Dysregulation of immune cell and cytokine signaling correlates with clinical outcomes in myelodysplastic syndrome (MDS)

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Novelty Statements

1. What is the NEW aspect of your work?

   This study utilizes correlative samples collected across 3 prospective clinical trials in MDS conducted in Australia (LEN-SCF, ALLG MDS3 and ALLG MDS4 clinical trials) and uses novel methods for analyzing immune cell subsets which correlated with clinical outcomes in MDS.

2. What is the CENTRAL finding of your work?

   The main findings from this study are that dysregulation in innate immunity and inflammatory signaling play central roles in the pathogenesis of MDS and correlated with clinical responses and maintenance of clinical benefit after 12 months of treatment.

3. What is (could be) the SPECIFIC clinical relevance of your work?

   The genetic and immunological biomarkers identified in this study can help define early markers of resistance to treatment with Azaciditine +/- IMiDs in MDS and future studies should focus on a biomarker-driven approach to guide treatment decisions in patients who may benefit from early escalation of treatment with novel therapies to improve their prognosis.

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Dysregulation of immune cell and cytokine signaling correlates with clinical outcomes in myelodysplastic syndrome (MDS)

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Running title: Immune and cytokine dysregulation in MDS

Abstract

Objectives

Myelodysplastic syndromes (MDS) are characterized by ineffective hematopoiesis. Although hypomethylating agents (HMA) have improved survival in higher-risk MDS, most patients eventually succumb to progressive disease. Utilizing samples collected

prospectively from three MDS clinical trials, we analyzed genetic and immunological biomarkers and correlated them with clinical outcomes.

Methods

154 samples were analyzed from 133 *de novo* MDS patients for T-cell and myeloid cell immunophenotyping and gene expression analysis. Treatments were with HMA or immunomodulatory drug (IMiD) alone or in combination.

Results

We observed differences in immune cell subsets between lower and higher risk IPSS groups with NKT cells, MDSCs, intermediate-proinflammatory and non-classical monocytes being higher in the latter group while naïve CD4+ T-cells were reduced. Intermediate-proinflammatory monocytes were increased in non-responders and those failing to achieve at least a hematological improvement. Pro-inflammatory NKT cells were increased at diagnosis for patients failing to derive clinical benefit after 12 months of treatment. Gene expression analysis of paired bone marrow (BM) colony-forming units (CFUs) from diagnosis and 4 cycles post-treatment confirmed that genes involved in cytokine signaling were downregulated in C4 normal colonies.

Conclusions

These findings support the central roles of dysregulation in innate immunity and inflammatory signaling in the pathogenesis of MDS which correlated with clinical outcomes post-treatment.

Keywords: Myelodysplastic syndromes, immunity, cytokine signaling, inflammation

Introduction

Myelodysplastic syndromes (MDS) are defined by ineffective hematopoiesis leading to cytopenias and dysfunctional blood cells. Based on the IPSS and IPSS-R prognostic scoring system, higher-risk MDS has an increased risk of transformation to acute myeloid leukemia (AML) and inferior overall survival (OS) (1, 2). Treatment of higher-risk MDS with the hypomethylating agent Azacitidine has shown a median OS benefit of 9.5 months; however, complete remission rates remain suboptimal at 10-20% with a median duration of response

of 13.1-13.6 months (3, 4) and the vast majority of responders eventually relapsing within 1-2 years (5). Survival outcomes after hypomethylating agent failure is dismal with an expected 2-year OS of 15% (6), and due to the high median age at diagnosis of 70 years, intensive chemotherapy is usually not offered at time of diagnosis or disease progression due to other comorbidities and the expected toxicities arising from intensive treatments. Newer targeted agents such as the anti-CD47 antibody, magrolimab and anti-TIM3 antibody, MBG453 show early promising augmentation in complete response rates in higher-risk MDS and AML when given in combination with Azacitidine but longer follow-up period is required to ascertain durability of these responses (7, 8).

The Australasian Leukemia and Lymphoma Group (ALLG) is a cooperative blood cancer clinical trials group that conducts trials across multiple sites within Australia and New Zealand. The ALLG MDS3 trial *(Trial ID: ACTRN12607000283471)* was a Phase II trial investigating the use of Azacitidine and Thalidomide (AZA+THAL) combination therapy in clinically advanced *de novo* MDS, chronic myelomonocytic leukemia (CMML) and low blast count AML (9) while the ALLG MDS4 trial (*Trial ID: ACTRN12610000271000)* was a Phase II randomised trial investigating the efficacy of Azacitidine in combination with Lenalidomide (AZA+LEN) in comparison with Azacitidine (AZA) alone in the treatment of *de novo* higher risk MDS, CMML and low blast count AML (10). The LEN-SCF trial *(Trial ID: NCT00434239)* was a single centre Phase II trial investigating combination therapy of lenalidomide and stem cell factor in lower risk MDS patients with anemia. All patients had no prior exposure to hypomethylating agents nor immunomodulatory drugs.

The MDS3 trial enrolled 80 patients from 15 sites. IPSS scoring was low in 9%, Int-1 in 42%, Int-2 in 35% and high-risk in 14% of patients. The median number of AZA cycles administered was 9 (range 1-24+) with 16% requiring dose reduction while the median duration of THAL treatment was 6.1 months (range 0.5-13.3). Overall response rate was achieved in 63% (complete response (CR) as best response 26%, partial response (PR) 5%, marrow CR 19%, hematological improvement (HI) 14%), median response duration was 26.3 months and median overall survival 28.1 months. The median time to best response was 3.7 months and in those achieving CR, the median time to CR was 4.5 months (9). In the MDS4 trial, 160 patients were enrolled across 30 sites. IPSS scoring was low in 14%, Int-1 in

47%, Int-2 in 24% and high in 15% of patients. The median number of AZA cycles delivered per patient was 11 in both arms (AZA and AZA+LEN) with only 2.6% cycles dose-reduced and for patients on AZA+LEN, the median duration of LEN treatment was 9 cycles (range 1-12) with only 2.8% of cycles dose-reduced. Overall response rate was 57% in the AZA arm and 69% in AZA+LEN arm with no difference in clinical benefit at 12 months (defined as alive and progression/relapse free at 12 months (+/- 1 month) post-commencement of treatment, including patients with stable disease), progression-free survival and overall survival between the two arms (median OS AZA 38.8 months vs AZA+LEN 29.2 months). The median time to best response for those achieving HI or better was 5.5 months for AZA and 4.8 months for AZA+LEN arms (10). In the LEN-SCF trial, 9 of the 24 patients (38%) had durable responses with 6 patients (25%) achieving CR. The median time to best response was 3 months and there was no difference in response rates between 5q- and non-5q- MDS (D. Ritchie, personal communication, unpublished data).

Previous studies have shown that abnormal clonal populations are not completely eradicated from the bone marrow of patients even in those achieving a complete remission, contributing to disease recurrence and resistance (11, 12). Moreover, immune profiling in MDS has demonstrated clonal expansion of CD8+/CD57+/CD28- effector T-cells across all IPSS risk groups (13) but the clinical significance of the role of NKT cells is unclear as impairment of NK cytotoxic function has been reported in association with disease progression (14) while we have not demonstrated similar findings in a longitudinal follow-up study of MDS patients treated on Lenalidomide; in our study, the NKT cell frequency and function remained stable throughout treatment and there was no correlation with clinical response (15). Therefore, using correlative samples collected prospectively from the LEN-SCF, MDS3 and MDS4 trials, we aimed to characterize further the genetic and immunological markers using a recently developed analytical platform, to predict inferior clinical outcomes in these cohorts of MDS patients.

Material and methods

Patients and samples

MDS patients from the three clinical trials as described above, who consented to correlative analyses had their samples processed. Immunological biomarker analysis was undertaken using flow cytometry for identification of immune cell population subsets of interest. Genetic biomarker analysis was undertaken using gene expression analysis from BM colonies pre- and post-treatment. This study was approved by the Melbourne Health Human Research Ethics Committee (HREC Reference No: 2014.023).

Flow cytometry

Peripheral blood was collected at study enrolment (baseline). Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells were isolated using Ficoll-Paque Plus (GE Healthcare, Chicago, IL, USA) and cryopreserved until required for analysis.

PBMC were stained using Live/Dead aqua fixable cell stain (Thermo Fisher Scientific, Waltham, MA, USA) prior to staining with specific antibodies. Antibodies used for T cell phenotyping were; CD3 BV786, CD4 APC-Cy7 AF700, CD27 BV711, CD45RO AF700, TRAV1-2 PE , CD161 BV605, CD28 PECy7 (BioLegend, San Diego, CA, USA), CD8a BV650, CD8β APC, TCRgd FITC (BD Biosciences), CD45RA PerCP Cy5.5, CCR7 PE-Cy7 (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), hCD1d-PBS44 BV421 (gift from Godfrey Laboratory, University of Melbourne). For monocyte phenotyping; CD14 APC, CD11b BV711, CD16 PerCP Cy5.5, CD45 AF700, CD33 PE-Cy7 AF700, CCR2 BV421, CX3CR1 PE, CD62L BV605 and CD15FITC (BioLegend, San Diego, CA, USA) were used.

BM MNC were stained using Live/Dead aqua fixable cell stain (Thermo Fisher Scientific, Waltham, MA, USA) prior to staining with specific antibodies. Antibodies used for BM monocyte phenotyping were; HLA-DR APC-H7, CD34 PerCP-Cy5.5 (BD Bioscience), CD14 APC, CD16 BV650, CD11b BV711, CD45 AF700, CD33 PE-Cy7 AF700, CCR2 BV421, CD15 FITC, CX3CR1 PE and CD62L BV605 (BioLegend, San Diego, CA, USA).

PBMC and BM MNC were stained in Live/Dead cell stain for 20 minutes in PBS at RT, washed in FACS buffer (PBS + 2% FCS), and blocked in Human Fc block (BD Bioscience) for 5 minutes at RT. Cell staining was performed in FACS buffer for 30 minutes at 4°C, followed by 2 washes in FACS buffer, and fixation in 2% paraformaldehyde (PFA, Electron Microscopy

Sciences). Samples were acquired on a LSR Fortessa (BD Bioscience) flow cytometer, and analyzed using FlowJo software (BD Bioscience). Populations were gated on single, viable cells.

Analysis of the cell populations was then performed using the Vortex package (16) as per the developer's guidelines. T cell data and myeloid cell data were clustered individually using the x-shift cluster algorithm. 30 iterations were performed for each dataset with incremental K values. Elbow point validation was used to select for the k value with the most appropriate clustering. Whole data was visualized as force-directed plots in the Vortex package to determine cluster integrity/validity. Individual cluster phenotype was determined representing specific T-cell and myeloid subsets and shown using the brickplots visualization (17). There were nine T-cell populations and twelve myeloid cell populations (out of 77 and 73 unique clusters respectively) that were substantively represented and were therefore assessed further for the clinical comparisons.

Cluster frequency was compared between clinical groups to determine relevance of each population. Where graphs compare two sets of data, statistical significance was determined by a Mann Whitney test of statistical significance. Where graphs compare more than two groups of data, statistical significance was determined by Kruskall Wallace and subsequent Dunn's multiple comparisons test.

BM Colony Assays

BM MNC from baseline patient samples and after 4 cycles of treatment with Azacitidine +/- Lenalidomide or Thalidomide (C4) were cultured in a methylcellulose-based medium with recombinant cytokines for human cells MethoCult H4034 Optimum, at 37°C and 5% CO_2 for 14 days to allow colony formation. C4 was chosen for analysis of post-treatment gene expression samples as previous clinical studies have shown that the median time to response with Azacitidine was 3-4 months (9, 10).

Once colonies were established a minimum of 40 colonies were picked from the baseline samples and for cycle 4 samples, 40 colonies of normal morphology and 40 abnormal colonies were picked. Normal colonies were not picked at baseline as abnormal colonies predominated at this timepoint and there were too few normal ones to select from.

Colonies from an individual patient were pooled at their specific timepoints. The study researcher picking the colonies was blinded to the patients' treatment responses so that there were no biases introduced during the colony selection process. Samples were stored at -80°C for later RNA extraction and gene analysis.

Gene expression analysis

Total RNA from BM MNC colonies were isolated using ReliaPrep RNA cell mini prep system (Promega, Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was determined using NanoString PanCancer Human Pathways Panel V1 (Nanostring Technologies, Seattle, WA, USA). All sample raw data was reviewed and all samples in downstream analyses had no QC flags and detection of at least 20% of probes in the sample. All experiments were normalized and analyzed using nCounter Advanced Analysis version 2.0.115 (NanoString Technologies). Gene expression is reported as $\log_2$ relative expression. Pathway signature and cell score was determined using proprietary algorithms (NanoString Technologies).

Statistical analysis

Genes expressed above background level in 25% of samples were included for statistical analyses and counts were normalized using TMM normalization (18). Differential expression analysis was performed using the R limma package (19) to fit a linear model for each gene. Patient effects were modelled with a random effect factor and each sample was weighted using limma's voomWithQualityWeights function. Empirical Bayes moderated t-statistics were used to identify differentially expressed genes. For testing between colonies, a fold-change cut-off of 1.5 was used with limma's treat function. P-values were adjusted using the Benjamini-Hochberg method for multiple hypothesis testing.

Clinical correlation

Any significant biomarkers identified were then correlated against the clinical outcomes of:

1. Clinical benefit at 12 months (defined as being alive with no progression or relapse of disease at this timepoint/ maintenance of stable disease response or better as defined by the IWG MDS response criteria (20))
2. Best response achieved during treatment (CR, PR, hematological improvement (HI), stable disease (SD), progressive disease (PD))
3. Overall survival

Results

The numbers of samples analyzed for each correlative analysis are shown in Table 1. Patient demographics for the three clinical trials are also listed in Table 1 based on the cohort analyzed for lymphoid and myeloid immunophenotyping studies as this had the largest number of patient samples available.

TABLE 1

Sample numbers analyzed	**LEN-SCF (n)***	**AZA+THAL (MDS3) (n)****	**AZA (MDS4) (n)****	**AZA+LEN (MDS4) (n)****	**TOTAL (n)**
Immunophenotyping (T-cell and myeloid)	25	23	44	40	132
BM Paired Colony Assays (Gene Expression Analysis)	0	4	10	8	22
Total	25	27	54	48	154
Immunophenotyping (T-cell and myeloid) cohort	**LEN-SCF (n=25)**	**AZA+THAL (MDS3) (n=23)**	**AZA (MDS4) (n=44)**	**AZA+LEN (MDS4) (n=40)**	**TOTAL (n=132)**
Median age at diagnosis (range in years)	65.5 (43-88)	68 (42-82)	68.8 (45.5-	72.3 (54-87.2)	68.4 (42-88)

			85.9)		
Sex					
• Male	17 (68)	15 (65.2)	28 (63.6)	28 (70)	88 (66.7)
• Female	7 (28)	8 (34.8)	16 (36.4)	12 (30)	43 (32.6)
• N/A	1 (4)	0 (0)	0 (0)	0 (0)	1 (0.8)
IPSS (n/%)					
• Low	12 (48)	1 (4.3)	6 (13.6)	1 (2.5)	20 (15.2)
• Int-1	9 (36)	8 (34.8)	20 (45.5)	22 (55.0)	59 (44.7)
• Int-2	1 (4)	10 (43.5)	12 (27.3)	10 (25.0)	33 (25.0)
• High	0 (0)	2 (8.7)	6 (13.6)	7 (17.5)	15 (11.4)
• N/A	3 (12)	2 (8.7)	0 (0)	0 (0)	5 (3.8)
IPSS (n/%)					
• Lower risk	21 (84)	9 (39.1)	26 (59.1)	23 (57.5)	79 (59.8)
• Higher risk	1 (4)	12 (52.2)	18 (40.9)	17 (42.5)	48 (36.4)
• N/A	3 (12)	2 (8.7)	0 (0)	0 (0)	5 (3.8)
IPSS-R (n/%)					
• Very Low	2 (8)	1 (4.3)	0 (0)	0 (0)	3 (2.3)
• Low	13 (52)	4 (17.4)	13 (29.5)	7 (17.5)	37 (28.0)
• Intermediate	7 (28)	4 (17.4)	13 (29.5)	16 (40.0)	40 (30.3)
• High	0 (0)	5 (21.7)	7 (15.9)	6 (15.0)	18 (13.6)
• Very high	0 (0)	3 (13.0)	9 (20.5)	7 (17.5)	19 (14.4)
• N/A	3 (12)	6 (26.1)	2 (4.5)	4 (10.0)	15 (11.4)
WHO MDS Subtype (n/%)					
• MDS-SLD	1 (4)	0 (0)	1 (2.3)	0 (0)	2 (1.5)
• MDS-MLD	6 (24)	1 (4.3)	17 (38.6)	9 (22.5)	33 (25.0)
• MDS-RS	3 (12)	1 (4.3)	3 (6.8)	2 (5.0)	9 (6.8)
• MDS with isolated del(5q)	5 (20)	0 (0)	0 (0)	0 (0)	5 (3.8)
• MDS-EB1	4 (16)	8 (34.8)	4 (9.1)	8 (20.0)	24 (18.2)
	0 (0)	4 (17.4)	11 (25.0)	9 (22.5)	24 (18.2)
	1 (4)	0 (0)	0 (0)	1 (2.5)	2 (1.5)

• MDS-EB2	1 (4)	8 (34.8)	4 (9.1)	7 (17.5)	20 (15.2)
• MDS-U	1 (4)	0 (0)	0 (0)	0 (0)	1 (0.8)
• CMML	0 (0)	1 (4.3)	4 (9.1)	4 (10.0)	9 (6.8)
• MDS/MPN					
• Transformed MDS to AML	3 (12)	0 (0)	0 (0)	0 (0)	3 (2.3)
• N/A					

*Len-SCF cohort only analyzed for T-cell and myeloid immunophenotyping studies

**MDS3 and MDS4 cohort numbers differed for BM colony assay studies due to availability of paired samples at baseline and after 4 cycles of treatment

Immunological biomarkers

1. *T-cell lymphoid and myeloid cell populations differ between treatment groups prior to commencing treatment*

   We first assessed the lymphoid and myeloid cell populations at baseline and compared these between treatment groups. The median expression of all assessed myeloid and T-cell lymphoid markers for the MDS samples are shown in Supplemental Figure 1.

   Myeloid populations: Comparing the LEN-SCF cohort with AZA+/-LEN and AZA+THAL cohorts, the intermediate-proinflammatory monocytes (cluster 4447) was increased in the LEN-SCF cohort in comparison to the other treatment groups and this population was not enriched for either sex, so therefore the observed difference is unlikely due to sex bias (Figs 1A-B and Table 2). The intermediate CD15+CD62L+ and non-classical monocyte populations (clusters 4450 and 4451 respectively) were significantly increased in the AZA+/-LEN cohorts in comparison to the AZA+THAL

cohort (Figs 1C-D and Table 2). Myeloid-derived suppressor cell populations (MDSCs, cluster 4398) were also observed to be increased in the AZA cohort in comparison to the AZA+THAL cohort (Fig 1E and Table 2).

T-cell populations: Comparing the LEN-SCF cohort with AZA+/-LEN cohorts, there was an increase in peripheral memory-like CD4+ T-cells (cluster 2238) and a decrease in CD161+ naïve-like CD4+ T-cells (cluster 2276) in the LEN-SCF cohort (Table 2 and Figs 2A-B).

When comparing AZA+/-LEN to the AZA+THAL cohorts, increases in CD161+ naïve-like CD4+ and effector CD4+ T-cells (clusters 2276 and 2267 respectively) were observed while natural killer T-cells (clusters 2277) and pro-inflammatory NKT cells (cluster 2281) were reduced in the AZA+/-LEN cohorts (Table 2 and Figs 2B-E).

TABLE 2

	Cluster number	**Cell surface markers***	**Sub-population**
Myeloid Cell Populations	4447	HLA-DR, CX3CR1, CD33, CD62L, CD14, CD11b	Intermediate-proinflammatory monocytes
	4450	HLA-DR, CD11b, CD16, CD33, CD14, CD62L, CD15, CX3CR1	Intermediate CD15+CD62L+ monocytes
	4451	HLA-DR, CX3CR1, CD33, CD11b, CD16, CD14, CD62L	Non-classical monocyte
	4449	CCR2, HLA-DR, CX3CR1, CD11b, CD16, CD33, CD14, CD62L	Intermediate CD15-CD62L+ monocytes
	4398	HLA-DR, CD33, CX3CR1,	MDSC

		CD11b	
Lymphoid Cell Populations	2238	CD4, CD45RO, CD27, CCR7, CD8a, CD8b, CD45RA	Peripheral memory-like CD4+ T-cell
	2276	CD4, CD45RA, CD27, CD45RO, CCR7, CD8a, CD8b, CD161	CD161+ naïve-like CD4+ T-cell
	2267	CD4, CD45RO, CD27, CD45RA, CD8b, CCR7	Effector CD4+ T-cell
	2277	CD4, CD45RO, CD27, CCR7, CD45RA, NKT.Tet, CD8a, CD8b	Natural killer T-cells
	2281	CD8a, CD27, CD8b, CD45RO, CD161, NKT.Tet, CD4, CD45RA, CCR7	Pro-inflammatory NKT cell
	2270	CD4, CD45RO, CD45RA, CD27, CD8a, CD8b, CCR7	Naïve CD4+ T-cell
	2226	CD4, CD27, CCR7, CD45RO, CD45RA, CD8a, CD8b, NKT.Tet	CD4+CD8+ double-positive NKT cell

*****cell surface markers listed in order of level of expression (based on brick size)

2. *T-cell lymphoid and myeloid cell populations differ between different IPSS risk groups*

We observed that there were differences in the distribution of IPSS between the treatment groups likely influenced by the differing enrolment criteria specified for the three clinical trials (Supplemental Figure 2). There was a predominance of lower risk IPSS for the Len-SCF trial (48% low risk, 36% Int-1), while in the Phase II MDS3 trial (AZA+THAL), there was a predominance of Int-2 IPSS risk patients (43.5%), followed by Int-1 IPSS risk group (34.8%). In the randomised Phase II MDS4 trial,

there was a predominance of Int-1 risk patients for both the AZA and AZA+LEN arms (45.5% and 55% respectively), followed by Int-2 risk groups (27.3% and 25% respectively) and high risk groups (13.6% and 17.5% respectively).

Based on differences in the IPSS scores and immune cell subsets between treatment groups, we next assessed the T-cell and myeloid populations and correlated these with the IPSS risk groups. There was an increase in the naïve CD4 T-cell population (cluster 2270) in the Int-1 vs Int-2 IPSS risk group (Table 2 and Supplemental Figures 3A-B). In the myeloid cell population, there were increases in the intermediate CD15+CD62L+ and non-classical monocyte populations (clusters 4450 and 4451 respectively) and MDSCs (cluster 4398) in the high risk vs low/ Int-1 IPSS risk groups (Table 2 and Supplemental Figures 3C-E).

When comparing lower risk (Low + Int-1) vs higher risk (Int-2 + High) IPSS groups, NKT cells (clusters 2226 and 2281) (Table 2), intermediate-proinflammatory and non-classical monocytes (clusters 4447 and 4451 respectively) and MDSCs (cluster 4398) (Table 2) were higher in the higher risk groups while naïve CD4+ T-cells (cluster 2270) were higher in the lower risk group.

As these cluster phenotypes were similar to those identified with differential expression between treatment groups and as IPSS distribution differed between the three different clinical trial populations, further analysis of the frequency of patient's cells in each cluster for each treatment arm was undertaken which showed similar trends of frequency between lower and higher risk disease for each treatment (Supplemental Figure 4). As such, we postulate that the variation in immune cell populations between the IPSS risk groups likely reflects the differing biology of the underlying MDS at diagnosis.

3. *Variation in T-cell lymphoid and myeloid repertoire at baseline predict clinical benefit and treatment outcomes*

We then investigated the effect of the different T-cell and myeloid populations on clinical outcomes using treatment responses as defined by the IWG MDS response criteria and clinical benefit at 12 months.

T-cell lymphoid populations: There were no significant T-cell clusters identified that predict clinical responses. However, the pro-inflammatory NKT cell population expressing CD161, CD27 and CD45RO (cluster 2281) was reduced at diagnosis in patients who derived clinical benefit from treatment with AZA+/-THAL/LEN in comparison to those that did not (Fig 3A).

Myeloid populations: There was an increase in intermediate-proinflammatory monocytes (cluster 4447) in non-responders vs responders and in those who achieved stable disease (SD)/ progressive disease (PD) as their best response during treatment in comparison to those achieving at least a hematological improvement (HI) to complete responses (CR) (Figs 3B-C). However, we did not observe any correlation of myeloid clusters at diagnosis which predict for clinical benefit at 12 months.

There was also no correlation observed for any of the T-cell nor myeloid clusters with age.

Genetic biomarkers

4. *Differentially expressed genes at baseline vs post-treatment in MDS correlate with clinical benefit and treatment responses and predict for clinical outcomes*

To assess changes in progenitor cells, gene expression in colonies from bone marrow at baseline and at C4 were analyzed, with C4 colonies separated into colonies that showed abnormal vs normal morphology. All 22 patients had paired samples at baseline and at C4 - 19 patients had samples from baseline, C4 normal and C4 abnormal colonies while the remaining 3 patients had samples from baseline and C4 abnormal colonies only as there were too few normal colonies to pick from at the C4 timepoint (total number of

samples = 63). However, 10 samples were excluded from the final biostatistical analysis as <25% of probes were above the background threshold – in total, 53 samples were utilized (9 patients with all 3 paired samples at baseline, C4 normal and C4 abnormal, while the others had 2 paired samples each - 3 patients with baseline and C4 normal, 5 patients with baseline and C4 abnormal and finally, 5 patients with C4 normal and C4 abnormal samples).

516 genes were included for statistical analyses. Comparing C4 normal (N) to baseline (B) colonies, 77 genes were identified with significant differential expression ($p<0.05$). Comparing C4 normal to C4 abnormal (A) colonies, 161 genes were identified with significant differential expression ($p<0.05$). There was no significant differential gene expression between baseline vs C4 abnormal colonies and baseline vs combined C4 colonies (A+N).

Comparing C4 normal to baseline colonies, the top 5 differentially expressed genes were *MAPK12, FGFR1, IL10, PLAU* and *FLNA*, downregulated in C4 normal colonies (Fig 4A and Supplemental Table 1). Additionally, for patients with maintained hematological improvement (HI) or better at 12 months, *MYD88* and *PIK3R5* were downregulated ($p<0.001$) and for patients who achieved at least a PR as best response, *NFKB1, SYK* and *TGFBR2* were also downregulated in the C4 normal colonies ($p<0.001$).

Comparing C4 normal to C4 abnormal colonies, the top 5 differentially expressed genes were *PLAU, PIK3R5, MYD88* and *IL10* downregulated and *BCL2L1* upregulated in C4 normal colonies ($p<0.001$) (Supplemental Table 2).

At baseline, *MYC* was upregulated in patients with inferior survival (Fig 4B and Supplemental Figure 5). For patients deriving clinical benefit at 12 months, *RFC3* and *LTBP1* were upregulated in the C4 normal colonies (Fig 4C). In patients who did not achieve at least a PR, *FN1* was upregulated in the C4 abnormal colonies (Fig 4D).

Using KEGG and Gene Ontology (GO) analyses, the top 10 upregulated pathways at baseline compared to C4 normal colonies are listed in Supplemental Tables 3 and 4. The top 3 upregulated pathways in KEGG were malaria, viral protein interaction with cytokine and

cytokine receptor and hematopoietic cell lineage while in GO, these were plasma membrane, cell periphery and receptor complex. These pathways were consistent in demonstrating that genes involved in cytokine signaling were upregulated at baseline in comparison to C4 normal colonies; predominantly within the Toll-like receptor (TLR) signaling pathway, IL-10, IL-6, TNF, TGF-B and IL-1 families (Supplemental Figures 6-8) (red colour denotes positive N vs B comparison, green denotes negative). Overall, these results show that colonies with normal morphology at C4 have differential gene expression patterns compared to baseline and persistently abnormal colonies post-treatment.

Discussion

In this study of MDS patients, we demonstrate differences in the immunological and genetic biomarker profiles at baseline and post-treatment with Lenalidomide or Azacitidine +/- IMiDs (Thalidomide/ Lenalidomide) which also correlate and are predictive of clinical outcomes. We observed that T-cell and myeloid cell populations differed at diagnosis amongst the different IPSS risk groups with higher numbers of intermediate and non-classical monocyte subsets and MDSCs in the higher risk MDS cohort while naïve CD4+ T-cells were reduced in comparison to lower risk IPSS groups. These monocyte populations are known to produce more of the inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) compared to the classical monocytes, through the TLR7-TLR8-MYD88-MEK pathway (21, 22). Consistent with these findings, Pollyea *et al* (23) have demonstrated that LPS-induced IL-6 and TNF-α protein production are positively correlated with an increasing peripheral blood monocyte count. MDSCs are known to play a central role in the pathogenesis of MDS and contribute to cytopenias through the production of immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β) via the S100A9/CD33/TLR4 axis (24). These findings are in keeping with disordered innate immune and inflammatory signaling in MDS being key drivers in its underlying pathogenesis (25, 26).

The differences observed in immune cell subsets between the different treatment groups at baseline prior to initiation of treatment is postulated to relate to differences in IPSS risk grouping and therefore, reflecting differences in underlying disease biology and aggressiveness. Previous studies have demonstrated that lower-risk MDS have higher

numbers of cytotoxic (CD8+) and helper (Th17) T-cells and NK cells with fewer T-regulatory lymphocytes. On the contrary, in higher-risk MDS lower numbers of cytotoxic, helper T-cells and NK cells are found with increased numbers of T-regs (27, 28). This increase in T-reg effector memory cells is associated with anemia, higher blast counts and impacts on survival outcomes only in higher-risk patients as assessed by the MDAS scoring system (29). Similar to these findings, we observed that CD161+ naïve-like CD4+ T-cells are reduced in the LEN-SCF cohort (84% lower-risk IPSS) whilst the effector CD4+ T-cells were increased in the AZA+/-LEN vs AZA+THAL cohorts (41.7% vs 52.5% higher-risk IPSS). The median OS was however longer in the AZA+/-LEN cohorts due to the higher predominance of lower-risk patients (58.3%) vs 39.1% in the AZA+THAL cohort. The important role of CD4+/FOXP3- conventional T-cells (Tcons) is further highlighted in a recent publication in which AZA-induced downregulation of IL-6/STAT3 signaling in Tcons correlated with improved responses and survival while in non-responders, IL-6/STAT3 signaling remained upregulated and disorganised in both Tcons and Treg cells (30).

Consistent with dysregulation in immune signaling, we also observed perturbations in inflammatory signaling at diagnosis. Genes involved in cytokine signaling, namely TLR2/4, IL-10, IL-6, TNF, TGF-B and IL-1 families were upregulated at baseline in comparison normal CFUs following 4 cycles of treatment. In MDS, TLR signaling in combination with IL-1 receptors ultimately lead to NF-κβ and MAP kinase activation through MYD88/ TLR3/ interleukin receptor-associated kinases (IRAKs) and TNF receptor-associated factors (TRAFs). These complex interactions subsequently result in apoptosis, pro-survival, impaired differentiation and self-renewal of hematopoietic stem cells and progenitors (HSPCs), contributing to cytopenias, a hallmark of MDS (25, 26). Recent evidence shows that the MDS HSPCs are conferred a growth advantage in comparison to normal HSPCs in response to inflammation, through non-canonical TLR-TRAF6 mediated NF-κβ signaling (31). IL-10 expression is known to inversely correlate with CD8+ T-cells numbers with higher expression in high-risk MDS (28). In keeping with published data, we observed downregulation of *MAPK12, IL10, MYD88, NFKB1* and *TGFBR2* in normal CFUs of patients deriving clinical benefit at 12 months and "good responders" (at least a PR as best response).

Higher pro-inflammatory NKT cell population was observed in patients who did not obtain clinical benefit from treatment at 12 months and similarly higher pro-inflammatory monocyte populations were seen in non-responders. The CD4+ pro-inflammatory NKT cells are known to make both Th1 and Th2 cytokines including IFN-γ, TNF, IL-1, IL-10 and IL-13 (32) contributing to disordered hematopoiesis. S100A8 expression is increased in pro-inflammatory monocytes inducing differentiation blocks of specific lineages depending on spatial location within the marrow; a predominant erythroid differentiation block results when present in erythroblastic islands (33).

There were several limitations encountered during our study. Firstly, the sample population in this study included patients enrolled across three different clinical trials and as such, this represented a heterogeneous group with regards to IPSS scores and WHO MDS subtypes. However, all patients were *de novo* MDS presentations without prior treatment and we utilized this heterogeneity to further understand the differences in immunological and genetic biomarkers between the different IPSS risk groups. There were similar trends in cluster frequency between lower and higher risk IPSS groups across the different treatment groups although the expression levels were not identical. This could have related to uneven distribution and small cohort numbers in specific IPSS risk groups which differed between the 3 clinical trials. Secondly, specific datasets including cytogenetic abnormalities, full white cell count differential and molecular mutational analysis was not provided or unavailable. As such correlations of gene expression and immune profiling could not be performed against these variables. Thirdly, our study focused on analyzing MDS patient samples only although it would have been useful to compare the biomarker analyses against healthy normal controls.

Previous work by Epling-Burnette *et al* (13) has demonstrated clonal T-cell expansion in MDS patients (50% vs 5% of healthy donors) associated with WHO category refractory anemia (RA), cytogenetic abnormalities of monosomy 20 and monosomy 7 and were phenotypically CD8+ cytotoxic T-cells with increased CD57, NKG2D and CD244 expression and reduced CD28 and CD62L expression. Notably, there was no significant association with peripheral blood counts including lymphocyte count, gender nor age. There was a trend towards increasing clonal T-cell expansion with IPSS risk categories but this was not

statistically significant. Another larger study on Trisomy 8 MDS (n=34) demonstrated significant CD8+ T-cell expansion with good response to immunosuppressive therapy with ATG and normalization of T-cell repertoire (34). This finding is further supported by gene expression profiling of CD34+ hematopoietic progenitor cells from MDS patients with Trisomy 8 which shows upregulated genes involved in immune and inflammatory responses and downregulation of anti-apoptosis genes while CD34+ cells in monosomy 7 demonstrated differential upregulation in cell cycle and proliferation genes and downregulation of genes involved in cell growth and maintenance (35).

In addition, with regards to molecular mutations, a recent publication by Wang *et al* looked at MDS-Immune-Risk (MIR) scores derived from 3 signature scores incorporating CD103+, immature dendritic cells and NK-cell with $CD56^{bright}$ and found correlations of high MIR scores with higher BM blast percentages, higher IPSS-R risk groups, presence of ASXL1, RUNX1 and TP53 mutations and inferior leukaemia-free and overall survival (36). The impact of high MIR scores on survival still persisted after treatment with hypomethylating agents or standard chemotherapy but was abrogated by allogeneic hematopoietic stem cell transplantation.

In conclusion, our study has confirmed the central roles of dysregulation in innate immunity and inflammatory signaling in the pathogenesis of MDS and that downregulation of these pathways correlate with improved clinical outcomes of treatment response and maintenance of clinical benefit after 12 months of treatment. These genetic and immunological biomarkers can help define early markers of resistance to treatment after 4 cycles of Azaciditine +/- IMiDs and identify patients in whom early escalation of treatment with novel therapies in MDS are required to improve their prognosis. The immune dysfunction in monocytic, MDSC, CD4+ T-cells and NKT cells warrant further investigation in therapeutic agents that target key signaling pathways through inhibition of TLR, IL-1β, NLRP3 inflammasome and MDSCs in both HMA-failure and HMA-naïve patients and a combination of lower and higher-risk MDS subgroups (26). Future studies should focus on a biomarker-driven approach to guide treatment decisions and to inform us of the benefits of concurrent use of these novel agents with current approved treatments for MDS including HMAs and the optimal timing and sequence of use of these therapeutic agents.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Fig 1: (A) Brick plot showing myeloid cluster phenotype for 4447 (intermediate-proinflammatory monocytes). Each brick represents the labelled marker. The size of each brick is indicative of the level of expression of that marker on that cluster. The absence of a brick indicates no expression of that marker in that cluster. The location of the brick is based on the rate of co-expression with the other markers. Overlapping bricks may shift slightly from their locations, otherwise all bricks are in the same location. The low range in CCR2, CD11b and CD16 means that a small brick for these markers is actually indicative of negative expression rather than low expression. **(B)-(E)** Dot graphs showing significant changes in myeloid cluster phenotypes for 4447, 4450 (intermediate CD15+CD62L+ monocytes), 4451 (non-classical monocytes) and 4398 (MDSC) respectively, between the different treatment groups as indicated. *p 0.01-0.05, **p=<0.01, ***p< 0.001

Fig 2: (A-E) Dot graphs showing significant changes in T-cell lymphoid cluster phenotypes for 2238 (peripheral memory-like CD4+ T-cells), 2276 (CD161+ naïve-like CD4 T-cells), 2267 (effector CD4+ T-cells), 2277 (natural killer T-cells) and 2281 (pro-inflammatory NKT cells) respectively, between the different treatment groups as indicated. *p 0.01-0.05, **p=<0.01, ***p< 0.001

Fig 3: Dot graphs showing **(A)** lower expression of the pro-inflammatory NKT-cell (cluster 2281) at baseline for patients deriving clinical benefit at 12 months following AZA+/-IMiDs treatment **(B)** increase in intermediate-proinflammatory monocytes (cluster 4447) in non-responders compared to responders and **(C)** also in those who only achieved stable/ progressive disease as best response vs those achieving at least a hematological improvement.

Fig 4: (A) Box and whisker plots showing the top 4 downregulated genes in the C4 normal (N) group following 4 cycles of AZA+/-IMiDs in comparison to baseline (B). The abnormal colonies after 4 cycles of treatment are marked as (A). Horizontal bar within boxplots

indicates median value for the indicated groups. **(B)** *MYC* is upregulated at baseline (B) in patients with inferior overall survival. **(C)** In normal colonies (N) after 4 cycles of treatment with AZA+/-IMiDs, *RFC3* is upregulated in patients deriving clinical benefit at 12 months. **(D)** In abnormal colonies (A) after 4 cycles of treatment with Azaciditine +/- IMiDs, *FN1* is upregulated in patients failing to achieve at least a PR.

TABLE LEGENDS

Table 1: Sample numbers analyzed for each correlative analysis and patient demographics

Table 2: Cluster numbers and their corresponding cell surface markers and sub-populations

Dysregulation of immune cell and cytokine signaling correlates with clinical outcomes in myelodysplastic syndrome (MDS)

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TABLES

TABLE 1

Sample numbers analyzed	LEN-SCF (n)*	AZA+THAL (MDS3) (n)**	AZA (MDS4) (n)**	AZA+LEN (MDS4) (n)**	TOTAL (n)
Immunophenotyping (T-cell and myeloid)	25	23	44	40	132
BM Paired Colony Assays (Gene Expression Analysis)	0	4	10	8	22
Total	25	27	54	48	154

Immunophenotyping (T-cell and myeloid) cohort	**LEN-SCF (n=25)**	**AZA+THAL (MDS3) (n=23)**	**AZA (MDS4) (n=44)**	**AZA+LEN (MDS4) (n=40)**	**TOTAL (n=132)**
Median age at diagnosis (range in years)	65.5 (43-88)	68 (42-82)	68.8 (45.5-85.9)	72.3 (54-87.2)	68.4 (42-88)
Sex					
• Male	17 (68)	15 (65.2)	28 (63.6)	28 (70)	88 (66.7)
• Female	7 (28)	8 (34.8)	16 (36.4)	12 (30)	43 (32.6)
• N/A	1 (4)	0 (0)	0 (0)	0 (0)	1 (0.8)
IPSS (n/%)					
• Low	12 (48)	1 (4.3)	6 (13.6)	1 (2.5)	20 (15.2)
• Int-1	9 (36)	8 (34.8)	20 (45.5)	22 (55.0)	59 (44.7)
• Int-2	1 (4)	10 (43.5)	12 (27.3)	10 (25.0)	33 (25.0)
• High	0 (0)	2 (8.7)	6 (13.6)	7 (17.5)	15 (11.4)
• N/A	3 (12)	2 (8.7)	0 (0)	0 (0)	5 (3.8)
IPSS (n/%)					
• Lower risk	21 (84)	9 (39.1)	26 (59.1)	23 (57.5)	79 (59.8)
• Higher risk	1 (4)	12 (52.2)	18 (40.9)	17 (42.5)	48 (36.4)
• N/A	3 (12)	2 (8.7)	0 (0)	0 (0)	5 (3.8)
IPSS-R (n/%)					
• Very Low	2 (8)	1 (4.3)	0 (0)	0 (0)	3 (2.3)
• Low	13 (52)	4 (17.4)	13 (29.5)	7 (17.5)	37 (28.0)
• Intermediate	7 (28)	4 (17.4)	13 (29.5)	16 (40.0)	40 (30.3)
• High	0 (0)	5 (21.7)	7 (15.9)	6 (15.0)	18 (13.6)
• Very high	0 (0)	3 (13.0)	9 (20.5)	7 (17.5)	19 (14.4)
• N/A	3 (12)	6 (26.1)	2 (4.5)	4 (10.0)	15 (11.4)
WHO MDS Subtype (n/%)					
	1 (4)	0 (0)	1 (2.3)	0 (0)	2 (1.5)
• MDS-SLD	6 (24)	1 (4.3)	17 (38.6)	9 (22.5)	33 (25.0)
• MDS-MLD	3 (12)	1 (4.3)	3 (6.8)	2 (5.0)	9 (6.8)

• MDS-RS	5 (20)	0 (0)	0 (0)	0 (0)	5 (3.8)
• MDS with isolated del(5q)	4 (16)	8 (34.8)	4 (9.1)	8 (20.0)	24 (18.2)
• MDS-EB1	0 (0)	4 (17.4)	11 (25.0)	9 (22.5)	24 (18.2)
• MDS-EB2	1 (4)	0 (0)	0 (0)	1 (2.5)	2 (1.5)
• MDS-U	1 (4)	8 (34.8)	4 (9.1)	7 (17.5)	20 (15.2)
• CMML	1 (4)	0 (0)	0 (0)	0 (0)	1 (0.8)
• MDS/MPN	0 (0)	1 (4.3)	4 (9.1)	4 (10.0)	9 (6.8)
• Transformed MDS to AML	3 (12)	0 (0)	0 (0)	0 (0)	3 (2.3)
• N/A					

*Len-SCF cohort only analyzed for T-cell and myeloid immunophenotyping studies

**MDS3 and MDS4 cohort numbers differed for BM colony assay studies due to availability of paired samples at baseline and after 4 cycles of treatment

TABLE 2

	Cluster number	**Cell surface markers***	**Sub-population**
Myeloid Cell Populations	4447	HLA-DR, CX3CR1, CD33, CD62L, CD14, CD11b	Intermediate-proinflammatory monocytes
	4450	HLA-DR, CD11b, CD16, CD33, CD14, CD62L, CD15, CX3CR1	Intermediate CD15+CD62L+ monocytes
	4451	HLA-DR, CX3CR1, CD33, CD11b, CD16, CD14, CD62L	Non-classical monocyte
	4449	CCR2, HLA-DR, CX3CR1, CD11b, CD16, CD33,	Intermediate CD15-CD62L+ monocytes

		CD14, CD62L	
	4398	HLA-DR, CD33, CX3CR1, CD11b	MDSC
Lymphoid Cell Populations	2238	CD4, CD45RO, CD27, CCR7, CD8a, CD8b, CD45RA	Peripheral memory-like CD4+ T-cell
	2276	CD4, CD45RA, CD27, CD45RO, CCR7, CD8a, CD8b, CD161	CD161+ naïve-like CD4+ T-cell
	2267	CD4, CD45RO, CD27, CD45RA, CD8b, CCR7	Effector CD4+ T-cell
	2277	CD4, CD45RO, CD27, CCR7, CD45RA, NKT.Tet, CD8a, CD8b	Natural killer T-cells
	2281	CD8a, CD27, CD8b, CD45RO, CD161, NKT.Tet, CD4, CD45RA, CCR7	Pro-inflammatory NKT cell
	2270	CD4, CD45RO, CD45RA, CD27, CD8a, CD8b, CCR7	Naïve CD4+ T-cell
	2226	CD4, CD27, CCR7, CD45RO, CD45RA, CD8a, CD8b, NKT.Tet	CD4+CD8+ double-positive NKT cell

*cell surface markers listed in order of level of expression (based on brick size)

Dysregulation of immune cell and cytokine signaling correlates with clinical outcomes in myelodysplastic syndrome (MDS)

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FIGURES

FIGURE 1

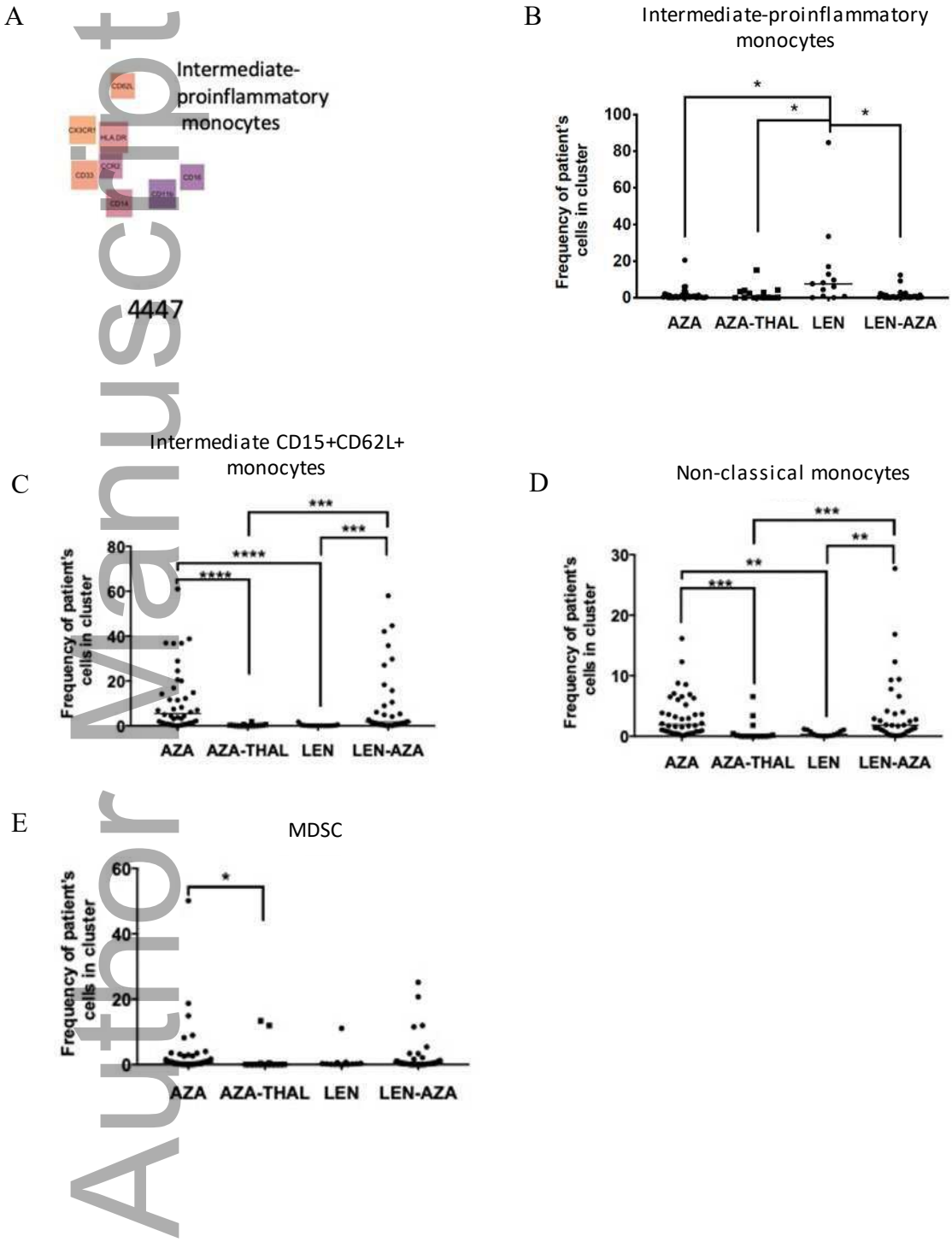

FIGURE 2

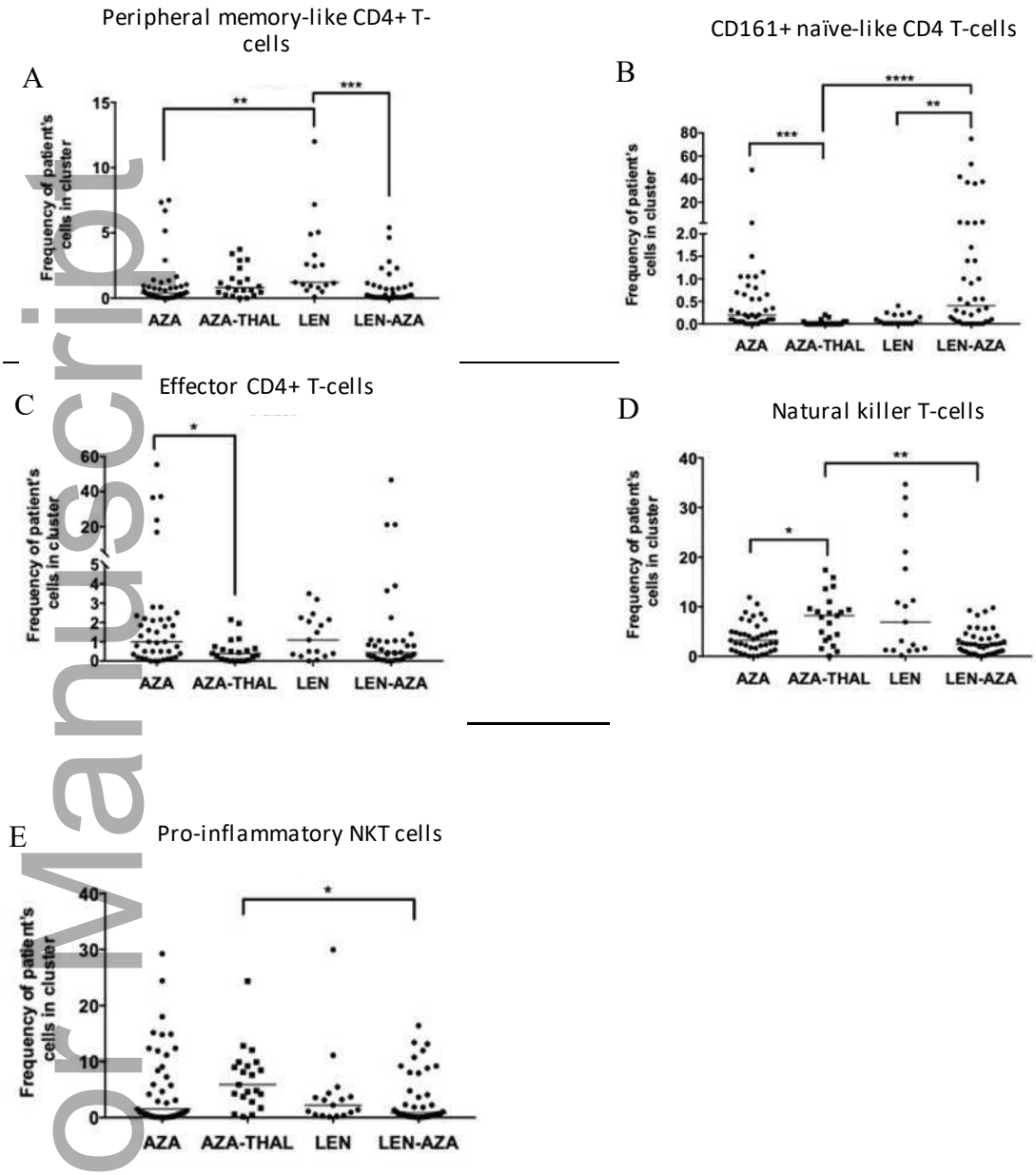

FIGURE 3

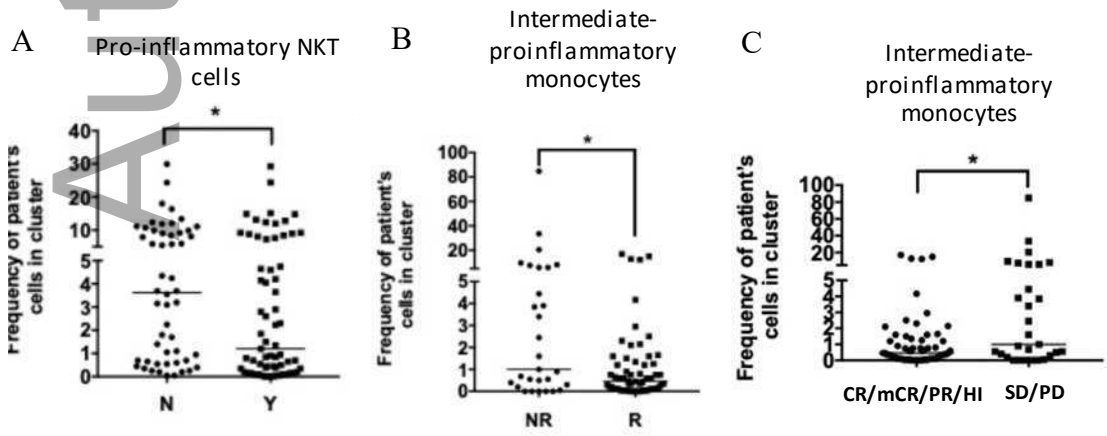

FIGURE 4

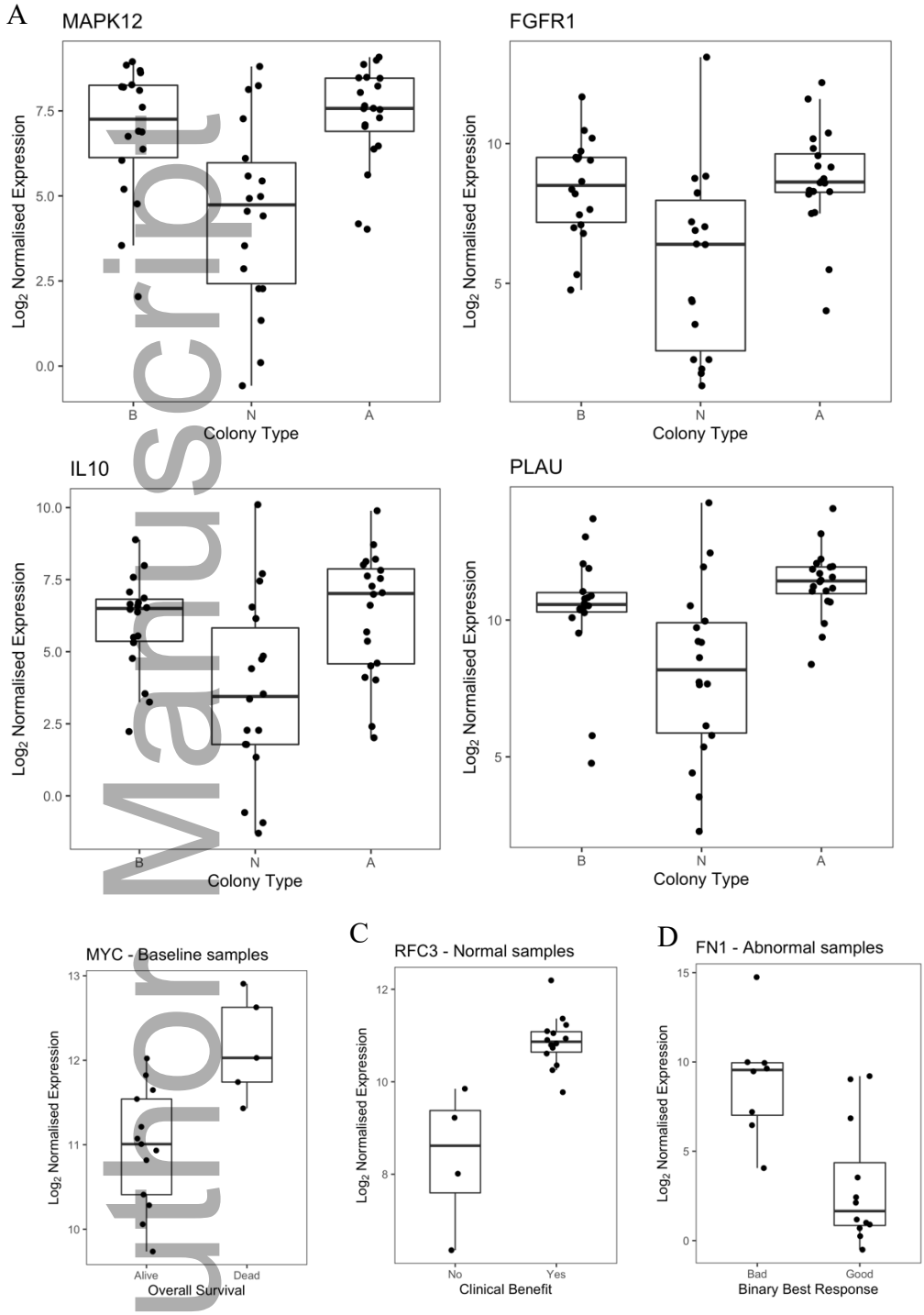

FIGURE LEGENDS

Fig 1: (A) Brick plot showing myeloid cluster phenotype for 4447 (intermediate-proinflammatory monocytes). Each brick represents the labelled marker. The size of each brick is indicative of the level of expression of that marker on that cluster. The absence of a brick indicates no expression of that marker in that cluster. The location of the brick is based on the rate of co-expression with the other markers. Overlapping bricks may shift slightly from their locations, otherwise all bricks are in the same location. The low range in CCR2, CD11b and CD16 means that a small brick for these markers is actually indicative of negative expression rather than low expression. **(B)-(E)** Dot graphs showing significant changes in myeloid cluster phenotypes for 4447, 4450 (intermediate CD15+CD62L+ monocytes), 4451 (non-classical monocytes) and 4398 (MDSC) respectively, between the different treatment groups as indicated. *p 0.01-0.05, **p=<0.01, ***p< 0.001

Fig 2: (A-E) Dot graphs showing significant changes in T-cell lymphoid cluster phenotypes for 2238 (peripheral memory-like CD4+ T-cells), 2276 (CD161+ naïve-like CD4 T-cells), 2267 (effector CD4+ T-cells), 2277 (natural killer T-cells) and 2281 (pro-inflammatory NKT cells) respectively, between the different treatment groups as indicated. *p 0.01-0.05, **p=<0.01, ***p< 0.001

Fig 3: Dot graphs showing **(A)** lower expression of the pro-inflammatory NKT-cell (cluster 2281) at baseline for patients deriving clinical benefit at 12 months following AZA+/-IMiDs treatment **(B)** increase in intermediate-proinflammatory monocytes (cluster 4447) in non-responders compared to responders and **(C)** also in those who only achieved stable/ progressive disease as best response vs those achieving at least a hematological improvement.

Fig 4: (A) Box and whisker plots showing the top 4 downregulated genes in the C4 normal (N) group following 4 cycles of AZA+/-IMiDs in comparison to baseline (B). The abnormal colonies after 4 cycles of treatment are marked as (A). Horizontal bar within boxplots indicates median value for the indicated groups. **(B)** *MYC* is upregulated at baseline (B) in

patients with inferior overall survival. **(C)** In normal colonies (N) after 4 cycles of treatment with AZA+/-IMiDs, *RFC3* is upregulated in patients deriving clinical benefit at 12 months. **(D)** In abnormal colonies (A) after 4 cycles of treatment with Azaciditine +/- IMiDs, *FN1* is upregulated in patients failing to achieve at least a PR.