

# Elotuzumab directly enhances NK cell cytotoxicity against myeloma via CS1 ligation: evidence for augmented NK cell function complementing ADCC

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**Abstract** Elotuzumab is a monoclonal antibody in development for multiple myeloma (MM) that targets CS1, a cell surface glycoprotein expressed on MM cells. In preclinical models, elotuzumab exerts anti-MM efficacy via natural killer (NK)-cell-mediated antibody-dependent cellular cytotoxicity (ADCC). CS1 is also expressed at lower levels on NK cells where it acts as an activating receptor. We hypothesized that elotuzumab may have additional mechanisms of action via ligation of CS1 on NK cells that complement ADCC activity. Herein, we show that elotuzumab appears to induce activation of NK cells by binding to NK

cell CS1 which promotes cytotoxicity against CS1(+) MM cells but not against autologous CS1(+) NK cells. Elotuzumab may also promote CS1–CS1 interactions between NK cells and CS1(+) target cells to enhance cytotoxicity in a manner independent of ADCC. NK cell activation appears dependent on differential expression of the signaling intermediary EAT-2 which is present in NK cells but absent in primary, human MM cells. Taken together, these data suggest elotuzumab may enhance NK cell function directly and confer anti-MM efficacy by means beyond ADCC alone.

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## Introduction

Therapeutic monoclonal antibodies (mAbs) have revolutionized treatment of many cancers [1]; however, none are yet approved for multiple myeloma (MM) [2]. The most promising candidate to date is elotuzumab, a humanized IgG<sub>1</sub> mAb that targets CS1 expressed on MM cells [3, 4]. Preclinical models have shown that elotuzumab acts through antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells [3, 4].

CS1 (also called SLAMF7, CS2-like receptor-activating cytotoxic cells or CRACC, CD319) is a type I membrane glycoprotein in the CD2 family of receptors [3, 4]. CS1 expression has been observed to be highly expressed on normal and malignant plasma cells as well as NK cells and some T cells and appears to be a self-ligand [4–7]. While the function of CS1 on MM cells remains unclear, CS1 is also expressed on NK cells where it acts as an activating receptor via interaction with the signaling intermediary

EAT-2 [5–7]. We hypothesized that elotuzumab may have additional mechanisms of action via ligation of CS1 on NK cells. Herein, we demonstrate that elotuzumab activates NK cells and promotes tumor cell-directed cytotoxicity independent from and in addition to ADCC. Elotuzumab enhances NK cell activation and cytokine production and may facilitate homotypic CS1 interactions yet does not facilitate cytotoxicity against fraternal CS1(+) NK cells. Elotuzumab promotes association of EAT-2 with CS1 in NK cells, as well as the activation of extracellular signaling-regulated kinase (ERK) in NK cells but not in MM cells.

## Methods

### Primary cells and cell lines

Cells were cultured in RPMI1640 media with 10 % FBS (Gibco) at 37 °C in 5 % CO<sub>2</sub>. Primary human NK cells were isolated from leukopaks (American Red Cross, Columbus, OH), peripheral blood mononuclear cells (PBMC), and bone marrow aspirates obtained from MM patients under institutional review board-approved protocols. CD138(+) cells were separated from whole marrow aspirates as previously described [8]. The CS1(+) MM cell lines L363 and OPM-2 were obtained from DSMZ (Braunschweig, Germany); K562, NK92, and Ba/F3 were obtained from ATCC (Manassas, VA).

### Antibodies and reagents

Elotuzumab, elo-G2M3 (a form of elotuzumab with an IgG<sub>2</sub> backbone and Fc region mutations which minimize CD16 binding), elotuzumab F(ab')<sub>2</sub>, and isotype controls were produced at Abbvie Biotherapeutics; all reported experiments were conducted using 100 µg/mL. This standard dose was utilized for experiments in the present work as this concentration is readily achieved in vivo in serum and bone marrow of patients participating in clinical trials of elotuzumab [9–11]. Anti-CD56-APC and anti-CD16-PE were purchased from Beckman Coulter (Brea, CA). Anti-CD69-PE-Cy7 was purchased from eBioscience (San Diego, CA). Anti-CD3-PerCP-Cy5.5, anti-CD138-FITC, anti-CD107a-FITC, anti-CD16-APC-Cy7, anti-HLA-ABC-PE, anti-CD38-PE-Cy7, 7AAD, anti-CD38-APC, and isotype controls were from BD Pharmingen (San Jose, CA).

### Cytotoxicity and activation assays

Interferon-γ (IFN-γ) and granzyme B (GrB) secretion (as an effector-based cytotoxicity assay) was measured by enzyme-linked immunosorbent spot (ELISPOT) as

previously described [8, 12, 13]. Spots were visualized and counted using an Immunospot Imaging Analyzer (Cellular Technology Ltd, Cleveland, OH). As a target-dependent cytotoxicity assay, primary human-enriched NK effector cells were plated at  $1 \times 10^6$  cells/ml in RPMI supplemented with 20 % FBS and treated with either 100 µg/ml elotuzumab or isotype control antibody for 24 h prior to killing. K562 target cells were plated at  $0.4 \times 10^6$  cells/ml in RPMI supplemented with 10 % FBS and treated with either 100 µg/ml elotuzumab or isotype control antibody for 24 h before use as targets in a 4 h <sup>51</sup>Cr release assay previously described [14]. Target cells were labeled with 100 µCi of <sup>51</sup>Cr in a total volume no greater than 150 µl for 75 min. Unbound antibodies from the treatment are washed out during multiple wash steps of the <sup>51</sup>Cr release protocol. Neither elotuzumab nor isotype antibodies were added to the plate during the killing incubation. To assay the proper effector–target cell ratios (E:T), effector cells were plated and serially diluted in triplicates while a constant number of target cells were added to every well. Spontaneous killing was less than 12 %.

CD69 mean fluorescent intensity (MFI) at baseline and after incubation in mAbs was evaluated on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Version 8.8.6; Tree Star Inc, Ashland, OR).

### CD107a expression

As a second, effector-based cytotoxicity assay, whole MM marrow aspirates were incubated 24 h in isotype mAb or elotuzumab. The percentage of CD56<sup>+</sup>CD16<sup>+</sup>CD3<sup>-</sup>CD107a<sup>+</sup> cells in the lymphocyte gate was determined by flow cytometry.

### Immunoprecipitation/Western blotting

EAT-2 and IgG isotype Abs were incubated with protein-G agarose beads in buffered 5 % BSA. NK cells were incubated 5 min in elotuzumab or isotype mAb, then lysed. Total protein (120 µg) was added to beads and incubated at 4 °C overnight. Immunoblotting for CS1 (AF1906, R&D Systems, Minneapolis, MN), EAT-2 (SC-21572, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK (4370, Cell Signaling Technology, Beverly, MA), and total ERK (4695, Cell Signaling Technology) were performed as previously described [15].

### Real-time polymerase chain reaction

CD138(+) MM cells were separated from whole marrow aspirates using the EasySep Human Whole Blood CD138 Selection Kit (StemCell Technologies, Vancouver, Canada)

according to manufacturer's instructions. Immediately afterward, mRNA was obtained by lysing an equal number of cells from the resulting CD138(+) and CD138(−) fractions. RNA was isolated using the RNeasy Micro Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized according to manufacturer's instructions using MMLV reverse transcriptase kit (Invitrogen, Grand Island, NY). Real-time PCR was performed on an ABI Prism 7900HT (Applied Biosystems, Grand Island, NY) using Taqman primer/probe set for SH2D1B (aka EAT-2) purchased from Applied Biosystems. Gene expression levels were normalized to an 18S internal control and then analyzed by the DD Ct method [16]. Fold change in EAT-2 mRNA expression in the CD138(+) fraction is depicted relative to level of EAT-2 detected in the CD138(−) fraction.

### Statistical considerations

Student's *t* test or one-way ANOVA were used to evaluate differences between conditions with  $p < 0.05$  considered to be statistically significant. The mean relative fluorescent intensity (MRFI) was calculated as described previously [8]. Nonparametric inferential statistics were used to evaluate data obtained in assays utilizing patient-derived effector cells and autologous MM targets.

## Results

### Elotuzumab activates NK cells and induces IFN- $\gamma$ production

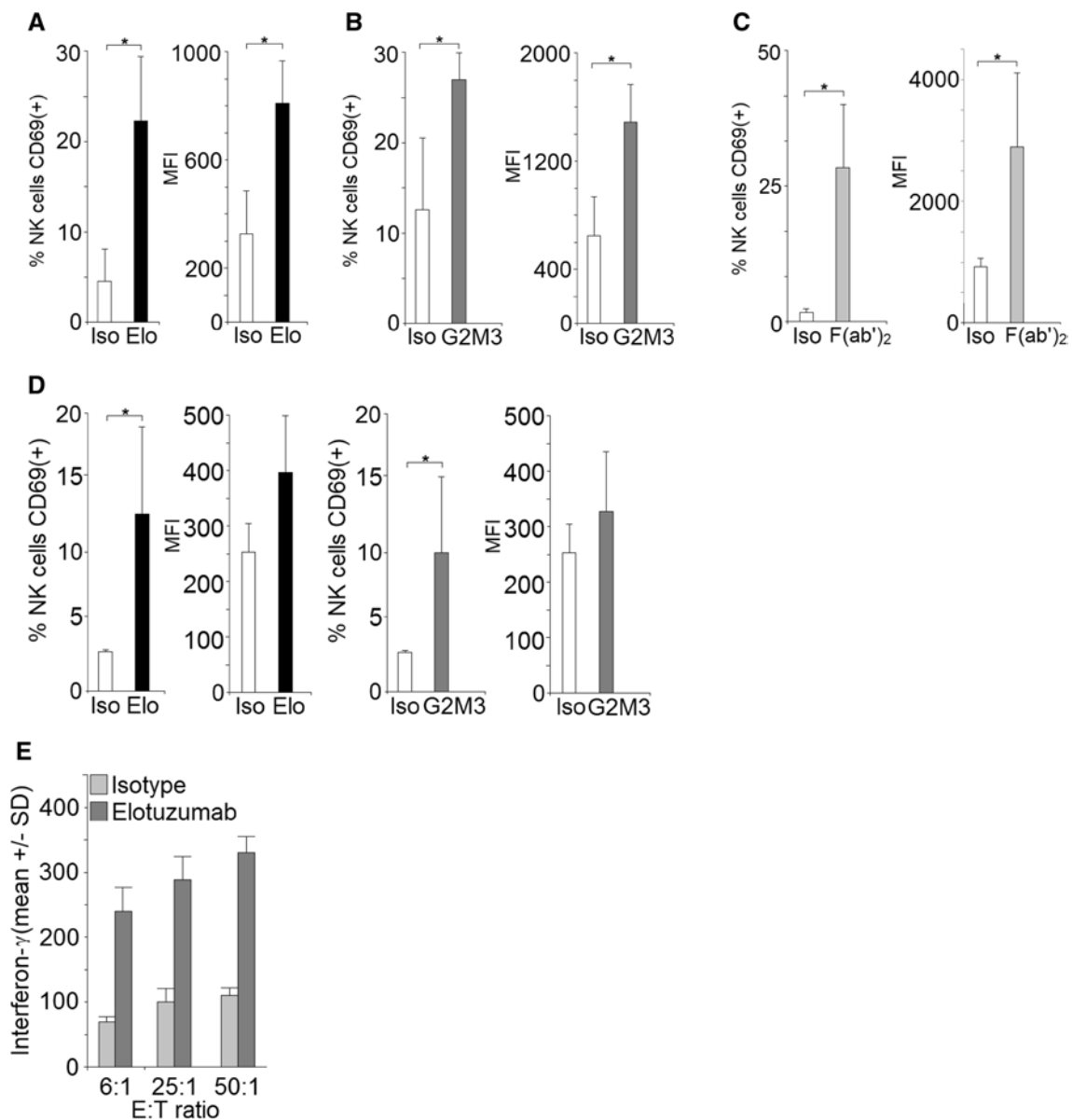
We observed CS1 expression beginning at stage 3 of NK cell development and on CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets (data not shown) [17, 18]. Elotuzumab increased the percentage of NK cells expressing CD69 as well as CD69 MFI on fresh, healthy donor NK cells in the absence of MM targets ( $4.5 \pm 7.1$  vs.  $22.3 \pm 3.6$  %,  $p = 0.019$ , MFI:  $326 \pm 162$  vs.  $809 \pm 159$ ,  $p = 0.021$ , Fig. 1a). To verify that this effect was due to elotuzumab ligating CS1 directly on NK cells and not mediated through Fc-binding of elotuzumab by CD16, experiments were conducted in parallel with elo-G2M3, an elotuzumab variant with reduced CD16 binding as well as with elo-F(ab')<sub>2</sub>. Increase in CD69 on NK cells was observed in response to elo-G2M3 ( $12.6 \pm 8$  % vs.  $26.7 \pm 3$  %,  $p = 0.04$ , MFI:  $650 \pm 289$  vs.  $3,572 \pm 410$ ,  $p = 0.02$ , Fig. 1b) and in response to elo-F(ab')<sub>2</sub> stimulation ( $29 \pm 15$  vs.  $1.83 \pm 0.7$  %,  $p = 0.035$ , MFI:  $929 \pm 144$  vs.  $2,901 \pm 1,227$ ,  $p = 0.051$ , Fig. 1c). We then verified this effect in NK cells from  $n = 3$  patients with MM ( $12.2 \pm 6$  vs.  $2.6 \pm 0.01$  % for elotuzumab,  $p = 0.001$ , vs.  $10 \pm 5$  % for elo-G2M3,  $p = 0.04$ , Fig. 1d). We also conducted activation experiments with

lower doses of elotuzumab. Nineteen percent ( $\pm 17$ ) of NK cells expressed CD69 in response to  $10 \mu\text{g/mL}$  and 22 % ( $\pm 16$ ) expressed CD69 in response to  $50 \mu\text{g/mL}$  (data not shown). Attempts were made to show this finding in the NK92 cell line as well, but were unsuccessful perhaps related to the line's dependence on interleukin-2 for viability and baseline expression of CD69. Elotuzumab also increased NK cell IFN- $\gamma$  production 2.5–3.4-fold (all pairwise comparisons  $p < 0.05$ ) over isotype control against CS1(+) L363 MM cell line targets (Fig. 1e).

Elotuzumab ligation of CS1 on NK cells directly enhances granzyme B release against CS1(+) MM cells and CS1(−) tumor cell targets but not against autologous CS1(+) NK cells

Healthy donor, primary NK cells, and/or the CS1(+) L363 MM cell line were cultured independently in the presence of elotuzumab, elo-G2M3, or isotype control. Using ELISPOT-based production of GrB as an effector-based cytotoxicity assay with an E:T ratio of 25:1 [12], we first confirmed ADCC as an elotuzumab mechanism leading to GrB release against MM cells in vitro (Fig. 2a “\*\*”). Isotype-treated NK cells produced an average of 50 GrB spots/well ( $\pm 4$  SEM) against isotype-treated MM cells. As expected, against elo-G2M3-treated targets, no enhancement of GrB release was observed (mean  $55 \pm 2$  spots/well,  $p = \text{n/s}$  compared to isotype-treated targets). ADCC was verified in comparing isotype-treated NK cell GrB production against elotuzumab-treated targets ( $127 \pm 6$  spots/well,  $p = 0.001$ ) to control conditions. Interestingly, pre-treatment of NK cell effectors with elotuzumab ( $117 \pm 7$ ,  $p < 0.05$ ) or elo-G2M3 ( $84 \pm 3$ ,  $p < 0.05$ ) also increased NK cell GrB degranulation against isotype-treated MM targets compared to control conditions (Fig. 2a, “\*\*\*” and “\*\*\*\*”) suggesting that CS1 ligation on NK cells directly promotes NK cell cytotoxicity. GrB release was greatest when both NK cells and MM targets were pre-treated with elotuzumab ( $150 \pm 10$ , Fig. 2a, far right bar). In addition, the experiment was repeated with higher E:T ratios (Fig. 2b) with similar results, except that direct ligation of NK cell CS1 by elo-G2M3 appears to provide a complementary increase in GrB release to ADCC conferred through elotuzumab-treated MM targets (Fig. 2b, arrow). These results suggest that ligation of CS1 on NK cells provides an additional mechanism by which elotuzumab enhances NK cell cytotoxic potential against MM cells.

We then co-cultured patient-derived NK cells with autologous CD138(+) MM targets. Independent pre-treatment of NK cells or MM cells or both with elotuzumab resulted in enhanced NK cell GrB production against MM targets (“\*” each condition  $p < 0.05$  pair-wise versus control, Fig. 2c). Additionally, elotuzumab enhanced NK cell



**Fig. 1** Elotuzumab activates NK cells **a** Elotuzumab, **b** elo-G2M3, and **c** elo-F(ab')<sub>2</sub> enhance healthy donor NK cell and **d** patient-derived NK cell activation in the absence of MM targets as measured

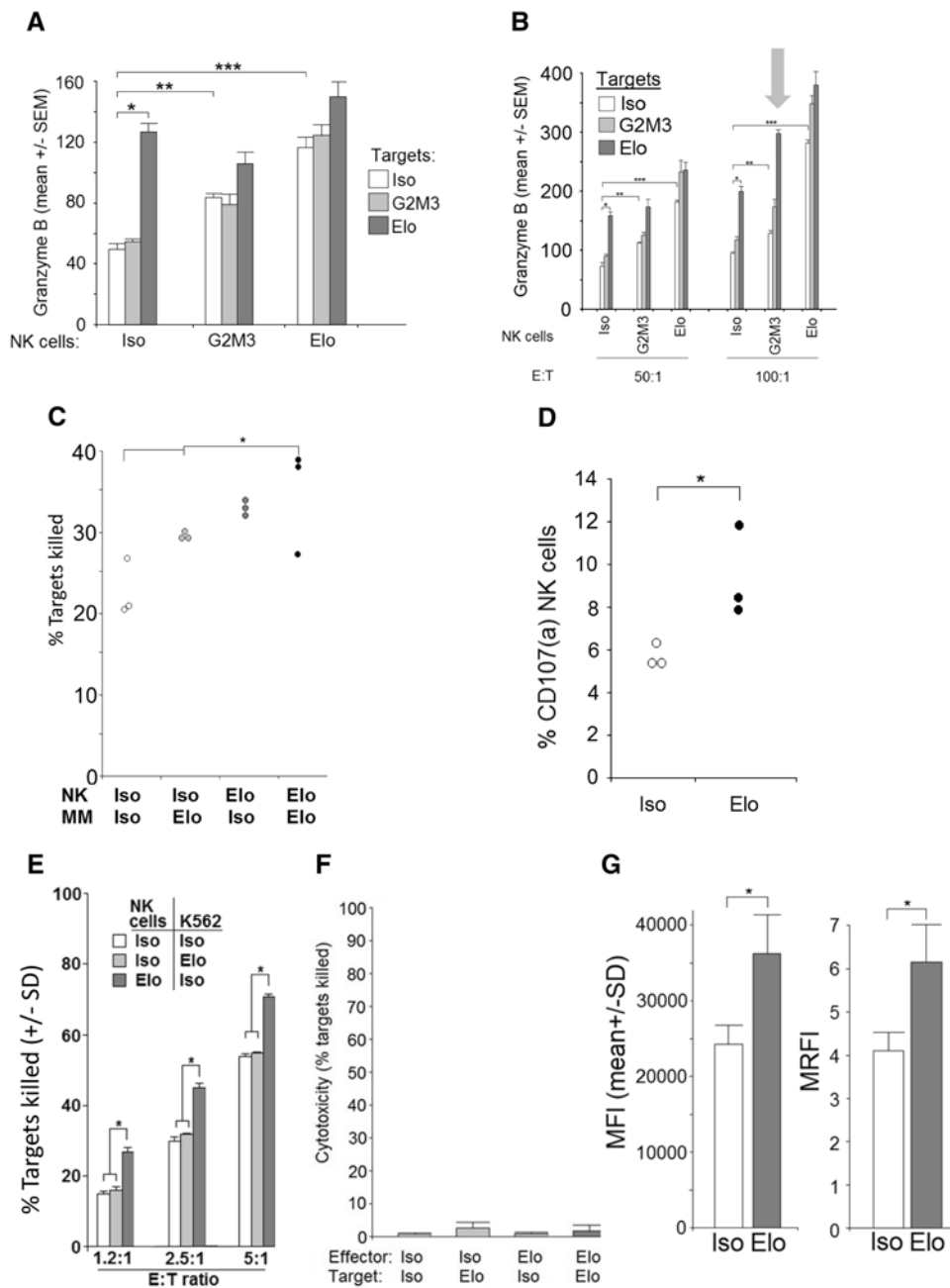
by CD69 expression. **e** Elotuzumab increased NK cell IFN- $\gamma$  production 2.5–3.4-fold (all pair-wise  $p < 0.05$ ) against CS1(+) L363 MM cells (results represent  $n = 3$  independent experiments)

degranulation in freshly isolated, whole MM bone marrow aspirates ( $p = 0.04$ ,  $n = 3$  independent experiments, Fig. 2d).

Although it has been shown previously that elotuzumab does not mediate ADCC against CS1(–) targets, we next questioned whether elotuzumab could directly potentiate NK cell cytotoxicity against CS1(–) tumor cell targets by binding to CS1 on NK cells. Primary, healthy donor NK cells or CS1(–) K562 targets were incubated for 72 h in isotype or elotuzumab. Following 4 h co-culture, cytotoxicity was determined by chromium release assay. Consistent with prior data, elotuzumab

treatment of CS1(–) K562 cells did not enhance NK cell killing. However, CS1(+) effector NK cells pre-treated with elotuzumab showed enhanced killing of CS1(–) K562 targets (Fig. 2e,  $p < 0.05$  for all indicated pair-wise comparisons).

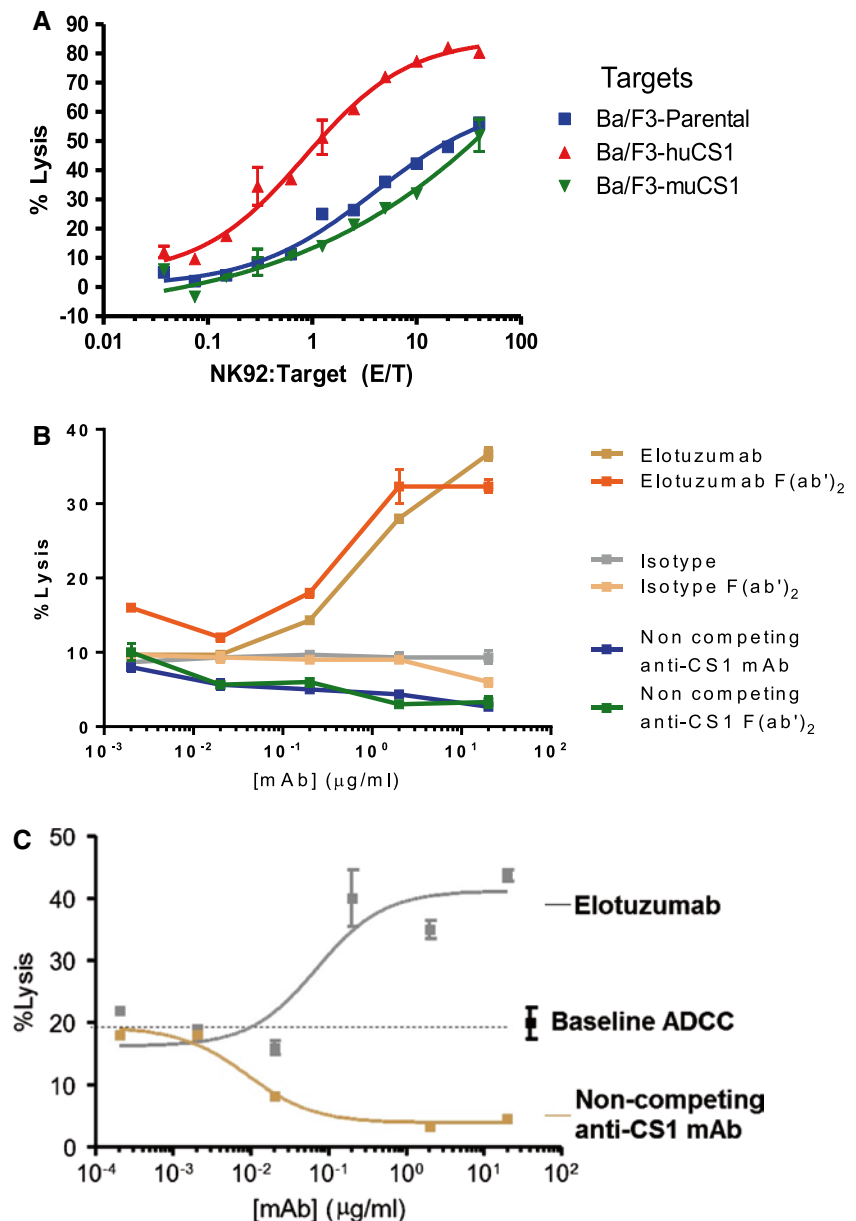
We next questioned whether elotuzumab could increase NK cell cytotoxicity against autologous CS1(+) NK cells. Pre-treatment of effector and/or target NK cells did not enhance NK cell killing of autologous NK cell targets (overall ANOVA  $p = n/s$ ,  $n = 3$  independent donor experiments, Fig. 2f). Interestingly, elotuzumab enhanced NK cell MHC Class I surface expression which serves as ligands to



**Fig. 2** Elotuzumab augments tumor-directed NK cell killing by ADCC and by direct NK cell activation **a** Isotype-treated NK cells produced an average of GrB 50 spots/well ( $\pm 4$  SEM) against isotype-treated MM targets. Against G2M3-treated MM tumor cells, as expected, no enhancement of killing was observed (GrB mean  $55 \pm 2$ ,  $p = n/s$  compared to isotype-treated targets); however, against elotuzumab-treated MM targets, isotype-treated NK cells produced  $127 \pm 6$  GrB spots/well, confirming ADCC as a mechanism of killing (“\*”,  $p = 0.001$ ). Elo-G2M3-treated NK cells showed evidence of enhanced cytotoxicity against isotype-treated MM tumor cells (“\*\*\*”, mean GrB =  $84 \pm 3$ ,  $p < 0.05$ ) as did elotuzumab-treated NK cells (“\*\*\*\*”, mean GrB =  $117 \pm 7$ ,  $p < 0.05$ ). **b** These results were validated at higher E:T ratios where elo-G2M3 treatment of NK cells enhanced killing of elotuzumab-treated MM targets, suggesting

complementary effects of CS1 ligation on NK cells and MM cells by elotuzumab. **c** Utilizing patient-derived NK cells as effectors, and purified, CD138(+) autologous MM cells as targets, pre-treatment of either effectors or targets or both with elotuzumab enhanced killing as compared to isotype control (“\*” each condition  $p < 0.05$  pair-wise versus control,  $n = 3$  independent experiments). **d** In whole marrow aspirates, elotuzumab enhanced NK cell degranulation measured by CD107a expression ( $p = 0.04$ ,  $n = 3$  independent experiments). **e** Pre-treatment of NK cells with elotuzumab for 72 h enhanced killing of CS1(-) K562 targets at multiple E:T ratios ( $p < 0.05$  for all “\*” comparisons indicated). **f** Elotuzumab did not enhance CS1(+) NK cell cytotoxicity against autologous CS1(+) NK cells, perhaps due in part to **(g)** upregulation of MHC class I molecules on NK cells in response to elotuzumab, MFI:  $p = 0.02$ , MRFI:  $p = 0.01$

**Fig. 3** Elotuzumab facilitates NK cell killing by modulating CS1–CS1 interaction **a** The CS1(+), C16(–) NK cell line NK92 shows increased cytotoxicity against CS1(+) Ba/F3 cells as compared to parental Ba/F3 or Ba/F3 expressing murine CS1. **b** Elotuzumab or elo-F(ab')<sub>2</sub> enhanced NK92 killing of CS1(+)-Ba/F3 targets as compared to relevant isotype or non-competing controls. **c** Increasing concentrations of elotuzumab enhanced NK92 killing of CS1(+)-Ba/F3 cells as compared to a non-competing, anti-CS1 control

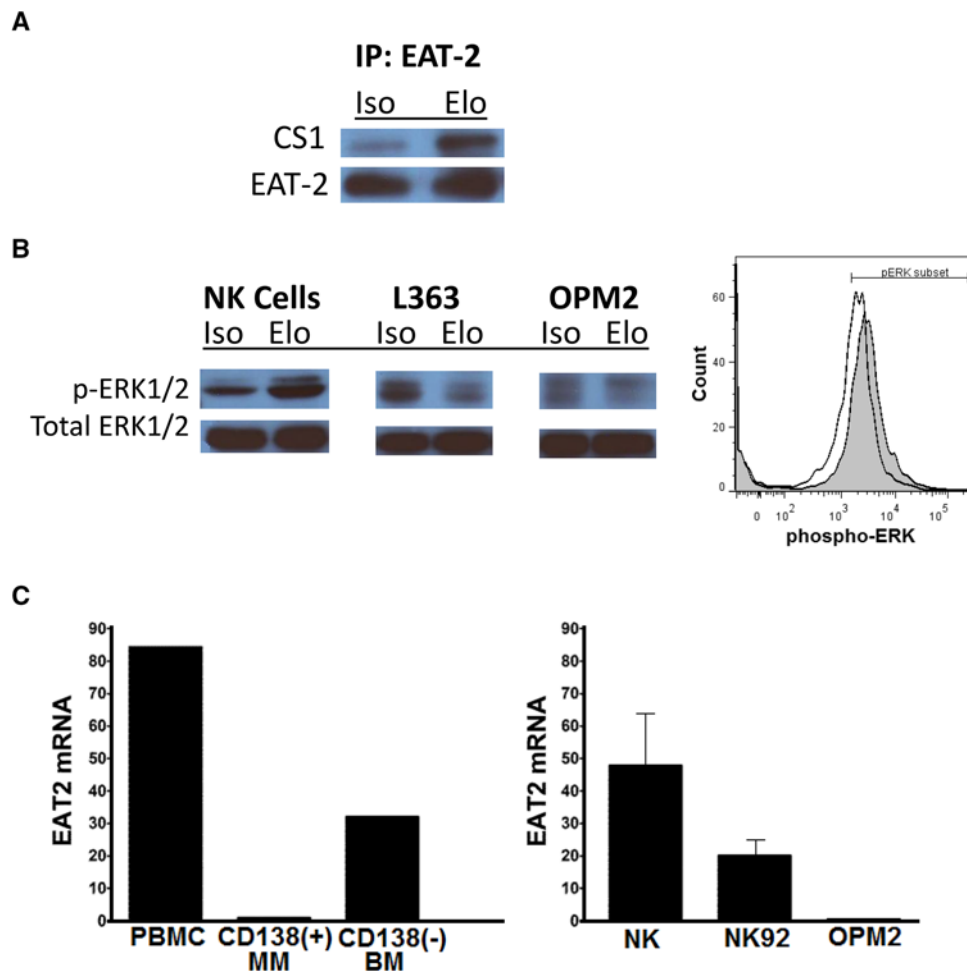


NK cell inhibitory killer immunoglobulin-like receptors, MFI  $24,433 \pm 2,482$  versus  $36,266 \pm 5,200$ ,  $p = 0.02$ , MRFI  $4.1 \pm 0.4$  versus  $6.2 \pm 0.8$ ,  $p = 0.01$ ,  $n = 3$  independent donor experiments, Fig. 2g). However, elotuzumab did not induce similar modulation of MHC class I expression on MM cells (data not shown).

Elotuzumab augments NK cell cytotoxicity against CS1(+) tumor cell targets by promoting CS1–CS1 interaction

CS1 is a homotypic adhesion receptor that promotes NK cell interactions with CS1(+) tumors in mouse models [6]. We compared the activity of elotuzumab, which recognizes the membrane-proximal IgC2-like domain with

a non-competing anti-CS1 mAb which binds the membrane distal IgV-like domain, on CS1–CS1 interactions. The CS1(+),CD16(–) NK92 NK cell line was utilized as effector cells, and target cells were either parental or CS1(+) Ba/F3 cells. NK92 cells showed higher cytotoxicity against Ba/F3-CS1(+) cells than the parental Ba/F3 cells (Fig. 3a). Elotuzumab and Elo-F(ab')<sub>2</sub> also promoted cytotoxicity (Fig. 3b). This activity was dependent on the CS1 epitope recognized by the antibody, as the non-competing anti-CS1 mAb inhibited cytotoxicity in this model (Fig. 3c). These data are consistent with structural studies on the homotypic binding of other SLAM family members, where interactions occur through the IgV-like domain [19]. Elotuzumab may stabilize these interactions, whereas



**Fig. 4** Elotuzumab recruits EAT-2 to CS1 in NK cells and induces downstream signal transduction **a** Elotuzumab facilitates association of CS1 with EAT-2. Lysates from NK cells incubated in either isotype or elotuzumab were immunoprecipitated with an EAT-2 antibody and immunoblotting was performed with an anti-CS1 antibody. Results show that elotuzumab increased co-localization of CS1 with EAT-2 (EAT-2 immunoblot shown as loading control, results representative of  $n = 2$  independent experiments in independent donors. The com-

plementary experiment was attempted using the CS1 antibody to immunoprecipitate and EAT-2 to blot, but this was not successful as the CS1 antibody was not able to function in this manner.) **b** Elotuzumab also appeared to enhance ERK phosphorylation in NK cells (*left panel*) but not in CS1(+) MM cell lines. **c** Neither EAT-2 protein nor transcript was observed in freshly isolated, primary CD138(+), human MM cells (*center bar*) as compared to peripheral blood mononuclear cells (*left bar*) or CD138(–) marrow elements (*right bar*)

the mAb against the membrane distal domain may block these interactions.

Elotuzumab promotes colocalization of CS1 with EAT-2 and activates downstream signaling in NK cells but not MM cells

To verify whether the functional effects described above were in fact related to elotuzumab binding and signaling through CS1 on NK cells, NK cells were treated with isotype or elotuzumab and colocalization of CS1 and EAT-2 was evaluated. Elotuzumab facilitated EAT-2 interaction with CS1 in primary NK cells (Fig. 4a), a phenomenon previously demonstrated in the NK cell line, NK92.<sup>7</sup> In

addition, elotuzumab also enhanced phosphorylation of ERK in NK cells (Fig. 4b, left) [15, 20]. A similar finding was observed in the NK cell line, NK92, where phospho-ERK was demonstrated in 37.3 % of isotype-treated cells (MFI 1,137) as compared to 52.5 % of elotuzumab-treated cells (MFI 2,230, Fig. 4b, right panel). Interestingly, although EAT-2 was detected by Western blot in the U266 MM cell line (but not other MM cell lines), no colocalization occurred between EAT-2 and CS1 upon elotuzumab stimulation of MM cell lines (data not shown) nor did elotuzumab lead to increased ERK phosphorylation in MM cell lines (Fig. 4b, center panels). In freshly isolated, primary human MM samples, the presence of EAT-2 could not be detected at protein (not shown) or message level (Fig. 4c).

## Discussion

A phase I trial has demonstrated elotuzumab to be well tolerated [9], and phase II studies in combination with bortezomib or lenalidomide (agents shown to exert anti-MM effects, in part, through enhancement of the NK cell versus MM effect) [21, 22] have shown promising results, which await confirmation in ongoing phase 3 studies [10, 11]. Interestingly, in the phase I single-agent trial, some patients experienced acute infusion reactions which may have been mediated by direct effects of elotuzumab on immune effectors, but these have been mitigated with premedication in subsequent studies [9–11]. These reactions appear to have correlated with early increases in circulating levels of pro-inflammatory cytokines [23], a phenomenon we have demonstrated *in vitro* with exposure of patient and healthy donor PBMC to elotuzumab, as well [24]. In addition, prior work by members of our group has also demonstrated that elotuzumab in combination with agents known to exert anti-MM activity, in part, through augmentation of NK cell function also leads to enhancement of MM cell killing over elotuzumab or other therapies alone [25, 26]. For example, whereas lenalidomide increases T cell IL-2 production, the combination of elotuzumab and lenalidomide results in decreased IL-2 levels due to a parallel increase in CD25 expression on NK cells, which further drives NK cell activation by the combination. This results in enhancement of IFN- $\gamma$  production and greater MM cell killing than observed with either agent alone [26].

These observations, in combination with the present findings, suggest that elotuzumab may exert anti-MM effects by means beyond ADCC alone which has already been characterized as a mechanism of action in several studies [3, 4]. First, elotuzumab appears to induce NK cell activation through binding to CS1 expressed on NK cells. This process appears to be mediated by recruitment of the membrane-proximal signaling intermediary EAT-2 and is associated with phosphorylation of downstream signal transduction proteins implicated in NK cell activation and cytotoxicity [15]. Primary, human MM cells appear to lack EAT-2 expression which may be a reason for differential effects of CS1 ligation by elotuzumab on NK cells and MM cells. Second, CS1 is known to be a homotypic receptor, and elotuzumab may facilitate CS1–CS1 interaction between effector NK cells and CS1(+) target cells to promote target recognition through other receptor–ligand systems and facilitate cytotoxicity. These processes appear to be independent of ADCC as demonstrated with CD16(–) effector cell populations and with elotuzumab variants (Elo-G2M3 and Elo-F(ab')<sub>2</sub>) which are not recognized by CD16. Additionally, elotuzumab may promote the secretion of cytokines from NK cells to indirectly modulate T cell function, as well.

Taken together, these results open up the possibility of considering the use of elotuzumab as therapy against CS1(–) but NK cell-sensitive tumors. These results provide novel data on possible, additional mechanisms by which elotuzumab promotes the NK cell versus MM effect to complement killing mediated by ADCC and inform ongoing, rational development of the agent as a promising therapeutic mAb for the treatment of patients with MM.

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**Conflict of interest** Don M Benson has served in an advisory capacity to and received research funding from Bristol Myers Squibb; Gary C Starling is a former employee of Abbott Laboratories; Hakju Kwon and Audie Rice are employees of Abbvie Biotherapeutics Inc. No other authors have conflicts of interest to disclose.

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