The efficacy of combination treatment with elotuzumab and lenalidomide is dependent on crosstalk between natural killer cells, monocytes and myeloma cells

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Abstract

Patients with refractory relapsed multiple myeloma respond to combination treatment with elotuzumab and lenalidomide. The mechanisms underlying this observation are not fully understood. Furthermore, biomarkers predictive of response have not been identified to date. To address these issues, we used a humanized myeloma mouse model and adoptive transfer of human natural killer (NK) cells to show that elotuzumab and lenalidomide treatment controlled myeloma growth, and this was mediated through CD16 on NK cells. In co-culture studies, we showed that peripheral blood mononuclear cells from a subset of patients with refractory relapsed multiple myeloma were effective killers of OPM2 myeloma cells when treated with elotuzumab and lenalidomide, and this was associated with significantly increased expression of CD54 on OPM2 cells. Furthermore, elotuzumab- and lenalidomide-induced OPM2 cell killing and increased OPM2 CD54 expression were dependent on both monocytes and NK cells, and these effects were not mediated by soluble factors alone. At the transcript level, elotuzumab and lenalidomide 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 multiple myeloma patients require elotuzumab- and lenalidomide-mediated upregulation of CD54 on autologous myeloma cells, in combination with NK cells and monocytes to mediate an effective anti-tumor response. Furthermore, our data suggest that increased myeloma cell CD54 expression levels could be a powerful predictive biomarker for response to elotuzumab and lenalidomide treatment.

Introduction

Multiple myeloma (MM) is an incurable plasma cell malignancy of the bone marrow and is the second most common hematologic malignancy in Western countries.[1] To address the need for new and effective treatment options, monoclonal antibodies targeting myeloma cells have been investigated for their potential to treat MM and enhance current therapies.[2] Elotuzumab is a humanized IgG1 antibody that 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] Elotuzumab exerts its cytotoxic effects by binding to SLAMF7 on the MM plasma (target) cell and in turn activating natural killer (NK) cells via their FcγRIII (CD16a), inducing antibody-dependent cell-mediated cytotoxicity of the target cell.[3,4] SLAMF7 may also control cellular activation due to the cytoplasmic domain comprising an immunoreceptor tyrosine-based

switch motif. 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 elotuzumab to SLAMF7 expressed on NK cells.[7] Patients with refractory relapsed MM (RRMM) treated with a combination elotuzumab, lenalidomide and dexamethasone in the ELOQUENT-2 clinical study showed improved objective response rates of 79% and improved overall survival compared to those treated with elotuzumab alone.[8-10] Despite these encouraging outcomes, the mechanisms by which the combination of elotuzumab and lenalidomide improves disease control have not been resolved. Furthermore, there is currently no reliable biomarker to predict which patients will respond to elotuzumab and lenalidomide with long-term remission.

To address these issues, we explored NK cell functional responses and gene expression profile changes in untreated newly diagnosed and RRMM patients in the context of elotuzumab and lenalidomide treatment. We also explored whether other immune cell types were required to support the elotuzumab- and lenalidomide-induced increase in myeloma cell cytotoxicity. Prior studies have shown that macrophages phagocytose myeloma targets in response to elotuzumab 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 in the peripheral blood and bone marrow.[12] In this study, we explored whether monocytes were required for the elotuzumab- and lenalidomide-induced increase in myeloma patients' NK cell cytotoxic responses. We also investigated elotuzumab and lenalidomide treatment-induced changes in the myeloma cells at both the transcript and protein levels. Our study revealed for the first time the mechanisms through which elotuzumab and lenalidomide treatment acts, involving complex interactions between myeloma cells, NK cells and monocytes. In addition, our study revealed a novel potential predictive biomarker for the response to combination elotuzumab and lenalidomide treatment in patients with MM.

Methods

Cell cultures

Jurkat (human T lymphoblast line, from the American Type Culture Collection), LCL (an Epstein-Barr virus-transformed lymphoblastoid B-cell line) and human multiple myeloma cell lines, OPM-2 and RPMI8226.hSLAMF7 cells (Kerry Campbell, Fox Chase Cancer Center), were maintained in RPMI-1640 media (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 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 ethics approval granted by the Peter MacCallum Cancer Centre Human Research and Ethics Committee (HREC# 01/14). Peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient (Ficoll-Paque Plus, GE Healthcare Life Science). Blood samples from MM patients were obtained from either Peter MacCallum Cancer Centre patients enrolled in the LITVACC (newly diagnosed MM) or REVLITE (RRMM) 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 patients' PBMC were covered by Peter MacCallum Cancer Centre ethics committee approval (HREC# 11-51).

PBMC were maintained in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 µM 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin containing 25 U/mL recombinant human (rh) interleukin (IL)-2.

Purification and culture of primary human natural killer 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 fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 µM 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin containing 25 U/mL rhIL-2.

Expansion of natural killer 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 fetal calf serum, 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.

Humanized myeloma mouse model

Female NOD-*scid* IL2R$\gamma^{-/-}$ (NSG) mice were bred in-house and were inoculated with $2x10^6$ RPMI8226.hSLAMF7 cells in the lower right flank. Two doses of expanded human NK cells ($1x10^7$ cells/dose) were transferred via intravenous injection, 24 h apart, when tumors measured 10 mm^2 in area (length x width, where length was the longest side of the tumor). Mice received exogenous cytokines ($5x10^5$ U rhIL-2

and 2.5 µg rhIL-15) intraperitoneally and were treated with 5 mg/kg elotuzumab, 50 mg/kg lenalidomide, 5 mg/kg pomalidomide or vehicle control, given intraperitoneally 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 humanized myeloma model, the human NK cells and myeloma cells (RPMI8226.hSLAMF7) were the only cells capable of responding to lenalidomide or pomalidomide via human cereblon.[13]

The *Online Supplementary Materials and Methods* section includes details on cytotoxicity assays (flow cytometry and Cr-release), reagents and antibodies, cytokine bead array assays, immune cell depletion, RNA sequencing analysis, statistics and bioinformatics analyses.

Results

Elotuzumab induces natural killer cell killing of myeloma targets and its effects are enhanced *in vivo* by lenalidomide

In prior clinical trials in patients with RRMM, compared to treatment with elotuzumab alone, treatment with a combination of elotuzumab and lenalidomide led to enhanced survival and disease control.[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 elotuzumab or elotuzumab and lenalidomide treatment. To do this, we developed a human NK cell expansion protocol based on prior studies[14,15] (Figure 1A, *Online Supplementary Figure S1*). Expanded (day 21) NK cells were $CD56^{hi}CD16^{+/-}$ and the $CD16^{+}$ subset expressed NK activation receptors ($NKp30^{hi}Nkp44^{hi}NKp46^{+}NKG2D^{+}$), inhibitory receptors ($KIR^{+}NKG2A^{hi}TIGIT^{+}$) and were $CD69^{hi}$, indicating activation (*Online Supplementary Figure S1A, B*). Expanded NK cells induced significantly higher killing of OPM2 cells, in the presence or absence of elotuzumab, compared to day 0 NK cells (*Online Supplementary Figure S1B*). To assess the *in vivo* efficacy of elotuzumab and lenalidomide treatment, NSG mice were inoculated subcutaneously with human SLAMF7-expressing RPMI18226 (RPMI8226.hSLAMF7) myeloma cells. RPMI8226 cells are innately resistant to direct lenalidomide-induced cytotoxicity,[13] allowing us to control for the effect of lenalidomide on NK activity. To explore the

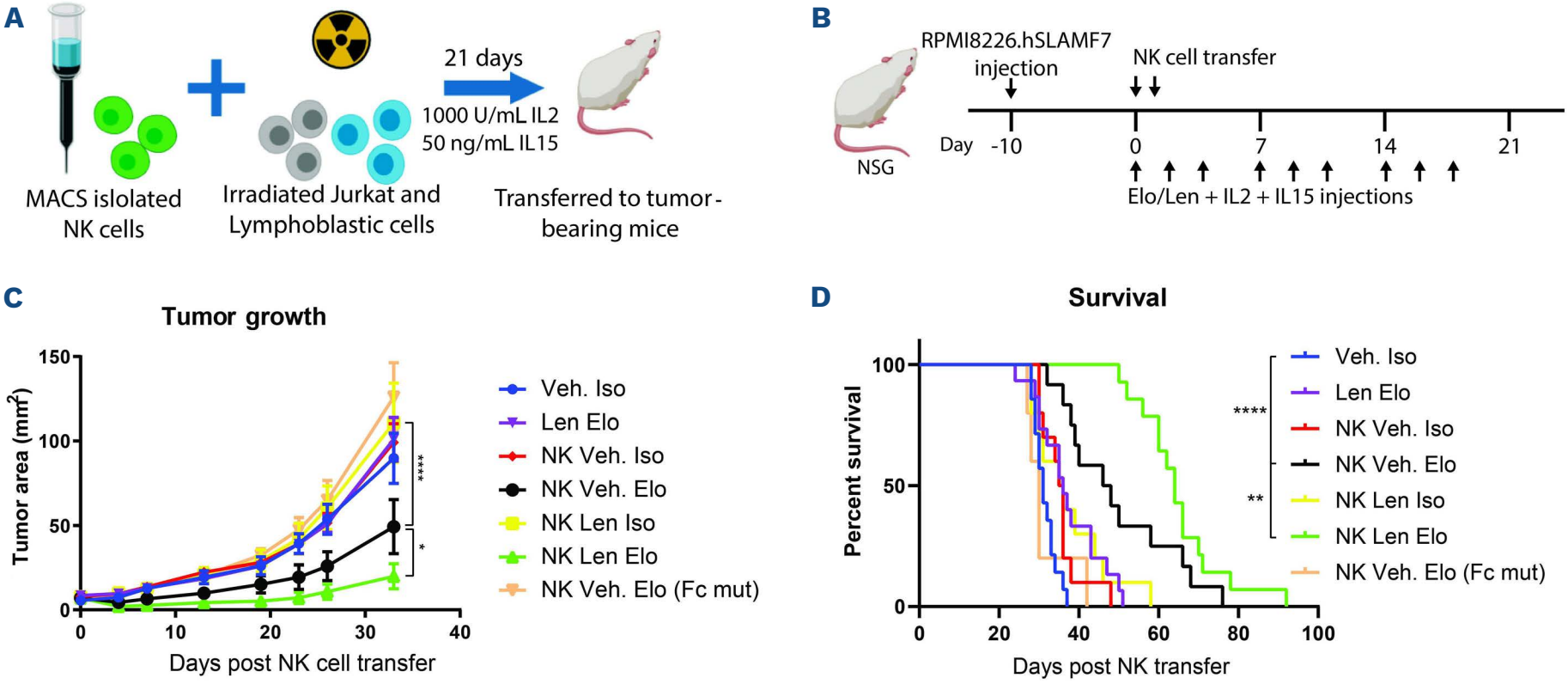

Figure 1. Combined treatment with elotuzumab and lenalidomide enhances natural killer-cell anti-myeloma activity *in vivo*. Natural killer (NK) cells were isolated from healthy donor peripheral blood mononuclear cells by magnetic activated cell sorting (MACS). (A) Schematic outlining the expansion of NK cells before *in vivo* transfer. MACS-isolated NK cells were co-cultured with 100 Gy-irradiated Jurkat cells and Epstein-Barr virus-transformed lymphoblastic cells at a 1:1:1 ratio, and were passaged every 2-3 days for 21 days with 1000 U/mL interleukin 2 (IL2) and 50 ng/mL interleukin 15 (IL15). (B) Timeline for NK-cell adoptive transfer treatment of a humanized mouse myeloma model. NSG mice were injected subcutaneously with $2x10^6$ RPMI8226.hSLAMF7 tumor cells in the right flank. When tumors reached 10 mm^2 (width x length), $10x10^6$ expanded NK cells were transferred intravenously on day 0 and day 1. Mice received 5 mg/kg elotuzumab/ human IgG1 isotype control and 50 mg/kg lenalidomide/vehicle (12.5% dimethylsulfoxide), CS1.2-IgG1.1f (elotuzumab CD16a binding mutant) and $5x10^4$ U IL2 and 2.5 µg IL15 via intraperitoneal injection, three doses per week for 3 weeks. (C) Representative tumor growth curves (6 mice/group, N=3, two-way analysis of variance, *P<0.05, ****P<0.0001). (D) Kaplan-Meier survival curve with data compiled from three experiments (log-rank test, ****P<0.0001, **P<0.01). MACS: magnetic activated cell sorting; Veh: vehicle; Elo: elotuzumab; Len: lenalidomide: Iso: human IgG1 isotype control.

importance of NK cell CD16 for the elotuzumab and lenalidomide anti-tumor effect, we treated established tumors with expanded NK cells plus either elotuzumab or elotuzumab FcγR mutant (which does not bind CD16), in the presence or absence of lenalidomide (Figure 1B). The results showed that transferred NK cells and the combination of elotuzumab and lenalidomide treatment resulted in the most effective control of myeloma tumor growth and led to improved mouse survival, compared to NK cells treated with either lenalidomide or elotuzumab alone (Figure 1C, D). The transfer of NK cells along with elotuzumab FcγR mutant had no effect on the inhibition of tumor growth, indicating an important role for NK cell CD16 in tumor control mediated by elotuzumab (Figure 1C, D). Pomalidomide is a second generation immunomodulatory drug,[16] and elotuzumab and pomalidomide combination treatment mediated improved myeloma control in RRMM patients.[17] We tested pomalidomide in combination with elotuzumab in our humanized myeloma NK cell adoptive therapy model. In this experiment, we treated myeloma-bearing mice with expanded NK cells, elotuzumab and lenalidomide or elotuzumab and pomalidomide (50 mg/kg lenalidomide *vs.* 5 mg/kg pomalidomide). NSG mice treated with adoptively transferred NK cells and combined elotuzumab and lenalidomide or elotuzumab and pomalidomide had significantly better myeloma tumor control compared to those treated with NK cells and elotuzumab alone (*Online Supplementary Figure S2*). Taken together, the results indicate that elotuzumab combination treatment with either lenalidomide or pomalidomide controlled myeloma tumor outgrowth via NK cell-mediated antibody-dependent cell-mediated cytotoxicity and required NK-cell CD16 expression.

Natural killer cells from refractory relapsed multiple myeloma patients have divergent cytotoxic responses 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 elotuzumab and lenalidomide treatment *in vitro* and kill myeloma cells. PBMC from healthy donors, newly diagnosed MM patients and RRMM patients were co-cultured with OPM2 tumor cells and treated with elotuzumab or elotuzumab and lenalidomide for 24 hours. Flow cytometry was used to assess PBMC phenotypes (Figure 2A), as well as the level of cytotoxicity directed towards OPM2 myeloma cells (Figure 2B). The cytotoxicity of PBMC from healthy donors and newly diagnosed MM patients increased in response to elotuzumab alone or elotuzumab and lenalidomide, but not with lenalidomide alone (Figure 2B). Interestingly, PBMC from 7/11 RRMM patients had very poor or no cytotoxic response to elotuzumab and elotuzumab and lenalidomide treatment, but PBMC from the other 4/11 RRMM patients showed significantly increased killing of OPM2 myeloma cells (Figure 2B). These samples are highlighted in red in Figure 2B and in the remainder of the figures of this work. With the exception of these highly cytotoxic RRMM PBMC, our results demonstrate the loss of NK-cell cytotoxic capacity on MM disease progression (Figure 2C, *Online Supplementary Figure S3*) and are consistent with our prior data.[18]

We next investigated potential biomarkers that identified RRMM patients with high *versus* low cytotoxic PBMC in order to stratify patients for elotuzumab and lenalidomide 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 tumor cells plus elotuzumab and lenalidomide treatment, NK cells from healthy donors lost significantly more surface CD16 than those from newly diagnosed MM or RRMM patients (Figure 2D, *Online Supplementary Figure S3B, C*). Supernatants taken from these co-cultures were then analyzed for interferon gamma (IFNγ), tumor necrosis factor (TNF) (Figure 2E, F), MIP-1α, MIP-1β and RANTES (*Online Supplementary Figure S4*). IFNγ and TNF levels were significantly increased in NK cells from healthy donors, compared to their levels in NK cells from newly diagnosed MM and RRMM patients, when PBMC and OPM2 cells were co-cultured and treated with elotuzumab, or elotuzumab and lenalidomide (Figure 2E, F). In the same co-culture system and with the same drug treatment combinations, similar data were observed for MIP-1α and MIP-1β (*Online Supplementary Figure S4*). In addition, PBMC from RRMM patients with a high cytotoxicity response against OPM2 cells also secreted high levels of RANTES (CCL5), the equivalent of that observed for PBMC from healthy donors (red triangles in *Online Supplementary Figure S4*).

To further identify differences in NK cell responses to elotuzumab and lenalidomide treatments between healthy donors and MM patients, cell surface expression of CD54, CD107a and CD69 was measured and compared to that of untreated NK cells (by difference in mean fluorescence intensity) (Figure 3A). CD54 (ICAM1) is a surface glycoprotein with an important role in NK cell adhesion to 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, NK cells from healthy donors significantly upregulated CD54 in response to elotuzumab alone, lenalidomide alone, and their combination, with the highest NK cell expression of CD54 resulting from treatment with the elotuzumab and lenalidomide combination (Figure 3B). No significant difference in CD54 expression was observed between treatment groups in NK cells from newly diagnosed and RRMM patients (Figure 3B), and expression levels were significantly lower than those for healthy donor NK cells (Figure 3B).

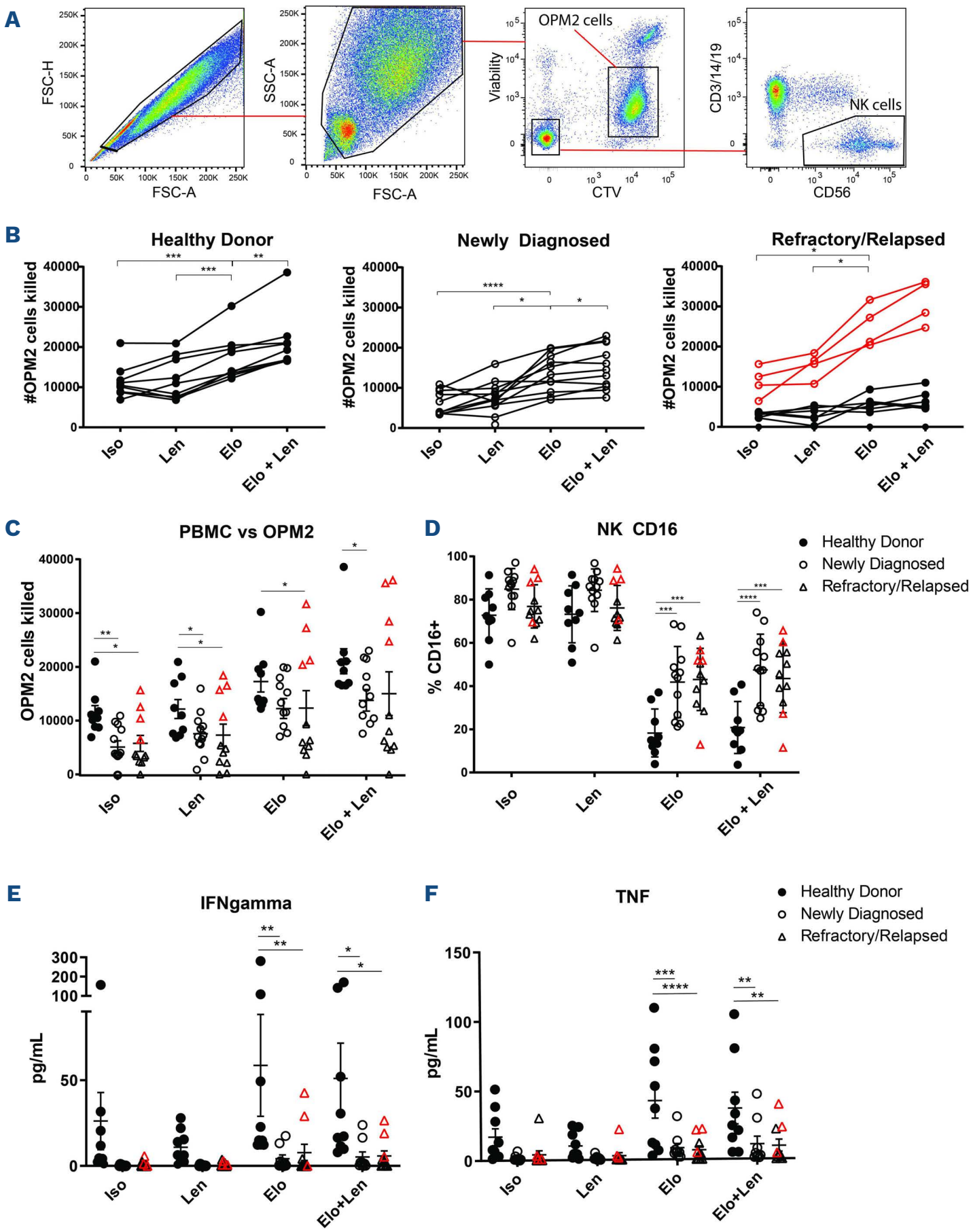

Figure 2. Elotuzumab- and lenalidomide-induced anti-myeloma activity of peripheral blood mononuclear cells from healthy donors and myeloma patients. Peripheral blood mononuclear cells (PBMC) from healthy donors (HD, n=9), patients with newly diagnosed multiple myeloma (NDMM, n=12) or refractory relapsed multiple myeloma (RRMM, n=11) were co-cultured with CTV-labeled OPM2 cells at a 2:1 ratio for 24 hours in the presence of 10 µg/mL human IgG1 isotype control, 10 µM lenalidomide, 10 µg/mL elotuzumab or elotuzumab + lenalidomide. (A) Flow cytometry gating strategy identifying viable CTV-labeled OPM2 myeloma cells, natural killer (NK) cells and NK phenotype markers. (B) OPM2 cell killing data were compared within individual cohorts (HD, NDMM and RRMM) for different drug treatments across the 24-hour co-culture period. (repeated measure one-way analysis of variance [ANOVA]). (C) Flow cytometry analysis identified the number of OPM2 cells killed during the 24-hour period of co-culture. OPM2 killing data were compared between patient cohorts for individual drug treatments, and RRMM patients with high cytotoxicity are highlighted in red (two-way ANOVA, mean ± standard error of mean [SEM]). (D) HD, NDMM and RRMM patients' 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. The graphs show comparative changes between cohorts (HD, NDMM and RRMM patients) for (E) interferon gamma and (F) tumor necrosis factor secretion (two-way ANOVA, mean ± SEM). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. FSC: forward scatter; SSC: side scatter; Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide: IFN: interferon; TNF: tumor necrosis factor.

However, when myeloma cell death was significantly increased, the RRMM patients' NK cells (Figure 2B, highlighted in red) upregulated CD54 in response to elotuzumab and lenalidomide, with levels of expression similar to those for healthy donor 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 elotuzumab and lenalidomide therapy in the clinic. In contrast, NK cell CD69 and CD107a expression was not associated with increased killing of OPM2 myeloma cells. We found that the early activation marker CD69 was significantly upregulated in response to elotuzumab in all three cohorts of patients (Figure 3C). However, elotuzumab-induced CD69 expression on NK cells from RRMM patients was significantly lower than that on NK cells from newly diagnosed MM patients and healthy donors, despite the divergence in RRMM NK cell cytotoxicity. Additionally, treatment with lenalidomide reduced the expression of CD69 (Figure 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. Surpris-

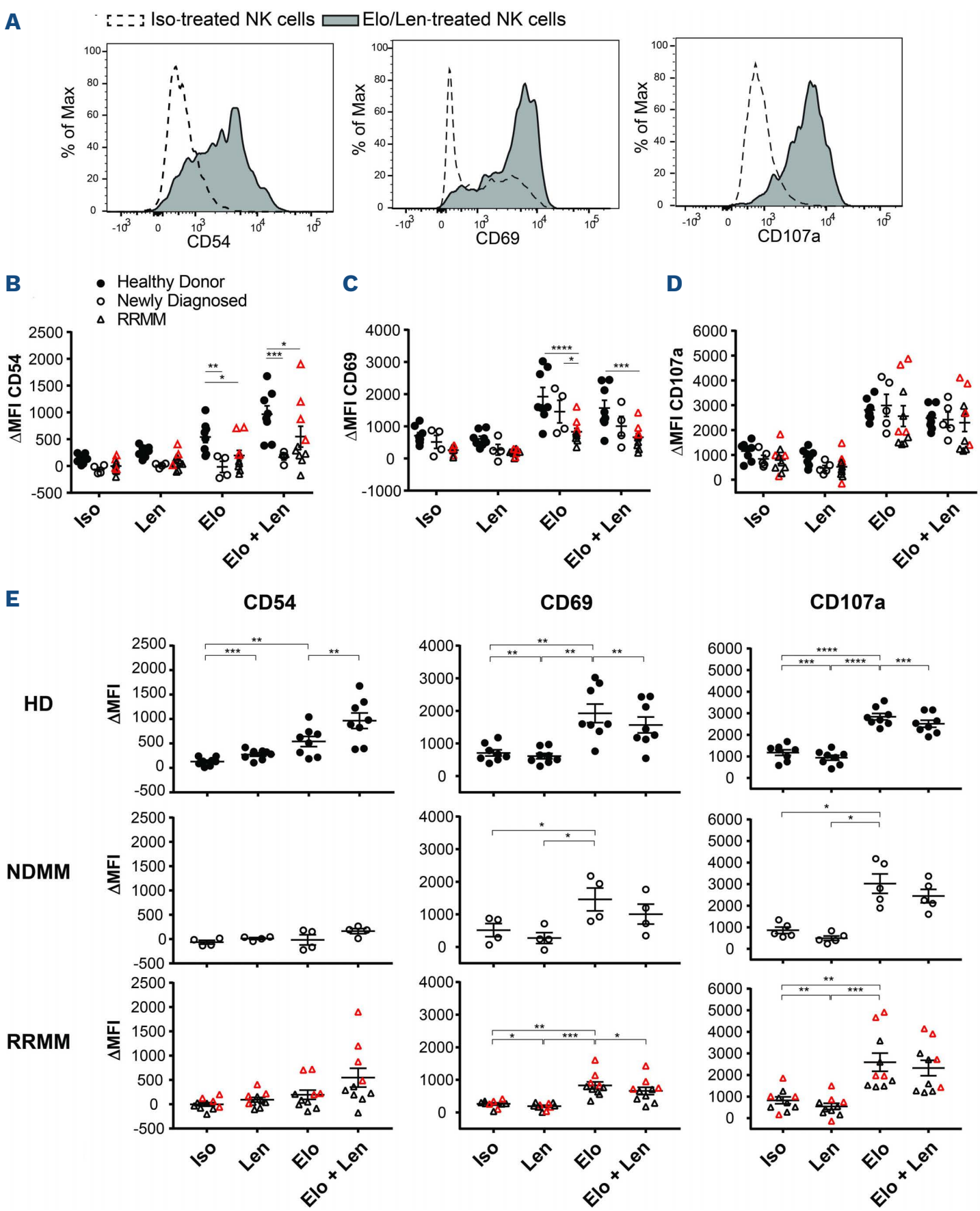

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Figure 3. Elotuzumab and lenalidomide treatment upregulated CD54 expression on natural killer cells. Peripheral blood mononuclear cells were co-cultured for 24 hours with OPM2 target cells (2:1), 10 μg/mL elotuzumab and 10 μM lenalidomide then analyzed by flow cytometry. (A) Representative overlay histograms show elotuzumab + lenalidomide-induced upregulation of CD54, CD69 and CD107a expression on natural killer (NK) cells from healthy donors (HD). (B-E) CD54, CD69 and CD107a expression was compared between NK cells from HD, patients with newly diagnosed multiple myelom (NDMM) and patients with refractory relapsed multiple myeloma (RRMM) in response to elotuzumab, lenalidomide or elotuzumab + lenalidomide treatment. Each data point represents the change in mean fluorescence intensity from baseline expression. Red triangles correspond to RRMM patients with high cytotoxic activity, as shown in Figure 2B. (B-D) Comparison between patients' cohorts for NK cell CD54, CD69 and CD107a expression changes induced by drug treatments (two-way analysis of variance [ANOVA], mean ± standard error of mean [SEM]). (E) Comparison within each cohort for NK cell CD54, CD69 and CD107a expression changes induced by different drug treatment(s) (repeated measures one-way ANOVA, mean ± SEM). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. MFI: mean fluorescence intensity; Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide.

ingly, all patients' NK cells were capable of elotuzumab-induced degranulation, with no significant difference observed between cohorts (Figure 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 surrogately by CD107a exposure, does not equate to myeloma target cell death. It was also observed that, as for CD69, the addition of lenalidomide resulted in reduced CD107a expression (Figure 3E). It is known that the interaction between CD54 and LFA1 is essential for the microtubule-organizing center and lytic granules to polarize to the immune synapse between 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 cell samples, may result in unpolarized, indirect 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]

Natural killer cells from healthy donors and myeloma patients 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, PBMC from healthy donors or patients were first treated with control immunoglobulin, lenalidomide, elotuzumab or the combination of lenalidomide plus elotuzumab (n=3 subjects in each group) (Figure 4A). We also performed a cytotoxicity assay, using PBMC from the same healthy donor controls and myeloma patients, to simultaneously evaluate OPM2 myeloma cell killing (Figure 4B). Once again, we observed that healthy donor PBMC induced increased myeloma cell killing and responded more effectively to elotuzumab and lenalidomide treatment.

To explore these differences at the transcriptional level, we first analyzed differential expression of genes to find genes upregulated by the different treatments. We identified that donor NK cells exhibited a large number of changes, particularly in response to elotuzumab alone and elotuzumab plus lenalidomide treatment (2,054-2,265 differentially expressed genes), and fewer following lenalidomide treatment alone (144 differentially expressed genes) (*Online Supplementary Figure S5A*). Conversely, RRMM patients responded much less, with 22 differentially expressed genes following treatment with elotuzumab alone, or elotuzumab and lenalidomide treatment, and only one following lenalidomide treatment alone. Approximately half of these differentially expressed genes in RRMM were also conserved with healthy donors' NK cells, indicating a shared molecular response to the treatments. Principle component analysis of the expression level of all genes showed that, despite variation between the donor sources, the treatments had predictable effects on healthy donor NK cells (Figure 4C). However, this was not observed in the RRMM patients' NK cells, with no clear organized response to any of the treatments. We also observed that patients' NK cells exhibited dramatically different expression profiles when directly compared (*Online Supplementary Figure S5B*) to healthy donors' NK cells. We hypothesized that changes in canonical NK cell functional responses to the treatments were fundamentally different between NK cells from healthy donors and patients. To explore this, we used gene set enrichment analysis of canonical functional NK cell pathways and identified that patients' NK cells were generally less stimulated by treatments but with little difference between the pathways (*Online Supplementary Figure 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 demonstrated 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 gene

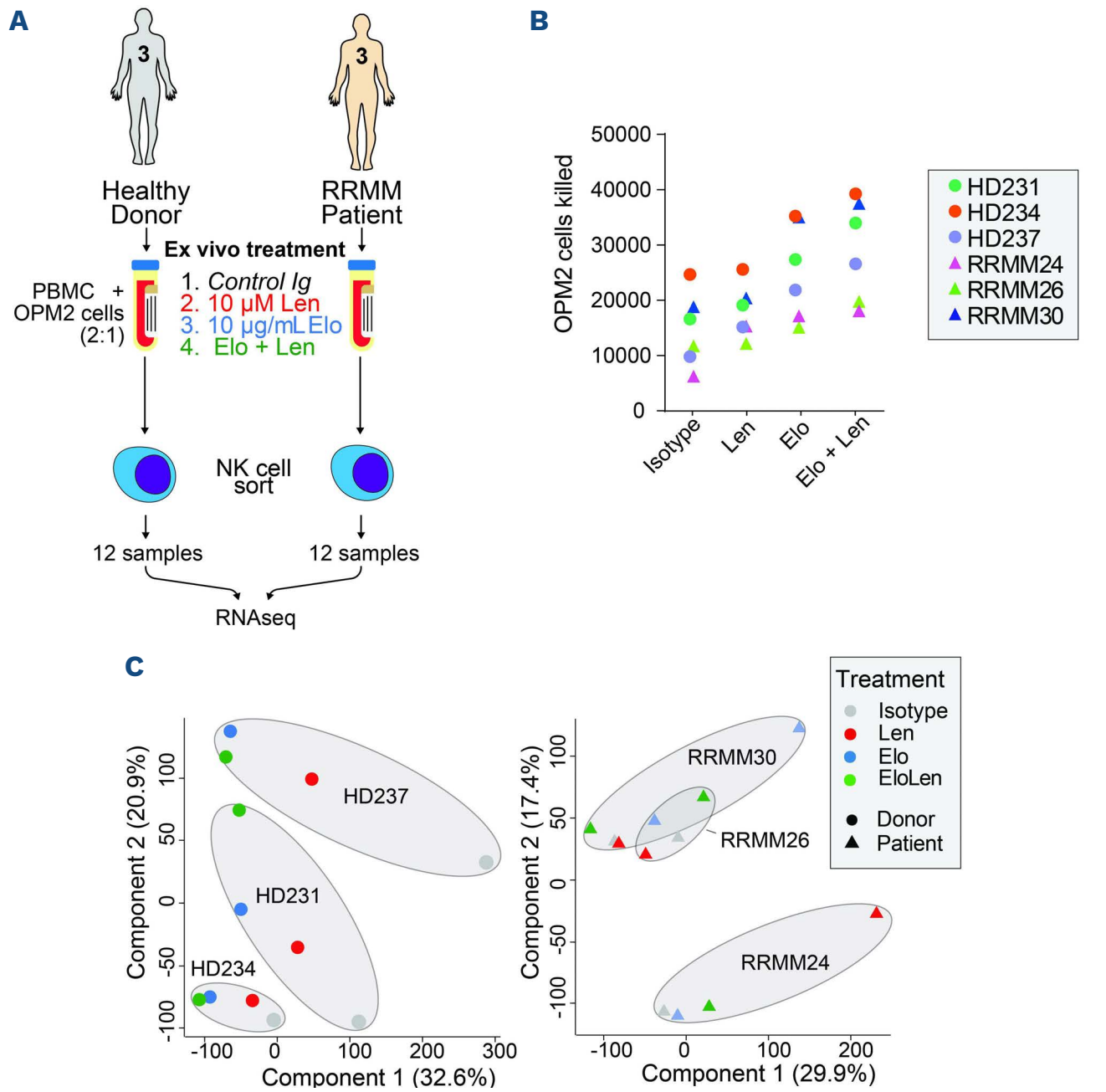


Figure 4. Transcriptomic analysis of natural killer cells from patients with refractory relapsed multiple myeloma reveals that these cells respond differently to elotuzumab and lenalidomide combination treatment. (A) Schematic overview of the experimental RNA-sequencing strategy. Peripheral blood mononuclear cells (PBMC) from donors and patients (n=3 each group) were treated with lenalidomide, elotuzumab or their combination prior to fluorescence activated cell sorting of natural killer (NK) cells and whole-transcriptomic RNA sequencing. (B) OPM2 cell killing by PBMC from healthy donors and patients in the presence of isotype antibody, lenalidomide, elotuzumab, or elotuzumab + lenalidomide. (C) Principle component analysis of RNA expression in 24 samples of NK cells from healthy donors (circles: left panel) or patients with refractory relapsed multiple myeloma (triangles: right panel) following treatment. RRMM: refractory relapsed multiple myeloma; Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide; HD: healthy donor.

set enrichment scores. Finally, we compared the expression levels of the individual genes within the gene ontology regulation of NK cell activation pathways between healthy donors and RRMM patients (Figure 5C). We observed that NK cells from RRMM patients exhibited different expression patterns overall compared to NK cells from healthy donors, even in the absence of any treatment, suggesting that NK cells from RRMM patients have fundamentally different NK activation states. Despite this, elotuzumab treatment (either as monotherapy or in combination) led to increased expression of many genes in this pathway. Interestingly, patient RRMM24 appeared not to exhibit this response to elotuzumab. In summary, elotuzumab and lenalidomide treatment induced expression of genes enriched in the cytotoxicity and activation pathways in NK cells from healthy donors but not in those from RRMM patients.

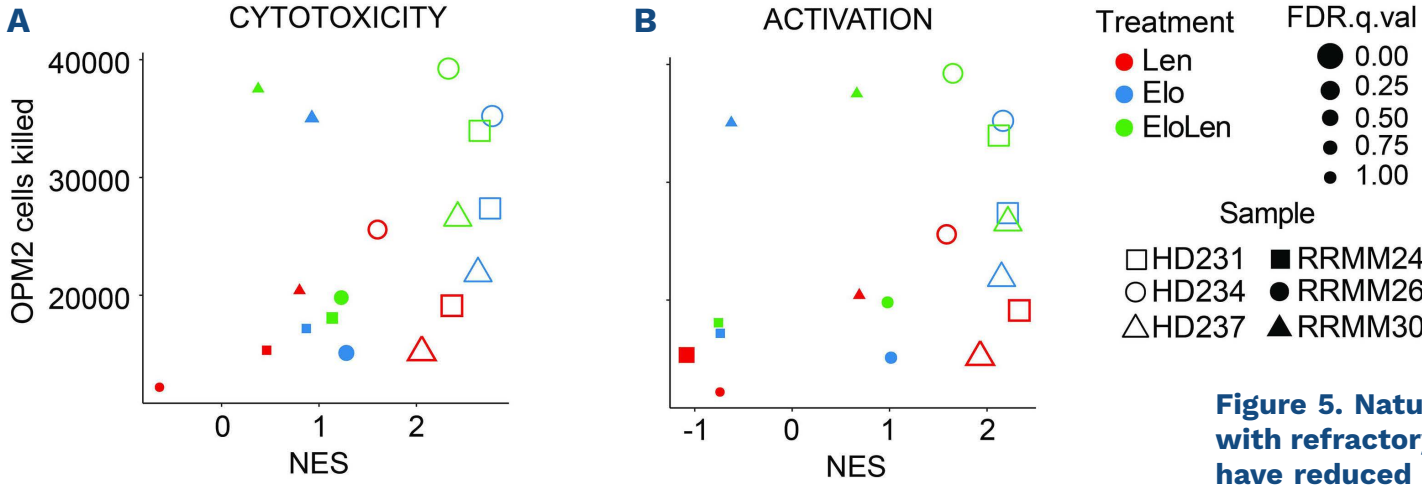
Elotuzumab- and lenalidomide-induced upregulation of OPM2 cell CD54 requires natural killer cells and monocytes

We next explored the effect of elotuzumab and lenalidomide treatment on OPM2 myeloma target cells. When PBMC from healthy donors were co-cultured with OPM2 cells and various drugs, the OPM2 cells had significantly greater expression of CD54 when treated with elotuzumab and lenalidomide than when treated with elotuzumab alone (Figure 6A). Interestingly, this effect was also observed in the RRMM patients' PBMC with high levels of OPM2 killing (red triangles Figure 6A). Furthermore, OPM2

killing correlated with increased expression of CD54 on OPM2 target cells when co-cultured with RRMM patients' PBMC in the context of elotuzumab and lenalidomide treatment (Figure 6B). CD11a was also upregulated on OPM2 cells in response to treatment with elotuzumab alone, and was further increased when combined treatment with elotuzumab and lenalidomide was used, indicative of similar mechanisms of regulation (*Online Supplementary Figure 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 elotuzumab and lenalidomide 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 in whom 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 elotuzumab and lenalidomide treatment, and as TNF secretion was not further enhanced by lenalidomide (Figure 2E, F), we hypothesized that additional mechanisms contributed to this effect.

To explore this further, we investigated which immune cell subsets were important for the induction of increased OPM2 myeloma cell killing and CD54/CD11a expression after elotuzumab and lenalidomide treatment. PBMC depleted of specific cell subtypes were co-cultured with OPM2 target cells and treated with elotuzumab and lenalidomide. Depletion of either $CD8^+$ or $CD4^+$ T cells had no effect on the level of OPM2 killing or on the expression of CD54 on both NK and OPM2 cells (*Online Supplementary Figure S7*). This was unexpected because lenalidomide 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 (Figure 6C). Lack of $CD14^+$ monocytes reduced the level of killing of OPM2 cells after treatment with elotuzumab and lenalidomide and also abrogated upregulation of CD54/CD11a on the tumor cells (Figure 6D, *Online Supplementary Figure S8A*). To investigate whether $CD14^+$ cells were directly responsible for increased OPM2 CD54 and CD11a expression in response to elotuzumab and lenalidomide, $CD14^+$ cells isolated from PBMC were co-cul-



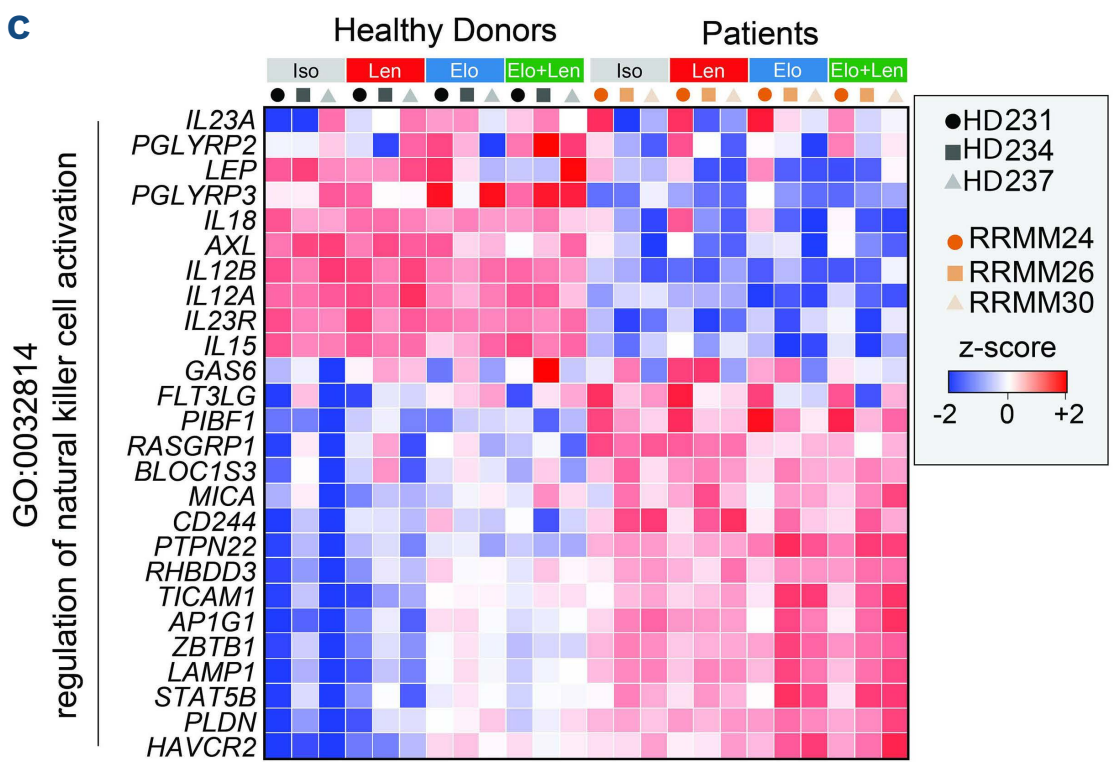


Figure 5. Natural killer cells from patients with refractory relapsed multiple myeloma have reduced gene signatures for cytotoxicity, activation and impaired regulation of activation. Scatterplots comparing *in vitro* killing capacity for individual natural killer (NK) cell sources with normalized enrichment scores for (A) KEGG Natural Killer Cell Mediated Cytotoxicity and (B) GO Regulation of Natural Killer Cell Activation. Colors indicate three treatment groups: lenalidomide, elotuzumab, and elotuzumab + lenalidomide. Size indicates false discovery rate q-value. All comparisons were made relative to isotype control treatment. Pearson correlation coefficients and *P* values are indicated. (C) Normalized z-score heatmap of GO:0032814: Regulation of NK cell activation pathway genes according to treatment for samples from both healthy donors and patients with refractory relapsed multiple myeloma. NES: normalized enrichment score; FDR: false discovery rate; Elo: elotuzumab; Len: lenalidomide; HD: healthy donor; RRMM: refractory relapsed multiple myeloma.

tured with OPM2 cells. This revealed that CD14⁺ cells alone did not induce an increase in OPM2 CD54/CD11a expression in the context of elotuzumab and lenalidomide treatment. However, low levels of OPM2 killing were observed, supporting a role for monocytes in the killing of OPM2 cells (Figure 6E). Indeed antibody-dependent cellular phagocytosis has been described previously as a mechanism through which elotuzumab acts.[11] When NK cells were depleted from healthy donors' PBMC, this reduced the overall level of OPM2 cell killing and CD54/LFA1 expression on OPM2 cells after combined elotuzumab and lenalidomide treatment (Figure 6C, D). Furthermore, when NK cells alone were co-cultured with OPM2 cells and treated with elotuzumab and lenalidomide, OPM2 killing was significantly increased compared to that induced by monocytes, but significantly less than that induced by whole PBMC (Figure 6E). This suggests that both monocytes and NK cells were required to induce optimal OPM2 myeloma cell

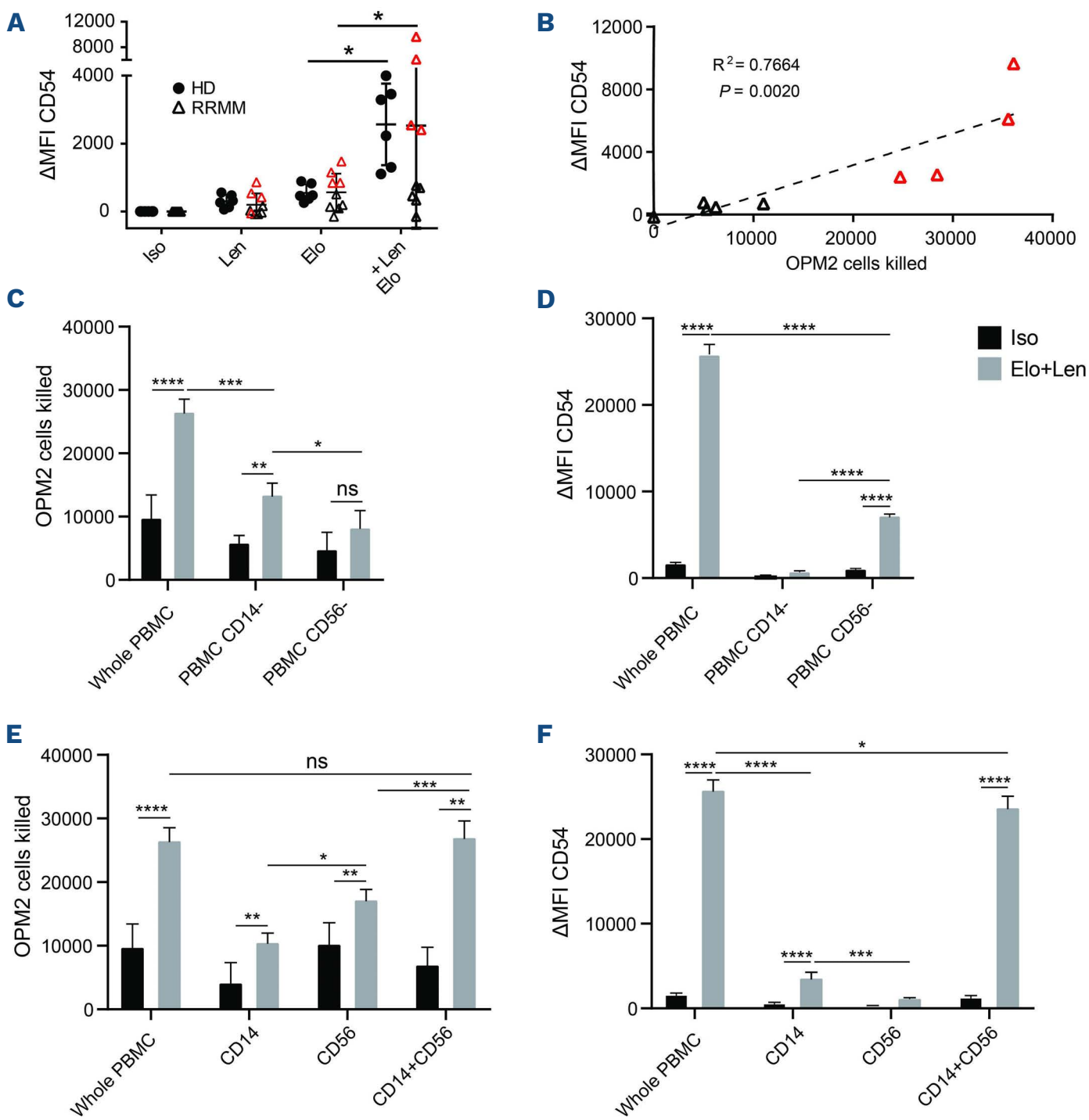


Figure 6. Elotuzumab and lenalidomide treatment upregulated CD54 on OPM2 cells and the treatment effects were dependent on both natural killer cells and monocytes. (A, B) Peripheral blood mononuclear cells (PBMC) and OPM2 cell co-cultures (2:1) were treated with elotuzumab and/or lenalidomide and analyzed via flow cytometry. Red data points indicate cells from refractory relapsed multiple myeloma (RRMM) patients with high cytotoxicity from Figure 2B. (A) Change in CD54 expression on OPM2 target cells when co-cultured with PBMC from healthy donors or RRMM patients, standardized 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 elotuzumab and lenalidomide treatment OPM2 cells were co-cultured for 24 hours with either whole PBMC (PBMC:OPM2 2:1), PBMC depleted of CD14⁺ monocytes (PBMC CD14⁻) or NK cells (PBMC CD56⁻). The graphs show the number of OPM2 target cells killed (C), and the change in OPM2 target CD54 expression level (D). (E, F) OPM2 cells were co-cultured for 24 hours with either whole PBMC (PBMC:OPM2 2:1), isolated CD14⁺ cells (CD14), isolated NK cells (CD56) or CD14⁺ and NK cells (CD14⁺CD56). The graphs show the number of OPM2 target cells killed (E), and the change in OPM2 target CD54 expression level (F). Two-way analysis of variance, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, n=3. MFI: mean fluorescence intensity; Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide.

killing following combination therapy. To further validate this, when NK cells were combined with $CD14^+$ monocytes, complete recovery of elotuzumab- and lenalidomide-induced target cell CD54/CD11a expression and target killing was observed, demonstrating crucial crosstalk between these cell types and the target OPM2 myeloma cells (Figure 6E, F, *Online Supplementary Figure S8B*). Finally, we observed that soluble mediators alone were unable to mediate these changes in CD54 expression on the OPM2 myeloma cells (*Online Supplementary Figure S8C*), so it can be inferred that increased OPM2 CD54 expression requires cell-to-cell interactions. These results imply that patients lacking functional monocytes may not benefit from this mechanism of elotuzumab + lenalidomide-induced priming of target cells for more efficient NK cell lysis.

Combination treatment with elotuzumab and lenalidomide stimulates cytokine and chemokine activity in OPM2 myeloma cells

Our analysis of transcriptional changes in RRMM patients' NK cells (see Figures 4 and 5) suggests that neither cytotoxicity, nor NK activation pathways were more sensitive to the combination treatment with elotuzumab and lenalidomide over elotuzumab alone. We therefore hypothesized that differential responses among other cell types (i.e., tumor cells or monocytes) may be responsible for driving differences in killing capacity. We therefore compared the transcriptional response in these cells to that of healthy donor cells to identify differences between their responses that may explain our data. 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 (Figure 7A). We previously showed that elotuzumab and lenalidomide treatment induced increased CD54 expression on both NK cells and OPM2 cells (Figures 3B and 6A). Here, we confirmed that elotuzumab and lenalidomide combination treatment increased *ICAM-1* expression on OPM2 cells over that produced by monotherapy, but this was not observed in monocytes (Figure 7B). We used an unbiased gene ontology enrichment strategy to identify novel pathways stimulated by the combination treatment. To use as input for pathway enrichment analysis, we first identified differentially expressed genes increased by each treatment (relative to control treatment) in the three cell types. The results, shown in Figure 7C, revealed that cells treated with elotuzumab alone, or elotuzumab and lenalidomide had the highest number of differentially expressed genes overall, but dramatically fewer differentially expressed genes were found in monocytes and OPM2 cells than in NK cells. In addition, many differentially expressed genes were unique to each cell type, suggesting that there are some unique effects that are dependent on the recipient cell. To further explore these differentially expressed genes, we used ontological enrichment within the *GO: Molecular Function* database to identify novel functional changes in monocytes and OPM2 cells (Figure 8A, B, *Online Supplementary Figure S9A, B*). The results revealed that cytokine and chemokine pathways were increased in monocytes in response to both elotuzumab and combination treatment with elotuzumab and lenalidomide. 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 changes in the same cytokine- and chemokine-related pathways. Interestingly, we found that MHC class II pathways, mostly comprising HLA-related genes, were strongly upregulated by the combination treatment (*Online Supplementary Figure S9B*).

As gene ontology analysis 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 Figure 8C, revealed that changes in these genes were observed broadly in response to treatment with elotuzumab, or elotuzumab and lenalidomide, 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 (Figure 8D, *Online Supplementary Figure S9D, E*) and are known to be IFN-γ-responsive genes.[25] Many of the other genes also responded to combination treatment, but the response appeared to be additive to the existing stimulation by elotuzumab alone. Considering all the data together, RNA-sequencing analysis of myeloma cells (purified from elotuzumab- and lenalidomide-treated PBMC co-cultures) showed significantly increased gene transcript levels for chemokines and receptors, adhesion molecules, MHC class II molecules and NK receptor ligands.

Discussion

Patients with RRMM respond to combination treatment with elotuzumab and lenalidomide, with a reported overall response rate of 83% and 17% achieving durable remissions.[8,9] Despite this, there are no reliable biomarkers to predict durable responses to combination treatment with elotuzumab and lenalidomide. In this study, we explored the mechanisms whereby combination elotuzumab and lenalidomide treatment is effective. To do this, we used PBMC collected from patients enrolled in two unique clinical trials on patients with newly diagnosed MM *versus*

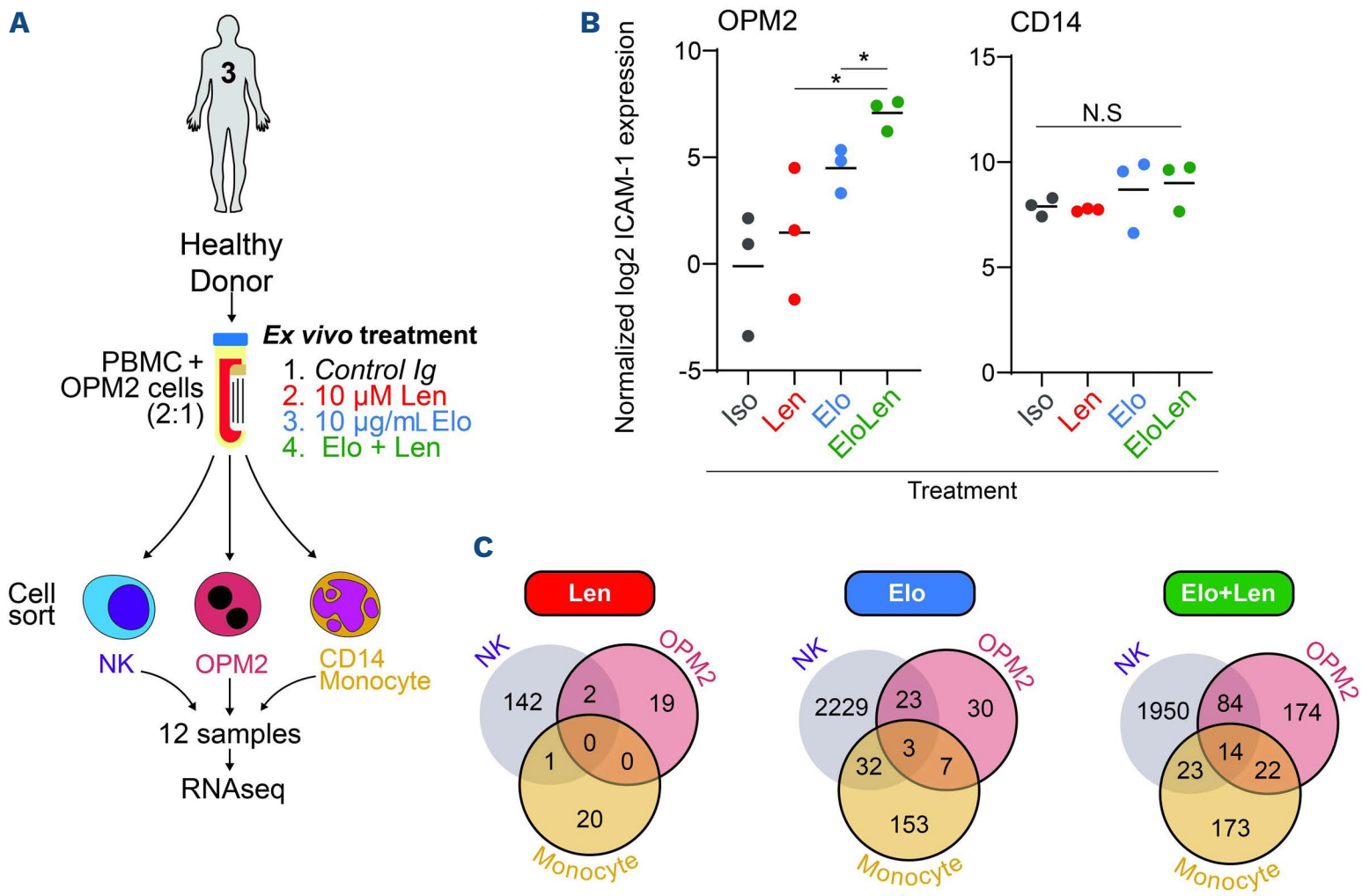


Figure 7. Transcriptome analysis reveals that natural killer cells, monocytes and OPM2 myeloma cells change their gene expression differentially in response to drug treatments. (A) Schematic overview of the experimental RNA-sequencing strategy. Peripheral blood mononuclear cells from healthy donors (n=3) were treated with lenalidomide, elotuzumab or their combination prior to fluorescence activated sorting of natural killer 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 cells and $CD14^+$ monocytes. (C) Venn diagrams of overexpressed genes (log_2 fold change >2; *P* value < 0.05) in each cell type and treatment group. PBMC: peripheral blood mononuclear cells; Control Ig and Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide, N.S.: not statistically significant; NK: natural killer.

RRMM. We identified a subset of RRMM patients whose PBMC killed myeloma targets and found that these patients had 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; the effects could not be produced by soluble factors alone (*Online Supplementary Figure S10*). The monocytes used in this study included classical ($CD14^{++}CD16^-$) and intermediate ($CD14^{++}CD16^+$) monocytes.[26] Prior mechanistic studies utilized healthy donor cells and showed that lenalidomide enhanced elotuzumab-induced myeloma antibody-dependent cellular cytotoxicity. 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, lenalidomide was shown to augment synaptic actin remodeling and reduce the threshold of NK cell activation. Lenalidomide decreased the EC_{50} for NK cell activation via CD16a and NKG2D stimulation, and also increased IFN-γ production.[28] Importantly, lenalidomide did not induce IFN-γ production without concurrent NK cell stimulation, thus enhancing clinical responses without inappropriate NK cell activation.[28] However, because MM patients' NK cells are dysfunctional[18] it was not known whether their NK cells would respond to elotuzumab and lenalidomide treatment in the same manner as NK cells from healthy donors. Our work builds on these observations using patient-derived PBMC, 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, elotuzumab and lenalidomide 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 hypothesize that this is a key immune escape mechanism. CD54 is important for immune effector cell adhesion with target cells and stabilizes the immune synapse enabling directed degranulation into the immune synapse.[22] Our studies show that elotuzumab and lenalidomide treatment induced increased myeloma cell CD54 expression and that this was associated with increased killing by RRMM pa-

tients' PBMC. Thus, the expression of CD11a/CD54 on NK cells and myeloma cells in patients represents a potential new predictive biomarker for selecting responders to lenalidomide and elotuzumab therapy.

In our investigations of underlying mechanisms of action of elotuzumab and lenalidomide combination therapy, we also performed RNA-sequencing analysis of purified NK cells, monocytes and myeloma cells from elotuzumab- and lenalidomide-treated co-cultures. Interestingly, myeloma cells showed the highest number of differentially expressed genes in response to the combination treatment, whereas NK cells and monocytes responded better to single-agent elotuzumab therapy. We showed that 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 found by RNA-sequencing analysis are concordant with our data showing increased CCL5 (RANTES) in PBMC myeloma co-culture supernatant in response to elotuzumab and lenalidomide 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 thereby allowing more effective cell killing. Interestingly, the increased expression of chemokines and chemokine receptors may be impor-

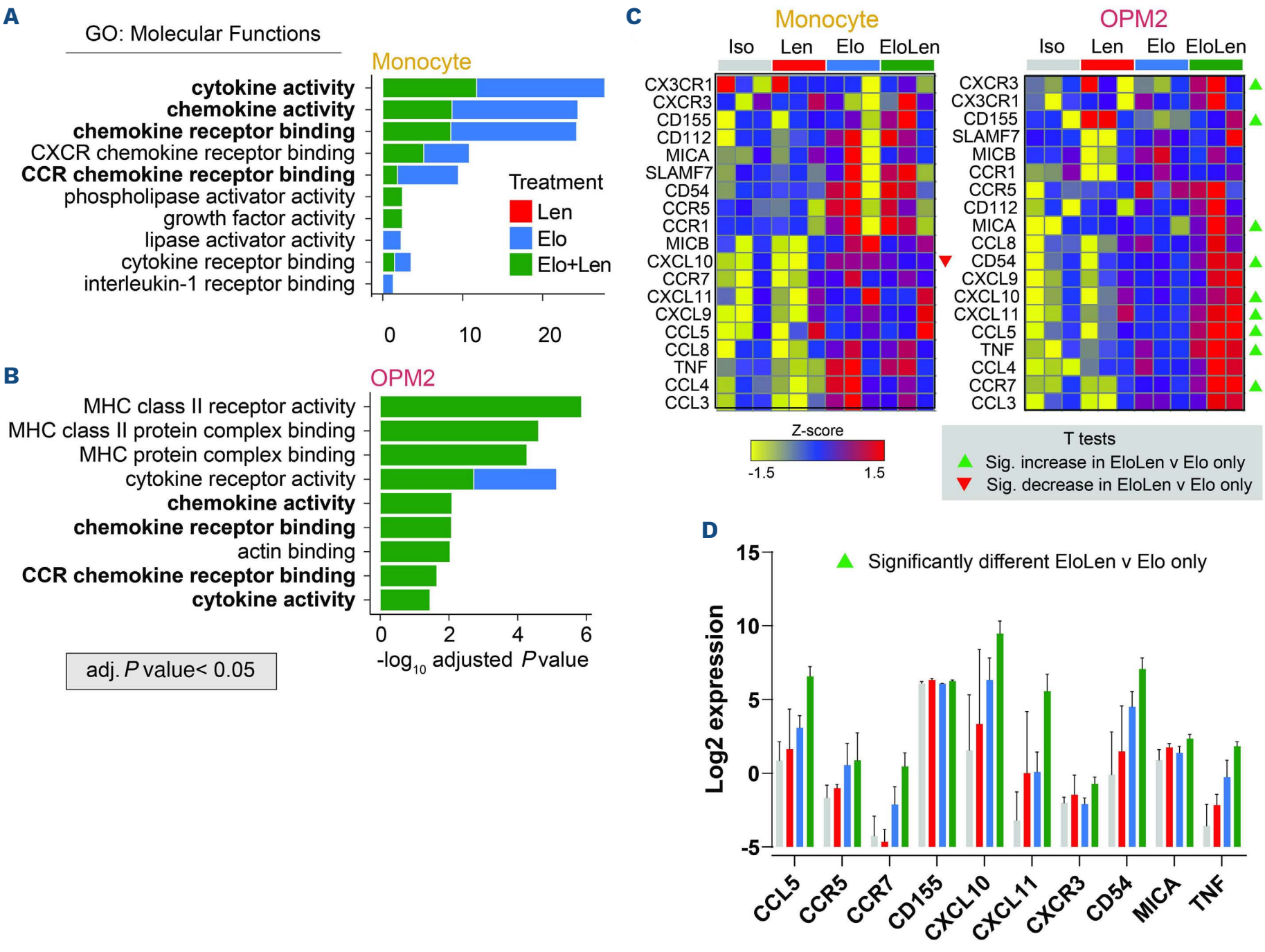


Figure 8. Ontological analysis reveals increased expression of effector cell trafficking signals, adhesion and MHC class II molecules in tumor cells in response to elotuzumab and lenalidomide combination therapy. (A, B) Ontological enrichment analysis within the *GO: Molecular Functions* database using overexpressed genesets for either monocytes (A) or OPM2 cells (B). 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 showing normalized relative mRNA expression of transcripts encoding chemokines, cytokines and receptors important for natural killer-cell activation and functionality on monocytes (left panel) and OPM2 cells (right panel). Arrows indicate significance for the elotuzumab + lenalidomide and elotuzumab treatment groups with direction of change indicated. (D) OPM2 cell $\log_2$ mRNA expression of genes showing significantly different expression when treated with the elotuzumab + lenalidomide combination *versus* elotuzumab treatment alone. GO: gene ontology; Iso: human IgG1 isotype control; Elo: elotuzumab; Len: lenalidomide; MHC: major histocompatibility complex.

tant 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 elotuzumab and lenalidomide treatment, we now know which immune cell types and molecular mechanisms should be targeted to potentially convert patients' PBMC from low to high myeloma killing. We also propose a potential predictive biomarker in which a patient's PBMC 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.

NK cells from patients with RRMM are known to be dysfunctional[18,29] and in our study the patients' PBMC whose myeloma killing increased in response to elotuzumab and lenalidomide treatment also had enhanced NK cell function in the absence of treatment. NK cells from patients with monoclonal gammopathy of unknown significance and smoldering multiple myeloma are known to have better function than those from patients with newly diagnosed MM or RRMM.[30] Therefore, applying elotuzumab and lenalidomide treatment earlier in myeloma disease (monoclonal gammopathy of unknown significance and smoldering multiple myeloma), when NK cells are more responsive, would be more likely to produce a better anti-tumor 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) could be useful.[24] Monocytes are known to induce myeloma cell antibody-dependent cellular phagocytosis in the context of elotuzumab treatment.[11] A subset of RRMM patients is known to have increased aberrant monocytes/monocytic-monocyte-like myeloid-derived suppressor cells,[12] which are less able than conventional monocytes to perform antibody-dependent cellular phagocytosis. In this context, inhibiting or depleting these suppressive monocyte-like myeloid-derived cells should generate a better anti-myeloma immune response along with elotuzumab and lenalidomide 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 next-generation immunomodulatory drugs, the cereblon E3 ligase modulators.[32]

In summary, our mechanistic studies showed that: (i) in the presence of elotuzumab CD16 (FcγRIII) signaling is required for effective tumor cell killing by NK cells; (ii) RRMM patients' NK cells have reduced cytotoxicity, as shown by lower activation and loss of CD16 expression; (iii) in the presence of elotuzumab + lenalidomide there is an association between increased adhesion molecule expression on the NK and target cells and the levels of target cell killing; (iv) monocytes may play a role in improving the efficacy of elotuzumab and/or lenalidomide treatment; and (v) elotuzumab + lenalidomide treatment induced a significant increase in myeloma-derived chemokines for trafficking of immune effectors to the tumor cells (*Online Supplementary Figure S11*).

In conclusion, our findings indicate that increased CD54 expression on myeloma cells would be a useful predictive biomarker for response to elotuzumab and lenalidomide treatment. This could be evaluated in myeloma co-cultures with patients' PBMC treated with elotuzumab and lenalidomide. The mechanistic studies indicate that cross-talk between NK cells, monocytes and myeloma cells is critical for this response.

Disclosures

PJN received research project funding from Roche Genentech, Allergan, Compugen, Advaxis, MSD, and Crispr Therapeutics.

Contributions

KJR designed and performed in vitro and in vivo studies, prepared figures, and wrote and edited the manuscript. SPK performed the bio-informatics analysis, prepared figures, and wrote and edited the manuscript. JJZ, DM, CDS, SM and KT performed experiments, and wrote and edited the manuscript. KC provided key research reagents, academic input and edited the manuscript. MR and NAB provided academic input into the project direction, and read and edited the manuscript. HQ, DSR, SJH and HMP all provided MM patients' samples from their clinical trials, and read and edited the manuscript. JAT provided academic direction, and read and edited the manuscript. MRJ, PAB, PKD and PJN supervised the project direction, experiments and manuscript writing. PJN and HMP also provided financial support for the project.

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Data-sharing statement

Data are available on reasonable request, as per our institute's policy.

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