SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS and METHODS

Preparation of human serum amyloid P and human serum albumin and *in vivo* treatment protocols. Human serum amyloid P component (hSAP, Cat. No: 970549) and human serum albumin (HSA, Cat No. 12667) used for labeling reactions were purchased from EMD Chemicals, Inc. (Gibbstown, NJ 08027), and extensively dialyzed into Ca^{++} free PBS. Protein labeling kits were purchased from Invitrogen Corp. (Carsbad, CA 92008, Alexa Fluor 488 kit, Cat. No. A10235; Alexa Fluor 594 kit, Cat. No. A10239). The labeling of hSAP and HSA was conducted following the instructions provided by the manufacturer. Briefly, 50 μL of 1 M bicarbonate was added to 0.5 mL of a 2 mg/mL protein solution, and then transferred to the vial of reactive dye. The reaction mixture was stirred for 1 hour at room temperature and then mixed gently by rotation overnight at 4^0 C. After the reaction, the reaction mixture was carefully loaded onto the freshly made purification column. The mixture was allowed to enter the column resin, and then 5mL of elution buffer was added. The first colored band, which contained the labeled protein, was collected. The concentration of the labeled protein was determined by UV spectrophotometry and Bradford assay. The intensity of labeling was determined using a Tecan Infinite M200 spectrophotometer and the degree of the labeling was calculated for each protein. The labeling degrees were ~2 moles dye per mole of protein for both hSAP and HSA. For *in vivo* experiments HSA solution (Baxter Inc.) and hSAP

were diluted in sterile PBS prior to use. hSAP (20mg/kg or 2mg/kg) or human serum albumin (20mg/kg) was given IP 2 hr before surgery and either every 48h or every 24h thereafter until sacrifice.

Patient cohort, data and sample collection. The Brigham chronic kidney disease (CKD) cohort is a prospective observational cohort, established in 2004, designed to study the influence of specific modifiable factors on the risk of progression of CKD and the risk of CVD in patients with CKD. Inclusion criteria include patients aged ≥18 years and chronic kidney disease defined as glomerular filtration rate (GFR) < 60mlmin/1.73m^2 or GFR≥ 60mlmin/1.73m^2 with microalbuminuria. Estimated GFR (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) II equation (*1*). Exclusion criteria included unwillingness to provide informed consent, patients who were hospitalized or had a thrombotic event or cardiovascular event one month prior to enrollment, those with a life expectancy <6 months, an active neoplastic disease and patients on cytotoxic treatment. The study was approved by the institutional review board at Partners Healthcare (IRB protocol 2003P002133) and an informed consent was taken from each patient. Accordingly, citrated plasma stored at -80°C until analysis, was used to analyze SAP levels in 58 patients with stage with kidney disease. Human kidney biopsy fresh frozen sections were obtained during routine biopsy collection for pathological analysis during the course of routine clinical practice (IRB protocol 2007P000268). Following complete analysis, unused remaining sections were used for this study with no patient information attached, except pathological diagnosis.

Molecular cloning and retroviral transduction, and adenoviral preparation. The open reading frame (ORF) of mFcγRI, mFcγRIII, mFcγRIV and FcRγ-chain were cloned with Kozak consensus sequences from C57BL6 mouse macrophage cDNA into pGEM easy (Promega) using standard techniques (*2*). mFcγRIIB was purchased from Open Biosystems (MMM1013-7514050). ORFs were ligated into the retroviral vector pMX puro at EcoR1 digestion sites. Amphotropic retroviruses were generated (pMX-puro, pMX-mFcγRI, II, III, IV and pMX-γchain) in 293T cells using pCLampho (Imgenex) packaging vector. 293T17 cells (50% confluence in 100mm dishes) were transfected with equimolar pMX vector and packaging vector DNA in polyethylenimine (PEI) complexes using methods modified from previous studies (*3*). Retroviruses were harvested 16h later, filtered through a 0.45 micron filter, and stored at -80^{o}C in DMEM/F12/10% FBS. 3T3 mouse fibroblast cell line (ATCC Number: CCL-92) was retrovirally transduced in 6 well plates. In brief in 1mL of full medium, retrovirus was added (1:3 dilution) and polybrene 10μg/mL. Plates were centrifuged (30 mins, 1000 X *g* at 37 ˚C). Cells were returned to the incubator. After 16h the medium was removed and replaced with full medium. After 48h, 3μg/ml puromycin was added for 72h to select cells. For co-expression of FcRγ-chain with FcγRs, 3T3 FcRγ-chain-expressing cells were repeatedly spinfected (>3 times) and tested for FcγR cell surface expression (see below). Once 100% of cells were expressing FcγR on the surface, no further spinfections were performed. Ad-IL10 and Ad-Mock were generated and purified as previously described (*4*). Briefly, the mouse *Il10* open reading frame cDNA sequence was cloned into the pAdhPGK vector. Adenoviruses were then generated by homologous recombination

followed by Adenovirus production in 293 cells. Ad-Mock was generated from the pAdhPGK vector without *Il10* ORF. Virus was purified from cells by freeze thaw cycling and successive banding on Cesium Chloride density gradient centrifugation. Adenoviral titers were calculated as plaque-forming units (pfu) on 293 cells.

In vitro experiments. Primary kidney fibroblasts were purified by FACS sorting from the single cell preparations of d7 UUO kidney of Coll-GFP mice, as described above. Coll1a1-GFP+ fibroblasts were sorted by FACSaria, and $2x10^5$ cells transferred to 1.3cm diameter wells and further cultured in DMEM/10% FBS, 1X ITS (Gibco). Cells were passaged (1:3) at 80% confluence and used between P3 and P7.

Binding experiments: $2x10^5$ PBMCs, $2x10^5$ bone marrow monocytes, $2x10^5$ bone marrow derived Mϕs, or 5 $x10^5$ kidney single cells, were washed in PBS three times, then incubated in 100 μL of HBSS/1%BSA, at 4 ˚C, for 30 min. with either hSAP-488 (25μg/mL), HSA-488 (25μg/mL) or no added protein. Cells were washed three times and fixed with 1% PFA for 1 min. prior to flow cytometric analysis. In some experiments leukocytes were simultaneously incubated with anti-CD11b-APC, or anti-CD11b-PE (1:500, Ebioscience) or anti-Ly6C-PE (1:400, ABD serotec) antibodies. In others 7-aminoactinomycin D (7-ADD, 1:1000, Molecular Probes) was added immediately prior to fixation. To test the specificity of binding of hSAP-488, BMD Mϕs were incubated with hSAP-488 (10μg/mL) and unlabeled hSAP (400μg/mL) or vehicle for 30min. at 4˚C, then washed three times with HBSS and fixed prior to analysis. In other

experiments, 10μg/mL of hSAP-488 was first incubated with BMD Mφs for 30mins at 4˚C, washed two times with PBS, then incubated with unlabeled 400μg/mL hSAP or vehicle for 30mins at 4˚C, followed by washing three times with HBSS and fixation prior to analysis. 3T3 cells lines singly expressing FcγRs or FcγRs and γ-chain, were incubated with hSAP-488 or HSA-488 identically as described above. In some experiments, 7-AAD (1:1000) was added immediately prior to fixation. Cells were washed and fixed as described above. In experiments to test binding of SAP to fibroblasts, $2x10^5$ primary fibroblasts were incubated with hSAP-596 or HSA-596 using the same methods as above. As a positive control, BMD Mφs were incubated in parallel. To study the binding of hSAP to apoptotic cells, human Jurkat T lymphocytes (ADCC) in serum free medium (RPMI) were exposed to UV irradiation (8W, 20min) and 2 $X10^5$ aliquots were incubated at the indicated timepoints following UV irradiation with hSAP-488 or HSA-488 as described above, then analyzed by flow cytometry for binding. All binding experiments were repeated at least 3X in independent experiments and generated similar to those presented.

Functional Experiments: Purified mouse bone marrow monocytes were cultured overnight in DMEM/F12 with 10% FCS or medium with mIFNγ (500 U/mL) in PTFE wells. After 16h monocytes were washed three times in serum free medium. $2x10^5$ of these bone marrow monocytes were transferred to wells of round bottom 96 well plates in 200μl of serum free DMEM/F12, in the presence of no additive, 25μg/mL hSAP or 25μg/mL HSA. In some experiments wells were pre-coated with 100μg mIgG, for 16h in PBS, and washed three times with PBS prior to addition of medium and monocytes (*5*). In other experiments, autologous apoptotic thymocytes were generated from thymus. In

brief, thymocytes were released from the thymus as described previously (*6*), then washed three times in serum free medium (SFM), exposed for 20min to UV irradiation (8W) then incubated for 16h. At 16h, complete apoptosis was confirmed (AnexinV-FITC, Propidium Iodide (*6*), data not shown). Apoptotic thymocytes were washed again then incubated with 25μg/mL hSAP for 1h or vehicle only. After further washing they were added to wells containing mouse monocytes as above (5:1 ratio). Control wells with thymocytes alone were used as a control. After 24h of incubation, monocytes were harvested for analysis. All binding experiments were repeated at least 3X in independent experiments and generated comparable results. Human PBMCs purified through ficoll hypaque and washed X3 in PBS, were cultured $5x10^5$ in 1cm diameter wells in serum free Fibrolife medium (Lifeline Cell technology, Walkersville, MD) containing 1% BSA,ITS-3 and P/S. hSAP (5μg/ml) or HSA was added to wells, and cells were incubated. Supernatants were collected at 12h, 24h, 48h, 72h, 96h and 120h. Supernatants were analyzed for secreted proteins including IL-10 by multi-analyte profiling (Rules-Based Medicine, Austin, TX). Human, mouse, rat (Sprague Dawley) and Cynomolgus monkey PBMCs purified through ficoll hypaque and washed X3 in PBS ($5X10^4$) were cultured in 0.3cm diameter wells in Fibrolife medium with ITS-3 and P/S for 120h, or in medium containing SAP (0.5-25μg/ml) (*7*). After fixing and staining wells fibrocytes are defined morphologically as adherent cells with an elongated spindle-shape and the presence of an oval nucleus, and counted in 20 randomly selected fields as described (5). 3T3 cell lines expressing FcRγ-chain alone, or FcRγ-chain with FcγRI, II, III or IV were incubated in SFM in 1.2 cm wells. Apoptotic thymocytes were labelled with CMFDA (Molecular Probes) as previously described (*2*) and preincubated with 25μg/ml hSAP or no additive

for 1 hour prior to incubation with 3T3 cell lines (5:1 ratio). Co-cultures were incubated for 4h at 37 ˚C, in DMEM/F12 containing 0.1% Ig-free BSA. Cells were washed three times with 5mM EDTA to dissociate non-phagocytosed cells, then fixed with 1% PFA in FACS buffer, , and analyzed by flow cytometry for phagocytosis, as described (*2*) Experiments were repeated at least 3X and gave comparable results. In other studies monocytes were purified from γ-chain$^{-/-}$ mice and compared with strain matched control mouse monocytes. $2x10^5$ leukocytes were incubated with IFNγ or no cytokine for 16h, then transferred to 96 well plates uncoated or precoated with immobilized IgG as described above, or incubated with 10 ng/mL LPS. Samples were harvested for analysis 24h later.

Coll-GFP primary kidney fibroblasts 70% confluence in 3cm diamteter wells were incubated in full medium with recombinant mIL10 (Pierce) or mIL10 from adenoviral supernatants, at doses from 1ng/ml to 50ng/ml for 8h, 24h and 48h. Cells were harvested and assessed for GFP fluorescence by flow cytometry.

Detection of Proteins

Western Blotting: Western Blots were performed using standard Methods as previously reported (*2*). For IL10 detection, SDS-PAGE, 40μg of protein from whole kidney lysates per lane was applied, and blots were incubated with rat anti-mouse IL10 (1:1000 R&DSystems MAB417) in blocking buffer rabbit followed by goat anti-rat –HRP conjugated antibodies (1:10,000). To detect hSAP, on blots from whole kidney lysates, anti-hSAP antibodies (1:1,000,000 dilution, Abcam ab 45151) were incubated overnight,

followed by HRP conjugated goat anti rabbit antibodies. Blots were stripped and βactin was detected as described.

ELISA: To detect, IL10, ELISA was performed on mouse plasma samples (diluted 1:1000 with reagent diluent) using a mouse IL10 ELISA kit from R&D Systems according to manufacturers instructions. To detect hSAP in human plasma samples, all plasma samples were collected in citrate tubes, separated from cells and stored at -80C. All samples underwent a single freeze thaw cycle only. 96 well plates (Corning Incorporate COSTAR 3590) were incubated with 100μl of capture antibody solution (mouse monoclonal to SAP Ab 27313 1mg/ml/10 ml in PBS pH 7.3-7.4) overnight 4C. After washing (0.05% Tween in 1X TBS pH 7.4), and blocking (4% BSA in 1X TBS+0.1% Sodium azide), plasma samples (duplicates) (100μl/well) diluted 1:5,000 or 1:20,000 with sample buffer (4% BSA in 1X TBS+0.1% Sodium azide) were applied and incubated 2h RT. After washing, detection antibody (Polyclonal Rabbit α-human SAP Ab (Calbiochem, 565191, 1:5000) was applied in 100μl blocking buffer 2h RT, then after further washing, anti-rabbit-HRP was applied in 100μl of blocking buffer (Peroxidase Conjugated Goat Anti Rabbit Jackson Immuno Research 111-035-144) 0.8 ng/ml) 1h RT. The composite sandwich in each well was then incubated with TMB solution (100μl, Promega, Cat # G7431),), for up to 5min until sufficient color had developed. The reaction stopped with hydrochloric acid (50μl) and the plate read at 450nm by Spectrophotometer. Samples were compared with a standards prepared from purified hSAP (hSAP-Calbiochem, 565190: 2000ng/ml top standard) in sample buffer. All samples were assessed on twice on separate plates for internal consistency.

Surface plasmon resonance for binding affinities. All surface plasmon resonance experiments were conducted using a Biacore X100 instrument using Biacore CM5 chips. Human FcγRs (R&D Systems) were recombinant forms of the extracellular domains of each receptor. For experiments using FcγRs or human SAP immobilized to the CM5 chip, Biacore's standard amine coupling procedure and reagents were used. For experiments using human SAP bound to the chip CM5 dextran, SAP capture followed preincubation at 0.5 mg/ml in 10 mM Hepes, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.4, and capture and kinetics experiments used the same buffer, with 0.1% (w/v) HSA added to dilutions of the 'analyte' (the binding partner in solution). Single-cycle kinetics methods and analysis on the Biacore X100 were used to obtain on-rate and off-rate constants and affinity of SAP binding to the dextran. For experiments to obtain affinities for SAP binding to the different FcγRs, dextran binding of SAP was stabilized by a very short immobilization using amine coupling (Biacore reagents). HSA covalently coupled to the same level as either FcγR or SAP on the test flow cell was used as the negative control flow cell in FcγR / SAP kinetic experiments. Single-cycle kinetics methods and analysis on the Biacore X100 were used to obtain affinities of SAP binding to each FcγR. With single-cycle kinetics, the need to find conditions to regenerate the chip surface between 'analyte' applications is obviated. Affinity calculations were obtained using the Biacore X100 software and best fit analysis.

Branched-chain DNA amplification quantification of mRNA. Cells were lysed with a proprietary cell lysis solution containing 50% Lysis Mixture (Panomics, Inc., Freemont, CA) and 1 mg/mL proteinase K at a ratio of 400 cells/μL (Panomics, Inc.) and to a final volume of up to 160 μL. The mixture was incubated at 50 °C for 30 min. and cells were then mechanically lysed. The lysate was stored at –80 °C. mRNA transcripts were analyzed in duplicated in 96 well plates. Target gene probe sets specific to the designated genes were designed and tested for sensitivity and specificity prior to use by the manufacturer (Panomics, Inc.). Gene probe sets containing capture extender probes (CE), label extender probe, and probe-specific fluorescently conjugated magnetic beads were added (up to 20 different gene targets per well) and hybridized for 18h at 54 ˚C in a shaker incubator (600rpm, Labnet Vortemp 56). Following hybridization, samples were transferred to a filter plate, washed three times in wash buffer, then hybridized with 2.0 preamplifier working reagent in 100 μL, for 1h at 50 ˚C, 600rpm. After washing twice, a further amplifying hybridization was performed with 2.0 amplifier working reagent for 1h at 50˚C, 600rpm. Following further washes a final hybridization with 100 μL label extender probe, which adds biotinylated sites to the amplified hybridization sandwich, was performed for 1h at 50˚C, 600rpm. Next, after washing, hybridized beads were incubated with 100μL Streptavidin-conjugated R-Phycoerythrin for 30 min at 25˚C, 600rpm. Two further wash steps were performed before beads were resuspended in 130 μL buffer. Beads in each well were analyzed using a Luminex Instrument and the Bio-Plex 5.0/Bio-Rad platform. At least 100 beads in each well for each gene of interest were assessed. In general the CV for each gene for each well was < 5%. The intensity of PE fluorescence signal associated with individual capture beads was detected by the

Luminex flow cytometer (Bio-Rad). The signal is reported as mean fluorescence intensity and is proportional to the number of target RNA molecules present in the sample. The linear range for fluorescence intensity for these muliplex beads is 10-4000. Samples were presented normalized to housekeeping gene HPRT-1. For quantification of *Il-10* transcripts qPCR of cDNAs generated from mRNA from isolated kidney Mϕs was performed using methods previously described (*8*). *Il-10* samples were normalized to GAPDH. b-DNA data and q-PCR data represent duplicate experiments analysed in duplicate. Studies were repeated and gave comparable results.

Statistics for human studies. Continuous data was analyzed with non-parametric test and categorical data was analyzed with Chi-square statistic in the analysis of demographic characteristics. The level of SAP, serum creatinine, GFR, urine albumin and deviated from normal distribution and hence the median was reported along with mean and standard deviation. Scatter plots were created for pairs of continuous variables and correlation was calculated using Spearman correlation coefficient. A *P*-value less than 0.05 were considered significant. SPSS version 17 (SPSS Inc, Chicago, IL) was used to analyze the data.

SUPPLEMENTARY REFERENCES

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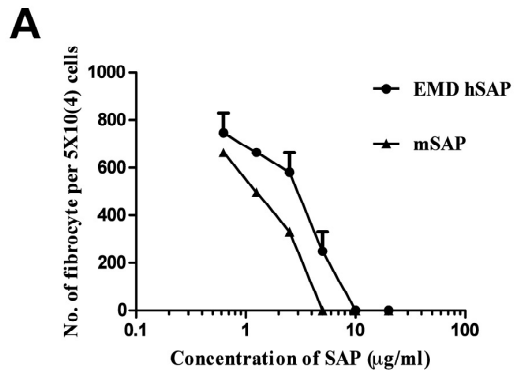
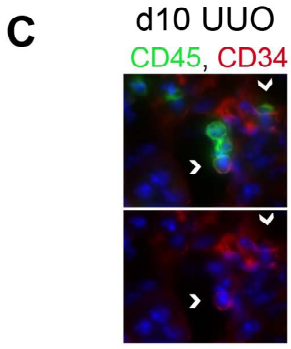
SUPPLEMENTARY FIGURES



B

Estimated IC50 (µg/ml)

Species	hSAP (EMD)	Mouse SAP	Rat SAP
Human	0.15	-	-
Cyno	0.2	-	-
Rat	0.2	-	0.2
Mouse	3.0	2.0	-



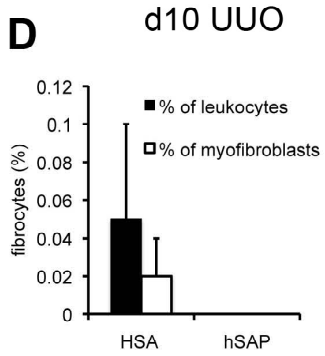


Fig S1. Fibrocyte characterization and response to SAP *in vitro* and *in vivo* in mouse kidney UUO disease. (**A**) *In vitro* culture (5days) of mouse monocytes in fibrolife medium results in fibocyte morphology which is significantly inhibited by hSAP and mouse SAP at concentrations of >5µg/ml. (**B**) Table showing the IC_{50} of mouse, rat, human, on inhibition of fibrocyte morphology *in vitro*. (**C-D**) Photomicrographs (**C**) showing fibrocytes (detected by co-expression of CD45 and CD34) in the d10 UUO kidney of C57BL6 mice treated with HSA. (**D**) % of fibrocytes expressed as percentage of CD45+ leukocytes or percentage of myofibroblasts detected in d10 UUO kidneys treated with HSA or hSAP.

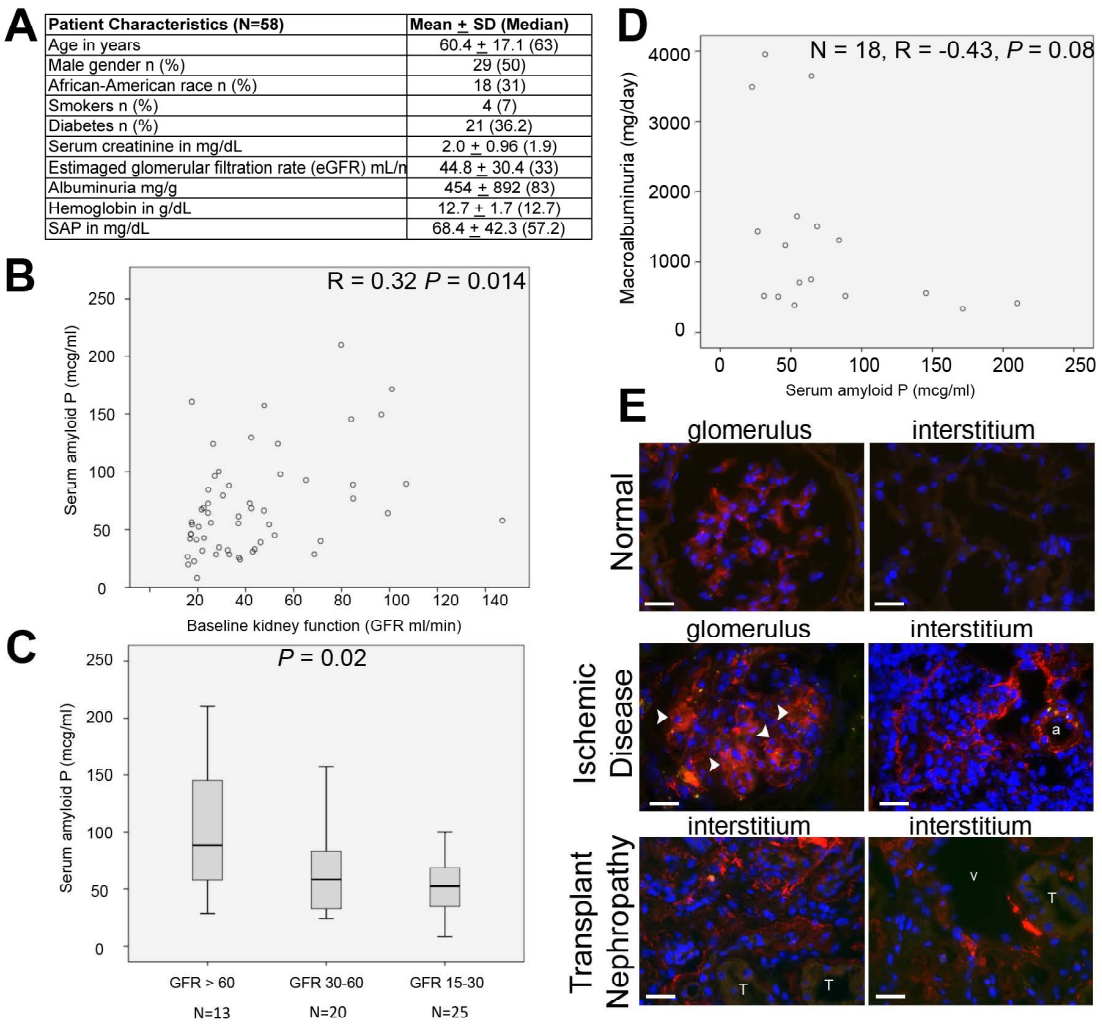


Fig S2. SAP plasma levels correlate with kidney function and inversely with protein leak in a cohort of patients with kidney disease. (**A**) Chart showing characteristics of patients. (B) Scatter plot of plasma SAP concentration at presentation with kidney function expressed as glomerular filtration rate (GFR). (**C**) Plasma SAP concentration (median, 25th and 95th CI) in tertiles according to kidney function >60ml/min normal, 30-60ml/min moderately reduced, and 15-30ml/min severely reduced. (**D**) Scatter plot of plasma SAP concentration in those patients with albumin leak detected in urine (macroalbuminuria). (**E**) Photomicrographs of human kidney biopsies from normal

kidney and patients with ischemic nephropathy or transplant nephropathy showing SAP deposition in normal glomerular basement membrane, but none in normal kidney interstitium. However, SAP was seen associated with areas of sclerosis/fibrosis of the diseased glomerulus (arrows), interstitial debris and pathological tubular basement membrane in the diseased interstitium and areas of sclerosis in glomeruli.

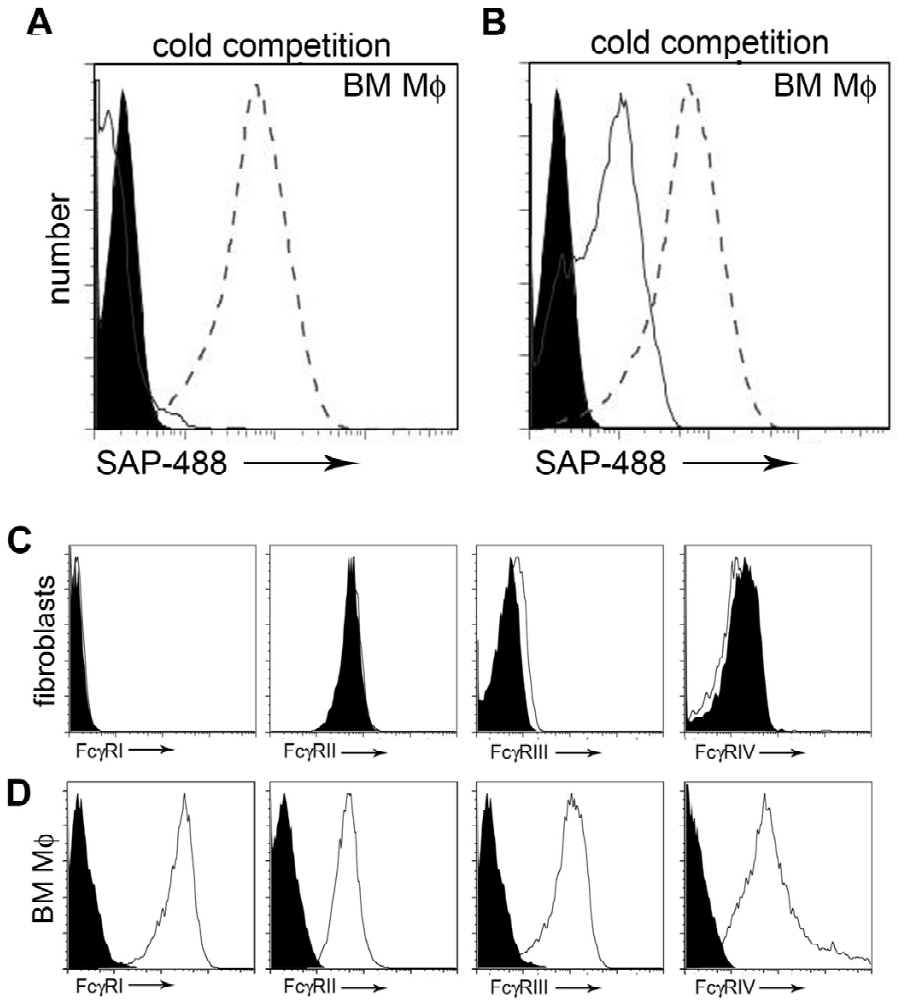


Fig S3. Characterization of binding SAP-Alexa488, to macrophages. (**A**) Histogram showing that 40 fold excess of unlabeled hSAP (gray unfilled) completely inhibits binding of SAP-488 (dotted unfilled) to d7 cultured BMD Mϕs. HSA-488 binding is shown (black solid). (**B**) Histogram showing that 40 fold excess of unlabeled hSAP (solid, unfilled) applied after SAP-488 has bound (dotted, unfilled) to BMD Mϕs promotes partial dissociation of SAP-488 from the Mϕ cell surface. HSA-488 binding is shown (black solid). (**C**) Histograms showing FcγR expression by cultured mouse fibroblasts (specific antibody unfilled vs control antibody black) (**D**) Histograms showing

FcγR expression by mouse d7 BM derived Mϕs (specific antibody unfilled vs control antibody black).

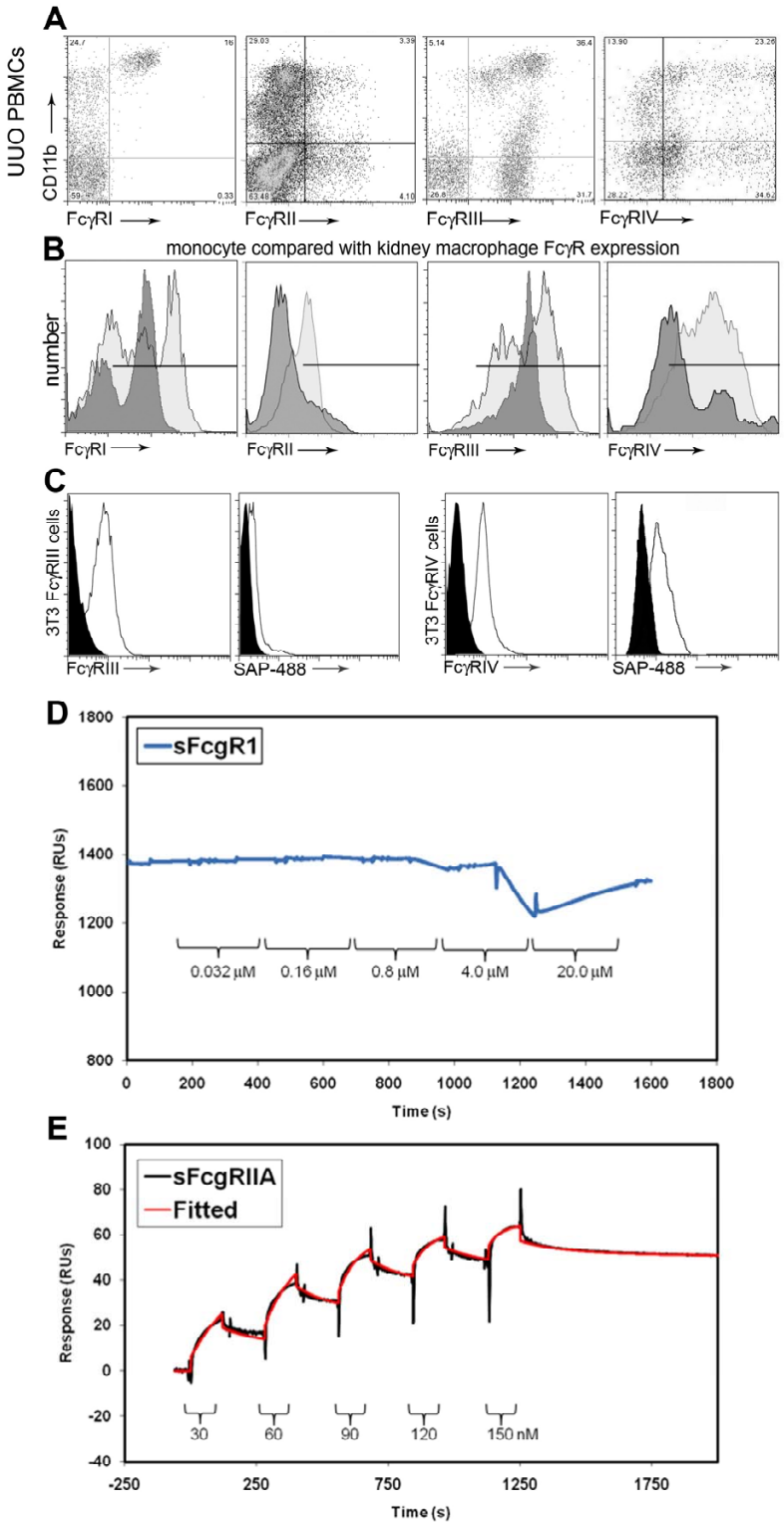

A
UUO PBMCs
CD11b
FcγRI
FcγRII
FcγRIII
FcγRIV
B
monocyte compared with kidney macrophage FcγR expression
number
FcγRI
FcγRII
FcγRIII
FcγRIV
C
3T3 FcγRIII cells
FcγRIII
SAP-488
3T3 FcγRIV cells
FcγRIV
SAP-488
D
sFcgR1
Response (RUs)
0.032 µM
0.16 µM
0.8 µM
4.0 µM
20.0 µM
Time (s)
E
sFcgRIIA
Fitted
Response (RUs)
30
60
90
120
150 nM
Time (s)

Fig S4. Expression of Fcγ receptors by monocytes and macrophages during kidney disease and binding characteristics of Serum Amyloid P to Fcγ receptors. (**A**) FACS plots of PBMCs from UUO d7 mice showing separation of leukocytes by CD11b and FcγRI, II, III, or IV. CD11b high cells are monocytes. (**B**) Histogram plots comparing expression of FcγRI, II, III, or IV by CD11b+ inflammatory monocytes (dark gray) with purified kidney Mϕs (light gray) collected d7 after UUO. Marker denotes positive signal compared with isotype control. (**C**) FcγRIII and FcγRIV cell surface expression, and SAP-488 binding in 3T3 fibroblast cells in absence of FcRγ chain. (**D**) Biacore affinity sensorgram of hFcγR1 ectodomain for amine coupled SAP SAP to the CR5 chip. (**E**) Sensorgram of single-cycle kinetic analysis of hFcγRIIA binding to chip-bound hSAP oriented through Ca^{++}-dependent binding to the CM5 dextran. Five different receptor concentrations injected in order of increasing concentration were used to obtain data for affinity calculation. Association time was 180s and the final long dissociation was 3100s for hFcγRIIA (attenuated for presentation). Both raw data (black) and data fitted for 1:1 binding (red) are shown on the same sensorgram and represent data after background subtraction. Off-rate is very slow and drives the high affinity of interaction we observed. Results are representative of three independent experiments for each FcγR.

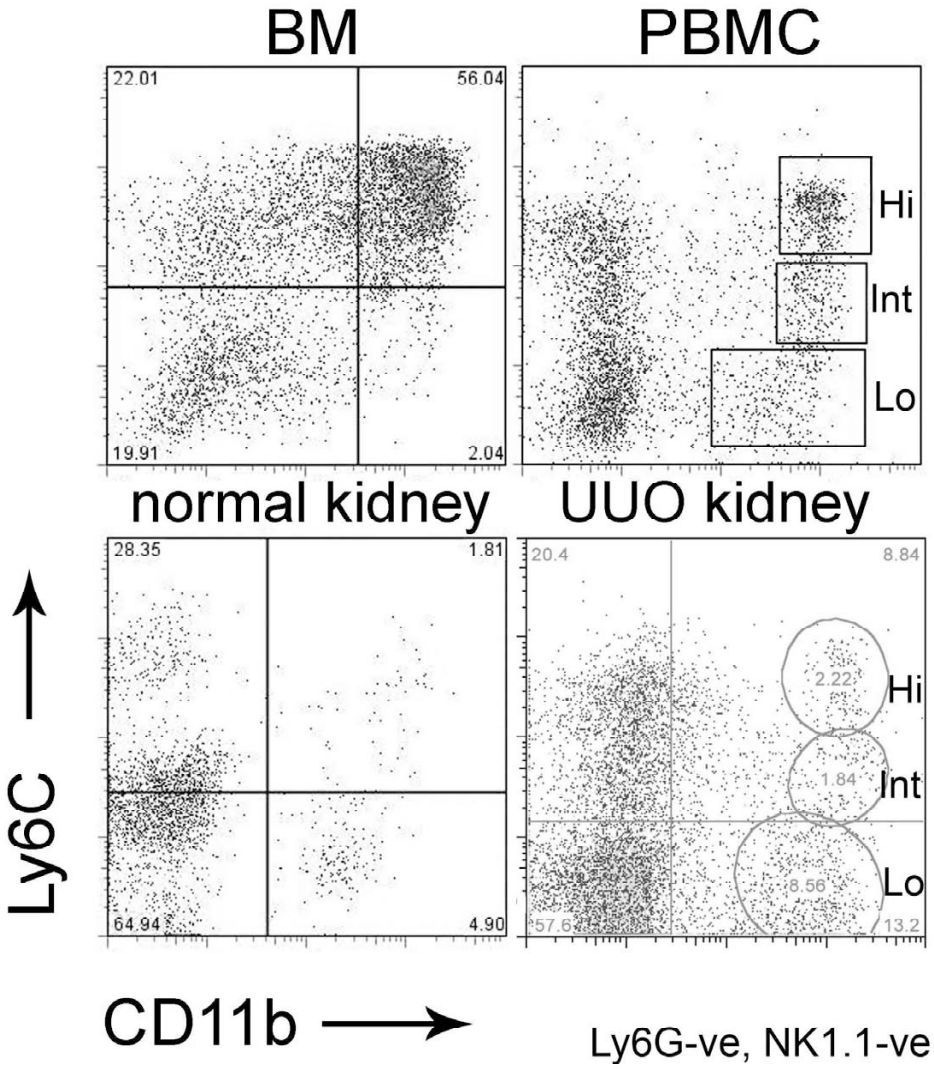


Fig S5. Characterization of monocytes and macrophage subpopulations by Ly6C expression. FACS plots of leukocytes from bone marrow (BM), blood (PBMC), normal and UUO diseased kidney separated by Ly6C and CD11b expression. Leukocytes are pre-gated to be negative for expression of Ly6C or NK1.1. Note high, intermediate and low level expression of Ly6C is seen in PBMCs and also in kidney inflammatory macrophages.

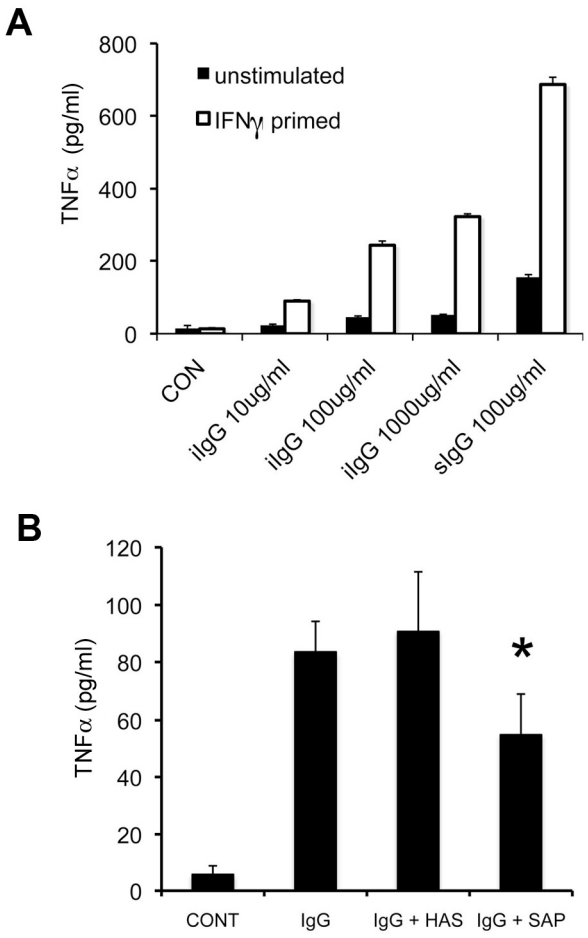


Fig S6. Soluble IgG activates monocyte/macrophages and SAP alone weakly inhibits mouse monocyte/macrophage activation. (A) d7 cultured BMD Mϕs unprimed or primed with IFNγ 16h prior to the experiment were activated with immoblilized or soluble IgG and supernatants were assessed for TNFα secretion (ELISA). Note sIgG in addition to iIgG activates Mϕs. (B) d7 cultured BMD Mϕs activated with iIgG (100μg/ml) and soluble HSA or hSAP (25μg/ml) were assessed 24h later for Tnfα secretion (ELISA). Note hSAP specifically inhibits Tnfα release.