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Enhanced SLAMF7 homotypic interactions by elotuzumab improves NK cell killing of multiple myeloma

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Abstract

Elotuzumab (Elo) is an IgG₁ monoclonal antibody targeting SLAMF7 (CS1, CRACC, CD319), which is highly expressed on multiple myeloma (MM) cells, natural killer (NK) cells, and subsets of other leukocytes. By engaging with FcγRIIIA (CD16), Elo promotes potent NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) and macrophage-mediated antibody-dependent cellular phagocytosis (ADCP) toward SLAMF7⁺ MM tumor cells. Relapsed/refractory MM patients treated with the combination of Elo, lenalidomide, and dexamethasone have improved progression-free survival. We previously showed that Elo enhances NK cell activity via a co-stimulation mechanism, independent of CD16 binding. Here, we further studied the effect of Elo on cytotoxicity of CD16-negative NK-92 cells. Elo, but not other SLAMF7 antibodies, uniquely enhanced cytotoxicity mediated by CD16-negative NK-92 cells toward SLAMF7⁺ target cells. Furthermore, this CD16-independent enhancement of cytotoxicity required expression of SLAMF7 containing the full cytoplasmic domain in the NK cells, implicating co-stimulatory signaling. The CD16-independent co-stimulation by Elo was associated with increased expression of NKG2D, ICAM-1, and activated LFA-1 on NK cells, and enhanced cytotoxicity was partially reduced by NKG2D blocking antibodies. In addition, an Fc mutant form of Elo that cannot bind CD16 promoted cytotoxicity of SLAMF7⁺ target cells by NK cells from most healthy donors, especially if previously cultured in IL-2. We conclude that in addition to promoting NK cell-mediated ADCC (CD16-dependent) responses, Elo promoted SLAMF7-SLAMF7 interactions in a CD16-independent manner to enhance NK cytotoxicity towards MM cells.

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Keywords

Multiple Myeloma; SLAMF7; Elotuzumab; NK cells

Introduction

Multiple myeloma (MM) is a bone marrow plasma cell neoplasm and second most common hematological malignancy (1). Immunomodulatory drugs (thalidomide, lenalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib, ixazomib), and autologous stem cell transplantation have significantly improved patient survival (2-8). However, multiple myeloma remains largely incurable, as most patients relapse and become refractory to existing treatments (9).

Elotuzumab (Elo) is an IgG₁ monoclonal antibody targeting signaling lymphocytic activation molecule family member 7 (SLAMF7), which is expressed on tumor cells in 95% of multiple myeloma (MM) cases (10-12). SLAMF7 is also found on immune cells, including natural killer (NK) cells, monocytes, and macrophages (13-16). Elo generates effective responses toward MM in pre-clinical studies *in vitro* and *in vivo* (10,11,17) and improves progression free survival (PFS) of relapsed/refractory (RR)MM patients when administered as an immunotherapeutic in combination with lenalidomide/dexamethasone (17, 18). Elo plus pomalidomide/dexamethasone also significantly improves PFS compared to pomalidomide/dexamethasone alone (19). Anti-tumor effects result from several innate immune cell activation mechanisms: 1) NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) through Fc γ RIIIA (CD16), 2) Fc γ R-dependent macrophage-mediated antibody-dependent cellular phagocytosis (ADCP), and 3) CD16-independent co-stimulation of NK cells through direct interaction with SLAMF7 (10,11,14,16,20-22).

The efficacy of ADCC-inducing antibodies, such as rituximab, in hematological malignancies is enhanced in patients homozygous for the high affinity polymorphic variant of CD16 [valine at position 176 (or position 158 if leader sequence is subtracted)] compared to patients with one or two alleles encoding the low affinity variant with phenylalanine (F) at position 176 (23, 24, 25). Accordingly, in a randomized phase II clinical trial of Elo plus bortezomib and dexamethasone, 176V/V homozygous patients have higher progression-free survival compared to 176F/F patients (26).

Like most members of SLAM family receptors, SLAMF7 serves as a self-ligand (27), but it has unique co-stimulatory function in NK cells (28). SLAMF7 contains an intracellular immunoreceptor tyrosine-based switch motif (ITSM), which can recruit the cytosolic EAT-2 adaptor protein (29). NK cells express EAT-2, which mediates intracellular co-stimulatory signaling by SLAMF7, but plasma and MM cells do not express EAT-2 and thereby lack SLAMF7 signaling (29-31). Tyrosine phosphorylated EAT-2 recruits PLC- γ 1, resulting in calcium mobilization, ERK activation, and enhanced functional responses by NK cells (29,32). SLAMF7 can also physically interact with Mac-1 to trigger activation signaling in macrophages (13). Alternative mRNA splicing generates SLAMF7-long (L) and SLAMF7-short (S) isoforms (33). SLAMF7-S lacks the ITSM, interaction with EAT-2, and activation signaling.

Previous work showed that Elo promotes cytotoxicity by NK cells independent from ADCC (22) by causing CD16-independent co-stimulation of NK cells through SLAMF7 (16). Here, we demonstrated that CD16-independent enhancement of cytotoxicity by Elo required SLAMF7 expression on both NK and target cells and required expression of SLAMF7-L in the NK cells. Elo had unique capacity among several SLAMF7 antibodies to enhance cytotoxicity by promoting SLAMF7-SLAMF7 interactions between NK and MM cells. In addition, a Fc mutant form of Elo lacking CD16-binding properties promoted cytotoxicity of MM target cells by primary NK cells from most healthy donors, especially when the NK cells were cultured with IL-2.

Methods

Cells and cell lines

NK-92 cells were obtained from ATCC in 2005 and cultured in complete α -MEM medium as described (34), supplemented with 100U/ml of human recombinant IL-2 (teceleukin, Hoffman-La Roche Inc.). Cells were passed with fresh medium and IL-2 every 3–4 days. The cDNAs encoding either high affinity (176V; GenBank: [BC017865.1](#)) or low affinity (176F; GenBank: [NM_000569.6](#)) variants of Fc γ RIIIA and human SLAMF7-L (GenBank: [NM_021181.4](#)) or SLAMF7-S (GenBank: [NM_001282592.1](#)) were subcloned into pBMN-NoGFP retroviral vector (35,36). NK-92 parental cells retrovirally transduced to express either CD16 variant were previously described (34,37) and always obtained from master stocks. NK-92 SLAMF7 knockout cells were generated using a doxycycline-inducible CRISPR/Cas9 system as previously described (38). Specific guide RNA sequences [sg1 5'-AAAGAGCTGGTCGGTTCCTG-3', sg2 5'-GAGACACAGGAGGACCATGG-3' (39) (Integrated DNA Technologies)] were cloned into pLRG to generate lentivirus in combination with pCMV-dR8.74PAX2 and pMD2.G (from Dr. Neil Johnson, FCCC) in 293T cells. NK-92 cells were infected twice with doxycycline-inducible blasticidin-resistant Cas9 lentivirus, viable blasticidin-resistant clones were single cell sorted, and clones with the highest induced Cas9 expression were selected. These were infected with lentivirus containing sgRNA and GFP, GFP⁺ cells were sorted and cultured with doxycycline for 48 hrs, and SLAMF7-deficient cells were sorted. NK-92 SLAMF7 KO cells were then transduced with retrovirus encoding CD16 176V, SLAMF7-L, or SLAMF7-S and sorted for SLAMF7 expression (34).

Multiple myeloma cell lines were cultured in complete RPMI-1640 medium (supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, sodium pyruvate, 10mM HEPES, and 50 μ M 2-mercaptoethanol). MM.1R and RPMI-8226 cells were obtained from ATCC at the beginning of the project and reauthenticated in 2018. RPMI-8226 were retrovirally transduced to express higher levels of SLAMF7-L (RPMI-8226+SLAMF7), and zinc finger nucleases (product number CKOZFND19392-1KT; Sigma-Aldrich) were introduced by nucleofection to disrupt SLAMF7 expression in MM.1R cells, followed by sorting for SLAMF7 expression, as previously described (16). SKOV3 cells were obtained from the Fox Chase Cancer Center Cell Culture Facility, authenticated in 2015, cultured in complete RPMI-1640 medium, and retrovirally transduced with SLAMF7-L cDNA in pBMN-IRES-NoGFP vector to obtain SLAMF7⁺ cells after sorting.

A hSLAMF7-TCR- ζ reporter cell line was engineered by transfecting 3A9 mouse T cells with a construct encoding fusion of human (h)SLAMF7 extracellular domain and mouse CD247 (TCR- ζ) transmembrane and intracellular domains in UCOE vector (detailed in Supplemental Figure S1). Cells were cultured in complete RPMI-1640 supplemented with 10% FBS and 800ug/ml of hygromycin B. SLAMF7 engagement was assayed by measuring mouse IL-2 secretion with ELISA according to the manufacturer (BD OptEIA, #55148).

Cell lines were authenticated using genetic short tandem repeat profiling analysis by IDEXX Bioresearch (Columbia, MO). Cell lines were thawed fresh every 2 months and found to be free of mycoplasma at least yearly in assays by the FCCC Cell Culture Facility. Primary NK cells were enriched from healthy donor PBMCs using the EasySep NK cell negative selection kit (StemCell Technologies) and confirmed to be >95% purity (CD45⁺CD56⁺CD3⁻; see Supplemental Figure S2). Healthy donor volunteers provided written informed consent using HIPAA-compliant procedures approved by the Institutional Review Board of FCCC and experiments were performed in accordance with the Declaration of Helsinki principles.

Reagents

SLAMF7 antibodies: Elotuzumab (Elo), Elo with Fc domain mutations that prevent recognition by CD16 (Elo Fc mut) (16), Elo Fab fragments (produced as recombinant proteins), Elo F(ab')₂ fragments (produced by pepsin cleavage), and PDL241 (40) were provided by Bristol-Myers Squibb (BMS; Princeton, NJ). The SLAMF7 antibody, ChLuc90, which consists of chimerized human IgG₁ Fc fused to mouse CDR variable regions, was provided by AbbVie Inc. (North Chicago, IL) (11). Elo Fc mut antibody (provided by BMS) was expressed by the CHO-S cell line co-transfected with vectors pICOFSCneoK (containing a cDNA encoding Elo variable regions) and pODpurIgG1.1f (containing a cDNA encoding IgG1 heavy chain constant region with L234A-L235E-G237A-A330S-P331S mutations, which abolish interaction with CD16, as previously described (16)). Comparative binding of Elo and Elo Fc mut to myeloma cells is shown in Supplemental Figure S3. The m24 antibody targeting activated LFA-1 and NKG2D blocking antibody, 1D11, were from Biolegend. The DNAM-1 blocking antibody was from R&D Systems (AF666; polyclonal goat IgG).

Flow cytometry

SLAMF7 expression was assayed by staining with biotinylated Elo conjugated antibody (EZ-Link NHS-Biotin, ThermoFisher #20217) and APC-streptavidin (Biolegend, #405207), SLAMF7 antibody conjugated with APC or DL650 (Biolegend, clone 162.1; (41)), or isotype control (Biolegend, #400330). Surface expression of 176V and 176F CD16 was assessed with GRM1 antibody (Southern Biotech, #9570-31), although CD16 176V was sometimes assayed with 3G8-APC (Biolegend, #302012). SLAMF7 antibody competition experiments were performed using the CHO cell line transfected to express high levels of SLAMF7-L. Cells (100,000 cells/condition) were incubated with various concentrations of SLAMF7 antibodies (Elo, PDL241, 162.1) on ice, washed 2x with staining buffer (0.1% Na azide and 1% FBS in HBSS) and incubated with conjugated SLAMF7 antibodies (162.1-PE, Biolegend; Elo-AF647, PDL241-AF647) on ice, washed x2 with staining buffer and 100

ng/ml of propidium iodide (Invitrogen) in the final wash, and then subjected to analysis on a BD LSR II or Aria II flow cytometer. Data were analyzed using FlowJo software (BD).

Cytotoxicity assay

NK cell cytotoxicity was assessed using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, #G1780), which is a colorimetric assay that measures lactate dehydrogenase released when target cells are lysed, according to the manufacturer's protocol.

xCELLigence assay

The xCELLigence Real-Time Cell Analyzer (RTCA; ACEA Biosciences) detects electrical impedance of adherent cells over time and can be used as a sensitive measure of kinetic cytotoxicity responses by NK cells. The upward trajectory of plots over time is characteristic of growing adherent cells and downward plots upon addition of NK cells are characteristic of adherent target cell death (Supplemental Fig. S4). SKOV3 cells (5,000/100µl of complete RPMI-1640) were added into each well of E16 xCELLigence plates (ACEA Biosciences) and cultured overnight at 37°C with 5% CO₂. The next day complete medium was added to control wells, plates were inserted into an xCELLigence RTCA DP unit (ACEA Biosciences) at 37°C with 5% CO₂ and baseline impedance was measured. NK-92 cells or purified donor NK cells (100µl of 0.5×10⁶/ml) and SLAMF7 antibodies (Elo, Elo Fc mut, Elo F(ab')₂, Elo Fab, PDL241, ChLuc90, 162.1; 10µg/ml) were added to desired wells. Data were recorded at 10-minute intervals for up to 24 hours. Every condition containing NK cells was performed in duplicate wells within each experiment and a total of 3–6 experiments were performed for each condition. Total lysis (100%) was determined by adding lysis buffer (20mM Tris HCl pH=8, 2mM EDTA pH=8, 150mM NaCl, 0.2% SDS, 10% Triton X-100) to a well of target cells alone and the percent cytotoxicity for each experimental condition was normalized to this 100% value at the 10-hour time point (Supplemental Fig. S4). All kinetic plots represent the lines generated from means of all values from every experiment at individual time points. Statistical analysis was performed using Student's t tests in GraphPad Prism on mean values from the duplicate wells for the percent cytotoxicity at 10 hours within each experiment performed for each condition, as detailed in figure legends, with significance defined as p<0.05.

Results

ADCC of SLAMF7⁺ MM cells by elotuzumab is influenced by CD16 polymorphism

We tested the effect of Elo on cytotoxicity of MM cell lines with various SLAMF7 expression levels by NK-92 cell lines expressing high (CD16–176V) and low (CD16–176F) affinity FcγRIIIA (Fig.1A). Two matching pairs of MM target cell lines were genetically modified as previously described (16): RPMI8226 cells expressing low endogenous levels of SLAMF7 were retrovirally transduced to express high SLAMF7 levels (RPMI8226+SLAMF7), and MM.1R cells, which constitutively express high levels of SLAMF7, were treated with zinc finger nucleases to knock out SLAMF7 expression (MM.1R SLAMF7 KO cells) (Fig. 1B). Adding low concentrations of Elo (0.001–0.1µg/ml) to CD16-bearing NK-92 cell lines in the presence of MM.1R cells promoted higher cytotoxicity by CD16–176V NK-92 cells, as compared to CD16–176F NK-92 cells. Using

higher concentrations of Elo (1–300 µg/ml) stimulated similar maximal response by NK cells expressing the high or low affinity polymorphism (Fig. 1C). A similar trend was observed when NK cells were co-cultured with RPMI8226+SLAMF7 cells, although the difference was not statistically significant (Fig. 1D). Elo did not appreciably stimulate cytotoxicity toward MM cells lacking (MM.1R KO) or expressing low levels of SLAMF7 (parental RPMI8226). These results demonstrate that low concentrations of Elo promote more potent NK cell-mediated ADCC toward SLAMF7⁺ MM cells if the NK cells express high affinity (176V) CD16, although the response plateaus to a level of cytotoxicity equivalent for CD16–176F at concentrations above 1 µg/ml of Elo.

Elotuzumab enhances natural cytotoxicity of NK cells in a CD16-independent manner.

To follow up studies showing CD16-independent enhancement of NK cell-mediated natural cytotoxicity by Elo (10,11,14,16,20–22), we utilized NK-92 cells lacking endogenous CD16 but expressing SLAMF7. In the presence or absence of Elo, knocking out SLAMF7 on MM.1R decreased cytotoxicity by NK-92 cells and overexpressing SLAMF7 on RPMI8226 boosted natural cytotoxicity by NK-92 cells (Fig. 2A), suggesting that SLAMF7-SLAMF7 interactions potentiate cytotoxicity. Interestingly, adding Elo to NK-92 further enhanced cytotoxicity of RPMI8226+SLAMF7 in a dose-responsive manner (Fig. 2A, bottom). Although it is unclear why this effect was not observed for MM.1R targets, the enhancement toward RPMI8226+SLAMF7 targets by Elo suggests that this effect may occur toward only a subset of SLAMF7⁺ MM target cells and further indicates that Elo promotes SLAMF7-SLAMF7 interactions between NK and myeloma cells.

Sensitive kinetic measurement of adherent target cell cytotoxicity can be performed using xCELLigence RTCA assays (see Methods). Unfortunately, available MM cell lines are minimally adherent and could not provide a sensitive enough signal in the xCELLigence system, even when wells were coated with adhesion ligands, such as fibronectin. Therefore, we utilized the adherent human ovarian cancer cell line, SKOV3, which undergoes minimal natural cytotoxicity by NK-92. We further generated SKOV3 cells that stably expressed SLAMF7 [SKOV3(+SLAMF7)] (Fig. 2B). Adding Elo at 0.1–1 µg/ml with CD16-expressing NK-92 cells significantly enhanced cytotoxicity of SKOV3(+SLAMF7) target cells to a similar level of cytotoxicity upon addition of trastuzumab, an ADCC-inducing antibody that binds the SKOV3 surface marker, Her2 (Supplemental Fig. S4A–B). Adding Elo to a co-culture of parental NK-92 cells lacking CD16 and parental SKOV3 cells lacking SLAMF7 expression did not impact target cell death (Fig. 2C, top, 2D, left). Similarly, trastuzumab did not promote cytotoxicity by parental NK-92 cells, due to their lack of CD16 (Fig. 2C, top). In contrast, Elo significantly enhanced killing of SKOV3(+SLAMF7) cells by parental NK-92 cells, but trastuzumab had no effect (Fig. 2C, bottom, 2D, right). To confirm the Fc-independent enhancement of cytotoxicity, the addition of a mutant form of Elo (Elo Fc mut), which has five amino acids altered in the Fc region to abrogate binding to CD16 (see (16)), also increased cytotoxicity of SKOV3(+SLAMF7) cells by CD16-deficient NK-92 cells (Supplemental Fig. S5, bottom panels), and this effect required SLAMF7 expression on the SKOV3 cells (Supplemental Fig. S5, top). Therefore, in the absence of CD16 interactions, Elo still enhances NK cell cytotoxicity apparently by promoting SLAMF7-SLAMF7

interactions between NK and target cells (Fig. 2E), analogous to our results with RPMI8226+SLAMF7 target cells (Fig. 2A, bottom).

Elotuzumab efficacy required dimeric F(ab')₂ composition and SLAMF7-expressing NK cells

To further investigate the observed effect of Elo to promote NK cell cytotoxicity toward SKOV3(+SLAMF7) cells, we generated SLAMF7-knockout NK-92 cells (SLAMF7 KO, CD16⁻) using a doxycycline-inducible CRISPR/Cas9 system (see Methods). The loss of SLAMF7 expression was confirmed by flow cytometry (Fig. 3A). Elo had no effect on the cytotoxicity of SLAMF7 KO NK-92 cells toward SKOV3(+SLAMF7) target cells (Fig. 3B-C, top). In contrast, adding Elo to NK cells that expressed SLAMF7 [NK-92(SLAMF7⁺,CD16⁻)] significantly enhanced the killing of SKOV3(+SLAMF7) cells (Fig. 3B-C, top), consistent with our previous results toward RPMI8226+SLAMF7 cells (Fig. 2A, bottom). Elo Fc mut had a similar effect on enhancing NK cell cytotoxicity (Figs. 3B-C, bottom). To further rule out a role for the Fc domain of Elo in the response, we generated bivalent F(ab')₂ fragments, which had similar capacity to stimulate cytotoxicity as intact Elo (Fig. 3D). Alternatively, monovalent Fab fragments of Elo were incapable of stimulation, demonstrating a requirement for bivalent association with SLAMF7 to stimulate cytotoxicity (Fig. 3D). These results showed that enhanced NK-mediated killing of SLAMF7⁺ tumor cells by Elo required SLAMF7 expression on NK cells and the bivalent structure of Elo, but was independent of the Fc domain (summarized in Fig. 3E).

Elotuzumab uniquely promotes SLAMF7-SLAMF7 interactions

To further study whether Elo promotes SLAMF7-SLAMF7 interactions, a mouse reporter T cell line expressing a chimeric receptor consisting of human SLAMF7 extracellular domain and mouse CD247 (TCR- ζ) intracellular domain was generated. The hSLAMF7-TCR- ζ reporter cells produced robust levels of mouse IL-2 (mIL2) when exposed to plate-bound Elo or three other plate-bound SLAMF7 antibodies (ChLuc90, 162.1, and PDL241; Fig. 4A). We found that Elo, PDL241, and 162.1 bind to distinct epitopes on SLAMF7, since they do not compete (Supplemental Fig. S6). To test for the capacity of these antibodies to mediate SLAMF7-SLAMF7 interactions, the reporter cell line was added to culture wells containing plate-bound recombinant human SLAMF7 in the presence of varying concentrations of each antibody. Elo concentrations of 0.1–3 μ g/ml were highly effective in inducing mIL2 secretion by the hSLAMF7-TCR- ζ reporter cells, whereas the three other SLAMF7 antibodies (ChLuc90, 162.1 and PDL241) induced very low levels of mIL2 at concentrations of 1–3 μ g/ml (Fig. 4B). In contrast, incubating hSLAMF7-TCR- ζ reporter cells with Elo did not affect mIL2 secretion in plates coated with a negative control recombinant protein (mesothelin) or if soluble recombinant hSLAMF7 was added (Fig. 4C). These data demonstrate that Elo has unique capacity among these antibodies to orient SLAMF7-TCR- ζ in an appropriate oligomerized form to induce cell signaling through the chimeric reporter construct, apparently by promoting SLAMF7-SLAMF7 interactions with the plate-bound SLAMF7 molecules.

The four SLAMF7 antibodies (Elo, ChLuc90, 162.1 and PDL241) were also compared in xCELLigence assays for their capacity to co-stimulate NK-92 cells through interaction with

SLAMF7 and enhance cytotoxicity of SLAMF7⁺ SKOV3 target cells. Again, only Elo promoted CD16-independent NK cell-mediated cytotoxicity (Fig. 5A, Supplemental Fig. S7A). In fact, the other SLAMF7 antibodies slightly decreased cell death of SKOV3(+SLAMF7) by NK-92 cells (although not to a statistically significant degree). In contrast, the Elo-induced enhancement of cytotoxicity was not evident with SLAMF7 KO NK-92 cells (Fig. 5B, Supplemental Fig. S7B). Taken together, our results indicated that Elo has unique capacity among SLAMF7 antibodies to promote SLAMF7-SLAMF7 interactions between NK cells and target cells, which resulted in enhanced cytotoxicity responses.

Adhesion molecules and NKG2D contributed to Elotuzumab-induced NK cell-cytotoxicity

To better understand the mechanism by which Elo enhances cytotoxicity in a CD16-independent manner, we tested for upregulation of a variety of activation markers and receptors on NK-92 cells when cultured for 24 hours with SLAMF7⁺ SKOV3 cells \pm Elo. The expression of CD69, NKG2D, CD25, ICAM-1, and the activated form of LFA-1 were significantly increased under these conditions (Supplemental Figure S8A), demonstrating NK cell activation. Most of these increases required or were more pronounced in the presence of SLAMF7⁺ SKOV3 target cells. The increased expression of ICAM-1 and activated LFA-1 suggests improved target cell adhesion upon Elo treatment, although we could not detect enhanced conjugation by Elo of NK-92 cells cultured with either MM1.R or RPMI8226+SLAMF7 target cells (Supplemental Fig. S8B). Nonetheless, upregulation of adhesion molecules by Elo may be playing a modest role in the effect. The increase in NKG2D levels by Elo was also intriguing, particularly when we found that SKOV3 target cells expressed ligands for both NKG2D (ULBPs) and DNAM-1 (Nectin 2 and PVR) (Supplemental Fig. S8C). Therefore, we tested whether blocking either NKG2D or DNAM-1 with antibodies could disrupt Elo-induced cytotoxicity. Whereas NKG2D blockade partially reduced the cytotoxicity, DNAM-1 blocking antibodies were ineffective (Fig. 5C-D and Supplemental Fig. S7C-D). Taken together, the CD16-independent enhancement of cytotoxicity by Elo appears to be mediated through multiple mechanisms, including the activation of LFA-1, upregulation of ICAM-1 and NKG2D, and potentially upregulation of CD69 and CD137, which can act as activating receptors on NK cells (42,43).

Elotuzumab enhances NK cell cytotoxicity only if SLAMF7 contains the full cytoplasmic domain

At least two differentially spliced isoforms of SLAMF7 are found in humans, which differ based on long (-L) or short (-S) cytoplasmic domains that contain or lack the ITSM, respectively. SLAMF7-S does not transduce activation signals (33,44). We reconstituted NK-92 (SLAMF7KO,CD16⁻) cells with these SLAMF7-L or SLAMF7-S isoforms and tested the ability of Elo to stimulate cytotoxicity toward SKOV3+SLAMF7 cells using the xCELLigence assay. Although SLAMF7-S was expressed at slightly lower levels than SLAMF7-L (Fig. 6A), Elo significantly increased cytotoxicity by NK-92(+SLAMF7-L,CD16⁻) cells, whereas Elo was entirely incapable of potentiating cytotoxicity by NK-92(+SLAMF7-S,CD16⁻) or NK-92(SLAMF7KO,CD16⁻) cells (Fig. 6B-C). The results suggest that the potentiated cytotoxicity required co-stimulatory signaling within the NK cells, since the cytoplasmic ITSM signaling motif that recruits EAT-2 was only present in the SLAMF7-L isoform.

Co-stimulation of donor NK cell cytotoxicity by Elo Fc mut depends on NK cell activation

To validate our results from NK-92 cells, we used purified healthy donor NK cells to test the CD16-independent effect of Elo Fc mut on promoting natural killing of parental SKOV3 and SKOV3(+SLAMF7) target cells in xCELLigence assays. Neither Elo nor Elo Fc mut was able to promote healthy donor NK cell-mediated killing of parental SLAMF7-deficient SKOV3 cells (Supplemental Fig. S9). In contrast, Elo promoted complete lysis of SKOV3(+SLAMF7) cells within 8–10 hours by purified donor NK cells from healthy donor #1 (HD#1) (Fig. 7A). On the other hand, Elo Fc mut had no effect on cytotoxicity of SKOV3(+SLAMF7) cells by NK cells from HD#1 (Fig. 7A). After screening seven healthy donors, we observed that Elo Fc mut promoted an increase in cytotoxicity by NK cells from only three of the donors, assessed as percent cytotoxicity at 10 hours in xCELLigence assays (Fig. 7B, left plots), with HD#8 being a representative example (Fig. 7C, left graph). Therefore, Elo Fc mut was able to potentiate cytotoxicity of fresh primary human NK cells in a subset of donors (3 of 7).

The finding that Elo is therapeutically ineffective in treating RRMM as a single agent (18), yet shows significant efficacy in combination with lenalidomide and dexamethasone (17) suggests that coordinated immune stimulation is important to achieve optimal anti-tumor effect by Elo. Lenalidomide is an immunomodulatory drug that stimulates NK and T cell activation, in part by increasing IL-2 production by T cells (21,45). Furthermore, our NK-92 cells are routinely cultured in IL-2. Therefore, we hypothesized that culturing NK cells in the presence of IL-2 might enhance the capacity of Elo Fc mut to stimulate cytotoxicity. When purified NK cells from HD#3 and HD#8 were assayed after culture for 48hrs in the presence of 100U/ml recombinant human IL-2, Elo Fc mut more potently increased cytotoxicity toward SKOV3(+SLAMF7) cells, as compared to freshly isolated NK cells from these donors (Figs. 7C-D, left vs. right). As summarized in Fig. 7B, whereas Elo Fc mut potentiated cytotoxicity by freshly prepared primary NK cells in only 3 of 7 healthy donors, cytotoxicity was boosted in 5 of 7 healthy donors after culture of the NK cells in IL-2. Hence, the CD16-independent co-stimulation of cytotoxicity by Elo engagement with SLAMF7 can be demonstrated in primary human NK cells, most prominently when NK cells are activated by IL-2.

Discussion

We identified an additional mechanism by which Elo promotes NK cell cytotoxicity toward MM cells independent of CD16-mediated ADCC. SLAMF7 functions as a co-stimulatory receptor on NK cells that mediates intracellular signaling via recruitment of cytosolic EAT-2 adaptor protein (12,29). Our previous work demonstrates that Elo can co-stimulate calcium mobilization responses triggered by the aggregation of NKp46 ± NKG2D activating receptors (16). Here, we demonstrated that Elo can also promote SLAMF7-SLAMF7 interactions between NK and MM cells to enhance NK cell-mediated cytotoxicity through co-stimulation in addition to facilitating ADCC.

At lower concentrations, Elo (1ng/ml–1µg/ml) promoted higher cytotoxicity by NK cells expressing the high affinity CD16 176V polymorphism vs. NK cells expressing the low affinity 176F polymorphism. In contrast, at higher concentrations of Elo (up to 300µg/ml;

similar to the observed plasma concentrations in Elo-treated patients, which are routinely above 70µg/ml (46)), no effect of CD16 polymorphism on NK cell ADCC was observed. Despite the lack of differential response at high Elo concentrations, CD16 176V/V homozygous patients respond better to Elo plus bortezomide/dexamethasone compared to 176V/F or 176 F/F patients (26). These results are consistent with better ADCC by NK cells and improved clinical efficacy in response to rituximab in follicular lymphoma patients possessing the CD16 176V/V genotype (23,47). It is currently unclear if the high affinity CD16 polymorphism is also beneficial in patients treated with Elo in combination with lenalidomide/dexamethasone.

We further demonstrated that Elo can significantly enhance the natural cytotoxicity of SLAMF7-expressing target cells by CD16-deficient NK-92 cells, and this effect required SLAMF7 expression on both the NK and target cells. This result is consistent with the work of Collins et al., showing that Elo induces CD16-independent target cell killing by NK-92 that requires SLAMF7 expression on the target cells (22). Our work extended their results by showing a requirement for expression of signaling-competent SLAMF7 in the NK cells, indicating a co-stimulatory signaling mechanism. The requirement for SLAMF7 expression on both effector and target cells suggests that Elo is capable of either 1) bridging SLAMF7 between the two cells or 2) enhancing the SLAMF7-SLAMF7 interactions between the two cell types. This enhancement only occurred toward SLAMF7⁺ RPMI8226 and SLAMF7⁺ SKOV3, but not SLAMF7⁺ MM.1R myeloma target cells. We hypothesize that Elo engagement with SLAMF7 effectively co-stimulates additional NK cell activating receptors engaging with ligands expressed on RPMI8226, but absent on MM.1R. This hypothesis is supported by our previous work showing that Elo engagement with SLAMF7 cannot induce calcium signaling in NK cells on its own, but Elo can potentiate calcium signaling triggered by engagement of the ITAM-linked NK cell receptor, NKp46, either with or without co-engagement of NKG2D (16). Consistent with our previous work, we showed here that NKG2D plays at least a partial role in the enhanced cytotoxicity induced by Elo. Of note, the CD16-independent effect occurs at Elo concentrations in the range of 0.1–300µg/ml, whereas ADCC responses are observed in the range of 1ng/ml–1µg/ml. These concentrations are both well below the high serum concentrations achieved for Elo at therapeutically effective dosing regimens (46).

Surprisingly, of four SLAMF7 antibodies tested, only Elo enhanced SLAMF7-SLAMF7-dependent activation responses. This indicates that Elo binds to a unique epitope on SLAMF7, whereby it is either better oriented to effectively bridge the receptor between effector and target cell or the binding induces a conformational change that enhances homotypic interactions between SLAMF7 molecules on opposing cells. Furthermore, we showed that the co-stimulatory impact of Elo requires bivalent binding to SLAMF7, since F(ab')₂ is effective, but Fab fragments are not. All four tested antibodies were found to bind distinct epitopes on SLAMF7. Elo reportedly binds to the membrane proximal C2 domain of SLAMF7, within an epitope encompassing amino acids 170–227 (U.S. patent 7842293B2). In contrast, PDL241 and ChLuc90 are reported to interact with the V domain (U.S. patent 7842293B2 and (40)), which mediates homotypic interactions between SLAM family members (48). These two antibodies may thereby block SLAMF7-SLAMF7 interactions, consistent with their capacities to slightly reduce cytotoxicity in our experiments. We could

not detect enhanced conjugation between NK-92 cells and MM target cells by Elo or Elo Fc mut using standard flow cytometry-based assays (49). This result is consistent with a previous report that SLAMF7 signaling in YT-S NK cell line could potentiate killing of SLAMF7⁺ K562 target cells, but not enhance conjugate formation (50). Although the exact mechanism by which Elo promotes SLAMF7 interactions will require further experimentation, Elo has unique capacity to mediate this biological effect. It was shown that Elo can also facilitate macrophages to mediate ADCP towards MM cells in a Fc-dependent manner (14). As SLAMF7 is also highly expressed on subsets of macrophages (notably M1 macrophages (15)), it is possible that Elo could also contribute to macrophage-mediated killing of SLAMF7⁺ tumors through a CD16-independent mechanism by promoting SLAMF7-SLAMF7 interactions.

We further demonstrated that CD16-independent co-stimulation by Elo requires the ITSM-containing cytoplasmic domain of SLAMF7 in NK cells, since Elo enhanced cytotoxicity by NK-92 cells reconstituted with SLAMF7-L, but not with the truncated receptor lacking ITSM (SLAMF7-S). Although we cannot rule out that another motif in the cytoplasmic domain is mediating the co-stimulation, our data suggest that the ITSM is responsible. Previous work showed that NK cell-mediated natural cytotoxicity toward SLAMF7⁺ target cells is potentiated via tyrosine phosphorylation of the ITSM motif on SLAMF7, which recruits EAT-2 (50) to recruit phospholipase C γ -1, resulting in intracellular calcium signaling (50). This is consistent with the calcium signaling co-stimulation by Elo that we previously observed (16). Our results also suggest that extensive SLAMF7-S isoform expression on NK cells from MM patients may impact their clinical outcomes.

We used an Fc mutant form of Elo (Elo Fc mut) to demonstrate CD16-independent enhancement of natural cytotoxicity by NK cells from healthy donors toward SLAMF7⁺ target cells. Elo Fc mut enhanced cytotoxicity of fresh NK cells from three out of seven healthy donors. Interestingly, Elo Fc mut boosted NK cell cytotoxicity of five of these healthy donors if the cells were cultured for 48 hours in IL-2, demonstrating that the CD16-independent effect of Elo on natural cytotoxicity is dependent upon the state of NK cell activation. This finding is of significant interest, since Elo is ineffective as a single agent in MM patients, but highly effective when co-administered with lenalidomide and dexamethasone (17). The immunomodulatory agent lenalidomide binds the E3 ubiquitin ligase cereblon and targets the degradation of the IKZF1 and IKZF3 transcription factors, resulting in increased production of IL-2 by T cells (51). Therefore, it is interesting to speculate that lenalidomide co-administration with Elo may, at least in part, improve Elo activity toward MM cells by elevating IL-2 levels to activate NK cells and thereby increase their susceptibility to Elo-mediated promotion of SLAMF7-SLAMF7 interactions. This is consistent with previous reports demonstrating lenalidomide can enhance Elo-mediated killing of MM cell lines in co-culture with PBMCs and this activity can be partially blocked by antibodies neutralizing the effects of IL-2 (anti-CD25/IL2R) on NK cells (21). Consistent with this, we also showed that Elo can induce IL-2R α (CD25) on NK cells, which may also contribute to enhanced function by lenalidomide in patients.

Our data further elucidate the mechanism of action of Elo beyond the previously established mechanism of ADCC. We showed that Elo co-activates NK cells and enhances cytotoxicity

towards SLAMF7⁺ tumor cells by promoting SLAMF7-SLAMF7 interactions. Furthermore, these findings are specific to Elo compared to other SLAMF7 antibodies tested demonstrating the unique mechanistic attributes of NK co-activation and ADCC dually mediated by Elo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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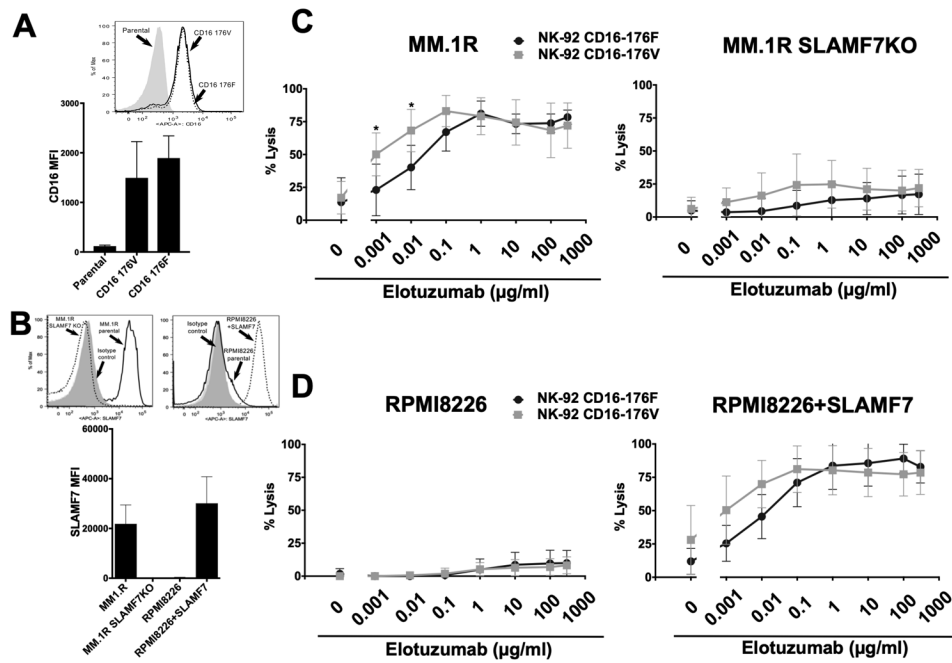


Figure 1. Elotuzumab promoted maximal ADCC if NK cells expressed high affinity CD16 and required SLAMF7 expression on MM cells.

A) Comparison of CD16 expression (GRM1 antibody, Southern Biotech) on parental NK-92 cells (CD16⁻; grey) and NK-92 cells transduced to express 176V (solid line) or 176F (dashed) polymorphic variants of CD16, and mean GMFI \pm S.D. from 5 experiments (bottom). B) SLAMF7 expression before and after the genetic modification of two human MM cell lines (Elo + anti-IgG1 secondary stain). MM.1R cells were transfected with SLAMF7 zinc finger nucleases (ZFN) to genetically deplete SLAMF7 (MM.1R SLAMF7KO; left), and RPMI8226 cells were stably transduced with SLAMF7 cDNA (RPMI8226+SLAMF7; right), and mean GMFI \pm S.D. from 5 experiments (bottom). Cells were stained with biotinylated Elo plus secondary streptavidin-APC. C,D) Effect of SLAMF7 expression on NK cell mediated ADCC in the presence of various concentrations of Elo (0.001–300 μ g/ml). Values are mean \pm S.D. Asterisks indicate statistical significance comparing cytotoxicity by NK-92 cells expressing 176V or 176F variants of CD16 using Welch's unequal variance t test. ** - $P < 0.01$, * - $P < 0.05$, $n = 5$ independent experiments.

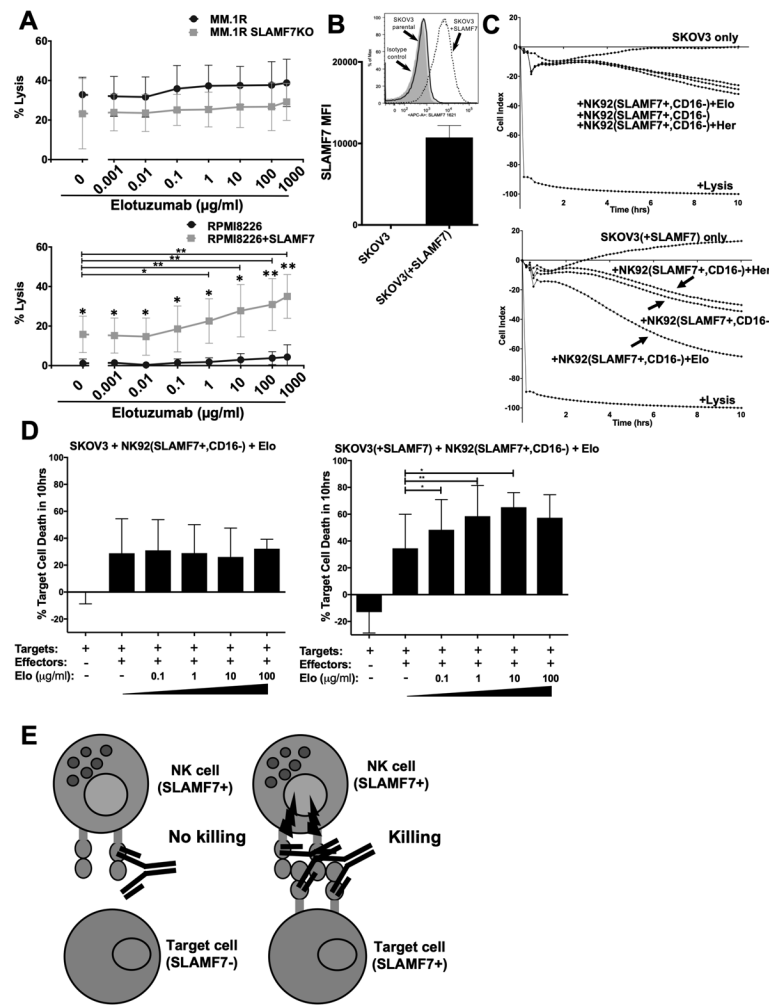


Figure 2. Elotuzumab enhances natural cytotoxicity by NK cells in a CD16-independent manner.

A) Effect of Elo (0.001–300ug/ml) on natural cytotoxicity of parental NK-92 cells expressing SLAMF7 and lacking CD16 (SLAMF7+, CD16–), toward MM.1R (top) and RPMI8226 (bottom) cells. Grey squares are genetically-modified MM.1R SLAMF7KO (top panel) or RPMI83226+SLAMF7 (bottom panel) targets. Values are mean \pm S.D. Overhead bars mark statistical significance between untreated and different Elo concentration groups using paired Student's t test, ** - $P < 0.01$, * - $P < 0.05$, $n = 5$ independent experiments. B) SLAMF7 expression on parental SKOV3 cells (solid line) and SKOV3 cells that were stably transduced with SLAMF7 cDNA (dashed line) and stained with the 162.1 SLAMF7 antibody (Biolegend), and mean GMFI \pm S.D. from 5 experiments (bottom). C) Kinetics of parental SKOV3 (top graph) or SKOV3(+SLAMF7) (bottom graph) target cell death by parental NK-92 cells (SLAMF7+, CD16–) analyzed in xCELLigence assays., $n = 4-6$. D) Mean, S.D., and statistical analysis for percentage target cell death (compared to detergent lysis conditions as 100%) at 10 hours of xCELLigence assays from 4-6 independent experiments. Overhead bars mark statistical comparison between indicated groups using paired Student's t test, ** - $P < 0.01$, * - $P < 0.05$. E) Schematic of Elo effect on cytotoxicity of NK cells against SLAMF7+ and SLAMF7– targets.

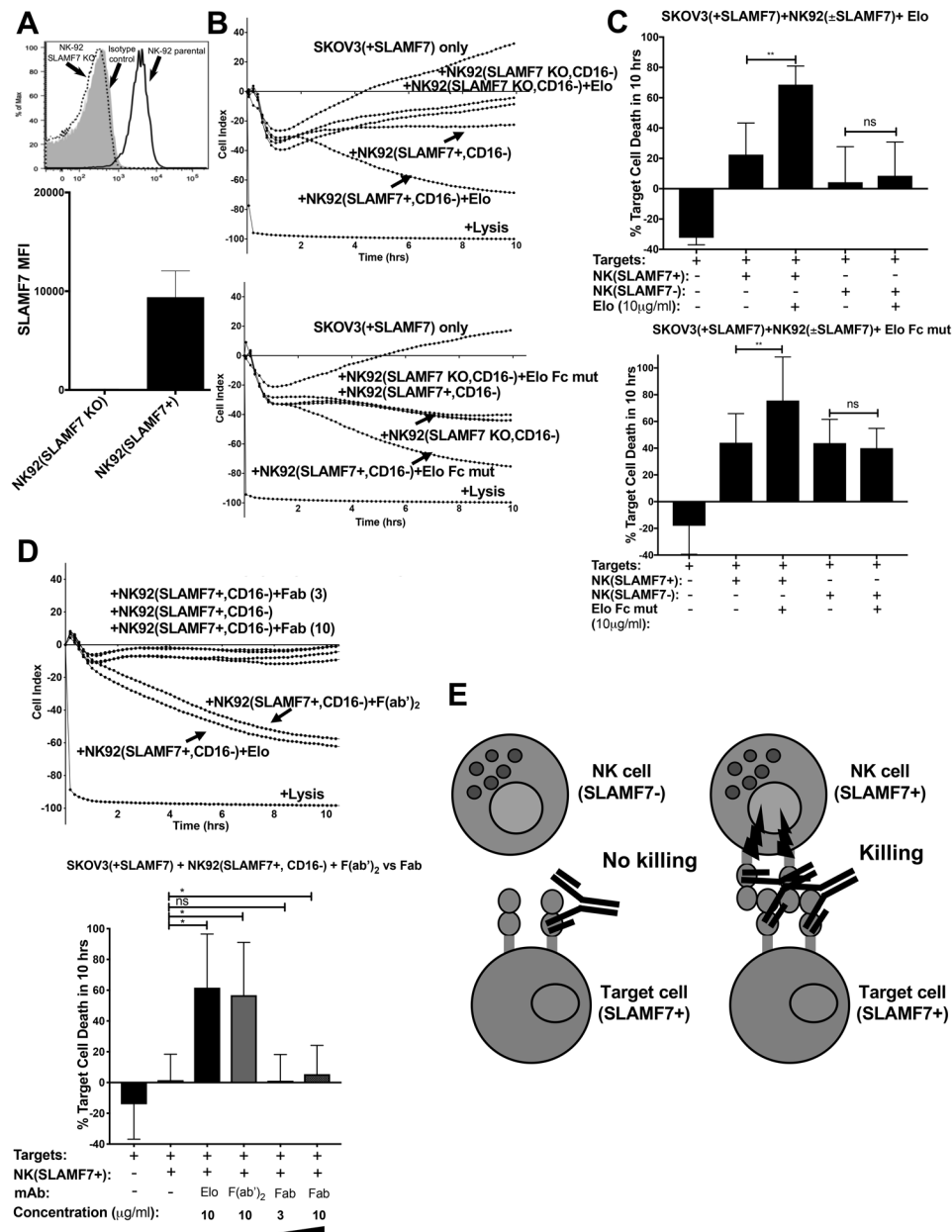


Figure 3. Elotuzumab is unable to enhance natural cytotoxicity of NK cells lacking expression of both SLAMF7 and CD16.

A) SLAMF7 expression [162.1 SLAMF7 antibody (Biolegend)] on NK-92 control (GFP knockout, SLAMF7+, CD16-; solid line) and NK-92 SLAMF7 KO (CD16-; dashed line) cells generated using CRISPR/Cas9, and mean GMFI \pm S.D. from 5 experiments (bottom).

B) Cytotoxicity toward SKOV3(+SLAMF7) target cells by NK-92 (SLAMF7+, CD16-) and NK-92 (SLAMF7 KO, CD16-) cells studied over time in xCELLigence assays with or without Elo (10 μ g/ml; top panel) or Elo Fc mutant (Elo Fc mut; 10 μ g/ml; bottom panel). C) Mean \pm S.D. target cell death at 10-hours from xCELLigence assays for Elo (top panel) and Elo Fc mut (bottom panel) toward SKOV3(+SLAMF7) target cells from 4-5 independent experiments. Overhead bars mark statistical comparison between indicated groups using

paired Student's t test, ** - $P < 0.01$, * - $P < 0.05$. D) Cytotoxicity toward SKOV3(+SLAMF7) target cells by NK-92 (SLAMF7+, CD16-) cells studied over time in xCELLigence assays (top panel) and mean \pm S.D. target cell death at 10-hours (bottom panel) for Elo vs Elo F(ab')₂ vs Fab (3 and 10 μ g/ml). Overhead bars mark statistics as in panel C. n = 4 independent experiments. E) Schematic showing requirement for SLAMF7 expression on NK cells for CD16-independent NK cell-mediated cytotoxicity.

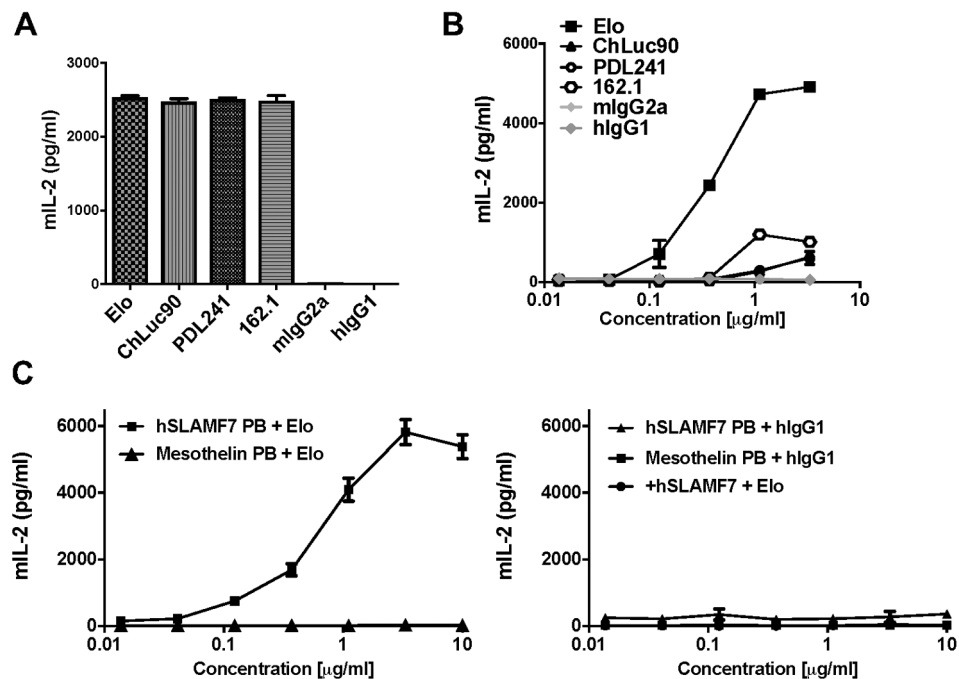


Figure 4. Elotuzumab uniquely promotes SLAMF7-SLAMF7 interactions.

A) SLAMF7-TCR ζ reporter cell line was cultured with four different plate-bound SLAMF7 antibodies (Elo, ChLuc90, PDL241 and 162.1; coated overnight at 10 μ g/ml) and mouse (m)IL-2 was assayed in culture supernatant. B) Indicated SLAMF7 antibodies or isotype controls (0.01 - 3 μ g/ml) were added to 3A9 SLAMF7-TCR ζ reporter cell line in plates coated with recombinant human(h) SLAMF7 overnight and mIL2 secretion was assayed. C) Soluble Elo (left panel) or hIgG1 isotype control (right panel) were added to SLAMF7-TCR ζ reporter cell line on plate-bound (PB) recombinant hSLAMF7 or PB mesothelin control protein, or hSLAMF7 was added as soluble protein (+hSLAMF7; right panel). Plotted as mean \pm SEM. Asterisks mark statistical comparisons for Elo Stimulated group using paired Student's t test, ** - $P < 0.01$, * - $P < 0.05$.

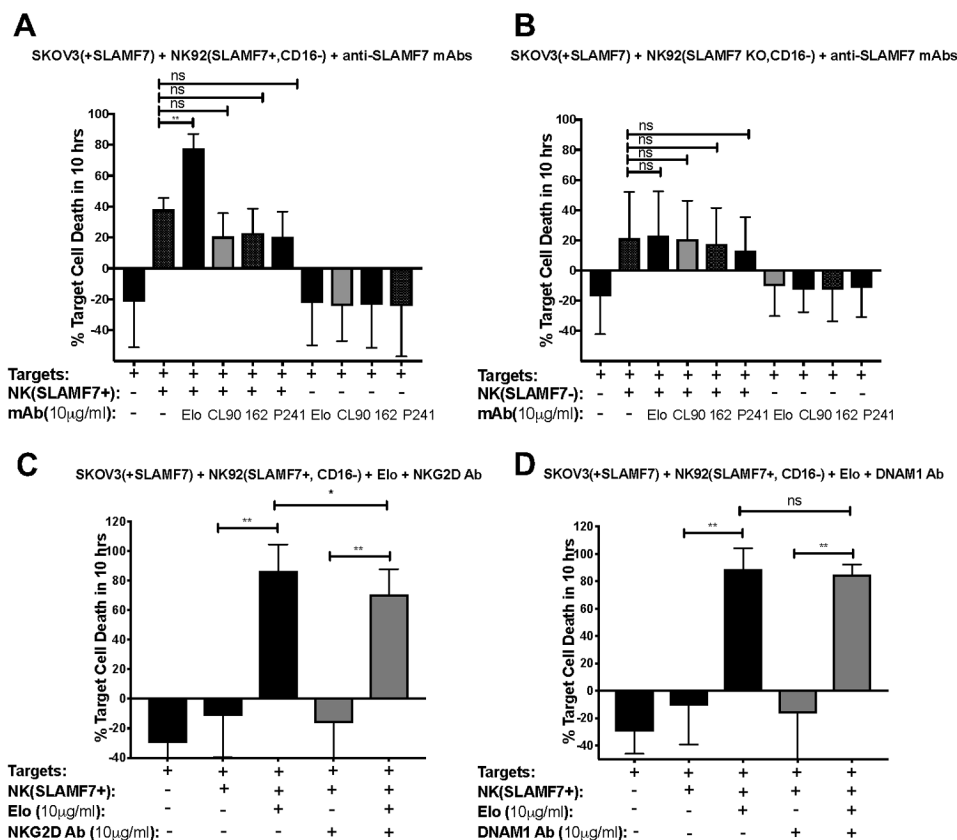


Figure 5. Elotuzumab promotes co-stimulation-mediated cytotoxicity.

Impact of various SLAMF7 antibodies (10 µg/ml each) on cytotoxicity by NK-92(SLAMF7+, CD16-) cells (A) or SLAMF7-deficient NK-92 (SLAMF7 KO, CD16-) cells (B) toward SKOV3(+SLAMF7) target cells in xCELLigence assays. Mean, S.D., and statistics for target cell death are shown at 10 hours from 3-4 independent xCELLigence assays. Overhead bars mark statistical significance between indicated groups using paired Student t test, ** $P < 0.01$, * $P < 0.05$. Elo = Elotuzumab, CL90 = ChLuc90, 162 = 162.1, P241 = PDL241. C), D) Test of the impact of blocking NKG2D (C) and DNAM1 (D) antibodies on Elo-induced cytotoxicity by NK-92(SLAMF7+, CD16-) cells toward SKOV3(+SLAMF7) target cells in xCELLigence assays. Mean, S.D., and statistics for target cell death at 10 hours from 5 independent assays using blocking antibodies at 10 µg/ml each. Overhead bars mark statistics as in panels A and B. Kinetic plots for all of these xCELLigence assays can be found in Supplemental Figure S5C-D.

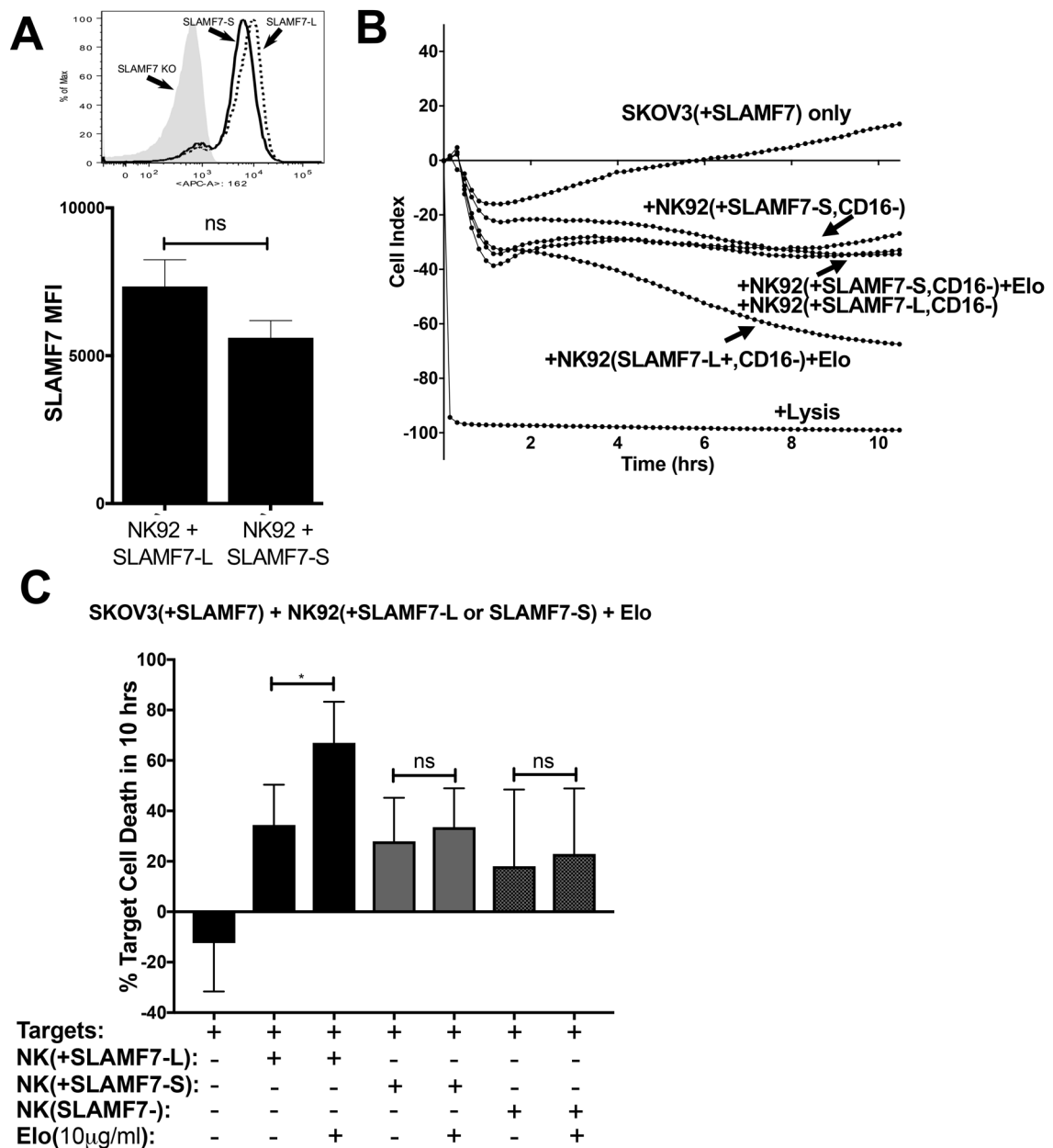


Figure 6. Elotuzumab enhances NK cell cytotoxicity only if SLAMF7 contains the full cytoplasmic domain.

A) Representative SLAMF7 expression [162.1 antibody (Biolegend)] on NK-92 SLAMF7 KO cells (grey) and reconstituted with SLAMF7-L (dashed) or SLAMF7-S (solid line) isoforms, and mean GMFI \pm S.D. from 5 experiments (bottom). B) Impact of Elo (10 μ g/ml) on cytotoxicity by NK-92(SLAMF7 KO,CD16 $^{-}$) cells reconstituted with either SLAMF7-L or SLAMF7-S toward SKOV3(+SLAMF7) target cells studied in xCELLigence assays. C) Mean, S.D., and statistics for target cell death at 10 hours from 5 independent assays. Overhead bars mark statistical comparisons between indicated groups using paired Student's t test, ** - $P < 0.01$, * - $P < 0.05$.

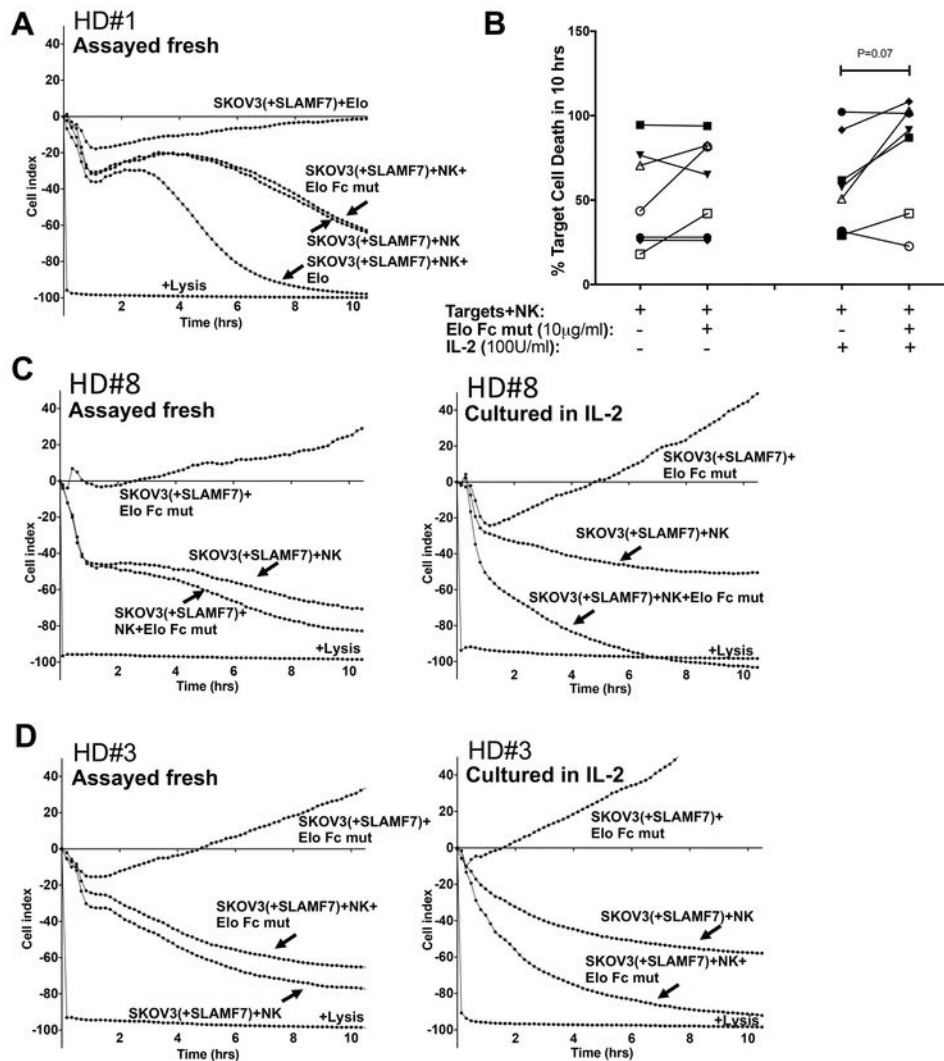


Figure 7. Co-stimulation of healthy donor NK cell cytotoxicity by Elo Fc mut depends on NK cell activation status.

A) Elo or Elo Fc mut (10 μ g/ml) were added to SKOV3(+SLAMF7) target cells in the presence or absence of freshly purified NK cells from healthy donor (HD) #1 in xCELLigence assays. B) Compilation of cytotoxicity data for n=7 healthy donors. Percentage target cell death at the 10 hour time point from xCELLigence assays in the absence (–) or presence (+) of Elo Fc mut by purified NK cells toward SKOV3(+SLAMF7) target cells on the day of the purification (left plots) or after cultured in presence of recombinant human IL-2 (100U/ml; +) for 48 hrs (right plots). Each patient is designated by a unique icon and results with or without antibody addition are connected with lines. Overhead bars mark statistical comparison between indicated groups using Wilcoxon matched-pairs signed rank test. C,D) Representative time course data in which purified NK cells from HD#3 and HD#8 were assayed fresh (left) or after cultured in presence of recombinant human IL-2 (100U/ml) for 48 hrs (right).