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Mechanisms by which SGN-40, a Humanized Anti-CD40 Antibody, Induces Cytotoxicity in Human Multiple Myeloma Cells: Clinical Implications

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ABSTRACT

CD40 is expressed on B-cell malignancies, including human multiple myeloma (MM) and a variety of carcinomas. We examined the potential therapeutic utility of SGN-40, the humanized anti-CD40 monoclonal antibody, for treating human MM using MM cell lines and patient MM cells ($CD138^{++}$, $CD40^{+}$). SGN-40 (0.01–100 μg/ml) induces modest cytotoxicity in MM cell lines and patient MM cells. In the presence of *de novo* protein synthesis inhibitor cycloheximide, SGN-40 significantly induced apoptosis in Dexamethasone (Dex)-sensitive MM.1S and Dex-resistant MM.1R cells and in patient MM cells. SGN-40-mediated cytotoxicity is associated with up-regulation of cytotoxic ligands of the tumor necrosis factor family (Fas/FasL, tumor necrosis factor-related apoptosis-inducing ligand, and tumor necrosis factor α). SGN-40 treatment also induces a down-regulation of CD40 dependent on an endocytic pathway. Consequently, pretreatment of MM cells with SGN-40 blocked sCD40L-mediated phosphatidylinositol 3′-kinase/AKT and nuclear factor κB activation. Importantly, pretreatment of MM.1S and MM.1R cells with SGN-40 inhibited proliferation triggered by interleukin 6 (IL-6) but not by insulin-like growth factor-I. In addition, SGN-40 pretreatment of MM.1S cells blocked the ability of IL-6 to protect against Dex-induced inhibition of DNA synthesis. This was associated with a 2–4-fold reduction of IL-6 receptor at protein and mRNA levels in SGN-40-treated MM.1S cells and patient MM cells. Taken together, these results provide the preclinical rationale for the evaluation of SGN-40 as a potential new therapy to improve patient outcome in MM.

INTRODUCTION

CD40 is a M_r 50,000 transmembrane protein in the tumor necrosis factor (TNF) receptor superfamily, which includes CD30 and CD95 (Fas/Apo-1). CD40-CD40 ligand (CD40L) interactions play a critical role in the regulation of humoral and cellular immune responses. Activation of CD40, using CD40L transfectants, soluble CD40L (sCD40L), or anti-CD40 monoclonal antibodies (mAbs), is critical for normal B-cell growth, differentiation, and isotype switching. We and others have demonstrated that CD40 is expressed on the majority of primary multiple myeloma (MM) cells (1–3). Specifically, triggering human MM cells via CD40 induces increased homotypic and heterotypic cell adhesion, up-regulation of various cell surface markers (4), translocation of Ku86/Ku70 to the cell surface (5, 6), and increased interleukin 6 (IL-6) secretion (4, 7). Ligation of CD40 with sCD40L or an anti-CD40 mAb also induces vascular endothelial growth factor (3) and urokinase-type plasminogen activator (8), suggesting its role in MM homing and migration. Several studies show that CD40 stimulation also suppresses human MM cell growth (9–12). Although the mechanism by which CD40 triggers growth arrest and apoptosis in MM cells is not delineated, wild-type p53 function is involved in these processes (9).

Because human MM remains incurable, novel biologically based therapies urgently are needed. In other cancers, mAbs and mAb-based reagents have shown clinical efficacy (13). For example, immunotherapy using antibody-targeting CD20 (rituximab), alone and in combination with chemotherapy, has been effective for management of B-cell lymphoproliferative diseases (14, 15). However, few MM patients express CD20 (16). The mAbs targeting CD40 on patient tumor cells represent an attractive therapeutic strategy for MM, and a variety of carcinomas also highly express CD40, broadening its potential therapeutic application. Many investigators have reported that anti-CD40 mAb can enhance antitumor activity and immunity. For example, murine anti-CD40 mAb blocked IL-6 secretion induced by MM cell adhesion to bone marrow stromal cells; because IL-6 is a key growth and survival factor for human MM cells, blockade of IL-6 secretion abrogates MM cell growth in the bone marrow milieu (4). In addition, anti-CD40 immunotoxin can effectively kill B-lineage acute lymphoblastic leukemia and non-Hodgkin's lymphoma (17). *In vivo* treatment of severe combined immunodeficiency mice bearing human B lymphomas with anti-CD40 antibodies inhibited tumor progression and enhanced survival (18, 19). Funakoshi *et al.* (20) demonstrated that murine anti-CD40 mAb was more effective at killing tumors in human B-cell lymphoma-xenografted severe combined immunodeficiency mice, in the absence of Fc receptor-bearing effector cells, than was anti-CD20 mAb to kill tumors. Subsequently, van Mierlo *et al.* (21) reported that systemic *in vivo* administration of agonistic anti-CD40 antibodies resulted in tumor eradication mediated via dendritic cell-induced $CD8^{+}$ T-cell responses. Honeychurch *et al.* (22) most recently showed that anti-CD40 mAb in combination with irradiation results in CD8 T-cell-dependent immunity against B-cell lymphoma in mice, suggesting that combining irradiation with anti-CD40 mAb may provide a more potent therapeutic approach.

In the present study, we evaluated the direct impact of SGN-40 humanized anti-CD40 mAb on MM cell lines and patient cells ($CD40^{+}CD138^{++}$). SGN-40 was engineered from SGN-14 mouse mAb, which showed significant antitumor activity against human HS-Sultan and IM-9 cell line-xenografted severe combined immunodeficiency mice, without adverse effects on human normal B cells (23). We recently showed that SGN-40 triggered antibody-dependent cell-mediated cytotoxicity against CD40-expressing MM cell lines and patient cells, supporting its potential therapeutic use in human MM (24). Moreover, the tumoricidal effects of SGN-40 could not be solely attributed to enhancing effector functions of antibody-dependent cell-mediated cytotoxicity. To examine for direct induction of apoptosis, as is induced in malignant B lymphocytes by rituximab, we specifically examined the effects of SGN-40 on $CD40^{+}$ and $CD138^{+}$ MM.1S and MM.1R cell lines and on patient MM cells (25). SGN-40-induced cytotoxicity against MM cells is associated with up-regulation of cytotoxic ligands of the TNF family FasL, TNF-related apoptosis-inducing ligand (TRAIL), and TNF-α. Moreover, SGN-40

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suppressed IL-6 receptor (IL-6R) expression at mRNA and protein levels, associated with inhibition of IL-6-mediated, but not insulin-like growth factor I (IGF-I)-mediated, MM cell growth and survival. These data provide the preclinical framework for the evaluation of SGN-40 as a novel therapy to improve patient outcome in MM.

MATERIALS AND METHODS

Cell Culture and Treatments. The $CD40^+$ and $CD138^{++}$ human MM-derived cell lines MM.1S and MM.1R were maintained as described previously (8). Freshly isolated tumor cells ($CD40^+$, $CD138^{++}$) from MM patients were obtained after informed consent and purified as described previously (8); 75% (Patient 1) and 85% (Patient 2) $CD138^+$ cells expressed CD40. For signaling experiments, MM.1S cells were washed with RPMI and cultured in serum-deprived RPMI and 0.2% BSA overnight. For treatment with sCD40L (Alexis Biochemicals, San Diego, CA), SGN-40, or isotype control immunoglobulin (Seattle Genetics, Seattle, WA), MM cells were incubated with RPMI containing these agents. To determine whether SGN-40 inhibits sCD40L-mediated activation or other pathways, cells were preincubated with SGN-40 or isotype control immunoglobulin overnight and then treated with sCD40L or SGN-40 for an additional 7 min. To determine whether down-regulation of CD40 by SGN-40 occurs by the proteasome pathway, cells were pretreated with proteasome inhibitor PS341 at a nontoxic dose (2 nM) for 2 h before treatment with sCD40L or SGN-40. To define the effect of a lysomotropic agent on SGN-40-mediated down-regulation of CD40, cells were pretreated with 10 mM ammonium chloride (Sigma, St. Louis, MO) for 2 h before treatment with sCD40L or SGN-40. To determine whether SGN-40 inhibits IL-6-induced downstream signaling and down-regulation of IL-6R, MM.1S cells were preincubated with SGN-40 overnight and then treated with IL-6 (50 ng/ml; Peprotech Inc., Rocky Hill, NJ).

Cytotoxicity Assay. MM cells (4×10^4 cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of SGN-40 for 6 h and then cocultured with or without cycloheximide (CHX; 0.2 μg/ml; Sigma) for an additional 36 h. Cells were pulsed with [^{3}H]thymidine (0.5 μCi/well; NEN Products, Boston, MA) during the last 8 h of 48-h cultures, harvested onto glass filters, and counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). To assay for inhibition of IL-6-induced cell proliferation by SGN-40, MM.1S and MM.1R cells were pretreated with 20 μg/ml of SGN-40 or control immunoglobulin overnight. They then were cultured with IL-6 (0, 50, or 200 ng/ml) or IGF-I (0, 20, or 200 ng/ml) for 36 h before pulsing with [^{3}H]thymidine. For IL-6-induced survival experiments, MM.1S cells pretreated with SGN-40 or control immunoglobulin were incubated with Dex (0–10 μM), in the presence or absence of IL-6 (50 ng/ml) or IGF-I (100 ng/ml). All of the experiments were performed in triplicate. Cell viability also was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (Chemicon International, Temecula, CA) assay, according to manufacturer's instructions.

Reagents. The humanized anti-CD40 mAb SGN-40 (IgG1) and control human monoclonal IgG1 were provided by Seattle Genetics. CHX (0.2 μg/ml) and phosphatidylinositol 3′-kinase inhibitor LY 294002 (5 μM) were obtained from Sigma. SB203580 (5 μM) was obtained from Calbiochem (San Diego, CA). Mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 inhibitors PD98059 (10 μM) and U1026 (1 μM) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). All of the antibodies for immunoblotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) except for anti-AKT and anti-pAKT antibody (Cell Signaling) and anti-α-tubulin mAb (Sigma).

Immunoblotting and Immunoprecipitation. MM.1S cells and patient MM cells were treated as described previously. Total cell lysates were subjected to 8–10% SDS-PAGE and transferred onto membranes; immunoblot analysis was performed as reported previously (8). For immunoprecipitation, 1 mg of lysates was incubated with anti-IL-6R antibody (4 μg) in lysis buffer for 2 h at 4°C.

Reverse Transcription-PCR. Total RNA samples were isolated using RNeasy kit (Qiagen, Valencia, CA). The first-strand cDNA was synthesized from 2 μg of total RNA priming by oligo(dT) using Superscript reverse-transcription kit (Invitrogen, Carlsbad, CA). Primers were for FasL, 5′-ggtccatgcctctggaatgg-3′ and 5′-cacatcgcccagtagtgca-3′; for TRAIL (Apo-2L), 5′-agacctgcgtgctgatcgt-3′ and 5′-gaccagttcaccattcctc-3′; for TNF-α, 5′-cttctgcctgctgcactttgga-3′ and 5′-tcccaaagtagacctgcccaga-3′; and for CD40L, 5′-cacctctctgccagaagataccatttcaa-3′ and 5′-ttatgaagactcccagcgtcacgtccacca-3′. IL-6R cDNA was amplified by reverse transcription-PCR using a primer pair (5′-cagctgagaacgaggtgtcc-3′ and 5′-gcagcttccacgtcttcttga-3′) flanking the transmembrane coding region of IL-6R. The primer pair for gp130 is 5′-atactggagtgactggagtg-3′ and 5′-catcttgtgagagtcacttc-3′. Templates initially were denatured at 95°C for 5 min, and then a cycle at 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min was repeated 25 times, followed by a final extension at 72°C for 5 min. The number of amplification cycles chosen for each reaction was determined to be within the linear range of the assay. PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GADPH) from the same reverse-transcribed cDNA template under the same conditions served as an internal control, with the primer pair of 5′-ccatggagaaggctgggg-3′ and 5′-caaagttgtcatggatgacc-3′. Real-time reverse transcription-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems). Primers for β-actin are 5′-acgtggacatccgcaaag-3′ and 5′-tgcatcctggtcggcaatg-3′.

RESULTS

Cytotoxic Effect of SGN-40 against CD40-Expressing MM Cells. Dex-sensitive MM.1S and Dex-resistant MM.1R cells and two patient MM cells were treated with increasing concentrations (0–100 μg/ml) of SGN-40 for 48 h. DNA synthesis was measured by [^{3}H]thymidine uptake. As shown in Fig. 1*A*, SGN-40 did not stimulate proliferation of MM.1S and MM1R cells and CD40-expressing tumor cells from two MM patients ($P > 0.1$). To further define the cytotoxic effect of SGN-40 against MM cells, MM.1S and MM.1R cells were

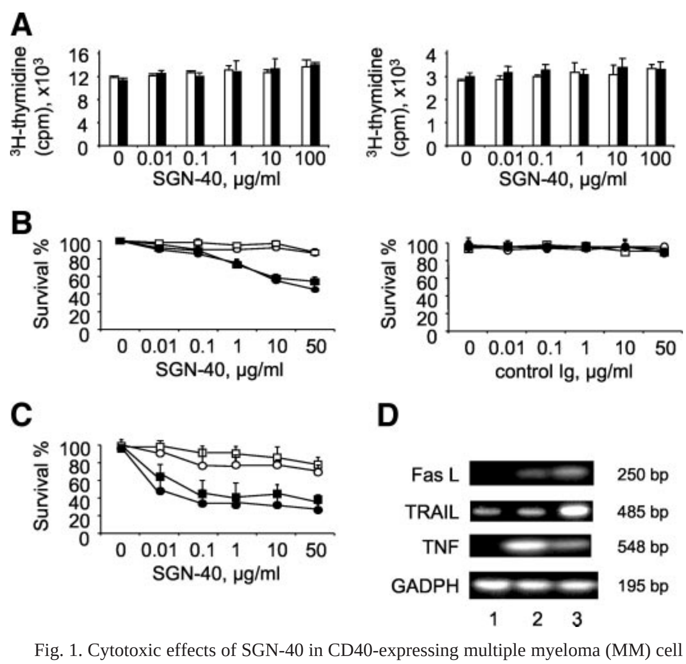

Fig. 1. Cytotoxic effects of SGN-40 in CD40-expressing multiple myeloma (MM) cell lines and patient MM cells. *A*, Dex-sensitive MM.1S and Dex-resistant MM.1R cell lines (*left*, □, MM.1S; ■, MM.1R) and two patient MM cells (*right*, □, MM#1; ■, MM#2) were incubated with SGN-40 at 0–100 μg/ml. After 36 h, cells were pulsed with [^{3}H]thymidine for 8 h (MM.1S and MM.1R cell lines) or overnight (patient MM cells), and DNA synthesis was measured. *B*, MM.1S (squares) and MM.1R (circles) were plated on 96-well plates in triplicate and treated with SGN-40 (0–50 μg/ml, *left*) or isotype control immunoglobulin (0–50 μg/ml, *right*) in the presence (solid symbols) or absence (open symbols) of protein synthesis inhibitor cycloheximide (CHX; 0.2 μg/ml). Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide assay. *C*, cell viability of two patient MM cells (circles, MM#1; squares, MM#2) treated with SGN-40 (0–50 μg/ml) in the presence (solid symbols) or absence (open symbols) of CHX (0.2 μg/ml). *D*, RNA was isolated from MM.1S cells treated with SGN-40 for 0 h, 3 h, or 24 h (*Lanes 1–3*, respectively) and subjected to reverse transcription-PCR analysis for the expression of FasL, tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*; Apo-2L), and tumor necrosis factor α (*TNF-α*). GADPH serves as an internal control.

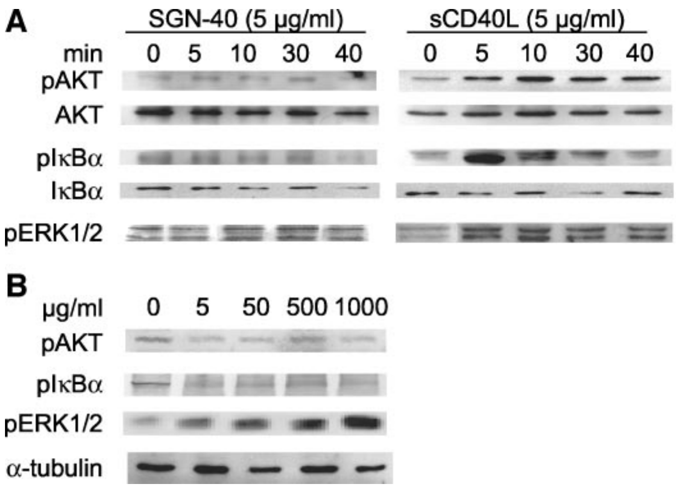

Fig. 2. SGN-40 activates neither AKT nor nuclear factor κB in MM.1S cells. *A*, serum-starved MM.1S cells were incubated with SGN-40 (5 μg/ml) or sCD40L (5 μg/ml) for the indicated time intervals. Samples were collected and evaluated by immunoblot analysis with antiphosphorylation-specific antibodies. Detection of total AKT on the same blot was used to determine equal loading of samples. *B*, serum-starved MM.1S cells were stimulated with SGN-40 at increasing concentrations (0–1000 μg/ml). Samples were collected and evaluated by immunoblot analysis with antiphosphorylation-specific antibodies. α-Tubulin was used as a loading control.

treated for 6 h with SGN-40 (10 μg/ml) and then cocultured with the *de novo* protein synthesis inhibitor CHX (0.2 μg/ml) for an additional 48 h. Cell viability was assayed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide. SGN-40 and CHX triggered 50–60% cell killing in both cell lines (Fig. 1*B, left*). Treatment with isotype control immunoglobulin alone or with CHX also did not induce cytotoxicity (Fig. 1*B, right*). SGN-40 triggered 20–30% cell cytotoxicity in two patient MM cells. In the presence of CHX at a nontoxic dose (0.2 μg/ml), SGN-40-induced cell cytotoxicity was enhanced significantly (Fig. 1*C*). These results suggested that endogenous production of cytotoxic cytokines might mediate SGN-40-induced cell death in CD40-expressing MM cells; therefore, we investigated whether SGN-40 treatment modulated expression of FasL and other cytotoxic members of the TNF superfamily, *i.e.,* TNF-α and TRAIL (Apo-2L). Expression of FasL, TNF-α, and TRAIL increased after SGN-40 treatment, although the kinetics of induction of each gene varied (Fig. 1*D*).

SGN-40 Does Not Alter sCD40L-Induced Phosphorylation of AKT, IκBα, and ERK in MM.1S Cells. We next examined the signaling pathways induced by SGN-40 in MM.1S cells. Serum-starved MM.1S cells were treated with either SGN-40 or sCD40L (5 μg/ml for 0–40 min). Cell lysates were subjected to immunoblot analysis to detect activation of AKT, inhibitor of nuclear factor-κBα (IκBα), and extracellular signal regulated kinase (ERK)-1/2 using phospho-specific antibodies. CD40 activation by sCD40L induces phosphorylation of AKT, IκBα, and ERK (Fig. 2*A, right*) as shown previously (8). In contrast, SGN-40 did not activate either AKT or IκBα phosphorylation. Phosphorylation of ERK was observed following SGN-40 stimulation, less potent than triggered by sCD40L. Little, if any, phosphorylation of AKT and IκBα was observed, even at increasing concentrations of SGN-40 (up to 1000 μg/ml; Fig. 2*B*).

We next studied whether combination treatments with these agents altered downstream signaling. MM.1S cells were treated with SGN-40 or sCD40L, alone or together, for 7 min. SGN-40 (5 μg/ml) did not induce phosphorylation of AKT and IκBα, whereas sCD40L either alone or together with SGN-40 (5 and 10 μg/ml) induced activation of these signaling proteins (Fig. 3). Phosphorylation of ERK-1/2 was similarly induced by SGN-40, either alone or with sCD40L. MM.1S cells then were pretreated with increasing concentrations (5–1000 μg/ml) of SGN-40 for 30 min before stimulation with sCD40L (5 μg/ml). As shown in Fig. 3*B*, SGN-40 at either 50 or 1000 μg/ml did not significantly inhibit sCD40L-induced phosphorylation of AKT.

Pretreatment of MM Cells with SGN-40 Inhibits sCD40L-Mediated Phosphorylation of AKT/IκBα and ERK Pathways. We next measured the levels of CD40 following treatment of MM.1S cells with SGN-40 *versus* sCD40L. Cells were treated with sCD40L (5 μg/ml), SGN-40 (20 μg/ml), or both for the indicated time intervals. CD40 levels were measured by immunoblot analysis using anti-CD40 antibody. No changes in CD40 levels were noted at 20 min to 24 h in untreated cells (labeled *Lane 1* for each time point; Fig. 4*A*). sCD40L treatment for 20 min to 24 h also did not alter levels of CD40 (Fig. 4*A*, *Lane 2* compared with *Lane 1*). In contrast, SGN-40 triggered down-regulation of CD40 after 4 h (Fig. 4*A*, *Lane 3*) and significant inhibition of CD40 expression by 24 h.

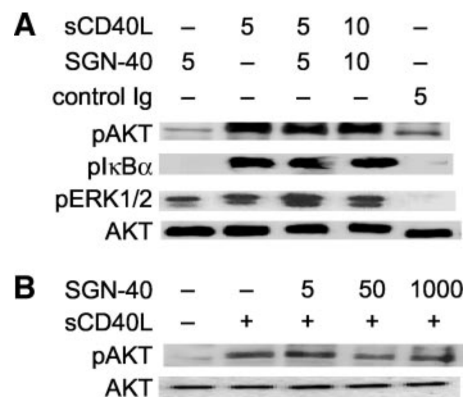

Fig. 3. Nuclear factor κB/AKT activation induced by sCD40L is not altered by SGN-40. *A*, serum-starved MM.1S cells were incubated with SGN-40 (5–10 μg/ml) for 7 min in the presence or absence of sCD40L (5–10 μg/ml). Isotype control immunoglobulin (5 μg/ml) was used as a control. Cell lysates were subjected to immunoblot analysis using anti-pAKT, pIκBα, and pERK1/2 antibodies, and with anti-AKT antibody as a loading control. *B*, serum-starved MM.1S cells were pretreated with various concentrations (5–1000 μg/ml) of SGN-40 for 30 min before stimulation with 5 μg/ml sCD40L (+) for 7 min. Cellular proteins then underwent immunoblot analysis with anti-pAKT and with anti-AKT as a loading control.

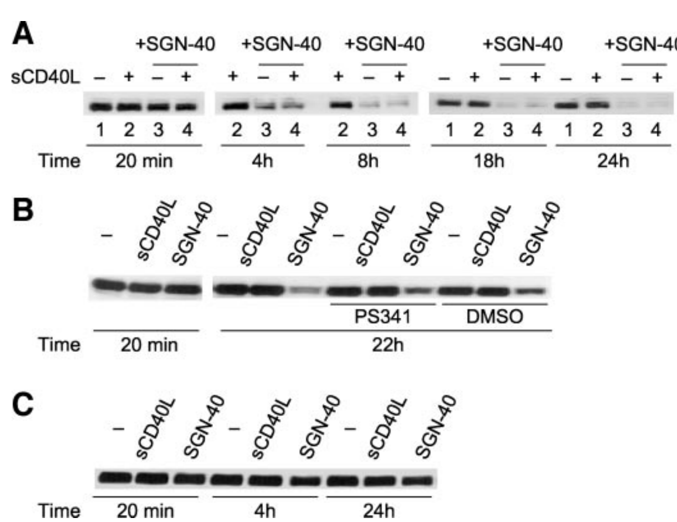

Fig. 4. SGN-40 causes down-regulation of CD40 via the endocytic pathway. *A*, MM.1S cells were left untreated (*lanes labeled 1*) or treated with 5 μg/ml of sCD40L (*lanes labeled 2*), 20 μg/ml of SGN-40 (*lanes labeled 3*), or sCD40L+SGN-40 (*lanes labeled 4*) for the indicated time intervals. *B*, cells were pretreated with 2 nM of PS341 in DMSO or DMSO alone for 2 h and then treated with sCD40L or SGN-40 for 20 min or 22 h. *C*, cells were pretreated with 10 mM ammonium chloride for 2 h before treatment with sCD40L or SGN-40 for indicated time intervals. Cell lysates then underwent immunoblot analysis for total levels of CD40 (M_r ~50,000) using anti-CD40 antibody.

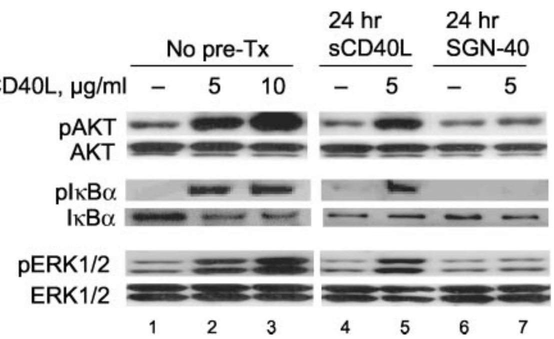

Fig. 5. Pretreatment with SGN-40 inhibits phosphorylation of AKT, inhibitor of nuclear factor-κBα (*IκBα*), and extracellular signal regulated kinase (*ERK*) induced by sCD40L. MM.1S cells were preincubated with either 5 μg/ml of sCD40L (*Lanes 4* and *5*) or 20 μg/ml of SGN-40 (*Lanes 6* and *7*) for 24 h. After this treatment, cells were either left untreated (–; *Lanes 4* and *6*) or stimulated with 5 μg/ml of sCD40L for 7 min (*Lanes 5* and *7*). *Lanes 1–3* show that sCD40L phosphorylates AKT, IκBα, and ERK-1/2. Immunoblot analysis also was performed with anti-AKT, anti-IκBα, and anti-ERK-1/2 antibodies. *Tx*, treatment.

Because many signaling molecules are regulated by ubiquitination followed by degradation by the proteasome, we next investigated whether the mechanism by which SGN-40 down-regulates CD40 is via the proteasome pathway. Cells were pretreated for 2 h with a nontoxic dose (2 nM) of proteasome inhibitor PS341, followed by sCD40L or SGN-40 for 22 h. As shown in Fig. 4*B*, PS341 did not block down-regulation of CD40 by SGN-40 compared with cells treated with only SGN-40 or DMSO vehicle control. This suggests that down-regulation of CD40 induced by SGN-40 does not occur via proteasomal degradation. To determine whether down-regulation of CD40 was mediated by an endocytic pathway, cells then were preincubated with ammonium chloride (10 mM for 2 h) and then treated with sCD40L or SGN-40 (26). Ammonium chloride increases pH of endosomes, thereby inhibiting the endocytic pathway. Pretreatment of cells with ammonium chloride blocked SGN-40-induced down-regulation of CD40 (Fig. 4*C*). This result suggests that down-regulation of CD40 triggered by SGN-40 occurs via a lysosomal and endocytic pathway.

We next examined whether MM.1S cells respond to sCD40L stimulation after antibody-induced down-regulation of CD40. Cells were pretreated with either sCD40L or SGN-40 for 24 h and then either untreated or treated with additional sCD40L (5 μg/ml). Phosphorylation of AKT, IκBα, and ERK was induced by sCD40L (Fig. 5, *Lanes 1–3*) as shown previously. In cells pretreated with sCD40L for 24 h and then treated with additional sCD40L for 7 min, AKT, IκBα, and ERK were phosphorylated (Fig. 5, *Lanes 4* and *5*). In contrast, in cells pretreated with SGN-40 followed by treatment with sCD40L for 7 min, phosphorylation of AKT, IκBα, and ERK was undetected (Fig. 5, *Lanes 6* and *7*). Cells pretreated with SGN-40 and re-treated with additional SGN-40 did not show enhanced AKT, IκBα, and ERK phosphorylation (data not shown). Thus, 24-h pretreatment of MM.1S cells with SGN-40 blocks sCD40L-induced phosphorylation of AKT and IκBα and activation of ERK. In contrast, cells treated with sCD40L for 24 h were stimulated by subsequent treatment with additional sCD40L. These data suggest that down-regulation of CD40 by SGN-40 blocked the ability of sCD40L to trigger phosphatidylinositol 3′-kinase/AKT, nuclear factor κB, and ERK signaling.

Pretreatment of MM Cells with SGN-40 Renders Them Refractory to IL-6-, but not IGF-I-, Mediated Proliferation and Antiapoptosis. Because IL-6 and IGF-I are key MM growth and survival factors, we next asked whether SGN-40-treated MM cells respond to these cytokines in a fashion similar to control immunoglobulin-treated cells. Dex-sensitive MM.1S and Dex-resistant MM.1R cells were pretreated with SGN-40 (20 μg/ml) or control immunoglobulin for 24 h. Cells were washed, and DNA synthesis was measured after 36-h culture with IL-6 or IGF-I (0–200 ng/ml). As shown in Fig. 6*A* (*left*), IL-6-induced cell proliferation is significantly blocked in MM.1S and MM.1R cells pretreated with SGN-40 ($P < 0.01$) compared with the 2.5–3-fold increase in cell proliferation induced by IL-6 in control immunoglobulin-treated cells (Fig. 6*A*, *left*). In contrast, IGF-I induces cell proliferation to a similar extent in SGN-40- and control immunoglobulin-treated MM.1S and MM.1R cells. We next tested whether pretreatment with SGN-40 alters IL-6-induced protection against Dex-induced apoptosis in MM.1S cells. Cells were pretreated with SGN-40 or control immunoglobulin for 24 h, washed, and then incubated with Dex (0–10 μM) for 36 h before assay for [^{3}H]thymidine incorporation. IL-6 (50 ng/ml) completely rescues Dex-induced cell death in control immunoglobulin-treated cells, whereas IL-6-induced protection against Dex was blocked significantly in SGN-40-pretreated cells ($P < 0.023$; Fig. 6*B*, *left*). In contrast and as shown in Fig. 6*B* (*right*), IGF-I-induced MM cell antiapoptosis was observed in SGN40-treated and control immunoglobulin-treated cells. These results indicate that SGN-40 pretreatment inhibits IL-6-, but not IGF-I-, mediated MM cell growth and survival.

We next examined downstream IL-6 signaling in SGN-40-treated *versus* control immunoglobulin-treated MM.1S cells. MM.1S cells were preincubated with SGN-40 (20 μg/ml) or isotype control immunoglobulin overnight, washed, and then stimulated with IL-6 for 0–10 min. Phosphorylation of downstream IL-6 signaling molecules (*i.e.*, signal transducers and activators of transcription 3, ERK, and AKT) was examined by immunoblot analysis. As seen in Fig. 7, activation of these IL-6 downstream signaling molecules was reduced in SGN-40-pretreated MM.1S cells, thereby inhibiting IL-6-induced growth and survival.

SGN-40 Suppresses IL-6R (gp80) Expression at mRNA and Protein Levels. To delineate the mechanism whereby SGN-40 treatment decreases IL-6 and IL-6R signaling, we first examined whether SGN-40 treatment alters IL-6R (gp80) expression. MM.1S and a patient's MM cells (MM#1) were treated with 20 μg/ml of SGN-40

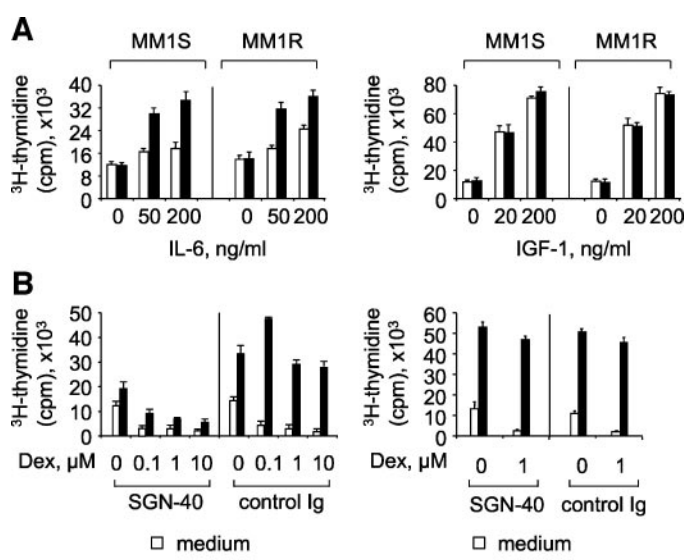

Fig. 6. Pretreatment with SGN-40 decreases interleukin 6 (*IL-6*)-induced growth and survival effects. *A*, MM.1S and MM1R cells were pretreated with 20 μg/ml of SGN-40 (□) or isotype control immunoglobulin (■) overnight. Cells then were treated with IL-6 (0–200 ng/ml; *left*) or insulin-like growth factor (*IGF*)-I (0–200 ng/ml; *right*) for 36 h and pulsed with [^{3}H]thymidine for 8 h, and DNA synthesis was measured. *B*, MM.1S cells were preincubated with SGN-40 or isotype control immunoglobulin as in (*A*) overnight. Cells then were treated with Dex (0–10 μM) in the presence (■) or absence (□) of IL-6 (50 ng/ml) for 48 h. [^{3}H]thymidine uptake then was measured. Cells also were treated with Dex (0, 1 μM) in the presence (■) or absence (□) of IGF-I (100 ng/ml; *right*).

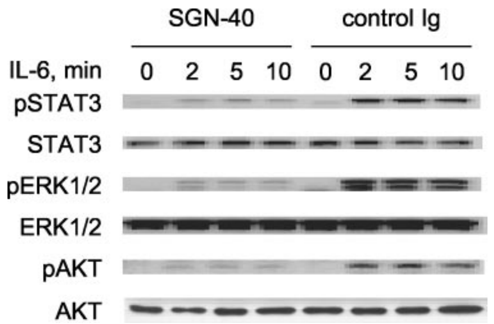

Fig. 7. Interleukin 6 (*IL-6*)-induced downstream signaling is inhibited by pretreatment with SGN-40. MM.1S cells were preincubated with 20 μg/ml of SGN-40 or isotype control immunoglobulin overnight and then stimulated with IL-6 (50 ng/ml) for 0–10 min. Cell lysates were subjected to immunoblot analysis using anti-pSTAT3, anti-pERK, and anti-pAKT, and anti-signal transducers and activators of transcription 3 (*STAT3*), extracellular signal regulated kinase (*ERK*)-1/2, and AKT antibodies as loading controls.

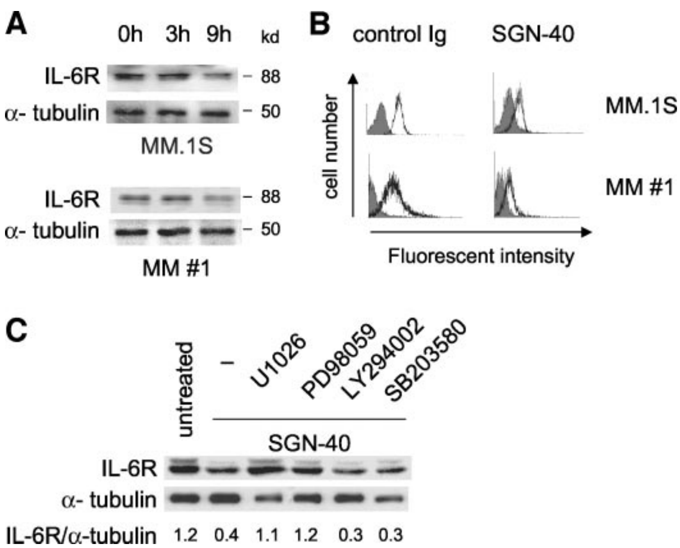

Fig. 8. Interleukin 6 receptor (*IL-6R*; gp80) expression in multiple myeloma (MM) cells treated with SGN-40. *A*, MM.1S cells (*top*) and patient MM cells (MM#1; *bottom*) were treated with 20 μg/ml of SGN40 for 0–9 h; expression of IL-6R then was evaluated by immunoblot analysis in MM.1S cells and by immunoprecipitation followed by immunoblot analysis in MM#1 cells. *B*, flow cytometric analysis (open histogram) of surface IL-6R expression is shown on MM.1S and patient MM cells (MM#1) treated with 20 μg/ml of either SGN40 or control immunoglobulin overnight. Shaded histogram is isotype control immunoglobulin for anti-IL-6R antibody. *C*, MM.1S cells were pretreated for 1.5 h with U1026 (1 μM), PD98059 (10 μM), LY294002 (5 μM), and SB203580 (5 μM) before overnight treatment with SGN-40. IL-6R was assayed by immunoblot analysis.

for 3 h and 9 h, and IL-6R levels then were measured using immunoprecipitation, followed by immunoblot analysis with anti-IL-6R antibody. As shown in Fig. 8*A*, a 2–4-fold suppression of IL-6R was observed in MM.1S and a patient's MM cells following 9 h of treatment with SGN-40. To confirm specific down-regulation of IL-6R expression, MM.1S and patient MM cells (MM#1) were incubated with 20 μg/ml of SGN-40 or control immunoglobulin overnight, followed by assessment for IL-6R by flow cytometry. Down-regulation in IL-6R expression was seen only in SGN40-treated, but not in control immunoglobulin-treated, MM.1S cells (Fig. 8*B*). In addition, SGN-40-induced down-regulation of IL-6R expression was prevented by mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2 inhibitor U0126 and PD98059 (Fig. 8*C*) but unaffected by inhibitors of either phosphatidylinositol 3′-kinase or p38 mitogen-activated protein kinase (Fig. 8*C*). These results suggest that mitogen-activated protein kinase/ERK signaling mediates SGN-40-induced inhibition of cell surface IL-6R expression.

To explore whether transcriptional mechanisms are involved in down-regulation of IL-6R induced by SGN-40, we next analyzed IL-6R mRNA in MM.1S cells following treatment with SGN-40 (20 μg/ml) or control immunoglobulin. IL-6R reverse transcription-PCR shows two specific products with a 94-bp size difference in all of the samples (Fig. 9*A*). The smaller species lacks 94 bp (exon 2), including the coding region of the transmembrane domain of the IL-6R (27). As shown in Fig. 9*A*, SGN-40 treatment induced time-dependent suppression of both species of IL-6R transcripts in MM.1S cells (*Lanes 4–6*) and two patients' MM cells (*Lanes 7–9* and *Lanes 10–12*), whereas no difference in IL-6R transcripts was found in control immunoglobulin-treated MM.1S cells (Fig. 9, *Lanes 1–3*). The gp130 and GADPH mRNAs were unchanged by treatment with either SGN-40 or control immunoglobulin. IL-6 mRNA suppression following SGN-40 treatment also was confirmed by real-time reverse transcription-PCR (Fig. 9*B*).

DISCUSSION

We report here that humanized anti-CD40 mAb SGN-40 treatment of $CD40^+$ and $CD138^+$ MM cell lines and patient MM cells significantly induces growth arrest and apoptosis in the presence of the *de novo* protein synthesis inhibitor CHX. SGN-40-induced cytotoxicity is associated with up-regulation in cytotoxic TNF ligands (*i.e.*, FasL, TRAIL, and TNF-α). In addition, SGN-40 suppresses expression of IL-6R protein and mRNA associated with inhibition of growth and survival of MM cells induced by IL-6 but not by IGF-I. These data support a new treatment strategy using SGN-40 to improve patient outcome in human MM.

We show that SGN-40 induces apoptosis of $CD40^+$ and $CD138^+$ MM cell lines and patient MM cells in the presence of CHX, in addition to its previously reported tumoricidal activity via antibody-dependent cell-mediated cytotoxicity (24). SGN-40-mediated MM cell death is associated with up-regulation of Fas/FasL because the expression of FasL was induced following SGN-40 treatment. Fas-induced apoptosis has been reported in MM.1S cells (28). We also found that SGN-40-induced cell death occurs through a caspase-8-dependent pathway (data not shown), consistent with Fas engaging a proapoptotic cascade leading to caspase activation (28–30). Furthermore, SGN-40-induced apoptosis in MM.1S and MM.1R lines is

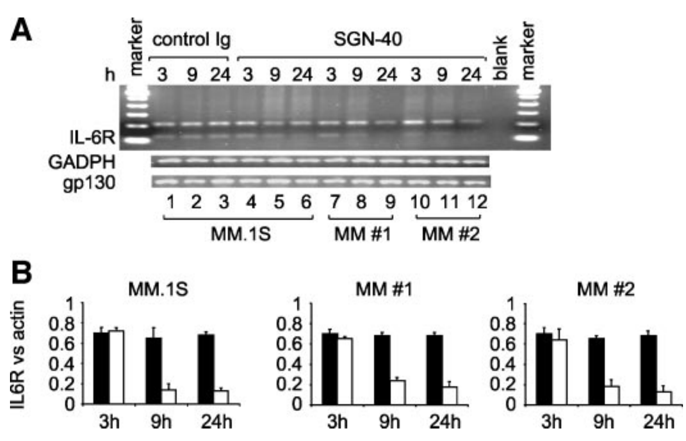

Fig. 9. Interleukin 6 receptor (*IL-6R*) mRNA is down-regulated following treatment with SGN-40. *A*, MM.1S cells (*Lanes 1–6*) and two patients' MM cells (MM#1, *Lanes 7–9*; MM#2, *Lanes 10–12*) were treated with SGN-40 or isotype control immunoglobulin for the indicated time intervals. Total RNA was prepared for reverse transcription-PCR to assay IL-6R (gp80) and gp130 transcripts. Reverse transcription-PCR for GADPH served as an internal control. The derived reverse transcription-PCR products of IL-6R include a 219-bp fragment of the transmembrane encoding region of IL-6R and a 125-bp fragment of an alternative spliced variant (94-bp deletion in exon 2). *B*, real-time reverse transcription-PCR analysis of IL-6R from control immunoglobulin (■) and SGN-40 (□)-treated cells. Bar graphs represent mRNA expression levels for IL-6R related to actin.

partially inhibited by neutralizing anti-FasL antibody NOK1 (30% ± 5%; data not shown). However, the inability of neutralizing anti-FasL antibodies to abolish completely SGN-40-mediated cytotoxicity suggests additional death signals. Exposure of MM.1S cells to SGN-40 also induced transcriptional activation of other cytotoxic members of the TNF family, such as TRAIL (Apo-2L) and TNF-α, although with different kinetics. TRAIL induces MM apoptosis through the rapid activation of caspase-8, caspase-9, and caspase-3 (31). Moreover, an individual neutralizing reagent only partially protects against SGN-40-induced cell death, confirming that SGN-40-mediated MM cell apoptosis occurs via targeting more than one cytotoxic ligand of the TNF family. Previous studies in human monocytes and normal plasma cells demonstrate the presence of high intracellular levels of FasL or TRAIL, which rapidly translocates to the cell surface in response to various stimuli (32–34). Whether SGN-40 treatment regulates the redistribution of intracellular cytoplasmic pools of these cytotoxic ligands, in addition to their *de novo* transcription and translation, remains to be elucidated.

SGN-40-induced cytotoxicity in MM cells is enhanced by inhibition of protein synthesis. In particular, CHX blocks the production of protective antiapoptotic proteins (*i.e.,* FLIP), thereby unmasking the cytotoxic potential of SGN-40. CHX-induced reduction of the short-lived antiapoptotic FLIP protein is associated with enhanced SGN-40-induced apoptosis in MM.1S cells (data not shown). Although the molecular basis for SGN-40-dependent sensitization to apoptosis in MM cells is not understood completely, this requirement of protein synthesis inhibition for efficient killing reveals a mechanism whereby MM cells, via activation of antiapoptotic cascades including AKT, Bcl-2, and Mcl-1, may escape CD40-mediated cytotoxicity. Although the ability of these pathways to block CD40-mediated cell death remains to be verified, previous studies have implicated Bcl-2, Bcl-xL, and AKT in suppression of TRAIL- and Fas-induced apoptosis in MM (35, 36).

In vitro studies have demonstrated internalization of CD40 after ligation by mAb (37, 38). Our data indicate that SGN-40 caused down-regulation of CD40 via the endosomal endocytic, but not the proteasome-ubiquitin, pathway. It is possible that SGN-40 causes increased endocytosis without allowing for receptor recycling to the membrane, resulting in a net decrease in cell surface levels over time. An increase in receptor turnover leading to down-modulation of HER-2/c-*erb*B-2 or IGF-I receptor has been reported with specific mAbs against these receptors (39, 40). In addition, rituximab therapy leads to down-regulation of CD20 expression at protein and mRNA in cells from treated patients (41). This down-modulation of targeted receptors may have important implications for scheduling and dosing, not only of anti-CD20 but also of potential novel anti-CD40 therapies.

We found down-regulation of IL-6R by SGN-40, correlating with decreased IL-6 downstream signaling and function. Because IL-6 is the major growth and survival factor for human MM, decreased IL-6R induced by SGN-40 treatment would inhibit MM cell growth and survival in the bone marrow milieu. These results are consistent with recent studies demonstrating that IFN-α (42) or IFN-γ (43, 44) significantly inhibits membrane IL-6 binding IL-6R (gp80) protein and mRNA in human plasma cell lines. Since IFN-γ up-regulates CD40 expression, it may also result in additional inhibition of IL-6R expression induced by SGN-40. In contrast to its effects on IL-6 signaling, SGN-40 did not alter the proliferative and antiapoptotic effects induced by IGF-I. The mechanism by which SGN-40 treatment induces differential responses of MM cells to IL-6 and IGF-I may, at least in part, be explained by differential changes of their cognate receptors because we could not detect any changes of IGF-I receptor at either protein and mRNA levels induced by SGN-40 treatment (data not shown).

In summary, the humanized anti-CD40 mAb SGN-40 has direct anti-MM effects that are independent of antibody-dependent cell-mediated cytotoxicity as we reported previously (24). SGN-40 mediates apoptosis in human MM cells in the presence of CHX, and SGN-40 up-regulates cytotoxic ligands belonging to the TNF family. In addition to regulating CD40-mediated cytotoxicity, these ligands and/or their receptors also are important in apoptosis induced by a broad spectrum of stimuli, including chemotherapy and radiation. Importantly, inhibition of IL-6R expression by SGN-40 renders MM cells refractory to IL-6-induced proliferative and protective effects, thereby inhibiting MM cell growth and survival in the bone marrow microenvironment. These results provide the framework for the clinical evaluation of SGN-40 to improve patient outcome in MM.

REFERENCES

1. Pellat-Deceunynck C, Bataille R, Robillard N, et al. Expression of CD28 and CD40 in human myeloma cells: a comparative study with normal plasma cells. Blood 1994;84:2597–603.
2. Hatada E, Niesvizky R, Meeus S, Chen-Kiang S. Two NF-κB activation pathways mediate CD40 signals to promote primary myeloma cell survival. Hematol J 2003; 4:S144.
3. Tai YT, Podar K, Gupta D, et al. CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. Blood 2002; 99:1419–27.
4. Urashima M, Chauhan D, Uchiyama H, Freeman GJ, Anderson KC. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. Blood 1995;85:1903–12.
5. Teoh G, Urashima M, Greenfield EA, et al. The 86-kD subunit of Ku autoantigen mediates homotypic and heterotypic adhesion of multiple myeloma cells. J Clin Investig 1998;101:1379–88.
6. Tai YT, Podar K, Kraeft SK, et al. Translocation of Ku86/Ku70 to the multiple myeloma cell membrane: functional implications. Exp Hematol 2002;30:212–20.
7. Westendorf JJ, Ahmann GJ, Lust JA, et al. Molecular and biological role of CD40 in multiple myeloma. Curr Top Microbiol Immunol 1995;194:63–72.
8. Tai YT, Podar K, Mitsiades N, Lin B, et al. CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-κB signaling. Blood 2003; 101:2762–9.
9. Teoh G, Tai YT, Urashima M, et al. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. Blood 2000;95:1039–46.
10. Tong AW, Seamour B, Chen J, et al. CD40 ligand-induced apoptosis is Fas-dependent in human multiple myeloma cells. Leuk Lymphoma 2000;36:543–58.
11. Bergamo A, Bataille R, Pellat-Deceunynck C. CD40 and CD95 induce programmed cell death in the human myeloma cell line XG2. Br J Haematol 1997;97:652–5.
12. Pellat-Deceunynck C, Amiot M, Robillard N, Wijdenes J, Bataille R. CD11a-CD18 and CD102 interactions mediate human myeloma cell growth arrest induced by CD40 stimulation. Cancer Res 1996;56:1909–16.
13. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 2001;1:118–29.
14. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235–42.
15. Sarris AH, Jiang Y, Tsimberidou AM, et al. Quantitative real-time polymerase chain reaction for monitoring minimal residual disease in patients with advanced indolent lymphomas treated with rituximab, fludarabine, mitoxantrone, and dexamethasone. Semin Oncol 2002;29:48–55.
16. Treon SP, Pilarski LM, Belch AR, et al. CD20-directed serotherapy in patients with multiple myeloma: biologic considerations and therapeutic applications. J Immunother 2002;25:72–81.
17. Uckun FM, Gajl-Peczalska K, Myers DE, Jaszcz W, Haissig S, Ledbetter JA. Temporal association of CD40 antigen expression with discrete stages of human B-cell ontogeny and the efficacy of anti-CD40 immunotoxins against clonogenic B-lineage acute lymphoblastic leukemia as well as B-lineage non-Hodgkin's lymphoma cells. Blood 1990;76:2449–56.
18. Murphy WJ, Funakoshi S, Beckwith M, et al. Antibodies to CD40 prevent Epstein-Barr virus-mediated human B-cell lymphomagenesis in severe combined immune deficient mice given human peripheral blood lymphocytes. Blood 1995;86:1946–53.
19. Francisco JA, Schreiber GJ, Comereski CR, et al. In vivo efficacy and toxicity of a single-chain immunotoxin targeted to CD40. Blood 1997;89:4493–500.
20. Funakoshi S, Longo DL, Murphy WJ. Differential in vitro and in vivo antitumor effects mediated by anti-CD40 and anti-CD20 monoclonal antibodies against human B-cell lymphomas. J Immunother Emphasis Tumor Immunol 1996;19:93–101.
21. van Mierlo GJ, den Boer AT, Medema JP, et al. CD40 stimulation leads to effective therapy of CD40(−) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. Proc Natl Acad Sci USA 2002;99:5561–6.
22. Honeychurch J, Glennie MJ, Johnson PW, Illidge TM. Anti-CD40 monoclonal antibody therapy in combination with irradiation results in a CD8 T-cell-dependent immunity to B-cell lymphoma. Blood 2003;102:1449–57.
23. Francisco JA, Donaldson KL, Chace D, Siegall CB, Wahl AF. Agonistic properties and in vivo antitumor activity of the anti-CD40 antibody SGN-14. Cancer Res 2000;60:3225–31.

24. Hayashi T, Treon SP, Hideshima T, et al. Recombinant humanized anti-CD40 monoclonal antibody triggers autologous antibody-dependent cell-mediated cytotoxicity against multiple myeloma cells. Br J Haematol 2003;121:592–6.
25. Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood 1998;91:1644–52.
26. Mellman I, Fuchs R, Helenius A. Acidification of the endocytic and exocytic pathways. Annu Rev Biochem 1986;55:663–700.
27. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. FASEB J 2001; 15:43–58.
28. Mitsiades N, Mitsiades CS, Poulaki V, et al. Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood 2002;99:4525–30.
29. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17:1675–87.
30. Chu P, Deforce D, Pedersen IM, et al. Latent sensitivity to Fas-mediated apoptosis after CD40 ligation may explain activity of CD154 gene therapy in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99:3854–9.
31. Mitsiades CS, Treon SP, Mitsiades N, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 2001;98:795–804.
32. Kiener PA, Davis PM, Rankin BM, et al. Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation. J Immunol 1997;159:1594–8.
33. Ursini-Siegel J, Zhang W, Altmeyer A, et al. TRAIL/Apo-2 ligand induces primary plasma cell apoptosis. J Immunol 2002;169:5505–13.
34. Martinez-Lorenzo MJ, Alava MA, Gamen S, et al. Involvement of APO2 ligand/ TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. Eur J Immunol 1998;28:2714–25.
35. Mitsiades N, Mitsiades CS, Poulaki V, et al. Molecular sequelae of proteasome inhibition in human multiple myeloma cells. Proc Natl Acad Sci USA 2002;99:14374–9.
36. Derenne S, Monia B, Dean NM, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood 2002;100:194–9.
37. Press OW, Farr AG, Borroz KI, Anderson SK, Martin PJ. Endocytosis and degradation of monoclonal antibodies targeting human B-cell malignancies. Cancer Res 1989;49:4906–12.
38. Sieber T, Schoeler D, Ringel F, Pascu M, Schriever F. Selective internalization of monoclonal antibodies by B-cell chronic lymphocytic leukaemia cells. Br J Haematol 2003;121:458–61.
39. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 1985;41:697–706.
40. Li SL, Kato J, Paz IB, Kasuya J, Fujita-Yamaguchi Y. Two new monoclonal antibodies against the a subunit of the human insulin-like growth factor-I receptor. Biochem Biophys Res Commun 1993;196:92–8.
41. Jilani I, O'Brien S, Manshuri T, et al. Transient down-modulation of CD20 by rituximab in patients with chronic lymphocytic leukemia. Blood 2003;102: 3514–20.
42. Jelinek DF, Aagaard-Tillery KM, Arendt BK, Arora T, Tschumper RC, Westendorf JJ. Differential human multiple myeloma cell line responsiveness to interferon-α. Analysis of transcription factor activation and interleukin 6 receptor expression. J Clin Invest 1997;99:447–56.
43. Francisco JA, Kiener PA, Moran-Davis P, Ledbetter JA, Siegall CB. Cytokine activation sensitizes human monocytic and endothelial cells to the cytotoxic effects of an anti-CD40 immunotoxin. J Immunol 1996;157:1652–8.
44. Portier M, Zhang XG, Caron E, Lu ZY, Bataille R, Klein B. γ-Interferon in multiple myeloma: inhibition of interleukin-6 (IL-6)-dependent myeloma cell growth and downregulation of IL-6-receptor expression in vitro. Blood 1993;81:3076–82.