Hematopoietic niche drives FLT3-ITD acute myeloid leukemia resistance to quizartinib *via* STAT5- and hypoxia-dependent upregulation of AXL

Pierre-Yves Dumas,[1,2*] Cécile Naudin,[3*] Séverine Martin-Lannerée,[3] Brigitte Izac,[3] Luana Casetti,[3] Olivier Mansier,[4] Benoît Rousseau,[5] Alexandre Artus,[3] Mélody Dufossée,[1] Alban Giese,[6] Pierre Dubus,[6,7] Arnaud Pigneux,[1,2] Vincent Praloran,[1,2] Audrey Bidet,[4] Arnaud Villacreces,[1] Amélie Guitart,[1] Noël Milpied,[1,2] Olivier Kosmider,[3,8] Isabelle Vigon,[1] Vanessa Desplat,[1] Isabelle Dusanter-Fourt,[3] and Jean-Max Pasquet[1**]**

[1]Université de Bordeaux, Institut National de la Santé et de la Recherche Médicale INSERM U1035, F-33000 Bordeaux; [2]CHU Bordeaux, Service d'Hématologie Clinique et Thérapie cellulaire, F-33000, Bordeaux; [3]Université de Paris, Institut Cochin, Institut National de la Santé et de la Recherche Médicale INSERM U1016, Centre National de la Recherche Scientifique CNRS UMR8104, F-75014 Paris; [4]Service de Biologie des Tumeurs and Laboratoire d'Hématologie Biologique, Centre Hospitalo-Universitaire CHU Bordeaux, F-33000, Bordeaux; [5]Service Commun des Animaleries, Animalerie A2, Université de Bordeaux, Bordeaux; [6]Institut National de la Santé et de la Recherche Médicale INSERM U1218, and UMS005 TBM Core, Plateforme d'Histopathologie Expérimentale, Université de Bordeaux, F33000 Bordeaux; [7]Institut National de la Santé et de la Recherche Médicale, INSERM U1053, F33000 Bordeaux and [8]Service d'Hématologie Biologique, Assistance Publique-Hôpitaux de Paris, Hôpitaux Universitaires Paris Centre, Paris, France

**P-YD and CN contributed equally to this work. **ID-F and J-MP contributed equally as co-senior authors.*

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Correspondence: *JEAN-MAX PASQUET* - jean-max.pasquet@u-bordeaux.fr

ISABELLE DUSANTER-FOURT - isabelle.dusanter@inserm.fr

SUPPLEMENTAL DATA

SUPPLEMENTAL TABLES

Table S1. shRNA sequences

AXL	STAT5	GAS6
AGAACAGCGAGATTTATGACTA	GTACTACACTCCTGTGTG	GCTGTAATGAAGATCGCGGTA
GGTACCGGCTGGCGTATCA	GCAACAGGCCCATGACCTA	CCTGGCACTGATGGAAATCAA

Table S2. Clinical and biological features of primary AML samples

Num	Gender	Age	WBC	FAB	Status	Blasts	Karyotype	NPM1	FLT3
#1	F	75	51	2	*de novo*	68	46,XY [20]	No	ITD
#2	F	74	150	4	*de novo*	83	46,XY [25]	Yes	ITD
#3	F	53	58	2	*de novo*	97	46,XX [25]	Yes	ITD
#4	F	46	260	1	*de novo*	70	46,XX [25]	No	ITD
#5	M	71	150	2	*de novo*	97	46,XY [25]	Yes	ITD
#6	F	50	36	1	*de novo*	96	46,XX [25]	No	ITD
#7	M	27	114	2	*de novo*	95	46,XY [25]	No	ITD
#8	M	37	95	5	*de novo*	78	46,XY [25]	Yes	ITD
#9	F	49	118	5	*de novo*	89	46,XY [25]	Yes	ITD
#10	M	82	15	5	Post MDS	60	46,XY [25]	No	ITD
#11	M	54	47	4	Post MPN	30	NA	Yes	ITD
#12	F	87	1.5	1	*de novo*	82	46,XX [25]	Yes	negative
#13	F	73	3.2	5	Post MDS	66	46,XX [25]	Yes	ITD
#14	F	57	280	4	*de novo*	43	46,XX [25]	No	negative
#15	F	74	17	1	*de novo*	96	46,XX [25]	Yes	ITD
#16	M	78	118	5	*de novo*	57	46,XX [25]	Yes	ITD
#17	F	57	60	4	*de novo*	98	46,XX [25]	Yes	ITD
#18	M	68	7.3	2	*de novo*	58	46,XX [25]	No	negative
#19	F	20	21.5	6	*de novo*	21	46,XY [25]	No	negative
#20	M	57	19.2	5	*de novo*	70	46,XY [25]	Yes	negative

Age: in years, M: male, F: female, WBC: white blood cell count (G/L), FAB: French American British classification, Status: *de novo* or secondary AML, MDS: Myelodysplastic syndrome, MPN:

myeloproliferative neoplasm, ITD: internal tandem duplication, blasts: medullar blasts (%), NPM1: mutation yes/no, NA: not available.

Table S3. Gene expression change in human $CD34^+$ $CD38^-$ HSPC upon STAT5 knockdown

Sorted $CD34^+$ $CD38^-$ cells were transduced with STAT5A or control luciferase shRNA and GFP co-encoding lentiviral vectors as previously described (1). Total RNA was isolated from sorted cord blood (CB) $CD34^+GFP^+$ cells at day 5 post transduction, using Trizol reagent following manufacturer's instructions. Genome wide expression analysis was performed on Affymetrix IVT3' Human Gene U133 plus 2.0 arrays. Two independent transduction experiments with two independent lots of $CD34^+CD38^-$ HSPC were performed and used for microarray screening. Data were analyzed using expression console software and common targets in the two independent experiments were selected considering targets with a p value below 0.1. The whole list of genes was ordered according to fold change (FC) of expression in shSTAT5- compared to shCtrl-expressing cell (Gene Expression Omnibus (GEO) number GSE97552). Then, transmembrane containing protein encoding genes were selected for and listed up to AXL expression FC. Dimly highlighted rows focus on the two regulated tyrosine kinase receptors; darker row indicates STAT5A down-regulation.

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p-value	FC	Symbol	Gene Name
5.53E-02	-1.707	AXL	AXL receptor tyrosine kinase
1.13E-01	1.717	KLRB1	killer cell lectin like receptor B1
5.11E-01	1.721	TAS2R50	taste 2 receptor member 50
4.37E-01	1.725	LPAR4	lysophosphatidic acid receptor 4
4.83E-01	1.730	TRPC3	transient receptor potential cation channel subfamily C member 3
2.53E-01	1.742	KCNV2	potassium voltage-gated channel modifier subfamily V member 2
2.14E-01	1.743	PCDH19	protocadherin 19
1.33E-01	-1.746	CD1C	CD1c molecule
5.83E-01	1.771	TMC1	transmembrane channel like 1
4.28E-01	1.773	RSPO3	R-spondin3
2.27E-01	-1.781	CD36	CD36 molecule
3.37E-01	1.785	CMTM5	CKLF like MARVEL transmembrane domain containing 5
8.52E-04	-1.794	GPNMB	glycoprotein nmb
4.55E-01	1.800	PTPRG	protein tyrosine phosphatase, receptor type G
1.68E-01	-1.807	LILRA4	leukocyte immunoglobulin like receptor A4
5.00E-01	1.824	SLC47A1	solute carrier family 47 member 1
2.96E-01	1.830	DCSTAMP	dendrocyte expressed seven transmembrane protein
1.57E-01	-1.832	TMEM31	transmembrane protein 31
7.51E-02	-1.838	SIT1	signaling threshold regulating transmembrane adaptor 1
1.54E-01	1.845	ADRB2	adrenoceptor beta 2
1.24E-01	-1.860	BTLA	B and T lymphocyte associated
2.73E-01	-1.863	RHAG	Rh-associated glycoprotein
6.26E-01	1.870	ERBB4	erb-b2 receptor tyrosine kinase 4
4.82E-01	-1.908	KEL	Kell blood group, metallo-endopeptidase
3.80E-01	-1.913	KCNH2	potassium voltage-gated channel subfamily H member 2
5.05E-01	-1.918	XK	X-linked Kx blood group
3.84E-01	1.945	KLRC4	killer cell lectin like receptor C4
1.73E-01	-2.033	CD24	CD24 molecule
6.40E-01	2.084	LRFN5	leucine rich repeat and fibronectin type III domain containing 5
2.29E-02	-2.089	CLEC4C	C-type lectin domain family 4 member C
5.72E-01	2.104	SCN2A	sodium voltage-gated channel alpha subunit 2
4.57E-01	2.274	ADGRL4	adhesion G protein-coupled receptor L4
6.27E-01	2.435	GABRB2	gamma-aminobutyric acid type A receptor beta2 subunit
5.45E-01	-2.469	GYPB	glycophorin B (MNS blood group)
3.74E-01	-2.495	DEFA4	defensin alpha 4
3.39E-03	-2.509	STAT5A	signal transducer and activator of transcription 5A
4.55E-01	2.775	ADAMDEC1	ADAM like decysin 1
5.47E-01	3.319	TPTE	transmembrane phosphatase with tensin homology
1.45E-01	-3.693	LY6G5B	lymphocyte antigen 6 family member G5B

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Table S4. Antibodies for Western Blot analysis

Primary antibody	**Clone**	**Company**
AXL	C44G1	Cell Signaling Technologies
pY_{779}AXL	713610	R&D
STAT5	sc-835	Santa Cruz Biotech
pY_{694}STAT5	9351	Cell Signaling Technologies
β actin	AC-15	Sigma
HSP60	SMC-110A	Stressmarq
pY_{591}FLT3	33G6	Cell Signaling Technologies
FLT3	8F2	Cell Signaling Technologies
pTyr	4G10	Millipore
ERK	L34F12	Cell Signaling Technologies
pT_{202}/Y_{204}ERK	D13.14.4E	Cell Signaling Technologies
AKT	9272	Cell Signaling Technologies
$pSer_{473}$AKT	D9E	Cell Signaling Technologies
GAS6	A-9	Santa Cruz Biotech
Secondary antibody	**Clone**	**Company**
Rabbit IgG-HRP	7074	Cell Signaling Technologies
Mouse IgG-HRP	7076	Cell Signaling Technologies
Goat IgG-HRP	sc-2020	Santa Cruz Biotech
Immunoprecipitation	**Clone**	**Company**
AXL	C44G1	Cell Signaling Technologies
RNA polymerase II	N-20, sc-899	Santa Cruz Biotech
STAT5	C-17, sc-835	Santa Cruz Biotech
Rabbit IgG	sc-2027	Santa Cruz Biotech
Goat IgG	0109-01	Southern Biotech

Table S5. Primer sequences for qPCR

		Forward Primer sequences	**Reverse Primer sequences**
CHIP	**AXL-P1**	CCTAGTCTCCCTCAGCTTGG	GCCTCTCCTTCTCACTACCC
	AXL-P2	GGGCAGATTCAAGGGGTCTA	TGTTTCCAGCAGAGTTCCCA
Other	**AXL**	TTCAACTCCTGCCTTCTCGT	AAGGTGGAGTTTTCCCCAGT
	GUS	GTTTCACCAGGATCCACCTC	CTCGTCGGTGACTGTTCAGT

	GAPDH	GGAGGAGTGGGTGTCGCTGTT	GGGAAAGTGTGGCGTGAT

Supplementary Methods

Animal models for *in vivo* studies

NOD Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ immunodeficient mice (NSG) were bred at the University of Bordeaux's animal facility for experiments validated by the French ministry (N°00048.2 authorization). Animals were included in protocols at eight weeks old and were monitored weekly for body weight. For subcutaneous xenografts, prior to implantation, cells were resuspended in 25% Matrigel (BD Biosciences) and 75% culture medium without FCS. Female NSG mice received injections into their hind flanks with 1,75x10^6 cells. Tumor growth was measured three times a week. When tumors reached 400-500mm^3, treatments began. At the indicated times, the mice were euthanized by cervical dislocation and tumors weighed. For hematopoietic engraftment, female NSG mice were conditioned with intraperitoneal injections of busulfan (Fabre) 20 mg/kg/day for two days, and then injected intravenously with 10^6 shCtrl (MV4-11 shCtrl) or shAXL (MV4-11 shAXL) luciferase-transduced cells (10^6 cells/100µL) at day 0. At day 7, engraftment was analyzed by whole bioluminescent imaging (BLI) before treating mice with vehicle or AC220 (5 mg/kg/day body weight) by daily oral gavage until the times indicated. At day 14, mice were injected intraperitoneally with firefly luciferase substrate D-luciferin (150 mg/kg) before imaging using a photon bioimager and M3Vision software (BIOSPACE LAB). Bones, spleen and liver were collected at sacrifice and analyzed by BLI *ex vivo* or fixed in 10% formalin and processed for hematoxylin, eosin and immunohistochemical labeling.

Immunoblotting, immunoprecipitation, chromatin immunoprecipitation assay, ELISA and quantitative PCR

The antibodies are listed in Supplemental Table S4. Total cell lysates were prepared by incubating cells for 30min at 4°C in a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 10% Glycerol) supplemented with a protease inhibitor cocktail (Roche), sodium orthovanadate (1 mM) and NaF (10 mM) phosphatase inhibitors. Immunoprecipitations (IP) were performed with 500 µg proteins and 5µg of antibody or 20-50 µg of whole cell lysates were loaded onto SDS-PAGE gels and transferred onto nitrocellulose or PVDF membranes (Amersham-Biosciences). Detection was performed using ECL (Amersham-Biosciences). Images were captured using a CCD camera, Fuji-LAS4000 (Fujifilm). ELISA experiments were performed according to manufacturer's instructions for mouse GAS6 (Duoset®, R&D system) and human GAS6 (Duoset®, R&D system). For chromatin immunoprecipitation (IP) assays, cells were processed as previously described (Supplementary reference 1). Briefly, cells ($5x10^6$) were stimulated for 30min with or without TPO (20 nM), washed and cross-linked with 1% formaldehyde (10^6 cells/ml) at room temperature for 5 min before adding glycine (0.125 M). IP of cell lysates were performed overnight with the indicated antibodies before adding salmon-sperm DNA pre-saturated protein G-dynabeads (Invitrogen) for 1h at 4°C. Immunoprecipitates were eluted with 1% SDS in 0.1 M NaHCO3. Formaldehyde cross-linking was reversed by heating for 1 at 45°C with proteinase K (40 µg/ml). DNA was extracted and analyzed by real-time quantitative PCR experiments. Two independent pairs of primers that encompassed the conserved STAT-responsive element (SRE) TTCN3GAA of human AXL intron 16 were used (Supplemental Table S5). All PCR assays were performed in triplicate, and the average value obtained for each sample was normalized to the amount of AXL chromatin DNA added to the IP reaction ("input DNA"). 1:10 of beads/immunoprecipitated complexes were removed to conduct an immunoblotting analysis. For AXL mRNA quantification, complementary DNA (cDNAs) were

82 synthetized from 1 µg of RNA, using the First Strand cDNA synthesis kit (Roche®, Meylan, France)
83 according to manufacturer's instructions. The real time quantitative PCR (qRT-PCR) was performed
84 using Brilliant SYBR® Green QPCR kit as previously described with primers in Supplemental Table S5
85 (13).

SUPPLEMENTAL FIGURES

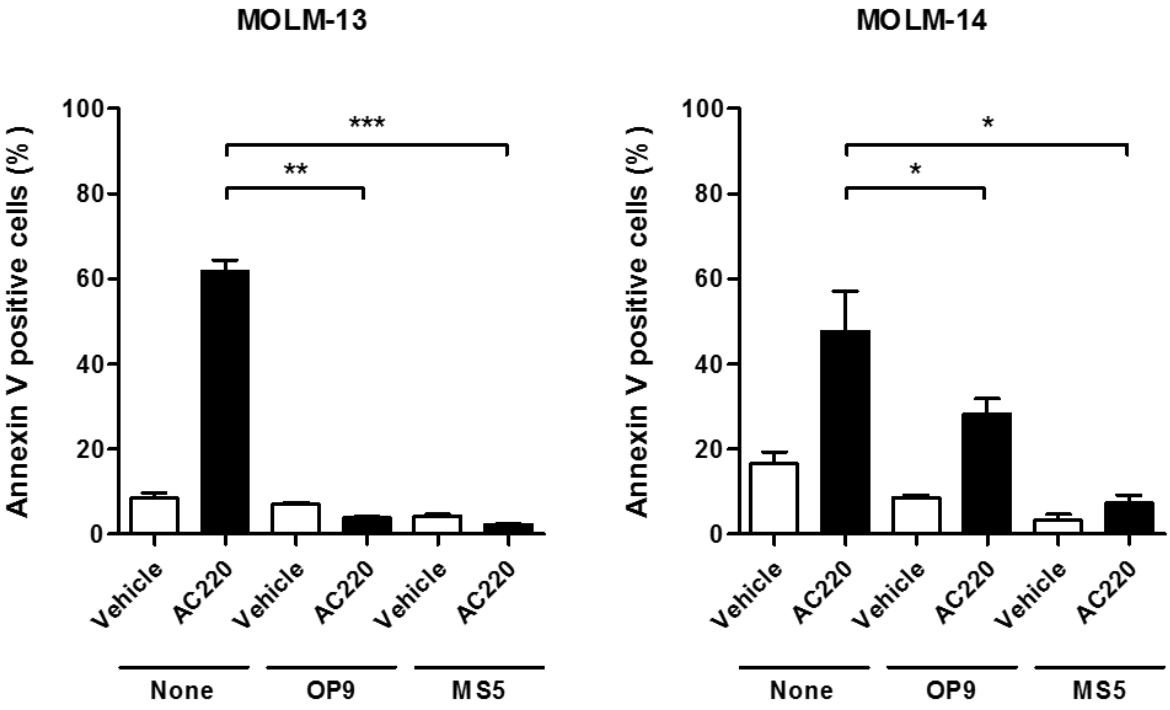


Figure S1A. Stromal cells protect FLT3-ITD AML cells from apoptosis upon AC220 treatment

MOLM-13 or MOLM-14 FLT3-ITD AML cells were cultured for 48 hours in the absence (Vehicle) or presence of AC220 (3 nM) and in the absence (None) or presence of stromal cell lines (OP9 or MS5), as indicated. Apoptosis was assessed by Annexin V/DAPI labeling and flow cytometry analysis. Data are expressed as mean ± SEM, n ≥ 3; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant.

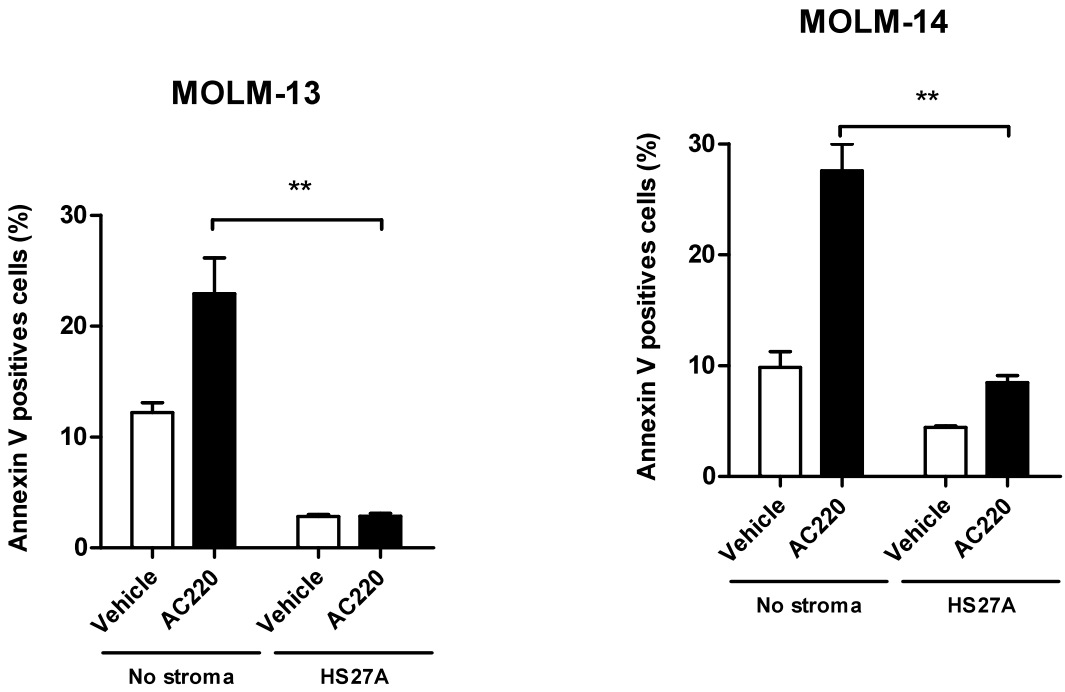


Figure S1B. Human stromal cells protect FLT3-ITD AML cells from apoptosis upon AC220 treatment

MOLM-13 or MOLM-14 FLT3-ITD AML cells were cultured for 48 hours in the absence (Vehicle) or presence of AC220 (3 nM) and in the absence (No stroma) or presence of HS27a stromal cell lines, as indicated. Apoptosis was assessed by Annexin V/DAPI labeling and flow cytometry analysis. Data are expressed as mean ± SEM, n ≥ 3; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant.

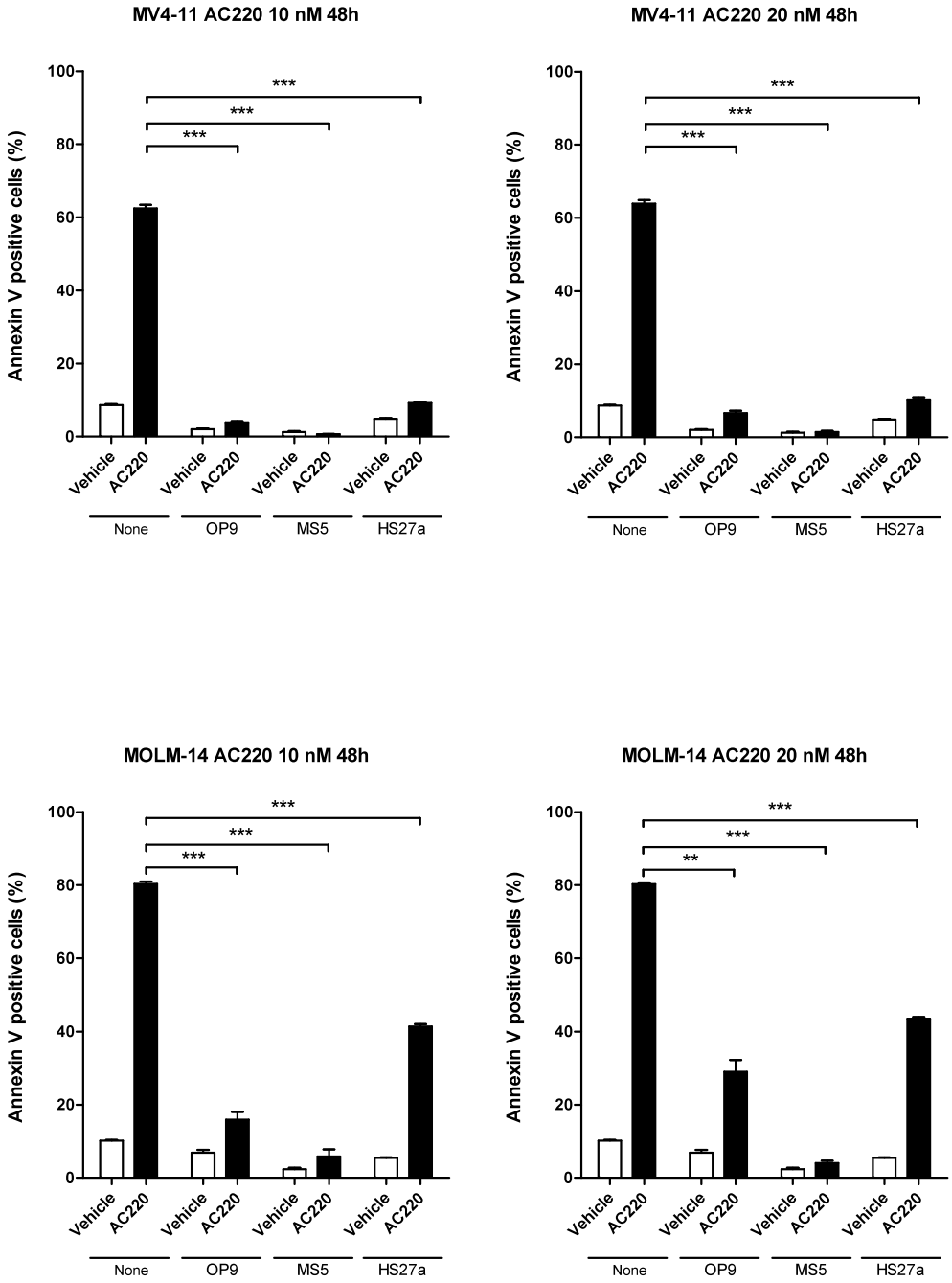


Figure S1C. Stromal cells protect FLT3-ITD AML cells from apoptosis upon AC220 treatment

MV4-11 or MOLM-14 FLT3-ITD AML cells were cultured for 48 hours in the absence (Vehicle) or presence of AC220 (10 and 20 nM) and in the absence (None) or presence of stromal cell lines (OP9, MS5 or HS27a), as indicated. Apoptosis was assessed by Annexin V/DAPI labeling and flow cytometry

analysis. Data are expressed as mean ± SEM, n ≥ 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, non-significant.

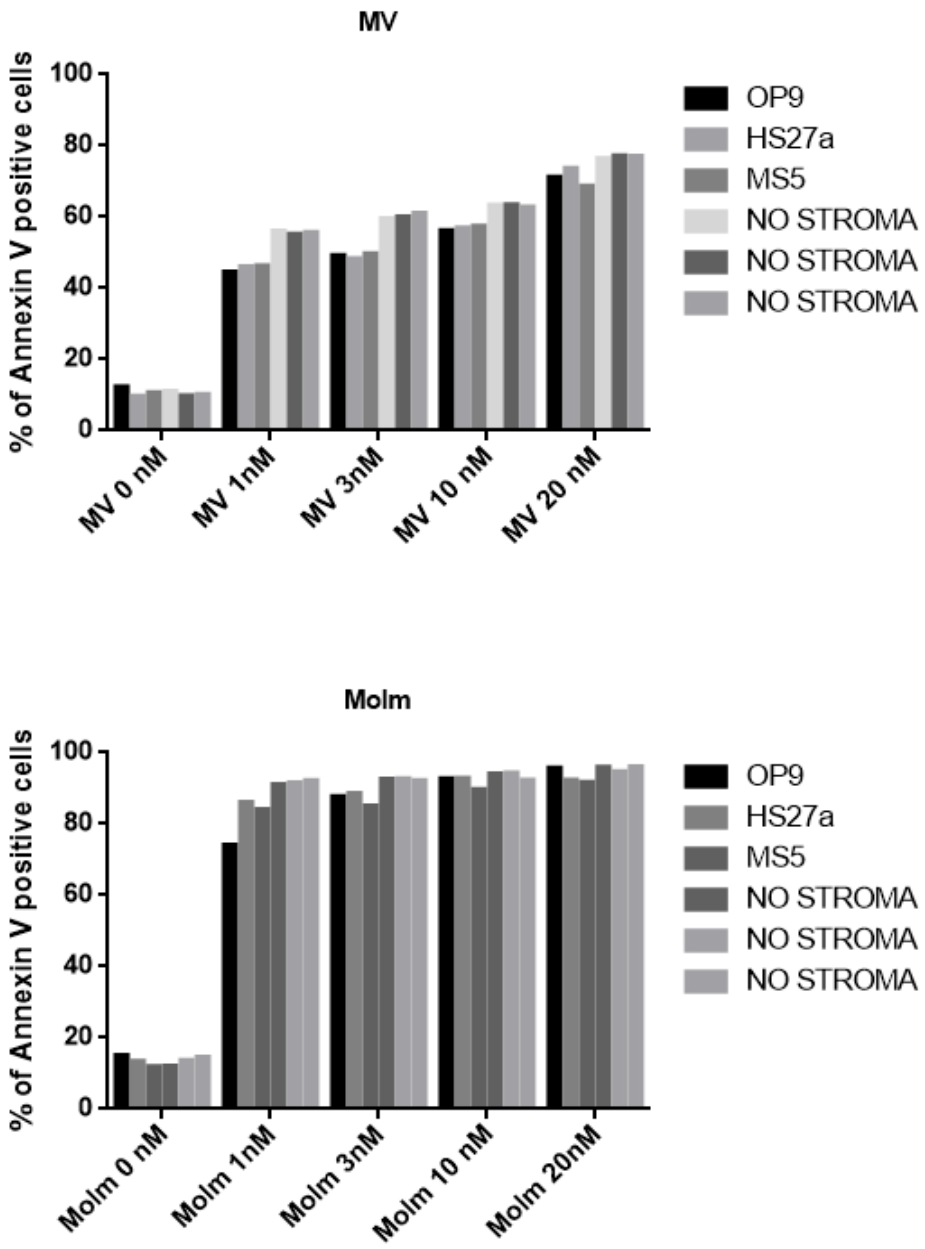


Figure S1D. Stromal cells protection is not correlated to AC220 degradation

Stromal cells (OP9, MS5 and HS27) were incubated with the indicated concentrations of AC220 for 48 h. Then, MV4-11 or Molm14 were incubated either with the culture media from these stromal cells as indicated, or with fresh medium (no stroma) supplemented with the indicated concentrations of AC220. After 48h, MV4-11 and Molm14 were labeled with annexin V and the

percent of annexin V positive cells was quantified by flow cytometry. Results are from one experiment representative of 3

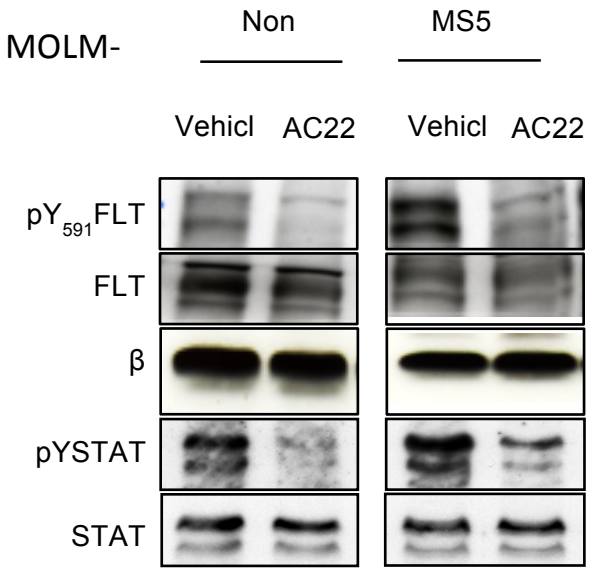


Figure S1E. Stromal cells protection of MOLM-14 against AC220 correlates with enhanced STAT5 activation

MOLM-14 cells were incubated in the absence (Vehicle) or presence of AC220 (1 nM), without (none) or with MS5 stromal cells (Stroma) for 48 hrs. Immunoblot analysis of the indicated protein, each of protein-dedicated immunoblot without (none) or with stroma (MS5) have been performed on the same membrane. Results shown are representative of 4 experiments.

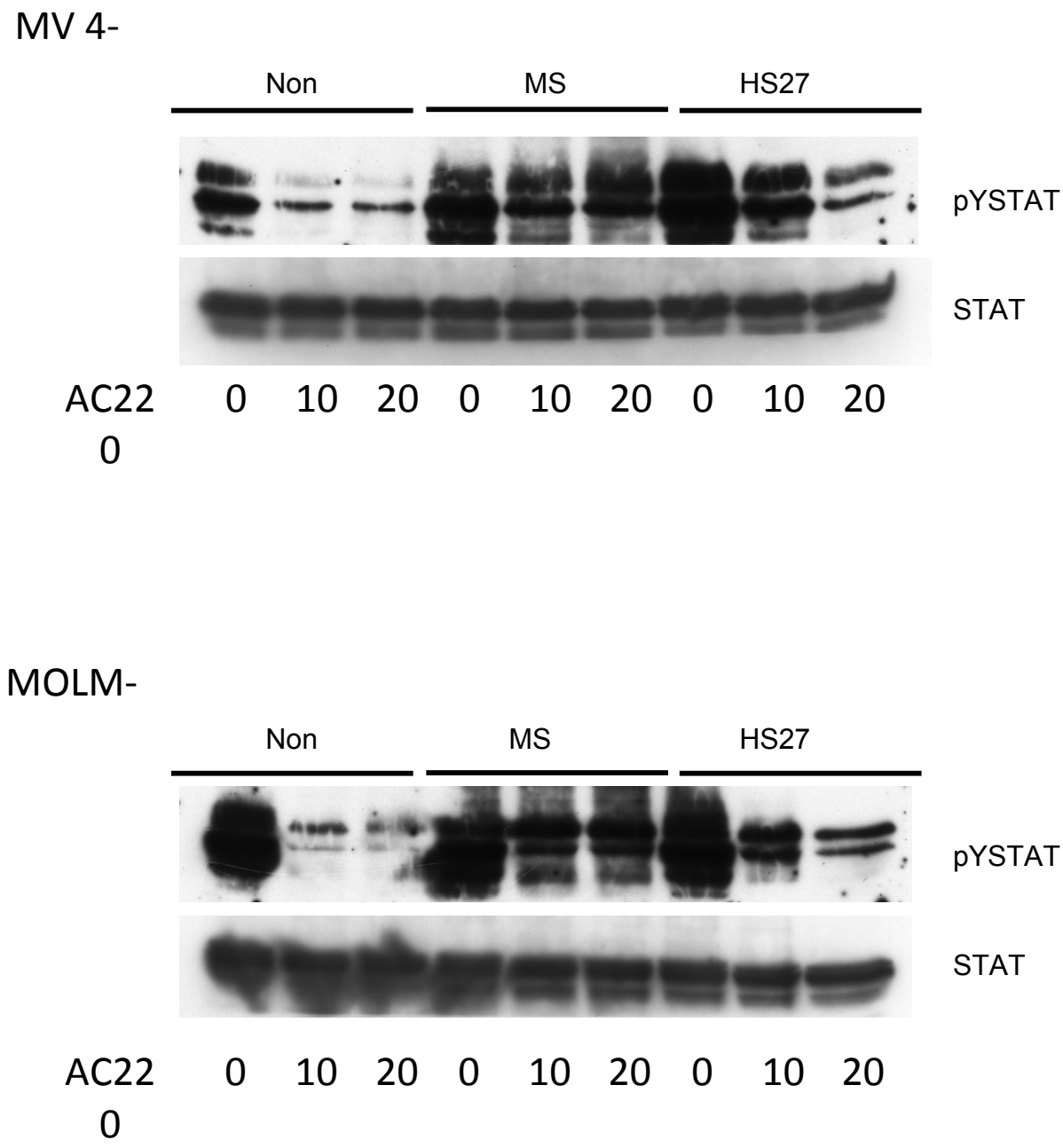


Figure S1F. Stromal cells protection of MV4-11 and MOLM-14 cells against AC220 correlates with enhanced STAT5 activation

MV4-11 and MOLM-14 cells were incubated in the absence or in the presence of AC220 (10 or 20 nM), without (none) or with MS5 or HS27 stromal cells for 48 hrs. Immunoblot analysis of the indicated protein, each of protein-dedicated immunoblot has been performed on the same membrane. Results shown are representative of 3 experiments.

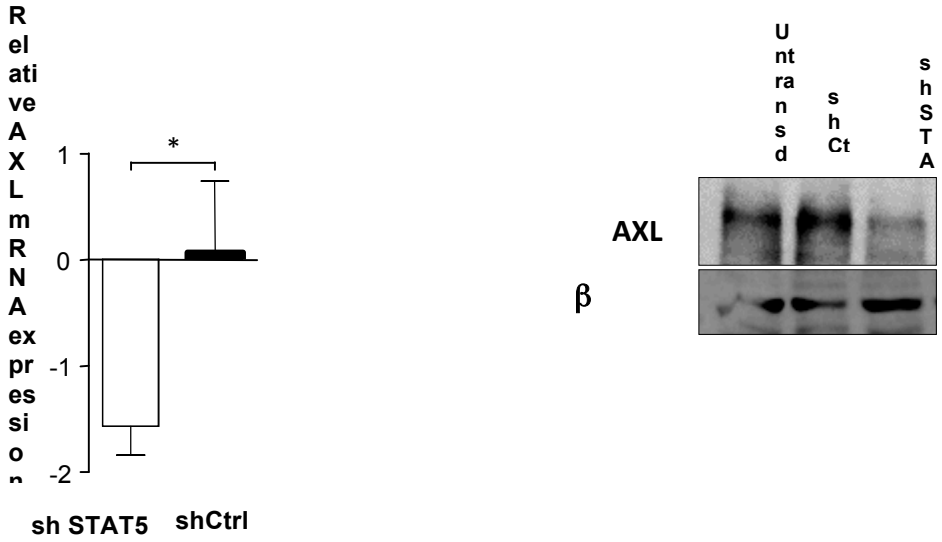


Figure S2A. Knock down of STAT5 inhibits AXL expression in human HSPCs

$CD34^+$ CB cells were transduced without (Untransduced) or with control luciferase (shCtrl) or STAT5 (shSTAT5) shRNA encoding lentiviral vectors and were collected 5 days later. (A) RT-qPCR analysis of AXL mRNA in the indicated cells. Results are normalized to GAPDH expression and expressed relative to untransduced cells (n=3). (B) Immunoblot analysis of the indicated proteins in the indicated cells. β actin is used as a loading control. Data are expressed as mean ± SEM, n ≥ 3; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant.

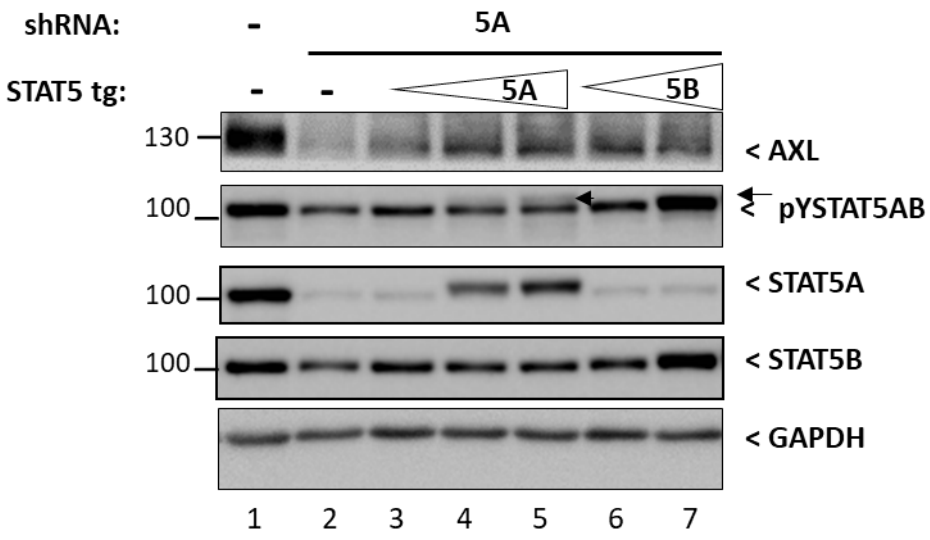


Figure S2B. Both STAT5A and STAT5B regulate AXL expression of myeloid cells.

UT7 AML cells were first transduced with inducible HA-tagged murine STAT5A (5A) or Flag-tagged murine STAT5B (5B) encoding vectors and cultured in absence of inducer for a few days before transducing them with or without shSTAT5A-encoding vector. Cells were then cultured in absence (-) or presence of increasing concentration (◁) of doxycycline to induce transgene (tg) expression and incubated for 8hrs before cell lysis. Expression of the indicated proteins was analyzed by immunoblotting. GAPDH served as a loading control; mol.wt (kDa) of appropriate markers is indicated on the left. Arrow points at slower migrating HA-tagged pYSTAT5A transgene compared to whole endogenous pYSTAT5, as revealed by anti-pY_{694}STAT5A/pY_{699}STAT5B antibodies (pYSTAT5).

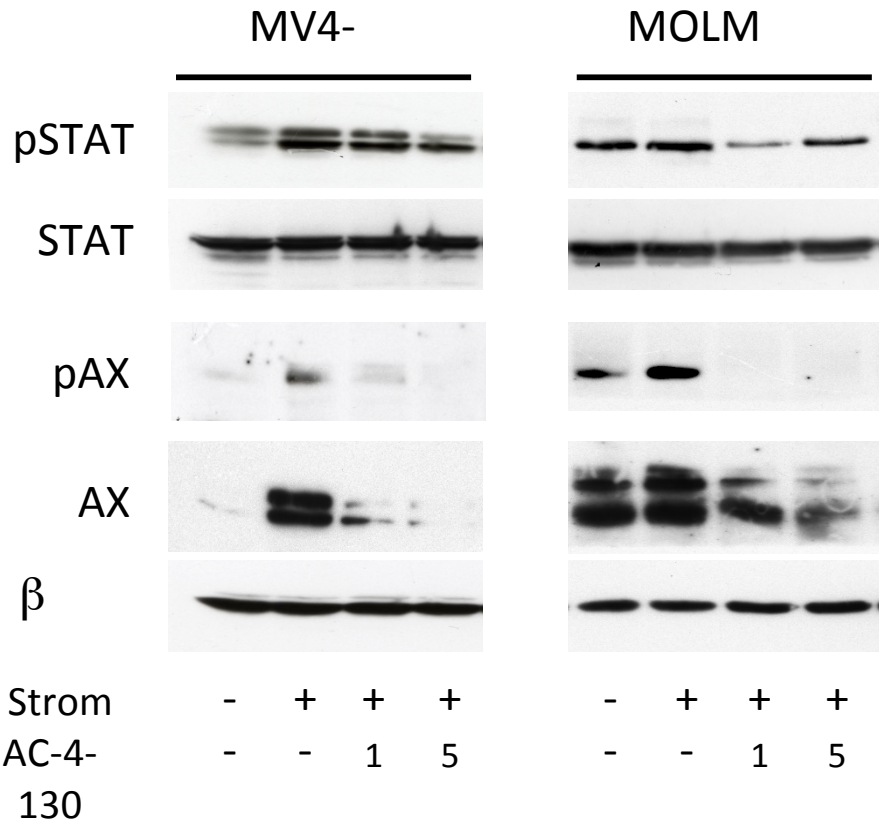


Figure S2C. Inhibition of STAT5 prevent stroma-induced AXL expression and activation ($pTyr_{779}$ phosphorylation)

MV4-11 and MOLM-14 cells were incubated without (-) or with HS27 stromal cells (+) in the absence or in the presence of AC-4-130 1 or 5 μM for 48 hrs. Immunoblot analysis of the indicated protein, each of protein-dedicated immunoblot has been performed on the same membrane. Results shown are representative of 2 experiments.

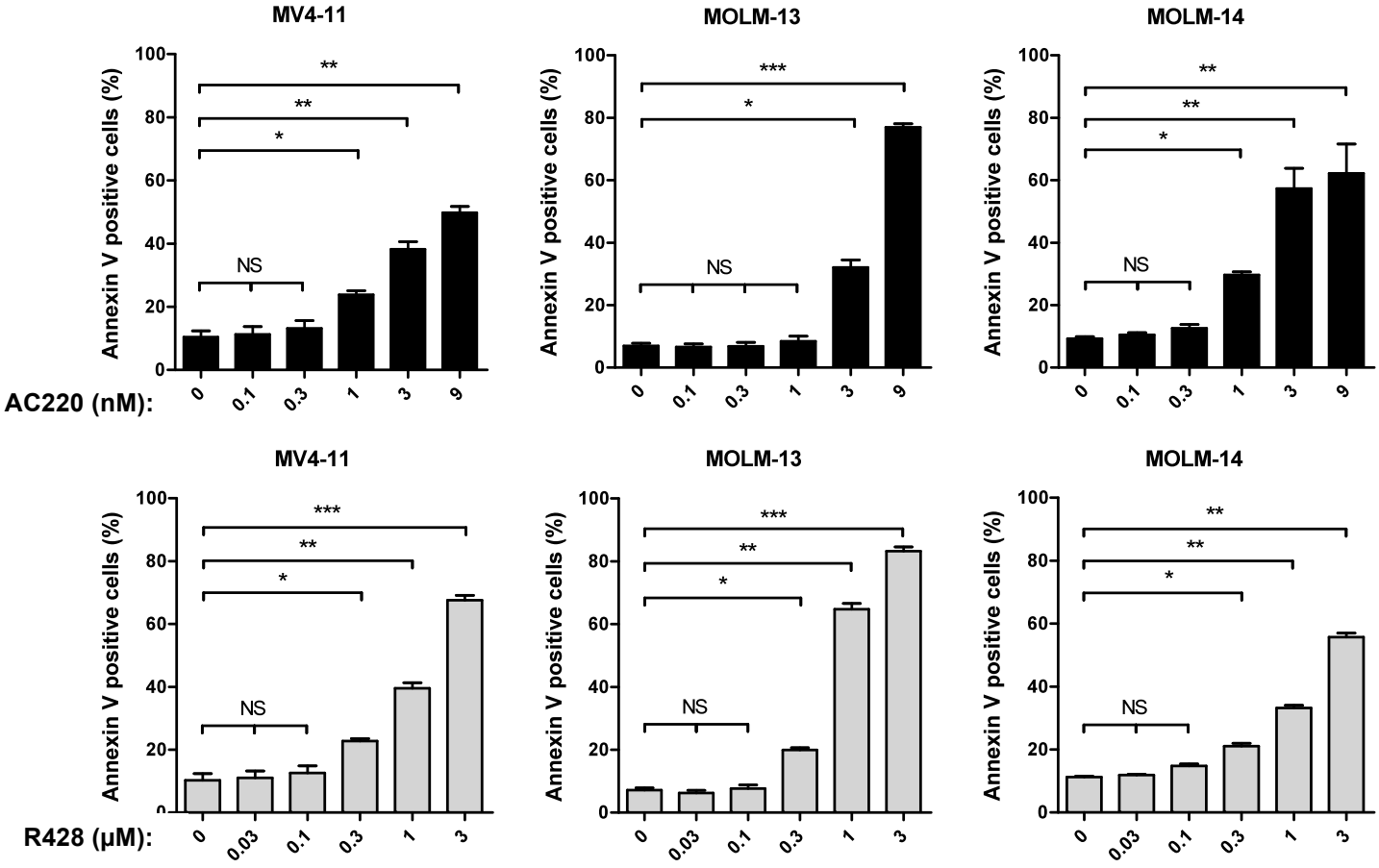


Figure S2D. AXL activity contributes to FLT3-ITD AML cell survival

FLT3-ITD AML cell lines MV4-11, MOLM-13 and MOLM-14 were treated in the absence or presence of AC220 (top panel) or R428 (bottom panel) at increasing doses for 48 hours. Apoptosis was then assessed by Annexin V/DAPI labeling and flow cytometry analysis. Data are expressed as mean ± SEM, n ≥ 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, non-significant.

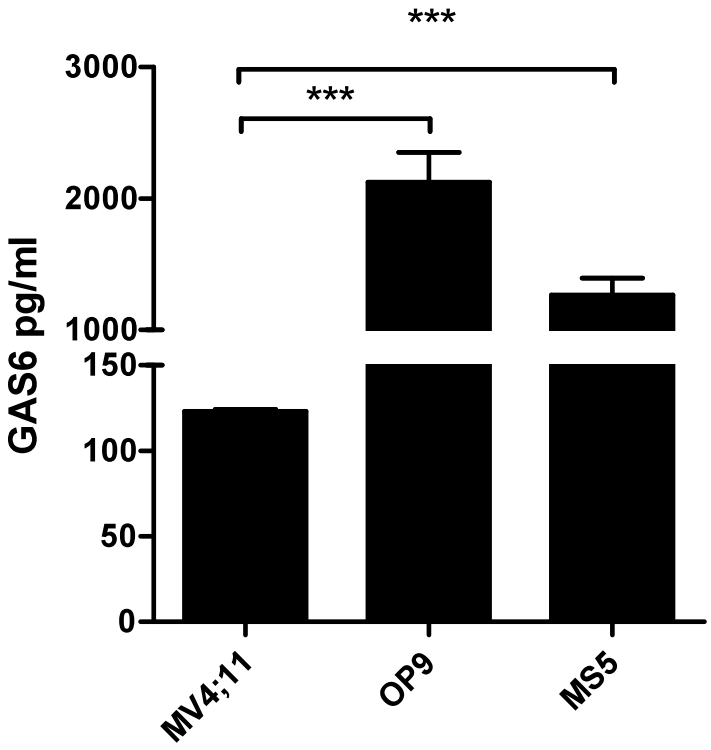


Figure S2E. Species-specific ELISA assay of GAS6

Human MV4-11 AML cells and murine OP9 and MS5 stromal cells were cultured for 24h. GAS6 was quantified in the supernatant by ELISA upon 24h of culture.

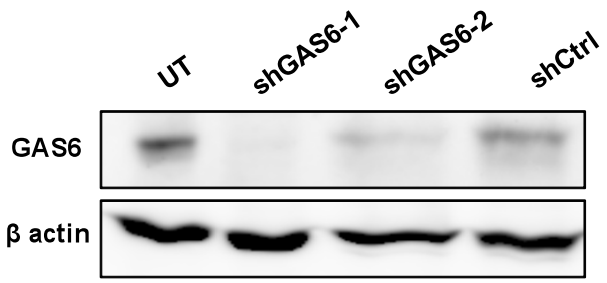


Figure S2F. Transduction of stromal cells with GAS6 shRNA-encoding lentiviral vectors inhibits GAS6 expression

Human stromal OP9 cells were either untransduced (UT) or transduced with GAS6 (shGAS6-1, shGAS6-2) or scramble (shCtrl) shRNA encoding lentiviral vector and collected 14 days later. Protein expression was assessed by immunoblot analysis. β actin serves as a loading control.

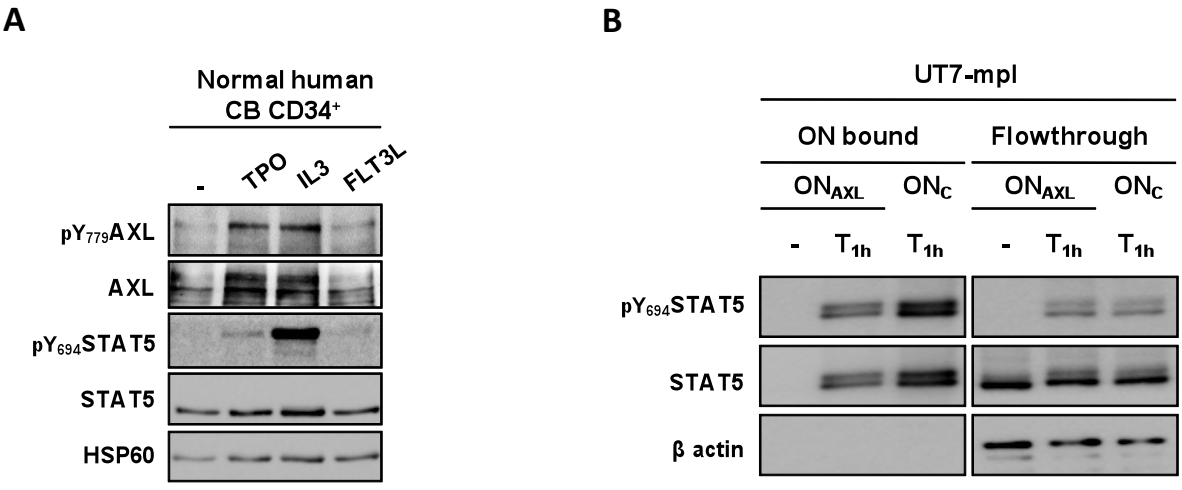
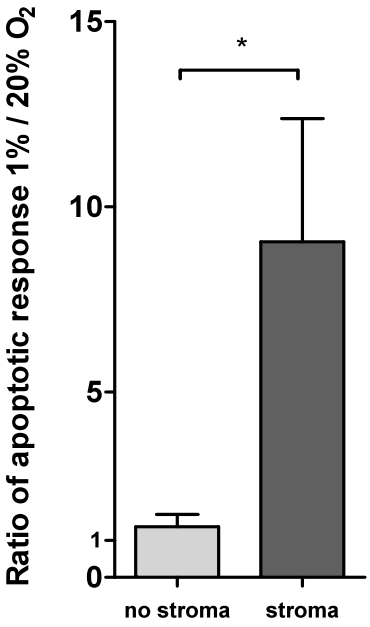


Figure S3. STAT5 activation enhances AXL expression through STAT5 binding to AXL gene sequence

(A) Human cord blood (CB) $CD34^+$ cells were incubated in absence of the four-exogenous cytokines/growth factors cocktail for 18h before being incubated for 8 hr in the absence (-) or presence of TPO (20 nM), IL-3 (50 ng/ml) or FLT3 ligand (FLT3L, 100 ng/ml) and lysed. Immunoblot analysis of the indicated proteins; HSP60 serves as a loading control. (B) UT7-mpl cells were growth-factor deprived for 18h before being incubated in the absence (-) or presence of TPO (20 nM) for 1 hr (T_{1h}). Cells were lysed and oligonucleotide (ON) pulldown assays were performed with biotinylated-AXL (ON_{AXL}) or -consensus (ONc) SRE from IRF1 promoter sequence. ON-bound and -unbound (flowthrough) proteins were analyzed by immunoblotting using the indicated antibodies. β actin is used as a loading control.



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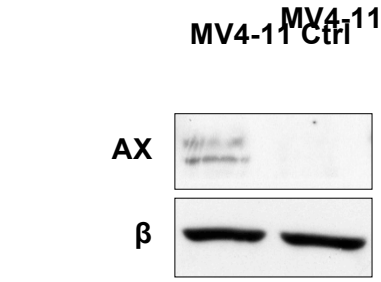
315 **Figure S4. Low O_2 enhanced apoptotic response to AXL inhibition in presence of stroma**

316 MV4-11 cells were incubated in the presence of R428 (1 µM) for 48 hrs, without (no stroma) or
317 with MS5 (stroma) at 20% or 1% O2. Apoptosis was assessed by Annexin V/DAPI labeling and
318 flow cytometry analysis. Results are expressed as the ratio of the apoptotic response at 1% on
319 the apoptotic response at 20%. Graphs show the mean ± SEM of results of at least three
320 independent experiments with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant.



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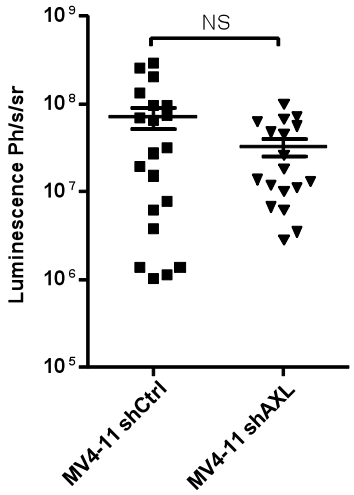

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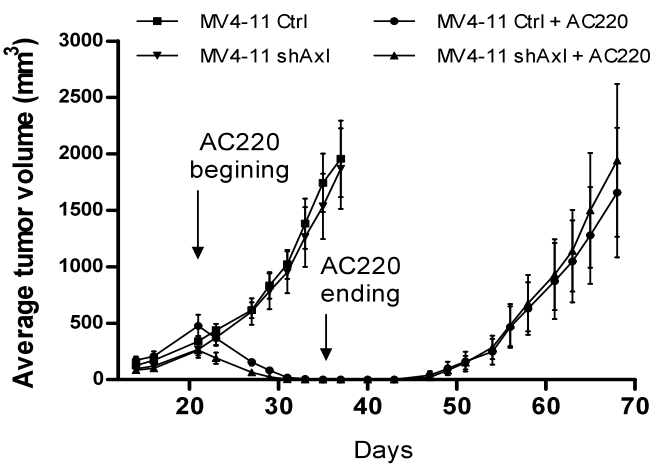

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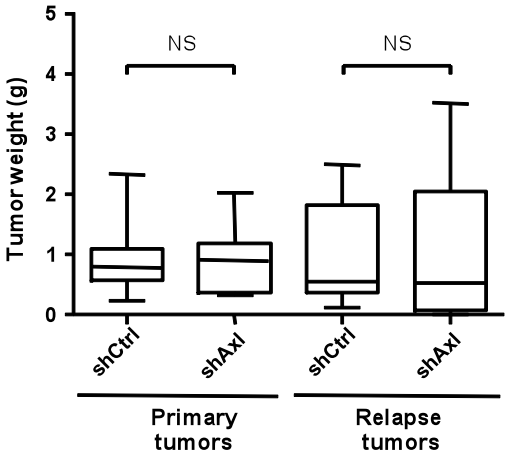

Figure S5. Loss of AXL does not impair leukemia cell growth upon sub-cutaneous injection

(A) MV4-11 were transduced with shCtrl or shAXL encoding lentiviral vector. Cells were maintained in culture for 7 days before being sorted for GFP expression and lysed. Proteins were analyzed by immunoblotting with the indicated antibodies. β actin serves as a loading control. (B) Quantification of photon flux bioluminescent signals from the whole body of the indicated

mice (n=40) at day 7 post injection of luciferase-labelled MV4-11 shCtrl or MV4-11 shAXL cells. Ph stands for photon, s for second and sr for steradian. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant. (C) Control (MV4-11 shCtrl) or shAXL expressing (MV4-11 shAXL) MV4-11 cells were subcutaneously injected into the limbs of NSG mice at day 0 and treated or not with AC220 (5 mg/kg/day), as indicated. Tumor volume was measured at the indicated days (mean ± SEM, n=20). (D) Weights of collected tumors from NSG mice injected subcutaneously with the indicated shRNA transduced MV4-11 cells. Upon leukemia cell injection, tumors grew and when tumor volume were about 400-500 mm^3, half of mice were treated with AC220 (5 mg/kg/day) for 14 days. Relapse tumors reappeared upon AC220 discontinuation. For primary tumors, animals were maintained until ~2 cm^3 tumor volume before necropsy (n=10). Relapse tumors were collected at day 35 post AC220 withdrawal (n=10). Data reported are the mean ± SEM (n=40); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, non-significant.

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347 **SUPPLEMENTAL REFERENCE**

348

349 1. Rouyez MC, Lestingi M, Charon M, Fichelson S, Buzyn A, Dusanter-Fourt I. IFN regulatory factor-2
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351 activity. J Immunol. 2005;3948-3958.