 0.2% aqueous phosphomolybdic acid. Sections were then immersed for 60 min in 0.1% solution of PSR F3BA in saturated aqueous picric acid, pH 2. Sections were washed in 0.01 N HCl for 2 min to remove excess stain, rapidly dehydrated through a graded series of ethanol solutions, cleared through xylenes, and coverslipped with a xylene-based mounting media.

Stained sections were examined and photomicrographs captured with a BX-51 fluorescence microscope using a 10x (0.4 N.A.) objective, TRITC filter set (excitation 545 nm, LP570 nm dichroic mirror, 610 nm emission filter) and DP80 camera (Olympus). For each subject, 3 images from the outer cortex were captured (one from each pole and one from the medial third of the organ). Images analysis was subsequently performed using ImageSense™ software (Olympus). TI fibrotic percentage was defined as the percentage of positive stained area above an empirically derived detection threshold, but excluding the area occupied by glomeruli and blood vessels. The same parameters for microscopy and image analysis were uniformly applied to all images. The mean TI fibrosis percentage derived from the three images served as the value for each subject.

To determine the distribution of the FnEDA mAb in the kidneys, immunohistochemistry was performed using an Alexa Fluor® 488-conjugated donkey anti-human IgG (Jackson ImmunoResearch, West Grove, PA). Briefly, sections were deparaffinized in xylenes, then hydrated through a graded series of ethanol solutions to water. Antigen retrieval was achieved through enzymatic digestion (1 mg/ml pepsin in PBS adjusted to pH 2.3 with HCl, 20 min). Nonspecific staining was blocked using 10% normal donkey serum for 30 min, followed by a 90-min incubation with anti-human IgG (1:500). DAPI (4',6-diamidino-2-phenylindole) was included in the first of three 15-min PBS washes as a counterstain, then sections were dehydrated through a graded series of ethanol solutions, cleared through xylenes,

and coverslipped with a fluorescence-preserving mounting media. The stained sections were photographed using a 20x (0.75 N.A.) objective, FITC filter set (excitation 475 nm, 530 nm dichroic mirror, 505 nm emission filter), and the mean area fraction derived from the three images served as the value for each subject.

SPECT imaging

A SPECT scanner with built-in CT (computed tomography) dedicated for small animals (Bioscan Poway, California) was used for two *in vivo* imaging studies to assess whole-body distribution of the different mAbs and DVD-Ig molecules. Antibodies (FnEDA and TGF-β) and the TGF-β + FnEDA DVD-Ig were conjugated with diethylenetriamine-pentaacetic acid (DTPA) followed by radiolabeling with ^{111}In. For the first imaging study (Figure 3), the ^{111}In-labeled FnEDA mAb or ^{111}In-IgG control was injected at a dose of 1.1 mg/kg (7.5 μmol/kg, i.v.) in a volume of 130 μl immediately after UUO or sham surgery. There were 7 rats in each of the UUO groups, 6 in the sham ^{111}In-FnEDA group and 4 in the sham ^{111}In-IgG group. For this study, a separate group of UUO animals (n = 6) were co-administered ^{111}In-FnEDA and the "cold" FnEDA (unlabeled) mAb (1.1 mg/kg and 35 mg/kg, respectively) in order to create competition for the FnEDA isoform. For the second imaging study (Figure 6, n = 6 per group), the ^{111}In-TGF-β + FnEDA DVD-Ig, ^{111}In-FnEDA mAb, or ^{111}In-TGF-β mAb was injected at 1.1 mg/kg, i.v., (7.5 μmol/kg) in a volume of 130 μl immediately following UUO surgery. For both SPECT studies, the 1.1 mg/kg (i.v.) dose was chosen in order to find a balance between minimizing perturbation of the system and sufficient material to detect the radioligand. The goal with this dose was to minimize downstream reactions by the mAb or DVD-Ig so that the target environment is relatively preserved, while ensuring that the dose is sufficient to allow good detection of the radiolabeled mAb or DVD-Ig over 6 days, taking into account labeling efficiency and radiation decay over the time.

Imaging data were collected 6 days after the UUO procedure. Two mice at a time were positioned on a heated bed (Minerve, Esternay, France) and imaged simultaneously under

isoflurane/O_2 (2%) anesthesia. Whole-body helical CT and whole-body SPECT images were then acquired continuously over 60 min or less. Images were reconstructed and coregistered using vendor-provided software. Regions of interest including ipsilateral kidney, contralateral kidney, heart, and muscle were defined using customized segmentation routines. Pixel-by-pixel radioactivity uptake was measured from the acquired SPECT images using VivoQuant (inviCRO, Boston, MA), then averaged over the regions to obtain tissue uptake. The calculated tissue uptake, divided by the total injected activity and the weight of the tissue, was converted to percent injected dose per gram of tissue (%ID/g), an index for relative tissue distribution of the ^{111}In-mAbs or ^{111}In-TGF-β + FnEDA DVD-Ig. The activity measured from the heart that was defined as blood pool was used as blood activity uptake. After subtracting the blood activity contribution from the tissue uptake based on blood volume fraction of each organ (42), the relative tissue distribution was calculated and presented as ratio of tissue to blood activity uptake.

Statistical analysis

Statistical significance was tested using a one-way or two-way ANOVA followed by a Fisher's LSD post-hoc test if significance was achieved in the ANOVA. T-tests were conducted if there were only 2 groups for comparison. All data are shown as mean ± SEM, and were considered significant if it reached a p value < 0.05.