

Immunomodulatory Drug Lenalidomide (CC-5013, IMiD3) Augments Anti-CD40 SGN-40–Induced Cytotoxicity in Human Multiple Myeloma: Clinical Implications

Yu-Tzu Tai,¹ Xian-Feng Li,¹ Laurence Catley,¹ Rory Coffey,¹ Iris Breitzkreutz,¹ Joeun Bae,¹ Weihua Song,¹ Klaus Podar,¹ Teru Hideshima,¹ Dharminder Chauhan,¹ Robert Schlossman,¹ Paul Richardson,¹ Steven P. Treon,¹ Iqbal S. Grewal,² Nikhil C. Munshi,¹ and Kenneth C. Anderson¹

¹Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts and ²Seattle Genetics, Inc., Bothell, Washington

Abstract

SGN-40, a humanized immunoglobulin G1 (IgG1) anti-CD40 monoclonal antibody, mediates cytotoxicity against human multiple myeloma (MM) cells via suppression of interleukin (IL)-6–induced proliferative and antiapoptotic effects as well as antibody-dependent cell-mediated cytotoxicity (ADCC). Here, we studied the clinical significance of an immunomodulatory drug lenalidomide on SGN-40–induced cytotoxicity against CD138⁺CD40⁺ MM lines and patient MM cells. Pretreatment with lenalidomide sensitized MM cells to SGN-40–induced cell death. Combined lenalidomide and SGN-40 significantly induced MM apoptosis, evidenced by enhanced cleavage of caspase-3/8/poly(ADP-ribose)polymerase and increased sub-G₀ cells, compared with either single agent at the same doses. Pretreatment of effector cells with lenalidomide augmented SGN-40–induced MM cell lysis, associated with an increased number of CD56⁺CD3[−] natural killer (NK) cells expressing CD16 and LFA-1. Importantly, pretreatment with lenalidomide or lenalidomide and SGN-40 markedly enhanced NK-cell-mediated lysis of autologous patient MM cells triggered by SGN-40. Lenalidomide also up-regulated CD40L on CD56⁺CD3[−] NK cells, facilitating IL-2–mediated activation of NK cells. In addition, lenalidomide induced the CD56^{dim} NK subset, which are more potent mediators of ADCC against target MM cells than the CD56^{bright} NK subset. Finally, pretreatment of both effector and target MM cells with lenalidomide markedly enhanced SGN-40–mediated ADCC against CD40-expressing MM cells. These studies, therefore, show that the addition of lenalidomide to SGN-40 enhances cytotoxicity against MM cells, providing the framework for combined lenalidomide and SGN-40 in a new treatment paradigm to both target MM cells directly and induce immune effectors against MM. (Cancer Res 2005; 65(24): 11712–20)

Introduction

Clinical efficacy of monoclonal antibody (mAb) and mAb-based therapies has been shown in both hematologic and solid tumors; for example, anti-CD20 rituximab, the first Food and Drug

Administration–approved mAb for the treatment of cancer, is now broadly used to treat non-Hodgkin's lymphoma and other B-cell lymphoproliferative diseases. This type of passive immunotherapy can be effective even if the host immune system is compromised. To date, however, there are few reports of mAb-based therapies for human multiple myeloma (MM). Although only a small percentage of MM patients express CD20, rituximab treatment achieved 32% responses in heavily pretreated patients whose MM cells were CD20⁺ (1). Because CD40 is expressed on a majority of primary MM patient cells (2, 3) and a wide spectrum of B-cell malignancies, mAbs targeting CD40 have potential therapeutic application for MM and other rituximab-resistant B-cell diseases. Cytotoxicity of SGN-40 humanized anti-CD40 mAb against human MM cell lines and patient MM cells (CD138⁺CD40⁺; ref. 4). SGN-40 is composed of the human IgG1 class constant region, as in anti-CD20 rituximab, coupled with humanized murine variable regions against CD40 (4, 5). It binds to CD40 on MM cells and triggers antibody-dependent cell-mediated cytotoxicity (ADCC) against CD40-expressing MM cell lines and patient cells (5), as well as down-regulates interleukin (IL)-6 receptor on MM cells, thereby inhibiting IL-6–mediated survival and growth signals (4). SGN-40 also up-regulates cytotoxic ligands of the tumor necrosis factor (TNF) family (i.e., FasL, TNF-related apoptosis-inducing ligand, and TNF- α). These preclinical data support a potential therapeutic use of SGN-40 to improve patient outcome of MM.

Thalidomide and the potent immunomodulatory derivatives of thalidomide (IMiD), including IMiD1 (CC-4047) and lenalidomide (IMiD3, CC-5013, Revlimid), have been shown to overcome resistance of human MM to conventional therapy (6). In previous *in vitro* studies, we have shown that IMiDs have more potent antitumor activity than thalidomide (7, 8). IMiDs induce apoptosis via caspase-8 activation in MM1S cells (9), trigger apoptosis or growth arrest even in resistant MM cell lines and patient cells (7), decrease production of MM cells to bone marrow stromal cells (10), inhibit production in the BM milieu of cytokines (IL-6, vascular endothelial growth factor) mediating growth and survival of MM cells (10), block angiogenesis (11, 12), and stimulate host anti-MM natural killer (NK) cell immunity (8). In phase I, II, and III clinical trials, lenalidomide has shown a favorable side-effect profile and achieved responses, including some complete responses even in patients with MM refractory to and/or relapsed after high-dose therapies as well as thalidomide (6, 13). The evaluation of lenalidomide, either alone or in combination, to treat patients with MM at earlier stages of disease, is currently ongoing.

Requests for reprints: Kenneth C. Anderson, Department of Medical Oncology, Dana-Farber Cancer Institute, M557, 44 Binney Street, Boston, MA 02115. Phone: 617-632-2144; Fax: 617-632-2140; E-mail: kenneth_anderson@dfci.harvard.edu.

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Cell-mediated cytotoxicity is an important immune response to cancer mediated by direct cell-to-cell contact between an effector cell and a target tumor cell. It has been reported that NK cells are responsible for tumor regression and elimination of blood-borne metastasis (14). Although the mechanisms mediating *in vivo* anti-MM activity triggered by IMiDs are undefined, their immunomodulatory effects on T cells and natural killer (NK) cells through modulation of IL-2, IFN- γ , and IL-12 are the main focus of recent studies. IMiDs stimulate T-cell proliferation as well as increase IL-2 and IFN- γ production following CD3 ligation (8, 15). IMiDs also enhance *in vitro* NK cell-mediated lysis of both MM cell lines and patient MM cells; an increase in NK cell number correlates with response to thalidomide therapy (8). In addition, lenalidomide activates CD28 and overcomes CTL antigen 4-immunoglobulin blockade, confirming that drug-induced costimulation is mediated via B7-D28 pathway (15). Most recently, our *in vitro* studies validated the role of lenalidomide-induced IL-2 induction by T cells in augmenting NK cell-mediated MM cell lysis (16), thereby further supporting their therapeutic use in MM.

In view of the lack of overlapping toxicities and the diverse mechanisms of action of both reagents, we here characterized the role of the interaction of lenalidomide and/or SGN-40 in inducing anti-MM activity to provide the preclinical basis for combination therapy to improve patient outcome.

Materials and Methods

Cell lines and cultures. MM cell lines were maintained as described previously (3, 4). Freshly isolated tumor cells (CD138⁺) from MM patients were obtained after informed consent and purified by CD138 MACS microbeads (Miltenyi Biotec, Auburn, CA) according to the protocol of the manufacturer. They were examined for expression of CD138 and CD40 by flow cytometric analysis.

***In vitro* cell proliferation assay.** CD40-expressing MM cell lines and patient MM cells were incubated with a humanized anti-CD40 SGN-40 mAb (Seattle Genetics, Bothell, WA) at various concentrations in 96-well plates for 3 days; cells were pulsed with [³H]thymidine (0.5 μ Ci/well for cell lines and 1 μ Ci/well for patient MM cells) for 8 hours (cell lines) or 24 hours (patient MM cells), harvested, and counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). CD40-expressing MM cell lines and patient MM cells were also incubated with dilutions of lenalidomide (0-50 μ mol/L, Celgene, Warren, NJ) in the presence of SGN-40 (10 μ g/mL) or control IgG1 for 2 days. To determine whether IL-6 protects MM cells pretreated with SGN-40 against lenalidomide, dexamethasone-sensitive MM.1S and dexamethasone-resistant MM.1R lines were pretreated with either control IgG1 (20 μ g/mL) or SGN-40 (20 μ g/mL) overnight, and then incubated with or without lenalidomide (1 μ mol/L for MM1S and 10 μ mol/L for MM1R) in the presence or absence of IL-6 (Peprotech, Rocky Hill, NJ) for 2 days. DNA synthesis was measured by [³H]thymidine incorporation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric survival assay. To examine whether pretreatment with lenalidomide sensitizes MM cells to SGN-40-induced cell death, cells were pretreated with lenalidomide (1 μ mol/L for MM1S and 10 μ mol/L for three other MM lines) for 24 hours before addition of SGN-40 (2, 20 μ g/mL); survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays as previously described (4). Cell survival was estimated as a percentage of the value of untreated controls. All experiments were repeated at least thrice and each experimental condition was repeated in triplicate wells in each experiment.

Cell cycle analysis. 12BM and 28BM MM lines were cultured for 3 days in medium alone, with cross-linked SGN-40 mAb (5 μ g/mL), with lenalidomide (10 μ mol/L), and with combined SGN-40 and lenalidomide. Cells were then harvested, washed with 1 \times PBS, fixed with 70% ethanol, and pretreated with 10 μ g/mL of RNase (Roche, Indianapolis, IN). After staining

with propidium iodide (5 μ g/mL, Sigma), cell cycle profile was determined using M software on a Coulter Epics XL (Beckman Coulter, Miami, FL).

Immunoblotting analysis. To determine whether SGN-40 mAb induces apoptotic signaling in CD40-expressing MM lines, 12BM and 28BM cells were incubated with SGN-40 mAb (50 μ g/mL) and a cross-linking secondary goat anti-human Fc antibody (GAH, 20 μ g/mL). Cell lysates were immunoblotted (4) using anti-caspase-3, anti-caspase-8, poly(ADP-ribose)polymerase (PARP), anti-p53, and anti-p21 antibodies with anti- α -tubulin mAb as a loading control. To assess whether lower concentrations of combined SGN-40 and lenalidomide potentiates activation of apoptotic signaling, 12BM cells were incubated overnight with SGN-40 (5 μ g/mL) and a cross-linking secondary GAH, with lenalidomide (2 μ mol/L), or with the combination.

Flow cytometric analysis. Direct immunofluorescence flow cytometric analysis was done using a Coulter Epics XL with data acquisition software (Cytomics FC500-CXP; Beckman Coulter). Peripheral blood mononuclear cells (PBMC) or NK cells treated with drugs alone or in combination were washed, followed by dual immunostaining with specific anti-FITC-, or anti-phycoerythrin (PE)-conjugated mAbs (Beckman Coulter). NK cells were purified using RosetteSep NK cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC, Canada). The purity of CD56⁺CD3⁻ NK cells (>88% purity) was monitored by dual immunofluorescence using anti-CD56-PE and anti-CD3-FITC mAbs. To assess CD16 or LFA-1 expression on NK cells following drug treatment, purified treated NK cells were immunostained with anti-CD16-FITC or anti-LFA-1-FITC mAbs. To assess CD40L expression on drug-treated or IL-2-activated NK cells, PBMCs were incubated for 3 days with drugs or with IL-2 (200 IU/mL). Purified NK cells were then washed and incubated with an anti-CD40L-FITC mAb and anti-CD56-PE mAb.

Antibody-dependent cell-mediated cytotoxicity assays. PBMCs were used as effector cells and calcein-AM-labeled MM lines as targets. PBMCs were separated from leukopheresis products from normal donors ($n = 3$) by suspension in PBS at a 1:1 volume ratio and layering over Ficoll-Hypaque solution (density = 1.077 g/L) at room temperature after informed consent. Cells were centrifuged for 30 minutes in a Beckman J6-MC centrifuge at 1,600 rpm. The mononuclear layer was then resuspended in PBS and washed twice before viability assessment. Effector cells were used immediately or were cultured for 3 days at 37°C in complete media (10% human serum/X-VIVO medium), with SGN-40 (2 μ g/mL), with lenalidomide (2 μ mol/L), or with both reagents. Target cells were labeled with calcein-AM for 0.5 hour at 37°C, washed thrice, and plated in triplicate in 96-well plates (5,000 cells per well). ADCC was done in the presence of SGN-40 or human control IgG1 (2 μ g/mL) at various effector-to-target (E/T) ratios (5:1, 10:1, and 40:1). The 96-well plates were centrifuged at 400 rpm for 2 minutes at room temperature followed by a 4-hour incubation at 37°C. Culture supernatants were transferred to a Black ViewPlate-96 plate and read on Wallac VICTOR2 using 492/520 nm filter set (Perkin-Elmer). This assay was valid only if $\{(\text{mean maximum release} - \text{medium control release}) / (\text{mean spontaneous release} - \text{medium control release})\} > 7$. Spontaneous release is the cpm in the supernatant from wells containing target cells alone. Maximum release is the supernatants of wells containing target cells and Triton X-100. Experimental release is obtained from the supernatant of wells containing effector cells, target cells, and antibody. Calculation of percentage specific lysis from triplicate experiments was done using the following equation:

$$\% \text{ specific lysis} =$$

$$100 \times \frac{(\text{mean experimental release} - \text{mean spontaneous release})}{(\text{mean maximal release} - \text{mean spontaneous release})}$$

Experiments were also done in which target MM cells and effector cells, alone or together, were pretreated with lenalidomide before ADCC assays. MM targets were pretreated with lenalidomide (0.5 μ mol/L for MM1S; 2 μ mol/L for 28BM, 28PE, 12BM, and MM1R; and one patient MM cells) for 24 hours, whereas effectors were preincubated with lenalidomide (2 μ mol/L) for 3 days. All cells were counted and assessed for viability (>90%) by means of trypan blue staining before ADCC assays. Cells were

suspended in complete media and plated at an E/T ratio of 20:1. Results are reported in terms of specific lysis, with the mean, SD, and *P* values determined from three samples per condition using the Student's *t* test for independent samples. All experiments were repeated at least twice.

Results

Effect of SGN-40 and lenalidomide on multiple myeloma cell growth and survival. The clinical relevance of the interaction of anti-CD40 SGN-40 mAb with lenalidomide in MM remains to be determined. We previously reported that SGN-40 induces growth arrest and apoptosis only in the presence of the *de novo* protein synthesis inhibitor cyclohexamide (4), which is unlikely to be used clinically because of its general toxicity. We here first evaluated the effect of SGN-40 and lenalidomide, alone or in combination, on [³H]thymidine incorporation by CD40-expressing MM lines and freshly isolated patient MM cells. SGN-40 alone did not alter DNA synthesis in MM1S, MM1R, 12BM, and 28BM MM cell lines and two patient MM cells (CD138⁺CD40⁺; Fig. 1A), consistent with our previous report (4). Simultaneous addition of SGN-40 and lenalidomide only modestly increased growth inhibitory effect by lenalidomide in the same four CD40-expressing MM lines and 2 patient MM cells (Fig. 1B). We next determined whether lenalidomide enhanced SGN-40-induced MM cell death by MTT assay. Percentage of specific cell death was calculated as the following formula: % specific cell death = 100 – (absorbance in cells treated

with lenalidomide and SGN-40) / (absorbance in cells treated with lenalidomide alone). Pretreatment of MM.1S cells with 1 μmol/L of lenalidomide overnight significantly enhanced sensitivity to cell death induced by SGN-40 (*P* = 0.048 at 2 μg/mL; *P* = 0.04 at 10 μg/mL; Fig. 1C). In three other MM cell lines, pretreatment with lenalidomide (10 μmol/L) also promoted cell killing following SGN-40 treatment in a dose-dependent manner (*P* = 0.04 at 2 μg/mL and *P* = 0.0235 at 10 μg/mL in MM1R; *P* = 0.036 at 2 μg/mL and *P* = 0.02 at 10 μg/mL in 12BM; and *P* = 0.045 at 2 μg/mL and *P* = 0.0334 at 10 μg/mL in 28BM).

SGN-40 pretreatment abrogates protective effect of interleukin-6 against lenalidomide. The known role of IL-6 as a growth factor and inhibitor of lenalidomide-induced MM cell apoptosis (7), coupled with the IL-6 receptor down-regulation induced by SGN-40 (4), provided the rationale to determine whether IL-6 overcomes the inhibition of DNA synthesis triggered by lenalidomide in SGN-40-pretreated MM cells. Dexamethasone-sensitive MM.1S cells and dexamethasone-resistant MM.1R cells were pretreated with SGN-40 (20 μg/mL) overnight followed by addition of lenalidomide in the presence or absence of IL-6; DNA synthesis was determined by [³H]thymidine uptake. As in our previous reports (7), IL-6 triggered dose-dependent cell protection of MM1S and MM1R cells against lenalidomide (Fig. 2). In contrast, pretreatment with SGN-40 abrogated this protective effect of IL-6. Therefore, SGN-40 treatment mitigates IL-6-induced growth and

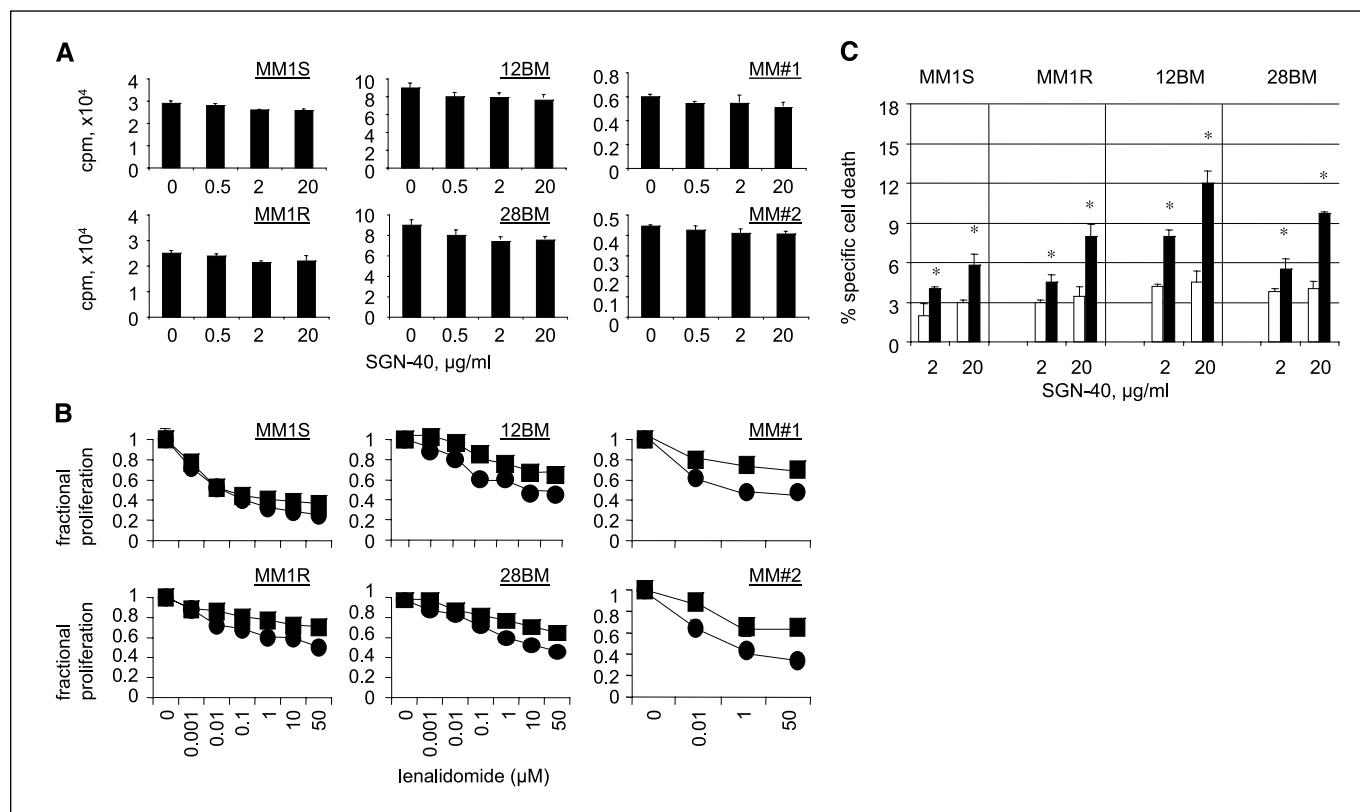
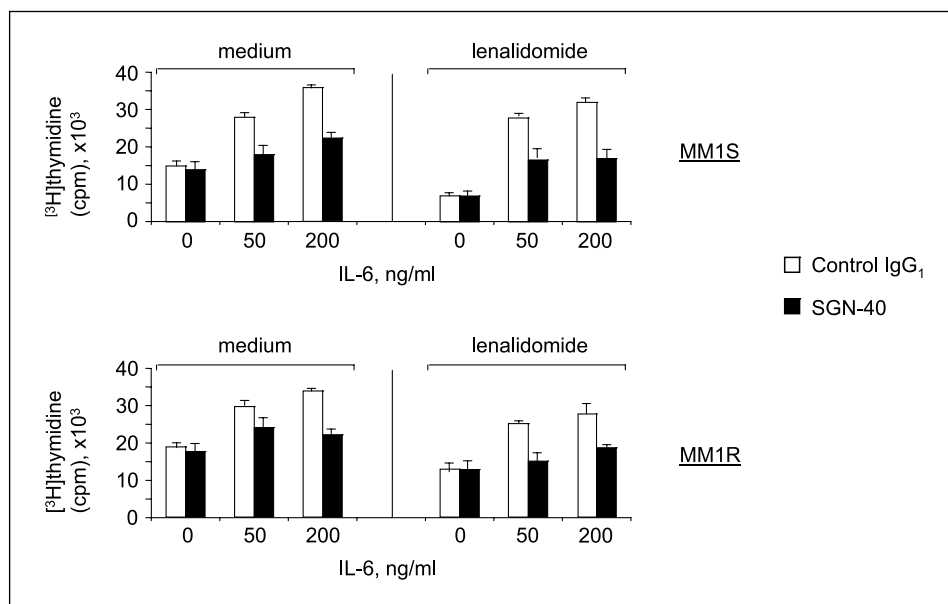


Figure 1. Effect of SGN-40 and lenalidomide on DNA synthesis and cell death. **A**, the growth inhibitory effect of SGN-40 on four CD40-expressing MM cell lines and two patient MM cells was measured by quantitating [³H]thymidine incorporation. SGN-40 treatment (0–20 μg/mL) did not alter constitutive DNA synthesis of these CD40-expressing MM cells. *P* > 0.5 for each line. **B**, growth inhibition by combined lenalidomide and SGN-40. Lenalidomide dose-response curves in the presence of SGN-40 (●) or control IgG1 (■) were determined by measuring [³H]thymidine uptake. Results are expressed as cpm of treated cells relative to control (no drug) cells. Representative of three experiments. **C**, lenalidomide promotes SGN-40-induced cell death. MM lines were pretreated with (black columns) or without lenalidomide (white columns) overnight before addition of SGN-40 (2 and 20 μg/mL). Survival was assessed by MTT assay. Percentage specific cell death = 100 – (absorbance in cells treated with lenalidomide and SGN-40) / (absorbance in cells treated with lenalidomide alone). Columns, mean of two experiments; bars, SD. *, *P* < 0.05 compared with control.

Figure 2. IL-6 does not rescue lenalidomide-induced inhibition of proliferation in SGN-40–pretreated MM cells. MM cells were pretreated with 20 μg/mL of SGN-40 (black columns) or control IgG1 (white columns) followed by addition of lenalidomide (1 μmol/L for MM1S and 10 μmol/L for MM1R) in the presence or absence of IL-6 (0, 50, and 200 ng/mL). After 48 hours, cells were pulsed with [³H]thymidine incorporation for 8 hours for measurement of DNA synthesis.



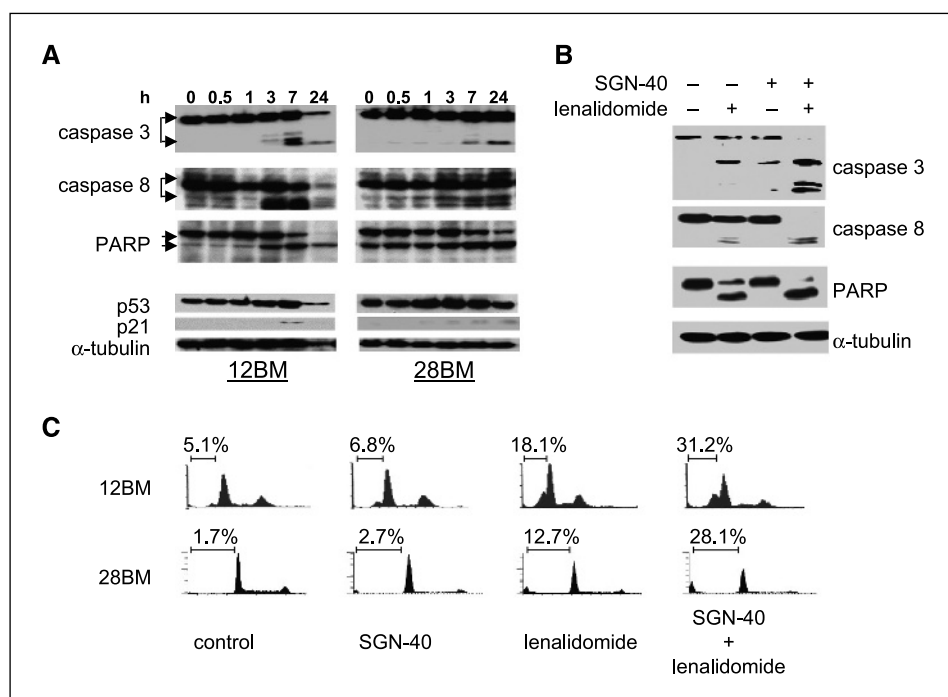
survival effects against lenalidomide, confirming the functional significance of SGN-40–induced decreased IL-6 receptor on MM cells (4).

Combined lenalidomide and SGN-40 enhance apoptotic signaling induced by either agent alone. Ligation of mAb with a cross-linking agent GAH, as reported for anti-CD20 mAb and anti-CD74 mAb, is required to cause inhibition of cell proliferation and induce apoptosis *in vitro* (17, 18). Therefore, we next determined whether cross-linking SGN-40 activates apoptotic signaling in MM cells. 12BM and 28BM MM cells were incubated with SGN-40 (50 μg/mL) and a cross-linking secondary GAH (20 μg/mL), followed by immunoblotting of cell lysates using specific antibodies against apoptosis-related molecules. Caspase-3, caspase-8, and

PARP cleavage were clearly observed in both 12BM and 28BM MM cells after 7-hour incubation with cross-linked SGN-40 mAbs (Fig. 3A). Cleavage of these proteins in 28BM cells was further enhanced after 24 hours. Both p53 and p21 were also induced by cross-linking SGN-40 mAbs in these MM cells (Fig. 3A).

We have previously shown that lenalidomide-induced apoptosis in MM cells is caspase-8 dependent (9) and, therefore, next asked whether low concentrations of SGN-40 and lenalidomide augment caspase-3, caspase-8, as well as PARP cleavage more than either single agent alone. 12BM cells were incubated with cross-linked SGN-40 (5 μg/mL) or lenalidomide (10 μmol/L) or the combination overnight. Cell lysates were then prepared and subjected to immunoblotting using indicated antibodies. SGN-40 (5 μg/mL)

Figure 3. Lenalidomide and SGN-40 enhance apoptotic signaling induced by either agent alone. **A**, 12BM (left) and 28BM (right) MM cell lines were incubated with SGN-40 (50 μg/mL) and a cross-linking secondary GAH antibody (20 μg/mL) at indicated time intervals; cell lysates were immunoblotted with anti-caspase-3, anti-caspase-8, PARP, anti-p53, and anti-p21 antibodies, with anti-α-tubulin mAb as a loading control. **B**, 12BM MM cells were incubated with cross-linked SGN-40 (5 μg/mL), lenalidomide (10 μmol/L), or the combination. Immunoblotting of cell lysates was done using the indicated antibodies. **C**, cell cycle profile by propidium iodide staining and flow cytometric analysis defined sub-G₀ apoptotic fraction in MM cells after 48 hours culture with control medium (control), cross-linked SGN-40 (5 μg/mL), lenalidomide (10 μmol/L), or both reagents.



rarely induces cleavage of any apoptotic proteins, whereas lenalidomide (10 $\mu\text{mol/L}$) only weakly triggers cleavage of these apoptotic proteins (Fig. 3B). Importantly, combined SGN-40 and lenalidomide induces greater cleavage of caspase-3, caspase-8, and PARP than either agent alone. We next did cell cycle analysis following 48-hour incubation with either combined cross-linked SGN-40 (5 $\mu\text{g/mL}$) and lenalidomide (10 $\mu\text{mol/L}$) or each agent alone. As shown in Fig. 3C, the combination of these reagents significantly increased sub- G_0 fraction in both MM lines. These results support the potential therapeutic utility of combining these agents in MM.

Lenalidomide enhances SGN-40-induced lysis of CD40-expressing multiple myeloma lines. Because lenalidomide and its analogues enhance *in vitro* NK cell-mediated lysis of both MM cell lines and autologous patient MM cells (8), we next determined whether lenalidomide augments SGN-40-mediated ADCC against CD40-expressing MM cells. ADCC was done using calcein-AM release assay (19, 20). Calcein-AM-labeled 12BM and 28BM MM target cells were added to freshly isolated PBMC effectors at E/T ratios of 5:1, 10:1, and 40:1, with control medium, human control IgG1 (2 $\mu\text{g/mL}$), SGN-40 (2 $\mu\text{g/mL}$), lenalidomide (2 $\mu\text{mol/L}$), or SGN-40 and lenalidomide. After 4 hours, supernatants were harvested by centrifugation to determine percentage specific lysis. SGN-40, but not control medium or control IgG1, specifically triggered lysis of CD40-expressing 12BM and 28BM MM cells

($P < 0.005$ and $P < 0.0005$ at E/T of 10:1 and 40:1, respectively; Fig. 4A). Because specific lysis curves induced by SGN-40 alone and combined SGN-40 with lenalidomide are almost superimposable, lenalidomide does not significantly increase SGN-40-mediated MM lysis in 4-hour incubation ADCC assays when the two reagents are added concomitantly. We next preincubated PBMC effectors with either individual agent alone or in combination, followed by ADCC assay induced by SGN-40. As shown in Fig. 4B, preincubation of PBMC effectors with lenalidomide significantly enhanced SGN-40-mediated ADCC against two CD40-expressing MM cells ($P < 0.005$), whereas preincubation of PBMCs with SGN-40 or control IgG1 mediates similar SGN-40-triggered MM lysis as in complete medium alone. Preincubation of PBMC effectors with the combination of SGN-40 and lenalidomide further augments SGN-40-triggered ADCC compared with pretreatment of PBMC effectors with lenalidomide alone. This is associated with an increased number of $\text{CD56}^+\text{CD3}^-$ NK cells following preincubation of PBMCs with lenalidomide or combined SGN-40 and lenalidomide (Fig. 4C). Lenalidomide alone induces a 1.5- to 1.8-fold increase in number of $\text{CD56}^+\text{CD3}^-$ NK cells, consistent with our previous reports (8, 16). Combined SGN-40 and lenalidomide pretreatment further increases the $\text{CD56}^+\text{CD3}^-$ NK population by >2-fold when compared with medium control. Because CD16 and LFA-1 play an important role in NK cell-mediated ADCC, we next further analyzed their expression on $\text{CD56}^+\text{CD3}^-$ NK cells using dual

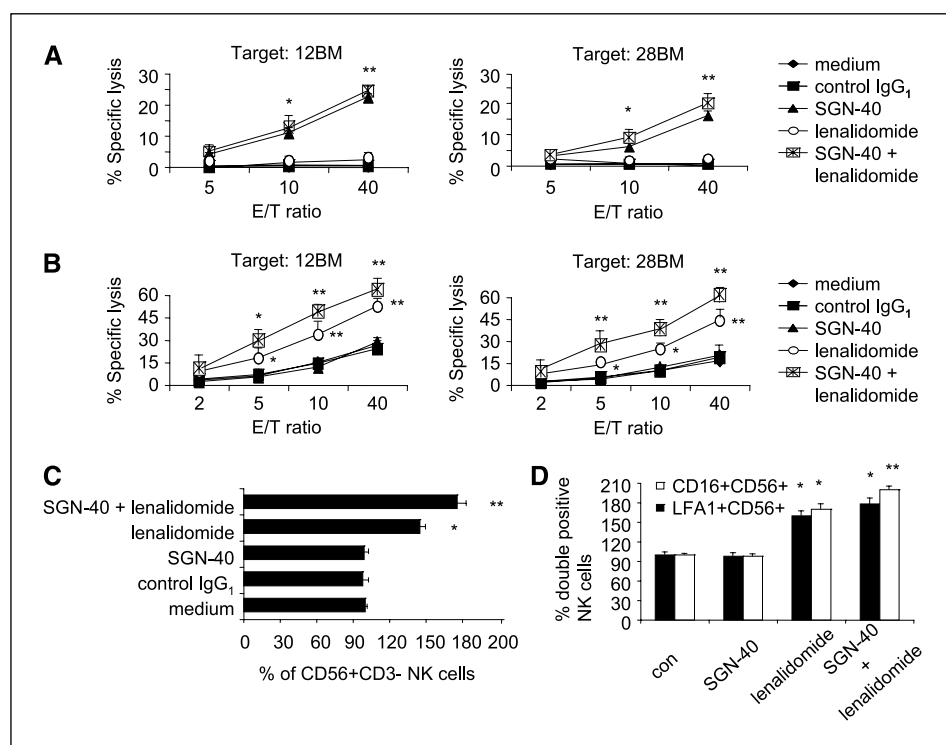
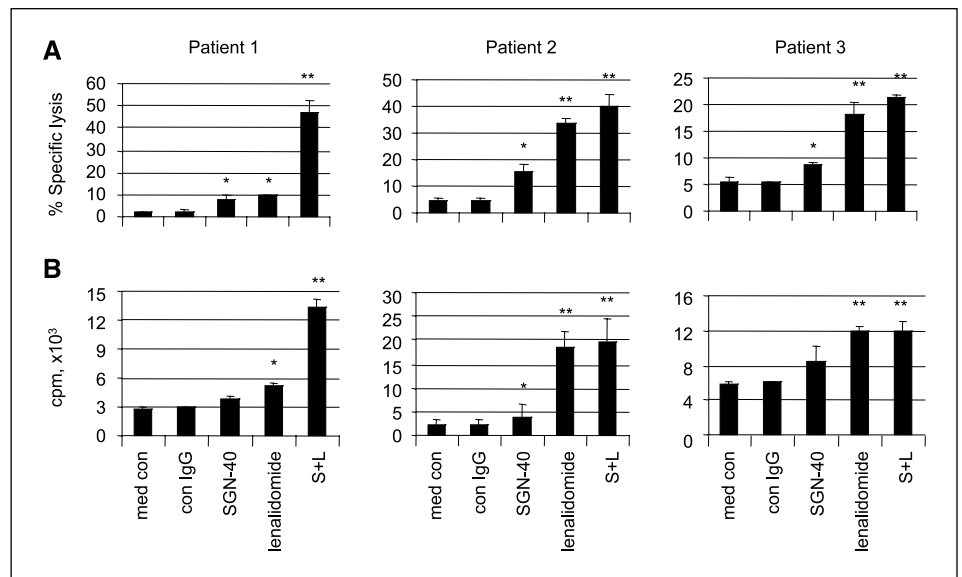


Figure 4. Lenalidomide increases SGN-40-induced ADCC against CD40-expressing MM targets. *A*, MM target cells [12BM (*left*) and 28BM (*right*)] labeled with calcein-AM were cocultured with normal donor PBMCs at various E/T ratios for 2 hours at 37°C with complete medium alone (◆), control IgG₁ (2 $\mu\text{g/mL}$, ■), SGN-40 (2 $\mu\text{g/mL}$, ▲), lenalidomide (2 $\mu\text{mol/L}$, ○), or SGN-40 and lenalidomide (2 $\mu\text{g/mL}$ + 2 $\mu\text{mol/L}$, ☒). Cells were centrifuged and absorbance in supernatants was then measured. Percentage specific lysis was calculated using $S_p - S / M - S$, where S_p is experimental lysis caused by antibody, S is the spontaneous lysis, and M is maximum cell lysis in Triton X-100. $\{(M - \text{medium control release}) / (S - \text{medium control release})\} > 7$ validated each experiment. Representative of three experiments. Points, mean of triplicate wells; bars, SE. *B*, PBMCs from normal donors were preincubated with complete medium alone (◆), control IgG₁ (2 $\mu\text{g/mL}$, ■), SGN-40 (2 $\mu\text{g/mL}$, ▲), lenalidomide (2 $\mu\text{mol/L}$, ○), or SGN-40 and lenalidomide (2 $\mu\text{g/mL}$ + 2 $\mu\text{mol/L}$, ☒) for 3 days before ADCC assay was done. MM target cells labeled with calcein-AM were then cocultured with control and treated PBMC, at various E/T ratios for 2 hours at 37°C, in the presence of SGN-40 (2 $\mu\text{g/mL}$). *C*, $\text{CD56}^+\text{CD3}^-$ NK cells were quantified in PBMCs after 3-day incubation with each treatment. Data were presented as normalized (to control) percentages. *D*, dual immunofluorescence analyses using CD56-PE/CD16-FITC, CD56-PE/LFA-1-FITC, or isotype control antibodies were done to quantitate NK cells ($\text{CD56}^+\text{CD3}^-$) in PBMCs after each treatment. Percentages of double-positive NK cells ($\text{CD16}^+\text{CD56}^+$ or $\text{LFA-1}^+\text{CD56}^+$) are quantitated and normalized to control percentages from three different experiments using three different donors. *, $P < 0.005$; **, $P < 0.0005$.

Figure 5. Treatment of patient PBMCs with lenalidomide triggers increased SGN-40-mediated lysis of autologous MM cells. **A**, PBMCs from three patients with MM were cultured for 3 days with medium control (*med con*), control IgG1 (*con IgG*, 1 μ g/mL), SGN-40 (1 μ g/mL), lenalidomide (2 μ mol/L), or SGN-40 + lenalidomide (*S+L*). Lysis of autologous CD138⁺CD40⁺ MM cells by effector cells was measured in an ADCC assay described above in the presence of SGN-40 (1 μ g/mL) at a 10:1 E/T ratio. *, $P < 0.05$; **, $P < 0.005$. **B**, proliferation of PBMCs from these three MM patients was assayed by [³H]thymidine incorporation assay.



immunofluorescence flow cytometry. We found that double-positive NK cells (CD16⁺CD56⁺CD3⁻ as well as LFA-1⁺CD56⁺CD3⁻) were increased ~2-fold by lenalidomide or the combination of SGN-40 and lenalidomide relative to complete medium alone, SGN-40, or control IgG1 (Fig. 4D). Therefore, although simultaneous addition of SGN-40 and lenalidomide did not significantly increase SGN-40-mediated MM lysis, preincubation of PBMC effectors with lenalidomide or combined lenalidomide and SGN-40 significantly enhanced ADCC against MM triggered by SGN-40. Importantly, this increment correlates with increased NK cells, as well as increased CD16 and LFA-1 expression.

Treatment of patient peripheral blood mononuclear cells with lenalidomide induces increased lysis of autologous multiple myeloma cells. We further asked whether treatment of patient PBMCs with either SGN-40 or lenalidomide, alone or in combination, also resulted in increased SGN-40-induced killing of autologous MM cells. As was observed with MM cell lines, treatment of MM patient PBMCs with lenalidomide resulted in increased autologous MM cell lysis triggered by SGN-40 ($P = 0.014$ for patient 1, $P = 0.0028$ for patient 2, and $P = 0.0008$ for patient 3; Fig. 5A). The addition of SGN-40 to lenalidomide treatment modestly further augmented lysis of autologous MM cells induced by SGN-40 in patients 2 and 3. Of note, treatment of PBMCs from MM patient 3 with combined SGN-40 and lenalidomide significantly increased autologous MM lysis triggered by SGN-40 ($P < 0.0001$). Proliferation of PBMCs from these three patients was assayed by [³H]thymidine uptake. Lenalidomide increased DNA synthesis of PBMCs ($P = 0.012$ for patient 1, $P = 0.0024$ for patient 2, and $P = 0.009$ for patient 3), confirming previous reports (8, 16). Importantly, addition of SGN-40 to lenalidomide further enhanced proliferation of PBMCs from patient 1 ($P = 0.00058$). The lack of adverse effects from either agents, alone or in combination, suggests a potential clinical application in MM.

CD40L is induced on CD56⁺CD3⁻ natural killer cells following lenalidomide treatment. CD40-CD40L interaction has been shown to regulate NK cell cytotoxicity and activation (21, 22). We next, therefore, characterized the effects of lenalidomide on CD40L expression on NK cells, in the presence or absence of SGN-40. PBMCs from normal donors were isolated and pretreated

with SGN-40, lenalidomide, SGN-40 and lenalidomide, or IL-2 for 3 days. CD56⁺CD3⁻ NK cells were then purified, followed by analysis for CD40L (CD154) expression using flow cytometry and reverse transcription-PCR (RT-PCR). As a positive control, CD56⁺CD3⁻ NK cells were purified from PBMCs treated with IL-2. Increased CD40L expression was induced on the CD56⁺CD3⁻ NK cells by preincubation with lenalidomide, but not control medium or SGN-40 (Fig. 6A). No binding of anti-CD40L mAb was observed on NK cells pretreated with control IgG1. Combined treatment with SGN-40 and lenalidomide induced CD40L expression on CD56⁺CD3⁻ NK cells to a similar extent as did lenalidomide treatment alone. This induction of CD40L following lenalidomide or combined lenalidomide and SGN-40 was further confirmed using RT-PCR of RNA isolated from pooled NK cells from three donors (Fig. 6B). In the presence of neutralizing anti-IL-2R antibody, lenalidomide-induced CD40L expression on NK cells was significantly inhibited, implicating IL-2 in CD40L induction. These results show that CD40L is induced on CD56⁺CD3⁻ NK cells by lenalidomide treatment, thereby enhancing an activation pathway for NK cells.

Lenalidomide alters the ratio of CD56^{bright} to CD56^{dim} natural killer subset. All human NK cells express CD56, but the density of surface expression of CD56 can classify functionally and developmentally distinct NK cell subsets (23). CD56^{bright}CD16^{dim} NK cells mediate low or no cytotoxicity, proliferate in response to low levels of IL-2, and produce high levels of inflammatory cytokines. In contrast, CD56^{dim}CD16^{bright} cells are potent mediators of cytotoxicity (23, 24). We, therefore, next asked whether lenalidomide effects CD56^{bright} and/or CD56^{dim} NK cell subsets, which may provide further evidence for enhanced ADCC activity triggered by lenalidomide. We first showed that 10% of normal donor CD56⁺ NK cells highly express CD56^{bright}, whereas the majority express low CD56 (CD56^{dim}; Fig. 7A). In the presence of IL-2, CD56^{bright} NK cells are the majority of CD56⁺ cells, consistent with prior reports that IL-2 induces expansion of CD56^{bright} NK cells *in vitro* (25). We next purified NK cells following 4-day incubation with IL-2 and cultured these cells for 24 hours with control medium, SGN-40, lenalidomide, or combined SGN-40 and lenalidomide, followed by flow cytometric analysis to determine

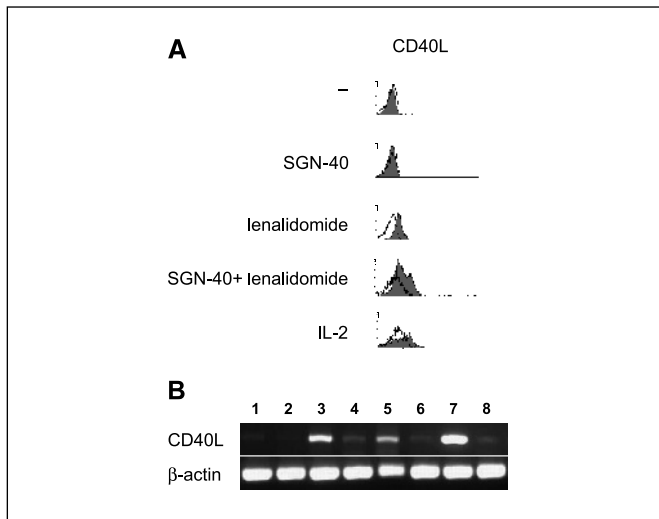


Figure 6. CD40L expression on CD56⁺CD3⁻ NK cells. *A*, CD56⁺CD3⁻ NK cells were purified from PBMCs cultured with control medium (-), SGN-40 (2 μg/mL), lenalidomide (2 μmol/L), or SGN-40 and lenalidomide (2 μg/mL + 2 μmol/L) or IL-2. Cells were then washed and immunostained with anti-CD56-PE, anti-CD3-FITC, and CD154-FITC mAbs. Results were shown as CD40L expression (solid histogram) on CD56⁺CD3⁻ cells (>90% purity) from one donor. Induction of CD40L on NK cells following lenalidomide or combined lenalidomide and SGN-40 was seen in two additional donors. *Open histogram*, isotype control antibody. *B*, RNA was prepared from purified CD56⁺CD3⁻ NK cells from pooled PBMC of three donors following different treatments and subjected to RT-PCR (0.95 kb) for CD40L. Lane 1, control medium; lane 2, SGN-40; lane 3, IL-2; lane 4, IL-2 + neutralizing anti-IL-2 receptor antibody (α-IL-2R Ab); lane 5, lenalidomide; lane 6, lenalidomide + α-IL-2R antibody; lane 7, lenalidomide+SGN-40; lane 8, lenalidomide+SGN-40 + α-IL-2R antibody. RT-PCR for CD154 on purified NK cells from normal PBMCs stimulated with IL-2 (lanes 3 and 4) was included as a positive control. RT-PCR for β-actin (0.62 kb) was used as an internal control.

the fraction of CD56^{bright} to CD56^{dim} NK cell subsets. The majority of NK cells in control medium containing low concentration of IL-2 express high levels of CD56 (CD56^{bright}; Fig. 7A). Interestingly, CD56^{dim} NK subset was clearly induced in the presence of lenalidomide or combined lenalidomide with SGN-40; conversely, CD56^{bright} NK subset was proportionally decreased (Fig. 7B).

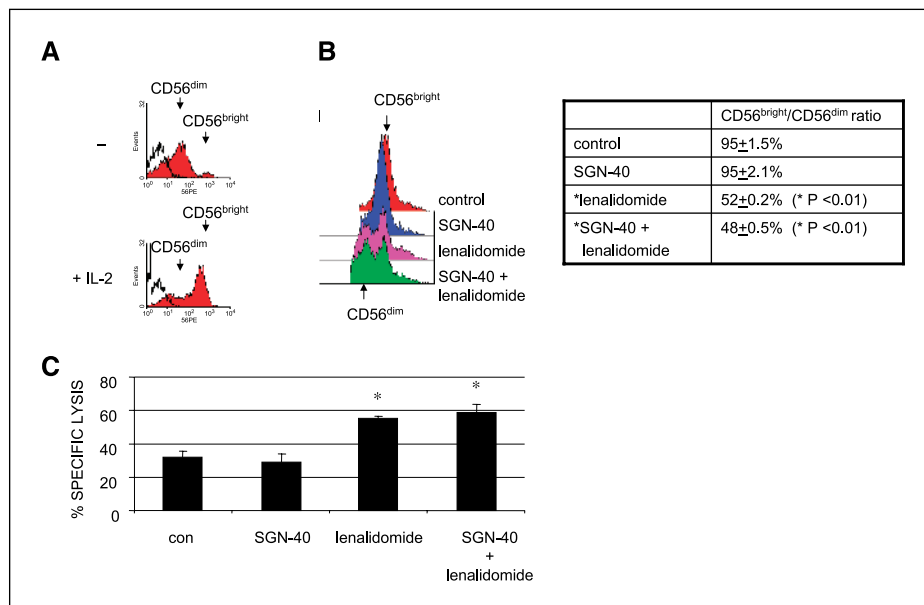
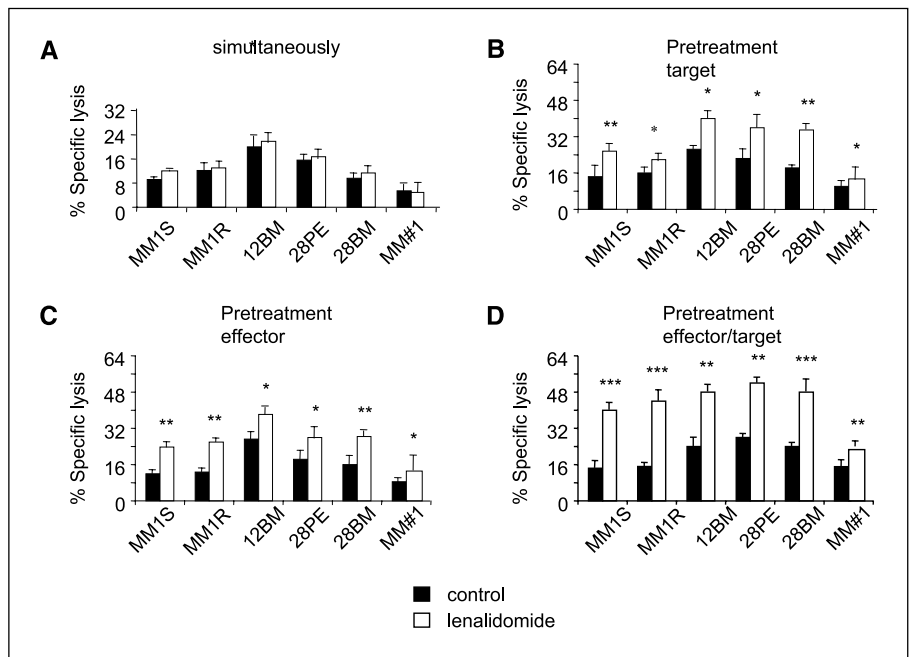


Figure 7. Lenalidomide alters ratio of CD56^{bright} to CD56^{dim} NK subsets. *A*, purified CD56⁺CD3⁻ NK cells were incubated with control 10% human serum/X-VIVO medium alone (-) or with IL-2 (40 unit/mL, +) for 4 days. CD56 expression was analyzed by flow cytometric analysis. *Solid histograms*, CD56 antigen; *open histograms*, isotype control. *B*, CD56⁺CD3⁻ NK cells following 4-day incubation with IL-2 as in (*A*) were incubated with control 10% human serum/X-VIVO medium, SGN-40 (2 μg/mL), lenalidomide (2 μmol/L), or SGN-40 and lenalidomide (2 μg/mL + 2 μmol/L). IL-2 (20 units/mL) was included in all treatments. Histogram of CD56 expression for each treatment and the ratio of CD56^{bright} (*left*) to CD56^{dim} NK (*right*) subsets. *, *P* < 0.01. *C*, SGN-40-mediated ADCC were done using 12BM target MM cells and NK effector cells following each treatment described in (*B*) at an E/T ratio of 10:1. *, *P* < 0.01.

SGN-40 alone did not alter the ratio of CD56^{bright} to CD56^{dim} NK subset because >95% of NK cells are CD56^{bright} as in control medium. Further flow cytometric analysis also confirmed high expression of CD16 on CD56^{dim} NK cells, whereas the majority of CD56^{bright} NK cells are CD16^{dim} (data not shown), as in prior reports that most CD56^{bright} NK cells (70%) lack expression of CD16 and have low-density expression of FcγR (23). Finally, SGN-40-mediated ADCC against MM cells was measured using NK effectors generated in the presence of complete medium control, SGN-40, lenalidomide, or SGN-40 plus lenalidomide. The 4-hour ADCC using calcein-AM-labeled target MM cells were done in the absence of IL-2. A significant increase of specific MM lysis induced by SGN-40 was observed when lenalidomide-treated NK cells were used as effectors (*P* < 0.01; Fig. 7C). Thus, lenalidomide increases the CD56^{dim} NK subset that mediates potent cytotoxic activity and lysis of target MM cells. These results further confirm increased cytotoxic activity of effector NK cells induced by lenalidomide mediating lysis of target MM cells.

Lenalidomide pretreatment of effector and target cells further augments SGN-40-induced antibody-dependent cell-mediated cytotoxicity. Because both MM target cells and effectors would be exposed to lenalidomide *in vivo*, simultaneous pretreatment of both effector and target cells with lenalidomide is the most clinically relevant assay. We, therefore, asked whether pretreatment of MM target cells with lenalidomide could affect SGN-40-mediated ADCC of MM cells. All MM cell lines and CD40-expressing patient MM cells were sensitive to SGN-40-induced ADCC. The addition of lenalidomide to MM target cells and effector cells simultaneously neither enhanced nor reduced specific lysis in any cell lines and patient MM cells (Fig. 8A). Pretreatment of either target cells or effector cells increased specific lysis in all MM cell lines and patient MM cells (Fig. 8B and C). Pretreatment of both target and effector cells markedly enhanced specific lysis triggered by SGN-40 in all MM lines and patient MM cells (*P* < 0.001 for MM1S, MM1R, and 28BM; *P* < 0.01 for 12BM, 28PE, and MM1). Therefore, lenalidomide treatment of target MM cells increased their sensitivity to SGN-40-mediated ADCC and pretreatment of both target and effector cells with lenalidomide further enhanced MM lysis induced by SGN-40.

Figure 8. Lenalidomide pretreatment of effectors and target cells further augments SGN-40-induced ADCC. *A*, the simultaneous addition of lenalidomide (2 $\mu\text{mol/L}$) and SGN-40 (2 $\mu\text{g/mL}$) weakly increases MM lysis in any target cells including 1 CD138⁺CD40⁺ patient MM cells (*MM1*). *B*, ADCC following preincubation of target MM cells with lenalidomide (2 $\mu\text{mol/L}$ for 3 days) resulted in increased lysis in MM cells triggered by SGN-40. *C*, ADCC assays done following preincubation of effector cells with lenalidomide (2 $\mu\text{mol/L}$ for 2 days) resulted in significant increase of ADCC against MM cells induced by SGN-40. *D*, preincubation of both effector and target cells with lenalidomide (2 $\mu\text{mol/L}$) for 2 days resulted in significant increase of SGN-40-induced ADCC against MM cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



Discussion

MM remains incurable and new therapeutic approaches are urgently needed. Most recently, phase III clinical trials of lenalidomide/dexamethasone versus dexamethasone treatment of patients with relapsed MM were unblinded because the time to progression was markedly prolonged in the lenalidomide-treated cohort (26, 27). Additionally, anti-CD40 mAb also holds great promise for MM therapy. We here report our studies of combined lenalidomide and SGN-40 to enhance cytotoxicity against MM cells through both direct and indirect mechanisms. Although SGN-40 alone did not alter MM cell proliferation, it modestly enhanced lenalidomide-induced growth inhibition when these two drugs were added simultaneously. More notably, pretreatment with lenalidomide sensitized MM cells to SGN-40-induced cell death. Importantly, increased cleavage of caspase-3, caspase-8, and PARP, as well as an increased sub-G₀ fraction, was induced when lenalidomide was added to cross-linked SGN-40 relative to treatment with either reagent alone. We then showed that pretreatment of effector cells with lenalidomide increased SGN-40-mediated ADCC against MM cells, associated with an increased number of CD56^{dim}CD3⁻ NK cells expressing both CD16 and LFA-1. Importantly, treatment of patient PBMCs with lenalidomide or combined SGN-40 and lenalidomide significantly increased lysis of autologous MM cells. CD40L is also induced on lenalidomide-treated NK cells in the presence or absence of SGN-40, mainly due to IL-2 secretion because neutralizing anti-IL2R antibody significantly inhibited CD40L up-regulation. Moreover, lenalidomide further increases the CD56^{dim}CD3⁻ NK subset, thereby enhancing ADCC and MM cell lysis. Finally, pretreatment of both effector cells and target MM cells with lenalidomide further enhanced specific MM cell lysis triggered by SGN-40 than relative to pretreatment of either effector cells or target cells alone. Together, these studies suggest that using both drugs in combination may both target MM cells directly and augment immune effectors against MM.

We here showed that lenalidomide could sensitize MM cells to SGN-40-induced cell death. Lenalidomide directly down-regulates nuclear factor- κ B transcriptional activity, which protects against apoptosis in MM cells by up-regulating caspase inhibitors

(i.e., cIAP-2 and FLIP; ref. 9). Down-regulation of these caspase inhibitors by lenalidomide may contribute both to the sensitization to SGN-40-induced apoptosis and the induction of caspase-8 activity. In addition, SGN-40-mediated MM cell death is associated with up-regulation of Fas/FasL and TNF-related apoptosis-inducing ligand (4). SGN-40-induced cell death occurs via a caspase-8-dependent pathway, consistent with Fas engaging a proapoptotic cascade leading to caspase activation (28). These observations may account, at least in part, for the increased efficacy of SGN-40 to induce MM cell death when combined with lenalidomide.

Efforts are ongoing to improve upon the success of mAb treatments for B-cell malignancies and solid tumors, including combining mAbs with IL-2 to modulate NK effector cells. For example, IL-2 and trastuzumab is a well-tolerated outpatient therapy for patients with HER-2-positive metastatic breast cancer, with increased efficacy and favorable side-effect profile (29). Concomitant IL-2 cytokine therapy with rituximab therapy to treat relapsed or refractory follicular non-Hodgkin's lymphoma was well tolerated and did not exacerbate antibody-related infusional toxicity (30). Gluck et al. (31) showed recently that addition of IL-2 to rituximab therapy in non-Hodgkin's lymphoma is safe and, using thrice weekly IL-2 dosing, results in NK cell expansion that correlates with response. Thus, this combination treatment regimen merits additional evaluation in a randomized clinical trial. To further support these results and our previous *in vitro* and *in vivo* immunofunctional studies on lenalidomide (6-8, 13), we here confirmed that lenalidomide, by up-regulating IL-2 production, enhanced NK effector cell-mediated MM lysis triggered by anti-CD40 SGN-40 mAb. This is consistent with our recent studies showing that lenalidomide augments both NK natural cytotoxicity and ADCC, dependent on IL-2 induction from T cells (16). In this study, we further showed induction of CD40L on CD56⁺CD3⁻ NK cells triggered by lenalidomide, confirming an additional IL-2-dependent activation pathway for NK cells triggered by lenalidomide. We also showed that lenalidomide has a direct effect on NK effector function by altering the NK CD56^{bright} to CD56^{dim} ratio, thereby further enhancing ADCC function in addition to

IL-2–dependent increases in NK cell number. Specifically, the majority (>95%) of CD56⁺CD3⁻ NK cells are CD56^{bright} cells following incubation with low-dose IL-2; the cytotoxic CD56^{dim} NK subset was further induced by lenalidomide in the presence or absence of SGN-40 but not in complete medium control. CD56^{dim} NK subset is also CD16^{bright}, the receptor that triggers NK cell ADCC. Importantly, an increase of the CD56^{dim}CD16^{bright}CD3⁻ NK subset induced by lenalidomide is correlated with enhanced SGN-40–mediated ADCC against MM cells. These results are consistent with previous reports that CD56^{dim} NK cells are more potent mediators of ADCC and natural cytotoxicity than CD56^{bright}CD3⁻ NK cells (23). To our knowledge, this is the first report to show that lenalidomide augments NK cytotoxicity by increasing CD56^{dim}CD3⁻ subset, in addition to inducing IL-2 in T cells. Together, these results further provide the cellular and molecular basis for the use of lenalidomide as an adjuvant in immunotherapeutic strategies of mAb-based therapies, such as anti-CD40 mAbs, in human MM.

Of the immune effector mechanisms triggered by SGN-40, ADCC is thought to be the most important mechanism of antibody activity *in vivo*. We here observed the greatest enhancement of SGN-40–mediated ADCC against MM cells when both effector and target tumor cells were pretreated with lenalidomide. Although the precise mechanism of further sensitization of MM cells to immunomediated mechanisms of cell destruction by SGN-40 remains undefined, a direct effect of lenalidomide on MM cells may render them more susceptible to immune effectors. For example, IMiDs sensitize MM cells to Fas-mediated apoptosis, a major mechanism of cell-

mediated cytotoxicity. On the other hand, specific MM lysis by SGN-40–induced complement-dependent cytotoxicity is insignificant *in vitro* (data not shown); therefore, complement-dependent cytotoxicity is not likely to be a dominant mechanism of SGN-40–induced cytotoxicity against MM cells *in vivo*. Because levels of MM cell surface expression of the complement-regulatory proteins CD55, CD59, and CD46, as well as CD40 were unchanged following lenalidomide treatment (data not shown), it is unlikely that lenalidomide sensitizes MM cells to SGN-40–mediated complement-dependent cytotoxicity. In fact, we did not observe any SGN-40–induced complement-dependent cytotoxicity either in the presence or absence of lenalidomide (data not shown).

In summary, we show that the addition of lenalidomide to SGN-40 enhances cytotoxicity against MM cells, mediated via both direct antiproliferative and apoptotic effects, as well as increased sensitivity of MM cells to ADCC by functional cytotoxic NK effector cells. These studies establish the framework for the development of lenalidomide and anti-CD40 mAb SGN-40 in a new treatment paradigm to both target MM cells directly and induce immune effectors to improve patient outcome in MM.

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