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Received: October 4, 2021.
Accepted: June 21, 2022.

Citation: Kelden Richardson, Simon P. Keam, Joe Jiang Zhu, Deborah Meyran, Criselle D’Souza, Sean Macdonald, Kerry Campbell, Michael Robbins, Natalie A. Bezman, Kirsten Todd, Hang Quach, David S. Ritchie, Simon J. Harrison, H. Miles Prince, Joseph A. Trapani, Misty R. Jenkins, Paul A. Beavis, Phillip K. Darcy, and Paul J. Neeson.
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Title: Combination elotuzumab and lenalidomide treatment efficacy is dependent on crosstalk between NK cells, monocytes and myeloma cells

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Running Title (50 chars):

Elo and Len induced mechanisms of myeloma killing
Key Words:
Multiple myeloma, natural killer cells, elotuzumab, SLAMF7, immunomodulatory drugs

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Disclosures:
PJN received research grant funding from BMS for this study. He also received research project funding from Roche Genentech, Allergan, Compugen, Advaxis, MSD, Crispr Therapeutics.

Data availability statement: Data are available on reasonable request, and will be available on request as per our institute policy.
Abstract

Patients with refractory relapsed multiple myeloma (RRMM) respond to Elotuzumab (Elo) and lenalidomide (Len) combination treatment. The mechanisms underlying this observation are not fully understood. Furthermore, predictive biomarkers of response have not been revealed to date. To address these issues, we used a humanized myeloma mouse model and adoptive transfer of human NK cells to show that Elo and Len treatment controlled myeloma growth, and this was mediated through CD16 on NK cells. In co-culture studies, we showed that PBMCs from a subset of RRMM patients were effective killers of OPM2 myeloma cells when treated with Elo and Len, and this was associated with significantly increased expression of CD54 expression on OPM2 cells. Furthermore, Elo and Len induced OPM2 cell killing and increased OPM2 CD54 expression was dependent on both monocytes and NK cells, and was not mediated by soluble factors alone. At the transcript level, Elo and Len treatment significantly increased OPM2 myeloma cell expression of genes for trafficking and adhesion molecules, NK cell activation ligands and antigen presentation molecules. In conclusion, our findings suggest that MM patients require Elo and Len-mediated upregulation of CD54 on the autologous myeloma cells, in combination with NK cells and monocytes to mediate an effective anti-tumour response. Further, our data suggest that increased myeloma cell CD54 expression levels could be a powerful predictive biomarker for response to Elo and Len treatment.
Introduction

Multiple Myeloma (MM) is an incurable plasma cell malignancy of the bone marrow and is the second most common hematological malignancy in Western countries (1). To address the need for new and effective treatment options, monoclonal antibodies (mAbs) targeting myeloma cells have been investigated for their potential to treat MM and enhance current therapies (2). Elotuzumab is a humanised IgG1 antibody which targets SLAMF7 (also known as CS1, CRACC or CD319), which is a homotypic adhesion molecule highly expressed on >90% of MM plasma cells (3, 4). Elo exerts its cytotoxic effects by binding to SLAMF7 on the MM plasma (target) cell and in turn activating NK cells via their FcγRIII (CD16a), inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of the target cell (3, 4). SLAMF7 may also control cellular activation due to the cytoplasmic domain comprised of an immunoreceptor tyrosine-based switch motif (ITSM). The presence of the adaptor protein EAT-2 allows transduction of an activation signal upon SLAMF7 ligation (5, 6). NK cells express both SLAMF7 and EAT-2 and have shown enhanced activation and activity upon direct binding of Elo to SLAMF7 expressed on NK cells (7).

In clinical trials, RRMM patients treated with combination Elo, Len and dexamethasone in the ELOQUENT-2 study showed improved objective response rates of 79% and improved overall survival compared to Elo therapy alone (8-10). Despite these encouraging outcomes, the mechanisms whereby combination Elo and Len treatment improves disease control have not been resolved. Furthermore, there is currently no reliable biomarker to predict which patients will respond to Elo and Len with long term remission.

To address this issue, we explored NK cell functional responses and gene expression profile (GEP) changes in untreated newly diagnosed and refractory relapsed MM (RRMM) patients in the context of Elo and Len treatment. We also explored whether other immune cell types were required to support this Elo- and Len-induced increase in myeloma cell cytotoxicity. Prior studies have shown that macrophages phagocytose myeloma targets in response to Elo activation (11). However, it is
also known that MM patients have an irregular monocyte population with a high proportion of monocyte-like myeloid derived suppressor cells (MDSCs) in the peripheral blood and bone marrow (12). In this study, we explored whether monocytes were required for the Elo and Len induced increase in myeloma patient NK cell cytotoxic responses. We also investigated Elo and Len treatment-induced changes in the myeloma cells at both the transcript and protein level. Our study revealed for the first time the mechanisms whereby Elo and Len treatment is effective - involving complex interactions between myeloma cells, NK cells and monocytes. In addition, our study revealed a novel potential predictive biomarker for combination Elo and Len treatment response in myeloma patients.
Methods

Cell culture

Jurkat (human T lymphoblast line, ATCC), LCL (EBV-transformed lymphoblastoid B-cell line) and human multiple myeloma cell lines, OPM-2 and RPMI8226.hS1AMF7 cells (FCC), were maintained in RPMI-1640 media (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Healthy normal donor blood buffy coats were obtained from the Australian Red Cross Blood Service under the Ethics approval granted by the Peter MacCallum Cancer Centre Human Research and Ethics committee (HREC# 01/14). Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient (Ficoll-Paque Plus, GE Healthcare Life Science). Multiple myeloma patient blood samples were obtained from either Peter MacCallum Cancer Centre patients enrolled in the LITVACC (newly diagnosed MM) or REVLITE (refractory/relapsed MM) clinical trials (Trial Numbers 12613000344796 and NCT00482261 respectively; details available at www.anzctr.gov.au) or directly from consenting patients, after approval from the Peter MacCallum Cancer Centre human ethics committee. The studies using patient PBMCs were covered under the Peter MacCallum Cancer Centre HREC approval number 11-51. PBMCs were maintained in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM nonessential amino acids, 50 µM 2-ME, and 100 U/mL penicillin, and 100 µg/mL streptomycin containing 25 U/mL human rIL-2.

Purification and culture of primary human NK cells

NK cells were isolated from human peripheral blood by negative selection using the MACS human NK Isolation Kit (130-092-657, Miltenyi) and LS columns (MACS, Miltenyi Biotec). NK cells were maintained at 10^6 cells/mL in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated FCS, 2
mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 μM 2-ME, and 100 U/mL penicillin, and 100 μg/mL streptomycin containing 25 U/mL human rIL-2.

**Expansion of NK cells**

Purified NK cells were co-cultured with irradiated LCL and Jurkat feeder cells (100 gy, X-RAD 320) at a 1:1:1 ratio and maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 10% heat-inactivated human AB-serum (Lonza), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 1000 U rhIL-2 and 50 ng/ml rhIL-15. Cell cultures were split, and cytokines were replenished every 2-3 days for 3-4 weeks.

**Humanised myeloma mouse model**

Female NOD-scid IL2Rγc−/− (NSG) mice were bred in-house and were inoculated with 2e6 RPMI8226.hSLAMF7 cells in the lower right flank. Two doses of expanded human NK cells (1e7 cells/dose) were transferred via intravenous injection, 24 hrs apart, when tumours measured 10 mm² in area (Length X Width, where Length is the longest side of the tumour). Mice received exogenous cytokines (5e5 U human recombinant IL-2 and 2.5 μg human recombinant IL-15) i.p. and were treated with 5 mg/kg Elo, 50 mg/kg Len, 5 mg/kg Pomalidomide or vehicle control i.p., 3 times per week for 3 weeks. All in-vivo experiments were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee (AEEC number E602). In this humanised myeloma model, the human NK cells and myeloma cells (RPMI8226.hSLAMF7) were the only cells capable of responding to Len or Pom via human cereblon (13).

**Supplementary materials and methods** include details for cytotoxicity assays (flow cytometry and Cr-release), reagents and antibodies, cytokine bead array assay, immune cell depletions, RNA sequencing analysis, Statistics and Bioinformatics analyses.
**Results**

**Elotuzumab induced NK cell killing of myeloma targets and is enhanced in vivo by Lenalidomide.**

In prior clinical trials in patients with relapsed/refractory MM, treatment with a combination of Elo and Len led to enhanced survival and disease control compared to Elo alone (9, 10). In this study, we explored the mechanisms underlying these observations by first performing *in vivo* adoptive therapy of myeloma-bearing NSG mice in the presence of Elo or Elo and Len treatment. To do this, we developed a human NK cell expansion protocol based on prior studies (14, 15) (Fig 1A, Fig S1). Expanded (day 21) NK cells were CD56^{hi}CD16^{\pm} and the CD16^{+} subset expressed NK activation receptors (NKp30^{hi}Nkp44^{hi}Nkp46^{hi}NKG2D^{+}), inhibitory receptors (KIR^{+}NKG2A^{hi}TIGIT^{+}) and were CD69^{hi} indicating activation (Fig S1A-B). Expanded NK cells induced significantly higher killing of OPM2 cells, in the presence or absence of Elo, compared to day 0 NK cells (Fig S1B). To assess the *in vivo* efficacy of Elo and Len treatment, NSG mice were inoculated subcutaneously with human SLAMF7 expressing RPMI18226 (RPMI8226.hSLAMF7) myeloma cells. RPMI8226 cells are innately resistant to direct Len-induced cytotoxicity (13), allowing us to control for the effect of Len on NK activity. To explore the importance of NK cell CD16 for the Elo and Len anti-tumour effect, we treated established tumours with expanded NK cells plus either Elo or Elo FcγR mutant (does not bind CD16), in the presence or absence of Len (Fig 1B). This data showed that transferred NK cells and combination Elo and Len treatment resulted in the most effective control of myeloma tumour growth and led to improved mouse survival, compared to NK cells with Len or Elo alone (Fig 1C-D). The transfer of NK cells along with Elo FcγR mutant had no effect on tumour growth inhibition, indicating an important role for NK cell CD16 in tumour control mediated by Elo (Fig 1C-D).

Pomalidomide (Pom) is a 2nd generation IMiD (16), and Elo and Pom combination treatment mediated improved myeloma control in refractory relapsed myeloma patients (17). We tested Pom in combination with Elo in our humanized myeloma NK cell adoptive therapy model. In this experiment, we treated myeloma-bearing mice with expanded NK cells, Elo and Len or Elo and Pom.
NSG mice treated with adoptively transferred NK cells and combination Elo and Len or Elo and Pom had significantly better myeloma tumor control compared to NK cells and Elo alone (Fig S2). Taken together, Elo combination treatment with either Len or Pom controlled myeloma tumor outgrowth via NK cell mediated ADCC and required NK cell CD16 expression.

**NK cells from refractory relapsed MM patients have divergent cytotoxic response to combination Elotuzumab and Lenalidomide treatment.**

We previously showed that NK cell cytotoxic function is poor in RRMM patients with advanced disease (18). We next explored whether RRMM NK cells can respond to Elo and Len treatment *in vitro* and kill myeloma cells. PBMC’s from healthy donors (HD), newly diagnosed MM (NDMM) patients and RRMM patients were co-cultured with OPM2 tumour cells and treated with Elo or Elo and Len for 24 hours. Flow cytometry was used to assess PBMC phenotypes (Fig 2A), as well as the level of cytotoxicity directed towards OPM2 myeloma cells (Fig 2B). Healthy donor and NDMM PBMC’s increased cytotoxicity in response to Elo alone or Elo and Len, but not with Len alone (Fig 2B). Interestingly, 7/11 RRMM PBMC’s had very poor or no cytotoxic response to Elo and Elo and Len treatment, but 4/11 RRMM PBMC’s significantly increased killing of OPM2 myeloma cells. These samples are highlighted in red for the remainder of the study (Fig 2B). With the exception of these highly cytotoxic RRMM PBMC’s, these results demonstrate the loss of NK cell cytotoxic capacity on MM disease progression (Fig 2C and Fig S3) and is consistent with our prior data (18). We next investigated potential biomarkers, which identified RRMM patients with high versus low cytotoxic RRMM PBMC’s to stratify patients for Elo and Len treatment. Loss of CD16 expression by NK cells indicates early activation via engagement with the antibody Fc domain, and this CD16 loss is mediated by ADAM-17 (19). In the presence of OPM2 tumour cells plus Elo and Len treatment, HD NK cells lost significantly more surface CD16 than NDMM or RRMM NK cells (Fig 2D and Fig S3B-C). Supernatants taken from these co-cultures were then analyzed for IFNγ, TNF (Fig 2E-F), MIP-1α,
MIP-1β and RANTES (Fig S4). In HD NK cells, IFNγ and TNF levels were significantly increased compared to NDMM and RRMM NK cells, when co-cultured PBMCs and OPM2 cells were treated with Elo, or Elo and Len (Fig 2E-F). In the same co-culture system and drug treatment combinations, similar data was observed for MIP-1α and MIP-1β (Fig S4). In addition, RRMM patient PBMCs with a high cytotoxicity response against OPM2 cells also secreted high levels of RANTES (CCL5), the equivalent of that observed in HD PBMC’s (red triangles in Fig S4). To further identify differences in HD and MM patient NK cell responses to Elo and Len treatments, cell surface expression of CD54, CD107a and CD69 was measured and compared to untreated NK cells (ΔMFI) (Fig 3A). CD54 (ICAM1) is a surface glycoprotein with an important role in NK cell adhesion with target cells. Upon activation, NK cell CD54 is upregulated and assists in the formation of the immune synapse through its engagement with LFA1 (20). In our co-culture assays, HD NK cells significantly upregulated CD54 in response to Elo alone, Len alone, or Elo and Len treatment with the highest NK cell expression of CD54 resulting from treatment with the Elo and Len combination (Fig 3B). No significant difference in CD54 expression was observed between treatment groups in NDMM and RRMM NK cells (Fig 3B), and expression levels were significantly less than on HD NK cells (Fig 3B). However, when myeloma cell death was significantly increased, the RRMM patient NK cells (Fig 2B, highlighted in red) upregulated CD54 in response to Elo and Len, with expression levels similar to HD NK cells. We therefore hypothesized that CD54 upregulation would increase NK cell cytotoxic potential and may serve as a biomarker for NK cell responsiveness to Elo and Len therapy in the clinic. In contrast, NK cell CD69 and CD107a expression did not associate with increased killing of OPM2 myeloma cells. We found that the early activation marker CD69 was significantly upregulated in response to Elo in all three patient cohorts (Fig 3C). However, Elo-induced RRMM NK cell CD69 expression was significantly lower than NDMM and HD NK cell expression, despite the divergence in RRMM NK cell cytotoxicity. Additionally, treatment with Len reduced the expression of CD69 (Fig 3E). CD107a is a transmembrane protein expressed on secretory vesicles and traditionally serves as a marker for the exocytosis of cytolytic granules at the plasma membrane cell surface. Surprisingly, all patient NK cells
were capable of Elo-induced degranulation, with no significant difference observed between cohorts (Fig 3D), regardless of the degree of cytotoxicity. This confirms recent data from Pazina et al. (21) and suggests that in myeloma, NK cell degranulation, as measured by surrogate CD107a exposure, does not equate to myeloma target cell death. It was also observed that, like CD69, the addition of Len resulted in reduced CD107a expression (Fig 3E). It is known that the interaction between CD54 and LFA1 is essential for the microtubule-organizing centre (MTOC) and lytic granules to polarize to the immune synapse between the effector and target cells, prior to delivery and target cell death (22). While CD107a indicates degranulation in all samples, low levels of CD54, as seen in the poorly cytotoxic RRMM NK samples, may result in unpolarized, non-direct secretion of lytic granules into the media instead of the target cells. There is prior evidence that this can indeed occur. In an in vitro model system, human NK cells degranulated when activated via NKG2D and 2B4 simultaneously but did not induce killing of target cells. Rather target cell killing by NK cells required simultaneous CD11A, NKG2D and 2B4 co-receptor engagement (23).

**Healthy donor and myeloma patient NK cells have distinct gene signatures in response to combination elotuzumab and lenalidomide treatment**

To gain insight into the functional differences between NK cells derived from RRMM patients, we used whole transcriptome RNA sequencing on sorted NK cells from either RRMM patients (RRMM24, RRMM26 and RRMM30) or healthy donors (HD231, HD234 and HD237) and explored changes which could explain our observations. Prior to NK cell sorting, RNA extraction, and whole-transcriptomic RNA sequencing, HD or patient PBMC’s were first treated with control Ig, Len, Elo or combination Len plus Elo (n=3 each group) (Fig 4A). We also performed a cytotoxicity assay, using PBMCs from the same healthy donor controls and myeloma patients, to simultaneously evaluate OPM2 myeloma cell killing (Fig 4B). Once again, we observed that healthy donor PBMCs induced increased myeloma cell killing and responded more effectively to Elo and Len treatment. To explore these differences at
the transcriptional level, we first used differential expression gene (DEG) analysis to find genes upregulated by the different treatments. We identified that donor NK cells exhibited a large number of changes, particularly in response to Elo alone and Elo plus Len treatment (2054-2265 DEGs), and fewer following Len treatment alone (144 genes) (Fig S5A). Conversely, RRMM patients responded much less, with 22 DEGS in Elo alone, and Elo and Len treatments, and only 1 following Len treatment alone. Approximately half of these RRMM DEGs were also conserved with donor NK cells, indicating a shared molecular response to the treatments. Principle component analysis of the expression level of all genes identified that despite variation between the donor sources, the treatments had predictable effects on healthy donor NK cells (Fig 4C). However, this was not observed in the RRMM patient NK cells, with no clear organised response to any of the treatments. We also observed that patient NK cells exhibited dramatically different expression profiles when directly compared (Fig S5B) to healthy donor NK cells. We hypothesized that changes in canonical NK functional responses to the treatments were fundamentally different between donor and patient NK cells. To explore this, we used gene set enrichment analysis (GSEA) of canonical functional NK cell pathways and identified that patient NK cells were generally less stimulated by treatments but with little difference between the pathways (Fig S5C). We next selected gene signatures of NK cytotoxicity or regulation of activation, and associated changes in the activity of these specific signatures, relative to control treatment, and the killing ability of the cognate NK cells—as shown in Figure 5A and B, respectively. The results showed that healthy donor NK cells were overall more enriched for both cytotoxicity and activation functions compared to RRMM NK cells. However, the different treatments did not correlate with the overall stimulation of either cytotoxic or regulation of activation GSEA scores. Finally, we compared the expression levels of the individual genes within the GO regulation of NK cell activation pathways between healthy donors and RRMM patients (Fig SC). We observed that RRMM patient NK cells exhibited different expression patterns overall compared to healthy donor NK cells even in the absence of any treatment, suggesting that NK cells from RRMM patient possess fundamentally different NK activation states. Despite this, Elo treatment (either as
monotherapy or in combination) led to increased expression of many genes in this pathway. Interestingly, patient RRMM24 appeared to not exhibit this response to Elo. In summary, Elo and Len treatment induced expression of genes enriched in the cytotoxicity and activation pathways in healthy donor but not RRMM NK cells.

**Elotuzumab and Lenalidomide induced upregulation of OPM2 cell CD54 requires NK cells and monocytes**

We next explored the effect of Elo and Len treatment on OPM2 myeloma target cells. When HD PBMCs were co-cultured with OPM2 cells and drug treatments, the OPM2 cells had significantly increased expression of CD54 in Elo and Len treated compared to Elo treatment alone (**Fig 6A**). Interestingly, this effect was also observed in the RRMM patient PBMCs with high levels of OPM2 killing (red triangles **Fig 6A**). Furthermore, OPM2 killing correlated with increased expression of CD54 on OPM2 target cells when co-cultured with RRMM patient PBMCs in the context of Elo and Len treatment (**Fig 6B**). CD11a was also upregulated on OPM2 cells in response to Elo alone, and was further increased when combined with Elo and Len treatment, indicative of similar mechanisms of regulation (**Fig S6**). The presence of adhesion and activation molecules on effector and target cells is essential to form a stable immune synapse to enable effective killing. The upregulation of CD54, and its receptor CD11a, on both NK and target cells, particularly in response to Elo and Len treatment indicates greater potential for conjugate formation and activation. This is also consistent with observed levels of OPM2 cell killing by NK cells following combination therapy in RRMM patients where CD54 was robustly upregulated. The inflammatory cytokine TNF is known to increase CD54 expression on myeloma cells (24). In our study the greatest changes in expression of CD54 on OPM2 cells occurred following Elo and Len treatment, and as TNF secretion was not further enhanced by Len (**Fig 2E-F**), we hypothesized that additional mechanisms contributed to this effect.
To explore this further, we investigated which immune cell subsets were important to induce increased OPM2 myeloma cell killing and CD54/CD11a expression after Elo and Len treatment. PBMCs depleted for specific cell subtypes were co-cultured with OPM2 target cells and treated with Elo and Len. Depletion of either CD8⁺ or CD4⁺ T cells had no effect on the level of OPM2 killing or the expression of CD54 on both NK and OPM2 cells (Fig S7). This was unexpected because Len is known to enhance IL2 secretion from CD4⁺ T cells, which in turn further activates NK cells (18). The greatest change to killing of OPM2 cells occurred when CD14⁺ monocytes were depleted (Fig 6C). Lack of CD14⁺ monocytes reduced the level of killing of OPM2 cells after treatment with Elo and Len and also abrogated upregulation of CD54/CD11a on the tumour cells (Fig 6D, Fig S8A). To investigate if CD14⁺ cells were directly responsible for increased OPM2 CD54 and CD11a expression in response to Elo and Len, CD14⁺ cells isolated from PBMC were co-cultured with OPM2 cells. This revealed that CD14⁺ cells alone did not induce an increase in OPM2 CD54/CD11a expression in the context of Elo and Len treatment. However, low levels of OPM2 killing were observed supporting a role for monocytes in the killing of OPM2 cells (Fig 6E). Indeed antibody-dependent cellular phagocytosis has been described previously as a mechanism for Elo (11). When NK cells were depleted from HD PBMCs, this reduced the overall level of OPM2 cell killing and CD54/LFA1 expression on OPM2 cells after combined Elo and Len treatment (Fig 6C-D). Furthermore, when NK cells alone were co-cultured with OPM2 cells with Elo and Len treatment, OPM2 killing was significantly increased compared to that induced by monocytes, but significantly less than that induced by whole PBMCs (Fig 6E). This suggests both monocytes and NK cells were required to induce optimal OPM2 myeloma cell killing following combination therapy. To further validate this, when NK cells were combined with CD14⁺ monocyte cells, a complete recovery of Elo and Len induced target cell CD54/CD11a expression and target killing was observed, demonstrating crucial cross-talk between these cell types and the target OPM2 myeloma cells (Fig 6E-F, Fig S8B). Finally, we observed that soluble mediators alone were unable to mediate these changes in CD54 expression on the OPM2 myeloma cells (Fig S8C), inferring that increased OPM2 CD54 expression required cell-to-cell interactions. These results
imply that patients lacking in functional monocytes may not benefit from this mechanism of Elo + Len induced priming of target cells for more efficient NK cell lysis.

**Combination Elotuzumab and lenalidomide stimulate cytokine and chemokine activity in OPM2 myeloma cells**

Our analysis of transcriptional changes in RRMM patient NK cells (see Fig 4-5) suggests that neither cytotoxicity, nor NK activation pathways were sensitive to the combination Elo and Len treatment over Elo alone. We therefore hypothesised that differential responses amongst other cell types (i.e. tumour cells or monocytes) may be responsible for driving differences in killing capacity. We therefore investigated the transcriptional response in these cells from healthy donor cells to identify differences between their responses that may explain our data. Similarly to Figure 4, we performed whole transcriptome analysis of NK cells, monocytes (CD14⁺) and OPM2 tumor cells sorted from healthy donor PBMC and OPM2 co-cultures treated with the three drug combinations (Fig 7A). We previously showed Elo and Len treatment induced increased CD54 expression on both NK cells and OPM2 cells (Fig 3B and 6A). Here, we confirmed that Elo and Len treatment increased ICAM-1 expression on OPM2 cells over monotherapy, but this was not observed in monocytes (Fig 7B). We used an unbiased gene ontology enrichment strategy to identify novel pathway stimulated by the combination treatment. To use as input for pathway enrichment analysis, we first identified DEGs increased by each treatment (relative to control treatment) and in the three cell types. The results, shown in Fig 7C, revealed that Elo alone, or Elo and Len treatment had the highest number of DEGs overall, but dramatically fewer DEGs were found in monocytes and OPM2 cells when compared to NK cells. In addition, many DEGs were unique to each cell type, suggesting that there are some unique effects that are dependent on the recipient cell. To further explore these DEGs, we used ontological enrichment within the GO: Molecular Function database to identify novel functional changes in monocytes and OPM2 cells (Fig 8A-B, Fig S9A-B). The results revealed that cytokine and chemokine pathways were increased in monocytes in response to both Elo or Elo and Len
combination treatment. Lenalidomide alone did not have any significant effects. In contrast, OPM2 cells exhibited most significant changes in response to the combination only (shown in green), including in the same cytokine- and chemokine-related pathways. Interestingly, we did identify that MHC Class II pathways were strongly upregulated by the combination treatment, mostly comprised of HLA-related genes (Fig S9B). As gene ontology suggested that cytokine and chemokine expression is affected in both OPM2 and monocytes, we next explored the role of a custom list of NK receptors and ligands for expression differences in response to the different treatments. The results, shown in Fig 8C, revealed that changes in these genes were observed broadly in response to treatment with Elo, or Elo and Len, in monocytes, but only in response to the combination in OPM2 cells. This suggests that combination treatment only had a significant impact on NK ligand/receptor pathways in OPM2 cells. To further identify the key genes involved, we inspected the significant genes for absolute expression change, and identified that CCL5, CXCL10 and CXCL11 responded specifically to combination treatment (Fig 8D and Fig S9D-E) and are known to be IFN-γ responsive genes (25). Many of the other genes also responded to combination treatment, but appeared to be additive to existing stimulation by Elo alone. Taken together, RNAseq analysis of myeloma cells (purified from Elo and Len treated PBMC co-cultures) showed significantly increased gene transcript levels for chemokines and receptors, adhesion molecules, MHC-II and NK receptor ligands.
Discussion

Patients with RRMM respond to combination treatment with Elo and Len, with 83% ORR and 17% durable remission (8, 9). Despite this, there are no reliable biomarkers to predict durable responses to combination Elo and Len treatment. To address these issues, we explored the mechanisms whereby combination Elo and Len treatment is effective. To do this, we used PBMCs collected from patients enrolled in two unique clinical trials on patients with newly diagnosed MM vs RRMM. We identified a subset of RRMM patients whose PBMCs killed myeloma targets, this observation was associated with increased expression of adhesion molecules (CD11a/CD54) on myeloma cells. The killing of myeloma cells and increased CD54 expression on myeloma cells required the combination of NK cells and monocytes as effectors, this effect could not be conferred by soluble factors alone (Fig S10). The monocytes used in this study included (CD14$^+$CD16$^-$) and intermediate (CD14$^+$CD16$^+$) monocytes (26).

Prior mechanistic studies utilized healthy donor cells and showed Len enhanced Elo-induced myeloma antibody dependent cellular cytotoxicity (ADCC). This effect was associated with increased expression of the synaptic adhesion molecule CD54 (ICAM-1) on both NK cells and myeloma cells (27). In another study, Len was shown to augment synaptic actin remodelling and reduce the threshold of NK cell activation. Len decreased the EC$_{50}$ for NK cell activation via CD16a and NKG2D stimulation, and also increased IFN-γ production (28). Importantly, Len did not induce IFN-γ production without concurrent NK cell stimulation, thus enhancing clinical responses without inappropriate NK-cell activation (28). However, because MM patient NK cells are dysfunctional (18) it was not known whether MM patient NK cells will respond to Elo and Len treatment in the same manner as healthy donor NK cells. Our work builds on these observations using patient derived PBMCs, demonstrating that when RRMM patients NK cells upregulated CD11a/CD54 this was associated with increased myeloma killing and increased levels of TNF, as well as IFN-γ, MIP-1α and RANTES in the culture supernatant. In RRMM patients with low myeloma cell killing, Elo and Len treatment did not alter myeloma cell CD11a/CD54 expression or cytokine/chemokine levels in the
co-culture supernatant. As myeloma cells are known to express CD54 at low levels (18), we hypothesise that this is a key immune escape mechanism. CD54 is important for immune effector cell adhesion with target cells and stabilises the immune synapse enabling directed degranulation into the immune synapse (22). Our studies show that Elo and Len treatment induced increased myeloma cell CD54 expression and this was associated with increased killing by RRMM patient PBMCs. Thus, the expression of CD11a/CD54 on NK cells and myeloma cells in patients represents a potential new predictive biomarker for selecting responders to Len and Elo therapy.

In our investigations of underlying mechanisms for Elo and Len combination therapy, we also performed RNAseq analysis of purified NK cells, monocytes and myeloma cells from Elo and Len treated co-cultures. Interestingly, myeloma cells showed the highest number of DEG in response to the combination treatment, whereas NK cells and monocytes responded better to single agent Elo therapy. We showed the myeloma cells upregulated genes for trafficking (CXCR3, CXCL10, CXCL11, CCR7, CCL5), adhesion (CD54), NK cell activation ligand (CD155) and antigen presentation (MHC-II). The increased OPM2 CCL5 levels by RNAseq analysis agrees with our data showing increased CCL5 (RANTES) in PBMC myeloma co-culture supernatant in response to Elo and Len treatment. In conjunction with the data demonstrating upregulated CD54/CD11a on myeloma cells, this suggests that the combination treatment restores adhesion between the NK cells and myeloma target cells allowing more effective cell killing. Interestingly, the increased expression of chemokines and chemokine receptors may be important for trafficking of immune effector cells to the myeloma cells. Finally, we show that the myeloma cells upregulate NK activating ligands (MICA) suggesting an additional mechanism for NK cell activation.

Having revealed mechanisms of response to Elo and Len treatment, we now know which immune cell types and molecular mechanisms should be targeted to potentially convert patient PBMCs from low to high myeloma killing. We also propose a potential predictive biomarker in which patient
PBMCs at baseline would be co-cultured with OPM2 myeloma cells, and increased OPM2 cell CD11a/CD54 expression would be associated with increased myeloma cell killing.

RRMM patient NK cells are known to be dysfunctional (18, 29) and in our study the patients PBMCs who increase myeloma killing in response to Elo and Len treatment also have enhanced NK cell function in the absence of treatment. MM patient NK cells are known to have better function in MGUS and smouldering myeloma compared to newly diagnosed MM and RRMM (30). Therefore, applying Elo and Len treatment earlier in myeloma disease (MGUS or smouldering) when NK cells are more responsive would be more likely to derive a better anti-tumour response and outcome. Alternatively, incorporating therapies that enhance NK cell function such as IL-15 (31) or that upregulate CD54 expression on NK cells and myeloma cells (e.g. TNF) (24). Monocytes are known to induce myeloma cell ADCP in the context of Elo (11). A subset of RRMM patients is known to have increased aberrant monocytes/Mo-MDSC (12), which are less able to perform ADCP than conventional monocytes. In this context, inhibiting or depleting these suppressive MDSC should generate a better anti-myeloma immune response along with Elo and Len treatment (12). Finally, a more direct approach may involve a targeted therapy to specifically upregulate CD54 expression on immune effector cells and myeloma cells using the next generation IMiDs, CELMoDs (32).

In summary, our mechanistic studies showed (1) in the presence of Elo that CD16 (FcyRII) signaling is required for effective tumor cell killing by NK cells (2) RRMM patient NK cells have reduced cytotoxicity, as shown by lower activation and loss of CD16 expression (3) in the presence of Elo+Len there is an association between increased adhesion molecule expression on the NK and target cells and the levels of target cell killing (4) monocytes may play a role in improving the efficacy of Elo and/or Len treatment (5) Elo+Len treatment induced a significant increase in myeloma-derived chemokines for trafficking of immune effectors to the tumor cells (Fig S11).
In conclusion, our findings indicate that increased CD54 expression on myeloma cells would be a useful predictive biomarker for response to Elo and Len treatment. This could be evaluated in myeloma co-cultures with patient PBMCs treated with Elo and Len. The mechanistic studies indicate that cross-talk between NK cells, monocytes and myeloma cells is critical for this response.
References


**Figure legends**

**Figure 1. Elotuzumab and Lenalidomide enhances NK cell anti-myeloma activity in vivo.** NK cells were isolated from healthy donor PBMCs via MACS. (A) Schematic outlining the expansion of NK cells before in-vivo transfer. MACS isolated NK cells were co-cultured with 100 Gy irradiated Jurkat cells and EBV-transformed lymphoblastic cells at 1:1:1 ratio, and were passaged every 2-3 days for 21 days with 1000 U/ml IL2 and 50 ng/ml IL15. (B) Timeline for NK adoptive transfer treatment of a humanised mouse myeloma model. NSG mice were injected s.c. with 2e6 RPMI8226.hSLAMF7 tumour cells in the right flank. 10^6 expanded NK cells were transferred intravenously when tumours reached 10 mm^2 (width x length) on day 0 and day 1. Mice received 5 mg/kg Elo/Iso and 50 mg/kg Len/Vehicle (12.5% DMSO), CS1.2-IgG1.1f (Elo CD16a binding mutant) and 5e4 U IL2 and 2.5 μg IL15 via intraperitoneal injection, 3 doses per week for 3 weeks. (C) Representative tumour growth curves (6 mice/group, N=3, 2 way ANOVA, * p<0.05, **** p<0.0001) and (D) Kaplan Meyer survival curve with data compiled from 3 experiments (Log-rank test, **** p<0.0001, ** p<0.01).

**Figure 2. Elotuzumab and Lenalidomide induced anti-myeloma activity of healthy donor and patient PBMCs.** PBMCs from healthy donors (HD, n=9), patients with newly diagnosed multiple myeloma (NDMM, n=12) or refractory relapsed multiple myeloma (RRMM, n=11) were co-cultured with CTV labelled OPM2 cells at a 2:1 ratio for 24 hours in the presence of 10 μg/ml human IgG1 isotype control (Iso), 10 μM Len, 10 μg/ml Elo or Elo + Len combination. (A) Flow cytometry gating strategy identifying viable CTV labelled OPM2 MM cells, NK cells and NK phenotype markers. (B) OPM2 cell killing data was compared within individual patient cohorts (HD, NDMM and RRMM) for different drug treatments across the 24 hour co-culture period. (RM one-way ANOVA). (C) Flow cytometry analysis identified the number of OPM2 cells killed during the 24 hour period of co-culture. OPM2 killing data was compared between patient cohorts for individual drug treatments, and RRMM patients with high cytotoxicity are highlighted in red. (2-way ANOVA, mean ± SEM). (D) HD, NDMM and RRMM patient NK cell CD16 expression changes in response to different drug treatments during the 24 hour co-culture (2-way ANOVA, mean ± SEM). (E-F) Supernatants from
triplicate wells of PBMC and OPM2 co-cultures were taken at 24-hours for cytokine bead array analysis. Shown are comparative changes between patient cohorts (HD, NDMM and RRMM patients) for (E) IFNγ and (F) TNF secretion. (2-way ANOVA, mean ± SEM) * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001.

Figure 3. Elotuzumab and lenalidomide upregulated CD54 expression on NK cells. PBMCs were co-cultured for 24 hours with OPM2 target cells (2:1), 10 μg/ml Elo and 10 μM Len then analysed by flow cytometry. (A) Representative overlay histograms show Elo + Len induced upregulation of CD54, CD69 and CD107a expression on HD NK cells. (B-E) CD54, CD69 and CD107a expression was compared between HD, NDMM and RRMM NK cells in response to Elo, Len or Elo + Len treatment. Each data point represents the change in MFI from baseline expression. Red triangles correspond to RRMM patients with high cytotoxic activity, as shown in Figure 2B. (B-D) Comparison between patient cohorts for NK cell CD54, CD69 and CD107a expression changes induced by drug treatments (2-way ANOVA, mean ± SEM). (E) Comparison within each patient cohort for NK cell CD54, CD69 and CD107a expression changes induced by different drug treatment/s (RM one-way ANOVA, mean ± SEM) * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001.

Figure 4: Transcriptomic analysis of natural killer cells from RRMM patients reveals they respond differently to Elotuzumab and lenalidomide combination treatment. (A) Schematic overview of experimental RNAseq strategy. Donor and patient PBMCs (n=3 each group) were treated with Len, Elo and combination treatments prior to FACS sorting NK cells and whole-transcriptomic RNA sequencing. (B) OPM2 cell killing by healthy donor and patient PBMCs in the presence of isotype antibody (Iso), Len, Elo, Elo+Len. (C) Principle component analysis of RNA expression in 24 samples from either donor (circles: left panel) or RRMM patients (triangles: right panel) NK cells following treatment.

Figure 5. RRMM patient NK cells have reduced gene signatures for cytotoxicity, activation and impaired regulation of activation
Scatterplots comparing in vitro killing capacity for individual NK cell sources with normalised enrichment score (NES) for (A) KEGG Natural Killer Cell Mediated Cytotoxicity and (B) GO Regulation of Natural Killer Cell Activation. Colors indicate three treatment groups: Len, Elo, Elo and Len. Size indicates false discovery rate q-value. All comparisons were made relative to isotype control treatment. Pearson correlation coefficients and p-values indicated. (C) Normalised z-score heatmap of GO:0032814: Regulation of NK cell activation pathway genes according to treatment for both donor and RRMM patient samples.

Figure 6. Elo and Len treatment upregulated CD54 on OPM2 cells and was dependent on both NK cells and monocytes. (A-B) PBMC and OPM2 cell co-cultures (2:1) were treated with Elo and/or Len and analysed via flow cytometry. Red data points indicate RRMM patient cells with high cytotoxicity from Figure 2B. (A) Change in CD54 expression on OPM2 target cells when co-cultured with PBMCs from HD or RRMM patients, standardised to human IgG1 isotype control treated cultures. (B) Pearson correlation between OPM2 CD54 expression change and RRMM cytotoxicity ($R^2$ and $P$ values are shown). (C-D) In the presence of Elo and Len treatment OPM2 cells were co-cultured for 24hrs with either whole PBMCs (PBMC:OPM2 2:1), PBMCs depleted of CD14$^+$ monocytes (PBMCs CD14$^-$) or NK cells (PBMCs CD56$^-$). Shown is (C) number of OPM2 target cells killed, and (D) change in OPM2 target CD54 expression level. (E-F) OPM2 cells were co-cultured for 24hrs with either whole PBMCs (PBMC:OPM2 2:1), isolated CD14$^+$ cells (CD14), isolated NK cells (CD56) or CD14$^+$ and NK cells (CD14+CD56). Shown is (E) number of OPM2 target cells killed, and (F) change in OPM2 target CD54 expression level. 2-way ANOVA, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, n=3.

Figure 7: Transcriptome analysis reveals NK cells, monocytes and OPM2 myeloma cells change their gene expression differentially in response to drug treatments.

(A) Schematic overview of experimental RNAseq strategy. Donor PBMCs (n=3 donors) were treated with Len, Elo and combination treatments prior to FACS sorting NK cells (CD56$^+$), monocytes (CD14$^+$) and OPM2 cells and subsequent whole-transcriptomic RNA sequencing. (B) Dotplot of normalized
ICAM-1 (CD54) expression in treatment groups in OPM2 and CD14+ monocyte cells. (C) Venn diagrams of overexpressed genes (log2 fold change >2; p-value < 0.05) in each cell type and treatment group.

Figure 8. Ontological analysis reveals increased expression of effector cell trafficking signals, adhesion and MHC-II in tumor cells in response to elotuzumab and lenalidomide combination therapy.

Ontological enrichment analysis within GO: *Molecular Functions* database using overexpressed genesets for either (A) monocytes, or (B) OPM2 cells. Only significantly enriched ontologies are shown (adjusted p-value < 0.05). Genes contributing to each ontology are shown. No enrichments for lenalidomide treatment were identified. (C) Heatmaps shown normalised relative mRNA expression of transcripts encoding chemokines, cytokines and receptors important for NK cell activation and functionality on monocytes (left panel) and OPM2 cells (right panel). Arrows indicate significance in Elo + Len versus Elo groups with direction of change indicated. (D) OPM2 cell log2 mRNA expression of genes showing significantly different expression difference in Elo + Len combination versus Elo treatment alone.
**A**

MACS isolated NK cells + Irradiated Jurkat and Lymphoblastic cells → Transferred to tumour bearing mice

- 1000 U/ml IL2
- 50 ng/ml IL15

**B**

RPMI8226.hSLAMF7 injection → NK cell transfer

Day -10 → 0 → 7 → 14 → 21

NSG

- Elo/Len + IL2 + IL15 injections

**C**

**Tumour growth**

- Veh. Iso
- Len Elo
- NK Veh. Iso
- NK Veh. Elo
- NK Len Iso
- NK Len Elo
- NK Veh. Elo (Fc mut)

Days post NK cell transfer

**D**

**Survival**

- Veh. Iso
- Len Elo
- NK Veh. Iso
- NK Veh. Elo
- NK Len Iso
- NK Len Elo
- NK Veh. Elo (Fc mut)

Percent survival

Days post NK transfer
A Healthy Donor

PBMCs + OPM2 cells (2:1)

Ex vivo treatment
1. Control Ig
2. 10 μM Len
3. 10 μg/ml Elo
4. Elo + Len

Cell sort

NK
OPM2
CD14
Monocyte

12 samples
RNAseq

B

Normalised log2 ICAM-1 expression

OPM2
CD14

Treatment

Iso Len Elo Elo+Len

* N.S.

C

Len

Elo

Elo+Len

NK
OPM2
Monocyte

142 2 19
2229 23 30
1950 84 174

20 1 0
32 3 7
23 14 22

153

173
**A**

GO: Molecular Functions

- cytokine activity
- chemokine activity
- chemokine receptor binding
- CXCR chemokine receptor binding
- CCR chemokine receptor binding
- phospholipase activator activity
- growth factor activity
- lipase activator activity
- cytokine receptor binding
- interleukin-1 receptor binding

![Bar chart for Monocyte treatment](image)

**B**

- MHC class II receptor activity
- MHC class II protein complex binding
- MHC protein complex binding
- cytokine receptor activity
- chemokine activity
- chemokine receptor binding
- actin binding
- CCR chemokine receptor binding
- cytokine activity

![Bar chart for OPM2](image)

adj. pvalue < 0.05

**C**

Monocyte and OPM2 expression heatmaps

- CX3CR1
- CX3CR3
- CD155
- CD112
- MICA
- SLAMF7
- CD54
- CCR1
- CCR5
- CCR7
- CXCL10
- CXCL11
- CXCL9
- CXCL8
- TNF
- CCL5
- CCL4
- CCR7
- CCL3

![Heatmap](image)

- T tests
  - Sig. increase in EloLen v Elo only
  - Sig. decrease in EloLen v Elo only

**D**

Log2 expression graph

- CCL5
- CCR5
- CCR7
- CD155
- CXCL10
- CXCL11
- CXCL9
- CD54
- MICA
- TNF

- Significantly different EloLen v Elo only

![Graph](image)
Supplementary Methods

Flow cytometry based cytotoxicity assay
PBMCs obtained from healthy donors or patients were seeded in a 96-well plate at 100,000 cells per well and co-cultured with 50,000 OPM2 cells pre-labelled with 1:1000 CellTrace™ Violet (Life Technologies). Co-cultures were treated with 10 μg/ml Elo or IgG1 isotype. Len dissolved in DMSO was added at 10 μM and cocultures were incubated for 24 hours at 37°C at 5% CO2. Cells were pelleted at 400 G for 5 minutes and supernatants were transferred to a new 96-well plate and stored at -80°C until analysed by cytokine bead array (CBA). Cells were washed and resuspended in FACS buffer for immunostaining. Counting beads (Quantibrite™ Beads, BD) were added to each well to enumerate remaining target cells for cytotoxicity. Flow cytometry based cytotoxicity assays were acquired on the LSRFortessa X-20 and analysed with FlowJo software.

Chromium release cytotoxicity assay
For labelling target cells with chromium, cells were incubated with 100 μCi Cr51 at 37ÅãC at 5% CO2, in 500 μl of complete RPMI1640 media for 1 hour. Cells were then washed 3x with complete RPMI1640 media. Effector cells were then seeded in a 96-well plate, and serially diluted in complete media to acquire the indicated effector-target ratio. Target cells were added to each well at 10,000 cells/well to reach a 200 μl final volume. Co-cultures were incubated at 37ÅãC for 4 hours in the presence of 10 μg/ml Elo or IgG1 isotype and 10 μM Len or DMSO control. All conditions were set up in triplicate. Cells were then pelleted and 150 ul of supernatants were transferred to microtitre tubes for gamma counting.

Reagents and Antibodies
Elotuzumab and Elo mutant Fc (Elo Fc-neg, CS1.IgG1.1F) were provided by BMS and reconstituted in PBS. This Elo mutant has been described in detail in a prior study by Pazina et al (1). For in vitro experiments, Lenalidomide was reconstituted at 100 μM in DMSO and appropriately diluted in PBS. For in vivo experiments, Lenalidomide was reconstituted in DMSO and diluted with PBS to make a 12.5% DMSO solution at 12.5 mg/ml Lenalidomide. Pomalidomide was similarly reconstituted for in vivo experiments, with a final concentration of 1.25 mg/ml.

Fluorescent-conjugated antibodies used to identify surface molecules for flow cytometry were anti-CD56-PE (5.1H11, Biolegend), anti-CD56-BV395 (NCAM16.2, BD), anti-CD16-BV786 (3G8, Biolegend), anti-CD11a-BV650 (HI111, BD), anti-CD54-ACP (HA58, BioLegend), anti-CD54-BV650 (HA58, BD), anti-CD69-BV510 (FN50, BioLegend), anti-CD107a-AF488 (H4A3,BioLegend), anti-CD25-PE (M-A251, BD), anti-CD3-APC-H7 (SK7, BD), anti-CD14-FITC (M5E2,BD), anti-CD14-ACP-Cy7 (MφP9, BD), anti-CD19-ACP (SJ25C1, BD), anti-NKp30-AF488 (210845, R&D Systems), anti-NKp44-PE (253415, R&D Systems), anti-NKp46-BV605 (9E2,BioLegend), anti-NKG2D-AF700 (149810, R&D Systems), anti-NKp30-AF488 (143211, R&D Systems), anti-NKG2A-PE (131221, R&D Systems), anti-TIGIT-APC (MBSA34, eBioscience), anti-KIR2DL1-AF488 (143211, R&D Systems), anti-KIR2DL2/3-PE-Cy7 (DX27, BioLegend), anti-KIR3DL1-BV421 (DX9, BioLegend), and anti-KIR3DL2-PE (539304, R&D Systems). CellTrace Violet™ (CTV) (Life Technologies) was used to stain target cells at 1:1000. Zombie RedTM fixable viability dye (Biolegend) was used to assess cell viability.

Cytometric bead array (CBA) assays
Cytokine and chemokines were measured in 10 μL of supernatant from human PBMC and OPM-2 co-cultures, incubated at a 2:1 effector:target ratio with Isotype, Elo, Len or combined Elo and Len for 24 hours. Cytokine and chemokine concentration was measured using CBA Flex sets (BD) according to the manufacturer’s instructions. CBA Flex sets used are Human IFN-g, TNF, CCL3 (MIP-1a), CCL4 (MIP-1b) and CCL5 (RANTES). Samples were analysed using a FACSVerse cytometer (BD) and FCAP Array software V3 (BD BioSciences).

**Immune cell depletion assays**

PBMCs were freshly isolated from healthy donors and were labelled with antibodies to CD14 (FITC), CD56 (PE), CD3 (APC-H7), CD19 (ACP-H7) and Zombie Red fixable viability dye. Cells were sorted directly into 90% FCS/10% RPMI1640 media with a FACSaria Fusion 3 (BD), for CD14+ cells, CD14 depleted PBMCs and NK cells. OPM2 cells were labelled with 1:1000 CellTrace Violet™ (Life Technologies) and seeded in a 96-well plate at 5e4 cells per well. 1e5 whole PBMCs, or an equivalent number of CD14 depleted PBMCs, isolated CD14+ cells or NK cells were added to each well. The number of isolated/depleted cells added to each well was equal to the expected number of cells found in whole PBMCs, based on cell percentages in the PBMC phenotype. Cells were co-cultured for 24 hours and were treated with Isotype or 10 μg/ml Elo and 10 μM Len. Flow cytometry was used to analyse killing activity and cell phenotype was analysed on the LSRFortessa™ X-20, BD. 5e3 counting beads (Quantibrite™ BD) were used to enumerate OPM2 cells, and to calculate killing, standardised to spontaneous cell death. For cell phenotypes, cells were stained for CD56 (PE), CD16 (BV786), CD3/19 (APCH7), CD14 (FITC), CD54 (APC), CD11a (BV650) and Zombie Red™ fixable viability dye.

**RNA sequencing analysis**

3-4e6 PBMCs were cocultured with 1e6 CTV labelled OPM2 cells for 24 hrs in a 24-well plate, and were treated with 10 μg/ml Elo and 10 μM Len. For cell sorting, cells were labelled with antibodies to CD56 (PE), CD16 (BV786), CD3/19 (APCH7), CD14 (APC) and Zombie Red™ viability dye (BioLegend.). Cells were sorted using the ARIA Fusion 3, directly into 90% FCS/10% RPMI1640, pelleted and snap frozen at -80ÅãC. RNA extraction was performed as per manufacturer’s instructions (15596026, Invitrogen, USA). cDNA libraries were prepared using either the SMART-seq v4 low input RNA kit for NK cell preparations (Takara Bio USA), or NEBNext Ultra II (New England Biolabs, USA) for monocyte and OPM2 cells preparations. Libraries were sequenced on the NextSeq 550 sequencing system (Illumina, USA) at the Molecular Genomics Facility at the Peter MacCallum Cancer Centre. Reads were aligned using seqliner v0.7.1 to HG19 reference genome and quantified using Htsel v0.6.1 software. Normalisation and differential expression analysis was performed with Limma-Voom in Rv3.3.3.

**Statistics and Bioinformatics analyses**

All data were analysed using GraphPad Prism software Version 8 using either ANOVA with post-hoc test or 2-tailed Student t-test, and significance levels are denoted in the figure legends. All experimental data are shown as mean (SD) or median (interquartile range) in the text as well as in the figures. RNAseq data visualization, principle component analysis and t-test volcano plots were performed using Perseus computational platform (version 1.5.6) from the Max Planck Institute of Biochemistry (2). 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) according to established protocols (3). An FDR of $<0.2$ was considered significant for GSEA pathway analysis. Ontology analysis of pre-defined gene lists was performed using Enrichr software (23586463, 23586463) according to standard protocols. Visualisations were performed within R using standard ggplot2 package.

References
Supplementary figures:

Supplementary Figure 1. In vitro expanded NK cells have an activated phenotype.
NK cells were isolated from healthy donor PBMCs and cultured for 21 days with irradiated feeder cells (Jurkat and LCLs) at a ratio of 1:1:1. In (A) day 0 and expanded NK cells were assessed for CD56 and CD16 expression, CD69, and NK activation and inhibitory receptors (B). Representative chromium release assays for NK cells from days 0, 7 and 21 of expansion. NK cells were co-cultured for 4hrs with OPM2 targets in the presence of 10 μg/ml Elotuzumab (Elo) or an isotype control antibody (Iso), where ns = not significant, * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001, (Student’s T-test, n=3).

Supplementary Figure 2. Combination elotuzumab and IMiD treatment significantly controls myeloma tumor growth.
NSG mice were injected s.c. with 2e6 RPMI8226.hSLAMF7 tumour cells in the right flank. 10e6 expanded NK cells were transferred intravenously when tumours reached 10 mm² (width x length) on day 0 and day 1. Mice received 5 mg/kg Elo/Iso, and 50 mg/kg Len/Vehicle (12.5% DMSO), 5 mg/kg pom/vehicle (12.5% DMSO), and 5e4 U IL2 and 2.5 μg IL15 via intraperitoneal injection, 3 doses per week for 3 weeks. Shown are tumour growth curves representative of three separate experiments (6 mice/group, N=3, 2-way ANOVA, * p<0.05, *** p<0.001 **** p<0.0001).

Supplementary Figure 3. RRMM patient PBMCs are distinct for efficient myeloma killers.
Data from Figure 2C was reformatted to show (A) statistical differences between RRMM patient PBMCs characterised with efficient killing activity vs patient PBMCs with minimal killing. 2 way ANOVA with Tukey’s test, * p<0.05, **, p<0.01, *** p<0.001, **** p<0.0001. (B) NK cells were analysed for treatment induced changes in NK cell CD16 expression levels using HD, NDMM or RRMM PBMCs. RRMM PBMCs nominated as efficient killers are highlighted in red. RM one-way ANOVA, * p<0.05, *** p<0.001, **** p<0.0001. (C) a representative histogram overlay depicting the change in NK cell CD16 expression when treated with either an isotype control or Elo+Len.

Supplementary Figure 4. Elotuzumab and lenalidomide treatment increased RANTES secretion in myeloma co-cultures with RRMM PBMCs.
PBMCs from healthy donors (HD, n=9), patients with newly diagnosed multiple myeloma (NDMM, n=12) or refractory relapsed multiple myeloma (RRMM, n=11) were co-cultured with OPM2 cells at a 2:1 ratio for 24 hours in the presence of 10 μg/ml human IgG1 isotype control (Iso), 10 μM Len, 10 μg/ml Elo or Elo + Len combination. Supernatants from triplicate wells of PBMC and OPM2 co-cultures were taken at 24-hours for cytokine bead array analysis. Shown are comparative changes between patient cohorts (HD, NDMM and RRMM patients) for (A) MIP-1a (B) MIP-1b (C) RANTES secretion. 2-way ANOVA, * p<0.05.

Supplementary Figure 5: Transcriptomic analysis of natural killer cells from myeloma patients.
(A) Venn diagram of overlap in significantly upregulated genes (p-value < 0.05, log2 fold-change>2) in two sample types (healthy donor and patient) sorted by three treatments groups (Elo, Len, Elo and Len) compared to isotype control. Boxes indicate gene sharing between relevant groups. (B) Principle component analysis of RNA expression in all NK cell
samples following treatment. (C) Gene set enrichment analysis of highly enriched NK cell-related canonical pathways using triplicate data. Only significant enrichments are shown (FDR<0.2). Normalised z-score heatmaps of GO:0032814:Regulation of NK cell activation pathway genes according to treatment for (D) healthy donor and (E) RRMM patient samples.

Supplementary Figure 6. Elo or Elo plus Len treatment upregulates CD11a on OPM2 and NK cells.
OPM2 cells were co-cultured 1:2 in the presence or absence of PBMCs from healthy donors and RRMM patients (efficient killers red triangles in Fig 2B). Co-cultures were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len and analysed via flow cytometry for change in CD11a expression following drug treatment on (A) OPM2 cells and (B) NK cells. (2-way ANOVA, ns = not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, n=3).

Supplementary Figure 7. Elo and Len treatment upregulated CD54 on OPM2 cells was not dependent on T cells.
PBMC (or CD4+ or CD8+ T cell depleted PBMCs) and OPM2 cell co-cultures (2:1) were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len and analysed via flow cytometry for (A) the % killing of OPM2 cells, and (B) change in OPM2 myeloma cell CD54, (C) CD11a expression level following drug treatment. (2 way ANOVA, * p<0.05, **, p<0.01, ***p<0.001, **** p<0.0001, n=3). Representative histogram overlays depicting Elo+Len-induced changes in CD54 and CD11a (E).

Supplementary Figure 8. Elo and Len treatment upregulated CD11a on OPM2 cells was dependent on the presence of monocytes and NK cells.
PBMC (or CD4+ or CD8+ T cell depleted PBMCs) and OPM2 cell co-cultures (2:1) were treated for 24 hours with 10 μg/ml human IgG1 isotype control (Iso) or 10 μg/ml Elo and 10 μM Len. OPM2 cell CD11a expression level was analysed via flow cytometry in (A) following depletion of monocytes of NK cells (B) following the addition of purified NK cells, monocytes or combination NK cells + monocytes. In (C) OPM2 CD54 expression after treatment for 24 hours with conditioned media harvested from Elo+Len treated PBMC:OPM2 co-cultures. (2 way ANOVA, * p<0.05, **, p<0.01, ***p<0.001, **** p<0.0001, n=3). Representative histogram overlays depicting Elo+Len-induced changes in OPM2 CD54 (D) and CD11a (E).

Supplementary Figure 9: Drug treatment changes in myeloma, NK cells and monocyte transcript levels and gene ontology analysis.
Related to Figure 6D and 6E. Ontological enrichment analysis using GO: Molecular Functions database of overexpressed genesets for either (A) monocytes, or (B) OPM2 cells. Only significantly enriched ontologies are shown (adjusted p-value < 0.05). Genes contributing to each ontology are shown. No enrichments for lenalidomide treatment were identified. Genes contributing to each group are shown in boxed. Heatmaps of absolute and relative expression differences between four treatment groups in triplicate samples for (C) NK cells, (D) monocytes and (E) OPM2 cells.
Supplementary Figure 10. Cross talk between NK cells, monocytes and myeloma target cells is required for optimal anti-tumour activity in response to Elo and Len.
A schematic illustrating the mechanisms for cross talk between NK cells, monocytes and myeloma cells. This conceptual figure is divided into panels depicting molecular interactions between (A) NK and myeloma cells treated with Len (B) NK cells, monocytes and myeloma cells (C) NK cells and myeloma cells treated with Elo or Elo and Len (D) NK cells and myeloma cells treated with Elo in the presence of monocytes (E) NK cells and myeloma cells treated with Elo and Len in the presence of monocytes. Changes in NK cell CD11a/CD54 and CD69 levels were observed when Elo was present (C), whereas increased myeloma cell CD11a/CD54, NK activating ligands (CD155 and MIC-A), or chemokines and receptors (CCR7, CXCR3, CCL5, CXCL10, CXCL11) were only observed in condition (E).

Supplementary Figure 11. Graphical abstract.
Co-culture of myeloma patient PBMCs and myeloma cells with Elo+Len treatment increased myeloma cell MHC-II, CD11a/CD54 and CXCL10-11, CCL5 expression. This change in myeloma cell profile was associated with myeloma cell death. In this context, myeloma cell death required monocytes and NK cells which collectively secreted increased IFN-γ, TNF and CCL5. In contrast, if no change in myeloma cell profile occurred, the myeloma cells were resistant to NK and monocyte effector mechanisms in the presence of Elo+Len treatment.