Targeting the A2A adenosine receptor counteracts immunosuppression *in vivo* in a mouse model of chronic lymphocytic leukemia

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Supplemental Information

Supplemental Methods

Adoptive transfer TCL1 mouse model

Disease progression was monitored by periodic bleeding. Early engraftment was defined by <20% of leukemic cell infiltration in the spleen, which was usually observed within 15-20 days after injection. Mice euthanized >4 weeks after injection were considered "late engraftment" independently of leukemic cell infiltration. Spleens from euthanized mice were partly formalin-fixed and paraffin-embedded and partly dismantled to obtain a single cell suspension. Bone marrows (BM) were flushed from femurs. Peripheral blood (PB) was separated using Ficoll-Hypaque (Sigma-Aldrich) to isolate PBMC.

Immunohistochemistry and confocal microscopy

Briefly, upon antigen retrieval with 10mM citrate buffer pH 6, slides were processed either for immunohistochemistry (IHC) or for confocal microscopy analyses. For IHC, cells were stained with hematoxylin/eosin or with rabbit polyclonal anti-CAIX (Novus Biological). HRP-conjugated secondary antibodies and 3,3'-diaminobenzidine (EnVision™ System, Dako) were used to visualize the reaction. Slides were analyzed using an AXIO Lab.A1 (Zeiss), equipped with a Canon EOS600D reflex camera and the images were acquired using the ZoomBrowserEX software (Canon). Quantification of brown signal was measured with the LAS Application Suite software (Leica Microsystems).

For confocal microscopy analysis, slides were stained with the following primary antibodies: goat anti-CD3ε (Santa Cruz), rat anti-B220 (Abcam), rabbit polyclonal anti-A2A, rabbit polyclonal anti-CD73 (both by Novus Biological) and rabbit polyclonal anti-PIM (Hypoxyprobe). Tissue sections were incubated with anti-rat-Alexa594, anti-goat-Alexa488, anti-rabbit-Alexa647 secondary antibodies and mounted in SlowFade Gold reagent (all from ThermoFisher Scientific).

Immunofluorescence studies were performed using a TCS SP5 laser scanning confocal microscope equipped with 4 lasers, a 20x/0.5, oil immersion 40x/1.25 and 63x/1.4 objective lenses (Leica Microsystems). Images were acquired using the LAS AF version Lite 2.4 software (Leica Microsystems) and processed with Adobe Photoshop (Adobe Systems). Pixel intensities were analyzed using the ImageJ software (downloadable at http://rsbweb.nih.gov/ij/).

HPLC measurement of adenosine production

2 x 10^6 leukemic cells purified from the spleen of TCL-1 mice were stimulated with AMP (400 μM, 1h, 37°C) alone or with pre-treatment with adenosine deaminase inhibitor EHNA hydrochloride (3 or

10 μM, 30 min, 37°C). At the end of the incubation time, supernatants were ultra-filtered and 50 μl of each sample were injected into the HPLC system. Chromatographic analysis was performed using a Waters Alliance 2965 Separation Module equipped with silica-based, reverse-phase Atlantis dC18 column (5 μm, 4.6 x 150 mm) and Atlantis dC18 Guard column (5 μm, 4.6 x 20 mm; all from Waters). Separation of AMP, adenosine and inosine was carried out using a method based on a binary mobile phase which consists of 7 mM ammonium acetate (Buffer A), pH 3, and acetonitrile (Buffer B) with a flow rate of 1 ml/min and UV detection set at 260 nm. Peak identities were confirmed by using standard compounds and concentrations were calculated by comparing the peak area of the samples with calibration curves for the peak areas of each standard compound.

Real-time PCR

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Thermo Scientific, USA). qRT-PCR reactions were done in triplicate from the same cDNA reaction (technical replicates). The data were analyzed with the $2^{-\Delta Ct}$ method, to calculate the relative expression of the gene under analysis {Arruga, 2019 #490}. For each gene, copy number values were computed as the difference (ΔCt) between the target gene Ct and *Actin* (*Actb*) Ct.

ELISA assay for PKA activity

Cells from TCL-1 mice spleen (2 x 10^6 cells) were re-suspended in 250 μL of AIM V serum-free medium (ThermoFisher Scientific). Cells were stimulated with 1 μM CGS21680 for 2 minutes at 37°C. Where indicated, cells were pretreated with 1 μM SCH58261 and treatment was maintained during stimulation. Cells were washed twice with ice-cold PBS, lysed and ELISA assay to measure PKA activity (Arbor Assays) was performed following manufacturers' instructions. Absorbance was measured at 450 nm using a Microplate reader (BioRad). PKA concentration (U/L) was calculated with an internal calibration curve.

Cells degranulation and cytokines release assay

10^6 cells freshly isolated from spleens of TCL-1 or WT mice were cultured overnight in RPMI-1640 medium with 10% fetal calf serum (FCS) and penicillin/streptomycin (all from Sigma-Aldrich).

Cells were then collected in a FACS tube and treated with Protein transport inhibitor (eBioscience, 1:500) alone or in combination with PMA (20 μg/mL) and Ionomycin (1 μg/mL) for 6 hours. Where indicated, anti-CD107a-APC (LAMP-1, BioLegend) was added to the cells in culture.

After stimulation, cells were stained with anti-CD8-BB515 and anti-CD4-PerCP-Cy5.5 (both by BD Biosciences) before fixation with 4% PFA and permeabilization with 0.1% saponin. Intracellular staining was performed with anti-IFNγ-PE or anti-IL-2-PE (both by BD Biosciences).

Phosflow assay

10^6 cells collected from spleens of TCL-1 or WT mice were stained with anti-B220-PECy7, anti-CD4-PerCP-Cy5.5 or anti-CD8-BB515 for 15 minutes at room temperature. Cells were then stimulated with 10 μM CGS21680 (TCL-1 cells for 2 minutes and WT cells for 30 minutes). Where indicated, cells were pretreated for 30 minutes with 10μM SCH58261. Stimulation with PMA (20 ng/mL) and Ionomycin (1 ng/mL) was used as positive control. Cells were washed twice with ice-cold PBS before fixation with 4% PFA and permeabilization with 0.1% saponin. Cells were stained with anti-phosphoCREB-PE (pS133)/ATF-1 (pS63) for 1h at room temperature.

Apoptosis assay

10^6 cells collected from TCL-1 mice were cultured *in vitro* in the presence or in the absence of 10μM fludarabine (Selleckchem) for 48 hours. Where indicated, cells were supplied with 50μM adenosine every 12 hours or treated with 10μM SCH58261, starting from the beginning of the experiments and without refilling it for the entire duration of the treatment. At the end of the treatment, cells were collected and apoptosis was measured using the Annexin V-APC Apoptosis Kit (EBioscience, Thermofisher). Samples were acquired at FACSCelesta flow cytometer (BD Biosciences) and analyzed using the DIVA software.

Supplemental Table 1. Flow Cytometry multiparametric panels

Subsets	MARKERS	Fluorophore	Manufacturer
B leukemic cells	B220	PECY7	BD Biosciences
	CD5	BB515	BD Biosciences
Monocytes	CD19	APC	BD Biosciences
	CD3	APC	BD Biosciences
	LY6G	APC	Miltenyi
	NK1.1	APC	BD Biosciences
	CD11b	PerCP-Vio770	Miltenyi
	F4/80	PE	BD Biosciences
	I-A/I-E	BB515	BD Biosciences
	LY6C	APC CY7	BD Biosciences
	CD43	BV421	BD Biosciences
Tregs	CD4	BV510	BD Biosciences
	CD25	PE	BD Biosciences
	CD39	APC (eFluo660)	eBioscience
Degranulation and cytokines	CD4	PerCP-Cy5.5	BD
	CD8	BB515	BD
	CD107	APC	BioLegend
	IL-2	PE	BD
	IFN-γ	PE	BD

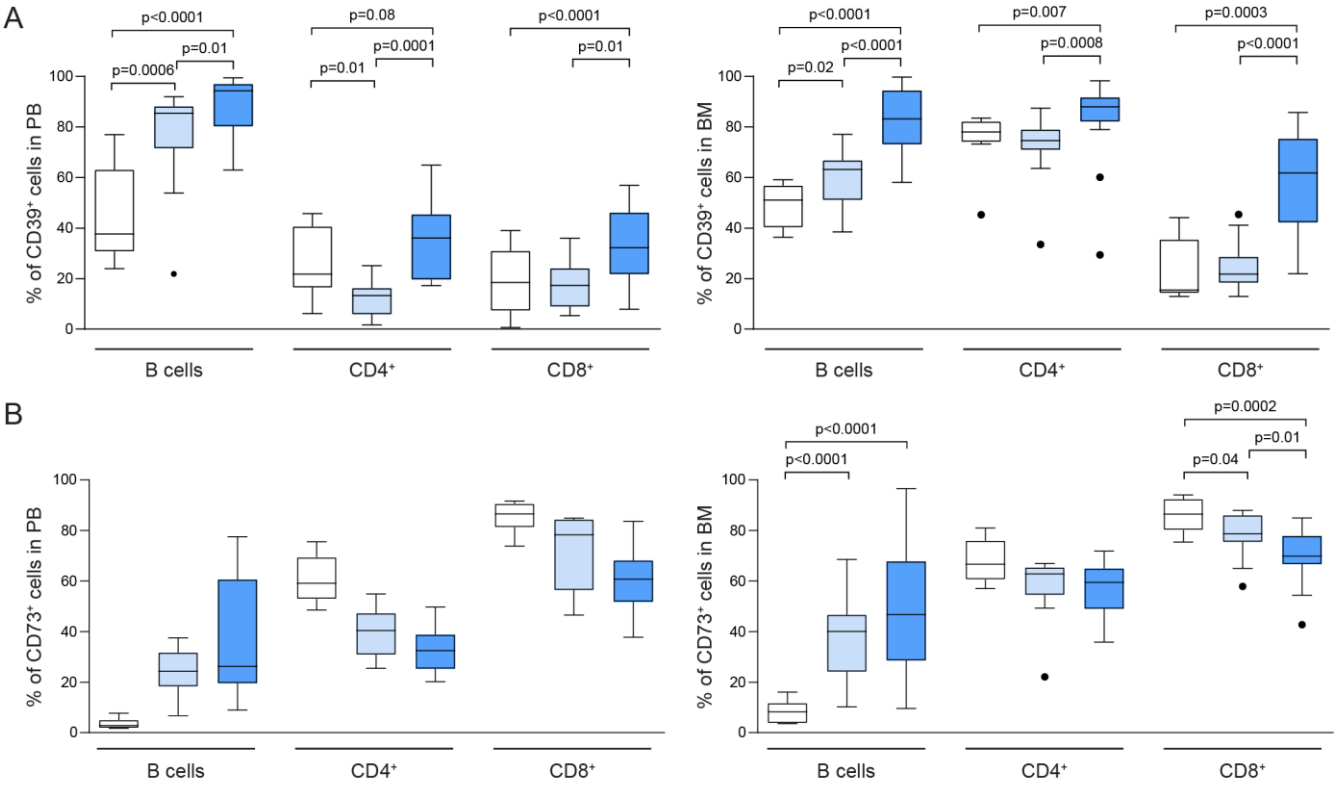
Supplemental Figure Legends

Supplemental Figure 1. (**A**) Flow cytometry analysis of surface CD39 expression on B cells, CD4+ and CD8+ T cells collected from PB (left panel) or BM (right panel) of TCL1 mice at early and late time points after leukemia injection and compared to WT mice. (**B**) Flow cytometry analysis of surface CD73 expression on B cells, CD4+ and CD8+ T cells collected from PB (left panel) or BM (right panel) of TCL1 mice at early and late time points after leukemia injection and compared to WT mice.

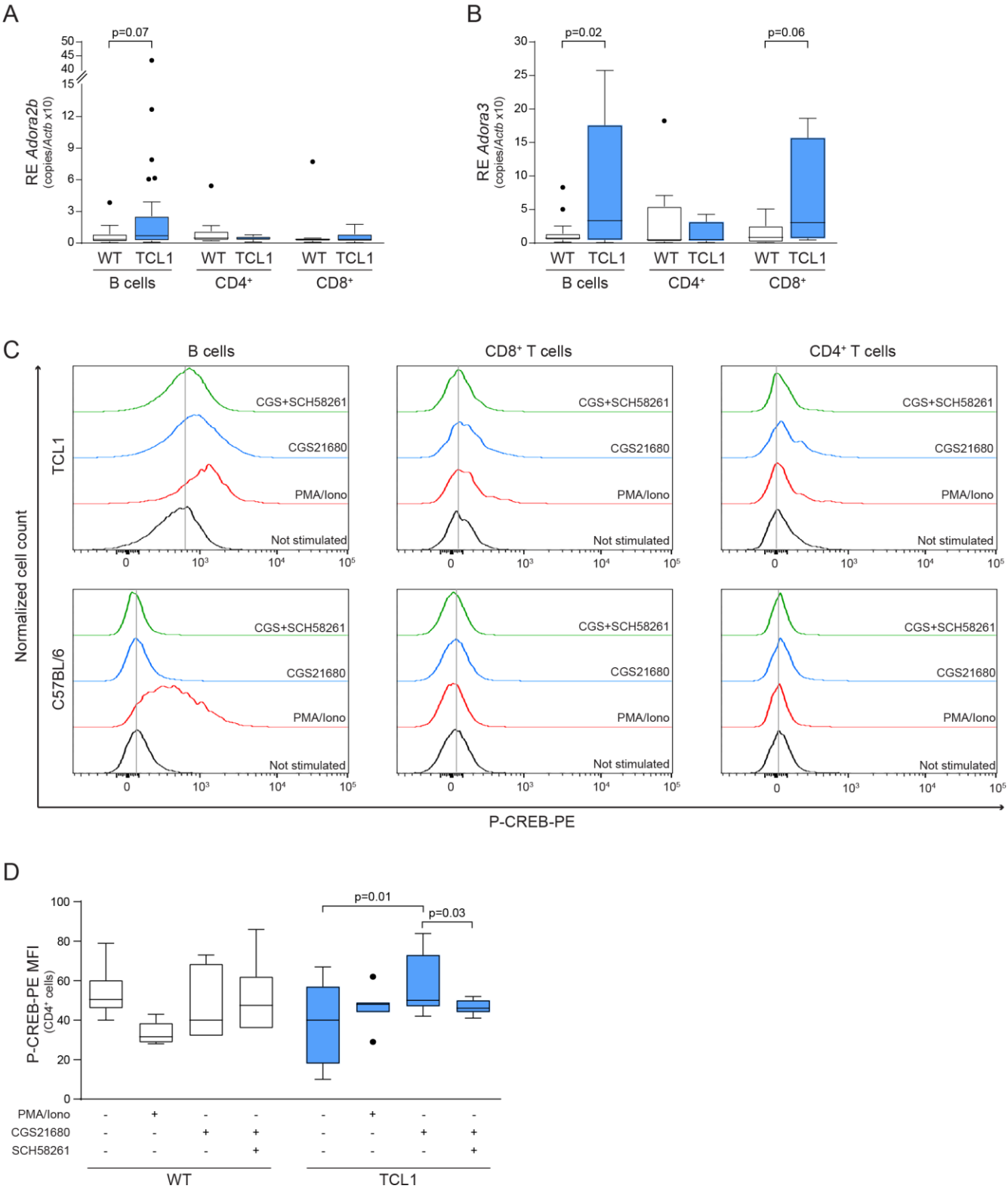
Supplemental Figure 2. (**A**) qRT-PCR analysis of *ADORA2B* expression in terminally ill TCL1 mice compared to WT animals. RE relative expression. (**B**) qRT-PCR analysis of *ADORA3* expression in terminally ill TCL1 mice compared to WT animals. RE relative expression. (**C**) Representative plots of the phosflow analysis of CREB phosphorylation performed on B cells, CD8+ and CD4+ T lymphocytes collected from TCL1 mice and compared to WT C57BL/6 not injected mice. (**D**) Phosflow analysis of CREB phosphorylation in $CD4^+$ cells upon treatment with A2A agonist CGS21680 or A2A antagonist SCH58261.

Supplemental Figure 3. Representative plots of the flow cytometry analyses of immune cell populations in TCL1 mice at different time points or treated with SCH58261 and compared to WT not injected mice. (**A**) Tregs were gated out of the $CD4^+$ T cells and were defined as the $CD39^+$ cells out of the total $CD4^+/CD25^+$ cells. (**B**) Analysis of the percentages of $CD107^+$, $IFN\gamma^+$ and $IL\text{-}2^+$ cells out of the total $CD8^+$ T lymphocytes. Cells from TCL1 or WT mice were either unstimulated or treated with PMA/Ionomycin for 6 hours before evaluating the degranulation capacity (CD107) and the production of IFNγ and IL-2 by flow cytometry. (**C**) Representative flow cytometry plot of monocytes subpopulations in TCL1 mice at different time points or treated with SCH58261 and WT mice. Monocytes were gated as the $F4/80^+$ cells out of the $CD11b^+$ cells (not shown) and the inflammatory, intermediate and patrolling subpopulations were defined according to LY6C and CD43 stainings.

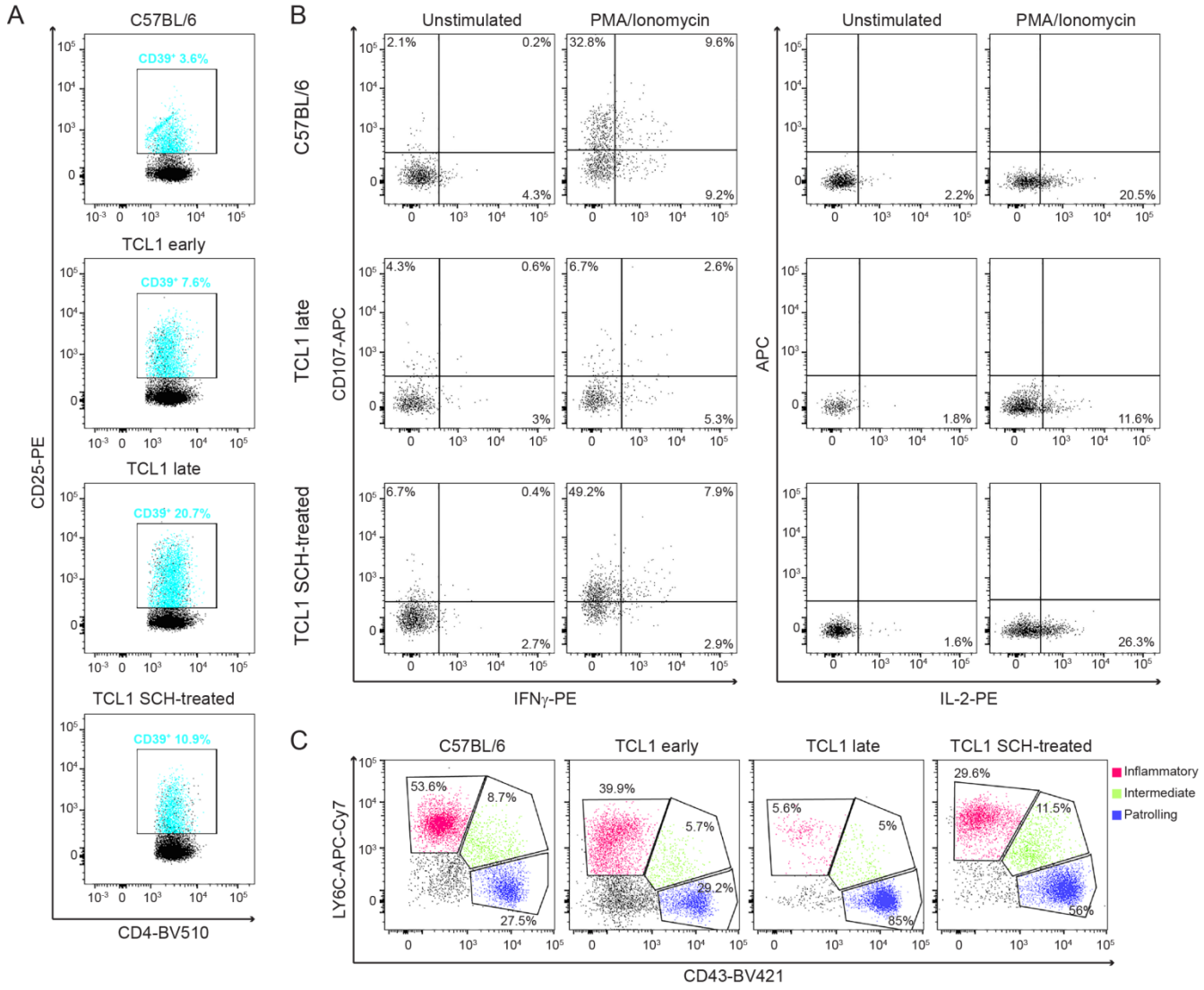
Supplemental Figure 4. Correlation between tumor burden (% of leukemic cells) in the spleen and (**A**) % of inflammatory monocytes, (**B**) % of intermediate monocytes, (**C**) % of patrolling monocytes and (**D**) % of Tregs.



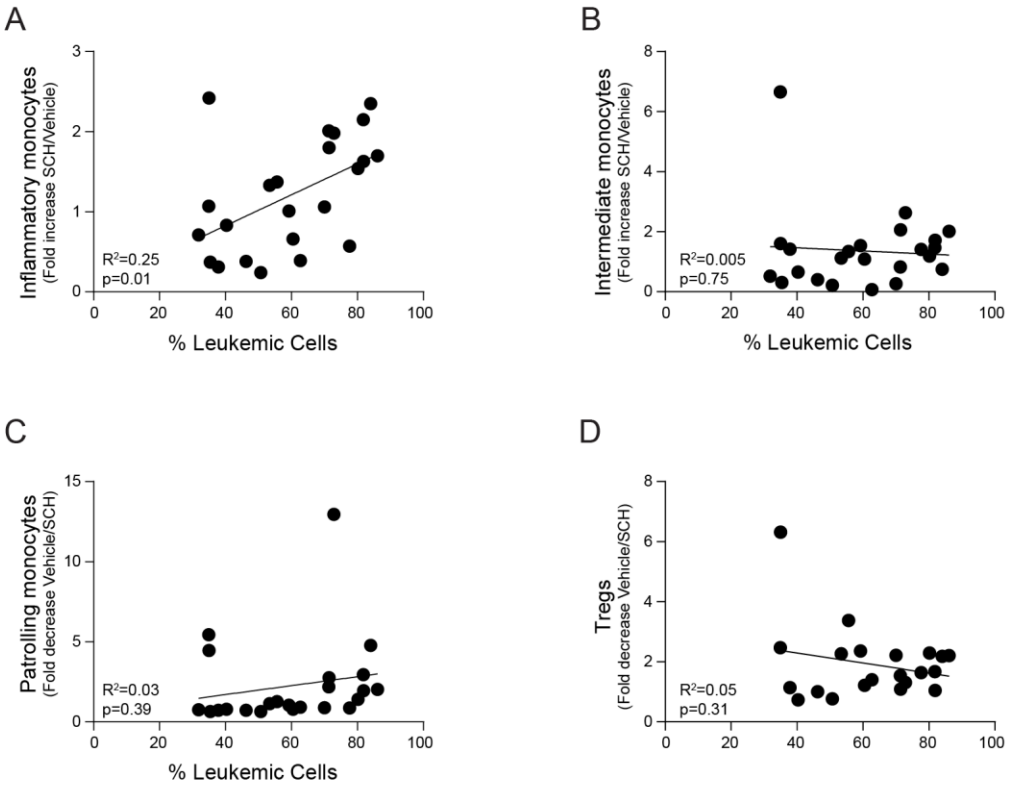
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4