Human Anti-CD40 Antagonist Antibody Triggers Significant Antitumor Activity against Human Multiple Myeloma

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Abstract

Monoclonal antibodies (mAb) directed against lineage-specific B-cell antigens have provided clinical benefit for patients with hematologic malignancies, but to date no antibody-mediated immunotherapy is available for multiple myeloma. In the present study, we assessed the efficacy of a fully human anti-CD40 mAb CHIR-12.12 against human multiple myeloma cells. CHIR-12.12, generated in XenoMouse mice, binds to CD138-expressing multiple myeloma lines and freshly purified CD138-expressing cells from >80% multiple myeloma patients, as assessed by flow cytometry. Importantly, CHIR-12.12 abrogates CD40L-induced growth and survival of CD40-expressing patient multiple myeloma cells in the presence or absence of bone marrow stromal cells (BMSC), without altering constitutive multiple myeloma cell proliferation. Immunoblotting analysis specifically showed that PI3-K/AKT, nuclear factor-κB (NF-κB), and extracellular signal-regulated kinase activation induced by CD40L (5 μg/mL) was inhibited by CHIR-12.12 (5 μg/mL). Because CD40 activation induces multiple myeloma cell adhesion to both fibronectin and BMSCs, we next determined whether CHIR-12.12 inhibits this process. CHIR-12.12 decreased CD40L-induced multiple myeloma cell adhesion to fibronectin and BMSCs, whereas control human IgG1 did not. Adhesion of multiple myeloma cells to BMSCs induces interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) secretion, and treatment of multiple myeloma cells with CD40L further enhanced adhesion-induced cytokine secretion; conversely, CHIR-12.12 blocks CD40L-enhanced IL-6 and VEGF secretion in cocultures of multiple myeloma cells with BMSCs. Finally, CHIR-12.12 triggered lysis of multiple myeloma cells via antibody-dependent cellular cytotoxicity (ADCC) but did not induce ADCC against CD40-negative multiple myeloma cells, confirming specificity against CD40-expressing multiple myeloma cells. These results provide the preclinical rationale for clinical trials of CHIR-12.12 to improve patient outcome in multiple myeloma. (Cancer Res 2005; 65(13): 5898-906)

Introduction

CD40, a member of the tumor necrosis factor receptor superfamily, is highly expressed in a variety of B-cell malignancies, including multiple myeloma. CD40 binding by its natural ligand (CD40L) leads to growth induction of both normal and malignant B cells. We and others have shown, using flow cytometry or immunoblotting analysis, that CD40 is widely expressed on primary multiple myeloma cells (1–5). Myeloma cells from patients with progressive multiple myeloma express high CD40 levels (4), suggesting that both disease activity and medullary homing are correlated with the expression of CD40 on tumor cells. A spectrum of diverse biological sequelae can be induced in multiple myeloma cells following CD40 activation, either by formalin-fixed human CD40 ligand (hCD40L) transfectants, soluble CD40 ligand (sCD40L), or an agonist mouse anti-CD40 G28.5 monoclonal antibody (mAb). First, studies of the interleukin-6 (IL-6)–dependent ANBL-6 multiple myeloma cell line suggest that CD40 stimulation may play a role in tumor cell expansion by inducing autocrine IL-6 secretion (3). Second, CD40 stimulation modulates adhesion of multiple myeloma cells to bone marrow stromal cells (BMSC) conferring growth, survival, and drug resistance in tumor cells due both to adhesion to extracellular matrix proteins and BMSCs, as well as induction of cytokine IL-6, vascular endothelial growth factor (VEGF), insulin-like growth factor-I secretion in BMSCs (6). We previously showed that CD40 activation of multiple myeloma cells also increased homotypic and heterotypic cell adhesion by up-regulating cell surface proteins (i.e., LFA-1, VLA-4; ref. 7) and translocating nuclear Ku86/Ku70 to the cell surface (8, 9). Third, stimulation of multiple myeloma cells and BMSC cocultures via CD40 up-regulates IL-6 and VEGF secretion in both populations (2, 6, 7, 10), supporting both autocrine and paracrine mechanisms of multiple myeloma cell growth triggered via CD40. Fourth, ligation of CD40 by CD40L induced multiple myeloma cell migration via activation of PI3K/AKT/nuclear factor-κB (NF-κB) signaling, supporting a functional role of CD40 activation in multiple myeloma homing (11). Because CD40/CD40L interaction triggers this broad array of functional sequelae in multiple myeloma, an inhibitory drug that abrogates these effects represent a promising novel therapeutic strategy to improve patient outcome in multiple myeloma.

Monoclonal antibodies directed against lineage-specific B-cell antigens have provided clinical benefit for patients with hematologic malignancies (12–15). The most successful to date is rituximab, a chimeric mAb targeting the B-cell–specific CD20 antigen. Despite the impressive responses to rituximab in low-grade, follicular lymphoma (12), responses to rituximab in multiple myeloma occur only in those 20% of patients whose tumor expresses CD20 (16). Ongoing efforts to improve rituximab treatments for B-cell malignancies include its use with other biological agents (17, 18), with chemotherapy (19, 20) and with mAbs that target antigens other than CD20. The majority of B-lineage tumors, including multiple myeloma, express CD40, suggesting that mAbs targeting CD40 may benefit

patients with multiple myeloma, as well as those with CD20^{+} B-cell malignancies resistant to rituximab. CD40 is also highly expressed on a spectrum of carcinomas (21), broadening its potential therapeutic application. We recently evaluated a humanized anti-CD40 mAb SGN-40 and showed that it mediates cytotoxicity against multiple myeloma cells by down-regulating IL-6 receptors and inhibiting IL-6–mediated survival and growth signals (22). However, SGN-40 did not block CD40L-induced signaling, even at concentrations as high as 1,000 μg/mL. SGN-40 is composed of the human IgG1 class constant region coupled with humanized murine variable regions against CD40 (23). A fully human anti-CD40 mAb CHIR-12.12 was recently generated in XenoMouse mice (Abgenix, Inc., Fremont, CA), a strain of transgenic mice expressing human IgG1 antibodies and selected based on its inhibition of CD40L-induced biological sequelae (24). CHIR-12.12 inhibits CD40L-induced proliferation of human peripheral blood lymphocytes, without perturbing baseline lymphocyte proliferation. It is of the IgG1 isotype and mediates *in vitro* killing of CD40-expressing lymphoma Daudi cells by antibody-dependent cellular cytotoxicity (ADCC). In addition, CHIR-12.12 shows more potent preclinical anti-human lymphoma activity *in vitro* and *in vivo* than rituximab (24). In this study, we characterize the cytotoxicity of CHIR-12.12 against multiple myeloma cells and its mechanisms of action to provide the framework for derived clinical trials.

Materials and Methods

Cell culture. The CD138^{+} human multiple myeloma–derived cell lines were maintained as described (22, 25). 12BM, 28BM, 28PE, and KMS18 cell lines were described previously (26). Freshly isolated tumor cells (CD138^{+}) from multiple myeloma patients were obtained after informed consent and purified by CD138 MACS microbeads (Miltenyi Biotec, Auburn, CA). More than 90% of purified multiple myeloma cells express CD138, evidenced by flow cytometric analysis. CHO/hCD40L transfectants and control vector transfectants were maintained in 0.4 μg/mL of G418/10% fetal bovine serum (FBS)/RPMI. In CHO/hCD40L transfectant stimulation experiments, the transfectants were washed in RPMI medium, fixed in 0.5% formaldehyde, and added to multiple myeloma cultures at CHO/hCD40L/multiple myeloma ratio of 2:1. BMSCs were derived from CD138-negative bone marrow fractions cultured for 1 month in 20% FBS/ISCOV medium. BMSCs were trypsinized, counted, and seeded in 96-well plates overnight before experiments.

Flow cytometric analysis. Direct immunofluorescence flow cytometric analysis was done as described previously (9) using a Coulter Epics XL with data acquisition software (Cytomics FC500-RXP; Beckman Coulter, Miami, FL). The expression of CD40 was monitored using FITC-labeled CHIR-12.12 (CHIR-12.12-FITC, human IgG1, Chiron, Emeryville, CA) or phycoerythrin (PE)-labeled anti-CD40 (Mab89, mouse IgG2, Beckman Coulter), with FITC-labeled anti-human IgG1 or anti-mouse IgG as isotype controls, respectively. The expression of CD138 and CD38 was determined using anti–CD138-PE and anti–CD38-FITC mAbs (Beckman Coulter), respectively.

Cytotoxicity assay. Multiple myeloma cells were incubated with CHIR-12.12 or human control IgG1 (0-100 μg/mL) for 1 hour, and then added in triplicate to 96-well plates for 2 days in the presence or absence of fixed CHO/hCD40L transfectants. Cells were pulsed with [³H]thymidine, harvested, and counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). Cell viability of CD40-expressing CD138^{+} patient multiple myeloma cells was also assessed by the yellow tetrazolium 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay (American Type Culture Collection, Manassas, VA). Absorbance of CD40L-induced cells at 570 nm was 100% cell viability, and absorbance of treated cells was divided by that of CD40L-induced cells to calculate the percentage cell viability.

Immunoblotting analysis. After overnight serum starvation, multiple myeloma cells (5×10^6/mL) were treated with or without 5 μg/mL of sCD40L (Alexis Biochemicals, San Diego, CA) in the presence of 5 μg/mL

of CHIR-12.12 or human control IgG1 (Chiron). Immunoblotting of cell extracts was done as described previously (22). All antibodies were obtained from Cell Signaling (Beverly, MA).

Cell adhesion assay. CD138^{+} patient multiple myeloma cells were resuspended in RPMI/0.2% bovine serum albumin (adhesion medium) and used directly after their isolation. Cells (5×10^6/mL) were labeled with calcein-acetoxymethyl (calcein-AM; Molecular Probes, Eugene, OR) for 30 minutes at 37°C, washed, and resuspended in adhesion medium. Multiple myeloma cells were pretreated with CHIR-12.12 or human control IgG1 at 0.1 to 10 μg/mL for 1 hour, and then added in triplicate to fibronectin (20 μg/mL)– or BMSC (30,000 per well)–coated 96-well plates at 37°C for 4 hours; unbound cells were then removed by four washes with RPMI medium. The absorbance of each well was measured using 492/520 nm filter set with a fluorescence plate reader (Wallac VICTOR2, Perkin-Elmer, Boston, MA).

ELISA. BMSCs derived from patient bone marrow aspirates were seeded (3×10^4 per well) in 48-well culture plates. Multiple myeloma cells (4×10^5/mL) were added to 48-well plates, in the presence or absence of BMSCs, with either fixed-vector transfectants (medium control) or CHO/hCD40L transfectants, as well as either CHIR-12.12 or human control IgG1 at 0 to 100 μg/mL. After 3-day incubation, supernatants harvested from these cultures were tested for IL-6 and VEGF levels by ELISA (R&D Systems, Minneapolis, MN). The minimum detectable levels of IL-6 and VEGF were 1 and 10 pg/mL, respectively.

Antibody-dependent cellular cytotoxicity. ADCC was measured by calcein-AM release assay (28, 29), with sensitivity similar to traditional Cr[51] assay (28, 29). Human natural killer (NK) cells (>85% CD56^{+}CD3^{−}) were isolated from leukopheresis products of normal donors using MACS NK cell isolation kit (Miltenyi Biotec). Target cells (10^6) were labeled with calcein-AM, washed, and plated in triplicate at 5×10^3 per well in round-bottomed 96-well plates. Increasing concentrations (0-10 μg/mL) of either CHIR-12.12 or human control immunoglobulin antibodies were added at effector-to-target (E/T) ratios of 10:1, in a final volume of 200 μL per well. After 4-hour incubation, 100 μL culture supernatants were transferred to a Black ViewPlate-96 plate and arbitrary fluorescent units (AFU) were read on Tecan SPECTRAFLUOR (485 nm excitation/535 nm emission). This assay is valid only if (AFU mean maximum release − medium control release) / (AFU mean spontaneous release − medium control release) > 7. Calculation of percentage of specific lysis from triplicate experiments was done using the following equation:

$$\text{\% specific lysis} = 100 \times \frac{(\text{AFU mean experimental release} - \text{AFU mean spontaneous release})}{(\text{AFU mean maximal release} - \text{AFU mean spontaneous release})}$$

where "AFU mean spontaneous release" is calcein-AM release by target cells in the absence of antibody or NK cells and "AFU mean maximal release" is calcein-AM release by target cells upon lysis by detergent. The results are shown as percentage of specific lysis at various concentrations of antibodies.

Statistics. Data are mean ± SE. Statistical analysis used the Student *t* test, with *P* < 0.05 considered significant.

Results

CHIR-12.12 binds to CD138-expressing multiple myeloma cell lines and patient multiple myeloma cells. We first tested the binding of human anti-CD40 mAb CHIR-12.12 to multiple myeloma cell lines and CD138^{+} patient multiple myeloma cells. CD40-expressing cell lines, previously identified with a PE-labeled mouse anti-CD40 Mab89 mAb, were stained with CHIR-12.12-FITC, anti-CD138-PE, anti-CD38-FITC, as well as Mab89-PE mAbs. One representative histogram of each indicated antigen for three CD40-expressing multiple myeloma lines is shown in Fig. 1*A*. All lines express CD138, CD40, and CD38 and are immunoreactive with CHIR-12.12. The mean fluorescence intensity (MFI) of CHIR-12.12
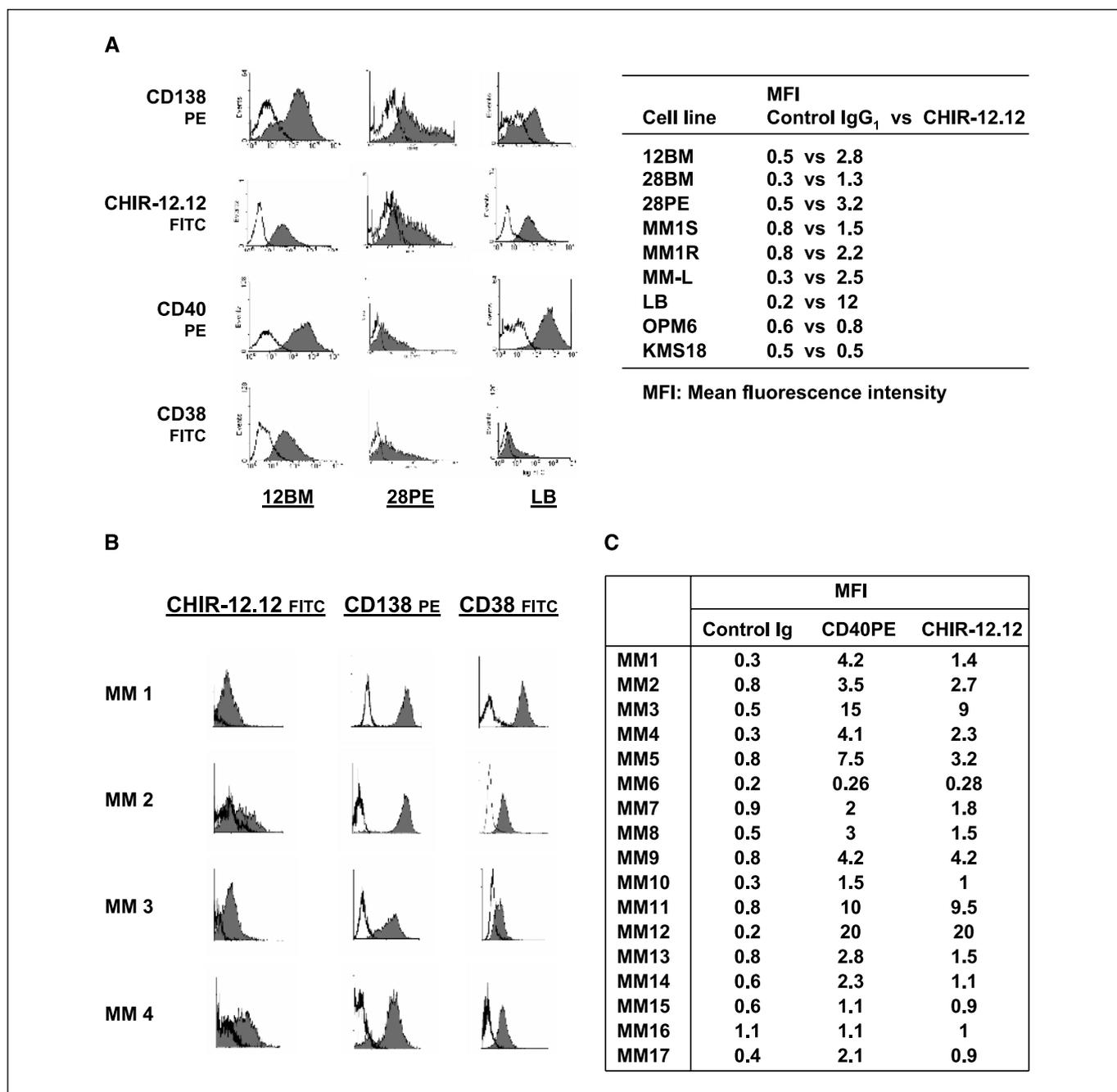
Figure 1. CHIR-12.12 binds to CD138-expressing multiple myeloma cell lines and patient multiple myeloma cells. *A,* binding of CHIR-12.12 in multiple myeloma lines. Cells were washed and immunostained with CHIR-12.12-FITC or human control IgG1-FITC. Immunostaining with CD138-PE, CD38-FITC, and CD40-PE (a mouse mAb Mab89) was also included. *Open histograms,* isotype control; *solid histograms,* indicated antigens. Three representative CD40-expressing multiple myeloma lines are shown in the left panel, whereas results, as MFI, are shown in the right panel. *B,* phenotyping of CD138-purified cells from multiple myeloma patients. Patient multiple myeloma cells were purified from bone marrow aspirates after informed consent, using MACS CD138 microbeads (Miltenyi Biotec). *Open histograms,* isotype control; *solid histograms,* CD138, CD40, or CD38. Four representative CD138-purified patient multiple myeloma cells are shown. *C,* CHIR-12.12-FITC staining in 17 CD138-expressing patient multiple myeloma cells. A mouse anti-CD40-PE mAb (Mab89) was used to confirm CD40 expression.

reactivity is 1.3 to 12, whereas MFI of isotype control is 0.3 to 0.8. Two CD40-negative lines OPM6 and KMS18 are not reactive with CHIR-12.12. CHIR-12.12-FITC staining of CD138$^+$ patient multiple myeloma cells is shown in Fig. 1*B*. All CD138$^+$ patient multiple myeloma cells express CD138 and CD38; >80% (14 of 17) of freshly isolated CD138$^+$ patient multiple myeloma cells also express CD40, although the level of CD40 expression varies among patients (Fig. 1*C*). These results are consistent with previously published

results and confirm that the majority of freshly isolated patient multiple myeloma cells express CD40 (2, 4).

CHIR-12.12 inhibits CD40L-induced viability and growth even in multiple myeloma cells adherent to bone marrow stromal cells. We next determined whether CHIR-12.12 prevents CD40L-induced multiple myeloma growth and viability. CD40-expressing patient multiple myeloma cells were treated with increasing concentrations (0-100 μg/mL) of either CHIR-12.12 or

human control IgG1 in the presence of CHO/hCD40L transfectants, and cytotoxicity of CHIR-12.12 was assayed by MTT. As shown in Fig. 2*A*, CD40L stimulates 1- to 1.5-fold increased patient multiple myeloma cell viability; conversely, CHIR-12.12 blocked this effect in a dose-dependent manner (Fig. 2*A*). This inhibition is specific because human control IgG1 does not alter CD40L-induced cell viability. Formalin-fixed vector transfectants were also used as controls, with viability similar to that observed in medium alone controls (data not shown), confirming the specificity of CD40L induction. In addition, CHIR-12.12 alone neither stimulates nor inhibits CD40-expressing patient multiple myeloma cells. Thus, CHIR-12.12 significantly blocked CD40L-induced growth and viability, but did not change constitutive multiple myeloma proliferation.

We subsequently determined whether CHIR-12.12 abrogates CD40L-induced multiple myeloma cell proliferation in cocultures with BMSCs. CD138-expressing patient multiple myeloma cells were added to 96-well plates, with or without BMSCs, in the presence of either CHIR-12.12 or human control IgG1. Coculture of CD138 patient multiple myeloma cells with BMSCs results in increased [³H]thymidine incorporation (Fig. 2*B*). Addition of fixed CHO/hCD40L transfectants further stimulated adhesion-induced multiple myeloma cell proliferation by 2.2-fold. Conversely, CHIR-12.12 mAb (1 μg/mL) decreased CD40-induced multiple myeloma

proliferation on BMSCs by 33%, with more complete blockade at increased concentrations (10 and 100 μg/mL) of CHIR-12.12. We did further similar experiments using CD40⁺CD138⁺ multiple myeloma cells from two additional patients using MTT assay. As shown in Fig. 2*C*, coculture of multiple myeloma and BMSCs triggered 1- to 2-fold increased multiple myeloma cell viability, and CD40L further increased this effect; conversely, CHIR-12.12 (10 μg/mL) completely blocked CD40L-induced cell survival in patient multiple myeloma cells cocultured with or without BMSCs. Interestingly, inhibition in multiple myeloma survival by CHIR-12.12 is even more pronounced when patient multiple myeloma cells are cocultured with BMSCs. These results suggest that CHIR-12.12 inhibits multiple myeloma cell growth in the bone marrow microenvironment.

CHIR-12.12 blocks CD40L-induced phosphorylation of AKT, IκBα, and extracellular signal-regulated kinase. We next determined whether CHIR-12.12 inhibits CD40L-stimulated signaling. Serum-starved 12BM cells were preincubated with either CHIR-12.12 or human control IgG1 mAbs, and then stimulated with sCD40L (5 μg/mL) for indicated time intervals. As shown in Fig. 3*A*, sCD40L triggers phosphorylation of AKT, IκBα, and extracellular signal-regulated kinase (ERK) as previously reported (11, 22); conversely, pretreatment with CHIR-12.12 completely blocks CD40-induced phosphorylation of AKT and IκBα and
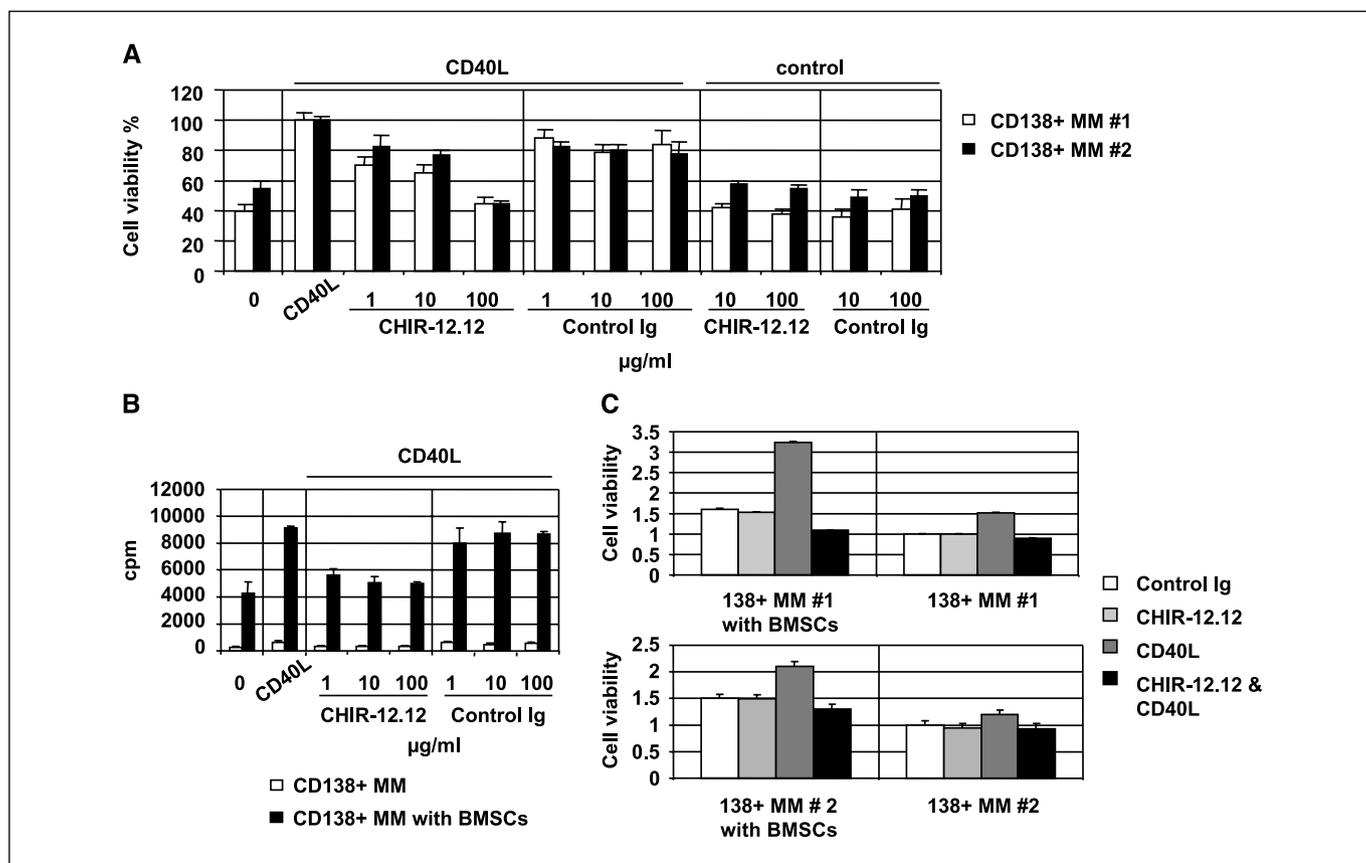

Figure 2. Effect of CHIR-12.12 on CD40-expressing multiple myeloma cells. *A,* CD40⁺CD138⁺ multiple myeloma cells from two patients were incubated with or without formalin-fixed CHO/hCD40L transfectants in the presence of CHIR-12.12 or control human IgG1 (control immunoglobulin) at 0 to 100 μg/mL. Cell viability was determined by MTT assay. Results shown are percentage of absorbance in treated cells relative toCD40L-induced cells. *B,* CD40⁺CD138⁺ patient multiple myeloma cells were stimulated with formalin-fixed CHO/hCD40L transfectants and cultured with (■) or without (□) BMSCs in the presence of either CHIR-12.12 or control immunoglobulin (10 μg/mL). After 3 days, cells were pulsed with [³H]thymidine overnight, and DNA synthesis was measured. *C,* CD40⁺CD138⁺ multiple myeloma cells from two additional patients were cultured with or without BMSCs in the presence of control immunoglobulin (10 μg/mL), CHIR-12.12 (10 μg/mL), formalin-fixed CHO/hCD40L transfectants (CD40L), or CHIR-12.12 and CD40L. Cell viability was determined by MTT assay and normalized to control cells cultured without BMSCs.
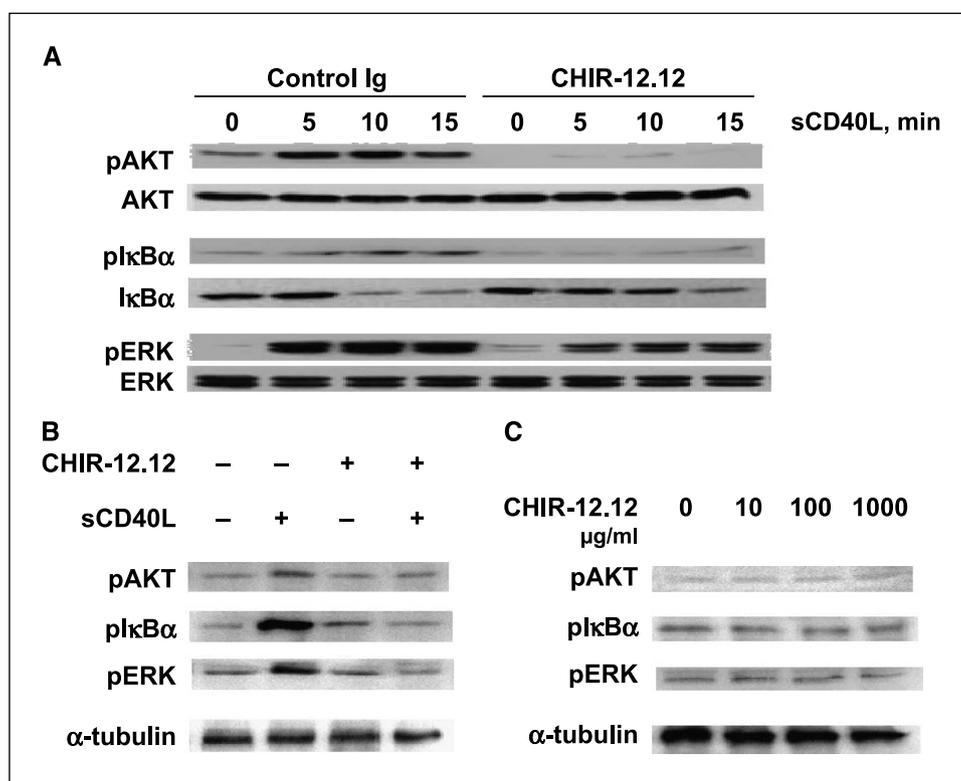
Figure 3. CHIR-12.12 blocks CD40L-induced signaling. *A,* pretreatment with CHIR-12.12. Serum-starved multiple myeloma cells were pretreated with either 5 μg/mL of CHIR-12.12 or control immunoglobulin, and then stimulated with sCD40L (5 μg/mL) for the indicated time intervals. Immunoblotting was done using anti-pAKT, pIκBα, IκBα, and pERK antibodies. Anti-AKT and anti-ERK antibodies were used as loading controls. *B,* simultaneous incubation with CHIR-12.12 and sCD40L. Cells were incubated with or without sCD40L in the presence or absence of CHIR-12.12 for 5 minutes. *C,* CHIR-12.12 alone. Cells were stimulated with CHIR-12.12 at 0 to 1,000 μg/mL for 5 minutes. In (*B*) and (*C*), immunoblotting was for pAKT, pIκBα, and pERK, with α-tubulin serving as a loading control.

inhibits CD40-induced phosphorylation of ERK. When CHIR-12.12 was added with sCD40L, no activation of CD40 signaling was seen (Fig. 3*B*). In addition, CHIR-12.12 itself did not activate AKT or nuclear factor κB (NF-κB) in multiple myeloma cells (Fig. 3*C*). In MM.1S and MM-L lines, CD40-activated signaling was similarly inhibited and CHIR-12.12 alone did not activate AKT and NF-κB (data not shown).

Effect of CHIR-12.12 on interleukin-6 and vascular endothelial growth factor secretion. We and others have previously reported that BMSCs from multiple myeloma patients express CD40 (7) and that IL-6 and VEGF secretion is significantly increased in CD40-activated multiple myeloma cells and BMSCs (2, 6, 7, 10). Therefore, we next asked whether CHIR-12.12 antagonized CD40L-induced cytokine secretion. BMSCs derived from patient bone marrow aspirates were seeded in 48-well culture plates a day before the experiments. Patient multiple myeloma cells were pretreated with either CHIR-12.12 or human control IgG1 for 1 hour. Multiple myeloma cells were added to BMSCs in the presence of either CHIR-12.12 or human control IgG1 as well as either fixed CHO/hCD40L transfectants or vector control transfectants (control). Supernatants from these cultures were collected and assessed for IL-6 and VEGF secretion by ELISA. Coculture of patient multiple myeloma cells with BMSCs dramatically increased IL-6 secretion from 9.5 ± 0.13 to 565 ± 45 pg/mL. Addition of CD40L to the coculture further increased IL-6 secretion by ~2.8 fold, from 565 ± 45 to $1,563 \pm 121$ pg/mL (Fig. 4*A*). CHIR-12.12 (1 μg/mL) decreased CD40L-induced IL-6 secretion by 75%, and higher concentrations of CHIR-12.12 mAbs (10 and 100 μg/mL) further blocked CD40L-induced IL-6 secretion in cocultures. Similarly, CD40L stimulated IL-6 secretion from BMSCs by 2.7-fold (from 276.5 ± 29 to $1,163 \pm 33$ pg/mL), which was inhibited by CHIR-12.12 mAb in a dose-responsive manner. Human control IgG1 (1, 10, and 100 μg/mL) has no effect on

CD40L-triggered IL-6 secretion in cocultures of multiple myeloma cells with BMSCs.

As shown in Fig. 4*B*, adhesion of patient multiple myeloma cells to BMSCs also triggers VEGF secretion from 125 ± 11 to $1,785 \pm 256$ pg/mL. CD40 activation triggered by fixed CHO/hCD40L transfectants further increased VEGF secretion in cocultures, from $1,785 \pm 256$ to $2,947 \pm 240$ pg/mL. In the presence of CHIR-12.12 (1 μg/mL), CD40-induced VEGF induction in cocultures was significantly blocked; higher CHIR-12.12 concentrations (10 and 100 μg/mL) completely blocked this induction. Addition of fixed CHO/hCD40L transfectants also enhanced VEGF secretion from BMSCs (from 802 ± 29.8 to $1,399 \pm 55$ pg/mL) or patient multiple myeloma cells (from 125 ± 16.4 to 224 ± 33 pg/mL) and addition of CHIR-12.12 blocked CD40L-induced VEGF secretion in either BMSCs or patient multiple myeloma cells. Control human IgG1 had no effect on CD40L-induced VEGF secretion, either from multiple myeloma cells or BMSCs alone or in cocultures. The blocking effects of CHIR-12.12 on IL-6 and VEGF secretion were also seen when CD40-expressing multiple myeloma lines (i.e., 28PE, MM-L) and BMSCs derived from different patients were used (data not shown). These results indicate that CHIR-12.12 specifically antagonizes CD40-induced IL-6 and VEGF secretion in CD40-expressing multiple myeloma cells and BMSCs, as well as in cocultures of multiple myeloma cells with BMSCs.

CD40L-induced multiple myeloma adhesion to fibronectin and bone marrow stromal cells was abrogated by CHIR-12.12. We next examined CD40L-induced multiple myeloma adhesion to fibronectin in patient multiple myeloma cells using calcein-AM adhesion assay. Fixed CHO/hCD40L transfectants were added to calcein-AM–labeled multiple myeloma cells in fibronectin-coated plates. After 4-hour incubation, nonadherent multiple myeloma cells were removed by washing and adherence was determined in a fluorescence plate reader. CD40 activation triggers 3- to 4.3-fold
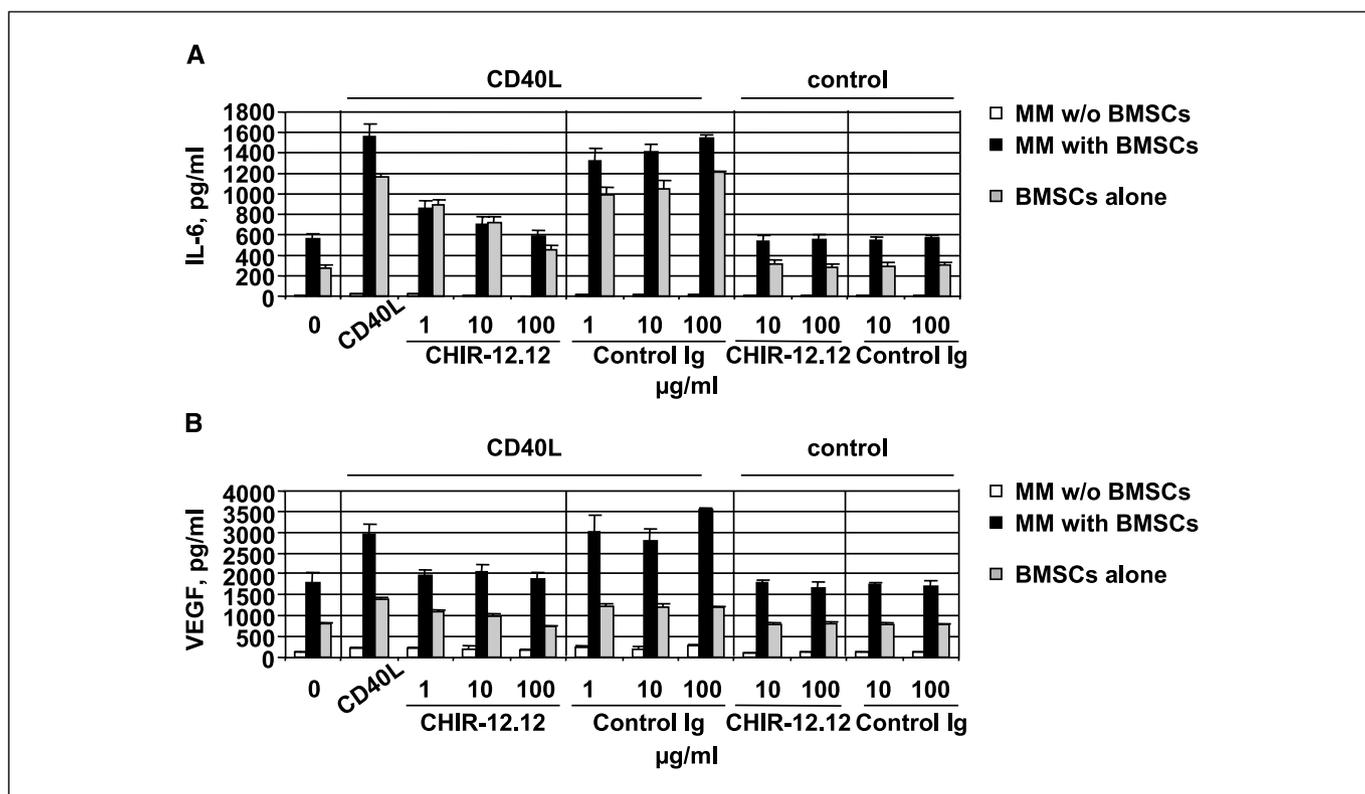
Figure 4. Cytokine secretion in coculture of multiple myeloma with BMSCs. CD40$^+$CD138$^+$ patient multiple myeloma cells were cultured with (■) or without (□) BMSCs and either formalin-fixed CHO/hCD40L transfectants (CD40L) or control medium (*control*) in the presence of either CHIR-12.12 or control immunoglobulin at 1 to 100 μg/mL. BMSCs alone (▨) were also cultured with either CD40L or control medium in the presence of either CHIR-12.12 or control immunoglobulin. IL-6 (*A*) and VEGF (*B*; ng/mL) levels in the supernatants from each culture were determined by ELISA. *Columns*, means of culture triplicates; *bars*, SE.

increased multiple myeloma cell adhesion to fibronectin (Fig. 5*A*). Multiple myeloma cell adhesion to fibronectin was also induced by sCD40L (1 μg/mL; data not shown). Conversely, CD40-induced multiple myeloma cell adhesion was completely blocked by CHIR-12.12 (0.1 μg/mL), but not by control IgG1. In medium control cultures without sCD40L, neither CHIR-12.12 nor human control IgG1 altered multiple myeloma adhesion to fibronectin. In similar experiments in BMSCs-coated plates, CHIR-12.12 also inhibited CD40-triggered multiple myeloma cell adhesion to BMSCs (Fig. 5*B*).

CHIR-12.12 induces antibody-dependent cellular cytotoxicity against CD40-expressing multiple myeloma cells. We next tested the ability of CHIR-12.12 to lyse multiple myeloma cells by ADCC, using calcein-AM release assay (28, 29). Calcein-AM–labeled multiple myeloma cells were added to freshly isolated human NK cells at an E/T ratio of 10:1, in the presence of serial dilutions of either CHIR-12.12 or human control IgG1 for 4 hours; after centrifugation, supernatants were harvested to determine %specific lysis. CHIR-12.12 activated ADCC against CD40-expressing multiple myeloma cells in cultures of CD56$^+$CD3$^-$ NK effectors from three different donors in a dose-responsive manner. Data from one representative experiment is shown in Fig. 6*A*. CHIR-12.12 lysed all multiple myeloma lines in a dose-dependent manner, with lytic activity starting at 0.001 μg/mL and reaching maximum lysis at 0.01 μg/mL. The ED$_{50}$ (antibody concentration to achieve half-maximal lysis) of CHIR-12.12 for these multiple myeloma lines is 28.7 pmol/L for 12BM, 15.66 pmol/L for 28BM, 15.95 for LB, 15.05 pmol/L for MM1R, and 37.21 pmol/L for MM-L cells. The highest and lowest specific maximum lyses were observed for LB and 28BM cell lines, respectively. In contrast, human control IgG1

did not induce any dose-responsive lysis in multiple myeloma cells (Fig. 6*B*). The susceptibility to CHIR-12.12–mediated lysis may depend either on the level of CD40 expression or other mechanisms, such as expression of ADCC resistance proteins. LB cells, which express the highest levels of CD40, are most sensitive to CHIR-12.12–mediated lysis. However, the differences in ADCC between 28BM and three other cell lines (12BM, MM-L, and MM1R) do not correlate with level of CD40 expression. To examine the specificity of CHIR-12.12–mediated ADCC against CD40-expressing target cells, an ADCC assay was done using CD40-negative multiple myeloma cell line KMM-1 and CD40-expressing lymphoma cell line Daudi as target cells. As shown in Fig. 6*C*, strong specific lysis of Daudi cells by CHIR-12.12 was observed whereas CHIR-12.12 showed no lytic activity against CD40-negative cell line. When other CD40-negative lines OPM6 and KMS18 were used as target cells, no CHIR-12.12–triggered ADCC was observed (data not shown). These data show that CHIR-12.12 induces ADCC specifically against CD40-expressing target multiple myeloma cells.

Discussion

The recent success of rituximab treatment for patients with non-Hodgkin's lymphoma has confirmed the remarkable potential of mAb-based therapy of cancers. Although tumor cells from only a minority (20%) of multiple myeloma patients express CD20, rituximab treatment achieved 32% responses in heavily pretreated patients whose multiple myeloma cells were CD20$^+$ (16). To date, no antibody-mediated therapy is available for the majority of patients with multiple myeloma, and mAbs targeting antigens more broadly
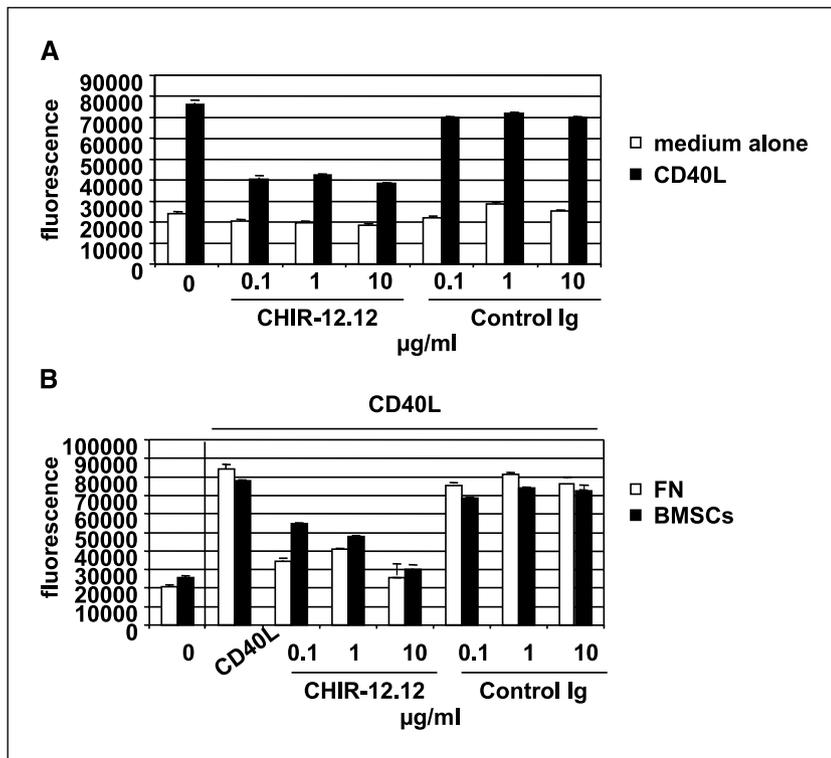
Figure 5. CHIR-12.12 abrogates CD40L-induced multiple myeloma cell adhesion to fibronectin and BMSCs. *A,* CD40+CD138+ patient multiple myeloma cells were labeled with calcein-AM, washed, and pretreated with either CHIR-12.12 or human control immunoglobulin at 0 to 10 μg/mL for 0.5 hour. Cells were added to fibronectin-coated 96-well plates with either formalin-fixed CHO/hCD40L transfectants (CD40L, ■) or medium alone (□). Nonattached cells were removed by washing, and adherence was measured in a fluorescence plate reader. *B,* CD40+CD138+ patient multiple myeloma cells were labeled with calcein-AM and treated with various concentrations of either CHIR-12.12 or control immunoglobulin. Cells were then added to fibronectin-coated (□) or BMSC-coated (■) plates in the presence of CD40L.

expressed on multiple myeloma cells are under development. In this report, we assessed the cytotoxicity of CHIR-12.12 against multiple myeloma cells and delineated several mechanisms by which CHIR-12.12 exerts anti–multiple myeloma activity.

The work presented here shows that the newly developed anti-CD40 antibody, CHIR-12.12, is a potent antagonist of CD40L-induced growth and survival signals for multiple myeloma cells. The antagonist activity was further confirmed biochemically in immunoblotting studies in which CHIR-12.12 down-regulated CD40L-induced phosphorylation of AKT, NF-κB, and ERK in multiple myeloma cells. More importantly, CHIR-12.12 blocked CD40L-induced biological sequelae in multiple myeloma cells in
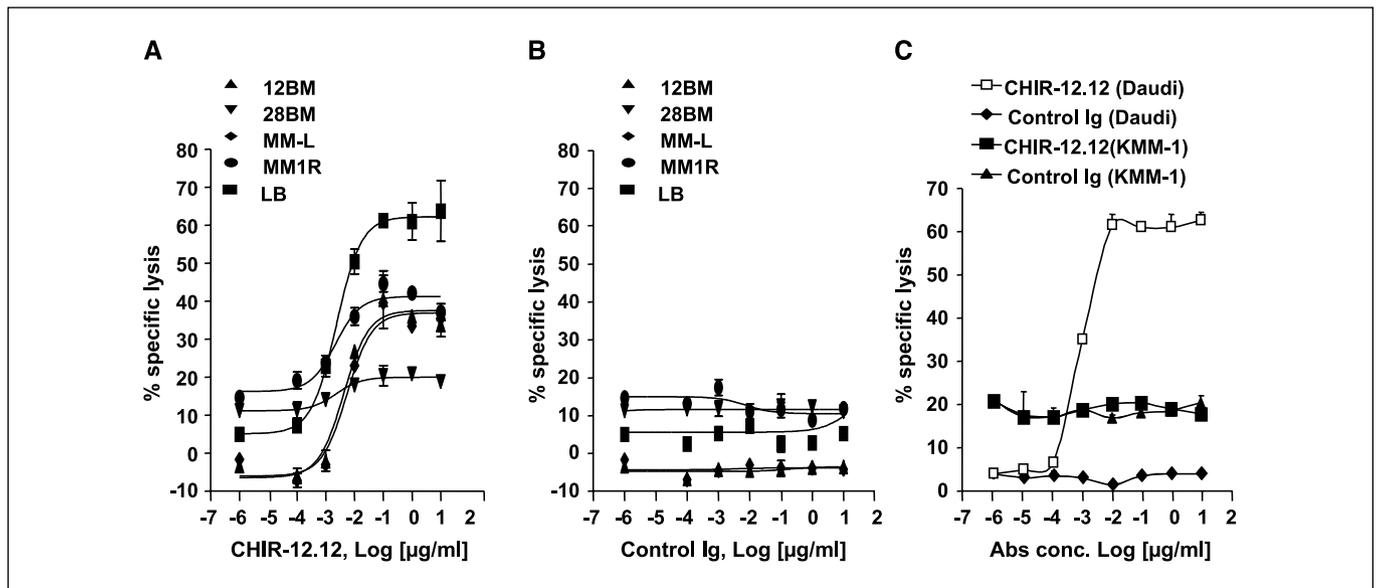

Figure 6. ADCC-mediated multiple myeloma cell lysis triggered by CHIR-12.12. Calcein-AM–labeled target multiple myeloma cells were incubated with human NK effector cells (CD56+CD3−) at an E/T ratio of 10:1 in the presence of varying concentrations of CHIR-12.12 for 4 hours at 37°C. Cells were centrifuged, 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 antibodies, 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. CHIR-12.12 induces %specific lysis of CD40-expressing multiple myeloma cells in a dose-dependent manner (*A*); background lysis by human control IgG1 is shown in (*B*). The data shown is representative of three experiments conducted with three different NK cell donors with similar results. *C,* CHIR-12.12 does not stimulate ADCC against CD40-negative KMM-1 multiple myeloma cells (■), whereas it significantly induces ADCC against CD40-positive Daudi target cells (□).

the bone marrow milieu; it inhibited multiple myeloma cell adhesion to fibronectin and BMSCs, binding-induced IL-6 and VEGF secretion in both multiple myeloma cells and BMSCs, as well as proliferation of multiple myeloma cells adherent to BMSCs.

These data, for the first time, show that antagonizing the CD40/CD40L interaction can result in effects that disrupt myeloma growth and survival. We have recently evaluated the potential application of another anti-CD40 mAb SGN-40, which does not inhibit CD40L-induced signaling (22). This is likely due to distinct epitopes recognized by SGN-40 and CHIR-12.12 antibodies. CHIR-12.12 competes off CD40L binding to CD40 without activating CD40 signaling pathways, whereas SGN-40 actually enhances CD40L binding to CD40 on the cell surface (27). CHIR-12.12 also mediated cytotoxicity against CD40-expressing multiple myeloma cells by the additional mechanism of ADCC, triggering NK-mediated lysis of CD40-expressing multiple myeloma cell lines with an ED50 of <40 pmol/L. CHIR-12.12 has previously been shown to mediate more potent ADCC than rituximab against CD20^{+}/CD40^{+} Daudi lymphoma cells (24).

We have confirmed that CD138^{+} multiple myeloma cells from >80% of multiple myeloma patients bind to CHIR-12.12, consistent with prior studies showing that a majority of patients express CD40 (2, 4). Recent studies suggest that there may be two compartments of multiple myeloma cells, the quiescent multiple myeloma plasma cells and the proliferating, clonogenic tumor "stem cells," phenotypically resembling mature B cells (30). Because CD40 is expressed both in proliferating B cells and in CD138^{+} multiple myeloma plasma cells in the majority of patients, CHIR-12.12 is likely to show activity against both of these multiple myeloma cell populations. We noted that CD40 expression in CD138^{+} multiple myeloma plasma cells is heterogeneous; however, CHIR-12.12 may have therapeutic effect even in those patients whose CD138^{+} plasma cells express low and heterogeneous levels of CD40 by inhibiting the survival or causing the death of CD138-negative malignant clonogenic multiple myeloma precursors. This hypoth-esis is strongly supported by a recent report by Matsui et al. (30) that clonogenic cells in multiple myeloma express CD20, but not CD138, and that clonogenic growth was inhibited by the anti-CD20 mAb rituximab. Multiple studies have shown that circulating B cells expressing CD20 and CD40 are clonally related to multiple myeloma plasma cells (31–33). In B-cell chronic lymphocytic leukemia, the relative CD20 expression does not influence the effectiveness of rituximab (34). Moreover, rituximab has achieved clinical responses in some multiple myeloma patients with CD20-negative tumor cells (35), further supporting this view. Taken together, these studies strongly support targeting anti-CD40 mAb by CHIR-12.12 as an immunotherapeutic approach in multiple myeloma.

In summary, CHIR-12.12 is a promising antibody-mediated immunotherapy to improve patient outcome in multiple myeloma. It effectively blocks hCD40L-induced multiple myeloma proliferation and survival and related AKT/NF-κB and ERK signaling. CHIR-12.12 further inhibited CD40L-induced multiple myeloma cell adhesion to fibronectin and BMSCs, binding-induced IL-6 and VEGF secretion in both multiple myeloma cells and BMSCs, and proliferation of multiple myeloma cells adherent to BMSCs, suggesting that it can inhibit multiple myeloma cell growth in the bone marrow milieu. Finally, CHIR-12.12 stimulates ADCC against CD40-expressing multiple myeloma cells. Our work, coupled with *in vivo* safety profile and bioactivity data in cynomolgus monkeys (36), supports clinical development of CHIR-12.12 to improve patient outcome of multiple myeloma.

Acknowledgments

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