# 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.

## Introduction

CD47 is a ubiquitous cell surface protein that serves as myeloid checkpoint and is the ligand for the macrophage receptor, SIRPα. The immune system relies on the binding of CD47-SIRPα to recognize cells as 'self' leading to the moniker of the 'don't eat me' signal.[1,2] The CD47-SIRPα is a myeloid check point and is highly conserved in mammalian species. Cancer cells aberrantly overexpress CD47 to evade immune detection and destruction. As CD47 expression is prevalent in hematologic malignancies, trials targeting CD47 with monoclonal antibodies have been a recently explored strategy in the clinic.[3,4]

Targeting CD47 has led to immune response against tumor tissues, but also led to opsonization of RBC, as older RBC with less CD47 are more susceptible to premature clearance by CD47 antagonists, leading to anemia. Previous groups have tried to address CD47 antagonist-induced hematotoxicity using dual targeting bispecific antibodies to CD47 and anti-SIRPα antibodies.[5-7] The novel CD47 inhibitor evorpacept (ALX148) was engineered with an inactive Fc binding subunit and a high affinity CD47 binding domain. The inactive Fc domain prevents a pro-phagocytic signal and consequent clearance of RBC to improve the overall tolerability of CD47-targeted therapy.[8] ALX90 is a small molecule inhibitor of CD47, with nanomolar level selectivity that serves as a tool compound for ALX148.[8-10] ALX90 did not exhibit hemagglutination activity when compared to other CD47 antibody clones in human, monkey and murine blood samples. Similarly, the clinical analog, ALX148, led to no changes to the hematological parameters in treated mice.[9]

Acute myeloid leukemia (AML) is hallmarked by the acquisition of somatic mutations and chromosomal structural abnormalities, the arrest of normal cellular differentiation,

and clonal expansion of immature myeloid cells, leading to ineffective hematopoiesis. In the past, standard of care therapy has been limited to cytotoxic chemotherapy such as cytarabine and anthracycline. However, since 2017 several targeted therapies were approved by the Food and Drug Administration as viable therapeutic options. The BCL-2 inhibitor VEN has demonstrated particular success, which has emerged as a critical component to the standard of care in multiple lymphoid and myeloid neoplasms. As a single agent, VEN demonstrates modest activity in relapsed/refractory AML,[11] yet when used in combination with a DNA methyltransferase inhibitor (DNMTi)[12] or low-dose cytosine arabinoside (LDAC) led to remission in 73% of newly diagnosed elderly patients.[13-15] Unfortunately, despite these encouraging response rates, most patients treated with VEN will eventually relapse. Previous studies have suggested mechanisms of resistance for both primary and acquired BCL-2 resistance include increased levels of MCL-1 as well as mutations in *TP53*.[12,16,17] Increasing and prolonging VEN efficacy in hematologic malignancies remains a priority.

We first sought to test CD47 inhibition in the context of an AML VEN-based regimen to determine if efficacy could be increased. We added ALX90 to a standard of care VEN/azacitidine (AZA) regimen in multiple AML *in vivo* models. In cell line-derived xenografts (CDX) models where medullary tumor burden is low (<50% cell of bone marrow [BM] cell volume at moribund) and native macrophages are present at endpoint found that CD47 inhibition led to a significant decrease in tumor burden as a monotherapy, and augments standard of care therapy with VEN/AZA. However, in patient-derived xenograft (PDX) models with high tumor burden and low native medullary macrophage constituency, CD47i failed to decrease medullary tumor burden as a monotherapy, despite almost complete clearance of tumor cells within the peripheral blood and spleen. Within the paucity of macrophages present in the marrow of PDX models, almost all were polarized toward M2. This implied that the small population of tumor-supportive M2 macrophages, and lack of M1 tumor-suppressive macrophages limited the potential of CD47 inhibition. Thus, in an attempt to increase medullary macrophage function in mice, we co-treated with poly(I:C), a toll-like receptor 3 (TLR3) agonist which mimics viral double stranded RNA and is known to stimulate macrophage differentiation and polarization to M1 macrophages.[18-20] As expected, in naïve NSGS mice, poly(I:C) transiently increased M1 macrophage populations in bone marrow, splenic, and blood compartments. Surprisingly, when poly(I:C) was combined with a VEN/AZA + ALX90 in PDX models it led not only to significant decreases in tumor burden in all compartments, but allowed for return to normal murine hematopoiesis and healthy BM. Poly(I:C) combined with ALX90 likewise reduced patient-derived leukemia in the BM of immunocompromised mice. These data suggest that in the setting of AML, the presence of active BM M1 macrophage populations is required to induce a meaningful response to CD47i. We demonstrate here that by increasing BM macrophage function in murine models of AML, a more effective immune microenvironment may be restored, thereby potentiating CD47i in AML.

# Methods

### Patient samples

Experiments were conducted on primary patient samples which were provided by the Vanderbilt-Ingram Cancer Center Hematopoietic Malignancies Repository and are in accordance with the tenets of the Declaration of Helsinki and approved by the Vanderbilt University Medical Center Institutional Review Board.

### Cell lines

AML cell lines MV-4-11 and THP-1 were purchased from the American Type Culture Collection (Manassas, VA). The MOLM-13 cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). ATCC and DSMZ cell lines are authenticated by short tandem repeat profiling and cytochrome C oxidase gene analysis. Cells were used for the experiments presented here within 10-30 passages from thawing. MV-4-11 cell line was grown in IMDM, and all other cell lines were cultured in RPMI and supplemented with 10-20% fetal bovine serum and 100 U/mL penicillin and 100 ug/mL streptomycin. Cells were kept at 37°C in a 5% $CO_2$ incubator.

### *In vivo* murine model

All animal studies were conducted in accordance with guidelines approved by the IACUC at Vanderbilt University Medical Center. Female NSGS [NOD/SCID/Tg(hSCF/hGM-CSF/hIL3)] mice, 6-8 weeks old were irradiated with 1 Gy microwave radiation. Twenty-four hours later, mice were injected with $1x10^6$ MV-4-11, $3x10^3$ MOLM-13 or $2x10^6$ AML leukapheresis primary patient cells via tail vein injection. Leukapheresis sample description is included in the *Online Supplementary Appendix*. After establishing microchimerism, 1-2 weeks post-transplant, mice were treated with either VEN and AZA (VEN 20 mg/kg 5 days [D] on 2 D off, AZA upfront Monday/Wedneday/Friday [M/W/F] 1.5 mg/kg), with and without ALX 90 (30 mg/kg M/W/F), the combination, or vehicle. Poly(I:C) (Invivogen) was dosed at 15 mg/kg M/W/F every 2 weeks, with 1 week off between cycles, for up to 8 weeks. Peripheral blood draws were taken weekly to assess chimerism.

### Flow cytometry

For flow cytometry of murine blood and tissues, red blood cells were lysed with EL Buffer on ice (Qiagen), with remain-

ing cells washed and resuspended in 1x phosphate-buffered saline (PBS) with 1% bovine serum albumin and stained for 15 minutes with the following antibodies: human CD45-APC, human CD33-PE-Cy7, murine CD45-PE and DAPI (Biolegend). For CD47 measurement in cell line experiments, human CD47-FITC (Biolegend) was used. As a background control, positive mean fluorescence intensity (MFI) from either control human IgG4-FITC or human IgG1-FITC was subtracted from CD47 MFI to estimate final value. Cells were washed and submitted for flow cytometric analysis using a 3-laser LSRII (Becton Dickinson).

### Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol before being embedding in paraffin and sectioned at 5 µm. The bone tissue was decalcified prior to being embedded in paraffin. Sections were dewaxed in Xylene and rehydrated in ethanol. Standard Mayer's Hematoxylin and Eosin staining was performed. Antigen retrieval using a standard pH 6 sodium citrate buffer (BioGenex) was performed and sections were stained with monoclonal mouse anti-human CD45 (Dako, M0701, dilution 1:200) using M.O.M. Kit (Vector).

### Statistics

Unless otherwise noted, data were summarized using the mean (+/- standard deviation). Per group sample sizes are presented in figures and results reported from two separate experiments, unless stated otherwise. In order to avoid normality assumptions, pairwise group comparisons were made using the non-parametric Mann-Whitney U test. The non-parametric Spearman correlation was used to assess pairwise variable associations. Data were analyzed using Graph Pad Prism 6.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). *$P$<0.05, **$P$<0.01 and ***$P$<0.001.

## Results

### ALX90 significantly conceals surface expression of CD47 on multiple acute myeloid leukemia cell lines

In order to ensure CD47 specificity of the CD47i ALX90, we incubated AML cell lines MV-4-11, MOLM-13, and THP-1 with ALX90 or magrolimab and stained for CD47. Flow cytometry revealed a significant decrease in detectable surface expression of CD47 MFI in the presence of ALX 90

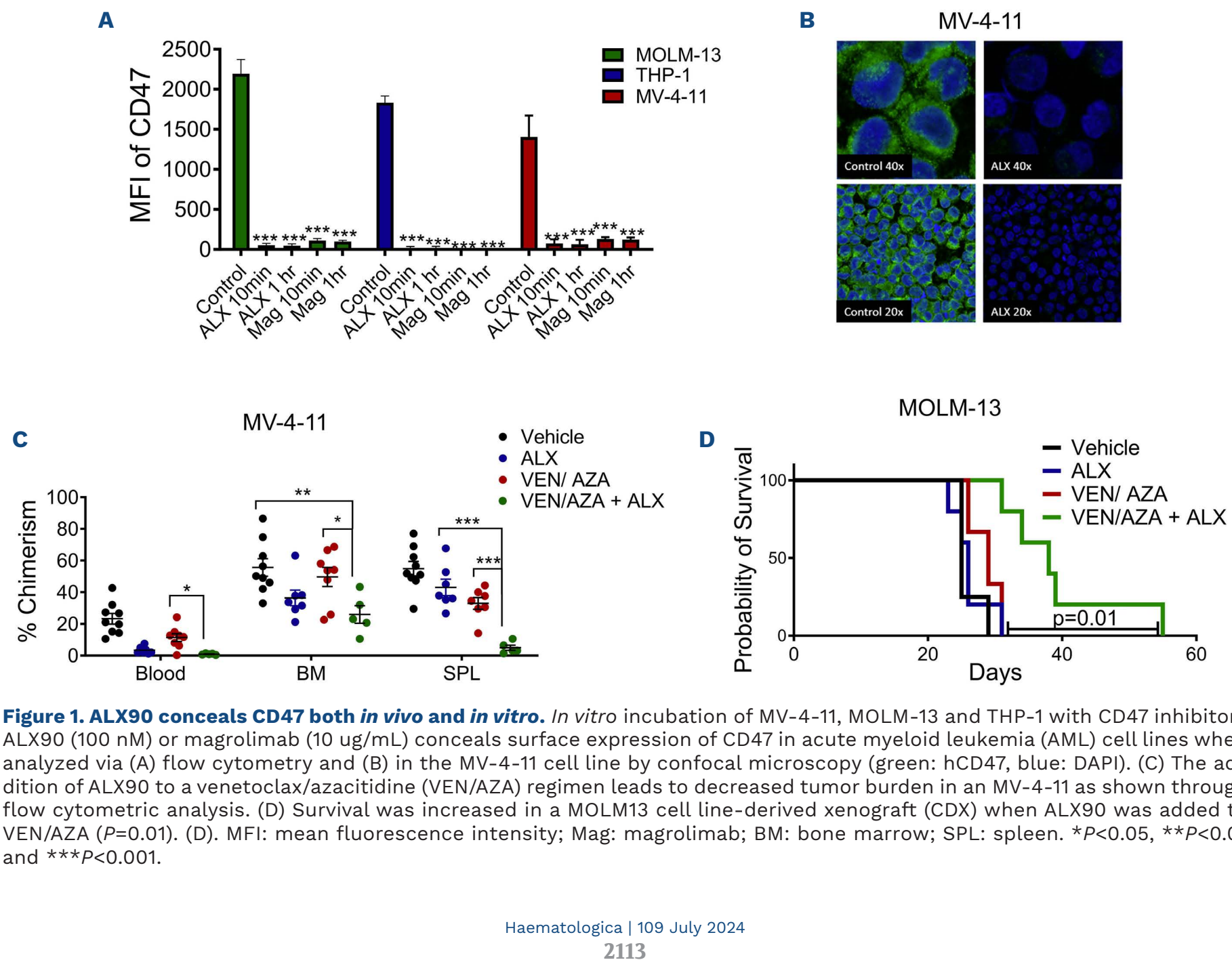


**Figure 1. ALX90 conceals CD47 both *in vivo* and *in vitro*.** *In vitro* incubation of MV-4-11, MOLM-13 and THP-1 with CD47 inhibitors ALX90 (100 nM) or magrolimab (10 ug/mL) conceals surface expression of CD47 in acute myeloid leukemia (AML) cell lines when analyzed via (A) flow cytometry and (B) in the MV-4-11 cell line by confocal microscopy (green: hCD47, blue: DAPI). (C) The addition of ALX90 to a venetoclax/azacitidine (VEN/AZA) regimen leads to decreased tumor burden in an MV-4-11 as shown through flow cytometric analysis. (D) Survival was increased in a MOLM13 cell line-derived xenograft (CDX) when ALX90 was added to VEN/AZA ($P$=0.01). (D). MFI: mean fluorescence intensity; Mag: magrolimab; BM: bone marrow; SPL: spleen. *$P$<0.05, **$P$<0.01 and ***$P$<0.001.

and magrolimab, representing coverage of CD47 protein (Figure 1A). Complete inhibition of CD47 by ALX90 was confirmed using immunofluorescence in MV-4-11 (Figure 1B) as well as MOLM-13 and THP-1 (*Online Supplementary Figure S1A*).

### The addition of CD47 inhibition to venetoclax/azacitidine treatment regimen leads to decreased tumor burden and lifespan prolongation in multiple *in vivo* cell line derived models of acute myeloid leukemia

In order to determine the efficacy of CD47 inhibition *in vivo*, NSGS mice were treated with a VEN/AZA, ALX90 or a combination. On day 28, when vehicle mice became moribund, tissues were collected for tricompartmental analysis, and human CD45/CD33$^+$ AML cells were counted relative to the amount of healthy murine CD45$^+$ cells. As shown in Figure 1C the addition of CD47i to a VEN/AZA regimen led to a significant decrease in AML when compared to a VEN/AZA alone. Furthermore, CD47i amended splenic weight, a result of reduced leukemia within the spleen and decreased extramedullary hematopoiesis, which is reflective of healthy BM (*Online Supplementary Figure S1B*), as well as body weight (*Online Supplementary Figure S1C*). Bone marrow and splenic tissues were stained for human CD45, and immunohistochemistry revealed a significant decrease of tumor cells in both bone marrow and spleen (*Online Supplementary Figure S2A*). In an additional MOLM-13 cell line xenograft, the addition of CD47 inhibition led to significant increases in lifespan compared to ALX or VEN/AZA treatments alone (Figure 1D).

### CD47i leads to decreased tumor burden in extramedullary compartments of multiple acute myeloid leukemia patient-derived xenograft models, but not within bone marrow

Given our observation that AML PDX models confluently involved the BM, we began measuring the expression of CD47 in various AML PDX models in an attempt to correlate surface protein expression as a biomarker. As shown in Figure 2A, varying levels of CD47 were noted between patient samples, which were not dependent on any detected mutations (*Online Supplementary Table S1*). In order to further elucidate the role of CD47 as a targetable biomarker, we explored the efficacy of monotherapy in PDX models with both high "++" (18-12-001) and low "+" (18-10-009) CD47 expression. Post-transplantation, peripheral chimerism was tested weekly in these models in search of circulating tumor cells, and leukemic engraftment was established

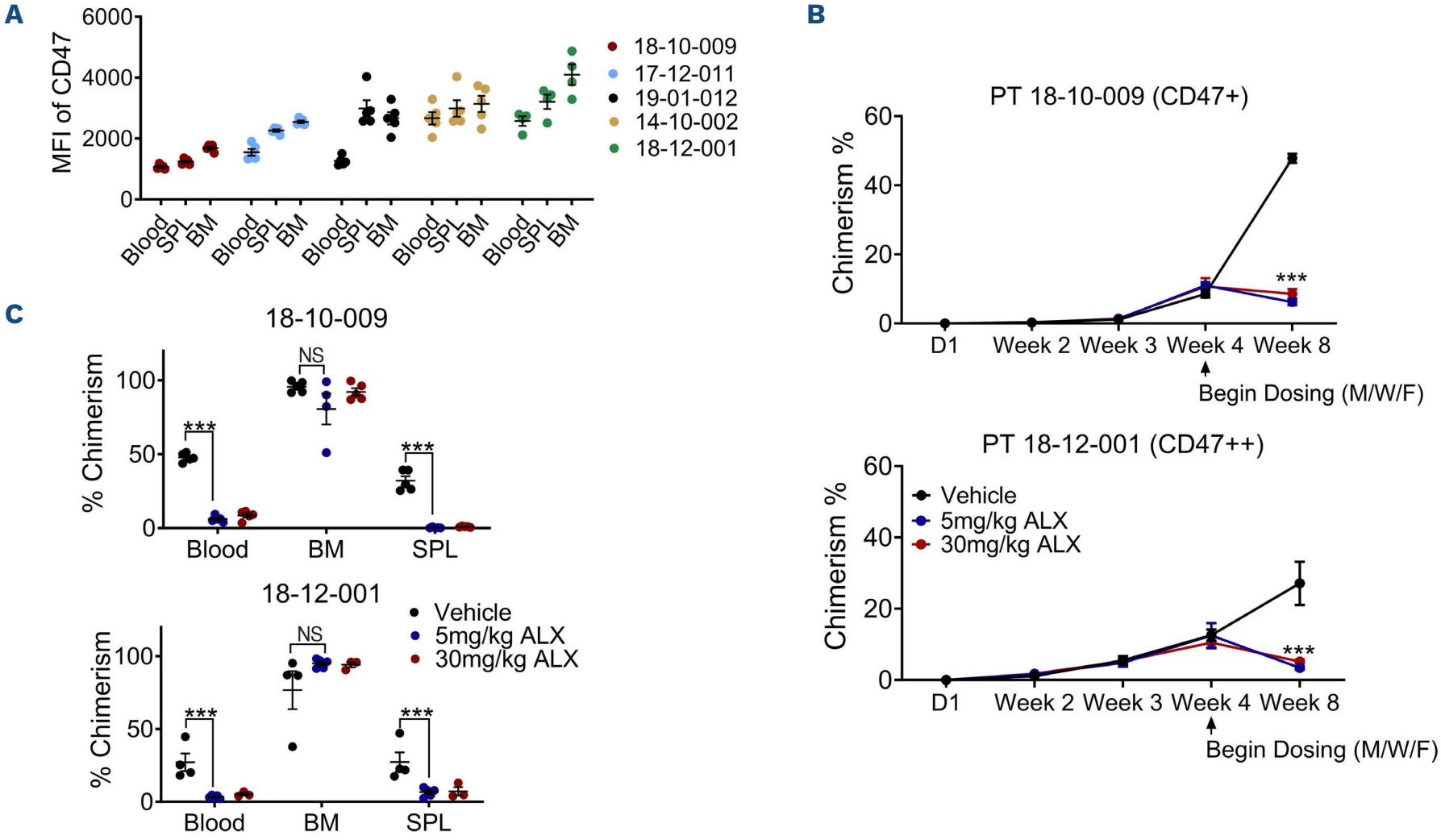


**Figure 2. CD47 inhibition monotherapy reveals minimal tumor clearance from bone marrow in a patient-derived xenograft model.** (A) Surface expression of hCD47 was measured by flow cytometry in multiple patient-derived xenograft (PDX) models. (B) CD47 inhibition leads to decreased leukemia in peripheral blood of PDX models with significant differences shown between 30 mg/mL and vehicle groups. (C) Chimeric analysis of PDX models reveals the effects of ALX in blood, spleen (SPL), and bone marrow (BM). MFI: mean fluorescence intensity; M/W/F: Monday/Wednesday/Friday; NS: not significant. *$P$<0.05, **$P$<0.01 and ***$P$<0.001.

at 4 weeks post-transplant. At this juncture, mice were treated with ALX90 monotherapy at a low 5 mg/kg and high 30 mg/kg dose (Figure 2B). Four weeks after treatment, mice were euthanized, and tissues were harvested for tricompartmental analysis. CD47i monotherapy led to a significant decrease in tumor burden in both the blood and spleen (Figure 2C). Contrary to CDX models, much of the BM remained unaffected in these PDX (Figure 2C). In order to confirm that ALX90 bound to CD47 antigen on PDX cells, we harvested human cells from the blood, BM and spleen from these mice, stained them for CD47, and quantified ALX90 bound human hematopoietic $CD45^+/CD33^+$ cells in a dose-dependent fashion (Figure 3A).

In order to determine if VEN/AZA increased efficacy of CD47i monotherapy, we treated the CD47-low AML PDX (18-10-009), with VEN/AZA and ALX90. Tumor kinetics were monitored peripherally through weekly measurements in blood chimerism, showing a significant decrease in chimerism in a regimen of all three drugs (*Online Supplementary Figure S2B*). Mice were sacrificed at 8 weeks, and VEN/AZA+ALX was found to lead to significant decreases of AML in both the blood and spleen of mice, with a more modest effect is more modest in the bone marrow (Figure 3B); however, the addition of CD47i failed to prolong the lifespan of VEN/AZA treated mice in this experiment (Figure 3C).

### CD47 inhibition in the bone marrow may be dependent upon the number and type of macrophages residing within the bone marrow during treatment

CD47 inhibition was detected on BM resident tumor cells in a PDX model of AML (Figure 3A), yet tumor chimerism levels remained unchanged in that compartment (Figure 3B). In order to understand this, we examined the resident macrophages responsible for phagocytosis of cells treated with CD47i. Using the remaining cells from our PDX models from the experiment in Figure 3, we calculated the number of macrophages residing within the compartments and compared with the number of macrophages found within a healthy, naïve NSGS mouse. In all treatment groups, macrophages were increased in the blood and significantly decreased in the other tissues, with the largest diminishment observed in the BM (Figure 4A-C).

Leukemia-associated macrophages (LAM) are a type of tissue-resident macrophages noted in the presence of leukemia. LAM are largely noted to be $CD206^+$ 'pro-tumoral', M2 macrophages. M2 macrophages are considered pro-tumoral due to their decreased phagocytotic ability and production of immunosuppressive cytokines.[21,22] Interestingly, CD206 expression has been noted as a negative prognostic factor in AML.[21] By contrast, M1 ($CD11b^+$) macrophages are associated with pro-inflammatory/anti-tumoral activity with increased phagocytosis and the production of inflammatory cytokines.[23] Treatments such as lipopolysaccharide and interferon-γ (IFN-γ) have been shown to promote proliferation and polarization of macrophages to an M1 status, supporting TH1 activation and tumoricidal activity.[24,25] While little is known about the effect that AML blasts have on the tissue-resident macrophages in murine PDX models, we began to look at these populations. To this end, we began characterizing the $F4/80^+$ resident macrophage populations in two additional PDX models 8 weeks post-transplantation (20-09-002 and 19-03-007),

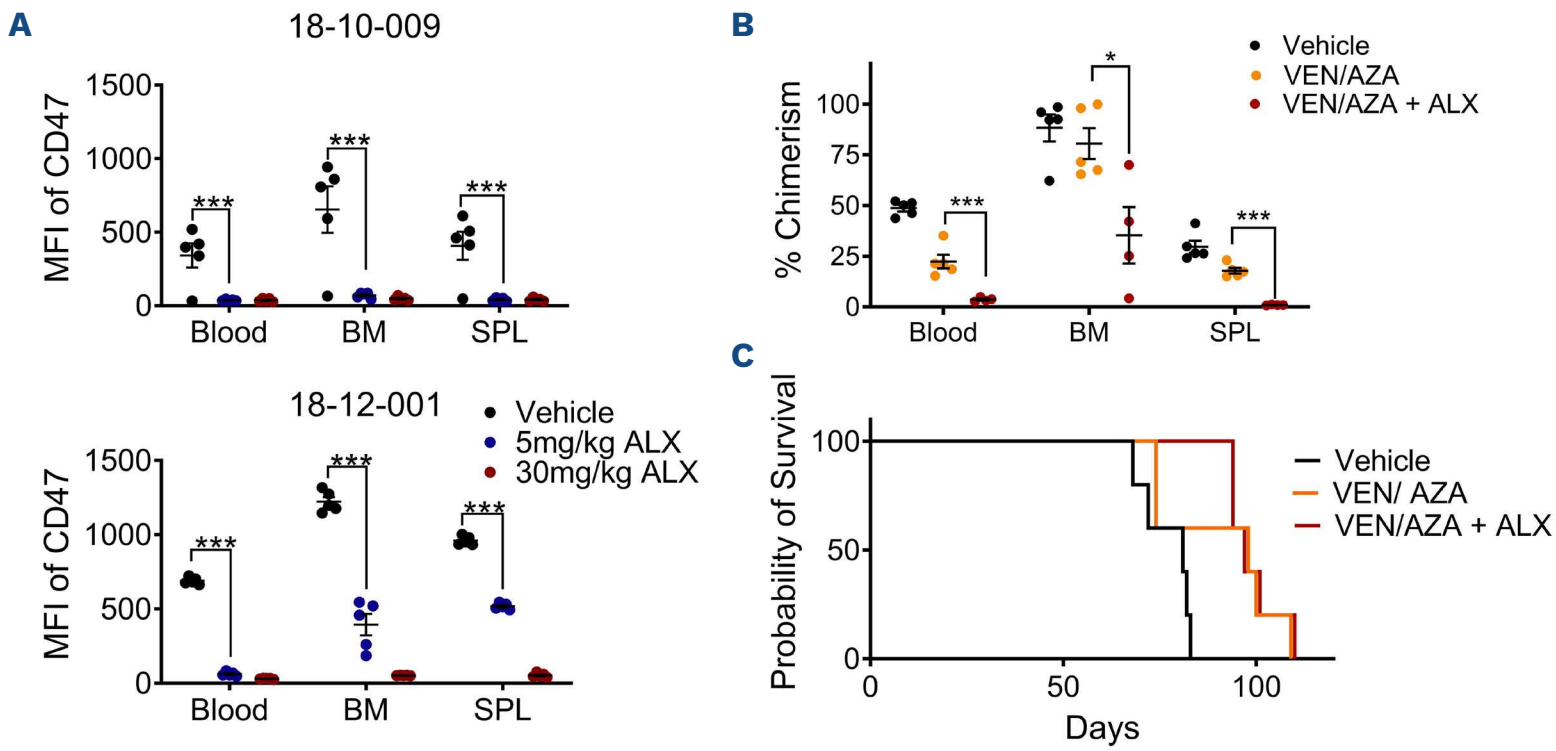


**Figure 3. The activity of CD47i in the bone marrow.** (A) Flow cytometric analysis denoting coverage of hCD47 epitope on human acute myeloid leukemia (AML) cells of 2 AML patient-derived xenograft (PDX) models. The addition of ALX90 to a venetoclax/azacitidin (VEN/AZA)-based regimen in AML PDX (18-10-009), leads to decreases in intercompartmental tumor burden (B) with Kaplan-Maier analysis of an VEN/AZA and VEN/AZA + ALX regimen showing failure to increase lifespan (C). MFI: mean fluorescence intensity; BM: bone marrow; SPL: spleen. *$P$<0.05, **$P$<0.01 and ***$P$<0.001.

comparing them to naïve NSGS mice. In the BM, the more tumor permissive M2 phenotype was dominant in PDX and undifferentiated M0 phenotypic macrophages were more common in native NSGS mice (*Online Supplementary Figure S3A*). These findings imply that the presence of AML blasts within the BM affects the relative number of macrophages, and polarizes those resident macrophages toward a tumor-permissive phenotype.

### TL3 agonism leads to the expansion of F4/80 macrophages in the bone marrow, spleen and blood of treated mice

Given to the historical application of IFN-γ in myeloid malignancies, and the role IFN-γ plays in macrophage differentiation, IFN-γ was proposed as potential therapeutic to artificially increase macrophage populations within the BM.[26-28] However, IFN-γ has only been shown to increase or activate macrophages with the addition of LPS.[29-31] Poly(I:C) is a TLR3 agonist which mimics viral double-stranded RNA in a sterile immune response known to stimulate macrophage differentiation and polarization to M1 macrophages.[18] Systemic administration of poly(I:C) in a TLR3-deficient mouse resulted in significant reductions in both macrophage activation and cytokine production compared to TLR3 wild-type.[32] Additionally, poly(I:C) has synergized previously with CD47 blockade in colorectal cancer models.[33] As variations of poly(I:C) are in use in over 13 clinical trials currently as an immunoadjuvant, we adapted use of this TLR3 agonist in our murine models.[34-37] Administering poly(I:C) over a 2-week period revealed significant shifts in macrophage phenotype, most notably the additional presence or increase in M1 macrophage populations (Figure 5A). Furthermore, the ratio of M1/M2 macrophages changed significantly between the treatment time points in these naïve NSGS mice, with dramatic shifts being noted after 2 weeks of poly(I:C) (Figure 5B). Additionally, levels of macrophages in of poly(I:C)-treated mice increased while receiving treatment (Figure 5C).

### TLR3 agonism potentiates CD47i by increasing active macrophages in a patient-derived xenograft model

Given the response of naïve NSGS mice to TLR3 agonism via poly(I:C) treatment, we attempted to increase BM macrophage populations to augment the effect of ALX90 in PDX models. In a AML PDX (18-12-001) mice were treated with ALX90 and or poly(I:C) to illustrate effects of TLR3 stimulation on CD47 inhibition. We found that poly(I:C) augmented the use of ALX90, but had negligible effects on the BM chimerism when used as a single agent (*Online Supplementary Figure S3B*). Neither PDX patient samples (18-12-001, 18-10-009, an additional sample 21-12-019) nor cell lines (MV-4-11, MOLM-13) were affected by poly(I:C) alone, suggesting that poly(I:C) is not inherently toxic to blast cells (*Online Supplementary Figure S4A*). In order to illustrate if addition of TLR3 agonism could improve upon triplet therapy (Figure 2), we transplanted PDX 18-10-009

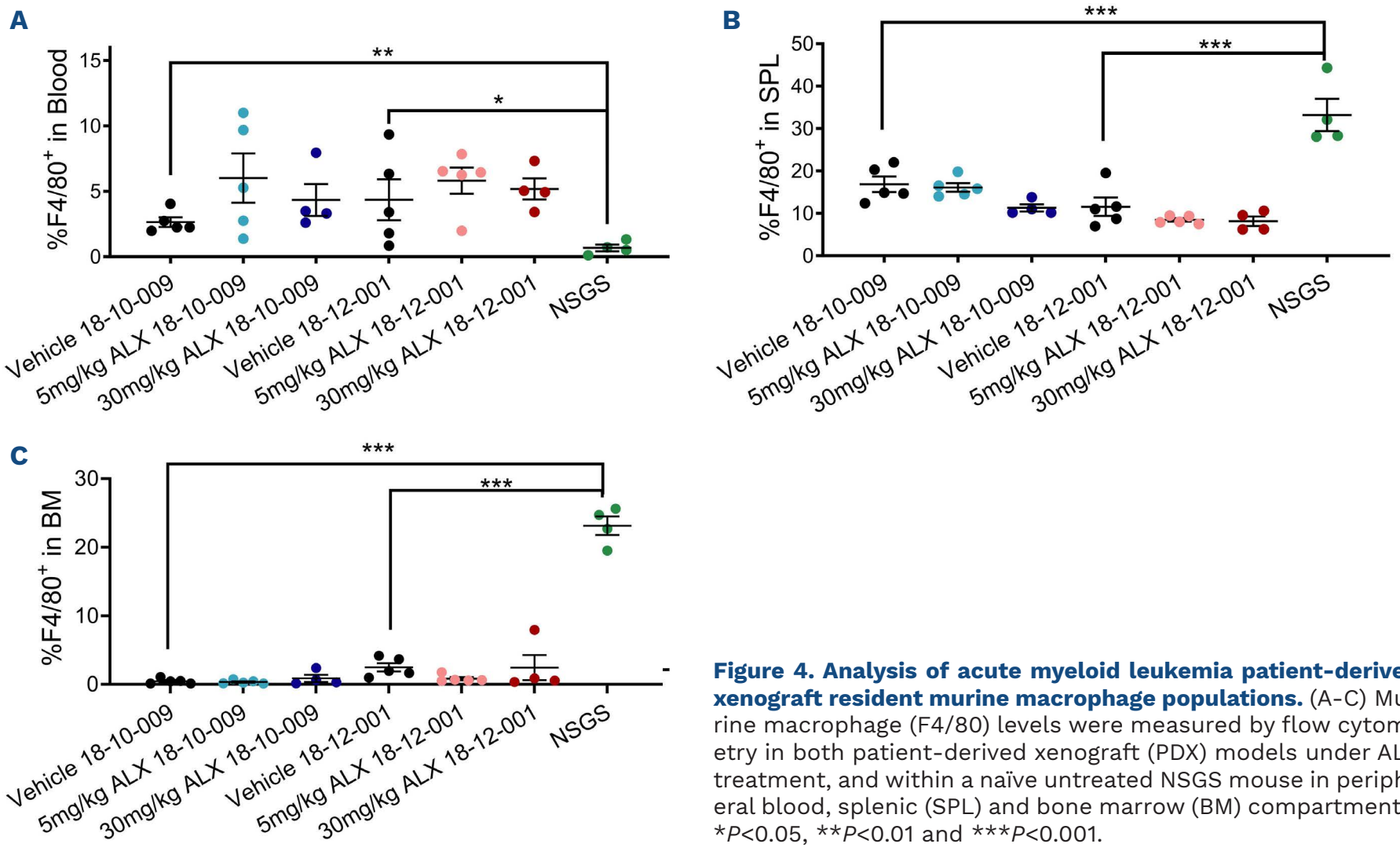


**Figure 4. Analysis of acute myeloid leukemia patient-derived xenograft resident murine macrophage populations.** (A-C) Murine macrophage (F4/80) levels were measured by flow cytometry in both patient-derived xenograft (PDX) models under ALX treatment, and within a naïve untreated NSGS mouse in peripheral blood, splenic (SPL) and bone marrow (BM) compartments. **P*<0.05, ***P*<0.01 and ****P*<0.001.

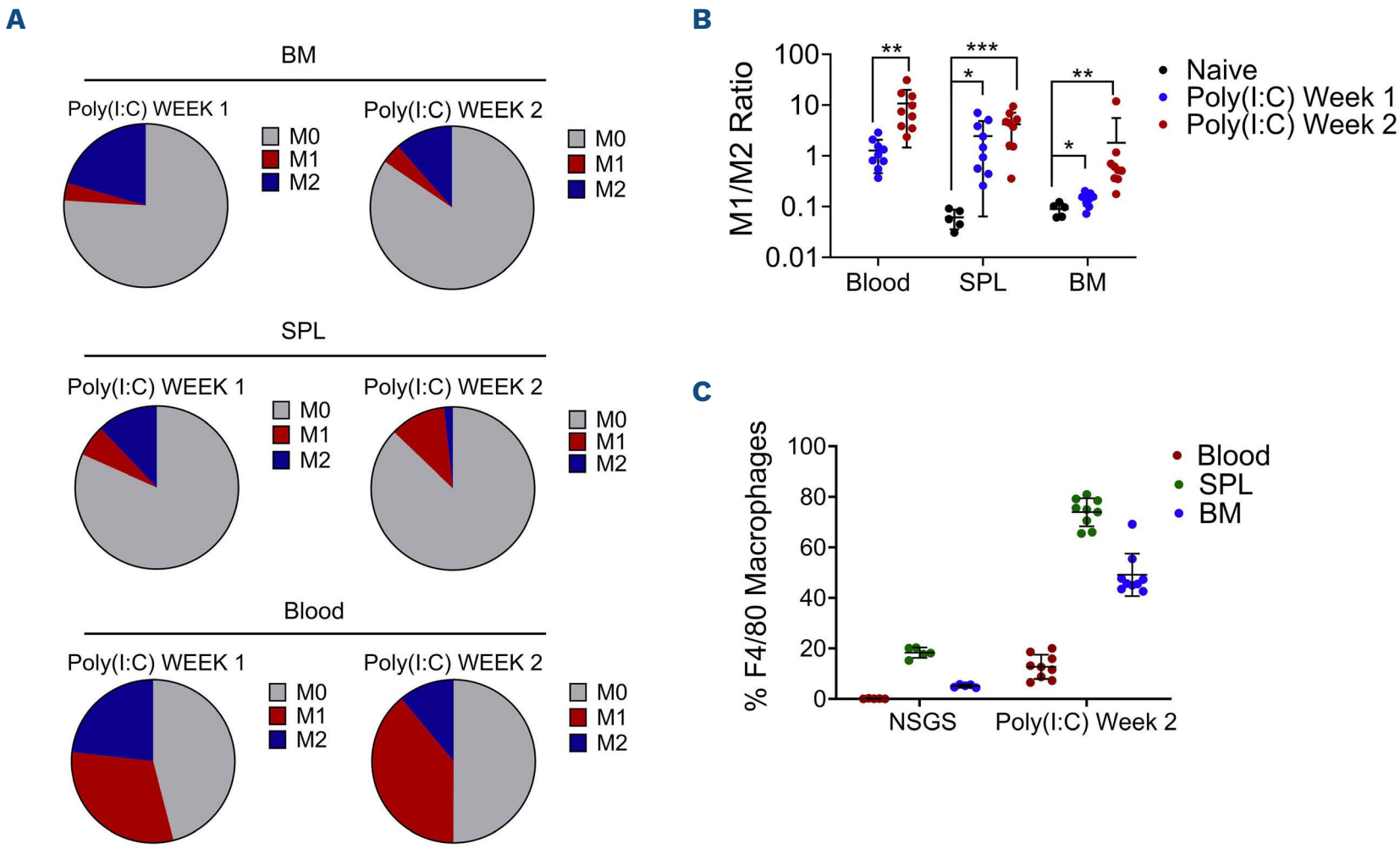


**Figure 5. The activity of polyinosinic:polycytidylic acid on murine macrophages.** (A) Polyinosinic:polycytidylic acid (poly(I:C)) leads to overall shifts in macrophage populations in the bone marrow (BM), spleen (SPL) and peripheral blood of treated mice. Shifts in M1/M2 ratio develop across compartments (B) while macrophage numbers increase (C) upon poly(I:C) administration. **P*<0.05, ***P*<0.01 and ****P*<0.001.

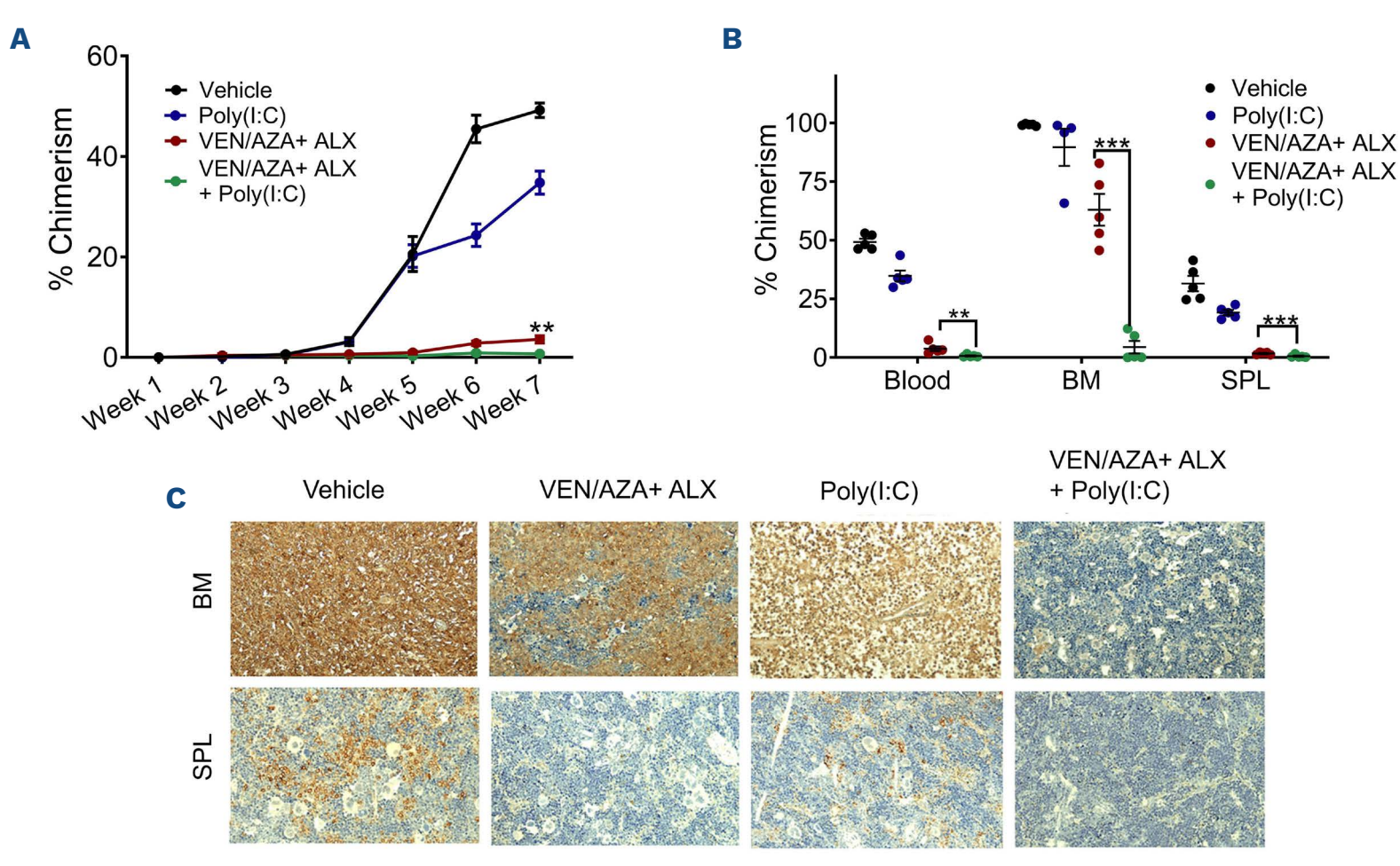


**Figure 6. The addition of polyinosinic:polycytidylic acid to a venetoclax/azacitidin regimen augments CD47 inhibition in a patient-derived xenograft model.** (A) Peripheral chimerism and (B) endpoint analysis of a venetoclax/azacitidin plus ALX (VEN/AZA + ALX) regimen with the addition of polyinosinic:polycytidylic acid (poly(I:C)). (C) Immunohistochemistry for hCD45 reveals visible decreases in human cells with the bone marrow (BM) and spleen (SPL) of patient-derived xenograft (PDX) mice treated with VEN/AZA + ALX + poly(I:C). ***P*<0.01 and ****P*<0.001.

and began treatment with VEN/AZA + ALX + poly(I:C). The "quadruplet therapy" of poly(I:C) together with VEN/AZA + ALX led to a drop in peripheral chimerism (Figure 6A). Again, at 8 weeks, mice were sacrificed and a significant decrease in leukemia was seen in the blood, spleen and, notably, BM in instances in which poly(I:C) was added to a Ven/AZA + ALX regimen (Figure 6B, C). We analyzed the quality of the remaining murine BM in this model for functional hematopoiesis. Erythropoiesis was noted through higher counts of TER119$^+$ cells with the BM, along with higher levels of myeloid (CD11b$^+$) and neutrophil (CD11b$^+$/Ly6g$^+$) levels (Figure 7A). Furthermore, the macrophage population within the BM of VEN/AZA+ALX-treated mice *versus* VEN/AZA + ALK + poly(I:C)-treated mice was significantly diminished. The resident macrophages within the BM of these VEN/AZA + ALX + poly(I:C)-treated mice included a greater portion with a M1 phenotype (Figure 4B). In cytospins, the the return to more normal hematopoiesis was evident (Figure 7B). Furthermore, the addition of poly(I:C) to a VEN/AZA + ALX regimen significantly extended survival in treated mice, even after ceasing treatment (Figure 7C). Finally, to confirm active phagocytosis we conducted an *ex vivo* phagocytosis assay with two additional AML primary patient samples. For this, naïve NSGS mice were again treated with poly(I:C) or control vehicle and after 2 weeks of treatment, the two BM groups were harvested and incubated with pHrodo-labeled patient samples pre-incubated with or without ALX90. Target cells labeled with pHrodo will only fluoresce when under the highly acidic condition of the phagolysosome. This fluorescence, when emitted from a macrophage, ensures that the engulfed cell was successfully phagocytosed and further incorporated into a functional phagolysosome. As shown in Figure 8A, F4/80$^+$ bone marrow macrophages displayed increased phago-lysosomal acidification following phagocytosis of patient samples when ALX is employed in combination with poly(I:C) treated BM *versus* BM from vehicle-treated mice. In order to similarly validate the cooperativity of ALX and poly(I:C) in human cells, peripheral blood monocytes were isolated from healthy donors, differentiated into macrophages and further subjected to macrophage stimulation with poly(I:C). These macrophages were incubated with pHrodo-treated AML cells which were then subsequently treated with CD47i (ALX90 or magrolimab). Baseline phagocytosis in these assays was measured in cultures where AML target cells were incubated with drug corresponding IgG antibodies. While there was expected variation between patients, consistently the greatest fold changes in phagocytosis were

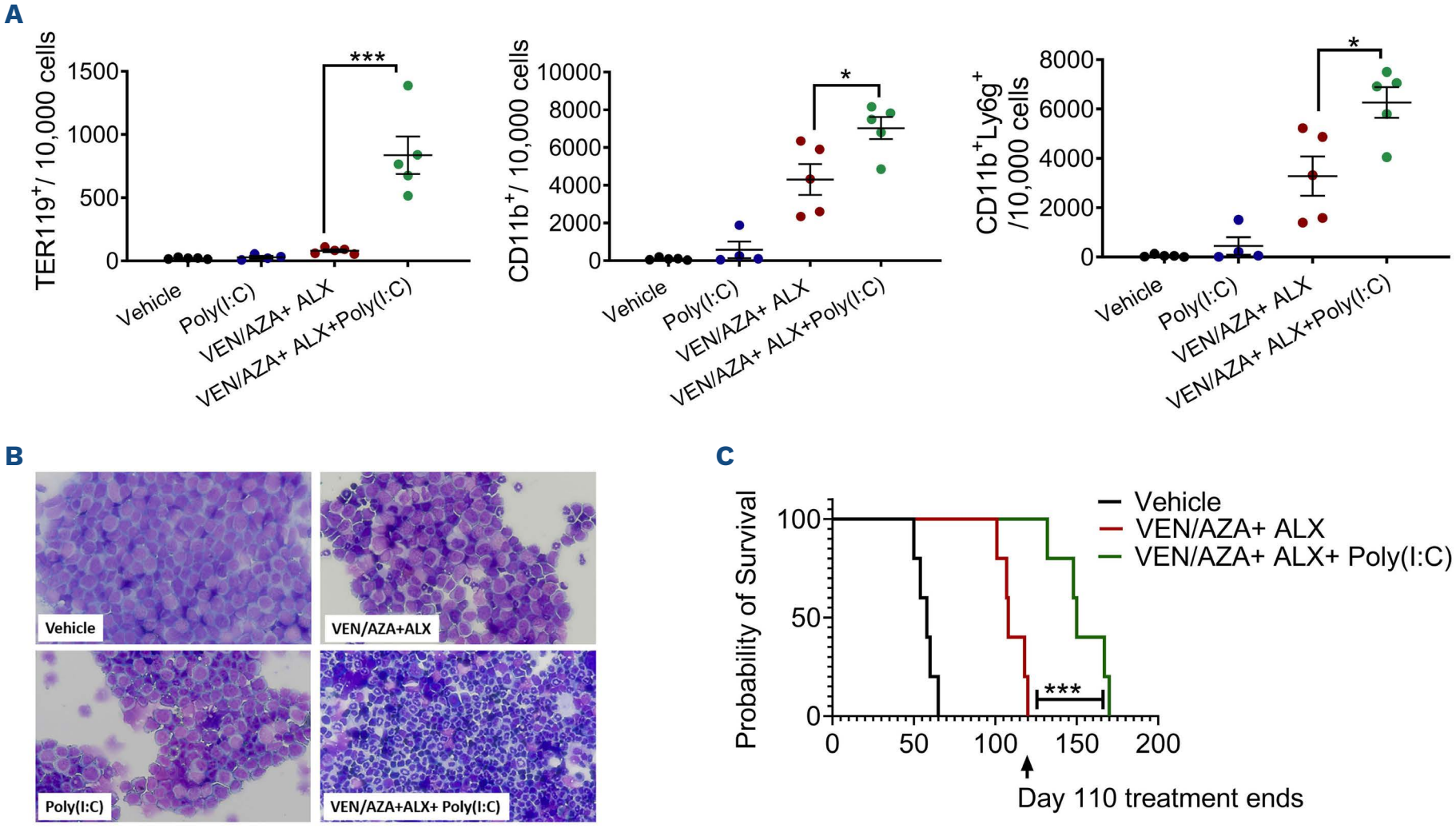


**Figure 7. Murine hematopoiesis and lifespan after a venetoclax/azacitidin plus ALX plus polyinosinic:polycytidylic acid regimen.** (A) Flow cytometry of remaining murine bone marrow (BM) in patient-derived xenograft (PDX) models reveals significant differences in erythroid, myeloid, and neutrophil populations. (B) Wright Giemsa staining of BM cytospins reveal decreased acute myeloid leukemia (AML) blast populations in venetoclax/azacitidin plus ALX plus polyinosinic:polycytidylic acid (VEN/AZA + ALX + poly(I:C))-treated PDX mice. (C) Kaplan Mayer analysis affirms increased lifespan when poly(I:C) is added to a VEN/AZA + ALX regimen.

noted in the assay populations where macrophages had been pretreated with poly(I:C) and AML samples had been treated with ALX90 or magrolimab (Figure 8B; *Online Supplementary Figure S5*). These data show that the capacity of CD47i is significantly enhanced when the appropriate macrophage populations are available.

## Discussion

The majority of clinically investigated immunotherapies in AML have been focused on the adaptive immune system, such as chimeric antigen receptor T-cell and T-cell immune checkpoint inhibitors. However, focusing on macrophage and innate immunity is proving a meaningful adjunct of immunotherapy. CD47, which functions as an anti-phagocytic signal, allows for CD47 expressing cells to escape phagocytosis by allowing for its cognate binding to SIRP-$\alpha$ on macrophages. However, many cancers overexpress CD47 as an evolutionary mechanism thereby evading phagocytosis. CD47 inhibition has real clinical potential, with CD47 inhibitors showing benefit in solid tumors and hematological cancers in early clinical trials. While the solid tumor microenvironment typical contains a plethora of macrophages,[38-40] limitations of clinical benefit in hematologic malignancies may be rooted in the availability of macrophages, or at least M0/M1 macrophages in the bone marrow microenvironment.

As shown in our studies, CD47 blockade greatly augments the clearance of peripheral AML blasts, but has less success in the BM despite sufficient CD47 present on the cell surface of BM blasts. Although peripheral blood in PDX models contains low numbers of macrophages, there are enough M0/M1 macrophages to clear the engrated AML from the blood with CD47i. We suspected that a loss of the majority of resident medullary macrophages may be responsible for this lack of phagocytic activity in the BM and while the resident macrophages were diminished in PDX models, they were also polarized as tumor-permissive M2 macrophages/LAM. With the induction of macrophage populations via poly(I:C), inhibition of CD47 was not only potentiated as a monotherapy for medullary reduction of tumor, but allowed for better response when combined with standard of care.

It has been previously shown that CD47 expression, while significantly higher on AML cells than in comparison to normal BM, was variable between AML patients.[41] Such findings might lead to the belief that high and low CD47 expression might determine drug efficacy, however, we found that CD47 intensity was not a biomarker of efficacy. The inter-patient variability of CD47 expression on AML blasts made no difference in effectivity of the drug in *in vitro* phagocytosis assays. Additionally, while MFI in our study reflected the quantity of CD47 expressed, the capacity to clear leukemia was no different between PDX models of high and low intensity CD47.

The concept of M2 macrophages driving tumor progression by sustaining an immunosuppressive environment is a phenomenon of growing interest. Additional studies are beginning to bring to light the role of tumor suppressive M2 macrophages in hindering necessary therapies such as immune checkpoint blockade, radiotherapy and chemotherapy.[42,43]

T cells interact with LAM to directly or indirectly effect response to tumor.[44] While the NSGS mice used in our modeling have negligible levels of T cells due to mutation in interleukin 2 receptor $\gamma$, M2 macrophages are dominant

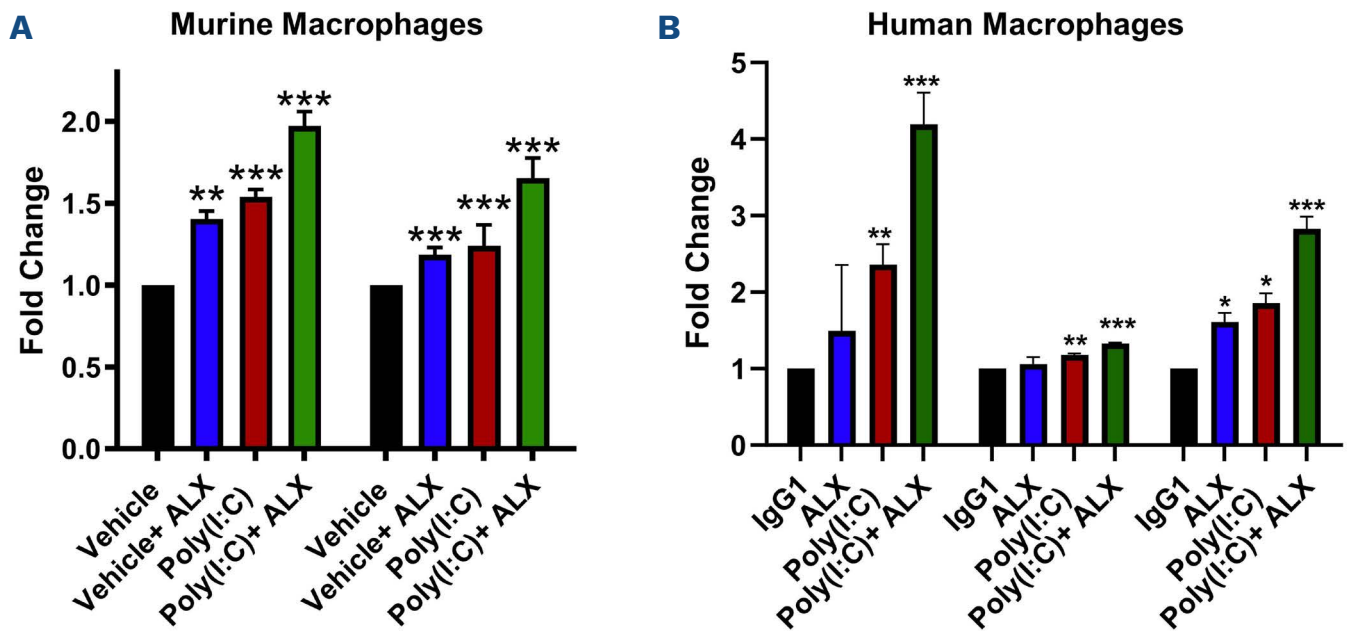


**Figure 8. Polyinosinic:polycytidylic acid enhances CD47 inhibitor-induced phagocytosis.** (A) Phagocytosis assays using macrophages from vehicle- or polyinosinic:polycytidylic acid (poly(I:C))-treated mouse bone marrow (BM) incubated with pHrodo-labeled cells from patient acute myeloid leukemia (AML) 18-12-001 and 19-03-007 reveal increases in phagocytosis in poly(I:C)-treated BM. (B) Healthy donor macrophages, when pretreated with poly(I:C) reveal increases in phagocytosis of CD47-treated AML (18-12-001) cells. Statistical comparisons in 3 individual donors shown here as ALX monotherapy, poly(I:C) monotherapy and combination against IgG1 control. Ig: immunoglobulin.

in AML xenografts. Our study may provide greater insight into the relationship between AML cells and monocyte/macrophage polarization which can be studied in maturational and mutational subsets for better understanding of how to utilize this type of immunotherapy for AML.

Furthermore, we suggest that this increased phagocytic ability of poly(I:C)-treated BM results from the concomitant increase of M1-primed macrophages after treatment. In previous studies, CD11c activation in macrophages through IFN-γ use was shown to increase broad spectrum phagocytosis, resulting in an enhancement of anti-tumor immunity via promotion of phagocytosis.[45] The increases of CD11c-positive macrophages in our study are consistent with this IFN-γ induced M1 priming, and does not require LPS administration required for response with IFN.

The ability to increase active macrophage populations within the BM of AML patients could potentiate the use of anti-CD47-based therapy, particularly when clonal leukemia has physically displaced functional hematopoietic cells, and polarized macrophages to a tumor permissive M2 subtype. Further, it may argue for incorporation of CD47 inhibition into maintenance therapy after establishing remission with VEN/AZA so normal macrophage constituency may reconstitute a healthy BM.

## Disclosures

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## Contributions

*Conception and design by HR and MS. Development of methodology by HR, AM, AG, PA, BNS and MS. Acquisition of data (provided animals, acquired and managed patient samples, provided facilities, etc.) by HR, BNS, AG, PA, LF and MS. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis) by HR, AM, AG, PA and MS. Writing, review, and/or revision of the manuscript by all authors. Administrative, technical, or material support (i.e., reporting or organizing data, constructing database) by all authors. Study supervision by MS.*

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## Data-sharing statement

*Protocols and original data from this manuscript are available upon request by contacting the corresponding author.*

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