TLR3 agonism augments CD47 inhibition in acute myeloid leukemia

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Abstract

CD47-SIRPa is a myeloid check point pathway that promotes phagocytosis of cells lacking markers for self-recognition. Tumor cells can overexpress CD47 and bind to SIRPa on macrophages, preventing phagocytosis. CD47 expression is enhanced and correlated with a negative prognosis in acute myeloid leukemia (AML), with its blockade leading to cell clearance. ALX90 is an engineered fusion protein with high affinity for CD47. Composed of the N-terminal D1 domain of SIRPα genetically linked to an inactive Fc domain from human immunoglobulin (Ig) G, ALX90 is designed to avoid potential toxicity of CD47-expressing red blood cells. Venetoclax (VEN) is a specific B-cell lymphoma-2 (BCL-2) inhibitor that can restore apoptosis in malignant cells. In AML, VEN is combined with azanucleosides to induce superior remission rates, however treatment for refractory/relapse is an unmet need. We questioned whether the anti-tumor activity of a VEN-based regimen can be augmented through CD47 inhibition (CD47i) in AML and how this triplet may be enhanced. Human AML cell lines were sensitive to ALX90 and its addition increased efficacy of a VEN plus azacitidin (VEN+AZA) regimen *in vivo*. However, CD47i failed to clear bone marrow tumor burden in PDX models. We hypothesized that the loss of resident macrophages in the bone marrow in AML reduced efficiency of CD47i. Therefore, we attempted to enhance this medullary macrophage population with agonism of TLR3 via polyinosinic:polycytidylic acid (poly(I:C)), which led to expansion and activation of medullary macrophages in *in vivo* AML PDX models and potentiated CD47i. In summary, the addition of poly(I:C) can enhance medullary macrophage populations to potentiate the phagocytosis merited by therapeutic inhibition of CD47.

Materials and Methods

Cell viability assay

Following the addition of poly(I:C), cells were pipetted into the 384-well plates at a concentration of between 2000 to 8000 cells per well in IMDM or RPMI media, as noted, supplemented with 10% FBS and incubated at 37 ° C, 5% CO2 in a tissue culture incubator. Plates were incubated for 48 hours, and cell viability was measured using the Cell Titer-Glo reagent (Promega Corp.). Percent viability was defined as relative luminescence units (RLU) of each well divided by the RLU of cells in DMSO control. Six-point dose response curves were generated with GraphPad Prism version 6.0h.

Flow Cytometric Analysis of Primary Patient Samples used in PDX Models

Leukapheresis samples used in AML PDX models both 18-12-001 and 18-10-009 were over 95% positive for myeloblasts, verified by clinical flow from the Vanderbilt University Medical Center. Leukapheresis sample 18-12-001 reported myeloblasts with the following abnormal immunophenotype: positive for CD4 (dim, subset), CD7 (heterogeneous), CD11b (heterogeneous), CD13 (heterogeneous), CD15 (heterogeneous dim), CD33 (bright), CD38 (heterogeneous), CD45 (moderate), CD64 (heterogeneous dim), CD117 (heterogeneous), and HLA-DR (heterogeneous); and negative for CD2, CD14, CD16, CD19, CD34, and CD56. and were negative for negative for CD2, CD14, CD16, CD19, CD34, and CD56.

Leukapheresis sample 18-10-009 reported myeloblasts the following abnormal immunophenotype: positive for CD13 (dim), CD33 (moderate), CD38 (bright), CD45 (dim), CD56, CD117 (heterogeneous dim), and CD45 (dim); and negative for CD2, CD4, CD7, CD11b, CD14, CD15, CD16, CD19, CD34, CD64, and HLA-DR.

hCD47 Immunofluorescence

An initial $2x10^6$ MV-4-11, MOLM-13 and THP-1 per sample were used. After an initial incubation in 100nM ALX90 , cell were washed with 1xPBS. Cytospins were fixed in 10% formalin, washed in PBS, blocked with 0.1% BSA and 5% normal gout serum, then incubated with anti-human CD47 (Invitrogen Cat# MA5-11895) 1:200 dilution for 1hr. Washed with 2X3min PBS. Then 10ug/ml of 2Ab (Invitrogen Cat# A32723) was used for 1hr. Then 5 min with Dapi (1:1000), washed again and mounted with Prolong Gold (Invitrogen cat#P36930). Vanderbilt Cell Imaging Shared Resource (CISR) was used to capture the images (Dr. Jenny C. Schafer).

Confocal images were acquired on a Zeiss LSM880 confocal microscope with the following parameters: Plan-Apochromat 63x/1.4 Oil DIC objective lens; 405 nm and 488 nm excitation lasers; frame size of 1024/1024 pixels (33.74 uM x 33.74 uM); scan zoom of 4.0; pixel dwell time of 1.02 uM; and line average of 2. XY scaling is set to 0.03 uM per pixel.

DAPI channel was acquired with 405 nm excitation and 410-498 nm emission. GREEN channel was acquired with 488 nm excitation and 498-598 nm emission. Both channels were acquired with spectral photomultiplier tube (PMT) detectors. Cytospins were fixed in 10% formalin, washed in PBS, blocked with 0.1% BSA and 5% normal gout serum, then incubated with anti-human Cd47 (Invitrogen Cat# MA5-11895) 1:200 dilution for 1hr. Washed with 2X3min PBS. Then 10µg/ml of 2Ab (Invitrogen Cat# A32723) was used for 1hr. Then 5 min with Dapi (1:1000), washed again and mounted with Prolong Gold (Invitrogen cat#P36930). VUMC Confocal Images Services were used to capture the images (Dr. Jenny C. Schafer).

Phagocytosis Assay

For detection of phagocytosis, pHrodo Red labeled AML primary patient samples were stained as per manufacturer's instructions (Invitrogen). For murine macrophage phagocytosis assays, AML patient cells were incubated in pHrodo and further subjected to a 10min incubation with 100nM ALX90 or vehicle. AML cells were then incubated with bone marrow from vehicle or POLY(I:C) treated mice for 3 hours at a 1:2 ratio. After incubation, cells were washed and analyzed by flow cytometric analysis for phagocytic activity, where murine $CD45^{+}$/$F4/80^{+}$ macrophage double positive with APC pHrodo labeled human cells were denoted as phagocytic. For human macrophage phagocytosis assays, 2×10^{6} mononuclear cells from peripheral blood of healthy donors was plated into a 12-well plate and incubated for 3 hours at 37°C in RPMI medium with 10% FCS, 10% heat-inactivated and filtered human serum (Thermo Fisher Scientific), with 1% P/S. Cells were incubated for 2 hours after which nonadherent cells were removed and the new medium was added with the addition of 50ng/ml M-CSF (Peprotech). Monocytes were differentiated into macrophages over 6 days, with one media change at day 3. At day 6, macrophages were stimulated with or without 20ug/ml poly(I:C) for an additional 24hrs. Macrophages were detached, and target AML Cells were stained with pHrodo and subsequently pre-incubated with ALX90 (100nM), magrolimab(10ug/ml), human IgG1 (10ug/ml) or human IgG4(10ug/ml)(Sellechem). Macrophages and target cells were added in a 1:2 ratio in a FACS tube and incubated for 3 hours at 37°C and 5% CO2. Cells were washed and stained for flow cytometric analysis after 3 hours of incubation.

Figure Legends

Figure S1: Confocal analysis of hCD47 after incubation with ALX90 in human AML cell lines MOLM-13 and THP-1(A). Splenic weights of MV-4-11 CDX model treated with VEN/AZA and ALX drug regimens (B) and body weight (C).

Figure S2: The addition of ALX90 to a VEN/AZA regimen leads to decreased tumor burden in an MV-4-11 as shown through Immunohistochemistry for human CD45(A). Peripheral AML kinetics in a PDX model reveals a significant decrease in tumor cells when ALX is added to a VEN/AZA regimen(B).

Figure S3: Macrophage phenotype was measured according to M0 (F4/80+/CD11b-/CD206-), M1(F4/80+/CD11b+/CD206-), or M2(F4/80+/CD11b-/CD206+), status in additional PDX and naïve NSGS mouse models (A). The combination of ALX and Poly(I:C) in an AML PDX(B).

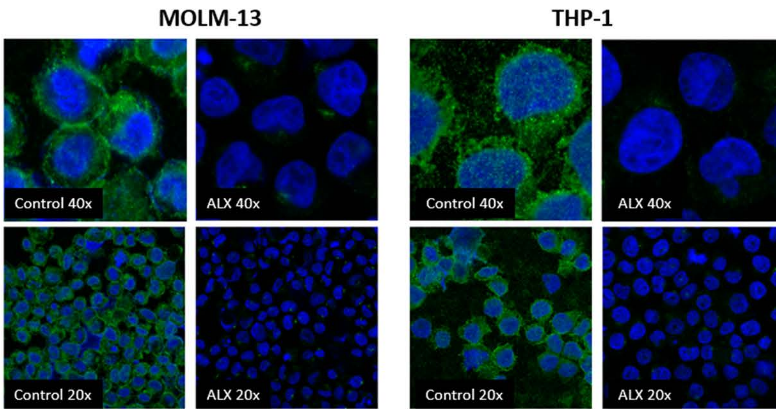
Figure S4: Cell viability analysis of AML primary patient samples and AML cell lines treated with poly(I:C) at 48 hours, as measured by cell titer-glo (A). Flow cytometric analysis of bone marrow macrophages from PDX mice treated with VEN/AZA+ALX and a VEN/AZA+ALX+poly(I:C) regimen (B).

Figure S5: *Ex vivo* phagocytosis assay shows increased efficacy of magrolimab in the presence of POLY(I:C) treated macrophages, in comparison to IgG4 treated AML cells alone.

Table S1: Characteristics of AML patient samples

Figure S1

A



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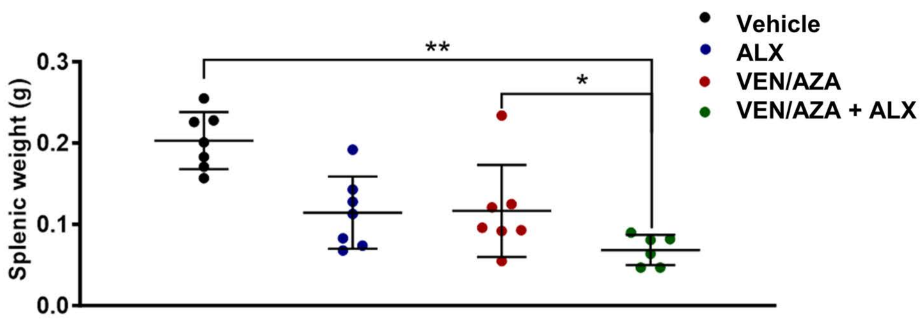

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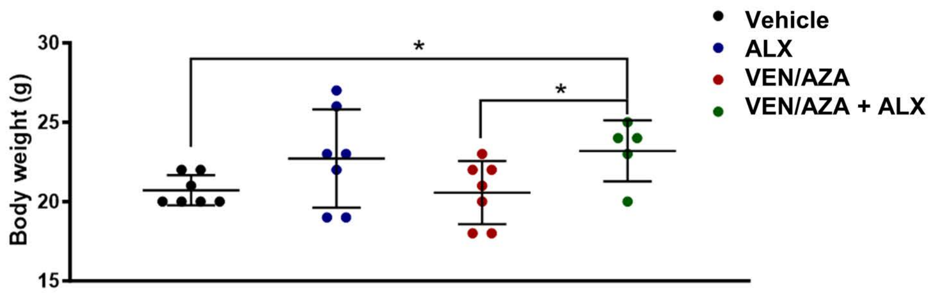

Figure S2

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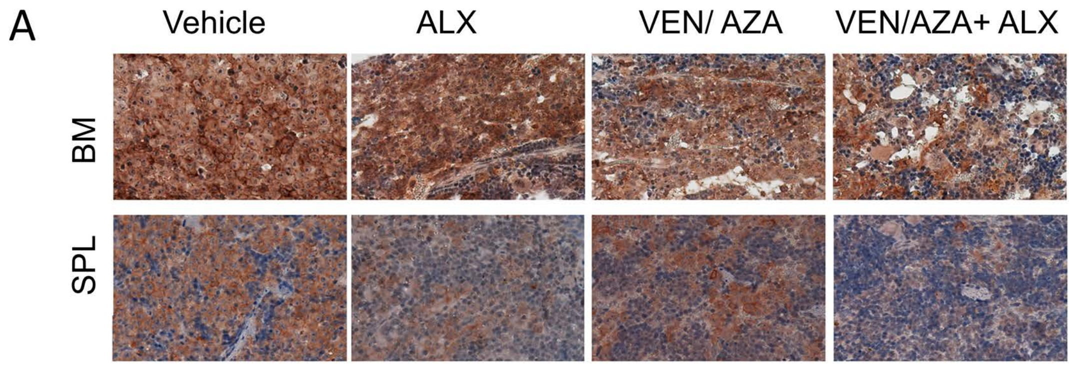

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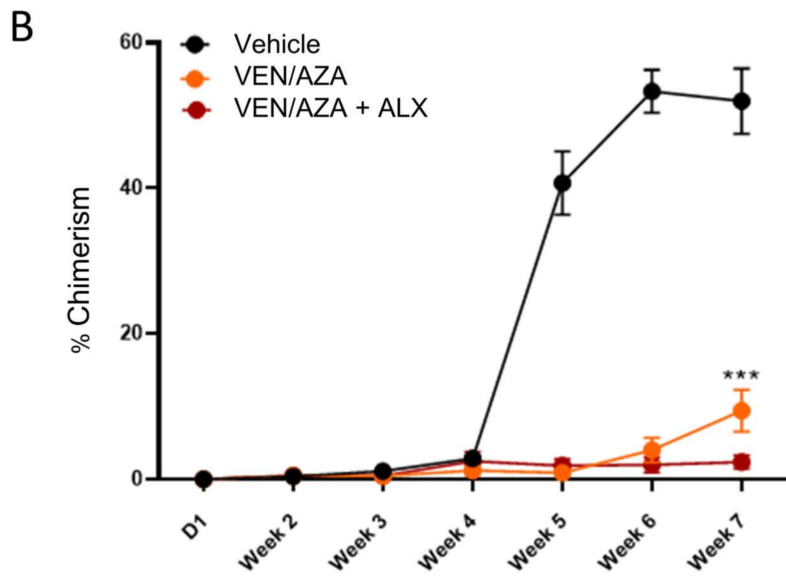

Figure S3

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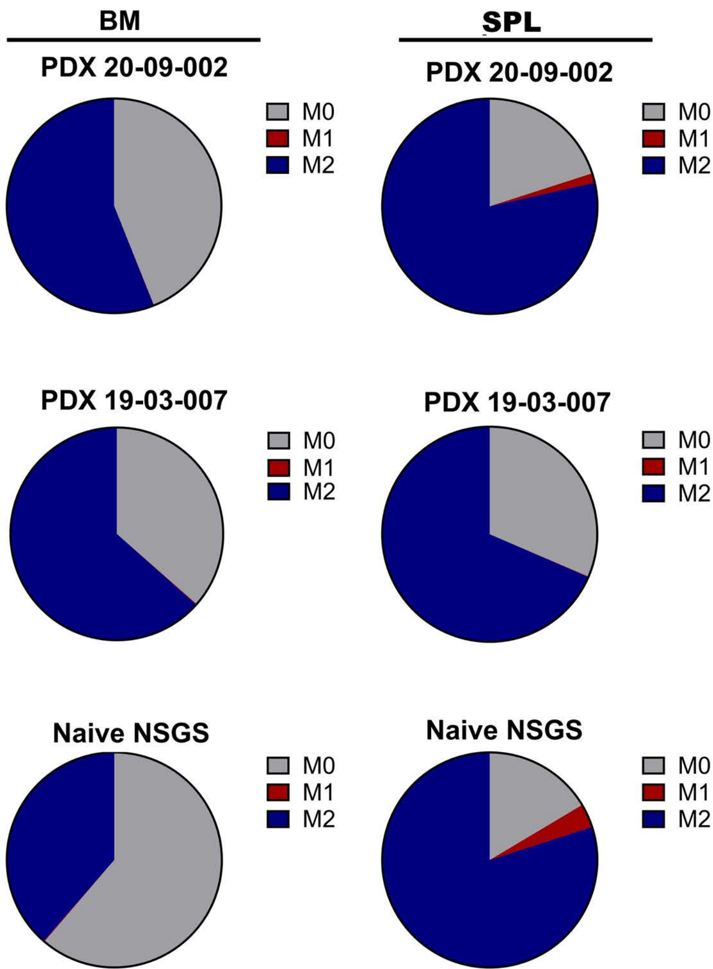

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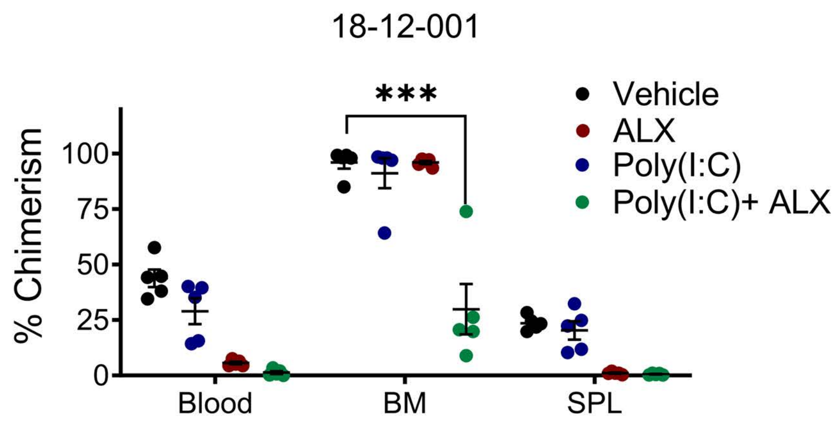

Figure S4

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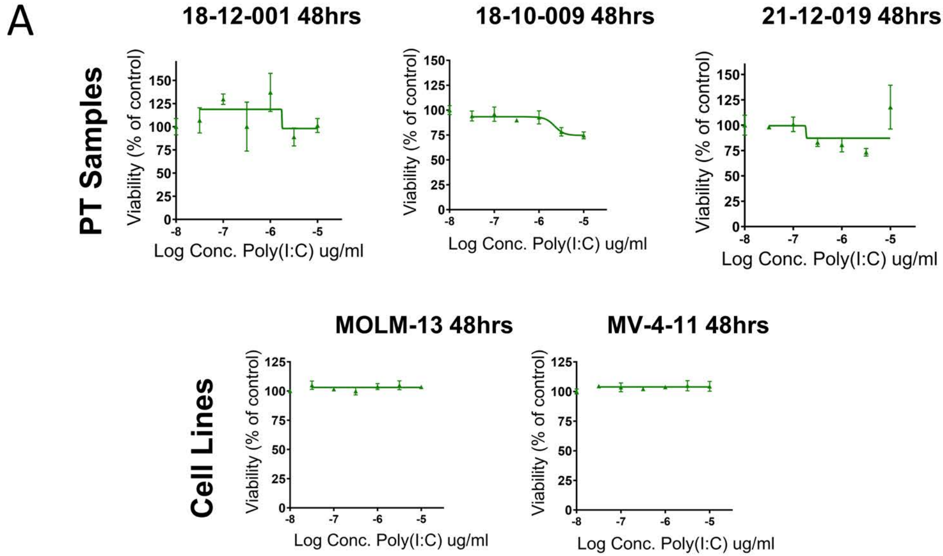

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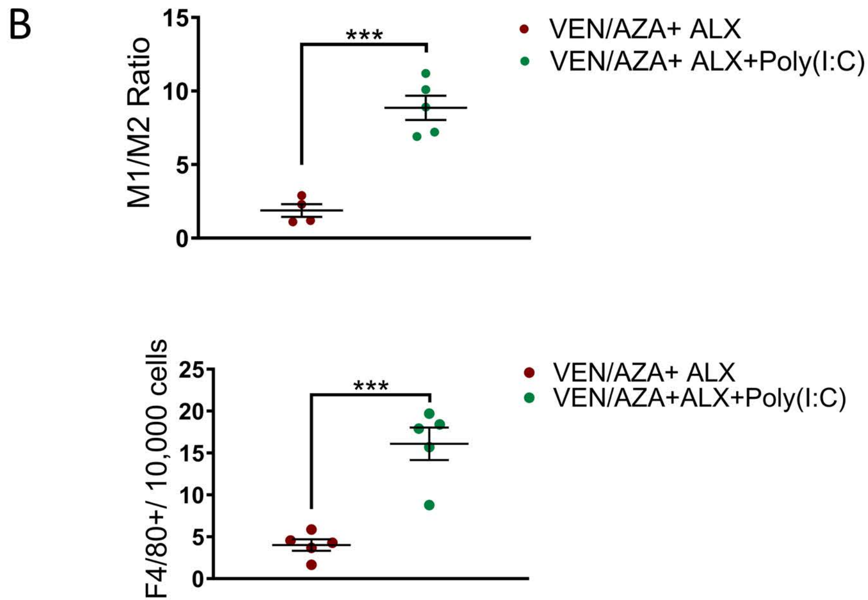

Figure S5

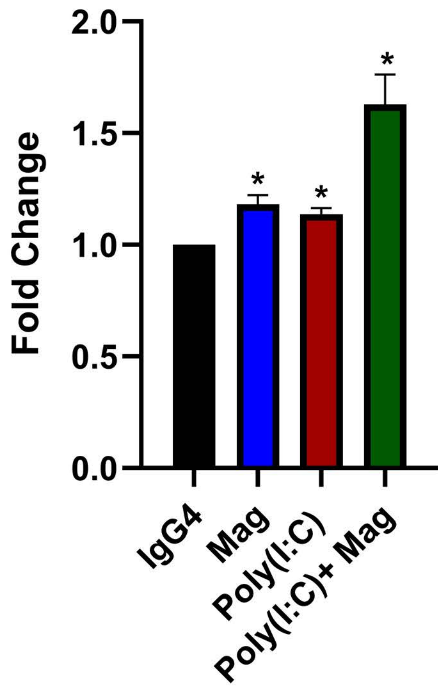

Table S1

Sample	Diagnosis	Source	Bone marrow Blast (%)	Pre-Treated (Y/N)	Karyotype	Mutation 1	Mutation 2	Mutation 3	Mutation 4	Mutation 5
18-12-001	AML	L	94	N	46, XX[20]	IDH2; c.419G>A; 48%	DNMT3A; c.2644C>T; 47%	NPM1; c.860_863dup;45%	FLT3-ITD; C.1738_1794dup; 32%	PTPN11;c.213 T>A
18-10-009	AML	L	93	Y	46, XY[20]	DNMT3A; c.2644C>T; 52%	FLT3-ITD; C.1837_1837_Ins65; 60%	IDH1; c.395G>A; 49%	NPM1; c.860_863dup; 42%	
20-09-002	AML	L	83	N	46, XX[20]	NPM1; c.860_863dup; 38%	FLT3-ITD; c.1784_1837+3dup; 52%	DNMT3A; VUS; c.1121A>C; 55%	DNMT3A (VUS); c.1584G>A; 48%	
19-03-007	AML	L	73	N	47, XX, +8, t(9;11)(p.22;q23)	FLT3; c.1775T>C; 46%				
14-10-002	AML	BM	90	N	46, XX, t(9;11)(p22;q23)[20]	None detected				
19-01-012	AML	L	80	Y	46, XY,+8[6]/46, idem,add(8)(p11.2)-21[cp14]	FLT3; c.2505T>G; 45%	TET2; c.3165del; 43%	TP53; c.839G>G; 8%	ASXL1; c.3865C>T; 48%	TP53; VUS; c.691_696dup; 55%
17-12-011	AML	L	95	N	46,XX,-16,add(17)(p11.2)[18]/46,XX[2]	FLT3; c.2508_2510del; 43%	TET2; c.2392G>T;47%	TP53; c.578A>T; 80%	TET2; c.4100C>A; 51%	
21-12-019	AML	L	n/a	N	46, XX[20]	NPM1; 44%	FLT3; 28%	ASXL1; 48%		

L= leukapheresis, BM= bone marrow