Oncogenic D816V-KIT signaling in mast cells causes persistent IL-6 production

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Supplementary Appendix

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Supplementary Methods

Inhibitors and antibodies

Anti-IL-6R tocilizumab (Actemra®) was obtained from Genentech (CA, SA). The PI3K inhibitor LY294002, was from Calbiochem EMD Millipore (CA, USA). Inhibitors of MAPK, (U0216), JAK (ruxolitinib phosphate), p38 (SB203580) and EGFR (gefitinib) were purchased from MCE MedChem Express (NJ, USA). The JAK2 inhibitor fedratinib (TG101348) and JAK3 inhibitor tofacitinib were from Selleckchem (TX, USA). Inhibitors of STAT3 (C188-9) and STAT5 (CAS285986-31-4), and the anti-β-actin antibody were from Millipore Sigma (MI, USA). Anti-pKIT(Y823), anti-JAK2, anti-ERK, anti-STAT4, anti-STAT5, anti-pSTAT5(S780) anti-CD3-QDOT605 and Fc receptor binding inhibitor polyclonal antibody were from ThermoFisher Scientific (IL, USA). Anti-KIT was from Santa Cruz Biotechnology (TX, USA). Anti-CD34-APC was from BD (CA, USA). Anti-KIT-BV421 and anti-FcεRI-FITC were from BioLegend (CA, USA). Anti-pJAK2(Y1007/1008), anti-AKT, anti-pAKT(Y308), anti-pERK(Y202/204), anti-STAT3, anti-pSTAT3(Y705), anti-pSTAT4(Y693) and anti-pSTAT5(Y694) were from Cell Signaling. Anti-gp130 was from R&D Systems (MN, USA). Antagonists for the S1P receptors $S1P_1$ and $S1P_3$ (VPC 23019), $S1P_2$ (JTE 013), and $S1P_4$ (CYM 50358) and the TGFβR antagonist SD208 were form Tocris (UK).

Cell cultures and cell lysates

The human mast cell lines HMC-1.1 and HMC-1.2 were kindly provided by Dr. JH Butterfield (Mayo Clinic, Rochester, MN, USA)[1] and cultured as described.[2] HMC-1.1 carries a variant in the juxta membrane domain of KIT (V560G) and HMC-1.2 harbors KIT with D816V plus the V560G variant [3]. An immortalized murine mast cell line that does not express mouse KIT, MCBS-1, was stably transfected with human WT and D816V-KIT and cultured as described.[4] The murine mastocytoma cell line P815 was from ATCC and cultured as specified by the provider. BM-derived mast cells (BMMCs) from C57BL/6 mice were cultured as described [5]. The human colorectal carcinoma cell lines HCT116 modified or not by CRISPR to introduce a D816V-KIT mutation, were purchased from ThermoFisher Scientific (CA, USA) and cultured in RPMI with 10% FBS, 2 mM L-Glutamine and 25 mM Sodium Bicarbonate. LAD2 human MCs were cultured in StemPro-34 Complete Medium (Invitrogen, NY, USA) with SCF (100 ng/mL) (R&D Systems, MN, USA), as described.[6]

To obtain lysates for western blots, $3x10^6$ cells were plated in 6 well plates and incubated with the indicated inhibitors for 2 h in serum-free media. Cells were then washed with PBS and lysed in lysis buffer [150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml leupeptin, complete protease inhibitor cocktail (Roche, Indianapolis, IN) and 1% NP-40] for 15 min as described.[7]

Stimulation of LAD2 cells by HMC-1.2 conditioned media

HMC1.2 conditioned media (serum-free) from overnight cultures under the conditions described below were collected after centrifugation at 1100 x rpm for 5 min and added to $2x10^6$ LAD2 cells plated in 6 well plates. LAD2 cells were previously starved in cytokine free media for 4 h at 37ºC. HMC-1.2 conditioned media (5 mL) were added to LAD2 cells for 30 min in the presence or absence of the neutralizing IL-6R antibody tocilizumab (100 µg/mL). Tocilizumab was added during the final 20 minutes during the starvation period as well as during the incubation with conditioned media. Recombinant IL-6 (50 ng/mL) was used as positive control. Plates were then transferred to ice for 2 min and cell pellets were obtained and washed once with cold PBS. Cells were lysed on ice in 80 µL of lysis buffer (100 µM Tris, 150 mM NaCL, 1% triton-X100, a cocktail of protease inhibitor (cOmplete™ ultra mini, EDTA free; Millipore-Sigma) and a cocktail of phosphatase inhibitors (Phosptop; Millipore-Sigma)). Protein content in clarified lysates was measured using Pierce BCA protein assay (Thermo Fisher) and 50 µg of proteins were loaded on to SDS PAGE gels. STAT3 and pSTAT3-Y705 contents were determined by Western blotting to evaluate IL-6R activation.

Measurement of IL-6 secreted into the media

HMC-1, BMMCs, P815, HCT116 or MCBS-1 cells ($3x10^6$), were plated in 6 well plates for 2 h to 24 h in 6 mL of serum-free media, as indicated in the figure legends. The experiments were performed in the absence of FBS to exclude the possibility that any extrinsic stimulant present in the serum would influence results. Aliquots of the supernatant were kept at -80ºC and the amounts of IL-6 measured by ELISA (R&D Systems). HCT116 cells were stimulated with 20 ng/mL PMA plus 1 µM ionomycin overnight and the supernatants then collected for IL-6 measurements. In some experiments, we determined the fraction of released IL-6 versus the amount remaining in cells in the presence or absence of various stimulants. HMC-1.2 cells (3×10^6) were cultured overnight in serum-free media containing a protease inhibitor cocktail (cOmplete™ ultra mini, EDTA free; Millipore-Sigma) alone or in combination with one of the following stimuli: the secretagogue compound 48/80 (C48/80) (500 ng/mL); complement component 5a (C5a) (500 ng/mL); PMA (20 ng/mL) and Ionomycin (1 µM); lipopolysaccharide (LPS) (10 µg/mL); IL-1β (100 ng/mL); or media containing 10% FBS. Cells were centrifuged at 1100 rpm for 5 min and IL-6 in the supernatants measured by ELISA. Cell pellets were washed and resuspended in 500 µL of cold PBS containing the protease inhibitor cocktail, freeze-thawed 5 times and clarified by centrifugation at 14,000 rpm. IL-6 amounts in the resulting supernatants (intracellular IL-6) were also determined by ELISA.

For measurement of IL-6 released by human BM cells, mononuclear cells from BM aspirates from subjects described in Supplementary Table 1 were cultured in StemPro-34 medium with human recombinant SCF (100 ng/mL) and supernatants collected after 2-4

days. In some experiments, the number of mast cells present in these cultures was determined by flow cytometry as indicated below.

Determination of single-cell IL-6 expression in cultures from bone marrow aspirates

Mononuclear cells from the BM aspirates were cultured ($1x10^6$/mL) as described above for 2-4 days. BM cells were then incubated with Brefeldin A for 4 h (37ºC) and washed with PBS+1% BSA. Fc receptors were blocked with Fc blocking antibody (1:100) for 8 min and cells were stained with 100 µL of an antibody cocktail containing anti-CD3-QDOT605, anti-CD34-APC, anti-KIT-BV605 and anti-FcεRI-FITC, for 30 min at room temperature. Cells were then fixed with 4% paraformaldehyde for 8 min at room temperature and permeabilized with 150 µL of BD Perm/Wash buffer (BD). Expression of IL-6 was detected by staining the cells with anti-IL-6-PE for 30 min, using a LSRII flow cytometer (BD Biosciences, Sparks, MD) and analyzed using FlowJo software (Tree Star, Ashland, OR). $CD3^-$/$CD34^-$/KIT^+/$FcεRI^+$ (mast cells) were gated, and IL-6 positive cells analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence staining of intracellular IL-6 in bone marrow specimens

Bone marrow trephine biopsies were fixed in B-5 fixative, decalcified in EDTA and embedded in paraffin using standard procedures. Sections were deparaffinized in xylene and rehydrated using an alcohol gradient. Following washes in water and PBS, samples were steamed for 20 minutes in antigen retrieval solution (Tris-HCl/EDTA; pH 9.0 (Vector Labs, Burlingame, CA, USA)). After washing with PBS, slides were blocked with immunofluorescent blocking buffer (0.2% Triton X-100, 0.2% BSA, 0.2% casein, 0.2% gelatin, and 0.02% sodium azide) containing 5% donkey serum. Slides were then probed with anti-mast cell tryptase (10 µg/mL; Abcam# ab2378) and anti-IL-6 (5 µg/mL; Abcam# ab214429) antibodies in immunofluorescent blocking buffer containing 1% donkey serum and incubated at 4ºC overnight. Slides were washed 3x in PBS and probed with the appropriate secondary antibodies (donkey anti-rabbit Alexa-488 and donkey anti-mouse Alexa-568, Molecular Probes, Thermo, Rockville, MD, USA) in immunofluorescent blocking buffer containing 1% donkey serum for 1 h at room temperature. Next, samples were washed 3x in PBS and stained using DAPI/PBS (1 µg/mL) for 20 minutes at room temperature, washed 3x PBS, and mounted in PermaFluor mounting medium (Thermo Scientific). Samples were visualized using a Zeiss 710 Meta confocal microscope using a 40x oil objective. Laser settings for the capture of images and adjustments of levels and brightness were identical among specimens. Imaging software used for visualization was Zeiss Zen (Carl Zeiss Microscopy, Thornwood, NY, USA) and Adobe Illustrator (Adobe Systems, San Jose, CA, USA).

Quantitative real-time PCR

HMC-1.2 cells ($3x10^6$) were plated in 6-well plates in 6 mL and incubated for 2 h in serum free media. RNA was then isolated from cells with RNeasy Plus Mini Kit (Qiagen, KY,

USA) following the manufacturer's protocol and cDNA was synthetized by reverse transcription using the SuperScript III First-Strand kit (Thermo Fisher Scientific, CA, USA) with random hexamers. The corresponding cDNA was amplified in a CFX96 Touch™ Real-Time detection system (Biorad®), using TaqMan® Gene Expression Master Mix and Taqman® Gene Expression Assays for IL-6 (Hs00985639_m1), STAT3 (Hs01047580_m1), STAT4 (Hs01028017_m1), STAT5A (Hs00559637_g1), STAT5B (Hs00560026_m1) or GAPDH (Hs99999905_m1) following the manufacturer's standard protocol (Thermo Fisher Scientific, CA, USA). Analysis of the data was performed using CFX Maestro Software and relative expression was calculated with the ΔCt method with GAPDH as the housekeeping gene.

Knockdown of STAT3 and STAT4 transcription factors by sh-RNA

Bacterial glycerol stocks expressing lentivirus plasmids (MISSION-pLKO-1-puro) with inserts of small hairpin RNA (sh-RNA) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). The lentiviral pLKO-1-puro plasmids contained sh-RNA sequences specific for STAT3 (TRCN0000020840), STAT4 (TRCN0000020894: construct #1; and TRCN0000020895: construct #3), STAT5A (TRCN0000019304, TRCN000019306 and TRCN0000019308), STAT5B (TRCN0000232141, TRCN0000232140 and TRCN0000232137), or non-target control vector (SHC002). These plasmids were isolated from the corresponding bacteria cultures using QIAprep Spin Miniprep Kit (Qiagen). To produce the lentiviral particles, HEK 293T ($4x10^6$ cells) were co-transfected with the corresponding purified lentiviral vectors (3.4 μg) and plasmids coding for key packaging and structural viral proteins (lentiviral packaging mix from Sigma-Aldrich) (34 μL) using the FuGENE6 transfection reagent (21 μL) (Roche, Indianapolis, IN) in 236 μL of RPMI. A day after transfection, the culture media was replaced with fresh media and collected 24 h later, when most of the viral production takes place. The media containing the viral particles was centrifuged at 2,000rpm for 10 min to eliminate cell remnants and then aliquoted and frozen at -80C until use.

For sh-RNA knockdown, $0.3x10^6$ HMC-1.2 cells were plated in 0.5 mL of media in 48-well plates and the lentivirus preparations (250 μl) were added. All lentiviral preparations were tittered for their effects on the gene target and 250 μL was usually within the optimal range. Two days after transduction, cells were washed, incubated for 2 h in serum-free media and tested for IL-6 expression. Of note, all lentiviral plasmids caused substantial reductions in the intended targets, except for the three constructs tested for each STAT5A and STAT5B which all proved ineffective in these cells.

Knockdown of STAT5 transcription factor by si-RNA

To knockdown STAT5 by small interference-RNA (si-RNA), the si-RNA constructs were introduced into the cells by electroporation using a nucleofector 2b device from Lonza (Cologne, Germany) (program T-020). HMC-1.2 cells ($2x10^6$) were washed with PBS and resuspended in 100 μL of nucleofector Kit V solution (Lonza) containing 2 μM of a si-

RNA "ON-TARGET" pool for human STAT5A (L-005169-00-0005), STAT5B (L-010539-00-0005), or a non-targeting si-RNA pool (D-001810-10-05) from Dharmacon (Lafayette, CO). Transfected cells were kept in culture at 37ºC in 5% CO_2 for 72 h. Cells were then washed, incubated in serum free culture media for 2 h and their cellular protein or RNA contents extracted.

Supplementary Table 1- D816V-KIT allelic frequency and tryptase levels in the patients used in this study

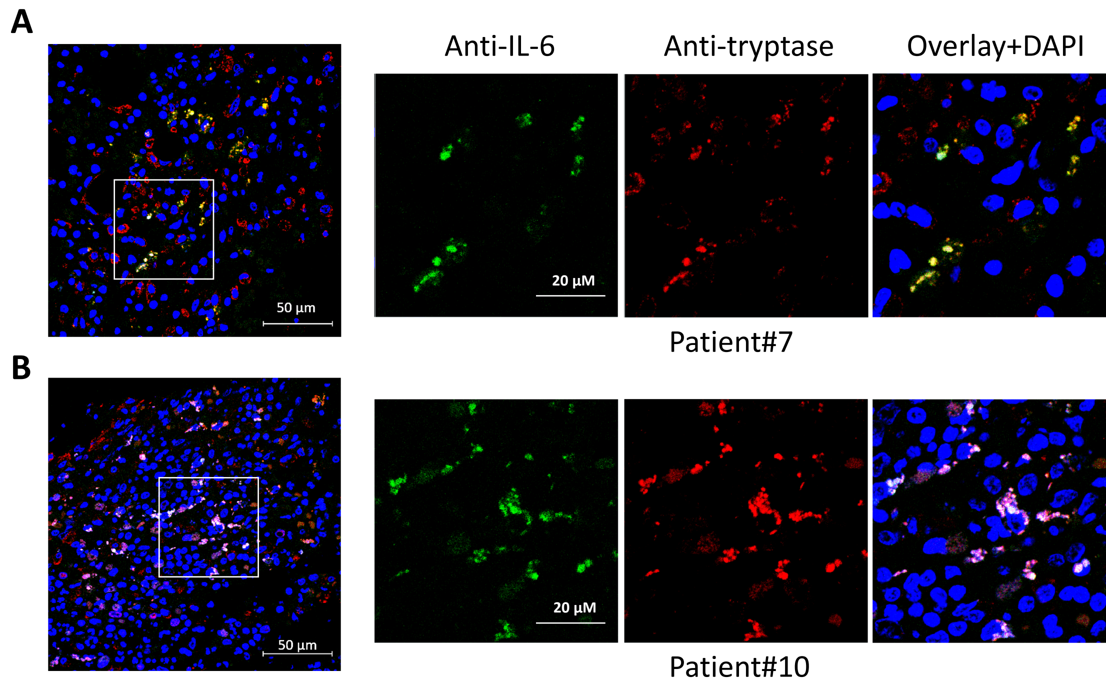
Patient number	Diagnosis	Sex	BM D816V-KIT Frequency (%)	Serum Tryptase (ng/mL)
1	IA*	F	Negative	4
2	ISM	M	2.74	35
3	ISM	M	5.50	182
4	ASM	F	1.41	479
5	ISM	F	0.35	105
6	ISM	F	31.08	293
7	ISM	M	13.44	291
8	ISM	M	0.06	14
9	ISM	M	0.03	10
10	SSM	M	44.66	590

*BM aspirates were obtained from this patient with idiopathic anaphylaxis (IA) during an evaluation for possible SM. SM was not diagnosed in this patient and thus findings were used as a control in this study. ISM: indolent systemic mastocytosis; SSM: smouldering systemic mastocytosis. Patients were classified according to the WHO criteria [8-11]

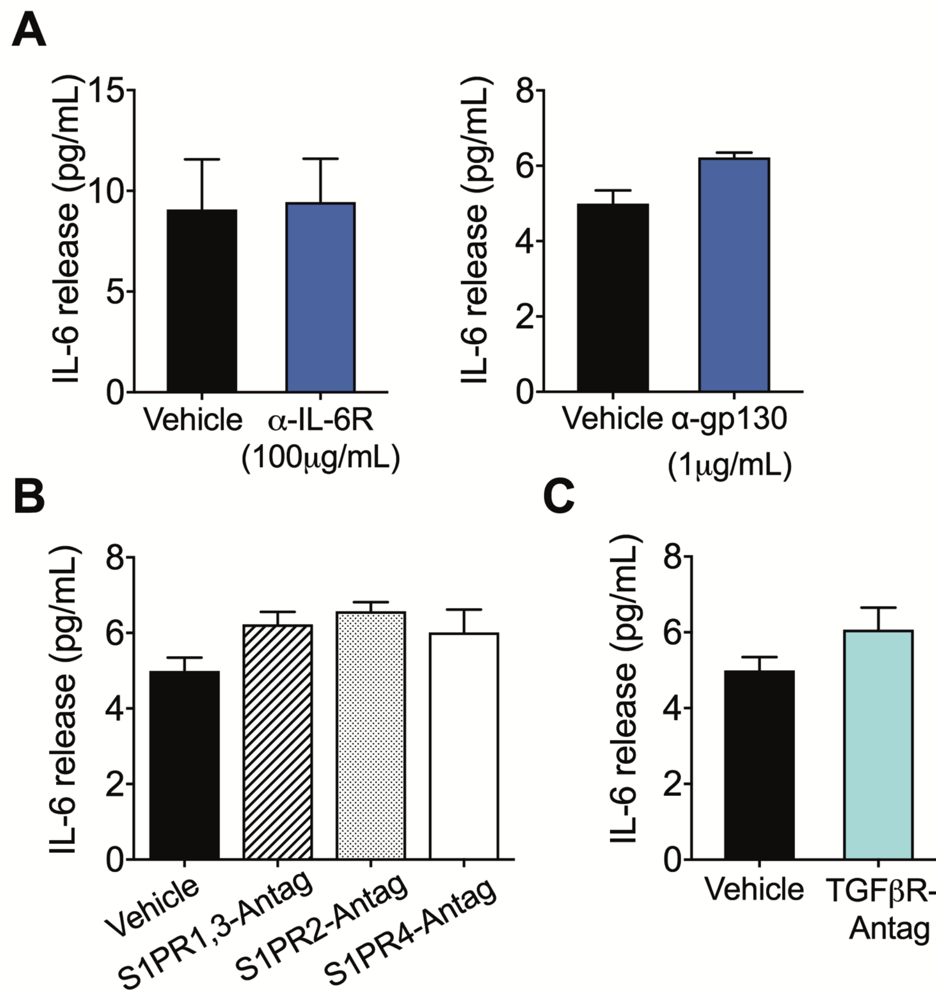
Supplementary Table 2- Intracellular IL-6 staining of bone marrow cells in patients with SM

Patient / D816V-KIT Frequency (%)	$KIT^+/Fc\varepsilon RI^+$		$KIT^+/Fc\varepsilon RI^-$		$KIT^-/Fc\varepsilon RI^+$		$KIT^-/Fc\varepsilon RI^-$	
	IL-6 MFI	% $IL6^+$cells	IL-6 MFI	% $IL6^+$cells	IL-6 MFI	% $IL6^+$cells	IL-6 MFI	% $IL6^+$cells
1/ (0.0%)	-	21	-	17	-	20	-	17
2/ (2.7%)	336	74	167	56	254	61	117	47
3/ (5.5%)	3371	98	768	88	524	86	522	73

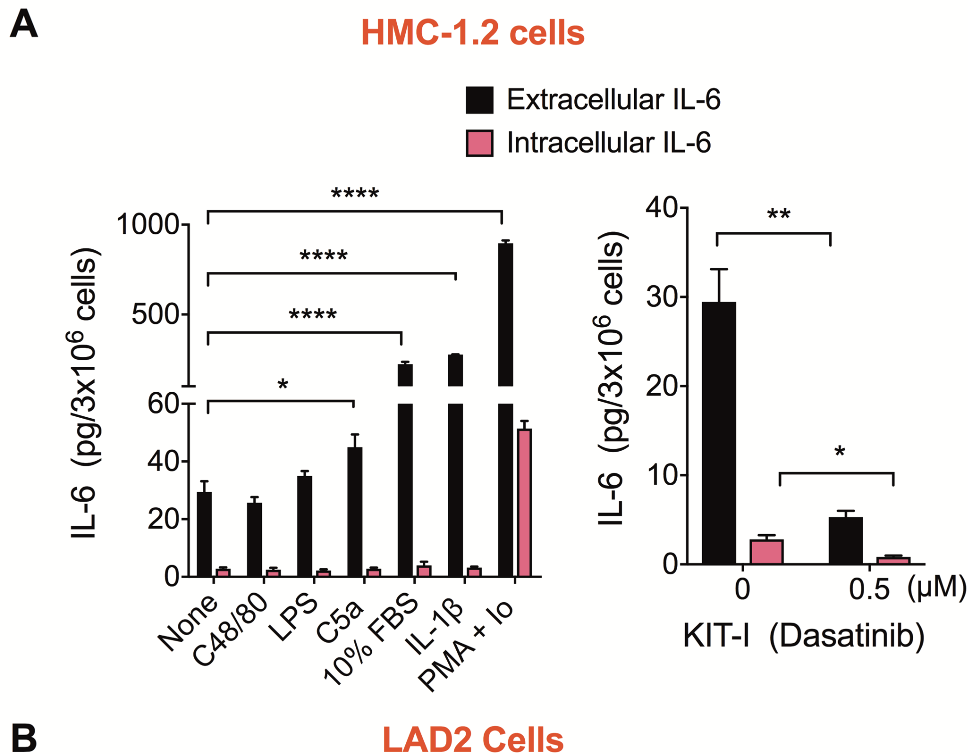
BM cells were cultured for 3-4 d and intracellular IL-6 staining in Brefeldin A- treated cells was analyzed by FACS. $CD3^-/CD34^-$ cells were gated and the mean fluorescence intensity (MFI) of intracellular IL-6 and the percentage of cells with IL-6^+ staining determined within each population ($KIT^+/Fc\varepsilon RI^+$, $KIT^+/Fc\varepsilon RI^-$, $KIT^-/Fc\varepsilon RI^+$ and $KIT^-/Fc\varepsilon RI^-$). Patients 1-3 are the same as shown in Figure 1C and as defined in Supplementary Table 1. Patient 1 had idiopathic anaphylaxis and did not meet criteria for SM but was used as a control.

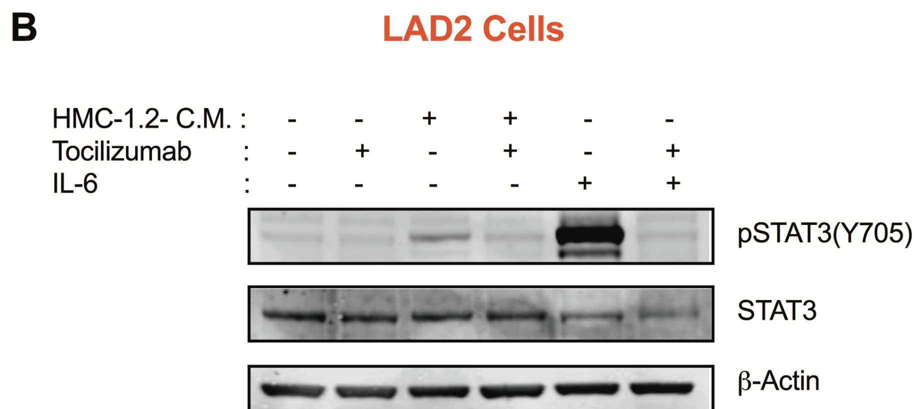


Supplementary Figure S1. Intracellular IL-6 in bone marrow specimens from patients with SM is mainly associated with mast cells. Bone marrow biopsies from patients # 7 (A) and #10 (C) defined in Supplementary Table 1 were prepared for immunofluorescence staining with anti-mast cell tryptase (red) and anti-IL-6 (green). Nuclei were identified by DAPI staining. In the overlay, proximal co-localization is shown in yellow or white depending on the intensity of the individual anti-tryptase and anti-IL-6 staining. Shown are areas with disperse mast cell infiltration for better visualization. Scale bars are 50 µM (left panels) and 20 µM (right panels). Right panels are the magnified areas indicated by white squares in the left panels.

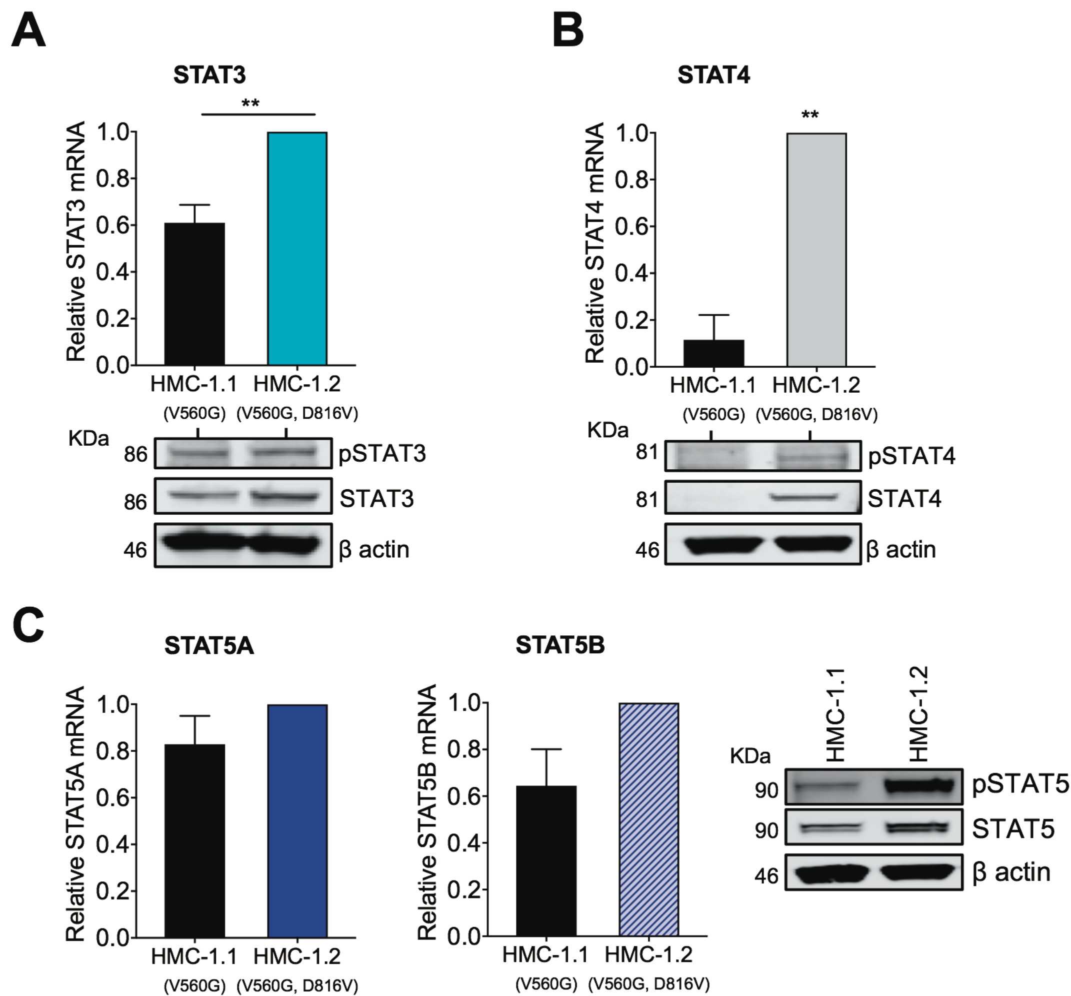


Supplementary Figure S2- Autocrine activation of IL-6, S1P and TGF-β receptors does not mediate the constitutive expression of IL-6 in HMC-1.2 cells. Effect of anti-IL-6 receptor (IL-6R) antibody (tocilizumab; 100 μg/mL) (A, left panel), anti-gp130 antibody (1 μg/mL) (A, right panel), S1PR1,3, S1PR2 and S1PR4 antagonists (VPC 23019; 1 μM, JTE 013; 1 μM and CYM 50358; 1 μM) (B) and a TGFβ-R antagonist (SD 208; 5 μM) (C), on IL-6 secretion by HMC-1.2 cells. The antibodies and antagonists were incubated for 24 h in serum free media. The data represent mean ± SEM of two experiments performed in duplicate.

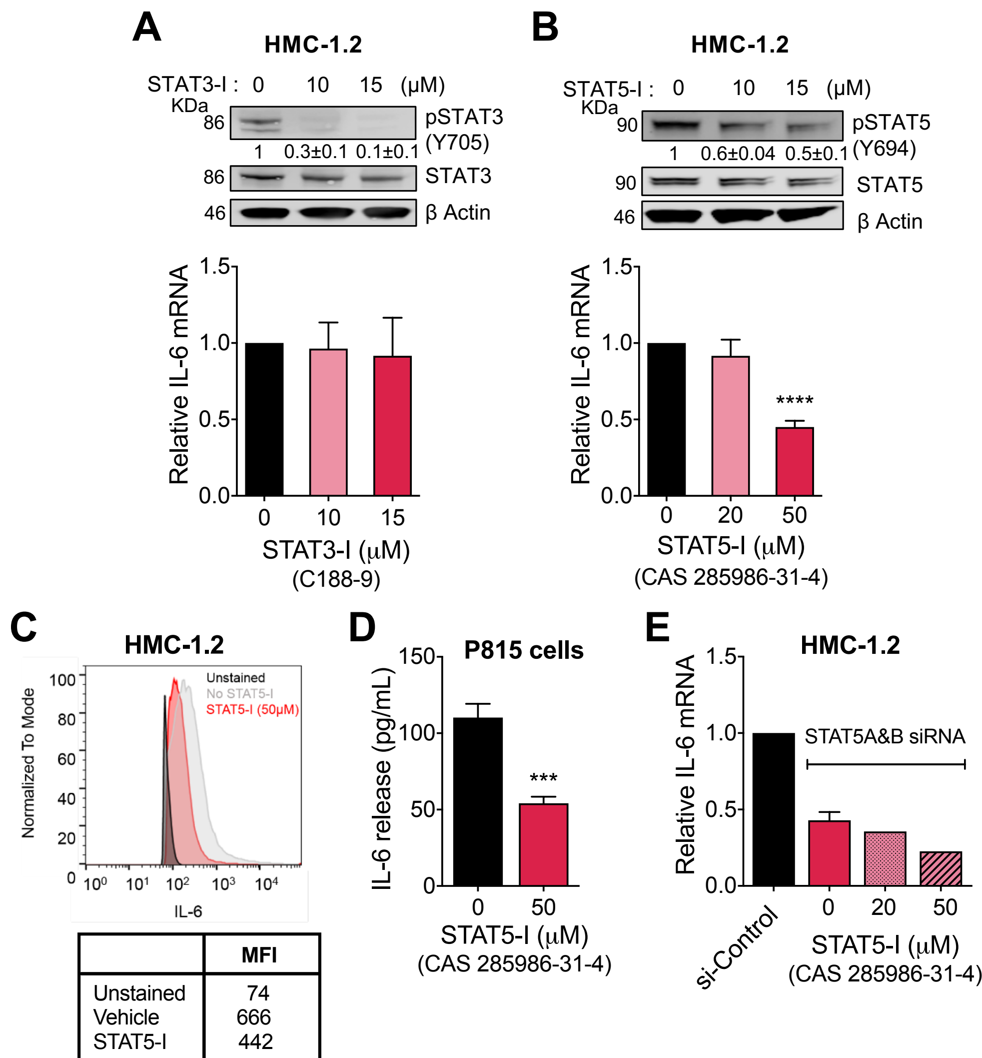




Supplementary Figure S3. Constitutive secretion of IL-6 by HMC-1.2 is enhanced by various stimuli; and secreted IL-6 is biologically active. (A) Total IL-6 released into the media by HMC-1.2 in comparison with total intracellular IL-6. Cells were incubated at 37°C overnight in serum free media with or without C48/80 (500 ng/mL), C5a (500 ng/mL), PMA (20 ng/mL) and Ionomycin (Io) (1 μM), LPS (10 μg/mL), IL-1β (100 ng/mL), or full media containing 10% FBS. In the right panel, the effects of the KIT inhibitor dasatinib (0.5 μM) on released IL-6 and intracellular IL-6 are shown. The percentages of intracellular compared to released IL-6 were 10% with or without dasatinib, or in the presence of C48/80; 6% after stimulation with C5a, LPS and PMA/Io; and 2% after incubation with IL-1β or 10% FBS. (B) IL-6 released by HMC-1.2 induces STAT3 phosphorylation in LAD2 cells. LAD2 cultures were treated for 30 min with conditioned media from HMC-1.2 (HMC-1.2-C.M.) in the presence or absence of the neutralizing IL-6R antibody tocilizumab (100 μg/mL) or incubated with serum-free media with or without 50 ng/mL recombinant IL-6. Cell lysates were obtained and phosphorylation of STAT3 determined by Western blotting.



Supplementary Figure S4. Baseline expression levels and phosphorylation state of STAT family members in HMC-1.2 compared to HMC-1.1 cells. (A) Expression levels of STAT3 mRNA (upper panel) and protein (lower panel) in HMC-1.1 and HMC-1.2 cells. (B) Expression levels of STAT4 mRNA (upper panel) and protein (lower panel) in HMC-1.1 and HMC-1.2 cells. (C) Expression levels of STAT5 mRNA (left panel, STAT5A; middle panel, STAT5B) and protein (right panel) in HMC-1.1 and HMC-1.2 cells. Both phosphorylated and total STAT family members are shown. Relative expression of STAT mRNA was obtained by comparing to the expression of GAPDH using the ΔCt method and the results were expressed as fold change compared to HMC-1.1 cells. Cells were incubated for 2 h in serum-free media before obtaining the cell lysates or RNA. The data represent mean ± SEM of three experiments performed in duplicate.



Supplementary Figure S5. Inhibition of STAT5 but not STAT3 reduces constitutive expression of IL-6 by HMC-1.2 cells. (A) Effect of the small STAT3 inhibitor, C188-9, at the indicated concentrations, on STAT3 phosphorylation (upper panel) and the expression of IL-6 mRNA (lower panel). (B-C) Effect of the STAT5 inhibitor CAS285986-31-4, at the indicated concentrations, on STAT5 phosphorylation (B, upper panel), the expression of IL-6 mRNA (B, lower panel) and IL-6 protein (C) in HMC-1.2 cells. The expression of IL-6 protein in C was evaluated by intracellular staining and FACS analysis as described in Methods. (D) Effect of the STAT5 inhibitor on IL-6 secretion by P815 cells. In A-D, cells were incubated in serum-free media with the corresponding inhibitors for 2 h (IL-6 mRNA expression) or 6 h (IL-6 release). (E) Combined effect of knocking down both STAT5A and B by si-RNA and incubation with the STAT5 inhibitor for 2 h, at the indicated concentrations, on the constitutive expression of IL-6 in HMC-1.2. Relative expression of IL-6 mRNA was obtained by comparing to the expression of GAPDH using the ΔCt method and the results were expressed as fold change compared to untreated or cell treated with si-non-target control. The data represent mean ± SEM of at least two individual experiments.

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