Supplementary Information

Figure and table legends

Figure S1.

Gating strategies for CD39 expression in BM-derived $CD138^+$ MM cells from patients and PC from healthy controls. (A) $CD138^+$ MM cells were enriched from BM aspirate from patients using anti-CD138 beads (Stem Cell Technologies) as described in Methods. The cells were stained with anti-CD38, -CD19 and -CD56. (The cells were not stained with anti-CD138 as these epitopes were already engaged by antibody used for enrichment). The CD39 expression was determined on gated $CD38^+$, $CD19^-$ and $CD56^+$ cells [1] using CD39-negative FMO controls. (B) As there were very few (2 – 5 %) PC in the bone marrow of healthy controls, there were not enough cells for $CD138^+$ bead enrichment. Crude BM aspirates were therefore stained with anti-CD138, -CD19, -CD38, -CD56 and -CD39 and analyzed as shown in Figure S1B. Healthy PC were defined as $CD138^+CD19^+CD38^+CD56^-$ cells. Figure shows CD39 expression on gated plasma cells on a representative sample of one out of 3 healthy controls. Gates were set on $CD39^-$negative FMO controls.

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Figure S2

AMP and adenosine generation by co-culture of myeloma cell lines and MSC. Mean±SD is shown.

(A) JJN-3 and HS-5 were cultured with 100 μM ATP separately for various time periods before measuring AMP in collected supernatants by HPLC-MS.

(B) IH-1 and MSC were incubated with 100 μM ATP for various time periods before adenosine was measured in supernatants by HPLC-MS.

(C) Enriched MM cells from 3 patients were incubated with 100 μM ATP for 1 hour before adenosine was measured in supernatants by HPLC-MS.

(D) Examples of chromatograms representing peaks for AMP (mass transition 348.1/136.1), adenosine (mass transition 268.1/136.1), and 13C5 adenosine (mass transition 273.1/136.1). (i) standard AMP peak. (ii) AMP peak in medium from JJN-3 cultures incubated with ATP for 5 min. (iii) AMP peak in medium from JJN-3 cultures incubated with ATP for 45 min. (iv) standard adenosine peak. (v) adenosine and 13C5 adenosine peaks in medium from JJN-3/HS-5 cultures incubated with ATP for 5 min. (vi) adenosine and 13C5 adenosine peaks in medium from JJN-3/HS-5 cultures incubated with ATP for 45 min.

Figure S3

ZM241385 restores T cell proliferation suppressed by A) CADO B) Adenosine generated from co-culture (CM). (C) AZD4635 restores T cell proliferation suppressed by CADO. Representative experiments are shown, where the horizontal bars indicate the CSFE levels retained by T cells that failed to undergo cell division. (D) Live/Dead cell staining on T cells isolated from BMMCs of MM patients cultured with or without CADO for 5 days.

Figure S4

ENTPD1 expression and prognosis

(A) Distribution of tumor samples among ISS risk groups ISSI (n=210), ISSII (n=240) ISSIII (n=205). Significantly higher percent of ENTPD1 high (TPM>10) myeloma cells in patients with ISS III score. (p=0.004, Chi-square test comparing non-expressers (TPM<2) vs high expressers (TPM>10)).

(B) Distribution (percentage) of translocation class types in tumors expressing no (TPM<2), intermediate (TPM 2-10) or high levels of ENTPD1 (TPM>10). Significant differences were found in percent tumors harbouring CCND1 (p=1.93e-9), HRD (p=1.93e-9) and MAF (p=0.03) translocations when comparing samples with no ENTPD1 (TPM<2) and high levels (TPM>10) of ENTPD1 (Chi-square test).

(C) ENTPD1 RNAseq expression levels (TPM) in myeloma tumors divided into different transcription class groups. The grouping was based on seqFISH data from the CoMMpass database (D) The two most significantly enriched pathways identified by GSEA in patients expressing ENTPD1 (TPM >2) (382 samples) vs non-expressers (TPM <2) (429 samples) (left), and when comparing patients expressing high levels of ENTPD1 (TPM >10) (76 samples) vs non-expressers (TPM <2) (429 samples) (right).

Figure S5

Impact of inhibiting CD39, CD73 and A2AR *in vivo* on immune cells.

(A) Treatment with combined POM-1 and AZD4635. Mice (n=11/group) were treated with AZD4635 and POM-1 separately or in combination as described in Methods. AZD4636 were gavaged daily from day 0. M-component in serum was determined as described after culling on day 21.

Gating strategies for Figure 7. Cells from spleen and BM from treated and un-treated mice were stained as described in Methods and analyzed by FACS. (B) IFNγ on spleen cells. Histograms of % IFNγ+ live cells gated on FSC and SSC is shown. Gate was set using isotype control. (C) Histograms of CD62L on gated CD3+CD4+ T cells. (D) FASL+ CD8 cells. Live gates were set on FSC and SSC. Dot plots of CD8 and FASL expression on CD3+ cells are shown. (E) Monocytes and granulocytes in spleen. Live gates were set on FSC and SSC. Dot plots of CD11b and Ly6G is shown. Histogram shows Ly6C expression of the gated

populations. (F) Activation of monocytes in the spleen. Live gates were set on FSC and SSC. Dot plots of CD11b and MHCII is shown. Ly6G vs Ly6C dot plot of gated $CD11b^{+}MHCII^{high}$ cells is shown. Histogram of $\%CD11b^{+}Ly6G^{-}MHCII^{high}$ cells of total spleen cells (G) Histograms of IFNγ expression on BM cells. % IFNγ+ live cells gated on FSC and SSC. Gate was set using isotype control. (H) Monocytes and granulocytes in BM. Live gates were set on FSC and SSC. Dot plots of CD11b and Ly6G is shown. Histogram shows Ly6C expression of the gated populations.

Table S1

IFN-γ concentration in serum	Controls (samples)	Treated (samples)
> 100 pg/ml	4	11
< 100 pg/ml	7	0

Table S1. Serum IFN-γ concentration from C57BL/KalwRij mice injected with 5T33MM cells, not treated (control) (n=11) and treated with POM-1, anti CD73 and AZD4635 (n=11).

Methods

Tumor cell lines and human bone marrow stromal cell line

Human myeloma cell lines IH-1, OH-2, RPMI8226, INA-6, JJN-3, KJON, ANBL-6 and U266 were used. OH-2, IH-1 and KJON were generated in our own lab.[2-4] INA-6[5] , JJN3[6] and ANBL-6[7] were kind gifts from Dr M. Gramatzki (University of Erlangen-Nurnberg, Erlangen, Germany), J. Ball (University of Birmingham, UK), and Dr D. Jelinek (Mayo Clinic, Rochester, MN, USA), respectively. RPMI-8226[8] and U266[9] were from ATCC (Rockville, MD, USA). INA-6 and ANBL-6 cells were grown in 10 % heat-inactivated fetal calf serum (FCS) in RPMI-1640 (RPMI) supplemented with 0.68 mM glutamine and interleukin IL-6 (1 ng/mL) (Biosource, Camarillo, CA, USA). JJN-3, RPMI-8226 and U266 were grown in RPMI with 10, 20 or 15% FCS, respectively. OH-2 and IH-1 were maintained in 10% heat-inactivated human serum (Department of Immunology and Transfusion Medicine, St. Olavs Hospital, Trondheim). HS-5, a human bone marrow stromal cell line (ATCC), was maintained in DMEM with 10% FCS.

Collection of peripheral blood mononuclear cells and T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy blood donors (blood bank of St. Olavs Hospital, Trondheim) using gradient centrifugation with Lymphoprep™ (Axis-Shield)[10]. T cells were subsequently purified from PBMCs using Dynabeads™ Untouched™ Human T Cells Kit (ThermoFisher). Then 1 x 10^7 T cells were incubated with 1 μM CFSE

(CellTrace™ CFSE Cell Proliferation Kit,Invitrogen) at 37ºC for 10 min, neutralized by FCS for 2 min, and then washed twice in PBS.

Bone marrow mononuclear cells (BMNC)

BMNCs were isolated from bone marrow aspirates after density centrifugation with Lymphoprep (Axis Shield 111544).

Purified CD138+ plasma cells

CD138+ plasma cells were isolated from mononuclear cells from BM aspirates using RoboSep automated cell separator and Human CD138 Positive Selection Kit (StemCell Technologies, Grenoble, France)[11]. The plasma cells were > 95 % pure.

T cell stimulation and proliferation

2 x 10^5 CFSE-labelled T cells were seeded per well in 96-well plates with and without 10 mM CADO, 0.3 μM ZM241385 and incubated in RPMI (10 % human serum), 20 U/mL rIL2 (Invitrogen) and 1:1 (beads:cells) T-Activator CD3/CD28 (Dynabeads,Invitrogen) for 5 days. The cells were then harvested, stained with anti-CD3 and analyzed on LSRII. For the experiments using AZD4635, CFSE-labelled T cells isolated from buffy coat were pre-treated with 3μM AZD4635 or DMSO control for 3 hrs at 37ºC. CADO (15μM) and T-activator CD3/CD28 were then added to the cells and incubated for 5 days. For the experiments using adenosine generated during the co-culture of myeloma cells and MSCs, a two-step method was devised to produce sufficient adenosine during T cell culture. In step 1, JJN-3 cells (2x10^5/well) were pretreated with or without 100 μM POM-1 in

96-well plates for 3 hrs at 37°C, then washed with PBS, and further cultured for another 1 hr in RPMI with20 U/mL rIL2 and 100μM ATP. Supernatants containing AMP were then collected for further use. Separately, in step 2, HS-5 cells (5 x 10^4/well) were pretreated with anti-CD73 or isotype-matched mAb for 3 hrs at 37°C before CD3/CD28-activated, CFSE-labelled T cells (2x10^5 /well) were added to the HS-5 cells. The AMP-containing supernatants from step1 were then added to the HS-5/T co-culture and the cells were further cultured for 2 days. Half of the medium in cell culture was replaced with supernatants from step1 on days 2 and 3. Cells were harvested on day 5, stained with anti-CD3 and analyzed by Flow cytometry. Division index was calculated using FlowJo to estimate the average number of divisions that the T cells go through during stimulation [12].

Multi/univariate analysis

We calculated the effect of CD39 gene expression in tumor cells on patient outcomes in the CoMMpass dataset (IA13 release) using univariate and multivariate Cox regression. Relative risk was expressed as hazard ratios (HR) with 95 % confidence intervals (CI), and two-sided Wald test p-values < 0.05 were considered statistically significant. In the full multivariate model, we adjusted for ISS stage, chromosome 14 translocations and hyperdiploidy, and stratified by whether the patient received induction treatment as part of first line treatment. Univariate survival analysis was visualized using the Kaplan-Meier method and log-rank test to compare survival distributions between groups. Analysis was done in R version 3.4.3 using the Survival package.

Relative ATP measurement

CellTiter-Glo Luminescent(CTG) Cell Viability Assay (Promega) was used to estimate the relative cell viability by measuring the content of ATP present in the wells according to instructions provided by the manufacturer. In summary, 1x 10^4 JJN-3 cells were seeded in a 96-well plate, incubated with or without 100 μM POM-1 for 3 hrs, the provided assay reagent was then added to the plates, after which the plates were agitated on a microplate shaker for 2 min, and kept at room temperature for 10 min before luminescence was determined. The luminescent signal was recorded with a Victor3 plate reader and Wallac 1420 Work Station software (PerkinElmer Inc.) [13].

References

1. Flores-Montero J, de Tute R, Paiva B, Perez JJ, Bottcher S, Wind H, Sanoja L, Puig N, Lecrevisse Q, Vidriales MB *et al*: **Immunophenotype of normal vs. myeloma plasma cells: Toward antibody panel specifications for MRD detection in multiple myeloma**. *Cytometry Part B, Clinical cytometry* 2016, **90**(1):61-72.
2. Hjertner O, Hjorth-Hansen H, Borset M, Seidel C, Waage A, Sundan A: **Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells**. *Blood* 2001, **97**(2):516-522.
3. Vatsveen TK, Borset M, Dikic A, Tian E, Micci F, Lid AH, Meza-Zepeda LA, Coward E, Waage A, Sundan A *et al*: **VOLIN and KJON-Two novel hyperdiploid myeloma cell lines**. *Genes, chromosomes & cancer* 2016, **55**(11):890-901.
4. Vatsveen TK, Tian E, Kresse SH, Meza-Zepeda LA, Gabrea A, Glebov O, Dai HY, Sundan A, Kuehl WM, Borset M: **OH-2, a hyperdiploid myeloma cell line without an IGH translocation, has a complex translocation juxtaposing MYC near MAFB and the IGK locus**. *Leukemia research* 2009, **33**(12):1670-1677.
5. Burger R, Guenther A, Bakker F, Schmalzing M, Bernand S, Baum W, Duerr B, Hocke GM, Steininger H, Gebhart E *et al*: **Gp130 and ras mediated signaling in human plasma cell line INA-6: a cytokine-regulated tumor model for plasmacytoma**. *The hematology journal : the official journal of the European Haematology Association* 2001, **2**(1):42-53.
6. Jackson N, Lowe J, Ball J, Bromidge E, Ling NR, Larkins S, Griffith MJ, Franklin IM: **Two new IgA1-kappa plasma cell leukaemia cell lines (JJN-1 & JJN-2) which proliferate in response to B cell stimulatory factor 2**. *Clinical and experimental immunology* 1989, **75**(1):93-99.

7. Jelinek DF, Ahmann GJ, Greipp PR, Jalal SM, Westendorf JJ, Katzmann JA, Kyle RA, Lust JA: **Coexistence of aneuploid subclones within a myeloma cell line that exhibits clonal immunoglobulin gene rearrangement: clinical implications**. *Cancer research* 1993, **53**(21):5320-5327.
8. Matsuoka Y, Moore GE, Yagi Y, Pressman D: **Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma**. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY)* 1967, **125**(4):1246-1250.
9. Nilsson K, Bennich H, Johansson SG, Ponten J: **Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient**. *Clinical and experimental immunology* 1970, **7**(4):477-489.
10. Li Y, Lund C, Nervik I, Loevenich S, Dollner H, Anthonsen MW, Johnsen IB: **Characterization of signaling pathways regulating the expression of pro-inflammatory long form thymic stromal lymphopoietin upon human metapneumovirus infection**. *Sci Rep* 2018, **8**(1):883.
11. Baranowska K, Misund K, Starheim KK, Holien T, Johansson I, Darvekar S, Buene G, Waage A, Bjorkoy G, Sundan A: **Hydroxychloroquine potentiates carfilzomib toxicity towards myeloma cells**. *Oncotarget* 2016, **7**(43):70845-70856.
12. Roederer M: **Interpretation of cellular proliferation data: avoid the panglossian**. *Cytometry Part A : the journal of the International Society for Analytical Cytology* 2011, **79**(2):95-101.
13. Abdollahi P, Vandsemb EN, Hjort MA, Misund K, Holien T, Sponaas AM, Ro TB, Slordahl TS, Borset M: **Src Family Kinases Are Regulated in Multiple Myeloma Cells by Phosphatase of Regenerating Liver-3**. *Molecular cancer research : MCR* 2017, **15**(1):69-77.

Figure S1

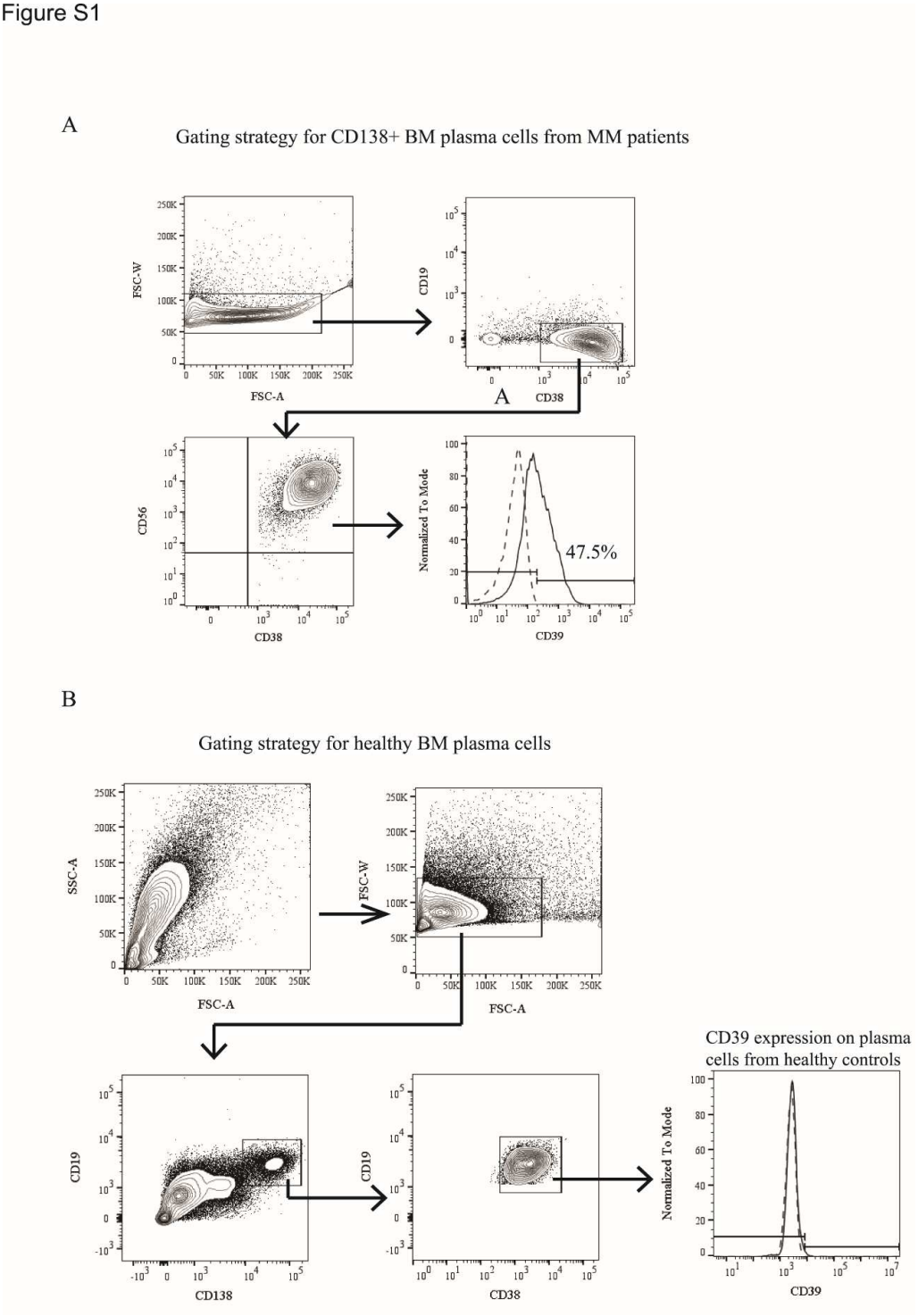

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Figure S2

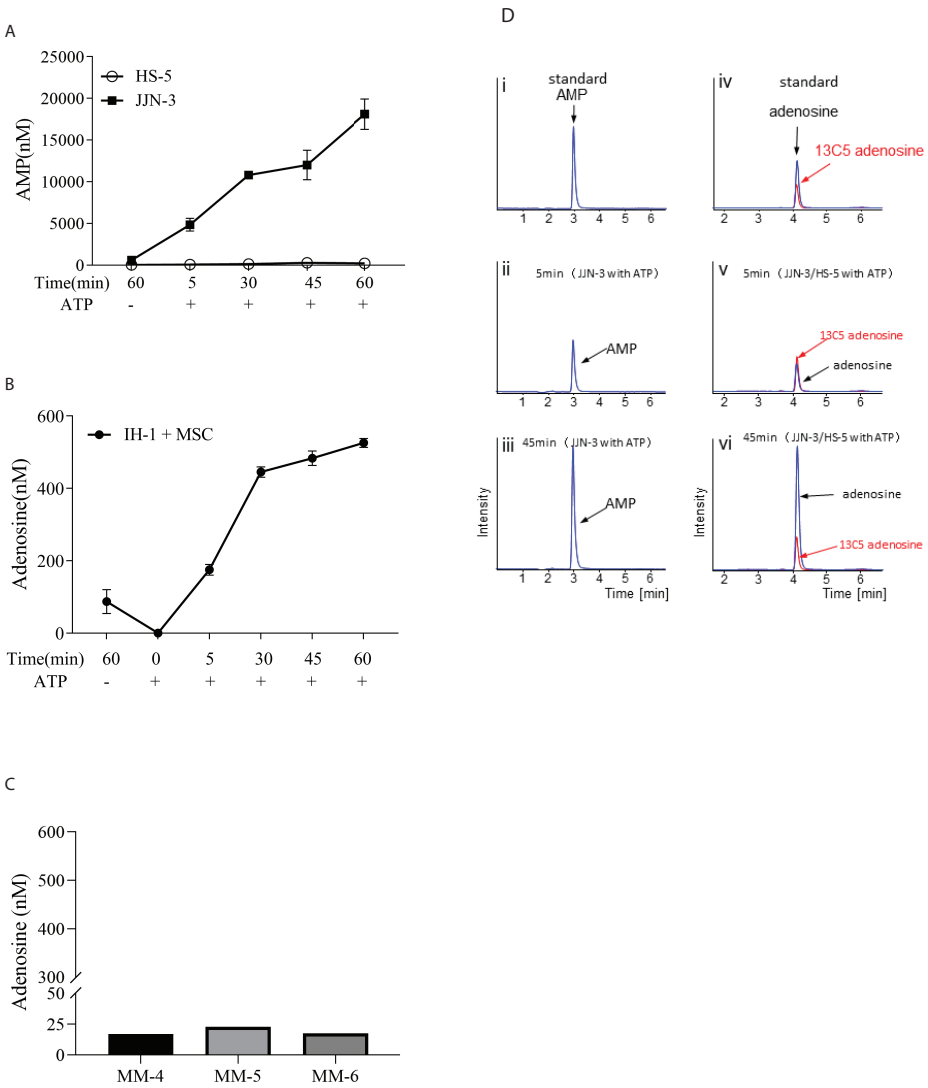

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Figure S3

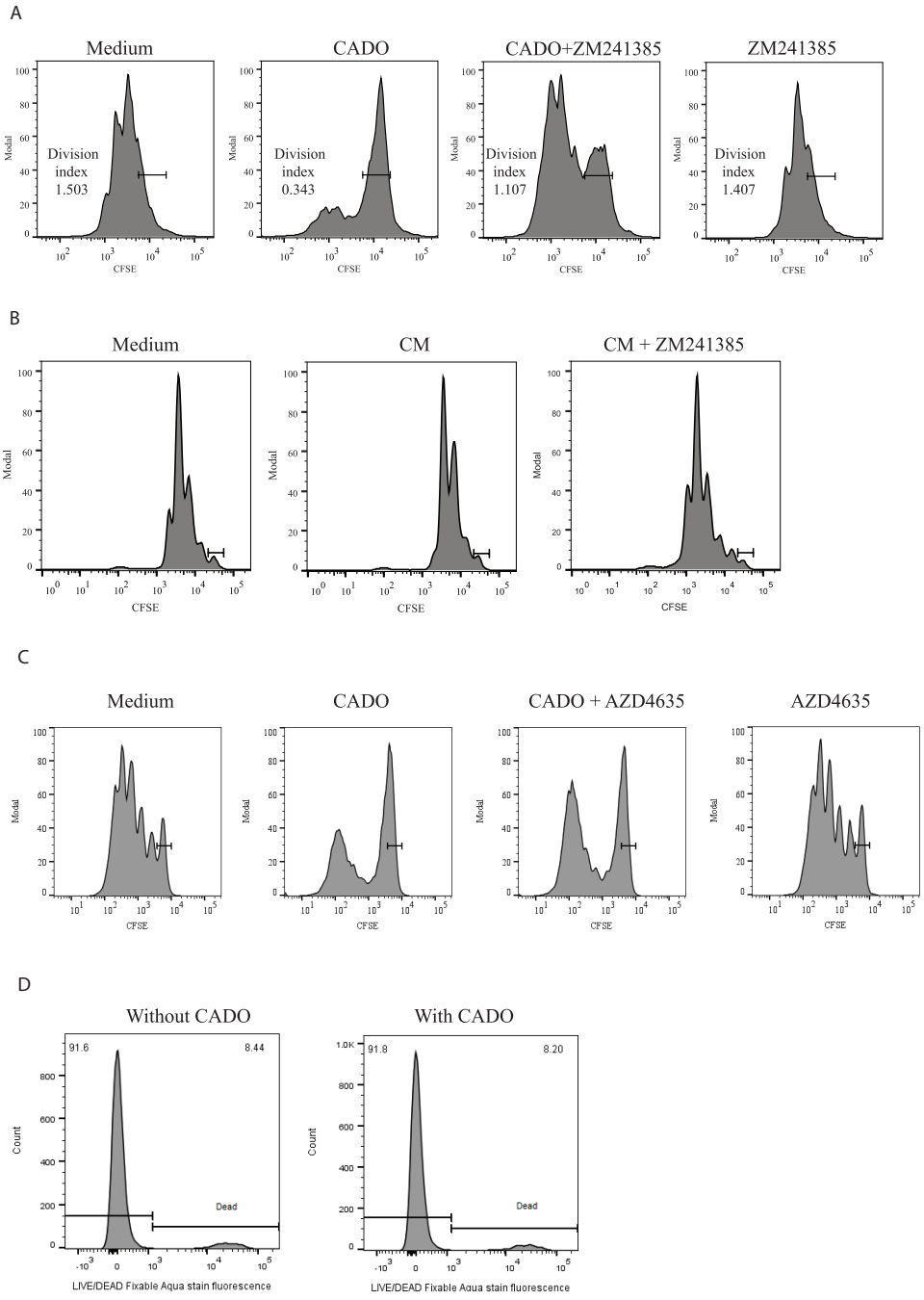

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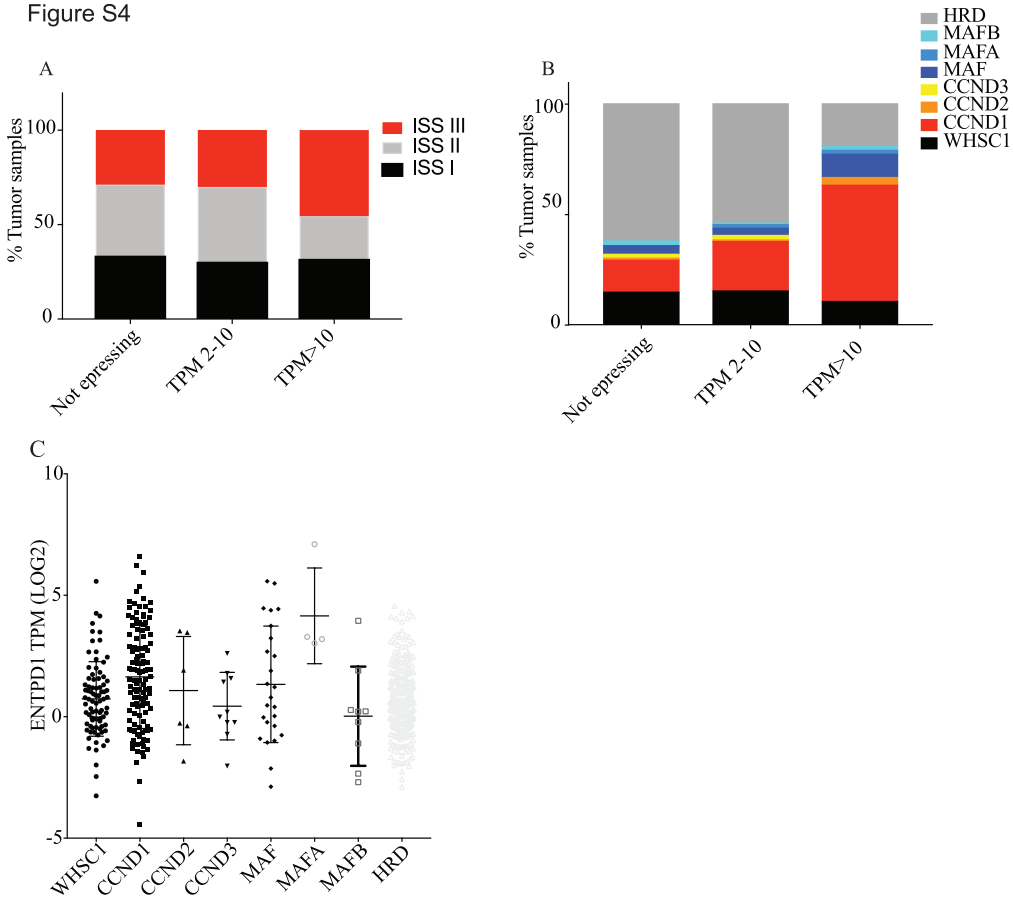
Figure S4



D

Top gene lists from Gene Enrichment Analysis

ETNTPD1 expressers (TPM>2)

ETNTPD1 high expressers (TPM>10)

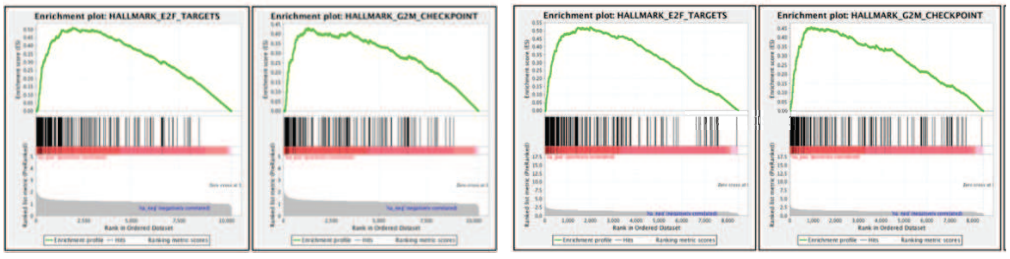


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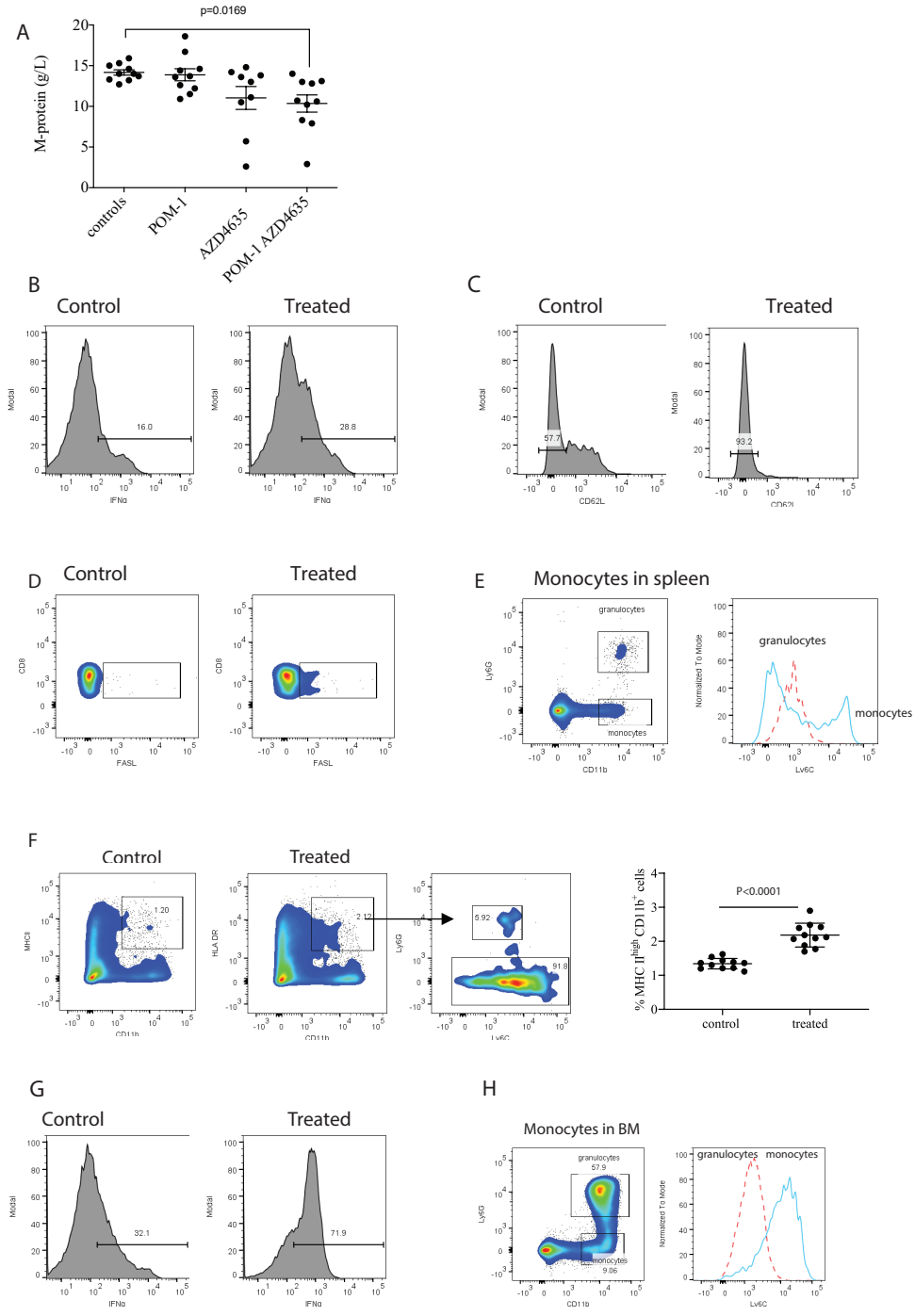
Figure S5



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