Immunomodulatory Drug CC-5013 or CC-4047 and Rituximab Enhance Antitumor Activity in a Severe Combined Immunodeficient Mouse Lymphoma Model

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Abstract   New thalidomide derivatives CC-5013 and CC-4047 (immunomodulatory drugs, IMiD) are up to 10,000 times more potent than Thalidomide. The biological effects of IMiDs are presumed to be mediated by (a) activation of some components of the innate [natural killer (NK) cells] or adoptive immune system (T cells), (b) modification of cytokine microenvironment in the tumor bed, or by (c) inhibition of angiogenesis. In this article, we tested an innovative combination strategy involving rituximab and IMiDs in aggressive lymphoma cell lines and human lymphoma xenografts. Treatment of non-Hodgkin's lymphoma cells with CC-5013 resulted in a 40% to 70% growth inhibition when compared with controls ($P < 0.05$). Exposure of lymphoma cells to CC-4047 resulted in a lesser degree of growth inhibition. Induction of apoptosis was shown in 10% to 26% of lymphoma cells 24 hours following exposure to either IMiD. *In vivo* studies in severe combined immunodeficient mice showed synergistic activity between CC-4047 (and to a lesser degree, CC-5013) plus rituximab. Animals treated with the CC-4047/rituximab combination had a median survival of 74 days ($P = 0.0012$) compared with 58 days ($P = 0.167$) in CC-5013/rituximab-treated animals compared with 45 days in rituximab monotherapy–treated animals. The synergistic effect between IMiDs and rituximab in our mouse model was attributed to NK cell expansion. The enhancement of rituximab activity by IMiDs was abrogated by *in vivo* depletion of NK cells. Augmenting NK cell function by CC-4047 or CC-5013 exposure may increase the antitumor effects of rituximab against B-cell lymphomas and warrants further exploration in the context of a clinical trial.

According to recently published cancer statistics, in 2004, ~19,470 lymphoma patients will die from their disease despite currently available treatment (1). Rituximab, an IgG1 chimeric monoclonal antibody (mAb) targeting the CD20 antigen, is the first of two mAbs approved by the Federal Drug Administration for the treatment of cancer (2). Results from phase II/III studies showed the ability of rituximab to achieve meaningful clinical responses in 40% to 50% of previously treated indolent lymphoma patients (3, 4). Therapeutic strategies combining rituximab with systemic chemotherapy have resulted in higher response rates and improvement in survival without adding significant toxicity (5, 6).

However, despite these promising results, not every patient responds to rituximab and many relapse after an initial response. It is postulated that the majority of previously rituximab-treated patients will eventually relapse with variable degrees of biologically resistant disease. Therefore, it is necessary to develop new strategies that will enhance the biological activity of currently available mAbs without adding significant toxicities.

Several biological effects have been postulated as rituximab's primary mechanism of antitumor activity, including antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, and induction of apoptosis/antiproliferation. A major area of research is the study of intracellular signals that result in apoptosis of lymphoma cells following binding of rituximab to the CD20 antigen and factors associated with activation of the innate immune system (7–18).

One of the most studied *in vitro* mechanisms of rituximab-associated antitumor activity is the activation of the innate immune system via complement-mediated cytoxicity or antibody-dependent cellular cytotoxicity. Recently published *in vivo* studies show that FcγR receptor expression is necessary to eradicate non-Hodgkin's lymphoma (NHL) in a murine animal model, suggesting that antibody-dependent cellular cytotoxicity plays a significant role in rituximab's activity (18). We have recently shown that effector cells [both natural killer (NK) cells and neutrophils] are necessary for optimal antitumor activity of rituximab, corroborating findings reported by other investigators (17, 18). Furthermore, specific polymorphisms in the *FcγRIIIa* gene have been associated with differences in the clinical and molecular response to anti-CD20 mAb therapy in patients with indolent NHL (19).

doi:10.1158/1078-0432.CCR-05-0577

Modulation of immune responses is also an attractive strategy to enhance the biological activity of mAbs.

Consistent with the attractive possibility of augmenting the biological effects of mAbs, several group of investigators have shown that various cytokines [i.e., interleukin-2 (IL-2), IFN-γ, or granulocyte colony-stimulating factor] can enhance the antitumor activity of rituximab using various preclinical systems (20–22).

Thalidomide derivatives Revimid (CC-5013) and Actimid (CC-4047) are a new class of anticancer agents known as immunomodulatory drugs (IMiDs). In general, IMiDs are 10,000 more potent that thalidomide and are known to be active against multiple myeloma (23). Thalidomide analogues are believed to have different therapeutically relevant effects such as inducing T-cell proliferation, increasing the production of IL-2/IFN γ by effector cells and angiogenesis inhibition (24–26).

Using a severe combined immunodeficient (SCID) mouse–disseminated lymphoma xenograft model, we evaluated the biological effects of rituximab in combination with CC-5013 or CC-4047. We show here that the addition of murine Actimid and to a lesser degree Remivid before each dose of rituximab results in synergistic antitumor activity and prolonged survival in lymphoma-bearing animals when compared with rituximab monotherapy. We also show that the administration of IMiDs results in expansion of murine NK cells and that depletion of NK cells results in a complete the lost in the synergistic antitumor effects of the rituximab-IMiD combination supporting the proposal that NK cell expansion is one mechanism by which IMiDs may augment rituximab antitumor.

Materials and Methods

Culture medium and reagents. The following culture media were used: RPMI 1640 (Sigma Chemical, St. Louis, MO) and RPMI 1640 supplemented with 10% heat-inactivated (60°C, 45 minutes) fetal bovine serum (Atlanta Biologicals, Norcross, GA), 5 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Corp., Grand Island, NY). Ficoll-Hypaque was purchased from Sigma Chemical. Sodium chromate[51] (^{51}Cr; Perkin-Elmer Life, Inc., Boston MA) and [^{3}H]-thymidine radioisotopes (Perkin-Elmer Life) were used in functional assays assessing antibody-associated cytotoxicity and cell proliferation, respectively. Triton X-100 was purchased from Sigma Chemical.

Cell lines. The Raji cell line is a well-characterized B-lymphoblastic cell line (phenotype: CD20^{+}, CD19^{+}, CD22^{+}) derived from a patient with Burkitt's lymphoma (obtained from the American Tissue and Cell Collection, Manassas, VA). The SU-DHL-4 and SU-DHL-10 cell lines derived from patients with diffuse large cell B-cell lymphomas and immortalized by concomitant EBV infection were a kind gift from Steven Treon (Dana-Farber Cancer Institute). The hybridoma TMβ-1 cell line was obtained from Dr. Julie Ostberg at Roswell Park Cancer Institute and used to produce the IL-2 mAb. The cells were cultured and maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 5 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cultures were free of *Mycoplasma* and pathogenic murine viruses.

Immunomodulatory drugs. CC-5013 (Revimid) and CC-4047 (Actimid) were obtained from Celgene, Inc. (Waren, NJ) and dissolved freshly in DMSO to make a 10 mg/mL solution. The compounds were then diluted directly into the tissue culture media at the required concentrations. For *in vivo* studies, IMiD stock solutions were diluted in sterile 0.9% normal saline to a final concentration of 1 mg/mL. The

final concentration of DMSO in all experiments was <0.01%, and all treatment conditions were compared with vehicle controls.

Antibodies. Human anti-mouse IL-2 receptor mAb was expanded in SCID mice. Ten- to 12-week-old SCID mice were inoculated with 1×10^7 TMβ1 cells via i.p. injection. Ascites fluid was collected after 2 weeks and sterilized by ultrafiltration. Individual 100-μL i.p. injections of sterile ascites containing anti-IL-2 receptor antibody induced NK cell inactivation before NHL tumor inoculation in SCID mice lasting ~30 days. *In vivo* depletion of NK cells was confirmed by flow cytometric analysis of peripheral blood (data not shown).

Rituximab (IDEC/Genentech, Inc., San Francisco, CA) was obtained from the Roswell Park Cancer Institute Pharmacy Department at a stock concentration of 10 mg/mL. The antibody was dosed at 10 mg/kg and diluted in sterile PBS (200 μg/100 μL) for tail vein injection into the SCID mice. Trastuzumab (Herceptin, Genentech) was used as an isotype control.

For phenotypic analysis of lymphoma cells, purified phycoerythin-conjugated monoclonal mouse anti-human CD19 and CD22 were obtained from Caltag (Burlingame, CA). Phycoerythin-conjugated mouse anti-human CD20 and CD59 as well as Cy-Chrome-conjugated mouse anti-human CD55 mAbs were purchased from BD PharMingen, Inc. (San Diego, CA). FITC-conjugated mouse anti human CD3 was used as isotype control (BD PharMingen, San Diego CA) and mouse IgG from Sigma was used to block Fc receptors.

For characterization of changes occurring in the phenotype of murine NK cells following *in vivo* IMiD stimulation, a phycoerythin-conjugated rat anti-mouse CD49b (DX5) was obtained from BD Biosciences (San Diego, CA). In addition, an FITC-conjugated rat anti-mouse CD3 (negative control) and rat anti-mouse CD16/CD32 mAb (mouse block) were purchased from BD Biosciences.

Animals. For the experiments with rituximab, 6- to 8-week-old SCID mice were bred and maintained at the Department of Laboratory Animal Resources facility at Roswell Park Cancer Institute. Older SCID mice (12 week old) were used for the production of the IL-2 receptor antibody.

The experiment design was approved by the Institutional Animal Care and Use Committee at Roswell Park Cancer Institute (protocols M821 and P966). All animals were housed and maintained in laminar flow cabinets or microisolator units and provided with sterilized food and water. Our laboratory facility has been certified by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulation and standards of the U.S. Department of Agriculture and the U.S. Department of Health and Human Services.

Apoptosis detection following in vitro *therapy with CC-5013/CC-4047 with/without monoclonal antibodies.* *In vitro* effects of either CC-5013 or CC-4047 as single agent or in combination with rituximab were evaluated by flow cytometric analysis. Lymphoma cell lines (1×10^6 cells) were exposed to either CC-5013 (5 μg/mL), CC-4047 (5 μg/mL), or vehicle control (DMRIE-C, 0.01%) alone or in combination with rituximab at a final concentration of 10 μg/mL. Following a period of incubation of 24 or 48 hours, apoptosis was assessed by staining-treated cells with FITC-labeled Annexin V and propidium iodine (Oncogene, Boston, MA). All samples were analyzed by multicolor flow cytometric analysis using a fluorescence-activated cell sorter/FACStar Plus (Becton Dickinson Immunocytometry, San Jose, CA) flow cytometer. Cells were scored as apoptotic if they were Annexin V–positive and propidium iodine–negative/positive (early and late apoptosis, respectively). The experiments were done in triplicate and the results were given as mean values with SE bars.

Assessment of cell proliferation after treatment with IMiDs, alone or in combination with monoclonal antibodies. Lymphoma cell lines were placed in 96-well plates (1×10^5 cells per well) and exposed to escalating concentrations of CC-5013, CC-4047 (2.5, 5, 10, 20, and 40 μg/mL), or vehicle control single agents or in combination with rituximab or trastuzumab (isotype), at a final antibody concentration of 10 μg/mL. The final concentration was adjusted to 200 μL with 10%

RPMI. The cell lines were incubated at 37°C and 5% CO_2 for 24 and 48 hours. Following 24 or 48 hours, 1 µCi per well of [³H]-thymidine (New England Nuclear Products, Boston, MA) was added and cells were incubated for 18 hours more. Cells were then harvested using the Harvest system (Tomac Cell Harvester, Tomtec, Hamden, CT) into the 96-well glass filters (Wallac, Inc., Turku, Finland) and [³H]-thymidine uptake was measured using an automated scintillation counter (Wallace, Gaithersburg, MD). Each experiment was done in triplicate at three different times; results are presented as the mean of counts per minute (cpm) at 24 and 48 hours ± SD. To study if a higher antitumor effect could be observed in a "low tumor burden setting," a second set of experiments was done using a lower number of tumor cells (1×10^4 cells per well).

CD20 expression following IMiD exposure in vitro. CD20 antigen expression among the NHL cell lines was confirmed by flow cytometric analysis. To correlate whether possible CD20 surface antigen changes correlate to incubation with specific IMiDs and leads to subsequent changes in rituximab-mediated antitumor activity, phenotypic analysis of NHL cells by multivariable flow cytometry were done at various time points.

Tumor cells were resuspended in RPMI 10 to a final concentration of 1×10^6 cells/mL. The cells were then incubated with either CC-5013 (5 µg/mL), CC-4047 (5 µg/mL), or RPMI 10 for 24 and 48 hours at 37°C and 5% CO_2. Immunophenotype analysis was done at baseline, after 24 and 48 hours of incubation with IMiDs. Changes of CD20 expression by NHL cell lines were studied by fluorescence-activated cell sorting analysis with antigen-specific FITC-conjugated mouse anti-human mAbs.

Lymphoma xenograft mouse model. These studies were carried out using a disseminated lymphoma-bearing SCID mouse xenograft model. Raji cells were harvested from confluent cultures and only suspensions with >90% viable cells were used for animal inoculation. Subsequently on day 0, SCID mice received 1×10^6 Raji cells via i.v. Untreated SCID mice inoculated by i.v. injection develop symptomatic central nervous system, pulmonary, and liver metastasis that result in death from massive tumor burden and central nervous system involvement after 17 to 21 days after inoculation. A second lymphoma mouse model was used to address the significance of NK cell expansion in the biological interactions observed between rituximab and IMiDs. The second mouse lymphoma xenograft consisted of SCID mice depleted of NK cells bearing Raji cells implanted via tail vein injection as described above.

In vivo *depletion of natural killer cells.* NK cells were depleted from SCID mice using the IL-2 receptor mAb. To determine the lowest effective dose, 8-week-old SCID mice were treated at single doses of 0 or 100 µL of pooled murine ascites containing the anti-IL-2 receptor mAb. Subsequently, blood samples were obtained on days +1, +10, +20, and +30 from the retro-orbital venous plexus. Fifty microliters of heparinized mouse blood were incubated on ice with 0.4 µg of anti-CD16/CD32 (FcγIII/IIR; BD PharMingen, San Diego CA) as a blocking step, followed by incubation on ice with 0.4 µg phycoerythin-conjugated anti-CD49 (BD PharMingen, St Louis, MO). RBC in the samples were then lysed with two rounds of 1× ack solution (15 mmol/L NH_4CL, 0.1 mmol/L $KHCO_3$, and 0.01 mmol/L Na_2EDTA) and all samples were washed in PBS before fixing in PBS/2% paraformaldehyde. Samples were then run on a FACScan (Becton Dickinson) flow cytometer.
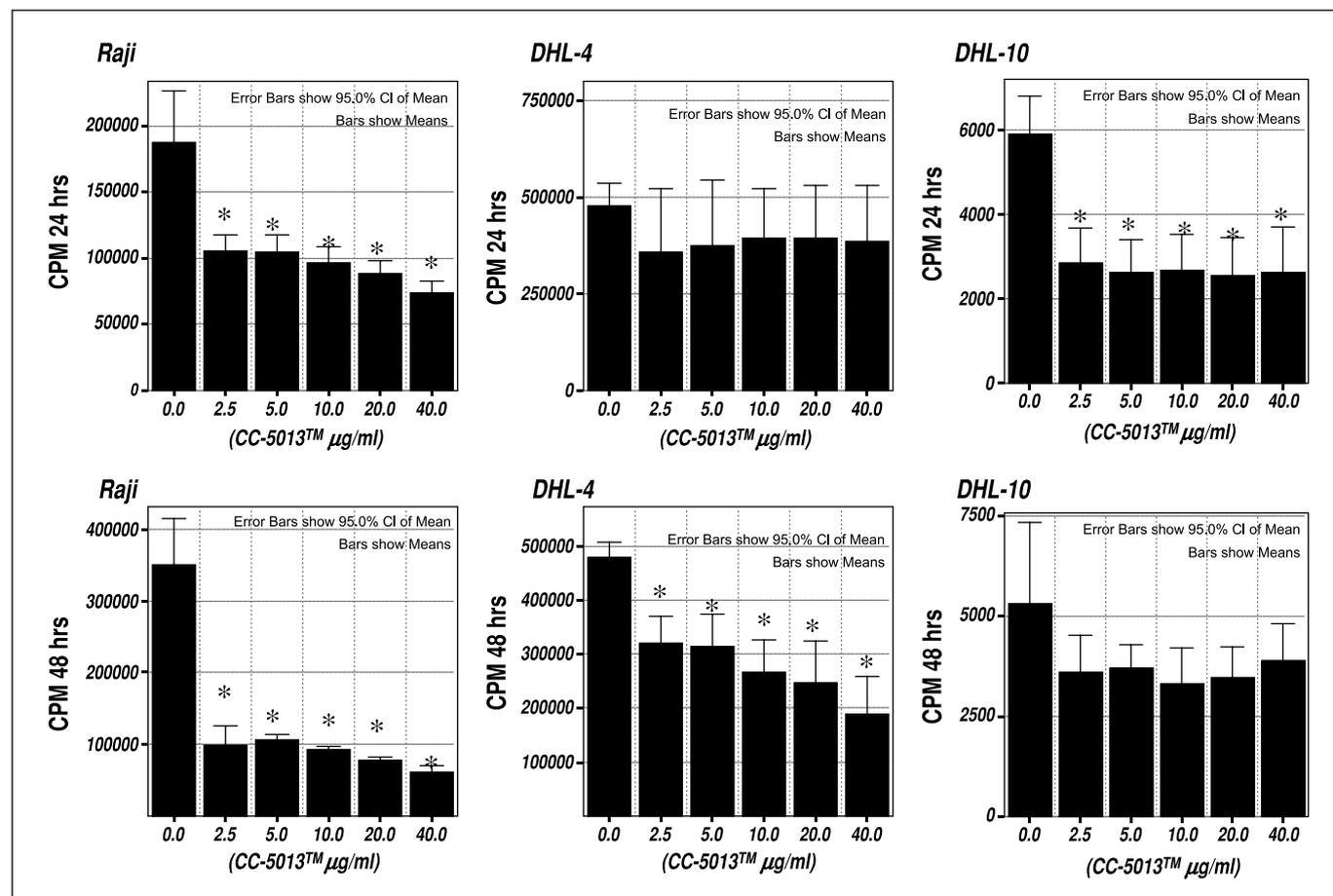

Fig. 1. *In vitro* exposure of NHL cell lines to various concentrations of CC-5013 (2.5-40 µg/mL) resulted in decrease DNA synthesis at 24 and/or 48 hours. *, $P < 0.05$. P value was calculated by χ^2 testing comparing vehicle-exposed cells with each dose level of CC-5013.
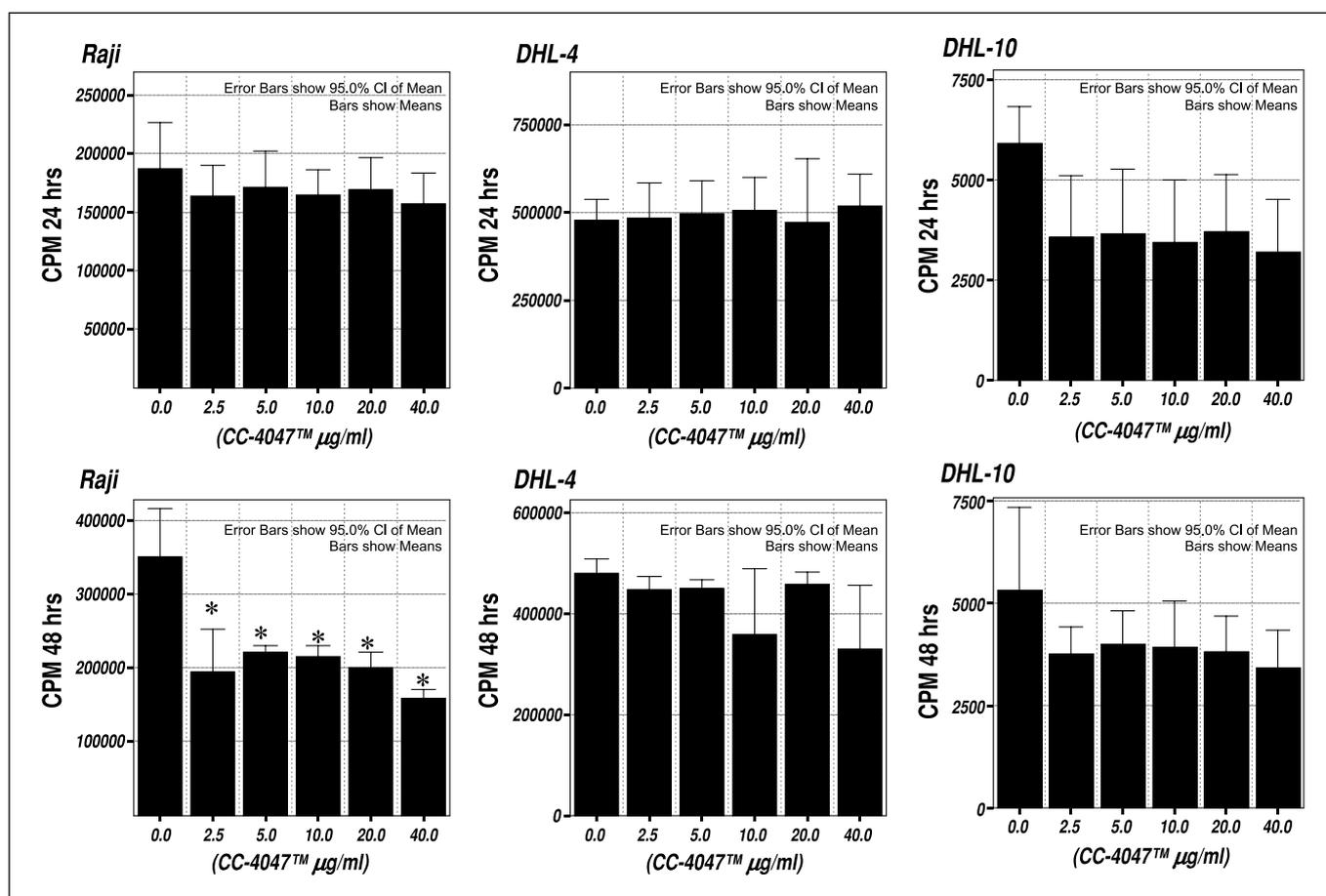
Fig. 2. *In vitro* exposure of NHL cell lines to various concentrations of CC-4047 (2.5-40 μg/mL) resulted in decrease DNA synthesis at 48 hours in Raji cells. *, $P < 0.05$. P value was calculated by χ^2 testing comparing vehicle-exposed cells with each dose level of CC-4047.

Treatment protocol to evaluate the effects of IMiDs in rituximab antitumor activity. Six- to 8-week-old SCID mice were used for this purpose. On day 0, all the animals received 1×10^6 Raji cells via tail vein injection. After 72 hours of tumor engraftment, the animals were divided into seven cohorts. The first cohort (group A) served as control and received no treatment. Groups B and C consisted of animals treated with either CC-5013 (0.5 mg/kg) or CC-4047 (0.5 mg/kg) given i.p. on days +3, +4, +8, +9, +13, +14, +18, and +19. Groups D and E were treated with rituximab or trastuzumab (isotype control) monotherapy given via tail vein injection at 10 mg/kg on days +5, +10, +15, and +20. Finally, groups F and G consisted of animals treated with rituximab in combination with CC-5013 (group E) or CC-4047 (group G). IMiDs were given i.p. for two consecutive days before each dose of rituximab. After completion of therapy, animals were observed for a period of 90 days. The end point of the study was survival defined as the time for the development of limb paralysis. Animals that reached the end point or survived after 3 months of observation were sacrificed by cervical dislocation. Pathologic examination of all organs (liver, lung, and brain) was done to detect any residual disease. The experiments were repeated in three separate occasions.

Flow cytometric analysis of changes in murine natural killer cells. To characterize significant changes in the number of NK cells and other effector cells, multicolor flow cytometric analysis was done from peripheral blood murine specimens. Specifically, after 2 days of IMiDs (0.5 mg/kg/dose) or vehicle administration, animals from groups A (placebo), B (CC-5013), and C (CC-4047) were anesthetized with Avertin [1.25% 2,2,2-tribromoethanol (Aldrich, St. Louis, MO) and 2.5% 2-methyl-2-butenol (Aldrich)]. Peripheral blood was collected thorough the retro-orbital sinus of each animal and collected into heparin-containing centrifuge tubes. Fifty microliters of heparinized mouse blood were incubated on ice with anti-CD16/CD32 (FcγIII/IIR; BD PharMingen, San Diego CA) as a blocking step followed by incubation on ice with primary phycoerythin-conjugated anti CD49b (DX5) or anti-CD3 (isotype control). RBC in the samples were then lysed with two to three rounds of 1× ack solution (15 mmol/L NH$_4$CL, 0.1 mmol/L KHCO$_3$, 0.01 mmol/L Na$_2$EDTA) and all samples were washed in PBS before fixing in PBS/2% paraformaldehyde. Finally, samples were then run on a FACScan (Becton Dickinson) flow cytometer.

In vivo depletion of natural killer cells to further define their role in the biological interactions between IMiDs and rituximab. Six- to 8-week-old SCID mice were depleted of NK cells 24 hours before tumor inoculation. On day 0, all the animals received 1×10^6 Raji cells via tail vein injection. After 72 hours of tumor engraftment, the animals were divided into six cohorts and treated with rituximab ± IMiDs as described above.

Statistical analysis. *In vitro* experiments were repeated in triplicates and the results are reported as the mean with 95% confidence intervals (95% CI). Statistical differences between treatment groups and controls were determined by χ^2 testing. In addition, differences in survival between treatment groups were calculated using Kaplan-Meier curves (SPSS 11.0 for Windows 2000 software). P values were calculated by log-rank test. Differences were considered significant when $P < 0.05$.

Results

In vitro *exposure of non-Hodgkin's lymphoma cell lines to IMiDs results in decrease cell growth and DNA synthesis.* Cell growth inhibition was observed in all the cell lines tested

following exposure to either CC-5013 and to a lesser degree CC-4047 at various dose levels (Figs. 1 and 2). No significant differences were observed between the different doses tested and antitumor activity was observed using IMiD doses raging from 2.5 µg/mL to the highest dose tested of 40 µg/mL (Figs. 1 and 2). However, differences in response and kinetics of response were observed between the lymphoma cell lines tested.

The decrease in DNA synthesis was more pronounced after 48 hours of drug exposure (IMiD dose of 5 µg/mL). Treatment of lymphoma cells with CC-5013 resulted in 40% to 85% growth inhibition. In addition, *in vitro* exposure of lymphoma cells to CC-4047 leads to 40% decrease in cell proliferation when compared with vehicle-treated controls (Fig. 2). The mean cpm value at 48 hours was significantly lower for SU-DHL-4 cells exposed to CC-5013 (mean, 314,072; 95% CI, 253,556-374,588 cpm) when compared with vehicle-treated cells (mean, 479,251; 95% CI, 450,761-507,740 cpm; vehicle versus CC-5013, $P < 0.001$; Figs. 1 and 2).

There was a 70% reduction in Raji cells exposed to CC-5013 when compared with placebo. The mean cpm for Raji cells treated with CC-5013 for 48 hours was lower (mean, 106,704 cpm; 95% CI, 70,225-125,610 cpm) than cells exposed to vehicle control (mean, 349,944; 95% CI, 284,642-415,246 cpm; $P = 0.014$). To a lesser degree, CC-4047 inhibited by 40%

the DNA synthesis of Raji cells ($P = 0.036$). The mean cpm counts following 48 hours of drug exposure was 215,115 cpm (95% CI, 212,727-229,285 cpm; Figs. 1 and 2). Similar results were observed in SU-DHL-10 cells (Figs. 1 and 2).

No antiproliferative effects were observed among the lymphoma cell exposed to rituximab. Moreover, no significant changes in the degree of cell growth inhibition were observed when rituximab was combined with either IMiDs when compared with CC-5013 or CC-4047 monotherapy (Fig. 3).

Similar results were observed when experiments were done with less tumor cells per well (low tumor burden setting; data not shown).

Exposure of non-Hodgkin's lymphoma cell lines to CC-5013 and CC-4047 induces a variable degree of apoptosis. NHL cell lines when exposed to varying concentrations of IMiDs show a variable degree of apoptosis. Induction of apoptosis was shown in 10% to 26% of lymphoma cells 24 hours following exposure to either CC-5013 or CC-4047 (data not shown).

Effects of IMiDs on CD20 antigen expression on non-Hodgkin's lymphoma cell lines. One possibility that could have explained the positive effects of IMiDs on rituximab's *in vivo* antitumor activity is that IMiDs may alter the expression and/or density of relevant target molecules on the surface of lymphoma cell lines. To test for this possibility, we analyzed changes in immunophenotype of the five NHL cell lines tested following exposure
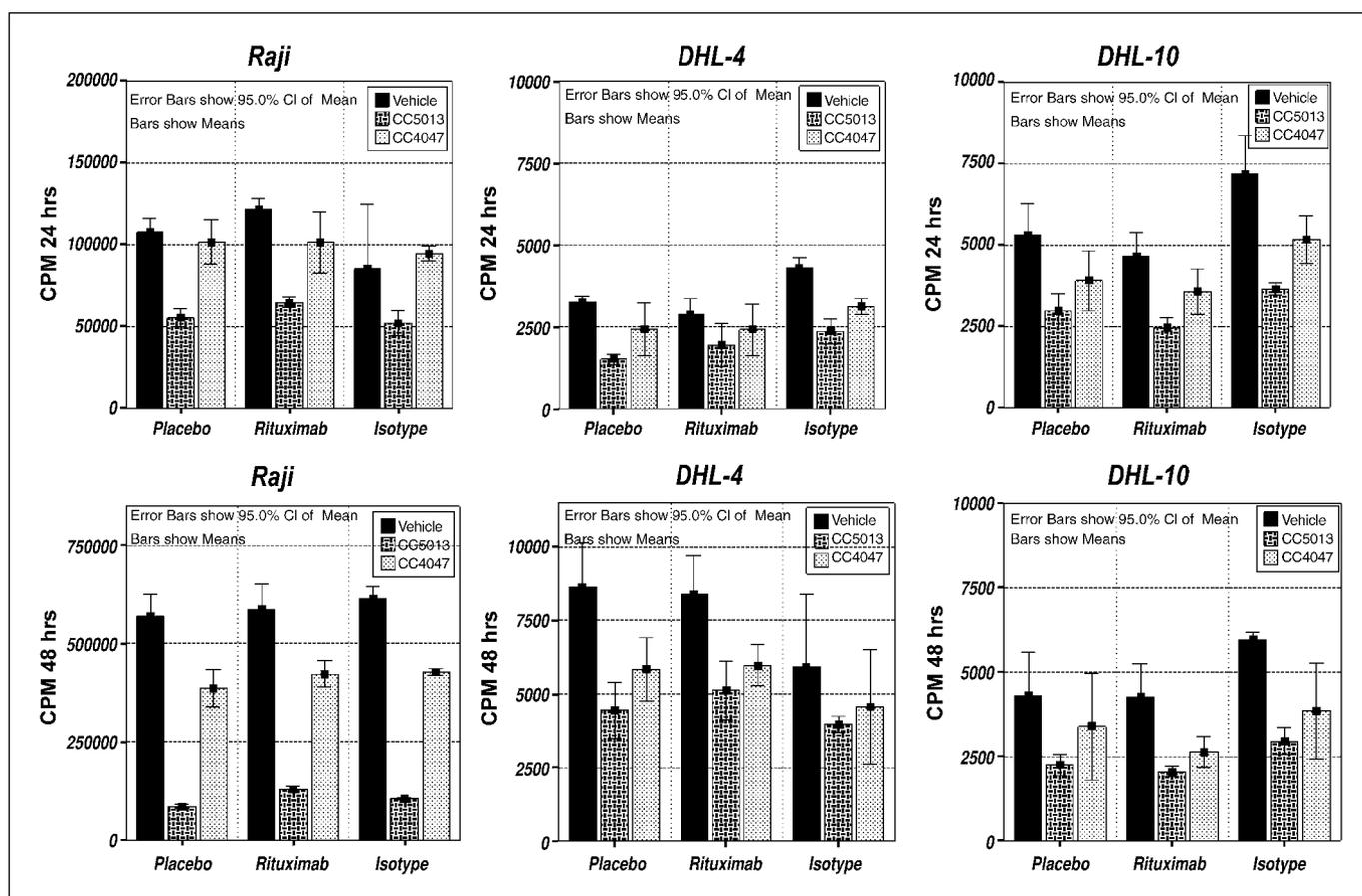
Fig. 3. Following *in vitro* exposure of Raji, SU-DHL-4, or SU-DHL-10 cell lines to IMiDs (CC-5013 or CC-4047) resulted in significant decrease in DNA synthesis and cell proliferation when compared with vehicle-treated cells as measured by [³H]-thymidine incorporation. No synergistic activity was observed in IMiD-treated cells subsequently exposed to each mAb. Lymphoma cell lines were initially exposed to CC-5013, CC-4047, or vehicle control for 24 or 48 hours in the presence of placebo, rituximab, or trastuzumab (isotype). Proliferation rate was determined by [³H]-thymidine incorporation.
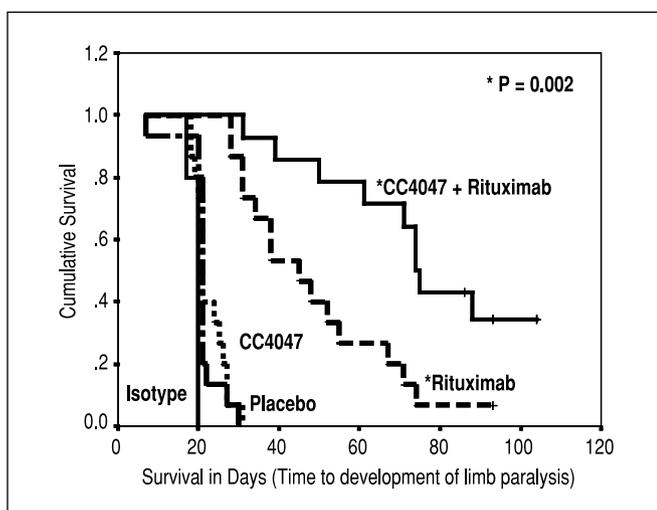
Fig. 4. Kaplan Meier analysis of cumulative survival of SCID mice bearing Raji lymphoma xenografts treated with rituximab and/or CC-4047. Groups of 15 SCID mice were inoculated with 1×10^6 Raji cells via tail vein i.v. injection (day 0) and treated either with placebo, rituximab, isotype (10 mg/kg i.v. on days +5, + 10, +15, and +20), CC-4047 (0.5 mg/dose on days +3, +4, +8, +9, +13, +14, +18, and +19), CC-4047 (0.5 mg/kg on days +3, +4, +7, +8, +11,+12, +15, and +16), or rituximab (10 mg/kg i.v. on days +5, + 10, +15, and +20) in combination with CC-4047 (0.5 mg/kg/d for two consecutive days before each rituximab dose) and analyzed for survival as function of time.
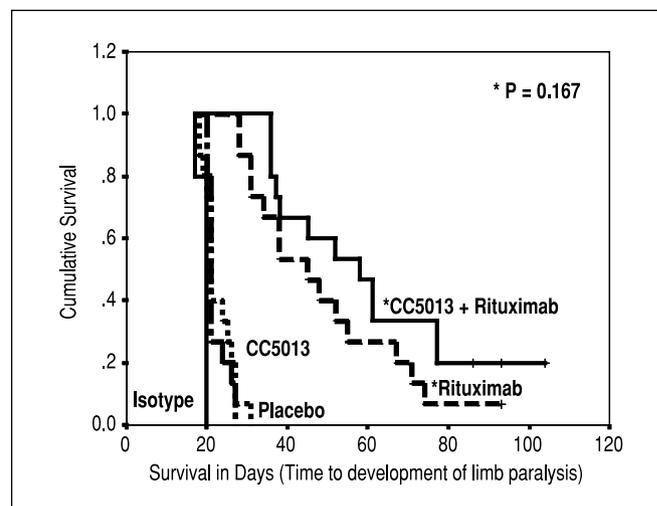
Fig. 5. Kaplan Meier analysis of cumulative survival of SCID mice bearing Raji lymphoma xenografts treated with rituximab and/or CC-5013. Groups of 15 SCID mice were inoculated with 1×10^6 Raji cells via tail vein i.v. injection (day 0) and treated either with placebo, rituximab, isotype (10 mg/kg i.v. on days +5, + 10, +15, and +20), CC-5013 (0.5 mg/dose on days +3, +4, +8, +9, +13, +14, +18, and +19), CC-5013 (0.5 mg/kg on days +3, +4, +7, +8, +11,+12, +15, and +16), or rituximab (10 mg/kg i.v. on days +5, + 10, +15, and +20) in combination with CC5013 (0.5 mg/kg/d for two consecutive days before each rituximab dose) and analyzed for survival as function of time.

with IMiDs. Incubation for up to 24 or 48 hours with either CC-5013 or CC-4047 (at 5 μg/mL final concentration) did not result in changes of surface CD20 (data not shown) on the NHL cell lines used in our experiments.

Concurrent administration of CC-4047, and to a lesser degree CC-5013, with rituximab is more effective in controlling lymphoma growth and in prolonging survival than rituximab monotherapy alone. Treatment of lymphoma-bearing SCID mice with rituximab resulted in prolongation in survival compared with placebo or isotype controls. The median survival time for rituximab-treated animals was 38 days (95% CI, 26-50) compared with a median survival of 21 days (95% CI, 20-22) for those animals receiving placebo (log-rank test, $P < 0.001$) or to a mean survival of 20 days (95% CI, 19-21) for those treated with an isotype control (log-rank test, $P < 0.001$). No significant antitumor activity was observed in animals treated with either CC-5013 or CC-4047 alone, and the median survival was similar to control mice [i.e., 21 days (95% CI, 20-22) for CC-5013-treated and 21 days (95% CI, 21-21) for CC-4047-treated animals; P = not significant; Figs. 4 and 5].

The administration of CC-4047 for two consecutive days before mAb therapy enhanced the antitumor activity of rituximab and doubled the median survival of lymphoma-bearing mice. Statistically, significant differences were observed between animals treated with rituximab versus CC-4047 + rituximab (Table 1). The median survival time of animals treated with CC-4047 and rituximab was longer (median survival, 74 days; 95% CI, 70-78) than those treated with rituximab monotherapy (median survival, 38 days; 95% CI, 26-50; log-rank test, P = 0.002; Fig. 4; Table 1).

To a lesser degree, a similar trend was observe among animals receiving rituximab in combination with CC-5013 (Table 2). The median survival for lymphoma-bearing SCID mice treated with rituximab and CC-5013 was longer (58 days; 95% CI, 43-73) than that of rituximab alone treated animals

(45 days; 95% CI, 32-58). Despite a trend toward an improve survival, differences between rituximab-treated animals and those receiving the concurrent administration of rituximab in combination with CC-5013 did not reach statistical significance (log-rank test, P = 0.167; Table 1).

After a follow-up period of 3 months, survival rates were the highest for animals treated with rituximab and CC-4047 (30%) when compared with animals treated with rituximab alone (5.3%) or in combination with CC-5013 (13.3%). Pathologic examination of all surviving animals at the end of the study failed to show any residual disease.

Natural killer cell expansion occurs in IMiD–treated lymphoma-bearing severe combined immunodeficient mice. The administration of CC-5013 or CC-4047 for two consecutive days led to a significant increase in the number of circulating NK cells as

Table 1. Antitumor activity was observed among animals treated with rituximab or the combination of rituximab and CC-4047

n = 15 mice per group	Median survival, d (95% CI)
Placebo	21 (20-22)
Rituximab	38 (26-50)
Isotype	20 (19-21)
CC4047	21 (21-21)
CC4047 + rituximab	74 (70-78)

NOTE: The administration of CC-4047 for two consecutive days before each mAb dose resulted in a statistically significant enhancement in the biological activity of rituximab. The median survival of rituximab-treated lymphoma-bearing mice was only 38 days. On the other hand, after a follow-up period of 3 months, the median survival time for animals treated with rituximab in combination with CC-4047 was 74 days (log-rank test, P = 0.002).
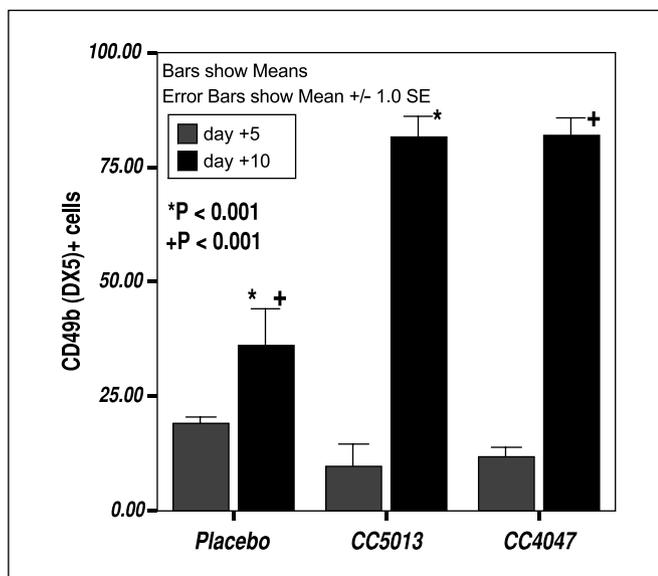
Table 2. Antitumor activity was observed among animals treated with rituximab or the combination of rituximab and CC-5013

n = 15 mice per group	Median survival, d (95% CI)
Placebo	21 (18-24)
Rituximab	45 (32-58)
Isotype	20 (19-21)
CC5013	21 (20-22)
CC4047 + rituximab	58 (43-73)

NOTE: The administration of CC-5013 for two consecutive days before each mAb dose, while improved rituximab activity, did not reach a statistically significance. The median survival of rituximab-treated lymphoma-bearing mice was only 45 days. On the other hand, after a follow-up period of 3 months, the median survival time for animals treated with rituximab in combination with CC-5013 was 58 days (log-rank test, P = 0.167).

shown by flow cytometry analysis, in lymphoma-bearing SCID mice (Fig. 6). The expansion of circulating NK cells was observed at day +10. No significant differences in CD49b (DX5)$^+$ cells were observed at earlier time points (Fig. 6). On day +10 and after four doses of IMiDs, we observed a nearly doubling in the number of CD49$^+$ cells. The percentage of NK cells in vehicle-treated animals was statistically significant lower (mean, 36 ± 11.62% SD) than CC-5013 (mean, 82 ± 6.2% SD) or CC-4047 (mean, 86 ± 2.5% SD; χ^2, P < 0.001).

Depletion of natural killer cells resulted in a complete abrogation on the biological effects of CC-4047 or CC-5013 in rituximab antitumor activity. In an attempt to define the role of NK cell expansion in the synergistic effects observed when rituximab is combined with CC-4047 or CC-5013, we did a

second set of *in vivo* experiments in which NK cells were depleted with a mAb targeting the IL-2 receptor antibody before lymphoma cell inoculation. A single dose of anti-IL2 receptor mAb given i.p. was sufficient to deplete NK cells from SCID animals as shown by flow cytometric analysis (data not shown).

Among untreated NHL-bearing SCID mice, tumor growth and survival (time to development of limb paralysis) was not significantly different between NK cell–depleted mice when compared with NK cell intact mice in the placebo group (data not shown). The median survival time for NHL-bearing NK cell deplete mice was 17 days (95% CI, 17-19 days) versus 21 days (95% CI, 18-24 days) for mice with intact neutrophil function (P = not significant).

Similarly to prior published reports, depletion of NK cells with rat anti-mouse IL-2 receptor mAb before rituximab therapy significantly impaired the outcome of NHL-bearing SCID mice. In addition, the synergistic effects observed by combining rituximab plus CC-4047 and to a lesser degree with CC-5013
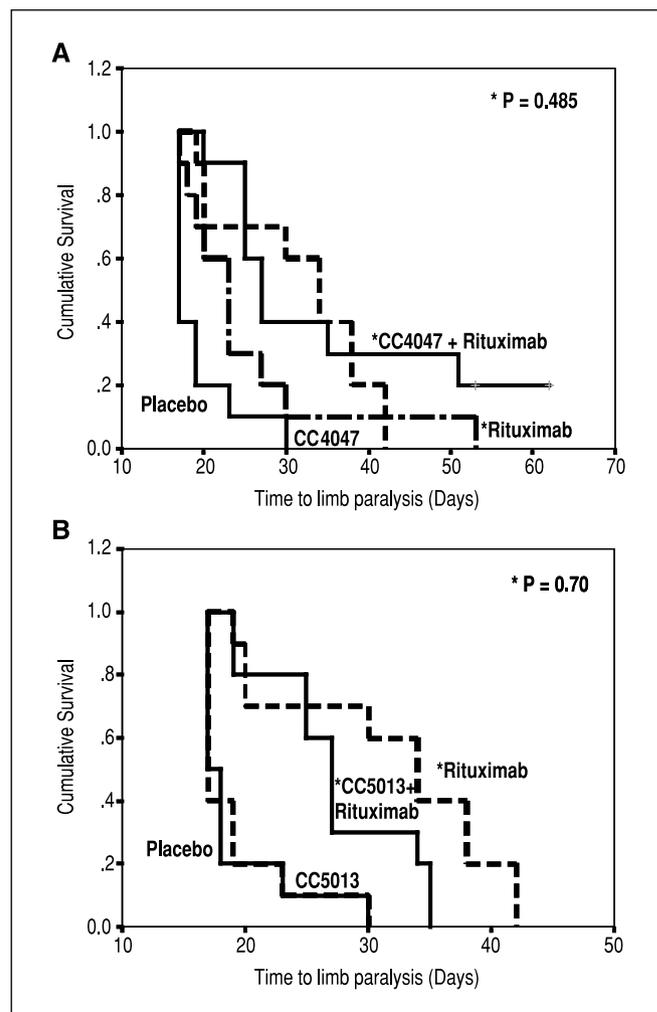

Fig. 6. Flow cytometric analysis of peripheral blood collected from SCID mice showed differences in the expression of the pan-NK cell marker CD49, suggesting *in vivo* expansion in NK cells following treatment with CC-4047 or CC-5013. No significant changes in the number of CD49b (DX5)$^+$ cells was observed between treatment groups at day +5. However, a significant higher number of NK cells (CD49b$^+$ cells) were observed among animals receiving murine CC-4047 and CC-5013 after 10 days of stimulation.

Fig. 7. Kaplan-Meier survival curves show that NK cell depletion dramatically reduces the synergistic effect of CC-4047 on rituximab in lymphoma-bearing SCID mice. Antitumor activity was observed in mice treated with rituximab or rituximab/CC-4047. *A*, however, in NK cell–depleted mice, the median survival between rituximab-treated (34 days; 95% CI, 28-40 days) and rituximab/CC-4047-treated (27 days; 95% CI, 24-30) animals was similar (P = 0.485). *B*, similar effects were observed in mice treated with rituximab ± CC-5013.

were lost following NK cell depletion. The mean survival for NK-depleted mice treated with rituximab of 34 days (95% CI, 28-40 days) was similar to those animals treated with rituximab/CC-5013 (27 days; 95% CI, 25-29 days) or rituximab/CC-4047 (27 days; 95% CI, 24-30 days; log-rank test, $P = 0.70$ and $P = 0.485$, respectively; Fig. 7).

Discussion

Whereas the use of rituximab has been integrated in combination with chemotherapy regimens into the standard management of various subtypes of B-cell lymphomas, a significant number of patients fail to respond or will eventually relapse as a consequence of acquired resistance. Current scientific efforts are based on developing strategies that can potentially enhance the antitumor activity of rituximab without the addition of significant toxicities.

Modulation of immune responses is an attractive strategy to enhance the biological activity of mAbs. CC-5013 and CC-4047, analogues of thalidomide, are best known as IMiDs. *In vitro* studies with these agents showed not only a higher antitumor activity when compared with thalidomide against myeloma cells but also a unique capacity to enhance the innate immune system (i.e., activation of NK cells and production of IL-2; refs. 26, 27). The antitumor activity of IMiDs is been studied in multiple clinical trials (28, 29). IMiDs inhibit tumor necrosis factor-α production by PBMC *in vitro* up to 50,000 more potently than thalidomide (24). Their exact mechanism of action is yet to be defined, although the immunomodulatory effects are thought to stem from the drug's inhibition of the proinflammatory cytokine tumor necrosis factor-α, inhibition of factors that promote tumor growth such as vascular endothelial growth factor, induction of apoptosis, and/or augment NK cell cytotoxicity against multiple myeloma (23, 25).

Of interest our data show that IMiDs are capable of decreasing cell proliferation and induce modest apoptosis in lymphoma cells *in vitro*. When used as single agents, IMiDs possess limited antitumor activity *in vivo* against lymphoma xenografts suggesting that these agents are cytostatics rather than cytotoxic. Induction of apoptosis and direct antiproliferative effects had been reported by other group of investigators in multiple myeloma cell lines (30). The exact signaling events occurring following IMiD exposure to susceptible cells *in vitro* are yet to be determined. A plausible hypothesis could be a decrease in the nuclear factor-κB activity in neoplastic cells. Recent published studies evaluating newer subclasses of thalidomide analogues had shown significant antitumor activity mediated by caspase-3 activation and down-regulation of antiapoptotic proteins such as Bcl-2 (31).

In addition, in our experiments, we were able to show that CC-4047 and to a lesser degree CC-5013 are capable to enhance the antitumor activity of rituximab *in vivo*. The enhancement in the antitumor activity of rituximab seems mediated by NK cell expansion occurring following IMiD therapy *in vivo* as we further showed in our NK cell depletion studies. The capacity of CC-4047TM or CC-5013 to produce *in vivo* NK cell proliferation and enhance rituximab activity could be the result of direct stimulation of IMiDs to the effector cells or by indirect costimulation of dendritic cells. Specifically, it has been shown that thalidomide or IMiDs trigger the proliferation of CD3^{+} cells only in the presence of anti-CD3 antibodies or dendritic cell priming, suggesting that this agents are costimulatory molecules rather than mitogenic drugs (32–34).

It is important to notice that whereas in our *in vivo* experiments CC-4047 was more potent in modulating the activity of rituximab, CC-5013 was more effective to inhibit the cell growth of lymphoma cells *in vitro*. In addition, the maximum tolerated dose of CC-5013 in cancer patients is 10 times higher that then maximum tolerated dose of CC-4047.

To our knowledge this is the first scientific report showing a significant biological interaction between CC-4047 and rituximab and serve as the basis for future clinical trials evaluating the combination of rituximab and IMiDs in CD20^{+} B-cell lymphomas.

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Immunomodulatory Drug CC-5013 or CC-4047 and Rituximab Enhance Antitumor Activity in a Severe Combined Immunodeficient Mouse Lymphoma Model

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