MUC1 Inhibition Leads to Decrease in PD-L1 Levels via Up-Regulation of miRNAs

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Running title: MUC1 Regulates PD-L1 Expression in AML

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Abstract

The PD-L1/PD-1 pathway is a critical component of the immunosuppressive tumor microenvironment in AML, but little is known about its regulation. We investigated the role of the MUC1 oncoprotein in modulating PD-L1 expression in AML. Silencing of MUC1 in AML cell lines suppressed PD-L1 expression without a decrease in *PD-L1* mRNA levels, suggesting a post-transcriptional mechanism of regulation. We identified the microRNAs miR-200c and miR-34a as key regulators of PD-L1 expression in AML. Silencing of MUC1 in AML cells led to a marked increase in miR-200c and miR-34a levels, without changes in precursor microRNA, suggesting that MUC1 might regulate microRNA processing. MUC1 signaling decreased the expression of the microRNA processing protein DICER, via the suppression of c-Jun activity. NanoString array of MUC1 silenced AML cells demonstrated an increase in the majority of probed microRNAs. In an immunocompetent murine AML model, targeting of MUC1 led to a significant increase in leukemia specific T cells. In concert, targeting MUC1 signaling in human AML cells resulted in enhanced sensitivity to T cell mediated lysis. These findings suggest MUC1 is a critical regulator of PD-L1 expression via its effects on microRNA levels and represents a potential therapeutic target to enhance anti-tumor immunity.

Introduction

Acute Myeloid Leukemia (AML) is a lethal hematologic malignancy in which the tumor microenvironment is characterized by an immunosuppressive milieu that fosters disease progression[1,2]. The PD-L1/PD-1 pathway confers a critical negative co-stimulatory signal that induces T-cell exhaustion and supports immune evasion by malignant cells[3–6]. In contrast, antibody blockade of PD-L1/PD-1 signaling results in the reversal of tumor-mediated immune suppression and durable responses in subsets of patients with solid tumors[7–9] and hematologic malignancies[10]. While PD-L1 expression in AML is dynamic, little is known about the mechanism(s) responsible for regulating PD-L1 expression in AML.

MUC1 is a heterodimeric oncoprotein aberrantly expressed in solid tumors and hematologic malignancies including AML, that supports critical aspects of the malignant phenotype including cell proliferation, self-renewal and resistance to apoptosis[11–16]. MUC1 interacts with the WNT/-catenin pathway and promotes the activation of WNT target genes[17,18], NFK-B[19–21] and STAT1/3[22,23], pathways critical for the proliferation and survival of tumor cells. In addition, MUC1 regulates pathways responsible for autonomous self-renewal[24] and is uniquely expressed on leukemia stem cells as compared to normal hematopoietic stem cells[25]. Inhibition of MUC1 using a cell-penetrating peptide (GO-203) that blocks MUC1-C homodimerization necessary for downstream signaling[26,27], abrogates leukemia engraftment and eradicates established disease in a xenogeneic leukemia model[25]. Given the critical function of MUC1, in supporting the

74 malignant phenotype of AML blasts and stem cells, we sought to explore the role
75 of MUC1 in mediating the immunosuppressive milieu of the tumor
76 microenvironment. Here, we demonstrate that silencing of MUC1 markedly
77 suppresses PD-L1 expression in AML cells. However, MUC1 suppression is
78 associated with a paradoxical increase in *PD-L1* mRNA, suggesting that MUC1
79 regulation of PD-L1 expression in AML occurs at the post-transcriptional level.

80 Noncoding RNAs epigenetically regulate critical aspects of the oncogenic
81 phenotype through the disruption of protein translation via selective binding and
82 degradation of target mRNAs[28]. The microRNAs miR-200c and miR-34a
83 demonstrate homology with the 3'UTR section of *PD-L1* mRNA[4,29]. MiR-200c
84 was recently shown to downregulate the expression of PD-L1 protein in a lung
85 cancer model[29], and miR-34a was shown to target PD-L1 in Acute Myeloid
86 Leukemia cell lines[4]. In the present study, we demonstrate that MUC1 negatively
87 regulates expression of miR-200c and miR-34a, which in turn controls PD-L1
88 expression in AML cells. Consistent with these findings, upregulation of miR-
89 200c or miR-34a via lentiviral transduction results in a corresponding decrease in
90 PD-L1 expression. Of note, silencing of MUC1 results in increased levels of
91 mature miR-34a and miR-200c while precursor microRNAs are unaffected.
92 Consistent with this observation, MUC1 inhibition resulted in increased
93 expression of DICER protein, which mediates the final splicing of precursor
94 miRNAs to their active form. Indeed, microRNA array of MUC1 silenced AML
95 cells demonstrated a profound global upregulation of microRNAs, consistent with
96 an increase in DICER expression. These findings strongly suggest MUC1 as a

key regulator of microRNA expression and demonstrate a critical mechanism by which MUC1 signaling exploits noncoding RNAs to elicit an immunosuppressive milieu in the bone marrow microenvironment.

Material and Methods:

Cell culture. The AML cells lines THP-1 and MOLM-14 and the murine cell line TIB-49 were purchased from ATCC, cultured at 37ºC in a humidified 5% CO_2 incubator and maintained in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with heat-inactivated 10% human serum albumin (Sigma, St. Louis, MO) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Cellgro, Manassas, VA). Cell lines were stably transduced with a lentiviral vector expressing a scrambled control shRNA (CshRNA, Sigma) or MUC1 shRNA (Sigma, St. Louis, MO) in the presence of 4-8 g/ml polybrene (Sigma, St. Louis, MO). Transduced cells were then selected using 2ug/ml puromycin. For the CRISPR edited cell line, sgRNAs targeting the first exon of the MUC1 gene were cloned into a lenti-plasmid (Genome Engineering Production Group, Harvard Medical School). MOLM-14 cells were transduced with viral vector containing the lenti-CRISPR plasmid and successfully transduced clones were selected for by limiting dilution and maintained in 2µg/ml Puromycin (Sigma, St. Louis, MO). Alternatively, cells were stably transduced with lentiviral vectors expressing pHR-GFP, miR-200c or miR-34a with a GFP selection marker. Transduced cells were selected by flow cytometric sorting for GFP positive cells. In some experiments, AML cells were treated once daily with 2µM MUC1-C inhibitor peptide (GO-203) or a control peptide (CP-2) (AnaSpec) for three days. In specific experiments, AML cells were treated for 30 mins with 100 or 400µM of c-Jun peptide inhibitor (Tocris) or control (PBS).

In vivo model

C57BL/6J mice were challenged with $100x10^3$ GFP transduced MUC1-C silenced TIB-49 syngeneic AML cells via retro-orbital injection. TIB-49 cells underwent lentiviral transduction with shRNA hairpin against MUC1-C or a control shRNA. Bone marrow and spleen cells were isolated on day 14 and assessed for PD-L1 expression by multichannel flow cytometric analysis staining for GFP+ AML cells and PD-L1 expression using mouse APC-conjugated anti PD-L1 antibody. Alternatively, MUC1 signaling was inhibited via daily subcutaneous injection of the MUC1 inhibitor, GO-203, (14 mg/kg) initiated 24 hours after tumor challenge.

AML specific T cells were quantified in bone marrow and spleen by flow cytometric analysis for intracellular IFN-γ expression following exposure to TIB-49 AML lysate. C57BL/6 mice were inoculated with $100x10^3$ GFP transduced syngeneic TIB-49 AML cells using retro-orbital injections. On day 14 following the inoculation bone marrow cells were harvested and stimulated ex-vivo with TIB-49 tumor lysate. Following 6 days of stimulation, CD4+ and CD8+ T cells underwent flow cytometric analysis for intracellular IFN-γ expression.

CTL assay

Lysis of AML cells by allogeneic T cells following MUC1 downregulation was assessed in a standard CTL flourochrome assay (OncoImmunin, Inc., Gaithersburg MD). Target cells were incubated in APC labeled PBS (1μL of

reconstituted TFL4 in PBS at 1:3000 ratio) at 1×10^6 cells/ml for 30 minutes at 37°C. Labeled cells were washed twice in PBS. Using MUC1 silenced or control MOLM-14 cell lines, healthy donor PBMCs were co-incubated with labeled target cells in the presence of a fluorogenic granzyme B substrate for 1-2 hours at 37°C. Cells were washed and analyzed by flow cytometry. Dead target cells are identified by cells that dually stain for granzyme B and APC label (right upper quadrant). As a negative control, Granzyme B positive tumor cells not cocultured with T cells, were quantified.

The impact of MUC1 inhibition on CTL mediated killing of patient derived AML cells, by T cells stimulated with a DC/AML fusion vaccine was examined. DC/AML fusions were generated as previously described[30]. Briefly, autologous Dendritic cells (DCs) were generated by culture of adherent peripheral mononuclear cells obtained from AML patients following remission, in the presence of GM-CSF, IL-4 and TNF-α. DCs were fused with autologous AML blasts, obtained at diagnosis, by coculture at a 1:1 ratio in the presence of polyethylene glycol (PEG). Autologous T cells were then stimulated with the DC/AML fusion vaccine for 6 days. Subsequently, vaccine stimulated T cells were cocultured with control autologous AML blasts or AML blasts exposed to daily treatment with 2.5 μM GO-203 for 3 days. The lysis of AML blasts, with and without treatment with GO-203, by vaccine stimulated T cells was detected using a standard CTL assay as described above.

FACS analysis. Cells were analyzed for MUC1 and PD-L1 expression by

multichannel flow cytometric analysis. Cells were incubated with monoclonal antibody (MAb) DF3 (anti-MUC1-N), anti-PD-L1 (Cell Signaling Technologies) or a control mouse IgG_1 for 30 min, followed by secondary labeling of the cells with phycoerythrin (PE)-conjugated goat anti-mouse IgG for an additional 30 min. The cells were then fixed in 2% paraformaldehyde. Stained cells were analyzed by flow cytometry using FACS Aria (BD Biosciences, San Jose, CA). Analysis of the obtained data was performed using Kaluza software (Beckman Coulter, Brea, CA).

The expression of IFN-γ was analyzed by intracellular flow cytometry. T cells were pulsed with GolgiStop (1 μg/mL; Pharmingen, San Diego, CA, USA) for 4–6 hrs at 37 °C prior to analysis. Cells were next harvested and labeled with CD4-PB and CD8-FITC. Cells were then permeabilized by incubation in Cyto-fix/Cytoperm plus™(Pharmingen, San Diego, CA, USA) containing formaldehyde and saponin for 30 min at 4 °C, washed twice in Perm/Wash™ solution (Pharmingen, San Diego, CA, USA), and incubated with PE-conjugated IFN-γ (Invitrogen, Camarillo, CA, USA), or a matched isotype control antibody for 30 min. Cells were washed in 1 × Perm/Wash™ solution and fixed in 2 % paraformaldehyde (Sigma, St. Louis, MO, USA) prior to analysis.

Immunoblotting

Whole cell lysates were prepared in RIPA lysis buffer and analyzed by immunoblotting with anti-MUC1-C (Thermo Scientific, Waltham, MA), anti-DICER, anti-Argo-2, anti-p-c-jun, anti-c-jun (Cell Signaling, Danvers MA) anti-PD-

L1 (Abcam, Cambridge MA) and anti-GAPDH (Sigma) as described[31]. Immune complexes were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Quantitative RT-PCR. Total RNA was isolated from cells using the RNeasy total RNA isolation kit (Qiagen). cDNA synthesis was performed with 1 µg of total RNA using the MiScript RT-PCR system (Qiagen). The SYBR green qPCR assay kit (Roche) was used with 2µl of diluted cDNA for each sample. The forward and reverse primers for qPCR of MUC1, PD-L1 DICER, c-Jun, miR-200c, miR-34a, pre-mir-200c, pre-mir-34a and GAPDH are listed in Supplementary Table S1. Statistical significant was determined by the student's *t* test.

mRNA Array

RNA was isolated from paired GO-203 treated or control peptide treated AML samples from 3 subjects (2.5µM GO-203 daily for 3 days). Affymetrix mRNA expression array, consisting of 47,321 probe features corresponding to 34,692 transcripts, was performed as described[32] and all arrays were included in the differential expression analysis. Standard normalization methods were applied, followed by paired T-test analysisfor Bonferroni-corrected significance, using the *Limma* Bioconductor package. Correction for multiple comparisons was performed using the False Discovery Rate of Benjamini and Hochberg[33].

miRNA Array

RNA was isolated from AML cells as described and run in triplicate on a NanoString (Seattle, WA) nCounter instrument using the human miRNA Expression Assay Kit v3, according to the manufacturer's instructions. Data obtained were then normalized to positive miRNA-ligation reaction controls and background noise was subtracted. . Correction for multiple comparisons was performed using the False Discovery Rate of Benjamini and Hochberg[33].

Results

MUC1 inhibition leads to decrease in PD-L1 expression

We examined the role of MUC1 in modulating expression of PD-L1, a critical mediator of T cell exhaustion and anergy. Downregulation of MUC1 expression in MOLM-14 and THP-1 cells, via lentiviral transduction with MUC1 specific shRNA, resulted in the marked suppression of PD-L1 expression as determined by flow cytometry (Figure 1A) and immunoblotting, (Figure 1B). To assess the effect of MUC1 silencing on PD-L1 expression *in-vivo*, C57BL/6 mice were inoculated with $100x10^3$ GFP transduced syngeneic TIB-49 AML control cells or TIB-49 cells that were silenced for MUC1 expression using lentiviral transduction with MUC1 specific shRNA. Leukemia engraftment, as manifested by the infiltration of GFP+ cells was noted 14 days after inoculation, in the bone marrow and spleen of animals challenged with AML cells (data not shown). PD-L1 expression was observed in the engrafted GFP+ wild type leukemic cells isolated from bone marrow or spleen. In contrast, leukemic engraftment was more modest following challenge with MUC1 silenced TIB-49 cells (data not shown) and the AML cells recovered from bone marrow and spleen showed decreased expression of PD-L1, suggesting a durable effect of silencing MUC1 on PD-L1 expression (Figure 1C). Interestingly, increased levels of *PD-L1* mRNA was observed in MUC1 silenced AML cells as determined by qPCR analysis (Figure 1D), suggesting that MUC1 regulation of PD-L1 expression was accomplished via a post-transcriptional mechanism.

MiR-34a and miR-200c regulate PD-L1 expression in AML cells

We hypothesized that MUC1 regulation of PD-L1 may be mediated by noncoding RNAs, which are known to epigenetically regulate cellular function via the binding and degradation of mRNAs with homologous sequences, providing a critical brake for protein translation. Consistent with this hypothesis, the PD-L1 3'UTR contains multiple binding motifs for miR-200c[29] and one for miR-34a[4] (Figure 2A). Remarkably, MUC1 silencing in both MOLM-14 and THP-1 cells led to a significant increase in miR-34a (Figure 2B) and miR-200c (Figure 2D), as determined by qPCR analysis. Accordingly, ectopic miR-34a expression in MOLM-14 and THP-1 human AML cells via lentiviral transduction of miR-34a-mimic, resulted in the near abrogation of PD-L1 expression (Figure 2C). Similar results were seen with ectopic overexpression of miR-200c which led to significant downregulation of PD-L1 expression in MOLM-14 cells, as demonstrated by western blot (Figure 2E), and flow cytometric analysis (Figure 2F). In summary, MUC1 negatively regulates expression of miR-34a and miR-200c attenuating their interference with PD-L1 translation, resulting in increased PD-L1 expression by AML cells. In contrast, silencing of MUC1 results in a corresponding increase in miR-200c and miR-34a, resulting in decreased expression of PD-L1.

MUC1 inhibition leads to increase in DICER expression via c-Jun

We then sought to determine the mechanism by which MUC1 signaling negatively regulates expression of miR-200c and miR-34a, as these microRNAs represent a diverse species of noncoding RNAs that do not share a common promoter region.

We first examined the effect of MUC1 silencing on microRNA processing. MUC1 silenced MOLM-14 and THP-1 cells were analyzed for precursor miR-200c and miR-34a levels, to determine if MUC1 signaling effects miR-200c and miR-34a maturation to their functional conformation. The results demonstrated that silencing of MUC1 did not result in alteration of levels of the precursor miRNAs pre-mir-34a (Figure 3A) or pre-mir-200c (Figure 3B), suggesting the regulatory effect of MUC1 on these microRNAs occurs at the post transcriptional stage.

We subsequently investigated whether MUC1 signaling impacted the expression of DICER, an RNAase III that forms a critical component of the RNA-induced silencing complex (RISC), required for the generation of functionally competent microRNAs. We demonstrated the novel finding that the MUC1 oncoprotein regulates DICER expression impacting activation of noncoding RNAs in a class wide effect. MUC1 silencing resulted in increased expression of DICER protein in the MOLM-14 and THP-1 AML cell lines (Figure 3C). Similarly, expression of DICER was increased following exposure of AML cells to the MUC1 inhibitor, GO-203. Of note, there was no change in the expression of the Argonaut 2 (Ago 2) protein, the “catalytic engine” of the RISC (Figure 3D). Interestingly, MUC1

281 silencing of both MOLM-14 and THP-1 cells lines resulted in the dramatic
282 increase in *DICER* mRNA levels by qPCR analysis (Figure 3E), consistent with
283 regulation at the transcriptional level.

284

285 c-Jun, a member of the AP-1 transcription factor family, has a binding site in the
286 *DICER1* gene promoter[34] and has been shown to mediate DICER expression in
287 Breast Cancer[35] and T cell Leukemia[34]. Immunoblots of MUC1 silenced AML
288 cells demonstrated an increase in total and phosphorylated forms of c-Jun
289 (Figure 4A). Consistent with this finding, MUC1 silenced AML cell lines
290 demonstrated increased levels of *c-Jun* mRNA by qPCR analysis (Figure 4B).
291 Furthermore, c-Jun was one of only 10 genes that demonstrated significant
292 differential expression in a microarray analysis of gene expression of three paired
293 GO-203 or control peptide treated AML patient derived samples, with MUC1
294 inhibition resulting in a statistically significant increase in *c-Jun* mRNA levels
295 (FDR-adjusted p=0.003) (Figure 4C). Finally, MUC1 silenced MOLM-14 and
296 THP-1 cells treated for 30 minutes with 100uM and 400uM of a peptide inhibitor
297 of c-Jun activity, demonstrated a dose dependent decrease in DICER protein
298 expression, confirming the central role of c-Jun in mediating MUC1 regulation of
299 DICER. (Figure 4D).

300

MUC1 inhibition leads to increase in microRNAs in AML cells

The finding that MUC1 regulates expression of DICER suggests that MUC1 exerts a class effect on the production of functionally mature miRNAs. To investigate this hypothesis, we performed an array to identify other microRNAs that were similarly impacted by silencing of MUC1. MicroRNA array of MUC1 silenced MOLM-14 and THP-1 cells demonstrated a profound global upregulation of a substantial proportion of microRNAs (Figure 5), including miR-34a and miR-200c. Of the panel of 801 miRNAs arrayed, MUC1 silenced MOLM-14 cells showed a statistically significant increase in 340 of 801 (42.4% at FDR of 0.05). Concordantly, MUC1 silenced THP-1 cells showed an increase in 154 of 801 (19.2%) microRNAs.

MUC1 exerts an immunoregulatory effect in AML cells *in-vivo* and *in-vitro.*

Having demonstrated that MUC1 regulates PD-L1 expression in AML cells, we subsequently examined whether targeting MUC1 enhances the susceptibility of AML cells to immune mediated killing. In an immunocompetent murine model, C57BL/6 mice were inoculated with control or MUC1 silenced syngeneic TIB-49 AML cells by retro-orbital injection (Figure 6A). Engraftment of MUC1 silenced AML cells was associated with an expansion of AML specific T cells as manifested by a statistically significant increase in bone marrow derived CD4+ cells which expressed IFN-γ upon exposure to autologous tumor lysate (Figure

6B). Similarly, animals challenged with TIB-49 cells and treated with GO-203 daily for 14 days showed a two-fold expansion of AML specific T cells upon *ex-vivo* exposure to leukemia derived antigens (Figure 6C).

We subsequently examined the impact of targeting MUC1 on the immunogenicity of human AML cells. MUC1 was silenced in MOLM-14 and THP-1 cells using a lentiviral shRNA and compared to MOLM-14 and THP-1 cells infected with control shRNA. Silencing of MUC1 resulted in enhanced susceptibility to immune mediated killing by alloreactive lymphocytes as determined by the cleavage of the tumor derived Granzyme B substrate in a standard cytotoxic T lymphocyte (CTL) assay (Figure 6D). We have developed a leukemia specific vaccine in which patient derived AML cells are fused with autologous DCs such that a broad array of tumor antigens are presented in the context of DC mediated costimulation[36]. To investigate the capacity of patient derived T cells to target autologous tumor, PBMCs were stimulated with DC/AML fusion vaccine. Consistent with the immunoregulatory effect of MUC1, exposure of patient derived AML cells to the MUC1 inhibitor GO-203, lead to enhanced susceptibility to immune mediated killing, by DC/AML fusion vaccine stimulated autologous T cells (Figure 6E).

Discussion

The bone marrow microenvironment in patients with AML is characterized by an immunosuppressive milieu that promotes tumor tolerance, immune escape, and disease growth[1,2]. PD-L1 expression by tumor cells is a critical contributor to the immunosuppressive environment[6]. Ligation of PD-1 on T cells in the tumor bed induces an exhausted T cell phenotype resulting in the loss of activation, expansion and functional capacity of tumor reactive lymphocytes[37]. PD-L1 is strongly expressed by AML cells[3,5,6] and is also expressed by other immunosuppressive elements in the bone marrow microenvironment[38–40]. Defining the mechanisms by which PD-L1 expression is regulated is vital to better understand the evolution of immune dysregulation in AML and as a basis for the design of novel therapies to restore immune function.

The *MUC1* oncogene is aberrantly expressed on solid tumors[41] and in hematological malignancies[11,42] including AML[25,43], and supports critical aspects of malignant transformation including resistance to apoptosis[44], cell proliferation[26], tissue invasion, and metastatic potential[41]. However, its role in immune regulation and tumor-mediated tolerance has not been well defined.

In the present study, we report on the novel finding that the *MUC1* oncogene exerts a potent immunomodulatory effect through its regulation of PD-L1 expression on AML cells. Suppression of MUC1 expression via MUC1 specific shRNA or CRISPR mediated gene deletion, results in the near abrogation of PD-L1 expression. In an immunocompetent murine model, *ex-vivo* silencing of

MUC1 in TIB-49 AML cells prior to tumor challenge, results in durable absence of PD-L1 in the engrafted leukemia cells. Of note, prior studies have shown that PD-L1 expression on AML cells is dynamic, likely regulated by factors in the microenvironment[6]. The present findings suggest that the MUC1 oncogene plays a critical role in regulating the immunosuppressive nature of the tumor microenvironment, via its effect on PD-L1. Of note, we have demonstrated that MUC1 expression by tumor cells may be upregulated through interactions between tumor cells and accessory cells in the microenvironment.

Interestingly, silencing of MUC1 is paradoxically associated with an increase in *PD-L1* mRNA expression, suggesting that MUC1 regulation of PD-L1 is likely mediated by a post-transcriptional mechanism. Noncoding RNAs have been identified as critical mediators of cellular function and may act as effectors of epigenetic regulation in malignant cells[29,45,46]. MicroRNAs bind to the 3'UTR sequence of candidate mRNAs leading to prevention of protein translation[28]. It was previously shown that miR-200c is complementary to the 3'UTR sequence of *PD-L1* mRNA in a lung cancer model[29]. In the present investigation, we demonstrate that that MUC1 negatively regulates miR-34a and miR-200c in AML and that the overexpression of these miRNAs in AML cells results in the near abrogation of PD-L1 expression.

The role of MUC1 in modulating multiple microRNA species raised the possibility of a common processing pathway being targeted by this oncogene. In the present study, we demonstrated that silencing MUC1 is associated with an

increase in levels of DICER, an protein critical for mature microRNA formation[47]. The generation of functionally active microRNAs is dependent on carefully scripted sequence of events beginning with the transcription of genomic DNA containing microRNA sequences, that are further processed into precursor-microRNAs (pre-microRNAs) by a nuclear protein complex[47–49]. Pre-microRNAs are then transported to the cytoplasm, loaded onto a protein complex called the RNA Induced Silencing Complex (RISC) composed of Dicer, Argonaute-2 (Ago-2), the Tar RNA Binding Protein (TRBP), and cleaved to their mature form by DICER[49]. Mature microRNAs are then capable of binding to target mRNAs preventing their translation to protein[28]. In the present study, we demonstrate that suppression of MUC1 is associated with increased levels of DICER, consistent with an increase in production of the functionally mature miR-200c and miR-34a. It has been previously shown that the transcription factor, c-Jun, acts as a primary regulator of DICER expression[34]. Accordingly, in the present study, we demonstrate that silencing or inhibition of MUC1 results in increased levels of phosphorylated c-Jun consistent with an associated increase in DICER expression. Conversely, the introduction of a c-Jun inhibitor reversed the effect of MUC1 silencing on DICER expression in a dose dependent fashion.

This is the first report of an oncogene regulating DICER as a means of governing oncogenic potential. We demonstrate that MUC1 regulation of DICER is associated with a downstream effect on miR-34a and miR-200c and resultant PD-L1 expression. While genetic deletion of DICER is lethal in utero[47] investigators have examined the selective deletion in subsets of cells and have

409 demonstrated that loss of DICER may be associated with loss of function and
410 blunting of differentiation in immune cells[50]. Conflicting data regarding the role of
411 DICER in malignancy has been observed, with some models demonstrating loss
412 of DICER associated with disease progression[51,52]. Similarly, microRNAs have
413 been shown to mediate diverse effects in tumor models with upregulation of
414 certain microRNA families associated with malignant transformation[53]. However,
415 given the role of microRNAs in disrupting protein translation, the loss of
416 microRNAs due to the oncogenic regulation may result in the increased
417 expression of critical oncoproteins. In the present study, as predicted by its
418 effect on DICER, we demonstrate that MUC1 silencing results in a broad class
419 effect on the generation of functionally active microRNAs. In fact, in MOLM-14
420 cells, MUC1 silencing was associated with an increase in 98% of microRNA
421 species.

422 Consistent with its role in modulating PD-L1 expression, we demonstrate that
423 MUC1 exerts a potent negative immunoregulatory effect in AML. In an
424 immunocompetent AML model, animals engrafted with MUC1 silenced AML cells
425 developed greater evidence of leukemia specific immunity, compared to control
426 cells, as manifested by increased levels of leukemia specific T cells detected in
427 the bone marrow, determined by the percent of cells expressing IFN-γ following
428 exposure to tumor lysate. While the higher levels of engraftment seen in animals
429 receiving control tumor may be associated a greater degree of immune
430 suppression, the emergence of leukemia reactive T cells also likely reflects the
431 greater immunogenicity of AML cells in the setting of MUC1 inhibition.

Consistent with this hypothesis, killing of human AML cells by alloreactive lymphocytes is enhanced in MUC1 silenced AML targets. Similarly, exposure to a MUC1 inhibitor renders AML cells more susceptible to lysis by T cells stimulated by leukemia vaccine generated by primary AML cells fused with autologous dendritic cells.

The observation that MUC1 modulation of miR-200c and miR-34a levels regulates PD-L1 expression in AML lends itself to several areas of clinical translation. Blockade of the PD-L1/PD-1 pathway has emerged as a major area of cancer therapeutics with profound and durable responses seen in a subset of patients with advanced melanoma[54], renal cell carcinoma[7], and non-small cell lung cancer[9]. The presumptive mechanism of this clinical effect is the breaking of tolerance of tumor reactive lymphocytes and the generation of tumor specific immunity. A recent report of patients with advanced Hodgkin's disease demonstrates that blockade of this pathway results in durable disease response in 87% of patients[55]. PD-L1 expression by tumor cells is of likely prognostic importance and potentially predictive of response to antibodies that provide checkpoint blockade. Expression is likely to be dynamic, arising out of the interactions with stromal elements in the bone marrow microenvironment. As such, staining characteristics of *ex-vivo* cells may not be reliable and *in-vivo* biomarker that correlates with PD-L1 expression is of potential great clinical significance.

We have developed a MUC1 inhibitor consisting of a cell penetrating peptide that

454 blocks MUC1 signaling by preventing dimerization necessary for translocation of
455 the MUC1-C component from the plasma membrane to the nucleus[56]. Exposure
456 to the MUC1 inhibitor results in downregulation of PD-L1 expression in primary
457 AML cells and potentially renders them more susceptible to T cell mediated
458 recognition and lysis. Investigators have begun to explore the use of microRNAs
459 as therapeutic agents[57]. Strategies to enhance miR-34a or miR-200c expression
460 or by the introduction of microRNA mimetics might similarly result in an enhanced
461 immunologic milieu.

462 We have developed a tumor vaccine in which patient derived AML cells are fused
463 with autologous dendritic cells such that a broad array of tumor antigens are
464 presented in the context of DC mediated costimulation[36,37]. Vaccination with
465 DC/AML fusions results in the expansion of leukemia specific T cells and
466 protection from relapse[36]. Vaccine efficacy is likely blunted by the presence of
467 PD-L1 expression on the hybridoma cells as well as its presence as a tolerizing
468 influence in the bone marrow microenvironment. In an immune competent AML
469 murine model, combining vaccination with GO-203 treatment, was shown to
470 enhance the vaccine potency (unpublished data). We are currently exploring the
471 use of the MUC1 inhibitor and microRNA mimetics to create an enhanced
472 platform for the development of tumor specific immunity using a leukemia cancer
473 vaccine.

474 In summary, we have identified a novel mechanism by which the MUC1
475 oncoprotein upregulates PD-L1 expression by AML cells through its effect on

476 microRNA species. We have identified a novel mechanism of tumorigenesis
477 through the downregulation of DICER and the loss of microRNA species that
478 regulate proteins with pro-oncogenic function such as PD-L1.

479
480
481 There are no conflicts of interest or funding sources to declare

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483

Supplemental Table 1: Table of PCR Primers

Primer	Company	Catalogue No.
18s	Qiagen	QF00451850
b-actin	Qiagen	QT01680476
DICER	Qiagen	QT00015176
GAPDH	Qiagen	QT00079247
JUN	Qiagen	QT00242956
miR-34a	Qiagen	MS00003318
precursor-mir-34a	Qiagen	MP00002044
miR-200c	Qiagen	MS00003752
precursor-mir-200c	Qiagen	MP00001414

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681

682

Figure Legends

Figure1: MUC1 inhibition leads to decrease in PD-L1 expression. MUC1 was silenced in MOLM-14 and THP1 AML cells using lentiviral shRNA hairpin against MUC1. As a control, MOLM-14 cells were infected with control shRNA. The cells were then evaluated for PD-L1 expression using **(A)** flow cytometry and **(B)** western blot analysis. For *in-vivo* evaluation of PD-L1 expression, C57BL/6 mice were inoculated retro orbitally with $100x10^3$ GFP transduced syngeneic TIB-49 AML control cells or TIB-49 cells that were silenced for MUC1 expression using lentiviral transduction. **(C)** 14 days after inoculation spleen and bone marrow cells were harvested and GFP+ AML cells were evaluated for PD-L1 expression (n=4). To evaluate *PD-L1* mRNA levels, *MUC1* gene was silenced in MOLM-14 cells using CRISPR/Cas9 technology, and using MUC1 specific shRNA in THP-1 cells. **(D)** A representative experiment showing *PD-L1* mRNA levels, evaluated using qPCR. Each condition was performed in triplicate ($p<0.05$).

Figure 2: miR-34a and miR-200c regulate PD-L1 expression in AML cells. **(A)** The seed sequences of miR-34a and miR-200c on the 3'UTR PD-L1, transcript variant 1, NM_014143.3, were identified by RegRNA: A Regulatory RNA Motifs and Element web server. MUC1 was silenced in MOLM-14 cells using the CRISPR/Cas9 technology and in THP-1 cells using transduction with MUC1 specific shRNA. **(B)** Relative levels of miR-34a were detected in MOLM-14 and THP-1 cells using qPCR. miR-34a was overexpressed in MOLM-14 and THP-1 cells using lentiviral transduction. **(C)** PD-L1 levels were evaluated using

705 western blot analysis in miR-34a overexpressed or control MOLM-14 and THP-1
706 AML cells. **(D)** Relative levels of miR-200c were detected in MOLM-14 and THP-
707 1 cells using qPCR. MiR-200c was overexpressed in MOLM-14 cells using
708 lentiviral transduction. PD-L1 levels were evaluated in control and miR-200c
709 overexpressed cells using **(E)** western blotting and **(F)** flow cytometry.

710 **Figure 3: MUC1 inhibition leads to increase in DICER expression.** MUC1
711 was silenced in MOLM-14 cells using CRISPR/Cas9 technology and in THP-1
712 cells using transduction with MUC1 specific shRNA. Relative levels of **(A)**
713 precursor mir-34a and **(B)** precursor mir-200c were detected using qPCR in
714 MOLM-14 and THP-1 cells with silenced MUC1 levels compared to control cells
715 (n=2). **(C)** DICER and Ago-2 levels were assessed using western blot analysis in
716 control and MUC1 silenced MOLM-14 and THP-1 cells (n=3). **(D)** MOLM-14 and
717 THP-1 cells were treated with 2μM GO-203 or control peptide daily for 3 days.
718 DICER and Ago-2 levels were detected using western blot analysis (n=2). **(E)**
719 *DICER* mRNA was detected using qPCR in MOLM-14 and THP-1 cells with
720 silenced MUC1 levels compared to appropriate control cells (n=2).

721 **Figure 4: Increase in DICER expression is mediated via c-Jun.** MUC1 was
722 silenced in MOLM-14 cells using CRISPR/Cas9 technology and in THP-1 cells
723 using transduction with MUC1 specific shRNA. **(A)** The cells underwent western
724 blot analysis for phospho c-Jun and c-Jun expression in MUC1 silenced and
725 control AML cells. **(B)** Relative *c-Jun* mRNA levels were evaluated using qPCR
726 analysis in MOLM-14 and THP-1 MUC1 silenced and control cells (n=2). **(C)** AML

cells were obtained from BM aspirates from patients with AML at diagnosis. The cells were then treated daily for 3 days with 2.5μM of GO-203 or control peptide. Affimetrix analysis was performed for mRNA expression and the relative expression of c-Jun is presented for three patients. **(D)** MUC1 silenced MOLM-14 and THP-1 cells were treated with 100μM and 400μM c-Jun inhibitor for 30 minutes. DICER levels were evaluated using western blot analysis (n=2).

Figure 5: MUC1 silencing leads to increase in microRNAs in AML cells. MUC1 was silenced in MOLM-14 cells using CRISPR/Cas9 technology and in THP-1 cells using transduction with MUC1 specific shRNA. RNA was isolated from the MUC1 silenced and control MOLM-14 and THP-1 AML cells and underwent NanoString™ analysis for microRNA expression in triplicates. Scatter plot of log2 count of microRNAs in MUC1 silenced vs. control cells are demonstrated.

Figure 6: MUC1 exerts an immunoregulatory effect in AML cells *in-vitro* and *in-vivo*. (A) MUC1 was silenced in TIB-49 mouse AML cells using MUC1 specific shRNA. C57BL/6 mice were inoculated retro-orbitally with $100x10^3$ GFP syngeneic TIB-49 AML cells transduced with control shRNA (CshRNA) or MUC1 silenced TIB-49 cells (MUC1shRNA). On day 14 following the inoculation bone marrow cells were harvested and stimulated *ex-vivo* with TIB-49 tumor lysate. Following 6 days of stimulation CD4+ T cells underwent flow cytometric analysis for intracellular IFN-γ expression. **(B)** The results are demonstrated as mean IFN-γ expression in 5 mice. C57BL/6 mice were inoculated with $100x10^3$ GFP

transduced syngeneic TIB-49 AML cells using retro-orbital injections. 24 hours after inoculation the mice were treated daily subcutaneously with 14 mg/kg GO-203. On day 14 following the inoculation bone marrow cells were harvested and stimulated ex-vivo with TIB-49 tumor lysate. **(C)** Following 6 days of stimulation CD4+ and CD8+ T cells underwent flow cytometric analysis for intracellular IFN-γ expression. MUC1 was silenced in MOLM-14 and TIB-49 cells. **(D)** MOLM-14 and THP-1 tumor cells were labeled with APC fluorochrome and co-cultured with allogeneic PBMCs in a ratio of 1:5. Tumor lysis was detected after the addition of FITC-granzyme B substrate as a percent of APC+FITC+ cells detected by flow cytometric analysis. Representative example of allogeneic cytotoxic T lymphocyte assay depicting allogeneic PBMC response against MUC1 silenced and control AML cells (n=2). **(E)** Tumor cells were obtained from bone marrow aspirate from patient with AML at diagnosis. Peripheral blood was obtained from the same patient at remission, DC were generated from the PBMCs and fused with the autologous tumor cells. The PBMCs were then stimulated with the fusion cells for 6 days in a 1:5 ratio and then co-cultured with autologous tumor cells that were either pre-treated with 2.5μM GO-203 for 3 days or control tumor cells. Tumor lysis was detected after the addition of FITC-granzyme B substrate as a percent of APC+FITC+ cells detected by flow cytometric analysis.

Figure 1

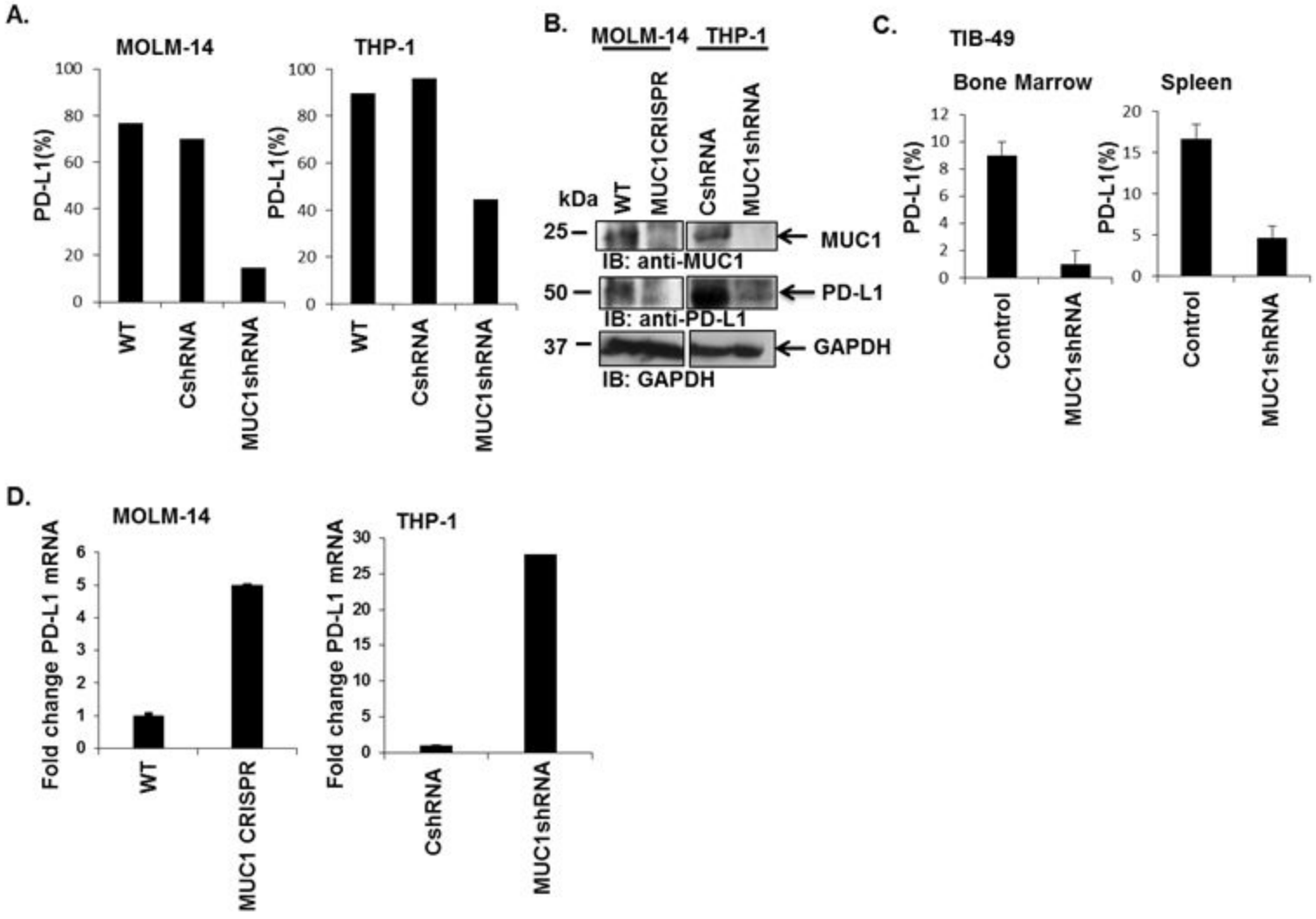

A.
MOLM-14
THP-1
PD-L1(%)
100
80
60
40
20
0
WT
CshRNA
MUC1shRNA
B.
MOLM-14
THP-1
kDa
WT
MUC1CRISPR
CshRNA
MUC1shRNA
25
MUC1
IB: anti-MUC1
50
PD-L1
IB: anti-PD-L1
37
GAPDH
IB: GAPDH
C.
TIB-49
Bone Marrow
Spleen
PD-L1(%)
Control
MUC1shRNA
D.
MOLM-14
THP-1
Fold change PD-L1 mRNA
WT
MUC1 CRISPR
CshRNA
MUC1shRNA

Figure 2

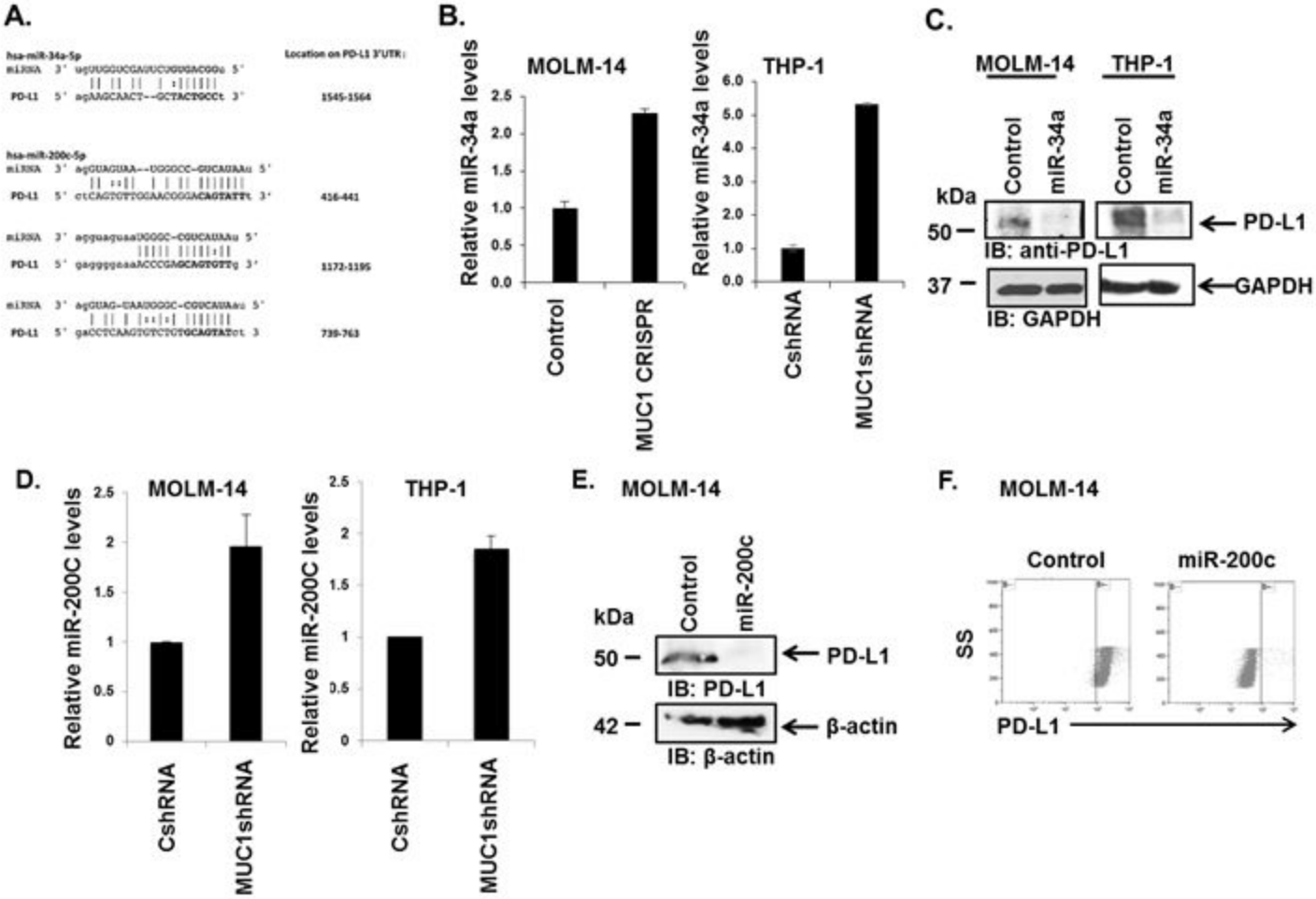

A.
hsa-miR-34a-5p
Location on PD-L1 3'UTR:
1545-1564
hsa-miR-200c-5p
416-441
1172-1195
739-763
B.
MOLM-14
THP-1
Relative miR-34a levels
Control
MUC1 CRISPR
CshRNA
MUC1shRNA
C.
MOLM-14
THP-1
Control
miR-34a
kDa
50
PD-L1
IB: anti-PD-L1
37
GAPDH
IB: GAPDH
D.
MOLM-14
THP-1
Relative miR-200C levels
CshRNA
MUC1shRNA
E.
MOLM-14
Control
miR-200c
kDa
50
PD-L1
IB: PD-L1
42
β-actin
IB: β-actin
F.
MOLM-14
Control
miR-200c
SS
PD-L1

Figure 3

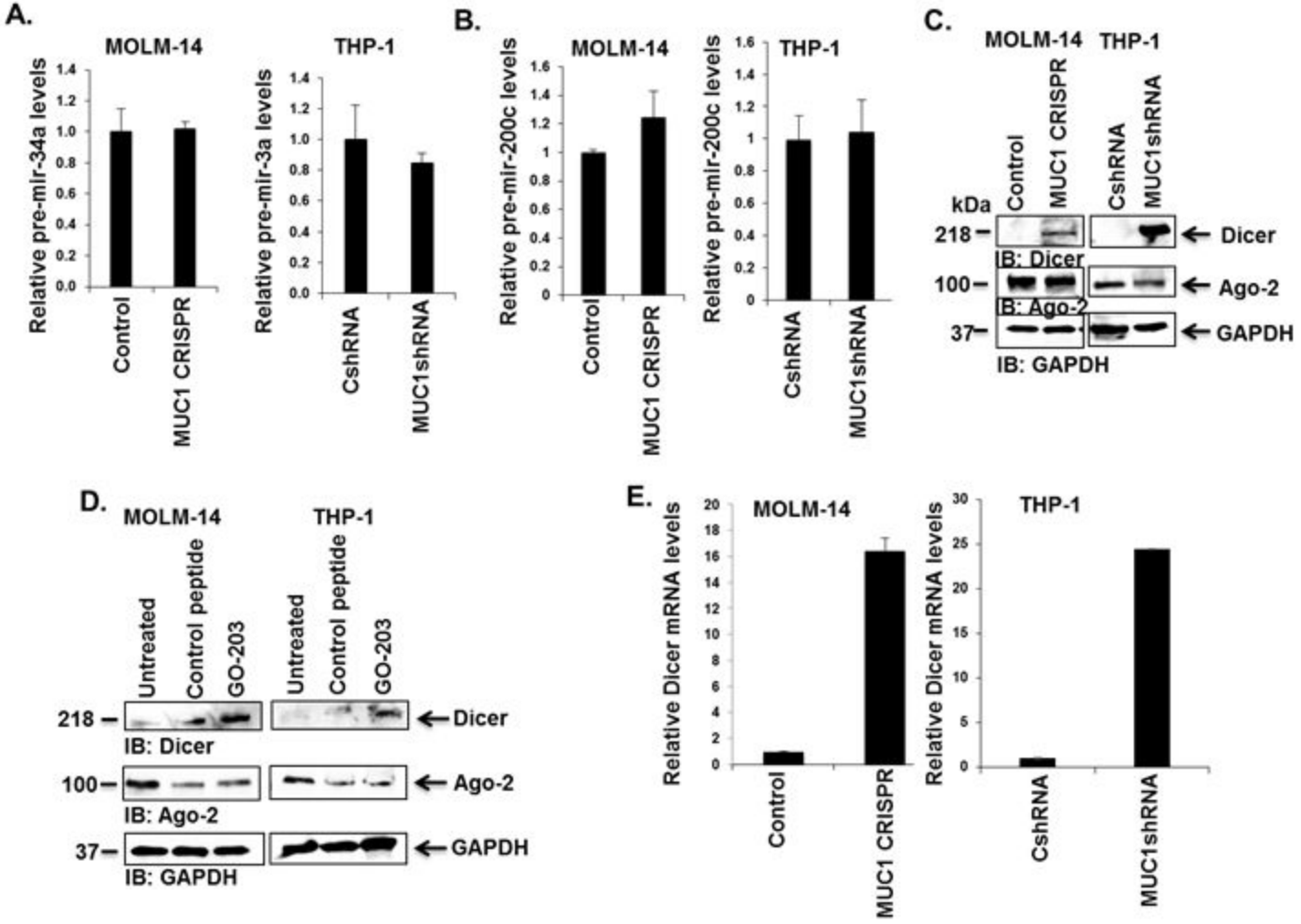
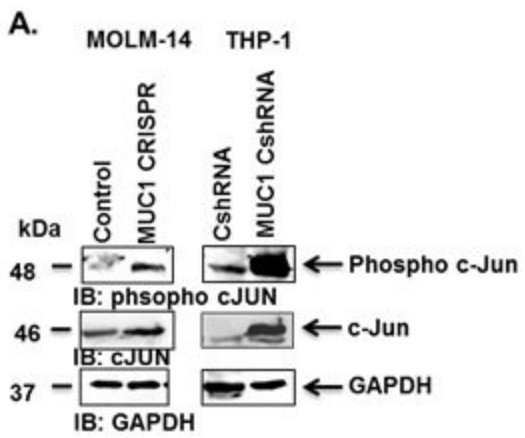
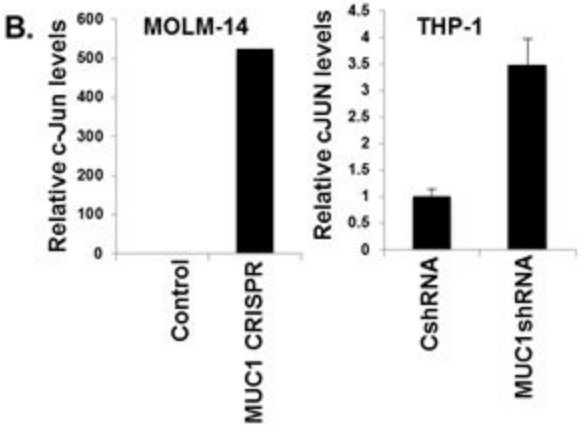
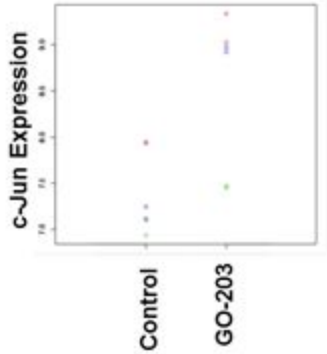

A.
MOLM-14
THP-1
Relative pre-mir-34a levels
Relative pre-mir-3a levels
Control
MUC1 CRISPR
CshRNA
MUC1shRNA
B.
MOLM-14
THP-1
Relative pre-mir-200c levels
Relative pre-mir-200c levels
Control
MUC1 CRISPR
CshRNA
MUC1shRNA
C.
MOLM-14
THP-1
Control
MUC1 CRISPR
CshRNA
MUC1shRNA
kDa
218
Dicer
IB: Dicer
100
Ago-2
IB: Ago-2
37
GAPDH
IB: GAPDH
D.
MOLM-14
THP-1
Untreated
Control peptide
GO-203
Untreated
Control peptide
GO-203
218
Dicer
IB: Dicer
100
Ago-2
IB: Ago-2
37
GAPDH
IB: GAPDH
E.
MOLM-14
THP-1
Relative Dicer mRNA levels
Relative Dicer mRNA levels
Control
MUC1 CRISPR
CshRNA
MUC1shRNA

Figure 4


A.
MOLM-14
THP-1
Control
MUC1 CRISPR
CshRNA
MUC1 CshRNA
kDa
48
Phospho c-Jun
IB: phsopho cJUN
46
c-Jun
IB: cJUN
37
GAPDH
IB: GAPDH


B.
MOLM-14
Relative c-Jun levels
600
500
400
300
200
100
0
Control
MUC1 CRISPR
THP-1
Relative cJUN levels
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CshRNA
MUC1shRNA


C.
c-Jun Expression
Control
GO-203

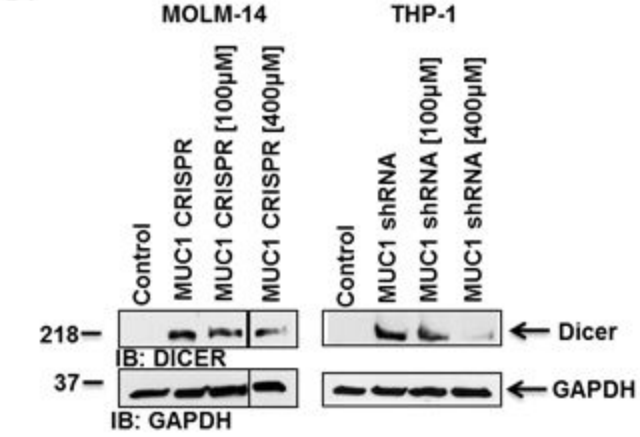

D.
MOLM-14
THP-1
Control
MUC1 CRISPR
MUC1 CRISPR [100μM]
MUC1 CRISPR [400μM]
Control
MUC1 shRNA
MUC1 shRNA [100μM]
MUC1 shRNA [400μM]
218
Dicer
IB: DICER
37
GAPDH
IB: GAPDH

Figure 5

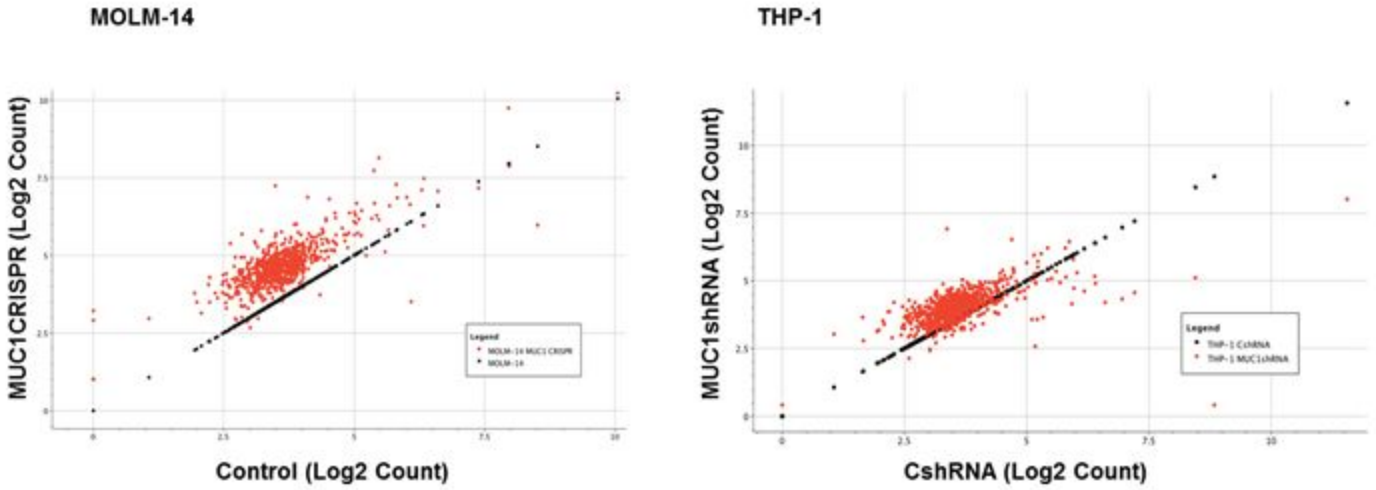
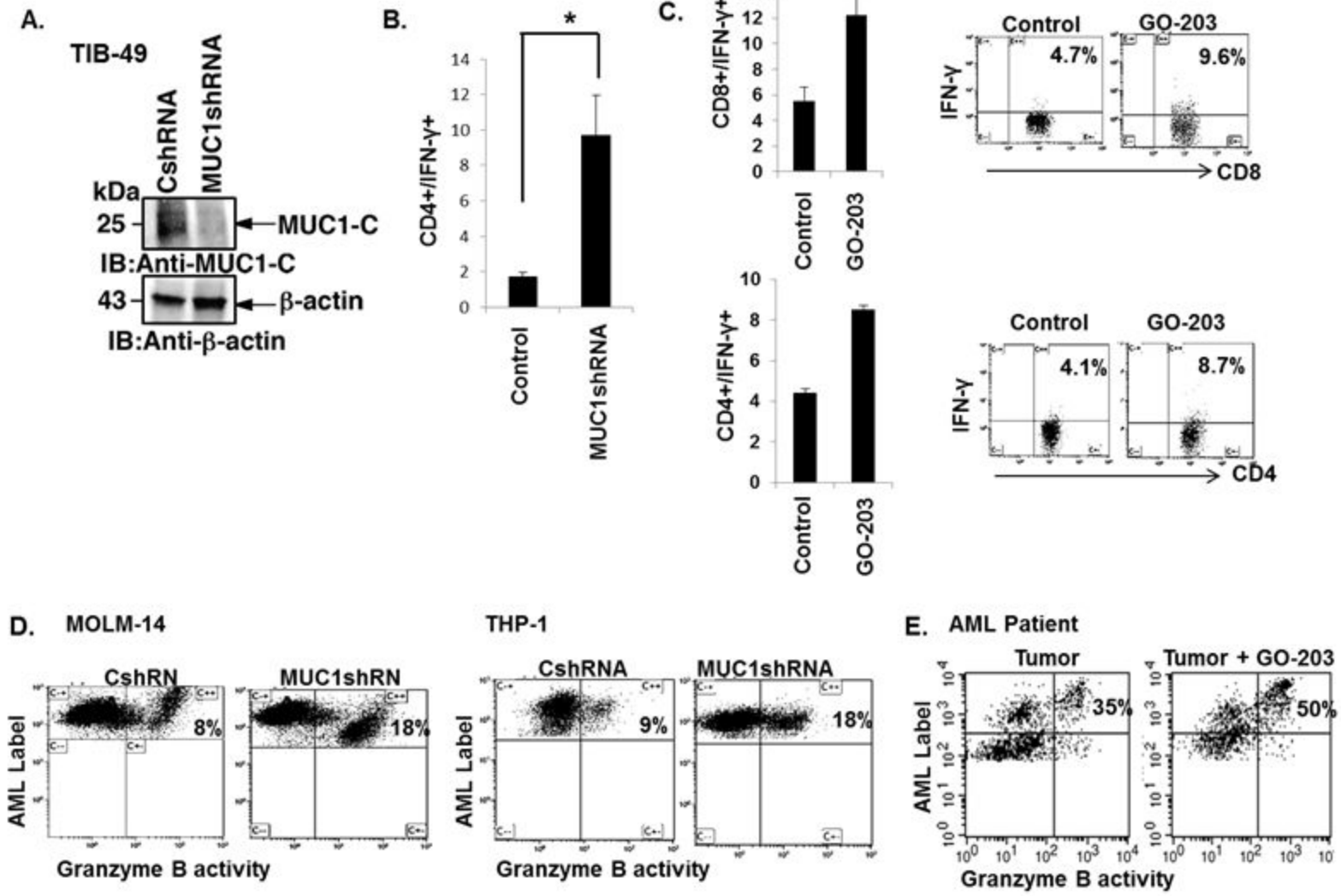

MOLM-14
MUC1CRISPR (Log2 Count)
Control (Log2 Count)
THP-1
MUC1shRNA (Log2 Count)
CshRNA (Log2 Count)

Figure 6


A.
TIB-49
CshRNA
MUC1shRNA
kDa
25
MUC1-C
IB:Anti-MUC1-C
43
β-actin
IB:Anti-β-actin
B.
*
CD4+/IFN-γ+
Control
MUC1shRNA
C.
CD8+/IFN-γ+
Control
GO-203
Control
GO-203
4.7%
9.6%
IFN-γ
CD8
CD4+/IFN-γ+
Control
GO-203
Control
GO-203
4.1%
8.7%
IFN-γ
CD4
D.
MOLM-14
CshRN
MUC1shRN
8%
18%
AML Label
Granzyme B activity
THP-1
CshRNA
MUC1shRNA
9%
18%
AML Label
Granzyme B activity
E.
AML Patient
Tumor
Tumor + GO-203
35%
50%
AML Label
Granzyme B activity