

Agonistic Properties and *in Vivo* Antitumor Activity of the Anti-CD40 Antibody SGN-14

Joseph A. Francisco, Karen L. Donaldson,¹ Dana Chace, Clay B. Siegall, and Alan F. Wahl²

Departments of Biochemistry and Molecular Biology, Seattle Genetics, Inc., Bothell, Washington 98021

ABSTRACT

Ligation of CD40 is essential for primary B-cell activation and expansion and yet has suppressive or apoptotic effects on some CD40-expressing neoplasia. SGN-14 is a monoclonal antibody that binds to the human CD40 receptor. Here we report that SGN-14, in the presence of interleukin 4, provided a modest level of stimulation of peripheral blood B cells, as measured by proliferation. Stimulation was greatly enhanced in the presence of nonproliferating CD40 ligand-expressing cells. The enhanced agonistic activity could be attributed to a dose-dependent increase in CD40L binding to CD40 in the presence of SGN-14. In contrast to its proliferative effect on primary B cells, SGN-14 inhibited the growth of B-cell-derived tumor lines *in vitro*, and this growth inhibition was enhanced in the presence of CD40L-expressing cells. *In vivo*, SGN-14 showed significant antitumor activity in treating human B-cell lymphoma and multiple myeloma xenografted severe combined immunodeficient mice. Antitumor activity was not diminished by blunting murine natural killer activity, suggesting that CD40 ligation contributes to the antitumor efficacy of SGN-14. On the basis of these activities, SGN-14 is being pursued for therapeutic use in treating patients with CD40-expressing hematological malignancies.

INTRODUCTION

Since the discovery of mAb³ technology (1), attempts have been made to use their exquisite specificity and affinity for the treatment of human diseases such as cancer. In recent years, significant advances have been made in the therapeutic use of mAbs, as demonstrated by the growing number that are gaining approval for clinical use. Hematological malignancies appear to be particularly well suited for mAb-based therapies, and numerous clinical studies have now demonstrated the efficacy of mAb therapies in hematological diseases. For example, clinical trials using anti-idiotypic mAb for the treatment of non-Hodgkin's lymphomas have resulted in a significant percentage of partial and complete remissions (2, 3). Radiolabeled anti-CD20 mAbs have shown promising results in a number of clinical trials (4, 5). More recently, the unlabeled chimeric mAb to CD20, Rituxan[®], has demonstrated clinical efficacy sufficient to become the first mAb approved by the United States Food and Drug Administration for the treatment of cancer (6, 7).

CD40 is a target of significant potential for the treatment of B-lineage hematological malignancies as well as certain carcinomas. This M_r 45,000–50,000 integral membrane glycoprotein was originally found on bladder carcinoma cells and later detected on both normal and malignant B cells (8). Subsequently, CD40 has been identified on a variety of other cell types including dendritic cells, monocytes, thymic epithelial cells, endothelial cells (9, 10), and on

carcinomas of the lung, colon, and breast (11). CD40 is a member of the TNF receptor superfamily and plays an important role in the growth, differentiation, and isotype switching of normal B cells (9). Importantly, ligation of CD40 via mAbs can result in inhibition and cell death on neoplastic B cells (12).

As a target for mAb-based therapy, CD40 complements CD20 and may have several advantages over CD20. Like CD20, CD40 is highly expressed in B-cell malignancies including leukemias, lymphomas, and Hodgkin's disease (13, 14). Importantly, CD40 is found on a higher percentage of multiple myelomas compared with CD20 (15). Unlike CD20, CD40 is also highly expressed on the surface of a variety of carcinomas, increasing the potential therapeutic application of mAbs targeted to CD40. In a recent study comparing the effects of an anti-CD20 and a ligand blocking anti-CD40 mAb in human B-cell lymphoma xenografted SCID mice, Funakoshi *et al.* (16) demonstrated that although both mAbs possessed potent antitumor activity in the presence of Fc receptor-bearing effector cells, the mAb targeted to CD40 was more efficacious when these effector cells were depleted.

In this study, we have examined the *in vitro* and *in vivo* antitumor activity of mAb SGN-14, an agonistic antihuman CD40 mAb. SGN-14, originally called S2C6, was the first mAb used to identify CD40 on bladder carcinoma cells (17). We show that SGN-14 enhances the interactions between CD40 and its ligand, CD40L. *In vitro*, this enhancement resulted in stimulation of primary B cells and modest antitumor activity in the presence of SGN-14 and CD40L. *In vivo*, through the use of human lymphoma xenografted SCID mice, we demonstrate that this antibody has significant antitumor activity even when the activity of natural killer cells has been inhibited. Additionally, SGN-14 was effective in treating SCID mice xenografted with human multiple myeloma.

MATERIALS AND METHODS

Reagents and Cell Culture. Ramos and HS-Sultan Burkitt's lymphoma and IM-9 multiple myeloma cell lines were purchased from American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μ g/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD). The BMS-2 cell line is a Jurkat derivative selected to express constitutive high levels of CD40L (18). Human peripheral blood B cells were isolated from a single donor by positive selection using immobilized antibodies against both CD19 and CD20. The final isolated cell population contained >85% B cells, as determined by flow cytometry.

Recombinant human IL-4 was purchased from Biosource (Camarillo, CA); FITC-labeled recombinant human CD40 ligand (CD40L), produced as a fusion protein with murine CD8 and was obtained from Research Diagnostics, Inc. (Flanders, NJ); soluble CD40-immunoglobulin, consisting of the extracellular domain of human CD40 fused to a human immunoglobulin tail, and G28-5 mAb were obtained from Bristol-Myers Squibb (Princeton, NJ); M3 mAb was obtained from Genzyme (Cambridge, MA); FITC-labeled F(ab')₂ goat antihuman immunoglobulin was purchased from Jackson ImmunoResearch (West Grove, PA); and anti-asialo G_{M1} was purchased from Wako Chemicals (Richmond, VA).

Antibody Preparation. The SGN-14 hybridoma, originally called S2C6 (17), was cultured at 37°C in complete HSFM media (Life Technologies, Inc.) supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin. When the cells reached a viability of \leq 50%, the culture was harvested by centrifugation.

Received 11/23/99; accepted 4/10/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Present address: Department of Immunology, Bristol-Myers Squibb Pharmaceutical Research Institute, P. O. Box 4000, Princeton, NJ 08543.

² To whom requests for reprints should be addressed, at Department of Biochemistry, Seattle Genetics, Inc., 22215 26th Avenue SE, Bothell, WA 98021. Fax: (425) 489-8888; E-mail: awahl@seagen.com.

³ The abbreviations used are: mAb, monoclonal antibody; TNF, tumor necrosis factor; SCID, severe combined immunodeficient; IL, interleukin; FBS, fetal bovine serum; NK, natural killer; TRAF, TNF receptor-associated factor; FACS, fluorescence-activated cell sorting.

gation, and the supernatant was collected by filtration through a 0.2 μm filter. Subsequently, the supernatant was diluted 1:1 with 3 M NaCl, 0.1 M NaBO_3 (pH 8.5) and loaded onto a Pierce GammaBind Sepharose column (Pierce, Rockford, IL), washed with PBS, and eluted with 0.1 M citrate (pH 3.0). Immediately upon elution, the mAb was neutralized with 1 M Tris-HCl (pH 8.0) and subsequently dialyzed into PBS and filter sterilized. SGN-14 preparations were verified as >99% monomeric by size exclusion chromatography and free of detectable endotoxin by LAL assay (BioWhittaker, Walkerville, MD) for use in these studies.

B-Cell Proliferation Assay. Human peripheral blood B cells were thawed and incubated in 96-well tissue culture plates at 1×10^5 per well in IMDM media plus 10% FBS in the presence of 5 ng/ml recombinant human IL-4 and various dilutions of the anti-CD40 mAbs SGN-14, G28-5, and M3. As a control, cells were incubated with IL-4 and a control mAb (anti-*Pseudomonas* exotoxin) EXA2-1H8. The plates were incubated at 37°C for 3 days and then pulsed for 16 h with 0.5 μCi [^3H]thymidine/well. Cells were harvested onto 96-well glass fiber filters using a Filtermate 196 Harvester (Packard Instruments, Meriden, CT), combined with scintillation fluid, and the extent of [^3H]thymidine incorporated into nascent DNA was measured by liquid scintillation counting in a Topcount LSC (Packard Instruments, Meriden, CT).

To evaluate B-cell proliferation in the presence of CD40L, BMS-2 cells were used as CD40L stimulator cells. B cells and IL-4 were initially combined with BMS-2 cells (2.5×10^4 per well), followed immediately by the addition of the anti-CD40 mAbs. To eliminate proliferation of the stimulator cells, BMS-2 cells were treated with mitomycin C, 50 $\mu\text{g}/\text{ml}$ in PBS for 20 min at 37°C, followed by three washes in PBS, prior to combining with B cells. mAbs were titrated with either a fixed concentration of stimulator cells, or stimulator cells were titrated with a fixed concentration of mAb.

CD40/CD40L Binding Assay. To evaluate the effect of anti-CD40 mAbs on CD40 binding to CD40L expressed on T cells, BMS-2 cells were used as target cells. BMS-2 cells were adjusted to a density of $2 \times 10^7/\text{ml}$ in 50- μl samples, and binding was performed in RPMI 1640 + 10% FBS. CD40-immunoglobulin (25 $\mu\text{g}/\text{ml}$; saturating level) was preincubated for 1 h on ice with increasing levels of either mAb SGN-14, G28-5, M3, or an irrelevant mAb to *Pseudomonas* exotoxin EXA2-1H8, as an isotype control, prior to adding to the BMS-2 cells for an additional 1 h on ice. CD40-immunoglobulin binding to BMS-2 cells was then detected by the addition of FITC-labeled antihuman immunoglobulin, and the resultant binding was evaluated using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

To evaluate the effect of anti-CD40 mAbs on CD40L binding to human B cells, Ramos cells were used as a target for the binding of FITC-labeled CD40L. Ramos cells (5×10^5) were preincubated for 1 h on ice with increasing levels of either mAb SGN-14, G28-5, M3, or control mAb EXA2-

1H8, prior to adding to the FITC-CD40L for an additional 30 min on ice. Cells were washed three times with complete media, and the resultant binding was evaluated by flow cytometry.

In Vitro Growth Inhibition. HS Sultan or Ramos cell lines were cultured in RPMI 1640 media containing 10% FBS at a density of 1×10^4 cells/well of 100 μl in 96-well flat-bottomed plates. Increasing concentrations of SGN-14 or control mAb were added, and cell viability was determined at 96 h by a tetrazolium viable dye assay, as described previously (19). To evaluate the effect in combination with CD40L, this assay was also carried out in the presence of nonproliferating CD40L-expressing BMS-2 cells at varying ratios and increasing concentrations of mAb. To block their proliferation, CD40L stimulator cells were pretreated with mitomycin C as described above.

In Vivo SCID Mouse Studies. Female CB-17 SCID mice (Taconic, Germantown, NY) were 6–8 weeks of age at the initiation of all studies. Mice were injected through the tail vein with 1×10^6 cells on day 0, and therapy with SGN-14 or control mAb was initiated on the indicated days. mAbs were administered s.c. with injections given every 4 days for a total of five injections. In some studies, mice were treated i.v. with anti-asialo G_{M1} , 200 μl of a 1:10 dilution, as described previously (12).

RESULTS

SGN-14 Promotes Soluble CD40 Interaction with CD40L-expressing T Cells. To differentiate the effects of mAb SGN-14 from those of mAb G28-5, a partial agonist and partial antagonist of CD40 (20) and mAb M3, which blocks CD40/CD40L interaction (21), we evaluated how the interaction between CD40 and CD40L was affected by the presence of each antibody. In a first approach, the effect of SGN-14 and other anti-CD40 mAbs on soluble CD40 binding to CD40L expressed on BMS-2 cells was evaluated. In the absence of mAb, titration with CD40-immunoglobulin showed receptor saturation at 25 $\mu\text{g}/\text{ml}$, which was used for these studies. Increasing concentrations of each anti-CD40 mAb were preincubated with 25 $\mu\text{g}/\text{ml}$ soluble CD40-immunoglobulin, followed by incubation of the complexes with CD40L-expressing BMS-2 cells. To prevent modulation, all incubations were performed on ice. The binding of soluble CD40 to CD40L on the target cells was then determined by flow cytometry using FITC-labeled goat antihuman immunoglobulin to detect CD40-immunoglobulin. SGN-14 complexed with CD40-immunoglobulin resulted in a dose-dependent increase, by 4-fold at 50 $\mu\text{g}/\text{ml}$ mAb, in CD40-immunoglobulin binding to CD40L (Fig. 1A). This is in con-

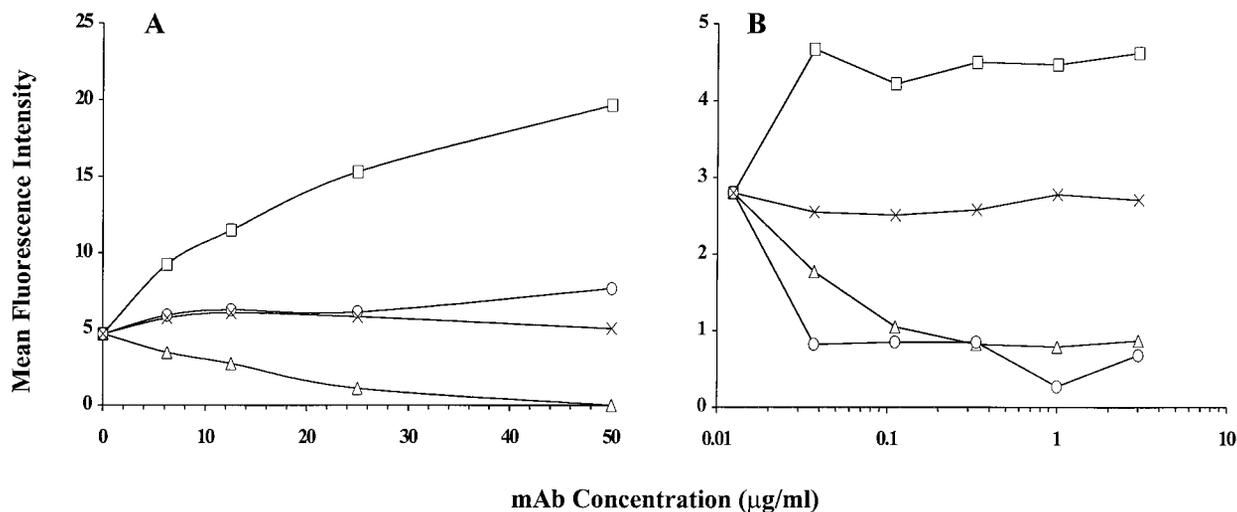


Fig. 1. SGN-14 enhances the interaction between CD40 and CD40L. A, CD40-immunoglobulin binding to CD40L-expressing BMS-2 cells. mAbs were preincubated for 1 h with CD40-immunoglobulin, followed by 1 h incubation with BMS-2 cells. CD40-immunoglobulin binding to target cells was then detected by FACS using FITC-labeled goat antihuman immunoglobulin. B, soluble CD40L binding to CD40 expressed on Ramos cells. Ramos cells were preincubated on ice for 1 h with anti-CD40 mAbs, followed by the addition of FITC-labeled CD40L. FITC-CD40L binding to target cells was then detected by FACS. mAbs used in these assays were SGN-14 (□), G28-5 (○), M3 (△), or a nonbinding control mAb EXA2-1H8 (X).

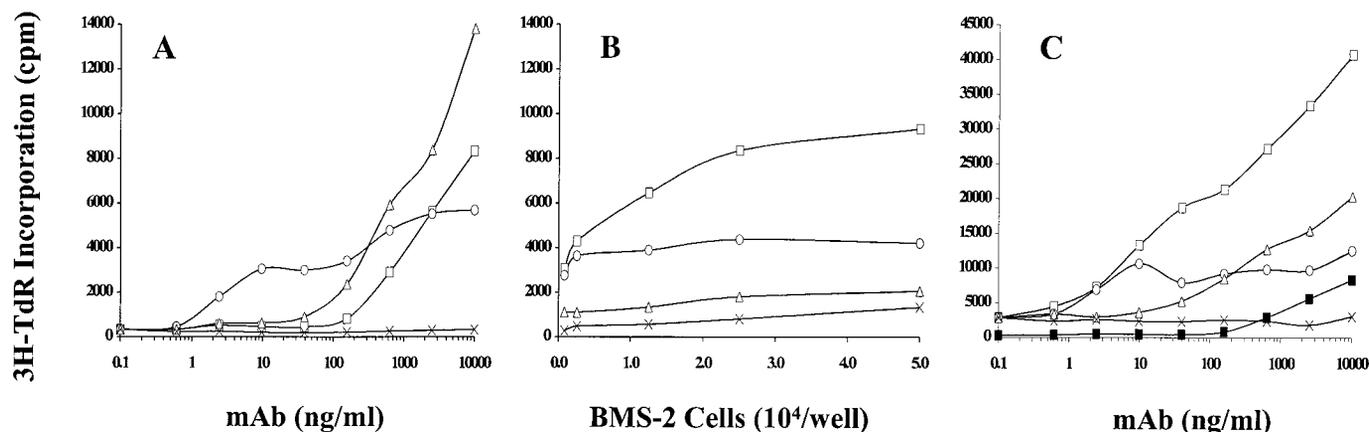


Fig. 2. Activation of human peripheral blood B cells by anti-CD40 mAbs. B cells, 1×10^5 /well in 96-well tissue culture plates, were combined with 5 ng/ml recombinant IL-4 and either increasing concentrations of mAbs alone (A), a fixed concentration (30 ng/ml) of mAbs and increasing concentrations of mitomycin C-treated BMS-2 cells (B), or a fixed concentration of BMS-2 cells (0.25×10^5) and increasing concentrations of mAbs (C). Cells were incubated for 72 h, followed by a 16-h pulse with [3 H]thymidine. The antibodies used in these assays were the anti-CD40 mAbs SGN-14 (\square), G28-5 (\circ), or M3 (\triangle), or a nonbinding control mAb EXA2-1H8 (X). In Fig. 2C, SGN-14 mixed with B cells in the absence of CD40L cells (\blacksquare) from A is included for reference of scale.

trast to a previous study that indicated that SGN-14 (S2C6) inhibited the binding of soluble CD40 to membrane-bound CD40L (22). A similar titration with the antagonistic mAb M3 blocked CD40/CD40L binding in a dose-dependent manner whereas mAb G28-5 showed little effect (Fig. 1A).

In a reciprocal assay, we evaluated the effect of anti-CD40 mAbs on the binding of soluble CD40L to membrane-bound CD40 expressed on the surface of Ramos cells. Titration with CD40L showed B-cell surface receptor saturation at 10 μ g/ml (data not shown), which was used for these comparisons. Increasing levels of various anti-CD40 mAbs were preincubated on ice with Ramos cells, followed by incubation of the cells with FITC-labeled soluble CD40L. The extent of CD40L binding to CD40 on Ramos cells was then determined by flow cytometry. Using saturating levels of soluble CD40L, SGN-14 resulted in an increase in CD40L binding to Ramos cells at all levels tested, ranging from 0.04 to 2 μ g/ml (Fig. 1B). Conversely, both mAbs G28-5 and M3 inhibited in a dose-dependent manner the binding of CD40L to CD40 on B cells.

SGN-14 and CD40L Costimulate Primary B-Cell Proliferation.

It has been demonstrated previously that agonistic anti-CD40 mAbs, including SGN-14 (S2C6), in combination with IL-4 will costimulate the proliferation of human B cells in culture (23). To confirm the agonistic activity of mAb SGN-14, human peripheral blood B cells were incubated in the presence of 5 ng/ml IL-4 and increasing concentrations of either SGN-14, mAb M3, mAb G28-5, or EXA2-1H8, an isotype matched non-binding control mAb. As shown in Fig. 2A, in the presence of IL-4, all three anti-CD40 mAbs stimulated primary B-cell proliferation in a dose-dependent manner. It should be noted that preparations of SGN-14 that contained varying levels of aggregate gave greater levels of stimulation than monomeric SGN-14 (data not shown). Even low levels of aggregate, as little as 2%, greatly enhanced the stimulatory activity, and care was taken to make certain that only monomeric SGN-14 was used in these studies. In parallel studies done in the absence of IL-4, SGN-14, G28-5, and M3 also stimulated the growth of peripheral B cells, although under these conditions the maximum level of stimulation for each was \sim 3-fold less than that seen in the presence of IL-4 (data not shown). As expected, control mAb did not provide any stimulation of B-cell growth, either in the absence or presence of IL-4.

We next evaluated the agonistic activity of SGN-14 on peripheral blood B cells in the presence of CD40L-expressing BMS-2 cells. Initially, peripheral blood B cells were combined with increasing

numbers of nonproliferating BMS-2 cells in the presence of a fixed concentration, 30 ng/ml, of SGN-14 and 5 ng/ml IL-4. The BMS-2 cells were pretreated with mitomycin C to prevent their proliferation, as described in "Materials and Methods." B-cell activation was determined at 72 h after this stimulus by measuring [3 H]thymidine incorporation. In the absence of CD40L-expressing cells, low levels of SGN-14 (<100 ng/ml) provided minimal costimulation of peripheral blood B cells (Fig. 2A). In contrast, in the presence of 30 ng/ml SGN-14, B-cell proliferation increased in a dose-dependent manner with increasing numbers of BMS-2 cells (Fig. 2B). Although G28-5 provided some stimulation in the absence of CD40L, the addition of CD40L-expressing cells only nominally increased B-cell proliferation (1.3-fold) over the level seen with G28-5 alone. M3, which showed the greatest proliferative response of the three mAbs on their own, demonstrated little additional response with the addition of the CD40L-expressing cells. This cooperative stimulation between SGN-14 and BMS-2 cells expressing high levels of CD40L was not seen using parental Jurkat cells expressing low levels of CD40L (data not shown).

In a variation of this assay, peripheral blood B cells and mitomycin-treated BMS-2 cells were combined at a fixed ratio of four B cells to one T cell (4:1) and incubated with a titration of anti-CD40 mAbs (Fig. 2C). Low level SGN-14 was significantly more active at inducing B-cell proliferation in combination with CD40L than by itself. In the presence of CD40L-expressing cells, the highest concentration of SGN-14 (10 μ g/ml) increased B-cell proliferation by >5 -fold over that seen with SGN-14 alone. In contrast, proliferation of primary human peripheral B cells increased by a maximum of 2-fold with mAb G28-5 when combined with CD40L cells and <2 -fold with mAb M3 when combined with CD40L cells compared with the respective mAb alone. Taken together, these data indicate that SGN-14 can promote the interaction between CD40 and CD40L, functionally resulting in a significant increase in the degree of primary B-cell proliferation.

In Vitro Growth Inhibition Activity of SGN-14. Ligation of CD40 on normal B cells is essential for activation, whereas its ligation on malignant B cells can inhibit proliferation (12). Having observed that SGN-14 and CD40L cooperate in inducing normal B-cell proliferation, we next investigated the effect of these agents on neoplastic B-cell lines. HS-Sultan and Ramos non-Hodgkin's lymphoma cell lines were cultured with increasing concentrations of SGN-14, M3, or a nonbinding control mAb, either in the absence or presence of

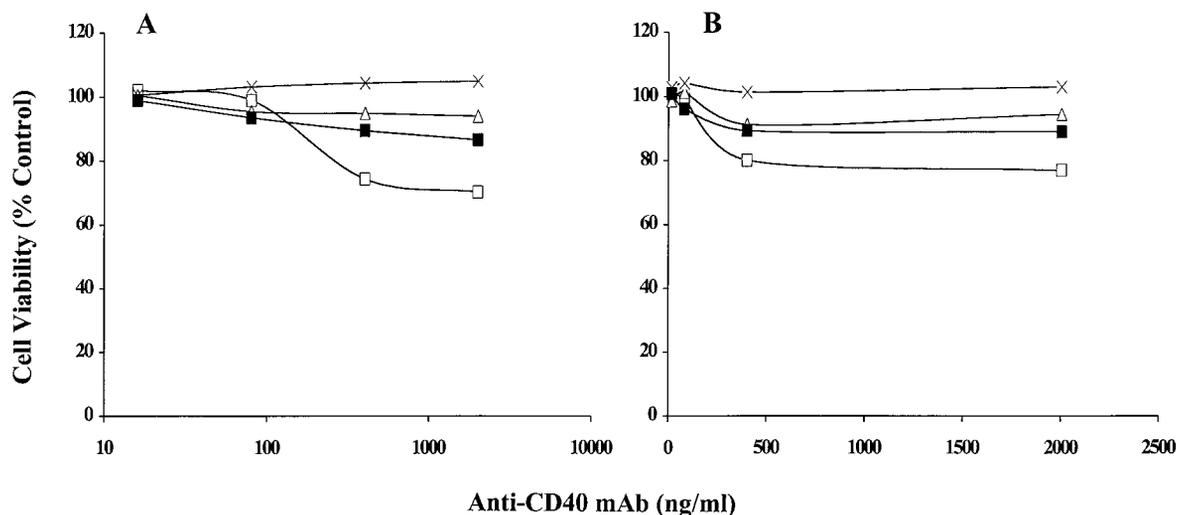


Fig. 3. *In vitro* cytotoxic activity of SGN-14. HS Sultan (A) or Ramos B (B) cell lines were cultured in the presence of nonproliferating CD40L-expressing BMS-2 cells and increasing concentrations of SGN-14 (□), M3 (Δ), or an irrelevant control mAb (X). Also shown is the effect of SGN-14 only in the absence of CD40L cells (■). Cell viability was determined at 96 h by tetrazolium dye assay.

nonproliferating, mitomycin C-treated, BMS-2 cells added at a ratio of 4:1 (B cell:BMS-2). Cell viability was determined at 96 h after treatment by the tetrazolium dye assay (19). This assay was selected because it measures cell viability, compared with thymidine incorporation, which does not discriminate between cytostasis and cytotoxicity. In the absence of CD40L⁺ cells, neither SGN-14 nor M3 had an effect on the growth of either HS-Sultan or Ramos cells. In contrast, SGN-14 inhibited the growth of these cell lines in a dose-dependent manner in the presence of the CD40L-expressing cells (Fig. 3). The inhibition of growth by SGN-14 became evident at concentrations >100 ng/ml with a maximum of 25% inhibition at 10 μg/ml. Under these conditions, a maximum of 10% inhibition was seen with mAb M3 combined with CD40L⁺ cells.

Antitumor Activity of SGN-14 in Human Lymphoma Xenografted SCID Mice. To address the potential therapeutic application of mAb SGN-14, we examined its antitumor activity in both Ramos and HS-Sultan non-Hodgkin's lymphoma xenografted SCID mice. In the first set of experiments, mice were implanted with 1×10^6 tumor cells injected through the tail vein. Mice left untreated develop a disseminated disease manifested by hind-limb paralysis and other neurological symptoms (24, 25). In these studies, untreated control mice or those treated with a control mAb became paralyzed and were sacrificed within 42 days (mean, 28 days) of implantation with Ramos cells and within 37 days (mean, 32 days) with HS-Sultan (Fig. 4). SGN-14 administered at 1 mg/kg/injection every 4 days for a total of five injections, starting either 1 or 5 days after tumor implantation, conferred a significant survival advantage in both disease models. SGN-14 was most effective in the Ramos model, where five of five animals receiving SGN-14 starting on day 1 after implant and four of five animals receiving it beginning on day 5 remained asymptomatic for the duration of the study (120 days; Fig. 4A). In the HS-Sultan-bearing animals, three of five mice receiving treatment on day 1 and two of five mice receiving treatment on day 5 survived for the length of the study (Fig. 4B). Importantly, even in mice that succumbed to these diseases, treatment with SGN-14 significantly delayed the onset of disease compared with untreated animals or those that received control mAb.

To assess the potential contribution of host effector cells, in particular NK cells, on the antitumor activity of SGN-14, mice were pretreated with anti-asialo G_{M1} 1 day prior to tumor implantation to blunt host NK activity. Anti-asialo G_{M1} has been used to eliminate

murine NK activity in mice carrying tumor xenografts (26, 27). As shown in Fig. 4C, all of the anti-asialo G_{M1}-treated mice bearing Ramos tumor cells and receiving SGN-14 therapy starting on day 5, and four of five animals starting SGN-14 treatment on day 1, survived for the duration of the experiment without developing any signs of disease. In a subsequent Ramos study aimed at maximizing NK depletion, anti-asialo G_{M1} was administered prior to each dose of SGN-14. This regimen yielded similar SGN-14 efficacy to that described above with 90% symptom-free survival for the duration of the study (data not shown). Thus, in the Ramos model, blunting of host NK activity did not detract from the antitumor efficacy of SGN-14. With the HS-Sultan tumor model (Fig. 4D), three of five animals receiving anti-asialo G_{M1}, followed by SGN-14 therapy starting on day 1, remained asymptomatic. This was comparable with results in mice that did not receive the anti-asialo G_{M1} pretreatment. When initiation of SGN-14 treatment was delayed until day 5, all of the mice that were pretreated with anti-asialo G_{M1} succumbed to the disease by day 105, with a mean paralysis time of 65 days. Under these conditions, SGN-14 still offered significant efficacy compared with untreated controls.

Antitumor Activity of SGN-14 in Multiple Myeloma Xenografted SCID Mice. In addition to its expression on non-Hodgkin's lymphoma, CD40 is also highly expressed in multiple myeloma. To determine whether SGN-14 was efficacious in treating multiple myeloma, SCID mice were implanted i.v. with IM-9 multiple myeloma cells. As with the non-Hodgkin's xenograft models, all of the untreated IM-9-bearing SCID mice developed a disseminated disease, primarily manifested by hind-limb paralysis and motor ataxia, and had to be sacrificed within 50 days of tumor implantation (Fig. 5). Treatment with SGN-14 at 1 mg/kg, every 4 days for a total of five injections, starting either 1 or 5 days after tumor inoculation, significantly increased the survival of all of the animals, with three of five remaining symptom free for 120 days after tumor implant in each group.

DISCUSSION

Ligation of CD40 is essential for primary B-cell activation and yet has suppressive or apoptotic effects on some CD40-expressing neoplasia. The reasons for this differential effect are unclear and may include autoinduction by TNF (28), inappropriate CD40/TRAF asso-

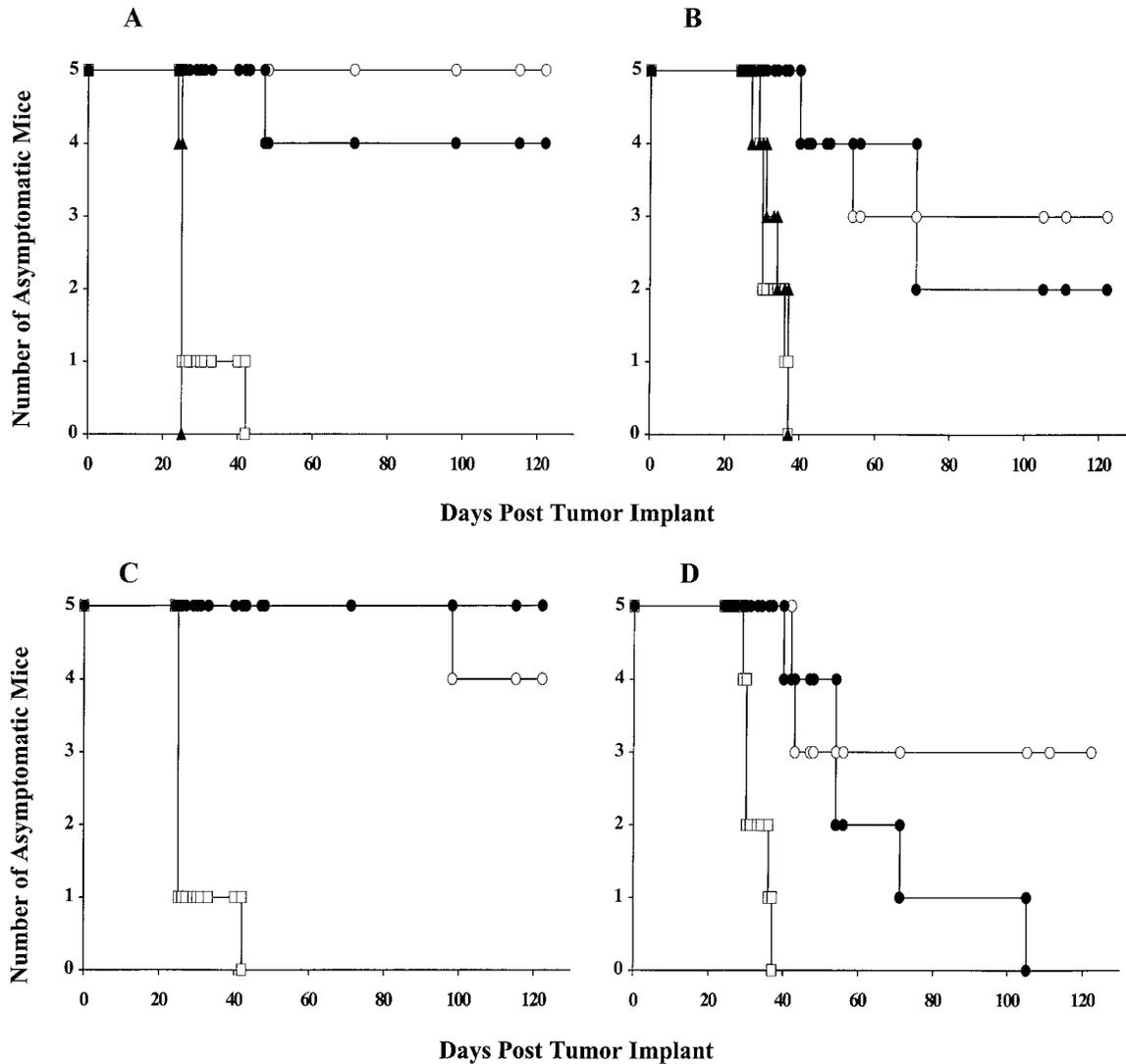


Fig. 4. Antitumor activity of SGN-14 in human B-cell lymphoma xenografts in SCID mice. Mice were implanted with 1×10^6 Ramos (A and C) or HS-Sultan (B and D) cells. A and B, groups of mice (five/group) either were left untreated (□), received 1 mg/kg/injection of a nonbinding control mAb starting 1 day after tumor inoculation (▲), or received 1 mg/kg SGN-14 starting 1 (○) or 5 (●) days after tumor inoculation. mAbs were administered i.p. every 4 days for five injections. C and D, to blunt host NK cell activity, animals were treated the same as above, except that anti-asialo G_{M1} was given as described in "Materials and Methods."

ciation (29), or the inability of tumor cells to attenuate receptor signaling, resulting in activation-induced cell death. Evidence for the latter is suggested from studies that show that the cytotoxicity of an anti-CD40 mAb is increased when it is immobilized prior to incubation with the target cells (12). Because CD40 internalizes after ligation by mAb or CD40L, immobilization could prevent the receptor from internalizing, thus preventing attenuation of CD40 signaling.

It has been shown previously that either soluble trimeric CD40 ligand or antagonistic mAbs that block ligand/receptor interaction inhibit the growth of B-lineage malignancies (12, 30) and CD40-expressing carcinomas (31). We show here that the agonistic anti-CD40 mAb, SGN-14, has potent antitumor activity against two aggressive human B-lymphoma cell lines, Ramos and HS-Sultan, as well as IM-9 multiple myeloma, in xenografted SCID mice. Left untreated, mice succumbed to disease without exception. In contrast, 90% of the Ramos and 50% of the HS-Sultan xenografted mice survived for the duration of the study, 120 days, after treatment with 1 mg/kg SGN-14. At the time of sacrifice, these surviving mice did not show any signs of disease. Because SGN-14 is a murine antibody and is thus able to engage host effector functions to eliminate tumor cells, we investigated the significance of host effector cells in antitu-

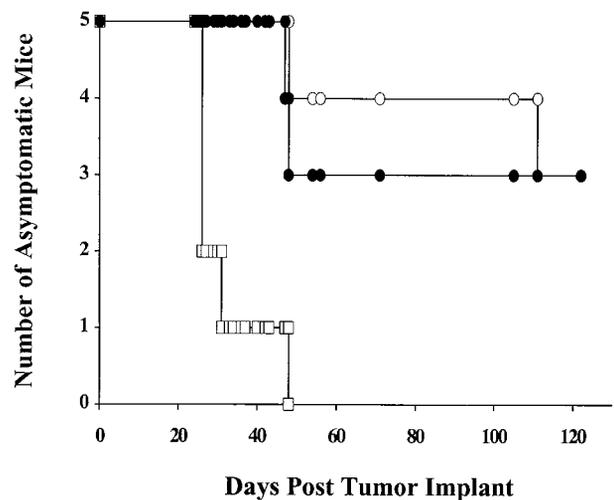


Fig. 5. Antitumor activity of SGN-14 in IM-9 multiple myeloma xenografted SCID mice. Groups of mice (five/group) were injected through the tail vein with 1×10^6 IM-9 cells and were either left untreated (□) or received 1 mg/kg/injection of SGN-14 starting 1 (○) or 5 (●) days after tumor inoculation. mAb was administered i.p. every 4 days for five injections.

mor activity by evaluating SGN-14 efficacy after blunting of host NK cells through the use of anti-asialo G_{M1}. Pretreatment with anti-asialo G_{M1} did not affect the activity of SGN-14 against Ramos xenografts, although it did impact long-term survival in the HS-Sultan model. Thus, although NK activity appears to play a role in the efficacy of SGN-14 *in vivo*, as demonstrated in the HS-Sultan model, the mAb itself possessed significant antitumor activity in the absence of these cells. Although it is informative to minimize the effect of host effector functions in xenograft models such as these, the ability to recruit effector functions, such as antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity, could help to increase the effectiveness of mAbs, such as SGN-14, in a clinical setting.

It is unclear why SGN-14 possesses potent antitumor activity *in vivo* and yet has only modest growth-inhibitory activity on these same cell lines *in vitro*. Other recent studies have had difficulty correlating the *in vivo* efficacy of mAbs with their relatively weak *in vitro* cell killing. In comparing the therapeutic effects of mAbs directed against a variety of surface antigens, including IgM, CD19, CD22, CD40, CD74, and MHC class II, as well as anti-idiotypic antibodies expressed on mouse B-cell lymphomas *in vivo*, Tutt *et al.* (32) demonstrated that the *in vitro* activity of these antibodies did not correlate with their activities *in vivo*. In fact, the *in vivo* activity appeared to be a function of the ability of the mAb to cross-link and signal via its target receptor. Additionally, other mAbs against CD40 that showed little cytotoxic activity *in vitro*, including the antibody M3 used in the present study, have had potent antitumor activity *in vivo* (12). Despite its potent antitumor activity *in vivo*, SGN-14 inhibited the growth of B-lymphoma cell lines *in vitro* by a maximum of 25%, and this was accomplished only in the presence of CD40L.

Because both soluble trimeric CD40L and SGN-14 possess antitumor activity *in vivo* and these agents cooperate to inhibit the growth of B-lymphoma cells *in vitro*, it will be interesting to evaluate the combined effect of ligand and mAb in CD40-expressing models of human cancer. This is particularly relevant because increased levels of soluble CD40L have been reported in some patients with B-cell malignancies (33). The mechanism by which SGN-14 augments the interaction of CD40L with CD40 is unclear. As with other TNF receptor family members, signaling via CD40 likely results from receptor and subsequent TRAF multimerization after interaction with the trimeric ligand (34). It is possible that SGN-14 induces a conformational change that stabilizes CD40 multimerization in the presence of CD40L. Alternatively, in the presence of ligand, SGN-14 may induce aggregation of ligand/receptor trimers. It has been shown that CD40 mutants defective in multimerization can be driven to TRAF-mediated signaling by aggregation (29).

The evolution of mAbs as therapeutics has taken many years to reduce to practice. mAb SGN-14 (S2C6) was originally characterized some 15 years ago as recognizing a tumor antigen displayed on bladder cell carcinoma, Bp50 (2), prior to its recognition as a key activating receptor on B cells. This report describes, for the first time, the ability of this mAb to eliminate B-cell disease in animal models. Although previous reports have shown that mAbs targeted to CD40 can be efficacious in treating mice with disseminated non-Hodgkin's lymphoma, the results presented here demonstrate that SGN-14 is effective in treating models of both non-Hodgkin's lymphoma and multiple myeloma and suggest that SGN-14 and other anti-CD40 mAbs should be evaluated in anti-cancer clinical trials.

As with other mAb-directed antitumor therapies, the expression of the CD40 on normal tissue in addition to neoplasia must be taken into account as clinical trials using SGN-14 are designed. CD40 is expressed on endothelial cells and other normal tissues. It is fortunate that antibodies against human CD40 cross-react with monkey CD40, which will allow for extensive toxicological analysis in non-human

primates. We have evaluated previously the toxicity of a single-chain immunotoxin targeted to human CD40 in cynomolgus monkeys and found that the immunotoxin was well tolerated with no signs of vascular toxicity (25). In the present study, soluble SGN-14 had little effect on the growth of normal B cells. We have also seen that SGN-14 has no effect on the growth of CD40-expressing fibroblasts *in vitro*.⁴ Taken together, these suggest that SGN-14 may be well tolerated in a clinical setting.

Currently, efforts are under way to generate engineered forms of SGN-14 for potential evaluation in clinical trials in CD40-expressing malignancies. The ability to target multiple myeloma, in addition to lymphoma, presents a significant opportunity for a chimeric or humanized form of SGN-14 because there are currently no durable therapeutic options for patients with multiple myeloma, and CD40 is one of just a few antigens that are available as targets for this disease (15). There is also the possibility that this mAb may be useful in treating CD40-expressing carcinomas, although the *in vitro* and *in vivo* effects of SGN-14 on solid carcinomas have not yet been examined.

REFERENCES

- Kohler, G., and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)*, 256: 495–497, 1975.
- Brown, S. L., Miller, R. A., Horning, S. J., Czerwinski, D., Hart, S. M., McElderry, R., Basham, T., Warnke, R. A., Merigan, T. C., and Levy, R. Treatment of B-cell lymphomas with anti-idiotypic antibodies alone and in combination with α interferon. *Blood*, 73: 651–661, 1989.
- Maloney, D. G., Brown, S., Czerwinski, D. K., Liles, T. M., Hart, S. M., Miller, R. A., and Levy, R. Monoclonal anti-idiotypic antibody therapy of B-cell lymphoma: the addition of a short course of chemotherapy does not interfere with the antitumor effect nor prevent the emergence of idiotype-negative variant cells. *Blood*, 80: 1502–1510, 1992.
- Press, O. W., Eary, J. F., Appelbaum, F. R., Martin, P. J., Nelp, W. B., Glenn, S., Fisher, D. R., Proter, B., Matthews, D. C., Gooley, T., and Bernstein, I. D. Phase II trial of ¹³¹I-B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B cell lymphomas. *Lancet*, 346: 336–340, 1995.
- Kaminski, M. S., Zasadny, K. R., Francis, I. R., Fenner, M. C., Ross, C. W., Milik, A. W., Estes, J., Tuck, M., Regan, D., Fisher, S., Glenn, S. D., and Wahl, R. L. Iodine-131-anti-B1 radioimmunotherapy for B-cell lymphoma. *J. Clin. Oncol.*, 14: 1974–1981, 1996.
- Maloney, D. G., Grillo-López, A. J., White, C. A., Bodkin, D., Schilder, R. J., Neidhart, J. A., Janakiraman, N., Foon, K. A., Liles, T. M., Dallaire, B. K., Wey, K., Royston, I., Davis, T., and Levy, R. IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood*, 90: 2188–2195, 1997.
- Leget, G. A., and Czuczman, M. S. Use of Rituximab, the new FDA-approved antibody. *Curr. Opin. Oncol.*, 10: 548–551, 1998.
- Paulie, S., Ehlin-Henriksson, B., Mellstedt, H., Koho, H., Ben-Aissa, H., and Perlmann, P. A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. *Cancer Immunol. Immunother.*, 20: 23–28, 1985.
- Banchereau, J., Bazan, F., Blanchard, D., Brière, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F., and Saeland, S. The CD40 antigen and its ligand. *Annu. Rev. Immunol.*, 12: 881–922, 1994.
- Karmann, K., Hughes, C. C., Schechner, J., Fanslow, W. C., and Pober, J. S. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc. Natl. Acad. Sci. USA*, 92: 4342–4346, 1995.
- Ledbetter, J. A., Clark, E. A., Norris, N. A., Shu, G., and Hellström, I. Expression of a functional B-cell receptor CDw40 (Bp50) on carcinomas. In: A. J. McMichael (ed.), *Leucocyte Typing III: White Cell Differentiation Antigens*, pp. 432–439. Oxford, United Kingdom: Oxford University Press, 1987.
- Funakoshi, S., Longo, D. L., Beckwith, M., Conley, D. K., Tsarfaty, G., Tsarfaty, I., Armitage, R. J., Fanslow, W. C., Spriggs, M. K., and Murphy, W. J. Inhibition of human B-cell lymphoma growth by CD40 stimulation. *Blood*, 83: 2787–2794, 1994.
- Clark, E. A., and Ledbetter, J. A. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc. Natl. Acad. Sci. USA*, 83: 4494–4498, 1986.
- Uckun, F. M., Gajl-Peczalska, K., Myers, D. E., Jaszcz, W., Haissig, S., and Ledbetter, J. A. 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*, 76: 2449–2456, 1990.
- Maloney, D. G., Donovan, K., and Hamblin, T. J. Antibody therapy for treatment of multiple myeloma. *Semin. Hematol.*, 36 (Suppl. 3): 30–33, 1999.

⁴ A. F. Wahl, unpublished observations.

16. Funakoshi, S., Longo, D. L., and Murphy, W. J. Differential *in vitro* and *in vivo* antitumor effects mediated by anti-CD40 and anti-CD20 monoclonal antibodies against human B-cell lymphomas. *J. Immunother.*, *19*: 93–101, 1996.
17. Koho, H., Paulie, S., Ben-Aissa, H., Jonsdottir, I., Hansson, Y., Lundblad, M. L., and Perlmann, P. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. I. Determination of the selectivity of six antibodies by cell ELISA and immunofluorescence. *Cancer Immunol. Immunother.*, *17*: 165–172, 1984.
18. Malik, N., Greenfield, B. W., Wahl, A. F., and Kiener, P. A. Activation of human monocytes through CD40 induces matrix metalloproteinases. *J. Immunol.*, *156*: 3952–3960, 1996.
19. Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.*, *48*: 4827–4833, 1988.
20. Ledbetter, J. A., Grosmaire, L. S., Hollenbaugh, D., Aruffo, A., and Nadler, S. G. Agonistic and antagonistic properties of CD40 mAb G28-5 are dependent on binding valency. *Circulatory Shock*, *44*: 67–72, 1995.
21. Fanslow, W. C., Clifford, K. N., Zappone, J., Alderson, M. R., and Armitage, R. J. CD40 mAbs M2 and M3 inhibit CD40L binding and function. *In: S. Schlossman (ed.), Leucocyte Typing V*, pp. 555–556. Oxford, UK: Oxford University Press, 1994.
22. Pound, J. D., Challa, A., Holder, M. J., Armitage, R. J., Dower, S. K., Fanslow, W. C., Kikutani, H., Paulie, S., Gregory, C. D., and Gordon, J. Minimal cross-linking and epitope requirements for CD40-dependent suppression of apoptosis contrast with those for promotion of the cell cycle and homotypic adhesions in human B cells. *Int. Immunol.*, *11*: 11–20, 1999.
23. Björck, P., and Paulie, S. CD40 antibodies defining distinct epitopes display qualitative differences in their induction of B-cell differentiation. *Immunology*, *87*: 291–295, 1996.
24. Ghetie, M.-A., Richardson, J., Tucker, T., Jones, D., Uhr, J. W., and Vitetta, E. S. Disseminated or localized growth of a human B-cell tumor (Daudi) in SCID mice. *Int. J. Cancer*, *45*: 481–485, 1990.
25. Francisco, J. A., Schreiber, G. J., Comerreski, C. R., Mezza, L. E., Warner, G. L., Davidson, T. J., Ledbetter, J. A., and Siegall, C. S. *In vivo* efficacy and toxicity of a single-chain immunotoxin targeted to CD40. *Blood*, *89*: 4493–4500, 1997.
26. Murphy, W. J., Bennett, M., Anver, M. R., Baseler, M., and Longo, D. L. Human-mouse lymphoid chimeras: host-vs.-graft and graft-vs.-host reactions. *Eur. J. Immunol.*, *22*: 1421–1427, 1992.
27. Yao, L., Sgadari, C., Furuke, K., Bloom, E. T., Teruya-Feldstein, J., and Tosato, G. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood*, *93*: 1612–1621, 1999.
28. Grell, M., Zimmermann, G., Gottfried, E., Chen, C. M., Grünwald, U., Huang, D. C. S., Lee, Y. H. W., Dürkop, H., Engelmann, H., Scheurich, P., Wajant, H., and Strasser, A. Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J.*, *18*: 3034–3043, 1999.
29. Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J., and Kehry, M. R. High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. *Biochemistry*, *38*: 10168–10177, 1999.
30. Dilloo, D., Brown, M., Roskrow, M., Zhong, W., Holladay, M., Holder, W., and Brenner, M. CD40 ligand induces an antileukemia response *in vivo*. *Blood*, *90*: 1927–1933, 1997.
31. Hirano, A., Longo, D. L., Taub, D. D., Ferris, D. K., Young, L. S., Eliopoulos, A. G., Agathangelou, A., Cullen, N., Macartney, J., Fanslow, W. C., and Murphy, W. J. Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. *Blood*, *93*: 2999–3007, 1999.
32. Tutt, A. L., French, R. R., Illidge, T. M., Honeychurch, J., McBride, H. M., Penfold, C. A., Fearon, D. T., Parkhouse, R. M. E., Klaus, G. G. B., and Glennie, M. J. Monoclonal antibody therapy of B cell lymphoma: signaling activity on tumor cells appears more important than recruitment of effectors. *J. Immunol.*, *161*: 3176–3185, 1998.
33. Younes, A., Snell, V., Consoli, U., Clodi, K., Zhao, S., Palmer, J. L., Thomas, E. K., Armitage, R. J., and Andreeff, M. Elevated levels of biologically active soluble CD40 ligand in the serum of patients with chronic lymphocytic leukaemia. *Br. J. Haematol.*, *100*: 135–141, 1998.
34. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. Crystallographic analysis of CD40 recognition and signaling by human TRAF2. *Proc. Natl. Acad. Sci. USA*, *96*: 8408–8413, 1999.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Agonistic Properties and *in Vivo* Antitumor Activity of the Anti-CD40 Antibody SGN-14

Joseph A. Francisco, Karen L. Donaldson, Dana Chace, et al.

Cancer Res 2000;60:3225-3231.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/60/12/3225>

Cited articles This article cites 31 articles, 17 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/60/12/3225.full#ref-list-1>

Citing articles This article has been cited by 7 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/60/12/3225.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/60/12/3225>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.