Antisense STAT3 Inhibitor Decreases Viability of Myelodysplastic and Leukemic Stem Cells

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Running Title: Inhibition of STAT3 in MDS and AML

Key Points

- STAT3 is overexpressed in highly purified AML and MDS stem and progenitor cells and its expression is associated with a worse prognosis
- Inhibition of STAT3 by an antisense oligonucleotide, AZD9150, leads to decreased viability of AML/MDS stem cells in in vitro and in vivo models

Abstract

Acute Myeloid Leukemia and Myelodysplastic Syndromes are associated with disease-initiating stem cells that are not eliminated by conventional therapies. Transcriptomic analysis of stem and progenitor populations in MDS and AML demonstrated overexpression of *STAT3* that was validated in an independent cohort. *STAT3* overexpression was predictive of a shorter survival and worse clinical features in a large MDS cohort. High *STAT3* expression signature in MDS CD34+ cells was similar to known pre-leukemic gene signatures. Functionally, STAT3 inhibition by a clinical, antisense oligonucleotide, AZD9150, led to reduced viability and increased apoptosis in leukemic cell lines. AZD9150 was rapidly incorporated by primary MDS/AML stem and progenitor cells and led to increased hematopoietic differentiation. STAT3 knockdown also impaired leukemic growth in vivo and led to decreased expression of MCL1 and other oncogenic genes in malignant cells. These studies demonstrate that STAT3 is an adverse prognostic factor in MDS/AML and provide a pre-clinical rationale for studies using AZD9150 in these diseases.

Statement of Significance

STAT3 is overexpressed in MDS/AML stem and progenitor cells and is a marker of poor prognosis. AZD9150, a clinical antisense oligonucleotide inhibitor of STAT3 that gets rapidly incorporated into MDS/AML stem cells, downregulates STAT3 and other important oncogenic genes and show in vitro and in vivo efficacy in leukemia models. This provides a strong pre-clinical rationale for STAT3 inhibition as a therapeutic strategy for MDS/AML.

Introduction

Myelodysplastic Syndromes (MDS) and Acute Myeloid Leukemia (AML) are malignant hematopoietic stem cell disorders that arise from a population of aberrant stem cells residing within the hematopoietic stem and progenitor cell (HSPC) compartments.(1-6) Precisely defined qualitative and quantitative alterations in HSPCs have recently been demonstrated in MDS and AML. FACS analysis using rigorous lineage depletion revealed that phenotypic hematopoietic stem cells (HSC) Long term (LT-HSC) and Short term (ST-HSC) and Granulocyte Monocytic Progenitor (GMP) compartments are expanded in high-risk MDS and AML and have been shown to contain leukemia initiating activity in various models. These aberrant HSPCs have been shown to persist through cytotoxic chemotherapy and expand at the time of relapse.(3, 7) Thus, curative strategies need to target these disease initiating and relapse causing stem and progenitor compartments.

We have previously demonstrated that the transcription factor Signal Transducer and Activator of Transcription 3 (*STAT3*) is over-expressed in marrow derived MDS CD34+ cells. (7, 8). The Janus Kinase (JAK) - STAT pathway, especially STAT3, has been an attractive therapeutic target in many cancer stem cell models, but has been hard to selectively inhibit therapeutically. AZD9150 is a generation 2.5 antisense oligonucleotide (ASO) that is a specific inhibitor of STAT3. It has previously demonstrated efficacy and safety in heavily pre-treated lymphoma and solid tumor patients.(9) Therapeutic antisense technology has greatly advanced since its inception 20 years ago and is approved for clinical use in various indications. These include approvals of Generation 2.0 ASOs for usage in homozygous familial hypercholesterolemia (Kynamro™) and infantile spino-muscular atrophy (Spinraza™).(10, 11). Gen 2.0 ASOs are incorporated predominantly in liver, kidney and adipose tissue when delivered systemically. To achieve potent activity in extra-hepatic tissues, including tumors, a next generation ASO

chemistry was developed. Gen 2.5 ASOs have higher affinity and greater intrinsic potency compared to Gen 2.0 and previous ASOs owing to an 8'-10' phosphorothioate modified deoxynucleotide "gap" flanked on either end with 2 to 3 cEt nucleotides.(12, 13) Furthermore, AZD9150 targets nucleotide sequences found only in the human *STAT3* gene and that are not present in the murine *STAT3* gene, underscoring the specificity of the drug. (9, 14).

In this study, we demonstrate that STAT3 is significantly overexpressed in highly purified AML and MDS LT-HSCs, ST-HSCs and GMPs when compared to healthy controls and is associated with poor prognosis. Functional studies show that inhibition of STAT3 with AZD9150 can inhibit leukemic growth in vitro and in vivo. These data indicate that the STAT3 pathway is frequently aberrantly activated in AML and MDS stem cells and that antisense oligo-mediated inhibition of STAT3 can serve as a novel way to impair MDS/AML stem cells.

Results

STAT3 is overexpressed in MDS and AML hematopoietic stem and progenitor cells (HSPCs) and is associated with an adverse prognosis: Leukemia and myelodysplasia disease initiating cells including pre-leukemic stem cells, reside in the lineage negative, phenotypic stem and progenitor compartments. To determine *STAT3* expression levels in highly purified AML and MDS stem and progenitor cells we examined gene expression profiles generated from FACS sorted LT-HSCs, ST-HSCs and GMPs from 12 AML/MDS samples with normal karyotype, del (chr7) and complex karyotype (Fig 1A) (GSE35008 and GSE35010). We observed that *STAT3* was significantly overexpressed in HSC and GMP populations, across normal karyotype, complex karyotype and deletion of chromosome 7 cases (Fig 1B-D). These results were validated in an independent cohort of samples by qRT-PCR. Two AML, three MDS and two healthy control samples were sorted and analyzed and were confirmed to have significant upregulation of *STAT3* in at least one of the 3 disease initiating populations examined in each disease sample when compared to controls. (Fig. 1E-F).

We next evaluated *STAT3* overexpression for prognostic impact in a large cohort of MDS CD34+ cells and observed that samples with higher expression (> median expression) had a significantly worse prognosis compared to low *STAT3* expressers (median overall survival of 2.61 years in high *STAT3* cases vs 5.75 years in low *STAT3* cases, Log rank P Value =0.001)(Fig 1G). Patients with high *STAT3* were found to present with worse disease phenotype, manifesting with lower hemoglobin levels (Fig 1H) and a higher percentage of transfusion dependence (40% for *STAT3* high vs 30% for *STAT3* low cases, P value <0.05) (Fig 1J). These patients also had a significantly higher percentage of myeloblasts in the marrow (Fig 1I), demonstrating STAT3 as an adverse prognostic factor in MDS. A multivariate analysis using IPSS as a variable was also conducted and demonstrated that high *STAT3* was an independent adverse prognostic factor (P Value=0.02, Multivariate Cox Proportional Model).

Gene expression signature of MDS HSPCs with high *STAT3* is similar to known preleukemic stem cell profiles and includes many important functional pathways

To determine the molecular pathways that were differentially activated in MDS HSPCs with high expression of *STAT3*, we identified 413 differentially expressed genes (with 312 higher, and 101 lower abundant transcripts) in samples with higher *STAT3* levels (using median *STAT3* expression as cutoff in a cohort of 183 MDS CD34+ samples, FDR<0.1) (Fig 2A). Pathway analysis revealed significant dysregulation of pathways involved in DNA replication, gene expression, cell death and survival in *STAT3* high samples, and also included many genes that play important roles in molecular leukemogenesis (Fig 2B, Supp Table 2). Next, we tested whether the high *STAT3* expression signature had any overlap with known preleukemic stem cell gene expression signatures. Gene set enrichment analysis with two recently published preleukemic stem cell signatures GSE6891(15) and GSEA12417(16), revealed highly significant enrichment, demonstrating that HSPCs from *STAT3* high MDS patients have a transcriptomic profile similar to than known pre-leukemic and leukemia-initiating cell populations (Fig 2C,D). Furthermore, we evaluated for correlation of high *STAT3* expression with mutational subgroups in 100 samples where targeted sequencing had been conducted.(17) We observed that high *STAT3* expression was significantly associated with *TP53* (12.5% in high *STAT3* group vs 0% in low *STAT3* group, P val=0.03) and *STAG2* (18% in high *STAT3* group vs 2% in low *STAT3* group, P val=0.03).

AZD9150 selectively inhibits STAT3, decreases proliferation and activates apoptosis in leukemic cells

AZD9150 is a next-generation, constrained ethyl-modified antisense oligonucleotide (Gen 2.5) targeting STAT3 that has been shown to be safe for human use in a Phase I trial in solid tumors (9). We determined that AZD9150 elicits specific target inhibition of STAT3 in hematopoietic

cells without affecting STAT5 when compared to a structural analogue control oligonucleotide in reporter assays. (Fig.3A) AZD9150 was also able to significantly reduce *STAT3* mRNA levels in several leukemic cell lines (Fig.3B) To determine the functional effects of STAT3 inhibition, we next treated several leukemia and MDS derived cell lines with AZD9150. In contrast to non-targeting control oligo treated cells, AZD9150 treatment led to significantly decreased viability in all cell lines examined (Fig 3C) and the inhibition observed was found to be dose dependent (Supp Fig 3). This decrease in viable cells was accompanied by increased apoptosis. (Fig.3D-G). Next, we treated one of the tested cell lines, CMK, with AZD9150 or the non-targeting control and xenografted them in immunodeficient non-obese diabetic scid gamma (NSG) mice. We observed that mice with AZD9150 treated leukemia cells showed a significantly improved median survival of 64 days compared to 43 days for the controls (p=0.028) (Fig 3H), demonstrating that STAT3 inhibition mediated by ASO leads to impaired leukemic cell growth ex vivo as well as in vivo.

AZD9150 is rapidly incorporated by primary MDS/AML stem and progenitor cells and leads to STAT3 inhibition

The increased potency of Gen 2.5 ASOs over previous ASO chemistries enables the delivery of sufficient amount of ASO to cells in culture or in vivo without the need for any type of delivery vehicle (9, 18). Thus, we evaluated the ability of AZD9150 formulated in saline, to be incorporated by primary stem and progenitor cells. We exposed MDS/AML (n=8) and healthy cord blood stem (n=5) and progenitors to AZD9150 and the control oligonucleotide in liquid culture and performed intracellular flow-cytometry with antibodies against the ASO backbone. We observed rapid uptake of ASOs (AZD9150 identified as ASO and the control oligonucleotide identified as NTC) by both stem (Lin-, CD34+/CD38-) and progenitor (Lin-, CD34+/CD38+) cells (Fig 4A-D). The MDS/AML HSPC samples showed a greater uptake when compared to healthy cord blood-derived control cells (Fig 4E). Moreover, when *STAT3* expression was evaluated

after 24 hours exposure to AZD9150, we determined a significant reduction in expression in MDS/AML samples when compared to healthy controls. (Fig.4F) These data demonstrate rapid uptake and on target effects by AZD9150 in primary stem and progenitor cells. AZD9150 incorporation into TP53 mutated MDS/AML stem cells leads to increased apoptosis in stem & progenitor populations in a dose-dependent manner compared to healthy controls. (Supplemental Fig.5) Additionally, STAT3 inhibition with AZD9150 also led to decreased replating efficiency of primary patient AML samples in colony assays further demonstrating efficacy against MDS/ AML stem and progenitor fractions (Supplemental Fig.1).

AZD9150 treatment leads to enhanced differentiation from primary MDS stem and progenitors

MDS and AML stem and progenitors are characterized by arrested as well as dysplastic differentiation.(19, 20) We treated a number of primary MDS patient samples with AZD9150 and control and grew them in methylcellulose assays supplemented with cytokines. Cells were harvested after 14 days and assessed for erythroid and myeloid differentiation by flow-cytometry. We observed an increase in erythroid as well as myeloid differentiation as measured by various Glycophorin A and CD11b differentiation markers respectively in most of the drug treated samples compared to control (Fig. 5A-C). Furthermore, examination of the colonies revealed larger and more differentiated colonies after treatment with AZD9150. (Fig.5D). Treatment of healthy controls with AZD9150 did not lead to any significant decreases in erythroid and myeloid colonies as well as in megakaryocytic colonies (Figure 5, Supp Fig. 4).

AZD9150 leads to decreased leukemic engraftment in primary MDS/AML xenografts

In vivo administration of antisense oligonucleotides with similar backbones can lead to significant uptake in spleen, liver and tumor tissues (Supp Fig.2) and so we wanted to determine

the efficacy of AZD9150 against primary AML/MDS xenografts. Peripheral blood or bone marrow derived MNCs from MDS/AML patients were transplanted into irradiated NSG mice. After confirmation of engraftment by serial bone marrow samples, the cohort was split and treated with either drug at a dose of 50 mg/kg or control oligonucleotide at the same dose for 4 weeks. (Fig.6A). AZD9150 treatment led to decreased MDS/AML burden in xenografted mice as compared to control (Fig 6B). This was seen in both low risk MDS patients (Fig 6C) as well as in high-risk MDS cases (Fig 6D). Since AZD9150 is specific inhibitor of only human STAT3, these data demonstrate inhibition against human xenografted cells in vivo.

AZD9150 treated leukemic cells show downregulation of MCL1 and other oncogenic genes

To determine the downstream genes affected by STAT3 knockdown by AZD9150 we treated CMK leukemia cells and performed RNA-seq analysis after 24 hours. We observed differential expression of numerous genes after AZD9150 treatment that belonged to many important oncogenic and regulatory pathways (Fig 7A,B, Supp Table 3). Differentially downregulated genes included known leukemogenic cell genes such as interleukin 1 receptor accessory protein (*IL1RAP*), Musashi 2 (*MSI2*) and anti-apoptotic myeloid leukemia cell differentiation (*MCL1*) transcripts(16, 21-23) (Fig.7A). Validation by qRT-PCR showed significant down regulation of *STAT3* along with known leukemia stem cell genes *IL8*, *CXCR2* and *IL1RAP* after treatment with AZD9150 (Fig.7C). Since we observed significant induction of apoptosis after AZD9150 treatment, we next evaluated MCL1 as a downstream target of STAT3 in leukemic cells. Gene expression analysis in a large MDS CD34+ transcriptomic dataset showed a strong correlation between *MCL1* and *STAT3* expression in 183 sorted MDS CD34+ samples (Fig.8A). Furthermore, we observed decreased levels of MCL1 and STAT3 protein levels after AZD9150 treatment (Fig 8B). Finally, chromatin immunoprecipitation using an antibody against STAT3 revealed enrichment of MCL1 promoter (Fig 8C), demonstrating MCL1 to be an important STAT3 regulated gene in leukemia cells.

Discussion

STAT3 has been an important oncogenic target in hematologic malignancies, but translating the utility of STAT3 inhibition from the bench to the bedside has proved to be challenging.(8, 24, 25) The development of antisense oligonucleotides have also been marred in the past by poor target engagement, inadequate biologic activity & significant off-target toxicities.(26) However, progress has been made in recent years, and targeting of transcription factors and other gene products with antisense oligonucleotides is becoming an important clinical tool for the treatment of multiple diseases, with several drugs having already made their way into the clinic.(11) AZD9150 is a generation 2.5 specific inhibitor of the human STAT3 and overcomes the problems faced by the earlier generations of oligonulceotides. (9)

We have shown that a high proportion of immature HSPCs harbor karyotypic abnormalities and demonstrate that STAT3 is significantly upregulated in HSPCs derived from MDS and AML patients. Interestingly, we found higher intra-cellular levels of AZD9150 in malignant stem cells, potentially due to higher amounts of *STAT3* mRNA inside these cells. We also show that high *STAT3* expression correlates strongly with an adverse prognosis and is associated with a worse overall survival, reduced mean hemoglobin levels, an increased transfusion burden and an increased percentage of blasts in MDS. These effects are potentially mediated by high STAT3 levels that lead to dysregulated cellular pathways that are crucial for the processes of proliferation and survival. The gene expression profiling of MDS CD34+ cells we performed was reflective of this hypothesis. Based on our data, targeting and inhibiting STAT3 in MDS/AML stem cells can potentially inhibit disease-initiating cells and induce durable remissions in these difficult to treat patients.

In our subsequent experiments, we demonstrate that AZD9150 is able to specifically inhibit STAT3 and induce apoptosis in multiple leukemic cell lines including the MDS-L cell line.(27) Primary MDS/AML derived HSPCs were able to incorporate the ASOs readily. Interestingly, the diseased stem cell populations had a greater uptake of the ASOs compared to the healthy stem cells and also demonstrated a greater inhibition of STAT3. This finding is of translational relevance and will aid clinical development of AZD9150 in MDS and AML. Our experiments with primary patient samples demonstrate that AZD9150 is able to relieve differentiation blocks in MDS/AML stem cells and improve erythroid and myeloid colony growth. We also observed induction of apoptosis in leukemia cell lines. Differentiation and apoptotic effects could be related to heterogeneity of samples examined. Cytopenias in patients with MDS lead to significant morbidity and mortality due to the need for high transfusion support and the increased risk of serious infections.(28, 29) If an improvement in erythroid and myeloid differentiation translates into an increased hemoglobin and neutrophil count, this may be of great clinical benefit for the MDS patient population.

Transcriptomic analysis demonstrated that *STAT3* expression can differentially regulate several important leukemic drivers that include *IL1RAP, MSI2, CXCR2, IL8* and *MCL1*. We observed a strong correlation between *MCL1* and *STAT3* expression levels with decreased expression of *MCL1* noted after treatment with AZD9150. MCL1 is a multi-domain anti-apoptotic protein belonging to the BCL2 family, which regulates the intrinsic apoptosis pathway important to regulating the processes of cell survival and death. It also plays an important role in mediating chemotherapy resistance. (30) MCL1 appears to be critical for the survival of human AML cells, as it has been reported that the removal of MCL1 without the blockade of BCXL, BCL2 or BCLW ameliorated AML in a murine model and inhibited transformed AML cells in culture.(31) Selective small molecule inhibitors of MCL1 are in early development. However, several such compounds have been reported to have significant off target activity or lack the reported cellular

effects. (32, 33). Downregulation of MCL1 with AZD9150 can lead to induction of apoptosis and provides a pre-clinical rationale for testing AZD9150 as a single agent or in combination with other agents in the treatment of MDS and AML.

Methods

Patient samples, cell lines and reagents

Specimens were obtained from patients diagnosed with MDS and AML after IRB approval by the Albert Einstein College of Medicine (Supp Table 1). The AML cell lines KG1a, KT-1, CMK,, MOLM13, MOLM 14, NB4, MV411, MDS-L & U937 were obtained from ATCC and were grown in RPMI supplemented with 10% FBS and 1% Penicillin/Streptomycin. AZD9150 (antisense STAT3 oligonucleotide) and a nontargeting antisense oligonucleotide (nt ASO) that has the same length, backbone, and base modifications as AZD9150 were provided by AstraZeneca Waltham, MA, USA and Ionis Pharmaceuticals Inc, Carlsbad, CA, USA. For in vitro studies, AZD9150 and nt ASO were dissolved in PBSS as a 4.6 mmol/L stock solution, respectively, and frozen in aliquots at -20C. For in vivo experiments, AZD9150 and ntASO were formulated in PBS, stored at 4C, and freshly made every week.

Cell Viability assays

Cell lines and primary samples were incubated at concentrations of 1uM-10uM of AZD9150. AZD9150 and control ntASO were introduced into the cell lines by electroporation with Amaxa Cell Line Nucleofector (Lonza Inc, Allendale, NJ). Primary samples were treated by free uptake in media. Cells and drug were incubated for 24, 48 and 72 hours. Viability was assessed by addition of Cell Titer Blue (Promega, Madison, WI) and measured via Fluostar Omega Microplate reader (BMG Labtech, Ortenberg, Germany).

Cell Lysis and Immunoblotting

Cells were lysed in phosphorylation lysis buffer as previously described (34). Immunoblotting was performed as previously described (34).

Multiparameter high-speed FACS of Stem and Progenitor Cells

Processing and sorting of hematopoietic stem and progenitor cells was performed as previously described (16). Briefly, mononuclear cells were isolated from BM aspirates and PB samples by density gradient centrifugation and then subjected to immunomagnetic enrichment of $CD34^+$ cells (Miltenyi Biotec, Bergish Gladbach, Germany). $CD34^+$ cells were stained with PE-Cy5 (Tricolor)-conjugated monoclonal antibodies against lineage antigens (CD2[RPA-2.10], CD3[UCHT1], CD4[S3.5], CD7[6B7], CD8[3B5], CD10[CB-CALLA], CD11b[VIM12], CD14[TueK4], CD19[HIB19], CD20[2H7], CD56[MEM-188], Glycophorin A[CLB-ery-1(AME-1)]), as well as fluorochrome-conjugated antibodies against CD34[581/CD34(class III epitope)], CD38 (HIT2), CD90 (5E10), CD45RA (MEM-56), and CD123 (6H6). Cells were sorted with a five-laser FACSAria II Special Order System flow cytometer (Becton Dickinson). Based on established surface marker characterization, we distinguished and sorted LT-HSCs (Lin^-, $CD34^+$, $CD38^-$, $CD90^+$), ST-HSCs (Lin^-, $CD34^+$, $CD38^-$, $CD90^-$), and GMPs (Lin^-, $CD34^+$, $CD38^+$, $CD123^+$, $CD45RA^+$)(35). Flow cytometry data were analyzed with BD FACSDiva (Becton Dickinson, Franklin Lakes, NJ) and FlowJo (TreeStar, Ashland, OR) software. After sorting, RNA was extracted with the RNeasy Micro kit (Qiagen, Hilden Germany).

RNA amplification and quantitative real-time RT-PCR

To measure STAT3 and STAT3 target gene expression upon ASO treatment quantitative real-time PCR (qRT-PCR) was used. Briefly, total RNA was isolated from cells after treatment as indicated using the RNAeasy Micro Kit (Qiagen) according to the manufacturer's recommendation. RNA quantity and quality was assessed using a Nanodrop 1000 (Thermo

Fisher Scientific, Waltham, MA). 1 µg of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). qPCR reactions were performed using the SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) using validated gene-specific primers (Supplementary table. 4). Transcript levels of genes of interest were normalized to a housekeeping gene (*GAPDH*) using the Pfaffl method. (36)

Clonogenic assays

For clonogenic assays, primary patient samples and healthy controls were plated in methylcellulose (Stem Cell Technologies H4435, Vancouver, CA) in 35mm dishes and incubated with the AZD-9150 and control at 10 µM. Colonies were counted after 14-17 days in culture. FACS analysis for differentiation was done on colonies.

Apoptosis analysis

Apoptosis analysis was performed using Annexin V and Propidium Iodide (Thermo Fischer Scientific, Waltham, MA). In brief, 1×10^6 AML cells were incubated at varying doses of AZD9150 and ntASO. After 48 hours, the cells were harvested and washed with PBS & binding buffer. They were then incubated with 5Ul of Annexin V for 15 minutes protected from light. Binding buffer was then added after another wash followed by 5 Ul of propidium iodide. The cell mixture was then incubated on ice at 2-8°C and analyzed by flow cytometry using a FACSAria II Special Order System (BD Biosciences, San Jose, CA).

Preparation of primary hematopoietic cells

Cord blood and patient-derived peripheral blood specimen were collected from patients with MDS and AML (Please refer to Supplementary table 1 for patient details). Mononuclear cells (MNCs) were isolated by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare) as published before.(7) CD34-positive hematopoietic stem and progenitor cells were isolated by

immuno-magnetic sorting according to the manufacturer's recommendation (MicroBead Kit human, (MiltenyiBiotec, Bergisch Gladbach, Germany) were cultured in serum-free growth medium (StemSpan, StemCell Technologies, Vancouver, BC) supplemented with 10 ng/ml recombinant human Interleukin-3 (Preprotech, Rocky Hill, NJ), 25 ng/ml recombinant human interleukin-6 (rhIL-6), 50 ng/ml recombinant human Stem Cell Factor (Gemini, West Sacramento, CA), 40 µg/ml human Low-density lipoprotein (Sigma, St. Louis, MO), and Primocin (InvivoGen, San Diego, CA) at 37°C and 5% CO_2. Cells were treated with 10 µM of a non-targeting control oligo, NTO), or ASO at the indicated concentrations for 24 and 48 hours.

Flow cytometric analysis of primary hematopoietic cells

We utilized cell surface marker detection to identify various hematopoietic cell populations as done before.(16, 37) Upon culture, primary cells were collected, washed once with 1x PBS (GE Healthcare Life Sciences, Logan, UT) and subsequently stained with PB-conjugated monoclonal antibody against human CD34 (581, 1:50, Biolegend, San Diego, CA), PE-Cy7 conjugated anti-human CD38 (HIT2, 1:50, eBioscience, San Diego, CA), PE conjugated anti-human CD33 (WM-53, 1:50, eBioscience, San Diego, CA), PE-Cy5 conjugated anti-human CD10 (eBioCB-CALLA, 1:50, eBioscience, San Diego, CA) and Pacific Orange conjugated anti-human CD4 (MCD0430, 1:50, Life Technologies, Carlsbad, CA) for 20 minutes at 4°C. Data were acquired using an ARIA II Special order System device (BD Biosciences, San Jose, CA). Data analysis was performed using FlowJo v. 10 (Treestar, Ashland, OR).

Quantification of intracellular antisense oligonucleotide uptake

Validation of cellular uptake of the antisense oligonucleotide was performed using and antibody raised against the ASO backbone and intracellular FACS analysis. Briefly, cultured primary cells were fixed with 1x Lyse/Fix Buffer (BD Biosciences, San Jose, CA) pre-warmed to 37°C. Cells were then permeabilized with Perm Buffer III (BD Biosciences, San Jose, CA) for 30 minutes on

ice. Cells were then incubated with 4% mouse serum (Thermo Fisher Scientific, Waltham, MA) to minimize unspecific binding of the antibody, after which 0.1 µg of an unconjugated anti-STAT3-ASO polyclonal antibody (provided by Ionis, Carlsbad, CA) in 100 µl PBS 1x (per 1×10^6 cells) was added. Cells were incubated for 20 minutes at 4°C and washed afterwards once with 1x PBS. Cells were then stained with an APC-labelled Donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) along with a FITC-conjugated anti-human pSTAT3 (serine 727) (49/p-Stat3, 1:50; BD Biosciences, San Jose, CA) and a PE-conjugated anti-human pSTAT3 (tyrosine 705) (4/P-Stat3, 1:50; BD Biosciences, San Jose, CA). Data acquisition was performed using an LSR II device (Becton Dickinson); data analysis was accomplished using BD FACSDiva (Becton Dickinson) or FlowJo v. 10 (Treestar) software packages.

Patient database and survival data and correlation with STAT3 gene expression:

Gene expression data from 183 MDS CD34+ samples and 17 controls was obtained from GEO (GSE19429)(38). Gene expression data on sorted LT-HSCs, ST-HSCs, and GMPs from AML/MDS patients and healthy controls is deposited in the GEO database (GSE35008 and GSE35010). GSEA analysis was performed by comparing the “STAT3 high” signature to two published preleukemic HSC signatures (GSE6891, (15) Ng et all and GSEA12417, (16).

Luciferase reporter assay for STAT3 and STAT5 binding

To measure STAT3 and STAT5 dependent luciferase activity, the STAT3-luc and STAT5-responsive NCAM2-luciferase constructs were transfected into CMK cells as previously described previously (39)

Leukemia Xenografts

NOD/SCID IL2Rgamma KO mice were injected with 10^6 CMK cells that had been transfected with AZD9150 and ntASO. Cells were injected into the mice approximately 2-4 hours after

transfection. Viability was assessed before injection and same number of viable cells (2.5 million) were injected from both groups. Overall survival was measured and analyzed by Kaplan Meier plotting and log-rank test.

Patient derived Xenografts (PDX)

NSG mice were irradiated (200 rads) 24 hours prior to injection. Peripheral blood mononuclear cells from primary MDS and AML patient samples were isolated by Ficoll separation. 2-5 $X10^6$ PBMNCs were administered via tail vein injection. Three weeks later, the mice underwent bone marrow aspiration and the aspirate was analyzed by flow cytometry for the rate of engraftment. Mice were considered to be engrafted if they showed 0.1% or higher human derived CD45+ cells. The engrafted mice were treated with subcutaneous injections of AZD9150 and ntASO at a dose of 50 mg/kg for five days / week for 4 weeks. After this, bone marrow aspiration and flow cytometry analysis for the above mentioned markers were repeated.

Statistics

R software version 3.4.1 was used for statistical analysis. The data presented in figures with box or bar plots has been represented as mean ± Standard error of mean (SEM) unless otherwise specified in the figure legend. The significance of the differences in Kaplan Meier survival curves has been computed by log rank test. The p-values for the significance of correlation have been generated from a Fisher Z transformation.

Study Approval

The studies reported in this manuscript were conducted under a tissue collection protocol (Protocol No. CCI-2005-536) approved by the Albert Einstein College of Medicine Institutional Review Board, Bronx, NY. The animal studies including patient derived xenografts were performed under an experimental protocol (Protocol No.20150703) approved by the Institutional

Animal Care and Use Committee (IACUC). There were no active human subjects enrolled into the study and hence there was no requirement to obtain informed consent.

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Conflicts of Interest: None

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Figure Legends:

Fig 1: STAT3 is overexpressed in MDS and AML HSCs and Progenitors and is associated with worse prognosis: (A) Gene expression data from sorted AML/MDS bone marrow samples was compared to healthy controls (Ctrl) and revealed significantly increased STAT3 expression in (B-D) LT-HSC (Lin-ve, CD34+, CD38-, CD90, N=12 AML/MDS, HC=4), ST-HSC (Lin-ve, CD34+, CD38-, CD90) and GMP (Lin-ve, CD34+, CD38+, CD90+, CD123+) (P Value<0.001, FDR<5%). (E-F)Cytogenetic abnormalities are depicted as NK=Normal Karyotype, CK= Complex Karyotype, -7= deletion of chr7). Crtl refers to healthy control sorted populations. qRT-PCR on independent cohort of sorted cells from controls, MDS and AML samples reveals increased expression of STAT3 in AML/MDS HSCs (LT/ST) and GMP. (G) Survival of 183 MDS patients was correlated with STAT3 expression in marrow derived CD34+ cells. Patients with higher STAT3 levels (> median) had a median survival of 2.6 years compared to 5.8 years for group with lower STAT3 (Log Rank P Value<0.01). (H-J) Pt with a high STAT3 expression also had significantly reduced mean hemoglobin levels, a higher blast percentage and increased transfusion dependence (Test of proportions, * P Value < 0.05, ** P<0.005).

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Fig 5: AZD9150 treatment leads to enhanced differentiation from primary MDS stem and progenitors: AZD9150 treatment leads to enhanced differentiation from primary MDS stem and progenitors : (A) Clonogenic assays from primary MDS MNC samples (N=10) were performed and cells were harvested after 14 days and assessed for differentiation by flow cytometry. An increase in erythroid (Glycophorin A) and myeloid (CD11b) differentiation was seen in samples when compared to controls. (B) A representative flow plot shows increased erythroid differentiation with increase in the pro and basophilic erythroblasts after AZD9150 treatment. (10uM) (C) The drug treated colonies were also larger in size at the same magnification.

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Figure 7. STAT3 downregulation by AZD9150 leads to downregulation of oncogenic and stem cell associated genes: (A) RNA-seq analysis of CMK leukemia cells treated with AZD9150 (10uM) and control (10uM) for 24 hours leads to differential expression of various

transcripts including known leukemia stem cell drivers, IL1RAP, MSI2 and MCL1. (B) Important gene pathways are regulated by STAT3 in CMK leukemia cells when analyzed by gene ontology analysis. (C) qRTPCR validation demonstrates downregulation of STAT3, IL8, CXCR2 & IL1RAP by AZD9150 treatment.

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Supplementary tables & figures

Table 1. Patient characteristics of samples used for primary patient assays, patient derived xenografts

Table 2. Genes associated with high STAT3 signature in sorted MDS CD34+ gene set

Table 3. RNA-seq data showing differential expression of genes after treatment with AZD9150

Table 4. Primers for RT-PCR

Figure 1. STAT3 inhibition with AZD9150 leads to decreased replating efficiency: 2 primary AML samples were treated with AZD9150 and non targeting control (NTC) and plated in methylcellulose assays. Total colonies were read after 2 weeks. In both samples, treatment with AZD9150 led to significantly decreased replating efficiency at the 2^{nd} (A) and 3^{rd} cycles (B) (P val<0.05). (C) Intracellular staining for pSTAT3 tyrosine (pY705 PE) measured in AML1 patient sample shows 12.4% positive HSCs (CD34+, CD38-) and 18.9% +ve progenitors (CD34+,CD38+) pretreatment. (D) MDS Stem & progenitor cells have a higher percentage of viable cells that express phospho-STAT3 com compared to cord blood controls.

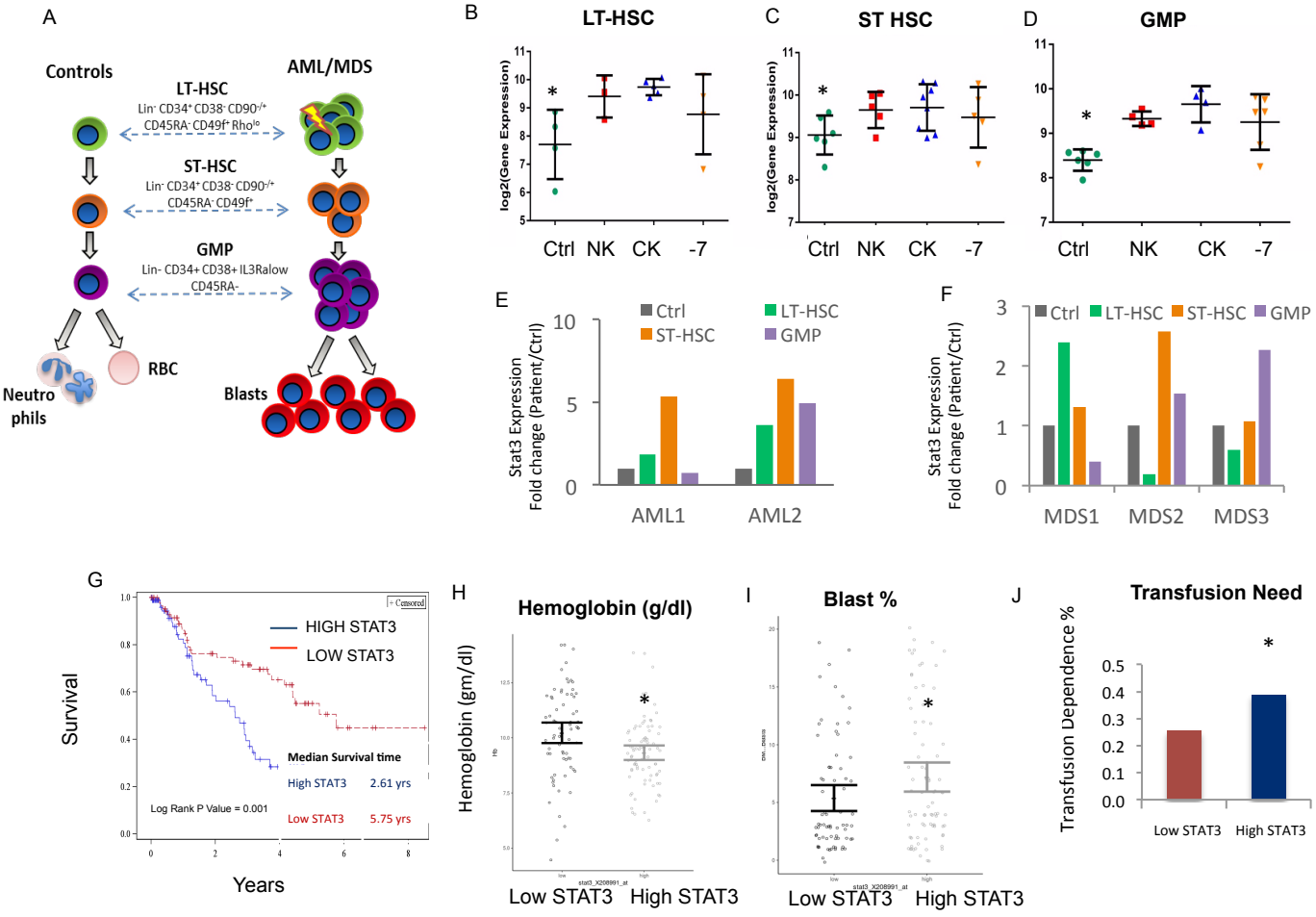
Figure 2. STAT3 ASO uptake is seen in Liver and Spleen in vivo. STAT3 ASO uptake in the spleen and the liver is demonstrated. **Fig.2A** shows Mouse MDSC's from the spleen of 4T1 breast cancer tumor bearing C57BL/6 mice after they were sacrificed, harvested and the spleen was homogenized. There is consistent inhibition of p-STAT3 and total STAT3 in the spleen of the mice treated with mouse STAT3 ASO compared to the controls. (Fig.2B) Shows A431epidermoid carcinoma tumor bearing mice with AZD9150 incorporated into the tumor and mouse STAT3 ASO in the liver. The data shows that there increased knockdown of STAT3

noted at higher drug concentrations in both the organs (liver & tumor) that were tested. Open symbols in Fig.2B are 25 mg/kg dose, closed symbols are 50 mg/kg dose.

Figure 3. Dose response relationship between STAT3 ASO treatment and STAT3 knockdown is seen in (A) Human CD8+T cells from peripheral blood mononuclear cells (PBMC) after treatment with AZD9150, (B) Mouse CD8+T cells from C57BL/6 spleen after treatment with mouse STAT3 ASO, where there is greater inhibition of pSTAT3 & Total STAT3 at the higher doses compared to control. (C) A higher dose of AZD9150 also resulted in more apoptosis in the CMK cell line in vitro. (* $p < 0.05$)

Figure 4. AZD9150 treatment does not lead to significant changes in megakaryocytic colony formation: Cord blood CD34+ cells were treated in megacult assay with AZD9150 and Non Targeting Control (NTC) at 10uM and various megakaryocytic colonies were counted after 14 days. These included Mature, Immature and Mixed Megakaryocytic (MK) colonies. No significant differences in colony numbers were seen after treatment with AZD9150 in two independent experiments. (Means + s.d are shown)

Figure 5. AZD9150 treatment lead to increased apoptosis in MDS/AML cells compared to healthy controls : AZD9150 incorporation in primary TP53 mutated MDS-AML stem cells led to increased apoptosis in stem and progenitor cell populations in a dose-dependent manner (A) as noted by the increase in the % of Annexin V positive cells in comparison to cord blood stem and progenitor cells (B)



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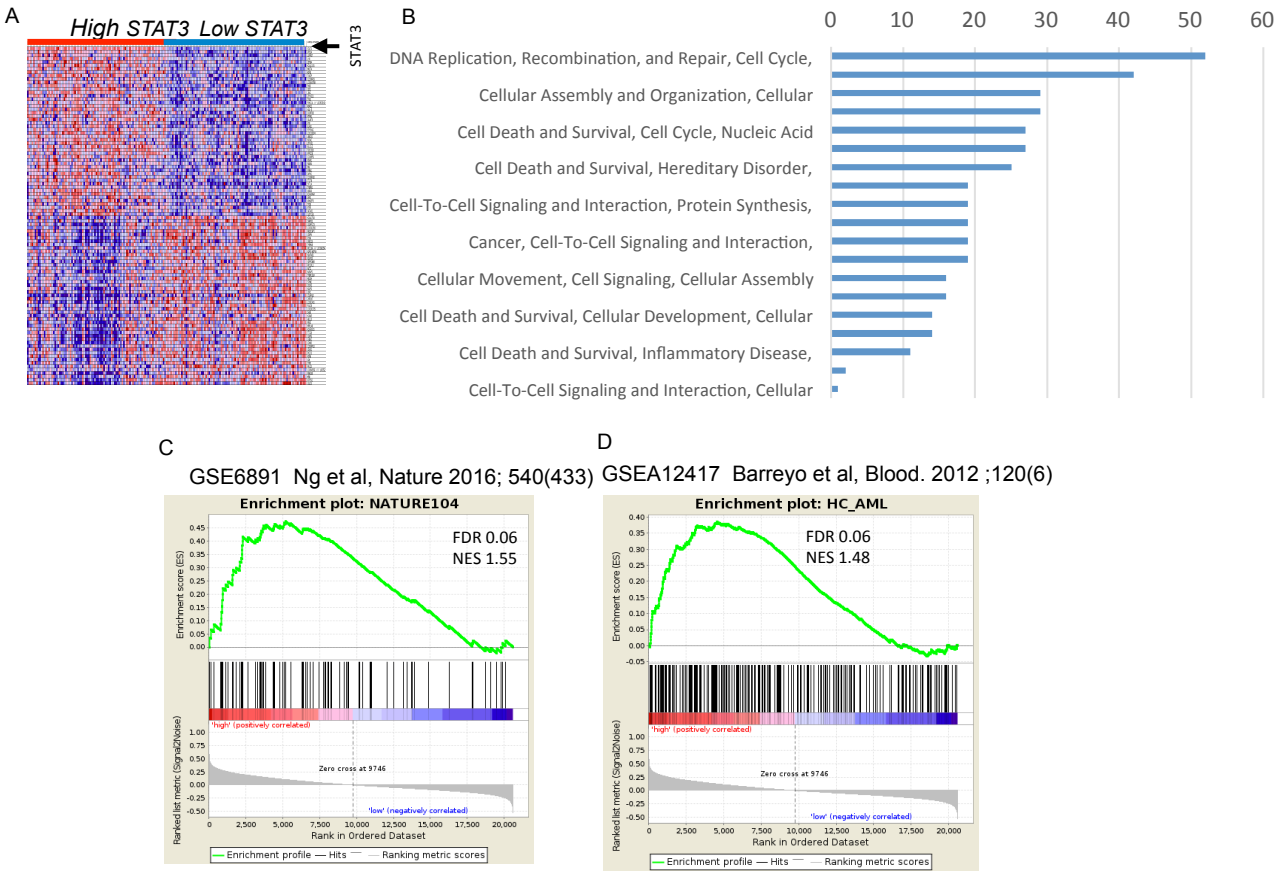

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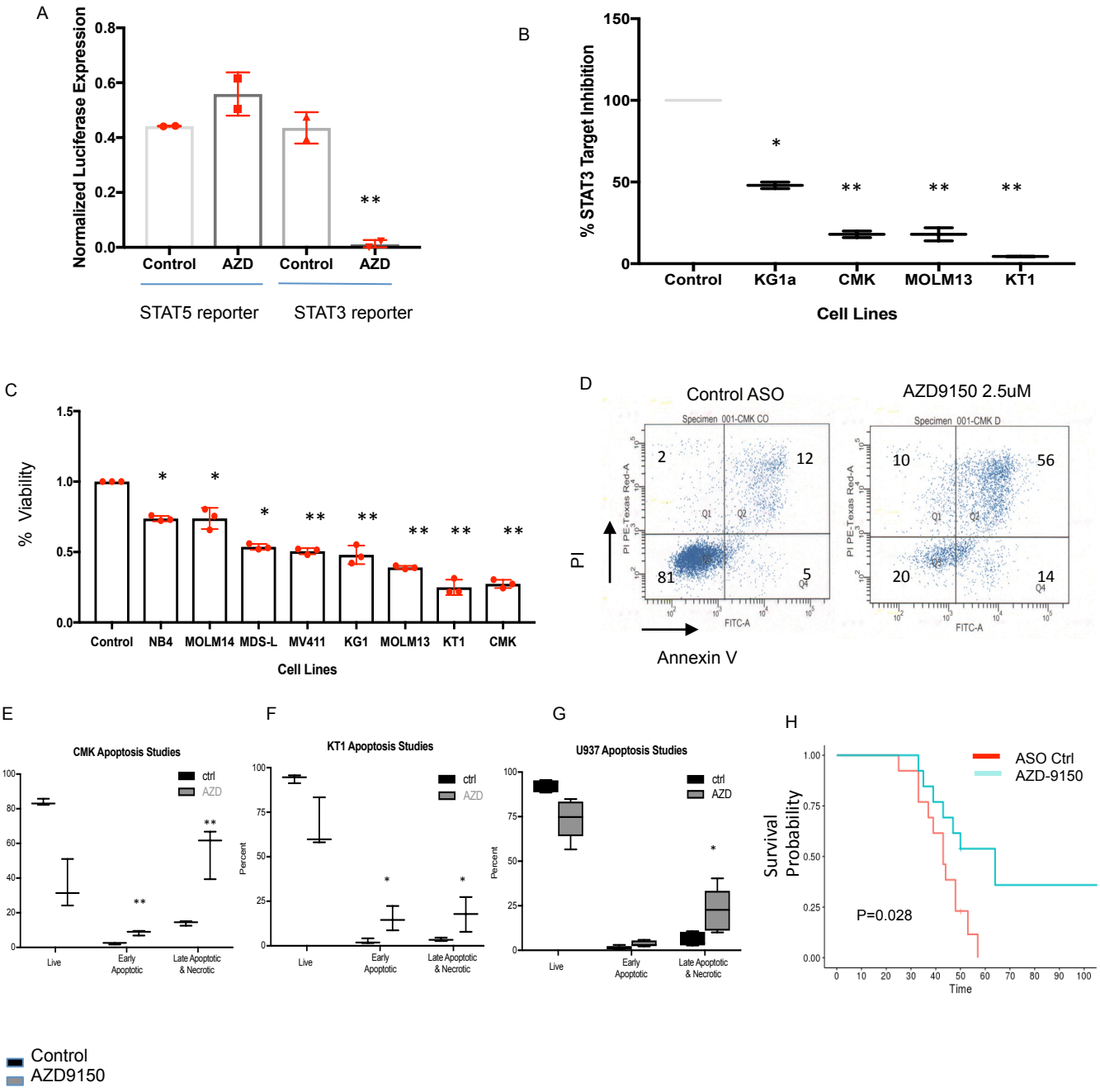

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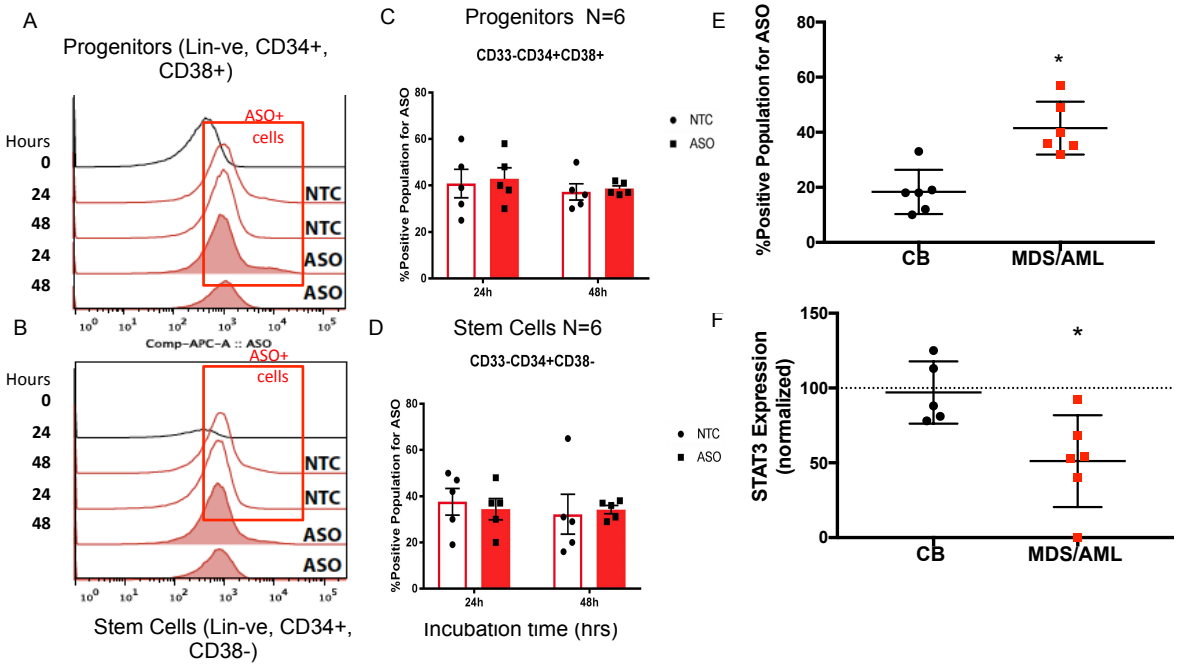

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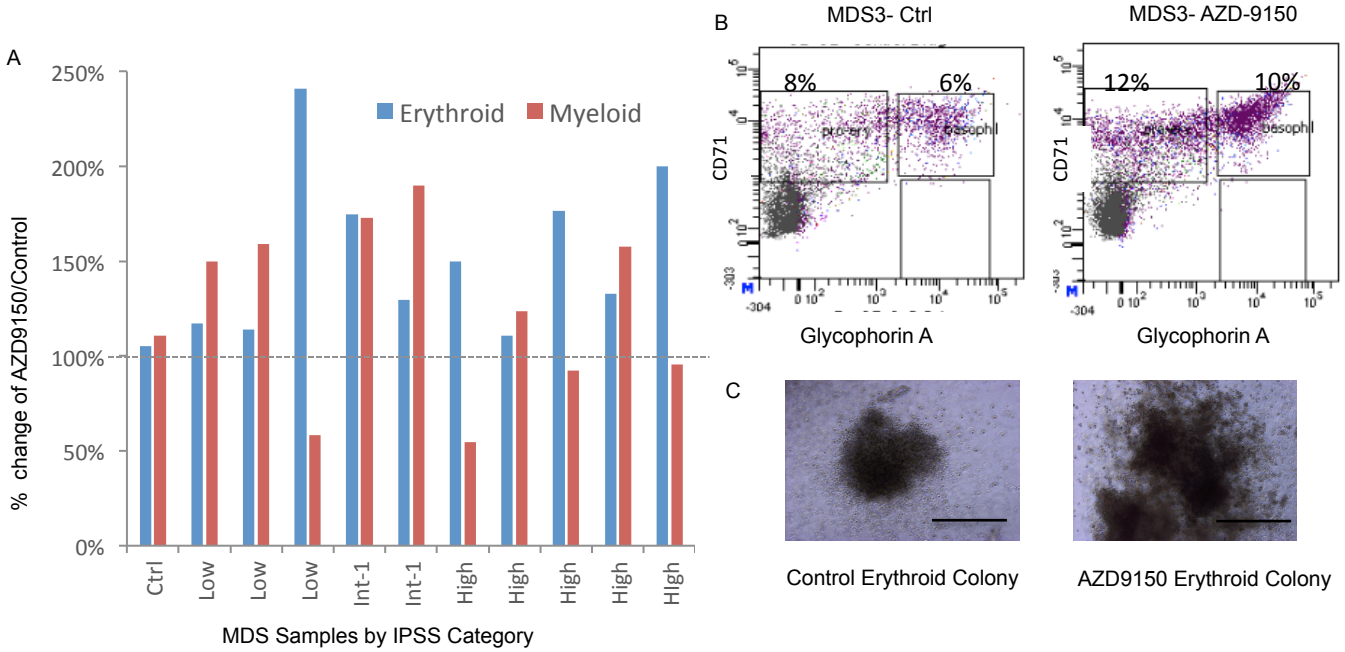

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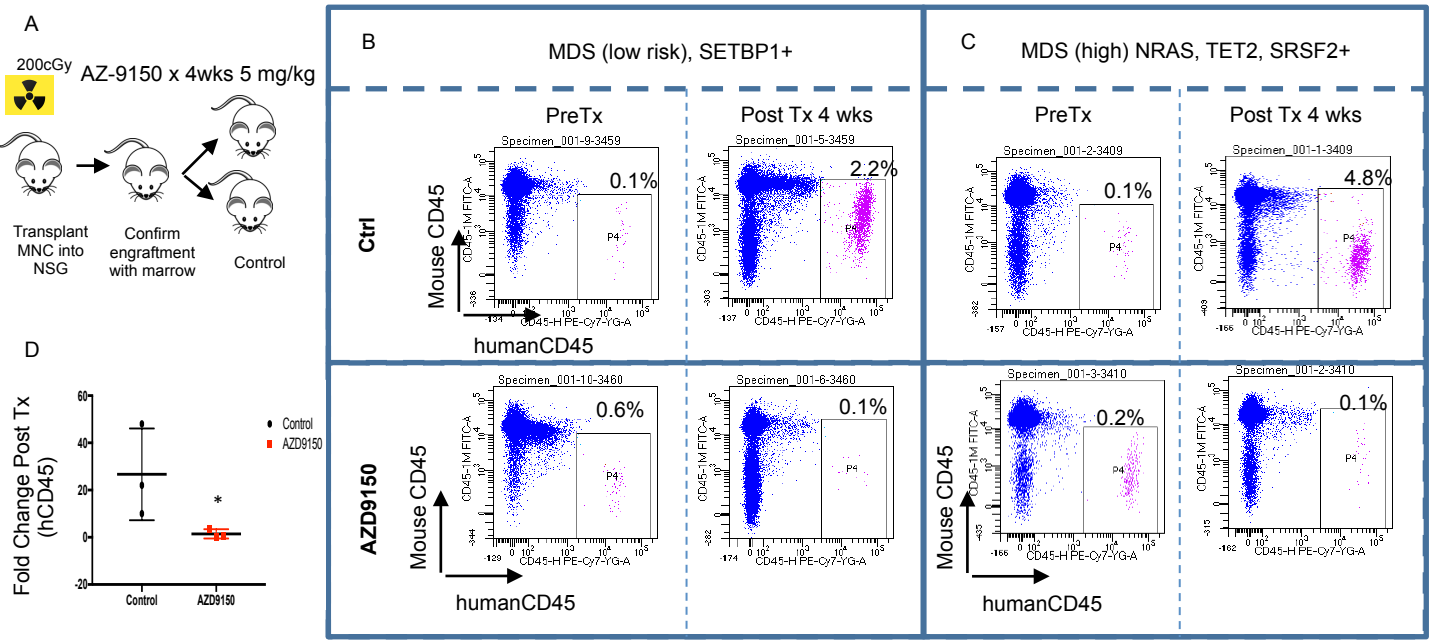

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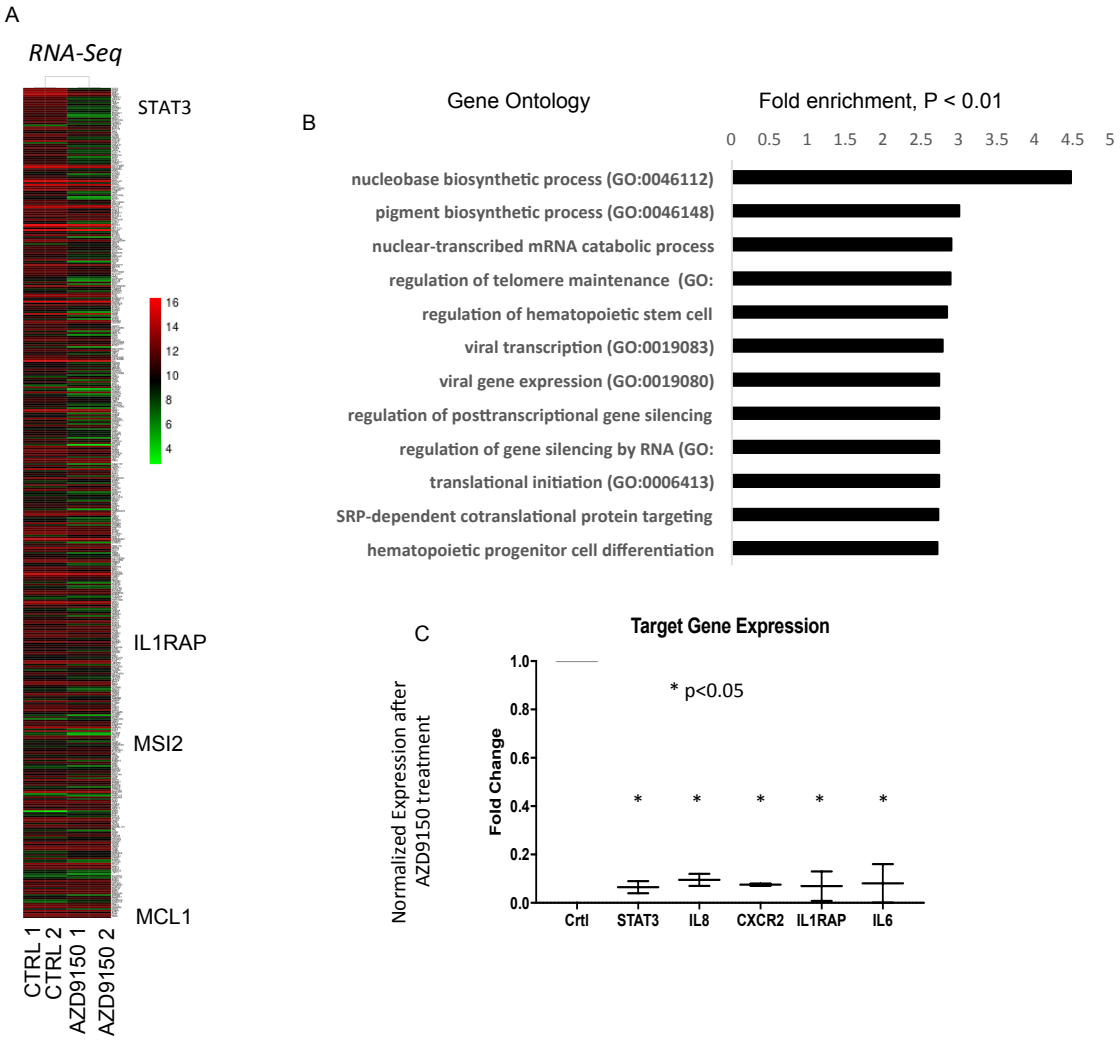

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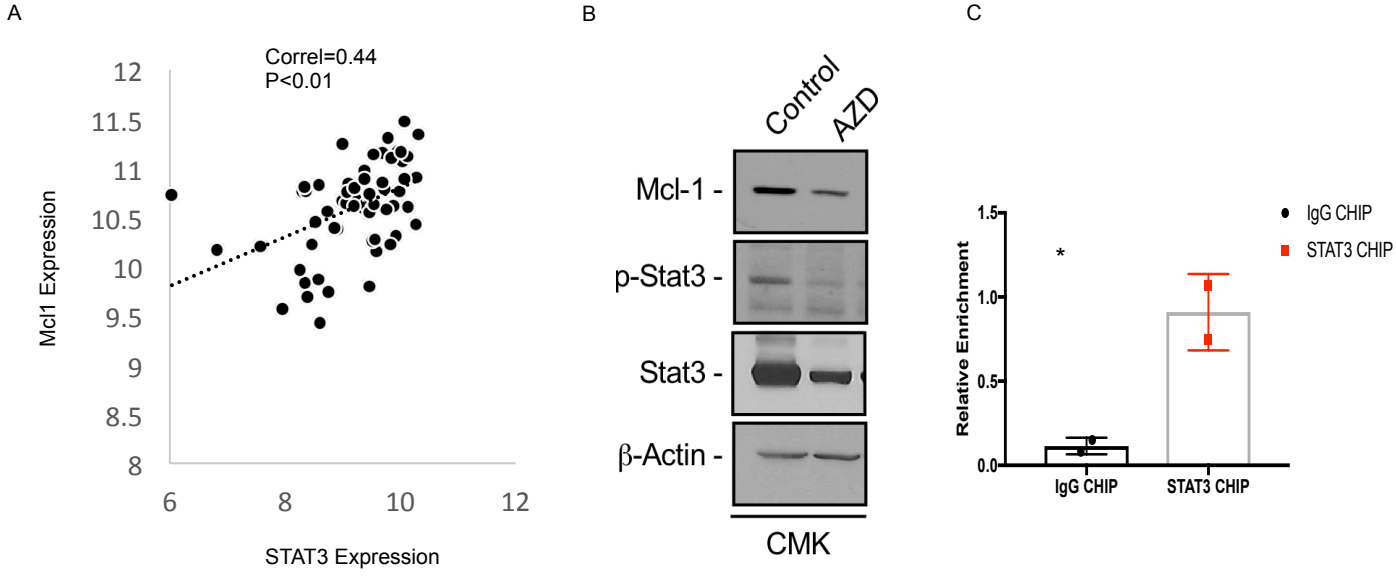

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