Myeloma natural killer cells are exhausted and have impaired regulation of activation

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Supplementary file

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Supplementary Figures: S1-S3

Supplementary figure legends:

Figure S1. Overarching schema of clinical trials gating strategy, and percentage of terminally differentiated NK cells. (A) An overarching schema of the two clinical trials. We examined a newly diagnosed MM (NDMM) patient cohort consecutively treated in the context of a prospective Phase II clinical trial [LITVACC trial (ACTRN12613000344796)][24]; patients were treated with lenalidomide (15-25mg daily for 21 of 28 day cycles) with low dose dexamethasone (0-20mg once weekly for three weeks on a 28 day cycle) prior to subsequent high dose melphalan (200mg/m^2) with ASCT followed by lenalidomide maintenance. Patients with refractory relapsed MM were treated in the context of the RevLite trial (trial number NCT00482261) [25] . Patients were treated with lenalidomide (15mg daily for 21 of 28 day cycles) and dexamethasone (20mg/day for 4 days per week for the first three weeks of each 28 day cycle) [25]. Details of the trials can be accessed from [www.clinicaltrials.gov.au]. Following Peter MacCallum Cancer Centre human ethics committee approval (11-51), peripheral blood was collected at pre-treatment, after 3 treatment cycles, and after 4 treatment cycles for both newly diagnosed (ND) and refractory relapsed (RR) MM patients. Black arrows indicate cycles of lenalidomide and dexamethasone treatment. Red arrows indicate timepoints of peripheral blood collection. In addition, newly diagnosed MM PB samples were investigated after autologous stem cell transplantation (ASCT) when the PB absolute lymphocyte count had reached >1.2x10^9/L. Prior to analysis, the cells were recovered overnight in complete RPMI Media (RPMI with 10% fetal bovine serum, penicillin, streptomycin and glutamax) supplemented with 20U/mL IL2. (B) Gating strategy to identify NK cells for figures 1, 2, 3 and supplementary figures S1, S2, and S3. NK cells were gated on cells that were $CD3^-$, $CD14^-$, $CD19^-$ (Lin^-) and identified as $CD56^{dim}CD16^+$ or $CD56^{hi}CD16^-$ or

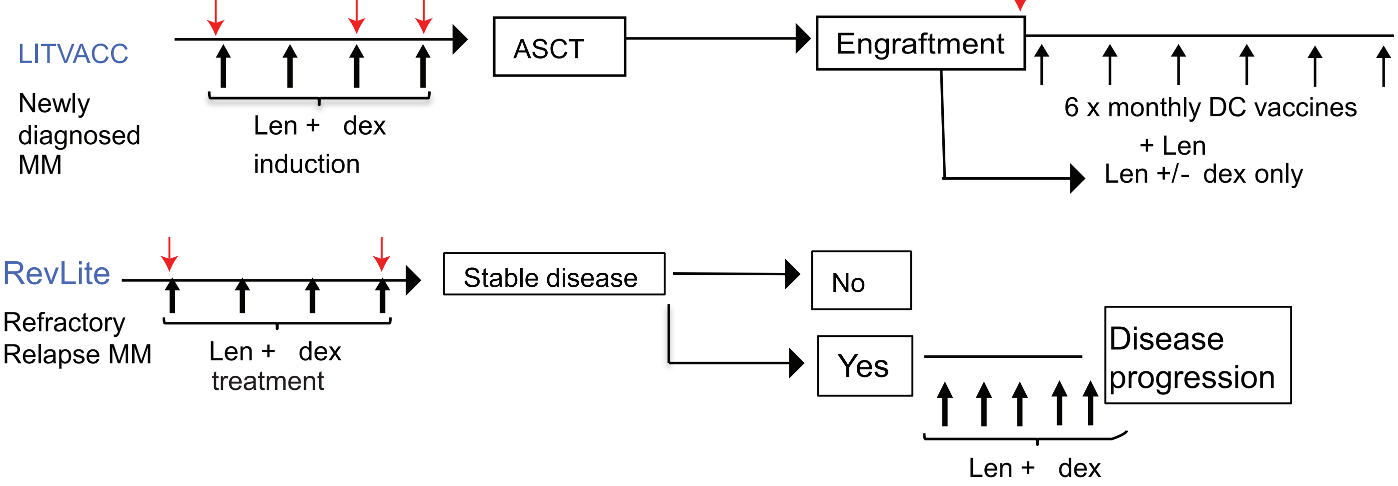
$CD56^{dim}CD16^{-}$. (C) Increased levels of terminally differentiated $CD57^{+}$ NK cells in bone marrow and peripheral blood NK cells. Gated on $CD3^{-}CD56^{+}$ NK cells, the proportion of $CD57^{+}$ NK cells in (C) PBMCs, (D) bone marrow cells of HD, NDMM and RRMM patients at pre-treatment (baseline). (n=5-11 per cohort). *P<0.05 (One way ANOVA with Dunnett's post-hoc test).

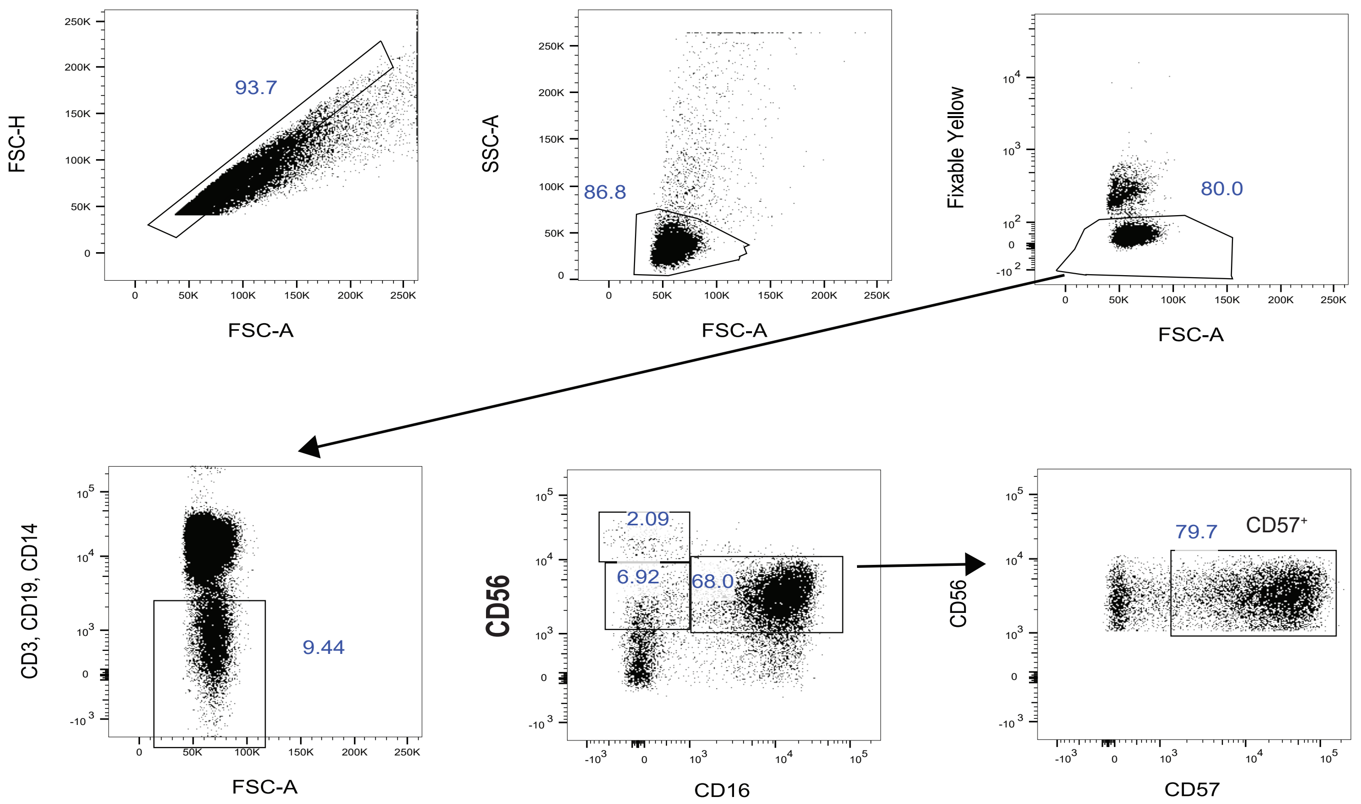
Figure S2. (A) Study schema for RNAseq analysis of NK cell subsets from refractory relapsed MM patients and healthy donors. RNA was extracted from FACS-sorted $CD57^{+}$ or $CD57^{-}$ NK cell subsets isolated from healthy donors (n=6) and refractory relapsed MM patients (n=6). (B) Volcano plot analysis for identification of DEGs in CD57+ NK cell subsets in either healthy donors (upper panel) or patients (lower panel). Gene encoding CD57 (*B3GAT1)* is indicated by arrow. FDR threshold was 0.05. (C) Venn diagram analysis of candidates from (B) to identify DEGs specific to CD57+ NK cells from patients. Venn diagrams indicate shared exclusive gene sets for healthy donor and patient $CD57^{+}$ NK cells. (D) Scatterplot of *ADAM17* transcript levels and mean NK activation gene expression score in healthy donor or RRMM patient samples. Pearson correlation coefficient and confidence interval indicated. (E) Expression levels for NK function related genes were compared between healthy donor and patient $CD57^{-}$ and $CD57^{+}$ NK cell subsets. Student's t-test * p<0.05, ** p<0.01, *** p<0.001 and ****p<0.0001. Volcano plot t-test analysis was performed using a two-sided t-test with 250 randomizations, a false discovery rate (FDR) of 0.05 and an S0 of 0.1. Candidate lists were comprised of genes with p-values < 0.05 and/or an FDR < 0.05. We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB). An FDR of <0.2 was considered significant for GSEA pathway analysis.

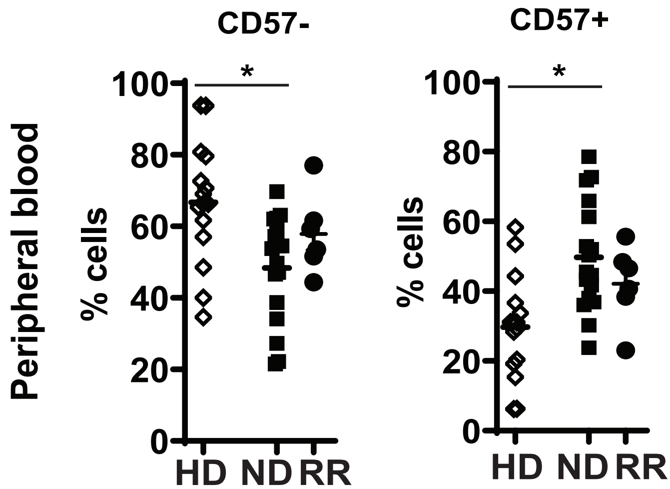
Figure S3: (A) Expression of SLAMF7 on NK cells at baseline, CD107a degranulation of NK cell subsets from HD and MM patients to elotuzumab-labeled myeloma cells, NDMM

patient NK cell responses pre and post ASCT and levels of CD16 on NDMM patient peripheral blood NK cells pre and post-treatment with decreased levels of terminally differentiated $CD57^+$ NK cells post-transplant. (A) SLAMF7 expression levels on NK cells from HD, NDMM and RRMM patients (n=10), One way ANOVA with Sidak's post-hoc test). (B) PBMCs from HD (n=9), and NDMM patients (n=10) or refractory relapsed MM (RRMM) patients (n=10) at baseline (pre-treatment) were cultured with cell trace violet labelled OPM2 target cells in the presence of 10 μg/ml elotuzumab (elo) or human IgG1 (iso) isotype control (at an E:T of 50:1 in the presence of anti-CD107a AF488 for 4 hours at 37°C). Collated data (n=9-10 per group) showing CD107a degranulation by $CD56^{hi}CD16^-$ and $CD56^{dim}CD16^+$ NK cell subsets. Data are pooled from 4 independent experiments. *$p<0.05$, (One-way ANOVA with Bonferroni post-hoc test). (C) Percentage $CD16^+$ NK cells gated on $CD56^+$ NK cells in PBMCs from NDMM patients at pre-treatment (baseline), end of induction (EOI) and post-ASCT (left panel) and percentage $CD57^+$ NK cells gated on $CD56^{dim}CD16^+$NK cells in PBMCs from NDMM patients at baseline, end of induction (EOI) and post-ASCT (right panel). (n=10 per cohort). *$P<0.05$ (One way ANOVA with Dunnett's post-hoc test). (D) PBMCs from HD (n=8), NDMM patients (post induction and post ASCT, n=10) were co-cultured with K562 target cells or OPM2 target cells at an E:T ratio of 2:1 in the presence of elotuzumab or human IgG1 isotype control and assessed for CD107a degranulation by flow cytometry. Graphs represent percentage of $CD107a^+$ cells gated on $CD57^+$ (left panel) and CD57- (right panel) NK cells. (E) Graphs represent amount of cytokines (CCL3, CCL2, CCL5, IFNγ, and TNF) released in supernatant under the same conditions as above quantified using a custom Legendplex assay kit (Biolegend). Data are pooled from 5 independent experiments. *$p<0.05$, Kruskal-Wallis test with Dunn's post hoc test.

Figure S1







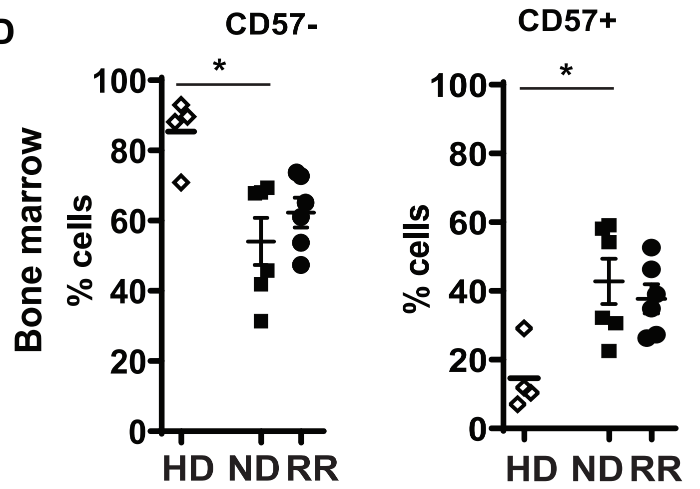
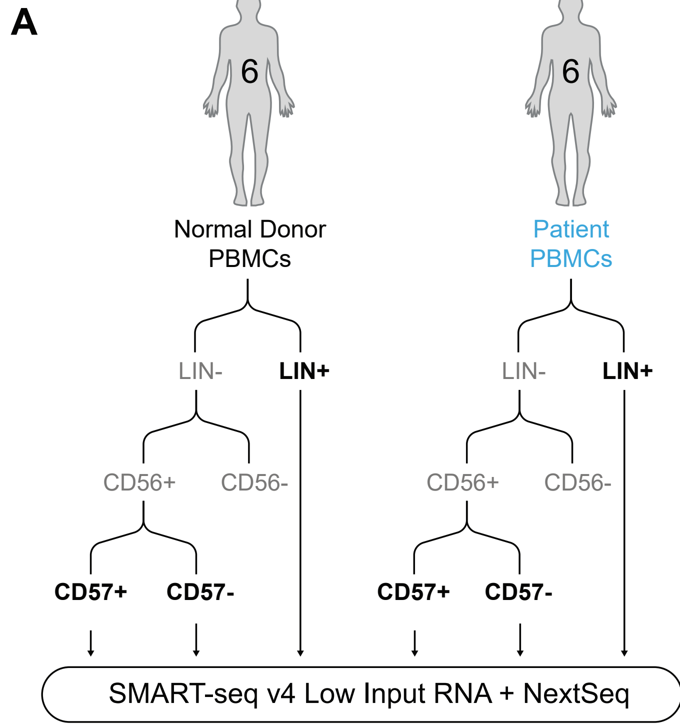
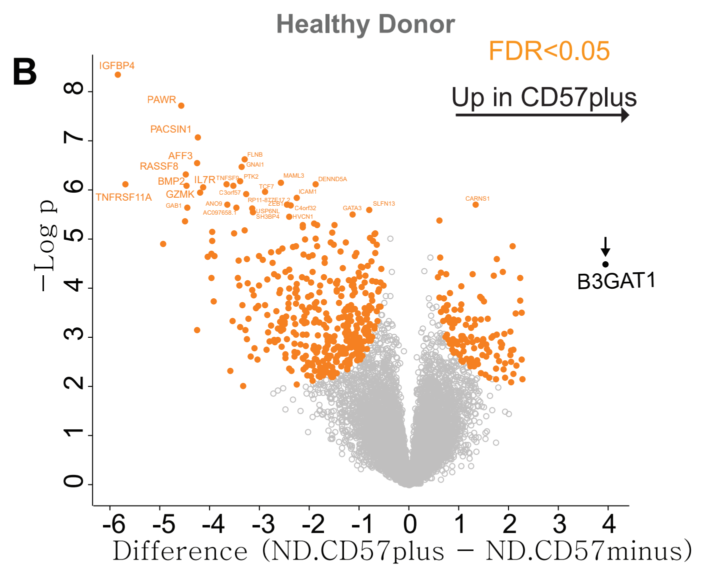
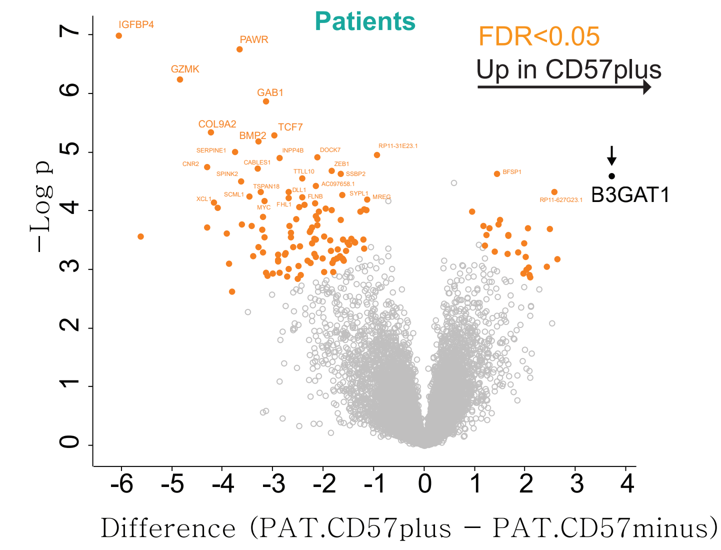
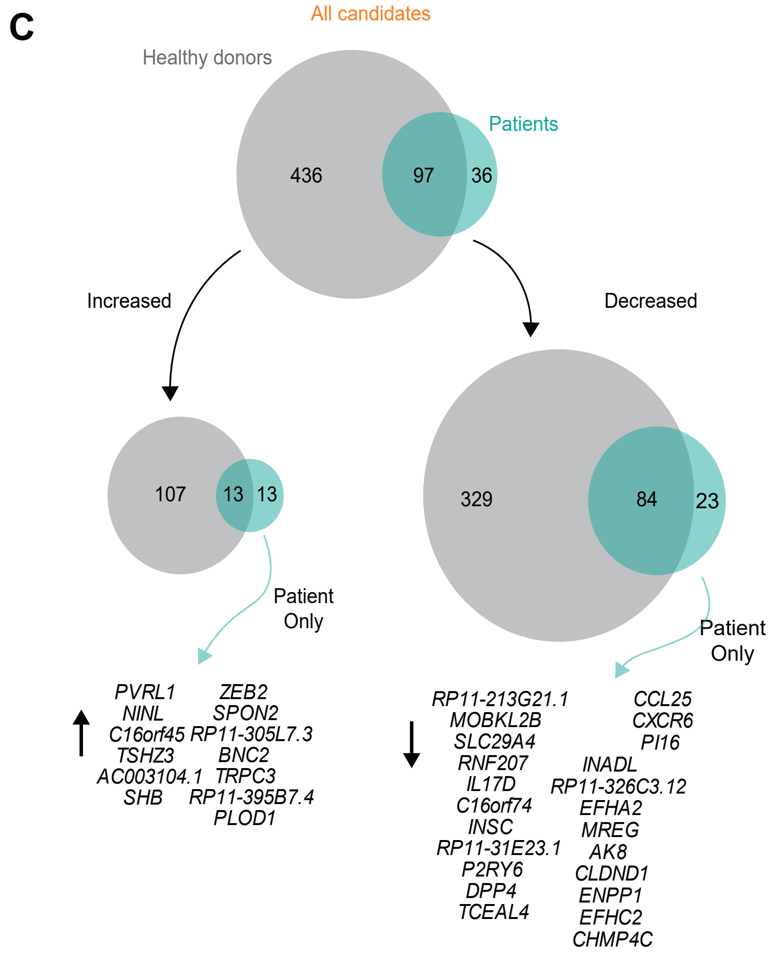


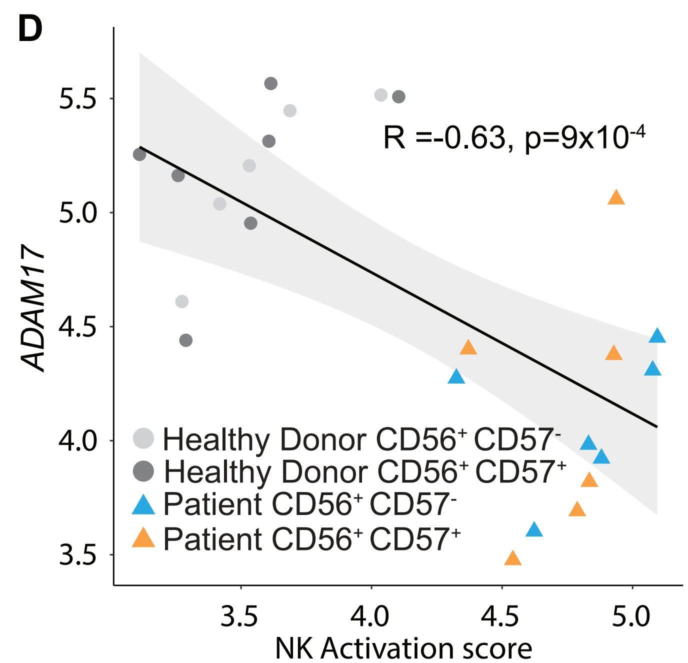
Figure S2

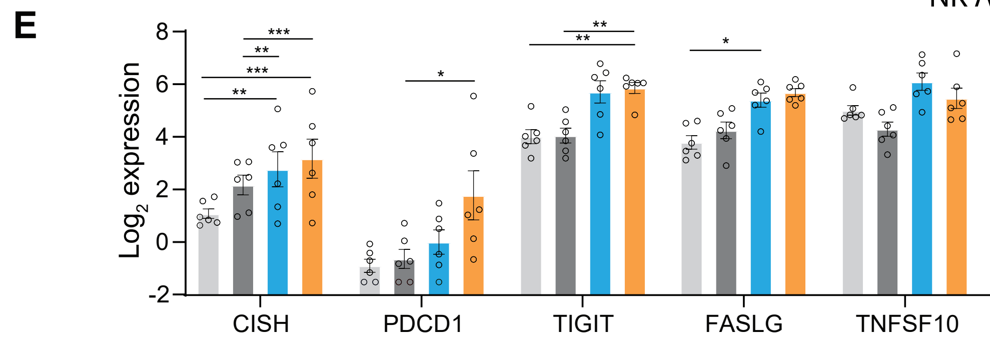












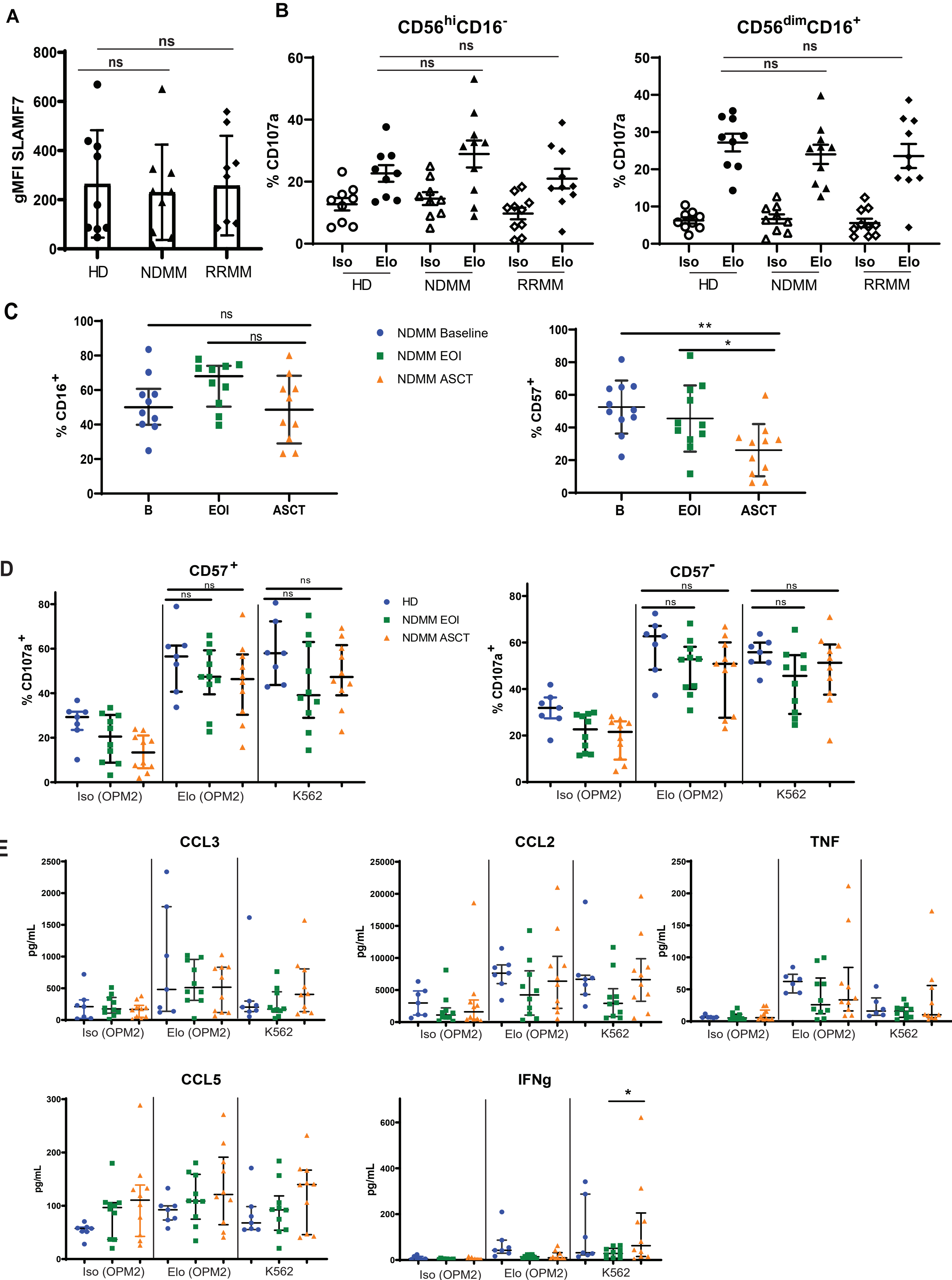


Figure S3
A
B
C
D
E
gMFI SLAMF7
HD
NDMM
RRMM
ns
CD56hiCD16-
CD56dimCD16+
% CD107a
Iso
Elo
% CD16+
% CD57+
NDMM Baseline
NDMM EOI
NDMM ASCT
B
EOI
ASCT
CD57+
CD57-
% CD107a+
HD
Iso (OPM2)
Elo (OPM2)
K562
CCL3
CCL2
TNF
CCL5
IFNg
pg/mL