Myeloma NK cells are exhausted and have impaired regulation of activation

by Criselle D'Souza, Simon P. Keam, Han Xian Aw Yeang, Michael Neeson, Kelden Richardson, Andy K. Hsu, Rachael Canfield, Natalie Bezman, Michael Robbins, Hang Quach, David S. Ritchie, Simon J. Harrison, Joseph A. Trapani, H. Miles Prince, Paul A. Beavis, Philip K. Darcy, and Paul J. Neeson

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Authorship contributions


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Multiple myeloma (MM) is an immunotherapy responsive disease. Treatment strategies including immune-modulatory drugs lenalidomide and pomalidomide, Bi-specific T cell Engagers (BiTE), and antibodies targeting myeloma surface proteins SLAMF7 (elotuzumab) or CD38 (daratumumab and isatuximab) and chimeric antigen receptor T cells have been effective. Currently, myeloma-targeting antibodies against CD38 and SLAMF7 mediate their effect in part, via natural killer cells as key effectors. However, Natural Killer (NK) cells from myeloma patients have decreased functional responses to myeloma in vitro. Despite this, myeloma targeting antibodies that are reliant on NK-cell mediated cytotoxicity have been successful in treating MM patients.

To understand this further, we explored NK cell differentiation and function in newly diagnosed MM patients (NDMM) and, for the first time, gene expression profiles of NK cell subsets from refractory relapsed MM (RRMM) patients. These analyses revealed underlying NK cell intrinsic properties explain this myeloma patient NK cell dysfunction. We also characterised whether NK cell dysfunction was rescued following induction therapy with lenalidomide and dexamethasone and post-autologous stem cell transplantation (ASCT) to understand whether myeloma-targeting antibodies such as elotuzumab could be used at these timepoints.

We compared peripheral blood and bone marrow NK cells from a NDMM patient cohort consecutively treated in the context of a prospective Phase II clinical trial [LITVACC trial (ACTRN1261300344796)] or RRMM patient cohort from the RevLite trial (trial number NCT00482261) to healthy donor (HD) NK cells. We first confirmed that RRMM and NDMM patients have a higher percentage of terminally differentiated CD57+ NK cells compared to HD both in peripheral blood and bone marrow. These RRMM NK cells are dysfunctional. In contrast, HD CD56dimCD16+KIR+CD57+ NK cells are highly cytotoxic and secrete increased levels of IFN-γ in response to contact with targets. To explore reasons for this difference, principal component analysis of RNA sequencing data showed RRMM patient NK cell gene expression profile (GEP) was distinct from HD NK cells. Differential GEP analysis revealed numerous genes either down- or up-regulated in patient or HD CD57+ NK cells. When CD57+ NK cells from myeloma patients and HD were compared, we revealed differentially expressed genes (DEG) (n=133 and 533 DEG respectively), where 97
DEG were common to both RRMM patients and HD (Figure S2C). Of the 36 DEG unique to patient CD57$^+$ NK cells, 13 were up-regulated and 23 were down-regulated (Figure S3C). When NK cell-specific genes were examined, we found decreased expression of genes associated with CD16 cleavage such as ADAM17 in RRMM patient NK cells, increased expression of genes associated with cytotoxicity and activation such as PRF1, GZMB, NCR1, NCR2, and increased expression of novel immune checkpoint genes, CISH and TIGIT (Figure 1B and Figure S2E). Cytokine-inducible SH2-containing protein (CIS, encoded by CISH) is a critical negative regulator of IL-15 signalling and inhibits cytotoxicity against tumour cells.$^9$

Gene set enrichment analysis (GSEA) revealed genes related to NK cell activation pathways were significantly up-regulated in RRMM patient NK cells compared to HD NK cells, suggesting that NK cells from patients are constitutively more activated (Figure 1C). This finding was also true when comparing NK cell activation pathways between RRMM patient and HD CD57$^-$ NK cells or CD57$^+$ NK cells (Figure 1C-D). However, genes related to pathways regulating NK cell activation (IL23A, IL23R, GAS6, IL1B, IL15, AXL, FLT3LG, TICAM1 and PLDN) were downregulated in CD57$^+$ NK cells from RRMM patients, suggesting dysregulation of patient NK cell activation (Figure 1C and 1E). GSEA enrichment plots highlight significantly increased MM patient NK cell activation, yet co-existing suppression of positive regulation of these activation pathways (Figure 1F). ADAM17 transcript levels also correlated negatively with NK cell activation in RRMM patients as compared to HD (Figure S2D). Taken together, these data indicate MM patient CD57$^+$ NK cells are constitutively more activated than their normal donor counterparts. However, they lack expression of key regulators of NK cell activation and have increased levels of the NK cell immune checkpoint molecules CIS and TIGIT, suggesting an ‘exhausted’ state.

We next explored whether NK cell chronic activation and low levels of ADAM17 observed in the GEP data in Figure 1 would affect the capacity of NK cells to respond via CD16 or SLAMF7 mediated signalling. To do this, peripheral blood mononuclear cells (PBMCs) from NDMM and RRMM patients and healthy donors were co-cultured with OPM2 myeloma targets and the anti-human SLAMF7 antibody, elotuzumab. In this context, activated NK cells were expected to down-regulate CD16 due to cleavage by ADAM17,$^{10}$ and this would be evident by a reduction in the %CD56$^{dim}$CD16$^+$ NK cells. Only HD NK cells significantly decreased the % of CD56$^{dim}$CD16$^+$ NK cells in response to elotuzumab (Figure 2A and 2B, left
panel), which was inhibited in the presence of an ADAM17 inhibitor (Figure 2C). Whilst there was a trend to decreased CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells in NDMM patients, this did not reach significance. We observed a similar result for terminally differentiated CD56\textsuperscript{dim}CD57\textsuperscript{+} NK cells. HD NK cells were responsive to activation via OPM2 cells and elotuzumab and significantly reduced the %CD56\textsuperscript{dim}CD57\textsuperscript{+} NK cells (Figure 2B, right panel). In the same conditions, untreated NDMM NK cells showed a trend for decreased %CD56\textsuperscript{dim}CD57\textsuperscript{+}CD16\textsuperscript{+} NK cells (p=0.051), whereas RRMM NK cells were relatively unresponsive. No difference was observed in % of CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells in RRMM patients in the presence of ADAM17 inhibitor (Figure 2C). Prior studies demonstrated no loss of NK cells in PBMC treated with elotuzumab at higher concentrations than used in our assays \textsuperscript{11}, suggesting fratricide was unlikely to occur. Our data supports this as the SLAMF7 levels on NK cells between MM patients and HD were similar (Figure S3A). Subsequently, NK cell subsets were examined for degranulation (CD107a\textsuperscript{+}) in the presence of OPM2 cells and elotuzumab. Of all NK cell subsets, only the CD56\textsuperscript{dim}CD16\textsuperscript{−} NK cells degranulated at significantly higher levels in healthy donors compared to both groups of MM patients (Figure 2C and Figure S3B). A similar trend was also observed for the HD vs myeloma patient CD57\textsuperscript{+} NK cells, but this did not reach significance. These results suggest that low levels of ADAM17 may lead to constitutive activation of NK cells via CD16, causing NK cell exhaustion in MM patients.

We then investigated whether NDMM patient NK cell cytotoxicity recovered post-induction treatment or post-ASCT and if they can be targeted with monoclonal antibody therapy. To reveal myeloma patient NK cell killing potential, we investigated their cytotoxicity against the MHC class I negative erythroid-leukemia cell line, K562 (Figure 3A), their antibody-dependent cellular cytotoxicity (ADCC) capacity against OPM2 myeloma cells with elotuzumab (Figure 3B), or an isotype control (Figure 3C). After induction treatment or ASCT, NK cells from newly diagnosed MM patients killed K562 cells at equivalent levels to healthy donor NK cells (Figure 3A). In contrast, NDMM patient NK cells were significantly less efficient at myeloma cell ADCC than HD NK cells, requiring higher numbers of NK effectors to achieve target lysis (Figure 3B); this reduced ADCC function was present after induction therapy, and after ASCT (Figure 3B). Finally, in the presence of an isotype control, myeloma patient NK cells were significantly less efficient than HD NK cells at killing myeloma
targets (Figure 3C). Taken together, this data suggests whilst myeloma patient NK cells have cytotoxic potential, they are unable to effectively kill myeloma targets.

There was no difference in CD16+ NK cells from pre- and post-induction treatment (Figure S3C); however, there were less mature CD57+ NK cells post-ASCT (Figure S3C). NK cell CD107a degranulation (although lower) was not significantly different in NDMM patients compared to HD at the post-induction or ASCT timepoints (Figure S3D). These findings reveal an apparent separation between NK cell degranulation and effective cytotoxicity against myeloma cells (but not against K562). This was previously observed in a model system where phospholipase \( \gamma \)2 signalling was impaired\(^\text{12}\) and also when adhesion was impaired between effector and target cells\(^\text{10}\). No apparent differences were observed in the level of cytokine TNF and chemokines (CCL3, CCL2 and CCL5) secreted by NK cells in the same co-culture conditions (Figure S3E).

A recent study also demonstrated that continual lenalidomide treatment of MM patients did not improve NK cell function with a lower ADCC response and decreased reactivity against K562 target cells\(^\text{13}\). These observations are similar to our findings suggesting that lenalidomide treatment alone is insufficient to rescue MM patient NK cell function \textit{in vivo}. In contrast, \textit{in vitro} lenalidomide-treated healthy donor NK cells up-regulate genes for IL2/STAT5, mTORC1 and TNF signalling pathway suggesting activation (data not shown).

In summary, our results showed that NK cells in MM patients are chronically stimulated with an increase in terminally differentiated NK cells and loss of regulation of activation. This scenario is plausible considering the bone marrow is a site of myeloma disease as well as NK cell development and maturation. Thus repetitive stimulation by the myeloma cells would impact NK cell maturation. In addition, we showed lenalidomide and dexamethasone combination treatment did not repair this intrinsic NK cell defect in MM. To address this issue, future combination immunotherapy approaches could use a tumour targeting antibody (eg Daratumumab, anti-CD38) with agonistic anti-CD137 mAb\(^\text{14}\) or anti-TIGIT\(^\text{15}\) to rescue NK cell dysfunction in MM.
References

Figure legends

Figure 1. Gene expression analysis of NK cell subsets from RRMM patient and donor PBMCs reveals increased activation but loss of regulatory pathways in myeloma patient CD57+ NK cells. Refractory relapsed multiple myeloma patient and healthy donor NK cells were FACS-sorted to CD57+ and CD57− subsets, RNA extracted and RNAseq performed using the SMART-seq v4 low input RNA kit (Takara Bio USA) and sequenced on the NextSeq 550 sequencing system (Illumina, USA). The 36 samples, each containing on average 14,496,483 reads, were aligned using seqliner v0.7.1 to hg19 reference genome and quantified using Htseq v0.6.1 software. Normalisation and differential expression analysis was performed with Limma-Voom in R v3.3.3 on a total of 20,850 genes. (A) Overarching differences in healthy donor and myeloma NK cell subset GEP are depicted in two-dimensional principal component analysis (PCA) of patient or healthy donor in four groups (n=6 per group). (B) Normalised Log2 counts-per-million (cpm) transcript levels of B3GAT1 (CD57), ADAM17, PRF1 (Perforin), GZMB (Granzyme B), FCGR3A (CD16), SLAMF7, KIR3DL2 and KIR2DL1. Protein products are indicated in parentheses. Statistical analysis performed using Student’s t-test * p<0.05, ** p<0.01, *** p<0.001 and ****p<0.0001. (C) Schema showing directionality of GSEA comparisons performed between the four NK cell groups (upper panel) and bubble chart of GSEA analysis NES and FDR scores when compared to curated NK-related gene sets from MSigDB (lower panel). Red arrows indicate analyses depicted in heatmaps and running enrichment score (ES) analysis. GSEA heatmaps for all replicates for (D) Patient CD57+ versus healthy donor CD57+ cells in NK Cell Activation pathways in GO, and (E) Patient CD57+ versus Patient CD57- cells in GO: Positive Regulation of NK cell activation pathway. (F) Running Enrichment Score (ES) analysis of panels (D) and (E).

Figure 2. CD56+ NK cells from MM patients are hypo-responsive to elotuzumab-labelled myeloma cells. PBMCs from healthy donors (n=9), and newly diagnosed MM (NDMM) patients (n=10) or refractory relapsed MM (RRMM) patients (n=10) at baseline (pretreatment) were cultured with OPM2 target cells in the presence of 10 μg/ml elotuzumab (elo) or human IgG1 (iso) isotype control. Shown in (A) histogram overlay of changes in CD16 expression on CD56dimCD16+ subset of NK cells. (B) Percentage distribution of NK cell
subsets (left panel) or %CD16+ on CD56dimCD57+ NK cells (right panel) in HD, newly diagnosed MM and RRMM patient PBMCs after treatment under the same conditions as above. (C) Percentage distribution of CD56dimCD16+ subset of NK cells in HD and RRMM patient PBMCs after treatment under the same conditions as above in the presence or absence of ADAM17 inhibitor (n=5 per group) (D) Collated data for healthy donor (HD), newly diagnosed (ND) MM and refractory relapsed (RR) MM patients (n=9-10 per group) showing CD107a degranulation by different NK cell subsets. Data are pooled from 4 independent experiments. *p<0.05, One-way ANOVA with Bonferroni post-hoc test.

Figure 3. NK cells from newly diagnosed MM patients show significantly lower myeloma ADCC response post-induction therapy and post-ASCT. A standard 4-hour chromium release assay was used to assess NK cell function, adapted from Hsu et al.\(^5\). PBMCs from healthy donors (n=8) and newly diagnosed MM at the timepoints end of induction (EOI) and post-autologous stem cell transplant (ASCT), n=10 per group, were co-cultured with K562 target cells to determine NK cell natural cytotoxicity levels (A), or with OPM2 myeloma target cells and 10 μg/ml elotuzumab (B) or human IgG1 isotype control (C) to determine ADCC capacity. Cytotoxicity was assessed by chromium (\(^{51}\)Cr) release assays and the data displayed as % target cell lysis (A-C, left panels). Each line represents a non-linear regression curve for healthy donors (blue line), or newly diagnosed MM at EOI (green line) and post ASCT (orange line) timepoints at the indicated effector: target cells ratios (normalised for the percentage of NK cells). Inserted bar graphs on the right for (A), (B) and (C) show the NK effector: target ratio required to achieve 40% target lysis (A, K562), 20% target lysis (B, OPM2 with elotuzumab) and 10% target lysis (C, OPM2 with isotype control) target lysis, extrapolated from the non-linear regression curves on the left. Each symbol represents an individual patient or healthy donor. Data are pooled from 5 independent experiments. ** p<0.01, * p<0.05, Student’s t test.
**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\(^{\text{dim}}\)CD16\(^+\) or CD56\(^{\text{hi}}\)CD16\(^-\) or
CD56<sup>dim</sup>CD16<sup>-</sup>. (C) Increased levels of terminally differentiated CD57<sup>+</sup> NK cells in bone marrow and peripheral blood NK cells. Gated on CD3<sup>-</sup>CD56<sup>+</sup> NK cells, the proportion of CD57<sup>+</sup> 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<sup>+</sup> or CD57<sup>-</sup> 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<sup>+</sup> 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<sup>+</sup> NK cells from patients. Venn diagrams indicate shared exclusive gene sets for healthy donor and patient CD57<sup>+</sup> 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<sup>-</sup> and CD57<sup>+</sup> 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 CD56hiCD16− and CD56dimCD16+ 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 CD56dimCD16+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

A

LITVACC
Newly diagnosed MM
Len + dex induction
ASCT
Engraftment
6 x monthly DC vaccines + Len Len +/- dex only

RevLite
Refractory Relapse MM
Len + dex treatment
Stable disease
No
Yes
Disease progression
Len + dex

B

93.7
68.0
6.92
2.09
80.0

CD3, CD19, CD14

CD56

CD57

C

CD57-
CD57+

Peripheral blood
Bone marrow

D

CD57-
CD57+

% cells
% cells
% cells
% cells
**Figure S2**

A. Normal Donor PBMCs

Healthy Donor PBMCs

SMART-seq v4 Low Input RNA + NextSeq

B. Healthy Donor

FDR<0.05

Up in CD57plus

-Log p

Difference (ND.CD57plus - ND.CD57minus)

C. Patients

FDR<0.05

Up in CD57plus

-Log p

Difference (PAT.CD57plus - PAT.CD57minus)

D. R =-0.63, p=9x10^-4

ADAM17

NK Activation score

E. Log expression

CISH, PDCD1, TIGIT, FASLG, TNFSF10