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

Multiple myeloma (MM) is an immunotherapy responsive disease. Treatment strategies including immunemodulatory 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.[1-4] Currently, myeloma-targeting antibodies against CD38 and SLAMF7 mediate their effect in part, via natural killer (NK) cells as key effectors.[1,2] However, NK cells from myeloma patients have decreased functional responses to myeloma *in vitro*.[5] Despite this, myeloma targeting antibodies that are reliant on NK-cell mediated cytotoxicity have been successful in treating MM patients.[2]

In order 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 that underlying NK-cell intrinsic properties explain this myeloma patient NK-cell dysfunction. We also characterized 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 time points.

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 LITVACC trial (clinicaltrials gov. Identifier: ACTRN12613000344796)[6] or RRMM patient cohort from the RevLite trial (clinicaltrials gov. Identifier: NCT00482261)[7] to healthy donor (HD) NK cells (for details see *Online Supplementary Figure S1*).

We first confirmed that RRMM and NDMM patients have a higher percentage of terminally differentiated $CD57^+$ NK cells compared to HD both in the peripheral blood and bone marrow (*Online Supplementary Figure S1C and D*). These RRMM NK cells are dysfunctional. In contrast, HD $CD56^{dim}CD16^+KIR^+CD57^+$ NK cells are highly cytotoxic and secrete increased levels of interferon-γ (IFN-γ) in response to contact with targets.[8] In order 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 (Figure 1A; *Online Supplementary Figure S2A*). Differential GEP analysis revealed numerous genes either down- or up-regulated in patient or HD $CD57^+$ NK cells (*Online Supplementary Figure S2B*). 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 (*Online Supplementary Figure S2C*). Of the 36 DEG unique to patient $CD57^+$ NK cells, 13 were up-regulated and 23 were down-regulated (*Online Supplementary 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; *Online Supplementary Figure S2E*). Cytokine-inducible SH2-containing protein (CIS, encoded by CISH) is a critical negative regulator of IL-15 signaling and inhibits cytotoxicity against tumor 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 and D). However, genes related to pathways regulating NK-cell activation (*IL23A, IL23R, GAS6, IL18, 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 E). 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 (*Online Supplementary 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 signaling. In order to do this, peripheral blood mononuclear cells (PBMC) from NDMM and RRMM patients and HD 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 percentage of $CD56^{dim}CD16^+$ NK cells. Only HD NK cells significantly decreased the percentage of $CD56^{dim}CD16^+$ NK cells in response to elotuzumab (Figure 2A and B, left panel), which was inhibited in the presence of an ADAM17 inhibitor (Figure 2C). Whilst there was a trend to decreased $CD56^{dim}D16^+$ NK cells in NDMM patients, this did not reach significance. We observed a similar result for terminally differentiated $CD56^{dim}CD57^+$ NK cells. HD NK cells were responsive to activation via OPM2 cells and elotuzumab and significantly reduced the percentage of $CD56^{dim}CD57^+$ NK cells (Figure 2B, right panel). In the same conditions, untreated NDMM NK cells showed a trend for decreased percentage of $CD56^{dim}CD57^+CD16^+$ NK cells (P=0.051), whereas RRMM NK cells were relatively unresponsive. No difference was observed in the percentage of $CD56^{dim}CD16^+$ 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,[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 (*Online Supplementary Figure S3A*). Subsequently, NK cell subsets were examined for degranulation ($CD107a^+$) in the presence of OPM2 cells and elotuzumab. Of all NK cell subsets, only the $CD56^{dim}CD16^-$ NK cells degranulated at significantly higher levels in HD compared to both groups of MM patients (Figure 2C; *Online Supplementary Figure S3B*). A similar trend was also observed for the HD *versus* myeloma patient $CD57^+$ NK cells, but this did not reach significance. These results suggest that low levels of ADAM17 may lead to consti-

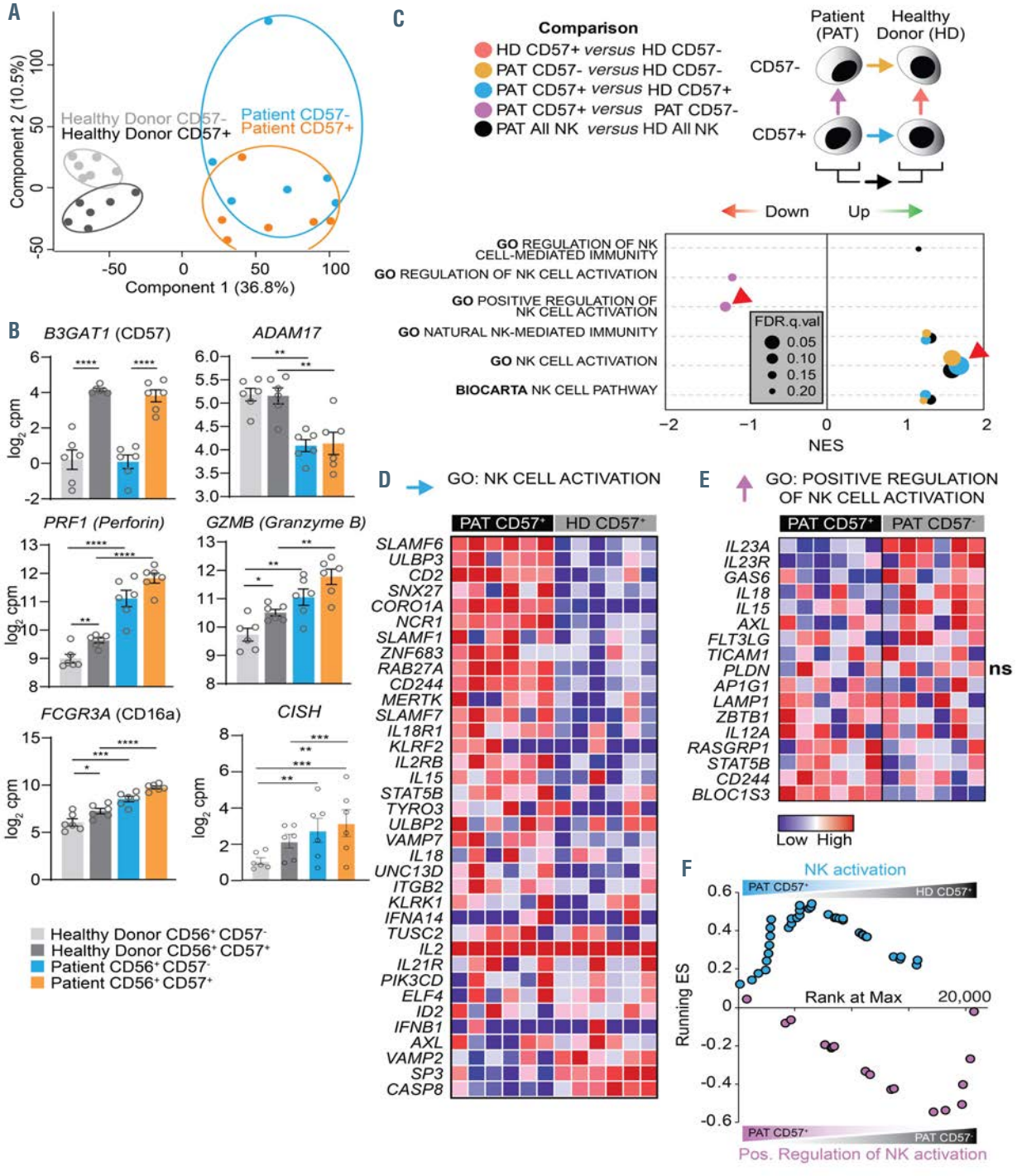

Figure 1. Gene expression analysis of natural killer (NK) cell subsets from refractory relapsed multiple myeloma patient and donor peripheral blood mononuclear cells reveals increased activation but loss of regulatory pathways in myeloma patient CD57⁺ NK cells. Refractory relapsed multiple myeloma (RRMM) patient and healthy donor (HD) NK cells were FACS-sorted to CD57⁺ and CD57⁻ subsets, RNA extracted and RNA sequencing 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. Normalization 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 HD and myeloma NK cell subset GEP are depicted in two-dimensional principal component analysis (PCA) of patient or HD in four groups (n=6 per group). (B) Normalized $\log_2$ 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⁺ *vs.* HD CD57⁺ cells in NK-cell activation pathways in GO, and (E) patient CD57⁺ *vs.* patient CD57⁻ cells in GO: positive regulation of NK-cell activation pathway. (F) Running enrichment score (ES) analysis of panels (D) and (E).

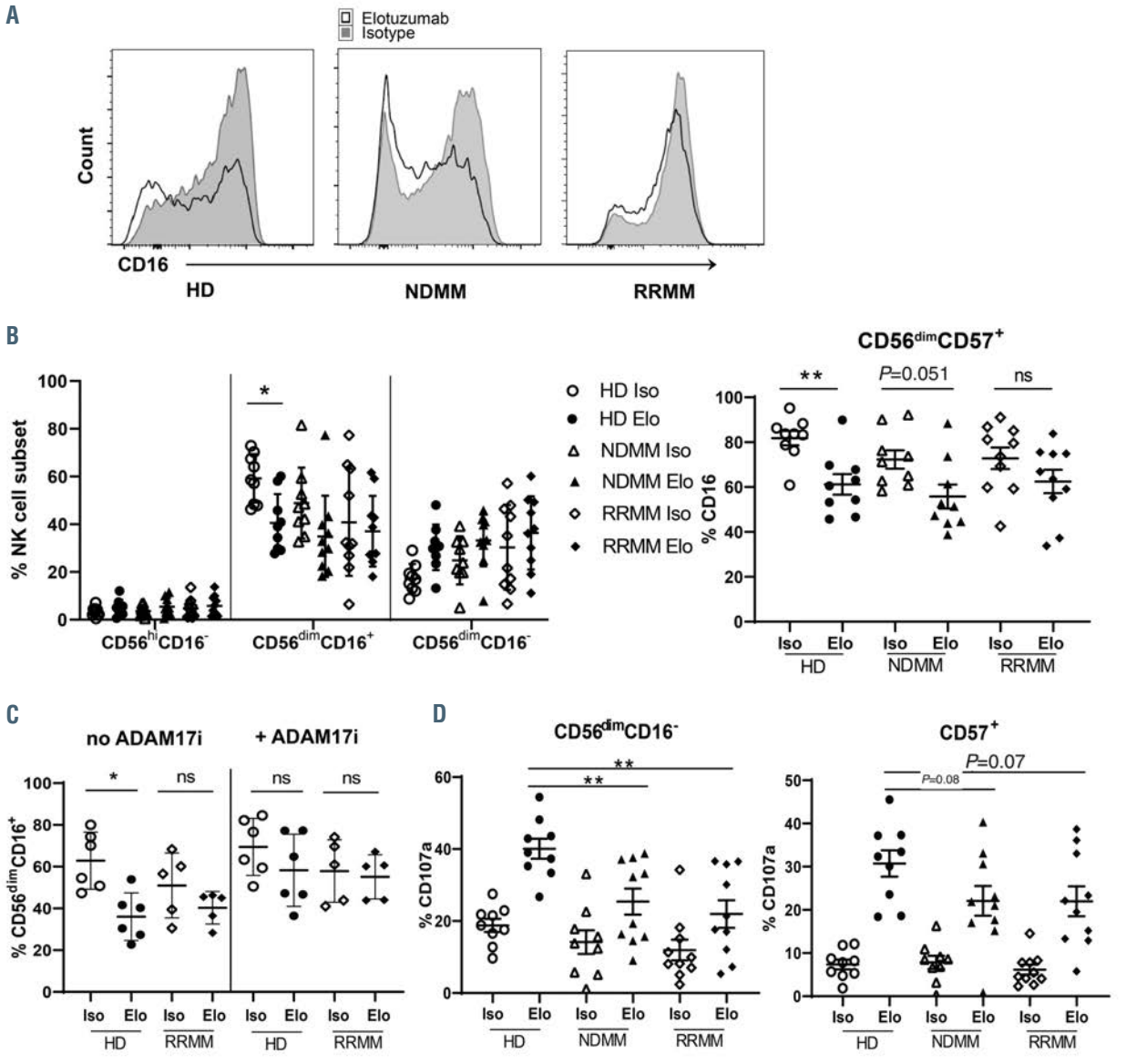

Figure 2. CD56^{+} natural killer cells from multiple myeloma patients are hypo-responsive to elotuzumab-labeled myeloma cells. Peripheral blood mononuclear cells (PBMC) from healthy donors (n=9), and newly diagnosed mulptiple myeloma (NDMM) patients (n=10) or refractory relapsed MM (RRMM) patients (n=10) at baseline (pre-treatment) 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 CD56^{dim}CD16^{+} subset of natural killer (NK) cells. (B) Percentage distribution of NK-cell subsets (left panel) or percentage of CD16^{+} on CD56^{dim}CD57^{+} NK cells (right panel) in healthy donor (HD), newly diagnosed MM and RRMM patient PBMC after treatment under the same conditions as above. (C) Percentage distribution of CD56^{dim}CD16^{+} subset of NK cells in HD and RRMM patient PBMC after treatment under the same conditions as above in the presence or absence of ADAM17 inhibitor (n=5 per group) (D) Collated data for HD, NDMM and refractory relapsed (RR) MM patients (n=9-10 per group) showing CD107a degranulation by different NK-cell subsets. Data are pooled from four independent experiments. *P<0.05, One-way ANOVA with Bonferroni *post-hoc* test.

tutive 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. In order to reveal myeloma patient NK cell killing potential, we investigated their cytotoxicity against the MHC class I negative erythro-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 HD 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 (*Online Supplementary Figure S3C*); however, there were less mature CD57^{+} NK

cells post-ASCT (*Online Supplementary 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 (*Online Supplementary 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 γ2 signaling was impaired,[12] and also when adhesion was impaired between effector and target cells.[10] No apparent differences were observed in the level of cytokine TNF and chemokines (CCL3,

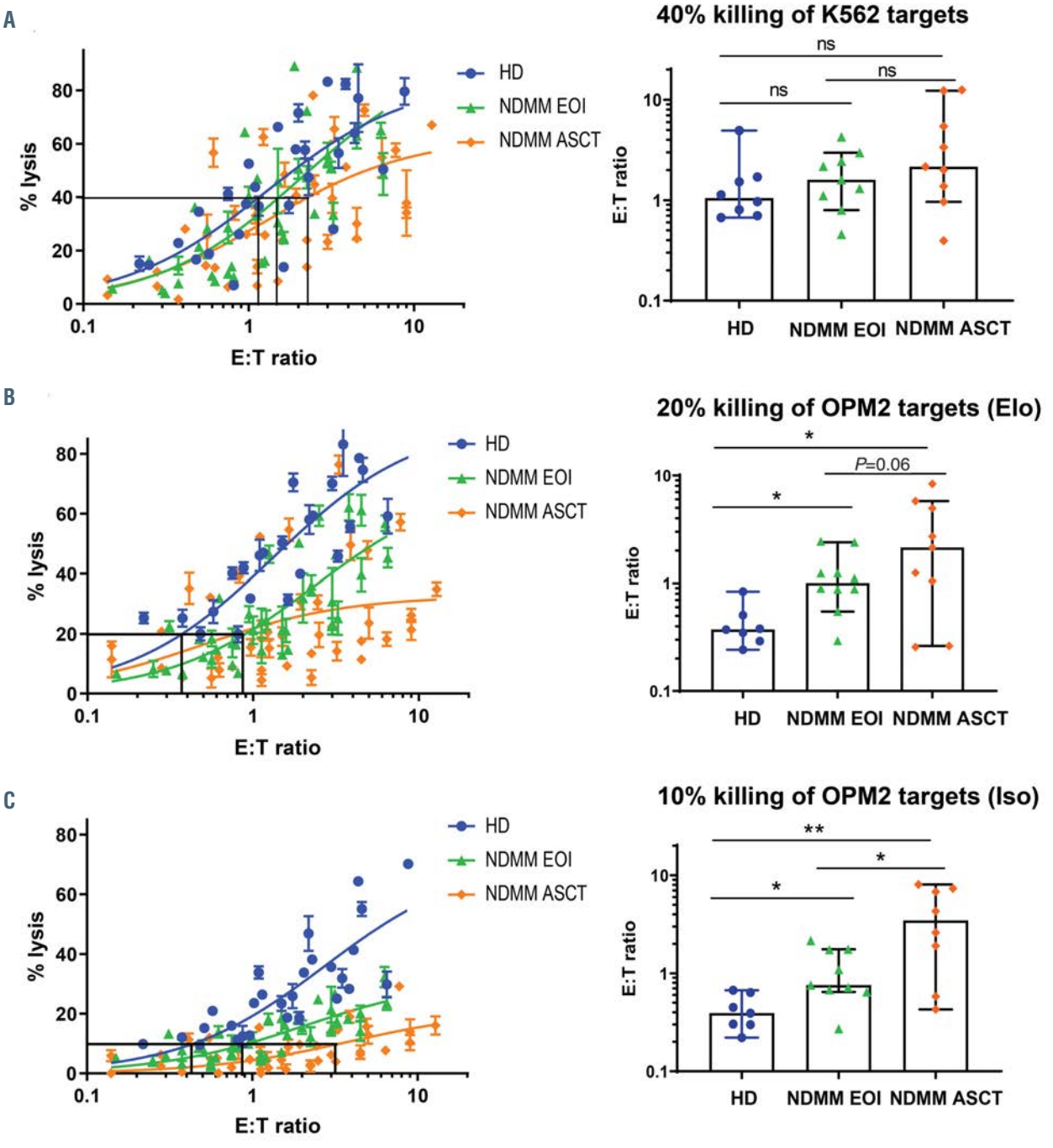

Figure 3. Natural killer cells from newly diagnosed multiple myeloma patients show significantly lower myeloma antibody-dependent cellular cytotoxicity response post-induction therapy and post-autologous stem cell transplant. A standard 4-hour chromium release assay was used to assess natural killer (NK) cell function, adapted from Hsu *et al*.[5] Peripheral blood mononuclear cells (PBMC) from healthy donors (HD) (n=8) and newly diagnosed multiple myeloma (NDMM) patients at the time points end of induction (EOI) and post-autologous stem cell transplant (post-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 (Elo) (B) or human IgG1 isotype (iso) control (C) to determine antibody-dependent cellular cytotoxicity (ADCC) capacity. Cytotoxicity was assessed by chromium (^{51}Cr) release assays and the data displayed as percentage of target cell lysis (A to C, left panels). Each line represents a non-linear regression curve for HD (blue line), or NDMM at EOI (green line) and post-ASCT (orange line) time points at the indicated effector:target (E:T) cells ratios (normalized for the percentage of NK cells). Inserted bar graphs on the right for (A), (B) and (C) show the NK E:T 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 HD. Data are pooled from five independent experiments. ***P*<0.01, * *P*<0.05, Student's *t*-test.

CCL2 and CCL5) secreted by NK cells in the same co-culture conditions (*Online Supplementary 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.[13] These observations are similar to our findings suggesting that lenalidomide treatment alone is insufficient to rescue MM patient NK cell function *in vivo*. In contrast, *in vitro* lenalidomide-treated HD 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. In order to address this issue, future combination immunotherapy approaches could use a tumor targeting antibody (e.g., Daratumumab, anti-CD38) with agonistic anti-CD137 mAb14 or anti-TIGIT15 to rescue NK-cell dysfunction in MM.

Criselle D'Souza,[1,2] Simon P. Keam,[1,2,3] Han Xian Aw Yeang,[1] Michael Neeson,[1] Kelden Richardson,[1] Andy K. Hsu,[1] Rachael Canfield,[1] Natalie Bezman,[4°] Michael Robbins,[5°] Hang Quach,[6,7] David S. Ritchie,[3,7,8] Simon J. Harrison,[3,8] Joseph A. Trapani,[1,2] H. Miles Prince,[1,2,8] Paul A. Beavis,[1,2] Phillip K. Darcy[1,2] and Paul J. Neeson[1,2*]*

[1]Cancer Immunology Program, Peter MacCallum Cancer Center, Melbourne, Victoria, Australia; [2]Sir Peter MacCallum Department of Oncology, University of Melbourne, Victoria, Australia; [3]Tumor Suppression and Cancer Sex Disparity Laboratory, Sir Peter MacCallum Cancer Center, Melbourne, Victoria, Australia; [4]Oncology Discovery Research, Bristol-Myers Squibb, Redwood City, CA, USA; [5]Translational Medicine, Bristol-Myers Squibb, Cambridge, MA, USA; [6]Department of Hematology, St Vincent's Hospital, Melbourne, Victoria, Australia; [7]Faculty of Medicine, University of Melbourne, Melbourne, Victoria, Australia and [8]Clinical Hematology, Sir Peter MacCallum Cancer Center and Royal Melbourne Hospital, Melbourne, Victoria, Australia

°NB current address: Arsenal Bio, San Francisco, CA, USA

°MR current address: io904 LLC, Jacksonville Beach, FL, USA

Correspondence: PAUL J. NEESON - paul.neeson@petermac.org

CRISELLE D'SOUZA - criselle.dsouza@petermac.org

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